# Molecular mechanism of action of the glucocorticoid receptor: Role of ligand-dependent receptor phosphorylation and half-life in determination of ligand-specific transcriptional activity.

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**Co-supervisor: Professor A. Louw**

# Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work (unless acknowledged otherwise) and that I have not previously in its entirety or in part submitted it at any university for a degree.

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Signature

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Date

# Dedication

Hierdie tesis word opgedra aan my ouers, Nellis en Petra Avenant. Sonder jul ondersteuning, geduld en absolute vertroue in my, sou hierdie tesis nie moontlik gewees het nie. Julle liefde, entoesiasme en aanmoediging om my drome na te jaag is sonder weerga en het my gedra tot hier waar ek nou is. Ek sal altyd dankbaar wees vir jul voorbeeld, integriteit, nederigheid en deernis. Dit het ’n onuitwisbare indruk op my lewe gemaak.

“The secret of life is not to do what you like, but to like what u do.”

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**Molecular mechanism of action of the glucocorticoid receptor: Role of ligand-dependent receptor phosphorylation and half-life in determination of ligand-specific transcriptional activity.**

**C. Avenant (August 2009)**

## Abstract

Glucocorticoids mediate their effects by binding to the glucocorticoid receptor (GR), resulting in modulation of transcription of target genes via direct binding to DNA or tethering via protein-protein interactions. A central question is what determines the rank order of ligand-selective transcription with different GR ligands for the same gene in the same cell. Using a panel of twelve GR ligands, including agonists, partial agonists and antagonists, the relationship between the extent of GR phosphorylation at S226, GR turnover and transcriptional response, was investigated using a variety of biochemical approaches. Using a phospho-S226-specific GR antibody, ligand-selective S226 phosphorylation was shown to occur in both COS-1 and U2OS cells, while GR phosphorylation at S226 was shown to inhibit maximal transactivation and transrepression efficacy. Attempts to identify the kinases responsible for this interaction were inconclusive but suggested a combination of kinases is responsible for the *in vivo* phosphorylation of the hGR in these cells. Similarly the rate of GR degradation was different for the different ligands. Interestingly, both ligand-selective GR phosphorylation and half-life were found to correlate with efficacy for transactivation and transrepression of model synthetic reporter genes, where agonists resulted in the greatest extent of phosphorylation and the fastest

rate of GR turnover, suggesting a link between these functions. Furthermore experiments where transcription was blocked suggest that GR turnover does not require transcription. However, using a S226A GR mutant, as well as in experiments where GR turnover was blocked, it was established that neither phosphorylation of the GR at S226 nor GR degradation rate determines the rank order of ligand-selective GR transactivation. The mechanisms whereby GR phosphorylation influence GR-mediated transcription was further investigated using a triple phosphorylation deficient mutant. It was shown that phosphorylation at one or more of residues S203/S211/S226 is required for transactivation of a MMTV promoter but does not affect unliganded or agonist-induced GR degradation and acetylation. Additionally, it was shown that phosphorylation at S203/S211/S226 is not the sole determinant of co-activator p300 recruitment to the GR. Interestingly, GR-mediated transrepression via AP-1 is less sensitive to GR phosphorylation than GR-mediated transactivation, indicating different mechanisms in the role of GR phosphorylation on transactivation vs. transrepression. Pull-down and chromatin immunoprecipitation assays showed that phosphorylation of the GR at one or more of these residues are required for interaction of the GR with the co-activator GRIP-1 *in vitro* and for maximal recruitment of GR and GRIP-1 to the MMTV promoter in intact cells. Cellular fractionation showed that phosphorylation at these residues is not however required for GR nuclear localisation. Taken together these results support the conclusion that phosphorylation at one or more of S203/S211/S226 of the hGR is required for maximal transactivation response to enable GRIP-1 recruitment to the hGR.

## List of abbreviations

| | |
|---|---|
| 3A | S203A/S211A/S226A triple phosphorylation mutant |
| ABCD | Avidin Biotin Complex DNA |
| AF1 | Activation region 1 |
| AF2 | Activation region 2 |
| AL438 | Compound Abbott-Ligand 438 |
| ald | Aldosterone |
| AP-1 | Activator protein-1 |
| AR | Androgen receptor |
| ATP | Adenosine triphosphate |
| cAMP | Cyclic adenosine monophosphate |
| CBP | CREB-binding protein |
| CDK | Cyclin dependent kinase |
| CHIP | Carboxy terminus of heat shock protein 70-interacting protein |
| ChIP | Chromatin Immunoprecipitation |
| CHX | Cycloheximide |
| cort | Cortisol |
| CpdA | 2(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammonium chloride |
| CRH | Corticotrophin releasing hormone |
| DBD | DNA binding domain |
| dex | Dexamethasone |
| DHT | Dihydrotestosterone |

| | |
|---|---|
| DNMT | Phenylethanolamine N-methyltransferase |
| DO6 | Abbott-Ligand 082D06 |
| DRIPs | Vitamin D interacting proteins |
| EC50 | Effective concentration 50% |
| ER | Estrogen receptor |
| FISH | Fluorescent in situ hybridisation |
| FKBP51 | FK506 binding protein 51 |
| FKBP52 | FK506 binding protein 52 |
| FRAP | Fluorescence Recovery After Photobleaching |
| GCs | Glucocorticoids |
| GFP | Green fluorescent protein |
| GILZ | GC-induced leucine zipper |
| GR | Glucocorticoid receptor |
| GRE | Glucocorticoid response element |
| GRE1/2 | GRE half site |
| GRIP-1 | GR-interacting protein 1 |
| GRU | Glucocorticoid response unit |
| GSK3β | Glycogen synthase kinase 3 β |
| HAT | Histone acetyltransferase |
| HDAC | Histone deacetylase |
| hGR | Human GR |
| HNF1 | Hepatocyte nuclear factor-1 |
| HPA | Hypothalamic-pituitary-adrenal |
| HREs | Hormone response elements |
| HSP70 | Heat-shock protein 70 |

| | |
|---|---|
| HSP90 | Heat-shock protein 90 |
| IC50 | Inhibitory concentration 50% |
| IGFBP1 | Insulin like growth factor binding protein-1 |
| IL | Interleukin |
| IRF8 | Interferon Regulatory Factor 8 |
| JNK | c-Jun N-terminal kinase |
| $K_d$ | Equilibrium dissociation constant |
| LAD1 | Leukocyte adhesion deficiency-1 |
| LBD | Ligand binding domain |
| MAPK | Mitogen-activated protein kinase |
| MG132 | Z-Leu-Leu-Leu-al |
| mGR | Mouse GR |
| MMTV | Mouse mammary tumor virus |
| MPA | 6α-Methyl-17αhydroxyprogesterone acetate |
| MR | Mineralocorticoid receptor |
| NCoR | Nuclear receptor corepressor |
| NES | Nuclear export signal |
| NET | Norethindrone acetate |
| NF-1 | Nuclear factor 1 |
| NfκB | Nuclear factor-κB |
| nGRE | Negative GRE |
| NLs | Nuclear localisation signals |
| NRIDs | Nuclear receptor interacting domains |
| NRs | Nuclear receptors |

| | |
|---|---|
| NTD | N-terminal domain |
| p300 | Adenovirus E1A-binding protein 300 |
| PAH | Phenylalanine hydroxylase |
| pCMV | Cytomegalovirus promoter |
| PMA | 4α-Phorbol 12-myristate 13-acetate |
| POMC | Pro-opiomelanocortin |
| PP5 | Protein phosphatase 5 |
| PPAR | Peroxisome proliferators-activated receptor |
| PR | Progesterone receptor |
| predn | Prednisolone |
| prog | Progesterone |
| P-S211 | phospho-serine 211 |
| P-S226 | phospho-serine 226 |
| RAR | Retinoic acid receptor |
| RBA | Relative binding affinity |
| rGR | Rat GR |
| RID | Receptor interacting domain |
| RU486 | Mifepristone |
| RXR | Retinoid X receptor |
| S203 | Serine 203 |
| S211 | Serine 211 |
| S226 | Serine 226 |
| SMRT | Silencing mediator for retinoic acid and thyroid hormone receptors |
| SRC | Steroid receptor coactivator |
| SUMO-1 | Small ubiquitin-related modifier-1 |

| | |
|---|---|
| TAT | Tyrosine aminotransferase |
| TGF | Transforming growth factor |
| TIF2 | Transcriptional intermediary factor 2 |
| TNF-α | Tumor necrosis factor-α |
| TPR | Tetratricopeptide repeat |
| TR | Thyroid receptor |
| TRAPs | Thyroid receptor associated proteins |
| TSG101 | Tumor suppressor gene |
| UDCA | Ursodeoxycholic acid |
| UPP | Ubiquitin/proteasome dependent protein degradation pathway |
| VDR | Vitamin D receptor |
| β-gal | β-galactosidase |

# Thesis outline

This thesis contains the following sections:

1. Chapter 1: **Literature review**. This chapter gives a detailed overview of the relevant knowledge currently available in the literature on GR function and transcriptional regulation, with particular focus on ligand-selectivity, phosphorylation and degradation of the GR.
2. Chapter 2: **Thesis Rationale, Aims and Hypotheses**. This chapter will briefly outline the relevant issues in the steroid receptor research field, explain the place of this thesis as being part of a larger investigation, and state the context, aims and hypotheses of this thesis.
3. Chapter 3: **Materials and Methods**. This chapter provides detailed information on the protocols used to obtain the results presented in chapters 4 to 7.
4. Chapter 4: **Ligand-selective GR phosphorylation (Results and Discussion)**. The first part of this study used a phospho-S226 GR-specific antibody to determine ligand-selective GR phosphorylation at S226 of transiently expressed hGR in COS-1, as well as stably transfected hGR in U2OS cells. Furthermore, ligand-selective GR phosphorylation was correlated to efficacy and potency for transactivation and transrepression (Addendum E) obtained with hGR in COS-1 cells. These efficacy and potency results were obtained by Elisabeth Stubsrud and Katharina Ronacher (previously from the same research group as the present author), as part of the larger investigation and are published (Stubsrud, 2005;Ronacher *et al*., 2009). Additionally, the role of GR phosphorylation on ligand-selective GR transcription is also presented in this chapter. Some of these results will

shortly be submitted for publication. The cell culture work was performed by the present author, except for plating and maintenance of stock cultures, which was done by Carmen Langeveldt.

5. Chapter 5: **Investigation into the kinases involved in GR phosphorylation (Results and Discussion).** This section contains results and discussion on the kinases involved in GR phosphorylation at S226 and S211. The ability of specific kinase inhibitors (JNK, p38 and CDK inhibitors), as well as over expression of wt and dominant negative kinases (JNK, p38 and CDK5), to inhibit GR phosphorylation at S226 and S211 was investigated in COS-1, LβT2 and/or END-1 cells. All the results presented in this chapter were obtained by the present author, and all the cell work was performed by the present author.
6. Chapter 6: **Ligand-selective GR degradation (Results and Discussion)**. This section contains results and discussion on ligand-selective GR degradation, as well as correlations thereof to ligand-selective transactivation and transrepression (Addendum E). Additionally, an investigation into the relationship between ligand-selective GR degradation and transactivation is presented in this chapter. These results will shortly be submitted for publication. All the results presented in this chapter were obtained by the present author, and all the cell work was performed by the present author.
7. Chapter 7: **Role of agonist-induced GR phosphorylation in GR mechanism of action (Results and Discussion)**. This section present results and discussion on the relationship between agonist-induced GR phosphorylation and transcription, as well as between GR phosphorylation and GR degradation. Additionally, this chapter presents results on the role of agonist-induced GR phosphorylation on additional GR functions, including acetylation and co-factor recruitment. The majority of these results have been submitted to the journal Biochemistry. All the results presented in this chapter were obtained by the present author, and all the cell work was performed by the present author.

8. Chapter 8: **Conclusions and future perspectives**. In this chapter, conclusions are discussed and drawn from the combined results in chapters 4 to 7. Some perspectives about future research are also included.
9. Addendum A: **Basic principles for evaluating transcriptional responses.** This section contains a discussion of the basic principles used in this study to evaluate the transcriptional responses, including potency, efficacy and ligand binding affinity. Additionally, this section contains an explanation of the basic principles used to perform the correlation analysis.
10. Addendum B: **Panel of GR ligands**. This section contains a detailed discussion of the available literature on each of the ligands used in this study.
11. Addendum C: **Antibodies**. This addendum contains a list, as well as the specific dilutions and incubation times used, for all the antibodies that were used in this study.
12. Addendum D: **Supporting data from the present author**. This addendum contains supporting data (relevant to chapter 4) not provided in results chapter 4 of the manuscript. GR dose-response curves with an agonist and partial agonist at different GR concentrations are shown. These results were obtained by the present author, and the cell work was performed by the present author.
13. Addendum E: **Supporting data from other researchers.** This addendum contains additional data that is used in this thesis for correlations and/or discussion, but that was performed by E. Stubsrud and K. Ronacher, as part of the larger investigation. Results on ligand-selective GR transactivation, phosphorylation at S211, as well as the role of GR phosphorylation at S211 on transactivation was obtained by E. Stubsrud (Stubsrud, 2005). Results obtained on ligand-selective GR binding, transrepression, as well as the effect of kinase inhibitors on transactivation and transrepression were obtained by K. Ronacher. Most of this data has already been published (Ronacher *et al*., 2009). In addition, the

present author worked closely together with E. Stubsrud in obtaining data on ligand-selective GR phosphorylation at S211, and also performed all the correlation analyses shown in this addendum.

14. **Ronacher *et al.* 2009**. The published research article that contains some of the data in addendum E. The present author contributed to this research article, in terms of intellectual planning of the experiments and interpretation of the data. Furthermore, all the correlation analyses were performed by the present author.

# Table of contents

# Chapter 1 Literature review

## 1.1 Nuclear Receptors

Nuclear receptors (NRs) are ligand activated transcription factors that regulate transcription through binding of small lipophilic molecules (ligands). Genes regulated by NRs can either be upregulated (transactivation) or down-regulated (transrepression) and play a role in most biological responses including the stress response, metabolism, immune function, growth, development and reproduction. Since the isolation of the estrogen receptor (ER) in 1961 and the cloning of the estrogen, glucocorticoid and thyroid hormone receptors in the 1980s, 48 known human NRs have been characterised (Tata, 2005;Gronemeyer *et al*., 2004;Zhang *et al.*, 2004). Based on evolutionary and sequence homology, NRs can be divided into six sub-families, which in turn can be divided up into different groups (Laudet, 1997;Nuclear Receptors Nomenclature Committee, 1999). The large subfamily one, denoted NR1, contains the thyroid hormone receptor (TR), retinoic acid receptor (RAR), the peroxisome proliferators-activated receptor (PPAR) and the vitamin D receptor (VDR) to name but a few (Fig. 1.1). Of most interest in the second subfamily (NR2), is the retinoid X receptor (RXR), which function as partners for NRs that bind DNA as heterodimers and thereby play an important role in NR signalling. Subfamily three (NR3) contains the steroid and sex hormone receptors, i.e. the glucocorticoid receptor (GR), mineralocorticoid receptor (MR), progesterone receptor (PR), androgen receptor (AR) and the estrogen receptor (ER). Subfamily four (NR4) and subfamily five (NR5) consist of the nerve growth factor IB-like and steroidogenic factor-like receptors, respectively, whereas subfamily six (NR6) contains the germ cell nuclear factor-like receptors. All nuclear receptors share a common

structure, consisting of three major domains, the highly variable N-terminal domain (NTD), the DNA binding domain (DBD) and the ligand binding domain (LBD). Small lipophilic ligands bind to the LBD of most NRs, which is proposed to result in a conformational change in the receptor. Most of these receptors (but not all) then bind to specific DNA sequences called hormone response elements (HREs), or other transcription factors, and regulate the transcription of a variety of genes (Reviewed in (Kumar and Thompson, 2005;Aranda and Pascual, 2001;Griekspoor *et al.*, 2007)).

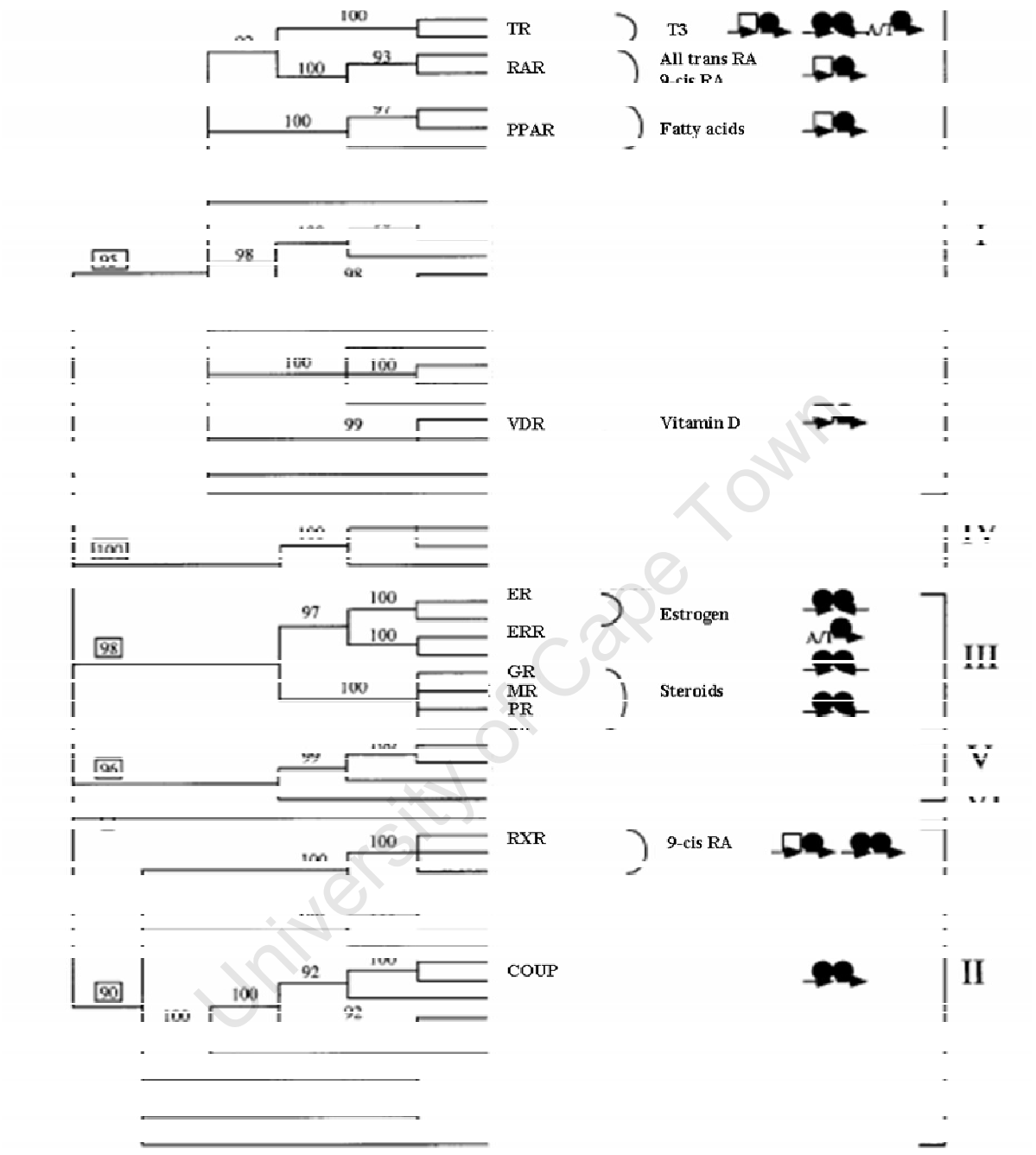





**Figure 1.1 Phylogenetic consensus tree of NRs.** Taken and modified from (Laudet, 1997). Black circle represent the NRs and the arrows represent the PuGGTCA NRE which can be a single unit, a palindrome or a direct repeat, whereas the open squares represent the RXR in heterodimeric complexes.

Nuclear receptors play a role in most physiological processes and are associated with a variety of diseases, including diabetes, obesity, cancer, atherosclerosis, cardiovascular diseases, osteoporosis and Alzheimer's disease, to name but a few (Kersten *et al.*, 2000;Shang *et al.*, 2000). Furthermore, nuclear receptor ligands can easily be exchanged with a drug of choice and therefore nuclear receptors and their ligands are ideal drug targets. Extensive research has been done on NRs and their ligands (Sladek, 2003).

## 1.2 Glucocorticoids

Glucocorticoids (GCs) are the most commonly prescribed anti-inflammatory drugs in the treatment of asthma and rheumatoid arthritis, as well as in treatment of autoimmune diseases and pulmonary diseases and have been used pharmacologically since the availability of cortisone about 50 years ago (Moutsatsou P, 2008). GCs suppress inflammation by down-regulating the expression of pro-inflammatory cytokines such as interleukin (IL) 1β, or by up-regulating cytokines such as transforming growth factor (TGF)-1β-3 and IL-10, which in turn suppress the production of pro-inflammatory mediators (Ashwell *et al.*, 2000;Galon *et al.*, 2002). Due to their anti-inflammatory, as well as their antiemetic, antiedema and palliative properties, GCs are given as co-medication with chemotherapy in cancer patients (recently reviewed in (Moutsatsou P, 2008;Lu *et al.*, 2006). However, long-term treatment with GCs causes serious side effects, including neural and physiological disturbances such as psychosis and depression. High levels of glucocorticoids, caused by chronic GC treatment, inhibit osteoblasts and collagen synthesis, as well as calcium absorption in the gastrointestinal tract and kidneys and thereby results in osteoporosis (Luengo *et al.*, 2001;Reid and Ibbertson, 1987;Robson *et al.*, 2002). The first direct

evidence for GCs negatively impacting bone formation came from a study that showed that local treatment with dexamethasone (dex) resulted in significant decrease in tibial growth, compared to the contralateral limb in rabbits (Baron *et al*., 1992). Furthermore, recent studies also indicate that the co-treatment of GCs with chemotherapy in breast cancer hampers the responsiveness of the cancer to the chemotherapy (Montecucco *et al*., 1992). GCs also induce sex hormone deficiency and alter vitamin D metabolism, leading to deleterious effects on growth and skeletal integrity (Montecucco *et al*., 1992).

By microarray analysis of GC-regulated genes, Galon *et al.* showed that GCs can have both positive and negative effects on inflammation and the immune response by either up-regulating or down-regulating a variety of genes, including pro-inflammatory and anti-inflammatory genes, as well as genes involved in cell trafficking and scavenger systems (Galon *et al.*, 2002). They, along with others hypothesize that the effect of GCs on inflammation and the immune response is context dependent and can be related to the state of disease, as well as by different sets of GR-recruited transcription factors, including different co-activators and co-repressors (Galon *et al*., 2002;Franchimont *et al*., 1999;Carroll, 1998;McKenna *et al*., 1999b).

The hypothalamic-pituitary-adrenal (HPA) axis controls the synthesis of GCs in the adrenal cortex. Endogenous GCs, e.g. cortisol (cort) in humans, are secreted into the blood stream in a continuous pulsatile pattern at a frequency of approximately 20 pulses per day (Fig. 1.2) which forms the basis of the diurnal rhythm in humans (Windle *et al*., 1998;Lightman *et al*., 2008). GCs can act in a negative feedback loop on the HPA axis, in different ways, involving both non-genomic as well as genomic responses (Hinz and Hirschelmann, 2000;Keller-Wood and Dallman, 1984). GCs circulate in the body bound to corticosteroid-binding globulin and serum albumin and mediate their action through binding of the GR, which in turn can regulate transcription of up

to 20% of the genes in the human genome (Galon *et al.*, 2002). The GR can repress the transcription of a variety of genes including pro-inflammatory genes, as well as transactivate the transcription of anti-inflammatory genes including IL-10 and annexin, which results in the desired anti-inflammatory effect of GCs. However, as discussed above, the GR can also transactivate gene expression of a variety of genes involved in the negative side-effects of prolonged or chronic GC treatment (Recently reviewed in (Lowenberg *et al.*, 2009)). A better understanding of the GR, as well as the development of GCs that can distinguish between the desired anti-inflammatory and the undesired side-effects, is therefore crucial for future pharmacological use of GCs.

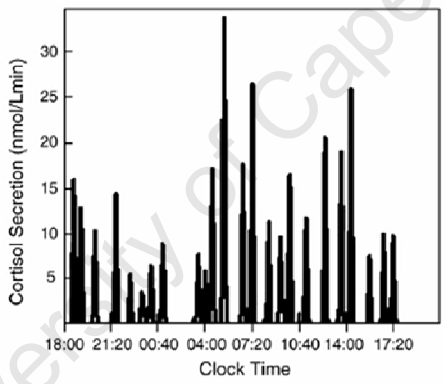



**Figure 1.2 Cortisol secretion in a healthy human female.** At a frequency of approximately 20 pulses per day with a pulse mass of 115.1 nmol/L/min. Taken from (Lightman *et al.*, 2008).



## 1.3 GR gene and protein structure

In 1989, the singular hGR gene was mapped to chromosome 5q31-32 and it was subsequently shown to comprise 10 exons spanning a 110 kb region (Francke and Foellmer, 1989;Theriault *et al.*, 1989;Encio and Detera-Wadleigh, 1991) (Fig. 1.3). Exon 1 comprises 184 nucleotides and

encodes the 5'-untranslated region, while exon 2 (1197 bp) encodes most of the N-terminal region (Fig. 1.3). Exon 3 (167 bp) and exon 4 (117 bp) each encodes a zinc-finger motif and together makes up the DBD, while exons 5, 6, 7, 8, 9α and 9β encode the LBD and the 3'-untranslated region (Reviewed in (Zhou and Cidlowski, 2005)). Alternative splicing of exon 9α and 9β results in two different GR proteins, namely GRα and GRβ, containing either exon 9α or 9β, respectively. These two proteins contain the same functional domains, however GRβ has a truncated LBD. In the absence of ligand, the classical GRα is mainly cytoplasmic and translocates to the nucleus upon ligand binding. In contrast, the GRβ isoform is mostly nuclear in the absence of ligand and has been shown to repress cytokine genes (IL5 and IL13) in HeLa cells in the absence of ligand through the recruitment of histone deacetylase 1 (HDAC1) (Kelly *et al.*, 2008). Recently microarray analysis has shown that overexpressed hGRβ in U2OS and COS-1 cells can both upregulate and repress several genes in the absence of ligand and that binding of RU486 to the GRβ diminishes this capacity (Lewis-Tuffin *et al.*, 2007). Additionally, the GRβ isoform has been shown to form heterodimers with the GRα isoform and act as a dominant negative to transcriptional regulation by GRα (Oakley *et al.*, 1999;Oakley *et al.*, 1996;Bamberger *et al.*, 1995). High expression levels of the human GRβ isoform have been reported in a variety of tissues, and more strikingly, increased GRβ expression has been identified in a variety of diseases related to GC resistance. For example 10 out of 12 patients with glucocorticoid-resistant colitis have been shown to contain high levels of hGRβ (Reviewed in (Lu and Cidlowski, 2004)). The increased expression of GRβ and its dominant negative effect on GRα could account for GC resistance associated with various diseases and is under intensive investigation.

Three additional GR splice variants, namely GRγ, GR-A and GR-P have also been identified. GRγ differs from GRα and GRβ by the addition of an arginine between exons 3 and 4, while GR-

A lacks exons 5, 6 and 7 and GR-P lacks exons 8 and 9. All three of the above mentioned splice variants (GRγ, GR-A and GR-P) have been found to be highly expressed in various GC resistant diseases, including GC resistance in childhood acute lymphoblastic leukaemia (Reviewed in (Lu and Cidlowski, 2004)). Apart from the above mentioned isoforms, due to alternative splicing, both GRα and GRβ mRNA can be differentially translated into multiple GRα and GRβ isoforms that are truncated at the N-terminal (Recently reviewed in (Moutsatsou P, 2008)). However, most of the above mentioned isoforms have a reduced (if any) transactivation potential as compared to GRα.

The classical GRα protein consists of 777 amino acids and can be divided into 3 independent domains, namely the NTD, the DBD and the LBD (Fig. 1.4). To date, the crystal structure of the whole GRα protein, or any other steroid receptor, has not been solved. For the GR, this is mainly due to an unordered NTD structure (Lu *et al.*, 2006). The NTD is the most variable in both size and sequence homology between different species of GR. Nevertheless, the NTD contains the transcriptional activation region 1 (AF1), which can act constitutively in the absence of the LBD and is required for maximal transcriptional activity (Dieken and Miesfeld, 1992;Godowski *et al.*, 1987;Giguere *et al.*, 1986). Interestingly, the AF1 region is not essential for life, as transgenic mice without the AF1 region still live (Mittelstadt and Ashwell, 2003). Furthermore, the AF1 region (comprising amino acids 77-262/282) binds selectively to several transcription factors, including TATA box-binding protein (TBP), CREB-binding protein (CBP) and steroid receptor co-activator 1 (SRC-1) (Kumar *et al.*, 2001;Kumar and Thompson, 2005). Additionally, the NTD is a major site for post-translational modifications such as phosphorylation and sumoylation.

**Figure 1.3 GR gene and multiple GR isoforms.** Modified from (Moutsatsou P, 2008) and (Zhou and Cidlowski, 2005).

The DBD (65 amino acids) is the most conserved region and is composed of two highly conserved zinc-fingers (Fig. 1.4). The crystal structure of the GRα DBD revealed that each zinc

atom is coordinated in a tethrahedral arrangement by four cysteines (Luisi *et al.*, 1991;Freedman *et al.*, 1988), with the resulting tertiary structure containing helices that interact specifically with DNA. The amino acids of the first zinc finger are responsible for specific binding of the GR to its glucocorticoid response element (GRE), whereas the amino acids of the second zinc finger stabilize the GR:GRE interaction. Additionally, the amino acids of the second zinc finger are also important for homodimerization of the GR (reviewed in (Kumar and Thompson, 2005)). The DBD also participates in interactions with other proteins, such as c-Jun and together with the LBD controls nuclear translocation, as both the DBD and the LBD contain nuclear localisation signal sequences (NLs) (Picard and Yamamoto, 1987) (Fig. 1.3). The DBD is connected to the moderately conserved LBD through a flexible hinge region.





**Figure 1.4 Structural Organization of the hGRα protein.** Taken and adapted from (Smoak and Cidlowski, 2004).

The LBD (approximately 250 amino acids) is located at the carboxy-terminal end of the receptor and is responsible for recognition and binding of the hormone ligands (Fig. 1.4). The crystal structure of the LBD of the GR, both in the presence of agonists or antagonists, as well as in the absence of hormone, indicates that the LBD of the GR consists of 12 α-helices and 4 small β strands that form a globular structure consisting of three sets of helices forming the sides, as well

as the top of the globule, thereby making a central hydrophobic ligand-binding pocket comprised of residues from helices 3, 4, 5, 6, 7, 10 and 12, as well as the β strands 1 and 2 (Bledsoe *et al.*, 2002). Additionally, the LBD, along with the DBD and possibly the hinge region, plays an important role in receptor homodimerisation. Two residues within the LBD, namely I628 and P625 have been shown to be important for homodimerisation of the GR LBD (Bledsoe *et al.*, 2002). The LBD has also been shown to play a role in binding to the heat-shock proteins, as well as in co-factor recruitment through its ligand-dependent transactivation domain 2 (AF2) (Reviewed in (Bledsoe *et al.*, 2004)).



## 1.4 GR mechanism of action

### 1.4.1 Ligand-binding and activation of the GR

In the absence of hormone, the inactive GR monomer forms part of a larger heteromeric protein complex that resides in the cytoplasm and maintains the GR in a conformation suitable for ligand-binding (Wikstrom *et al.*, 1987;Dalman *et al.*, 1989;Picard *et al.*, 1990) (Fig. 1.5). This heteromeric protein complex includes heat-shock protein 90 (HSP90), 70 (HSP70), other heat-shock proteins, immunophilins such as FK506 binding protein 51 (FKBP51), phosphatases such as protein phosphatase 5 (PP5) and p23 (Pratt and Toft, 1997;Wang *et al.*, 2007b;Kumar and Thompson, 2005;Zhou and Cidlowski, 2005;Moutsatsou P, 2008). Glucocorticoids are lipophilic hormones that passively diffuse over the plasma membrane. Once inside the target cell, pharmacologically administered glucocorticoids are metabolised by 11β-hydroxysteroid dehydrogenase that converts the inactive glucocorticoid into an active glucocorticoid, for

example cortisone into active cortisol (Reviewed in (Seckl, 2004;Tomlinson *et al.*, 2004). Additionally, active cortisol, secreted from the adrenals, also travels through the blood and can passively diffuse over the plasma membrane (Lu *et al.*, 2006).



**Figure 1.5 GR mechanism of action.** Taken and modified from (Gross and Cidlowski, 2008). TPR = Tetratricopeptide; Pol II = RNA polymerase II; P = phosphorylated

The active glucocorticoid can then bind to the GR. It is generally accepted that upon binding, different ligands result in different conformational changes of the GR. The crystal structure of the LBD of the GR bound by the agonist dex (Bledsoe *et al.*, 2002;Kauppi *et al.*, 2003), as well as the antagonist RU486 (Kauppi *et al.*, 2003) revealed that the final position of helix-12 is very different in the presence of a GR agonist versus a GR antagonist (Fig. 1.6). Helix-12 contains the AF2 domain and binds co-factors through their LXXLL motif. Agonist binding changes the position of helix-12 to fold over the ligand binding cavity and creates a favourable surface for the binding of co-activators, e.g. transcription intermediary factor 2, TIF2 (Fig. 1.6). Upon antagonist binding, the position of helix-12 changes into a position that covers the co-activator cavity, preventing co-activators from binding (Kauppi *et al.*, 2003). More recently, results obtained with hydrogen/deuterium exchange mass spectrometry (HXMS) analysis, showed that different GR conformations occur in the presence of dex vs. RU486 as well different GR conformations with dex-mediated recruitment of TIF2, versus RU486-mediated recruitment of NCoR (Frego and Davidson, 2006). These ligand-induced conformational changes are accompanied by GR phosphorylation, as well as the release of the GR from the heteromeric HSP protein complex, which exposes the nuclear localisation signals (NLs).

**Figure 1.6 The crystal structure of the GR LBD.** The crystal structure of the GR LBD bound with the agonist dex (a) with the co-activator TIF-2 and with the antagonist RU486 (b). Taken and adapted from (Kauppi *et al.*, 2003).



### 1.4.2 Nuclear translocation and dimerisation

The unliganded GR shuttles continuously between cytoplasm and nucleus, but is at any given moment mostly cytoplasmic (Wikstrom *et al.*, 1987;Hache *et al.*, 1999). For a long time it was believed that ligand binding, release from the heteromeric HSP complex and GR conformational change allows the GR to translocate to the nucleus, and that GR homodimerisation occurs on the DNA. However, more recent data supports a hypothesis that receptor dimerisation occurs before nuclear import and does not require DNA binding. Savory *et al.* showed that GR dimerisation occurs in the cytoplasm (Savory *et al.*, 2001). Additionally, mutation of residue I628 in the LBD, as well as residues in the DBD, which are important for GR homodimerisation, still allows for nuclear translocation and GR mediated transrepression, but not transactivation (Bledsoe *et al.*, 2002;Reichardt *et al.*, 1998). Furthermore, it was recently shown that a dissociated GC, Compound A (CpdA), does not lead to GR dimerisation, but still allows nuclear translocation and GR mediated transrepression, but not transactivation (Dewint *et al.*, 2008). Taken together,

recent data therefore suggest that GR dimerisation is ligand-specific, that it occurs in the cytoplasm and does not require nuclear translocation. However, dimerisation appears to be required for transactivation on some GREs (but not all), but not for transrepression via tethering.

The major GR NLs is located in the hinge region, with two additional NLs within the DBD (Wan *et al.*, 2001;Picard and Yamamoto, 1987). Interestingly, when the LBD of the GR is removed, the remainder of the GR is constitutively nuclear in the absence of hormone (Picard *et al.*, 1988). Additionally, an antibody that reacts with the major NLs can only react with the liganded, but not with the unliganded GR (Urda *et al.*, 1989). These two observations have lead to the hypothesis that the GR LBD itself, or the HSP90 bound to the LBD, masks the NLs and that the conformational change upon ligand binding and/or dissociation from the HSP90 complex exposes the NLs (Wan *et al.*, 2001). However, in the literature, there is increasing evidence indicating that HSP90 plays an important role in GR mobility within the nucleus (Elbi *et al.*, 2004). Additionally, a recent paper demonstrated that two specific tetratricopeptide repeat (TPR) proteins, namely FK506-binding protein 52 (FKBP52) and FK506-binding protein 51 (FKBP51) play a crucial role in GR nuclear translocation and that these two TPR proteins bind directly to HSP90 (Banerjee *et al.*, 2008). Banerjee *et al.* demonstrated that when the unliganded GR is bound to FKBP51 (indirectly through HSP90) it is mostly cytoplasmic, however when the unliganded GR is bound to FKBP52, it is mostly nuclear. Additionally, these authors have previously shown that ligand binding to the GR induces a switch from FKBP51 to FKBP52, which is accompanied by nuclear translocation (Davies *et al.*, 2002). Furthermore, FKBP52 has also been shown to bind the motor protein dynein, which moves along the microtubules (Silverstein *et al.*, 1999;Czar *et al.*, 1994). Additionally, GR movement to the nucleus in 3T3 mouse fibroblasts, has been shown to depend on FKBP52, dynein and microtubules (Harrell *et al.*, 2004;Galigniana *et al.*, 2001).

The active GR interacts with the importin system and translocates via the nuclear pore into the nucleus (Elbi *et al.*, 2004), where the receptor can then interact with critical regulatory sites of GC-responsive genes (Okamoto *et al.*, 1993;Pratt, 1993). The above mentioned nuclear import occurs very rapidly with a T½ of only 5 minutes after dex stimulation (Hache *et al.*, 1999;Yang *et al.*, 1997).

### 1.4.3 GR transcriptional regulation

Once in the nucleus, the active GR can either bind directly to the DNA through binding to GREs or bind to other transcription factors (called tethering). Thereafter the GR recruits the basal transcription machinery, co-factors (co-activators or co-repressors), as well as a variety of other transcription factors, including chromatin remodelling complexes, which facilitate GR mediated transactivation or transrepression (McKenna *et al.*, 1999b;McKenna *et al.*, 1999a).



#### 1.4.3.1 Different modes of DNA binding

1.4.3.1.1 Direct DNA binding

The DBD of the active GR binds to specific GRE sequences. Classically these GREs are defined as imperfect palindromes of hexameric half sites, separated by a three base pair spacer, 5'-GGTACAnnnTGTTCT-3' (Nordeen *et al.*, 1990;So *et al.*, 2007;Schoneveld *et al.*, 2004). This 15 bp core GR binding sequence varies slightly between GREs on different genes (mostly at the 5' half site), but is highly conserved between species for a specific GRE (So *et al.*,

2008;Schoneveld *et al.*, 2004). Activated GR binds to these 15 bp core sequences and activates the respective genes (Fig. 1.7 A). GC-responsive genes containing such simple-acting GREs include the serine/threonine protein kinase, tyrosine hydroxylase and tyrosine aminotransferase (TAT) genes (Schoneveld *et al.*, 2004;Schmid *et al.*, 1987). On the mouse mammary tumor virus (MMTV) promoter, the GR binding to four GREs, mediates transactivation, even though the promoter contains binding sites for octamer transcription factors and TBP (Hebbar and Archer, 2003).

In contrast to the above mentioned simple-acting GREs, many genes contain glucocorticoid response units (GRUs), in which transcription not only depends on GR binding to the GRE, but also requires the binding of other transcription factors, including activator protein 1 (AP-1) proteins and TBP, to adjacent binding sites (Fig. 1.7 B). On the proliferin gene, for example, GR binding to its GRE can either result in transactivation or transrepression, depending on the composition of the AP-1 proteins, c-jun and c-fos, on an adjacent AP-1 site (Miner and Yamamoto, 1992;Pearce *et al.*, 1998). Interestingly, many of the additional transcription factors required for transcription on composite GREs, are tissue specific, such as in the case of the hepatic genes involved in gluconeogenesis. For example, on the rodent phenylalanine hydroxylase (PAH) gene, GR mediated transactivation requires the presence of cAMP-activated CREB protein, as well as binding of hepatocyte nuclear factor-1 (HNF1) to the enhancer, in addition to GR binding to GREs (Bristeau *et al.*, 2001;Faust *et al.*, 1996). Additionally, these GRUs typically result in a higher level of induction than genes containing a simple GRE (Kassell and Herrlich, 2007;Schoneveld *et al.*, 2004).

The GR can also bind as a monomer to GRE half sites (GRE1/2) (Fig. 1.7 C). To date, no GC induced genes that contain simple GRE half sites have been found. However genes containing

composite GRE half sites, including the phenylalanine-hydroxylase (PAH) gene, as mentioned above, have been investigated and shown to bind GR monomers. Furthermore, genes lacking composite GRE half sites, but containing multiple GRE half sites, such as the phenylethanolamine N-methyltransferase (PNMT) gene, which encodes for an enzyme that converts noradrenaline to adrenaline, can induce transactivation via binding of GR monomers (Schoneveld *et al.*, 2004;Adams *et al.*, 2003;Aumais *et al.*, 1996;Sabban *et al.*, 1995).

GR binding to negative glucocorticoid response elements (nGREs), such as occur in the pro-opiomelanocortin (POMC), osteocalcin and prolactin promoters, results in transrepression of the respective genes (Drouin *et al.*, 1993;Meyer *et al.*, 1997a;Sakai *et al.*, 1988) (Fig. 1.7 E). A good example of a nGRE is in GR negative feedback on the HPA axis through the corticotrophin-releasing hormone (CRH) gene. The promoter of this gene contains 3 nGRE half-sites, as well as an adjacent AP-1 binding site and mutation of either site results in a loss of GR-mediated transrepression (Malkoski and Dorin, 1999). The nGRE has a similar recognition sequence to the well defined GRE, but contains more sequence variation (ATYACnnTnTGATCn). Simple nGREs, as well as nGRE half sites, such as occur in the serotonin receptor gene promoter, have been found in GC-responsive genes. Additionally, the GR can also repress transcription by competing with other transcription factors for DNA binding (Fig. 1.7 F). In this case, the nGRE overlaps with the binding site of other transcription factors and GR binding to the nGRE interferes with the binding of other transcription factors to their binding sites (Kassell and Herrlich, 2007;Schoneveld *et al.*, 2004;Meyer *et al.*, 1997b).

**Figure 1.7 Mechanisms of GR transcriptional regulation.** Taken and modified from (Schoneveld *et al.*, 2004).



1.4.3.1.2 Tethering

In certain genes, transcriptional regulation by the GR does not involve direct binding of the GR to DNA, but rather GR binding to other transcription factors on the DNA (Figs. 1.7 D and G). Examples of both GR-mediated transactivation (e.g. binding to Stat5 on the β-casein gene), as well as transrepression (e.g. binding to AP-1 or NFκB on the collagenase, IL6 and IL8 genes), have been reported (Kassell and Herrlich, 2007). Interestingly, the DBD of the GR is required for this interaction (Heck *et al.*, 1994;Liden *et al.*, 1997;De Bosscher *et al.*, 1997). Furthermore, few examples of transactivation via tethering have been shown and the most well studied GR tethering examples are for repression of genes involved in inflammation via AP-1- and NFκB-mediated gene expression (Kassell and Herrlich, 2007).

**1.4.3.2 Transactivation**

After DNA binding, the GR recruits ATP-dependent chromatin remodelling complexes, as well as histone acetyltransferases (HATs), resulting in disruption of the higher order chromatin organisation and less condensed chromatin (Deroo and Archer T.K., 2001;Muchardt and Yaniv, 1999;Lorch *et al.*, 1999). This process enables additional transcription factors to bind to their respective DNA binding sites. An example is the MMTV gene wherein binding of active GR to multiple GRE half sites, results in the recruitment of an ATP-dependent chromatin remodelling complex and subsequent binding of NF-1, Oct and TBP to their respective sites (Hebbar and Archer, 2003;Schoneveld *et al.*, 2004).

Genes that are transcribed by RNA polymerase II are recognised by the basal transcription machinery. Furthermore the NTD of the GR has been shown to interact with the general transcription factor TFIID (Ford *et al.*, 1997) and binding of the GR to its GRE thus facilitates the recruitment of the basal transcription machinery. Additionally, the GR can recruit several co-factors, including CBP or the adenovirus E1A binding protein 300 (p300) and members of the SRC family, through its LBD (Frego and Davidson, 2006). These co-factors can interact with the basal transcription machinery and act as transcriptional bridges between the GR and the basal transcription machinery, thereby regulating transcription (Reviewed in (Glass and Rosenfeld, 2000)). Some of these co-factors can acetylate and deacetylate the histones, thereby altering the higher order chromatin organisation and allowing additional transcription factors to bind the DNA and alter transcription (Vo and Goodman, 2001).

Genetic profiling indicates that 12% of genes in immune cells are up-regulated upon dex stimulation, whereas only 9% of genes are down-regulated (Galon *et al.*, 2002). However, GR

transactivation is usually associated with the undesirable side-effects of pharmacological GC treatment, although there are some exceptions, including some anti-inflammatory genes, such as IL-10 and annexin (Newton, 2000). The positive anti-inflammatory effects of GCs are generally thought to be mediated via transrepression of inflammatory genes (e.g. through tethering with AP-1 and NFκB).

#### 1.4.3.3 Transrepression

GR binding to a nGRE results in transrepression of the respective gene, as found in the human immunodeficiency virus type 1 and the neuronal serotonin receptor genes (Soudeyns *et al.*, 1993;Ou *et al.*, 2001). The ability of the GR to transrepress AP-1- and NFκB-mediated transcription of pro-inflammatory genes requires specific residues in the DBD of the GR. However, these pro-inflammatory genes do not contain nGREs and it has been shown that GR dimerisation is not needed for repression (Heck *et al.*, 1994). Instead, it is believed that specific residues in the DBD of the GR may be important for protein-protein interactions between the GR and the AP-1 and NFκB proteins. Furthermore, protein-protein interactions between the GR and members of the AP-1 protein family, as well as with NFκB proteins, have been shown on different GC-regulated genes (Reviewed in (Kassell and Herrlich, 2007)). For instance, GR interferes with AP-1 protein function by preventing the binding of AP-1 proteins to the human IL-2 gene promoter, resulting in repression thereof (Paliogianni *et al.*, 1993). However the precise mechanism by which the GR represses AP-1- and NFκB- regulated genes is still unclear and has been extensively studied in recent years (Adcock *et al.*, 2006;De Bosscher *et al.*, 2003;Itoh *et al.*, 2002;Ito *et al.*, 2006a)

## 1.5 Co-factor recruitment

Traditionally primary co-factors have been categorised as being either co-activators or co-repressors, depending on their effects on nuclear receptor driven transcription (Review by (van der Laan and Meijer, 2008)). Nuclear receptors interact directly with primary co-factors mostly through the AF2 region in the LBD of the receptor, but recruitment of co-factors through the AF1 in the NTD of the GR has also been reported (Bledsoe *et al.*, 2002;Warnmark *et al.*, 2000;Kumar *et al.*, 2001). Many nuclear receptor co-factors have been identified, including the vitamin D interacting proteins (DRIPs), that form part of a larger mediator complex, and the thyroid receptor associated proteins (TRAPs) (Reviewed in (McKenna *et al.*, 1999b)).

The most well known and most extensively studied co-activators are the SRC or p160 family. These co-activators contain three LXXLL (L=leucine and X=any aa) motifs in their receptor-interacting domains (RIDs), which are found in amphipathic helices on their surface via which they form direct ligand-dependent interactions with SRs (Heery *et al.*, 1997;Li and Chen, 1998;Voegel *et al.*, 1998;Onate *et al.*, 1998). The SRC family consists of SRC-1 (or NcoA1), SRC-2 (or TIF2 or GR interacting protein-1(GRIP-1)) and SRC-3 (or p/CIP, RAC3, ACTR or AIB1) (Carapeti *et al.*, 1998;Ning *et al.*, 1999;Anzick *et al.*, 1997). The importance of the p160 family of co-factors has been demonstrated in knockdown mice. SRC-1 -/- mice show partial resistance to hormone (estrogen, progesterone (prog), testosterone and thyroid hormones) and studies on these mice indicate that SRC-1 plays an important role in brain function and development (Weiss *et al.*, 1999;Xu *et al.*, 1998;Auger *et al.*, 2000). SRC-2 -/- mice showed significantly reduced male and female fertility, and results from these mice suggest that SRC-2 plays an important role in lipid metabolism and energy balance (Gehin *et al.*, 2002;Picard *et al.*,

2002). Additionally, overexpression of SRC-2 in frogs caused severe developmental defects such as a loss of head structures, suggesting that SRC-2 also plays an important role in development (De la Calle-Mustienes and Gomez-Skarmeta, 2000). SRC-3 -/- mice showed growth retardation and reduced adult body size, suggesting that SRC-3 is important for growth and development in mice. Furthermore, results from these mice suggest that SRC-3 plays an important role in the development and function of the female reproductive system (Xu *et al.*, 2000;Wang *et al.*, 2000).

SRC-1 is highly expressed in most tissues, including the brain, kidney and heart and has been shown to enhance transcriptional regulation of a broad range of nuclear receptors, including the PR, ER and GR (Torchia *et al.*, 1997;Xu *et al.*, 1998;Onate *et al.*, 1995;Sheppard *et al.*, 1998;Leo and Chen, 2000;Xu and Li, 2003). For instance, overexpression of SRC-1 in HeLa cells increased ligand-mediated GR transactivation on a MMTV promoter by 3 fold (Li *et al.*, 2002). TIF2 (found by Voegel *et al.*) and GRIP-1 (found by Hong *et al.*) are the human and murine equivalents, respectively, of SRC-2 (Voegel *et al.*, 1996;Hong *et al.*, 1996). Like SRC-1, SRC-2 is expressed in multiple tissues, including the brain, heart and kidney and has been shown to enhance AF-1 and AF-2 dependent AR transactivation in CV1 cells on the MMTV promoter (Torchia *et al.*, 1997;Ma *et al.*, 1999). Recruitment of secondary co-factors such as CBP/p300 via SRC-2 to the ER *in vitro* has been shown to be necessary for the formation of the pre-initiation complex, as well as the subsequent initiation of transcription (Kim *et al.*, 2001). SRC-2 has also been shown to enhance GR-mediated transactivation on a variety of different GC-regulated promoters, in different cells (Rogatsky I, 2001;Ding *et al.*, 1998;Grenier *et al.*, 2004;Szapary *et al.*, 1999). Furthermore, photobleaching experiments have shown dex-mediated recruitment of the GR and SRC-2 to the MMTV promoter in the 3134 cell line (Becker *et al.*, 2002). SRC-2 recruitment to the GR, in response to dex stimulation on the endogenous collagenase 3 promoter, has also been shown to occur in U2OS cells (Rogatsky I, 2001). On the

collagenase 3 promoter, SRC-2 was shown to enhance dex-mediated transrepression (Rogatsky I, 2001). In addition, *in vitro* recruitment of SRC-2 by the GR to a GRE, has been shown to be ligand-dependent and to be a major determinant of ligand-selective GR transactivation and transrepression (Ronacher *et al.*, 2009). SRC-3 has been shown to interact with various different transcription factors, including CBP and RAR (Reviewed in (Leo and Chen, 2000;Xu and Li, 2003)). Interestingly, the intracellular localisation and the recruitment of the SRC family members to the GR is cell specific. By comparing primary astrocytes and immortalized Schwann cells, Grenier *et al.* demonstrated that SRC-1 is predominantly found in the nucleus of astrocytes in the presence and absence of dex, whereas in Schwann cells, SRC-1 translocates to the nucleus only upon dex stimulation (Grenier *et al.*, 2006). In both cells, SRC-2 was found predominantly in the nucleus in the absence or presence of dex, whereas SRC-3 was found predominantly in the cytoplasm, with a small increase in nuclear SRC-3 upon dex stimulation. Additionally, by overexpression or RNA interference of the individual SRCs, Grenier *et al.* showed that in Schwann cells, the GR preferably recruited SRC-1 or SRC-3 to a $(GRE)_2$-TATA promoter-reporter and knockdown of SRC-1 or SRC-3 reduced GR-mediated transactivation on the promoter by ~60%. However, in astrocytes, the GR preferentially recruits SRC-2 or SRC-1 to the $(GRE)_2$-TATA promoter and knockdown of SRC-2 or SRC-1 reduced GR-mediated transactivation on the promoter by 40-60%. Additionally, stimulation with dex results in preferential GRIP-1 (SRC-2) versus SRC-1 recruitment to the GR, whereas prog-stimulated PR preferentially recruits SRC-1 over GRIP-1 (Li *et al.*, 2003). More recent data suggest that recruitment of GRIP-1 versus SRC-1 to the GR may also be ligand-selective, where the more potent agonists dex and prednisolone (predn) appear to preferentially recruit GRIP-1 while the partial agonists MPA and aldosterone (ald) appear to preferentially recruit SRC-1 (Ronacher *et al.*, 2009). Taken together, these results suggest that recruitment of the different members of the SRC family by the GR occurs in ligand-, cell- and receptor-specific manner.

The most well known and studied co-repressors are NCoR and silencing mediator for retinoic acid and thyroid hormone receptors (SMRT) (Horlein *et al.*, 1995;Chen and Evans, 1995;Sande and Privalsky, 1996). Both NCoR and SMRT contain three highly related nuclear-receptor-interaction domains (NRIDs) that can transfer active repression to the DBD of nuclear receptors, including the GR (Seol *et al.*, 1996;Zamir *et al.*, 1996;Ordentlich *et al.*, 1999;Park *et al.*, 1999;Chen and Evans, 1995;Horlein *et al.*, 1995). Each of these NRIDs have a L-XXX/XXX1/L motif, which is similar to the LXXLL motif found in co-activators (Nagy *et al.*, 1999;Perissi *et al.*, 1999;McInerney *et al.*, 1998). The importance of NCoR was shown with knockdown mice, wherein NCoR -/- mice died in midgestation (Jepson *et al.*, 2000). NCoR has also been implicated as a major contributor to Huntington's disease, by interacting with the gene product of the disease (Boutell *et al.*, 1999). Furthermore, mutations in the LBD of the thyroid hormone receptor, which results in enhanced ligand-independent recruitment of NCoR and/or SMRT, have been correlated with thyroid hormone resistance (Yoh *et al.*, 1997). These co-repressors are believed to associate with steroid receptors and DNA-bound complexes in the absence of hormone and to repress transcription (Chen and Evans, 1995;Horlein *et al.*, 1995). Indeed direct interactions between NCoR and SMRT with different HDACs, including HDAC 1 and HDAC 2, which deacetylate histones and result in more condense "inaccessible" chromatin, have been shown (Li *et al.*, 2000;Wen *et al.*, 2000;Jones *et al.*, 2001). Additionally specific domains of NCoR and SMRT, which have no intrinsic HDAC activity, have also been shown to interact directly with HDAC 4 and HDAC 5 (Huang *et al.*, 2000;Hu and Lazar, 1999). Recruitment of ligand-activated steroid receptors and associated co-activators, is believed to remove co-repressors from the DNA-complex (Leo and Chen, 2000). Additionally, both NCoR and SMRT have been shown to be recruited to a variety of ER- and PR- responsive genes in the presence of receptor antagonists, but not receptor agonists, and to induce repression of the specific genes (Jackson *et al.*, 1997;Lavinsky *et al.*, 1998;Smith *et al.*, 1997;Xu *et al.*, 1996;Shang *et al.*, 2000).

Recently, the recruitment of NCoR and SMRT by agonist stimulated GR, was shown to repress GR-mediated transactivation on the human MTIIa promoter, in COS-7 and HeLa cells (Hong *et al.*, 2009). There are increasing amounts of data showing that agonist-bound GR, PR and AR can recruit NCoR and SMRT (Ronacher *et al.*, 2009;Wang and Simons, Jr., 2005;Wang *et al.*, 2007a;Wang *et al.*, 2004;Yoon and Wong, 2006;Cheng *et al.*, 2002). It therefore seems that agonists do not recruit co-activators exclusively, while antagonists recruit only co-repressors. Furthermore, in both transactivation and transrepression models, recruitment of co-activators and co-repressors is possible. Taken together, it seems that co-factor recruitment is a complex process. To this extent, Lefstin and Yamamoto proposed that the specific GRE on a target gene functions as an allosteric "ligand", which triggers a structural change in the steroid receptor protein after DNA binding and that this change signals the recruitment of co-factors (Lefstin *et al.*, 1994;Lefstin and Yamamoto, 1998). In agreement with this hypothesis, Heneghan *et al.* recently showed, by statistical thermodynamic analysis of *in vitro* proteins that SRC-2 interacts only weakly with the PR in the absence of DNA, but strongly in the presence of DNA (Heneghan *et al.*, 2007). Furthermore, Rogatsky *et al.* showed that changes in the composition of the regulatory elements on the DNA can result in GRIP-1 acting as a co-activator or co-repressor in U2OS cells via an AP-1 promoter (Rogatsky I, 2001). Similarly, some reports in the literature suggest that SMRT can act as a co-activator and not as a co-repressor for the GR (Ronacher *et al.*, 2009;Song *et al.*, 2001). Taken together, these results indicate that the "classical" definition of co-activator vs. co-repressor needs to be revised and further studies are needed to better understand the precise role of the co-factors in transcriptional regulation of the GR.

Most of the co-factors described above, can recruit secondary co-factors to the DNA, including CBP, p300 and the mammalian homologs of yeast Sin3 (HDAC1/HDAC2) (Reviewed in (Glass and Rosenfeld, 2000). CBP and p300 contain HAT activity and are reported to acetylate

histones, resulting in a less condensed chromatin structure, thereby enhancing transcription. Recruitment of p300, by SRCs, to a MMTV promoter in *Xenopus* oocytes has been shown to be required for ligand-selective activation of the AR and thyroid hormone receptor (Huang *et al.*, 2003). Overexpression of p300 in HeLa cells increased GR ligand-induced transactivation on a MMTV promoter by three fold (Li *et al.*, 2002). Furthermore the HAT activity of p300 was shown to be required for this increase and more strikingly, when endogenous p300 was inhibited, an almost complete loss of ligand-mediated GR transactivation was found (Li *et al.*, 2002). In contrast, HDAC1 and HDAC2 have histone deacetyltransferase (HDAC) activity that deacetylates histones and results in a more condense chromatin structure and inhibition of transcription (Heinzel *et al.*, 1997;Nagy *et al.*, 1997). Taken together, the recruitment of primary co-factors, as well as secondary co-factors by specific steroid receptors appears to modulate transcription in a ligand-, cell- and gene-specific manner.



## 1.6 GR degradation/turnover

Both GR mRNA and GR protein levels are down-regulated in response to agonist (Dong *et al.*, 1988;Hoeck *et al.*, 1989;Webster *et al.*, 1997). It has been proposed that agonist-mediated GR degradation occurs after transcription and is due to ligand-dissociation from the GR which initiates GR dissociation from the DNA and subsequent GR degradation. Using live cell imaging and fluorescent in situ hybridisation (FISH) to directly visualise green fluorescent protein (GFP)-labelled GR binding to multiple MMTV promoters, Stavreva *et al.* demonstrated that stimulation with dex or cort results in GR recruitment to the MMTV promoters within 5 minutes (Stavreva *et al.*, 2004). In the same study the authors showed that the 19S subunit of the proteasome complex

is recruited to the MMTV promoter in response to agonist. These authors proposed that after transcription, the ligand dissociates from GR:DNA complex, which signals the recruitment of the proteasome to the DNA and degradation of the GR (Stavreva *et al.*, 2004). Additionally, when GR degradation is inhibited, by means of a proteasome inhibitor, GR-mediated transactivation is increased (Deroo *et al.*, 2002;Wallace and Cidlowski, 2001;Garside *et al.*, 2006). One interpretation of this data is that GR degradation occurs after GR-mediated transcription and ligand dissociation. However, in the literature there is a substantial amount of evidence showing that the unliganded GR is also degraded (Wallace and Cidlowski, 2001;Dong *et al.*, 1988;Webster *et al.*, 1997). If GR-mediated transcription was required for GR degradation, the unliganded GR, which is not transcriptionally active, should not be degraded. This implies that GR-mediated transcription is not required for GR degradation. However, whether GR-mediated transcription is required for agonist-induced GR degradation has not directly been shown. Furthermore, when FRAP was used to determine the nuclear mobility of ligands that covalently bind to the GR (Dex-Mes and Dex-Cl), it was found that both ligands showed nuclear mobilities similar to that of dex (Meijsing *et al.*, 2007). If ligand-dissociation was a prerequisite for GR dissociation from the DNA, and subsequent degradation of the GR, then these covalently bound GR ligands should restrict GR nuclear mobility, suggesting that ligand dissociation is not a prerequisite for GR dissociation from the DNA. Additionally, Meising *et al.* compared the rate of dex dissociation from the GR ($t_{1/2}$ ~ 20 minutes) to the rate of dex-GR dissociation from the DNA ( < 2 seconds) and further concluded that ligand-dissociation is not required for GR:DNA dissociation (Meijsing *et al.*, 2007). Taken together, these results suggest that ligand dissociation is not needed for GR dissociation from the DNA.

The liganded- and unliganded-GR has been shown to be degraded through the ubiquitin/proteasome-dependent protein degradation pathway (UPP) (Wallace and Cidlowski,

2001). Proteasomal degradation can occur either in the nucleus, or the GR can first be exported to the cytoplasm and then be degraded (Liu and DeFranco, 2000). Results with the rat GR have identified the calreticulin-mediated nuclear export (NES-CRM-1) pathway in playing a role in GR nuclear export, since when a classical nuclear export signal (NES) is cloned into the GR, nuclear export is greatly enhanced. However more recent work does not support the involvement of the NES-CRM-1 pathway in rat or human GR nuclear export (Holaska *et al.*, 2001;Holaska *et al.*, 2002;Walther *et al.*, 2003;Savory *et al.*, 1999;Liu and DeFranco, 2000).

By comparing saturation binding curves in HeLa cells, over different time points, Cidlowksi and Cidlowski observed that the GR protein levels decreased over time (Cidlowski and Cidlowski, 1981). This decrease was only observed with the GR agonists dex and cort, while prog only slightly decreased GR protein levels and the ER agonist estradiol and AR agonist dihydrotestosterone (DHT) did not reduce the GR levels at all. This result suggests that GR degradation occurs in a ligand-selective manner. Later Dong *et al.* used rat hepatoma cells to show that the unliganded GR protein is degraded slowly over time with a half-life of about 25 hours, while stimulation with dex dramatically decreased the half-life to 11 hours (Dong *et al.*, 1988). This result shows that the unliganded GR is degraded slowly over time and that stimulation with an agonist, results in a quicker rate of GR degradation. The half-life, or time it takes to degrade 50% of the GR protein, varies considerably in the literature, indicating potential cell- or species-specific differences in GR degradation. By using cycloheximide to stop *de novo* protein synthesis, Webster *et al.* was able to demonstrate that the half-life of the unliganded GR in COS-1 cells is roughly 20 hours, while stimulation with the GR agonist dex resulted in a dramatic increase in GR degradation with a half-life of only 9 hours (Webster *et al.*, 1997). Hoeck *et al.* used [$^{35}$S] methionine pulse chase experiments to show that the half-life of the unliganded GR in NIH3T3 cells was a mere 8 hours and that dex stimulation decreased the half-

life to 3 hours (Hoeck *et al.*, 1989). Interestingly, in the same study, Hoeck *et al.* showed that the dex induced degradation of the GR is dose-dependent and that the GR ligand RU486 was also capable of increasing the degradation of the GR, albeit to a lesser extent than dex. Taken together, it seems that GR degradation is ligand-dependent, wherein agonists results in the fastest and antagonists result in the slowest rate of GR degradation. However, the rate of GR degradation in response to other ligands, i.e. other than dex, cort, prog and RU486, has not been determined.

Like the other members of the steroid receptor family, the GR is degraded by the UPP, as mentioned above (Wallace and Cidlowski, 2001). In this pathway, proteins are covalently linked to a chain of ubiquitin moieties at specific lysine residues found in so-called PEST (amino acid sequence of Pro (P), Glu (E), Ser (S), and Thr (T)) degradation motifs by proteins with ligase activity (Hershko and Ciechanover, 1998;Dvorak *et al.*, 2005). The importance of the E3 ligase CHIP (carboxy terminus of heat shock 70-interacting protein E3 ligase) in GR degradation was shown in HT22 hippocampal cells naturally deficient in CHIP. These cells did not demonstrate dex-mediated GR degradation, but overexpression of CHIP restored dex-mediated GR degradation (Wang and DeFranco, 2005). Specific ubiquitin-binding subunits of the proteasome recognise these ubiquitin moieties and bind to them, resulting in degradation by proteolysis (Hershko and Ciechanover, 1998;Dvorak *et al.*, 2005). A recent study showed that calpain, which is a calcium-activated cysteine protease, is the protease responsible for degradation of the ligand-activated GR in COS-7 cells (Kim *et al.*, 2008).

The discovery of proteasome inhibitors has significantly enhanced our understanding of receptor degradation (Lee and Goldberg, 1998). Proteasomal inhibition results in a decrease in transcription of the PR, AR and ER (Lange *et al.*, 2000;Sheflin *et al.*, 2000) This suggests that proteasomal degradation of the PR, AR and ER is required for transcription of these receptors.

However, whether proteasomal degradation of the GR is required for transcription appears controversial. Proteasomal inhibition results in an increase in hGR- and mGR-mediated transcription on stable or transiently transfected MMTV and GRE-TATA-luc promoters in human breast cancer, COS-1 and HeLa cells (Deroo *et al.*, 2002;Wallace and Cidlowski, 2001;Garside *et al.*, 2006). This suggests that proteasomal degradation of the GR is not required for GR-mediated transcription, but rather acts as a "shut-down" mechanism to stop transcription and prevent over-stimulation. However in mouse hippocampal cell lines and primary rat hippocampal neurons, the endogenous GR is not degraded in a dex-dependent manner. Interestingly, in these cells proteasomal inhibition results in an increase in GR-mediated transactivation on an MMTV promoter, in a manner similar to the above mentioned studies. This increase in GR-mediated transcription is not due to inhibition of GR degradation, since the GR is not degraded in a dex-mediated manner in these cells (Wang *et al.*, 2002a). Proteasome inhibitors have been shown to directly influence the basal transcription machinery and chromatin structure (Kinyamu and Archer, 2007;Dantuma *et al.*, 2006), thereby increasing transcription. However whether the increase in GR-mediated transcription, in the mouse hippocampal cell lines and primary rat hippocampal neurons, is due to indirect effects of the proteasome inhibitor on the basal transcription machinery and chromatin structure, is not known. Proteasomal inhibition also reduces the mobility of the GR in the nucleus, the association of the GR with the nuclear matrix and reduces the clearance of the GR from the nucleus (Conway-Campbell *et al.*, 2007). This can also result in an increase in GR-mediated transcription, which can falsely be interpreted to imply that degradation "restricts" GR-mediated transcription. Nevertheless, an interesting study, on the MMTV promoter, in cell line 3617, showed that inhibition of the proteasome resulted in an increase in dex-mediated transactivation, with concomitant decrease in corticosterone-mediated transactivation (Stavreva *et al.*, 2004). This result implies that the relationship between transactivation and GR degradation may be ligand-specific. Ligand-selective ER proteasomal

degradation has been shown to correlate with the RBAs of the different ligands for the ER (Preisler-Mashek *et al.*, 2002). Additionally, a recent paper showed with microarrays that inhibiting the proteasome had similar effects on both the GR and the ER, in that several GR- and ER- responsive genes were upregulated and down-regulated, depending on the specific gene (Kinyamu *et al.*, 2008). The relationship between GR transcription and degradation is complex, possibly involving cell-, ligand- and gene-specific regulation, and warrants further investigation.

## 1.7 Post-translational modifications

The GR, as well as the other members of the SR family, undergoes several post-translational modifications, including ubiquitination, sumoylation, acetylation and phosphorylation (Fig. 1.8), which play an important role in modulating its biological actions (Reviewed in (Duma *et al.*, 2006;Faus and Haendler, 2006).



### 1.7.1 Ubiquitination

Ubiquitination is a post-translational covalent modification, which targets proteins for degradation by the UPP, as mentioned above (Wallace and Cidlowski, 2001). Ubiquitin is a 76 amino acid protein that is covalently linked to proteins, including all the steroid receptors, in a three step process. First, ubiquitin is activated by an E1 enzyme, where after E2 conjugating enzymes transfers the activated ubiquitin to a specific E3 ubiquitin ligase, which in turn covalently links the ubiquitin to lysine residues found in PEST degradation motifs. The human

GR contains a PEST motif at lysine 419 (K419) (Fig. 1.8) and mutation of the mouse analogue (K426) to the human K419, results in stabilisation of the mGR protein and increased transactivation on a GRE-TAT promoter in HeLa and COS-1 cells (Wallace and Cidlowski, 2001). Furthermore, overexpression of the E3 ligase CHIP has been shown to restore GC-mediated endogenous mGR degradation in HT22 cells, in which GC-induced GR degradation does not normally occur (Wang and DeFranco, 2005). Interestingly, overexpression of CHIP also resulted in reduced mGR mediated-transactivation on an MMTV promoter in HT22 cells, which is consistent with the current thinking that increased GR degradation, by ubiquitination, decreases GR-mediated transcription.



**Figure 1.8 Post-translational modifications of the human GR.** Taken and modified from (Faus and Haendler, 2006;Ismaili and Garabedian, 2004;Galliher-Beckley *et al.*, 2008). P: phosphorylation; A: acetylation; U: ubiquitination; S: sumoylation.



### 1.7.2 Sumoylation

Sumoylation is the covalent attachment of a small ubiquitin-related modifier-1 (SUMO-1) to proteins (Le Drean *et al.*, 2002). The process of sumoylation is similar to that of ubiquitination and involves substrate recognition and SUMO linkage to a lysine residue by a specific enzyme named Ubc9 (Duma *et al.*, 2006). Three sumoylation sites in the hGR have been identified, namely lysine 277 (K277), lysine 293 (K293) and lysine 703 (K703) (Fig. 1.8), which are

conserved in the rGR. Overexpression of SUMO-1 results in enhanced degradation of overexpressed rGR in COS-7 cells, indicating a role for sumoylation in GR stability (Le Drean *et al.*, 2002). The effect of GR sumoylation is likely promoter specific, since the simultaneous mutation of all three rat equivalent GR sumoylation sites did not affect rGR dex-mediated transactivation on the MMTV promoter (containing 4x GREs), but enhanced dex-mediated transactivation on a GRE promoter (containing 2x GREs), as well as on a 4xGRE-tk-luc promoter in COS-7 cells with expressed rGR at saturating concentrations of dex (Tian *et al.*, 2002;Le Drean *et al.*, 2002).



### 1.7.3 Acetylation

Acetylation is the addition of an acetyl group to a protein and is carried out by proteins that possess HAT activity. The removal of the acetyl group is done by proteins that contain HDAC activity, such as HDAC1 to HDAC17. Ligand-selective acetylation of both the ER and the AR has been shown to be required for maximal transcription on multiple promoters and is directly mediated by the HAT activity of p300 (Wang *et al.*, 2001;Kim *et al.*, 2006;Fu *et al.*, 2000;Fu *et al.*, 2004). Additionally, AR acetylation and phosphorylation have been shown to be functionally linked (Fu *et al.*, 2004). Mutation of an AR acetylation site reduces ligand-dependent AR phosphorylation, whereas mutation of the AR phosphorylation site, reduces HDAC-mediated deacetylation of the AR. Furthermore, AR acetylation has also been implicated in enhanced growth of human prostate cancer cells *in vivo* (Fu *et al.*, 2002). Results in the literature on GR acetylation and the effect thereof on GR-mediated transcription, has mainly been due to indirect effects such as HSP90 acetylation affecting GR-mediated transcription and not direct GR acetylation (Murphy *et al.*, 2005;Kovacs *et al.*, 2005). However, an acetylation motif

(KXKK/RXKK) in the GR that corresponds to amino acids 492-495 has been identified and recently dex-dependent acetylation of the endogenous hGR at K494 and K495 has been shown in A549 cells (Fig. 1.8) (Ito *et al.*, 2006b). Ito *et al.* also demonstrated that hGR binding to NFκB and subsequent repression of transcription on a reporter-promoter construct requires deacetylation of the hGR by HDAC2. Furthermore, overexpression of p300, which contains HAT activity and has been shown to interact with the GR, results in an increase in rGR mediated transactivation on the MMTV (4xGREs) promoter in HeLa cells (Li *et al.*, 2002). Additionally, by using p300 deletion mutants, Li *et al.* showed that the HAT activity of p300 was required for this increase in rGR mediated transactivation on the MMTV promoter in HeLa cells. However, a more recent paper showed that GR acetylation by p300 decreased GR-mediated transactivation on a 2xGRE-TATA and SV40-luc promoter in astrocytes, as well as Schwann cells (Fonte *et al.*, 2007), indicating cell- or promoter-specific differences on the effect of GR acetylation on GR-mediated transactivation. Additionally, Faus and Haendler recently showed that the effect of AR acetylation on AR-mediated transcription is promoter-specific (Faus and Haendler, 2008), indicating that apart from cell-specific differences, the effect of acetylation on AR function can also be promoter-specific. The effects of acetylation on steroid receptor transcription are complex and are further complicated by the difficulty in distinguishing between direct acetylation of steroid receptors and acetylation of histones or other proteins that affect transcription.



## 1.8 Phosphorylation

In the beginning of the 1980s, it was shown that the GR, PR and ER exist as phosphoproteins in intact cells (Housley and Pratt, 1983;Singh and Moudgil, 1985;Mendel *et al.*, 1986;Dalman *et al.*,

1988), suggesting that phosphorylation may be a common feature of all steroid receptors and indicating a functional role for phosphorylation.

### 1.8.1 Identification of phosphorylation sites

Several groups showed that the mouse (mGR), rat (rGR) and human (hGR) GR become hyperphosphorylated at multiple residues upon hormone treatment and that phosphorylation occurs only in the presence of agonists, while the antagonist RU486 does not result in phosphorylation of the GR (Dalman *et al.*, 1988;Housley and Pratt, 1983;Singh and Moudgil, 1985;Hoeck *et al.*, 1989;Orti *et al.*, 1989). Early speculations that the phosphorylation of the GR may play an important role in the transcriptional activation of GR-responsive genes (Hoeck *et al.*, 1989) encouraged many groups to find the specific sites where the GR is phosphorylated. By using proteases and chemical cleavage reagents, along with an assortment of other techniques including immunoprecipitation, [$^{32}$P] orthophosphate labeling and GR deletion mutants, it was demonstrated that dex-dependent phosphorylation of the GR occurs mainly in the amino-terminal (Dalman *et al.*, 1988;Hoeck and Groner, 1990). More specifically, phosphorylation occurs mainly on serine and threonine residues in the potent transactivation domain τ1 or enh2, of the rGR (Hoeck and Groner, 1990). Identification of the specific serine and threonine residues that are phosphorylated, was made possible with solid phase sequencing of the mouse GR (Bodwell *et al.*, 1991). Bodwell *et al.* were able to identify the location of seven *in vivo* phosphorylation sites on the mouse GR (S122, S150, S212, S220, S234, S315 and Thr159) and found that all seven phosphorylation sites were situated in the amino terminal of the mGR (Fig. 1.8). These seven phosphorylation sites are all conserved in the rat GR, whereas 6 are conserved in the human GR (see table 1), all of which reside in the AF-1 transactivation domain (Bodwell *et al.*, 1991;Ismaili

and Garabedian, 2004). More recently, another phosphorylation site at S404 on the hGR was identified (Galliher-Beckley *et al.*, 2008).

**Table 1. Residues phosphorylated on the GR**. The phosphorylation sites identified in the mouse GR (mGR) and their equivalent positions in the rat (rGR) and human (hGR) glucocorticoid receptor* (Bodwell *et al.*, 1991;Ismaili and Garabedian, 2004;Webster *et al.*, 1997;Galliher-Beckley *et al.*, 2008).



In addition to the phosphorylation sites being situated in the AF-1 transactivation domain, three of the serine residues (S212, S220 and S234, in mouse numbering) are situated in a highly acidic region on the mouse GR, which has been shown to be necessary for transactivation (Bodwell *et al.*, 1991). An more in-depth investigation of the seven phosphorylation sites in the mouse GR, revealed that hyperphosphorylation in response to dex treatment, occurred mostly at S212, S220 and S234 (Bodwell *et al.*, 1995). The development of phospho-specific antibodies that are capable of recognizing individual phosphorylated serine residues (P-S203, P-S211 and P-S226), greatly enhanced the *in vivo* analysis of the individual phosphorylation sites (Wang *et al.*, 2002b;Chen *et al.*, 2008). Using a panel of ligands consisting of three agonists (dex, predn and fluocinolone) and two antagonists (RU486 and ZK299), Wang *et al*. were able to show that agonists phosphorylated the hGR at S211, while antagonists caused only minimal phosphorylation (Wang *et al.*, 2002b). More recently it was shown that GR agonists (dex and cort), as well as prog and ald phosphorylate the hGR at S226 (Chen *et al.*, 2008). Furthermore, phosphorylation was found to be a dynamic process wherein the phosphorylation status on one serine residue influences the phosphorylation of another (Wang *et al.*, 2007b). Phosphorylation

* Indicating the only residue that is not conserved in the human GR.

at S211 is only slightly influenced by phosphorylation at S203 and/or S226, while the extent of phosphorylation at S226 depends on the phosphorylation status of S203 (Wang *et al.*, 2007b). S226 has a greater tendency to become phosphorylated when S203 is not phosphorylated, and vice versa. These authors concluded that phosphorylation on all three residues is a dynamic process in which the final steady-state phosphorylation status is determined by a combination of kinases and phosphatases (Wang *et al.*, 2007b).

### 1.8.2 Both kinases and phosphatases involved in GR phosphorylation

Endogenous protein kinases were implicated in the phosphorylation of the GR (Kaul *et al.*, 2002) and subsequent sequence analysis of the mouse, rat and human GR demonstrated that the phosphorylation sites are all followed by a proline residue (reviewed in (Ismaili and Garabedian, 2004)), making it the favoured context for cyclin-dependent kinases (CDKs) and mitogen-activated protein kinases (MAPKs), as shown in table 2. A subsequent *in vitro* study with the rat GR, showed that these two kinase families were indeed able to phosphorylate the rat GR, with CDKs phosphorylating S224 and S232 (human S203 and S211), and MAPKs phosphorylating S246, human S226 (Krstic *et al.*, 1997).

**Table 2. Kinases capable of phosphorylating the GR.** The kinase families capable of phosphorylating the hGR, as well as their consensus binding sequence and the residues they phosphorylate (in human numbering). Modified from (Ismaili and Garabedian, 2004)

A more in-depth study, using *in vitro* kinase overexpression assays, showed that c-Jun N-terminal kinase (JNK), a member of the MAPK family, is responsible for the *in vitro* phosphorylation of the overexpressed rGR at the rat equivalent to S226 (Rogatsky *et al.*, 1998b). The study also demonstrated that activation of JNK via UV treatment led to the inhibition of transcriptional activation from a GTCO-CAT promoter (2x GREs) construct in HeLa cells. A similar study on the hGR (Itoh *et al.*, 2002) confirmed that JNK phosphorylates the GR at S226 *in vitro* and that transcriptional activation is inhibited in COS-7 cells on a TK-luc promoter when JNK is activated with UV treatment. They also demonstrated that S226 phosphorylation, by JNK, was accompanied by enhanced nuclear export of the GR after dex withdrawal and proposed that this most likely leads to the down-regulation of GR transcriptional activity. Additionally, using *in vitro* kinase assays, it was shown that CDK and JNK phosphorylate the rGR *in vitro* at the rat equivalent of S211 and S226, respectively and that yeast strains devoid of CDK and JNK reduced and enhanced GR mediated transcription, respectively (Krstic *et al.*, 1997). However, in the above studies, whether the activation of JNK by UV treatment directly increased GR phosphorylation at S226 in mammalian cells was not investigated. Thereafter, Miller *et al.* showed in CV-1 cells, that mutation of the hGR S211 to alanine reduced transactivation on a pGRE-SEAP promoter and showed *in vitro* with purified kinases that p38 phosphorylates the hGR at S211 (Miller *et al.*, 2005). In the same study, inhibition of p38 by means of a specific p38 inhibitor, slightly decreased GR phosphorylation on S211. However, the total amount of GR was also decreased with the p38 inhibitor, which could account for the slight decrease in S211 phosphorylation (Miller *et al.*, 2005). More recently, the phosphorylation status of overexpressed hGR at S211 and S203 was increased by overexpressing CDK5 in HCT116 cells (Kino *et al.*, 2007). Furthermore, inhibition of CDK, by means of an inhibitor, resulted in increased or decreased transactivation, depending on the specific gene (Kino *et al.*, 2007). Taken together, *in vitro* data suggest that JNK phosphorylates the GR on S226, whereas p38 and possibly CDK

phosphorylate the GR on S211. However, there is no conclusive data directly linking an endogenous kinase, by means of kinase inhibition, with site specific phosphorylation of the GR on S211 and/or S226 in mammalian cells.

Interestingly, very little is known about the phosphatases involved in GR de-phosphorylation. However, it has recently been shown that inhibition of general serine/threonine protein phosphatases, as well as a reduction in protein phosphatase 5 (PP5) protein levels (by siRNA knock-down of PP5), enhanced GR phosphorylation at S203 and S226, but not S211 (Wang *et al.*, 2007b). These results suggest that PP5 plays an important role in de-phosphorylating the GR at S203 and S226. More recently, PP5 has also been implicated in de-phosphorylating the GR at S211 (Zhang *et al.*, 2009).



### 1.8.3 Transcriptional regulation by phosphorylation

In an attempt to clarify the role of these three serine residues in transcriptional transactivation, expression vectors for mutated mGR incapable of being phosphorylated on those specific residues, where the serine residues were replaced with an alanine (S212A, S220A and S234A in mouse numbering), were constructed (Mason and Housley, 1993). These mutated proteins were compared to wild type (wt) mGR in their ability to activate transcription of the complex MMTV promoter upstream of the chloramphenicol acetyltransferase (CAT) gene (MMTV-CAT) in COS-1 cells. The mutated proteins showed similar transactivation efficiency as the wt GR, suggesting that phosphorylation at those three residues is not necessary to activate transcription on this complex promoter. A similar study, where the hGR signaling system was reconstructed in yeast, showed that the phosphorylation mutants (S203A and S211A) had the same transactivation

efficiency as the wt hGR on a complex thymidine kinase promoter linked to two GREs (Almlof *et al.*, 1995). However, this study also indicated that the S226 mutant (S226A) had a transactivation efficiency higher than that of the wt, suggesting that S226 phosphorylation caused an inhibitory effect on transcriptional activation of this promoter. Webster *et al.*, looked at the ability of the three phosphorylation mutants to activate the complex MMTV-CAT promoter, as well as a simple tyrosine aminotransferase (TAT) promoter containing a minimal adenovirus E1b TATA sequence with two GREs ($GRE_2$-TATA-CAT) in COS-1 cells (Webster *et al.*, 1997). Consistent with the study of Mason and Housley, the phosphoryation mutants did not show a significant difference from the wt mGR in activating the complex MMTV-CAT promoter. However, two of the mutants (S212A and S220A in mouse numbering) demonstrated only 50-75% transactivation efficiency, compared to wt mGR, on the simple $GRE_2$-TATA-CAT promoter construct, suggesting that phosphorylation on those two sites are necessary for maximal transactivation on the simple promoter. The S234 mutant (mGR) did however not show any difference as compared to the wt receptor, even on the simple promoter. Additionally, the combined mutation of all three phosphorylation sites (S212A/S220A/S234A or 3A) of the mGR did not show any difference on the MMTV-CAT promoter, but led to an almost complete loss of dex-mediated transactivation on a simple GRE promoter in COS-1 cells (Webster *et al.*, 1997). In contrast, Kino *et al.* recently demonstrated that mutation of S203 (S203A) resulted in a 3-fold increase in efficacy (above wt hGR), upon dex stimulation, whereas mutation of either S211 (S211A) or S226 (S226A) resulted in a 2-fold increase and mutation of all three sites simultaneously (3A) led to a 4-fold increase in transactivation on the MMTV promoter in human colon carcinoma, HCT116 cells (Kino *et al.*, 2007). A possible explanation for some of the apparent inconsistencies in the above mentioned studies came when the effects of the two single mutants, S211A and S226A, were studied on endogenous genes in human bone cancer, U2OS cells. Looking at a variety of glucocorticoid-regulated genes, using increasing concentrations of

dex, Chen *et al*. demonstrated that phosphorylation at S226 of the hGR inhibits transactivation in a dose-dependent manner on the GILZ, LAD1, IGFBP1 and IRF8 genes, whereas phosphorylation at S211 was required for maximal transactivation efficacy and potency on the GILZ and LAD1 genes at low concentrations of dex, and on the IGFBP1 and IRF8 genes at low and high concentrations of dex (Chen *et al.*, 2008). Interestingly, at high concentrations of dex, phosphorylation at S211 inhibited transactivation on the GILZ and LAD1 genes. Furthermore, Chen *et al.* showed that phosphorylation at S211 or S226 is required for maximal GR-mediated repression on six different genes, including c-Jun and SMAD7. However on the PAC1 gene, both the S211A and S226A mutants increased transcription, whereas wt GR repressed the gene. Taken together, these results indicate that the effect of GR phosphorylation on transcription is cell-, gene-, as well as GC concentration-dependent (Chen *et al.*, 2008). Nevertheless, the underlying mechanisms whereby phosphorylation of the GR regulates GR-mediated transcription are still unclear.



### 1.8.4 Role of GR phosphorylation

In attempts to determine the mechanism whereby GR phosphorylation influences transcription, several steps in the GR signalling pathway have been implicated, including nuclear trafficking, protein stability and protein-protein interactions (recently reviewed in (Weigel and Moore, 2007). Wang *et al*. demonstrated that dex stimulation leads to the accumulation of GR phosphorylated at S211 in the nucleus, whereas GR phosphorylated at S203 remained cytoplasmic, suggesting a role for phosphorylation at S211 in nuclear import in U2OS cells (Wang *et al.*, 2002b). However, mutation of the mGR residue equivalent to the hGR S211, as well as the simultaneous mutation of eight phosphorylation sites of the mGR did not affect dex-mediated nuclear import of

the GR in COS-1 cells (Webster *et al.*, 1997). Furthermore, it has been shown that phosphorylation of the progesterone and estrogen receptors (PR and ER, respectively) influences receptor localisation in the absence of ligand and that phosphorylation of the liganded AR is required for nuclear export (Weigel and Moore, 2007;Pierson-Mullany and Lange, 2004). Similarly, phosphorylation of the hGR at S226 has been shown to play a role in nuclear export of the GR after dex withdrawal in COS-7 cells (Itoh *et al.*, 2002). However, nuclear import does not appear to be affected by agonist-induced phosphorylation of either the PR, AR or ER. Taken together these results suggest that the role of agonist-induced phosphorylation in steroid receptor subcellular trafficking may be receptor-, and cell-specific or depend on which particular serine residue(s) within a particular receptor is phosphorylated.

Several lines of evidence implicate receptor phosphorylation as playing a role in receptor protein degradation both in the absence and presence of ligand. Webster *et al.* showed that the combined mutation of the mGR residues equivalent to the hGR S203, S211 and S226 residues, resulted in a decrease in dex-mediated mGR degradation (Webster *et al.*, 1997). Furthermore, Webster *et al.* also showed that the simultaneous mutation of 7 or 8 phosphorylation sites completely abolished dex-mediated mGR degradation and slightly stabilized the unliganded GR protein, resulting in its slower degradation in COS-1 cells. More recently, Galliher-Beckley *et al.* showed that the mutation of a single phosphorylation site (S404) resulted in a complete loss of dex-mediated GR degradation (Galliher-Beckley *et al.*, 2008). Thus GR phosphorylation is strongly implicated in playing a role in stability of the unliganded and liganded receptor, where the effect on liganded receptor may be a mechanism to regulate transcription efficacy.

The concept of modulation of steroid receptor degradation via phosphorylation is not limited to the GR, as similar observations have been made for the PR (Lin H, 2002;Qiu and Lange, 2003).

However, phosphorylation of the PR at S400 enhances degradation of the unliganded PR (Pierson-Mullany and Lange, 2004), whereas phosphorylation at S294 enhances degradation of the liganded PR (Lange *et al.*, 2000), indicating a complex mechanism involving serine-specific phosphorylation on the stability of the unliganded or liganded PR. In contrast to the GR, agonist binding to the AR leads to increased stability of the receptor, which is believed to be mediated via agonist-induced AR phosphorylation (Chen *et al.*, 2006). Thus steroid receptor phosphorylation is implicated in playing a role in stability of the unliganded and liganded receptor, where the effect on liganded receptor may be a mechanism to regulate transcriptional efficacy. However, as for the effect of receptor phosphorylation on subcellular trafficking, these effects may be receptor-and cell-specific or also depend on which particular serine residue(s) within a particular receptor is phosphorylated.

Aside from the above mentioned nuclear translocation and degradation of the GR, recent studies demonstrate that differences in the phosphorylation status of the GR also influence the interaction of the GR with other proteins, including tumor suppressor gene protein (TSG101), components of the mediator complex and CBP/p300 (Ismaili *et al.*, 2005;Chen *et al.*, 2008;Kino *et al.*, 2007;Galliher-Beckley *et al.*, 2008). In U2OS cells, TSG101 binds preferentially to the unphosphorylated form of the unliganded GR and thereby stabilizes the unliganded GR against degradation (Ismaili *et al.*, 2005). Using a yeast two-hybrid screening, it was found that the vitamin D receptor-interacting protein 150 (DRIP150 or MED14), that forms part of the mediator complex, binds to the GR AF-1 domain and that this interaction enhances GR transactivation (Hittelman *et al.*, 1999). It was later shown that mutation of S211 resulted in a 50% decrease in MED14 binding to the GR (Chen *et al.*, 2008), indicating a possible role for GR phosphorylation at S211 in the recruitment of the mediator complex in U2OS cells. Chen *et al.* proposed a model whereby phosphorylation of S211 influences transcriptional activity via modulating the

interaction between the hGR and MED14, since mutation of S211 resulted in a 50% decrease in transcriptional activity on two MED14-dependent genes, IGFBP1 and IRF8, as well as inhibited the interaction between GR and MED14 to a similar degree (Chen *et al.*, 2008). However, this model does not account for differences seen on MED14-independent genes and indeed when two such genes, GILZ and LAD1, were investigated, they found that mutation of S211 resulted in a reduced transcriptional activity at low GC concentration, but an increased transcriptional activity at high GC concentrations. Furthermore, mutation of S226 did not change the interaction between the GR and MED14, but still resulted in an increased transcriptional activity on MED14-dependent and MED14-independent genes. Taken together these results indicate that GR phosphorylation affects GR interaction with additional factors, other than MED14, to modulate GR transcriptional activity in U20S cells. Additionally, over-expression of CDK5, known to hyperphosphorylate the GR *in vitro*, resulted in a decrease in dex-induced recruitment of the histone acetyltransferase co-activator, p300 to the GR bound on a MMTV-luciferase promoter in COS-1 and HCT116 cells (Kino *et al.*, 2007). In contrast, phosphorylation of the hGR at S404, by glycogen synthase kinase 3β (GSK3β), was shown to be required for the recruitment of p300 in U2OS cells (Galliher-Beckley *et al.*, 2008). Studies on the ER demonstrated that phosphorylation of the receptor is important for the recruitment of co-factors, wherein the phosphorylation status of one specific serine residue determines the recruitment of co-activator, SRC3, versus co-repressor, stromelysin-1 platelet-derived growth factor-responsive element-binding protein (Likhite *et al.*, 2006;Endoh *et al.*, 1999;Gburcik *et al.*, 2005;Weigel and Moore, 2007).

Taken together, these studies suggest that the mechanisms by which dex-induced phosphorylation of the GR modulates GR-mediated transcription may occur at multiple levels and be promoter- and cell-specific, as well as glucocorticoid dose-dependent. In the literature there are conflicting

results about whether phosphorylation plays a role in GR nuclear import, but some evidence that GR phosphorylation plays a role in stabilizing the unliganded and liganded GR protein. Additionally, phosphorylation of the GR has been linked to the binding of MED14, which forms part of the mediator complex, and hyperphosphorylation of the GR at S203, S211 and S226 has been shown to restrict the dex-dependent binding of p300, whereas dex-mediated phosphorylation at S404 is required for dex-dependent binding of p300.

# Chapter 2 Thesis rationale, Aims and Hypotheses:

## 2.1 Thesis Rationale

The GR plays a role in most inflammatory processes and GCs are currently used pharmacologically to treat a variety of diseases, including autoimmune diseases and pulmonary diseases (Moutsatsou P, 2008). However, long-term treatment with steroids results in a variety of side-effects including osteoporosis (Luengo *et al.*, 2001;Reid and Ibbertson, 1987;Robson *et al.*, 2002). A better understanding of how steroid receptors function, is therefore needed in order to design more effective drugs with fewer side-effects, for many different pharmacological applications.

A wide range of GCs bind to the GR resulting in an array of different biological responses in a ligand-, promoter- and cell-specific manner (Ronacher *et al.*, 2009). A central question in SR research is what determines the basis of ligand-selective biological responses. For example why do different ligands, all acting through the GR in the same cell, on the same promoter, display different potencies (the concentration of ligand needed for half the maximal response) and efficacies (maximal response)? This is in addition to cell-specific differences, i.e. differences obtained with the same ligand, on the same promoter in different cells, as well as promoter-specific differences, i.e. differences obtained with the same ligand, in the same cells, on different promoters (Ronacher *et al.*, 2009). In the GR transcriptional pathway, there are 8 different steps that have been hypothesised to exhibit ligand-selective differences and are therefore potentially

important steps in determining ligand-selectivity, namely (1) ligand-binding to the receptor (affinity and kinetics) (Koubovec *et al.*, 2005;Lind *et al.*, 2000;Selman *et al.*, 1996;Attardi *et al.*, 2004), (2) conformational changes induced by ligand-binding (Bledsoe *et al.*, 2002;Kauppi *et al.*, 2003), (3) phosphorylation of the liganded GR (Stubsrud, 2005;Wang *et al.*, 2002b;Chen *et al.*, 2008), (4) dimerisation (Savory *et al.*, 2001;Dewint *et al.*, 2008) and (5) nuclear translocation of the liganded GR (Schaaf and Cidlowski, 2003;Vicent *et al.*, 2002), (6) binding of the liganded GR to DNA (Garside *et al.*, 2004;Pandit *et al.*, 2002), (7) interaction with other transcription factors (Coghlan *et al.*, 2003;Garside *et al.*, 2004;Wang *et al.*, 2004;Cho *et al.*, 2005;Wang and Simons, Jr., 2005;Kroe *et al.*, 2007;Wang *et al.*, 2007a;Miner *et al.*, 2007;Tao *et al.*, 2008) and (9) degradation of the liganded GR (Cidlowski and Cidlowski, 1981;Dong *et al.*, 1988) (Fig. 2.1). However, most studies published to date that have attempted to investigate the basis of ligand-selective responses were performed with too few ligands to accurately determine the role of a particular step in GR (8) ligand-selective transcription.

**Figure 2.1 Steps in the GR transcriptional pathway potentially determining GR ligand-selectivity.** Taken and modified from (Gross and Cidlowski 2008).

Reports in the literature have shown different relative binding affinities (RBAs) and binding constants for different ligands for the GR (Koubovec *et al.*, 2005;Lind *et al.*, 2000;Selman *et al.*, 1996;Attardi *et al.*, 2004). However, most of these studies were done in different cells and only focussed on a few GR ligands. Ronacher *et al.* 2009 showed for the first time in a systematic approach with binding affinities and efficacy and potency for transactivation and transrepression, in the same cell system with a wide range of ligands, that the efficacy of a ligand for both

transactivation and transrepression, is not related to its relative affinity for the receptor ((Ronacher *et al.*, 2009;Koubovec *et al.*, 2005). In other words, ligand-selective efficacy is not determined by the different affinity of the ligands for the GR. In contrast, however, relative affinity for the receptor correlated to ligand-selective potency for transactivation, but not for transrepression.

It is generally accepted that different ligands induce different conformational changes of the GR upon binding. The crystal structure of the LBD of the GR in the presence of the agonist dex and antagonist RU486 revealed that the final position of helix 12 is very different in the presence of agonist vs. antagonist (Bledsoe *et al.*, 2002;Kauppi *et al.*, 2003). More recently, hydrogen/deuterium exchange measured by mass spectrometry experiments with dex-bound GR in complex with the co-activator TIF-2 and RU486-bound GR in complex with the co-repressor NCoR, has confirmed the crystal structure results (Frego and Davidson, 2006). These ligand-induced conformational changes are accompanied by GR phosphorylation and exposure of the nuclear localisation signals. Furthermore, these ligand-induced conformational changes are though to form different interacting surfaces and form the basis of differential co-factor recruitment (Kroe *et al.*, 2007). However, a direct link between different conformations and ligand-selective GR transcription with a large panel of ligands has not been established.

Ligand-selective GR phosphorylation at S211, with a panel of ligands consisting of three agonists (dex, predn and Fluocinolone) and two antagonists (RU486 and ZK299), showed that agonists, but not antagonists phosphorylated the hGR at S211 (Wang *et al.*, 2002b). Ligand-selective phosphorylation at S211 with a large panel of GR ligands was found to correlate with ligand-selective GR transactivation potency and efficacy ((Stubsrud, 2005); Table E3 in addendum E). Furthermore, mutation of S211 reduced ligand-mediated transactivation by 50%, indicating a

direct role for GR phosphorylation in transcription ((Stubsrud, 2005); Table E2 in addendum E). However, ligand-selective GR phosphorylation at S211 did not determine the rank order of ligand-selective GR transactivation, as a S211 phosphorylation deficient GR mutant still showed the same rank order of ligand-selective GR transactivation. More recently it was shown that GR agonists (dex and cort), and to a lesser extent prog and ald result in phosphorylation the hGR at S226 (Chen *et al.*, 2008). However, whether ligand-selective GR phosphorylation at S226 is required for ligand-selective GR transcription is yet to be determined.

The liganded, but not the unliganded GR, has been shown to form dimers in solution, in the absence of DNA (Savory *et al.*, 2001). Recently it was demonstrated that a dissociated GC, CpdA, does not lead to GR dimerisation, but still allows nuclear translocation and GR-mediated transrepression, but not transactivation (Dewint *et al.*, 2008). Taken together, these results indicate that GR dimerisation could be ligand-specific and possibly contribute to ligand-selective GR-mediated transcription. However, a direct link between ligand-selective GR dimerisation and transcription with a large panel of ligands has not been established.

The GR continuously shuttles between the cytoplasm and the nucleus and data in the literature suggest that the pathways of receptor trafficking and processing within the nucleus influence GR mediated transcription (DeFranco, 2002). Additionally, it has been shown that nuclear import of the receptor is influenced by ligand affinity and that differences in nuclear retention characteristics of agonist-activated GR may determine ligand-specific responses (Schaaf and Cidlowski, 2003;Vicent *et al.*, 2002). Taken together, these results indicate that GR nuclear import could be ligand-specific and possibly contribute to ligand-selective GR mediated transcription. However, a direct connection between ligand-selective GR nuclear import and transcription with a large panel of ligands, has not been established.

There is some evidence in the literature that the dissociation rate of liganded-GR from DNA is a measure of ligand potency (Pandit *et al.*, 2002). More recently, chromatin immunoprecipitation experiments in HeLa cells, showed ligand-selective recruitment of the GR to the IL-8 promoter, wherein more GR was recruited in the presence with dex, than in the presence of the antagonist RU486 (Garside *et al.*, 2004). Furthermore, these differences were not due to differential nuclear import (Garside *et al.*, 2004). Taken together, these results indicate that GR binding to DNA could be ligand-selective and contribute to ligand-selective GR-mediated transcription.

Different ligands have been shown to result in differential co-activator and co-repressor recruitment to the GR (Coghlan *et al.*, 2003;Garside *et al.*, 2004;Wang *et al.*, 2004;Cho *et al.*, 2005;Wang and Simons, Jr., 2005;Kroe *et al.*, 2007;Wang *et al.*, 2007a;Miner *et al.*, 2007;Tao *et al.*, 2008). Furthermore, there is evidence for a co-factor binding model, whereby the transcriptional response of the GR to agonists, partial agonists and antagonists are linked to the recruitment of co-activator, mixed co-activator/co-repressor and co-repressor complexes, respectively (Cho *et al.*, 2005). However, there is also substantial evidence refuting this model. For example, the GR agonist dex recruits the co-repressors NCoR and SMRT (Wang and Simons, Jr., 2005;Wang *et al.*, 2007a;Wang *et al.*, 2004). A critical evaluation of the literature reveals that there is little quantitative evidence for the model with no studies showing a direct correlation between the extent of recruitment of co-regulators by the liganded GR and the potency or efficacy for transactivation. In addition, most of the above mentioned studies used too few ligands to establish a general conclusion. Since most researchers have investigated the model in the context of transactivation, very little is known about the effects of ligand on differential co-factor recruitment in the context of transrepression. Ronacher *et al.* 2009 for the first time provided strong quantitative biochemical support for a model in which GR-mediated ligand-selective differential interaction with co-factors is a major determinant of ligand-selective and

promoter-specific differences in potency and efficacy, for both transactivation and transrepression (Ronacher *et al.*, 2009).

Many researchers have shown that the unliganded GR is degraded slowly over time, and that stimulation with dex significant enhances the rate at which the GR is degraded (Cidlowski and Cidlowski, 1981;Dong *et al.*, 1988). However, most studies have been conducted with only one or two ligands, most commonly with dex and RU486. Taken together, these results indicate that the rate of GR degradation is possibly ligand-specific and could determine the basis of ligand-selective GR mediated transcription. However, a direct link between ligand-selective GR degradation and transcription with a large panel of ligands, has not been shown.

This study forms part of a larger investigation which aims to investigate the role of each of the steps depicted in figure 2.1 in ligand-selective responses. The biological response of a large panel of ligands in transactivation and transrepression was previously determined by other researchers in the candidate's laboratory (E. Stubsrud and K. Ronacher; Table E4 in addendum E). The biological responses were measured as potency and efficacy derived from the dose-response curves for transactivation and transrepression. For the transactivation model, reporter assays were performed on a TAT-GRE promoter in COS-1 cells with the hGR ((Stubsrud, 2005); Table E5 Addendum E). For the transrepression model, reporter assays on were performed with two different promoters, the AP-1- and NFκB-luc promoters, in COS-1 cells with the hGR (K. Ronacher; Table E5 in addendum E).

The biological response of a particular ligand can be correlated with the behaviour of the liganded GR at any particular step in the GR transcriptional pathway. These correlations do not definitively establish a cause and effect, but rather indicate which steps could potentially

modulate ligand-selectivity. These steps can then be investigated further to establish which step in the GR transcriptional pathway determines ligand-selectivity.

A broad panel of GR ligands including the available endogenous and synthetic steroidal and non-steroidal agonists, full/partial agonists, partial agonists, dissociative glucocorticoids and antagonists will be used. This should provide a good basis for correlating ligand-selective effects at a specific step with transcriptional response. The panel of ligands used, as well as their relative efficacy for transcription on the three different promoters and their classification is shown in table 2.1. More detailed discussion of each individual ligand is given in addendum A ("Panel of GR ligands"). For the purposes of this study, for the particular gene and system being investigated, a ligand was classified as a full agonist if it induces the maximal possible response (efficacy $\geq$ 85% as compared to dex) and as partial agonist if it induces a response less than maximal (efficacy $\leq$ 85% as compared to dex). An antagonist was defined as a compound that binds to the receptor, induces no response of its own and has been shown to antagonize the transcriptional effects of dex. Furthermore, compounds that show full agonist activity on one promoter and partial activity on another promoter were classified as full/partial agonists, whereas ligands which show no activity on one promoter with partial of full activity on at least one other promoter were classified as dissociated GCs.

**Table 2.1 Relative efficacy for transactivation and transrepression via the GR and ligand classification.** Relative efficacy for transactivation via the TAT-GRE ((Stubsrud, 2005); Addendum E) and for transrepression via the NFκB-luc and AP-1-luc were determined in transiently transfected COS-1 cells with hGR (K. Ronacher; Addendum E; also (Ronacher *et al.*, 2009))[†].



## 2.2 Aims and Hypotheses

**1. Does hGR phosphorylation at S226 and/or GR degradation occurs in a ligand-selective manner?**

The hypothesis is that different ligands result in different extents of hGR phosphorylation at S226, wherein the agonists result in the most and the antagonists result in the least amount of GR phosphorylation at S226. This hypothesis will be tested by comparing the ability of a chosen panel of ligands, to phosphorylate the expressed hGR at S226, using an antibody that specifically recognises GR phosphorylated at S226 (P-S226), in COS-1 cells. Furthermore, it is hypothesised that hGR phosphorylation at S226 occurs in a GC-dose-dependent manner. Therefore, ligand-selective GR phosphorylation at S226 in response to saturating ligand concentrations (10 μM), as

[†] Na = no activity

well as sub-saturating ligand concentrations for some ligands (100 nM), will be determined. Additionally, since results in the literature suggest that there are cell-specific differences in GR phosphorylation, it was also hypothesised that ligand-selective hGR phosphorylation at S226 occurs in a cell-specific manner. This will be investigated by examining ligand-selective hGR phosphorylation in two different, COS-1 and U2OS, cell lines.

Similarly, it is hypothesised that GR degradation occurs in a ligand-selective manner, wherein the rate of degradation is faster with agonists than with antagonists. In order to determine if GR degradation occurs in a ligand-selective manner, the ability of different ligands to induce hGR protein degradation will be tested by inhibiting *de novo* protein synthesis, stimulating with the different ligands and examining the GR protein levels over time.

**2. Does the extent of hGR phosphorylation at S226 and/or hGR degradation determine ligand-selective efficacy and/or potency for transactivation and/or transrepression?**

The hypothesis is that the relative amount of GR phosphorylation at S226, induced by the different ligands, will determine the 'rank order' of ligand-mediated transactivation and transrepression. Firstly, correlation analyses between the potency and efficacy of the ligands in transactivation and transrepression, in the same cell system (E. Stubsrud and K. Ronacher; Addendum D), and the extent of ligand-selective phosphorylation at S226 will be performed to test the hypothesis that ligand-selective hGR phosphorylation at S226 could possibly determine the rank order of ligand-selective GR mediated transcription. Thereafter, if a positive correlation is obtained, the ability of a S226 phosphorylation-deficient, transiently transfected hGR mutant (S226A), to cause ligand-selective GR transactivation and transrepression, will be tested in COS-1 cells. If ligand-selective GR phosphorylation at S226 determines the 'rank order' of ligand-

mediated transactivation or transrepression, the phosphorylation mutant should not show ligand-selective transactivation or transrepression.

Similarly, the alternative hypothesis that ligand-selective GR degradation determines the 'rank order' of ligand-induced transactivation and transrepression will be tested. In other words, the rate at which the GR is degraded in response to a particular ligand may determine the transcriptional response of the particular ligand. First correlation analyses between the potency and efficacy of the ligands in transactivation and transrepression, in the same cell system (E. Stubsrud and K. Ronacher; Addendum E), and the extent of ligand-selective GR degradation will be performed to test the hypothesis that ligand-selective GR degradation could possibly determine the rank order of ligand-selective GR mediated transcription. The hypothesis will be further investigated by inhibiting hGR degradation with a proteasome inhibitor and examining the effect thereof on ligand-selective hGR transactivation of a TAT-GRE-luc reporter using transiently expressed hGR in COS-1 cells. If ligand-selective GR degradation determines ligand-selective transactivation, then when GR degradation is inhibited such that the GR levels are similar in the presence of all the ligands, they should all result in the same efficacy for transactivation. Furthermore, the alternative hypothesis that ligand-selective GR transactivation determines ligand-selective GR degradation will also be investigated. In other words, the transcriptional response with a particular ligand may determine the rate at which the GR is degraded with that particular ligand. This will be investigated by inhibiting GR-mediated transcription with an RNA polymerase inhibitor, and examining the effect thereof on ligand-selective GR degradation. If ligand-selective GR transactivation is required for ligand-selective GR degradation, then inhibition of GR-mediated transcription should result in a similar degradation rate for the hGR with all the ligands.

**3. Which kinases phosphorylate the hGR at S211 and S226 in intact cells?**

According to results in the literature with *in vitro* kinase assays, JNK and p38 are responsible for hGR phosphorylation at S226 and S211 in COS-1 cells, respectively. Therefore, it is hypothesised that JNK and p38 are responsible for phosphorylation of the transiently expressed hGR at S226 and S211, respectively in COS-1 cells. This will be tested by examining the effect of specific JNK and p38 inhibitors on hGR phosphorylation at S226 and S211. If JNK and p38 are responsible for phosphorylation of the GR at S226 and S211, respectively, then the relative amount of hGR phosphorylation should be inhibited or reduced in response to the specific inhibitors.

**4. What are the molecular mechanisms whereby agonist-induced hGR phosphorylation modulates transcription efficacy?**

In the literature there are conflicting results on the role of GR phosphorylation on GR degradation, and the effect thereof on GR-mediated transcription. The hypothesis that agonist-induced hGR phosphorylation at S211 and S226 modulates transcription via modulation of hGR degradation rate will be investigated in more detail. This will be done by directly comparing the degradation rate of transiently expressed wt hGR with phosphorylation hGR mutants, which can no longer be phosphorylated at S211 (S211A) or S226 (S226A), in the absence and presence of the agonist dex, in COS-1 cells. If agonist-induced hGR phosphorylation at S211 or S226 is required for enhanced GR degradation, then the phosphorylation mutants should have a slower rate of GR degradation, as compared to wt GR.

Furthermore, acetylation and phosphorylation of the androgen receptor (AR) have been functionally linked (Fu *et al.*, 2004). Additionally, direct acetylation of the AR by p300 has been shown to be important for AR-mediated transactivation (Fu *et al.*, 2000;Fu *et al.*, 2004). Therefore the hypothesis that GR phosphorylation at S211 and S226 is important for the recruitment of p300 and acetylation of the GR will be investigated. By comparing the ability of transiently expressed wt and phosphorylation mutant hGR to recruit p300 in *in vitro* pull down assays using COS-1 cell extracts, it will be determined if GR phosphorylation is required for the recruitment of p300. Furthermore, using an antibody raised against acetylated lysine, the acetylation status of expressed wt and phosphorylation mutant hGR will be determined. If hGR phosphorylation is required for hGR acetylation via p300, then the phosphorylation mutant should show reduced acetylation and should not be able to recruit p300.

In addition, GR phosphorylation has been shown to play a role in the recruitment of the secondary co-factor, p300 (Kino *et al.*, 2007;Galliher-Beckley *et al.*, 2008). However, the requirement of GR phosphorylation at S226 and/or S211 for recruitment of a primary co-factor, has not previously been shown. The hypothesis that hGR phosphorylation at S226 and/or S211 is required for the recruitment of the primary co-factor, GRIP-1, will thus be investigated. This will be done by examining the interaction between GRIP-1 and wt hGR, as well as the S211A and S226A phosphorylation mutants by immunoprecipitation assays (in the presence and absence of a GRE), as well as chromatin immunoprecipitation assays in COS-1 cells. If GR phosphorylation is required for GRIP-1 recruitment, then the mutants would not be able to recruit GRIP-1.

Understanding what determines ligand-selective potency and efficacy will further our understanding of the physiological responses to endogenous ligands and drugs and assist in the

design of more effective drugs with fewer side-effects, for many different pharmacological applications.

# Chapter 3 Materials and Methods

## 3.1 Cell Lines

Monkey kidney fibroblast cells (COS-1 and COS-7), human endocervical cells (END-1/E6E7) and human osteosarcoma cells (U2OS) were purchased from American Type Culture Collection (ATCC, USA) and mouse pituitary gonadotrope cells (LβT2) were kindly provided by Dr. Pamela Mellon from the University of California, San Diego, USA. COS-1, COS-7, U2OS cells and LβT2 cells were cultured in 75 $cm^2$ culture flasks (Greiner Bio-One International, Austria) in high glucose (1 g/ml) Dulbecco's Modified Eagles Medium (DMEM, Sigma-Aldrich, South Africa) supplemented with 10% (v/v) fetal calf serum (Delta Bioproducts, South Africa), 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco Invitrogen, Paisley, UK) at 37°C in a 5% $CO_2$ incubator. Human osteosarcoma U2OS cells stably transfected with HA-tagged human GR using FuGENE 6 (Roche, South Africa) were obtained from Dr. MJ Garabedian and were cultured under the same conditions as described above, however in the presence of 300 μg/ml Geneticin (Sigma-Aldrich, South Africa). END-1 cells were cultured in Keratinocyte serum free medium (Gibco Invitrogen, Paisley, UK) supplemented with 0.1 ng/ml EGF (Gibco Invitrogen, Paisley, UK), 50 μg/ml bovine pituitary extract (Gibco Invitrogen, Paisley, UK), 0.4 mM $CaCl_2$ (Gibco Invitrogen, Paisley, UK), 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco Invitrogen, Paisley, UK) at 37°C in a 5% $CO_2$ incubator.

The COS-1, COS-7 and U2OS cells stock cultures, in 75 $cm^2$ culture flasks (Greiner Bio-One International, Austria), were trypsinized in 2 ml trypsin for 5 minutes at 37°C in a 5% $CO_2$

incubator, before the addition of 8 ml DMEM, containing 10% fetal calf serum and antibiotics as described above. The COS-1, COS-7 and U2OS cells were maintained by regular splitting twice a week at a 1/10 dilution. The LβT2 cell stock cultures, in 175 $cm^2$ culture flasks (Greiner Bio-One International, Austria), were trypsinized in 2 ml trypsin for 3 minutes at 37°C in a 5% $CO_2$ incubator, before the addition of 8 ml DMEM, containing 10% fetal calf serum and antibiotics as described above. These cells were maintained by splitting at a 1/4 dilution only once a week. The END-1 cells, in 75 $cm^2$ culture flasks (Greiner Bio-One International, Austria), were trypsinized in 2 ml trypsin for 10 minutes at 37°C in a 5% $CO_2$ incubator, before the addition of 8 ml keratinocyte serum free medium (Gibco Invitrogen, Paisley, UK) supplemented with 0.1 ng/ml EGF (Gibco Invitrogen, Paisley, UK), 50 μg/ml bovine pituitary extract (Gibco Invitrogen, Paisley, UK), 0.4 mM $CaCl_2$ (Gibco Invitrogen, Paisley, UK), 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco Invitrogen, Paisley, UK). These cells grow very slowly and were maintained by splitting at a 1/4 dilution every second week.



## 3.2 Compounds and antibodies

Dexamethasone, cortisol (hydrocortisone), prednisolone, aldosterone, MPA (6α-Methyl-17αhydroxyprogesterone acetate), progesterone, NET (norethindrone acetate), RU486 (mifepristone) and UDCA (ursodeoxycholic acid) were purchased from Sigma-Aldrich, South Africa and made up in 100% ethanol. Compound A (2,4-acetoxyphenyl)-2-chloro-N-methyl-ethylammonium chloride) was synthesized in Stellenbosch as previously described (Louw *et al.*, 1997). All the above mentioned compounds were dissolved in EtOH. DO6 and AL438 were a generous gift from Dr J. Miner (Ligand Pharmaceuticals, San Diego, USA) and dissolved in

100% DMSO and a 50% DMSO, 50% EtOH mixture, respectively. The test compounds were added to the cells such that the final concentration of EtOH was less than 0.1%.

Tumor Necrosis Factor-α (TNF-α), 4α-Phorbol 12-myristate 13-acetate (PMA), Z-Leu-Leu-Leu-al (MG132), α-amanitin and cycloheximide (CHX) was purchased from Sigma-Aldrich, South Africa. The p38 inhibitor (SB203580), JNK inhibitor (SP600125) and CDK inhibitor (roscovitine) were purchased from Merck chemicals South Africa.

The anti-phospho-serine 226 (P-S226) hGR and anti-phospho-serine 211 (P-S211) hGR specific antibodies was a generous gift from Dr M.J. Garabedian (New York University, School of Medicine, USA). The GR (H300; sc-8992), MKP-1 (sc-370) and P300 (sc-32244) antibodies were purchased from Santa Cruz Biotechnology (USA). The β-actin (#4967), phospho-SAPK/JNK (Thr183/Thr185) (#9251), SAPK/JNK (#9252), p53 (#2524), phospho-p44/42 MAP kinase (Thr202/Tyr204) (#9101), p44/42 MAPK (#9102), phospho-p38 MAPK (Thr180/Tyr182) (#9211), p38 MAPK (#9212), GAPDH (#14C10) and acetylated lysine (#9441) antibodies were purchased from Cell Signaling Technology. The HA (12CA5) and histone H3 (#ab1791) antibodies were from Roche (South Africa) and Abcam (UK), respectively. The secondary anti-rabbit HRP conjugate (NA934VS) and anti-mouse HRP conjugate (NA931) antibodies were purchased from AEC Amersham (South Africa). The dilutions used for each specific primary antibody, as well as the corresponding secondary antibody and dilution thereof can be obtained from addendum B (Antibodies).

## 3.3 Plasmids

HA-tagged human GR wt (HA-hGRwt), as well as the S211A (HA-hGRS211A) and S226A (HA-hGRS226A) mutant expression vectors were obtained from Prof. M. J. Garabedian at New York University, School of Medicine, USA (Wang *et al.*, 2002b). The pTAT-GRE-E1b-luc (or TAT-GRE containing an E1b promoter and 2 copies of rat TAT-GRE) and the MMTV-luc (containing 4 GREs; MMTV) plasmids were gifts from Dr. G. Jenster at Erasmus University of Rotterdam, Rotterdam, the Netherlands (Sui *et al.*, 1999) and Gordon L Hager (National Cancer Institute, USA), respectively. The pRS human GR (pRS-hGRwt), pRS S203/211/226A (pRS-hGR3A), pRS S211A (pRS-hGRS211A) and pRS S226A (pRS-hGRS226A), as well as the wt and dominant negative CDK5 plasmids were a gift from Tomoshige Kino (NIH, Bethesda, USA). The 7x AP-1-luc (AP-1) and 5x NFκB-luc (NFκB) plasmids were obtained from Stratagene (Houston, Texas, USA), whereas the pcDNA3.1 (empty vector) plasmid was obtained from Invitrogen (UK). The pCMV-β-galactosidase plasmid (β-gal) was a gift from Dr G. Hageman (University of Gent, Belgium). HA-GRIP-1 plasmid was a gift from M.R. Stallcup (University of California, USA), while wt and dominant negative JNK and p38 plasmids were generously given by Prof. A. Moon (Duksung Women's University, Seoul, Korea).



## 3.4 Plasmid transformation and preparation

The pRS human GR constructs were transformed into HB101 competent cells and all the other plasmids were transformed into DH5α competent cells. The transformations were done by heat-shock as described in Sambrook *et al*. (Sambrook *et al.*, 1989). Briefly 1-10 ng DNA was mixed

with 100 µl competent cells and left on ice for 30 minutes. Thereafter the mixture was placed at 42°C for 2 minutes and back on ice for an additional 2 minutes before 900 µl SOC medium (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.05% (w/v) NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ and 20 mM glucose) (Sambrook *et al.*, 1989) was added and incubated for 1 hour at 37°C with shaking. The cells were plated on LB-AMP plates (1% (w/v) tryptone, 0.5% yeast extract, 1% NaCl and agarose containing 50 µg/ml ampicillin) (Sambrook *et al.*, 1989) and left overnight at 37°C. The next day, a positive colony was picked and grown in 50 ml LB medium (1% (w/v) tryptone, 0.5% yeast extract and 1% NaCl) (Sambrook *et al.*, 1989) containing 50 µg/mL ampicillin (Sigma-Aldrich, St. Louis, MO, USA) for at least 8 hours at 37°C with shaking. For glycerol stocks equal amounts (1 ml) of 80% (v/v) glycerol and cell suspension were mixed and stored at -80°C. For plasmid purification, LB medium containing 50 µg/ml ampicillin was inoculated and allowed to grow overnight at 37°C with shaking. The next day the plasmid DNA was purified with the Promega PUREYIELD plasmid maxiprep (Promega, Madison, WI, USA), according to the manufacturers' protocol. The integrity and purity of the plasmids were assessed by restriction enzyme digestion and agarose gel electrophoresis.



## 3.5 Transactivation

COS-1 cells were seeded into 10-cm dishes (NUNC, AEC Amersham) at a density of $2x10^6$ cells/dish in DMEM, containing 10% fetal calf serum and antibiotics as described above. The next day the cells were transfected with 10 µg of either HA-hGRwt, HA-hGRS226A, pRS–hGRwt, pRS-hGR3A, pRS-hGRS211A or pRS-hGRS226A and 3.75 µg TAT-GRE or MMTV using FuGENE 6 (Roche, South Africa) according to the manufacturer's instructions, using a

ratio of 1 μg DNA : 2 μl FuGENE 6, and incubated for 24 hours. On the morning of day three, the cells were washed with PBS (Sambrook *et al.*, 1989) and incubated with trypsin (Highveld Biologicals, South Africa) at 37°C before being re-plated into 24-well plates (NUNC, AEC Amersham) at a density of $5x10^4$ cells/well in DMEM, containing 10% fetal calf serum and antibiotics as described above. After a 24 hour incubation, the cells were washed in PBS and serum-free DMEM was added before the cells were treated with vehicle (EtOH) or the respective compounds for 24 hours. The cells were harvested by washing twice with PBS and lysed in 50 μl Reporter lysis buffer (Promega, Madison, WI, USA). Luciferase activity was measured using the Luciferase Assay System (Promega, Madison, WI, USA) and a Modulus microplate reader (Turner Biosystems, Sunnyvale CA, USA). The luciferase values obtained were normalized to protein content per well as determined by standard Bradford assay (Bradford, 1976). If the COS-1 cells were plated directly into 24-well plates, the cells were transfected with proportionally less DNA, keeping the ratio of 10 μg GR/ 2 X $10^6$ cells, as well as 0.025 μg β-gal. In this case the expression of β-galactosidase, which was measured using the GalactoStar Assay Kit (Tropix, Bedford MA, USA), was used to normalize for transfection efficiency in each well. If so, it is stated in the figure legend of the appropriate experiment. The normalised transactivation value for 10 μM dex with wt GR was set as 100 % and values for all the other samples were calculated relative to the maximal induction by 10 μM dex with wt GR. The relative efficacy is given as the maximal induction point and the $EC_{50}$ (nM) was obtained from fitting the dose-response curve in GraphPad Prism.

## 3.6 Transrepression

COS-1 cells were seeded into 24-well plates at a density of $5x10^4$ cells/well in DMEM, containing 10% fetal calf serum and antibiotics as described above. The following day, the cells were transfected with 0.125 μg HA-hGRwt, HA-hGRS211A, HA-hGRS226A, pRS-hGRwt, pRS-hGRS211A or pRS-hGRS226A and 0.25 μg of NFκB or AP-1 plasmids, as well as 0.025 μg β-gal using FuGENE 6 according to the manufacturer's instructions, using a ratio of 1 μg DNA : 2 μl FuGENE 6, and incubated for 24 hours. On day three the cells were washed with PBS and incubated with serum-free DMEM containing 20 ng/ml PMA and vehicle (EtOH) or the appropriate compounds for 24 hours. The following day the luciferase activity was measured using the Luciferase Assay System (Promega, Madison, WI, USA) and a Modulus microplate reader (Turner Biosystems, Sunnyvale CA, USA). The luciferase values obtained were normalized to the expression of β-galactosidase, which was measured using the GalactoStar Assay Kit (Tropix, Bedford MA, USA). Induction with PMA only was set to 100% and other conditions expressed as relative % thereof.



## 3.7 Western Blotting

### 3.7.1 Western blotting for ligand-selective phosphorylation (Chapter 3.1)

For the phosphorylation assays COS-1 were plated into 6-cm tissue culture dishes (NUNC, AEC Amersham) at a density of $7x10^5$ cells/dish and U2OS cells were plated into 12-well plates (NUNC, AEC Amersham) at a density of $2x10^5$ cells/well in DMEM, containing 10% fetal calf

serum and antibiotics as described above. COS-1 cells were transiently transfected with 3.5 μg HA-hGRwt DNA per dish, using FuGENE 6 (Roche, South Africa) according to manufacturer's instructions, using a ratio of 1 μg DNA : 2 μl FuGENE 6. The U2OS cells were not transfected, since they contain stably transfected HA-hGRwt (obtained from Dr M.J. Garabedian (New York University, School of Medicine, USA). After 24 hours, the COS-1 cells were washed with PBS, trypsinised and re-plated into 12-well plates (NUNC, AEC Amersham) at a density of $2x10^5$ cells/well in DMEM, containing 10% fetal calf serum and antibiotics as described above. Twenty four hours after re-plating, the COS-1 and the U2OS cells were washed with PBS and incubated with serum-free DMEM containing vehicle (EtOH), 100 nM or 10 μM of the appropriate compounds for 1 hour. Subsequently the cells were washed with PBS and lysed in 400 μl lysis buffer (20 mM Tris, 150 mM NaCL, 1 mM EDTA, 1 mM EGTA, 1% (v/v) TritonX-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM sodium fluoride) containing protease inhibitors (1 complete mini protease inhibitor cocktail tablet/10 ml, Roche, South Africa). The protein content of each sample was determined by Bradford assay before loading equal amounts of protein, typically 20 μg, in 1x SDS sample buffer (5x SDS sample buffer: 100 mM Tris-CL pH 6.8, 5% (v/v) SDS, 20% (v/v) glycerol, 5% β-mercaptoethanol and 0.1% (w/v) bromophenolblue) onto an 8% SDS-polyacrylamide gel. Rainbow marker (1.5 μl) (Amersham, South Africa) was also loaded on the gel. The separated proteins were transferred onto a Hybond-ECL Nitrocellulose membrane (Amersham, South Africa) for 1 hour at 180 mA, using the Mini Protean III blotting system (BioRad, South Africa) in Tris/Glycine transfer buffer (25 mM Tris, 250 mM glycine and 10% (w/w) methanol). The membranes were blocked overnight at 4°C in 4% blocking solution (4% (w/v) ECL Advance blocking powder (Amersham, South Africa) and Tris-buffered saline (TBS, 50 mM Tris, 150 mM NaCl) containing 0.1% (v/v) Tween, TBST). Next the membranes were incubated in 4%

blocking solution containing an anti-P-S226 specific antibody (previously described in (Chen *et al.*, 2008)) at a dilution of 1:10 000 for 1 hour at RT. Thereafter the blots were washed 1x 10 minutes and 2x 5 minutes in TBST at RT. As secondary antibody an anti-rabbit HRP conjugate antibody (NA934VS, Amersham, South Africa) was used at a dilution of 1:10 000 in a 5% milkpowder solution (5% (w/v) milkpowder, 1x TBST) for 1 hour at RT. The blots were washed 1x 10 minutes and 2x 5 minutes in TBST at RT and autoradiography was performed. Thereafter, the membranes were stripped for 30 minutes at 60 °C in stripping buffer (100 mM 2-mercaptoethanol, 2% (w/v) SDS, 62.4 mM Tris-Cl, pH 6.7). The blots were washed twice with TBST for 10 minutes, blocked overnight, as previously described, and quantification of total GR levels were performed on the same blot by reprobing with an antibody to total GR (H300, Santa Cruz Biotechnology, USA), followed by secondary antibody probing and autoradiography.



### 3.7.2 Western blotting for other applications (Chapter 3.3 – 5.4)

COS-1 cells transiently transfected with hGR, as well as U2OS, LβT2 or END-1 cells, containing endogenous GR were plated into 12- or 6-well plates at different amounts of cells/well, as indicated in the figure legends and treated with inducing compound or inhibitor and vehicle (EtOH) or test compound, as indicated in the figure legends. After ligand stimulation the cells were washed twice with PBS and harvested in 50 or 100 µl 2x SDS sample buffer, for 12- or 6-well plates respectively, and boiled for 5 minutes. Thereafter equal amounts were separated on a SDS-PAGE gel and transferred to membrane, as described above. The membranes were blocked in 4% blocking solution for 1 hour at RT and incubated with primary antibody, in 4% blocking solution, overnight at 4°C. The following day, the membranes were washed 1x 10 minutes and

2x 5 minutes at RT with TBST and incubated with secondary antibody, in 5% milkpowder solution for 1 hour at RT. Thereafter the blots were washed 1x 10 minutes and 2x 5 minutes in TBST at RT and autoradiography was performed. The dilutions used for each specific primary antibody, as well as the corresponding secondary antibody and dilution thereof can be obtained from addendum B (Antibodies).

### 3.7.3 Autoradiography and quantification

The proteins were visualized by the ECL advance Western blotting detection kit (Amersham, South Africa) or ECL normal Western blotting detection kit (Amersham, South Africa), according to the manufacturer's protocol, and Hyperfilm MP high performance autoradiography film (Amersham, South Africa). Bands on the X-ray film were scanned or the film was photographed, before quantification using AlphaEaseFC FluorChem 5500 (Alpha Innotech).



## 3.8 GR Half-life

COS-1 cells were plated into 10-cm dishes (NUNC, AEC Amersham) at a density of $2x10^6$ cells/dish in DMEM, containing 10% fetal calf serum and antibiotics as described above. The next day the cells were transfected with 10 μg HA-hGRwt, HA-hGRS211A, HA-hGRS226A, pRS-hGRwt or pRS-hGR3A. On day three the cells were washed with PBS and incubated with trypsin at 37°C before being re-plated into 6-well plates (NUNC, AEC Amersham) at a density of $4x10^5$ cells/well in DMEM, containing 10% fetal calf serum and antibiotics. After 24 hour incubation, the cells were washed in PBS and pre-treated with serum-free DMEM containing 1

μM cycloheximide for 1 hour. The cells were then treated with vehicle (EtOH) or 10 μM compound and harvested at various time points, before Western blot analysis was performed as described above, using an antibody to total GR and β-Actin as loading control. The amount of GR at the zero hour time point was set to 100% and the amount of GR at the other time points was calculated as percentage of the zero hour time point.

## 3.9 Inhibition of transcription

COS-1 cells were seeded into 24-well plates (NUNC, AEC Amersham) at a density of $5x10^4$ cells/well in DMEM, containing 10% fetal calf serum and antibiotics as described above, and incubated for 24 hours. The following day the cells were transfected with 0.047 μg TAT-GRE and 0.125 μg HA-hGRwt per well, using FuGENE 6 (Roche, South Africa) according to the manufacturer's instructions, using a ratio of 1 μg DNA : 2 μl FuGENE 6. After 24 hour incubation, the cells were pre-treated with either 1 μg/ml or 2.5 μg/ml of the RNA polymerase II inhibitor, α-amanitin (Sigma-Aldrich, South Africa), for 2 hours and subsequently stimulated with vehicle (EtOH) or 100 nM dex or MPA for 24 hours. The cells were harvested by washing twice with PBS and lyses in 50 μl Reporter lysis buffer (Promega, Madison, WI, USA). Luciferase activity was measured using the Luciferase Assay System (Promega, Madison, WI, USA) and a Modulus microplate reader (Turner Biosystems, Sunnyvale CA, USA). The luciferase values obtained were normalized to protein content per well as determined by standard Bradford assay. After the luciferase and Bradford assays were performed, equal amount of proteins were loaded onto a 8% SDS-PAGE and Western blot analysis were performed, as

described above, probing for total GR with anti-GR antibody (H300, Santa Cruz) and for β-actin with anti-β-actin antibody (Cell Signaling) as loading control.

## 3.10 Inhibition of degradation

COS-1 cells were seeded into 24-well plates (NUNC, AEC Amersham) at a density of $5x10^4$ cells/well in DMEM, containing 10% fetal calf serum and antibiotics as described above, and incubated for 24 hours. The following day the cells were transfected with 0.047 μg TAT-GRE and 0.125 μg HA-hGRwt per well, using FuGENE 6 (Roche, South Africa) according to the manufacturer's instructions, using a ratio of 1 μg DNA : 2 μl FuGENE 6. After 24 hour incubation, the cells were pre-treated with either 5 μM or 10 μM of the proteasome inhibitor, MG132 (Sigma-Aldrich, South Africa), for 2 hours and subsequently stimulated with vehicle (EtOH) or 100 nM dex or MPA for 24 hours. The cells were harvested by washing twice with PBS and lysed in 50 μl Reporter lysis buffer (Promega, Madison, WI, USA). Luciferase activity was measured using the Luciferase Assay System (Promega, Madison, WI, USA) and a Modulus microplate reader (Turner Biosystems, Sunnyvale CA, USA). The luciferase values obtained were normalized to protein content per well as determined by standard Bradford assay. After the luciferase and Bradford assays were performed, equal amount of proteins were loaded onto a 8% SDS-PAGE and Western blot analysis were performed, as described above, probing for total GR with anti-GR antibody (H300, Santa Cruz) and for β-actin with anti-β-actin antibody (Cell Signaling) as loading control.

## 3.11 JNK CASE assay

COS-1 cells were plated into 96-well plates (NUNC, AEC Amersham) at a density of $5x10^3$ cells/well in DMEM, containing 10% fetal calf serum and antibiotics as described above, and incubated for 24 hours. The following day, the cells were washed with PBS and serum-free DMEM was added to the cells, before being transfected with 0.05 μg HA-hGRwt. On day three the cells were pretreated with 0.02 μg/ml TNF-α for 1 hour before being stimulated with vehicle (EtOH) or 100 nM compound for an additional 1 hour. Subsequently the cells were fixed to the 96-well plate in a 4% cell fixing buffer (89% (v/v) PBS and 11% (v/v) 37% formaldehyde) for 20 minutes at RT. In order to determine the amount of phosphorylated JNK and total JNK in each well, the Superarray CASE JNK (T183/Y185) assay (FE-004, Super Array Bioscience Corporation) was performed according to manufacturers' protocol. Briefly, the amount of phosphorylated JNK (P-JNK) and total JNK, in each well, were determined by the use of phospho-JNK and total JNK specific antibodies (Super Array Bioscience Corporation). Furthermore the relative cell number in each well was determined and used to normalize the amount of phosphorylated and total JNK. Thereafter the ratio of P-JNK / total JNK was calculated for each compound.



## 3.12 Kinase and GR phosphorylation timecourse

COS-1 cells were seeded into 6-well plates (NUNC, AEC Amersham) at a density of $3x10^5$ cells/well in DMEM, containing 10% fetal calf serum and antibiotics as described above, and incubated for 24 hours. The following day, the cells were transfected with 3μg HA-hGRwt per

well and incubated for an additional 24 hours before being pretreated with 0.02 μg/ml TNF-α for 15 minutes. Subsequently, the cells were treated with vehicle (EtOH) or 100 nM dex and incubated for various times. At the appropriate time, the cells were harvested and equal amounts of cell extract were separated on an 8% SDS-PAGE. The Western blots were cut in half and the top part of the Western blots were probed with either an anti-P-S226 GR-specific or an anti-P-S211 GR-specific antibody (previously described in (Wang *et al.*, 2002b;Chen *et al.*, 2008)), whereas the bottom half of the Western blots were probed with either an anti-phospho-SAPK/JNK antibody (P-JNK; Cell Signaling Technology) or an anti-phospho-p38 (P-p38; Cell Signaling Technology) antibody. After developing, the blots were stripped, as described above, and re-probed for either total GR with anti-GR antibody (H300, Santa Cruz), total JNK with anti-JNK antibody (Cell signaling Technology) or for total p38 with anti-p38 antibody (Cell Signaling Technology).



## 3.13 Kinase inhibition

### 3.13.1 MAPK inhibition

COS-1 cells were seeded into 6-well plates (NUNC, AEC Amersham) at a density of $3x10^5$ cells/well in DMEM, containing 10% fetal calf serum and antibiotics as described above, and incubated for 24 hours. The following day, the cells were transfected with 3 μg HA-hGRwt per well and incubated for an additional 24 hours before being pre-treated with either 10 μM p38 inhibitor (SB203580), 50μM JNK inhibitor (SP600125) or no inhibitor for 16 hours in serum-free DMEM. Subsequently, the cells were treated with vehicle (EtOH) or 100 nM dex for 1 hour and

harvested. Equal amounts of protein were separated on an 8% SDS-PAGE and probed either for anti-P-S226 GR, anti-P-S211 GR, anti-P-JNK or anti-P-p38. After developing, the blots were stripped as described above and re-probed for either total GR with anti-GR antibody (H300, Santa Cruz), total JNK with anti-JNK antibody (Cell signaling Technology) or for total p38 with anti-p38 antibody (Cell Signaling Technology).

### 3.13.2 CDK inhibition

LβT2, END-1 cells and COS-1 cells were plated into 12-well plates at a density of $5x10^5$ cells/well, $5x10^5$ cells/well and $1.5x10^5$ cells/well, respectively. The following day, the COS-1 cells were transiently transfected with 1 μg HA-hGRwt and incubated for 24 hours. Thereafter the LβT2, END-1 cells and COS-1 cells were pretreated with 20 μM of the general CDK inhibitor, roscovitine, for 1 hour in serum-free DMEM, before stimulation with 100 nM dex or vehicle (EtOH). The cells were harvested and equal amounts of cell extract were separated on SDS-PAGE. Western blot analysis was performed using either an anti-P-S211 or an anti-P-S226 GR-specific antibody. Thereafter the membrane was stripped and reprobed with anti-GR antibody (H300, Santa Cruz) for total GR.

## 3.14 Overexpression of wt and dominant negative (DN$^-$) kinases

### 3.14.1 CDK5

LβT2, END-1 cells and COS-1 cells were plated into 12-well plates at a density of $2x10^5$ cells/well, $5x10^5$ cells/well and $1.5x10^5$ cells/well, respectively. The following day, the cells were transiently transfected with 1 μg p35 (to activate CDK) and 1 μg wt plasmid expressing CDK5 or DN$^-$ CDK5 (Kino *et al.*, 2007). The COS-1 cells were also transiently transfected with 1 μg HA-hGRwt. After a 24 hour incubation, the cells were stimulated with 100 nM dex or vehicle (EtOH) for 1 hour in serum-free DMEM. The cells were harvested and equal amounts of cell extract were separated on SDS-PAGE. Western blot analysis was performed using either an anti-P-S211 or an anti-P-S226 GR-specific antibody. Thereafter the membrane was stripped and reprobed with anti-GR antibody (H300, Santa Cruz) for total GR.

### 3.14.2 JNK and p38

LβT2 and COS-1 cells were plated into 12-well plates at a density of $2x10^5$ cells/well and $1.5x10^5$ cells/well, respectively. The following day, the cells were transiently transfected with 1 μg plasmid expressing wt or DN$^-$ JNK, or DN$^-$ p38 (Kang *et al.*, 2003). The COS-1 cells were also transiently transfected with 1 μg HA-hGRwt. After a 24 hour incubation, the cells were stimulated with 100 nM dex or vehicle (EtOH) for 1 hour in serum-free DMEM. The cells were harvested and equal amounts of cell extract were separated on SDS-PAGE. Western blot analysis was performed using either an anti-P-S211 or an anti-P-S226 GR-specific antibody. Thereafter the membrane was stripped and reprobed with anti-GR antibody (H300, Santa Cruz) for total GR.

## 3.15 Effect of α-amanitin and MG132 on GR and kinase phosphorylation

COS-1 cells were seeded into 6-well plates (NUNC, AEC Amersham) at a density of $3x10^5$ cells/well in DMEM, containing 10% fetal calf serum and antibiotics as described above, and incubated for 24 hours. The following day, the cells were transfected with 3 μg HA-hGRwt per well and incubated for 24 hours, before being pre-treated with either 2.5 μg/ml α-amanitin or 10 μM MG132 for 2 hours in serum-free DMEM. The cells were subsequently stimulated with 100 nM dex or vehicle (EtOH) and harvested. Western blot analysis were performed as described above, using either an anti-P-S226 GR, anti-P-S211 GR, anti-P-JNK or anti-P-p38 specific antibody. After developing, the blots were stripped as previously described, and re-probed with antibodies to either total GR (H300, Santa Cruz), total JNK (Cell signaling Technology), or total p38 (Cell Signaling Technology).



## 3.16 Co-immunoprecipitation

COS-1 cells were seeded into 10-cm dishes (NUNC, AEC Amersham) at a density of $1.5x10^6$ cells/dish in DMEM containing 10% fetal calf serum and antibiotics, as described above. After a 24 hour incubation, the cells were transfected with 3 μg HA-GRIP-1 and 3 μg of either pRS-hGRwt, pRS-hGR3A or pcDNA3.1 (empty vector) using FuGENE 6 (Roche, South Africa) according to the manufacturer's instructions. The following day, the cells were treated with 1 μM dex or vehicle (EtOH) for 1 hour in serum-free DMEM, washed once with PBS and lysed with 500 μl cytobuster (Novagene) containing protease inhibitors (1 complete mini protease inhibitor cocktail tablet/10 ml, Roche, South Africa). After removing 9 μl supernatant for inputs,

1.6 µg GR (H300, Santa Cruz) antibody was added to the remaining supernatant and incubated at 4°C rotating over night. The following day the antibodies were precipitated by addition of 28 µl of a 25% slurry pre-blocked protein A/G PLUS-Agarose beads (Santa Cruz) for 2 hours at 4°C while rotating, followed by 2x washes with PBS. The GR-bound proteins were released from the beads by boiling in 2x SDS-sample treatment buffer. The samples were separated on 6% SDS-PAGE and Western blotted as described above, probing with anti-GR (H300, Santa Cruz) and anti-HA (12CA5, Roche) antibodies.



## 3.17 Avidin Biotin Complex DNA (ABCD) assay

The ABCD assay was performed as previously described in Cho *et al.* with minor modifications (Cho *et al.*, 2005). This assay is a multistep assay, wherein seperate cytosols for the GR and the co-factor of interest are prepared. Thereafter, the GR cytosol is incubated with vehicle or hormone, before being incubated with beads containing GRE oligonucleotides, allowing the GR to bind to its GREs. Subsequently, the second cytosol (containing the co-factor of choice) is incubated with the GR-GRE-bead mixture, thereby allowing the co-factor of choice to bind to the GR. This complex on the beads is then pelleted by centrifugation, washed, eluted with SDS sample buffer and analysed by SDS-PAGE and Western blotting, probing with antibodies for the GR and the co-factor of choice is performed. After autoradiography, the relative amount of co-factor bound to the GR can then be measured.

COS-7 cells were seeded into 15-cm dishes (NUNC, AEC Amersham) at a density of $2x10^6$ cells/dish in DMEM, containing 10% fetal calf serum and antibiotics, as described above.

Twenty four hours later, the cells were transfected with 5 μg of either pRS-hGRwt, pRS-hGR3A, HA-GRIP-1 or pcDNA3.1 (empty vector) using FuGENE 6 (Roche, South Africa) according to the manufacturer's instructions and incubated for 48 hours. The cells were then harvested using either the TAPS method (GR and TAPS mock cytosols) or the cytobuster method (GRIP-1 and cytobuster mock cytosols).

For cytosol preparation using the "TAPS" method (Cho *et al.*, 2005), the cells were washed twice with ice cold PBS and incubated with 2 ml trypsin for 5 minutes at 37°C before the addition of 9 ml DMEM containing 10% fetal calf serum and antibiotics per dish. After scraping loose all the cells, they were transferred into a 50 ml tube and pelleted at 500 x g for 5 minutes at 4°C. After two PBS washes, the pellet was snap-frozen in an ethanol-dry ice bath and weighed. One pellet volume TAPS buffer (0.1 M TAPS pH 9.5) was added to the pellet (1 ml TAPS per 1 g pellet) and incubated on ice for 1 hour with vortexing every 10 minutes. The cellular debris was pelleted at 15 000 x g for 15 minutes at 4°C and aliquots of the supernatant were snap-frozen in an ethanol-dry ice bath and stored at -80°C.

For cytosol preparation using the "cytobuster" method (Cho *et al.*, 2005), the cells were washed twice with PBS and incubated with 2 ml cytobuster (Novagene) containing protease inhibitors (1 complete mini protease inhibitor cocktail tablet/10 ml, Roche, South Africa) per dish for 5 minutes at room temperature. Subsequently the cells were scraped and pelleted at 15 000 x g for 15 minutes at 4°C and aliquots of the supernatant were snap-frozen in an ethanol-dry ice bath and stored at -80°C.

To prepare the annealed GRE oligonucleotides, consensus GRE oligonucleotides (sense: 5'-Biotin-GAT CCT GTA CAG GAA TGT TCT AGC TACA-3'; antisense: 5'-Biotin-TGT AGC

TAG AAC ATT CCT GTA CAG GATC-3', Invitrogen) that are 5' biotinylated were annealed by mixing equimolar amounts (1.74 μg/μl) of each oligonucleotide, heating to 100°C for 5 minutes, then allowing to cool overnight. In order to determine whether annealing had occurred, the mixture and input single stranded oligonucleotides was electrophoresed on a 20% polyacrylamide-TAE gel at 120 V and the DNA was visualised by ethidium bromide staining.

On day one of the ABCD assay, 20 μl TAPS cytosol containing either hGRwt, hGR3A or TAPS mock cytosol were diluted to 100 μl with HEPES wash buffer (10 mM Hepes pH 7.5, 1 mM EDTA, 10% (v/v) glycerol) and stimulated with 1 μM dex or vehicle (EtOH) for 2.5 hours on ice before being heat activated for 30 minutes at 20°C. During that time, 5 μl of the annealed GRE oligonucleotides were immobilized on 100 μl of a 50% slurry Streptavidin affinity matrix (EZview Red Streptavivdin Affinity Gel (E5529) Sigma-Aldrich, South Africa) for 2 hours rotating at 4 °C. The activated GR cytosol (20 μl cytosol diluted to 100 μl with HEPES wash buffer) was incubated in the presence of 40 μl of the GRE/bead complex O/N at 4 °C with rotating, to allow DNA binding. The following day, the samples were centrifuged at 8 200 x g for 1 minute at 4 °C and supernatants were removed. To determine co-factor recruitment, the GR-DNA-bead complex pellet (20 μl actual beads) was incubated at 4 °C for 4 hours on the rotator with 200 μl GRIP-1 cytosol diluted 1:1.8 with mock (untransfected) cytobuster cytosol. Thereafter the beads were pelleted by centrifugation at 8 200 x g for 1 minute, washed with 1 ml HEPES buffer and vortexed for 10 seconds, before centrifugation (as above). The beads were washed three times with 1 ml HEPES buffer, as described above, and the protein complexes were eluted off the beads by boiling in 30 μl 2 x SDS sample buffer, vortexing and centrifugation at room temperature for 5 minutes at 20 000 x g. Thereafter the supernatant was collected, resolved

by 6% SDS-PAGE and Western blotted, probing with anti-GR (H300, Santa Cruz) and anti-HA (12CA5, Roche) antibodies, as described above.

## 3.18 Chromatin Immunoprecipitation (ChIP) assay

The ChIP assay was performed as previously described (Ma H, 2003;Shang *et al.*, 2000), with some minor modifications. This is a multistep assay, wherein DNA binding proteins in living cells are crosslinked to the DNA. Thereafter, the cells are harvested and the chromatin is sonicated to break it up into smaller pieces, before immunoprecipitation with an antibody of choice (i.e. the specific protein being investigated). The crosslinking is then reversed and the specific DNA to which the protein was bound is amplified by PCR, using primers specific for the DNA sequence of interest. Thereby the relative recruitment of the protein of choice to the specific DNA sequence can be determined.

COS-1 cells were plated into 15-cm dishes (NUNC, AEC Amersham, South Africa) at a density of $2x10^6$ cells/well, in DMEM containing 10% fetal calf serum and antibiotics, as described above. After a 24 hour incubation the cells were transfected with 5 μg HA-GRIP-1, 5 μg MMTV and 9 μg of either pRS-hGRwt, pRS-hGR3A or pcDNA3.1 (empty vector) using FuGENE 6 (Roche, South Africa) according to the manufacturer's instructions. The following day the cells were washed with PBS and serum starved for 2 hours in serum-free DMEM, before stimulation with vehicle (EtOH) or 1 μM dex for 1 hour. After stimulation, the proteins were cross-linked with 1% formaldehyde for 10 minutes at 37°C, before the reaction was stopped with 0.125 M glycine for 5 minutes at RT. Thereafter the cells were washed twice with PBS, scraped in 4 ml

PBS, containing protease inhibitors (1 Complete Mini Protease Inhibitor tablet per 20 ml), pelleted by centrifugation and resuspended in 500 µl nuclear lysis buffer (1% (w/v) SDS, 50 mM Tris pH 8.0, 10 mM EDTA, containing protease inhibitor tablet (one tablet per 10 ml nuclear lysis buffer)). The cross-linked DNA was fragmented into 100 – 400 bp fragments, by means of sonication (Misonix Sonicator 3000) with a microtip at 20 pulses (power setting 3), each pulse being 20 seconds, with resting phases in between each pulse of 40 seconds, on ice. After sonication, the mixture was centrifigated for 10 minutes at 15 000 x g and the supernatant (containing the fragmented DNA) was obtained for further analysis. To confirm the size of the DNA fragment in the sonicated chromatin, the DNA was purified, as described below, and electrophoresed on a 1% agarose gel and visualisation with ethidium bromide. The sonicated chromatin was either used immediately for further analysis or stored at 80°C.

In order to pre-block the protein A/G PLUS beads (sc-2003 Santa Cruz Biotechnology USA), 500 µl pure beads were incubated with 100 µg salmon sperm DNA (Invitrogen) and 1 mg/ml BSA (Roche) in 2 ml IP dilution buffer (0.01% (w/v) SDS, 20 mM Tris-HCl pH 8.0, 1.1% (v/v) Triton X-100, 167 mM NaCl, 1.2 mM EDTA and 1x protease tablet per 10 ml) for 1 hour at 4°C on a rotating wheel. Thereafter the beads were centrifuged at 5 500 x g for 1 minute at 4°C and resuspended as 50% slurry in IP dilution buffer.

DNA quantification of the sonicated chromatin was performed by means of optical density readings at 260 nm. For the input samples, 30 µg chromatin was made up to 30 µl with nuclear lysis buffer and diluted with 90 µl IP elution buffer (1% (w/v) SDS and 100 mM $NaHCO_3$). The cross-links were reversed and the input DNA was purified as described below. For the immunoprecipitation, 50 µg chromatin was made up to a final volume of 50 µl with nuclear lysis

buffer and diluted by adding 950 µl IP dilution buffer. Thereafter the sample chromatin (1 ml) was pre-cleared by adding 20 µl of a 50% slurry of pre-blocked protein A/G PLUS beads and incubated for 1 hour at 4°C on a rotating wheel to reduce non-specific binding. The pre-cleared chromatin was collected by means of centrifugation at 15 000 x g for 10 minutes, and incubated with 5 µg anti-GR (H300, sc-8992), anti-HA (12CA5) or anti-IgG antibody (sc-2350) overnight at 4 °C on a rotating wheel. The following day, the protein-DNA complexes were incubated with 40 µl of 50% slurry pre-blocked protein A/G PLUS beads for 6 hours at 4°C on a rotating wheel, followed by centrifugation at 5 500 x g for 1 minute at 4°C. The bead pellet, containing the protein-DNA complexes, was washed once with 1 ml wash buffer I (0.1% (w/v) SDS, 1% (v/v) Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0 and 150 mM NaCl), followed by washing with 1 ml wash buffer II (0.1% (w/v) SDS, 1% (v/v) Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0 and 500 mM NaCl) . Thereafter, it was washed with 1 ml wash buffer III (1% (v/v) NP-40, 1% (w/v) sodium deoxycholate, 500 mM LiCl, 1 mM EDTA and 10 mM Tris-HCl pH 8.0), followed by three washes with 1 ml TE (10 mM Tris pH 8.0 and 0.1 mM EDTA). In order to elute the protein-DNA complexes from the beads, containing the protein-DNA complexes, the beads were incubated twice with 150 µl IP elution buffer for 15 minutes at RT on a rotating wheel, followed by centrifugation at 15 000 x g for 10 minutes.

To reverse the cross-links, NaCl (to a final concentration of 300 nM) was added to the input and immunoprecipitated DNA, before overnight incubation at 65°C. The following day an appropriate volume of 0.5 M EDTA (to a final concentration of 15 nM EDTA) and 1 M Tris pH 6.5 (final concentration 125 nM Tris), as well as 1 µl (thus 10 µg) or 2 µl (thus 20 µg) proteinase K (10 µg/µl stock) (Roche, South Africa) were added to the input and immunoprecipitate, respectively, before incubation at 45°C for 1 hour. Thereafter, the DNA was purified using the

QIAquick PCR Purification Kit (Qiagen, USA) according to the manufacturer's instruction. Conventional and quantitative Real-Time PCR was performed using specific primers spanning the GRE region of the MMTV promoter (forward 5'-AAC CTT GCG GTT CCC AG-3'; reverse 5'-GCA TTT ACA TAA GAT TTG G-3') (Kino *et al.*, 2007).

Conventional PCR conditions :



Each conventional PCR reaction contained the following:

| | |
|---|---|
| Input DNA / precipitated DNA | 1 µl / 2 µl |
| Forward primer | 0.2 µM |
| Reverse primer | 0.2 µM |
| dNTP Mix | 0.05 µM of each dNTP final |
| $MgCl_2$ | 1 mM |
| 5x GoTaq buffer (Promega) | 1 x |
| GoTaq Flexi DNA polymerase (Promega) | 1.25U |
| PCR $H_2O$ | Make up to a final volume of 50 µl |

After conventional PCR, the amplified DNA was analysed on a 1.5% agarose gel by means of gel electrophoresis and visualised with ethidium bromide.

Quantitative Real-time PCR conditions:

Each Real-Time PCR reaction contained the following:

| | |
|---|---|
| Input DNA / precipitated DNA | 0.5 µl / 1 µl |
| Forward primer | 0.5 µM |
| Reverse primer | 0.5 µM |
| SensiMixdT (Quantace) | 12.5 µl |
| PCR $H_2O$ | Make up to a final volume of 25 µl |

In order to assess the relative amount of protein recruitment to the MMTV promoter, the "Fit Points" method, described by Pfaffl was used to normalise the inputs in the quantitative Real-time PCR (Pfaffl, 2001), using the following equation: Response (fold) = [$2^{(Ct\ EtOH - Ct\ Dex)\ IP}$ / $2^{(Ct\ EtOH - Ct\ Dex)\ Input}$]. Herein the relative amount of specific DNA immunoprecipitated, as measured by the crossing points (Ct) from quantitative real-time PCR, of the vehicle sample was subtracted from the dex-treated sample. Similarly, the difference between the vehicle input and dex-treated sample was calculated. Thereafter, the relative 'fold' dex-induction in the immunoprecipitate was normalised to the relative 'fold' difference in the input.

## 3.19 Subcellular fractionation

COS-1 cells were plated into 6-well plates (NUNC, AEC Amersham, South Africa) at a density of $3x10^5$ cells/well, in DMEM containing 10% fetal calf serum and antibiotics, as described above. After 24 hour incubation the cells were transfected with 1 μg of either pRS-hGRwt, pRS-hGR3A or pcDNA3.1 (empty vector) using FuGENE 6 (Roche, South Africa) according to the manufacturer's instructions. The following day the cells were treated with 100 nM dex or vehicle (EtOH) for 1 hour, washed once with PBS, resuspended in a low ionic strength buffer (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM DTT and 0.05% NP40) and incubated on ice for 10 minutes. For the inputs, 10 μl of each sample was removed and boiled in 1x SDS sample treatment buffer for 5 minutes. Thereafter the nuclear pellet and cytoplasmic fractions of the remaining cellular fractions were separated by centrifugation at 3000 rpm for 5 minutes and the supernatant removed (cytoplasmic fraction). Typically, 10% of the total volume of the nuclear fraction, as well as 10% of the total cytoplasmic fraction, was separated by SDS-PAGE. Western blotting was performed using anti-GR (H300, Santa Cruz Biotechnology, USA), anti-GAPDH (14C10, Cell Signaling, South Africa) and anti-histone H3 (ab1791, Abcam, UK) antibodies.



## 3.20 Statistical Analysis

Statistical analyses were carried out using GraphPad Prism software (version 5), using one-way ANOVA analysis of variance with either Bonferroni (when comparing all values to each other) or Dunnett (when comparing all to values to a single control) post tests. Correlation analyses were performed using GraphPad Prism software. Statistical significance is denoted by *, ** or ***, to

indicate $P < 0.05$, $P < 0.01$ or $P < 0.001$, respectively when differences were compared to a single control (Dunnett post test). Statistical significance, when comparing all values to each other (Bonferroni), is indicated by different lower-case letters, such that all the conditions with the same letter are not statistically significantly different from each other ($P > 0.05$), while those having different letters are statistically significantly different from each other ($P < 0.05$). Fractional occupancy of the GR for each compound was calculated using [ligand]/([ligand]+RBA), where the RBA was obtained from Ronacher *et al.* 2009 ((Ronacher *et al.*, 2009); Table E4 in addendum E).

# Chapter 4 Ligand-selective GR Phosphorylation (Results and Discussion)

Upon binding of dex, the GR becomes hyperphosphorylated and the active GR moves into the nucleus, where it is able to regulate transcription of GC-responsive genes (Hoeck *et al.*, 1989;Bodwell *et al.*, 1995;Webster *et al.*, 1997;Wang *et al.*, 2002b;Kino *et al.*, 2007). Studies have shown that dex-dependent phosphorylation occurs mainly at S203, S211 and S226 of the hGR and that dex-mediated GR phosphorylation regulates the transcription of GC responsive genes (Orti *et al.*, 1989;Bodwell *et al.*, 1995;Blind and Garabedian, 2008). However, few studies have examined the effect of a wide panel of GR ligands on S211 phosphorylation, using at most three GR agonists (dex, predn and fluocinolone) and two antagonists (RU468 and ZK299) (Wang *et al.*, 2002b), making it difficult to accurately determine whether GR phosphorylation on S211 correlates with biological activity. Furthermore, at the time this study was initiated, the effect of different ligands on S226 phosphorylation was still undetermined. However, recently a study has been published, using two agonists (dex and cort), one full/partial agonist (ald) and one partial agonist (prog) (Chen *et al.*, 2008).



The main aims of this section were to determine if GR phosphorylation at S226 occurs in a ligand-selective manner and whether ligand-selective phosphorylation at S226 determines the rank order of ligand-selective GR-mediated transcription. Ligand-selective efficacy for transactivation in COS-1 cells, with the HA-hGR and the panel of ligands, is shown in table E5 of addendum E and forms the basis upon which the biological activity of the ligands is defined. Previously, ligand-selective GR phosphorylation at S211, with the same panel of GR ligands, was

shown to occur in a ligand-selective pattern, in that agonists phosphorylated the GR the most and antagonists phosphorylated the GR the least at S211 ((Stubsrud, 2005); Table E1 in addendum E contains data from E. Stubsrud that will be used by the candidate for some analyses). Furthermore, phosphorylation at S211 was shown to be required for full transactivational efficacy, in that mutation of S211 resulted in a 50% decrease in transactivational efficacy, but was found not to determine the rank order of ligand-selective GR-mediated transactivation ((Stubsrud, 2005); Table E2 in addendum E). In other words, when S211 was mutated and could no longer be phosphorylated, the same relative pattern of ligand-selective transactivation efficacy was obtained. This thesis, will therefore not show results on ligand-selective S211 phosphorylation, and the transactivational response of the S211A mutant with different GR ligands and will refer to results obtained by E. Stubsrud. However, work done by the present author on S211 other than that performed by E. Stubsrud will be presented in this thesis.



## 4.1 Ligand-selective hGR phosphorylation

The effect of a wide panel of GR ligands on S211 phosphorylation has been shown ((Stubsrud, 2005); Table E1 in addendum E). Additionally, Wang *et al.* indicated that phosphorylation at S203 is only weakly increased upon dex stimulation and therefore this study focussed mainly on the effects of a wide panel of GR ligands on S226 phosphorylation (Wang *et al.*, 2002b). A phospho-S226-GR-specific antibody (P-S226) was used in the present study. To show the specificity of this antibody, COS-1 cells transiently transfected with hGR wt or S226A mutated GR were treated with dex for one hour. Thereafter the cells were harvested and Western blotting and probing for P-S226 and total GR was performed. The unliganded wt GR is basally

phosphorylated at S226 and becomes hyperphosphorylated at S226 upon stimulation with dex (Fig. 4.1). Both the unliganded and dex-stimulated S226A mutant GR did not show any S226 phosphorylation, indicating that the P-S226 antibody is highly specific and that there is no endogenous wt GR in the COS-1 cells detectable by this method.

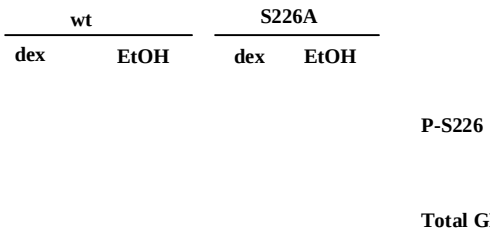



**Figure 4.1 Specificity of the P-S226 antibody.** COS-1 cells were plated in a 6-cm dish at a density of $7x10^5$ cells/dish and transfected with 3.5 μg HA-hGRwt or HA-hGRS226A per dish. After 24 hours, the cells were replated into 6-well plates at a density of $4x10^5$ cells/well and treated with 10 μM dex or vehicle (EtOH) for 1 hour. Whole cell extracts were prepared and equal amounts of protein (20 μg) was separated on a 10% SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane and analyzed by immunoblotting with P-S226, stripped and reprobed for total GR.

In order to determine whether S226 phosphorylation is GC dose-dependent, ligand-mediated S226 phosphorylation experiments were done using 10 μM compound, as well as 100 nM compound. The same panel of GR ligands, as part of the larger investigation (see Thesis Rationale, Aims and hypotheses table 2.1), was used in this study. Briefly, this panel consists of three full agonists (dex, cort and predn), two full/partial agonists (ald and MPA), two partial agonists (AL438 and prog), two dissociated ligands (RU486 and CpdA) and three antagonists (NET, DO6 and UDCA). Results show that the unliganded GR is basally phosphorylated, but becomes hyperphosphorylated upon stimulation with 10 μM (Fig. 4.2 A) or 100 nM (Fig. 4.2 B) agonist. Furthermore, it can clearly be seen that different ligands result in ligand-selective differences in GR phosphorylation at S226, where the agonists (dex, cort and predn) result in the greatest extent of phosphorylation, whereas the antagonists (NET and DO6) results in only

minimal phosphorylation of the GR. After quantification and normalisation, the results with 10 μM test compound (Fig. 4.2 C) show that the full agonists dex, cort and predn induced the most phosphorylation (100%, 122% and 85%, respectively), followed by the full/partial agonists ald and MPA (60% and 44%) and the partial agonists AL438 and prog (34% and 48%). The antagonists NET, DO6 and UDCA (5%, 0% and 8%), as well as the dissociated GC ligand CpdA (14%) resulted in little to no increase in S226 phosphorylation. Interestingly, the dissociated compound RU486 resulted in relatively high levels (44%) of S226 phosphorylation, similar to that found with the full/partial or partial agonists. Quantification and normalisation of the results obtained with 100 nM test compound also showed that the full-, full/partial- and partial agonists resulted in more phosphorylation that the dissociated compounds and the antagonists, however this was not as clear as with the 10 μM (Fig. 4.2 D).

When comparing figure 4.2 A and figure 4.2 B with the full agonists, it is obvious that the amount of S226 phosphorylation with cort (46%) at 100 nM, is significantly less than that observed with 10 μM test compound (122%). Additionally, the partial agonist prog did not result in any S226 phosphorylation at 100 nM. The differences between the phosphorylation results obtained with 10 μM and 100 nM could partly be explained by variations in the fractional occupancy or the percentage of GR molecules occupied by ligand. Due to the RBAs of these compounds to the GR (IC50 cort 152 nM; IC50 prog 274 nM; Table E4 in addendum E), the fractional occupancy of the GR with cort and prog at 100 nM is 83% and 73%, versus 99.8% and 99.6%, respectively at 10 μM. Accordingly, ald and NET (IC50 ald 1130 nM and IC50 NET 1688 nM; Addendum E) which have a fractional occupancy at 100 nM of 42% and 34%, should also result in less S226 phosphorylation at 100 nM, as compared to 10 μM where they have a fractional occupancy of 99% and 98%, respectively. However, due to the relatively large error in

S226 phosphorylation for ald with 10 μM and 100 nM, as well as for NET with 100 nM, it is difficult to accurately determine whether the amount of phosphorylation is less with 100 nM than with 10 μM.

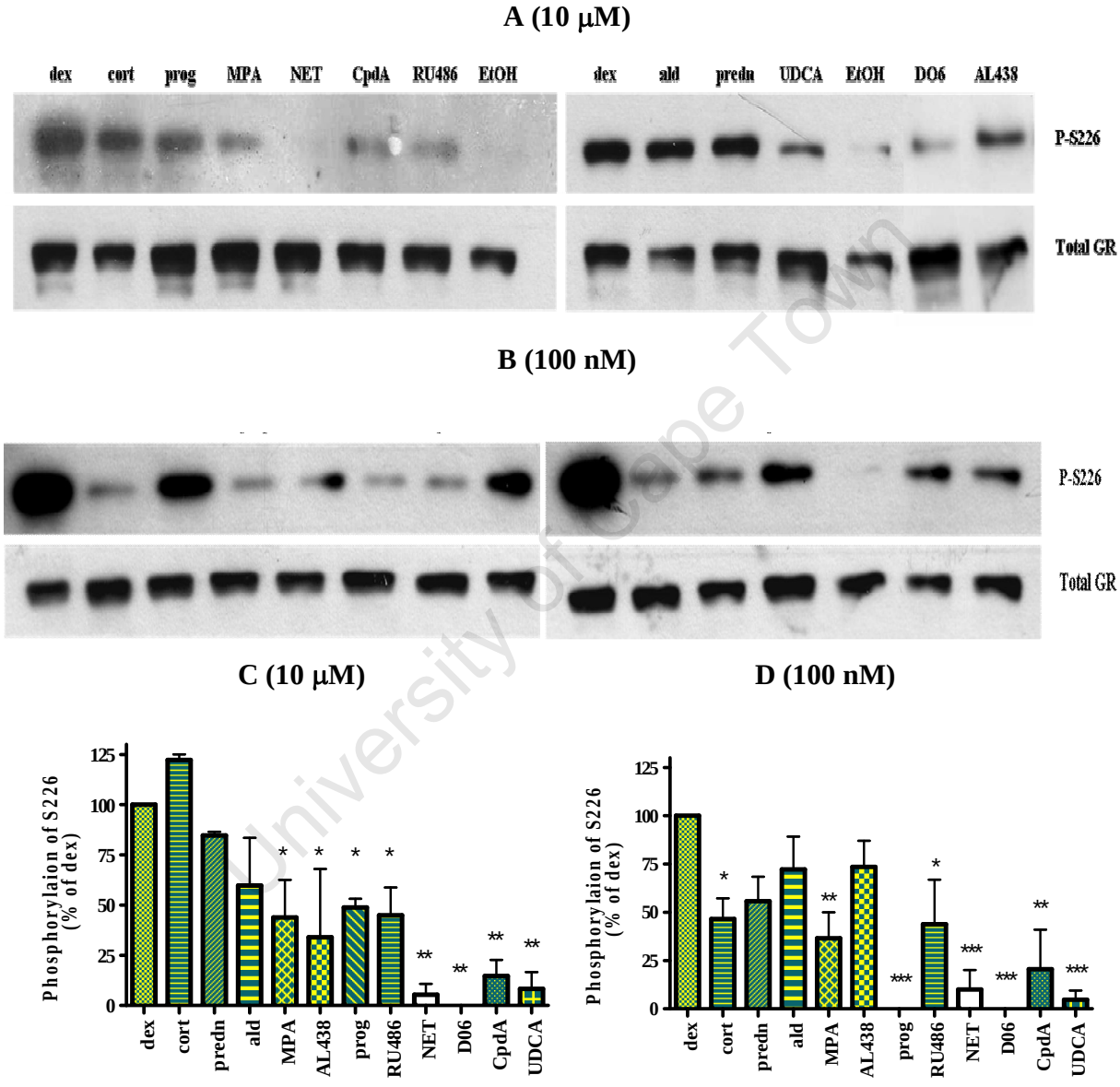



**Figure 4.2 Phosphorylation of the human GR at S226 in COS-1 cells**. COS-1 cells plated a 6-cm dishes were transiently transfected with 3.5 μg HA-hGRwt. After 24 hour incubation, the cells were replated into 12-well plates and incubated an additional 24 hours. Thereafter the cells were stimulated with 10 μM (A) or 100 nM (B) test compound, or vehicle (EtOH), for 1 hour. Whole cell extracts were prepared and equal amounts of protein (20 μg) were analyzed by Western blotting with P-S226 antibody,

before membranes were stripped and reprobed with an antibody detecting total GR. After quantification, the basal phosphorylation (EtOH control) was subtracted from each test compound, the phosphorylation induced by 10 μM (C) or 100 nM (D) dex was set as 100 % and the phosphorylation induced by the other test compounds was calculated relative to dex. The Western blots in (A) and (B) are representative figures of three independent experiments, whereas data presented in graphs (C) and (D) are presented as mean ± SD of three independent experiments. Statistical significance is denoted by *, ** or ***, to indicate $P < 0.05$, $P < 0.01$ or $P < 0.001$, respectively.

However, differences in fractional occupancy alone, cannot account for the differences seen between the 100 nM and 10 μM results. If the amount of S226 phosphorylation at 100 nM and 10 μM for cort and prog is plotted against their respective fractional occupancies, at those ligand concentrations, it is clear that the percentage decrease in S226 phosphorylation is much more than the decrease in fractional occupancy (Fig. 4.3). With prog the difference in S226 phosphorylation (52%) is roughly 2x the difference in fractional occupancy (26%), whereas the difference in S226 phosphorylation with cort (76%) is roughly 5x the difference in fractional occupancy (16%). This indicates that not only is the "relationship" between fractional occupancy and S226 phosphorylation not linear, but that the "relationship" is different for the respective ligands. Consequencely, something other than fractional occupancy, which occurs in a ligand-selective manner, is responsible for the differences between the amount of S226 phosphorylation at 100 nM vs. 10 μM.

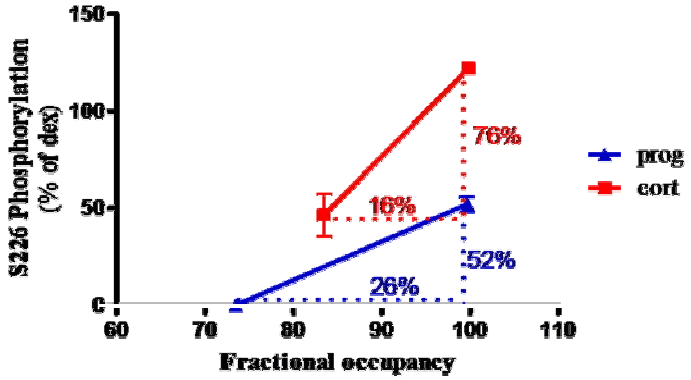



**Figure 4.3 Comparison between the amount of S226 phosphorylation and fractional occupancy with cort and prog.** The amount of S226 phosphorylation with cort and prog at 100 nM, as well as 10 μM, was plotted against the respective fractional occupancy of cort and prog at 100 nM and 10 μM.

The results with 10 μM compound show the effects on GR phosphorylation at S226 without the complicating effect of differential fractional occupancy, as all the ligands saturate the receptor at this concentration (except for DO6 with a fractional occupancy of 80.5%; Table E4 in addendum E). There is a general trend that the full agonists result in the most S226 phosphorylation, followed by the full/partial agonists, the partial agonists, the dissociated ligands and the antagonists.

Reports in the literature suggest that the effect of phosphorylation on GR transactivation could be cell-specific (Mason and Housley, 1993;Webster *et al.*, 1997;Chen *et al.*, 2008;Kino *et al.*, 2007). In order to determine whether there are cell-specific differences in S226 phosphorylation itself, the 10 μM S226 phosphorylation experiment was repeated in U2OS cells stably expressing hGR. As was found with the S226 phosphorylation results in COS-1 cells, the GR is basally phosphorylated in the absence of hormone and becomes hyperphosphorylated upon dex treatment (Fig. 4.4 A). Furthermore, the agonists dex and cort resulted in the highest amount of S226 phosphorylation, whereas the antagonists DO6 and UDCA showed the least amount of

phosphorylation, which is consistent with the results obtained in the COS-1 cells. However, after quantification and normalisation, it is evident that there are some differences (Table 4.1) from the results obtained in COS-1 cells (Fig. 4.4 B). For instance, 10 μM of the full agonist predn resulted in relatively less S226 phosphorylation in the U2OS cells as compared to the COS-1 cells (31% versus 75% as compared to dex). Another striking difference is that 10 μM of the full/partial agonist MPA and the antagonist NET resulted in relatively more S226 phosphorylation, compared to dex, in U2OS versus COS-1 cells, giving phosphorylation patterns expected of a full agonist and a full/partial agonist, respectively. However, the test compounds were classified by their relative efficacy for transactivation in COS-1 cells. Cell-specific differences, other than phosphorylation itself, could influence the ability of a ligand to transactivate. Cell-specific differences, such as GR protein levels, would therefore likely affect the relative efficacy of partial agonists for transactivation and in order to further examine the relationship between phosphorylation and transactivation in U2OS cells, one would have to compare phosphorylation in U2OS cells to the efficacy for transactivation in U2OS cells. For instance, the full/partial agonist MPA can switch from a partial agonist to a full agonist, depending on the relative amounts of GR (Fig. D1 in addendum D). In the literature, similar results have been obtained for MPA. The dissociated ligand RU486 has also been shown to switch to full agonist activity, depending on the amount of GR in the cell (Zhao *et al.*, 2003;Zhang *et al.*, 2007). However, a direct comparison between the relative amount of GR in the transiently transfected COS-1 cells versus the stably transfected U2OS cells was not done. Furthermore, differences in GR levels alone would not account for differences in S226 phosphorylation with the full agonist, predn. As mentioned earlier, the ligands were classified according to their efficacy for transactivation in COS-1 cells. Nevertheless, in U2OS cells, predn behaves as a full agonist for transactivation (Ronacher *et al.*, 2009). The above results are very

interesting, and suggest cell-specific differences, other than GR levels, such as differential kinase and/or phosphatase expression differentially influencing ligand-selective GR phosphorylation.

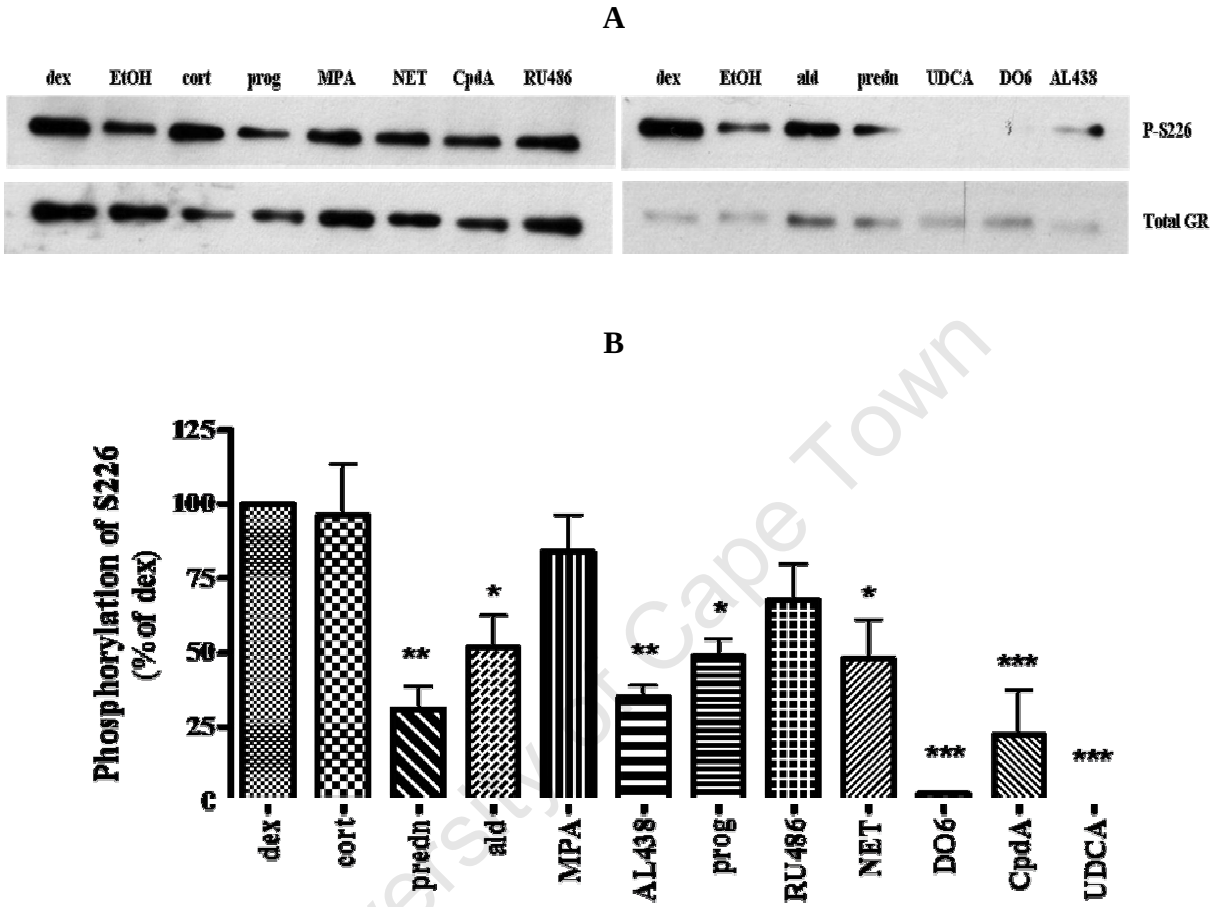





**Figure 4.4 Phosphorylation of the human GR at S226 in U2OS cells**. U2OS cells stably expressing HA-hGRwt were stimulated with 10 μM test compound or vehicle (EtOH), for 1 hour. Whole cell extracts were prepared and equal amounts of protein (20 μg) were analyzed by Western blotting with P-S226 antibody, as well as an antibody detecting total GR (A). After quantification, the basal phosphorylation (EtOH control) was subtracted from each test compound, the phosphorylation induced by dex was set as 100 % and the phosphorylation induced by the other test compounds was calculated relative to dex (B). The Western blot (A) is one representative of three independent experiments and data in histogram (B) is presented as mean ± SD of three independent experiments. Statistical significance is denoted by *, ** or ***, to indicate $P < 0.05$, $P < 0.01$ or $P < 0.001$.

**Table 4.1 Summary of S226 phosphorylation by the panel of ligands compared to dex.** The basal phosphorylation (EtOH) control is subtracted and dex was set to 100%.



Nevertheless, results show ligand-selective S226 phosphorylation at saturating concentrations for all ligands and sub-saturating concentrations for some ligands in COS-1 cells, as well as at saturating concentrations in U2OS cells. Furthermore, there is a general trend that the most efficacious ligands resulted in the most S226 phosphorylation and the least efficacious ligands resulted in the least amount of S226 phosphorylation in COS-1 cells (Table 4.1). This trend in ligand-selective GR phosphorylation is not simply due to differences in binding affinity of the ligand for the GR, since the same trend was observed at saturating concentrations of ligand. Additionally, there are some numerical outliers between the COS-1 and U2OS cells, suggesting cell-specific differences in S226 phosphorylation.

At the time this work was done, the effect of different ligands on GR phosphorylation at S226 was not known. However, hGR phosphorylation at S226 in response to dex, cort, prog and ald

has recently been published in U2OS cells (Chen *et al.*, 2008). The results presented in this section with dex, cort, prog and ald are consistent with those of Chen *et al*. and also show the effect of 8 additional ligands on S226 phosphorylation, where there is a general trend that the most efficacious ligands result in the most GR phosphorylation at S226. Furthermore, the effect of increasing amounts of dex on S226 phosphorylation has not previously been shown and this thesis shows for the first time, the effect of different ligand concentrations on GR phosphorylation at S226, with a whole panel of GR ligands. Results showed numerical differences between the different ligand concentrations, which was shown to partially reflect different fractional occupancy of the receptor. However, the same general ligand-selective pattern of GR phosphorylation at S226 was found at sub-saturating concentrations for some ligands, as well as saturating concentrations for all the ligands, indicating that ligand-selective GR phosphorylation at S226 is not only due to differences in receptor binding affinity. It is likely, that ligand-binding induces different conformational changes of the GR, with some conformations allowing for better interactions between the GR and kinases or phosphatases, thereby resulting in ligand-selective GR phosphorylation at saturating ligand concentrations. However this remains to be tested. Nevertheless, these results suggest that with any given GR ligand, at any sub-saturating ligand concentration, the amount of GR phosphorylation at S226 will reflect a combination of fractional occupancy, as well as a ligand-specific contribution to phosphorylation (e.g. resulting from a particular GR-ligand conformation).

In addition, although cell-specific differences in the extent of GR phosphorylation have previously been proposed (Rogatsky *et al.*, 1998a), this thesis shows for the first time in a comprehensive study with a wide panel of GR ligands, that there are cell-specific differences in the extent of S226 phosphorylation. These cell-specific differences are not due to species-specific differences, since hGR was used in both cells, or due to differences in the relative

amount of GR protein, as explained above, and therefore likely reflect cell-specific differences in the kinases and/or phosphatases involved in GR phosphorylation at S226.

## 4.2 Role of hGR phosphorylation in ligand-selective GR transcriptional activity

Having shown ligand-selective S226 phosphorylation in COS-1 cells, it was next determined whether ligand-selective S226 phosphorylation correlates with ligand-selective GR transcriptional activity in COS-1 cells. In order to determine this, correlation analysis between the 10 μM COS-1 phosphorylation results and results on GR transactivation and transrepression activity (Table E5 in addendum E) was performed.

### 4.2.1 Correlation analysis between ligand-selective GR phosphorylation at S226 and ligand-selective GR-mediated transcriptional activity

In order to correlate the S226 phosphorylation results with ligand-selective GR transactivation, transactivation data ((Stubsrud, 2005); Table E5 in addendum E), obtained in COS-1 cells using the same HA-hGR and TAT-GRE constructs, were used. Correlation analysis between GR phosphorylation at S226 and efficacy for transactivation, revealed a good and statistically significant correlation ($R^2$ = 0.73, p = 0.0004) for all of the test compounds (Fig. 4.5 A). The antagonists DO6, UDCA and NET do not transactivate, which correlates with no S226 phosphorylation. By excluding the antagonists for transactivation (compounds that do not transactivate) from the correlation analysis, in order to determine if there is a correlation for the compounds that do show transcriptional efficacy, a weaker but still statistically significant correlation is observed ($R^2$ = 0.61, p = 0.0135). This result indicates that there is a correlation between GR phosphorylation at S226 and transactivation for all the compounds that results in

GR-mediated transactivation. Correlation analysis of potency for transactivation and S226 phosphorylation also indicates a statistically significant correlation between potency for transactivation and S226 phosphorylation (Fig. 4.5 B). However, this correlation ($R^2 = 0.60$, $p < 0.0031$) is not as strong as between efficacy for transactivation and S226 phosphorylation. However, when the antagonists for transactivation (no transactivational potency) are excluded, no correlation was found, for the compounds that do transactivate.

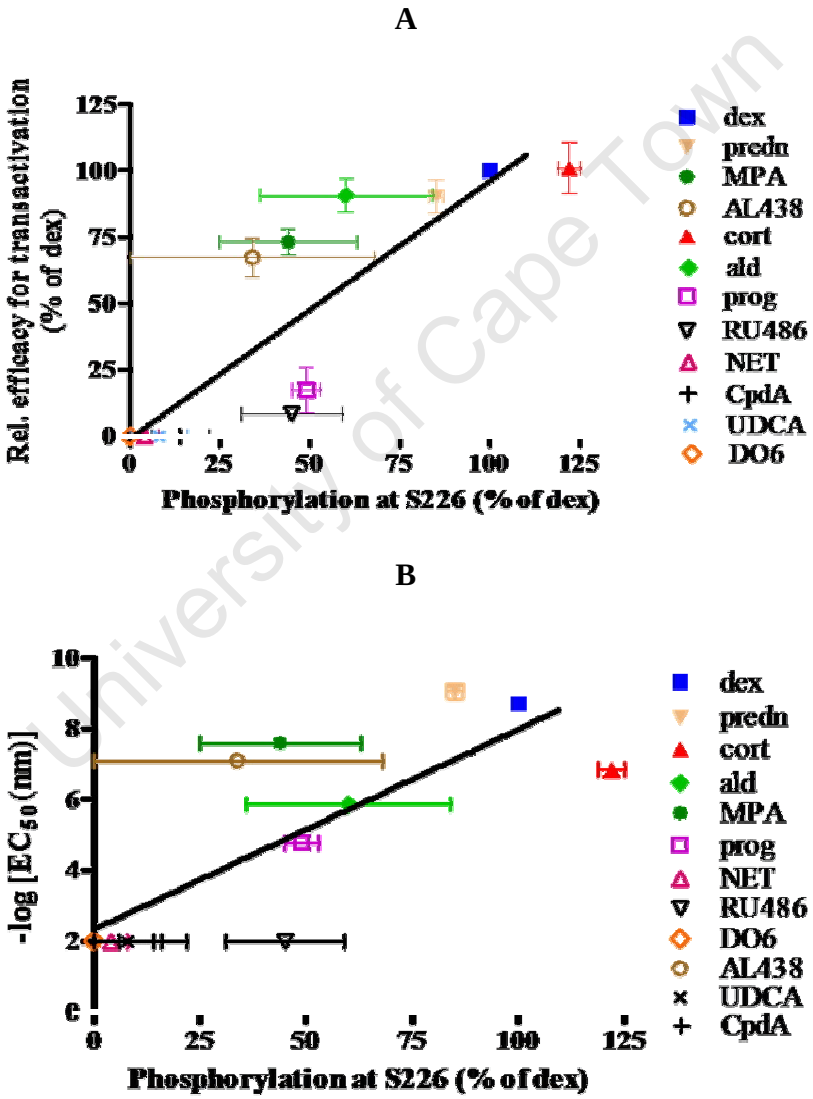





**Figure 4.5 Correlation between S226 phosphorylation with 10 μM compound and potency and efficacy for transactivation in COS-1 cells.** Data from the phosphorylation studies was correlated with

transactivation data obtained in COS-1 cells ((Stubsrud, 2005); Table E5 in addendum E). Phosphorylation is correlated with efficacy (A) and potency (B).

In order to accurately determine the role of S226 phosphorylation on hGR mediated transrepression, two different transrepression promoters (AP-1 and NFκB) were studied. To correlate the S226 phosphorylation results with ligand-selective transrepression via AP-1 and NFκB proteins, transrepression data from this lab, obtained in COS-1 cells with the same HA-hGR, as well as either AP-1 or NFκB promoter-reporter constructs, were used (K. Ronacher; Table E5 in addendum E). Correlation analysis between GR phosphorylation at S226 and efficacy for transrepression via AP-1, revealed a good and statistically significant correlation ($R^2$ = 0.71, p = 0.0006) for all of the test compounds (Fig. 4.6 A). Similar to the transactivation correlation, exclusion of the antagonists for transrepression (no transrepression) resulted in a slightly weaker, but still statistically significant correlation ($R^2$ = 0.51, p = 0.0299) (Table 4.3). This correlation shows, that the inclusion of the compounds that do not transrepress or phosphorylate the GR at S226 in the correlation with all the ligands, did not give a false positive correlation and that there is a correlation between GR phosphorylation at S226 and GR-mediated transrepression efficacy, for all the ligands that result in transrepression. Consistent with the transactivation correlation, correlation analysis of potency for transrepression on AP-1 and the degree of phosphorylation at S226 also indicates a statistically significant correlation (Fig. 4.6 B). However, this correlation ($R^2$ = 0.46, p = 0.0159) is not as strong as between efficacy for transrepression and S226 phosphorylation. Additionally, when the antagonists for transrepression (no transrepression potency) are excluded, no correlation was found, indicating, as was the case for transactivation, that for the compounds that do transrepress, there is not a correlation between GR phosphorylation at S226 and potency for transrepression. Furthermore, the inclusion of the

compounds that do not transrepress or phosphorylate the GR at S226 in the correlation with all the ligands, resulted in a “false” positive correlation.

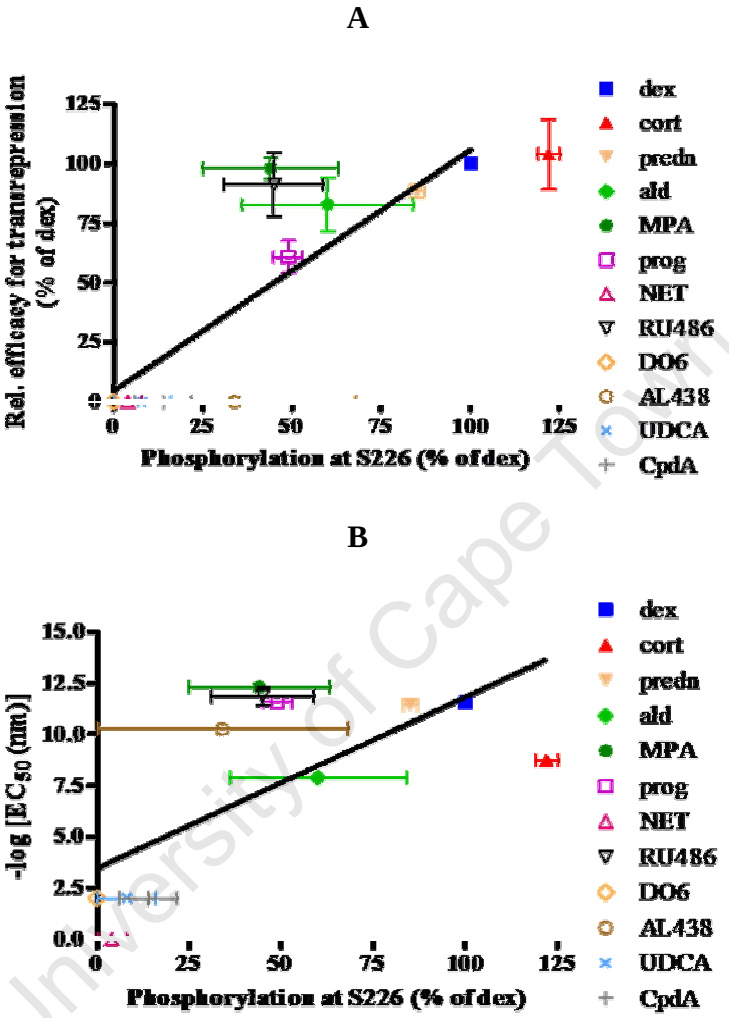





**Figure 4.6 Correlation between S226 phosphorylation with 10 μM compound and potency and efficacy for AP-1 transrepression in COS-1 cells.** Data from the phosphorylation studies was correlated with transrepression data obtained in COS-1 cells (K. Ronacher; Table E5 in addendum E). Transrepression efficacy (A) and potency (B) values were calculated by plotting dose-response curves with the different ligands, subtracting the vehicle background and the potency or efficacy for transrepression were calculated as –log EC50 (nM) or relative efficacy with wt dex set to 100%.

Similarly, statistically significant positive correlations between S226 phosphorylation and efficacy and potency for transrepression via NFκB for all the ligands were found (Table 4.3).

However, when the antagonists were excluded from the correlations, no correlation between GR phosphorylation and efficacy or potency for transrepression via NFκB was found. This is interesting and indicates that the compounds that do not transrepress via NFκB, led to a false positive correlation between GR phosphorylation and efficacy or potency for transrepression via NFκB. In addition, this correlation suggests that for all the compounds that do transrepress via NFκB, there is no statistically significant correlation between efficacy or potency for transrepression via NFκB and GR phosphorylation at S226.

**Table 4.3 Summary of S226 phosphorylation and transcription correlation analysis ($R^2$ values)[‡].**



Taken together these correlations indicate a positive correlation between GR phosphorylation at S226 with efficacy, but not potency for transactivation for all the ligands that result in GR-mediated transactivation. Similarly positive correlations between GR phosphorylation at S226 with efficacy, but not potency for transrepression via AP-1 were obtained while no correlation between GR phosphorylation at S226 and efficacy or potency for transrepression via NFkB was obtained, for all the ligands that result in GR-mediated transrepression.

[‡] Values were obtained from correlation analysis, with $R^2$ values shown and statistical significance denoted by *, ** and *** to indicate $P < 0.05$, $P < 0.01$ or $P < 0.001$, respectively.

One interpretation of these correlations is that ligand-selective GR phosphorylation at S226 influences or plays a determining role in ligand-selective efficacy, but not potency, for transactivation and transrepression (for AP-1, but not NFκB). On the other hand, these correlations could indicate that ligand-selective efficacy, but not potency, for transactivation and transrepression (for AP-1, but not NFκB) influences or plays a determining role in ligand-selective GR phosphorylation at S226. In other words, the degree of S226 phosphorylation per se could determine the transcriptional response, if phosphorylation occurs before transcription, or the transcriptional response could determine the extent of S226 phosphorylation, if phosphorylation occurs after transcription. Additionally, these correlations could simply indicate that both ligand-selective GR phosphorylation at S226 and efficacy, but not potency, for transactivation and transrepression (for AP-1, but not NFκB) are mediated by the same ligand-selective "determining step" and that ligand-selective GR phosphorylation and efficacy for transactivation and transrepression are not functionally linked to each other. Such a ligand-selective "determining step" could be a particular ligand-induced GR conformation, wherein, ligand-binding causes a particular conformational change in the GR (in agreement with literature on crystal structures) and these conformational changes determine both the extent of S226 phosphorylation and transactivation and transrepression (for AP-1, but not NFκB). Accordingly ligand-selective S226 phosphorylation and ligand-selective transcription would both be indicative of a particular GR conformation. Nevertheless, all of the above mentioned interpretations suggest a different mechanism of ligand-selective GR action on AP-1 vs. NFκB. Additionally, phosphorylation did not correlate with potency for transactivation or transrepression (minus antagonists). This is very interesting and suggests that another factor, other than phosphorylation, influences potency for transactivation and transrepression. Ronacher *et al.* showed that ligand-selective RBA correlated with potency, but not efficacy for transactivation

with the full and full/partial agonists (Ronacher *et al.*, 2009). It is therefore likely that ligand-selective RBAs are the major contributing factor to potency, but not efficacy, for transactivation and transrepression, explaining the lack of a correlation between ligand-selective GR phosphorylation and potency for transactivation and transrepression.

### 4.2.2 Role of ligand-selective GR phosphorylation in ligand-selective GR-mediated transactivation

In order to determine if ligand-selective GR phosphorylation at S226 determines the rank order of ligand-selective efficacy for transactivation, the ability of a S226 GR mutant (S226A), which can no longer be phosphorylated on S226, was compared with the ability of wt receptor to transactivate a TAT-GRE reporter-promoter construct. COS-1 cells transiently transfected with hGR wt or S226A, as well as a TAT-GRE-reporter construct, were stimulated with increasing amount of dex. After 16 hours, luciferase activity was measured and normalised to total amount of protein. Results show typical dex dose-response curves for both wt and S226A mutant (Fig. 4.7). When comparing wt and S226A mutant it can clearly be seen that the S226A mutant has a much higher efficacy (~ 2.8 fold more than wt), indicating that S226 phosphorylation inhibits transactivation efficacy. Interestingly, the wt and S226A mutant had similar potencies ($EC_{50}$s of $6.96e^{-10}$ M and $2.8e^{-9}$ M), which are not statistically significantly different from each other. This result is consistent with the correlations, wherein GR phosphorylation at S226 influences or plays a determining role in efficacy, but not potency, for transactivation. However, the correlation analysis gave a positive correlation, indicating that the more GR phosphorylation at S226, the higher the efficacy. In contrast, results obtained with the mutant receptor indicate that S226 phosphorylation inhibits efficacy in a statistically significant manner ($p < 0.0001$). This could

indicate that ligand-selective phosphorylation per se does not determine ligand-selective efficacy for transactivation, but rather reflects a particular conformation that determines ligand-selective transactivation.

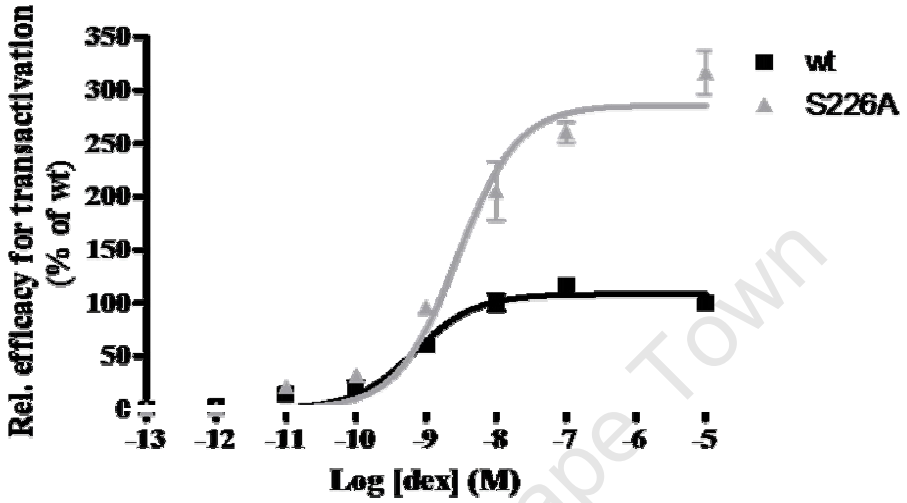



**Figure 4.7 Comparison between GR-dependent transactivation with wt receptor and the S226A mutant receptor in COS-1 cells.** COS-1 cells were plated in 10-cm dishes at a density of $2x10^6$ cells/dish and transfected with 10 µg HA-hGRwt or HA-hGRS226A and 3.75 µg TAT-GRE. After 24 hours, the cells were replated into 24-well plates and incubated for another 24 hours. Thereafter the cells were treated with vehicle (EtOH) or the indicated concentrations of dex. After 16 hours, luciferase activity was assayed. Bradford protein determination was performed to normalize for equal protein concentrations in each well. Dose-response curves with wt and the mutant receptor were performed three times with each value in triplicate plotted as means ± SEM, expressed as relative luciferase activity (10 µM dex with wt GR set as 100 %).



Having shown that S226 phosphorylation influences efficacy, but not potency for transactivation, the efficacy for transactivation of wt and the S226A mutant for the different ligands were determined. For wt receptor, results show that the agonists dex, cort and predn are the most efficacious (100%, 114% and 96%, respectively), followed closely by the full/partial agonists ald (91%) and MPA (89%) (Fig. 4.8). Thereafter it is the partial agonist prog (43%) and the dissociated ligand RU486 (7%). The dissociated CpdA and the antagonists NET and UDCA did

not transactivate. Consistent with the results in figure 4.7, the S226A mutant had a much higher efficacy (~ 2.5 fold) than wt when stimulated with dex. Interestingly, the S226A mutant had an efficacy of ~ 2.5 fold higher than wt efficacy for all the compounds that were able to transactivate. This resulted in the ligands having the same "rank order" in efficacy as with wt receptor. Since the S226A mutant cannot be phosphorylated on S226, but still shows the same ligand-selective efficacy for transactivation pattern as wt, and since the S226A mutant did not influence potency for transactivation, it can be concluded that ligand-selective S226 phosphorylation does not determine ligand-selective transactivation. In other words, the percentage of GR molecules phosphorylated at S226, with any particular ligand, does not determine the transactivational response of the particular ligand. However, GR phosphorylation at S226 does play a role in the maximal efficacy for transactivation, wherein phosphorylation at S226 inhibits maximal efficacy for transactivation for all the ligands.



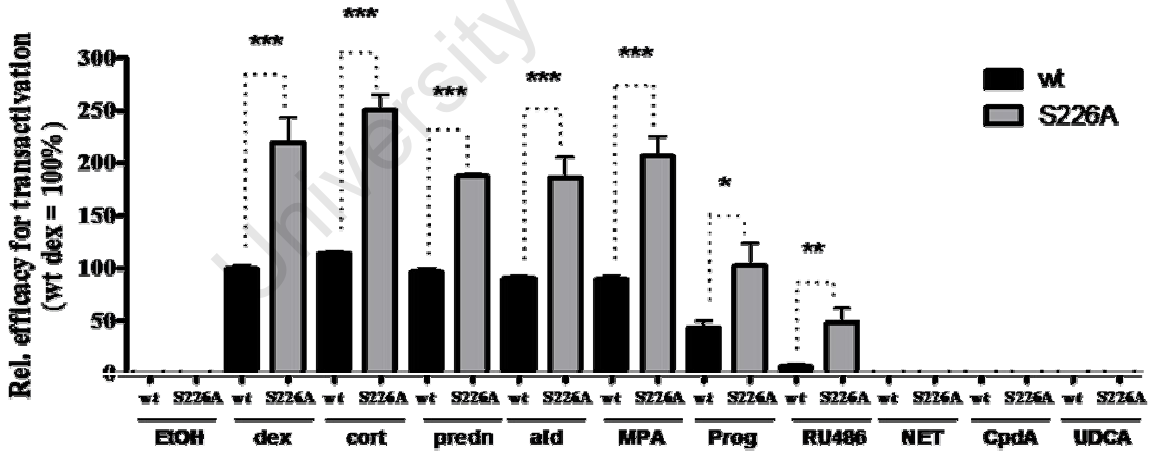



**Figure 4.8 Comparison between GR-dependent transactivation efficacy with wt receptor and the S226A mutant receptor in COS-1 cells.** COS-1 cells were plated in 10-cm dishes at a density of $2x10^6$ cells/dish and transfected with 3.75 µg TAT-GRE and 10 µg HA-hGRwt or HA-hGRS226A. After 24 hours, the cells were replated into 24-well plates and incubated for another 24 hours. Thereafter, the cells were treated with vehicle (EtOH) or 10 µM compound. After 16 hours, luciferase activity was assayed. Bradford protein determination was performed to normalize to protein concentrations in each well. The

graph represents the average of the means of three independent experiments, each performed in triplicate with each value plotted as mean ± SEM expressed as relative luciferase activity (with wt dex set as 100 %). Statistical analyses were carried out using GraphPad Prism software (version 5), using 2-tailed t-test, for selected pairs of columns, comparing wt and S226A GR mutant with different ligands. Statistical significance is denoted by *, ** or ***, to indicate $P < 0.05$, $P < 0.01$ or $P < 0.001$.

As mentioned earlier, phosphorylation at S211 was found to be required for maximal efficacy for transactivation, wherein mutation of S211 resulted in a 50% decrease in efficacy for transactivation with all the ligands tested ((Stubsrud, 2005); Table E2 in addendum E). It is therefore interesting to note, that inhibition of p38, the kinase reportedly responsible for phosphorylating the GR at S211 (Miller *et al.*, 2005) resulted in a 50% decrease in efficacy for transactivation on the same TAT-GRE reporter promoter, in the same cells (K. Ronacher; Figure E2 in addendum E). These results, together with the S211A transactivation results, are consistent with a model wherein p38-mediated phosphorylation of the hGR at S211 is required for maximal GR-mediated efficacy for transactivation. Furthermore, inhibition of JNK, the kinase reportedly responsible for S226 phosphorylation (Rogatsky *et al.*, 1998b) resulted in a ~2 fold increase in transactivation efficacy on the same TAT-GRE reporter promoter, in the same cells (K. Ronacher; Figure E2 in addendum E). These results, together with the S226A results, are consistent with a model where JNK-mediated phosphorylation of the hGR at S226, is inhibitory of maximal GR-mediated efficacy for transactivation. Therefore, although ligand-selective GR phosphorylation does not determine the rank order of ligand-selective transactivation per se, phosphorylation on S211 and S226 have opposite effects on efficacy for transactivation and modulate the maximal amount of transactivation.

Having shown that phosphorylation at S211 is needed for maximal transactivation efficacy ((Stubsrud, 2005); Table E2 in addendum E), whereas phosphorylation of S226 inhibits maximal

transactivational efficacy, as well as the fact that it has previously been suggested that the three main GR phosphorylation sites in the LBD can compensate for each other (Wang *et al.*, 2007b), the significance of their simultaneous mutation (S226A/S211A/S203A or 3A) on transactivational efficacy was next examined. The S203A mutation was included since earlier reports in the literature indicated that phosphorylation of S203 is also weakly increased upon dex stimulation (Wang *et al.*, 2002b). The absence of phosphorylation at S211 and S226 in this mutant construct was confirmed using GR phospho-serine-specific antibodies (Fig. 4.9).

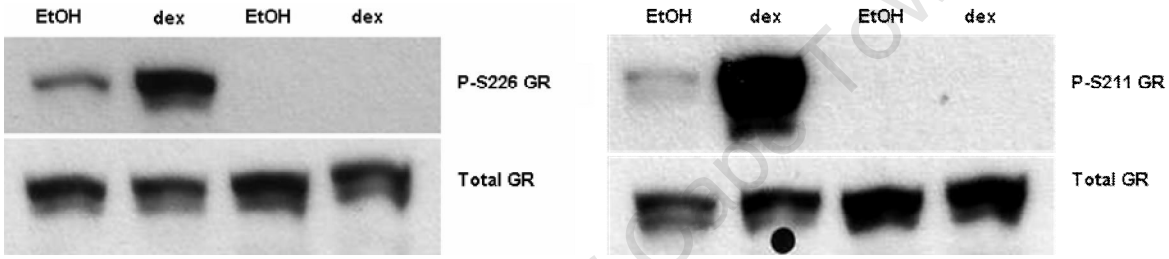



**Figure 4.9 Specificities of the P-S211 and P-S226 antibodies.** COS-1 cells transiently transfected with either pRS-hGRwt or pRS-hGR3A were treated with vehicle (EtOH) or 100 nM Dex for 1 hour. Western blot analysis was performed using either an anti-P-S226 GR-specific antibody (A) or an anti-P-S211 GR-specific antibody (B). Thereafter the membrane was stripped and reprobed with anti-GR antibody.

COS-1 cells transiently transfected with hGR wt, S211A, S226A, or 3A as well as a MMTV reporter-promoter and β-gal construct, were stimulated with dex for 16 hours, before luciferase and β-galactosidase activities were determined. As can be seen in figure 4.10, mutation of S211 resulted in a decrease, whereas mutation of S226 resulted in an increase in transcription efficacy on the MMTV promoter, which is consistent with the results shown on the TAT-GRE construct with S211A ((Stubsrud, 2005); Table E2 in addendum E) and S226A. Interestingly, the decrease seen with the S211 mutant is more prominent on the MMTV promoter, where it shows no statistically significant dex-induction, compared to the TAT-GRE construct where it decreased by only 50%. In contrast, the two fold increase found on the TAT-GRE with the S226 mutant, is

much weaker on the MMTV promoter (Fig. 4.10). These results show promoter-specific differences, between the TAT-GRE construct and the MMTV promoter, on the effect of phosphorylation at S211 and S226 on transcriptional efficacy, which is consistent with other reports in the literature (Webster *et al.*, 1997). On the MMTV promoter, the triple phosphorylation mutant showed a small, but not statistically significant dex-induction (Fig. 4.10), indicating that in this cell system, phosphorylation on S203, S211 and S226 is required for transactivation on this promoter.

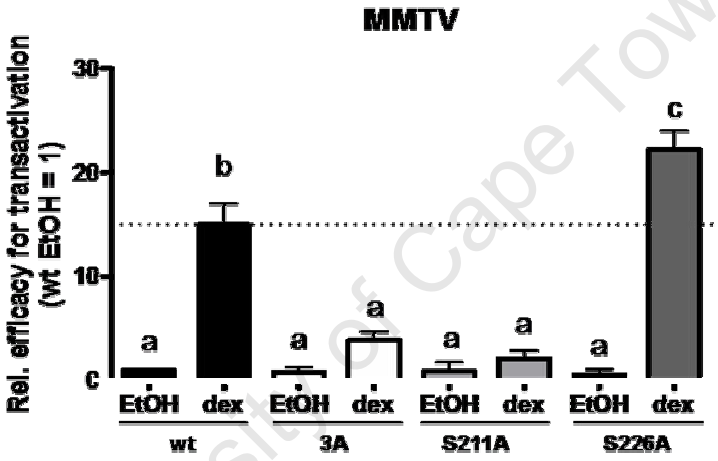



**Figure 4.10 Relative transactivation efficacy on the MMTV promoter.** COS-1 cells transiently transfected with either 0.125 μg pRS-hGRwt, pRS-hGR3A, pRS-hGRS211A or pRS-hGRS226A, as well as 0.047 μg MMTV reporter-promoter and 0.012 μg β−gal constructs, were treated with vehicle (EtOH) or 100 nM Dex for 24 hours. Luciferase activity in the cell lysates was normalised to β−gal activity per well. The histogram shows pooled results from two independent experiments, where each condition was performed in triplicate, and average values were plotted as means ± SEM, expressed as fold induction (relative to EtOH = 1). Statistical significance is indicated by different lower-case letters, such that all the conditions with the same letter are not statistically significantly different from each other ($P > 0.05$), while those having different letters are statistically significantly different from each other ($P < 0.05$).

Furthermore, the result with the S211A mutant is similar to that of the triple phosphorylation mutant, indicating that the requirement for S211 phosphorylation on transactivation efficacy is more dominant than the inhibitory effect of S226 phosphorylation on transactivation efficacy, on

this promoter. Having shown a functional role of GR phosphorylation on transactivation efficacy on two different promoters, the role of GR phosphorylation on ligand-selective GR transrepression was next examined.

### 4.2.3 Role of ligand-selective GR phosphorylation in ligand-selective GR-mediated transrepression

Ligand-selective S211 phosphorylation correlates with efficacy, but not potency for transrepression via AP-1 and NFκB, for all the ligands that result in GR-mediated transrepression (Table E3 in addendum E). Furthermore, results in this thesis showed that ligand-selective S226 phosphorylation correlates with efficacy, but not potency for transrepression via AP-1, but not via NFκB. In order to shed more light on these correlations and to determine whether ligand-selective GR phosphorylation at S226 and/or S211 determines ligand-selective GR-mediated efficacy for transrepression via AP-1 and/or NFκB, COS-1 cells, transiently transfected with either hGR wt, S226A or S211A, as well as either an AP-1 or NFκB reporter-promoter construct were stimulated with 10 ng PMA and increasing concentrations of dex. After 24 hours, cells were harvested and luciferase and β-galactosidase activity was measured. Dose-response curves with wt and mutant receptors were plotted and the potencies and efficacies for transrepression were calculated. Results on the AP-1 promoter show that both the S226A and S211A mutant had similar potencies to wt GR ($EC_{50}$ values: 10.15 nM, 10.67 nM and 11.67 nM, respectively) (Fig. 4.11 A). Similarly, on the NFκB promoter, the S226A and S211A mutants had EC50s of 10.89 nM and 10.91 nM, which are similar to the value of 10.68 nM found for wt dex (Fig. 4.11 B). These results are consistent with the correlations, wherein phosphorylation at both S226 and

S211 do not influence potency for transrepression on the AP-1 and NFκB promoters. However, on the AP-1 promoter, both the S226A and S211A mutants had relative efficacies higher than that of wt (122.5% and 153.25%, as compared to wt) (Fig. 4.11 C). Similarly, on the NFκB promoter, the S226A mutant had a higher efficacy (155%, as compared to wt), whereas the S211A mutant had a slightly lower efficacy (78%, as compared to wt). Taken together, these results show that phosphorylation at S211 and S226 inhibits efficacy for transrepression via AP-1, whereas phosphorylation at S226, but not S211 inhibits efficacy for transrepression via NFκB. However, due to the relatively large experimental error, these differences were not statistically significant. These results are not consistent with the positive correlation results between GR phosphorylation at S226 and efficacy for transrepression via AP-1 and NFκB, which indicated that the more phosphorylation, the higher the efficacy for transrepression. Similarly the positive correlation between phosphorylation at S211 and efficacy for transrepression via AP-1 indicated that the more S211 phosphorylation, the higher the efficacy for transrepression via AP-1. Nevertheless, these results indicate that GR phosphorylation at S211 and/or S226 plays a role in efficacy for transrepression on the AP-1 and/or NFκB promoter.



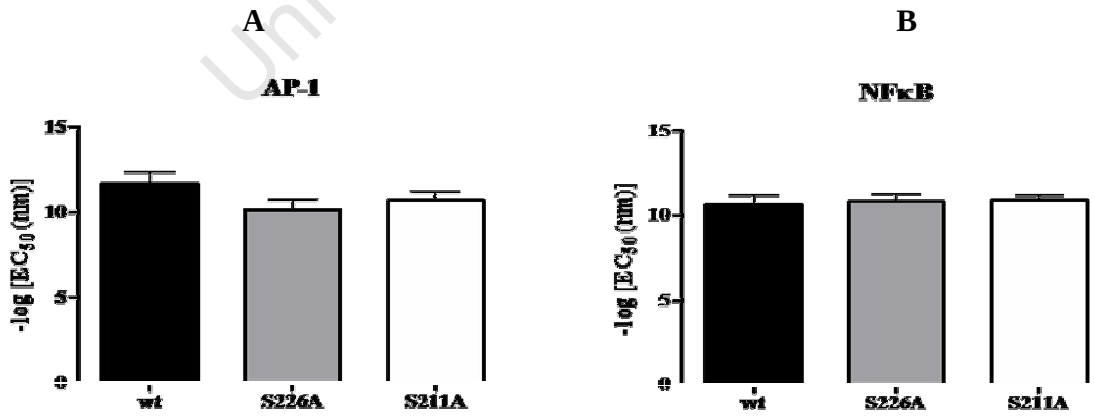



**Figure 4.11 Effect of mutating S211 and S226 on dex stimulated potency and efficacy for transrepression via the GR (continues on next page).**

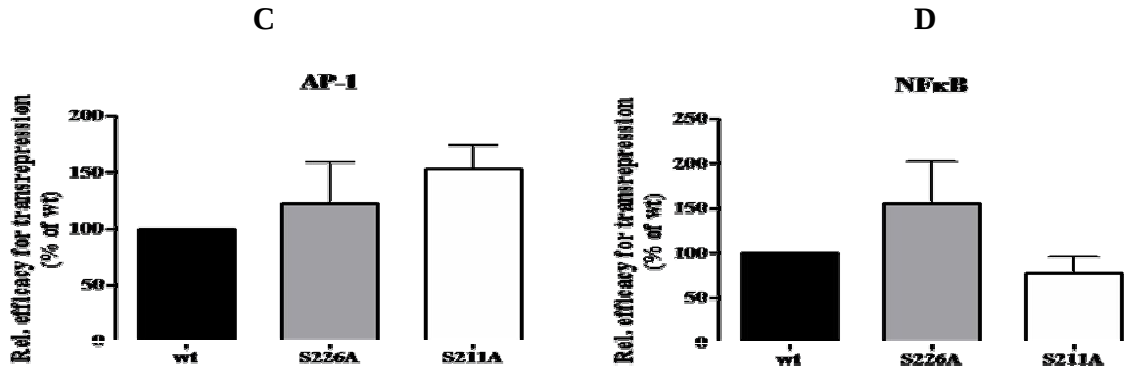



**Figure 4.11 Effect of mutating S211 and S226 on dex stimulated potency and efficacy for transrepression via the GR**. COS-1 cells were plated in 24-well plates at a density of $2.5x10^4$ and transfected with 0.125 μg HA-hGRwt, HA-hGRS211A or HA-hGRS226A, 0.25 μg AP-1 (A and C) or NFκB (B and D) reporter-promoter and 0.012 μg β-gal constructs per well. Twenty-four hours post-transfection the cells were treated with increasing concentrations of dex and 10 ng PMA/well. After 24 hours, luciferase and β-gal activity was assayed. β-gal values were used to normalize for transfection efficiency. Dose-response curves with wt and the mutant receptors were fitted, vehicle background was subtracted and the potency or efficacy for transrepression were calculated and plotted as –log EC50 (nM) (A and B) or relative efficacy with 10 μM dex for wt GR set to 100% (C and D). Graphs represent the average of three independent experiments, plotted as mean ± SEM.

In order to further investigate these potential differences in efficacy for transrepression, the transrepression efficacies for wt and mutant GR, in the presence of the panel of ligands, were determined under saturating ligand concentrations. Results with wt GR on the AP-1 promoter, show that RU486 (classified as a dissociated compound) is a full agonist for transrepression and resulted in the most transrepression of about ~ 60% (Figs. 4.12 A and B). The full/partial agonist MPA, as well as the full agonists dex, cort and predn also resulted in transrepression (~ 40%, 40%, 30% and 30% repression, respectively). The full/partial agonist ald and the partial agonist prog repressed very little (~ 20% and 10% repression), while the dissociated ligand CpdA and the antagonists NET and UDCA did not result in any transrepression. Furthermore, there is a general trend that both the S226A and S211A mutants repressed more than wt in a statistically significant manner for the full-, full/partial- and partial agonists, e.g. cort, predn, ald, MPA and prog (Fig.

4.12 A and B, respectively). However, a similar ligand-selective pattern as wt was found with both the mutants. Therefore, ligand-selective GR phosphorylation at S211 or S226, does not determine the rank order of ligand-selective GR transrepression via AP-1. Interestingly, with the dissociated ligand CpdA and the antagonists NET and UDCA, which do not cause transrepression by wt GR, the S226A mutant increased transcription in a statistically significant manner, while the S211A mutant did not. These results are not due to differential expression levels between wt and mutant GR, as expression levels were monitored throughout the thesis by means of Western blotting and were found to be similar (data not shown). The increase in transcription with the S226 mutant with CpdA, UDCA and NET is difficult to interpret, but suggests that there are some mechanistic differences in ligand-mediated GR transrepression via AP-1 between the agonists and partial agonists as compared to the dissociated ligand CpdA, as well as the antagonists NET and UDCA. Nevertheless, ligand-selective GR phosphorylation at S211A or S226A, plays an important role in mediating the efficacy for transrepression via AP-1, but does not determine the rank order of ligand-selective transrepression via AP-1.

On the NFκB promoter, very little repression with wt GR was obtained, e.g. with dex only 20% repression (Figs. 4.12 C and D). Nevertheless, RU486 resulted in the most repression (~ 40% repression), followed by dex, ald, predn and MPA (~ 20%, 15%, 16% and 10%), whereas cort, prog, NET, CpdA and UDCA did not result in any transrepression. Similar to the results obtained on the AP-1 promoter, results indicate a general trend wherein S226A and S211A repressed more than wt GR in a statistically significant manner for the full- and full/partial agonists, e.g. dex, predn, ald and MPA. Additionally on the NFκB promoter, with prog and RU486, S226A, but not S211A repressed more than wt GR. Contrary to results obtained on AP-1, S226A did not increase transactivation with CpdA, UDCA or NET, suggesting that there are

some promoter-specific differences in the mechanism of GR-mediated transrepression on AP-1 versus NFκB for these specific ligands. Taken together, these results indicate, that ligand-selective GR phosphorylation at S211 or S226 does not determine the rank order of ligand-selective transrepression via NFκB. Furthermore, these results with S226 are not consistent with the correlations, wherein no correlation between GR phosphorylation at S226 and efficacy for transrepression via NFκB, for the ligands capable of transrepression, was found. However, these results are consistent with the correlation between ligand-selective S211 phosphorylation and NFκB-mediated GR transrepression.

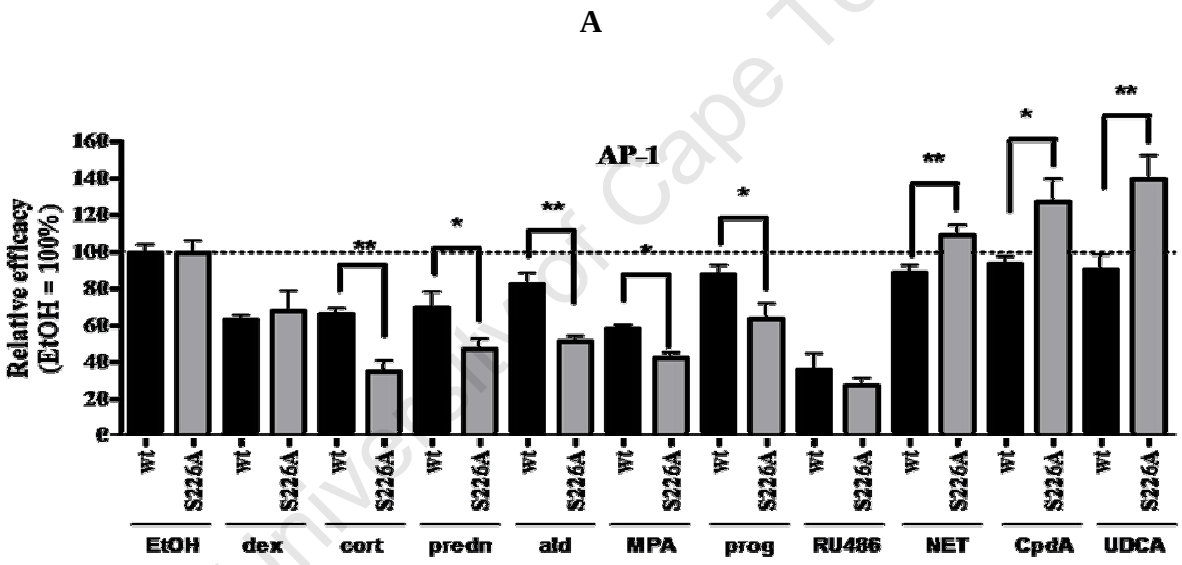
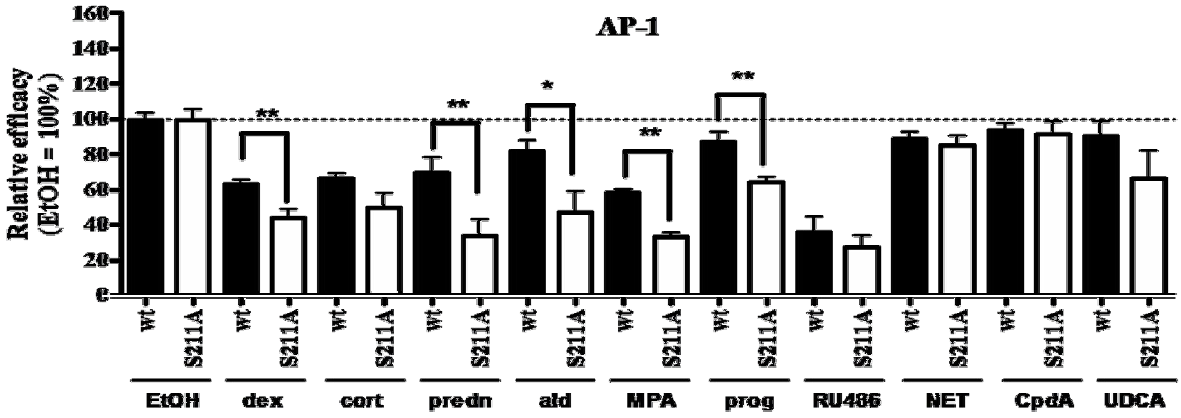
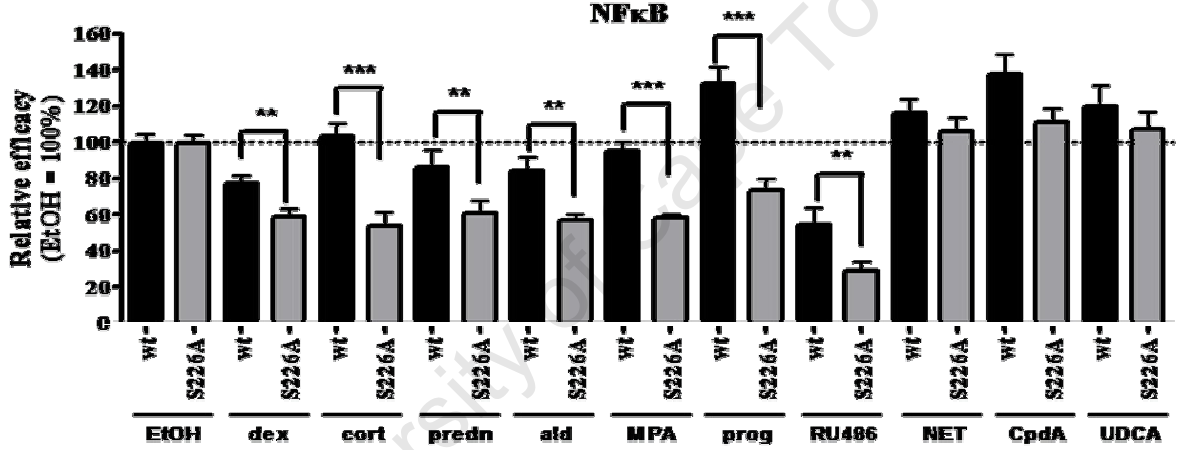



**Figure 4.12 GR-dependent AP-1 and NFκB efficacy for transrepression in COS-1 cells (continues on next page).**

**B**




**C**




**D**

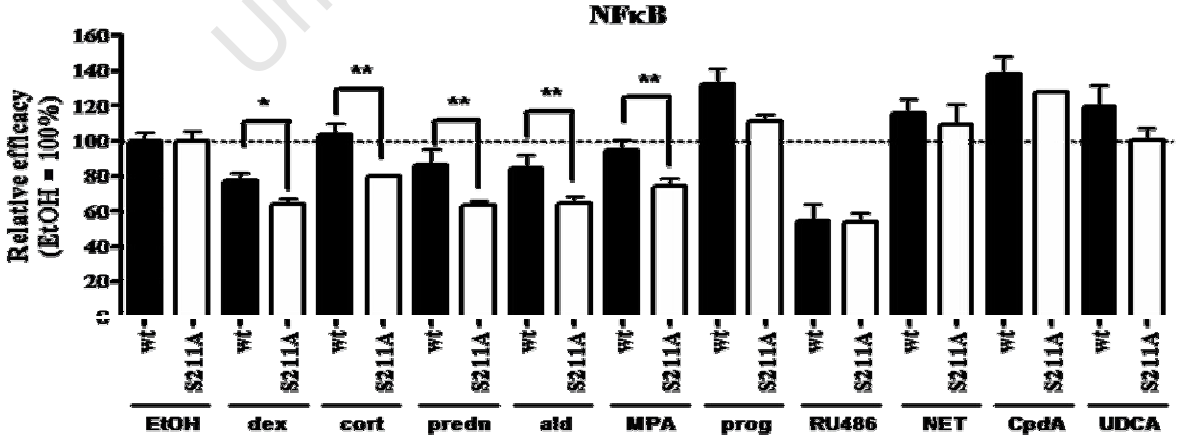





**Figure 4.12 GR-dependent AP-1 and NFκB efficacy for transrepression in COS-1 cells**. COS-1 cells were plated in 24-well plates at a density of $2.5x10^4$ and transfected with 0.125 µg HA-hGRwt, HA-

hGRS211A or HA-hGRS226A, 0.25 μg AP-1 or NFκB reporter promoter and 0.012 μg β-gal construct per well. Twenty-four hours post-transfection the cells were treated with 10 ng PMA/well and either 10 μM of the indicated compounds or vehicle (EtOH). After 24 hours, luciferase and β-gal activity was assayed. β-gal values were used to normalize for transfection efficiency. The AP-1 (S226A and S211A, A and B, respectively) and NFκB (S226A and S211A, C and D, respectively) efficacies for transrepression were calculated and plotted as relative efficacy, with vehicle wt set to 100%. Graphs represent the average of three independent experiments and are plotted as means ± SEM. Statistical analyses were carried out using GraphPad Prism software (version 5), using a two-tailed t-test, with selected pairs of columns, comparing wt and phosphorylation mutant GR with different ligands. Statistical significance is denoted by *, ** or ***, to indicate $P < 0.05$, $P < 0.01$ or $P < 0.001$.

Interestingly, inhibition of JNK, the kinase reportedly responsible for S226 phosphorylation, resulted in a statistically significant increase in dex-mediated efficacy for transrepression on the NFκB promoter (K. Ronacher; Figure E2 in addendum E). Inhibition of p38, the kinase reportedly responsible for S211 phosphorylation, resulted in a small but not statistically significant increase in dex-mediated efficacy for transrepression on the NFκB promoter (K. Ronacher; Figure E2 in addendum E). However, on the AP-1 reporter-promoter construct, inhibition of neither JNK nor p38 had any effect on dex-mediated efficacy for transrepression (K. Ronacher; Figure E2 in addendum E). These results are consistent with a model, wherein p38- and JNK-mediated GR phosphorylation on S211 and S226, respectively, inhibits maximal efficacy for transrepression via NFκB, but not AP-1. The result with the inhibitors are in agreement with the results for NFκB in figures 4.12 C and D, i.e. increased efficacy for transrepression with the phosphorylation mutants and is consistent with a model wherein p38- and JNK-mediated phosphorylation at S211 and S226 inhibits maximal efficacy for transrepression. However, the inhibitor results are not in agreement with the results for AP-1 in figures 4.12 A and B, since an increased efficacy for transrepression was also found with the phosphorylation mutants. The apparent differences between the results obtained with the

phosphorylation mutants vs. the kinases inhibitors on AP-1-mediated transrepression, could indicate that the kinases inhibitors are not completely inhibiting GR phosphorylation at S211 and S226 and that a complete loss of GR phosphorylation is needed to influence efficacy for transrepression via AP-1. However, this is not the case on NFκB-mediated transrepression, indicating slight differences between these two promoters, for example different proteins and pathways involved. On the other hand, these differences could indicate that the effect of the kinase inhibitors on NFκB is not directly mediated by a loss in GR phosphorylation, but rather by an indirect effect of the kinase inhibitors.

Having shown that phosphorylation at S211 and S226 inhibits maximal efficacy for transrepression via AP-1 and NFκB, and in light of a report suggesting that the GR phosphorylation sites can compensate for each other (Wang *et al.*, 2007b), the significance of their simultaneous mutation (3A) on transrepression efficacy was next examined. Due to the fact that very little transrepression was found with wt dex, only 20%, on the NFκB reporter-promoter versus 40% on the AP-1 promoter, the effect of GR phosphorylation on S226, S211 and S203 was only examined on the AP-1 reporter-promoter. Results show ligand-induced transrepression with wt, as well as the triple phosphorylation mutant GR, with the agonist dex giving the most transrepression (Fig. 4.13). Interestingly, for wt and 3A receptor, the partial agonist prog appeared to result in similar or even more repression than the full/partial agonist MPA, although these differences were not statistically significant. This is contrary to the previous findings on the AP-1 promoter (see figures 4.12 A and B, as well as Table E3 in addendum E), where MPA resulted in more transrepression than prog and could indicate methodological differences between these experiments. However, the only difference between these experiments is the specific hGR expression constructs that were used. It would be tempting to suggest that differences in the

relative expression levels of these two hGR constructs can account for these differences, however a direct comparison of the relative GR levels should first be performed. Nevertheless, contrary to the transactivation efficacy with the triple phosphorylation mutant (Fig. 4.10), as well as efficacy for transrepression via AP-1 with the single phosphorylation mutants (Figs. 4.12 A and B), no statistically significant differences between wt and triple mutant receptor in efficacy for transrepression on AP-1 were found when comparing all the ligands. However, there was a general trend with all the ligands investigated, that the triple phosphorylation mutant resulted in slightly more repression than wt receptor, and when only dex-mediated transrepression of wt and 3A mutant GR was compared to each other, a small but statistically significant difference was found (Fig. 4.13).



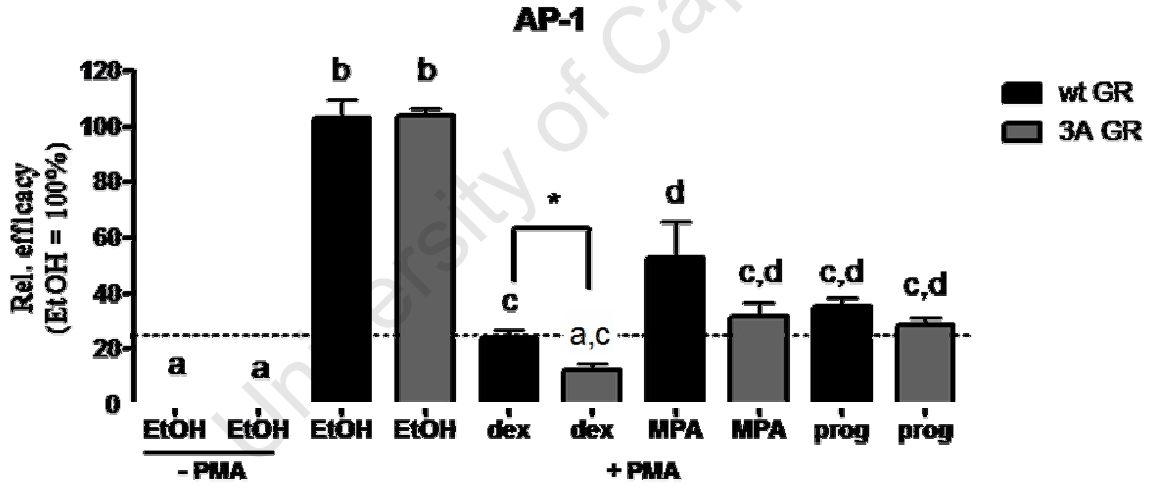



**Figure 4.13 GR-dependent AP-1 efficacy for transrepression in COS-1 cells**. COS-1 cells were plated in 24-well plates at a density of $2.5x10^4$ and transfected with 0.125 µg pRS-hGRwt or pRS-hGR3A, 0.25 µg AP-1 reporter-promoter and 0.012 µg β-gal construct per well. Twenty-four hours post-transfection the cells were treated with vehicle (EtOH) or 10 µM of the indicated compounds and 10 ng PMA/well. After 24 hours, luciferase and β-gal activity was assayed. β-gal values were used to normalize for transfection efficiency. The efficacy for transcription were calculated and plotted as relative efficacy (EtOH +PMA set to 100%). Values shown are from three independent experiments, plotted as means ± SEM. Statistical significance is indicated by different lower-case letters, such that all the conditions with the same letter are not statistically significantly different from each other ($P > 0.05$), while those having

different letters are statistically significantly different from each other ($P < 0.05$). Additional statistical analyses were carried out using GraphPad Prism software (version 5), using a two-tailed t-test, with selected pairs of columns, comparing wt and phosphorylation mutant GR with different ligands. Statistical significance is denoted by * , to indicate $P < 0.05$.

These results were unexpected and difficult to interpret. Since both the single phosphorylation mutants resulted in more transrepression than wt receptor, a bigger difference in 3A versus wt repression was expected. One interpretation of this data is that phosphorylation on either S211 or S226 already inhibits efficacy for transrepression to a "maximal" extent and that their combined phosphorylation cannot further inhibit efficacy for transrepression via AP-1. Additionally, in this experiment dex in the presence of wt GR resulted in more relative repression (~75%) as compared to the results for dex in the presence of wt GR shown in figures 4.12 A and B (~40%). This indicates that the hGR expression constructs used in figure 3.13 (pRS-hGR) expresses more GR than that in figure 4.12 (HA-hGR). The higher GR levels could also result in "maximal" transrepression with wt GR, preventing further repression with the triple phosphorylation mutant and repeat experiments with lower amounts of GR, could possibly result in a bigger difference between wt and triple phosphorylation mutant. However, this remains to be tested. On the other hand, since the triple phosphorylation mutant includes S203, it is possible that phosphorylation at S203 is required for maximal efficacy for transrepression. Therefore, the result obtained with the triple phosphorylation mutant could be the combination of inhibitory and enhanced effects on efficacy for transrepression with the individual phosphorylation sites. However, the role of S203 phosphorylation on efficacy for transrepression has not been investigated, herein or in the literature, and further experiments are needed to confirm a role for S203 phosphorylation in efficacy for transrepression.

Taken together, results suggest that GR phosphorylation at S211 and/or S226 inhibits efficacy for transrepression, but that the rank order of ligand-selective GR-mediated transrepression is not dependent on ligand-selective GR phosphorylation per se, as ligand-selective differences in efficacy for transrepression was still observed with the single phosphorylation mutants. Unfortunately, the results obtained with combined mutation of S226, S211 and S203 did not show any statistically significant differences and should be repeated with lower amounts of GR in order to determine whether ligand-selective transrepression is still obtained with the combined mutation of S226, S211 and S203.

Results presented in this section, represent for the first time a comprehensive study with a wide panel of GR ligands, on the role of ligand-selective GR phosphorylation at S226 and/or S211 on ligand-selective GR mediated transactivation and/or transrepression on different GR-regulated promoters. Ligand-selective GR phosphorylation at S226 and S211 correlates with ligand-selective efficacy, but not potency, for transactivation and transrepression. However, transactivation and transrepression with GR phosphorylation mutants, still showed ligand-selective transactivation and transrepression. This shows that ligand-selective GR phosphorylation at S226 or S211 does not determine the rank order of ligand-selective GR-mediated transactivation or transrepression. This leaves unanswered the question as to what determines ligand-selectivity, as well as why positive correlations between GR phosphorylation and efficacy for transactivation and transrepression were obtained. It is likely that both ligand-selective GR phosphorylation and ligand-selective transcription are mediated by the same ligand-selective "determining" step. Thereby, ligand-selective GR phosphorylation is an indirect consequence and only "mimics" ligand-selectivity. This would explain why the positive correlations were obtained between GR phosphorylation and transcription. In addition, the phosphorylation mutants displayed the same ligand-selective pattern of efficacy for

transactivation and transrepression, indicating that the same ligand-selective "determining" step is maintained in the phosphorylation mutants. This further indicates that ligand-selective GR phosphorylation is an indirect effect and only reflects ligand-selectivity, since the mutants cannot be phosphorylated but still resulted in ligand-selective transactivation and transrepression. For more detailed discussion on this, see Chapter 8 (Conclusions and future perspectives).

At the time this work was done, the effect of GR phosphorylation at S226 with different ligands on GR-mediated transactivation was not known, but recently GR phosphorylation at S226 with dex and prog has been shown to inhibit efficacy for transactivation on a MMTV reporter-promoter in U2OS cells (Chen *et al.*, 2008). Results presented in this section are consistent with those of Chen *et al.*, which showed that phosphorylation at S211 is required for, while phosphorylation at S226 inhibits maximal efficacy for transactivation and additionally show the effect of GR phosphorylation at S226 on efficacy for transactivation with 8 additional ligands. The results in this thesis are consistent with a model wherein dex-mediated GR phosphorylation at S211 is required for the recruitment of a co-activator (e.g. "X"), whereas dex-mediated phosphorylation at S226 is required for the recruitment of a co-repressor (e.g. "Y") (Fig. 4.14). This is consistent with a recent report in the literature, where the co-repressors NCoR and SMRT were shown to be recruited to an endogenous GC-regulated gene in response to dex (Hong *et al.*, 2009). According to the model shown below, wt GR bound with dex, that is phosphorylated at both S226 and S211, will recruit both co-activator (X) and co-repressor (Y). When S211 is not phosphorylated (i.e. with the S211A mutant), dex-bound GR would not be able to recruit the co-activator (X), but would still be able to recruit the co-repressor (Y) through phosphorylated S226. This combination would result in the S211A mutant having a reduced efficacy for transactivation, compared to wt, as was found on the MMTV promoter (Fig. 4.10). In contrast, when S226 is not phosphorylated (i.e. with the S226A mutant), dex-bound GR would not be able to recruit the co-

repressor (Y), but would still be able to recruit the co-activator (X) through S211, resulting in the increased maximal efficacy seen on the MMTV promoter in figure 4.10. This is consistent with the recruitment of NCoR and SMRT to an endogenous human metallothionein IIa (hMTIIa) gene in COS-7 and HeLa cells, where knock-down of NCoR or SMRT, by siRNA, resulted in a increase in dex-mediated transactivation on the hMTIIa gene (Hong *et al.*, 2009).



**Figure 4.14 Phosphorylation site-specific GR phosphorylation mediates co-activator vs. co-repressor recruitment.** Light blue square illustrates the GR bound with dex (small red triangle). While X = co-activator; Y = co-repressor; GRE = glucocorticoid response element; BTM = basic transcription machinery.

Furthermore, the inhibitory effect of phosphorylation at S226 on efficacy for transactivation, was much weaker on the MMTV- versus the TAT-GRE promoter. In contrast, the requirement of S211 phosphorylation for efficacy for transactivation on the MMTV promoter, as shown in this thesis, was greater than observed in results previously obtained on the TAT-GRE promoter ((Stubsrud, 2005); Table E2 in addendum E). These results indicate promoter-specific differences in the role of GR phosphorylation at S226 and S211 on transactivation efficacy, which is consistent with a previous report in the literature (Webster *et al.*, 1997). However, there are some inconsistencies between the promoter-specific differences obtained by Webster *et al.* and those obtained in this thesis. On a MMTV promoter, Webster *et al.* did not see any effect of phosphorylation of the mGR at the mouse equivalents to S211 or S226 on transactivation efficacy in COS-1 cells. However Webster *et al.* found that phosphorylation of the mGR at the mouse

equivalent to S211 was required for maximal transactivation efficacy and phosphorylation at the mouse equivalent to S226 had no effect on a TAT-GRE promoter in COS-1 cells. In contrast, results in this thesis show that phosphorylation of the hGR at S226 inhibited transcription efficacy on both the MMTV- and TAT-GRE promoters, albeit to different extents, while phosphorylation of the hGR at S211 is required for maximal transactivation efficacy on the MMTV promoter. Furthermore, on the MMTV promoter Webster *et al.* did not see any difference in transactivation efficacy between wt and the triple phosphorylation mutant, whereas a complete loss in transactivation efficacy with the triple phosphorylation mutant on the MMTV promoter was shown in this thesis. Other than methodological differences, possibly resulting in different GR expression levels, the different results obtained in this thesis as compared to those of Webster *et al*. could be due to species-specific differences (rat vs. human GR). To this extent Rogatsky *et al.* examined the role of phosphorylation of the rat GR at threonine 171, the only know phosphorylation site that is not conserved in humans, on GR-mediated transactivation and came to the conclusion that there are species-specific differences in the role of GR phosphorylation on transactivation (Rogatsky *et al.*, 1998a). Whether there are species-specific differences in the role of GR phosphorylation, can only be determined by a direct comparison of mGR vs. hGR and requires further investigation. Furthermore, the results obtained with the triple phosphorylation mutant on the MMTV promoter in this study, as well as by Webster *et al*., appears to be inconsistent with a recent report where a 4 fold increase, compared to wt, in hGR-mediated transactivation efficacy on the MMTV promoter was observed in HCT116 cells (Kino *et al.*, 2007). In the same study, Kino *et al*. also showed a 2 fold increase, compared to wt, in hGR-mediated transactivation efficacy on the MMTV promoter with the S211A or S226A mutant. The inconsistencies between the results obtained by Kino *et al.* and results presented in this thesis, both with the hGR, are most likely due to cell-specific differences in downstream proteins such as kinases or phosphatases, as well as co-factors and warrants further investigation.

Interestingly, with the dissociated ligand CpdA, no transrepression on AP-1 or NFκB was found for wt receptor. This is in agreement with recently published data on CpdA on the same AP-1 and NFκB reporter-promoter constructs in COS-1 cells (Ronacher *et al.*, 2009), but in contrast to another report in the literature, which showed GR-mediated transrepression via NFκB with CpdA (De Bosscher *et al.*, 2005). In the study by De Bosscher *et al*. CpdA was shown to repress TNF-α induced expression of the NFκB-regulated IL6 and E-selectin promoters in L929 and HEK293T cells. Apart from possible cell- and promoter-specific differences between the study of De Bosscher *et al.* and results represented in this thesis, as well as in Ronacher *et al.* 2009, there are also some methodological differences, including induction with TNF-α versus PMA. Furthermore, De Bosscher *et al*. stimulated with test compound first, before induction with TNF-α, whereas results presented in this thesis, as well as in Ronacher *et al*. 2009 was obtained by first inducing with PMA and then stimulated with test compound. Whether the above mentioned differences could account for the differences on the CpdA mediated transrepression via NFκB is very interesting, but remains to be tested.

At the time this work was done, the effect of GR phosphorylation on GR-mediated transrepression was not known. However, recently phosphorylation at S211 and S226 has been shown to be required for maximal dex-mediated efficacy for transrepression on 6 different GC-regulated endogenous genes, including c-Jun and SMAD17 in U2OS cells (Chen *et al.*, 2008). These results are in contrast to the results obtained in this thesis in COS-1 cells, where phosphorylation at S211 and S226 inhibited maximal efficacy for transrepression on both the AP-1 and NFκB promoter and indicate possible cell- and/or promoter specific differences. One possible explanation is that phosphorylation at S211 and S226 interferes with the ability of the GR to bind to the AP-1 and NFκB proteins. It has recently been shown that deacetylation of the

GR is required for transrepression (Ito *et al.*, 2006b), suggesting that post-translational modifications play an important role in GR-mediated transrepression. However, whether GR phosphorylation inhibits GR-mediated transrepression via interference with GR binding to AP-1 and NFκB remains to be tested. On the other hand, these results could indicate that GR phosphorylation at S211 and S226 is required for the recruitment of a co-activator to the AP-1 and NFκB promoters. Interestingly, on the collagenase 3 promoter, agonist induced GR recruits the co-activator GRIP-1 to an AP-1 promoter. However, in this study, GRIP-1 behaved more like a co-repressor and not as a co-activator (Rogatsky I, 2001). Whether GR phosphorylation at S211 and S226 is required for the recruitment of another co-activator, which behaves as a co-activator on the AP-1 and NFκB promoters, remains to be tested. However, in the literature there is no evidence that supports this hypothesis.

Furthermore, Chen *et al.* showed that wt GR repressed the PAC1 gene with dex in the absence of inducing compound (i.e. no TNF-α or PMA). However, the S211A mutant resulted in a 5 fold increase in transcription, whereas the S226A mutant increased transcription by 2 fold, in response to dex, in the absence of inducing compound (Chen *et al.*, 2008). These results are interesting and also indicate promoter-specific differences on the effect of GR phosphorylation at S211 and S226 on GR-mediated transrepression in the same cell. Additionally, these results by Chen *et al.* for dex are similar to results in this thesis showing an increase in transcription with the S226A mutant on the AP-1 promoter with the dissociated ligand CpdA and the antagonists NET and UDCA. This result is novel as it indicates that not only is the effect of GR phosphorylation on transrepression promoter-specific, but also ligand-specific. In other words, GR phosphorylation at S226, with different ligands, results in different transrepression responses in the same cells, on the same promoter.

To summarise, ligand-selective phosphorylation at S226 was shown to inhibit efficacy, but not potency for transactivation. Furthermore, ligand-selective phosphorylation at S226, as well as S211 was shown to inhibit efficacy, but not potency, for transrepression on AP-1 and NFκB. However, ligand-selective phosphorylation at neither S211 nor S226 determines the rank order of ligand-selective transactivation or transrepression via AP-1 or NFκB. This leaves the question of what determines ligand-selective GR transcription unanswered, and other possibilities will be investigated in Chapter 6.

# Chapter 5 Investigation into the kinases involved in GR phosphorylation (Results and Discussion)

According to the literature, S226 is predominantly phosphorylated by JNK, whereas S211 is primarily phosphorylated by p38 or CDKs (Rogatsky *et al.*, 1998b;Krstic *et al.*, 1997;Miller *et al.*, 2005;Itoh *et al.*, 2002;Kino et al., 2008). However, to date there is not one study that convincingly shows *in vivo*, which kinases phosphorylate the hGR at S226 and/or S211 in mammalian cells by inhibiting kinase activity. Most studies involved *in vitro* kinase assays, or were performed in yeast cells devoid of specific kinases.



## 5.1 The use of specific MAPK inhibitors on GR phosphorylation

Transactivation and transrepression data with GR phosphorylation deficient mutants presented in figures 4.8 and 4.12, as well as with JNK and p38 kinase inhibitors (Fig. E2 in addendum E), are consistent with a role for JNK and p38 in mediating phosphorylation of the GR at S226 and S211, respectively. In order to determine whether JNK and p38 phosphorylates the GR at S226 and S211, respectively, in COS-1 cells, these cells were transiently transfected with hGR and pre-treated with either a JNK or p38 inhibitor, before stimulation with dex and determination of the amount of S226 or S211 phosphorylation (Fig 5.1 A and B, respectively). Results show that inhibition of JNK results in a small decrease in the amount of basal, as well as dex induced S226 phosphorylation, whereas inhibition of p38 reduces the amount of basal and dex induced S226 phosphorylation even more (Fig. 5.1 C). Additionally, the combination of JNK and p38 inhibitors resulted in a statistically significant reduction in the total amount of basal and dex-induced S226

phosphorylation (Fig. 5.1 C). Inhibition of JNK did not significantly reduce the total amount of dex-induced S211 phosphorylation, whereas inhibition of p38 and the combined inhibition of p38 and JNK significantly reduced the total amount of basal and dex-induced S211 phosphorylation (Fig. 5.1 D). However, results show that inhibition of JNK and p38 slightly decreased the total amount of GR, whereas the simultaneous inhibition of JNK and p38 decreased the total amount of GR even more (Figs. 5.1 E and F). After quantification and normalisation to the total GR levels, results show that the fold dex-induced S226 phosphorylation was not reduced upon inhibition of JNK or p38, or the combination thereof (Fig. 5.1 G). Furthermore, inhibition of JNK alone or p38 alone did not reduce the fold dex-induced S211 phosphorylation, whereas the combination of JNK and p38 inhibitors slightly decreased the fold dex-induced S211 phosphorylation (Fig. 5.1 H).



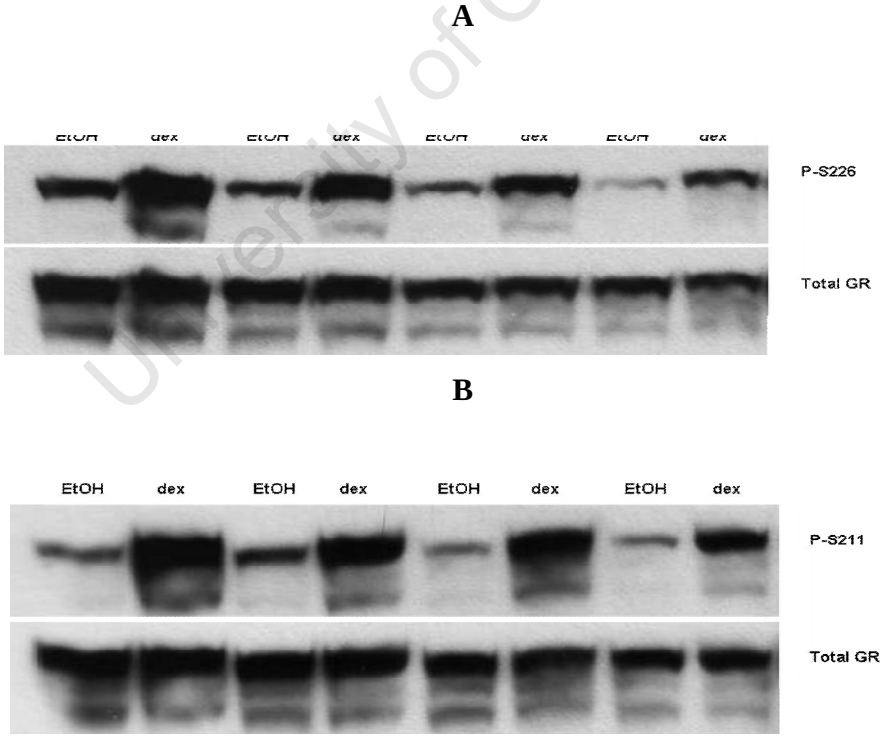



**Figure 5.1 Effect of JNK and p38 inhibitors on the phosphorylation status of the GR at S226 (A) S211 (B) (continues on next page).**

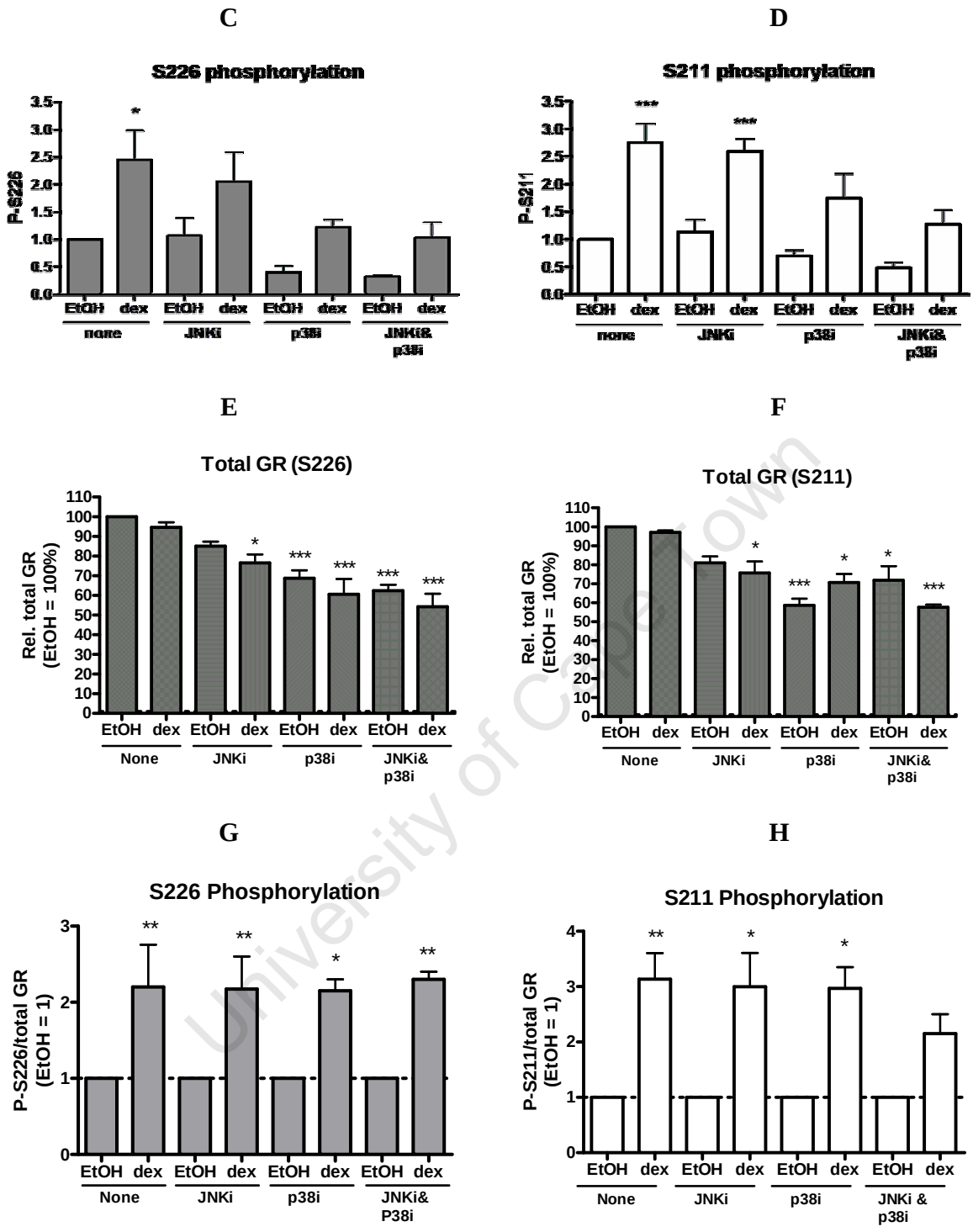



**Figure 5.1 Effect of JNK and p38 inhibitors on the phosphorylation status of the GR at S226 (A) S211 (B).** COS-1 cells were plated in 6-well plates at a density of $3x10^5$cells/dish and transfected with 3.5 μg HA-hGRwt per well. After 24 hour incubation, cells were pre-treated with 50 μM JNK (JNKi) or 10 μM p38 inhibitor (p38i), or a combination of them (JNKi & p38i) for 24 hours and then treated with vehicle (EtOH) or 100 nM dex for 1 hour. Whole cell extracts were prepared and equal volumes of

extracts (10 µl) were analyzed by Western blotting with either an anti-P-S226 (A) or anti-P-S211 antibody (B). After developing, the blots were stripped and re-probed with total GR (A and B). After quantification the amount of P-S226 (C), P-S211 (D) and total GR (E) and (F) was determined. Thereafter, the amount of phosphorylated/total protein was calculated and plotted relative to the EtOH no inhibitor sample set as one (G and H). The histograms (C-H) are the average of three independent experiments, plotted as the ave ± SEM, whereas the Western blots (A) and (B) is one representative blots of three independent experiments. Statistical significance, relative to EtOH no inhibitor, is denoted by *, ** or ***, to indicate $P < 0.05$, $P < 0.01$ or $P < 0.001$.

These results show that inhibition of JNK, which reportedly phosphorylates the GR at S226, does not inhibit or even reduce the fold dex-induced S226 phosphorylation. Similarly, S211 is reportedly phosphorylated by p38, but inhibition of p38 does not inhibit or even reduce the fold dex-induced S211 phosphorylation. The combination of the two inhibitors, did however reduce the fold dex-induced phosphorylation at S211, but not S226. Similar results on dex-mediated phosphorylation of the mGR with the JNK and p38 inhibitors was found in mouse LβT2 cells at the equivalent residues in the mGR (Kotitschke, 2009).

Due to apparent inconsistency between these results and reports in the literature that used *in vitro* kinases assays to show that JNK phosphorylates S226, while p38 phosphorylates S211, it was next determined whether the JNK and p38 inhibitors were active under the experimental conditions. Reports in the literature suggest that JNK and p38 have to be phosphorylated themselves in order to be active (Miller *et al.*, 2005;Rogatsky *et al.*, 1998b). The phosphorylation status of JNK and p38 was therefore used as a measurement of their activity. In the absence of any inducing compound (such as TNFα), JNK1 and JNK2/3 are only weakly phosphorylated (Fig. 5.2 A). Inhibition of JNK resulted in a small decrease in the amount of JNK1 and JNK2/3 phosphorylation, whereas inhibition of p38 did not reduce the amount of phosphorylated JNK1 or JNK2/3. However, it should be mentioned that since the amount of

phosphorylated JNK is very small (without prior stimulation with TNFα), the decrease seen is very small. Additionally, the combined inhibition of JNK and p38, also slightly decreased the amount of phosphorylated JNK1 and JNK2/3. On the other hand, whereas very low levels of phosphorylated p38 were visible in the control or with the JNK inhibitor, inhibition of p38 increased, rather than decreased the amount of phosphorylated p38 (Fig. 5.2 B). Similarly, the combined inhibition of JNK and p38 also increased the amount of phosphorylated p38. Furthermore, as with the total GR levels, after quantification it can be seen that the total amount of JNK and p38 decreased in response to the kinase inhibitors (Figs. 5.2 C, D and E). After quantification and normalisation to total amount of JNK or p38, it can be seen that inhibition of JNK slightly reduced phosphorylated JNK1 and reduced phosphorylated JNK2/3 (Figs. 5.2 F and G), while it increased the amount of phosphorylated p38 (Fig. 5.2 H). In contrast, inhibition of p38 increased the amount of phosphorylated JNK1, JNK2/3 and p38 (Figs. 5.2 F-H). Similarly, the combined inhibition of JNK and p38, also increased the amount of phosphorylated JNK1, JNK2/3 and p38. The results with the p38 inhibitor on p38 activity are very difficult to interpret, since the data suggest that the p38 inhibitor activated rather than de-activated p38. However, the p38 inhibitor was shown to inhibit p38 in mouse LβT2 cells, under similar conditions as described above (Kotitschke, 2009).

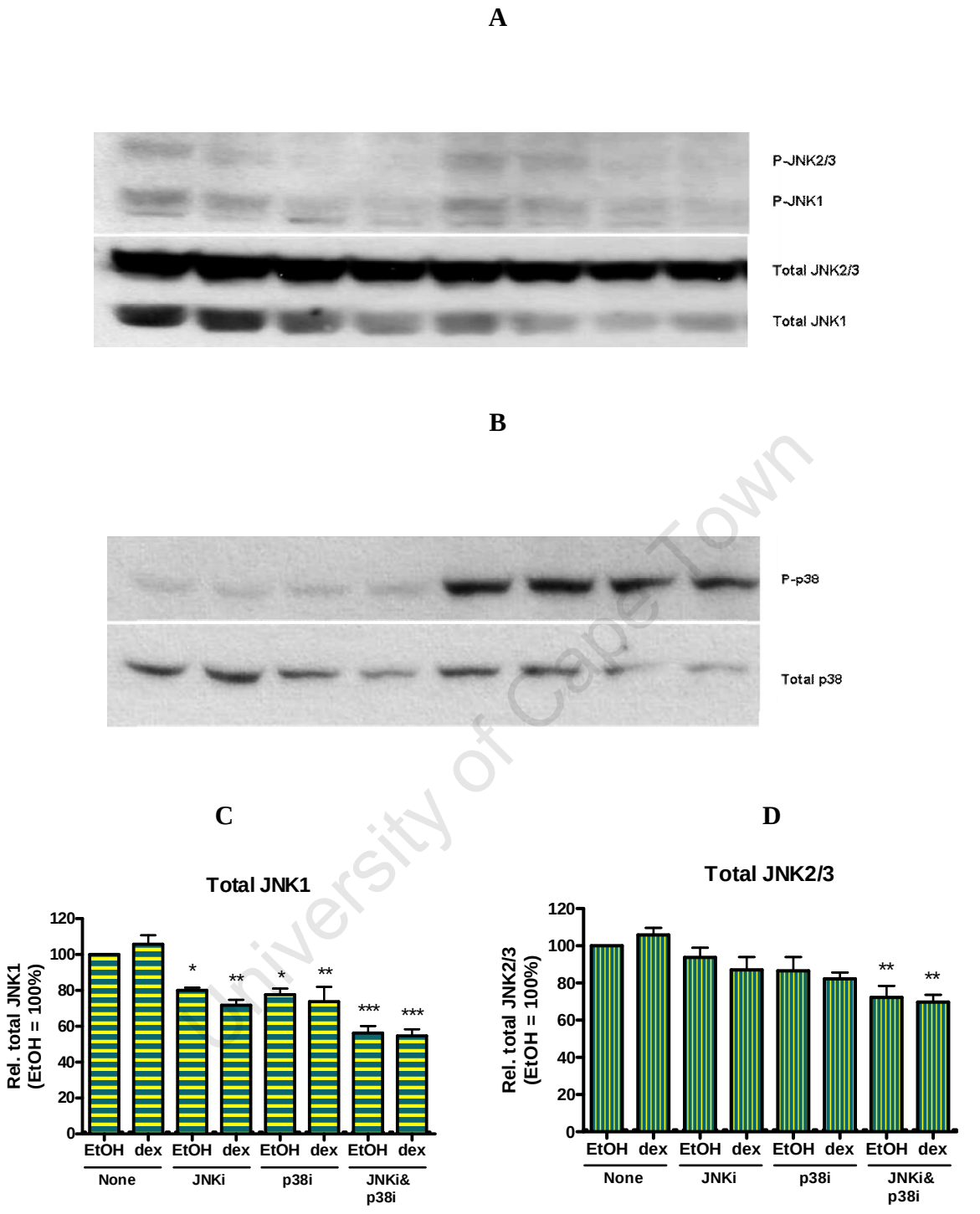





**Figure 5.2 Effect of JNK and p38 inhibitors on the phosphorylation status of JNK and p38 (continues on next page).**

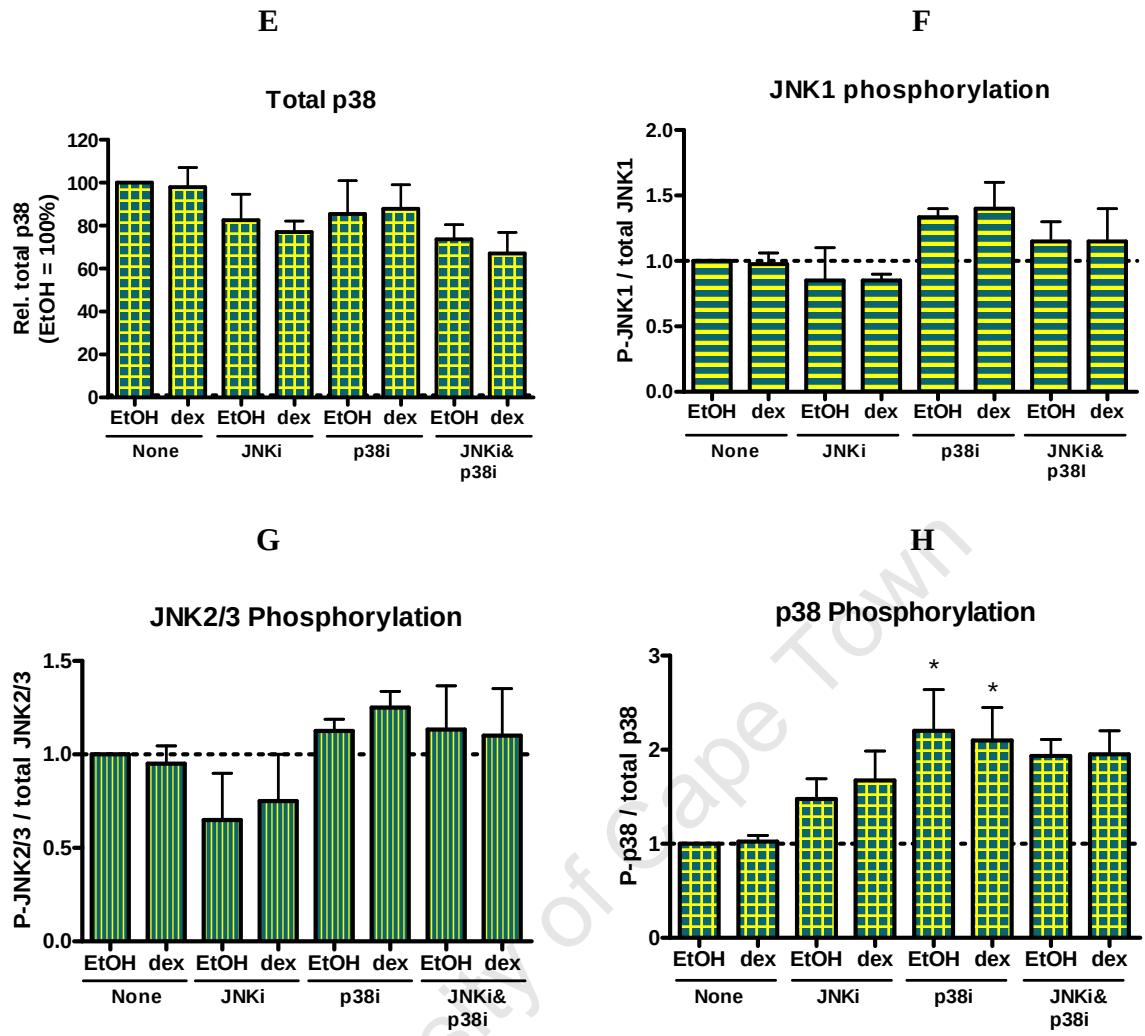



**Figure 5.2 Effect of JNK and p38 inhibitors on the phosphorylation status of JNK (A) and (B) and p38 (C).** COS-1 cells were plated in 6-well plates at a density of $3x10^5$cells/dish and transfected with 3.5 µg HA-hGRwt. After 24 hour incubation, cells were pre-treated with 50 µM JNK or 10 µM p38 inhibitor, or a combination of them for 24 hours and then treated with vehicle (EtOH) or 100 nM dex for 1 hour. Whole cell extracts were prepared and equal amounts of protein (10 µl) were analyzed by Western blotting with either an anti-P-JNK (A) or anti-P-p38 antibody (B). After developing, the blots were stripped and re-probed with total JNK (A) or p38 (B). After quantification the amount of total JNK (C and D) and p38 (E) was plotted with the EtOH no inhibitor sample set to 100%. Thereafter, the amount of phosphorylated/total JNK (F and G) and p38 (H) was calculated and plotted with the EtOH no inhibitor sample was set to one. The amount of total JNK (C) and (D) and total p38 (E), as well as histograms (F), (G) and (H) is representative of the average of three independent experiments, plotted as the ave ± SEM, whereas the Western blots (A) and (B) is one representative blot of three independent experiments.

Nevertheless, these results indicate that these kinase inhibitors cross-react and that the use of these kinase inhibitors in order to determine which kinase phosphorylates the GR *in vivo*, is not possible. The decrease in total protein levels for GR, JNK and p38 indicate that there is cell death or apoptosis occurring. This is in agreement with published data showing that the inhibition of JNK enhances apoptosis, but in contrast to data showing that the inhibition of p38 protects against apoptosis (Miller *et al.*, 2005). Interestingly, the use of the same p38 inhibitor, as in this study, by Miller *et al*. also resulted in a decrease in total GR levels in CEM-C7-14 cells. However, the authors did not comment on the decreased GR levels and further concluded that inhibition of p38 protected against apoptosis. It therefore seems that the concentrations of inhibitors and the time of incubation needed to see a decrease in GR phosphorylation, would result in cell death due to a requirement of these kinases for cell survival.



**5.2 Modulation of GR phosphorylation using MAPK expression constructs**

The use of JNK and p38 MAPK inhibitors to investigate GR phosphorylation at S226 and/or S211 in COS-1 cells was unsuccessful. Therefore overexpression of wt and/or dominant negative ($DN^-$) JNK and p38 protein to modulate GR phosphorylation at S226 and/or S211, was attempted as an alternative strategy. In order to investigate whether this strategy would be successful for both expressed GR in COS-1 cells and on endogenous GR, the effect of MAPK expression constructs on GR phosphorylation was determined in COS-1 cells transiently transfected with hGR, as well as in LβT2 cells expressing endogenous mGR. Results show that expression of $DN^-$ p38 (Fig. 5.3 A), as well as wt JNK (Fig. 5.3 B), resulted in increased total p38 and JNK1 protein levels, respectively. Both expressed and endogenous GR exhibited dex-induced phosphorylation at S211 and S226 for hGR and at the mouse equivalents thereof (Figs. 5.3 C and D), although the

fold dex-induced phosphorylation in the LβT2 cells was not as high as in COS-1 cells. Furthermore, overexpression of wt and $DN^-$ JNK and $DN^-$ p38, did not influence the total GR levels, as was observed when the kinases were inhibited (Figs. 5.3 E and F). This could indicate that overexpression of the $DN^-$ kinases does not completely block the effect of the endogenous kinases, and that enough endogenous kinases are still available to perform crucial functions required for cell survival. On the other hand, this could indicate that it is not the inhibition of the MAPKs that result in cell death per se, but rather that the inhibitors themselves are toxic to the cells by inhibiting or enhancing some other factors that are crucial for cell survival. After quantification and normalisation, it was found that expression of $DN^-$ p38 did not reduce the level of dex-induced GR phosphorylation of the human or mouse equivalent to S226 or S211 (Fig. 5.3 E and F, respectively). Furthermore, expression of wt and $DN^-$ JNK did not increase or decrease, respectively, GR phosphorylation on the human or mouse equivalent to S226 or S211 (Fig. 5.3 G and H, respectively). In retrospect, it would have been a good idea to determine if the total amount of phosphorylated p38 and/or JNK was decreased, i.e. to determine if the $DN^-$ constructs were working properly.

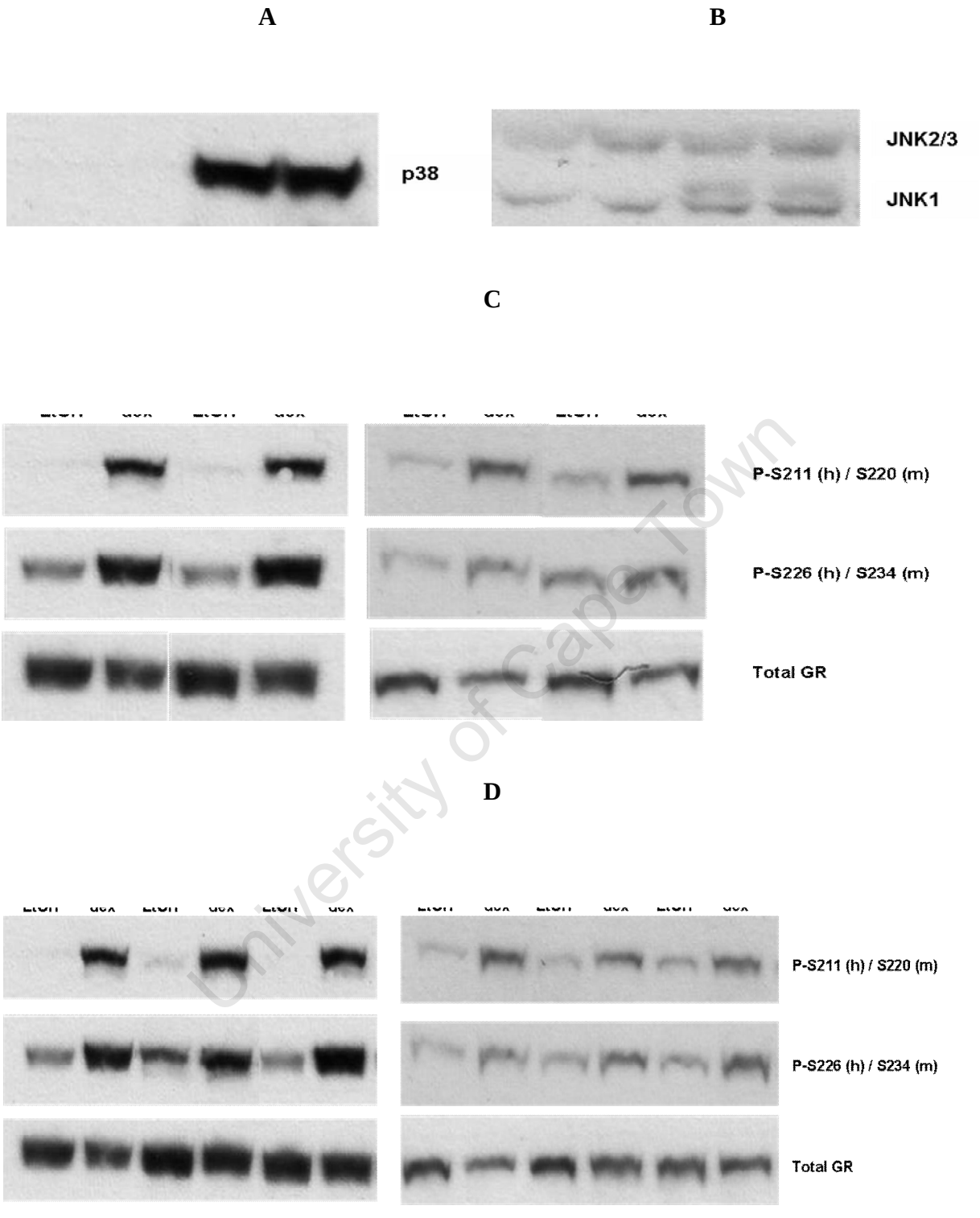





**Fig. 5.3 The effect of expression of wt and DN- JNK and/or p38 on S226 and S211, or mouse equivalent, hGR or mGR phosphorylation (continues on next page).**

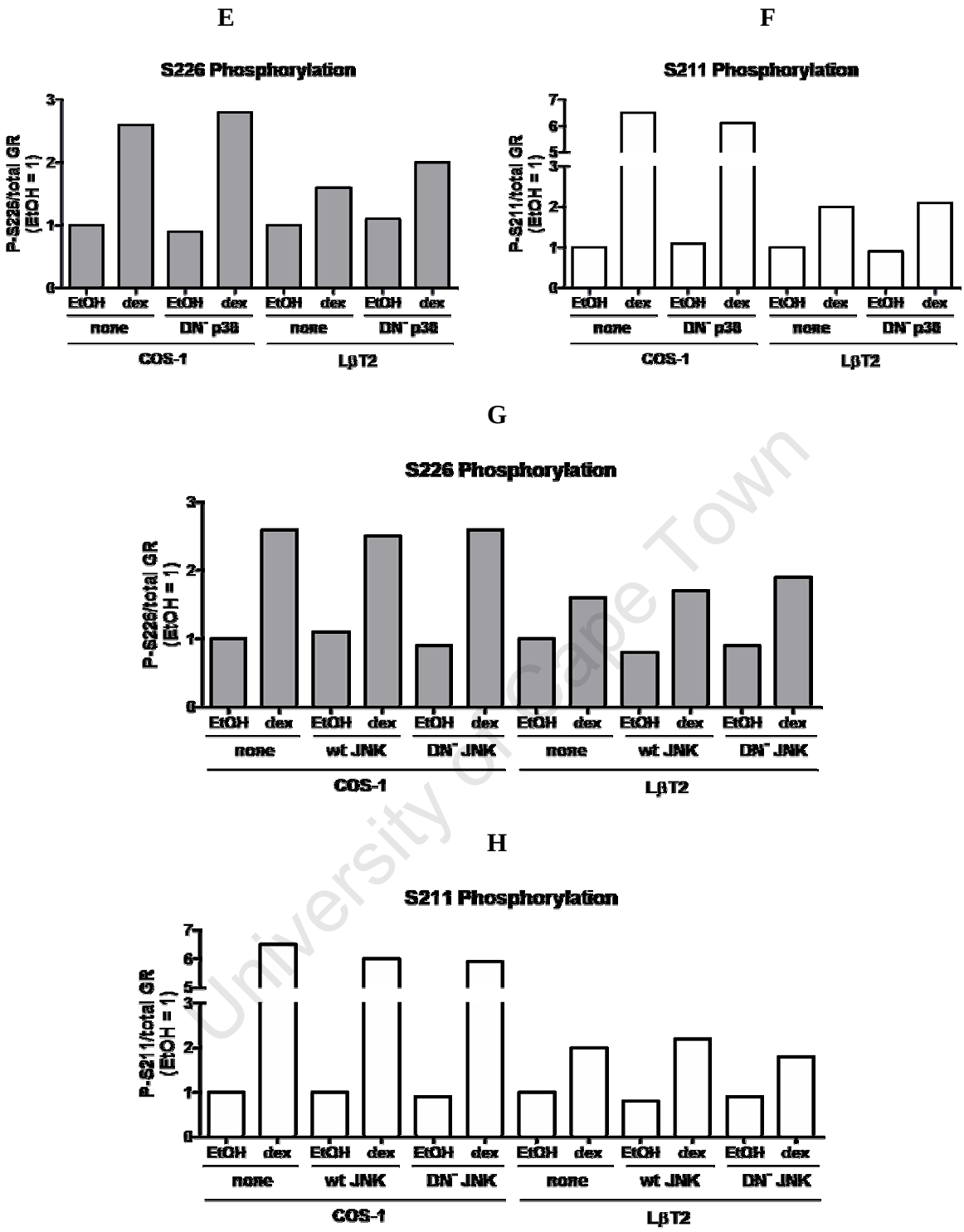





**Fig. 5.3 The effect of expression of wt and DN- JNK and/or p38 on S226 and S211, or mouse equivalent, hGR or mGR phosphorylation.** LβT2 or COS-1 cells transiently transfected with 1 μg HA-hGRwt were transfected with 1 μg DN- p38 (A and C), wt JNK or DN- JNK (B and D) and incubated for 24 hours. Thereafter the cells were treated with vehicle (EtOH) or 100 nM dex for 1 hour and cell lysates were harvested and separated on SDS-PAGE. Western blot analysis was performed using either an anti-P-

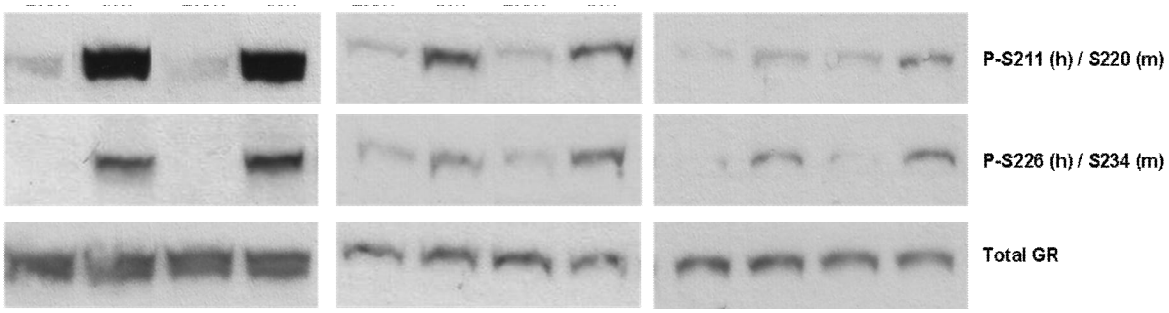
S211 GR-specific antibody or an anti-P-S226 GR-specific antibody. Thereafter the membrane was stripped and reprobed with anti-GR antibody. After quantification and normalisation to total GR, the amount of phosphorylated/total protein in the EtOH sample was set to one (E – H). The Western blots (A - D) and the graphs (E – H) are from a single experiment.

These results are similar to the inhibitor results, in that expression of wt and $DN^-$ JNK does not change the fold dex-induced S226 phosphorylation. Similarly, expression of $DN^-$ p38 does not change the fold dex-induced S211 phosphorylation. It should be noted that these expression experiments were only performed once and more repeats will have to be done to show this result in a statistically significant manner. However, due to the fact that the same result was obtained in both cell lines and that these results are in agreement with the inhibitor results, it was concluded that reports in the literature with *in vitro* assays, wherein JNK and p38 phosphorylates the GR at S226 and S211, respectively, may be misleading and that different kinases are likely to phosphorylate the GR *in vivo* in these cells. Alternatively, it is possible that these kinases can compensate for one another, wherein inhibition of one kinase results in activation of another kinase (as shown in Fig. 3.16), which in turn could phosphorylate the GR.

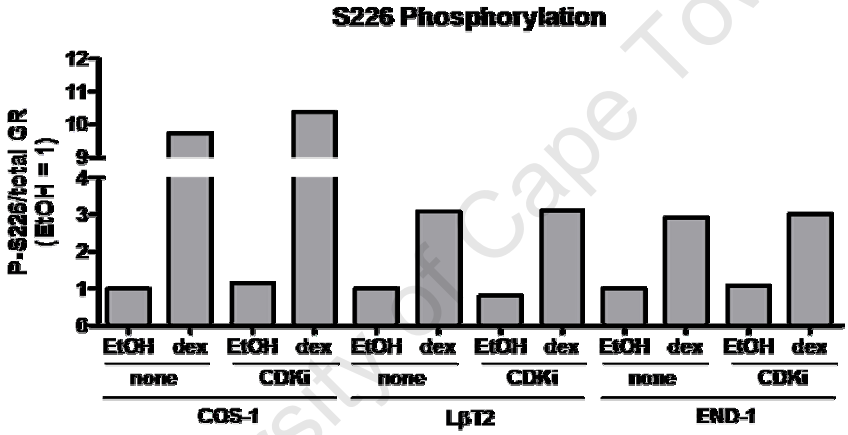
In the literature, there are two studies that indicate that CDKs can phosphorylate the GR at S211 in yeast and HCT116 cells (Krstic *et al.*, 1997;Kino *et al.*, 2007). In a last attempt to identify the kinases that phosphorylate the GR at S211 and/or S226 in mammalian cells *in vivo*, the effect of a general CDK inhibitor on S211 and S226 phosphorylation was examined. Human END-1 and mouse LβT2 cells, containing endogenous GR, as well as COS-1 cells transiently transfected with hGR were treated with a CDK inhibitor and dex, before determining GR phosphorylation at the human and mouse equivalents of S211 and S226. Similar to the results obtained with the MAPK expression constructs, all three cell lines showed dex-induced GR phosphorylation on the human or mouse equivalent to S211 and S226, while the endogenous GR in the LβT2 and END-1

cells showed a smaller fold dex-induced GR phosphorylation, than the expressed GR in COS-1 cells (Fig. 5.4 A). In contrast to the results obtained with the MAPK inhibitors, inhibition of CDKs did not decrease the total amount of GR. This could indicate that CDKs are not crucial for the survival of these cells, or that the inhibitor did not completely inhibit all the CDKs and that enough CDKs are still active to mediate crucial functions required for cell survival. After quantification and normalisation to total GR levels, it can be seen that inhibition of CDK did not inhibit GR phosphorylation of the human and mouse equivalents to S226 or S211 (Fig. 5.4 B and C, respectively) in any of the cell lines investigated. Furthermore, expression of wt and $DN^-$ CDK5 on GR phosphorylation at the human and mouse equivalents of S211 and S226 were also investigated in all three cell lines (Fig. 5.4 D) and similarly to the CDK inhibitor results, expression of $DN^-$ CDK5 did not change dex-induced GR phosphorylation at the human or mouse equivalents to S226 or S211 (Fig. 5.4 E and F, respectively).

**A**




**B**




**C**

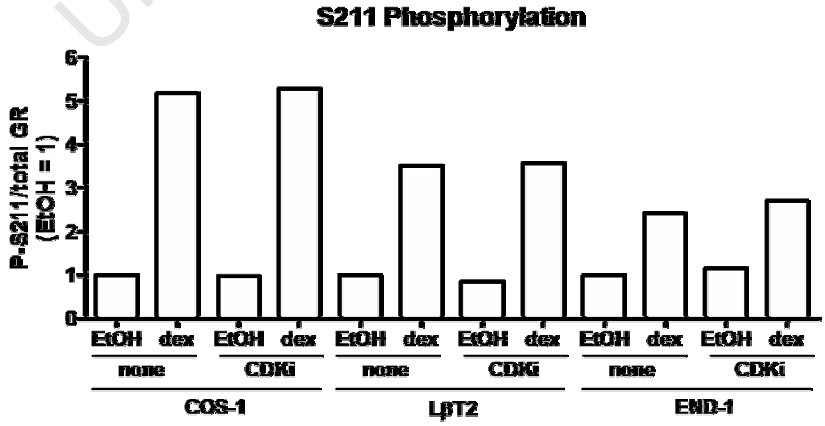
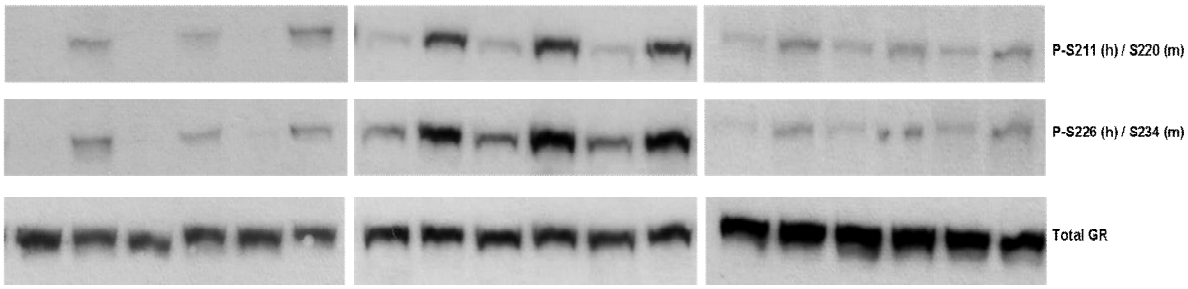


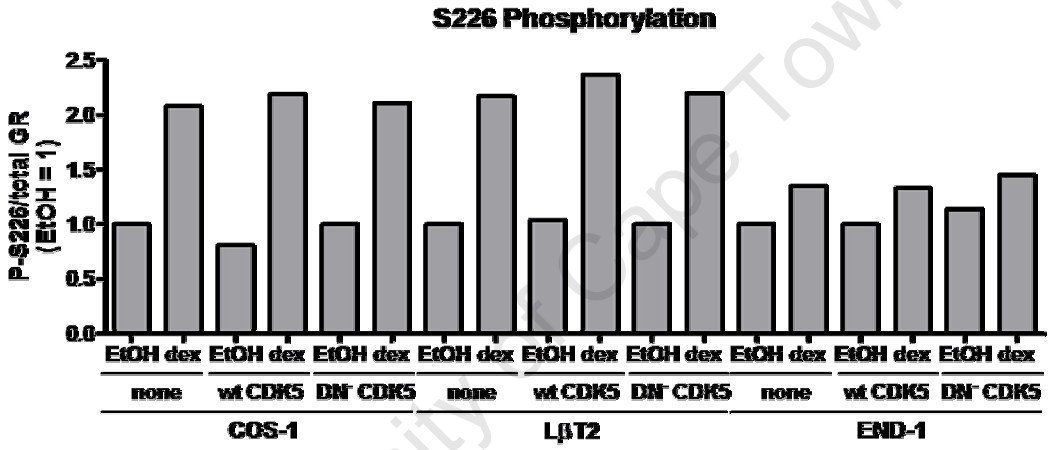



**Figure 5.4 Effect of an CDK inhibitor and expression constructs on GR phosphorylation (continues on next page).**

**D**




**E**




**F**

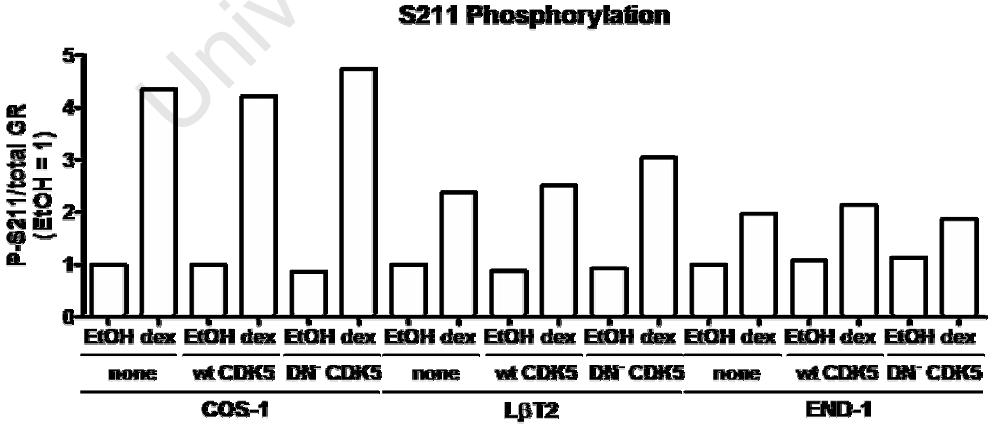





**Figure 5.4 Effect of a CDK inhibitor and expression constructs on GR phosphorylation.** LβT2 and END-1 cells or COS-1 cells transiently transfected with 1 μg HA-hGRwt were either pre-treated with 20 μM roscovitine for 1 hour, or transiently transfected with 1 μg p35 and 1 μg wt CDK5 or DN$^-$ CDK5 and

incubated for 24 hours, before stimulation with 100 nM dex or vehicle (EtOH). Thereafter the cells were harvested and equal amounts of cell extract were separated on SDS-PAGE. Western blot analysis was performed using either an anti-P-S211 GR-specific antibody or an anti-P-S226 GR-specific antibody (A and D). Thereafter the membrane was stripped and reprobed with anti-GR antibody. After quantification and normalisation to total GR, the amount of phosphorylated/total protein in the EtOH sample was set to one (B, C, E and F). The Western blots (A and D) and the graphs (B, C, E and F) are from a single experiment.

These inhibitor and expression experiments were only performed once and more repeats will have to be done to show these results in a statistically significant manner. However, due to the fact that the same result was obtained in all three cell lines and that inhibitor and expression results are in agreement with each other, it was concluded that CDKs do not play a major role in GR phosphorylation at S226 or S211 in these cells *in vivo*.

Having been unable to determine the kinases responsible for GR phosphorylation in COS-1, LβT2 and END-1 cells at the human or mouse equivalent to S211 and S226 by the above mentioned strategies, an alternative strategy was investigated.



**5.3 Kinetics of GR, JNK and p38 phosphorylation**

As an alternative strategy to investigate the kinases responsible for the *in vivo* phosphorylation at S226 and S211 in COS-1 cells, the kinetics of JNK and p38 phosphorylation, as well as GR phosphorylation at S226 and S211, was further examined. This was done in order to correlate an increase or decrease in kinase activity with an increase or decrease in GR phosphorylation. Reports in the literature suggest that JNK and p38 have to be phosphorylated themselves in order to be active and the phosphorylation status of JNK and p38 is therefore used as a measurement of

their activity (Miller *et al.*, 2005;Rogatsky *et al.*, 1998b). Additionally, earlier very low levels of phosphorylated JNK (Fig 5.2) were shown in the absence of inducing compound (e.g. TNF-α). In order to obtain a more detectable JNK phosphorylation signal, the following experiments were conducted in the presence of TNF-α. COS-1 cells, transiently transfected with hGR were pretreated with TNF-α for 15 minutes, before treatment with 100 nM dex. At various time points, cells were harvested and equal amounts of protein were separated on SDS-PAGE. Phosphorylated JNK and GR phosphorylated at S226 were detected by Western blotting with anti-P-JNK or anti-P-S226 antibodies, respectively. Thereafter the membranes were stripped and reprobed for total JNK or GR (Figs. 5.5 A and B). Results show that in the unliganded form the GR is basally phosphorylated at S226, while dex stimulation results in hyperphosphorylation (Fig. 5.5 A). GR phosphorylation at S226 is rapid, with high levels of dex-mediated phosphorylation already visible at 15 minutes and peaks at around 30 to 60 minutes. However due to experimental error no statistically significant differences were detected (Fig. 5.5 C). In the absence of TNF-α, very little phosphorylated JNK is observed (Fig. 5.2 A), while stimulation with TNF-α results in phosphorylation of JNK1 and JNK2/3 (Fig. 5.5 B). The amount of phosphorylated JNK1 and JNK2/3 is already maximal with TNF-α stimulation and 15 minutes to 30 minutes after addition of dex. There after the amount of phosphorylated JNK1 and JNK2/3 decreases rapidly over time and at 60 minutes no phosphorylated JNK1 or JNK2/3 was detected (Figs. 5.5 D and E). Total GR (Fig. 5.5 F) and total JNK (Figs. 5.5 G and H) were not affected by TNF-α induction and did not change significantly over the time examined. These results indicate that JNK is rapidly activated/phosphorylated by TNF-α (within 15 minutes), which correlates with GR phosphorylation at S226 (also within 15 minutes, but not max at 15 minutes). However after 15 to 30 minutes, the amount of phosphorylated JNK decreases rapidly in a dex-independent manner, whereas GR phosphorylation at S226 does not decrease accordingly.

Furthermore, dex-stimulation does not result in JNK phosphorylation, but does result in GR phosphorylation at S226 (in the absence of TNF-α). Taken together, these results are not consistent with a role for JNK in phosphorylating the GR at S226.

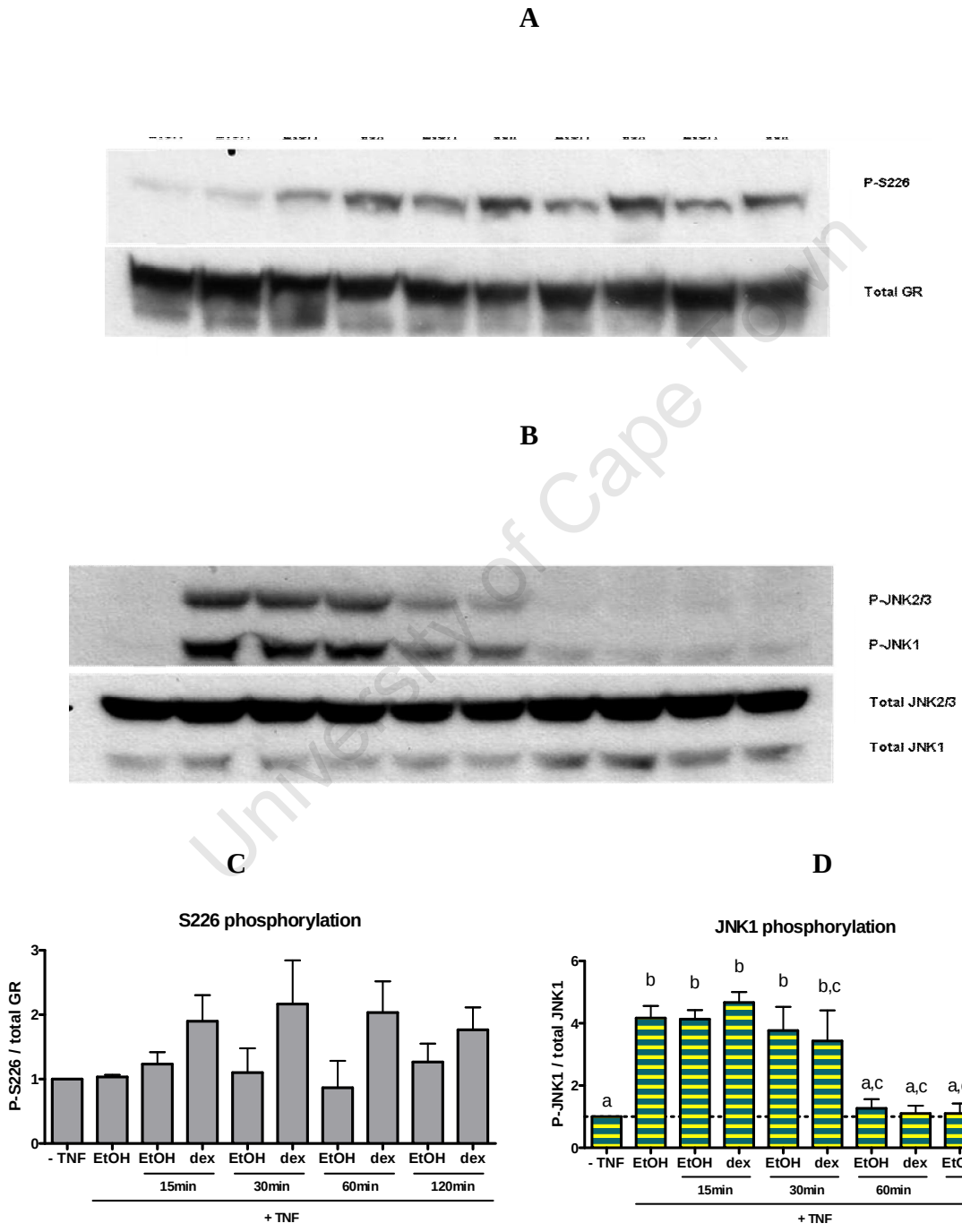





**Figure 5.5 Time-dependent phosphorylation status of the GR at S226 and JNK (continues on next page).**

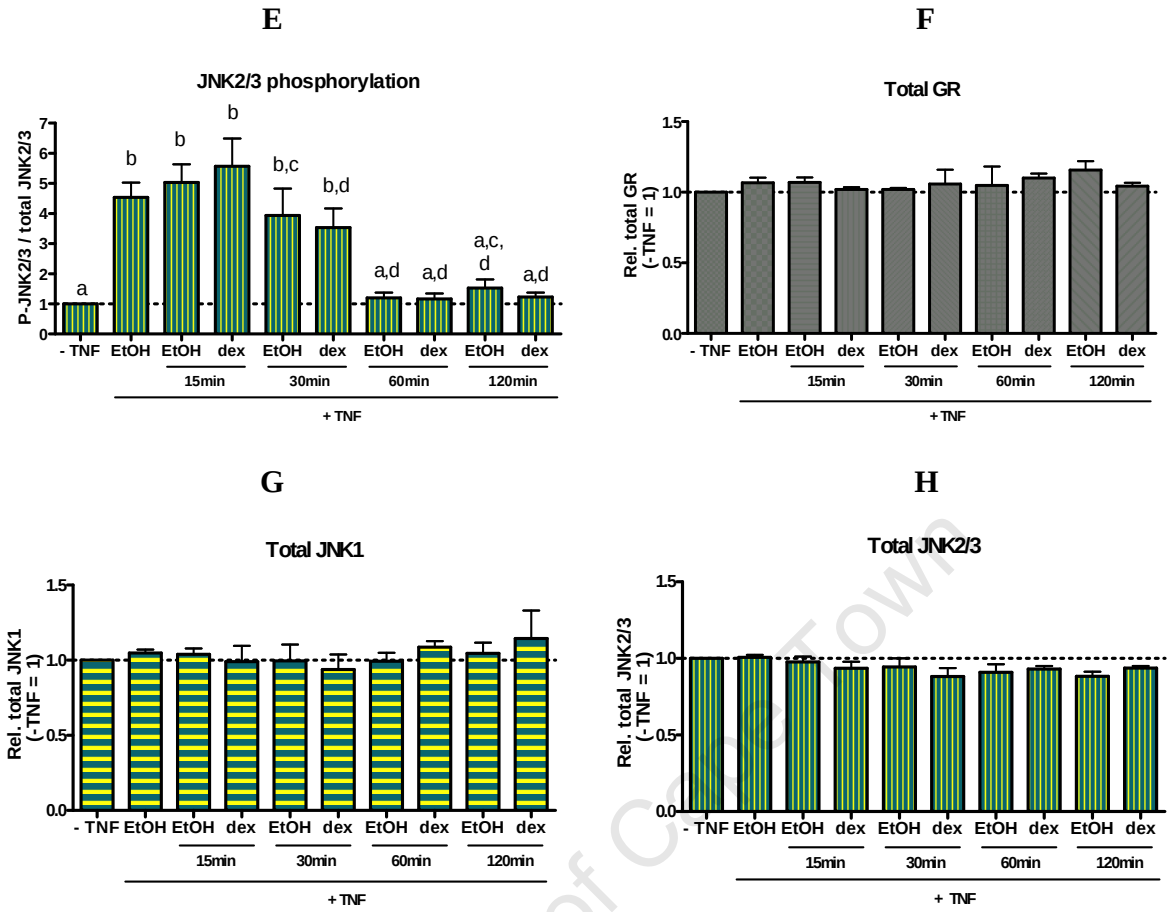



**Figure 5.5 Time-dependent phosphorylation status of the GR at S226 (A) and JNK (B).** COS-1 cells were plated in 6-well plates at a density of $3x10^5$cells/dish and transfected with 3.5 µg HA-hGRwt. After 24 hour incubation, cells were pre-treated with 0.02 µg/ml TNF-α for 15 minutes and then treated with vehicle (EtOH) or 100 nM compound. At the appropriate time, whole cell extracts were prepared and equal amounts of protein (10 µl) were analyzed by Western blotting with either an anti-P-S226 (A) or anti-P-JNK antibody (B). After developing, the blots were stripped and re-probed with total GR or total JNK antibody, respectively. After quantification and normalisation to total GR or JNK, the amount of P-S226 (C) and P-JNK (D and E) were plotted with EtOH –TNF set to one. The total amount of GR (F) or JNK (G and H) is shown with EtOH –TNF set to one. The histograms (C - H) are representative of the average of four independent experiments, plotted as the ave ± SEM, whereas the Western blots (A) and (B) is one representative blot of four independent experiments. Statistical significance is indicated by different lower-case letters, such that all the conditions with the same letter are not statistically significantly different from each other ($P > 0.05$), while those having different letters are statistically significantly different from each other ($P < 0.05$).

As mentioned earlier, phosphorylated/active p38 reportedly phosphorylates the GR on S211 (Miller *et al.*, 2005). Additionally, in the literature there is conflicting data on the role of GCs in p38 phosphorylation, with some results indicating that dex stimulation leads to phosphorylation of p38 in CEM-C7-14 cells (Miller *et al.*, 2005;Zhang *et al.*, 2000a), whereas others show that dex stimulation leads to de-phosphorylation of p38 in the presence of TNF-α in human pulmonary endothelial and A549 cells (Pelaia *et al.*, 2001;Jang *et al.*, 2007). In an attempt to determine what happens in COS-1 cells, the kinetics of p38 and GR S211 phosphorylation was examined (Figs. 5.6 A and B). Results show that the unliganded GR is basally phosphorylated on S211 and that stimulation with TNF-α did not lead to induction of S211 phosphorylation (Fig. 5.6 A). Stimulation with dex results in rapid S211 phosphorylation, which is apparent at 15 minutes and reaches maximum phosphorylation around 60 minutes (Fig. 5.6 C). Phosphorylation of p38 was not significantly induced by TNF-α, and did not increase or decrease significantly over time in the presence of TNF-α or in the presence of dex (Figs. 5.6 B and D). Similarly to the results shown for S226 and JNK, induction with TNF-α did not change the levels of total GR or p38 over the time investigated (Figs. 5.6 E and F). The results obtained with p38 are difficult to interpret, as it is neither phosphorylated nor de-phosphorylated in response to dex or TNF-α. These differences, with respect to the literature (Pelaia *et al.*, 2001;Jang *et al.*, 2007), could possibly be due to cell-specific or methodological differences, since the above mentioned studies were performed in human pulmonary endothelial or A549 cells. Furthermore, in the studies by both Pelaia *et al*. and Jang *et al.* the cells were first stimulated with dex and then treated with TNF-α, wherein results presented in this thesis were obtained by pre-treatment with TNF-α first and then stimulation with dex (Pelaia *et al.*, 2001). However, whether the effect of dex and/or TNF-α on p38 phosphorylation is cell-specific or whether these apparent inconsistencies are due

to the above mentioned methodological differences remains to be tested and warrants further investigation.

Taken together, S211 phosphorylation is rapid, being already visible at 15 minutes after hormone addition and reaches a max at about 60 minutes, whereas the phosphorylation status of p38 does not change in response to TNF-α, dex or within the time period investigated. The phosphorylation status of p38 does not correlate with an increase in GR phosphorylation at S211, which is consistent with a model wherein p38 does not phosphorylate the GR at S211. However, these results were all obtained in the presence of TNF-α and the possibility that dex stimulation results in an increase in p38 phosphorylation in the absence of TNF-α, as has previously been shown (Miller *et al.*, 2005;Zhang *et al.*, 2000a), can not be excluded and warrants further investigation.

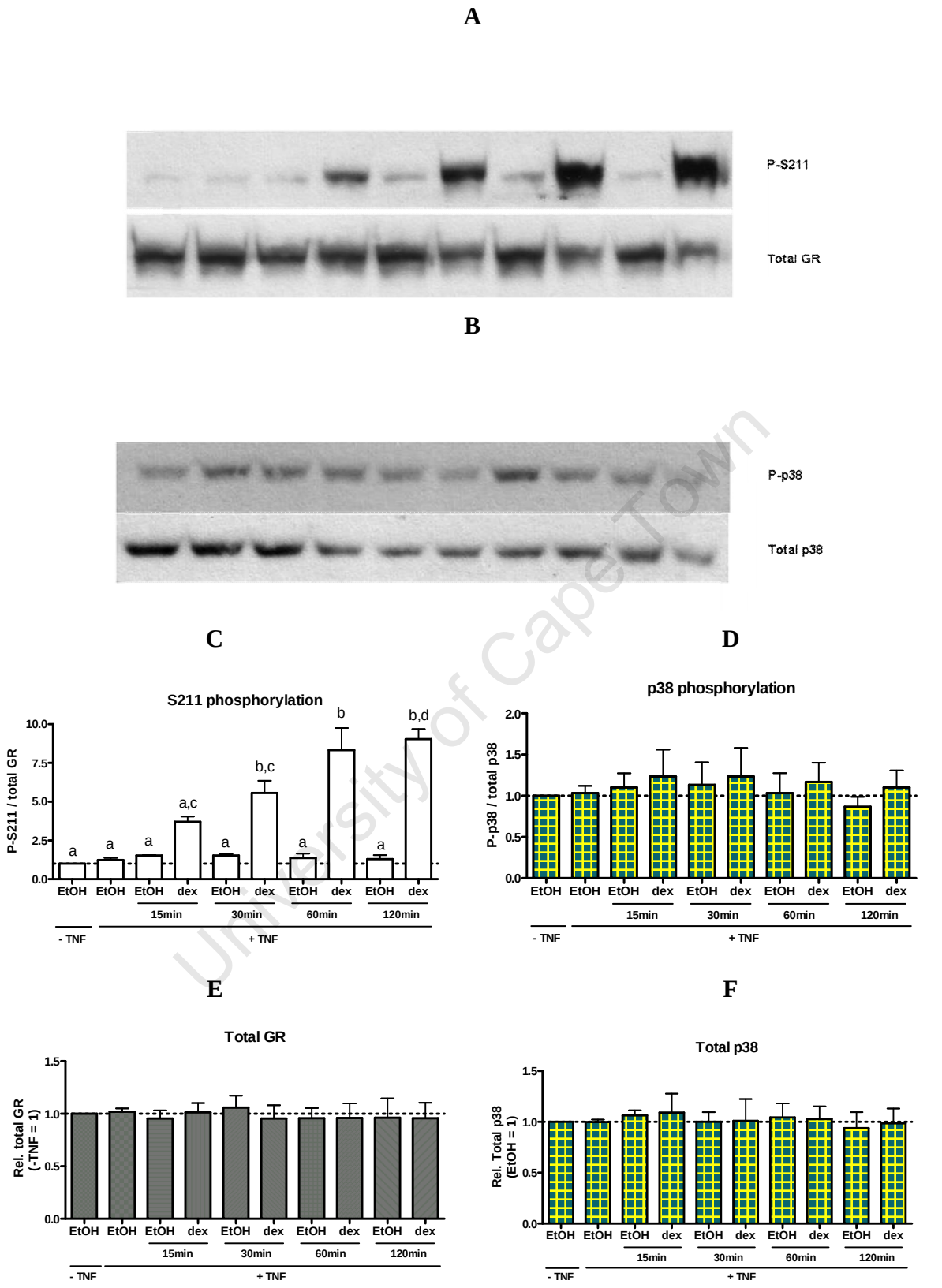





**Figure 5.6 Time-dependent phosphorylation status of the GR at S211 (A) and p38 (B).** COS-1 cells were plated in 6-well plates at a density of $3x10^5$cells/dish and transfected with 3.5µg HA-hGRwt. After

24 hour incubation, cells were pre-treated with 0.02 μg/ml TNF-α for 15minutes and then treated with vehicle (EtOH) or 100 nM compound. At the appropriate time, whole cell extracts were prepared and equal amounts of protein (10 μl) were analyzed by Western blotting with either an anti-P-S211 (A) or anti-P-p38 antibody (B). After developing, the blots were stripped and re-probed with total GR or total p38 antibody, respectively. After quantification and normalisation to total GR (C) or p38 (D), the amount of phosphorylated/total protein in the –TNF sample was set to one. The amount of total GR (E) and p38 (F), as well as histograms (C) and (D) is representative of four independent experiments, plotted as the ave ± SEM, whereas the Western blots (A) and (B) is one representative of four independent experiments. Statistical significance is indicated by different lower-case letters, such that all the conditions with the same letter are not statistically significantly different from each other ($P > 0.05$), while those having different letters are statistically significantly different from each other ($P < 0.05$).

Taken together, the kinetics of all the above results are not consistent with a role for JNK- or p38-mediated GR phosphorylation at S226 or S211.

In order to determine if JNK is phosphorylated/de-phosphorylated in this system, as well as whether this happens in a ligand-selective manner, a JNK CASE assay was performed. This assay is an ELISA based assay, which determines the amount of phosphorylated and total JNK as a percentage of total protein and is more sensitive than the JNK phosphorylation Western blots (as above). The JNK CASE assay was first performed in the absence of TNF-α, but no difference in the amount of phosphorylated JNK was observed in the absence or presence of dex (data not shown). This supports the Western blot results wherein dex stimulation did not activate JNK (Fig. 5.7). COS-1 cells transiently transfected with hGR, were pre-treated with TNF-α for 15 minutes, before treatment with 100 nM test compound for 30 minutes. Thereafter the CASE assay was performed, determining phosphorylated and total JNK, as well as the relative cell number. After normalisation to relative cell number, the amount of phosphorylated JNK over total JNK was determined and plotted. Results in figure 5.7 show that stimulation with TNF-α results in phosphorylated JNK, which is consistent with results obtained on JNK kinetics (Fig.

5.5), as well as other reports in the literature (Pelaia *et al.*, 2001). Furthermore, stimulation with dex statistically significantly decreases the amount of phosphorylated JNK by 60%, which is also consistent with reports in the literature (Pelaia *et al.*, 2001;Jang *et al.*, 2007), but not with results obtained in the JNK kinetics assay, where no statistically significant decrease was seen. However, as mentioned above, the CASE assay is more sensitive than the JNK phosphorylation Western blots. Furthermore, stimulation with the full/partial agonist MPA also resulted in a statistically significant decrease of 58% in phosphorylated JNK, whereas the partial agonist prog, the dissociated ligand RU486 and the antagonist NET also decreased the amount of phosphorylated JNK. However, due to relatively large experimental error, these decreases were not statistically significantly different.



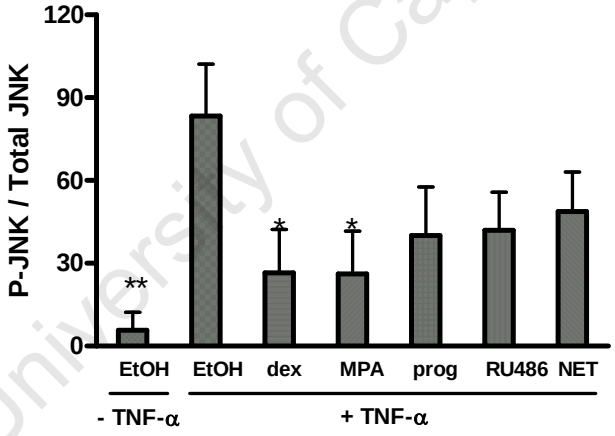



**Figure 5.7 Ligand-dependent de-phosphorylation of JNK.** COS-1 was seeded in a 96-well plate at a density of $5x10^3$ cells/well. The following day the cells were transfected with 0.05 µg HA-hGRwt per well and incubated for 24 hours. After 24 hour incubation, cells were pre-treated with 0.02 µg/ml TNF-α for 15 minutes and then treated with vehicle (EtOH) or 100 nM compound for 1 hour. Thereafter the cells were fixed to the wells and probed with an anti-P-JNK or total JNK antibody, as well as a cell staining buffer to determine the relative cell number in each well. After normalization to relative cell number, the amount of phosphorylated JNK over total JNK was determined and plotted. Values were determined in duplicate and plotted as an average of four independent experiments. Statistical significance, relative to EtOH +TNF, is denoted by * or **, to indicate $P < 0.05$ or $P < 0.01$.

Taken together, these results are consistent with a model whereby full agonists and full/partial agonists result in de-phosphorylation/de-activation of JNK, whereas partial agonists, dissociated GCs and antagonists only slightly (if at all) de-phosphorylate/de-activate JNK.

A careful look at the literature reveals that there are only four papers showing manipulations of GR phosphorylation in intact cells. In a key paper by Garabedian's group (Chen *et al.*, 2008), the authors tried to inhibit overexpressed hGR phosphorylation in U2OS cells at S211 and S226 using a variety of 19 different kinase inhibitors, including CDK, cAMP, tyrosine kinase, MEK1 and MEK2 and general kinase inhibitors, to no avail. This is consistent with results presented in this thesis, where inhibition of JNK, p38 and CDK did not reduce GR phosphorylation at S211 or S226. In the study by Chen *et al*., the only compound that significantly reduced GR phosphorylation at these residues was curcumin or turmeric, the yellow colouring agent found in curry powder. Miller *et al.* showed a decrease in S211 phosphorylation of endogenous hGR in the presence of p38 inhibitors in CEM-C7-14 cells, however they did not normalise to their decreasing total GR levels and did not show whether the p38 inhibitor is active which makes interpretation of the data difficult (Miller *et al.*, 2005). Since results in this thesis show that the p38 inhibitor did not inhibit GR phosphorylation on S211 or of p38 itself, the effect of expressing a dominant negative p38 on GR phosphorylation on S211 and S226 in COS-1 cells overexpressing hGR, as well as mouse LβT2 cells containing endogenous mGR, was examined. However, as with the p38 inhibitor, transfection of the dominant negative p38 construct where the protein was shown to be expressed, did not significantly reduce the amount of GR phosphorylation on S211 or S226. Similarly, overexpression of wt and dominant negative JNK in COS-1 cells overexpressing hGR, as well as mouse LβT2 cells containing endogenous mGR, did not significantly increase or decrease GR phosphorylation on human or mouse equivalent

S211 or S226. In another study the phosphorylation status of the overexpressed hGR at S211 and S203 was manipulated by overexpressing wt and dominant negative CDK5 in HCT116 cells (Kino *et al.*, 2007). However, the same wt and dominant negative CDK5 expression constructs did not cause a significant increase or decrease in GR phosphorylation on S211 or S226 in COS-1 cells overexpressing hGR, or human endocervical END-1 and mouse LβT2 cells containing endogenous hGR or mGR, respectively. Additionally, a general CDK inhibitor (roscovitine) did not significantly reduce S211 or S226 phosphorylation in COS-1, END-1 or LβT2 cells. The 4$^{th}$ paper used a GSKβ inhibitor to block endogenous and expressed hGR S404 phosphorylation in A549 and U2OS cells, stably expressing wt hGR (Galliher-Beckley *et al.*, 2008). These authors showed that some inhibitors can be used successfully, but that GSK is not substantially involved in S211 or S226 phosphorylation. This inhibitor would therefore not have been appropriate in this study.

Taken together, it seems that manipulating GR phosphorylation at S211 and S226 *in vivo*, is difficult. It is possible that an combination of kinases are involved in this process or that the kinases can compensate for each other, e.g. inhibition of one kinase leads to hyper activation of another kinase (as shown in figure 5.2), which in turn phosphorylates the GR at S211 and/or S226. However, transactivation and transrepression results with wt and S211A and S226A, as well as p38 and JNK inhibitors, are consistent with a role of p38 and JNK in phosphorylating the GR at S211 and S226, respectively. Furthermore, kinetics data of phosphorylated JNK, p38 and GR at S211 and S226, are not consistent with a role for JNK or p38, in mediating GR phosphorylation on S226 and/or S211. Additionally, JNK is de-activated/de-phosphorylated in a ligand-selective manner in a timeframe that correlates to increased ligand-selective GR phosphorylation, wherein the ligands that results in the most GR phosphorylation on S226 and

S211, also results in the most JNK dephosphorylation. Taken together, results in this thesis are not consistent with a direct role for JNK in phosphorylating the GR at S226 and/or S211 *in vivo*. It would be tempting to hypothesis that active JNK and p38 can indirectly modulate GR-mediated transcription (given the kinase inhibitor transcription results) in a process that does not involve direct GR phosphorylation at S226 or S211. The GR in turn, could result in ligand-selective deactivation of JNK as a means to prevent over-stimulation. However, further studies are required to elucidate the kinase(s) responsible for GR phosphorylation in COS-1 cells.

# Chapter 6 Ligand-selective GR degradation (Results and Discussion)

Although there are some inconsistencies in the literature, as discussed in the literature review, previous studies are consistent with the hypothesis that after dissociating from the DNA, the liganded-GR is exported to the cytoplasm, where it is degraded through the ubiquitin/proteasome-dependent protein degradation pathway (Bellingham *et al.*, 1992;Liu and DeFranco, 2000;Wallace and Cidlowski, 2001). Furthermore, degradation of the GR has been shown to restrict GR-mediated transactivation and it is hypothesised to occur after transcription and act as a "shut-down mechanism" to prevent over-stimulation (Deroo *et al.*, 2002;Wallace and Cidlowski, 2001;Kinyamu *et al.*, 2008). The down-regulation of GR protein levels in the absence of ligand, as well as in the presence of the GR agonist dex or antagonist RU486, has previously been shown (Cidlowski and Cidlowski, 1981;Hoeck *et al.*, 1989;Wallace and Cidlowski, 2001;Webster *et al.*, 1997). However, these studies used one or two GR ligands only, and the effect of a wide panel of GR ligands on GR mediated down-regulation remains unclear. Additionally, some of these studies did not inhibit transcription, which further complicates the interpretation of the data, since the results measured is most likely the combination of protein degradation, as well as new protein synthesis.



## 6.1 Ligand-selective GR degradation

To test the hypothesis that ligand-selective GR degradation determines the rank order of ligand-selective GR-mediated transcription, it was first determined whether different ligands cause

differential ligand-dependent GR degradation. Therefore, the half-life of the GR in response to a wide panel of ligands was investigated. In order to accurately determine the half-life of the GR, *de novo* protein synthesis needs to be inhibited. This was done by pre-treatment with cycloheximide (CHX), which acts specifically on the 60S subunit of eukaryotic ribosomes, thereby inhibiting protein translation (Clotworthy and Traynor, 2006), followed by measuring the amount of GR protein at various time points, in the absence and presence of ligand. It should be noted that this method is not considered to be the most accurate method in determining the exact half-life of the GR, where pulse-chase experiments are considered to be more accurate. However, in the present work the aim was determine relative GR degradation with a large panel of ligands, rather than the exact half-life of one ligand, which would have been very difficult and costly. In order to determine the optimal concentration of CHX, COS-1 cells were treated with increasing amounts of CHX. It was established that using amounts higher than 1 μM CHX caused cell death (data not shown). To determine if 1 μM CHX was sufficient to inhibit *de novo* protein synthesis, the levels of endogenous tumor suppressor protein 53 (p53), in the presence and absence of CHX, were examined over a period of 72 hours. p53 has a short half-life of 45-60 minutes and is usually expressed at low levels in cells (Utama *et al.*, 2006). COS-1 cells were treated with 1 μM CHX for up to 72 hours. Thereafter, equal amounts of cell extract were separated by SDS-PAGE and Western blotting with anti-p53 was performed. Figure 4.1 shows that in the absence of CHX, the levels of p53 gradually increase over time, whereas in the presence of CHX, no p53 was detectible (Fig. 6.1). The zero hour time point (0h) already included a one hour pre-treatment with CHX. It should be mentioned that ideally a protein loading control should have been included. Nevertheless, this result shows that in the presence of CHX, all the endogenous p53 protein was degraded within the first hour (pre-treatment with

CHX) and there after no new p53 protein was made, indicating that 1 μM CHX is sufficient to stop *de novo* protein synthesis of p53.

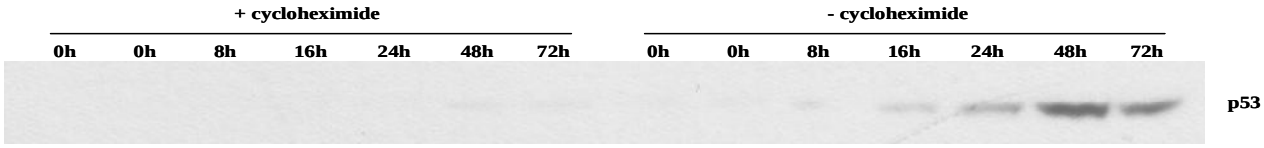
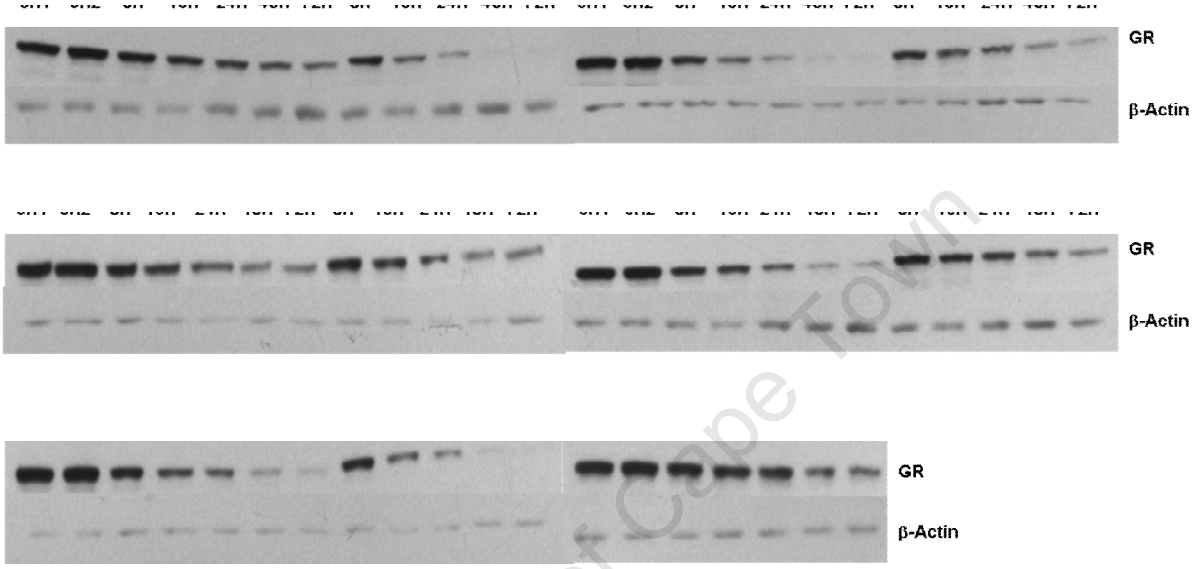



**Figure 6.1 p53 protein levels in the presence and absence of CHX.** COS-1 cells were treated with 1 μM CHX for up to 72 hours. Thereafter, equal amounts of cell extract were separated on SDS-PAGE and Western blotting with anti-p53 was performed.

In order to determine the half-life of the GR in response to the selected panel of ligands (as shown in figure 2.1 in Thesis Rationale, Aims and Hypothesis), COS-1 cells transiently transfected with hGR were pretreated with 1 μM CHX for 1 hour, before stimulation with 10 μM test compound for different times. Results show that the unliganded GR degrades slowly over time and that stimulation with dex dramatically increases the rate of degradation (Fig. 6.2 A). Moreover, all the ligands caused the GR to degrade at different rates. If it is assumed that eventually all the ligands will lead to the total degradation of all the GR proteins (0% GR remaining), the half-life of the GR or the time it takes to degrade 50% of the initial amount of GR, for all the different ligands can be determined (Fig. 6.2 B). In general the full agonists dex, cort and predn had the shortest half-life of 10, 12 and 9 hours, respectively, followed by the full agonists/partial agonists (ald and MPA) and the partial agonist (prog) (Fig. 6.2 C and Table 6.1). Note that the classification of the biological activity of the ligands is based on their efficacy for transcription in COS-1 cells with HA-hGR (see table E5 in addendum E). The dissociated ligand CpdA and the antagonist NET had a half-life of 42 and 37 hours, respectively, which is similar to 44 hours for the unliganded GR. However, within the partial agonist, dissociated and antagonist groups, several outliers were found. For instance, RU486, a dissociated glucocorticoid in this system, has a shorter half-life than the partial agonist progesterone (19 versus 28 hours,

respectively). Similarly, the antagonist NET had a shorter half-life than the dissociated ligand CpdA (37 versus 42 hours, respectively). Furthermore, a similar trend was observed for all the ligands for the relative amounts of GR remaining at the 72 hour time point (Fig. 6.2 D).

**A**




**B**

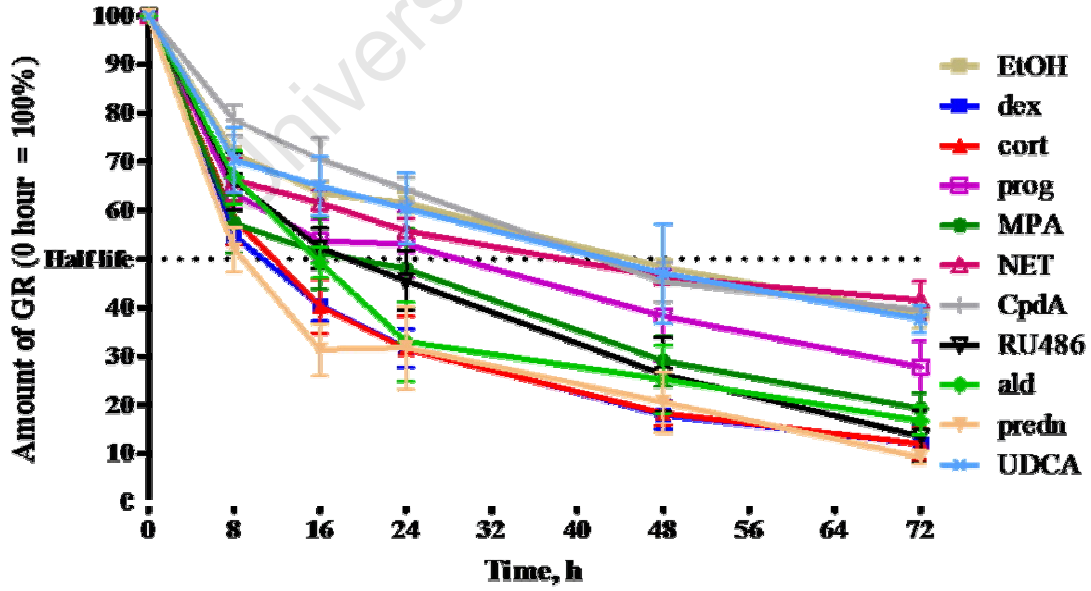



**Figure 6.2 Ligand-dependent GR degradation (half-life) (continues on next page).**

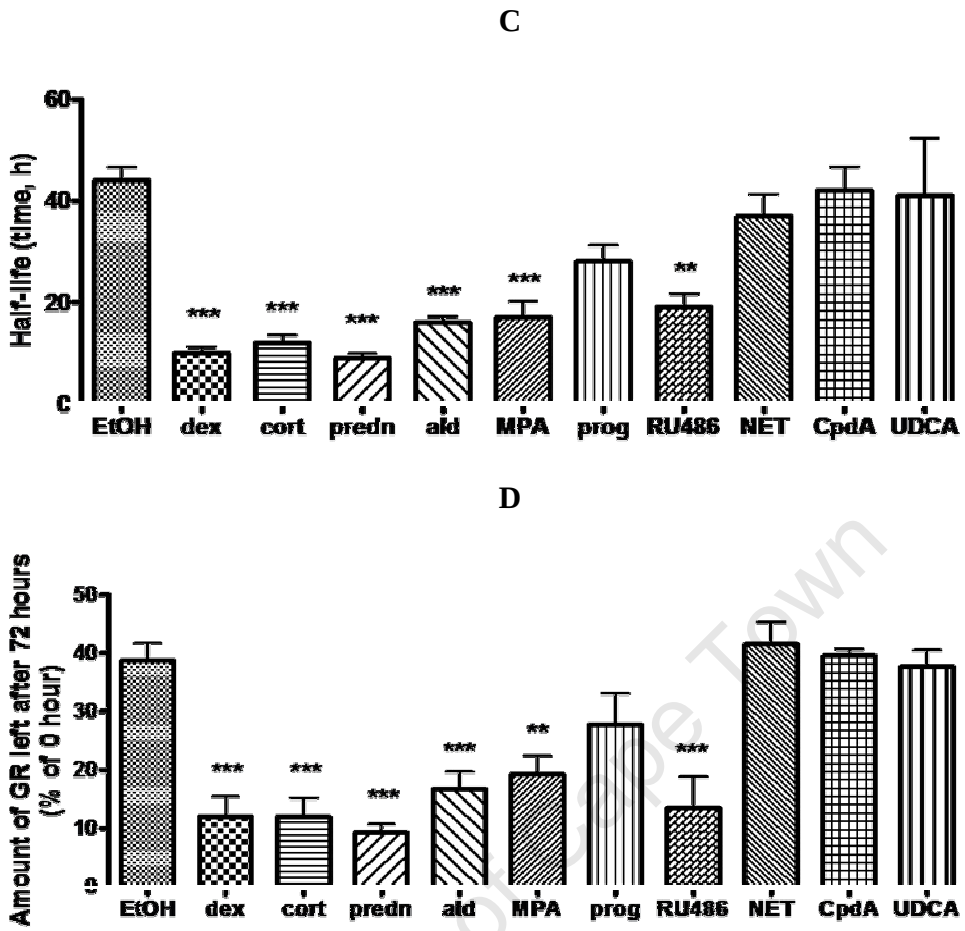



**Figure 6.2 Ligand-dependent GR degradation (half-life).** COS-1 cells were plated in 10-cm dish at a density of $2x10^6$cells/dish and transfected with 10 μg HA-hGRwt. After 24 hour incubation, cells were replated into 6-well plates at a density of $4x10^5$ cells/well. The next day the cells were pre-treated with 1 μM cycloheximide for 1 hour and then treated with vehicle (EtOH) or 10 μM compound. The zero hour time points (0h1 and 0h2) were taken at the time of stimulation (i.e. after the 1 hour pre-treatment with CHX). At the appropriate time, whole cell extracts were prepared and equal amounts of protein (7 μl) were analyzed by Western blotting with anti-GR and β-actin antibody (A). After quantification and normalization to β-actin, the amount of GR present at the two 0 hour time points (0h1 and 0h2) were averaged and was set to 100%. Thereafter the amount of GR present at the other time points was calculated as a percentage of the amount of GR at 0 hour (B). Thereafter the half-life (C) and the amount of GR remaining at 72 hours (D) were calculated and plotted. The data shown in graphs (B), (C) and (D) is representative of four independent experiments, plotted as ave ± SEM, and the Western blots (A) is one representative from four independent experiments. Statistical significance, relative to EtOH, is denoted by ** or ***, to indicate $P < 0.01$ or $P < 0.001$.

**Table 6.1 GR half-life values in response to different compounds, as calculated from figure 4.2.**

As mentioned above, there was a general trend that the more potent ligands for transactivation, had the shortest half-life. In an attempt to investigate the relationship between ligand-selective GR degradation and ligand-selective GR transcription, correlation analysis on the results obtained from the half-life studies, as well as transcription data from this lab (E. Stubsrud, K. Ronacher, table in E5 Addendum E) was performed.

In order to correlate the half-life results with GR transactivation, efficacy and potency for transactivation results obtained in the same cells (COS-1) and with the same GR construct, on the TAT-GRE, were used ((Stubsrud, 2005); Table E5 in addendum E). A very good and statistically significant correlation was obtained for both potency ($R^2 = 0.72$, $p < 0.01$) and efficacy ($R^2 = 0.78$, $p < 0.001$) (Fig. 6.3 A and B, respectively). When the antagonists for transactivation (showing no transactivation) were excluded from the correlation, an even better correlation for both potency and efficacy was obtained ($R^2 > 0.93$, $p < 0.001$). This is done to exclude the possibility that the ligands that do not lead to GR transactivation and/or degradation will give a false positive correlation and suggests that for the ligands that give a transcriptional response, there is a good correlation between GR degradation and transactivation. The correlation analysis indicates a potential relationship between transactivation and degradation of the GR.

In order to determine whether this correlation also holds for transrepression, correlation analysis between half-life and transrepression was also performed. Data obtained from two different transrepression constructs (AP-1 and NFκB) were used in the correlation analysis (K. Ronacher;

Table in E5 in addendum E). As for transactivation, a good and statistically significant correlation between GR half-life and AP-1 potency ($R^2 = 0.69$, $p < 0.01$), as well as efficacy ($R^2 = 0.87$, $p < 0.001$) was observed (Fig. 6.3 C and D, respectively). Furthermore, when the antagonists for transrepression (showing no transrepression) were removed, to prevent a false positive correlation with ligands that do not transrepress, a good and statistically significant correlation between GR half-life and AP-1 potency ($R^2 = 0.62$, $p < 0.001$), as well as efficacy ($R^2 = 0.72$, $p < 0.001$) was still observed. This result indicates that for those ligands that do cause GR-mediated transrepression, there is a good correlation with GR degradation. Additionally, on the NFκB promoter, an even better correlation was found for both transrepression efficacy ($R^2 = 0.96$, $p < 0.0001$) and potency ($R^2 = 0.87$, $p < 0.0001$) (Fig. 6.3 E and F, respectively) for all the ligands, as well as for efficacy ($R^2 = 0.94$, $p < 0.0001$) and potency ($R^2 = 0.83$, $p < 0.001$) when the antagonists (showing no transrepression) were removed.

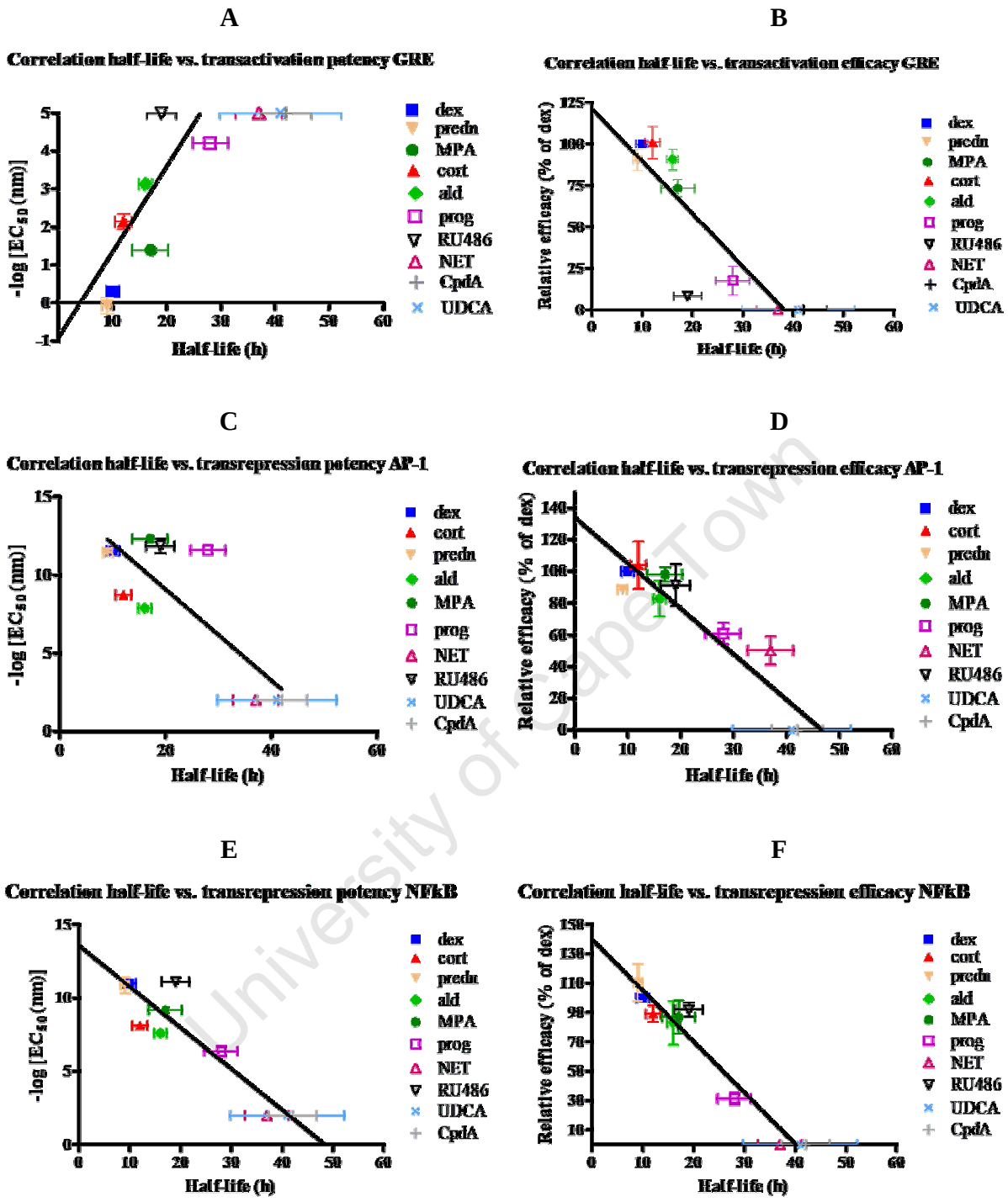



**Figure 6.3 Correlation analysis between half-life and transcription.** Transactivation potency (A) and efficacy (B) was measured using a TAT-GRE reporter-promoter in the same cells and with the same GR construct (HA-hGR) with which the half-life experiments was performed ((Stubsrud, 2005); Table E5 in addendum E). Transrepression potency (C) and efficacy (B) was measured using an AP-1-, as well as an NFκB reporter-promoter constructs (E and F), in the same cells and with the same GR construct that the

half-life experiments were performed (K. Ronacher; Table E5 in addendum E). The efficacies and potencies were calculated as a percentage of dex.

These correlations can be interpreted in different ways. Firstly, ligand-selective GR degradation determines the rank order of ligand-selective transcription, wherein the rate at which the GR is degraded, with any given ligand, will determine the extent of GR-mediated transcription of that particular ligand. Secondly, they can suggest the inverse of the above mentioned scenario, wherein ligand-mediated transcription of the GR, with any given ligand, determines the rate at which the GR is degraded in response to that particular ligand. Additionally, they can imply that both ligand-selective GR degradation rates and transcription activity, are simply a consequence of another ligand-selective "determining step" (e.g. different conformational changes) and that ligand-selective GR transcription and GR degradation are not directly functionally linked, i.e. do not have a direct cause and effect relationship.

Interestingly, when ligand-selective GR degradation was correlated with ligand-selective GR phosphorylation at S226 ($R^2$ = 0.78, $p = 0.0008$) and S211 ($R^2 = 0.89$, $p < 0.0001$), very good statistically significant correlations were found (Fig. 6.4 A and B, respectively). These correlations could simply imply a direct relationship between GR phosphorylation at S211 and/or S226 and GR degradation, wherein the amount of ligand-selective GR phosphorylation will determine the rate at which the GR is degraded, or vice versa. However, this remains to be tested and will be addressed in Chapter 7.

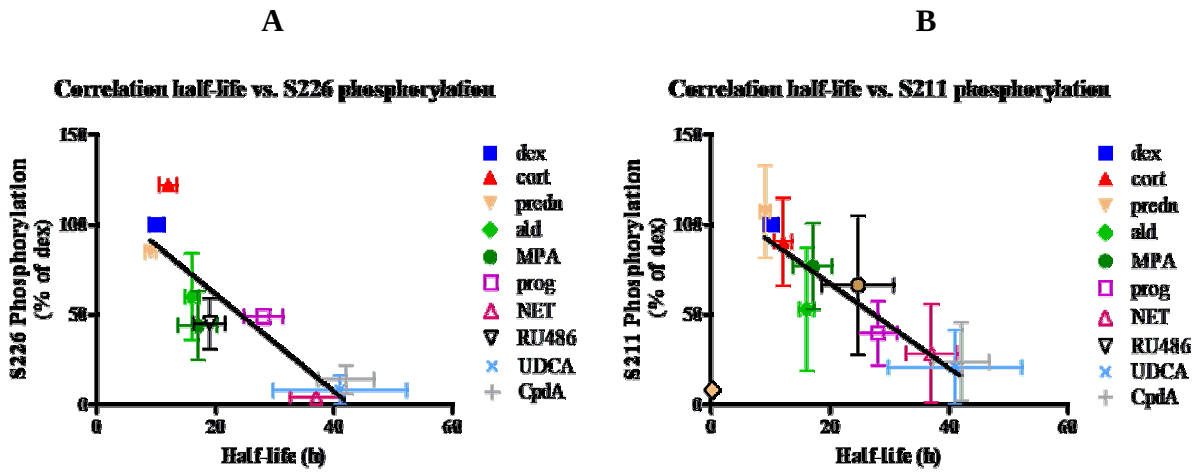



**Figure 6.4 Correlation between ligand-selective GR phosphorylation and half-life.** Ligand-selective GR phosphorylation at S226 (A) and S211 (B) was obtained from Chapter 3 and E. Stubsrud ((Stubsrud, 2005); Table E1 in addendum E) and correlated to ligand-selective GR half-life.

To summarize, results presented in this thesis shows ligand-selective GR degradation in response to a wide panel of GR ligands for the first time, wherein the more potent ligands results in the most rapid GR degradation. Furthermore, correlation analysis showed a strong correlation between GR half-life and GR phosphorylation, as well as ligand-selective transcription on three different promoters. These results indicate a possible relationship between GR ligand-mediated biological response (as measured by transactivation and transrepression on the three different promoters) and GR ligand-mediated protein degradation for both direct DNA binding (GRE) and tethering (AP-1 and NFκB) models. In order to further investigate this possible relationship, specific inhibitors of transcription or proteasome mediated degradation were used.

## 6.2 Relationship between GR half-life and transcription

In order to further investigate the relationship between transcription and GR degradation, the effect of MG132 (which blocks proteasomal degradation) and α-amanitin (which blocks RNA polymerase II transcription) on GR transcription and degradation, respectively, was determined.

### 6.2.1 Inhibition of degradation

Proteasomal inhibition has been shown to increase GR mediated transcription on stable or transiently transfected MMTV promoters in human breast cancer cells (Deroo *et al.*, 2002;Wallace and Cidlowski, 2001). Additionally, it has recently been shown with microarray analysis that proteasomal inhibition results in simultaneous up- and down-regulation of GR target genes, depending on the specific gene (Kinyamu *et al.*, 2008). In order to investigate what happens in this system, MG132 was used to block proteasomal degradation of the GR and the effect thereof on transcription was determined. In the absence of MG132, the GR is degraded in a dex-dependent manner, whereas using increasing amounts of MG132, blocks dex-mediated GR degradation (Fig. 6.5 A). After quantification and normalization of several experiments, it was determined that after 24 hours without MG132, dex resulted in a 70% decrease in GR protein levels relative to EtOH (Fig. 6.5 B). This result is consistent with GR half-life experiments that showed that the half-life of dex-bound GR is 9 hours. In the presence of MG132, even at the lower 5 μM MG132 concentration, no statistically significant dex-mediated GR degradation was detected, showing that GR degradation was inhibited (Fig. 6.5 B) and that GR degradation is mediated by proteasomal degradation.

After verifying that GR degradation was inhibited, the effect of MG132 on GR-mediated transcription was investigated. With increasing amounts of MG132, ligand-selective GR transactivation efficacy on the TAT-GRE reporter-promoter increased in a dose-dependent manner (Fig. 6.5 C). Unfortunately, the error in this experiment was large and no statistical significance could therefore be established. It is interesting that the transactivation efficacy of MPA did not increase as much as that of dex, indicating that the relationship between GR degradation and transcription is possibly ligand-selective. This is in agreement with results obtained on the MMTV promoter, where proteasomal inhibition increased dex-mediated transactivation, but decreased corticosterone-mediated transactivation (Stavreva *et al.*, 2004).

When looking at the effect of MG132 on a β–gal reporter-construct, a constitutively expressed promoter-reporter construct, inhibition of proteasomal degradation did not have any effect (Fig. 6.5 D). This is possibly due to the β–gal protein having a long half-life and that it is not degraded during the time of this experiment. Nevertheless, this result indicates that the increase in GR transactivation is not due to MG132 increasing transcription in general, e.g. resulting in more active RNA polymerase II.

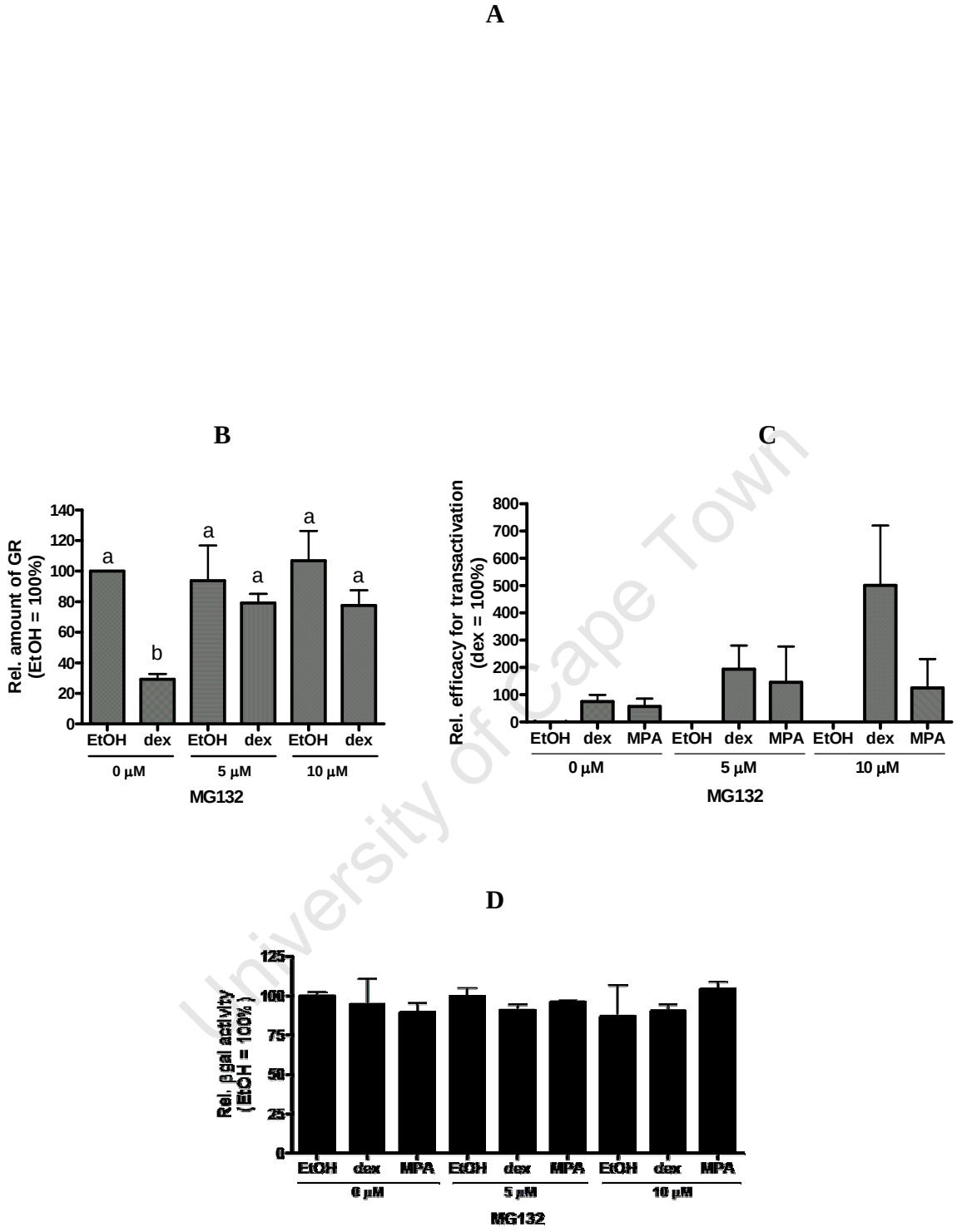





**Figure 6.5 The effect of blocking degradation on dex-mediated GR transactivation**. COS-1 cells were plated in 24-well plates at a density of $2.5x10^4$ and transfected with 0.125 µg HA-hGRwt and 0.047 µg TAT-GRE reporter-promoter and 0.012 µg β-gal construct per well. After 24 hours, the cells were pre-treated with MG132 for 4 hours before stimulation with vehicle (EtOH) or 100 nM Dex for an additional 24 hours. Thereafter luciferase activity was assayed and Bradford protein determination was performed to

normalize for equal protein concentrations in each well and set to 100% for dex in the absence of MG132 (C) and β-gal activity was measured (D). Equal amounts of samples were loaded onto an 8% SDS-PAGE and Western blotting was performed probing with an antibody detecting total amounts of GR and a β-actin antibody as loading control (A). After quantification and normalisation to β-actin, the amount of GR present in the EtOH 0 μM MG132 was set to 100% and the other samples plotted as percentage thereof (B). Graphs in (B), (C) and (D) are the average of four independent experiments with each value in triplicates plotted as means ± SEM, while the Western blot (A) shows one representative of four independent experiments. Statistical significance is indicated by different lower-case letters, such that all the conditions with the same letter are not statistically significantly different from each other ($P > 0.05$), while those having different letters are statistically significantly different from each other ($P < 0.05$).

Taken together, these results show that the inhibition of GR degradation results in an increase in ligand-mediated GR transactivation on the TAT-GRE reporter-promoter in COS-1 cells. This is consistent with other reports in the literature, showing that inhibiting degradation results in increased GR-mediated transactivation (Deroo *et al.*, 2002;Wallace and Cidlowski, 2001;Garside *et al.*, 2006), which suggests that GR degradation happens after GR mediated transcription (since blocking degradation still allows transactivation) and acts as a "shut-down" mechanism to prevent over stimulation. However this study, as well as the above mentioned studies, only looked at the effect of inhibiting proteasomal degradation on transactivation, but did not look at transrepression. It would be interesting to determine the effect of proteasomal inhibition on GR-mediated transrepression. An interesting study by Kinyamu *et al.* recently showed in microarray analysis that proteasomal inhibition results in both up- and down-regulation of GR-mediated transcription in a promoter-specific manner (Kinyamu *et al.*, 2008). It would be tempting to speculate that the genes found by Kinyamu *et al.*, to be down-regulated in response to proteasomal degradation, are genes regulated by GR-mediated transrepression. However, this remains to be tested.

Interestingly, results in figure 6.5 C show that inhibition of ligand-selective GR proteasomal degradation, still resulted in ligand-selective GR-mediated transactivation, wherein dex had a higher efficacy than MPA. This suggests that ligand-selective GR degradation does not determine the rank order of ligand-selective GR transactivation. However, inhibition of GR proteasomal degradation resulted in an increase in dex-mediated, but not MPA-mediated transactivation. This result is very interesting and indicates that although ligand-selective GR degradation does not determine the rank order of ligand-selective transactivation, that GR degradation influences transactivation of the GR in a ligand-specific manner, wherein ligand-induced GR degradation decreases transactivation with some ligands, but not all GR ligands. However, this study only investigated dex and MPA, and experiments with more ligands would have to be performed to get a better understanding thereof. This still leaves unanswered the question as to whether or not transcription is required for GR degradation.



### 6.2.2 Inhibition of transcription

Since α-Amanitin inhibits eukaryotic RNA polymerase II and III (Schultz and Hall, 1976), it was used to inhibit transcription in this study. Before investigating whether or not transcription is needed for GR protein turnover, a suitable concentration where α-amanitin inhibits transcription was first established. This was done by performing a transactivation reporter-promoter assay using increasing concentrations of α-amanitin. In the absence of α-amanitin, both dex and MPA induced transactivation in a ligand-dependent manner (Fig. 6.6 A). Increasing the amount of α-amanitin resulted in a decrease of both dex- and MPA-mediated GR transactivation. At the highest concentration of α-amanitin, there was still ligand-dependent transactivation (above

background), indicating that even though transcription was inhibited, it was not blocked completely. However, this ligand-dependent transactivation, at the highest concentration of α-amanitin, was not statistically significantly different from vehicle. Furthermore, as with GR-mediated transactivation, it can clearly be seen that with increasing amounts of α-amanitin the amount of β−gal activity decreases (Fig. 6.6 B). At the highest concentration of α-amanitin there was still some βgal activity, however this could possibly be due to β−gal protein being synthesized during the 24 hours post-transfection (before the addition of α-amanitin) and not being degraded due to a long half-life. It should also be mentioned that adding α-amanitin at concentrations higher than 2.5 μg/ml caused the cells to die (data not shown).

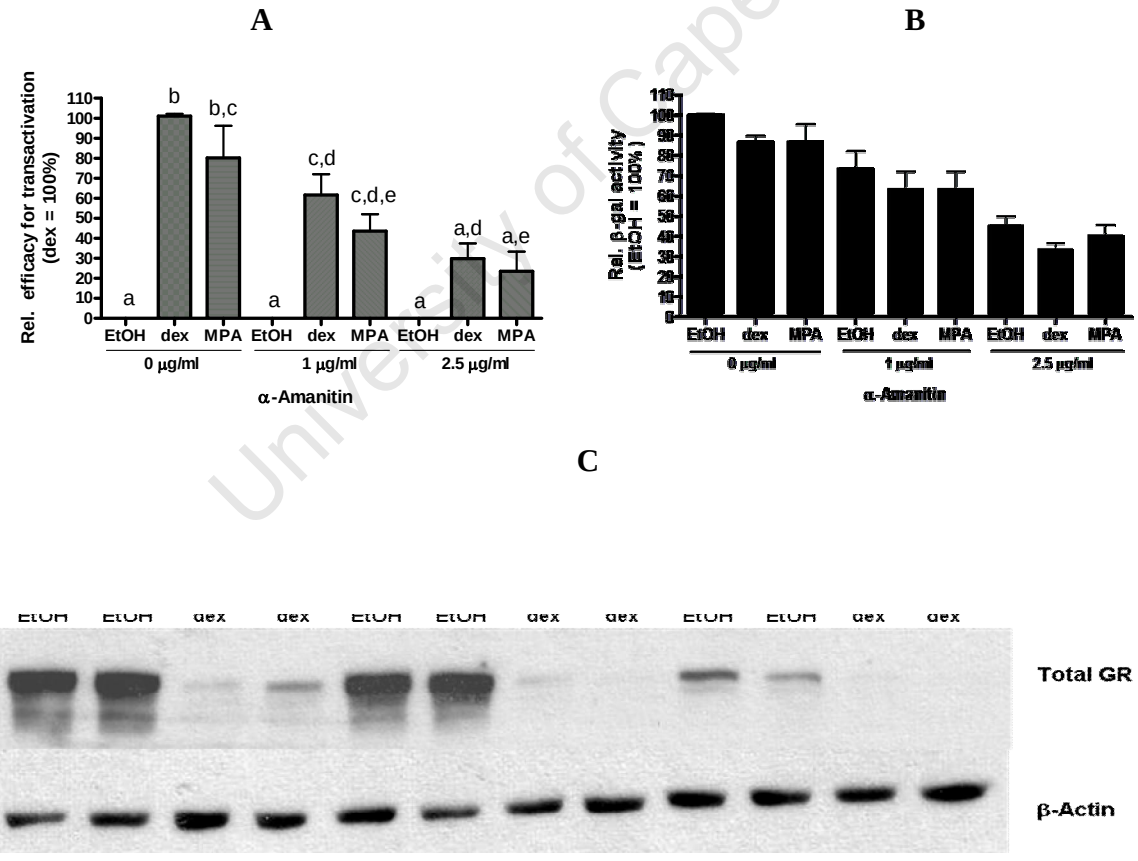



**Figure 6.6 The effect of inhibition of GR-mediated transcription on GR degradation (continues on next page).**

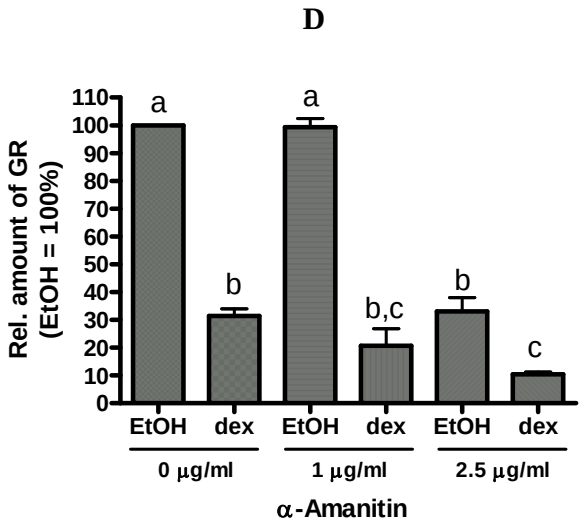



**Figure 6.6 The effect of inhibition of GR-mediated transcription on GR degradation.** COS-1 cells were plated in 24-well plates at a density of $2.5x10^4$ and transfected with 0.125 μg HA-hGRwt and 0.047 μg TAT-GRE reporter-promoter and 0.012 μg β-gal construct per well. After 24 hours, the cells were pre-treated with α-amanitin for 24 hours before stimulation with vehicle (EtOH) or 100 nM Dex for an additional 24 hours. Thereafter luciferase activity was assayed and Bradford protein determination was performed to normalize for equal protein concentrations in each well (A). After β-gal activity was measured (B), equal amounts of samples were loaded onto an 8% SDS-PAGE and Western blotting was performed probing with an antibody detecting total amounts of GR and an β-actin antibody as loading control (C). After quantification and normalisation to β-actin, the amount of GR present in EtOH 0 μg/ml α-amanitin was set to 100% and the other samples plotted as a percentage thereof (D). Graphs (A), (B) and (D) are the average of four independent experiments with each triplicate value plotted as the mean ± SEM, while the Western blot (C) shows one representative of four independent experiments. Statistical significance is indicated by different lower-case letters, such that all the conditions with the same letter are not statistically significantly different from each other ($P > 0.05$), while those having different letters are statistically significantly different from each other ($P < 0.05$).

Having shown that α-amanitin inhibits GR-mediated transactivation (Fig. 6.6 A) and β–gal expression (Fig. 6.6 B), in a dose-dependent manner, the effects of α-amanitin on dex-mediated GR degradation were examined (Fig. 6.6 C). In the absence of α-amanitin, dex-mediated GR degradation was observed, with the amount of GR protein levels decreasing by 70% in response to dex after 24 hours (Fig. 6.6 D). While pre-treatment with 1 μg/ml α-amanitin reduced dex-

mediated transactivation by 40% and β−gal activity by 30% (Figs. 4.6 A and B), it did not change the amount of dex-mediated GR degradation (~80% decrease in GR protein levels in response to dex). With the highest amount of α-amanitin, where no statistically significant transactivation was observed, the amount of dex-mediated GR degradation was only reduced by ~20%. However, the total amount of GR, in the absence of ligand, was also significantly less (~65% decrease) than without α-amanitin or with 1 μg/ml α-amanitin. The change in the amount of unliganded GR for 2.5 μg/ml α-amanitin versus untreated, is likely due to an additive effect of the degradation of the unliganded GR and the complete inhibition of transactivation, thereby not allowing the synthesis of new GR mRNA and hence protein translation.

Since the 40% reduction in dex-mediated GR transactivation, with 1 μg/ml α-amanitin, does not result in a loss of dex-mediated GR degradation, the results indicate that GR transactivation is not required for dex-mediated GR degradation. Furthermore, if ligand-mediated transactivation was required for ligand-mediated GR degradation, then the highest amount of α-amanitin where no statistically significant transactivation was observed should not show any dex-mediated GR degradation, which is not the case. Taken together, these results show that GR transactivation is not required for unliganded or ligand-mediated GR degradation.

The half-life results are in agreement with results obtained for dex with the mGR and rGR in COS-1 and rat hepatoma cells, which showed that dex treatment reduced the half-life of the mGR and rGR to 9 and 11 hours, respectively (Webster *et al.*, 1997;Dong *et al.*, 1988). However, results in this thesis show for the first time the half-life of the hGR in response to a wide panel of ligands and show a direct correlation with ligand-selective GR transcription, wherein the more potent GR ligands (as measured by biological response on three different promoters) results in

the quickest GR degradation. By inhibiting ligand-selective GR degradation, it was shown that GR degradation 'restricts' dex-mediated transcription. This is consistent with results in the literature that showed inhibition of proteasomal degradation, increases dex-mediated transcription (Deroo *et al.*, 2002;Wallace and Cidlowski, 2001;Garside *et al.*, 2006). Furthermore, the increase in transcription is not due to the proteasome inhibitor influencing the basal transcription machinery, as has also been shown by others (Kinyamu and Archer, 2007;Dantuma *et al.*, 2006), thereby increasing transcription, since transcription of a constitutively expressed β-gal reporter did not increase with proteasomal inhibition. These results suggest that GR degradation limits GR-mediated transcription, thereby acting as a "shut-down" mechanism to prevent over-stimulation of GR-mediated transcription.

Results in this thesis showed for the first time that when dex-mediated GR transactivation was inhibited, dex-mediated GR degradation was still observed, suggesting that transcription is not required for dex-mediated GR degradation, suggesting that GR degradation can occur before or after GR-mediated transcription. Furthermore, the half-life results with the unliganded GR, as well as the GR degradation results obtained when transcription was inhibited showed that the unliganded GR is also degraded. Since the unliganded GR is not transcriptionally active and does not translocate to the nucleus, these results suggest that GR degradation can also occur in the cytoplasm. This is consistent with an earlier study showing that GR degradation can occur in both the cytoplasm and nucleus (Liu and DeFranco, 2000). Taken together, the results obtained with dex, are consistent with a model, wherein dex-stimulated GR can degrade before of after initiating transcription and acts as a fine-tuning mechanism that restricts GR-mediated transcription. This model is unique for the GR, since proteasomal degradation of the PR, AR and ER has been shown to be required for transcription, wherein proteasomal inhibition results in a decrease in transcription (Lange *et al.*, 2000;Sheflin *et al.*, 2000).

In the results presented in this thesis, dex and MPA had similar efficacies (when degradation was not inhibited), making it difficult to determine if GR degradation determines the rank order of ligand-selective GR transcription. When GR degradation was inhibited, dex had a higher efficacy than MPA, suggesting that ligand-selective GR degradation does not determine the rank order of ligand-selective transactivation. In addition, inhibition of dex-mediated transactivation, still resulted in dex-mediated GR degradation, suggesting that the amount of transactivation does not determine the rate of GR degradation. However, further experiments with the whole panel of GR ligands are needed to confirm this hypothesis and to determine if it holds for all ligands. Nevertheless, inhibition of GR proteasomal degradation resulted in a larger fold-increase in dex-mediated, than MPA-mediated transactivation. This result is very interesting and indicates that although ligand-selective GR degradation does not determine the rank order of ligand-selective transactivation, that GR degradation influences transactivation of the GR in a ligand-specific manner, wherein ligand-induced GR degradation decreases transactivation with some ligands, but not all GR ligands. This suggests mechanistic differences in ligand-selective GR transactivation and degradation. However, this study only looked at dex and MPA, and experiments with more ligands would have to be performed to get a better understanding thereof.

# Chapter 7 Role of agonist-induced GR Phosphorylation in GR mechanism of action (Results and Discussion)

Phosphorylation of the hGR on S211 was previously shown to be required for maximal agonist-induced transactivation efficacy on a TAT-GRE reporter-promoter construct ((Stubsrud, 2005); table E2 in addendum E). Results in this thesis, showed that phosphorylation of the hGR on S226 inhibits maximal agonist-induced transactivation efficacy on a TAT-GRE reporter-promoter construct (Fig. 4.8 in Chapter 4). Similarly, on a MMTV reporter-promoter, phosphorylation at S211 was shown to be required for, whereas phosphorylation at S226 inhibited agonist-induced maximal transactivation efficacy (Fig. 4.10 in Chapter 4). Furthermore, phosphorylation at S203/S211/S226 was shown to be required for agonist-induced GR-mediated transactivation on a MMTV reporter-promoter (Fig. 4.10 in Chapter 4), indicating that phosphorylation plays an important role in the biological function of the GR. The specific aim of this section is to determine the role of agonist-induced GR phosphorylation at S211 and S226.



## 7.1 Relationship between phosphorylation and transcription and/or half-life

As mentioned above, GR phosphorylation was shown to be required for (S211), or inhibitory of (S226), maximal transactivational efficacy for transcription (Fig. 4.10 in Chapter 4). These results support the hypothesis that there is a functional link between GR phosphorylation and transactivation efficacy. However, whether GR phosphorylation is influenced by, or requires GR-mediated transcription, is unknown. Additionally, GR phosphorylation was shown to correlate

with GR degradation for all the ligands (Fig. 6.4 in Chapter 6), indicating a link between GR phosphorylation and degradation. In the literature, there is conflicting evidence on the role of GR phosphorylation on GR degradation (Hoeck *et al.*, 1989;Wallace and Cidlowski, 2001). However, whether GR phosphorylation is influenced by GR degradation, is unknown. In order to investigate in more detail the relationship between GR phosphorylation and transcription, as well as GR phosphorylation and half-life or degradation, and to determine whether GR phosphorylation is influenced by, or requires GR-mediated transcription and/or degradation, α-amanitin and MG132 was used to inhibit transcription and proteasomal degradation, respectively. COS-1 cells, transiently transfected with hGR wt, were pre-treated with 2.5 μg/ml α-amanitin or 10 μM MG132 (concentrations established to inhibit transcription and degradation, see figures 6.5 and 6.6 in Chapter 6) for 24 or 4 hours, respectively, before stimulation with 100 nM dex for 1 hour. Equal amounts of cell extract were separated on SDS-PAGE and Western blotting was performed probing for either anti-P-S226 (Fig. 7.1 A) or anti-P-S211 (Fig. 7.1 B). Interestingly, the amount of basal S226 phosphorylation increased when transcription was inhibited and increased even further when degradation was blocked. This result indicates that basal S226 phosphorylation does not require, but is influenced by GR-mediated transcription and/or degradation, wherein transcription and/or degradation restrict basal GR phosphorylation at S226. However, the amount of dex-induced S226 phosphorylation decreased slightly when transcription or degradation were inhibited, resulting in no statistically significant difference between basal and dex-induced S226 phosphorylation (Fig. 7.1 C). This result indicates that GR-mediated transcription and/or degradation is required for agonist-induced GR phosphorylation levels at S226.

In contrast, the amount of basal S211 phosphorylation did not increase when either transcription or degradation was inhibited (Fig. 7.1 D). Furthermore, inhibition of transcription did not influence dex-mediated S211 phosphorylation. This indicates the GR-mediated transcription is not required for agonist-induced GR phosphorylation at S211. However, when degradation was blocked, the amount of dex-induced S211 phosphorylation decreased in a statistically significant manner, indicating that GR degradation is required for dex-mediated GR phosphorylation at S211. During the one hour incubation with dex, neither α-amanitin nor MG132 resulted in a change in the total amount of GR protein levels (Fig. 7.1 E), showing that the changes in the amount of GR phosphorylation are not due to changes in total GR levels.

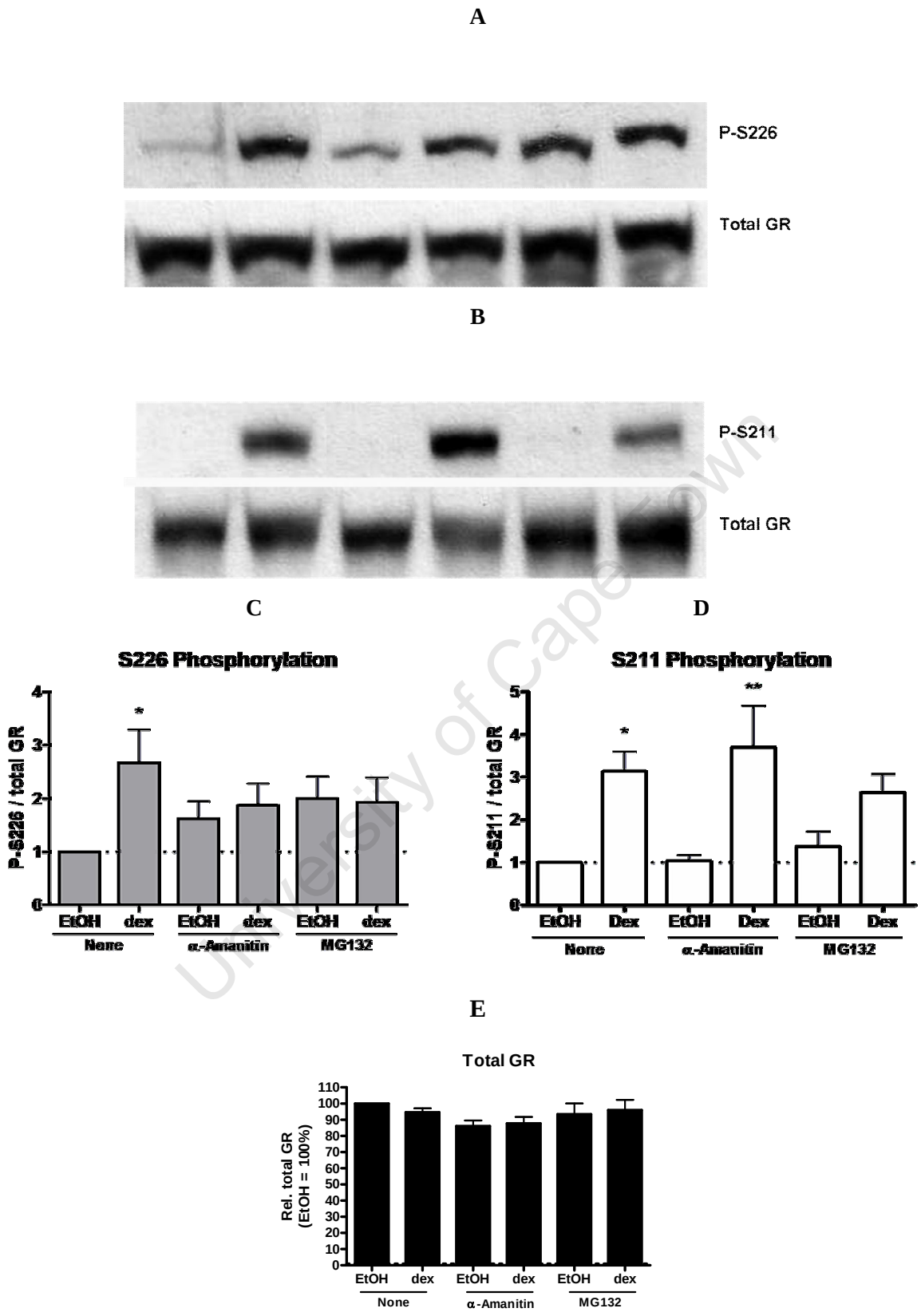





**Figure 7.1 Proteasomal degradation and RNA polymerase II activity are differentially required for agonist-mediated hGR phosphorylation at S226 and S211.** To test the effect of α-amanitin and

MG132 on GR phosphorylation, COS-1 cells transiently transfected with 1 μg HA-hGRwt were pre-treated with either 10 μM MG132 for 4 hours or 2.5 μg/ml α-amanitin for 24 hours before stimulation with vehicle (EtOH) or 100 nM Dex for an additional 1 hour. Equal amounts of samples were loaded onto an 8% SDS-PAGE and Western blotting was performed, probing with either an anti-P-S226 (A) or anti-P-S211 antibody (B). After developing, the blots were stripped and re-probed with total GR (E). The amount of phosphorylated S226 and S211 was normalised to total GR and plotted as the average of four independent experiments ± SEM (C) and (D). The histograms in C, D, and E also show pooled results from four independent experiments, plotted as means ± SEM, while Western blots in A and B each show results of one representative experiment. Statistical significance, relative to EtOH without inhibitor, is denoted by * or **, to indicate $P < 0.05$ or $P < 0.01$.



However, caution should be exercised when interpreting this data, since α-amanitin and MG132 are not specific for the GR. MG132 is known to activate JNK, the kinase reported to phosphorylate the GR at S226 (Meriin *et al.*, 1998). In order to determine whether α-amanitin and MG132 resulted in activation or inhibition of the kinases, JNK and p38, reportedly involved in GR phosphorylation at S226 and S211, respectively, the same cell extracts (as above) were separated on SDS-PAGE and Western blotting was performed probing for P-JNK (Fig. 7.2 A) or P-p38 (Fig. 7.2 B). According to the literature, TNF-α is known to activate JNK and was therefore included in this study as a positive control (Pelaia *et al.*, 2001). Results show that TNF-α stimulation induces phosphorylation of JNK1 and JNK2/3, which is inhibited by stimulation with dex (Figs. 7.2 C and D). Although these differences are not statistically significant, the results are consistent with other reports in the literature (Pelaia *et al.*, 2001;Zhou *et al.*, 2007), as well as the results found in the CASE assay (Fig. 5.7 in Chapter 5). Furthermore, stimulation with MG132 induced phosphorylation of JNK1 and JNK2/3, which is consistent with Meriin *et al*., whereas stimulation with α-amanitin did not induce or inhibit JNK1 and JNK2/3 phosphorylation (Meriin *et al.*, 1998). Interestingly, contrary to TNF-α induced JNK phosphorylation, stimulation with dex did not seem to inhibit MG132-induced phosphorylation of

neither JNK1 nor JNK2/3. This suggests the possibility of different mechanisms of activation of JNK by TNF-α versus MG132. Phosphorylation of p38 was also slightly increased upon TNF-α stimulation and increased very strongly upon stimulation with MG132 (Fig. 7.2 E). Furthermore, stimulation with dex did not inhibit TNF-α– or MG132- induced p38 phosphorylation. The total levels of JNK1 and JNK2/3 protein did not change upon stimulation with TNF-α, α-amanitin or MG132 (Figs. 7.2 F and G), whereas the total levels of p38 protein decreased slightly (Fig. 7.2 H). The decrease in total p38 does not change the above mentioned p38 phosphorylation results, as it was normalized to the total p38 levels.

If JNK phosphorylates the GR at S226, then the increase in phosphorylated or active JNK upon MG132 stimulation, could explain the increase in basal S226 phosphorylation with MG132 stimulation (Fig. 7.1 C). However, if this is the case, then the amount of dex-mediated S226 phosphorylation should also increase with MG132 stimulation. One possible explanation for the lack of an increase in dex-mediated S226 phosphorylation, is that dex-stimulation leads to "saturation" of S226 phosphorylation and whether or not there is more active JNK, with additional MG132 stimulation, the amount of S226 phosphorylation can not increase any further. Alternatively, degradation of a phosphatase could also be inhibited, thereby resulting in increased phosphatase protein levels and slightly less dex-mediated S226 phosphorylation, however this remains to be tested. It is interesting to note, that the increase in basal S226 phosphorylation was not due to an increase in kinase protein levels, but rather activation of the kinase (JNK) by MG132 and it would be interesting to determine if MG132 also results in activation of phosphatases or if it simply results in increased phosphatase protein levels. Interestingly, α-amanitin does not seem to increase the amount of phosphorylated/active JNK, but still results in an increased basal S226 phosphorylation, which is not accompanied by an increased amount of

total GR or total JNK. One possible explanation for the increase in basal S226 phosphorylation, is that α-amanitin blocks transcription of the phosphatase responsible for de-phosphorylating the GR at S226 and that the result seen is not due to increased phosphorylation of S226, but rather a "lack" of de-phosphorylation. However, stimulation with α-amanitin increased the amount of total mitogen-activated protein kinase phosphatase 1 (MKP-1) (data not shown). Whether MKP-1 plays a role in GR de-phosphorylation and/or whether α-amanitin inhibits transcription of another phosphatase responsible for de-phosphorylating S226, has yet to be tested.

Stimulation with MG132 significantly increased p38 phosphorylation and decreased dex-mediated S211 phosphorylation. This result is inconsistent with a model in which p38 phosphorylates the GR at S211 in COS-1 cells, as reported in the literature for different cells (Rogatsky *et al.*, 1998b), but is consistent with data presented in this thesis (Figs. 5.1 and 5.3 in Chapter 5) showing that p38 is not responsible for S211 phosphorylation in COS-1 cells. One possible explanation for the apparent inconsistency in the decreased S211 and increased p38 phosphorylation is that other kinases, such as CDKs plays a role in S211 phosphorylation. However, overexpression of wt and dominant negative CDK5 constructs, as well as the use of a general CDK inhibitor (roscovitine), did not increase or decrease S211 or S226 phosphorylation in three different cell lines (See figure 5.7 in Chapter 5). Additionally, as discussed above for the decrease in dex-mediated S226 phosphorylation upon MG132 treatment, degradation of a phosphatase, responsible for dephosphorylation of the GR at S211, can be blocked. However this remains to be tested.

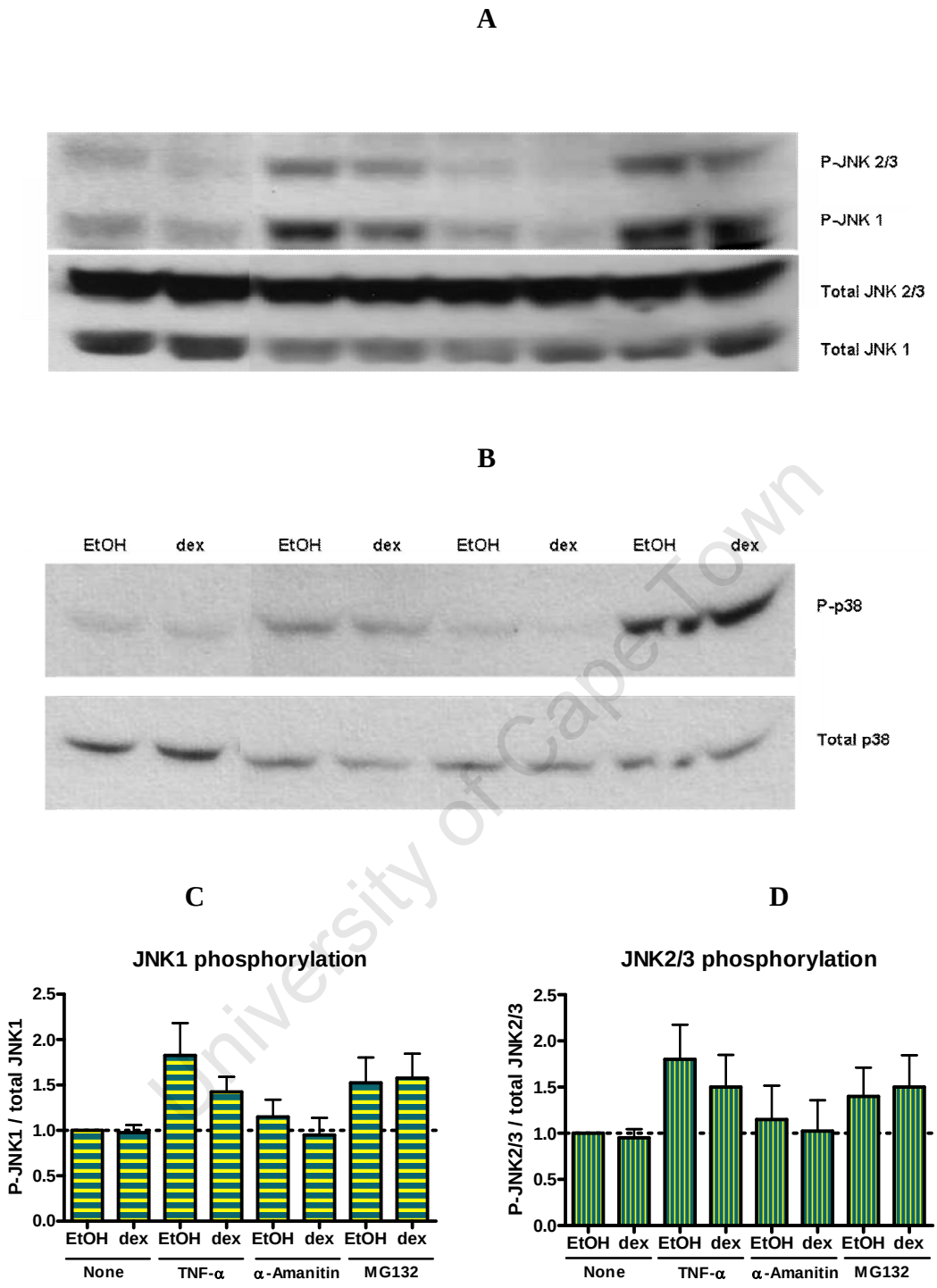





**Figure 7.2 Proteasomal degradation and RNA polymerase II activity influences phosphorylation of JNK and p38 (continues on next page).**

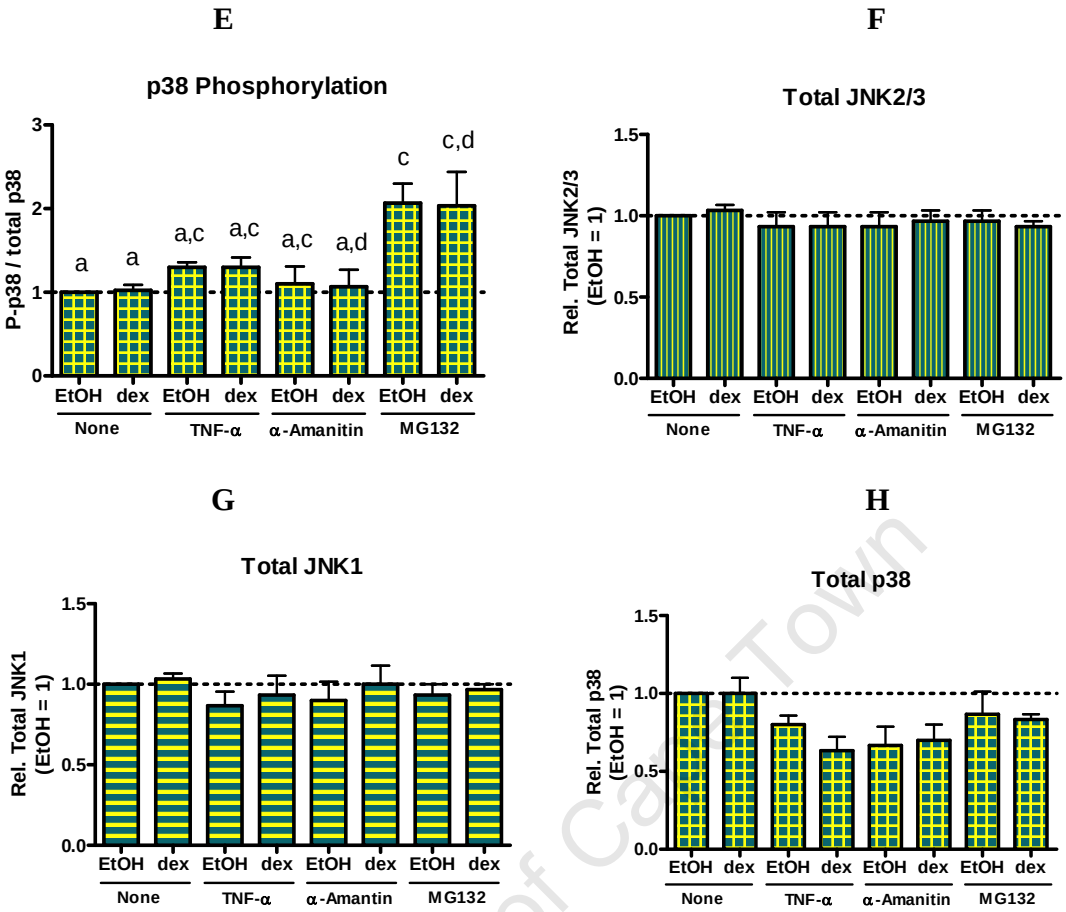



**Figure 7.2 Proteasomal degradation and RNA polymerase II activity influences phosphorylation of JNK and p38.** To test the effect of α-amanitin and MG132 on JNK and p38 phosphorylation, COS-1 cells transiently transfected with 1 μg HA-hGRwt were pre-treated with either 10 μM MG132 for 4 hours, 2.5 μg/ml α-amanitin for 24 hours or 0.02 μg/ml TNF-α for 15 minutes before stimulation with vehicle (EtOH) or 100 nM Dex for an additional 1 hour. Equal amounts of samples were loaded onto an 8% SDS-PAGE and Western blotting was performed, probing with either an anti-P-JNK (A) or anti-P-p38 antibody (B). After developing, the blots were stripped and re-probed with total JNK or p38. The amount of phosphorylated JNK and p38 was normalised to total JNK or p38 and plotted as the average of four independent experiments ± SEM (C and D) and (E). Total JNK and p38 is shown in (F and G) and (H), respectively. The histograms in C, D, E, F, G and H show pooled results from four independent experiments, plotted as means ± SEM, while Western blots in A and B each show results of one representative experiment. Statistical significance is indicated by different lower-case letters, such that all the conditions with the same letter are not statistically significantly different from each other ($P > 0.05$), while those having different letters are statistically significantly different from each other ($P < 0.05$).

The above mentioned effect of these inhibitors on kinases, and phosphatases, makes interpretation of the GR phosphorylation data and requirement of transcription and degradation, difficult. Nevertheless, taken together, these results suggest that protein degradation is required for dex-induced GR phosphorylation at S211, but inhibits basal GR phosphorylation at S211, as well as S226. The significance of this on GR function is unclear. One interpretation would be that in order for newly synthesized GR to be phosphorylated, the already phosphorylated GR needs to first be degraded. Additionally, different GR ligands result in faster or slower GR degradation (Fig. 6.2 in Chapter 6). If GR degradation is required for ligand-mediated GR phosphorylation, then the different rates of GR degradation with the different ligands, will result in differential GR phosphorylation, which is consistent with the ligand-selective GR phosphorylation results found in Chapter 8.

Phosphorylation is a dynamic process, wherein the amount of phosphorylation observed, is a combination of kinases and phosphatases (Wang *et al.*, 2007b). Accordingly, inhibition of protein degradation and transcription would also influence the relative kinase and/or phosphatase levels, thereby resulting in an indirect effect on GR phosphorylation. For example, without any inhibitors, the relative levels of the kinases responsible for phosphorylating the GR, may be higher than that of the phosphatases, resulting in the high levels of GR phosphorylation observed. If degradation is inhibited, less GR phosphorylation could be observed due to, the kinase-phosphatase balance being shifted towards less kinase and more phosphatase. Since degradation and transcription of both the kinase and the phosphatase would be inhibited, one interpretation of the current data is the presence of a phosphatase that is rapidly synthesized and slowly degraded, and a kinase that is slowly synthesized and rapidly degraded. When degradation is inhibited, the rapid synthesis of the phosphatase could result in relatively more phosphatase than kinase accumulating, resulting in relatively more phosphatase than kinase, and less GR phosphorylation

would be observed. Similarly, when transcription is inhibited the rapidly degrading kinase and the slow degrading phosphatase would also result in relatively more phosphatase than kinase being present and less GR phosphorylation would be observed. This hypothesis is consistent with results in figure 7.2 H, where inhibition of transcription and degradation resulted in slightly less p38 kinase. This hypothesis would also explain the effects of inhibition of degradation on dex-induced S226 phosphorylation, where a decrease in S226 phosphorylation is observed when transcription and degradation is inhibited. However, the amount of basal S226 phosphorylation increases when both transcription and degradation is inhibited. This is not consistent with the above mentioned model and could imply that different kinases and/or phosphatases are responsible for phosphorylation of the liganded and unliganded GR. However, further experiments are needed to test this hypothesis.

Results in figures 7.1 C and D show that transcription is required for dex-induced phosphorylation at S226, but not at S211. It therefore seems that the mechanism of ligand-mediated GR phosphorylation at S226, differs from that of ligand-mediated GR phosphorylation at S211. One interpretation is that ligand-mediated phosphorylation at S211 is much more 'stable' than that of S226. Therefore transcription of new GR molecules is required for a high level of ligand-mediated GR phosphorylation at S226, whereas ligand-mediated phosphorylation at S211 remains longer and does not require transcription of new GR molecules. To this extent dex-mediated GR phosphorylation at S211 has been shown to be more 'stable' than dex-mediated GR phosphorylation at S203 (Wang *et al.*, 2007b). In order to get a better understanding of the requirement of GR degradation and GR transcription on GR phosphorylation, would require more direct means of inhibiting only GR-mediated transcription and degradation specifically. For instance, GR mutants wherein the AF-1 and AF-2 domains are mutated and can no longer transactivate, and/or mutants that can no longer be degraded, would provide a better

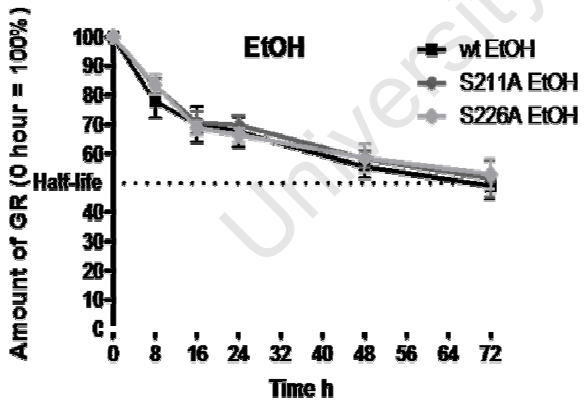
understanding of the requirement of GR transactivation and/or degradation for GR phosphorylation.

## 7.2 The role of GR phosphorylation on GR degradation

In order to more directly determine whether or not GR phosphorylation plays a role in ligand-mediated GR degradation and whether such effects could be correlated with the changes in transactivation efficacy observed in Chapter 4, GR half-life experiments comparing wt and phosphorylation mutants (S211A and S226A) were performed. COS-1 cells transiently transfected with hGR wt, S211A or S226A were pretreated with 1 μM cycloheximide for 1 hour before stimulation with compound. Equal amounts of cell extract were separated on SDS-PAGE and Western blot analysis, probing with anti-GR and anti-β-actin antibodies, was performed (Fig. 7.3 A). Results show no clear difference between the degradation of unliganded or liganded wt versus phosphorylation mutant receptors (Figs. 7.3 A-E). As with the previous half-life results (Chapter 6), the unliganded GR is degraded slowly over time (Fig. 7.3 B). Upon stimulation with the full agonist dex or full/partial agonist MPA or dissociated ligand RU486 (Figs. 7.3 C-E), the rate at which the GR is degraded increases.

**A**

**B**




**C**

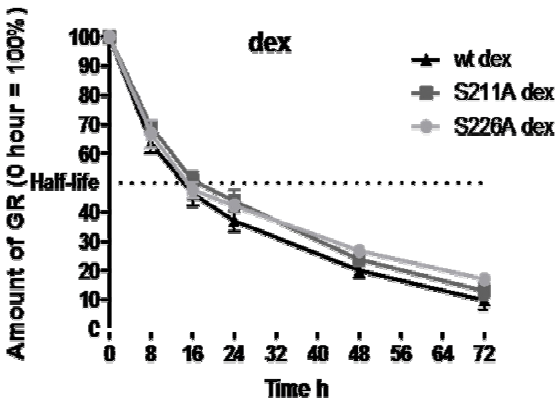





**Figure 7.3 Ligand-selective down-regulation of S211A and S226A GR (continues on next page).**

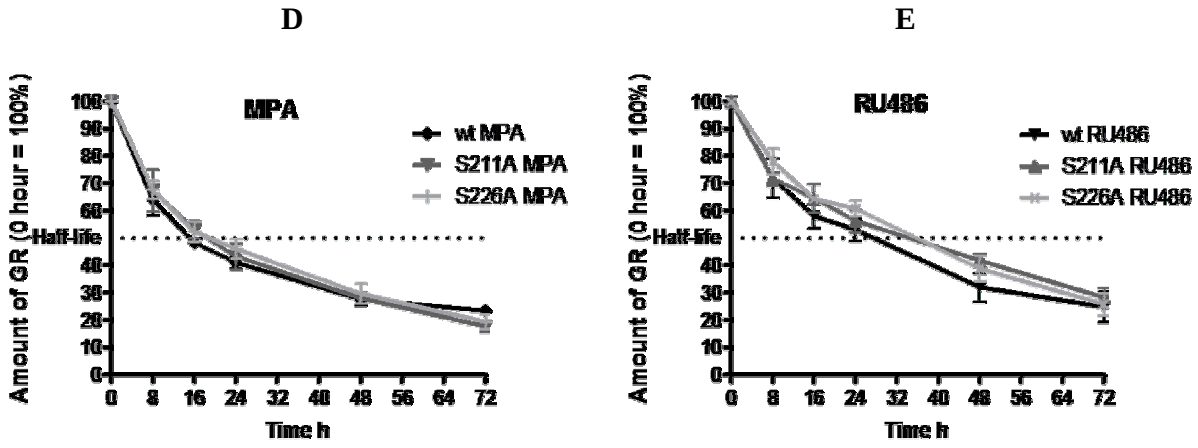



**Figure 7.3 Ligand-selective down-regulation of S211A and S226A GR**. COS-1 cells were plated in 10-cm dish at a density of $2x10^6$cells/dish and transfected with 10 μg of either HA-hGRwt, HA-hGRS211A or HA-hGRS226A. After 24 hour incubation, cells were replated into 6-well plates at a density of $4x10^5$ cells/well. The next day the cells were pre-treated with 1 μM cycloheximide for 1 hour and then treated with vehicle (EtOH) or 10 μM compound. At the appropriate time, whole cell extracts were prepared and equal amounts of lysate (7 μl) were analyzed by Western blotting with anti-GR and β-actin antibody (A). After quantification and normalization to β-actin, the amount of GR present at 0 hour was set to 100% and the amount of GR present at the other time points were calculated as a percentage thereof (B-E). The histograms in B, C, D and E show pooled results from four independent experiments, plotted as means ± SEM. while Western blots in A show results of one representative experiment



In order to determine if there is any statistically significant difference between the half-life of wt GR vs. the phosphorylation mutants, as well as the total amount of degradation over time (the amount of GR left at 72 hours), the half-life and end-point values were plotted as bar graphs and analysed (Figs. 7.4 A and B). From figures 7.4 A and B, it is evident that there is no statistically significant difference in the half-life or the total amount of degradation over time, in both the absence and presence of ligand, for wt versus the phosphorylation mutants. Furthermore, the same ligand-selective pattern of GR degradation, that is seen in Chapter 6 (Fig. 6.2), whereby agonists (dex) results in the quickest GR degradation, followed by the partial/full agonists (MPA) and then the dissociated ligand RU486, was found for wt and the mutant GRs. These results show that the decrease in transactivation on the MMTV promoter with the S211A mutant (Fig. 4.10 in

Chapter 4) is not due to an increased rate of GR degradation. Similarly, the increase in transactivation with the S226A mutant on the TAT-GRE and MMTV promoter (Figs. 4.8 and 4.10 in Chapter 4) is not due to a decreased rate of GR degradation.

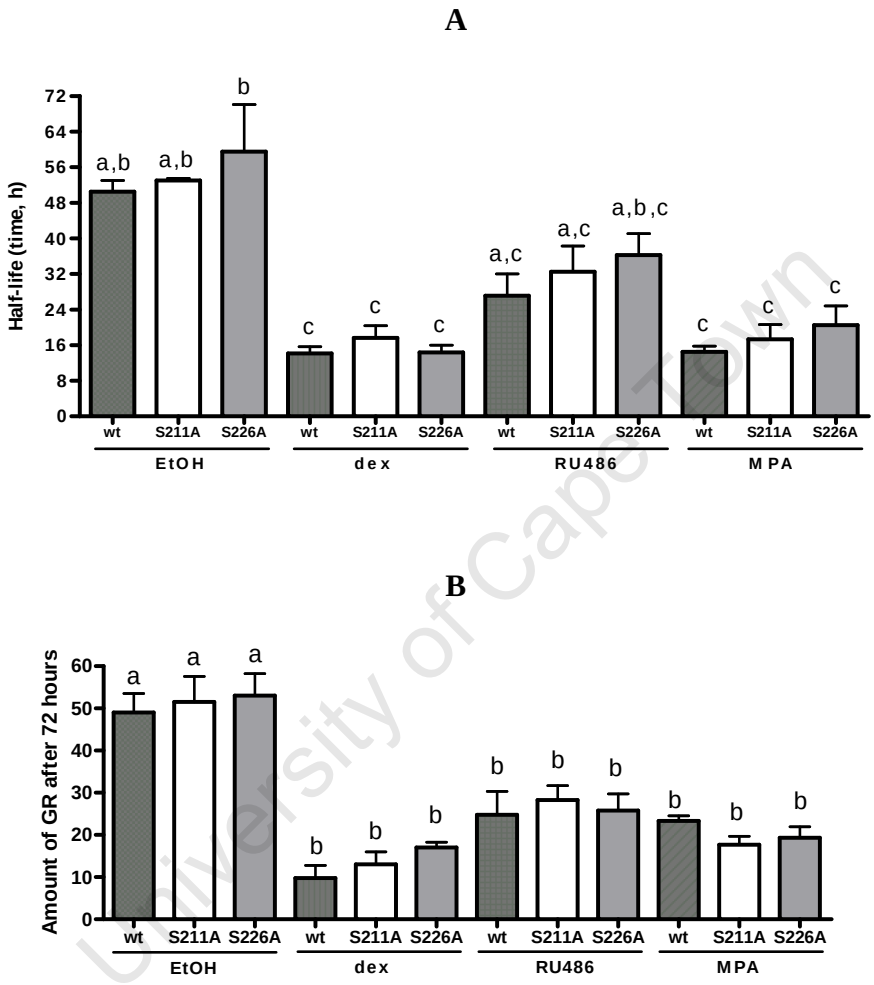





**Figure 7.4 Comparison between the half-life and total degradation of wt, S211A and S226A GR**. The respective half-life values (A) were calculated from figure 5.3 and the amount of GR left at the 72 hour time point (B) was taken from figure 5.3. The histograms in A and B show pooled results from four independent experiments, plotted as means ± SEM. Statistical significance is indicated by different lower-case letters, such that all the conditions with the same letter are not statistically significantly different from each other ($P > 0.05$), while those having different letters are statistically significantly different from each other ($P < 0.05$).

In order to rule out the possibility that phosphorylation site compensation could have lead to the false conclusion that GR degradation, in the presence or absence of ligand, is not determined by GR phosphorylation, half-life experiments comparing the triple phosphorylation mutant with wt GR was performed. Consistent with the results mentioned above, as well as those in Chapter 6 (Fig. 6.2), the unliganded GR degrades slowly over time and the rate of GR degradation is enhanced upon stimulation with the agonist dex (Fig. 7.5 A). Furthermore, the same pattern of ligand-selective GR degradation is found, since the dissociated ligand RU486 degrades slower than the full agonist dex and has a longer half-life. In addition, consistent with the results obtained with the S211A and S226A mutants, the half-life of the unliganded (Fig. 7.5 B) and liganded (Figs. 7.5 C and D) triple phosphorylation mutant is not different to that of wt. These results clearly show that unliganded and liganded GR degradation does not require GR phosphorylation at S203, S211 or S226 and that extent of ligand-selective GR phosphorylation does not determine the rate of ligand-selective GR degradation. Furthermore, the lack of dex-mediated GR transactivation with the triple phosphorylation mutant on the MMTV reporter-promoter construct (Fig. 4.10 in Chapter 4), is not due to phosphorylation increasing the rate of GR degradation.

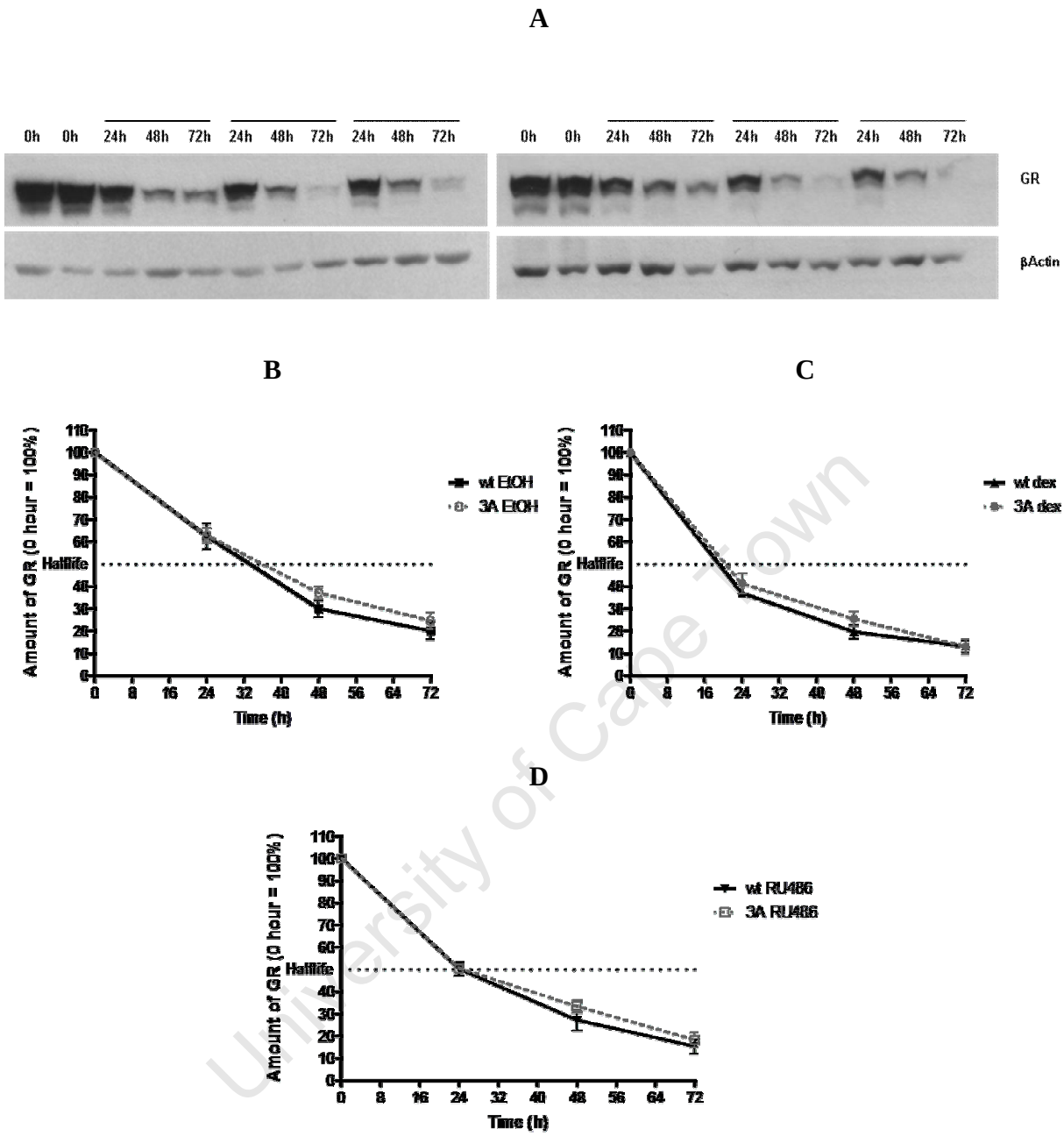





**Figure 7.5 Triple phosphorylation mutant degradation.** COS-1 cells were plated in 10-cm dish at a density of $2x10^6$cells/dish and transfected with either pRS-hGRwt or pRS-hGR3A. After 24 hour incubation, cells were replated into 6-well plates at a density of $4x10^5$ cells/well. The following day the cells were pre-treated with 1 μM cycloheximide for 1 hour and then treated with vehicle (EtOH) or 100 nM compound. At the appropriate time, whole cell extracts were prepared and equal amounts of protein were analyzed by Western blotting and probed with anti-GR and anti-β-actin antibody. A single representative Western blot is shown for each GR construct. (A). After quantification and normalization to β-actin, the amount of GR present at 0 hour was set to 100% and the amount of GR present at the other

time points was calculated as a percentage thereof (B), (C) and (D). Graphs show the average of four independent experiments, with values plotted as means ± SEM.

Taken together, these results show that GR degradation in the absence or presence of ligand is not dependent on GR phosphorylation at S203, S211 or S226. Furthermore, the extent of ligand-selective GR phosphorylation does not determine the rate of ligand-selective GR degradation. The correlation between ligand-selective GR phosphorylation and degradation (Fig. 6.4 in Chapter 6) therefore, does not indicate a direct relationship between GR degradation and phosphorylation. It is likely, that the correlation indicates that both GR phosphorylation and degradation is a consequence of the same "ligand-selective determining step", e.g. ligand-induced conformational change upon binding to the GR. For a more detailed discussion on this, see Chapter 8 (Conclusions and future perspectives).

Furthermore, the results obtained with the S211A and S226A mutants, are consistent with those of Webster *et al.* who showed in COS-1 cells, that phosphorylation of the individual residues of the mouse equivalent of S211 or S226 does not play a role in unliganded and liganded mGR degradation (Webster *et al.*, 1997). The triple phosphorylation mutant results presented in this thesis, are however in contrast to those of Webster *et al.* who showed that the combined mutation of the mouse equivalent S203, S211 and S226 residues resulted in a decrease dex-mediated mGR degradation. However, in these experiments, Webster *et al.* showed differential expression levels of wt and 3A mutant receptor, but did not show the degradation of the unliganded 3A mutant. In the above mentioned study, the 3A mGR could have a similar fold decrease in degradation of the dex-mediated vs. unliganded GR, as wt mGR. Additionally, Webster *et al.* did not use cycloheximide to stop *de novo* protein synthesis, making it difficult to distinguish between GR degradation and synthesis. A direct comparison between results obtained in this thesis and those

of Webster *et al.* is therefore difficult and would have to be done with mGR and hGR in parallel to obtain further insight into the apparent species-specific differences. Furthermore, Webster *et al.* also showed that the simultaneous mutation of 7 or 8 phosphorylation sites completely abolished ligand-selective mGR degradation. A more recent paper showed that the mutation of a single phosphorylation site, S404 resulted in a complete loss of dex-mediated hGR degradation (Galliher-Beckley *et al.*, 2008). Webster *et al.* did not include the single mutation of the mouse equivalent to S404 in his studies, making it difficult to directly compare these two studies. However, with the simultaneous mutation of 7 and 8 phosphorylation sites, Webster *et al.* included the mouse equivalent to S404. It is therefore likely, that the complete loss in dex-mediated mGR degradation with the GR mutated at 7 or 8 phosphorylation sites, was due to the mutation at S404. Additionally, in the same paper, Webster *et al.* showed that mutation of the mouse equivalent to S211 reduced GR-mediated transactivation on a simple TAT-GRE promoter-reporter construct, but not on a MMTV reporter-promoter construct, whereas, mutation of the mouse equivalent to S226 did not influence GR-mediated transactivation on the TAT-GRE or MMTV reporter-promoter construct (Webster *et al.*, 1997). The mouse triple mutant resulted in a complete loss in GR-mediated transactivation on the TAT-GRE reporter-promoter, but had no effect on the MMTV reporter-promoter (Webster *et al.*, 1997). These transactivation results are inconsistent with the proposed role of GR phosphorylation on GR-mediated transcription, by changing GR degradation. Furthermore, the apparent differences in transactivation, as well as in GR half-life obtained in this thesis to those of Webster *et al*. indicate that there are possible species-specific differences in the role of GR phosphorylation and warrants further investigation.

Results presented in this thesis, are the first to convincingly show with different GR ligands, by inhibiting *de novo* protein synthesis and with similar hGR expression levels, that hGR phosphorylation on S203, S211 and/or S226 does not determine the rate of hGR degradation with

the unliganded or liganded receptor. Furthermore, hGR phosphorylation on S203, S211 and/or S226 does not influence GR-mediated transcription via increasing or decreasing degradation of the unliganded- or liganded-GR and the mechanism of how GR phosphorylation influences GR-mediated transcription remains unanswered.

## 7.3 The role of phosphorylation at S203, S211 and S226 on GR acetylation and p300 recruitment

In order to investigate whether phosphorylation at S203, S211 and S226 is required for GR acetylation, and recruitment of p300, and thereby influences GR-mediated transcription, an *in vitro* DNA pull down ABCD assay using biotinylated GREs was performed. Results show that the unliganded GR bound the GREs to a similar extent as dex-stimulated GR and that slightly more triple phosphorylation mutant GR, versus wt GR, bound to the GREs (Fig. 7.6). However, this is likely due to more concentrated triple phosphorylation mutant cytosols in the input. Both wt and triple phosphorylation mutant resulted in the recruitment of endogenous p300, both in the absence and presence of dex. In two independent experiments the amount of p300 recruited with wt GR was similar in the dex stimulated and non-stimulated samples, indicating that in this *in vitro* assay, wt GR does not recruit p300 in a dex-dependent manner. Furthermore, the amount of p300 binding with the triple phosphorylation mutant was slightly less than that of wt GR, both in the absence and presence of dex. However, this was variable between the two independent experiments. Taken together, the small fraction of p300 in the pull down, versus the extremely high levels of endogenous p300 in the input, as well as the fact that p300 was not recruited in a dex-dependent manner, makes it unlikely that p300 plays a major role in dex-mediated GR transcription via a GRE. After stripping the GR Western blot and reprobing with an anti-acetyl-lysine antibody, a faint band corresponding to the size of the GR was detected (Fig. 7.6).

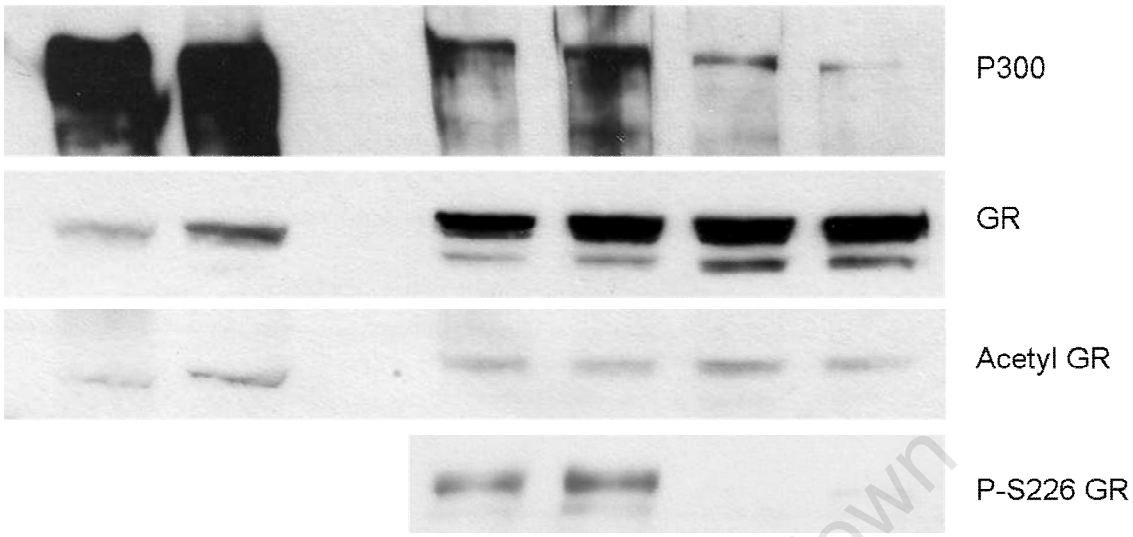



**Figure 7.6 GR acetylation and P300 recruitment.** COS-7 cytosols (20 µl) containing endogenous p300, as well as expressed pRS-hGRwt, pRS–hGR3A or empty vector (pcDNA3.1) containing hGR prebound with ethanol vehicle (EtOH) or 10 µM Dex were incubated with biotinylated GRE oligonucleotides attached to Streptavidin beads. Equal amounts of input cytosol, as well as DNA-bound GR or p300 were separated by SDS-PAGE and visualized by Western blotting with anti-p300 or anti-GR antibodies. Thereafter the GR blot was stripped and reprobed with anti-acetyl-lysine or anti-P-226 antibodies. The Western blots are one representative of two independent experiments.



Results show that wt and the triple phosphorylation mutant were both acetylated in the absence and presence of dex, with no clear difference between untreated or dex-stimulated GR, indicating that in this *in vitro* assay, the GR is not acetylated in a dex-dependent manner. Furthermore, the triple phosphorylation mutant was acetylated to a similar extent as wt, even though the triple mutant can not be phosphorylated, showing that phosphorylation of the GR at S203, S211 and S226 is not required for GR acetylation and does most likely not affect the extent of GR acetylation.

Recently, the HAT activity of overexpressed p300 has been shown to decrease GR mediated transactivation on a TAT-GRE and SV40 reporter-promoter construct in astrocytes and Schwann

cells (Fonte *et al.*, 2007). However, direct GR acetylation and p300 recruitment by the GR was not examined in this study. Furthermore, Li *et al.* showed that p300 interacts with the rGR in a dex-dependent manner, and that this interaction results in an increase in rGR mediated transactivation on a MMTV reporter-promoter construct in HeLa cells (Li *et al.*, 2002). Treatment with HDAC inhibitors, sodium butyrate and trichostatin A, decreased GR-mediated transactivation on the MMTV-promoter, as well as decreased the interaction between GR and p300, indicating that acetylation plays a role in the interaction of the GR and p300, as well as in GR transcription. However, direct GR acetylation was not examined in this study. The above mentioned studies indicate that p300 can either increase or decrease GR-mediated transcription in a process that seems to be cell-specific, i.e. increased transcription in HeLa cells and decreased transcription in astrocytes and schwann cells. Results presented in this thesis, suggest that p300 is not recruited to DNA-bound GR in a dex-dependent manner *in vitro* and is unlikely to play a major role in the complete loss in dex-mediated GR transcription found with the triple phosphorylation mutant. Although it seems that there is a small decrease in p300 recruitment with the triple phosphorylation mutant, this was highly variable between the experiments. Due to the variability of this result, it was concluded that phosphorylation of the GR at S203, S211 and S226 does not play a major role in the recruitment of p300. This is in contrast to results obtained by Kino *et al*. which showed that phosphorylation of the GR at S203, S211 and S226, inhibited the recruitment of p300, and thereby decrease GR-mediated transactivation on a MMTV promoter in HCT116 cells (Kino *et al.*, 2007). Apart from methodological differences, a major difference in the above mentioned study and results presented in this thesis, is that different cells were used. It is likely, that the relative abundance of specific co-factors in different cells differ, which could form the basis of these apparent cell-specific differences. Further investigation into the role of p300 on dex-mediated GR transcription on different genes and different cells is required.

Results presented above, showed that GR acetylation does not occur in a dex-mediated manner. These results are inconsistent with a previous report in the literature that showed dex-mediated GR acetylation in A549 cells (Ito *et al.*, 2006b). It is likely that cell specific differences, or methodological differences including the length of dex treatment (4 vs. 16 hours) can account for the different results obtained in the literature and the present study. In addition, whether p300 is directly responsible for acetylation of the GR has not been shown. Since the results presented in this study showed low levels of p300 recruitment *in vitro*, as well as low levels of GR acetylation, neither of which was dex-dependent, it was not determined whether or not p300 acetylates the GR. .

Nevertheless, results in this thesis, show for the first time, that phosphorylation of the GR at S203, S211 and S226 is not required for GR acetylation. Furthermore, in this cell system, GR phosphorylation at S203, S211 and S226 is not a major determinant of the recruitment of p300. Thus the mechanism whereby GR phosphorylation at S203, S211 and/or S226 influences GR-mediated transcription is unlikely to involve p300 recruitment or GR acetylation.

## 7.4 The role of phosphorylation on GRIP-1 recruitment

Previously a direct correlation between potency and efficacy for transactivation of the GR and GRIP-1 co-activator recruitment was shown (Ronacher *et al.*, 2009). Having shown that phosphorylation of the GR at S203, S211 and/or S226 is not required for GR degradation, p300 recruitment or GR acetylation, the requirement of GR phosphorylation in GRIP-1 recruitment was next examined. When the S226 and S211 phosphorylation results were correlated with GRIP-1 binding, as determined by a mammalian two-hybrid assay (data from (Ronacher *et al.*, 2009); Addendum E), a small but significant correlation for both S226 ($R^2 = 0.55$, $p < 0.03$) and S211 ($R^2 = 0.66$, $p < 0.01$) was found (Fig. 7.7).

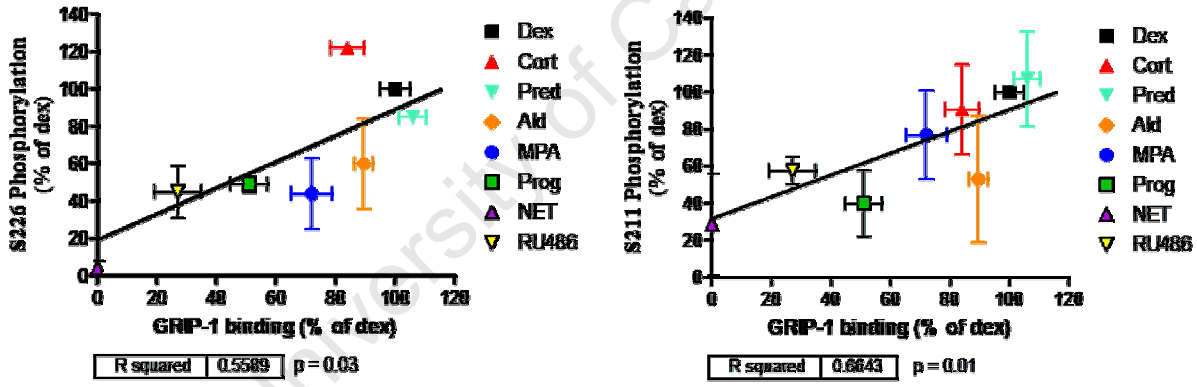


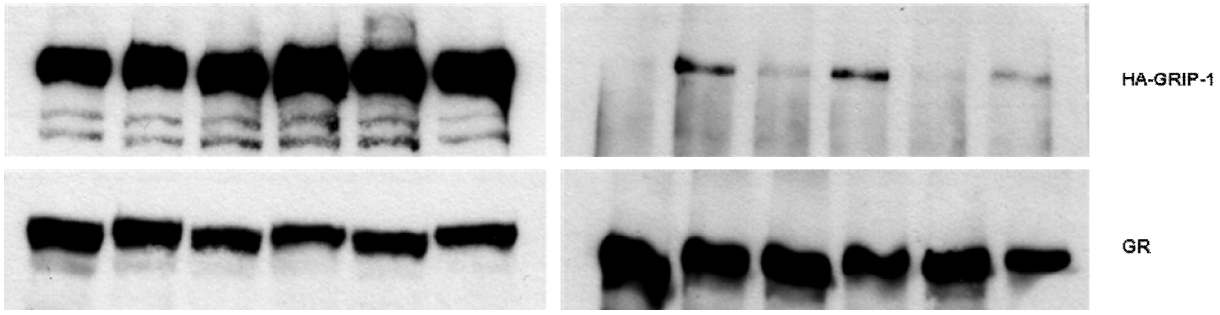

**Figure 7.7 Correlation between S226 and S211 phosphorylation and GRIP-1 binding.** Ligand-selective GR phosphorylation at S226 (Chapter 4) and at S211 ((Stubsrud, 2005), Addendum D) results were correlated with ligand-selective GRIP-1 binding to the hGR as measured by a mammalian two-hybrid assay ((Ronacher *et al.*, 2009)).

These correlations and the influence of GR phosphorylation on transactivation efficacy, as well as the direct correlation between efficacy and potency for transactivation of the GR and GRIP-1 recruitment (Ronacher *et al.*, 2009), indicates that phosphorylation of the GR could be required for GRIP-1 recruitment.

### 7.4.1 GRIP-1 binding to the GR *in vitro*

In order to determine whether GR phosphorylation at S211 or S226 is required for GRIP-1 binding to the GR, COS-1 cells transiently transfected with hGR wt, S211A or S226A, as well as HA-GRIP-1, were stimulated with 100 nM dex for 1 hour. After lysis, the cell extracts were immunoprecipitated with anti-GR antibodies and the bound proteins were separated on SDS-PAGE. After Western blotting with anti-GR and anti-HA antibodies, it can be seen that the unliganded GR does not bind GRIP-1, as no GRIP-1 was co-immunoprecipitated in the absence of ligand (Fig. 7.8 A). However, upon dex-stimulation, GRIP-1 bound to the GR and there seems to be less GRIP-1 immunoprecipitated with the S226A mutant than with wt GR. Furthermore, before the immunoprecipitation, all the cell extracts contained the same amount of GRIP-1 and GR, which excludes the possibility that less GRIP-1 was immunoprecipitated with the S226A mutant simply because it had a lower concentration of GRIP-1 or GR in the input lysate. After quantification and normalisation to the amount of precipitated GR, the S226A mutant (~ 1.5 fold), as well as the S211A mutant (~ 2 fold) (although to a lesser extent) showed less dex-induced GRIP-1 binding than wt (~ 2.5 fold) (Fig. 7.8 B).

**A**




**B**

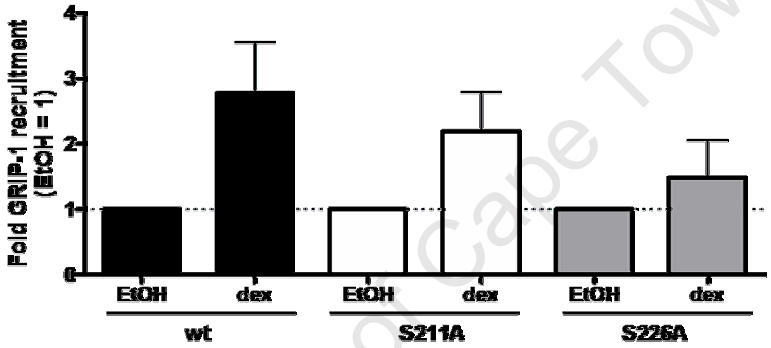





**Figure 7.8 The importance of S211 and S226 phosphorylation in GRIP-1 recruitment.** COS-1 cells transiently transfected with HA-GRIP-1, as well as either HA-hGRwt, HA-hGRS211A or HA-hGRS226A were treated with vehicle (EtOH) or 100nM Dex for 1 hour. After lysis, 1/50 of the cell lysate was subjected to SDS-PAGE and Western blotting and analysed for input levels of GR and HA-GRIP-1 (A). The remainder of the cell lysate was immunoprecipitated using anti-GR antibody and protein A/G beads. Proteins bound to the complex were analysed by Western blotting and probing for GR and HA-GRIP-1 (A). The amounts of HA-GRIP-1 and GR immunoprecipitated were normalised to their respective inputs, where after the amount of GRIP-1 was normalised to the amount of GR and plotted as the average ± SEM of between two to four independent experiments (B).

These results indicate that phosphorylation of the GR at S226 and S211, albeit to a lesser extent, plays a role in the interaction between the GR and the co-activator GRIP-1. However, due to relatively large experimental error and variability in the experiment, these results were not statistically significant. Since both the S226A and S211A mutant had a reduced ability to bind

GRIP-1, the co-immunoprecipitation experiments were repeated with the triple phosphorylation mutant in the hope that this mutant would have less experimental variability. Consistent with the results in figure 7.8, the unliganded wt GR did not bind GRIP-1, since no GRIP-1 was immunoprecipitated in the absence of ligand, whereas dex stimulation resulted in GRIP-1 binding (Fig. 7.9 A). However, contrary to the small decrease seen in GRIP-1 binding with the single phosphorylation mutants, the triple phosphorylation mutant had extremely low levels of GRIP-1 in the immunoprecipitation.



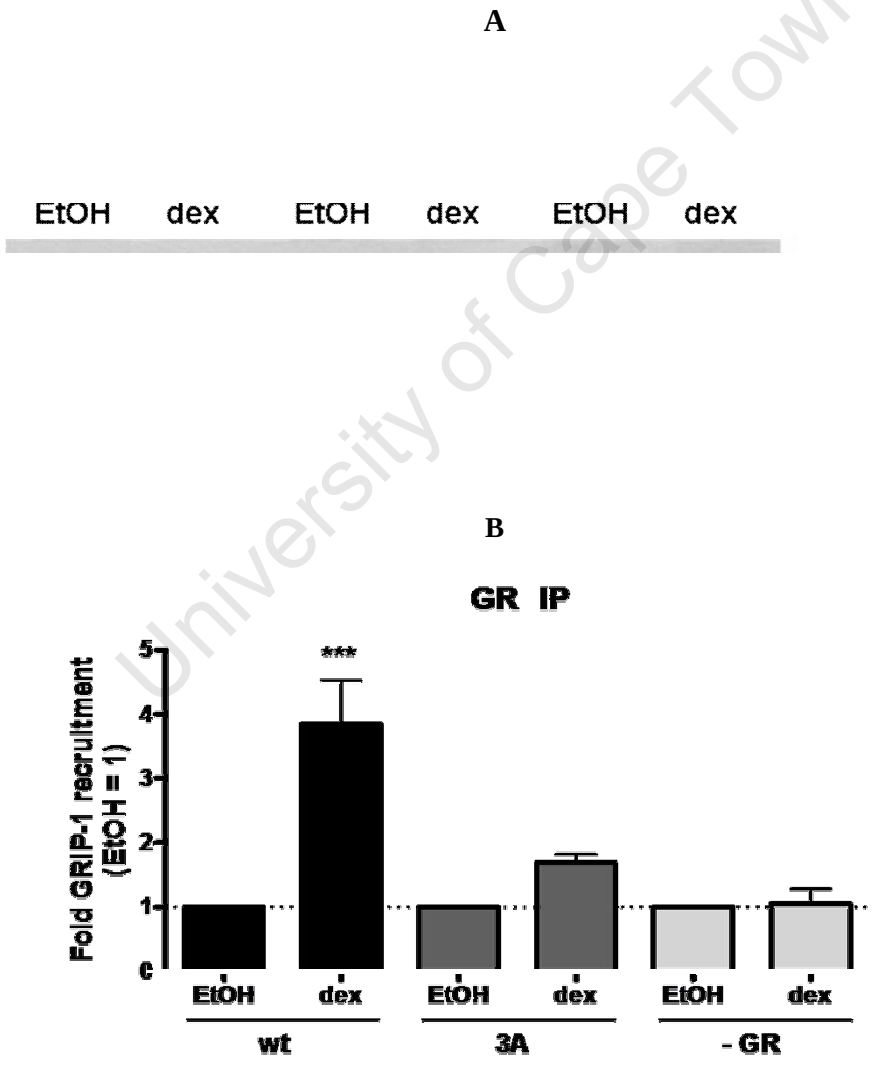



**Figure 7.9 GRIP-1 co-immunoprecipitation (continues on next page).**

**C**

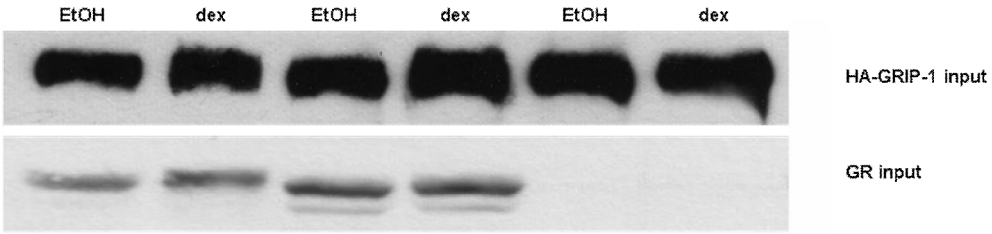



**Figure 7.9 GRIP-1 co-immunoprecipitation.** COS-1 transiently transfected with HA-GRIP-1, as well as either pRS-hGRwt, pRS-hGR3A or empty vector (pcDNA3.1) were treated with vehicle (EtOH) or 100 nM Dex for 1 hour. After lysis, 1/50 of the cell lysate was subjected to SDS-PAGE and Western blotting and analysed for input levels of GR and HA-GRIP-1 (C). The remainder of the cell lysate was immunoprecipitated using anti-GR antibody and protein A/G beads. Proteins bound to the complex were analysed by Western blotting and probing for GR and HA-GRIP-1 (A). The amounts of HA-GRIP-1 and GR immunoprecipitated were normalised to their respective inputs, and plotted as the average ± SEM of between two to four independent experiments (B). Statistical significance is denoted by ***, to indicate $P < 0.001$.

Quantification and normalisation to the immunoprecipitated GR shows that wt GR had a 4 fold increase in GRIP-1 binding upon dex stimulation, whereas the triple phosphorylation mutant showed a modest 1.5 fold induction in GRIP-1 binding (Fig. 7.9 B). This difference was not due to differential expression of GRIP-1 or GR, since the amount of GRIP-1 and GR was similar in the inputs (Fig. 7.9 C). Furthermore, GRIP-1 binding is specifically mediated by the overexpressed plasmids, since the minus GR control did not show any GRIP-1 binding in the absence or presence of dex (Figs. 7.9 A and B). These results clearly show that phosphorylation of the GR at S203, S211 and S226 is required for the interaction between the GR and the co-activator GRIP-1 *in vitro*.

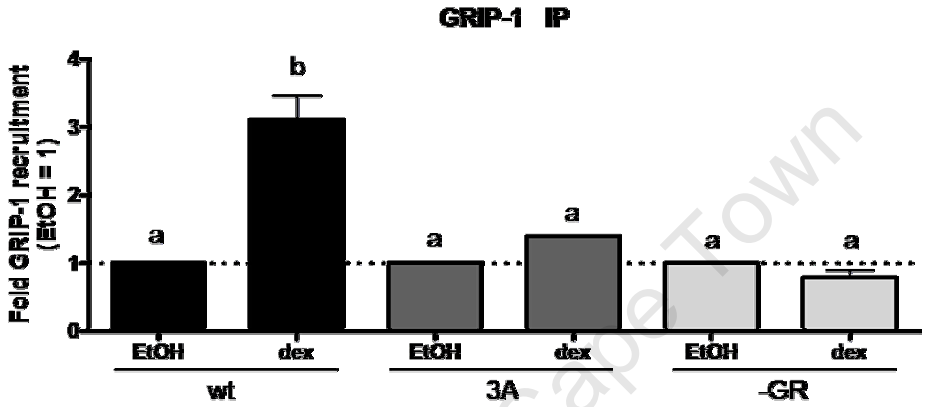
### 7.4.2 GRIP-1 and GR recruitment to the MMTV promoter *in vivo*

Having shown that GR phosphorylation at S203, S211 and S226 is required for the interaction between the GR and GRIP-1 *in vitro*, the requirement of phosphorylation at S203, S211 and S226 for the interaction between the GR and GRIP-1 on the MMTV promoter, *in vivo*, was next examined. COS-1 cells transiently transfected with HA-GRIP-1 and MMTV promoter, as well as either wt or 3A hGR, were stimulated with dex for 1h and a ChIP assay was performed, immunoprecipitating with an anti-HA antibody. Conventional PCR, using primers to the GRE region of the MMTV promoter, shows that all the input levels are the same (Fig. 7.10 A), which makes it possible to directly compare the samples with each other. Additionally, very little DNA was immunoprecipitated in the absence of over-expressed GR (-GR) and the IgG antibody control did not show any amplified DNA (Fig. 7.10 A). Results show that wt GR, bound to the GRE, recruited GRIP-1 in a dex-dependent manner, while the 3A GR did not show any dex-dependent effects on GRIP-1 recruitment (Fig. 7.10 A). Moreover, quantitative real-time PCR of the immunoprecipitated DNA revealed that the dex-mediated increase in GRIP-1 recruitment to wt GR is 3 fold, while 3A did not show any statistically significant dex-mediated increase in GRIP-1 recruitment (Fig. 7.10 B). These results show that phosphorylation of the GR is needed for dex-mediated GRIP-1 recruitment to the GRE of the MMTV-luc promoter *in vivo*.

To determine whether the triple phosphorylation GR mutant could still bind to the GRE, and whether the lack of GRIP-1 recruitment with the 3A mutant is due to less binding of the mutated GR to the GRE, a GR ChIP assay was performed. After the chromatin was immunoprecipitation with a GR antibody, it was found that dex stimulation led to the recruitment of wt GR to the GRE (Fig. 7.10 C). Interestingly, the triple phosphorylation mutant was also recruited in a dex-dependent manner, but to a lesser extent than wt GR.

**A**

**B**




**C**

**D**

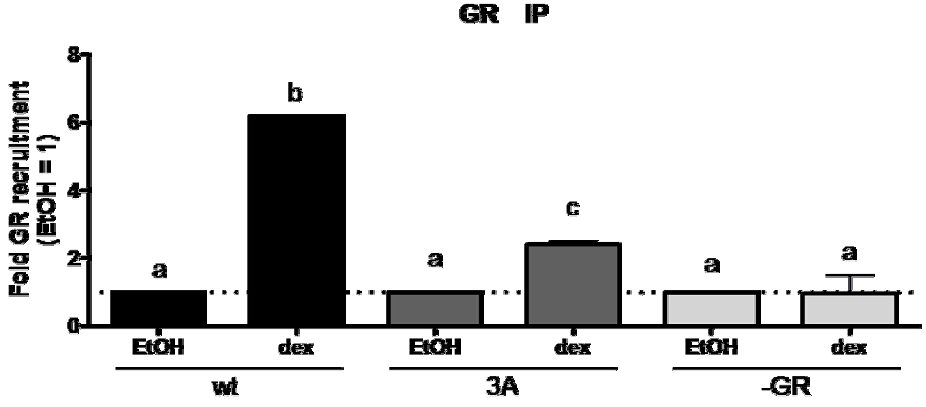





**Figure 7.10 GRIP-1 and GR recruitment to the MMTV promoter (continues on next page).**

**E**


**Figure 7.10 GRIP-1 and GR recruitment to the MMTV promoter.** COS-1 cells transiently transfected with HA-GRIP-1, MMTV reporter-promoter and either pRS-hGRwt, pRS-hGR3A or empty vector (pcDNA3.1) were treated with vehicle (EtOH) or 100 nM Dex for 1 hour. ChIP was performed using either an anti-HA antibody, an anti-GR antibody or an anti-IgG antibody. Conventional PCR amplification, using PCR primers encompassing the GRE region of the MMTV construct, was performed on input chromatin, as well as the chromatin precipitated with anti-HA antibody (A), or anti-GR antibody (C) and products were resolved on agarose gels. Quantitative real-time PCR on the DNA immunoprecipitated with the anti-HA (B) or anti-GR (D) antibodies was also performed. Expression of wt and 3A GR in the chromatin was analysed by Western blot probing for total GR (E). Results shown in B and D are from three independent experiments, with values plotted as the average ± SEM , normalised to input DNA and EtOH set to 1. NTC = no template control. The letters a, b and c are used to denote statistically significant differences, such that all the conditions with the same letter are not statistically significantly different from each other ($P>0.05$), while those having different letters are statistically significantly different from each other ($P<0.05$).

Quantitative real-time PCR of the immunoprecipitated DNA revealed that wt GR recruitment to the GRE increased 6 fold upon dex-stimulation, whereas the triple phosphorylation mutant only increased by 2 fold (Fig. 7.10 D). This difference in DNA recruitment was not due to less GR present in the input, since the amount of wt GR in the sonicated chromatin, as determined by Western blotting with a GR antibody, was found to be slightly less than the amount of 3A GR (Fig. 7.10 E).

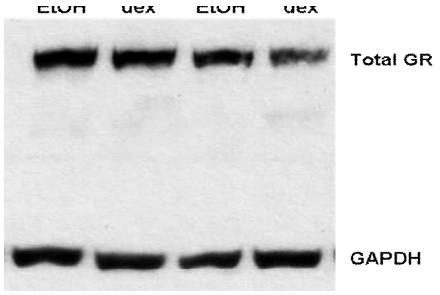
These results show that phosphorylation of the GR at S203, S211, and S226 is required for GRIP-1 and GR recruitment to the MMTV promoter in intact cells. The decreased association of the

mutated GR with the GRE could possibly explain the lack of significant detectable GRIP-1 recruitment observed with the 3A mutant.

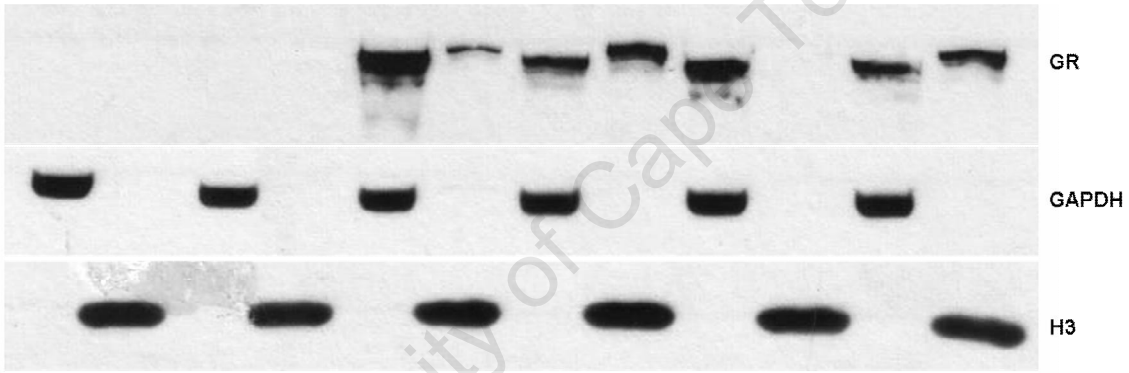
### 7.4.3 The role of phosphorylation at S203, S211 and S226 in GR subcellular localisation

Having shown less hGR and GRIP-1 recruitment to the MMTV promoter with the phosphorylation mutant after 1 hour dex treatment, it was next determined whether phosphorylation of the hGR plays a role in GR nuclear translocation. COS-1 cells, transiently transfected with hGR wt or 3A were stimulated with 100 nM dex or vehicle (EtOH) for 1 hour. Thereafter, cytoplasmic and nuclear fractions were prepared and separated on SDS-PAGE, followed by Western blotting with anti-GR, anti-GAPDH and anti-Histone H3 antibodies as controls. Wt and 3A GR were expressed similarly in the input (Fig. 7.11 A). The cytoplasmic GAPDH and nuclear H3 Western blots show that pure fractions were obtained (Fig. 7.11 B). In the absence of ligand, the hGR is predominantly cytoplasmic, whereas stimulation with dex results in GR nuclear translocation with ~50% of the hGR located in the nuclei (Fig. 7.11 C). Furthermore, no difference between wt and 3A GR subcellular localisation in the presence or absence of dex was detected. These results show that phosphorylation at S203/S211/S226 does not influence hGR nuclear translocation, suggesting that the decrease in GR and GRIP-1 recruitment to the MMTV promoter in the ChIP assay, is not due to deficient nuclear translocation of the triple phosphorylation mutant.

**A**




**B**






**C**

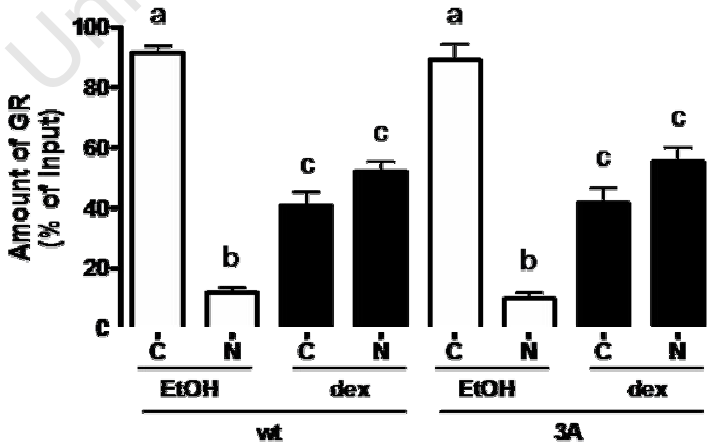


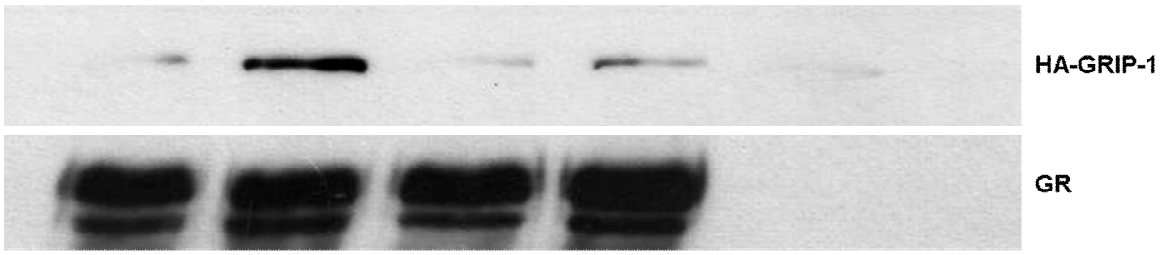

**Figure 7.11 Phosphorylation of the hGR at S203/S211/S226 does not influence GR nuclear translocation.** COS-1 cells transiently transfected with either pRS-hGRwt, pRS-hGR3A or empty vector

(-GR) were treated with vehicle (EtOH) or 100 nM dex for 1 hour. Western blotting with an anti-GR antibody was performed on the input samples (A), before cytoplasmic and nuclear fractions were prepared. Thereafter equal amounts of the fractions were separated on SDS-PAGE and Western blotting with anti-GR (GR), anti-GAPDH (GAPDH) and anti-Histone H3 (H3) was performed (B). After quantification, the amount of GR in the input was set to 100% and the amount of GR in the cytoplasmic and nuclear fractions was calculated as a percentage of input (C). The blots (A and B) are single representative figures from four independent experiments and the graph in C is pooled data from four independent experiments, with values plotted as the average ± SEM. In (C) the letters a, b and c are used to denote statistically significant differences, such that all the conditions with the same letter are not statistically significantly different from each other ($P>0.05$), while those having different letters are statistically significantly different from each other ($P<0.05$). C, cytoplasmic fractions; N, nuclear fractions

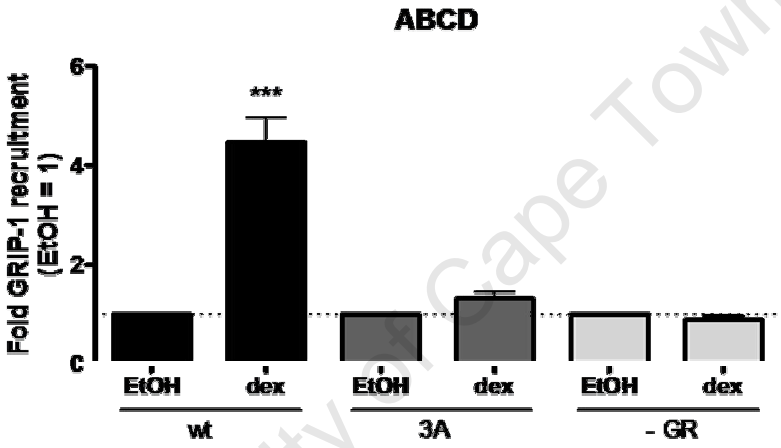
### 7.4.4 The role of phosphorylation at S203, S211 and S226 in DNA binding *in vitro*

In order to investigate whether mutation of the three serine residues results in a loss of GR DNA binding per se, an *in vitro* DNA pull down ABCD assay using biotinylated GREs was performed. These results showed that the same amount of GR was bound to the biotinylated GRE for wt and 3A mutated GR (Fig. 7.12 A). However, consistent with the ChIP and co-immunoprecipitation assays, stimulation with dex resulted in recruitment of more GRIP-1 for wt GR than for the 3A GR mutant. Normalisation and quantification revealed that dex stimulation led to a ~4.5 fold increase in GRIP-1 binding for wt GR while no significant GRIP-1 binding occurred for the 3A GR (Fig. 7.12 B). Additionally, no GRIP-1 binding was detected in the absence of ligand or in the absence of expressed GR, showing a requirement for the GR for GRIP-1 association with the DNA. Both wt and 3A GR were expressed at similar levels (Fig. 7.12 C) and the same GRIP-1 cytosols were used for wt and GR mutant pull down assays. These results show that phosphorylation of the GR at one of more of residues S203, S211 and S226 is required for GRIP-1 binding, but not for DNA binding *in vitro*.

**A**




**B**




**C**

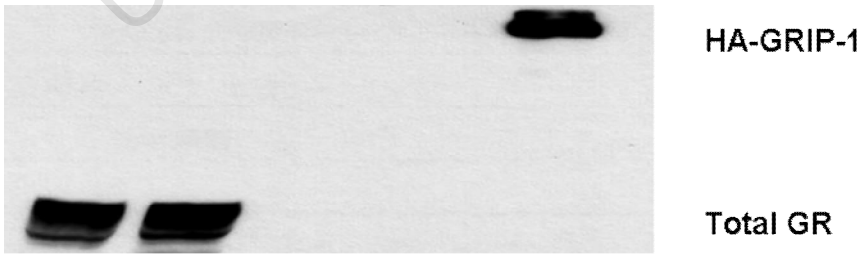





**Figure 7.12 GRIP-1 recruitment to the GRE in vitro.** COS-7 cytosols (20 µl) containing expressed pRS-hGRwt, pRS-hGR3A or empty vector (pcDNA3.1) containing hGR prebound with ethanol vehicle (EtOH) or 10 µM Dex were incubated with biotinylated GRE oligonucleotides attached to Streptavidin beads followed incubation with COS-7 cytosols containing overexpressed HA-GRIP-1. Equal amounts of

input cytosol (C), as well as DNA-bound GR or HA-GRIP-1 were separated by SDS-PAGE and visualized by Western blotting with anti-HA or anti-GR antibodies (A). After quantification and normalization to GR levels, the amount of HA-GRIP-1 bound to the GR:DNA complex was plotted (B). The graph shows results of three independent experiments, with averages thereof plotted as fold induction, relative to vehicle. Statistical significance is denoted by ***, to indicate $P < 0.001$.

Furthermore, GRIP-1 recruitment to the GR on the MMTV promoter, increases GR-mediated efficacy for transactivation (Fig. 7.13). The lack of GRIP-1 recruitment to the MMTV promoter with the GR triple phosphorylation mutant, is therefore likely to result in the complete lack of GR-mediated efficacy for transactivation observed with the triple phosphorylation mutant on the MMTV promoter (Fig. 4.10 in Chapter 4).



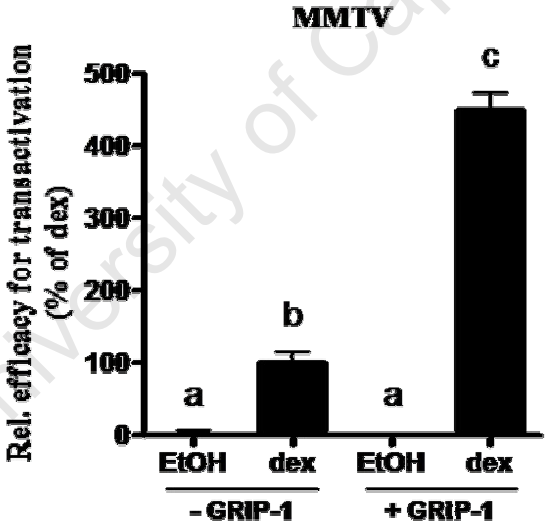



**Figure. 7.13 GRIP-1 recruitment to the MMTV promoter increases GR-mediated efficacy for transactivation.** COS-1 cells transiently transfected with 0.125 μg pRS-hGRwt, 0.047 μg MMTV reporter-promoter and 0.012 μg β−gal construct, in the presence or absence of 0.047 μg HA-GRIP-1 were treated with vehicle (EtOH) or 100 nM Dex for 24 hours. Luciferase activity in the cell lysates was normalised to β−gal activity per well. The histogram shows pooled results from two independent experiments, where each condition was performed in triplicates, and average values were plotted as means ± SEM, expressed as fold induction. The letters a, b and c are used to denote statistically significant differences, such that all the conditions with the same letter are not statistically significantly different from

each other (P>0.05), while those having different letters are statistically significantly different from each other (P<0.05).

It is possible that the decreased GR-DNA interaction observed for the triple mutant in the ChIP assay was due to changes in nuclear trafficking of the GR mutant. Working in intact cells, multiple factors including nuclear export and/or import could influence the ChIP result. Itoh *et al.* showed that phosphorylation of the GR at S226 enhances nuclear export of the GR (Itoh *et al.*, 2002). Accordingly, the 3A triple mutant which contains the S226A mutation would be predicted to exhibit a reduced nuclear export resulting in more nuclear accumulation of GR compared to wt. Increased nuclear accumulation of the GR could however not explain less GR-DNA interaction, making it unlikely that a reduced nuclear export could explain our results. Others have shown that dex-stimulation leads to the accumulation of phosphorylated S211 in the nucleus, which led Wang *et al.* to postulate that S211 phosphorylation could increase GR nuclear import (Wang *et al.*, 2002b). If S211 phosphorylation increases nuclear import in the current study, the 3A triple mutant that contains the S211A mutation should exhibit reduced nuclear import of the GR. This would be consistent with the reduced DNA binding found for the triple mutant in the ChIP assay. However, results presented in this thesis show that the wt and triple phosphorylation hGR mutant exhibit a similar degree of dex-mediated nuclear accumulation, consistent with another report for the mGR. Webster *et al*. showed that the combined mutation of the mouse residues equivalent to the human S203/S211/S226 GR residues did not influence mGR nuclear import, but still led to an almost complete loss of transactivation efficacy on a GRE-luciferase construct (Webster *et al.*, 1997). Thus one possible interpretation of these results is that phosphorylation of the hGR at S203/S211/S226, in addition to being required for GRIP-1 interaction, also plays a role in DNA binding in intact cells. If this is the case, then the *in vitro* ABCD data showing no effect on DNA binding with the triple mutant would suggest that this

loss of DNA binding cannot be mimicked *in vitro*. Another explanation for the apparent discrepancy between the GR DNA binding result in the ChIP compared to the *in vitro* ABCD assay could be that the GR-DNA binding affinity is similar in intact cells and *in vitro*, but that differences are observed due to different incubation times and differential kinetics of wt versus triple mutant hGR for binding to DNA Thus the relative amounts of phosphorylated versus unphosphorylated hGR bound to DNA and hence the transcriptional response may vary over time and represent another mechanism of fine tuning transcriptional responses. Nevertheless, taken together, these results clearly show that phosphorylation of the hGR at one or more of residues S211/226/203 is required for GR interaction with GRIP-1 in the absence and presence of a GRE *in vitro*, as well as on the MMTV promoter in intact cells.

Interestingly, results in figure 7.8 suggest that the S226A mutant seems to be the dominant site involved in GRIP-1 recruitment. However, this result is inconsistent with the transactivation result, where phosphorylation at S226 inhibits transactivation efficacy. One possible explanation for the apparent inconsistency is that apart from playing a role in GRIP-1 recruitment, phosphorylation on S226 might be involved in the recruitment of corepressors, such as NCoR or SMRT and thereby inhibits transactivation efficacy. However, in the literature, there is no evidence to suggest S226 phosphorylation is involved in co-repressor recruitment.

Results from Kino *et al.*, using the same triple hGR mutant construct, suggest that phosphorylation of S203/S211/S226 inhibits transcriptional activity of the hGR on a MMTV-promoter in HCT116 cells via inhibition of recruitment of the histone acetyltransferase (HAT) co-activator, p300, to the GR bound on a MMTV-luciferase promoter (Kino *et al.*, 2007). In contrast, here it is shown that phosphorylation of S203/S211/S226 is required for maximal transcriptional activity on the MMTV-promoter in COS-1 cells and that this is not due to

differential p300 recruitment, but most likely due to reduced GRIP-1 recruitment to the MMTV-promoter. Interestingly, phosphorylation of the hGR at S404, by glycogen synthase kinase 3β (GSK3β), was recently shown to be required for the recruitment of p300 (Galliher-Beckley *et al.*, 2008). It would thus appear that phosphorylation of the GR at specific selected serine residues may be a mechanism for rapid differential gene regulation via increasing or decreasing co-activator, HAT, and/or mediator complex recruitment in a cell- and promoter-specific manner, thereby increasing or decreasing transcription efficacy.

# Chapter 8 Conclusions and future perspectives

A central question in the steroid receptor field is how do different GR ligands elicit their differential physiological responses on the same gene in the same cell. In order to investigate what determines ligand-selectivity, the biological response of a large panel of ligands in transactivation and transrepression was determined (Ronacher *et al.*, 2009). The biological response of a particular ligand was then correlated with the behaviour of the liganded GR at a particular step in the GR transcriptional pathway. These correlations do not establish a cause and effect, but can be used as a guide to focus on specific steps that could potentially determine ligand-selectivity. This thesis concentrated on the role of two different steps in the hGR transcriptional pathway that could potentially determine ligand-selective GR-mediated transcription, namely GR phosphorylation at S226 and GR degradation.



## 8.1 Ligand-selective GR phosphorylation

Results presented in this thesis showed that GR phosphorylation at S226 occurs in a ligand-selective manner. At the time this study was initiated, the effect of different ligands on S226 phosphorylation was not know, but recently a study has been published with four GR ligands being investigated (Chen *et al.*, 2008). Results in this thesis are consistent with those of Chen *et al*., but showed ligand-selective GR phosphorylation at S226 with eight additional GR ligands. In addition, the present study showed for the first time that dose-dependent GR phosphorylation occurs at S226 with different GR ligands.

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After establishing that GR phosphorylation at S226 occurs in a ligand-selective manner, the second aim was to determine if the extent of GR phosphorylation at S226 determines the rank order of ligand-selective transcription. Results showed a good correlation between ligand-selective GR phosphorylation at S226 and S211 with efficacy, but not potency for transactivation. However, by examining the effect of GR phosphorylation at S226 on ligand-mediated GR transactivation, with 12 different GR ligands, this thesis showed for the first time that the extent of ligand-selective GR phosphorylation at S226 does not determine the rank order of ligand-selective transactivation. At the time this work was performed, the effect of GR phosphorylation by different GR ligands on GR-mediated transactivation was not known, but was recently shown with dex (Chen *et al.*, 2008). Although ligand-selective GR phosphorylation at S226 was shown not to determine ligand-selective transactivation, in this thesis, phosphorylation at S226 was shown to inhibit efficacy, but not potency for transactivation, on a TAT-GRE- and MMTV promoter. Furthermore, phosphorylation at S211 was shown to be required for full transactivational efficacy on the MMTV promoter. This result is consistent with previous reports in the literature, showing that phosphorylation at S211 influences maximal efficacy for transactivation (Stubsrud, 2005;Chen *et al.*, 2008).

The recruitment of co-factors via the GR, has previously been shown by others to modulate the maximal efficacy for transactivation (Li *et al.*, 2002;Rogatsky I, 2001;Ding *et al.*, 1998;Grenier *et al.*, 2006). A model to explain the results showing that phosphorylation at S226 inhibits transactivation, while phosphorylation at S211 is required for transactivation, is not easy to imagine and would involve the combined actions of both co-activators and co-repressors (Fig. 8.1). In order to explain the increase in transactivation efficacy with the S226A mutant observed in the present study (Fig. 4.8 in Chapter 4), it is hypothesised that GR phosphorylation at S226 is required for the recruitment of an unknown co-repressor (e.g. “Y” in figure 8.1). The lack of the

recruitment of this putative co-repressor Y with the S226A GR mutant, could explain the increase in efficacy for transactivation (Fig. 4.8 in Chapter 4). Consistent with this idea, Hong *et al.* recently showed that dex-stimulation resulted in the recruitment of NCoR and SMRT to a GRE-containing GC-responsive gene and that knock-down of NCoR or SMRT increased dex-mediated transactivation on this gene (Hong *et al.*, 2009). In contrast to the results for S226, phosphorylation at S211 was shown in the present study (Fig. 4.10 in Chapter 4) to be required for maximal efficacy for transactivation. One possible explanation for this result is that phosphorylation at S211 could be required for the recruitment of an unknown co-activator (e.g. "X" in figure 8.1), thereby increasing efficacy for transactivation.



**Figure 8.1 Proposed model to explain the role of GR phosphorylation at S211 and S226 in the recruitment of GRIP-1 for transactivation on the MMTV promoter.** Phosphorylation at S226 is hypothesised to be required for the recruitment of a co-repressor (Y), whereas phosphorylation at S211 is hypothesised to be required for the recruitment of a co-activator (X). Furthermore, results presented in this thesis showed that phosphorylation at S211 and S226 is important for GRIP-1 recruitment. For wt GR

stimulated with dex, both S226 and S211 are phosphorylated, resulting in the recruitment of GRIP-1, as well as possibly both a co-repressor (Y) and a co-activator (X). With the S211A mutant, stimulation with dex is proposed to result in the recruitment of co-repressor (Y), since S226 is still phosphorylated, but not the recruitment of co-activator (X). Furthermore, GRIP-1 is still recruited, albeit to a lesser extent than wt GR. This combination may explain the decrease in dex-mediated efficacy for transactivation with the S211A mutant versus wt GR. With the S226A mutant, stimulation with dex is proposed to result in the recruitment of co-activator (X), since S211 is still phosphorylated, but not in the recruitment of co-repressor (Y). Additionally, GRIP-1 is still recruited, albeit to a lesser extent than wt GR. This combination may explain the increase in efficacy for transactivation with the S226A mutant vs. wt GR. With the triple phosphorylation mutant (3A), neither S211 nor S226 is phosphorylated. Therefore, it is proposed that neither co-activator (X), co-repressor (Y) nor GRIP-1 is recruited. GRE = glucocorticoid response element; BTM = basic transcription machinery, PS226 = GR phosphorylated at S226; PS211 = GR phosphorylated at S211. X = unknown co-activator; Y = unknown co-repressor. The square indicates a specific conformation of the GR, bound by dex (the small triangle).

In support of the idea that phosphorylation at S226 is required for the recruitment of a co-repressor, whereas phosphorylation at S211 is required for the recruitment of a co-activator, studies on the ER demonstrated that the phosphorylation status of one specific serine residue determines the recruitment of co-activator (SRC-3) versus the co-repressor, stromelysin-1 platelet-derived growth factor-responsive element-binding protein (Weigel and Moore, 2007;Likhite *et al.*, 2006;Endoh *et al.*, 1999;Gburcik *et al.*, 2005). Interestingly, Rogatsky *et al.* showed that GRIP-1 can switch from a co-activator to a co-repressor, depending on the composition of the regulatory elements on the DNA (Rogatsky I, 2001). It would be tempting to speculate that GRIP-1 can switch between co-activator vs. co-repressor, depending on whether S211 or S226 is phosphorylated. For example, if GRIP-1 is recruited via phosphorylation at S211, it could act as a co-activator for transactivation, whereas if it is recruited via phosphorylation at S226, it could act as a co-repressor. The phosphorylation mutant transactivation results would be consistent with such a model. However, currently there is no evidence in the literature to support this hypothesis and it remains to be tested.

In order to test if the above mentioned model (Fig. 8.1) holds for two different promoters, the transactivation results with the phosphorylation mutants on the MMTV promoter were compared to those on a TAT-GRE promoter. Although the model holds for both promoters, there are some numerical differences in the extent to which phosphorylation influences maximal efficacy on the different promoters. The inhibitory effect of S226 phosphorylation on transactivation was more pronounced on the TAT-GRE (Fig. 4.8 in Chapter 4), than the MMTV promoter (Fig. 4.10 in Chapter 4), whereas the requirement for S211 phosphorylation for transactivation was much more on the MMTV (Fig. 4.10 in Chapter 4), than the TAT-GRE promoter ((Stubsrud, 2005); Table E2 in addendum E). These results indicate promoter-specific differences in the role of GR phosphorylation on transactivation. The TAT-GRE promoter, contains two GREs, where GR binding to the DNA alone initiates transcription (Reviewed in (Schoneveld *et al.*, 2004;Schmid *et al.*, 1987)). In contrast, the MMTV promoter is much more complex and GR binding to the four GREs results in the recruitment of an ATP-dependent chromatin remodelling complex and subsequent binding of TBP and additional transcription factors (Hebbar and Archer, 2003;Schoneveld *et al.*, 2004). It would be tempting to speculate that a putative co-activator (X) recruited by S211 is crucial for the recruitment and subsequent binding of TBP and/or additional transcription factors to the MMTV promoter. This would explain the almost complete loss of transcription with the S211A mutant on the MMTV promoter, while only a partial loss in efficacy is seen on the simpler TAT-GRE, which does not require binding of TBP or additional transcription factors. In contrast, on the TAT-GRE, the recruitment of a putative co-repressor (Y) via S226 would have a larger effect than on the more complex MMTV promoter, where multiple additional transcription factors mediate transactivation. Consistent with this idea, Ronacher *et al.* recently showed that differential co-factor recruitment is a major determinant of promoter-specific differences in potency and efficacy for transactivation and transrepression (Ronacher *et al.*, 2009). It is therefore likely that site-specific GR phosphorylation at S211 and S226 and

thereby differential recruitment of co-factors, can account for some these above mentioned promoter-specific differences.

Another aim was to determine if ligand-selective GR phosphorylation at S226 and/or S211 determines the rank order of ligand-selective transrepression. Similar to the transactivation results, results in this thesis showed good correlations between ligand-selective phosphorylation at S226 and S211 and ligand-selective efficacy for transrepression. However, by examining the effect of GR phosphorylation at S226 and S211 on ligand-mediated GR transrepression, with 12 different GR ligands, this thesis showed for the first time that the extent of ligand-selective GR phosphorylation at S226 and S211 does not determine the rank order of ligand-selective transrepression via AP-1 or NFκB. At the time this work was done, the effect of S226, as well as S211 phosphorylation on transrepression was also not known, but has recently been shown to inhibit transrepression on some but not all genes (Chen *et al.*, 2008).

Although ligand-selective GR phosphorylation at S226 and S211 was shown to not determine ligand-selective transrepression in this thesis, phosphorylation at S211 as well as S226 slightly inhibited maximal efficacy for transrepression on an AP-1 and NFκB promoter. This result is very interesting and could possibly indicate that phosphorylation at S211 and S226 inhibits binding of the GR to NFκB and AP-1 proteins. Results presented in this thesis also indicate that GR phosphorylation at one or more of residues S203, S211 and S226 are required for maximal DNA binding on the MMTV promoter (Fig. 7.10 in Chapter 7). This result is not consistent with a role for GR phosphorylation at S211 and S226 inhibiting indirect DNA binding via tethering to NFκB or AP-1 proteins. However, in the literature, deacetylation of the GR by HDAC2 has recently been shown to be required for GR binding to NFκB and subsequent transrepression (Ito

*et al.*, 2006b). Furthermore, AR phosphorylation and acetylation have been shown to be functionally linked (Fu *et al.*, 2004). Thus a possible hypothesis that could explain the inhibitory effect of GR phosphorylation on transrepression is that GR phosphorylation at S211 and S226 is required for GR acetylation, thereby inhibiting binding of the GR to NFκB and resulting in less transrepression. However, this thesis shows for the first time that phosphorylation of the GR on S226, S211 and S203 is not required for GR acetylation. Whether GR phosphorylation at S211 and/or S226 inhibits GR binding to NFkB and AP-1 proteins in a manner independent of acetylation remains to be tested.

Another possibility is that GR phosphorylation at S211 and S226 could also inhibit transrepression via the recruitment of a co-activator. In the present study GR phosphorylation at S211 and S226 was shown to be required for recruitment of the co-activator GRIP-1 to the MMTV-promoter. The lack of GRIP-1 recruitment with the single phosphorylation mutants could therefore explain the increase in transrepression observed on the AP-1 and NFκB promoter-reporter constructs (Fig. 4.12 in Chapter 4). To this extent Rogatsky *et al.* has shown agonist-mediated GRIP-1 recruitment, in U2OS cells with rGR, to the collagenase 3 gene, where GR-mediated repression is via tethering to AP-1 (Rogatsky I, 2001). On an AP-1 luciferase construct, GRIP-1 behaved as a co-repressor and recruitment of GRIP-1 increased GR-mediated transrepression in U2OS cells (Rogatsky I, 2001). This result of Rogatsky *et al.* is not consistent with a model wherein hGR phosphorylation at S211 and S226 is required for GRIP-1 recruitment to the AP-1 and NFκB promoter, where GRIP-1 acts as a co-activator (that is causing less repression) in COS-1 cells, indicating possible cell-specific differences. Further experiments are required to test whether the presence of GRIP-1 results in less transrepression on the AP-1 and NFκB promoters in COS-1 cells.

When comparing the role of GR phosphorylation on transactivation versus transrepression, the combined mutation of S211, S226 and S203 only slightly increased efficacy for transrepression on the AP-1 promoter (Fig. 4.13 in Chapter 4), whereas a complete loss of dex-mediated transactivation on the MMTV promoter (Fig. 4.10 in Chapter 4) was seen. These results indicate that GR-mediated transrepression via AP-1 is less sensitive to GR phosphorylation than GR-mediated transactivation, indicating different mechanisms in the role of GR phosphorylation on transactivation vs. transrepression. These differences could possibly include the recruitment of different co-factors to the GR when bound to DNA than when tethered to another transcription factor. Inhibiting the kinases responsible for GR phosphorylation might therefore distinguish between GR-mediated transactivation and transrepression and could be used as a strategy for anti-inflammatory drugs.



## 8.2 The kinases that phosphorylate the hGR at S211 and S226 in intact cells

In an attempt to determine which kinases phosphorylate the GR in intact cells, GR phosphorylation at S211 and S226 was manipulated *in vivo*, by means of MAPK and CDK inhibitors, as well as expression constructs for MAPKs and CDK5. In this study, no direct evidence for a role of JNK, p38 or CDKs in phosphorylating the GR at S226 and/or S211 was found, using the above mentioned strategies. Furthermore, although data on GR and kinase phosphorylation kinetics were not inconsistent with a role for JNK and p38 in mediating GR phosphorylation at S226 and S211, it did not provide direct evidence in support of such a model. Transactivation and transrepression data with the JNK and p38 inhibitors were consistent with a role for JNK and p38 in mediating GR phosphorylation at S226 and S211, respectively. However

since the inhibitors did not reduce GR phosphorylation, it is likely that these effects on transactivation are indirect and do not involve inhibition of GR phosphorylation. A careful look at the literature reveals that several authors have tried to inhibit GR phosphorylation, by the above mentioned strategies, mostly without substantial success (Chen *et al.*, 2008;Miller *et al.*, 2005;Kino *et al.*, 2007). Due to the difficulty of inhibiting GR phosphorylation *in vivo*, in this thesis, as well as in the literature, it seems that combinations of MAPKs and possibly CDKs are involved in this process or that the kinases can compensate for each other, e.g. inhibition of one kinase leads to hyper activation of another kinase (as shown in figure 5.2 in Chapter 5), which in turn phosphorylates the GR on S211 and/or S226. However, further investigation is needed to show that the kinases can compensate for each other, as well as to provide a means to inhibit phosphorylation of endogenous GR *in vivo*.



## 8.3 Ligand-selective GR degradation

Similar to ligand-selective GR phosphorylation at S226, another aim of this thesis was to determine whether GR degradation occurs in a ligand-selective manner. Results presented in this thesis showed ligand-selective GR degradation with a panel of 10 different GR ligands for the first time. By using this panel of GR ligands, it was possible to show that there is a general trend, wherein the most potent GR ligands result in the most rapid GR degradation. The half-life results presented in this thesis with hGR are in agreement with results obtained with the mGR and rGR in COS-1 and rat hepatoma cells, which showed that dex treatment reduced the half-life of the mGR and rGR from 20 and 25 hours to 9 and 11 hours, respectively (Webster *et al.*, 1997;Dong *et al.*, 1988).

After establishing that GR degradation occurs in a ligand-selective manner, the second aim was to determine if the extent of GR degradation determines the rank order of ligand-selective transcription. Results presented in this thesis showed a good correlation between ligand-selective GR turnover and transactivation, as well as transrepression. However, GR degradation was further shown to limit dex-mediated, but not MPA-mediated transactivation, indicating that GR degradation influences GR-mediated transactivation in a ligand-selective manner. However, when GR degradation was inhibited, dex-mediated efficacy for transactivation was still higher than MPA-mediated efficacy for transactivation, i.e. the same relative rank order of ligand-selective transactivation was maintained, suggesting that ligand-selective GR degradation does not determine the rank order of ligand-selective transactivation. However, further experiments with the whole panel of GR ligands are needed to confirm this hypothesis. Furthermore, when dex-mediated transactivation was inhibited, dex-mediated GR degradation was still observed, suggesting that dex-mediated GR degradation does not require dex-mediated transactivation.



## 8.4 Determinants of ligand-selective transcription

Statistically significant correlations between ligand-selective GR phosphorylation, and degradation, with ligand-selective GR-mediated transcription were obtained. However, as mentioned above, ligand-selective GR phosphorylation, or degradation, does not determine the rank order of ligand-selective transcription. One interpretation of the correlations is that the ligand-selective "determining step" influences GR phosphorylation, degradation and transcription to a similar extent, and that ligand-selective GR phosphorylation and degradation are a consequence of this determining step. It is generally accepted that ligand-selective maximal

responses by steroid receptors are due to different ligands resulting in different conformational changes of the GR (Bledsoe *et al.*, 2002;Kauppi *et al.*, 2003;Shiau *et al.*, 2002;Padron *et al.*, 2007;Kroe *et al.*, 2007). These conformational changes are believed to be important for the differential recruitment and binding of other proteins, e.g. co-factors (Ronacher *et al.*, 2009) and the basic transcription machinery (BTM), and can thereby form the basis of ligand-selective biological response (Fig. 8.2). Furthermore, according to the tripartite model, these conformational changes allow for allosteric effects on the whole GR-DNA and/or BTM complex (Katzenellenbogen *et al.*, 1996).



**Figure 8.2 Ligand binding induces different GR conformational changes**. It is generally accepted that ligand binding induces different GR conformations. These conformational changes, are proposed to affect the efficacy for transactivation via allosteric effects, for example the recruitment of BTM and other co-factors and form the basis of ligand-selectivity. The particular GR conformation induced with dex, is proposed to allow for more favourable interaction with the BTM or other transcription factors, than the particular GR conformation induced by ald, thereby resulting in a higher efficacy for transactivation. The large square is used to denote a particular conformation of the GR bound with dex (the small red triangle), whereas the large triangle is used to denote a different conformation of the GR bound with ald (small circle). Similarly, the large circle is used to denote a different conformation of the GR bound with RU486 (small square). GRE = glucocorticoid response element; BTM = basic transcription machinery;

Since the same rank order of ligand-selective phosphorylation at S211 and S226 as that for transcription was obtained, these ligand-selective conformational changes may determine the relative extent of S211 and S226 phosphorylation. This would explain the correlations between ligand-selective GR phosphorylation and efficacy for transactivation and transrepression. One possible explanation is that the different ligand-induced conformational changes allow for different extents of interaction between the GR and kinases and/or phosphatases. This in turn could influence the extent of GR phosphorylation in response to a particular ligand (Fig. 8.3). The particular conformation induced by dex may allow for better interaction with kinases (or worse interaction with phosphatases), resulting in more GR phosphorylation (or less GR de-phosphorylation). Accordingly, Bruna *et al.* showed that the extent of GR-JNK interaction is ligand-dependent, with dex-bound GR promoting interaction with JNK while RU486-bound GR fails to induce binding with JNK (Bruna *et al.*, 2003).



**Figure 8.3 Ligand-induced conformational changes are proposed to determine the interaction between the GR and kinases.** According to this model, the particular GR conformation induced by dex, is proposed to allow for a better interaction with kinases, than the particular GR conformation induced by ald, thereby resulting in more GR phosphorylation. In other words the affinity of the kinases of dex-GR is proposed to be higher than for ald-GR. In this example, only the proposed interaction between the GR and kinases is shown. However the model also allows for dex-GR to interact less efficiently with a

phosphatase, thereby having less de-phosphorylation. Similarly, only phosphorylation at S211 is shown, but the same is proposed to happen with GR phosphorylation at S226. Additionally, since the same ligand-selective transactivation pattern is obtained with the S211A mutant, as wt GR, the particular GR conformation induced by the different ligands, are maintained in the S211A mutant. The large square is used to denote a particular conformation of the GR bound with dex (the small red triangle), whereas the large triangle is used to denote a different conformation of the GR bound with ald (small circle). Similarly, the large circle is used to denote a different conformation of the GR bound with RU486 (small square). PS211 = GR phosphorylated at S211.

This thesis shows ligand-selective S226 phosphorylation with a wide panel of ligands, in both COS-1 and U2OS cells. Differences in the extent of ligand-selective phosphorylation on S226 between these two cell lines, with individual compounds, suggest that there are some cell specific differences in the extent of GR phosphorylation. This is the first time that cell-specific differences in S226 phosphorylation with 12 different GR ligands has been shown. The relative amount of specific kinases and/or phosphatases in different cells could modulate differences in GR phosphorylation and result in cell-specific differences. This could form the basis of cell-specific differences in response to GR phosphorylation.

Different conformational changes could also allow for different extents of interaction between the GR and the proteasome. This in turn would influence the extent of GR degradation in response to a particular ligand (Fig. 8.4). The particular conformation induced by dex may allow for better interaction with the proteasome, resulting in more rapid GR degradation. This would explain why a correlation between ligand-selective GR degradation and transcription was obtained although ligand-selective GR degradation does not determine the rank order of ligand-selective transcription, or vice versa. This model would be consistent with the finding that the S211A and S226A phosphorylation mutants have the same ligand-selective pattern of efficacy for transactivation and transrepression, as well as the same ligand-selective pattern of GR

degradation, as compared to wt GR. Furthermore, although it was found that ligand-selective GR phosphorylation correlates with ligand-selective GR degradation, the phosphorylation mutants degraded to a similar extent as wt GR. This implies that the ligand-induced conformational change is maintained in the mutants (Fig. 8.4), consistent with the model.



**Figure 8.4. Ligand-induced conformational changes are proposed to determine the extent of GR degradation.** The ligand-induced conformational changes are proposed to determine the extent of the GR interaction with the proteasome, thereby resulting in ligand-selective GR degradation. In this example, the particular GR conformation induced by dex, would allow for a better interaction between the GR and the proteasome, than the particular GR conformation induced by ald, thereby resulting in a faster rate of GR degradation. In other words the affinity of the proteasome for dex-GR is proposed to be higher than that for ald-GR. Additionally, since the GR phosphorylation mutants showed the same ligand-selective GR degradation, as wt GR, these conformational changes are proposed to be maintained in the GR mutants, thereby resulting in the same relative interaction with the proteasome. The large square is used to denote a particular conformation of the GR bound with dex (the small red triangle), whereas the large triangle is used to denote a different conformation of the GR bound with ald (small circle). Similarly, the large circle is used to denote a different conformation of the GR bound with RU486 (small square). The faded, dashed squares, triangles and circles are used to depict degraded proteins. PS211 = GR phosphorylated at S211; PS226 = GR phosphorylated at S226.

To summarise, different ligand-induced GR conformational changes are proposed to form the basis of ligand-selective transcription response. Thus dex-GR (i) has a higher efficacy for transactivation than ald-GR (ii), which in turn is higher efficacy for transactivation than RU486-

GR (iii) (Fig. 8.5), as is shown in table E5 in addendum E. Transactivation data with the S211A and S226A phosphorylation mutants, lead to the hypothesis that GR phosphorylation at S211 is required for the recruitment of a co-activator ("X"), whereas phosphorylation at S226 is required for the recruitment of a co-repressor ("Y"). This hypothesis is consistent with the S211A and S226A transactivation results with dex, where the S211A mutant had a lower maximal efficacy (iv), while the S226A mutant had a higher maximal efficacy for transactivation (vii) than wt GR (i) (Fig. 8.5). However, these conformations are proposed to be maintained in the phosphorylation mutants, for example with S226A GR, dex-GR (vii) has a higher efficacy for transactivation than ald-GR (iix), which in turn is higher efficacy for transactivation than RU486-GR (ix). This is in agreement with the ligand-selective transactivation results with the S226A GR, presented in this thesis.



**Figure 8.5 Ligand-induced conformational changes are proposed to be maintained in the phosphorylation mutants**. The recruitment of GRIP-1, the co-repressor (Y) or co-activator (X) by wt, S211A or S226A GR is the same as proposed in figure 8.1. The proposed ligand-induced conformational changes, as well as the interaction with the BTM and additional transcription factors to modulate ligand-

selective transactivation is explained in figure 8.2. The large square is used to denote a particular conformation of the GR bound with dex (the small red triangle), whereas the large triangle is used to denote a different conformation of the GR bound with ald (small circle). Similarly, the large circle is used to denote a different conformation of the GR bound with RU486 (small square). GRE = glucocorticoid response element; BTM = basic transcription machinery, PS226 = GR phosphorylated at S226; PS211 = GR phosphorylated at S211. X = unknown co-activator; Y = unknown co-repressor;

## 8.5 Role of agonist-induced hGR phosphorylation in co-factor recruitment

Results presented in this thesis indicate for the first time that phosphorylation of the GR at S211 and S226 is required for the interaction between the GR and the co-activator GRIP-1 (Fig. 7.4). Mutation of either S211 or S226 resulted in a decrease in GRIP-1 binding *in vitro*, while the combined mutation of S211 and S226 resulted in a complete loss in GRIP-1 binding *in vitro* and in whole cells. Whether phosphorylation at S211 is also required for the recruitment of another co-activator (i.e. "X" in the above mentioned model) or whether the decrease in GRIP-1 recruitment with the S211A mutant (Fig. 7.4) can account for the complete loss of dex-mediated transactivation on the MMTV promoter (Fig. 4.10 in Chapter 4), remains to be tested.

Interestingly, from results obtained with GRIP-1 recruitment with the single phosphorylation mutants (Fig. 7.8), it seems that the S226A mutant is the dominant site involved in GRIP-1 recruitment. However, this result is inconsistent with the transactivation data, wherein phosphorylation at S226 inhibits transactivation efficacy. One possible explanation for this inconsistency is that apart from playing a role in GRIP-1 recruitment, phosphorylation on S226 might also be involved in the recruitment of corepressors (i.e. "Y"), as proposed above, resulting in inhibition of transactivation efficacy.

Results from Kino *et al.*, suggest that phosphorylation at S203/S211/S226 inhibits transcriptional activity of the hGR on a MMTV-promoter in HCT116 cells via inhibition of recruitment of the histone acetyltransferase (HAT) co-activator, p300, to the GR bound on a MMTV-luciferase promoter (Kino *et al.*, 2007). In contrast, here it is shown that phosphorylation of S203/S211/S226 is not a major determinant of p300 recruitment, but is required for maximal transcriptional activity on the MMTV-promoter in COS-1 cells and does regulate the maximal efficacy for transactivation by possibly playing a role in GR DNA binding *in vivo*, as well as a major role in the recruitment of the co-factor GRIP-1. Interestingly, phosphorylation of the hGR at S404, by glycogen synthase kinase 3β (GSK3β), was recently shown to be required for the recruitment of CBP/p300 (Galliher-Beckley *et al.*, 2008). It would be interesting to speculate that differential GR phosphorylation, induced by different conformational changes and kinase interactions (as discussed above), is the underlying mechanism for differential co-factor recruitment. Phosphorylation of the GR at specific selected serine residues may be a mechanism for rapid differential gene regulation via increasing or decreasing co-activator, HAT, and/or mediator complex recruitment in a cell- and promoter-specific manner, thereby increasing or decreasing transcription efficacy.



## 8.6 Future perspectives

In this thesis two specific steps in the GR-regulation pathway, that was hypothesised to be involved in ligand-selective effects in gene transcription, were investigated. Although many interesting new finding were obtained, results presented in this thesis raised several new questions and hypotheses for future research.

Results in this thesis, showed cell-specific differences in the results obtained with the COS-1 vs. U2OS cells. When ligand-selective GR phosphorylation at S226 was compared in COS-1 vs. U2OS cells, there was a general trend in both cells that the agonists (classified in the respective cell lines) resulted in the most GR phosphorylation. However, with some ligands, the extent of phosphorylation was different in the two cell lines. For some of these numerical outliers, e.g. the partial agonist MPA, these differences could possibly be explained by differences in GR levels. Accordingly, if the U2OS cells (stably expressing hGR) contained more GR than the COS-1 cells (transiently transfected hGR), the high levels of GR in the U2OS cells could allow the partial agonist to shift to a full agonist, as can be seen in figure D1 in addendum D, as well as in the literature (Zhao *et al.*, 2003;Zhang *et al.*, 2007). This would explain the higher levels of GR phosphorylation in the U2OS cells than in COS-1 cells with these compounds. However, in this study, a direct comparison between the relative GR levels in the two cell lines was never done. Furthermore, some of the numerical differences could not be explained by different GR levels, for example the full agonist prednisolone showed very low levels of S226 phosphorylation in the U2OS cells, as compared to the COS-1 cells. This is very interesting and suggests that other cell-specific differences occur, such as differences in kinases and/or phosphatase expression levels. In addition, results presented in this thesis on dex- and TNF-α-mediated phosphorylation of p38 contradict previous results obtained in the literature in different cells (Pelaia *et al.*, 2001;Jang *et al.*, 2007), suggesting cell-specific differences in kinase activation. Taken together, it seems that GR phosphorylation at S226, as well as the crosstalk between the GR and kinase activation pathways are cell-specific and warrant further investigation. A direct comparison of GR phosphorylation, with similar GR levels, as well as the role of GR in the activation of different kinases, and the relative abundance of kinases and phosphatases in different cells would have to be done in order to determine the molecular mechanisms underlying these cell-specific differences in GR phosphorylation and kinase activity. Phosphorylation of the GR plays an

important role in the recruitment of co-factors and modulating transcription. These cell-specific differences in kinase activity and GR phosphorylation could potentially form the basis of cell-specific differences in GR-mediated transcription. Understanding these cell-specific differences in kinase activity and GR phosphorylation is therefore crucial to further our understanding of GR mechanism of action.

Results presented in this thesis, indicates a role of GR phosphorylation in the recruitment of co-factors. By investigating the role of GR phosphorylation in GR-mediated transactivation, phosphorylation at S226 was hypothesised to be required for the recruitment of a co-repressor to the TAT-GRE and MMTV promoters. Similarly, phosphorylation at S211 was hypothesised to be required for recruitment of a co-activator to the TAT-GRE and MMTV promoters. In this thesis phosphorylation at both S226 and S211 was shown to be required for the recruitment of the co-activator GRIP-1 to the MMTV promoter. However whether phosphorylation at S226 or S211 is required for the recruitment of additional co-repressors or co-activators has not been shown. ChIP experiments with the mutant receptors would have to be done in order to determine if phosphorylation at these two residues is required for the recruitment of additional co-factors, i.e. NCoR or SRC-1, to the TAT-GRE and MMTV promoters.

Another unlikely, but possible hypothesis to explain the effect of GR phosphorylation at S211 and S226 on transactivation, is that GRIP-1 could switch between co-activator and co-repressor depending on the phosphorylation status of S211 and S226. This hypothesis would have to be tested in transactivation studies (on the TAT-GRE and MMTV promoters) by increasing GRIP-1 expression or GRIP-1 knockdown and examining the effect thereof on the efficacy for transactivation via the S211A and S226A mutants. The recruitment of co-factors, via the GR, to

GC-regulated genes modulates the maximal GR-mediated transcriptional response (Stoney Simons, 2003). Understanding the molecular mechanisms underlying co-factor recruitment, and the role of phosphorylation therein, would increase our knowledge of the basic mechanism of GR action, which could lead to a better understanding of inflammation and potentially help with the design of better GC drugs.

In an attempt to determine how GR phosphorylation modulates transactivation, the role of GR phosphorylation in GR acetylation via p300, was investigated. Results presented in this thesis, showed that GR phosphorylation at one or more of residues S226, S211 and S203 is not required for GR acetylation and does not play a major role in the recruitment of p300. However, whether p300 is directly responsible for GR acetylation and the role thereof on GR-mediated transactivation and transrepression, was not determined. The use of HDAC inhibitors, as well as p300 siRNA on GR-mediated transcription and acetylation, as well as ChIP experiments showing p300 recruitment to the GR (in the presence and absence of HDAC inhibitors) should provide a more direct way of determining whether p300 acetylates the GR and whether this post-translation modification plays a role in GR-mediated transcription.



While investigating the role of GR phosphorylation on transrepression, results obtained with the single phosphorylation mutants on AP-1- and NFκB-mediated transrepression suggest that GR phosphorylation inhibits transrepression via a mechanism whereby GR phosphorylation inhibits GR binding to AP-1 and NFκB proteins. The ability of the phosphorylation mutants, as well as wt GR, to bind to NFκB and AP-1 proteins would therefore be interesting to investigate. This could be done by co-immunoprecipitation experiments with wt and mutant GR and NFκB and

AP-1 proteins. Additionally, ChIP experiments showing more phosphorylation mutant GR recruited to the AP-1 and NFκB promoters would also determine if phosphorylation inhibits GR binding to AP-1 and NFκB proteins, thereby resulting in less transrepression.

Another possible explanation for the inhibitory effect of GR phosphorylation at S226 and S211 on transrepression is that GRIP-1 is recruited as a co-activator. Thereby the loss of the co-activator GRIP-1 recruitment would result in more transrepression. However, as mentioned above, GRIP-1 has been shown to act as a co-repressor on an AP-1 luciferase construct (Rogatsky I, 2001), i.e. the presence of GRIP-1 results in more repression in the presence of dex. Therefore, transrepression studies on the AP-1 and NFκB promoters, with increasing amounts of GRIP-1 would first have to be investigated in order to determine if GRIP-1 acts as a co-activator or co-repressor in COS-1 cells on these promoters. Thereafter, recruitment of GRIP-1, by wt and the phosphorylation mutants, to the AP-1 and NFκB promoters would have to be investigated, by means of ChIP assays. Additionally, it is possible that phosphorylation at S211 and S226 is required for, or inhibitory of, the recruitment of other co-factors to the AP-1 and NFκB promoters, thereby inhibiting transrepression. For a better understanding of the role of GR phosphorylation on GR-mediated transrepression, recruitment of additional co-factors, e.g. SRC-1, could be investigated. Furthermore, all of these ChIP assays could be repeated on endogenous genes, in order to verify the results obtained on the synthetic reporter-promoters.

Interestingly, ligand-selective GR-mediated transrepression, as well as the role of GR phosphorylation in transrepression, seems to be dependent on the relative GR levels. To this extent, with wt HA-hGR on the AP-1 promoter, results presented in figure 4.12 showed less repression with prog than with dex. This is consistent with results in the literature (Ronacher *et*

*al.*, 2009). However on the same AP-1 promoter, results in figure 4.13 did not show a big difference between dex- and prog-mediated repression with wt pRS-hGR, indicating that the amount of repression with prog in figure 4.13 was already maximal and that dex could not result in more repression. In addition, in figure 4.12, phosphorylation at S211 or S226 was shown to inhibit maximal repression on the AP-1 promoter, while the combined mutation of S211 and S226 had only a minimal effect in transrepression. Taken together, these results could indicate that the relative GR levels in figure 4.13 (with pRS-hGR) were higher than in figure 4.12 (with HA-hGR), thereby resulting in maximal repression with wt and that an increase in repression with the combined mutant could not be obtained. However, a direct comparison between the relative expression levels of HA-hGR vs. pRS-hGR was not done and would have to be examined in order to determine if increased expression of GR resulted in maximal repression in figure 4.13. Thereafter, the effect of the combined mutation of S211, S226 and S203 on AP-1 mediated transrepression could be repeated at lower GR levels in order to determine more accurately the role of GR concentration in transrepression. The relative expression levels of endogenous GR have been shown to vary considerably between tissue or cell types (Miller *et al.*, 1998). Understanding the molecular mechanisms whereby GR levels influence ligand-selective GR-mediated transrepression, as well as the role of GR phosphorylation in GR-mediated transcription, would significantly enhance our understanding of cell-specific differences in GR-mechanism of action.

Promoter-specific differences in the role of GR phosphorylation in transactivation and transrepression have previously been shown (Webster *et al.*, 1997;Chen *et al.*, 2008). Results presented in this thesis also showed promoter-specific differences in the role of GR phosphorylation in transactivation and transrepression. Although GR phosphorylation at S211 and S226 mediated the same quantitative effect in transactivation via the TAT-GRE and the

MMTV promoter, the relative extent thereof was different on the two promoters. The requirement for S211 phosphorylation for transactivation efficacy, was much more on the MMTV promoter than on the TAT-GRE promoter. In contrast, the inhibitory effect of S226 phosphorylation on transactivation efficacy was much more on the TAT-GRE promoter. Furthermore, on the AP-1 promoter, mutation of S226 resulted in an increase in transcription with CpdA, UDCA and NET. This was not observed on the NFκB promoter, further suggesting promoter-specific differences in the role of GR phosphorylation at S226 in transrepression. In order to shed more light on these promoter-specific differences, a direct comparison with a range of different GC-regulated promoters (for both transactivation and transrepression) with a few selected GR ligands (e.g. a full-, full/partial- and partial agonist, as well as a dissociated ligand and an antagonist) could be performed. This should be done on the synthetic reporter-promoters (used in this study) and should also be confirmed on endogenous genes. Additionally, the composition of the promoters, e.g. 'simple-acting' vs. complex promoters, as well as the additional transcription factors and the co-factors recruited to these promoters, could be further investigated in order to determine the mechanistic details of these promoter-specific differences. This could most easily be performed using different GC-regulated promoters, where the composition has already been determined, and examining the effect of phosphorylation mutants on transcription. Additionally, the recruitment of additional transcription factors and co-factors could be determined by means of ChIP analysis.

When the role of GR phosphorylation at S211 and S226 on transrepression was investigated, it was found that wt GR simulated with CpdA did not result in transrepression (Fig. 4.12). This is contrary to the literature on different genes and in different cells (De Bosscher *et al.*, 2005). It is possible that CpdA-mediated transrepression might be promoter-specific, wherein the specific TATA box sequence in the promoter determines whether CpdA stimulation results in GR-

mediated transrepression or not. Furthermore, De Bosscher *et al.* used TNF-α to induce transcription of the genes, whereas results presented in this thesis were obtained with PMA. Furthermore, De Bosscher *et al.* pre-treated with CpdA before stimulation with TNF-α, whereas results obtained in this thesis were done by pre-treatment with PMA before stimulation with CpdA. These apparent inconsistencies are interesting and may indicate that CpdA can only repress genes that are induced by the TNF-α and not the PMA pathway. On the other hand, these differences could indicate differences in the molecular mechanism of CpdA repression, wherein stimulation with CpdA has to precede induction of the genes, in order for CpdA to result in repression thereof. Since CpdA is currently being investigated as a potential anti-inflammatory drug, because of its ability to distinguish between GR-mediated transactivation and transrepression, it is crucial to determine the basis of the above mentioned inconsistencies. This could easily be done by a direct comparison, in the same cells and on the same promoter, of the ability of CpdA to repress TNF-α or PMA induced genes, wherein induction of the genes precedes or follows CpdA stimulation.

Due to the fact that GR phosphorylation can result in the differential regulation of GC-responsive genes, as discussed above, it is important to determine which kinases is responsible for phosphorylation of the GR *in vivo*. Many publications have reported the use of *in vitro* kinase assays to identify the kinases responsible for phosphorylating the GR *in vitro*. However to date there are only a few publications that have, with limited success, inhibited phosphorylation of endogenous GR *in vivo*. Future studies could include the use of kinase inhibitors for different kinases, other than JNK, p38 and CDK and determining the amount of GR phosphorylation in the presence and absence of the inhibitors. Additionally, different JNK-, p38- and CDK inhibitors, i.e. different catalogue numbers or from different suppliers, could be used. Furthermore, knock-

down of the kinases, by siRNA could also be investigated as a means of inhibiting GR phosphorylation. Additionally, the physiological effects of GR phosphorylation mutants in mice (or in embryonic cells) could also be investigated. Only thereafter, can the role of GR phosphorylation in mediating the interaction between the GR and GRIP-1 (as well as other co-factors), be studied on endogenous genes, with endogenous GR and co-factors. Throughout the thesis most of the results were obtained by using transiently transfected proteins and reporter plasmids. This is not optimal, since it relies on the assumption that the multiple plasmids transfected co-distribute equally, which is not necessarily the case. It is therefore important that these results are confirmed on endogenous genes with endogenous proteins. Verifying the role of GR phosphorylation on co-factor recruitment and the effect on GR-mediated transcription would enhance our understanding of GR function and provide possible "tools" for developing better GC drugs.

The role of GR degradation in GR-mediated transcription was also investigated in this thesis. Results presented here with dex and MPA, as well as in the literature (Stavreva *et al.*, 2004), suggest that proteasomal degradation of the GR influences GR-mediated transactivation in a ligand-specific manner. Furthermore, results presented in this thesis showed various promoter-specific differences in transactivation and transrepression. In order to more accurately determine the effect of proteasome-mediated GR degradation on GR-mediated transcription, the effect of proteasomal inhibition on transcription, with a few selected GR ligands (e.g. a full-, full/partial- and partial agonist, as well as a dissociated ligand and an antagonist), on different GC-responsive transactivation and transrepression promoters would have to be investigated.

Interestingly, results obtained in this thesis on the role of hGR phosphorylation at S211 and S226 in transactivation on the TAT-GRE and MMTV promoters, as well as the role of GR

phosphorylation at S211, S226 and S203 in GR degradation, contradict previous results obtained for the mGR (Webster *et al.*, 1997). These differences do not appear to be due to cell- or promoter-specific differences, since COS-1 cells and the same TAT-GRE and MMTV promoters were used in both studies. The results rather suggest species-specific differences. Rogatsky *et al.* has previously suggested that there are species-specific differences in the role of GR phosphorylation on transactivation (Rogatsky *et al.*, 1998a). However, a direct comparison between the role of hGR phosphorylation at S211 and S226, and the mouse equivalent thereof, in transactivation has not been done. Similarly, the role of hGR and mGR phosphorylation in GR degradation has not been directly compared. These studies warrant further investigation by means of a direct comparison, in parallel, in the same cells and with similar methodological conditions in order to examine the role of species-specific differences in GR phosphorylation and the role thereof in transactivation and GR degradation.

Last, but not least, ligand-binding induces different conformational changes, as determined by GR crystal structures (Bledsoe *et al.*, 2002;Kauppi *et al.*, 2003) which are believed to be important for differential co-factor recruitment and ligand-selective biological responses. Results presented in this thesis are consistent with a model where these different conformational changes induce more and/or less favourable interactions between the GR kinases and/or phosphatases, as well as with the proteasome. Results suggest that these interactions, indirectly result in ligand-selective GR phosphorylation at S211 and S226, as well as ligand-selective GR degradation. Furthermore, results in this thesis show that GR phosphorylation is important for co-factor recruitment. It is likely that ligand-selective GR phosphorylation could form the basis for differential co-factor recruitment. However, whether ligand-induced conformational changes form different interactions between the GR and kinases and/or phosphatases, as well as the proteasome, thereby resulting in ligand-selective GR phosphorylation and degradation, has not

been shown. In order to accurately determine this, ligand-selective interactions between the GR and different kinases and/or phosphatases, as well as the proteasome, would first have to be shown. This can be done by means of co-immunoprecipitation experiments. Furthermore, these ligand-dependent interactions would then have to be shown to be a direct consequence of different ligand-induced GR conformational changes. This would require computer analysis of specific three dimensional crystal structures of the GR bound by the different ligands, as well as the kinases and/phosphatases, or the proteasome. Since the crystal structure of the whole GR protein has not been solved and crystal structures of the LBD of the GR in complex with only a few GR ligands are known, this would be more difficult and would require more investigation.

Taken together, variations in receptor and co-factor concentration, as well as GR phosphorylation, modulates GR-mediated transcriptional regulation, which has significant clinical implications (Reviewed in (Stoney Simons, 2003)). For endogenous hormones, variations in receptor and co-factor concentration, as well as GR phosphorylation are likely to be beneficial, as they could account for some of the promoter- and cell-specific differences seen on GR mechanism of action. These promoter- and cell-specific differences could provide for differential gene induction, in different cellular environments, by the same endogenous hormone (Reviewed in (Stoney Simons, 2003)). However, these promoter- and cell-specific differences in GR mechanism of action are likely to contribute to the various side-effects observed with pharmacologically administered GCs and complicate the design of better GC drugs. In order to better predict the biological outcome of pharmacological drugs, an improved understanding of the fundamental mechanisms and determinants of promoter-, cell- and ligand-specific differences in GR mechanism of action, is required.

# Addendum A Basic principles for evaluating transcriptional responses

To evaluate the transcriptional response mediated via steroid receptors and their ligands, some basic principles and terminology are used. Steroid receptor-ligand complexes are characterised by three parameters, namely affinity, potency and efficacy, each of which can be measured quantitatively in any given cell system.

The strength, with which a particular ligand binds to its receptor, is the affinity of the ligand for the receptor. The receptors bind their corresponding ligands according to the laws of mass action, which is a kinetic process in which ligand molecules bind to and dissociate from the receptor at defined rates (Fig. A1). The fraction of the receptor occupied by ligand at any given time is dependent on the relative rates of onset ($k_{on}$) and offset ($k_{off}$) of ligand attachment to the receptor. When the amount of ligand molecules that bind the receptor is equal to the amount of molecules that dissociate from the receptor, the reaction has reached equilibrium. At equilibrium, the concentration of ligand that results in half the receptors being occupied, is called the equilibrium dissociation constant, $K_d$ (Reviewed in (Neubig *et al.*, 2003)).



**Figure A1 Kinetics of ligand binding to the GR.** The affinity of a ligand for its receptor is determined by the association and dissociation rates as the $K_d$ equals the ratio of $K_{on}$ / $K_{off}$

The $K_d$ can be determined by different experimental procedures. For example, the $K_d$ can be determined via kinetic experiments, wherein the association ($K_{on}$) and dissociation ($K_{off}$) rates are determined. Specific binding of a fixed concentration of radiolabelled ligand is measured over time. It is important that the law of mass action applies. Therefore, it is more accurate to determine association rates at different radiolabelled concentrations. If the law of mass action applies, a linear increase in association rates will be observed. $K_d$ can then be calculated from the measured $K_{on}$ and $K_{off}$ values ($K_d = K_{on}/K_{off}$).

Additionally, the $K_d$ can be determined by saturation binding experiments (Fig. A2). Although more tedious and expensive, saturation binding curves are thought to be more accurate. In these experiments, specific binding of increasing amounts of radiolabelled ligand is measured at equilibrium. By subtracting the amount of non-specific binding (with very high levels of non-radiolabelled ligand) from the total amount of binding that is measured, the specific binding of the radiolabelled compound is determined. Thereafter the amount of specific binding is plotted against ligand concentration and the $K_d$ can be determined.

**Figure A2 Determining the Kd from saturation binding experiments.** The $K_d$ is taken as the concentration of ligand (nM) needed to result in 50% binding. Taken and modified from GraphPad Prism 5 manual.

Experimentally, it is also important to determine the $K_d$ of other ligands, of which no radiolabelled form is available, for the same receptor. Also called heterlogous competive binding, two ligands compete for binding on the same receptor where the radiolabelled ligand and the competing ligand are different. In these assays, increasing concentrations of the desired ligand (unlabelled) is used to compete with binding of the radiolabelled reference ligand. This type of experiment is used to determine relative affinities (these are not constants) (IC50 or Ki's) of various different types of ligands. The IC50 value is the concentration of unlabelled ligand that is needed to displace 50% of the radiolabelled ligand from the receptor (Fig. A3). Relative binding affinities (RBAs) are commonly used in the literature and are calculated as a percentage of the IC50 value of the reference ligand, often used [RBA = IC50 (ref ligand) / IC50 (ligand of interest) x 100]. However, these IC50 values are not true dissociation constants, since the values will vary depending on the concentration of radiolabelled ligand and receptors used in the study. When the concentration of radiolabelled ligand is less than half the IC50, it is possible to determine a true equilibrium dissociation constant Ki with a combination of homologous and

heterologous competition binding curves. The Ki value is the concentration of competing ligand that will bind to half the receptor at equilibrium (Cheng and Prusoff, 1973).

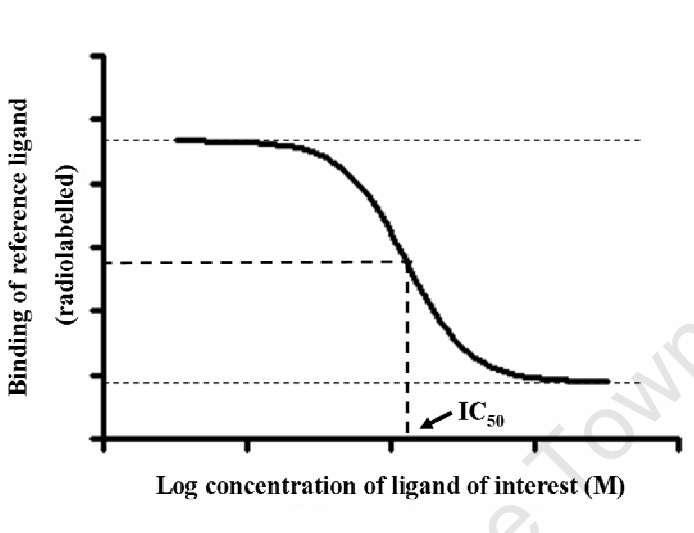



**Figure A3 A schematic representation of a competitive binding curve.** Binding affinity is expressed as the concentration of unlabelled ligand (of interest) needed to displace 50% of the radiolabelled ligand from the receptor and is measured in molar units (IC50 value), if the x-axis is given in M. Taken and modified from GraphPad Prism 5 manual.

Fractional occupancy describes the relative receptor occupancy for any given ligand at equilibrium as a function of the ligand concentration and the $K_d$ of the specific ligand (Fig. A4). When there is no ligand present the fractional occupancy of the receptor will be zero. At saturating concentrations of ligand, i.e. where the ligand concentration is much higher than the $K_d$, the fractional occupancy is close to 100%. Furthermore, when the ligand concentration used is equal to the $K_d$, the fractional occupancy will be 50%. Fractional occupancy is often used when comparing different ligands at sub-saturating concentrations of ligand.

**Figure A4 The equation used to calculate fractional occupancy.** This equation assumes equilibrium.

Efficacy is used to describe and quantify the maximal transcriptional response for a given ligand (Fig. A5). When a full agonist is used as a reference ligand and the efficacy of the other ligands are calculated as a percentage of the efficacy of the reference ligand (which is set to 100%), the term relative efficacy is used. In figure A5 ligand A is the reference ligand, a full agonist with a relative efficacy set to 100%. Ligand B has the same relative efficacy as ligand A and is therefore also a full agonist, whereas ligand C has a relative efficacy much lower than ligand A, but still gives a response, and is therefore a partial agonist (Reviewed in (Neubig *et al.*, 2003)).



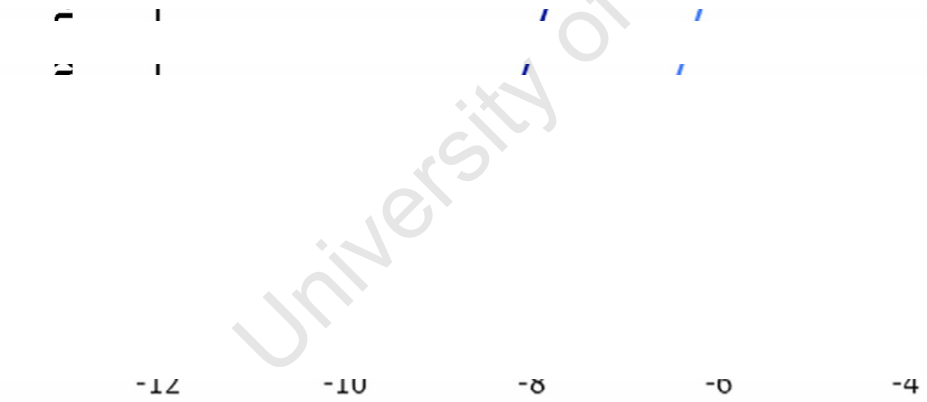



**Figure A5 Efficacy and potency**. Taken and modified from http://glutxi.umassmed.edu/lectures/dynamics.pdf

Potency is used to describe and quantify the molar concentration of ligand needed to elicit half of the possible maximal response (50% of maximal response) for that particular ligand (Fig. A5).

The lower the concentration of ligand needed to give half the maximal response, the more potent the ligand. Potency is also referred to as the EC50 value, when the potency is taken by reading the concentration of ligand, at 50% of the maximal response, from a sigmoidal dose-response curve (a $\log_{10}$ scale of ligand concentration plotted against percentage response). The relative potency is the ratio of the potency of a specific ligand to that of a standard reference ligand. In figure A5 ligand A has a smaller numerical EC50 value than ligand B, and is therefore more potent than ligand B, whereas ligand C has a similar numerical EC50 value, as ligand A, and is therefore as potent as ligand A (Reviewed in (Neubig *et al.*, 2003)).

For the purpose of this thesis, correlation analysis between the GR transcriptional response and other steps in the GR pathway will be performed. This is done by performing a Pearson's correlation analysis (GraphPad Prism 5 manual) of the numerical values (potency (expressed as – log [ligand in nM]) and efficacy (expressed as % relative to agonist dex) of the transcriptional response with the numerical values obtained for another step in the GR pathway. In figure A6, numerical data obtained for step A (e.g. efficacy for transactivation) is correlated with the numerical data obtained for step B (e.g. nuclear translocation of the receptor) using GraphPad Prism 5 correlation analysis. From the correlation analysis a $R^2$ value, or the coefficient of determination which ranges from zero to 1, is given. This is the fraction of variance that is shared in the two steps, for example a $R^2 = 0.88$ means that 88% of the variance in step A can be explained by variation in step B, or vice versa. In addition, correlation analysis also calculates the p value that indicates if the $R^2$ value is statistically significant. Furthermore, this correlation analysis does not assume linearity, however it is slightly influenced thereby (GraphPad Prism 5 manual). In the above mentioned example, if the numerical values from step A are correlated with the numerical values from $B^2$, an even better correlation with a $R^2 = 0.9143$ ($p < 0.0001$) is obtained.

**Figure A6 An example of the correlation analysis**. Numerical data obtained from step A is correlated with numerical data obtained from step B, using Pearson's correlation analysis by GraphPad Prism 5 software. The data used in this figure was not determined experimentally, but is hypothetical data to illustrate the point.

However, a good correlation (i.e. $R^2$ close to 1) between step A and step B, does not necessary mean that step A determines step B, or vice versa. It could also mean, that both step A and step B are equally influenced by another step, e.g. step X (ligand-induced conformational change of the receptor), and that step A and step B are not functionally linked to each other. In this example, the ligand-induced conformational change of the receptor could influence the ability of the receptor to result in nuclear translocation, as well as efficacy for transactivation. Therefore, since the same trend in ligand-induced conformational change is "mimicked" in nuclear translocation and efficacy for transactivation, a good statistically significant correlation between nuclear translocation and efficacy for transactivation will be obtained, even though the one does not determine or influence the other.

# Addendum B Panel of GR ligands

## A.1 Full agonists

### A.1.1 Dexamethasone (dex)

Dex is a potent, steroidal, synthetic GR agonist and since its synthesis in 1959, dex has been used in the treatment of a variety of diseases, including rheumatoid arthritis and cancer (Fig. B1) (Stein *et al.*, 1999;Cook *et al.*, 2004;Richardson *et al.*, 2005;Koutsilieris *et al.*, 2004). Dex is the most commonly used GR agonist, with a strong relative binding affinity for the GR, $K_d$ ranging between 5 and 10 nM, and a potent agonist for transactivation, EC50 ranging between 1 and 10 nM, depending on the cell system, promoter and the amount of GR used in the studies (Stubsrud, 2005;Driver *et al.*, 2001;Then Bergh *et al.*, 1999;Charmandari *et al.*, 2005;Hammer *et al.*, 2003;Attardi *et al.*, 2004;Muller *et al.*, 2004;Einstein *et al.*, 2004;Smit *et al.*, 2005). In addition, dex is also a potent agonist for transrepression, with an EC50 ranging between 0.05 nM and 15 nM (Stubsrud, 2005;Zhao *et al.*, 2003;Adcock *et al.*, 1999;Einstein *et al.*, 2004).

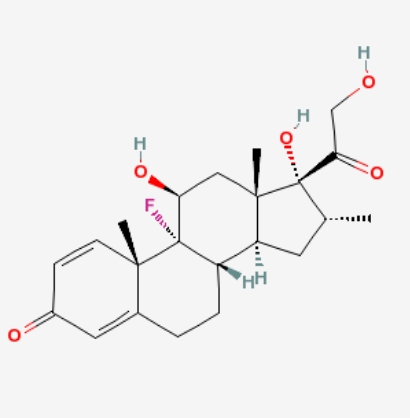


**Figure B1 Structure of dexamethasone** [(9-fluoro-11,17-dihydroxy-17-(2-hydroxyacetyl)-10,13,16-trimethyl-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydrocyclopenta[a]phenanthren-3-one)]. Structure from http://pubchem.ncbi.nlm.nih.gov

In COS-1 cells, with the hGR on the TAT-GRE promoter, dex has an EC50 of 0.23 nM and a relative binding affinity of 14 nM (IC50) ((Ronacher *et al.*, 2009); Tables E5 and E4 in addendum E). Furthermore, in the same cell system, dex has an EC50 of 0.005 nM and 0.003 nM on the NFκB and AP-1 promoters, respectively ((Ronacher *et al.*, 2009); Table E5 in addendum E). Additionally, dex bound-GR recruited the co-activators GRIP-1, TIF-2 and SRC-1 (~75%), as well as the co-repressors NCoR( ~25%) and SMRT (~150%), as compared to RU486 (100%), in a mammalian two-hybrid assay (Ronacher *et al.*, 2009).

### A.1.2 Cortisol (cort)

Cortisol is the endogenous GR ligand produced in humans and its production is under control of the HPA axis (Fig. B2). Cortisol is secreted into the blood stream in a continuous pulsatile pattern at a frequency of approximately 20 pulses per day which forms the basis of the diurnal rhythm in humans (Windle *et al.*, 1998;Lightman *et al.*, 2008). Excessive and insufficient production of cortisol causes Cushing's and Addison's disease, leading to diabetes and cardiovascular

disorders, respectively (Whitworth *et al.*, 2000;Lovas and Husebye, 2003). Cortisol has a high binding affinity for the GR and is a potent GR agonist for transactivation and transrepression, with an EC50 ranging between 10 and 40 nM, depending on the system being investigated (Stubsrud, 2005;Lind *et al.*, 2000;Mulatero *et al.*, 1997;Grossmann *et al.*, 2004;Lim-Tio *et al.*, 1997;Rebuffat *et al.*, 2004;Muller *et al.*, 2004;Zhao *et al.*, 2003).

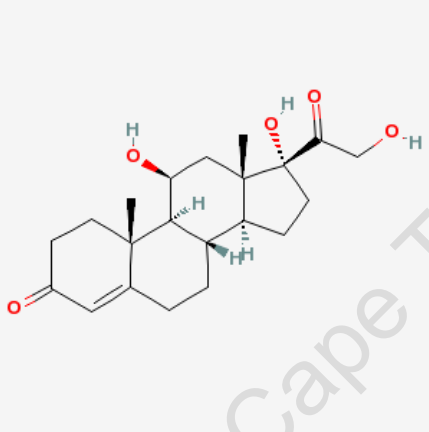


**Figure B2 Structure of cortisol** [11,17-dihydroxy-17-(2-hydroxyacetyl)-10,13-dimethyl-1,2,6,7,8,9,10,11,12, 13,14,15,16,17-tetradecahydrocyclopenta[a]phenanthren-3-one]. Structure from http://pubchem.ncbi.nlm.nih.gov

In COS-1 cells, with the hGR on the TAT-GRE promoter, cort has an EC50 of 16 nM for transactivation and a relative binding affinity of 152 nM (IC50) ((Ronacher *et al.*, 2009); Tables E5 and E4 in addendum E). Furthermore, in the same cell system, cort has an EC50 of 7 nM and 2 nM on the NFκB and AP-1 promoters, respectively ((Ronacher *et al.*, 2009); Table E5 in addendum E). Additionally, cort bound-GR recruited the co-activators GRIP-1 (~80%), TIF-2 (~80%), SRC-1 (~90%), as compared to dex (100%), while it recruited the co-repressors NCoR (~30%) and SMRT (~150%), as compared to RU486 (100%), in a mammalian two-hybrid assay (Ronacher *et al.*, 2009).

### A.1.3 Prednisolone (predn)

Predn is a potent, steroidal, synthetic GR agonist and is mainly used for treatment of asthma and rheumatic disorders (Fig.B3) (Qureshi *et al.*, 2001;Haugeberg *et al.*, 2004). Predn has a high binding affinity for the GR, with an IC50 ranging between 20 and 85 nM and is a potent GR agonist for transactivation and transrepression, with an EC50 ranging between 1 to 200 nM and 5 to 20 nM, respectively, depending on the system being investigated (Stubsrud, 2005;Ko *et al.*, 2000;Ko *et al.*, 2002;Schacke *et al.*, 2004;Lind *et al.*, 2000;Smit *et al.*, 2005;Ali A *et al.*, 20040;Grossmann *et al.*, 2004).



**Figure B3 Structure of prednisolone** [11,17-dihydroxy-17-(2-hydroxyacetyl)-10,13-dimethyl-6,7,8,9,10,11, 12,13,14,15,16,17-dodecahydrocyclopenta[a]phenanthren-3-one]. Structure from http://pubchem.ncbi.nlm.nih.gov

In COS-1 cells, with the hGR on the TAT-GRE promoter, predn has an EC50 of 0.1 nM and a relative binding affinity of 68 nM (IC50) ((Ronacher *et al.*, 2009); Tables E5 and E4 in addendum E). Furthermore, in the same cell system, predn has an EC50 of 0.05 nM and 0.002 nM on the NFκB and AP-1 promoters, respectively ((Ronacher *et al.*, 2009); Table E5 in addendum E). Additionally, predn bound-GR recruited the co-activators GRIP-1 (~100%), TIF-2 (~100%), SRC-1 (~110%), as compared to dex (100%), while it recruited the co-repressors

NCoR (~50%) and SMRT (~150%), as compared to RU486 (100%), in a mammalian two-hybrid assay (Ronacher *et al.*, 2009).

## A.2 Full/partial agonists

### A.2.1 Aldosterone (ald)

Aldosterone (ald) is the endogenous mineralocorticoid and was first isolated in 1953 (Fig. B4) (Reviewed in (Williams and Williams, 2003). Ald is produced by the adrenals and the level of circulating ald is monitored by the kidneys. Ald regulates the absorption of sodium and potassium, mostly in the kidney, and thus regulates blood pressure and heart function. Furthermore, ald can bind to, and act via, both the GR and the mineralocorticoid receptor (Williams and Williams, 2003;Heymes *et al.*, 2004). Ald has a low affinity for the GR, with a $K_d$ ranging from 15 to 290 nM, and has partial agonist activity for transactivation, with EC50s ranging from 150 to 500 nM (Stubsrud, 2005;Martinez *et al.*, 2005;Hellal-Levy *et al.*, 1999;Grossmann *et al.*, 2004;Rebuffat *et al.*, 2004).

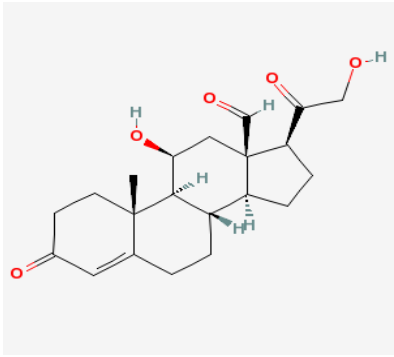


**Figure B4 Structure of aldosterone** [11-hydroxy-17-(2-hydroxyacetyl)-10-methyl-3-oxo1,2,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydrocyclopenta[a]phenanthrene-13-carbaldehyde]. Structure from http://pubchem.ncbi.nlm.nih.gov

In COS-1 cells, with the hGR on the TAT-GRE promoter, ald has an EC50 of 138 nM and a relative binding affinity of 1130 nM (IC50) ((Ronacher *et al.*, 2009); Tables E5 and E4 in addendum E). Furthermore, in the same cell system, ald has an EC50 of 27 nM and 18 nM on the NFκB and AP-1 promoters, respectively ((Ronacher *et al.*, 2009); Table E5 in addendum E). Additionally, ald bound-GR recruited the co-activators GRIP-1 (~90%), TIF-2 (~100%), SRC-1 (~130%), as compared to dex (100%), while it recruited the co-repressors NCoR (~50%) and SMRT (~125%), as compared to RU486 (100%), in a mammalian two-hybrid assay (Ronacher *et al.*, 2009).



### A.2.2 Medroxyprogesterone acetate (MPA)

MPA is a steroidal, synthetic progestin used in contraception, as the injectable Depo Provera, as well as in hormone replacement therapy (Fig. B5) (Kaunitz, 1998;Blossey *et al.*, 1984;Yamashita *et al.*, 1996;Affandi, 2002;Brunelli *et al.*, 1996;Irahara *et al.*, 2001;Taitel and Kafrissen, 1995). Both MPA and another synthetic progestin, NET, are used in preference to the natural progestin, progesterone, because of their increased half-life and bioavailability. However, the use of these

synthetic progestins have been shown to correlate with severe side-effects, such as such as increased risk of cardiovascular complications, stroke and breast cancer (Beral, 2003;McKenzie *et al.*, 2004;Rossouw *et al.*, 2002;Valdivia *et al.*, 2004). MPA has been shown bind to the GR and agonist or partial agonist activity for GR-mediated transactivation has also been shown for MPA, with a potency ranging from about 10 nM to 90 nM (Stubsrud, 2005;Koubovec *et al.*, 2005;Kontula *et al.*, 1983;Tabata *et al.*, 2003;Zhang *et al.*, 2000b;Koubovec *et al.*, 2004). In addition, MPA has also been shown to have agonistic activity in transrepression, with EC50 values varying from about 2.5 nM to 90 nM (Stubsrud, 2005;Koubovec *et al.*, 2004;Koubovec *et al.*, 2005;Zhao *et al.*, 2003).

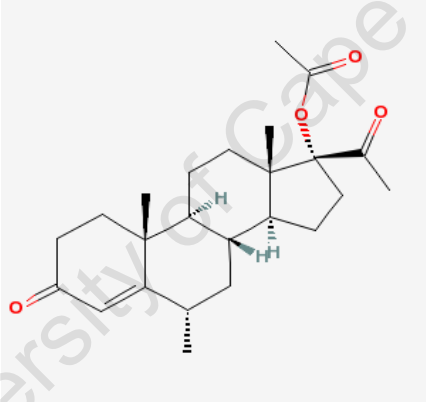


**Figure B5 Structure of MPA** [acetic acid (17-acetyl-6,10,13-trimethyl-3-oxo-1,2,6,7,8,9,10,11,12,13,14,15,16,17-tetra-decahydrocyclopenta[a]phenanthren-17-yl)ester]. Structure from http://pubchem.ncbi.nlm.nih.gov

In COS-1 cells, with the hGR on the TAT-GRE promoter, MPA has an EC50 of 2 nM and a relative binding affinity of 19 nM (IC50) ((Ronacher *et al.*, 2009); Tables E5 and E4 in addendum E). Furthermore, in the same cell system, MPA has an EC50 of 0.7 nM and 0.0005 nM on the NFκB and AP-1 promoters, respectively ((Ronacher *et al.*, 2009); Table E5 in addendum E). Additionally, MPA bound-GR recruited the co-activators GRIP-1 (~75%), TIF-2 (~75%), SRC-1 (~100%), as compared to dex (100%), while it recruited the co-repressors NCoR

(~25%) and SMRT (~25%), as compared to RU486 (100%), in a mammalian two-hybrid assay (Ronacher *et al.*, 2009).

## A.3 Partial agonists

### A.3.1 Abbott-Ligand 438 (AL438)

AL438 is a potent, synthetic, non-steroidal agonist for selective promoters, in both GR-mediated transactivation and transrepression (Fig. B6) (Coghlan *et al.*, 2003). AL438 has a strong binding affinity for the GR, Ki 2.5 nM and is a partial agonist for transactivation and transrepression, with an EC50 from 500 to 800 nM and 10 to 60 nM, respectively (Coghlan *et al.*, 2003). Additionally, AL438 bound-GR binds the co-activator GRIP-1 to a similar extent as the GR agonist prednisolone, whereas it binds the co-activator PGC-1 less effectively than prednisolone (Coghlan *et al.*, 2003).



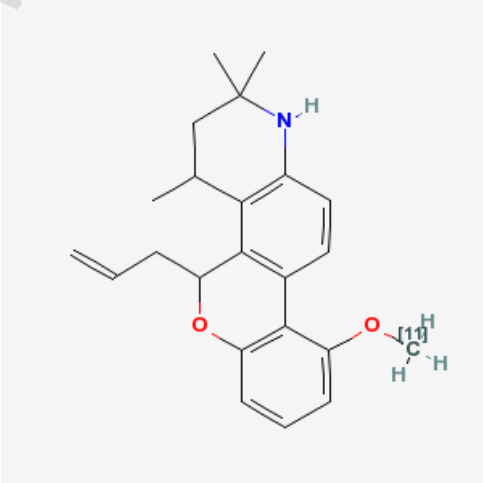



**Figure B6 Structure of AL438** [2, 5-dihydro-10-methoxy-5-(2-propenyl)-2, 2, 4-trimethyl-1H-[1] benzo- pyrano [3, 4-f] quinoline]. Structure from http://pubchem.ncbi.nlm.nih.gov

In COS-1 cells, with the hGR on the TAT-GRE promoter, AL438 has an EC50 of 8 nM and a relative binding affinity of 61 nM (IC50) ((Ronacher *et al.*, 2009); Tables E5 and E4 in addendum E). Furthermore, in the same cell system, AL438 has an EC50 of 13 nM and 0.05 nM on the NFκB and AP-1 promoters, respectively ((Ronacher *et al.*, 2009); Table E5 in addendum E). Additionally, AL438 bound-GR recruited the co-activators GRIP-1 only (~50%), TIF-2 (~30%), SRC-1 (~75%), as compared to dex (100%), while it recruited the co-repressors NCoR (~25%) and SMRT (~125%), as compared to RU486 (100%), in a mammalian two-hybrid assay (Ronacher *et al.*, 2009).

### A.3.2 Progesterone (prog)

Progesterone (Prog) is the endogenous hormone for the progesterone receptor (PR) and in women is produced primarily in the ovaries, adrenal glands and the placenta (during pregnancy) (Fig. B7). Prog plays an important role in maintaining the monthly menstrual cycle, pregnancy and the nervous- and cardiovascular systems (reviewed in (Panay and Studd, 1997). Recently, prog has also been implicated in playing a protective role in brain function and traumatic brain injury (Roof and Hall, 2000;Gibson *et al.*, 2008;Wright *et al.*, 2007;Pan *et al.*, 2007). Prog has a weak affinity for the GR, with a Ki ranging from 95 to 215 nM, and has been shown to switch between partial agonist activity and no activity for transactivation and transrepression, with EC50s between 280 to 930 nM and 26 to 470 nM, respectively, depending on the system and amount of GR in the assay (Stubsrud, 2005;Koubovec *et al.*, 2004;Koubovec *et al.*, 2005;Selman *et al.*, 1996;Bamberger *et al.*, 1999;Kurebayashi *et al.*, 2003).

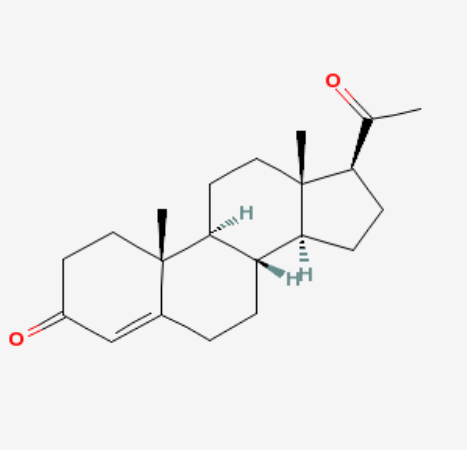


**Figure B7 Structure of progesterone** [17-acetyl-10,13-dimethyl-1,2,6,7,8,9,10,11,12,13, 14,15,16,17-tetra-decahydrocyclopenta[a]phenanthren-3-one]. Structure from http://pubchem.ncbi.nlm.nih.gov

In COS-1 cells, with the hGR on the TAT-GRE promoter, prog has an EC50 of 1688 nM and a relative binding affinity of 274 nM (IC50) ((Ronacher *et al.*, 2009); Tables E5 and E4 in addendum E). Furthermore, in the same cell system, prog has an EC50 of 384 nM and 0.004 nM on the NFκB and AP-1 promoters, respectively ((Ronacher *et al.*, 2009); Table E5 in addendum E). Additionally, prog bound-GR recruited the co-activators GRIP-1 only (~50%), TIF-2 (~60%), SRC-1 (~85%), as compared to dex (100%), while it recruited the co-repressors NCoR (~20%) and SMRT (~80%), as compared to RU486 (100%), in a mammalian two-hybrid assay (Ronacher *et al.*, 2009).

## A.4 Dissociated GCs

### A.4.1 Roussel-Uclaf 38486 (RU486)

RU486, also know as mifepristone, is a potent, steroidal antagonist or partial agonist, depending on the cell system, and has a very high binding affinity for the GR, as well as the PR (Fig. B8). By antagonizing the effects of progesterone, which is needed for pregnancy, RU486 terminates pregnancy and is therefore used in emergency contraception and abortions (Cadepond *et al.*, 1997). RU486 also has a very high binding affinity for the GR roughly 4 times as high as dex, $K_d$ 0.68, and is a strong agonist for transrepression, but not transactivation, with an EC50 for transrepression ranging from 0.3 to 1 nM (Stubsrud, 2005;Wagner *et al.*, 1999;Attardi *et al.*, 2004;Einstein *et al.*, 2004;Zhao *et al.*, 2003).

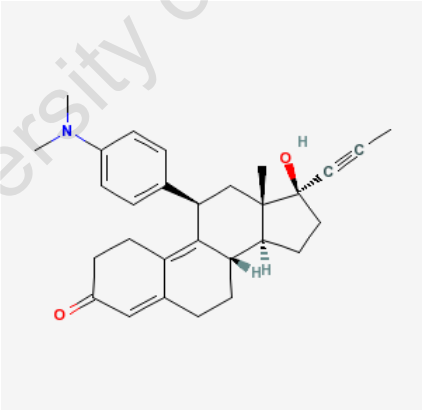



**Figure B8 Structure of RU486** [11-(4-dimethylaminophenyl)-17-hydroxy-13-methyl-17-prop-1-ynyl-1,2,6,7,8,11,12,13,14,15,16,17-dodecahydrocyclopenta[a]phenanthren-3-one]. Structure from http://pubchem.ncbi.nlm.nih.gov

In COS-1 cells, with the hGR on the TAT-GRE promoter, RU486 does not show any activity, but has a relative binding affinity of 1 nM (IC50) ((Ronacher *et al.*, 2009); Tables E5 and E4 in addendum E). Furthermore, in the same cell system, RU486 has an EC50 of 0.008 nM and 0.004

nM on the NFκB and AP-1 promoters, respectively ((Ronacher *et al.*, 2009); Table E5 in addendum E). Additionally, RU486 bound-GR did not recruit the co-activator TIF-2, while it recruited the co-activators GRIP-1 and SRC-1 only (~20%) and (~40%), respectively, as compared to dex (100%). The corepressor NCoR was very strongly recruited with RU486 bound-GR, while it recruited the co-repressor SMRT only (~60%), as compared to dex (100%), in a mammalian two-hybrid assay (Ronacher *et al.*, 2009).

### A.4.2 Compound A (CpdA)

CpdA, is a synthetic non-steroidal analogue of a plant derivative that was developed at the University of Stellenbosch in the Department of Biochemistry in 1997 (Fig. B9) (Louw *et al.*, 1997). CpdA interacts with steroidogenic enzymes and steroid-binding globulins and has been shown to have anti-androgenic and anti-progestogenic properties (Louw *et al.*, 2000;Louw A, 2000;Tanner *et al.*, 2003). More recently, CpdA has been shown to compete with dex for binding to the GR and initiate GR nuclear translocation, but not GR dimerisation, and to repress NFκB-mediated, but not AP-1, mediated gene expression in the presence of the GR (De Bosscher *et al.*, 2005;Dewint *et al.*, 2008). Furthermore, CpdA does not cause GR-mediated transactivation and is a potential anti-inflammatory drug (De Bosscher *et al.*, 2005;Dewint *et al.*, 2008).

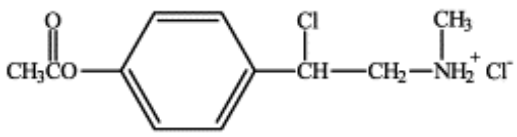



**Figure B9 Structure of CpdA** [2-(4-acetoxyphenyl)-2-chloro-*N*-methyl-ethylammonium chloride]. Structure from (Tanner *et al.*, 2003).

In COS-1 cells, with the hGR on the TAT-GRE promoter, CpdA does not show any activity, but has a relative binding affinity of 0.003 nM (IC50), and shows an atypical binding curve ((Ronacher *et al.*, 2009); Tables E5 and E4 in addendum E). Furthermore, in the same cell system, CpA has an EC50 of 1543 nM on the NFκB promoter and no activity on the AP-1 promoter ((Ronacher *et al.*, 2009); Table E5 in addendum E). Additionally, CpdA bound-GR did not recruit the co-activators GRIP-1, TIF-2 or SRC-1, and only minimally recruited the co-repressors NCoR and SMRT, in a mammalian two-hybrid assay (Ronacher *et al.*, 2009).



## A.5 Antagonists

### A.5.1 Norethisterone acetate (NET)

NET is a steroidal synthetic progestin and is widely used in contraception, as injectable Noristerat, and hormone replacement therapy (Fig. B10). It has previously been shown to compete with dex for binding to the GR (Koubovec *et al.*, 2005) and has a very weak affinity for the GR, Ki 270 nM, with no activity for transactivation and very weak partial activity for transrepression via the GR (Koubovec *et al.*, 2005). NET has also been shown to antagonise dex-mediated transactivation (D. Africander Unpublished data).

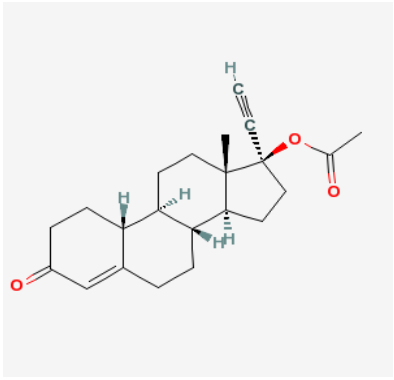


**Figure B10 Structure of NET** [acetic acid (17-ethynyl-13-methyl-3-oxo-1,2,6,7,8,9,10,11,12,13,14,15,16,17tetra-decahydrocyclopenta[a]phenanthren-17-yl)ester]. Structure from http://pubchem.ncbi.nlm.nih.gov

In COS-1 cells, with the hGR, NET does not show any activity in transactivation or transrepression on the TAT-GRE, NFκB- or AP-1 promoters, and has a very low relative binding affinity of 1688 nM (IC50) ((Ronacher *et al.*, 2009); Tables E5 and E4 in addendum E). Additionally, NET bound-GR did not recruit the co-activators GRIP-1, TIF-2 and SRC-1, or the co-repressors NCoR and SMRT, in a mammalian two-hybrid assay (Ronacher *et al.*, 2009).

### A.5.2 Abbott-Ligand 082D06 (D06)

DO6 is a synthetic non-steroidal ligand that binds the GR with low affinity (Ki 210 nM), has been shown to antagonize the effects of dex-mediated GR transactivation and transrepression, has no known agonist activity, does not induce GR nuclear translocation and does not induce DNA binding by GR *in vitro* or *in vivo* (Fig. B11) (Miner *et al.*, 2003). D06 has also been shown to antagonise dex-mediated transactivation (Miner *et al.*, 2003).

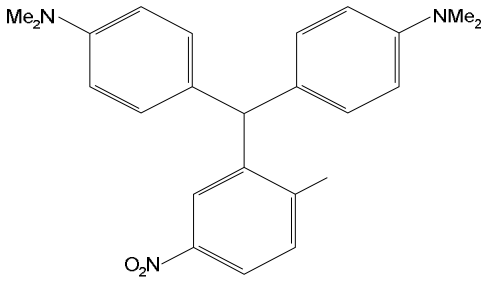



**Figure B11 Structure of D06** [bis (4-N, N-dimethylaminophenyl) (2-chloro-5-nitrophenyl) methane]. Taken from (Ronacher *et al.*, 2009).

In COS-1 cells, with the hGR DO6 does not show any activity in transactivation or transrepression on the TAT-GRE, NFκB- or AP-1 promoters, and has a very low relative binding affinity of 6500 nM (IC50) ((Ronacher *et al.*, 2009); Tables E5 and E4 in addendum E). Additionally, DO6 bound-GR did not recruit the co-activators GRIP-1, TIF-2 and SRC-1, or the co-repressors NCoR and SMRT, in a mammalian two-hybrid assay (Ronacher *et al.*, 2009).

In this study, DO6 was used for the initial ligand-selective S226 phosphorylation studies, but due to limited availability, not all experiments in this thesis included DO6.

### A.5.3 Ursodeoxycholic acid (UDCA)

UDCA is a hydrophilic bile acid used for dissolution of gallstones and chronic liver diseases (Fig. B12). UDCA behaves like a weak partial agonist in GR-mediated transactivation with an efficacy of only 10% as compared to dex (Weitzel *et al.*, 2005;Tanaka *et al.*, 1996). However UDCA behaves like a full agonist for GR-mediated transrepression with an efficacy of 100% as compared to dex (Miura *et al.*, 2001).

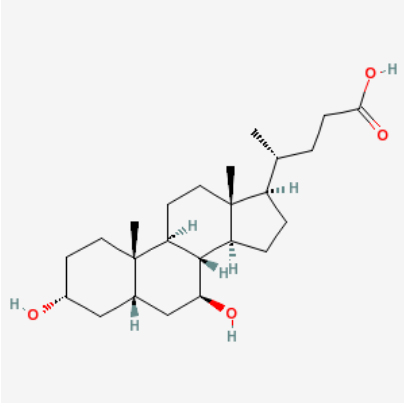


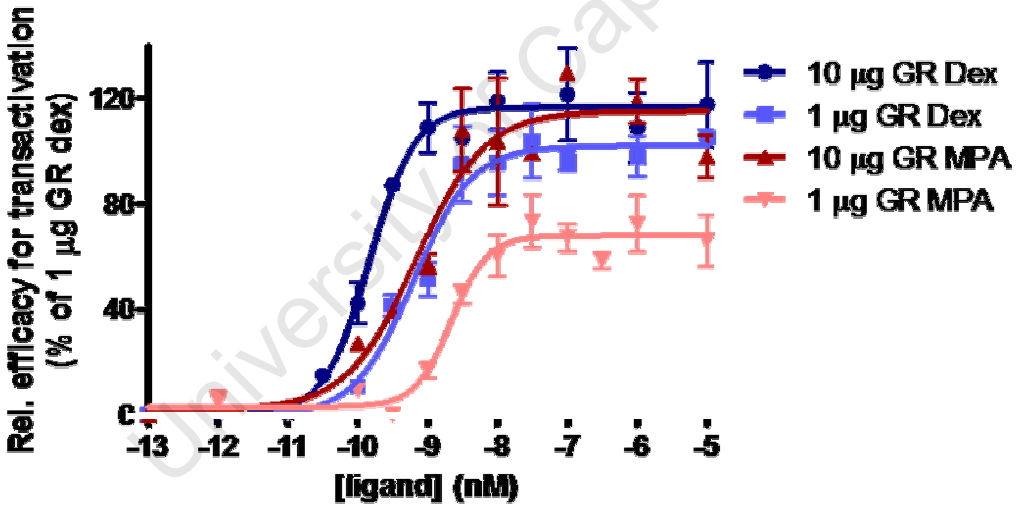
**Figure B12 Structure of UDCA** [4-[(3,7-dihydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17-hexadecahydro-1H-cyclopenta[a]phenanthren-17-yl)]pentanoic acid]. Structure from http://pubchem.ncbi.nlm.nih.gov

In COS-1 cells with the hGR, UDCA did result in GR-mediated transactivation, transrepression or co-factor recruitment (Stubsrud 2005; K. Ronacher; J. Hapgood; unpublished data) and was therefore classified as a GR antagonist in this cell system. UDCA was included in the initial ligand-selective GR phosphorylation at S226 work, but has recently been shown not to bind directly to the GR ((Weitzel *et al.*, 2005); K. Ronacher and J. Hapgood unpublished data), suggesting an interesting mechanism of action, not via the classical GR mechanism of action and was therefore excluded from the other experiments.

# Addendum C Antibodies

# Addendum D Supporting data from the present author

**Figure D1 The amount of GR shifts partial agonist activity to that of a full agonist.** COS-1 cells were plated in 10-cm dishes at a density of $2x10^6$ cells/dish in DMEM, containing 10% fetal calf serum and antibiotics. After 24 hours, the cells were transiently transfected with TAT-GRE, as well as either 10 µg or 1 µg HA-hGRwt and incubated for an additional 24 hours. The following day, the cells were re-plated into 24-well plates at a density of $5x10^4$ cells/well and incubated for 24 hours, before stimulation with vehicle or increasing amounts of test compound for 24 hours. Thereafter luciferase activity was determined and normalised to protein levels, as determined by the standard Bradford protein determination assay. The amount of luciferase activity with the vehicle control was subtracted and the amount of 1 µg GR with 10 µM dex was set to 100% and the values for the rest calculated as a percentage thereof. The graph represents pooled results from three independent experiments; each performed in triplicate and is shown as the mean ± SEM.



# Addendum E Supporting data from other researchers

**Table E1 Ligand-selective GR phosphorylation at S211**. COS-1 cells transiently transfected with HA-hGRwt were treated with vehicle (EtOH) or 10 mM test compound for 1 hour. Equal amounts of protein (typically 20 μg) were separated on 10% SDS-PAGE and Western blotting probing with an anti-phospho-S211-specific (P-S211) antibody was performed. Thereafter the amount of basal phosphorylation (vehicle) was subtracted and the amount of phosphorylation with dex set to 100%. Data is presented as the average or three independent experiments and is presented as the average ± SEM. Results were obtained by E. Stubsrud (Stubsrud, 2005).

**Table E2 Phosphorylation at S211 is required for maximal efficacy in transactivation**. COS-1 cells transiently transfected with HA-hGRwt or HA-hGRS211A, as well as TAT-GRE reporter-promoter construct were treated with vehicle (EtOH) or test compound for 16 hours. Thereafter luciferase activity was measured and normalised to the amount of protein, as determined by the standard Bradford protein determination method. The relative efficacy for transactivation of each ligand with wt receptor was set to 100% and the S211A mutant results calculated as a percentage thereof. Results present the average of three independent experiments, each performed in triplicate and values are given as the average ± SEM. Results were obtained by E. Stubsrud (Stubsrud, 2005).



**Table E3 Correlation analysis of S211 phosphorylation with transcription.** The S211 phosphorylation results were correlated with the efficacy and potency for transactivation and transrepression. Transactivation data was obtained by E. Stubsrud (Stubsrud, 2005) and the transrepression results were obtained by K. Ronacher. $R^2$ values are shown, while statistical significance is denoted by *, ** and ***, to represent $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

**Table E4 Ligand-selective GR binding affinity and fractional occupancy[§].** Results were obtained by performing competitive binding with [3H] dex in COS-1 cells transiently transfected with HA-hGRwt in the presence and absence of increasing amounts of test compound. The IC50 values were obtained separately from curves of the individual experiments and the mean ± SEM calculated from several experiments performed by K. Ronacher and is already published (Ronacher *et al.*, 2009). The fractional occupancy was calculated according to the following equation: fractional occupancy = [ligand] / ([ligand] + Ki).



[§] a = Estimated by GraphPad Prism due to incomplete curve; n/a = no activity

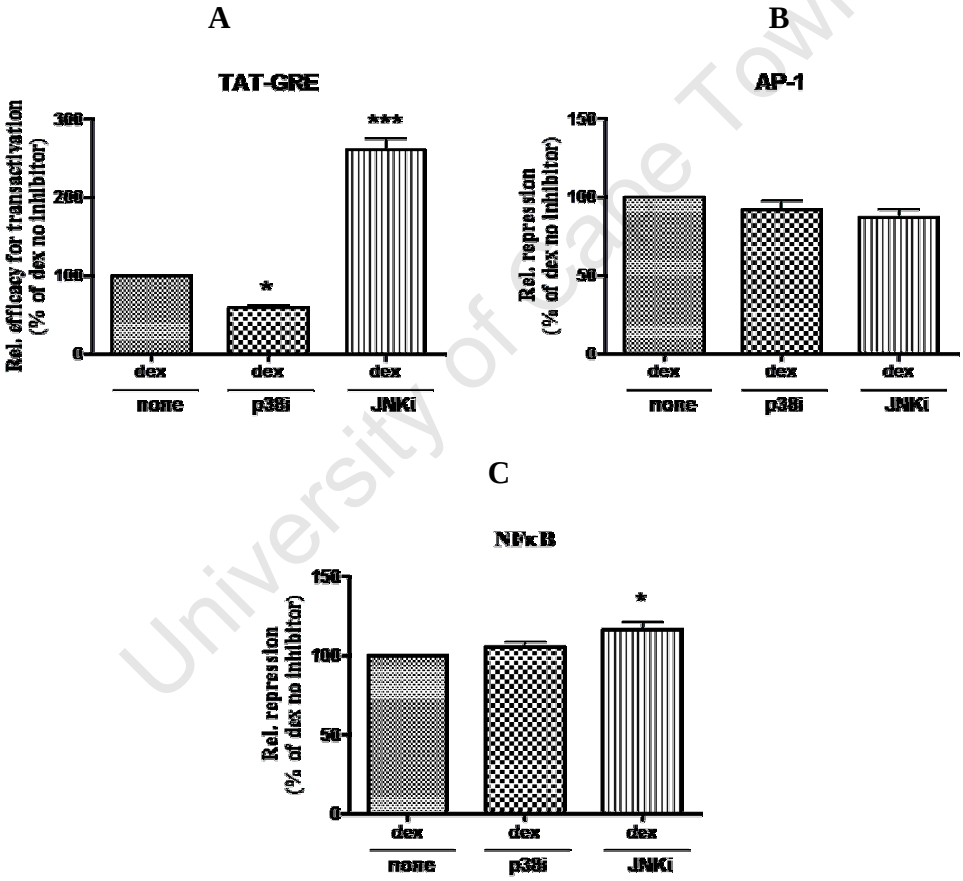
**Table E5 Efficacy and potency for transactivation and transrepression by the panel of ligands compared to dex expressed in relative efficacy and relative potency (-logEC50) in nM.**[**] Results were obtained in COS-1 cells transiently transfected with HA-hGRwt, as well as TAT-GRE, AP-1 or NFκB reporter-promoter constructs. Values are given as relative efficacy and potency (nM) as compared to dex and are from three independent experiments, each performed in triplicate. Transactivation results were obtained from E. Stubsrud (Stubsrud, 2005) and transrepression results were obtained by K. Ronacher. These results are already published (Ronacher *et al.*, 2009).



| | | | | | | |
|---|---|---|---|---|---|---|
| UDCA | na | na | na | na | na | na |

[**] na = no activity

**Figure E1 Effect of kinase inhibitors on transactivation and transrepression**. COS-1 cells were transiently transfected with HA-hGR and either TAT-GRE (A), AP-1- (B) or NFκB- (C) reporter-promoter constructs and pre-treated for 15 minutes with 10 μM p38- or 50 μM JNK inhibitor and then stimulated with vehicle or 10 μM dex for 24 hours in the presence of the inhibitors. Thereafter luciferase activity was measured. The amount of luciferase activity with no test compound was subtracted, the amount of dex induction without inhibitor was set to 100% and the rest was calculated as a percentage thereof. Graphs represent the average of three independent experiments, each performed in triplicate and is given as the mean ± SEM. p38i = p38 inhibitor; JNKi = JNK inhibitor. These experiments were performed by K. Ronacher. Statistical significance is denoted by *, ** and ***, to represent $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.



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**SUPPLEMENTARY FIGURES (Ronacher *et al.* 2009)**

**Figure 1**

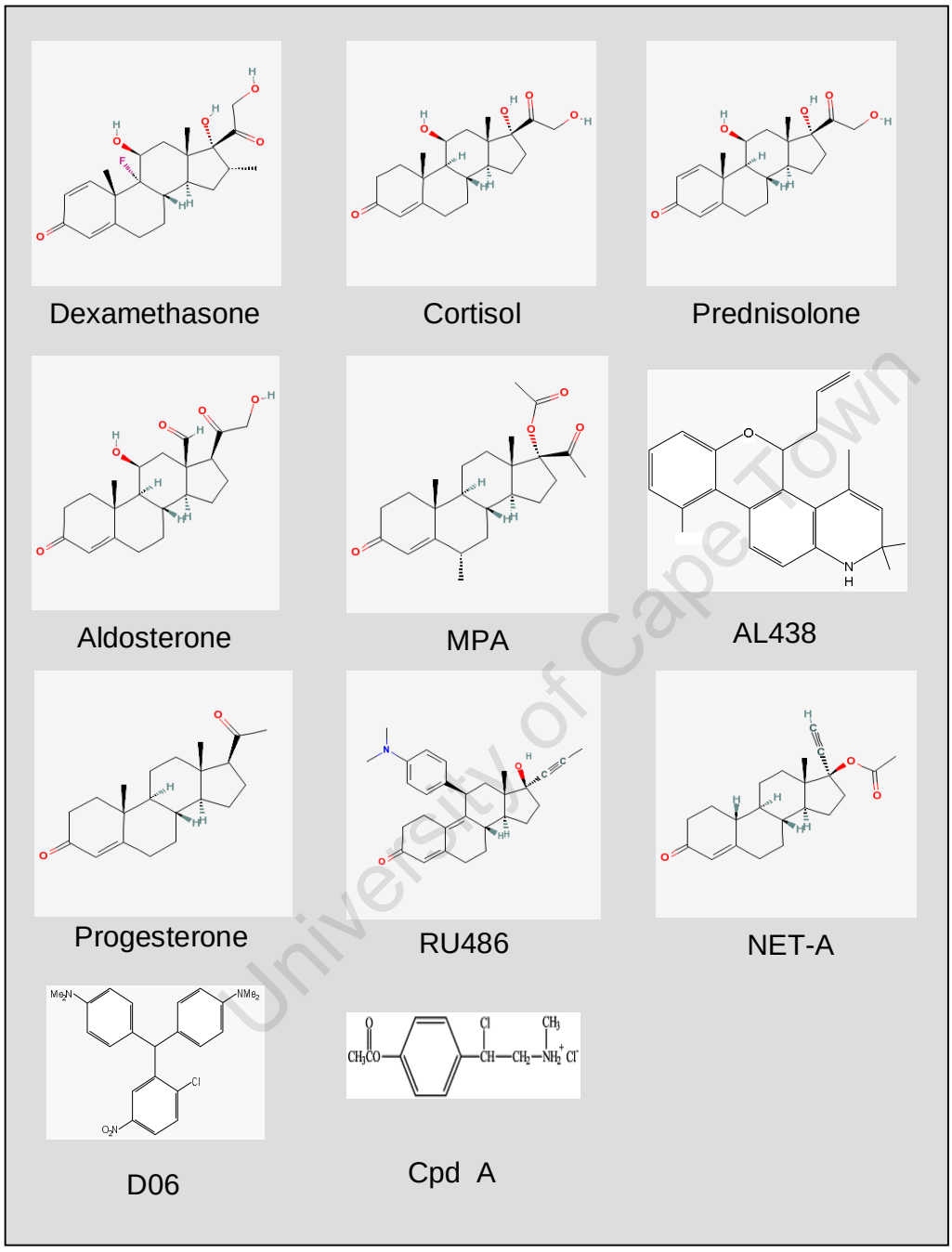




**Fig 1. Structures of the panel of GR ligands**