An investigation into the role of acetylation and ligand-dependent nuclear localisation in glucocorticoid receptor transcriptional regulation

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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 submitted any part of it at any university for a degree.

Signature..... Date.....

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ABSTRACT

The glucocorticoid receptor (GR) is a ligand-activated transcription factor which, due to its central role in anti-inflammatory responses, is a target of many therapeutically prescribed drugs. The GR undergoes multiple post-translational modifications, including phosphorylation and acetylation; however the role of GR acetylation in transactivation is unclear. The functional consequences of GR acetylation at K494/495 were investigated at several steps in the hGR pathway, using expressed hGR mutants as compared to wild-type GR. Results of reporter transactivation assays and immunofluorescence microscopy indicated that acetylation plays a role in transactivation and nuclear translocation of the GR. Mutation of acetylation sites also differentially affected GR phosphorylation status. Competitive whole cell binding assays suggest that GR acetylation at these residues is required for ligand binding by the GR. Chromatin immunoprecipitation assays on the endogenous GRE-containing GILZ gene support a model in which interaction of the immunophillin FKBP52 with acetylated hGR plays an important role in GR function by acting as a GR coactivator. Taken together, these results suggest that modulation of GR acetylation at K494/K495 represents an attractive physiological mechanism for regulation of glucocorticoid sensitivity and transactivation efficacy.

GR ligands vary enormously in terms of potency and efficacy for transactivation and transrepression. Using an endogenous model system and a panel of 8 different ligands differential regulation of five endogenous model genes was investigated, and differences in ligand efficacy were observed. In order to investigate the molecular mechanism by which ligand-specific differences arise, subcellular fractionation and immunofluorescence microscopy were used to examine the extent of GR nuclear localisation. It was found that the extent of GR nuclear localisation differed for different ligands, and that these values correlated with the maximal transactivation efficacy of the GR, and GR phosphorylation at Serine-211. Taken together, these results indicate that ligand-selective nuclear import is a key step in modulating ligand-selective transcriptional responses by the GR.

LIST OF ABBREVIATIONS

Ald	Aldosterone
ALL	Acute lymphoblastic leukaemia
ANOVA	Analysis of variance
AP-1	Activator protein-1
AR	Androgen receptor
bp	base pair
BMAL1	Brain-muscle-arnt-like protein 1
CARM1	coactivator associated arginine methyltransferase 1
CBP	CREB binding protein
CDK	Cyclin dependent kinase
ChIP	Chromatin immunoprecipitation
Cort	Cortisol
CREB	cAMP response element binding protein
DBD	DNA binding domain
dex	Dexamethasone
DHT	Dihydroxy testosterone
DMEM	Dulbecco's modified Eagle medium
ECL	Enhanced chemiluminescence
ER	Estrogen receptor
EtOH	Ethanol
FCS	Fetal calf serum
FKBP51	FK506 binding protein 51
FKBP52	FK506 binding protein 52
FRAP	Fluorescence recovery after photobleaching
GC	Glucocorticoid
GILZ	GC induced leucine zipper
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
GRIP1	GR interacting protein 1
HA	Hemagglutanin
HAT	Histone acetyl transferase
HDAC	Histone deacetylase

hGR	human GR
HRE	Hormone response element
Hsp70	Heat shock protein 70
Hsp90	Heat shock protein 90
IgG	immunoglobulin G
IL	interleukin
LBD	Ligand binding domain
MAPK	Mitogen-activated protein kinase
MMTV	Mouse mammary tumour virus
MPA	6α-methyl-17αhydroxyprogesterone acetate
MR	Mineralocorticoid receptor
mRNA	messenger ribonucleic acid
NCoR	Nuclear receptor corepressor
NET	Norethindrone acetate
NFκB	Nuclear factor kappa B
nGRE	negative glucocorticoid response element
NLS	Nuclear localisation signal
NR	Nuclear receptor
p300	Adenovirus E1A-binding protein p300
PBS	Phosphate buffered saline
pCMV	Cytomegalovirus promoter
PMA	4α-Phorbol 12-myristate 13-acetate
PP5	Protein phosphatase 5
PR	Progesterone receptor
Pred	Prednisolone
Prog	Progesterone
PTM	Post-translational modification
RU486	Mifepristone
S203	Serine 203
S211	Serine 211
S226	Serine 226
SDS	Sodium dodecyl sulfate
SGK	Serum/glucocorticoid regulated kinase

SMRT	Silencing mediator for retinoic acid and thyroid hormone receptors
SR	Steroid receptor
SRC1	Steroid receptor coactivator 1
Stat	Signal transducer and activator of transcription
SUMO	Small ubiquitin-related modifier
TAT	Tyrosine aminotransferase
TE	Tris-EDTA buffer
TNFα	Tumour necrosis factor α
TPR	Tetratricopeptide repeat
β-gal	β-galactosidase

Thesis outline

The thesis contains the following sections

1. **Chapter 1: Factors affecting ligand- and cell-specific transcriptional responses by the GR**

This chapter provides a brief introduction to the GR and its mechanism of action. It then gives an overview of the available literature on GR function, with a focus on mechanisms eliciting ligand- and cell-selective transcriptional differences.

2. **Chapter 2: Aims and hypotheses**

This chapter will cover the aims of the present work, and hypotheses on which the direction of the research was based.

3. **Chapter 3: Critical role of lysines 494/495 in GR ligand binding and function: functional implications of GR acetylation**

This chapter takes the form of a scientific paper. As such, it includes an abstract, an introduction covering relevant literature in the field of SR acetylation, materials and methods used to generate the data, results and a thorough discussion of the results in the context of available literature. All the work presented in this chapter was performed by the candidate, except for the site-directed mutagenesis and one transactivation experiment performed by another laboratory member and duly acknowledged. A modified version of this chapter is in preparation for submission, to include data generated by other researchers in the laboratory.

4. **Chapter 4: Differential nuclear import of the GR plays a role in ligand-selective transcriptional responses**

This chapter takes the form of a paper. As such, it includes an abstract, an introduction covering relevant literature in the field of GR nuclear import, materials

and methods used to generate the data, results and a thorough discussion of the results in the context of available literature. All the work presented in this chapter was performed by the candidate. Some of these results have been previously published in Ronacher *et al.,* 2009. An abridged version of this thesis chapter is in preparation for submission.

5. **Chapter 5: Future perspectives**

In this chapter possible prospects for further research are discussed.

6. **REFERENCES** for all the above chapters and addendums to follow

7. **Appendix A:** Ligands

This addendum shows the chemical structures of all the ligands used, as well as their relative binding affinity as obtained by others in the laboratory, and GR fractional occupancy, with a brief discussion of the calculation and relevance of these parameters.

8. **Appendix B:** Dilutions and diluents of primary and secondary antibodies used in Western blot

A table is included summarising the antibody conditions used for Western blotting

9. **Appendix C:** Supplementary results pertaining to Chapter 3

10. **Appendix D:** Supplementary results pertaining to Chapter 4

11. **Appendix E:** Optimisation of experimental techniques

12. **Ronacher, K., Hadley, K., Avenant, C., Stubsrud, E., Simons, S. S., Louw, A., and Hapgood, J. (2009) Ligand-selective transactivation and transrepression via the glucocorticoid receptor: role of cofactor interaction. *Molecular and Cellular Endocrinology*, *299* (2), 219-31**

This research article includes results from other members of the research group as well as some of the results presented in this thesis. The present author also contributed to the analysis and interpretation of results, and assisted with writing of the paper. It is included here to illustrate the context of this thesis as part of a broader research effort by the group.

Contents

Chapter 1

Literature review

Factors affecting ligand- and cell-specific transcriptional responses by the GR

The glucocorticoid receptor (GR) is a transcription factor belonging to the nuclear receptor superfamily, which also includes the androgen (AR), estrogen (ER), mineralocorticoid (MR), and progesterone receptors (PR). These steroid receptors (SRs) are all ligand-activated transcription factors, and share a high degree of homology (reviewed in Heitzer *et al.,* 2007). This class of receptors makes an attractive target for rational drug design due to the central role played by steroid receptors in gene regulation and their control of a wide array of physiological processes (Adcock, 2000).

Endogenous glucocorticoids (GCs) (cortisol in humans, and corticosterone in rats) are synthesised in the adrenal cortex. Release of GCs is pulsatile and follows distinct diurnal and ultradian patterns. Physical or emotional stress can also trigger the release of GCs via the hypothalamic- pituitary- adrenal (HPA) axis (Lightman *et al.,* 2008). In response to stress, the hypothalamus releases corticotrophin releasing hormone (CRH). CRH acts on the anterior pituitary, stimulating the release of adrenocorticotrophic hormone (ACTH), which in turn stimulates the release of cortisol from the adrenal cortex into the bloodstream. Cortisol suppresses the activity of both CRH and ACTH, providing a negative feedback loop and ensuring tight regulation of GC production (reviewed in Papadimitriou & Priftis, 2009). Endogenous GCs modulate a wide range of physiological functions by binding to and activating the GR, and are essential for homeostasis. Metabolic effects of cortisol include increase in blood glucose levels, stimulation of gluconeogenesis by the liver, and mobilisation of amino- and fatty acids (Buckingham, 2006). Cortisol is also essential for lung development and erythroid cell proliferation. However, the most important

activity of GCs from a pharmacological point of view is their effect on components of the immune system regulating inflammation (reviewed in Buckingham, 2006).

GCs have been in use as anti-inflammatory drugs for more than 50 years (Barnes, 2006). However, while GCs are therapeutically useful, they also cause many side-effects, including immune suppression, osteoporosis, diabetes, hypertension, thinning of the skin and Cushing's syndrome (Stanbury & Graham, 1998). These side-effects limit the usefulness of GCs for long-term treatment of chronic inflammatory diseases and are generally due to activation of GR in a non-target organ or cell-type, or its activity on non-target genes. Therapeutically administered GCs have been observed to exhibit effects that are highly patient-, and cell-specific. For instance, in treatment of acute lymphoblastic leukaemia (ALL), the effect of GCs is highly cell-specific in promoting apoptosis of lymphocyte cells, while simultaneously protecting cells of the endometrium, ovarian follicle, and mammary epithelium against apoptosis (reviewed in Gross & Cidlowski, 2008). Thus, a better understanding of the molecular mechanisms that govern transcriptional activation by the GR is crucial for the design of drugs with greater potency and reduced side-effects. It would be advantageous for such drugs to be selective for the desired target tissue, cell type and gene(s) (Schäcke *et al.,* 2005).

GR ligands can be broadly categorised as agonists (those that increase the transcriptional response by GR, either transactivation or transrepression) or antagonists (which bind GR but do induce a transcriptional response). It has been widely reported that the beneficial effects of GCs are a result of transrepression of inflammatory genes, while their transactivation activity is responsible for the negative side-effects. Exceptions to this model have been identified, such as transactivation of the anti-inflammatory response mediators, mitogen activated protein kinase phosphatase 1 (MKP1) (Kassel *et al.,* 2001) and glucocorticoid induced leucine zipper (GILZ) (Eddleston *et al.,* 2007). However, this model has stimulated interest in the discovery of GCs which cause only transrepression via the GR, without eliciting transactivation. GCs with such activity are termed "dissociated GCs" or "selective GR agonists" (SEGRAs). The development of drugs with selective anti-inflammatory

activity would represent a significant improvement in the treatment of a range of inflammatory and autoimmune diseases (reviewed in Adcock, 2000). Furthermore, different drugs may elicit the same type of response (e.g. transactivation of a certain gene) in the same cell type with differences in potency and efficacy. Understanding what causes these differences in drug activity is crucial to the design of drugs that are more efficacious in the target tissue, with less activity in non-target tissues (reviewed in Simons, 2003).

In this chapter, a brief outline of the structure and mechanism of action of the GR will be given, followed by an overview of each of the steps at which ligand-specific effects may manifest. The mechanisms by which the same GR ligand can cause completely different effects on transcription of the same gene in different cell types will be highlighted, as well as possible reasons why different GR ligands can cause different transcriptional responses of the same promoter, in the same cell, under the same conditions. Although the main focus will be on the human GRα (hGRα), where necessary, examples will be drawn from rat GR (rGR) and mouse GR (mGR) and other steroid receptors within the class.

1.1 Structure of the GR protein

Although several isoforms of GR have been identified, which will be discussed later in the section on ligand and tissue specificity of GR, GRα is the most abundant and best-studied isoform. The structure of the GRα protein has been described as “modular” because it comprises distinct domains with different functions, as shown in figure 1.1 (Revollo & Cidlowski, 2009). At the N-terminus is the variable transactivation domain, consisting of activation function-1 (AF-1), which has constitutive transcriptional activity and is associated with the basal transcription machinery. All the major sites of phosphorylation reside in this domain (reviewed in Kumar & Thompson, 2005). The centrally located DNA binding domain (DBD) is made up of two highly conserved zinc fingers, which allow the protein to recognise and bind to its target motif in DNA. The first zinc finger contains a three amino acid

“P box” motif responsible for specific binding sequence recognition. The second zinc finger contains the so-called “D box” which is involved in dimerization of the GR monomers (Luisi *et al.,* 1991). A short hinge region adjacent to the DBD gives GRα flexibility, allowing for changes in conformation on ligand and DNA binding. The C-terminal domain harbours the ligand-binding domain (LBD). It contains binding sites for cofactors and heat shock protein 90 (Hsp90), and the AF-2 domain, which confers ligand-dependent transcriptional activity (reviewed in Kumar & Thompson, 2005). Two nuclear localisation signals (NLSs) are also present, and are exposed on ligand binding, thereby facilitating nuclear translocation of the molecule (Picard & Yamamoto, 1987).

Figure 1.1. Graphical representation of the modular structure of hGRα. The C-terminal LBD is shown in grey. Nuclear localisation signals are represented by black bars. Transcription is carried out by the activation function domains AF-1 (constitutive) and AF-2 (ligand-dependent), represented by ovals. (Modified from Revollo & Cidlowski, 2009).

1.2 Mechanisms of action of GR

In its unliganded state, the GR resides in the cell cytoplasm in a complex with Hsp proteins, and cochaperones (reviewed in Pratt & Toft, 1997). Upon ligand binding, a conformational change takes place, and the GR undergoes a number of post-translational modifications, the best-studied of which is phosphorylation (Faus & Haendler, 2006). The GR subsequently translocates to the nucleus, where it can bring about an increase (transactivation) or a decrease (transrepression) in transcription by binding to the promoter regions of target genes. The mechanisms by which GR functions can be divided into two categories for the purposes of simplicity:

either direct DNA-binding by the GR or protein-protein interactions of the GR with other transcription factors, which is known as “tethering”, as shown in figure 1.2. Either of these mechanisms can bring about transactivation or transrepression (reviewed in Smoak & Cidlowski, 2004). These mechanisms are discussed below with a few examples; however it is important to note that depending on the cellular context, different mechanisms may act in concert (reviewed in Newton, 2000).

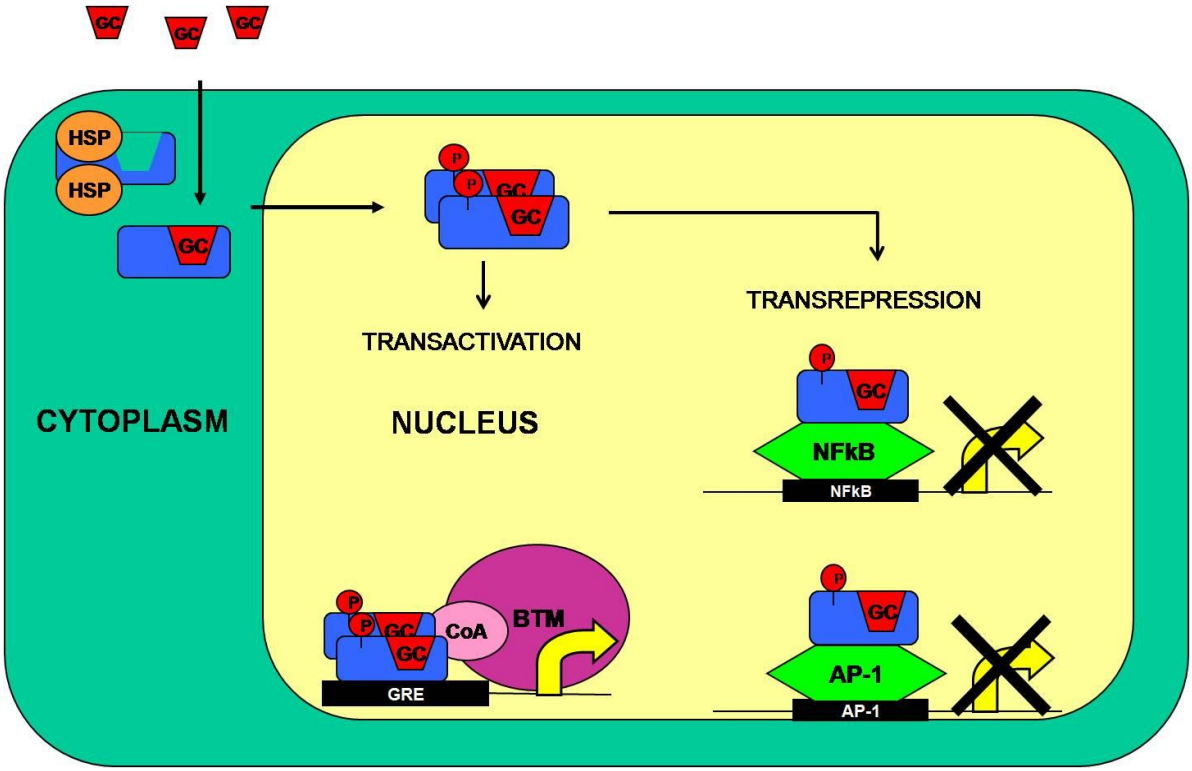


Figure 1.2. Mechanisms of action of GR. In the absence of ligand, GR resides in the cytoplasm in a complex with chaperones, including heat shock proteins (HSPs). On binding to GCs, GR undergoes post-translational modifications (P), and translocates to the nucleus, where it can facilitate transactivation or transrepression. Transactivation occurs by direct DNA binding, and recruitment of cofactors (CoA) and the basal transcription machinery (BTM), while transrepression occurs by tethering to other transcription factors such as AP-1 and NFκB.

GR transactivation generally involves binding of ligand-activated GR to a glucocorticoid response element (GRE) in the promoter region of a target gene. The canonical GRE is a palindromic sequence (5’-GGTACAnnnTGTTCT-3’) (Beato *et al.,*

1989), to which the activated GR binds as a homodimer (Tsai *et al.,* 1988). Dimerization of the GR requires a five amino acid region called the D-loop within the DBD (Luisi *et al.,* 1991). The GR homodimer recruits cofactors to the promoter, some of which facilitate chromatin remodelling because of their intrinsic histone acetyl transferase (HAT) activity. The relaxed chromatin structure allows RNA polymerase II and TATA box-binding protein (TBP) access to the promoter to initiate transcription (Horwitz *et al.,* 1996). Examples of promoters with GREs include those of the metabolic enzymes tyrosine aminotransferase (TAT), and phosphoenolpyruvate carboxykinase (PEPCK) (reviewed in Revollo & Cidlowski, 2009).

Some promoters have a less well-conserved sequence that is also recognised and bound by GR homodimers: the negative GRE (nGRE). Binding of GR to an nGRE results in transrepression of the gene, usually by exclusion of another transcription factor or member of the transcriptional machinery from its response element (reviewed in Necela and Cidlowski, 2004). For example, an nGRE is found in the promoter of the osteocalcin gene. Binding of ligand-activated GR to this site prevents binding of the TATA binding protein (TBP) to its recognition sequence, thereby repressing transcription of the gene (Strömstedt *et al.,* 1991; Meyer *et al.,* 1997).

The phenomenon of GR tethering is best illustrated by genes under transcriptional control of nuclear factor kappa B (NFκB) and activator protein-1 (AP-1). The AP-1 transcription factor consists of a dimer composed of Jun, Fos or Activating transcription factor (ATF) subunits. While Jun family members can form homodimers or heterodimers with other AP-1 proteins via a leucine zipper motif, the Fos subunit is incapable of forming homodimers (Vesely *et al.,* 2009). Genes under the transcriptional control of AP-1 encode several tissue-damaging proteins, such as collagenase and stromelysin, which contribute to the inflammatory response (reviewed in Revollo & Cidlowski, 2009). Ligand-activated GR disrupts this response by binding to AP-1, preventing it from recruiting cofactors and initiating transcription, and thereby results in transrepression. This tethering mechanism does not require GR binding to DNA, but rather occurs via a protein-protein interaction between DNA-bound AP-1 and GR (Schoneveld *et al.,* 2004).

NFκB is a ubiquitous heterodimeric transcription factor. The predominant heterodimer involved in transactivation consists of p65 and p50 subunits (reviewed in Simmonds & Foxwell, 2008). Under normal physiological conditions NFκB exists in the cytoplasm bound to and inactivated by inhibitor kappa B (IκB). Activation of the tumour necrosis factor α (TNFα) signalling pathway, by injury or infection, results in phosphorylation of IκB, which allows it to be ubiquitinated and targeted for degradation. The liberated NFκB translocates to the nucleus and binds DNA at specific response elements in the promoter region of a range of target genes, thereby initiating transcription of these pro-inflammatory genes (Kassel & Herrlich, 2007). On ligand-activation, GR interacts with the p65 subunit of NFκB, preventing it from activating transcription of inflammatory genes (Nissen & Yamamoto, 2000).

GR tethering can also serve to increase the transcription of genes, such as in the case of signal transducer and activator of transcription 5 (Stat5). Stat5 and GR interact synergistically to transactivate the β-casein gene, in the absence of a GRE (Stoecklin *et al.,* 1997). Interactions between GRα and other members of the Stat family have been identified, and may result in transactivation or transrepression of target genes (reviewed in Rogatsky & Ivashkiv, 2006). GR tethering to AP-1 has also recently been found to have a positive effect on gene transcription of certain target genes (Maya-Núñez & Conn, 2003; Rani *et al.,* 2009; Kotitschke *et al.,* 2009).

In addition to its well-known genomic effects, the GR is increasingly being implicated in alternative, non-genomic effects of GC treatment, and ligand-independent transcriptional regulation. These effects are rapid, and insensitive to inhibitors of transcription and translation. Although non-genomic effects appear to occur at the membrane, it has not been established whether the classical cytosolic GR is responsible, or a distinct membrane-bound isoform of the GR. It has been reported that GR also takes part in cross-talk with membrane bound receptors such as G-protein-coupled receptors, resulting in a wide range of intracellular effects. Although the subject of non-classical mechanisms of GR activity will not be discussed in detail

here, these complex signalling pathways are the subject of a recent review from the Hapgood laboratory (Dr A. Kotitschke, PhD thesis, 2009).

1.3 Tissue- and ligand-specificity of GR activity

The cellular activity of GCs involves a complex multistep process, from ligand-binding to the ultimate biological response, as shown in figure 1.2.1 below, with a variety of factors causing differences in responses. Selectivity of action is introduced by the different GCs themselves, and also by variation in receptor isoform expression. The popular tripartite model of steroid hormone as proposed by Katzenellenbogen *et al.,* (1996) includes a third factor: that of the effector site, which refers to the tissue or cell type in which the activity takes place. Together these three factors (ligand-receptor-effector site) regulate the response to GC, and will be discussed in more detail below.

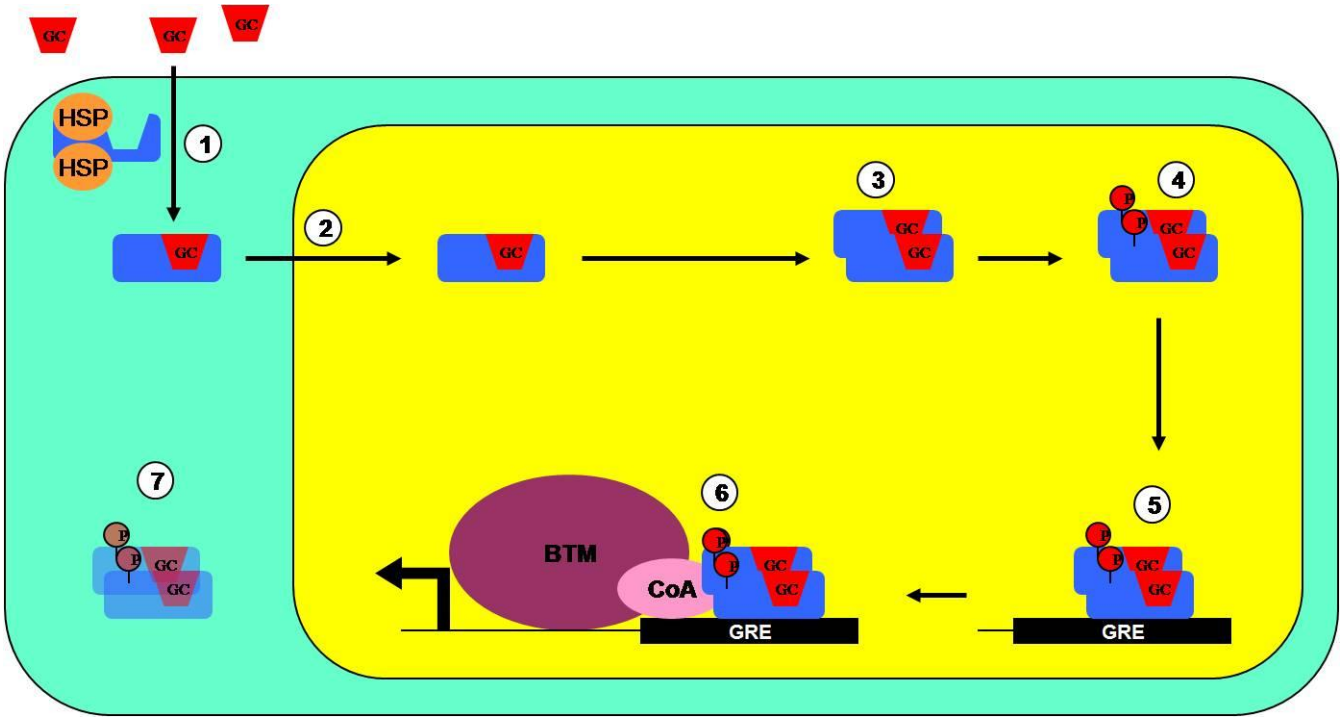


Figure 1.2.1. GR transcriptional regulation is a multistep process. (1) cytoplasmic GR, held in a ligand-receptive conformation by a complex of proteins including heat shock proteins (HSPs), binds GC. (2) GR then translocates to the nucleus, (3) dimerizes, and (4) undergoes post-translational modifications such as phosphorylation and acetylation (P).

(5) GR interacts with gene promoters, either directly as depicted here, or indirectly via other transcription factors and (6) facilitates recruitment of cofactors (CoA) and basal transcription machinery (BTM). (7) Liganded GR is rapidly degraded by the proteasome.

1.3.1 Different isoforms of GR allow for GC response to be specific for different cell and tissue types, and physiological states

The gene for human GR has been mapped to chromosome 5 (Gehring *et al.,* 1985). Unique binding sites for 15 transcription factors have been identified in the promoter region of this gene, and their differential activity plays a role in the tight cell- and tissue-specific regulation of GR expression (reviewed in Gehring *et al.,* 1985). The 5'UTR also governs the splicing of the mRNA, which can result in further variation through alternative promoter usage (Turner *et al.,* 2006). The two main splice variants of human GR are termed α and β, which differ in their splicing of exon 9. The α isoform is the classical 777 amino acid GR, while the β isoform is a 742 amino acid protein with a shorter ligand binding domain, and therefore reduced ligand binding and transcriptional activity (reviewed in Nicolaides *et al.,* 2010).

GRβ is located in the nucleus and cytoplasm (Kino *et al.,* 2009), and its role there is controversial. GRβ has been shown to exert a dominant negative effect on transactivation by the α isoform on the MMTV promoter in several cell lines, as a result of formation of transcriptionally inactive α-β heterodimers (Oakley *et al.,* 1999) and prevention of coactivator complex formation (Charmandari *et al.,* 2005). The physiological relevance of such activity is disputed, as the cellular concentrations of the β isoform would appear to be too low to allow a dominant negative effect to occur *in vivo*. However, elevated expression levels of GRβ isoform have been reported in some GC resistant disorders, which may indicate a role for this isoform in tissue-specific GC resistance (reviewed in Lu & Cidlowski, 2006). One report has indicated that GRβ resides in the cytoplasm in the absence of ligand (Lewis-Tuffin *et al.,* 2007). Exogenous GRβ was reported to bind the antagonist RU486, and subsequently translocate to the nucleus in COS-1 and U2OS cell lines, and to regulate gene expression of more than 5000 genes in stably transfected U2OS cells

(Lewis-Tuffin *et al.,* 2007). Further experimentation in HCT116 cells failed to confirm this observation (Kino *et al.,* 2009). However, GRβ has recently been reported to positively and negatively regulate expression of a number of genes in a ligand-independent, GRE-independent manner in HCT116 cells (Kino *et al.,* 2009). Further work therefore needs to be done to clarify the physiological role of GRβ (reviewed in Kino *et al.,* 2009).

Alternative translation start sites are present in the mRNA for GRα. Leaky ribosomal scanning results in generation of at least 8 different isoforms which differ at the amino terminal. GRαA is translated from Methionine 1, and GRαB is translated from Methionine 27, while GRαC1,2,3 and D1,2,3 are several amino acids shorter (reviewed in Duma *et al.,* 2006). Both A and B forms have been found endogenously in several cell lines, and Yudt and Cidlowski (2001), have demonstrated that while GRαA and GRαB have similar transrepression activity, expressed GRαB exhibits a two-fold greater transactivation activity than GRαA in COS-1 cells on various synthetic promoters. Although the physiological relevance of the A and B isoforms is not clear, this may present an additional mechanism determining cell-type specific effects of GCs.

Three other isoforms of hGR have been described: GR-P, hGR-A, and hGRγ, which are present at much lower concentrations than GRα, and most have reduced transactivation activity (reviewed in Zhou & Cidlowski, 2005). All the alternative isoforms add to the complexity and diversity of GR signalling in different cell and tissue types. For instance, differences in expression levels and subcellular distribution of GRβ have been implicated in the different GC responses between human monocytes and T-cells (Li *et al.,* 2006). However, whether different ligands elicit ligand-specific effects via GR isoforms other than GRα has not been established.

In addition to the different isoforms identified, variation within the population is further increased by a number of different GR polymorphisms which have been shown to have an impact on GR function (reviewed in Gross & Cidlowski, 2008). For instance,

the ER22/23K polymorphism, found in about 3% of the population (Derijk & de Kloet, 2008), is associated with decreased transactivation compared to WT GR, and has been postulated to be involved in GC resistance (Russcher *et al.,* 2005). The wide variety of GR protein isoforms strongly suggests that gene regulation by GR is a highly complex process. The variety of possible post-translational modifications, and the implications thereof, increases this complexity even further. These will be discussed in the section on post translational modifications. However, for the rest of the thesis, only the GRα isoform will be examined.

1.3.2 Bioavailability of GR ligand

Under normal conditions, about 80-95% of cortisol in the bloodstream is bound to corticosteroid-binding globulin (CBG), and thus is not free to diffuse across cell membranes to bind GR (Buckingham, 2006). Only a small percentage of total cortisol is thus biologically active. It has also been suggested that CBG plays a role in tissue-specific delivery of GCs (Torpy & Ho, 2007). Bioavailability of cortisol is further controlled by the activity of 11-β hydroxysteroid hydrogenase enzymes type 1 and 2. These enzymes regulate intracellular levels of cortisol by catalysing its conversion to or from the inactive form, cortisone (Seckl & Walker, 2001). The tissue-specific distribution of these two enzymes presents a mechanism to control the cellular response to systemic GCs (Gross & Cidlowski, 2008). Whether differential bioavailability of ligands is ligand-specific has not been described.

1.3.3 GR concentration and binding affinity of ligand

It is well-established that endogenous GR concentrations vary in different cells and tissues of the body (Miller *et al.,* 1998), and this presents an attractive way to modulate the transcriptional effects of endogenous GCs. In a thorough analysis of the effect of hGR receptor levels in COS-7 cells, Zhao *et al.,* (2003) reported that increasing GR levels increased the potency and efficacy of transrepression by dex, but interestingly this was not the case for all GR ligands. The authors examined the

effect of overexpressing different levels of GR on the transrepressive effect of RU486, 6α-methyl-17αhydroxyprogesterone acetate (MPA), budesonide and cortisol, and found that, in contrast to budesonide and cortisol, both MPA and RU486 behaved as agonists at high receptor density, but as antagonists at low receptor density. Plausible explanations proposed for this phenomenon include cooperative dimerization, which increases with receptor density, or differential recruitment of cofactors being more thermodynamically favourable at higher receptor density (Zhao *et al.,* 2003). Besides causing tissue-specific effects, differential expression of GR could also be responsible for ligand-selective effects, since only a subset of ligands exhibited altered biocharacter in response to the variation in GR levels. The observation that varying GR levels can shift a GC response curve has been corroborated in the Hapgood laboratory (unpublished data).

Different ligands display different binding affinities for the GR depending on their particular chemical structure, and their three-dimensional fit into the GR ligand binding pocket. The binding affinity of a ligand for the GR determines the fractional occupancy, or the ratio of liganded to unliganded GR, at a given concentration (see Appendix A for more details). The concentration of GR present does not determine the fraction that will be occupied by ligand at a given concentration, since fractional occupancy is dependent only on the rate of association and disassociation of GR:ligand complexes at a given concentration of ligand. However, Schaaf and Cidlowski (2003) reported that ligand affinity correlates with subnuclear mobility of liganded GR, and may therefore have important implications for the transcriptional effect. For agonists and partial agonists, binding affinity has been shown to correlate with potency for transactivation, but not transrepression (Ronacher *et al.,* 2009). However the reason for this correlation has not yet been established.

1.3.4 Ligand-binding alters GR conformation

It is generally accepted that ligand-selective maximal responses by steroid receptors are due to the induction of ligand-specific conformations of the liganded receptor

(Rosen & Miner, 2005). Different conformations of GR LBD bound to different ligands have been identified by X-ray crystallography (Kauppi *et al.,* 2003) and hydrogen-deuterium exchange mass spectroscopy (Frego & Davidson, 2006). Consistent with this, full-length GR has been shown to yield different patterns upon partial proteolytic digestion when bound to different ligands, indicating differences in protein conformation (Vicent *et al.,* 2002).

The rank order of GR transcriptional efficacy has been shown to have a strong correlation with the extent of GR phosphorylation at S211 and S226 (Avenant *et al.,* manuscript in preparation). In turn, phosphorylation at S211 and S226 has been implicated in recruitment of the cofactor GRIP-1 to the GR (Avenant *et al.,* 2010), and recruitment of GRIP-1 also correlates strongly with GR transactivational efficacy (Ronacher *et al.,* 2009). Thus a central question is what upstream step brings about the differential phosphorylation of the GR, as this is likely the basis for ligand-selectivity at several other steps in the GR transcriptional regulation pathway. One possibility is differences in the conformation of liganded GR which cause differential exposure of serine target residues to kinases. A second possibility which will be discussed shortly is GR nuclear translocation, which indeed could also arise from differences in GR conformation.

Differences in GR conformation have not been examined with a large panel of ligands. This would allow investigation whether different ligands cause quantifiable differences in conformation along a continuum of possible conformations. If so, and the rank order of ligands along the continuum matched the rank order of their biological activity, this would strongly support the hypothesis that the differences in biological activity were a direct result of GR conformation induced upon ligand binding.

1.3.5 The composition of the Hsp-heterocomplex

In its unliganded state, the GR resides in the cytoplasm and is bound in a complex of proteins consisting of Hsps and a variety of cochaperones (Pratt, *et al.,* 2004). Formation of the heterocomplex is an ATP-dependent process, involving an initial reaction of GR with Hsp70 and the cochaperone Hsp40 (Grad & Picard, 2007). This complex is primed for interaction with Hsp90 and the cochaperone Hsp organising protein (Hop). The latter complex is stabilised by a dynamic interaction with p23, a small ubiquitous cochaperone (reviewed in Pratt *et al.,* 2004). The association of GR with this heterocomplex is crucial for its folding, maturation to a ligand-binding conformation, trafficking and degradation (reviewed in Grad & Picard, 2007).

Early observations of GR transformation *in vitro* indicated that dissociation of the GR from Hsps on ligand binding was a key step in regulation of GR activity, allowing subsequent DNA binding (Mendel *et al.,* 1986; Pratt & Toft, 1997). However, more recent reports contest this model, suggesting an interaction between GR and Hsp90 inside the nucleus (Elbi *et al.,* 2004; Stavreva *et al.,* 2004), and showing an interaction between liganded GR and Hsp90 (Fang *et al.,* 2006). Furthermore, Hsp90 and p23 are ligand-dependently recruited to GRE-containing promoters similarly to the GR (Freeman & Yamamoto, 2002). It has recently been shown that upon ligand binding, the MR translocates to the nucleus with the Hsp90 heterocomplex intact (Galigniana *et al.,* 2010).

Hsp90 contains a tetratricopeptide repeat (TPR) acceptor site at its C-terminal domain, to which one of a number of TPR proteins binds (Young *et al.,* 1998). TPR sequences are degenerate 34 amino acid repeats which are involved in a number of protein-protein interactions. The main TPR proteins found in the GR heterocomplex are the immunophilins, so named because they bind to immunosuppressant drugs such as FK506 or Cyclosporin A. Immunophilins include FK506 binding protein 51 (FKBP51), FK506 binding protein 52 (FKBP52) and Cyclophilin 40 (Cyp40). Another important TPR protein is the immunophilin homolog protein phosphatase 5 (PP5)

(Pratt *et al.,* 2004). Although a subject of some debate, it is now generally accepted that Hsp90 dimers contain only one TPR binding site for which the TPR proteins compete (Pratt & Toft, 1997). The specific TPR protein occupying this binding site has functional implications for the GR heterocomplex.

FKBP52 has been shown to potentiate GR signalling by increasing its hormone binding affinity (Riggs *et al.,* 2003), while FKBP51 has been reported to decrease GR ligand binding and transactivation (Denny *et al.,* 2000). Furthermore, FKBP52 has been shown to interact with the motor protein dynein, while FKBP51 does not (Wochnik *et al.,* 2005). Hormone binding to the GR has also been observed to correlate with a switch from FKBP51 to FKBP52 association on the heterocomplex (Davies *et al.,* 2002). This observation lead to the current model of GR nuclear import, in which ligand binding induces association of FKBP52 with the heterocomplex, allowing association with dynein microtubules, and therefore nuclear import (Davies *et al.,* 2005), as shown in figure 1.2.

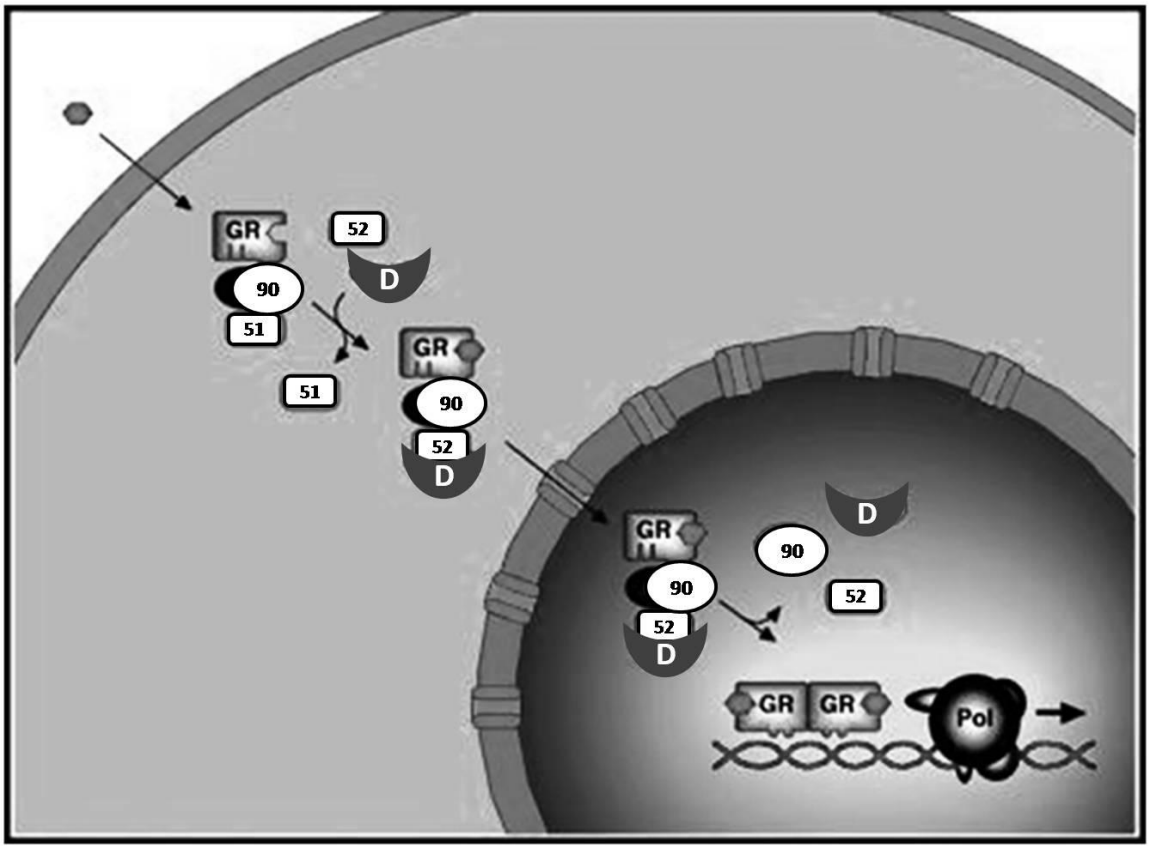


Figure 1.2. Model for role of immunophilins in nuclear translocation of hormone-activated GR (Davies *et al.,* 2002). Immunophilin swop of FKBP51 for FKBP52 on ligand binding allows interaction with dynein and subsequent nuclear translocation (90= Hsp90, 51= FKBP51; 52= FKBP52; D= dynein; Pol= RNA polymerase)

Levels of FKBP51, FKBP52 and PP5 have been shown to vary between different cell lines, and this has direct implications for the subcellular localisation of the GR (Banerjee *et al.,* 2008). Studies with knockout mice deficient in various TPR proteins have lead to the general conclusion that the required TPR protein for appropriate GR activity varies between different tissue types (reviewed in Ratajczak *et al.,* 2009). Furthermore, high levels of endogenous FKBP51 have been implicated in GC resistance of new world primates (Reynolds *et al.,* 1999), indicating that species-specific differences in TPR expression levels can impact on GR function.

The association of different TPR proteins with the GR heterocomplex has also been shown to depend on the nature of the ligand to which GR is bound. Croxtall and co-workers (Croxtall *et al.,* 2003) used a panel of four different GR ligands to demonstrate that the switch of FKBP51 for FKBP52 in association with GR was ligand-selective. Interestingly, no correlation between the amount of FKBP52 in the complex and GR nuclear translocation was seen. For instance, methyl-prednisolone resulted in a strong GR: FKBP52 interaction, but little nuclear translocation, while GR liganded to mometasone exhibited a weaker interaction with FKBP52 (predominantly remaining complexed to FKBP51), and a greater extent of nuclear translocation. Thus, while it is plausible that the different conformations of GR when complexed to different ligands are responsible for the differential association with TPR proteins, the role of differential TPR association in the resulting GR transcriptional response is still unclear.

1.3.6 GR Dimerization

The palindromic nature of GRE sequences strongly suggests that gene regulation by direct DNA binding is to be mediated by dimers, and GR has indeed been shown to bind its response element as a homodimer (Tsai *et al.,* 1988). GR molecules dimerize through specific contacts on the second zinc finger within the DBD (Luisi *et al.,* 1991). GR dimerization has been reported to depend on DNA binding, such that DNA binding by one liganded monomer would induce dimerization and DNA binding by the second GR molecule to the second half-site of the palindrome (Dahlman-Wright *et al.,* 1990; Mikuni *et al.,* 2007). However, more recent reports indicate that GR dimerization occurs in the cytoplasm (Savory *et al.,* 2001). It has also been observed that nuclear translocation deficient mutant GR can be induced to translocate when co-expressed with wild-type GR, indicating that translocation of the mutant occurs as a dimer with a wild type partner (Savory *et al.,* 1999).

The commonly accepted view is that transactivation requires dimerization, while transrepression by protein tethering is mediated by GR monomers (reviewed in Nicolaides *et al.,* 2010). This was shown in experiments where mutations to the GR D-loop, responsible for dimerization, abrogated transactivation, but did not affect transrepression (Tuckermann *et al.,* 1999; Heck, 1994). Dimerization thus presents an attractive step for drug targeting, as drugs preventing dimerization could potentially have greater transrepressive activity, and reduced transactivation (reviewed in Newton, 2000). However, exceptions to this rule of thumb have been identified, and the ability of dimerization deficient mutants to transactivate appears to depend on the promoter context (reviewed in Newton & Holden, 2007; Meijsing *et al.,* 2009). Several endogenous promoters have been identified which contain half GRE sites, and are positively regulated by binding of GR monomers (reviewed in Schoneveld *et al.,* 2004)

The GR can also function as a heterodimer with other SRs such as MR (Liu *et al.,* 1995; Savory *et al.,* 2001) and AR (Yu *et al.,* 1997). It has been suggested that GR:MR heterodimerization may play a role in mediating tissue-specific effects of GCs which activate both receptors (e.g. cortisol and aldosterone) (Savory *et al.,* 2001), especially in light of the fact that MR and GR are only co-expressed in a

subset of tissues (Lu *et al.,* 2006). Furthermore, dimerization of GR appears to be ligand-selective as the dissociated GC CpdA abrogates GR dimerization (Dewint *et al.,* 2008; Robertson *et al.,* 2009). However, whether the extent of GR dimerization elicited by other GR ligands is ligand-selective, and the affect this may have on ligand-selective gene regulation by GR, has not been elucidated. The relative amount of homo- vs. heterodimers formed may also be ligand-selective.

1.3.7 GR nuclear localisation

In order to elicit its transcriptional effects, the GR must translocate to the nucleus in response to ligand. Translocation occurs through a large multiprotein complex called the nuclear pore complex (NPC) (reviewed in Kaffman & O'Shea, 1999). This is a GTP-dependent process, involving formation of a complex between the cargo protein and an importin protein to facilitate docking of the cargo at the NPC (reviewed in Kaffman & O'Shea, 1999).

The GR has two NLSs, designated NL1 (in the DBD/hinge region) and NL2 (in the LBD) (Picard & Yamamoto, 1987). Although NL1 is required for ligand-dependent nuclear import, when incorporated into a fusion protein, this signal caused constitutive nuclear localisation. NL2 on the other hand, was found to mediate ligand-dependent nuclear import (Picard & Yamamoto, 1987). While NL1 was found to facilitate nuclear import through interaction with importin α, NL2-mediated nuclear import was independent of importin α (Savory *et al.,* 1999). Subsequently, a variety of importin proteins have been implicated in nuclear localisation of the GR in different cell systems (Echeverría *et al.,* 2009; Tao *et al.,* 2006; Freedman & Yamamoto, 2004), suggesting that the precise importin, or combination of importins, involved may be cell-type specific.

Few studies have addressed the question of ligand-specific differences in nuclear translocation of the GR. While one study identified differences in nuclear

translocation between cortisol and RU486, cortisol was used at a sub-saturating concentration, so the reduced nuclear GR nuclear translocation in response to cortisol could be a due to the reduced fractional occupancy of GR with cortisol compared to GR with RU486 (Peeters *et al.,* 2008). Another study which used sub-saturating concentrations of GR ligand was that of Croxtall *et al.,* (2003) in A549 cells (e.g. 10nM methyl-prednisolone). Another study showed no significant difference in GR nuclear localisation between cells treated with saturating concentrations of dex and RU486 in mouse L929 cells (Pariante *et al.,* 2001). These studies suffer either the weakness of use of sub-saturating GC concentrations, or that of the use of too few ligands (generally only full agonists and antagonists) to allow the formulation of robust conclusions about the general effect of different ligands on GR nuclear translocation. Many questions thus remain unanswered with regard to ligand-selective GR nuclear localisation.

As mentioned in the section on GR conformation, the rank order of GR transcriptional efficacy has been shown to have a strong correlation with the extent of GR phosphorylation at S211 and S226 (Avenant *et al.,* manuscript in preparation). Furthermore, GR phosphorylated at S211 and S226 has been reported to be predominantly localised to the nucleus (Wang *et al.,* 2002). This observation could indicate that phosphorylation at S211 and S226 is required for nuclear import. However this has been shown not to be the case by use of site directed GR mutants (Avenant *et al.,* 2010*)*. An alternative explanation is that nuclear import is required for phosphorylation at S211 and S226, while yet another possibility is that GR phosphorylation and nuclear translocation are mutually independent but both modulated by a common upstream event, such as the conformation of ligand-bound GR. However the reason for the strong correlation between phosphorylation and nuclear localisation presents another unanswered question regarding the molecular mechanism of GR activity. Ligand-selective nuclear localisation of the GR definitely warrants further investigation with a large panel of ligands used at saturating concentrations, to elucidate the impact of nuclear translocation on the biological activity of different GR ligands, with different biocharacters.

1.3.8 GR mobility within the nucleus

The original model of GR transcriptional regulation involved fairly static promoter occupancy by the GR for a period of minutes or hours (Biddie & Hager, 2009). However, the currently accepted model portrays a highly dynamic association, either by direct DNA binding or protein tethering, between the GR and target promoter, and suggests that promoter interactions only last a matter of seconds (reviewed in George *et al.,* 2009; Biddie & Hager, 2009*)*. Although the evolutionary benefit conferred by the rapid cycling of GR on the chromatin is not understood, it is thought that this allows faster responses to changes in ligand concentration than would be allowed by a longer-term static GR:chromatin interaction (reviewed in Biddie & Hager, 2009).

Fluorescence recovery after photobleaching (FRAP) analysis and the use of tandem mouse mammary tumour virus (MMTV) long terminal repeat (LTR) arrays has greatly enhanced understanding of the molecular dynamics of nuclear GR (Biddie & Hager, 2009). When fluorescently tagged GR associated with a chromatin array was subjected to FRAP photobleaching, fluorescence was recovered within a matter of seconds, indicating that unbleached GR molecules are highly mobile, and swiftly replace the photobleached molecules at the promoter (McNally *et al.,* 2000). Subsequent reports have confirmed this finding, and in addition, have shown that nuclear mobility of the GFP-rGR requires Hsp90 (Elbi *et al.,* 2004) and ATP (Fletcher *et al.,* 2002).

The mobility of GR has been shown to vary between different cell types (Schaaf & Cidlowski, 2003), and the cell-type specific characteristics responsible for these differences in GR nuclear mobility remain to be determined. Furthermore, GR nuclear mobility has been found to be ligand-specific, and there is a negative correlation between GR mobility and ligand affinity (Schaaf & Cidlowski, 2003). The GR has also been found to adopt different distribution patterns within the nucleus

when bound to different ligands (Vicent *et al.,* 2002; Schaaf *et al.,* 2005). In COS-1 cells, the subnuclear distribution of GR ranged from discrete, punctuate foci, to a diffuse random distribution depending on the ligand. Using a panel of 13 GR ligands, it was found that high affinity (mostly synthetic) ligands caused a more punctuate distribution, and a greater reduction in nuclear mobility of GR than lower affinity (mostly naturally occurring) ligands. This could suggest that more potent or efficacious ligands induce stronger interactions with chromatin, however this hypothesis is controversial (Schaaf *et al.,* 2005). Consistent with this finding, Stavreva and co-workers (2004) noted a positive correlation between GFP-rGR residency at a promoter and transcriptional output (Stavreva *et al.,* 2004). On the other hand, Schaaf *et al.,* (2005) reported that GR mobility was lower in the subnuclear regions with higher concentrations of GR, and van Steensel *et al.* (1995) showed that subnuclear regions of high GR concentration do not colocalise with RNA polymerase or newly synthesised RNA. This would suggest that the reduction in GR mobility at concentrated foci is not due to GR transactivation activity, although it may relate more to GR transrepression. Thus the role of ligand-specific differential nuclear mobility of GR remains to be fully elucidated.

1.3.9 DNA binding

Since the GR is accepted to adopt different conformations on binding to different ligands, it makes sense that the extent and kinetics of DNA binding would also be affected by the differences in conformation. However, few studies have specifically addressed this issue. Wang *et al.* (2006) examined the transcriptional effect of a panel of structurally similar arylpyrazole GR ligands, and further went on to examine GR promoter occupancy at several endogenous promoters in A549 human lung epithelial cells. Interestingly, promoter- and ligand-specific differences in GR recruitment were identified, but recruitment of GR to the promoter did not appear to correlate with the fold change in transcription observed for any of the three genes and six compounds tested. Thus the precise role of differential DNA binding in ligand-specific transcription is unclear.

It has been shown that the sequence of the GRE to which GR binds can have a profound influence on its DNA-binding affinity and transactivational activity. This was confirmed by X-ray crystallography of the GR DBD complexed with different DNA sequences, showing slightly different GR conformations (Meijsing *et al.,* 2009). It has also been reported that DNA interactions by the DBD induce conformational changes in the AF-1 domain (Kumar *et al.,* 1999). Collectively, this led to the suggestion that DNA functions as an allosteric modulator of GR conformation, and can thereby alter recruitment of cofactors in a promoter-specific fashion (Meijsing *et al.,* 2009; Lefstin *et al.,* 1994). This could have implications for tissue-specific GR activity in certain types of cancers, which exhibit a high rate of mutation, as even a single base pair mutation in a regulatory region could deregulate GR recruitment and activity.

1.3.10 Protein-protein interactions and cofactor recruitment

GR tethering to other promoter-bound transcription factors has been found to be ligand-specific. Garside *et al.,* (2004) noted ligand-induced differences in the interaction of GR with the p65 subunit of NFκB, resulting in significantly less GR recruitment to the NFκB-regulated interleukin 8 (IL8) promoter in the presence of RU486 than in the presence of dex, in HeLa cells. Unfortunately the use of only two ligands prevents the formulation of a general conclusion of the role of differential interaction with p65 in GR-mediated transrepression, and this remains to be investigated with a broader panel of ligands. Moreover, to the best of the present author's knowledge, no reports of ligand-selective differential GR interaction with promoter-bound AP-1 subunits or other DNA-binding transcription factors exist in the literature.

Once bound to chromatin, or tethered to other transcription factors, steroid receptors recruit a range of cofactors to elicit transactivation or transrepression (Horwitz *et al.,* 1996). These cofactors have traditionally been designated as either "coactivators" or "corepressors", but it is becoming increasingly clear that a single cofactor can elicit either an increase or decrease in transcription depending on the promoter context

(Rogatsky *et al.,* 2002; Peterson *et al.,* 2007). These definitions have therefore been avoided here. The GR interacts with a large number of transcriptional coregulators in order to carry out its biological activity, with possibly the best known interaction partners being members of the p160 family of transcriptional coregulators: steroid receptor coactivator 1 (SRC1), glucocorticoid receptor interacting protein 1 (GRIP1) and amplified in breast cancer 1 (AIB1) (reviewed in Xu *et al.,* 2009). These three coregulators share a high level of homology and are roughly 160 kDa in size. In addition to SRs, the p160s can also potentiate transcriptional responses of other transcription factors such as AP-1, NFκB and CREB (reviewed in Xu & Li, 2003). Although members of the p160 family can compensate for one another to a certain extent, several reports of specific differences in recruitment and activity have been reported (Kotitschke *et al.,* 2009; reviewed in Xu *et al.,* 2009). Cofactors which are primarily involved in transrepression include silencing mediator of retinoic and thyroid hormone receptors (SMRT) and nuclear receptor corepressor (NCoR), which are highly homologous (reviewed in Collingwood *et al.,* 1999).

Cofactors mediate their effects by modifying chromatin structure via two main mechanisms: either by their own enzymatic activity or by recruitment of other factors with enzymatic activity. The intrinsic HAT or HDAC activity of cofactors may play a role in modulating acetylation of histones or other proteins to alter the transcriptional response. All p160s have been found to possess intrinsic HAT activity (Goel & Janknecht, 2004), (Spencer *et al.,* 1997; Chen *et al.,* 1997), while NCoR and SMRT have intrinsic histone deacetylase (HDAC) activity that removes acetyl groups from histones, causing compaction of the chromatin structure, and reducing transcription from the promoter (reviewed in Privalsky, 2004). Cofactors such as the p160 family may also act as “scaffolds” for recruitment of secondary coregulators such as adenovirus E1A-binding protein p300 (p300), CREB-binding protein (CBP), p300/CBP associated factor (pCAF), and coactivator associated arginine methyltransferase 1 (CARM1) (reviewed in Rosenfeld & Glass, 2001). These secondary coregulators possess chromatin modifying properties, and induce a more relaxed chromatin conformation in which the promoter of the target gene is more accessible to RNA polymerase or basal transcription machinery (reviewed in Xu & Li, 2003). NCoR and SMRT, acting in a corepressive context, recruit other HDAC

proteins, which cause chromatin compaction and reduce transcription (reviewed in Rosenfeld & Glass, 2001).

Different ligands have been shown to result in differential coregulator recruitment for the GR (Kroe *et al.,* 2007; Wang *et al.,* 2004; Wang & Simons, 2005; Wang *et al.,* 2007b; Coghlan *et al.,* 2003; Garside *et al.,* 2004; Cho *et al.,* 2005; Miner *et al.,* 2007; Tao *et al.,* 2008). Strong evidence exists for a steroid-dependent cofactor binding model whereby the transcriptional response of a steroid receptor to agonists and antagonists is linked to the recruitment of cofactors which activate or repress gene transcription, respectively, while partial agonists recruit a mixture of these cofactors (Kang *et al.,* 2004). In support of such a model, it has been shown that agonist-bound GR binds GRIP-1 to a greater extent than partial agonist-bound GR (Cho *et al.,* 2005). However, Wang *et al.* (2007b) found that GR recruited equal amounts of TIF2 (GRIP-1) when liganded to the agonist dexamethasone (dex) and the antagonist RU486.

A recent investigation by Ronacher *et al.,* (2009) in COS-1 cells provided strong biochemical support for a model in which GR-mediated ligand-selective differential interactions with GRIP-1, SRC1, NCoR and SMRT is a major determinant of ligand-selective and promoter-specific effects for both transactivation and transrepression. Interestingly, it was found that the relative preference of liganded-GR for GRIP-1 versus SRC-1A is clearly dependent on the ligand. Previous investigators have shown that on the MMTV promoter, the PR liganded to progesterone interacts preferentially with SRC-1 rather than GRIP-1 (Li *et al.,* 2003). Similarly, the MR when liganded to aldosterone, shows preferential interaction with SRC-1-4a peptides but not with GRIP-1 peptides (Hultman *et al.,* 2005), while the GR liganded to dex interacts preferentially with GRIP-1 rather than SRC-1 (Li *et al.,* 2003). The finding that the GR cofactor selectivity shifts towards a preference for SRC-1A rather than GRIP-1 when liganded to progesterone, MPA or aldosterone suggests that the ligand plays a role in cofactor selectivity independent of the receptor, within the MR, PR and GR family (Ronacher *et al.,* 2009). This would be consistent with crystal

structure studies suggesting that the GR and PR share a common mechanism of cofactor selectivity (Bledsoe *et al.,* 2002).

Besides mediating ligand-specific effects, differential cofactor recruitment provides a mechanism to elicit tissue specific effects to systemic GCs. The requirement for different p160 cofactors by GR has been shown to be different in astrocytes of the central nervous system, and Schwann cells of the peripheral nervous system (Trousson *et al.,* 2007; Grenier *et al.,* 2006). This could depend on the relative endogenous levels of coregulators present in different cell types, which have been shown to vary between different cell lines (Zhang *et al.,* 2004) and tissues (Meijer *et al.,* 2000). An interesting report by Fryer and co-workers (2000) demonstrates ligand- and tissue-specific effects of cofactor recruitment on gene transactivation. It was found that exogenous GR liganded to RU486 recruited the chromatin remodelling factor BRG1, and elicited subsequent transactivation of an MMTV promoter, in an osteosarcoma cell line, but not in a breast cancer cell line, while other GR antagonists failed to elicit an effect in either cell line. Thus differential recruitment of cofactors is an important step in mediating both ligand- and cell type-specific effects of GR ligands.

1.3.11 Ligand-dependent turnover of GR

GR levels have been found to be ligand-dependently downregulated (Bellingham *et al.,* 1992; Hoeck *et al.,* 1989), by a mechanism involving both decreased transcription of the GR gene, and increased receptor turnover (reviewed in Oakley & Cidlowski, 1993). While protein degradation can occur via ubiquitin-dependent and –independent pathways, it has been shown that the GR is ubiquitinated (Wallace & Cidlowski, 2001), and that the proteasome is recruited to activated GR, which is required for rapid GR cycling at promoter sites (Stavreva *et al.,* 2004). Proteasomal degradation of proteins is a tightly-regulated process involving a series of fairly well-characterised steps. Proteins must be tagged with a poly-ubiquitin tag, which occurs at conserved lysines via an ATP-dependent three-step enzyme cascade, to identify them as degradation targets (Kinyamu *et al.,* 2005). Following ubiquitination, the

target protein is degraded by a multi-protein complex called the 26S proteasome (reviewed in Glickman & Ciechanover, 2002; Kinyamu *et al.,* 2005).

Several groups have identified ligand-specific differences in the rate of ligand-mediated turnover of GR (Cidlowski & Cidlowski, 1981; Hoeck *et al.,* 1989). Most recently, a thorough study of GR turnover with a wide panel of ligands has identified a positive correlation between the half-life of liganded GR and the potency and efficacy of transcription elicited by a particular ligand (Dr C. Avenant, PhD thesis). This observation could indicate that proteasomal degradation of activated GR acts as a negative feedback mechanism to modulate the response to GCs. However, the molecular mechanism underlying ligand-dependent GR turnover is not fully understood.

1.3.12 GR ligand-independent signalling

As mentioned previously, a number of non-GC signalling molecules have been identified which modulate GR activity (Dr A. Kotitschke, PhD thesis, 2009). The relative levels of such molecules in different tissues can have an impact of the specific activity of GR in a given cell-type. For instance, gonadotropin releasing hormone (GnRH), the peptide ligand of the GnRH receptor (GnRHR), has recently been found to activate GR, causing phosphorylation, nuclear translocation and binding to the GnRHR gene promoter (Kotitschke *et al.,* 2009). Because this response is mediated by the GnRHR itself, this signalling pathway would only occur in cells which express endogenous GnRHR, such as pituitary gonadotropes and reproductive tissues (Kotitschke *et al.,* 2009).

Non-GC signalling molecules may also augment a GC response, as in the case of GnRH and dex, which together elicit a synergistic effect on transcription (Kotitschke *et al*., 2009). It has not been determined whether such interactions may be ligand-selective, for instance co-treatment of cells with GnRH and another GR ligand may

not result in a synergistic effect on gene transcription. This possibility warrants further investigation.

1.3.13 Post-translational modifications of the GR

In this section, known post-translational modifications (PTMs) of GR will be discussed. It is becoming increasingly evident that cross-talk occurs between different residues of the GR, such that different post-translational modifications can modulate each other. For instance, Davies and co-workers reported cross-talk between GR sumoylation and phosphorylation (Davies *et al.,* 2008); while preliminarily evidence suggests that nitration at tyrosine residues may enhance ubiquitination of GR, resulting in its degradation by proteasomes (Ito, 2007). Furthermore, a complex relationship between GR phosphorylation at different sites has been identified (Wang *et al.,* 2007a). This interplay between GR post-translational modifications adds to the complexity of regulation of GR transcriptional effects.

1.3.13.1 Phosphorylation

The GR, like all steroid hormone receptors, is constitutively phosphorylated and undergoes hyperphosphorylation upon ligand binding (Faus & Haendler, 2006). Most of the phosphorylation sites identified thus far are serine residues in the N-terminal domain, namely S113, S141, S203, S211 and S226 (Ismaili & Garabedian, 2004), while S404 near the DBD has also been identified as a phosphorylation site (Galliher-Beckley, Williams, Collins, & Cidlowski, 2008). GR phosphorylation appears to form part of a complex regulatory mechanism controlling GR transcriptional activity. The phosphorylation status of one site can affect the phosphorylation at other sites in a particular GR molecule, as well as transcriptional efficacy (Wang *et al.,* 2002), and these effects are gene-specific (Chen *et al.,* 2008). GR phosphorylation has been implicated in regulating its interaction with the cofactor GRIP1 (Avenant *et al.,* 2010), TSG101, a mediator of ubiquitin-dependent

proteolysis and DRIP150, a subunit of the mediator complex (Ismaili & Garabedian, 2004). Furthermore, phosphorylation of hGR at S404 has been shown to be required for recruitment of p300 (Galliher-Beckley *et al.,* 2008).

The three best characterised phosphorylation sites are S211, S226 and S203, which are conserved between human, mouse and rat GRs. GR phosphorylation at S211 appears to be closely linked with the transcriptional activity of the GR, since this site is rapidly phosphorylated in the presence of dex, and GR phosphorylated at S211 is located mainly inside the nucleus (Wang *et al.,* 2002). However introduction of an S211A mutation to abolish phosphorylation resulted in increased transcription on some promoters, and decreased transcription on others (Chen *et al.,* 2008). S226 is also rapidly phosphorylated in the presence of dex and GR phosphorylated at S226 is also located primarily in the nucleus (Wang *et al.,* 2002), but mutation of this site to abolish phosphorylation (S226A) increases the potency and efficacy of GR transcription on a number of endogenous genes (Chen *et al.,* 2008), indicating that this site may be required to negatively modulate the GR response. In contrast, S203 exhibits higher basal phosphorylation than S211 or S226, and a smaller dex-mediated increase in phosphorylation in U2OS-hGR and A549 cells (Chen *et al.,* 2008). This phospho-isoform is also located mainly in the cytoplasm (Wang *et al.,* 2002), and consistent with this, GR S203P was not immunoprecipitated on a variety of endogenous promoters (Blind & Garabedian, 2008). S211 and S226 phospho-GR isoforms have been shown to be recruited to the promoters of tyrosine aminotransferase (tat) and sulfonyltransferase-1A1 (sult) genes in rat hepatoma cells, and the glucocorticoid-induced leucine zipper (GILZ) gene in human U2OS cells, and differences in the kinetics of recruitment to different promoters have been identified, adding another layer of complexity to the role of differential GR phosphorylation (Blind & Garabedian, 2008). Although the phosphorylation status of different residues has been found to correlate with the subcellular localisation of the GR, this could indicate that phosphorylation determines the subcellular localisation, or that the subcellular localisation determines the phosphorylation status of the GR. Another possibility is that another upstream factor (e.g. GR conformation) determines both the phosphorylation status of the GR and its subcellular localisation. A recent finding that mutation of hGR to abolish phosphorylation at S203, S211 and S226 did not affect nuclear translocation of the GR (Avenant *et al.,* 2010) points

towards one of the latter two possibilities. However further work is needed to fully elucidate the role of nuclear translocation in GR phosphorylation.

Mitogen activated protein kinases (MAPKs), cyclin dependent kinases (CDKs) and casein kinase II have been implicated in GR phosphorylation (reviewed in Faus & Haendler, 2006). Several reports have suggested that the serine-threonine phosphatase, PP5 is responsible for dephosphorylation of the GR (Ismaili & Garabedian, 2004). PP5 is a TPR protein which can associate with the GR-Hsp-heterocomplex. Dissociation of PP5 from the Hsp-heterocomplex has been suggested to be a crucial step enabling GR phosphorylation upon ligand binding (Wang *et al.,* 2007a). PP5 was also recently shown to mediate dephosphorylation GR at S211 within the nucleus, resulting in reduced GR-mediated transactivation of MKP1 and serum/glucocorticoid regulated kinase (SGK) genes (Zhang *et al.,* 2009).

Chen *et al.,* (2008) elegantly demonstrated that GR phosphorylation is both cell-type and ligand-specific. Distinct temporal patterns and kinetics of GR phosphorylation were identified between stably transfected U2OS-hGR and A549 cells. In particular, dex mediated phosphorylation at S226 was found to be lower in A549 than in U2OS cells. The reason for these cell-type specific differences remains to be elucidated, but could include differential expression of kinases or phosphatases. Using a panel of 9 ligands, Chen *et al.,* (2008) observed differential phosphorylation of S211, S226, and S203 of stably expressed GR in U2OS cells. Furthermore, the ratio of S211P:S226P was found to vary with different ligands. Another study found that while phosphorylation at S226 is ligand-selective and correlates with transcriptional efficacy for transcription on three different promoters, mutation of S226 to abolish phosphorylation caused the same relative increase in transcription for all ligands, indicating that phosphorylation at S226 inhibits maximal transcription, but does not determine the rank order of ligand-selective transcription (Dr C. Avenant, PhD thesis). Taken together, this strongly suggests that differential GR phosphorylation is an important regulator of the ligand-selective efficacy of GR responses, although GR phosphorylation alone does not determine the relative transcriptional response to a specific ligand.

1.3.13.2 Acetylation

Acetylation of lysine residues plays an important role in the activity of several transcription factors (reviewed in Glozak *et al.,* 2005). To date, only two reports investigating the effect of GR acetylation have been published (Ito *et al.,* 2006; Nader *et al.,* 2009). Ito *et al.,* (2006) found that the overall acetylation status of GR increased on co-incubation with dex and IL1β. Although ligand-dependency was not demonstrated, this report showed that residues K494/495 were acetylated in the presence of dex. The authors further report that GR acetylation at K494/495 reduces its ability to interact with the p65 subunit of NFκB, and thus its ability to repress gene expression. Furthermore, HDACs 1 and 3 were shown to deacetylate the GR *in vitro*. Nader *et al.,* (2009) implicate the circadian rhythm modulator CLOCK in GR acetylation at several lysine residues within the hinge region. Overexpression of CLOCK and its heterodimerisation partner, brain-muscle-arnt-like protein 1 (BMAL1), in HeLa cells was found to decrease DNA-binding by the GR, and thereby reduce transactivation.

The possibility of SR acetylation as a mechanism of control of tissue specific effects is both plausible and intriguing. Ito *et al.,* (2006) showed that knockdown of specific HDACs could affect the acetylation status of the GR. Therefore, the relative ratio of endogenously expressed HAT and HDAC proteins could fine-tune the GR response for a particular setting, since HAT and HDAC expression and subcellular distribution varies in different cell and tissue types (Meijer *et al.,* 2000; Petrie *et al.,* 2003; Van den Wyngaert *et al.,* 2000; Igarashi-Migitaka *et al.,* 2005). However, to the best of the author's knowledge, there are no reports investigating differences in acetylation levels or acetylated residues of SRs when activated by different ligands.

1.3.13.3 Other PTMs: ubiquitination, sumoylation, nitration and methylation

Ubiquitin is a highly conserved 76 amino acid polypeptide, which is generally tagged onto a protein to target it for proteasomal degradation by a carefully controlled series of stepwise reactions (reviewed in Kinyamu *et al.,* 2005). It is attached to serine residues within conserved Proline-Glutamate-Serine-Threonine (PEST) motifs (reviewed in Duma *et al.,* 2006). The GR has been shown to become ubiquitinated, and K419 of hGR, found within a conserved degradation motif, has been implicated, as mutation of the corresponding residue in mGR results in a decrease in degradation of the protein (Wallace & Cidlowski, 2001).

Sumoylation refers to the addition of a small ubiquitin-related modifier (SUMO) at lysine residues. SUMO is an 11 kDa (98 amino acid) polypeptide, which appears to play a role in a wide range of cellular functions, from subcellular localisation to protein stability (Faus & Haendler, 2006). GR has been shown to be sumoylated *in vitro* (Poukka *et al.,* 2000) and *in vivo* on overexpression of SUMO-1 (Le Drean *et al.,* 2002). Three sumoylation consensus sites have been identified, and mutation studies indicate that sumoylation of GR enhances its activity in a promoter-specific fashion (Tian *et al.,* 2002), while overexpression studies indicate a role for sumoylation in increasing GR turnover (Le Drean *et al.,* 2002). Since sumoylation is influenced by environmental cues, such as stress, this presents a possible mechanism to fine-tune GR activity to suit a cell's particular physiological state (reviewed in Duma *et al.,* 2006).

Proteins can further be modified by nitration of tyrosine residues. GR has been reported to undergo tyrosine nitration on binding to NCX-1015, a member of a family of NO-donating ligands, termed nitrosteroids (Paul-Clark *et al.,* 2003). GR nitration increased ligand-binding, nuclear translocation and transcriptional activity (Paul-Clark *et al.,* 2003).

Protein methylation is a relatively common mechanism of regulating protein function. The methyltransferase, CARM1, is recruited to ER-regulated promoters through a GRIP1 interaction domain, and has been shown to methylate CBP/p300 (Chevillard-

Briet *et al.,* 2002) leading to synergistic transcriptional activation (Chen *et al.,* 2000; Lee *et al.,* 2002). Methylation of GR has not been demonstrated, so it is still unclear whether this particular post-translational modification may regulate GR activity (reviewed in Stallcup *et al.,* 2003).

1.4 Conclusion

An appropriate, cell-specific response to a systemic GC is crucial for homeostasis. Various systems have evolved to ensure that GR responses are carefully controlled in different cell and tissue types in the body. These mechanisms often involve varying the expression levels of endogenous GR, or GR interacting partners, to modulate the response. When these control mechanisms go awry, problems such as GC insensitivity may result, and have a significant physiological impact.

The central role of GR in a number of biological processes has made it an excellent drug target for a number of disorders. However, the wide range of physiological effects and side-effects elicited by different GR ligands has made it clear that GR activation is far from a simple “on/off switch”. Subtle differences in ligand structure can cause GR responses to vary. Although the basis of ligand-specific effects seems to be differences in the conformation of the ligand-activated GR, these differences impact on several different steps in the GR transcriptional regulation pathway.

This chapter has given a broad overview of the steps at which both ligand- and tissue specific GC effects can manifest. The specific DNA sequence at which the GR acts can also have a profound influence on the biological outcome of GR activity, adding to the complexity of GC responses. A deeper understanding of the molecular mechanisms behind these variations is critical to the design of more specific drugs, or treatment plans more tailored to individual patients. As highlighted in this review, GR-mediated gene regulation is an extremely complex process, regulated at many levels. Therefore the development of GC drugs with a high therapeutic index and negligible side-effects will be enormously challenging. However, given the

importance of GC therapy for a wide range of diseases, this lofty aspiration is certainly worth striving towards.

Chapter 2

AIMS AND HYPOTHESES

Unravelling the molecular mechanisms of GR activity and uncovering the mechanisms responsible for ligand-specific responses of GR is an area of intensive research by many laboratories. The broad aims of the Hapgood laboratory include contributing to understanding these mechanisms. In order to achieve this aim, the process of GR transcriptional regulation has been broken down into individual steps, several of which have been the focus of intensive research by laboratory members. Important findings have recently been published on phosphorylation (Avenant *et al.,* 2010) and cofactor recruitment (Ronacher *et al.,* 2009) by ligand-activated GR, as discussed in Chapter 1. This study therefore formed part of a wider research effort.

Although acetylation of other SRs appears to play an important role in their function, there is a paucity of literature on GR acetylation. The first part of this thesis (Chapter 3) aimed to elucidate the role of GR acetylation in transactivation. The hypotheses on which this work was based are:

- that GR is acetylated at K494/495 when bound to and activated by ligand, when endogenously expressed or transiently overexpressed in COS-7 cells.
- that GR acetylation at K494/495 plays a functional role in transactivation through modulation of one or several of the following steps: ligand binding, nuclear translocation, phosphorylation, DNA binding, or cofactor recruitment.
- that FKBP52 plays a role in modulating the biological activity of acetylated GR.

Chapter 1 highlights the lack of understanding of the role of ligand in determining the extent of nuclear localisation of the GR. Few reports exist in the literature investigating ligand-selective nuclear translocation of GR, and the results appear contradictory. Thus the second aim of this project (results reported in Chapter 4) was

to investigate the relationship between ligand-selective differences in transcriptional regulation by the GR and differential nuclear localisation of the GR when bound to different ligands. The following hypotheses were formulated and subsequently investigated:

- that different ligands at saturating concentrations will result in different transcriptional responses via the GR on promoter-reporter constructs and endogenous genes in U2OS cells.
- that different ligands at saturating concentrations will cause differences in the extent of nuclear localisation of endogenous GR in U2OS cells.
- that different ligands at saturating concentrations will cause different extents of phosphorylation at serine-211 of activated endogenous GR in U2OS cells.
- that the extent of ligand-specific differential nuclear localisation of GR and/or phosphorylation at serine-211 will correlate with the biological response of different ligands.

Chapter 3

Critical role of lysines 494/495 in GR ligand binding and function:

Functional implications of GR acetylation

The glucocorticoid receptor (GR) and other steroid receptors undergo multiple post-translational modifications, including phosphorylation and acetylation, which play an important role in modulating their biological effects. GR has previously been shown to be acetylated at residues K494 and K495. However the role of GR acetylation in transcriptional activation is unclear. The functional consequences of GR acetylation were investigated at several steps in the GR transactivation pathway, using expressed hGR mutants as compared to wild-type GR (WT hGR). The acetylation deficient K494/495A hGR mutant showed a loss of GR nuclear translocation, ligand-dependent phosphorylation, *in vitro* DNA-binding and transactivation activity on a synthetic glucocorticoid response element (GRE). This is consistent with the finding that this mutant exhibits undetectable ligand-binding, although its expression levels and cytoplasmic immunofluorescence staining pattern are indistinguishable from that of WT hGR. Results with the K494/495Q acetylation mimic mutant strongly support the argument that acetylation of the GR at K494/495 is important for ligand-binding and the resulting downstream actions of the GR. Compared to WT hGR, the K494/495Q mutant has an enhanced ligand binding capacity and transactivation efficacy on a GRE reporter gene. While *in vitro* DNA-binding activity is unaltered, the K494/495Q mutant surprisingly displays reduced nuclear translocation and phosphorylation at S211 and S226 compared to WT hGR. Chromatin immunoprecipitation (ChIP) on the endogenous GRE-containing GILZ gene supports a model in which interaction of the immunophillin FKBP52 with acetylated K494/495 of the hGR plays an important role in GR function by acting as a GR coactivator. Taken together, the results suggest that modulation of GR acetylation at K494/K495 represents an attractive physiological mechanism for regulation of glucocorticoid sensitivity and transactivation efficacy.

The GR is a ligand-activated transcription factor that regulates expression of a wide range of genes (reviewed in Zhou & Cidlowski, 2005; Nicolaides *et al.,* 2010). It belongs to the family of steroid receptors encompassing the androgen, mineralocorticoid, estrogen, and progesterone receptors (AR, MR, ER, and PR respectively). Unliganded GR resides largely in the cell cytoplasm, in a complex with heat shock proteins (Hsps) 40, 70, 90 and the Hsp 90-binding tetratricopeptide (TPR) containing immunophilin, FK506-binding protein 51 (FKBP51) (Davies *et al.,* 2002). Upon ligand binding the complex undergoes a functional exchange of FKBP51 for FK506-binding protein 52 (FKBP52) (Davies *et al.,* 2002). Ligand binding also results in a conformational change, hyperphosphorylation and nuclear translocation of the GR, where it can either positively or negatively regulate gene expression by direct DNA binding, or interaction with other DNA binding proteins (reviewed in Zhou & Cidlowski, 2005).

FKBP52 is an immunophilin cochaperone that has been found in Hsp heterocomplexes of AR, MR, PR and GR (Tai *et al.,* 1986; Gallo *et al.,* 2007; Barent *et al.,* 1998). FKBP52 has been shown to promote GR activity at several steps in the transcriptional activation process. Firstly, its presence in the Hsp heterocomplex improves the ligand binding ability of the GR (Riggs *et al.,* 2003; Riggs *et al.,* 2007). After ligand binding, FKBP52 interacts with dynein to facilitate movement of the GR heterocomplex along the microtubules into the nucleus (Harrell *et al.,* 2004; Galigniana *et al.,* 2002). However, a significant portion of FKBP52 resides in the nucleus (Czar *et al.,* 1994; Perrot-Applanat *et al.,* 1995). Nuclear FKBP52 has recently been shown to play a gene-specific role in transactivation by GR, since targeted ablation of FKBP52 significantly reduces expression of GILZ, but has no effect on GR-mediated SGK expression (Wolf *et al.,* 2009). Furthermore, overexpression of FKBP52 has been shown to increase agonist-mediated transactivation potency of the GR (Riggs *et al.,* 2003). However the molecular mechanism of modulation by FKBP52 of GR-mediated transcription in the nucleus remains to be determined, and whether or not FKBP52 is recruited to a GRE promoter has not previously been established. Interestingly, FKBP52 has been shown to interact directly with the GR between amino acids 465 and 500 *in vitro* (Silverstein *et al.,* 1999). Although FKBP52 primarily docks to a site on the Hsp 90

dimer (Young *et al.,* 1998), it contains a conserved sequence of 8 amino acids with 6 negatively charged residues which are electrostatically complementary to the GR NLS (490-RKTKKKIK-497) (Czar *et al.,* 1995). Furthermore lysine residues within this region of the GR have been implicated as acetylation targets (Ito *et al.,* 2006), suggesting that GR acetylation at residues between amino acids 465 and 500 may regulate FKBP52-mediated transcriptional effects on the GR.

The GR and other steroid receptors have been shown to undergo multiple post translational modifications, such as phosphorylation, ubiquitinylation, sumoylation and acetylation, which play an important role in modulating their biological actions (reviewed in Faus & Haendler, 2006). Although acetylation has not been reported for the MR or PR, several studies have investigated the functional role of lysine acetylation for the AR, ER and GR. The AR, GR, MR and PR share a highly conserved KXKK motif (where K= lysine and X= any amino acid), within a nuclear localisation signal (NLS) in their DNA binding domains (DBD) (Kim *et al.,* 2006). Lysine residues within this region have been shown to be acetylated for the AR and GR, and results support a functional role for these acetylated lysines in transcriptional regulation (Ito *et al.,* 2006; Fu *et al.,* 2000; Nader *et al.,* 2009). Lysine residues in the DBD of both the AR and ERα have been shown to be acetylated directly by p300 *in vitro*, which resulted in increased DNA binding for ERα (Kim *et al.,* 2006; Fu *et al.,* 2000). Acetylation of the AR at residues within the KXKK motif has been shown by mass spectrometry and Edman degradation, and has been shown to be ligand-independent (Fu *et al.,* 2000). AR acetylation appears to play a key role in its nuclear translocation, cofactor recruitment and transactivation (Gaughan *et al.,* 2002; Fu *et al.,* 2002; Fu *et al.,* 2004; Fu *et al.,* 2003). Acetylation of ERα in the DBD has also been shown by ^{3}H-acetyl CoA incorporation assays under basal conditions with a small increase being observed in the presence of ligand (Kim *et al.,* 2006). The GR has also been reported to undergo an increase in overall acetylation on ligand binding, while lysine residues 494 and 495 have been shown by immunoprecipitation experiments to be acetylated in the presence of dex and the proinflammatory cytokine IL1β in A549 cells. This acetylation resulted in reduced association with p65-NF-κB, lifting the transrepressive effect of the GR (Ito *et al.,* 2006). The deacetylation of the GR by histone deacetylase 2 (HDAC-2) increased its

binding affinity for p65. Another report in HCT116 cells showed an increase in dex-mediated GR acetylation at K494/495 was only observed in the presence of overexpression of the CLOCK/BMAL1 heterocomplex, which possesses HAT activity (Nader *et al.,* 2009). The hGR undergoes dex-mediated phosphorylation at S226 and S211 which influences maximal efficacy for transactivation (Chen *et al.,* 2008; reviewed in Duma *et al.,* 2006; Ismaili & Garabedian, 2004). The relationship between GR phosphorylation and acetylation is not known, but results with the ER (Cui *et al.,* 2004) and AR (Fu *et al.,* 2004) suggest that these modifications may modulate each other.

In the current study, the role of acetylation of the hGR at lysines 494 and 495 was investigated in transactivation, nuclear translocation, phosphorylation of the GR at S211 and S226, DNA-binding and ligand-binding. Furthermore, the role of FKBP52 in modulation of transactivation by the acetylated GR was explored.

MATERIALS AND METHODS

Cell lines and test compounds COS-7 monkey kidney and A549 human lung fibroblast cells (ATCC) were cultured in high glucose (1 g/ml) Dulbecco's Modified Eagles Medium (DMEM) (Sigma-Aldrich) supplemented with 10% (v/v) fetal calf serum (FCS) (Delta Bioproducts), 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco Invitrogen) (complete medium) at 37°C in a water jacket incubator (90% humidity and 5% CO_2). Dexamethasone ((11β,16α)-9-Fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione) (dex) and 4α-Phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich, dissolved in absolute ethanol, and stored at -20˚C. Trichostatin A (TSA) was purchased from Sigma-Aldrich.

Plasmids The pTAT-GRE-E1b-luc reporter plasmid containing two copies of the Glucocorticoid response element (GRE) from the tyrosine amino transferase (TAT) promoter has been described previously (Sui *et al.,* 1999). The hemagglutanin (HA) tagged WT hGR (WT HAhGHR) in pCMV vector was a gift from Dr M.J. Garabedian (New York University, USA). pcDNA3 WT hGR (WT hGR) was a gift from Dr D. Ray (University of Manchester, UK), and the pcDNA3 K494/495A hGR (K494/495A hGR) mutant was donated by Dr K. Ito (Imperial College, London). The HA-tagged K494/495Q HAhGR (K494/495Q HAhGR) mutant was generated with pCMV-HA-hGRwt as a template. Briefly, the mutant was generated by PCR using overlapping sense (GGAAGCTCGAAAAACAcAGcAAAAAATAAAAGGAAT) and anti sense (ATTCCTTTTATTTTTTgCTgTGTTTTTCGAGCTTCC) primers (mutated bases are indicated in lowercase) using Accusure™ Bioline Taq polymerase (BIO 20168) according to the manufacturer's specifications using the following conditions: 95°C for 10 min followed by 18 cycles of 95°C for 45 sec, 55°C for 45 sec and 68°C for 14 min. The PCR product was purified and concentrated using the Zymogen clean and concentrator kit (Zymogen Research, D 4003). The template DNA was subsequently removed by digestion with *Dpn* I (Fermentas). DNA was then re-purified, concentrated and transformed into *Escherichia coli* (*E.coli*) DH5α according to a standard protocol. The mutation in the GR plasmid was confirmed by sequencing.

Acetylation experiments in A549 cells Cells were plated at a density of 1 x 10^5 cells/ml in 6 cm dishes in complete medium. Cells were treated for 24 h with 1 ng/ml PMA or vehicle (ethanol) in serum-free DMEM. Dex was added to a final concentration of 100 nM, for 1 h. Cells were lysed and harvested in 200 µl cytobuster (Novagen) with protease inhibitors 1 mM PMSF, 2 µg/ml aprotinin, 5 µg/ml leupeptin and 10 µg/ml TSA. After centrifugation at 15000g for 15 min, 1 µg anti-GR H300 (Santa Cruz Biotechnology) was added to the supernatant. After 1 h incubation at 4˚C on a rotating wheel, protein A/G beads (sc-2003, Santa Cruz Biotechnology) (20 µl) were added, for a further 2 h. Beads were then washed in PBS, resuspended in 30 µl 2 x SDS sample buffer, and analysed by Western blotting as described below. Membranes were probed with an antibody towards acetylated lysine, before being stripped and reprobed for total GR.

Transactivation promoter-reporter assay COS-7 cells were seeded into 10 cm dishes at a density of 1×10^6 cells per dish. After 24 h the cells were transfected with 5 µg of plasmid constructs encoding either WT hGR, K494/495A hGR, WT HAhGR or K494/495Q HAhGR and with 5 µg pTAT-GRE-E1b-luc, using the DEAE-Dextran method as previously described (Ausubel, 1999) with modifications. Briefly, cells were incubated for 1 h in a transfection mix consisting of 100 µM chloroquine diphosphate, 0.1 mg/ml DEAE dextran and DNA in serum-free DMEM. Transfection mix was then replaced with 1 volume of 10% DMSO in PBS for 3 min. Cells were then returned to the incubator in DMEM containing 10% FCS. The following day cells were replated into 24 well plates, and allowed to adhere for a further 24 h. The following day, cells were treated with 100 nM dex in serum free DMEM for 24 h. Cells were then harvested in 50 µL reporter lysis buffer (Promega) and 10 µL of the lysate was used for the luciferase assay (Luciferase Assay System, Promega), which was read using a Modulus microplate reader (Turner Biosystems). The protein concentration of the lysate in each well was determined using a standard Bradford assay (Pierce). The values obtained from the luciferase assay were normalised to protein concentration of the lysate and values and were expressed relative to WT hGR dex (100%).

Immunofluorescence COS-7 cells were plated into 10 cm dishes at a density of 1×10^6 cells per dish, and transfected with 5 µg of plasmid constructs encoding either WT hGR, K494/495A hGR, WT HAhGR or K494/495Q HAhGR, as described above. After 24 h, cells were replated onto coverslips, and allowed to adhere for a further 24 h. Cells were treated for 1 h with 1 µM dex, before being fixed and permeabilised in methanol at -20˚C for 15 min. Coverslips were blocked with 3% bovine serum albumin (BSA) in PBS for 1 h, and then incubated with primary antibody (anti-GR, H300, Santa Cruz Biotechnology; 1:500 in 5% BSA/PBS) for 1 h. Coverslips were then washed and incubated in secondary antibody (anti-Rabbit, Alexa 488 (Invitrogen), 1:500 in 5% BSA/PBS) for 1 h. Hoechst nuclear stain was used to counterstain nuclei, before mounting coverslips on glass slides using Mowiol mounting medium (13% (w/v) Mowiol, 33% glycerol in 0.2 M Tris pH 8.5). Slides were incubated at room temperature overnight to allow the mounting medium to set, and then examined on a Zeiss Axiovert 200 M fluorescent microscope and analysed with AxioVision Rel 4.7 software. Confocal microscopy was performed on a Zeiss LSM510 META NLO Inverted microscope, using an Argon laser with 488 nm excitation line.

Western blotting and antibodies COS-7 cells were transfected, replated and treated as described above. Cells were washed with PBS, harvested in 2 x SDS sample buffer, and boiled at 100˚C for 5 min. Proteins were separated on an 8 or 10% polyacrylamide gel by SDS-PAGE at 200V. Proteins were transferred from the gel to Hybond ECL membrane (Amersham Biosciences), by electroblotting at 180mA for 1 h. Membranes were then blocked in 5% non-fat milk powder in Tris-buffered saline containing 0.1% Tween-20 (TBST), and probed with specific antibodies overnight at 4˚C. Membranes were then washed three times for 5 min each in TBST, incubated in secondary antibody for 1 h at room temperature, and washed again as previously. Blots were developed using Amersham ECL Western blotting detection reagents (GE Healthcare). After probing, blots were stripped by incubation in stripping buffer (62.5 mM Tris pH6.5, 1% SDS, 0.7% (v/v) β-mercaptoethanol) at 65˚C for 30 min, before being blocked and probed as previously. Antibodies included anti-GR (H-300, sc-8992, Santa Cruz Biotechnology), anti-β tubulin (T4026, Sigma

Aldrich), anti-ε acetylated lysine (#9441, Cell Signalling Technology), anti-GR phospho-S211 and phospho-S226 (Dr M.J. Garabedian, New York University, USA).

Whole cell binding Competitive whole cell binding assays were performed as previously described (Koubovec *et al.,* 2005) with minor modifications. Briefly, COS-7 cells were seeded into 10 cm tissue culture dishes at 1 x 10^6 cells per dish. On day two, cells were transiently transfected with 5 μg of various GR constructs per well using FuGENE 6 (Roche) according to the manufacturer's instructions. On day three the cells were replated into 24 well plates. On day four, cells were washed twice with prewarmed PBS and incubated with DMEM containing 10 nM [^{3}H]- dex (50 Ci/mmol, AEC-Amersham) and 10 μM excess unlabelled dex or vehicle for 90 min at 37°C. Thereafter cells were placed on ice and washed three times for 15 min with ice cold PBS containing 0.2% (w/v) BSA. Cells were lysed with 100 μl reporter lysis buffer (Promega) and total binding was determined by liquid scintillation counting.

***In vitro* DNA binding assay** The DNA binding assay was performed essentially as previously described (Ronacher *et al.,* 2009) with minor modifications. COS-7 cells were transfected with WT or mutant GR constructs as described above, and incubated for 48 h. Cytosols were prepared from COS-7 cells overexpressing WT hGR or mutant GR by trypsinising and pelleting cells. The pellet was snap frozen at -80˚C, and resuspended in TAPS buffer (0.1 M TAPS, pH9.5). Cells were incubated on ice for 1 h with vigorous vortexing every 10 min. Cellular debris was pelleted by centrifugation at 15 000 g for 10 min at 4˚C. The supernatant was aliquotted and stored at -80˚C. The GRE oligonucleotides (sense: biotin-GAT CCT GTA CAG GAA TGT TCT AGC TACA; Antisense: biotin-TGT AGC TAG AAC ATT CCT GTA CAG GATC) were annealed by mixing equimolar amounts, heating to 100˚C for 5 min and allowing to cool slowly overnight. The annealed biotinylated oligonucleotides were incubated with streptavidin coated agarose beads (E5529, Sigma Aldrich) for 2 h at 4˚C to facilitate binding of oligonucleotides to beads. Cytosols containing liganded-GR complexes were activated by heating at 20°C for 30 min, followed by overnight incubation with biotinylated DNA on streptavidin-agarose beads. Beads were then washed with 1 ml HEPES wash buffer (10 mM HEPES pH7.5, 1 mM EDTA, 10% (v/v) glycerol) and bound proteins were eluted in 30 μl 2 x SDS sample buffer. GR

levels were detected by Western blotting as described above, using an antibody towards GR (H300, Santa Cruz Biotechnology).

Quantitative real time PCR COS-7 cells were plated in 6-well plates at a density of 0.5 x 10^5 cells per well, and transfected with 2.5 µg per well of either empty vector (pcDNA 3.1), WT HAhGR, or K494/495Q HAhGR. After 24 h the medium was replaced with serum-free DMEM and after 2 h of serum starvation, 1 µM dex was added for a further 2 h. The cells were then washed with PBS, and RNA was extracted using Tri Reagent (Sigma-Aldrich) according to the manufacturer's protocol. RNA (0.5 µg) was reverse transcribed with Oligo-dT priming, using the Transcriptor First Strand cDNA synthesis kit (Promega, Madison, WI), and an equal volume of each cDNA synthesis reaction (1 µl) was used as template for real time PCR, using the Sensimix dT Kit (Quantace). Quantitative PCR was carried out using QuantiTect primers (QT00091035, Qiagen) for Glucocorticoid-induced leucine zipper (GILZ). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for normalization (F: 5' TGAACGGGAAGCTCACTGG 3'; R: 5' TCCACCACCCTGTTGCTGTA 3') (Ishibashi *et al.,* 2003). Standard curves were used to determine the efficiency of each primer set, and the relative expression of GILZ in each sample was calculated according to the Pfaffl mathematical model (Pfaffl, 2001).

Chromatin immunoprecipitation (ChIP) ChIP was performed as previously described (Ma *et al.,* 2003) with some modifications. COS-7 cells were seeded at 1 x 10^6 cells/ dish in 15 cm dishes and transfected with WT HAhGR or K494/495Q HAhGR. After 24 h each 15 cm dish was replated into two 10 cm dishes to ensure equal transfection efficiency between treatment conditions. Expression of WT HAhGR and K494/495Q HAhGR was compared by Western blotting as described above. Cells were serum starved for 2 h and treated for 2 h with 1 µM dex before being crosslinked with formaldehyde (1% final concentration) for 10 min at 37˚C. Crosslinking was stopped by addition of glycine (0.125 M final concentration) for 5 min at room temperature. Cells were scraped in 3 ml PBS, centrifuged and resuspended in 300 µl nuclear lysis buffer (1% SDS, 50 mM Tris-HCl pH8, 10 mM EDTA, 1 mM PMSF, 2 µg/ml aprotinin, 5 µg/ml leupeptin). Cells were sonicated in a

bioruptor (Diagneode), (30 sec on, 30 sec off for 10 cycles) at 4˚C and debris was pelleted by centrifugation. Concentration of sonicated chromatin was measured on a nanodrop, and samples were diluted to an equal concentration with NLB before being aliquotted and stored at -80˚C. For input samples, 15 – 30 µg of chromatin were aliquotted. For immunoprecipitation equal amounts (50-100 µg) of DNA were diluted to 1 ml with IP dilution buffer (0.01% SDS, 20 mM Tris-HCl pH8, 1.1% Triton X 100, 167 mM NaCl, 1.2 mM EDTA, 1 mM PMSF, 2 µg/ml aprotinin, 5 µg/ml leupeptin). Protein A/G beads (sc-2003, Santa Cruz Biotechnology) were pre-blocked by incubation with salmon sperm DNA (0.2 mg/ml) and bovine serum albumin (BSA) (1 mg/ml) for 1 h on a rotating wheel at 4˚C. The beads were resuspended in IP dilution buffer (0.01% SDS, 20 mM Tris-HCl pH8, 1.1% Triton X 100, 167 mM NaCl, 1.2 mM EDTA, 1 mM PMSF, 2 µg/ml aprotinin, 5 µg/ml leupeptin) as a 50% slurry, and stored at 4˚C. The chromatin was precleared by incubation with preblocked protein A/G agarose beads (Santa Cruz Biotechnology) for 1 h. The supernatant was incubated with 2 µg of antibody overnight at 4˚C on a rotator. Exogenously expressed GR was immunoprecipitated using anti-HA (y-11) (Santa Cruz Biotechnology). Endogenous FKBP52 was immunoprecipitated using anti-FKBP52 (N-17) (Santa Cruz Biotechnology). Negative control immunoprecipitations were performed in parallel, using 2 µg anti-rabbit-HRP, or anti-mouse-HRP (sc2313 or sc2005, respectively, Santa Cruz Biotechnology).

The following day 40 µl of pre-blocked beads were added, and the mixture was incubated for 6 h. Beads were then washed sequentially with 1 ml each of wash buffer I (0.1% (w/v) SDS, 1% (v/v) Triton X-100, 2 mM EDTA, 20 mM Tris-Cl pH8, and 150 mM NaCl), wash buffer II (0.1% (w/v) SDS, 1% (v/v) Triton X-100, 2 mM EDTA, 20 mM Tris-Cl pH8, and 500 mM NaCl), and wash buffer III (1% (v/v) NP-40, 1% (v/v) sodium deoxycholate, 500 mM LiCl, 1 mM EDTA, 10 mM Tris-Cl pH8), followed by three 1 ml washes with TE (10 mM Tris pH8, 0.1 mM EDTA). Immunoprecipitated complexes were eluted from beads in 300 µl elution buffer (1% SDS, 100 nM $NaHCO_3$) for 30 min at room temperature. For input, 30 µl of chromatin solution was stored at -20˚C overnight, before addition of 90 µl elution buffer, and processing in parallel with immunoprecipitated samples.

NaCl was added to input and IP eluate to a final concentration of 300 mM, and eluates were incubated at 65˚C overnight to reverse crosslinks. Following proteinase K treatment, DNA was isolated using a QiaQuick PCR purification column (Qiagen). The purified DNA was subjected to quantitative real time PCR, using specific primers annealing to the promoter of the endogenous monkey GILZ gene, spanning the equivalent of GREs 3-6 of the human GILZ promoter (Chen, Rogatsky, & Garabedian, 2006) (GILZ F 5'-AGT TAA GCT CCT GAT TTA AGA AG-3'; GILZ R 5'-CCC GAT CTC AGG ACA TTC-3'), and based on homology between the human, chimp and rhesus monkey GILZ promoter sequences (Avenant *et al.*, 2010). Real time PCR on input and immunoprecipitated samples was performed using Sensimix dT Kit (Quantace).

Statistical analysis Statistical analysis was performed using GraphPad Prism software, with tests as indicated in figure legends. When comparing several different treatment conditions, one way Anova was used, with a Dunnet's or Tukey's post test for comparison of all values to a control value, or comparison of all values to each other, respectively. For comparison of only two treatment conditions, a student's t-test was used. The symbols *, **, and *** indicate $p<0.05$, 0.01 and 0.005, respectively.

RESULTS

Endogenous hGR is acetylated in the absence and presence of ligand

To investigate whether endogenous hGR is acetylated in the absence or presence of agonist, A549 cells were incubated in the absence or presence of 1 μM dex and in the absence or presence of 1 ng/ml PMA. PMA was included to mimic a state of inflammation, and investigate whether this would modulate the GR response in any way. Endogenous GR was immunoprecipitated with an anti-GR antibody in the presence of the HDAC inhibitor TSA, followed by Western blotting and probing for GR and acetyl-lysine. The results show that GR was precipitated from the lysates in equal amounts irrespective of whether the cells were untreated or treated with dex. When the same blot was stripped and reprobed with an acetyl lysine antibody, a band with similar intensity that co-migrated with the GR was detected both in the absence and presence of dex (figure 3.1). This result suggests that the hGR is basally acetylated in a dex-independent manner. Similar results were obtained when the experiments were performed in the absence of PMA in A549 cells and in U2OS cells (data not shown). No signal was detected when immunoprecipitation was performed using a non-specific antibody (data not shown). These results show that endogenous hGR is basally acetylated in A549 and U2OS cells, in the absence and presence of PMA, and that addition of dex does not change the GR acetylation levels under the experimental conditions used here.

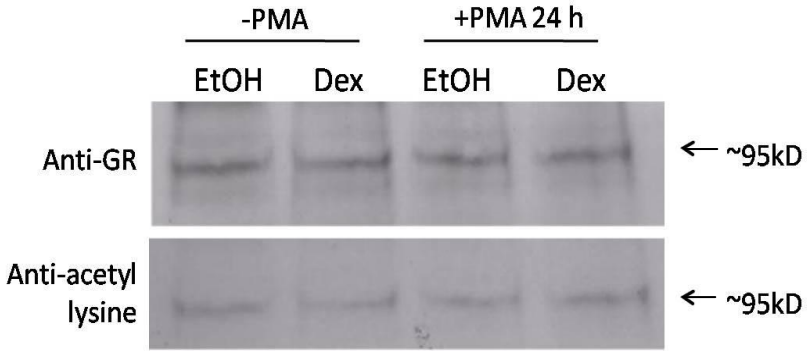


Figure 3.1. Endogenous GR is acetylated in the absence and presence of ligand. A549 cells were treated with PMA or vehicle (-PMA) for 24 h followed by 1 h treatment with 100 nM dex or vehicle (EtOH). GR was immunoprecipitated in the presence of 10 µM TSA, and immunoprecipitated proteins were analysed by Western blot, probing for acetyl lysine and then GR.

Mutation of K494/495 to abrogate or mimic acetylation affects transactivation efficacy, suggesting that acetylation of K494/495 is required for hGR-mediated transactivation of a GRE

In order to investigate the effect of GR acetylation at K494/495 on transactivation, two GR mutants were exploited. The K494/495 to A (K494/495A hGR) mutant cannot be acetylated because it does not have an amide side chain, which is required for transfer of the acetyl group from acetyl CoA. The K494/495Q HAhGR mutant contains glutamine substitutions that mimic the charge and size of an acetyl lysine group. The transactivation activity of these mutants was tested using a cell-based reporter gene assay. Briefly, COS-7 cells were transfected with expression vectors for WT hGR, K494/495A hGR, WT HAhGR and K494/495Q HAhGR, and a luciferase reporter construct containing the minimal tyrosine amino transferase (TAT) promoter, which contains two GREs. After 24 h of stimulation with 100 nM dex, whole cell lysates were prepared and luciferase activity was determined (figure 3.2A and B). As expected, WT hGR efficiently transactivated the reporter gene in a ligand-dependant manner (figure 3.2A and C). However, the hGR K494/495A mutant failed to transactivate the reporter gene above untransfected levels in the absence or presence of dex (figure 3.2A). Interestingly the K494/495Q HAhGR mutant, which mimics acetylated GR, resulted in a 2-fold increase in reporter gene activity relative to WT HAhGR (figure 3.2C). These differences could not be explained by different expression levels, since similar levels of GR were expressed with the different constructs (figure 3.2C-F).

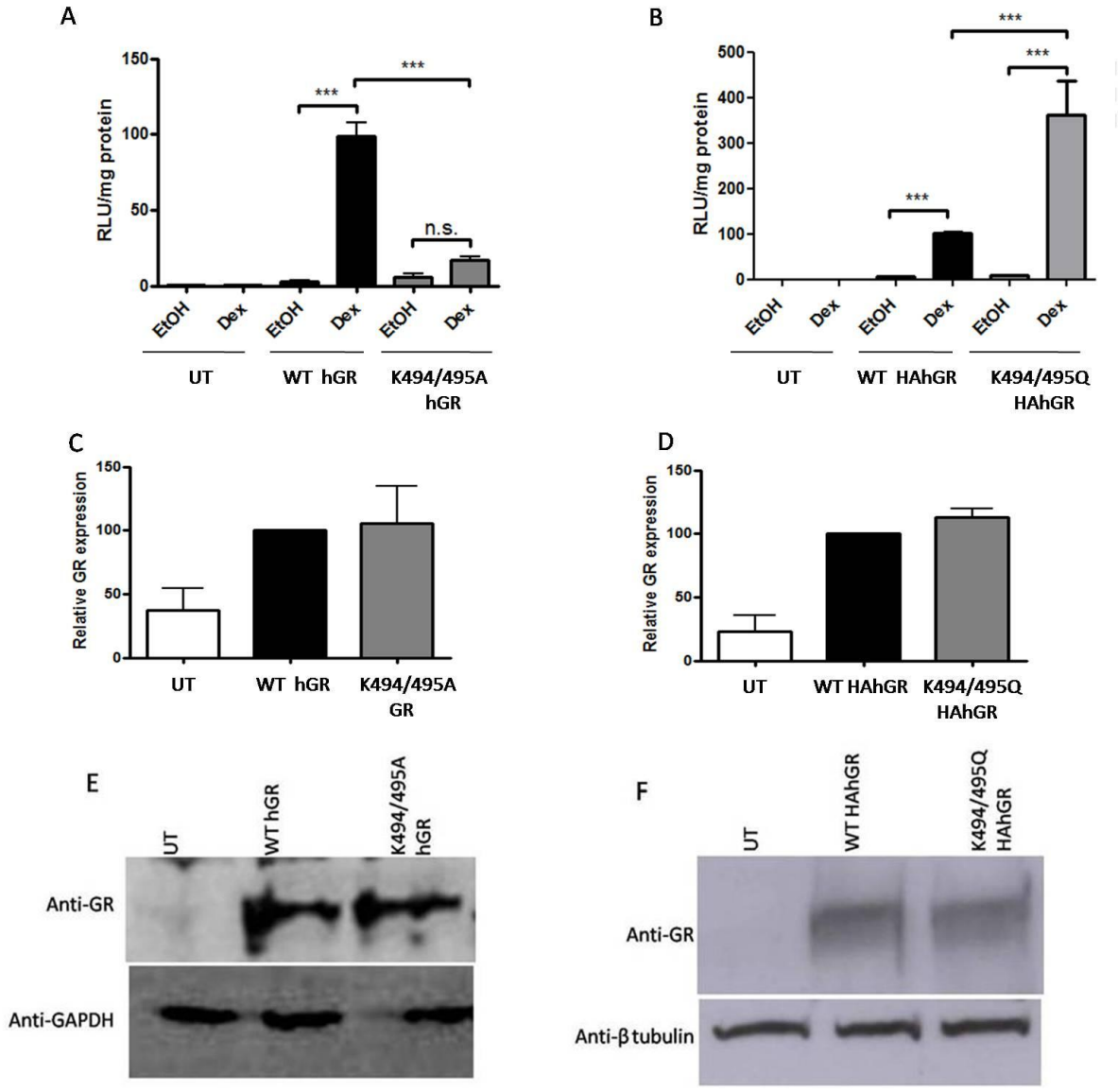


Figure 3.2. The K494/495A GR mutant exhibits no transactivation efficacy while the K494/495Q GR mutant exhibits increased dex-induced transactivation. COS-7 cells were transfected in 10 cm dishes with WT hGR and K494/495A hGR (A) or WT HAhGR and K494/495Q HAhGR (B), as well as a GRE-luciferase reporter plasmid. Cells were subsequently replated into 24 well plates. Cells were then treated for 24 h with 1 µM dex or vehicle control (EtOH). Luciferase activity was measured and normalised to total protein in each well as determined by a standard Bradford assay. Graph shows pooled results of three (A) or two* (B) independent experiments, each performed in triplicate analysed by one way Anova with Tukey's multiple comparison test. (C) and (D) show pooled relative expression levels of each construct, measured by Western blotting, for each of the experiments relative to GAPDH (C) or β-tubulin (D) with WT GR being set as 100% in each case. (E) and (F) show representative Western blots of expression levels of each construct.

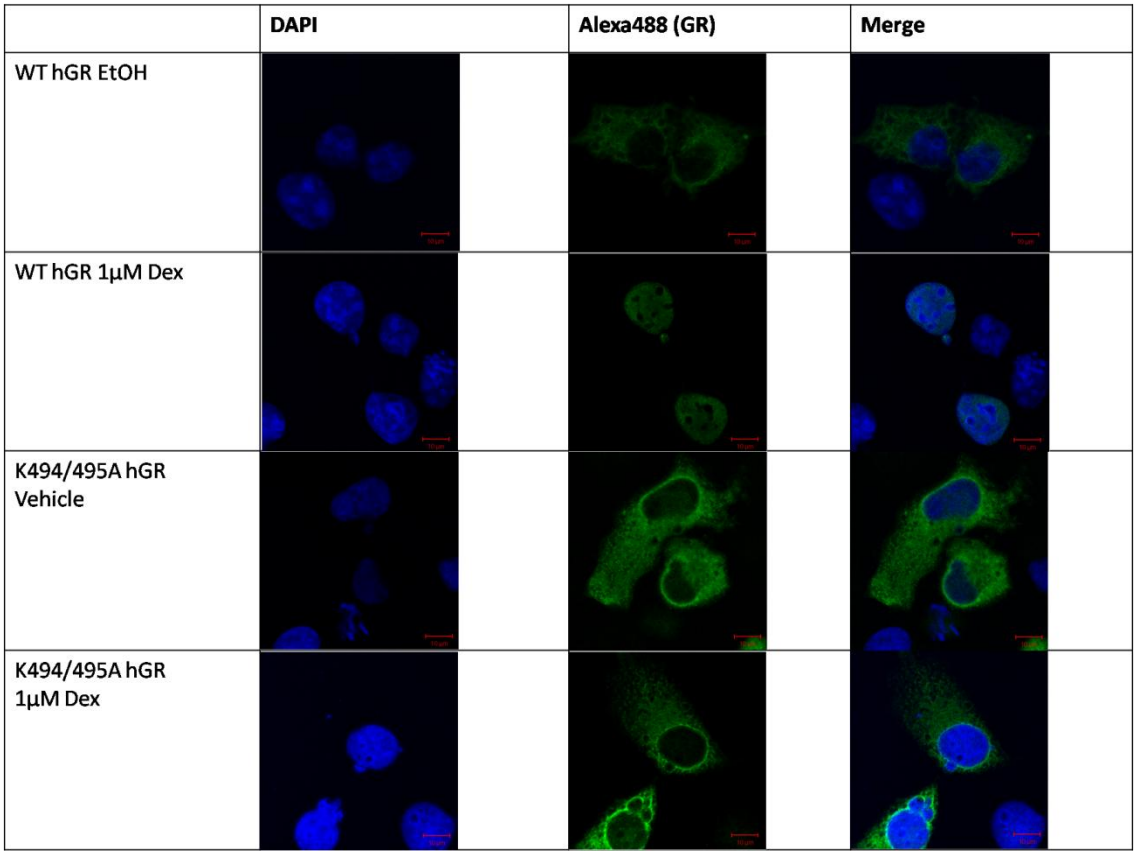
*One of these independent experiments was performed by Dr M. Tomasicchio.

Mutation of K494/495 to alanine almost abolishes, while mutation to glutamine reduces GR ligand-dependant nuclear translocation

The lack of transactivational ability displayed by the K494/495A hGR mutant compared to WT hGR could be a result of decreased nuclear translocation ability. Similarly, the significant increase in transactivation observed for the GR acetylation mimic, K494/495Q HAhGR, versus WT HAhGR may be a result of increased nuclear translocation. To test this hypothesis COS-7 cells were transfected and incubated with 1 µM dex for 1 h. On microscopic examination of fluorescently stained GR, while WT hGR translocated to the nucleus upon dex treatment, the K494/495A hGR mutant remained localised in the cytoplasm (Fig 3.3A). The even distribution of hGRwt and mutant GR in the cells indicates that the expressed proteins are not targeted to the proteasomes for degradation, suggesting they are correctly folded, and the mutation does not create a null mutant.

The K494/495Q HAhGR mutant did exhibit dex-dependent nuclear translocation, but to a lesser extent than WT HAhGR (Fig 3.3B). K494/495Q HAhGR showed a strong fluorescent signal in the cell cytoplasm, even after dex treatment. In order to investigate whether the reduced nuclear translocation by both mutants was time-dependent (i.e. slower kinetics of nuclear import relative to WT GR), GR localisation was also examined after 16 h of dex treatment. The results looked similar to the 1 h treated cells (Appendix C, figure C.1); indicating that the difference between WT and mutant GRs was not simply due to slower nuclear import. These results were confirmed by subcellular fractionation by another member of the Hapgood laboratory (data not shown). Taken together the biochemical fractionation and immunofluorescence results show that the K494/495A hGR mutant is unable to enter the nucleus, consistent with a loss of transactivation efficacy. However K494/495Q HAhGR, while still able to enter the nucleus, exhibited less nuclear translocation than WT HAhGR. Thus the increase in transactivation by K494/495Q HAhGR acetylation mimic cannot be attributed to increased nuclear translocation ability.

A



B

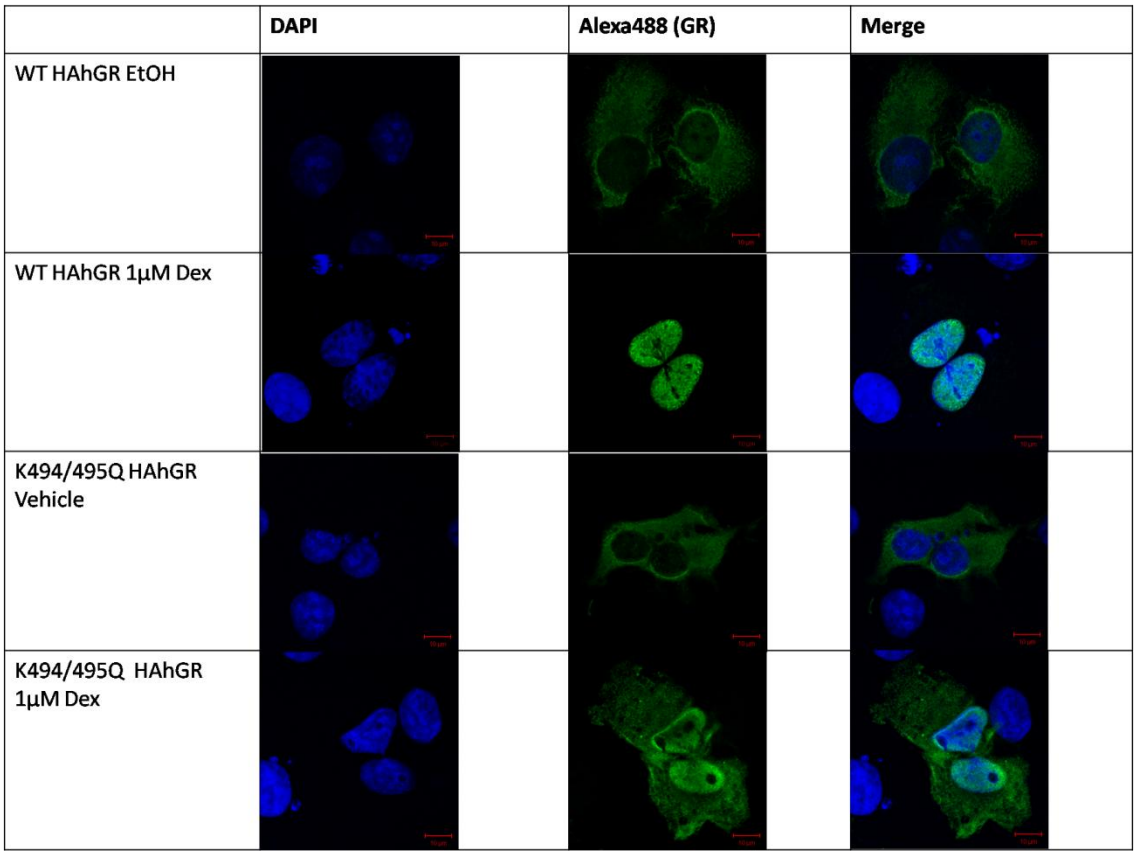


Figure 3.3 legend on following page

Figure 3.3. K494/495A hGR and K494/495Q HAhGR exhibit less nuclear translocation than WT GR, but the same even distribution. COS-7 cells were transfected in 10 cm dishes with WT hGR and K494/495A hGR (A) or WT HAhGR and K494/495Q HAhGR (B) constructs. Cells were replated onto coverslips, and 24 h later, treated for 1 h with 1 µM dex or vehicle (EtOH), before being fixed with methanol and immunostained for GR. Results shown are representative of at least 100 cells per condition from three (A) or two (B) independent experiments.

The uniform distribution of the WT and mutant GR in the cells, and the absence of GR protein aggregates, indicates that the protein expressed in each case is not targeted to the proteasome for degradation, suggesting it is correctly folded, and that the mutation does not create a null mutant. Even the presence of dex does not induce a speckling pattern indicative of proteasomal degradation or misfolding (Thomas *et al.,* 2004).

Mutation of K494/495 to alanine abolishes, while mutation to glutamine reduces hGR ligand-dependant phosphorylation at Serines 211 and 226*.*

GR phosphorylation at serines 211 (S211) and 226 (S226) is a well-established marker of GR activation by ligand (reviewed in Weigel & Moore, 2007). To determine the ligand-dependent phosphorylation of these two serine residues in WT and mutant GR molecules, GR phospho-serine specific antibodies were used. As expected, both WT GR constructs showed a statistically significant increase in phosphorylation at S211 and S226 upon dex treatment (figure 3.4). However, phosphorylation of K494/495A hGR was completely abolished at both S211 (figure 3.4A and B) and S226 (figure 3.4C and D). Even the relatively low levels of phosphorylated S211 observed in the absence of ligand was lost in the K494/495A hGR mutant (figure 3.4A, lane 1 vs. lane 3). Experiments in the absence of expressed GR showed that phosphorylation of K494/495A hGR was not above background levels of endogenous GR (data not shown). Although not statistically significant, a small, reproducible dex-dependent increase in S211 (figure 3.4E and F) and S226 (figure 3.4G and H) phosphorylation was observed for K494/495Q HAhGR.

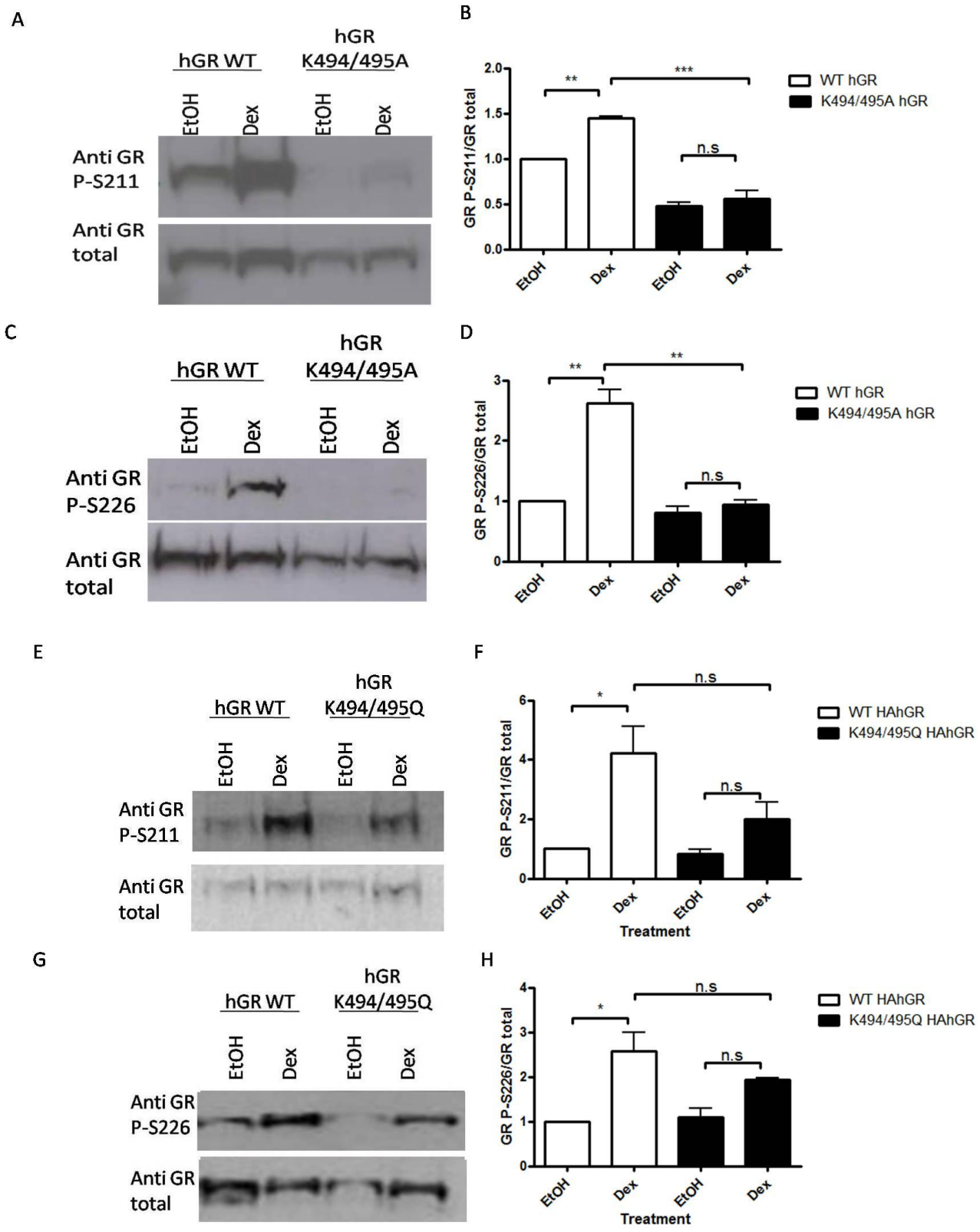


Figure 3.4. Mutation of K494/495 to alanine abolishes, while mutation to glutamine decreases phosphorylation of hGR at S211 and S226.

COS-7 cells were transfected with WT hGR and K494/495A hGR (A-D) or WT HAhGR and K494/495Q HAhGR (E-H) constructs in a 10cm dish before being replated into 12 well plates. Cells were treated for 1 h with vehicle (EtOH) or 100 nM dex before being lysed and analysed by Western blot for GR phospho-S211 (A, B, E, F) or GR phospho-S226 (C, D, G, H) and total GR. Representative Western blots are shown (A, C, E, G). Bands on phospho- and total GR Western blots were quantified using Alphaease software, normalised to total GR levels, and plotted relative to WT hGR EtOH. Results from three independent experiments were pooled and analysed by one way Anova, repeated measures, with Tukey's post-test (B, D, E, H).

Mutation of K494/495 to alanine abolishes, while mutation to glutamine increases, ligand binding of the GR

The observations that K494/495A hGR neither became phosphorylated nor moved to the nucleus, caused uncertainty as to whether this mutant was capable of binding ligand. Thus, it was decided to compare the ligand binding capacity of both the GR mutants to WT GR, using a competitive whole cell binding assay with tritiated dex.

Competitive binding assays in COS-7 cells showed that K494/495A hGR bound significantly less dex than the corresponding WT construct. In fact, the ligand binding capacity of this mutant GR was barely above background (figure 3.5A). Conversely, K494/495Q HAhGR consistently bound slightly more dex than the WT HAhGR construct (although not statistically significantly different from WT HAhGR) (figure 3.5B). In order to ensure that observed differences in GR ligand binding capacity were not due to differences in GR expression levels, GR expression was quantified in parallel by Western blot for each experimental repeat, and the average expression of three independent experiments shows little variation between WT and mutant GR constructs (figure 3.5C and D). However, transfection of a WT GR construct resulted in significantly higher expression of GR than in untransfected cells. These results strongly suggest that the acetylation status of GR K494/495 determines its ligand-binding capacity.

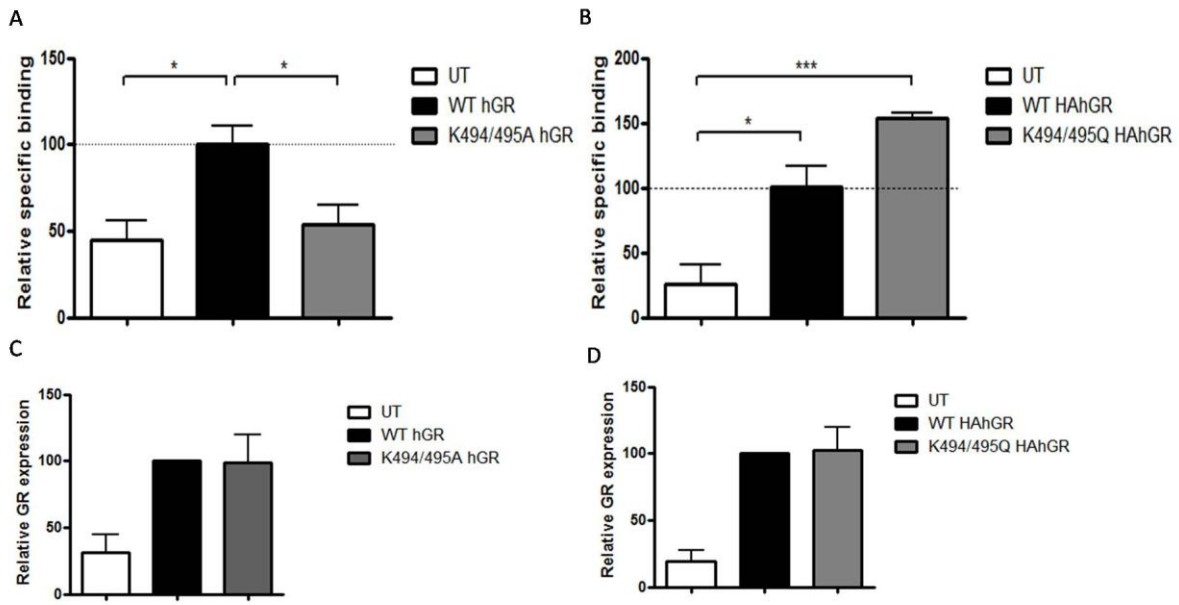


Figure 3.5. Mutation of K494/495 to alanine abolishes, while mutation to glutamine increases the ligand binding capacity of the GR. COS-7 cells were plated in 10 cm dishes and transfected with WT hGR and K494/495A hGR (A) or WT HAhGR and K494/495Q HAhGR (B), before being replated in 24 well plates. 24 h later, cells were incubated for 90 min with 10 nM [^{3}H]-dex, in the absence (total binding) or presence of 10 µM unlabelled dex (non-specific binding) before being washed and lysed. Specific binding (total binding minus non-specific binding) normalised to the total protein per well as measured by a standard Bradford assay, is shown. Pooled results of four (A) or three (B) experiments performed in triplicate are shown, analysed by one-way Anova, with a Tukey's post-test comparison. (C) and (D) For each experimental repeat, expression of each GR construct was compared to untransfected (UT) COS-7 cells by Western blot, quantified, and plotted in histograms.

Mutation of K494/495 to alanine results in a decrease, while mutation to glutamine does not affect DNA-binding *in vitro*

Since K494/495 are in the DNA binding domain of the GR, and mutation of lysines in the ERα DBD has been reported to affect DNA binding (Kim *et al.,* 2006), the effect of mutation of these residues on the DNA binding capacity of the GR was investigated. Having previously shown that mutation of these residues causes differences in ligand binding (figure 3.5) and nuclear import (figure 3.4), an *in vitro* DNA binding assay was employed. Since this assay requires neither ligand binding

nor nuclear translocation (as it is performed on whole cell lysates) (Cho *et al.,* 2005) differences in ligand binding or nuclear translocation between WT and mutant GR would not obscure differences in DNA binding, as measured by this assay.

As shown in figure 3.6A, WT hGR bound the GRE sequence in the absence and presence of dex, and only background levels of WT hGR bound the beads in the absence of GRE oligonucleotides. The fact that there is no dex-dependent increase in GRE binding is expected, since in this assay, GR is activated by heat-shock, which is independent of ligand (Cho *et al.,* 2005). K494/495A hGR does not give a signal above background in the presence of GRE oligonucleotides, indicating that this mutant has reduced DNA binding capacity compared to WT hGR (figure 3.6A, C), over and above its ligand binding and nuclear import deficiencies. Since the K494/495Q HAhGR mutant elicited higher maximal transactivation than WT HAhGR, one might expect it to exhibit more efficient DNA binding than WT HAhGR. However the results of the *in vitro* DNA binding assay revealed this is not the case (figure 3.6B, D). This could indicate that mutation to Q does not affect DNA binding by GR, or could indicate the limitations of the assay setup, such as possible limiting concentration of oligonucleotides.

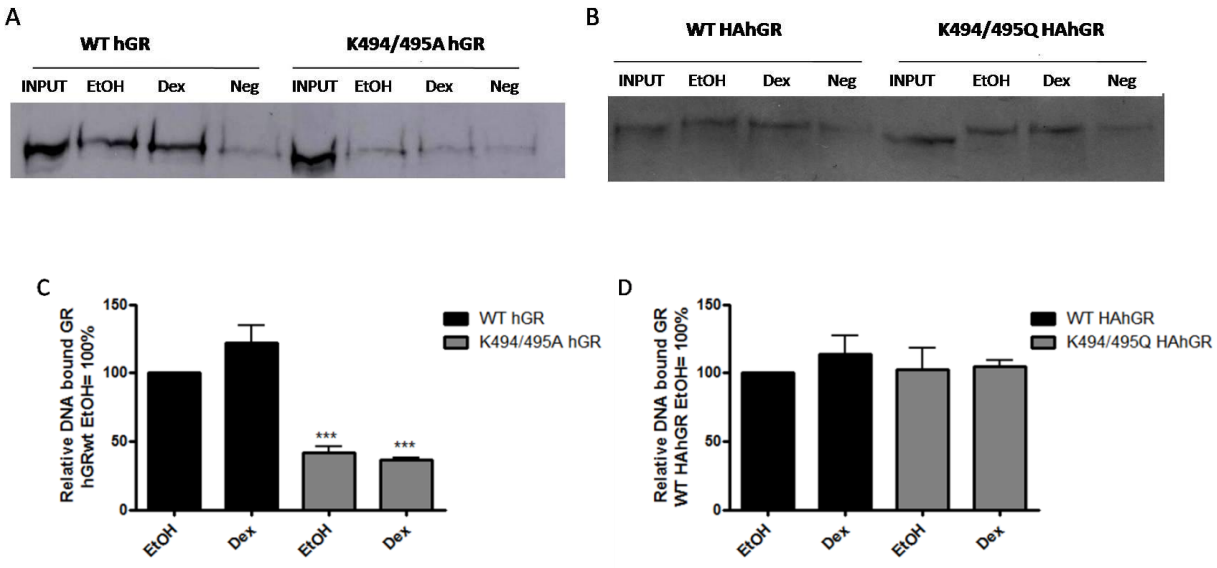


Figure 3.6. Mutation of K494/495 to alanine results in a decrease, while mutation to glutamine does not affect DNA-binding *in vitro.* Whole cell lysates of COS-7 cells overexpressing WT hGR or K494/495A hGR (A, C) or WT HAhGR or K494/495Q HAhGR (B, D) were treated with 10 µM dex or vehicle, heat activated, and incubated with agarose beads coated with DNA

oligonucleotides encoding the GRE sequence, or uncoated beads (Neg). The beads were subsequently washed and bound proteins were analysed by Western blot. Representative Western blots of four (A) or two (B) experiments are shown. The relative intensities of WT and mutant GR bands were quantified and pooled histograms of four (C) or two (D) experiments are shown, analysed by one way Anova with Dunnett's post test relative to WT EtOH.

WT HAhGR and K494/495Q HAhGR mutant both upregulate the endogenous GILZ gene, but an increase in GR recruitment to the GILZ promoter is only apparent for WT HAhGR

The results thus far are consistent with a role for GR acetylation in ligand binding. However the mechanism of increased transactivation activity of the glutamine acetylation mimic is still unclear, although it could be partly due to increased ligand binding. Towards gaining further insight into this mechanism, it was decided to pursue a ChIP approach in intact cells, on an endogenous promoter. Before examining the recruitment of GR to the endogenous GILZ promoter in COS-7 cells, it was necessary to confirm that this gene is indeed regulated by GR in these cells. Real time PCR confirmed that GILZ expression increased in the presence of dex, and that this effect was greater in the presence of overexpressed WT HAhGR or K494/495Q HAhGR than in cells transfected with empty vector (figure 3.7A).

ChIP analysis of the GILZ promoter showed a reproducible 2 fold increase in promoter occupancy by WT HAhGR in response to dex treatment. However, no dex-dependent increase in K494/495Q HAhGR was observed (figure 3.7B). Western blotting confirmed the expression levels of WT and K494/495Q HAhGR were similar for the real time and ChIP experiments (figure 3.6 C and D). The apparent lack of recruitment of GR to the GILZ promoter was perplexing and given the observed dex-dependent upregulation of the gene in the presence of exogenous K494/495Q HAhGR indicating that this mutant must be active on the GILZ promoter to a similar extent as WT HAhGR, although it was not detectable by the ChIP technique. This was not due to different GR expression levels (figure 3.7C and D), nor enhanced receptor degradation of K494/495Q HAhGR in the presence of dex, since Western blot confirmed that K494/495Q HAhGR levels were the same as, or slightly higher than, WT HAhGR levels after 24 h dex treatment (Dr Tomasicchio, data not shown).

Furthermore, *in vitro* DNA binding assays indicated that K494/495Q HAhGR was capable of efficient binding to a minimal GRE sequence (figure 3.6B and D). Therefore, the data suggest that a different complex may be formed by K494/495Q HAhGR at the promoter, preventing access of the antibody to the DNA-bound GR. This phenomenon has previously been reported for the ERα, the presence of which is masked by GRIP1 on the TNFα promoter (Cvoro *et al.,* 2006).

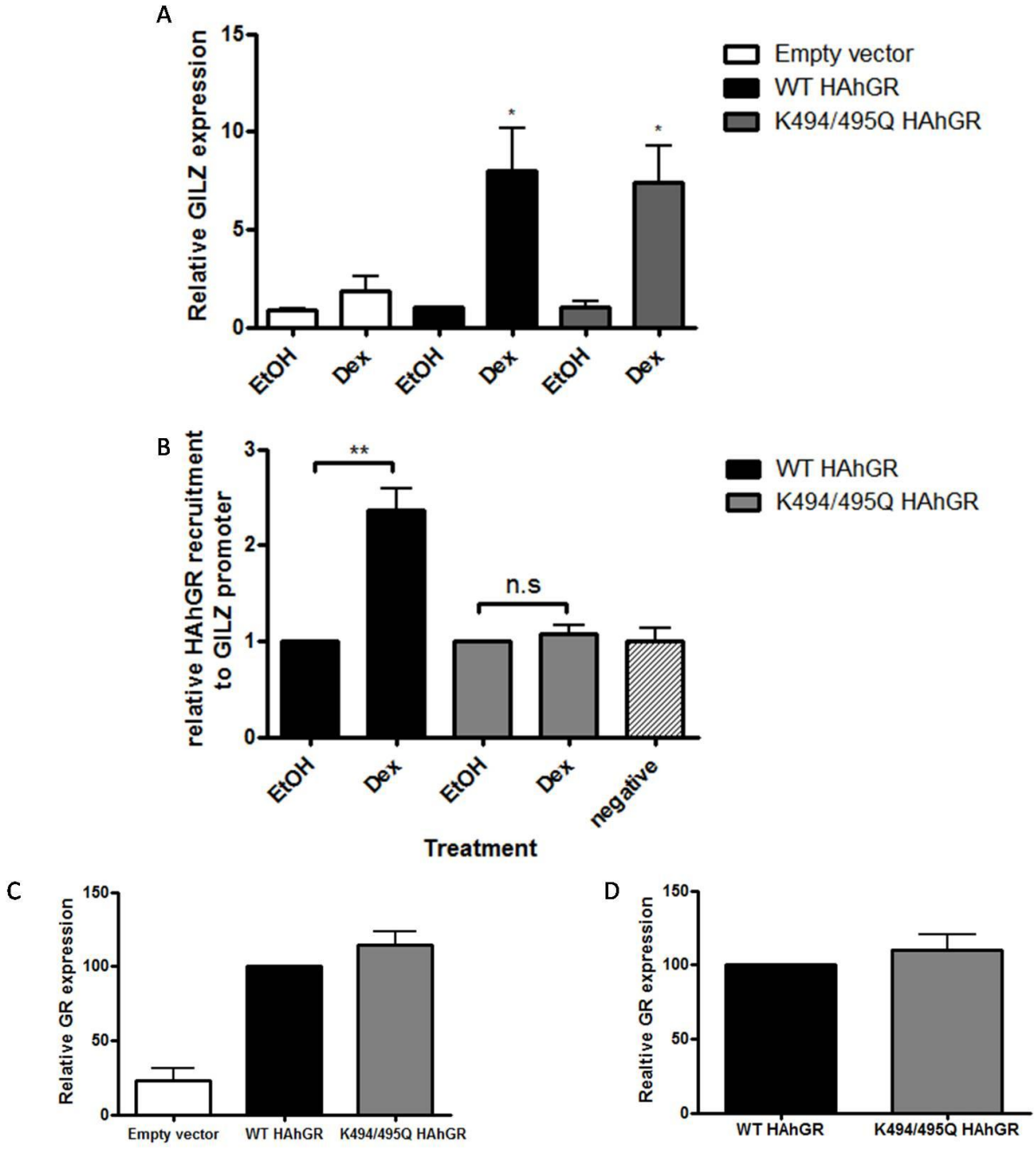


Figure 3.7. WT HAhGR and K494/495Q HAhGR mutant regulate the endogenous GILZ gene, but an increase in GR recruitment to the GILZ promoter is only apparent for WT hGR (A) COS-7 cells were transfected with WT HAhGR or K494/495Q HAhGR, and treated for 2 h with 1 μM dex or vehicle. After treatment, RNA was extracted, reverse transcribed, and endogenous GILZ expression was measured by real-time PCR, normalising to GAPDH expression levels. Pooled results from three independent experiments are plotted, and analysed by one-way Anova with Dunnet's post-test relative to WT HAhGR EtOH (B) COS-7 cells transfected with WT or K494/495Q HAhGR, were treated for 1 h with 1 μM dex or vehicle (EtOH). ChIP was carried out using an anti-HA antibody to immunoprecipitate exogenous GR, and primers specific for the endogenous GILZ promoter for PCR amplification. Negative refers to immunoprecipitation with non-specific IgG. Pooled results of three anti-HA immunoprecipitations or four negative control immunoprecipitations are shown. Statistical significance is determined by a paired t-test. Prior to ligand treatment, GR expression was analysed by Western blot for each experiment, quantified and plotted relative to WT HAhGR for real time expression analysis (C) or ChIP (D).

FKBP52 is dex-dependently recruited to the GILZ promoter in the presence of the hGR K494/495Q mutant

Given multiple lines of evidence implicating FKBP52 as a direct interaction partner for the GR DNA binding domain (Silverstein *et al.,* 1999), and as playing a role in GR-mediated transcription of the GILZ gene (Wolf *et al.,* 2009), it was decided to investigate recruitment of FKBP52 to the GILZ promoter. ChIP was used to determine whether FKBP52 was dex-dependently recruited to the GRE-rich region of the endogenous GILZ promoter. A small, non-significant increase in FKBP52 was observed in the presence of WT HAhGR (Fig 3.8). However, in the presence of overexpressed acetylation mimic, K494/495Q HAhGR, a significant increase in FKBP52 recruitment ($p<0.05$) was observed upon dex treatment (Fig 3.8). This increase in FKBP52 at the promoter may be responsible for the apparent lack of increase in the K494/495Q HAhGR mutant levels on the GILZ promoter, by forming a complex with GR that blocks access of the anti-HA antibody to the HA-tagged K494/495Q HAhGR mutant. The increased recruitment of FKBP52 by the glutamine mutant as compared to WT HAhGR could be due to an increase in the % of GR in the "acetylated" form for the mutant as compared to WT HAhGR. These results

suggest that GR acetylation at K494 and/or K495 recruits FKBP52 which acts as a coactivator for transactivation.

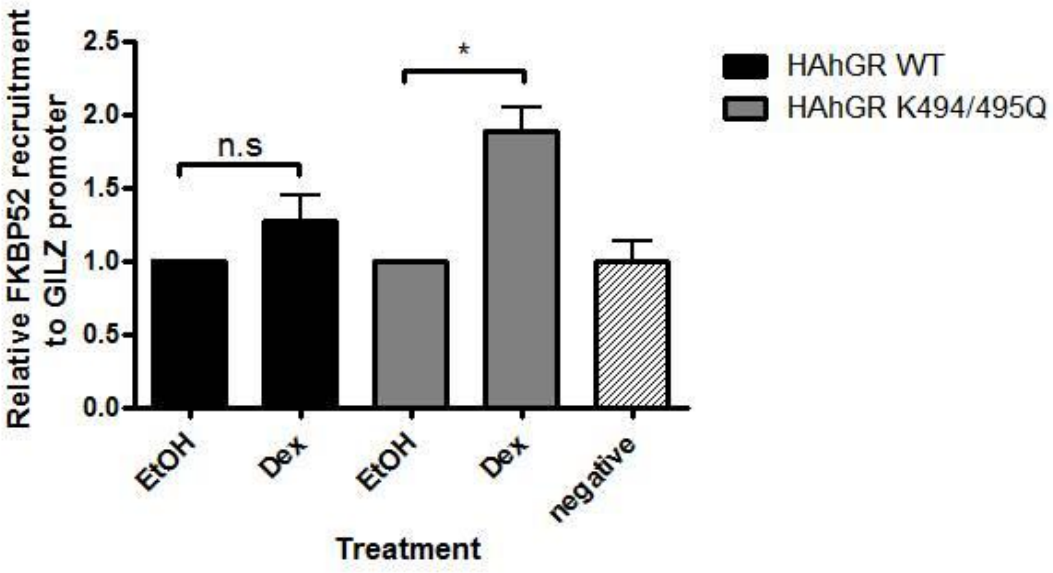


Figure 3.8. Endogenous FKBP52 is dex-dependently recruited to the GILZ promoter in COS-7 cells overexpressing the K494/495Q HAhGR mutant. COS-7 cells transfected with WT or K494/495Q HAhGR, were treated for 1 h with 1 µM dex or vehicle. ChIP was carried out using an anti-FKBP52 antibody to immunoprecipitate endogenous FKBP52, and primers specific for the endogenous GILZ promoter were used for PCR amplification. Negative refers to immunoprecipitation with non-specific IgG. Pooled results of three anti-FKBP52 immunoprecipitations, or four negative control immunoprecipitations are shown. Statistical analysis performed was a paired t-test.

Determination of levels of association between FKBP52 and WT GR was attempted using a co-immunoprecipitation approach, but unfortunately this approach was not successful, as shown in Appendix E (figure E.3.2). This experimental technique requires further optimisation.

DISCUSSION

Several studies suggest a functional role of lysine acetylation for the AR, ER and GR, while acetylation has not been reported for the MR or PR (Faus & Haendler, 2006). These studies collectively suggest a role for steroid receptor acetylation at one or more steps in the receptor pathway, including nuclear translocation, DNA-binding and transactivation efficacy, although many issues remain unclear. The present study investigated the functional consequences of GR acetylation in COS-7 cells using two expressed GR mutants as compared to WT hGR. Results with the acetylation deficient K494/495A hGR mutant, showed a loss of transactivation on a synthetic GRE (figure 3.2A) and also for the first time, a loss of nuclear translocation (figure 3.3A), and a loss of ligand-dependent phosphorylation at S211 and S226 (figure 3.4A-D). These results are all consistent with the novel finding that this mutant does not bind ligand (figure 3.5A). Results of the *in vitro* DNA binding assay strongly suggest the K494/495A hGR also displays reduced DNA binding capacity compared to WT hGR (figure 3.6A). However, it is possible that the mutation affects the ability of the GR to respond to heat shock. Therefore, more experimentation would be required to conclusively show reduced DNA binding ability. However, since this mutant neither binds ligand, nor translocates to the nucleus, the *in vitro* assay was a useful strategy to examine DNA binding in the absence of these confounding factors.

Results with the K494/495Q HAhGR acetylation mimic mutant strongly support the argument that acetylation of the GR at these residues is important for ligand binding and resulting downstream actions of the GR. It is further demonstrated that this mutant has an enhanced transactivation efficacy via a synthetic GRE reporter gene (figure 3.2B) and an endogenous gene (figure 3.7A) and exhibits increased ligand-binding, as compared to WT HAhGR (figure 3.5B). However it displays reduced nuclear translocation (figure 3.3B) and phosphorylation at S211 and S226 (figure 3.4 E - H), as compared to WT HAhGR.

It is well-established that acetylation occurs within the DBD of the AR, ER and GR. However whether this occurs in a ligand-dependent manner appears to be

controversial. Although a dex-dependent increase in GR acetylation was not observed in A549 cells (figure 3.1), the results suggest that the endogenous hGR is basally acetylated. The A549 result differs from a previous report that the hGR is acetylated in a ligand-dependent manner in the presence of both dex and IL-1β in A549 cells (Ito *et al.,* 2006). A possible reason for this difference could be that the latter experiments were conducted in the presence of IL1-β, presumably to mimic a state of chronic inflammation, and allow investigation of transrepression by the GR. While no increase in GR acetylation was observed in A549 cells in the presence of dex plus a similar pro-inflammatory compound, PMA, (figure 3.1) it is possible that the ligand-dependent increase in acetylation observed by Ito *et al.* was dependent on the presence of IL1β. Results in the absence of IL1β, in HCT116 cells with overexpressed hGR indicate that no GR acetylation was detectable in the absence of overexpressed circadian rhythm controlling proteins CLOCK (a HAT) and BMAL1 (Nader *et al.,* 2009). However, in the presence of overexpressed CLOCK and BMAL1, GR became dex-dependently acetylated at several lysine residues in the hinge region, including K494 and K495 (Nader *et al.,* 2009). Interestingly, acute systemic inflammation has been shown to increase expression of the Bmal1 gene *in vivo* (Murphy *et al.,* 2007). Furthermore, in experiments performed to demonstrate ligand-dependent acetylation of GR at K494/495 of the conserved KXKK motif, preincubation of cells with the HDAC inhibitor TSA was employed, prior to treatment with dex (K. Ito, personal communication). This may increase the apparent ligand-dependent acetylation of the GR. However, the physiological relevance of this approach is yet to be established. Variation in experimental conditions and biological responses may make it difficult to establish whether ligand-dependent acetylation is a general feature for steroid receptors under physiological conditions. Factors could include a dynamic equilibrium between acetylated and unacetylated forms, critically dependent on the relative concentrations of acetylases and deacetylases, which is dependent on the cell type and physiological state. In addition, only a small percentage of the receptors may become acetylated in a ligand-dependent manner (Kim *et al.,* 2006) and there may be cell-specific and receptor-specific differences in the role and mechanisms of acetylation. Moreover, localizing acetylation residues may be challenging by immunoprecipitation techniques since ligand-dependent increases in acetylation of particular residues may be masked by the steady level of

acetylation of other lysine residues. Nevertheless the results presented here show that endogenous GR is basally acetylated in U2OS and A549 cells, in the absence and presence of PMA, and there is no detectable change in overall GR acetylation upon ligand stimulation under these conditions. This result does not, however, exclude the possibility that under physiological conditions GR acetylation levels are modulated at particular lysine residues in response to ligand.

For the AR, early reports indicated that lysine acetylation was ligand independent (Fu *et al.,* 2000; Gaughan *et al.,* 2002), however later reports hinted at ligand dependent acetylation of the same residues (Fu *et al.,* 2003; Fu *et al.,* 2006). A shortcoming of these later papers is that they do not include loading controls, making the results inconclusive, since DHT is known to increase total AR levels (Grad *et al.,* 1999). Fu *et al.,* (2003) comment on the possibility that the observed increase in AR acetylation following DHT treatment is simply a result of increased AR levels. Interestingly they also found that TSA treatment in the absence of AR ligand was sufficient to cause an increase in AR acetylation, suggesting that ligand is not necessary for acetylation of these residues. In a later report, Fu *et al.,* (2006) once again report inconclusive findings, showing an increase in AR Ac-K after 24 h treatment with DHT, but failing to show input levels of total AR. Thus, although there is currently no consensus on this issue, available data seem to point towards ligand independent acetylation of the AR at the conserved KXKK motif. So, while the effect of SR ligand binding on acetylation requires further investigation, it is important to note that at least some basal acetylation has been reported at the conserved lysines at K494/495 of the GR DBD (Ito *et al.,* 2006; Nader *et al.,* 2009), or equivalent positions for AR (Fu *et al.,* 2000). The effect of acetylation of these residues on GR activity was therefore investigated.

This report is the first to directly investigate the effect of GR receptor acetylation on ligand binding. Results showing a loss of ligand binding with K494/495A hGR mutant and an increase in ligand binding with the K494/495Q HAhGR mutant strongly support a requirement for acetylation at these residues for ligand-binding by the hGR. These results would at first appear to be inconsistent with the finding by Ito *et al.* (2006), that the same K494/495A hGR mutant is dex-responsive for

transrepression in the presence of IL1β. However several results are consistent, with the report by Ito *et al.* (2006), who also found that K494/495A hGR did not give a response above background for transactivation of an endogenous gene. The observed effect of the alanine mutant by Ito *et al.* (2006) on transrepression and ligand-dependent p65 interaction, would suggest that sufficient ligand-binding does occur for nuclear translocation and transrepression. One possible explanation for these results is that the alanine mutant exhibits very low affinity for ligand binding, sufficient at high receptor concentrations to facilitate some nuclear translocation and transrepression. Since agonist-activated GR has about a tenfold higher potency for transrepression than for transactivation (Ronacher *et al.,* 2009), it is possible that such a low affinity could result in a measurable response for transrepression of some genes, but not transactivation. An alternative explanation is one of ligand-independent activation. Results from the Hapgood laboratory have recently shown that endogenous GR can be activated in a ligand-independent manner via a G-protein coupled receptor (Kotitschke *et al.,* 2009). Thus it is possible that the alanine mutant undergoes ligand-independent activation via the IL1β receptor since the experiments were performed in the presence of dex and IL1β (Ito *et al.,* 2006). Yet another possible explanation is that the observed interaction between K494/495A hGR and p65 occurs in the cytoplasm, and the reduced transactivation is thus a result of cytoplasmic sequestration of the p65. Since p65 has also been shown to be acetylated (Furia *et al.,* 2002), it is also possible that pretreatment with HDAC inhibitors could modify the acetylation status of p65, thereby confounding the results.

While there are some discrepancies, several of the findings presented here concur with aspects of a recent study on GR acetylation by Nader *et al.,* (2009). Nader and co-workers found that a K494/495A hGR mutant exhibited slightly reduced transactivation compared to WT hGR. This drop in transactivation would be consistent with reduced ligand binding by the alanine mutant, although the authors did not investigate ligand binding. Furthermore, ChIP analysis of the GILZ promoter in HeLa cells under conditions which favour GR acetylation at K494/495 indicated no apparent increase in GR recruitment, similar to what was observed in COS-7 cells with K494/495Q HAhGR in this study (figure 3.7B). Unfortunately, the authors did not investigate expression of the GILZ gene under these conditions, so it is unclear

whether this apparent lack of GR recruitment correlated with reduced GILZ expression. Taken together, results by Nader and co-workers (2009) lead the authors to conclude that GR acetylation reduced DNA binding and transcriptional activity of the GR, which is inconsistent with their results showing decreased transactivation with the alanine mutant compared to WT GR, and is also not in agreement with results presented here. Differences in the experimental strategy (vis. overexpression of HAT proteins versus use of site-specific mutants in different cell lines) may be responsible for this apparent discrepancy.

AR acetylation mutants have been widely used, and the results from different groups do not always concur. For instance, Fu *et al.,* (2002) generated a K632/633A mutant of the AR, and showed that this mutant exhibited greatly reduced transactivation compared to WT AR in DU145 cells. Thomas *et al.,* (Thomas *et al.,* 2004) showed that in HeLa cells this mutant responded to ligand by misfolding and forming subcellular aggregates. The K632/633A mutant AR has also been shown to exhibit a decrease in ligand-dependent phosphorylation compared to WT AR (Fu *et al.,* 2004) and differential cofactor recruitment compared to WT AR, while the AR conformation, sumoylation and DNA binding capacity reportedly remained unchanged (Fu *et al.,* 2002). Cutress and co-workers (2008) generated a triple lysine mutant, K630/632/633A AR, and found that ligand responsiveness (transactivation and nuclear translocation) was completely ablated in COS-7 cells, which would be consistent with results presented here on the ligand binding capacity of the corresponding hGR mutant K494/495A hGR (figure 3.5A). Shiota *et al* (2009) generated a triple arginine mutant AR (K630/632/633R), and also found that it was unresponsive to ligand for nuclear translocation in PC-3 cells. Although this mutant is reported to transactivate a reporter plasmid in both PC-3 cells and LNCaP cells, to a lesser extent than WT cells, no indication of background transactivation in the absence of overexpressed AR is given. Thus it is not clear whether this mutant can cause transactivation above background levels.

Shiota *et al* (2009) also used a triple glutamine AR mutant (K630/632/633Q AR) to mimic acetylation at all three conserved lysines. This mutant exhibited largely the same subcellular localisation as WT AR. While it did not exhibit greater ligand-

dependent transactivation than WT, K630/632/633Q AR demonstrated higher transactivation than WT AR in the absence of ligand in both PC-3 and LNCaP cells. This could indicate greater sensitivity of the acetylation mimic AR to low levels of androgens present in the growth medium, which would be consistent with results presented here that an acetylation mimic HAhGR construct binds ligand more efficiently that WT HAhGR. Indeed, while direct ligand binding has not been investigated, mutation of K630 to Q has been shown to increase hormone sensitivity of the AR, while also increasing transactivation activity, and cell proliferation (Fu *et al.,* 2003).

A recent study by Faus and Haendler (Faus & Haendler, 2008) highlighted the specificity of acetylation at different sites in the AR KXKK motif. Single, double and triple K→A mutations all had different effects on transcriptional efficacy of the AR response. Furthermore, this effect was promoter-specific, and thus independent of the effect of each mutation on ligand binding and nuclear import. This could certainly explain some of the discrepancies identified in the literature, and suggests that steroid receptor acetylation is a precisely regulated process with promoter-specific, and possibly also cell type-specific effects. While many parallels can be drawn between the AR and GR in terms of the highly conserved KXKK motif, residues around this motif are not conserved between AR and GR (Kim *et al.,* 2006). Therefore it is not clear whether this hypothetical interaction site for FKBP52 on the GR would function similarly in the AR. A putative site for a direct interaction between FKBP52 and the surface region of the AR hormone binding domain has recently been identified, and small molecule inhibitors of this interaction are currently under development (Meneses De Leon *et al.,* 2009). The development of specific modulators of SR:FKBP52 interactions could have important pharmacological applications.

Determination of levels of association between FKBP52 and WT GR was attempted using a co-immunoprecipitation approach, but unfortunately this approach was not successful. The conditions required for co-immunoprecipitation of immunophilins with the GR-Hsp complex are extremely mild, as this interaction is quite labile (Davies *et al.,* 2002; Echeverría *et al.,* 2009). However, in the present author's hands, only very

weak association was detected, and no ligand-dependent change in levels of co-immunoprecipitated FKBP52 were observed using two previously published methods in A549 cells (Appendix E, figure E.3.2). Failure to observe the expected swap of FKBP51 for FKBP52 upon ligand binding, which was a positive control for the assay, indicated that the co-immunoprecipitation assay was not working, and thus differences between WT and mutant GR could unfortunately not be examined. These results do not exclude an involvement of a GR: FKBP52 interaction that is sensitive to GR acetylation levels in modulating ligand binding, since ligand-dependent acetylation was also not observed in the A549 model system (figure 3.1), despite having been shown in A549 cells under similar conditions (Ito *et al.,* 2006). Thus, although experimental error could account for the failure to observe ligand-dependent GR acetylation and immunophilin exchange, both of which have been reported in A549 cells (Ito *et al.*, 2006; Croxtall *et al.*, 2003), an alternative explanation may be an unknown difference in the experimental system used here, compared to those of previous studies.

Failure to observe changes in levels of GR-associated FKBP52 was not due to changes in total FKBP52 levels. Although FKBP52 expression has not been shown to be regulated by steroid receptors FKBP51 expression has been shown to be regulated by the PR (Hubler *et al.,* 2003) and by GCs (Baughman *et al.,* 1997). Since the levels of FKBP52 recruited to the GRE-containing region of the GILZ promoter differed between WT and mutant hGR, experiments were conducted to assess whether the WT and mutant GR differentially regulated FKBP52 expression, but after 1 h dex treatment (time point at which ChIP was performed), there was no difference in FKBP52 levels (Appendix C, figure C.3). However, since FKBP52 activity is known to be modulated by casein kinase II phosphorylation (Miyata *et al.,* 1997), the possibility that FKBP52 activity may be differentially modulated by phosphorylation cannot be excluded here.

Another interesting line of research would be to determine the effects of K494/495Q HAhGR on transrepression. Although Ito *et al.,* (2006) suggested GR has to be deacetylated at these sites in order to interact with p65 and transrepress gene expression at NFκB containing promoters, they failed to demonstrate this using the

K494/495Q mutant. They did however show that the acetylation deficient GR mutants (K494/495A and K494/495N) behaved similarly to WT hGR in a p65 immunoprecipitation assay in the presence of IL1β, but differently to WT hGR in the presence of TSA. It is possible that if acetylation status governs FKBP52 association with the GR heterocomplex, this could also affect transrepression in a gene specific manner.

In this report, promoter-reporter assays indicated that K494/495Q HAhGR caused a three times greater transcriptional response than WT HAhGR (figure 3.2B), while transcription of the endogenous GILZ gene was activated to a similar extent by both WT HAhGR and K494/495Q HAhGR (figure 3.7A). This discrepancy is most likely due to the differences in experimental protocols followed for each assay. Since a reporter assay requires translation of a transcript, a longer time is needed before the transcriptional response is measured than an assay in which mRNA levels are measured. The luciferase reporter assay was incubated for 24 h before activity was measured, while mRNA levels of GILZ were measured after just 2 h of incubation with dex. Thus, it is possible that the transactivation by K494/495Q HAhGR is more sustained than WT HAhGR, but that this is not evident after a short incubation. This possibility could be further investigated by performing a time course analysis of reporter activity elicited by WT and K494/495Q HAhGR.

It was noteworthy that the K494/495Q HAhGR mutant gives a remarkably strong transcriptional response, despite its reduced nuclear import compared to WT HAhGR. This suggests that the transcriptional activation per molecule of nuclear GR is much greater for the K494/495Q HAhGR mutant than for WT HAhGR. It was thus postulated that the complex formed by the K494/495Q HAhGR mutant on the promoter could differ from that formed by WT HAhGR.

ChIP results of the endogenous GILZ promoter are consistent with this theory, since no increase in K494/495Q HAhGR recruitment was observed upon dex treatment (figure 3.7B), although gene transcription was increased (figure 3.7A). This indicates that whatever complex of proteins and coregulators was recruited to the GILZ promoter was able to block access of the immunoprecipitation antibody to its antigen

on the mutant, but not the WT HAhGR. It was subsequently found that FKBP52 is recruited to the GILZ promoter upon dex treatment, but this was only significant in the presence of the K494/495Q HAhGR mutant (figure 3.8). Since FKBP52 has been shown to be required for the maximal dex response on the GILZ gene by WT hGR (Wolf *et al.,* 2009), it is likely that the presence of FKBP52 with WT HAhGR on the promoter may be masked by recruitment of other cofactors such as GRIP1, which has been shown to be recruited to this promoter by WT HAhGR (Avenant *et al.,* 2009). It would be interesting to examine the relative recruitment of other cofactors, such as p160s and p300, by WT versus K494/495Q HAhGR.

The data generated in this study, together with available literature, support a model in which acetylation of K494/495 mediates the interaction of GR with FKBP52, which increases the efficiency of ligand binding by the GR, so that for K494/495A hGR mutant, ligand binding is impaired due to a weaker interaction with FKBP52, resulting in a loss of GR nuclear translocation, phosphorylation and transactivation. Conversely, according to such a model, the acetylation mimic K494/495Q HAhGR would be expected to exhibit a strong interaction with FKBP52, resulting in the observed increase in ligand binding over WT hGR. However, a strong interaction with FKBP52 (and thus dynein and microtubules) may actually retard nuclear translocation, resulting in less dex-mediated nuclear localisation of K494/495Q HAhGR than WT HAhGR. If one were to further speculate that GR phosphorylation at S211 and S226 occurs on or after nuclear import, the reduction in nuclear translocation may be responsible for the corresponding reduction in dex-mediated phosphorylation of K494/495Q HAhGR compared to WT HAhGR.

In conclusion, it has been shown for the first time that mutations of K494 and K495 of the hGR that prevent or mimic acetylation, can abolish or increase ligand-binding by the hGR, to decrease or increase transactivation, respectively. Results presented here are consistent with a model in which basal GR acetylation is required for ligand-binding and associated downstream effects such as increased GR phosphorylation at S211 and S226, nuclear translocation, DNA binding and transactivation. These results do not exclude the possibility of a role for interaction of acetylated GR with FKBP52 in regulating ligand binding and nuclear translocation, which is furthermore

supported by the literature. Chromatin immunoprecipitation data on the endogenous GRE-containing GILZ gene strongly support a model in which interaction of FKBP52 with acetylated residues at K494 and K495 of the GR plays an important role in GR function by acting as a cofactor in transactivation. These results suggest that modulation of GR acetylation at K494/K495 represents an attractive physiological mechanism for regulation of glucocorticoid sensitivity.

Chapter 4

Differential nuclear localisation of the GR

plays a role in its ligand-specific transcriptional responses

The glucocorticoid receptor (GR) is a ligand-activated transcription factor, for which a number of ligands, both endogenous hormones and synthetic compounds, exist. A panel of eight different ligands was used, and cells were treated with saturating concentrations of each to control for differences in the GR binding affinity of the different ligands. It was found that different GR ligands elicit different maximal responses for gene transactivation and transrepression, via endogenous GR in U2OS cells, on synthetic and endogenous promoters. In order to find out how these ligand-specific differences in transcriptional efficacy arise, the extent of nuclear translocation elicited by different GR ligands, was examined using subcellular fractionation and immunofluorescence microscopy. It was found that the extent of GR nuclear localisation differed for different ligands, and correlated with the maximal biological activity of the GR as well as the extent of GR phosphorylation at Serine-211. These results suggest that nuclear import may be the rate-limiting step for both GR phosphorylation and transactivation, or that a common upstream event modulates all of these processes, resulting in the same rank order for different ligands.

The glucocorticoid receptor (GR) is a ligand-activated transcription factor that regulates transcription of many target genes via several mechanisms. These include transactivation via binding of liganded GR to consensus glucocorticoid-response elements (GREs) as well as transrepression via tethering mechanisms where the GR mutually interferes with other transcription factors such as activator protein-1 (AP-1) and nuclear factor kappa B (NFκB) (Hayashi *et al.,* 2004). A wide range of ligands bind to the GR resulting in a range of transcriptional responses for both

transactivation and transrepression in a ligand-, promoter-, and cell-specific manner. In order to effect its biological responses, the liganded GR must translocate from the cytoplasm to the nucleus via the nuclear pores. However, the extent to which nuclear localisation contributes to ligand-selective biological responses has not been determined for steroid receptors. The GR and its endogenous ligand play a key role in many physiological processes and synthetic glucocorticoids are extensively used in treating several diseases. It is thus important to understand the biochemical basis for ligand-selective transcription via the GR, with a view to developing new drugs with fewer side-effects (Rhen and Cidlowski, 2005).

It is generally accepted that ligand-selective maximal responses by steroid receptors are due to the induction of ligand-specific conformations of the liganded receptor, exposing different interacting surfaces. There is substantial evidence that different ligands induce different conformations in the ligand-binding domain of the GR (Kauppi *et al.,* 2003); (Kroe *et al.,* 2007) and that these result in differential recruitment of coregulators by liganded GR (Coghlan *et al.,* 2003; Wang *et al.,* 2004); (Garside *et al.,* 2004; Cho *et al.,* 2005; Wang & Simons, 2005); (Kroe *et al.,* 2007); (Wang, *et al.,* 2007b; Miner *et al.,* 2007; Ronacher *et al.,* 2009). However, the effect that these different conformations of GR have on its nuclear translocation is not understood. Only a few studies have addressed the issue of differential nuclear localisation of the GR when bound to different ligands. Of these studies some show no difference in GR nuclear localisation between ligands (Pariante *et al.,* 2001; Schaaf *et al.,* 2005), while others report ligand-selective differences (Vicent *et al.,* 2002; Croxtall *et al.,* 2003; Peeters *et al.,* 2008). Unfortunately these reports suffer weaknesses such as the use of concentrations of ligands which result in differences in the fractional occupancy of GR, thus confounding the results (Croxtall *et al.,* 2003; Peeters *et al.,* 2008), or differences in treatment times, which prevents quantitative comparison between different ligands (Schaaf *et al.,* 2005). In addition, most of the above studies use too few ligands (two to four ligands, usually only full agonists and antagonists) to establish a general conclusion (Croxtall *et al.,* 2003; Peeters *et al.,* 2008; Vicent *et al.,* 2002; Pariante *et al.,* 2001).

A critical evaluation of the literature reveals that there are no studies investigating a direct relationship between the extent of nuclear localisation by liganded GR and the potency (ligand concentration for half maximal response) or efficacy (maximal response) for transactivation. In this study the quantitative relationship between the extent of nuclear localisation elicited by saturating concentrations of different ligands, phosphorylation of GR at serine-211, and the efficacy of the transcriptional effect for both transactivation and transrepression was investigated. A model system consisting of endogenously expressed GR in U2OS cells, and panel of eight ligands, including agonists, partial agonists and antagonists, were used.

MATERIALS AND METHODS

Cell lines and test compounds U2OS cells (ATCC) were cultured in high glucose (1 g/ml) Dulbecco's Modified Eagles Medium (DMEM) (Sigma-Aldrich) supplemented with 10% (v/v) fetal calf serum (FCS) (Delta Bioproducts), 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco Invitrogen) at 37°C in a 5% CO_2 incubator. Dexamethasone (dex) ((11β,16α)-9-Fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione), cortisol (cort) (11β,17α,21-Trihydroxypregn-4-ene-3,20-dione-17-Hydroxycorticosterone), prednisolone (pred) (1,4-Pregnadiene-11β,17α,21-triol-3,20-dione), progesterone (prog) (4-Pregnene-3,20-dione), MPA (6α-Methyl-17αhydroxyprogesterone acetate), NET-A (NET) (Norethisterone-17-acetate), aldosterone (ald) (11β,21-Dihydroxy-3,20-dioxo-4-pregnen-18-al), RU486 (Mifepristone, 11β-(4-Dimethylamino)phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one), dihydroxy testosterone (DHT) and R5020 were purchased from Sigma Aldrich. R1881 and mibolerone were purchased from Perkin Elmer Life and Analystical Science. Test compounds were dissolved in absolute ethanol and stored at -20˚C. The final concentration of each GR ligand was calculated to give a fractional GR occupancy of more than 96.5%, according to RBA data reported in Ronacher *et al.* (2009) (100 nM dex, 1 μM pred, 1 μM cort, 100 nM MPA, 10 μM ald, 1 μM prog, 100 nM RU486, and 10 μM NET). More details of these calculations are given in Appendix A.

Plasmids and antibodies Plasmids encoding human steroid receptors GR, GRβ, PR, AR and MR (pCMV-HAhGR, pCMV-hGRβ, pMT-hPRβ, pSV AR_o, and pRS.hMR) were kind gifts from Dr M. Garabedian (New York University School of Medicine), Dr. J. Cidlowski (National Institute of Environmental Health Sciences, North Carolina), Dr S. Okret (Karolinska Institute, Sweden), Dr F. Classens (University of Leuven, Belgium), and Dr R. Evans (Salk Institute, La Jolla) respectively. pCMV-β gal was purchased from Clontech, while pAP-1-Luc (containing 7x AP-1 site upstream of a TATA box) and pNFκB-Luc (containing 5x NFκB site upstream of TATA box) were purchased from Stratagene. The pTAT-

GRE-E1b-Luc plasmid containing two copies of the hormone response element (HRE) from the TAT gene was a kind gift from Dr G. Jenster (Erasmus University, Rotterdam) and has been described previously (Sui *et al.,* 1999). Antibodies towards the AR (sc-7305), MR (sc-11412), PR A/B (sc-810), and GR (sc-8992) were purchased from Santa Cruz Biotechnology. The antibody towards GRβ (PA3-514) was purchased from Affinity Bioreagents. The anti-GR phosphoserine 211 antibody was a kind gift from Dr M. Garabedian (New York University School of Medicine). The anti-β tubulin antibody (T4026) was from Sigma Aldrich. Markers for subcellular fractionation were anti-GAPDH (14C10, Cell Signalling) and anti-histone H3 (ab1791, Abcam). The antibody towards FKBP52 (sc-1803) was purchased from Santa Cruz Biotechnology. Secondary antibodies used were anti-rabbit-HRP, or anti-mouse-HRP (sc2313 or sc2005 respectively, Santa Cruz Biotechnology). Details of the concentrations at which different antibodies were used can be found in Appendix B.

Reporter assays For transactivation assays U2OS cells were seeded into 24 well plates at a density of 0.5 x 10^5 cells per well. The next day the cells were transfected with 0.25 µg pTAT-GRE-E1b-luc and 0.05 µg p-CMV-β gal using Fugene 6 (Roche) according to the manufacturer's instructions. In cases where a particular steroid receptor was overexpressed, 125 ng of plasmid DNA encoding the receptor of interest was included in the transfection mix. After 24 h, the cells were stimulated with different compounds for 24 h in serum-free DMEM. Thereafter the cells were washed with PBS and lysed in 50 µl per well of Reporter lysis buffer (Promega). Luciferase activity in the lysate was measured using the Luciferase Assay System (Promega) and a Veritas microplate luminometer (Turner Biosystems). The values obtained were normalised to expression of β-galactosidase to normalize for transfection efficiency between wells, which was measured using the GalactoStar Assay Kit from Tropix.

siRNA siRNA transfection conditions were optimised as shown in Appendix E.4. U2OS cells were plated in 12-well plates at a density of 0.8 x 10^5 cells per well. After 8 h, cells were transfected with 10 nM siRNA (Qiagen), directed against either the

GR (GR6), or a validated non-silencing control sequence (NSC) using HiPerfect transfection reagent (Qiagen). 16 h later, cells were transfected with the pTAT-GRE-E1b-luc reporter plasmid using Fugene 6 transfection reagent. Cells were left for a further 24 h, before being treated with ligands and reporter activity was analysed as above. Parallel samples were analysed by Western blot as described below to check for successful GR knockdown. Blots were probed with antibodies towards GR and β-tubulin which served as a loading control.

Real time PCR quantification of endogenous gene expression U2OS cells were plated in 12-well plates at a density of 2.5 x 10^5 cells per well. After 24 h the cells were treated. For the glucocorticoid-induced leucine zipper (GILZ) gene, medium was replaced with serum-free DMEM and after 2 h of serum starvation, the different GR ligands were added for 2 h. For interleukin 8 (IL8) and collagenase 3 (COL3) gene expression analysis, cells were serum starved for 2 h followed by a 2 h simultaneous treatment with 25 ng/ml PMA (Sigma Aldrich) and ligand. For osteocalcin (OCN) gene expression analysis, cells were serum starved for 2 h followed by a 5 h simultaneous treatment with 25 ng/ml PMA (Sigma Aldrich) and ligand. For tumour necrosis factor α (TNFα) gene expression analysis, cells were pre-treated with GC for 30 min before stimulation with 20 ng/ml TNFα (Sigma Aldrich) for a further 3.5 h. Following treatment, the cells were washed with PBS, and RNA was extracted using Tri Reagent (Sigma-Aldrich). RNA was analysed by formaldehyde gel electrophoresis for integrity, before being reverse transcribed with Oligo-dT priming, using the Transcriptor First Strand cDNA synthesis kit (Promega). An equal volume of each cDNA synthesis reaction was used as template for real time PCR, using the Sensimix dT Kit (Quantace). Quantitative PCR was carried out using QuantiTect primers (Qiagen) for GILZ, COL3 and IL8. OCN primers were described in Diefenderer *et al.* (2003). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for normalization (F: 5' TGAACGGGAAGCTCACTGG 3'; R: 5'ATTCGTTGTCATACCAGG 3'). Standard curves were used to determine the efficiency of each primer set, and the relative expression of transcript of interest in each sample was calculated according to the Pfaffl mathematical model (Pfaffl, 2001).

Nuclear fractionation U2OS cells were plated in 6 cm dishes at a density of 8 x 10^5 cells per dish. After 24 h, cells were serum starved for 2 h and treated for 2 h with different ligands at GR-saturating concentrations. Cells were washed with PBS and scraped in 100 µl of 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. Thereafter, the nuclear pellet and cytoplasmic fractions were separated by centrifugation at 3000 g for 5 min at 4˚C. The nuclear pellet was washed with 1 ml PBS before being resuspended in 80 µl DNaseI buffer (40 mM Tris pH 7.9, 10 mM NaCl, 6 mM $MgCl_2$, 10 mM $CaCl_2$, 0.25 units/µl DNaseI) and incubated at 37˚C for 10 min before addition of 20 µl 5x SDS sample buffer. Equal amounts for cytoplasmic and nuclear fractions were separated by 10% SDS-PAGE, and analysed by Western blot as described below.

Indirect immunofluorescence U2OS cells were plated on glass coverslips at a density of 5 x 10^4 cells/ml, in DMEM 10% FCS. The following day, cells were serum starved for 2 h, before treatment with saturating concentrations of different ligands for 2 h. Cells were fixed and permeabilised by incubation in methanol at -20˚C for 15 min. Coverslips were blocked in 3% BSA/PBS for 1 h, and then incubated in primary antibody (anti-GR H300 (Santa Cruz Biotechnology), 1:500 in 5% BSA/PBS) for 1 h. Coverslips were then washed and incubated in secondary antibody (anti-Rabbit, Alexa 488 (Invitrogen), 1:500 in 5% BSA/PBS) for 1 h. Hoechst DNA stain (1:1000, Sigma Aldrich) was used to counterstain nuclei, before mounting coverslips on glass slides using mowiol mounting medium (13% (w/v) mowiol, 33% glycerol in 0.2 M Tris pH 8.5). Slides were incubated at room temperature overnight to allow mowiol to set, and then examined on a Zeiss fluorescent microscope. Quantification of nuclear fluorescence was performed using Zeiss Axiovision Rel 4.7 densitometry software, which measures the intensity of the signal within a selected region of interest. At least 40 randomly selected nuclei were quantified per condition, per experiment.

Western blot analysis of phosphorylated GR U2OS cells were treated exactly as described above for indirect immunofluorescence. Cells were then washed with PBS, harvested in 2x SDS sample buffer (100 mM Tris-HCl, pH 6.8, 5% (v/v) SDS, 20% (v/v) glycerol, 5% β-mercaptoethanol, and 0.1% bromophenol blue) and boiled for 5

min. Proteins were separated by electrophoresis on an 8% polyacrylamide gel by SDS-PAGE, at 200V. Proteins were transferred from the gel to Hybond ECL membrane (Amersham Biosciences) by electroblot at 180 mA for 1 h, and blocked for 1 h in 4% ECL advance blocking powder (Amersham Biosciences) in Tris-buffered saline containing 0.1% Tween-20 (TBST). Membranes were incubated on a shaker at 4˚C overnight in primary antibody solution in TBST. The following day, membranes were washed 3 x 5 min in TBST on a shaker, and incubated in secondary antibody diluted in 5% non-fat milk powder for 1 h, before washes were repeated. Dilutions at which primary and secondary antibodies were used are included in Appendix B. Blots were developed using Amersham ECL Western blotting detection reagents (GE Healthcare). For consecutive detection steps, membranes were stripped by incubation in stripping buffer (100 mM β-mercaptoethanol, 2% (v/v) SDS, 62.5 mM Tris-Cl, pH 6.8) for 30 min at 65˚C (Sambrook *et al.,* 1989).

Chromatin Immunoprecipitation (ChIP) assay ChIP was performed as described in Ma *et al.* (2003), with modifications. U2OS or A549 cells were plated at 2 x 10^6 cells per dish in 15 cm dishes and grown for 48 h. Cells were then washed and incubated in serum-free DMEM for 2 h, before treatment with 100 nM dex or vehicle for 1 h. Cells were crosslinked for 10 min at 37˚C by addition of formaldehyde to a final concentration of 1%. Crosslinking was stopped by addition of glycine to a final concentration of 0.1 mM for 5 min at room temperature. Cells were scraped in PBS, pelleted by centrifugation at 1200 g for 10 min, and resuspended in 300 µl nuclear lysis buffer. Chromatin was sheared by sonication in a Bioruptor (Diagenode) (30 second on/off x 10 cycles), before centrifugation at 15000 g for 10 min. The concentration of DNA in the supernatant was quantified by measuring absorbance at 260 nm, and the different samples were diluted to equal concentration with nuclear lysis buffer. This chromatin was then aliquotted and frozen at -80˚C. Separate aliquots were made for analysis of input samples.

Protein A/G beads (sc-2003, Santa Cruz Biotechnology) were pre-blocked by incubation with salmon sperm DNA (0.2 mg/ml) and bovine serum albumin (BSA) (1

mg/ml) for 1 h on a rotating wheel at 4˚C. The beads were resuspended in IP dilution buffer (0.01% SDS, 20 mM Tris-HCl pH8, 1.1% Triton X 100, 167 mM NaCl, 1.2 mM EDTA, 1 mM PMSF, 2 µg/ml aprotinin, 5 µg/ml leupeptin) as a 50% slurry, and stored at 4˚C. For immunoprecipitation, 100 µl of chromatin (50-100 µg of DNA) was diluted with 900 µl IP dilution buffer. This solution was precleared with 10 µl of pre-blocked protein A/G agarose beads (sc-2003, Santa Cruz Biotechnology) for 1 h at 4˚C. Beads were spun down, and 2 µg anti-GR (H300) (Santa Cruz Biotechnology) was added to the supernatant, which was incubated on a rotator at 4˚C overnight. The following day, 30 µl of preblocked protein A/G beads were added, and tubes were incubated on a rotator for 6 h at 4˚C. Beads were washed with 1 ml each of wash buffer I (0.1% (w/v) SDS, 1% (v/v) Triton X-100, 2 mM EDTA, 20 mM Tris-Cl pH8, and 150 mM NaCl), II (0.1% (w/v) SDS, 1% (v/v) Triton X-100, 2 mM EDTA, 20 mM Tris-Cl pH8, and 500 mM NaCl), and III (1% (v/v) NP-40, 1% (v/v) sodium deoxycholate, 500 mM LiCl, 1 mM EDTA, 10 mM Tris-Cl pH8). Beads were then washed three times with 1 ml TE buffer (10 mM Tris pH8, 0.1 mM EDTA). Proteins were eluted from the beads by addition of 300 µl elution buffer (1% SDS, 100 nM $NaHCO_3$) for 30 min at room temperature on a rotating wheel. For input, 30 µl of chromatin solution was stored at -20˚C overnight, before addition of 90 µl elution buffer, and processing in parallel with immunoprecipitated samples.

Of a 5 M NaCl stock solution, 18 µl or 7.2 µl was added to eluate and input samples respectively, to yield a final concentration of 300 nM NaCl. Tubes were then incubated at 65˚C overnight to reverse crosslinks. The following day EDTA, Tris-HCl pH 6.5 and proteinase K (Roche) were added. For eluate samples 10 µl of a 0.5 M EDTA stock (for a final concentration of 15 nM EDTA), 40 µl of a 1 M Tris pH 6.5 stock (for a final concentration of 125 nM Tris) and 2 µl of a 10 mg/ml proteinase K stock (for a final concentration of 0.67 ng/µl proteinase K) were added. For input samples 5 µl of a 0.5 M EDTA stock, 20 µl of a 1 M Tris pH 6.5 stock, and 1 µl of a 10 mg/ml proteinase K stock were added to yield the same final concentrations as the eluate samples. All samples were then incubated at 45˚C for 1 h. DNA was purified using Qiagen PCR cleanup columns. Real time PCR was performed on a Corbett Rotorgene, using the Sensimix dT Kit (Quantace), which measures SYBR

Green fluorescence. Primers corresponding to the promoter region of human GILZ were used (Wang *et al.,* 2004).

Quantification and statistical analysis Densitometric analysis of bands on Western blots was performed using AlphaEase software. Statistical analyses were carried out using GraphPad Prism software, using one-way analysis of variance with either Tukey's or Dunnett's post tests. Correlations were carried out using two-tailed Pearson correlation analysis. Statistical significance of differences are denoted by *, ** or ***, to indicate $P<0.05$, $P<0.01$ or $P<0.001$, respectively.

RESULTS

Endogenous GR in U2OS activates a GRE reporter gene, but no other steroid receptors induce transactivation

Since this study aimed to compare a large panel of steroid receptor ligands, it was important to characterise the steroid receptors expressed in the selected model system. Several of the ligands in the panel are capable of activating other members of the steroid receptor family, in addition to the GR. U2OS human osteosarcoma cells are widely believed not to express GR (Rogatsky *et al.,* 1997). However, when reporter assays were performed in the cells in the absence of overexpressed GR, a significant induction of transcription by the GR-specific agonist dexamethasone (dex) was observed (figure 4.1A). Specific agonists for the AR (mibolerone (mib)), MR (aldosterone (ald)), and PR (R5020) did not elicit a transcriptional response on the hormone response element (HRE)-containing promoter (figure 4.1A). While mib and R5020 were used at 1 µM, ald was used at 10 nM. This was because ald exhibits weak binding to the GR, and would saturate approximately 87% of any endogenous GR present at 1 µM. At 10 nM, ald would only saturate about 6% of the GR (for more information see Appendix A, table A.1). When used at these concentrations, all of the above-mentioned receptor-specific agonists did give a response on this reporter construct when the specific steroid receptor was co-transfected (Appendix D, figure D.1), indicating that the assay was working. Results shown in figure 4.1A indicate that U2OS cells endogenously express a functional GR, but no detectable AR, MR or PR.

In order to confirm that the observed transcriptional response to dex was due to the GR, specific siRNA was transfected to knock down the GR. This experimental technique was first optimised as shown in Appendix E.4. The Western blot (figure 4.1 B) shows that expression levels of β-tubulin were not affected by transfection of siRNA, which is in agreement with microscopic examination of cells, indicating siRNA transfection had no adverse effect on cell growth (data not shown). However GR expression levels were efficiently reduced by transfection of siRNA (GR6) compared to NSC. GR knockdown did not affect the basal transcriptional response in

the absence of dex, but resulted in a loss of the dex-mediated transcriptional response, confirming a requirement for the GR in this response (figure 4.1C).

The results of the reporter assay (figure 4.1A) were confirmed using a Western blot approach (figure 4.1D). COS-1 cells were transiently transfected with constructs encoding each SR, to serve as positive controls for antibody specificity and technique. It was found that PR, GRβ and MR were undetectable in U2OS cells, while a band corresponding in size to the positive control was detected with the anti-AR antibody, indicating that endogenous AR is expressed in U2OS cells. Since this was not in agreement with the reporter assay, which indicated no response to the AR-specific ligand mib, further reporter assays were carried out to investigate whether endogenous AR would give a response to other AR-specific ligands (figure 4.1E). All of the AR-specific ligands tested (mibolerone, DHT and R1881) induced a strong transcriptional response in the presence of transiently overexpressed AR, but showed no activity above background in WT U2OS cells. This strongly suggested that the AR band detected on the Western blot (figure 4.1D) is transcriptionally incompetent AR, or that the levels of endogenously expressed AR are too low to elicit a response.

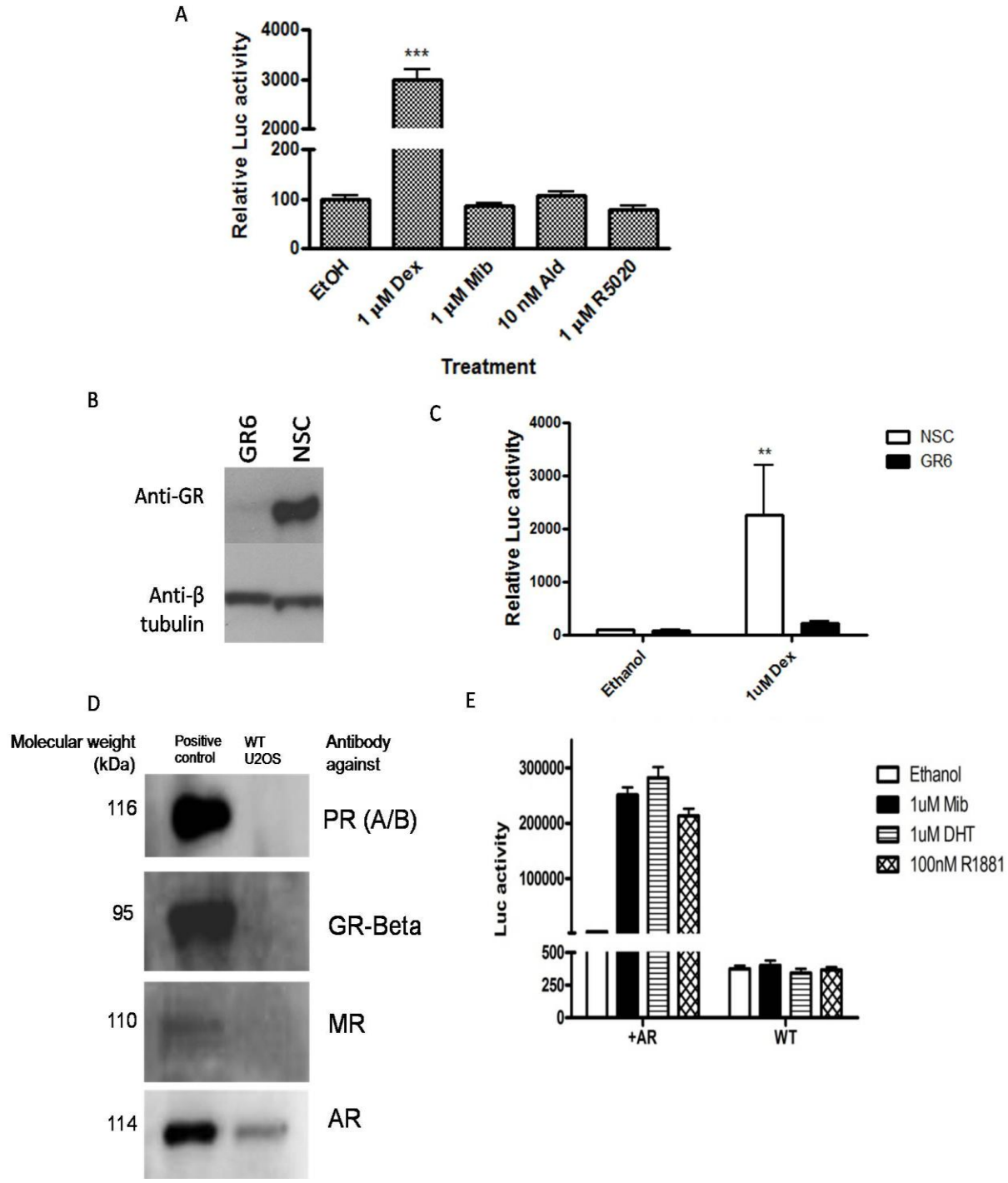


Figure 4.1. Only GR is expressed in U2OS cells, and not AR, MR, PR or GRβ. (A) U2OS cells were transfected with pCMV β-galactosidase reporter and the HR responsive luciferase reporter construct pTAT-GRE-E1b-luc. Cells were treated with vehicle (EtOH) or 1 µM dex, 1 µM mib, 10 nM ald, or 1 µM R5020 for 24 h. Luciferase activity of cell lysates was monitored and normalised to β-galactosidase activity for each well to control for transfection efficiency. Histogram shows pooled results of three

independent experiments each performed in triplicate, analysed by one way Anova with Dunnett's post-test. (B) A representative Western blot, showing the efficiency of knockdown by GR6 siRNA compared to NSC in U2OS cells is shown. (C) Reporter assays were conducted in U2OS as in A, with cotransfection of GR siRNA (GR6), or a non-silencing control (NSC). Cells were then treated with vehicle (EtOH) or 1 μM dex for 24 h, before luciferase activity of cell lysates was monitored and normalised to β-galactosidase activity for each well to control for transfection efficiency. The histogram shows pooled results of two independent experiments each performed in triplicate, analysed by one way Anova with Dunnett's post-test. (D) A Western blot is shown in which lysates from cells overexpressing PR-B, GR-β, MR and AR (positive controls) were analysed in parallel with WT U2OS cell lysate. (E) Luciferase reporter assays were performed as described in A, in the presence or absence of overexpressed AR in U2OS cells. Cells were treated with different AR agonists for 24 h before being lysed and the luciferase activity of the lysates were analysed, and normalised to β-galactosidase activity to normalise for transfection efficiency. The graph shows the result of a single experiment performed in triplicate.

At saturating concentrations, different GR ligands give different maximal transcriptional responses, via endogenous GR on a GRE-driven reporter gene

Since only GR, and not MR, PR or AR, gave a detectable transcriptional response in U2OS cells (figure 4.1A), it was concluded that all transcriptional responses to steroidal ligands were elicited by the GR. A panel consisting of eight different GR ligands (see appendix A for structures) was compared for their transcriptional effect elicited on a GRE-containing promoter, via endogenous GR in U2OS cells. The panel was selected to include full agonists (dex, pred, cort), partial agonists (MPA, ald, prog), an antagonists (NET), and a dissociated glucocorticoid (RU486), as defined by Ronacher *et al.,* (2009). The K_i values published in Ronacher *et al.,* (2009), measured in COS-1 cells overexpressing HAhGR, were used to determine the concentration of each ligand which would cause occupation of at least 97% of the endogenous GR in U2OS cells, and these concentrations were used in subsequent assays in the present study (these calculations are shown in Appendix A). Although the K_i values were calculated in COS-1 cells, and absolute binding of

different ligands may thus differ from the U2OS cell system, this allowed normalisation for differences in relative binding affinity between different ligands.

As shown in figure 4.2, dex, a full agonist, gave the highest response of approximately 50-fold increase in reporter luciferase activity, while RU486, a GR antagonist, gave no response. It was interesting that NET caused a small response in this cell system. NET has been reported to be a GR antagonist, which competes with dex for GR binding, but causes no transactivation in COS-1 cells overexpressing GR (Ronacher *et al.*, 2009). It is possible that this ligand could act differently at different intracellular concentrations of GR, as shown by Zhao *et al.,* (2003) for a subset of steroid ligands, as GR concentrations in WT U2OS are likely to be much lower in WT U2OS cells than transiently transfected COS-1 cells overexpressing GR.

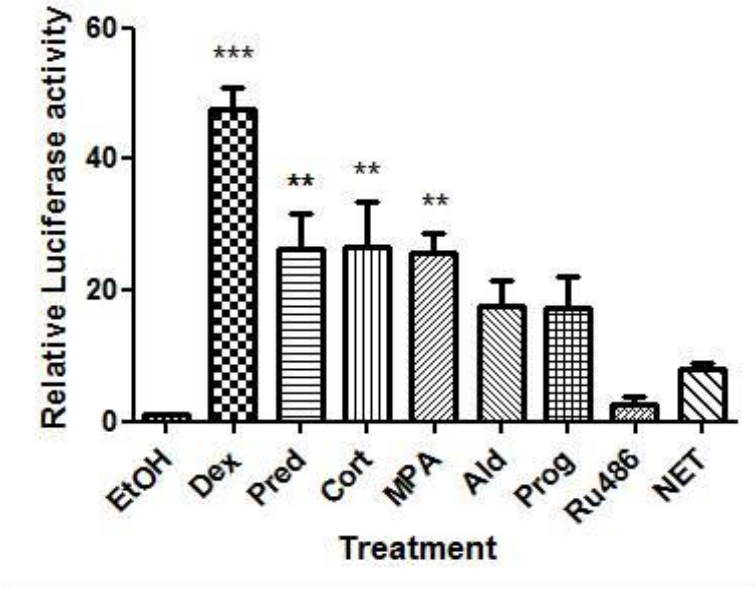


Figure 4.2. Ligand-specific transcriptional maxima observed in reporter assay. U2OS cells were transfected with pCMV β-galactosidase reporter and the GR responsive luciferase reporter construct pTAT-GRE-E1b-luc. Cells were treated with vehicle (EtOH) or saturating concentrations of GC ligands (100 nM dex, 1 µM pred, 1 µM cort, 100 nM

MPA, 10 µM ald, 1 µM prog, 100 nM RU486, and 10 µM NET) for 24 h. Luciferase activity of cell lysates was monitored and normalised to β-galactosidase activity for each well to control for transfection efficiency. Histograms show pooled results of three independent experiments plotted relative to EtOH (1 ± SEM), analysed by one way Anova with Dunnett's post-test, relative to EtOH.

At saturating concentrations, different GR ligands give different maximal transcriptional responses, via endogenous GR on the endogenous GRE-driven gene GILZ

In order to determine whether the results observed in the reporter assay shown in figure 4.2 were representative of the effect on an endogenous gene, GR-mediated transactivation of the endogenous GILZ gene was measured by real-time PCR. GILZ is a negative regulator of NFκB in airway epithelial cells and plays a major role in anti-inflammatory response (Eddleston *et al.,* 2007). The promoter of the GILZ gene contains a number of GRE sequences (Wang *et al.,* 2004).

The results show clear differences in maximal transcription between the different ligands, ranging from a significant 6-fold increase in GILZ mRNA upon dex stimulation, to no change in mRNA levels for the antagonists RU486 and NET (figure 4.3). This is in contrast to the reporter assay, in which NET resulted in a small increase in transactivation. Only full agonists (dex, pred and cort) elicited a significant increase in transcription. However, similarities were observed between endogenous gene and synthetic reporter expression, in the rank order of ligand efficacy.

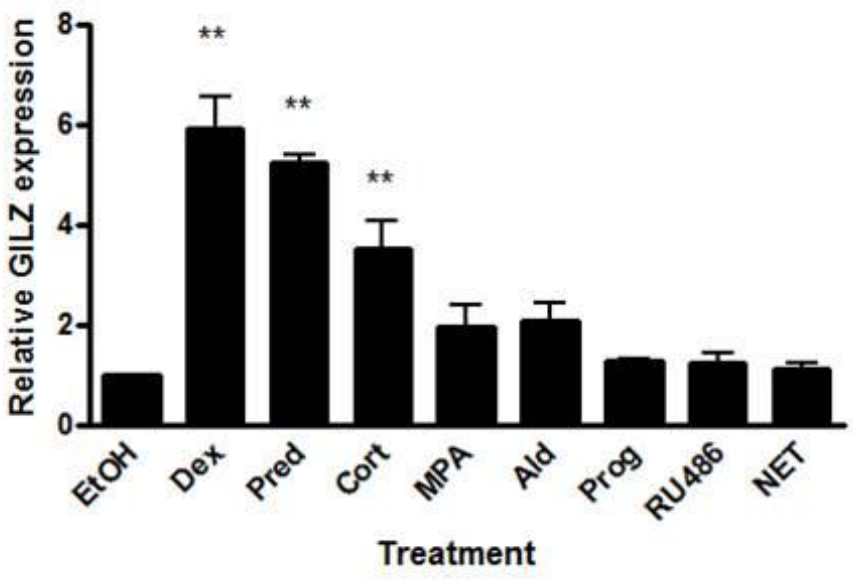


Figure 4.3. Regulation of the endogenous GILZ gene by GR ligands. U2OS cells were serum starved for 2 h, before treatment with saturating concentrations different ligands (100 nM dex, 1 µM pred, 1 µM cort, 100 nM MPA, 10 µM ald, 1 µM prog, 100 nM RU486, and 10 µM NET) for 2 h. RNA was harvested, reverse transcribed and subjected to real time PCR analysis, using primer sets specific for GILZ and GAPDH. Levels of GILZ mRNA transcripts were normalised to GAPDH levels for each sample. The graph shows pooled results of three independent experiments +/- SEM. One way Anova was performed, with Dunnet's post-test relative to EtOH.

The differential responses to different ligands observed on the GILZ gene are mediated by endogenous GR

In order to confirm that transcription of the endogenous GILZ gene measured in response to different ligands was mediated by the GR, GR knockdown was performed. In U2OS cells transfected with siRNA targeting the GR mRNA expression of endogenous GR was reduced by approximately 60- 70% compared to cells transfected with a non-silencing control RNA sequence (figure 4.4C).

The reduction in GR expression coincided with an almost complete loss of GILZ induction (figure 4.4A), thus confirming a requirement for the GR in this transcriptional response. Although the dex-induced fold induction in GILZ in this experiment was slightly lower than in the previous figure (approximately 4.5-fold vs. approximately 6-fold), quantification of the Western blot shows that GR levels in cells transfected with NTC were slightly lower than in untreated cells. Although not statistically significant, this difference could account for the slight discrepancy in maximal efficacy of dex in the two experiments.

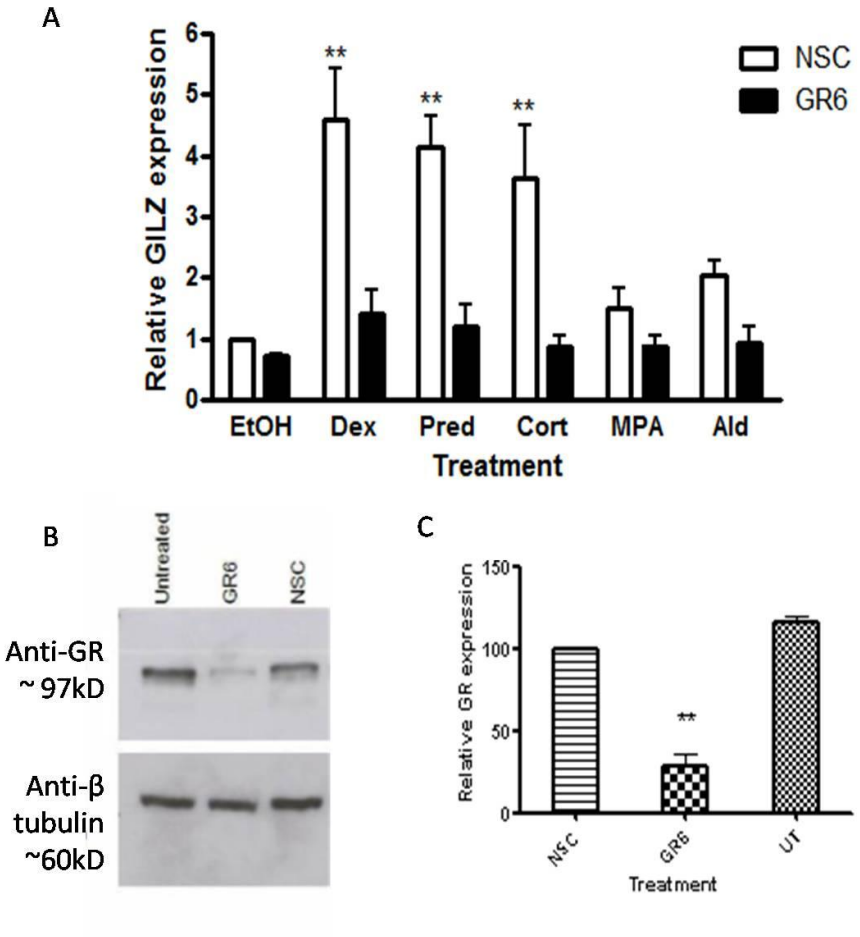


Figure 4.4. GR is required for the observed response to GR ligands on the GILZ gene. (A) U2OS cells were transiently transfected with 10 nM siRNA encoding GR (GR6) or an equivalent amount of NSC, for 48 h. Cells were then serum starved and treated with saturating concentrations of different ligands (100 nM dex, 1 µM pred, 1 µM cort, 100 nM MPA, 10 µM ald) for 2 h. RNA was harvested, reverse transcribed, and GILZ expression was quantified by means of real time PCR, normalising to

GAPDH for each sample. Results from 3 independent experiments were pooled and the graph represents mean +/- SEM, analysed by one-way Anova with Dunnet's post-test relative to NSC EtOH. (B) A representative Western blot showing GR levels relative to β-tubulin is shown. (C) Whole cell lysate samples harvested in parallel with RNA were analysed by Western blot. Relative GR levels in untreated (UT), NSC transfected, and GR6 transfected cells were quantified, and results of three independent experiments were pooled, and shown as mean +/- SEM. Results were plotted relative to NSC, and analysed by one-way Anova, with Dunnett's post-test relative to NSC.

Different ligands induce transrepression of promoter-reporter plasmids to different extents

Having confirmed that different GR ligands elicit different extents of transactivation via the GR, the question of whether the same applies to transrepression was next investigated. Transrepression in U2OS via endogenous GR was first examined on transiently transfected promoter-reporter constructs. The promoters contain multiple copies of the AP-1 response element or NFκB response element. Promoter activity was induced by PMA treatment in order to provide a high background against which to visualise GR-mediated transrepression.

Promoter reporter assays showed that transrepression by endogenous GR was ligand-selective and promoter-specific. However, the trend observed for transactivation (i.e. full agonists give higher maximal efficacy than partial agonists or antagonists) was not observed. On the AP-1 promoter (figure 4.5A), only dex, cort and ald caused significant repression, with NET causing a significant increase in promoter activity. On the NFκB promoter, none of the ligands tested resulted in significant repression of promoter activity (figure 4.5B).

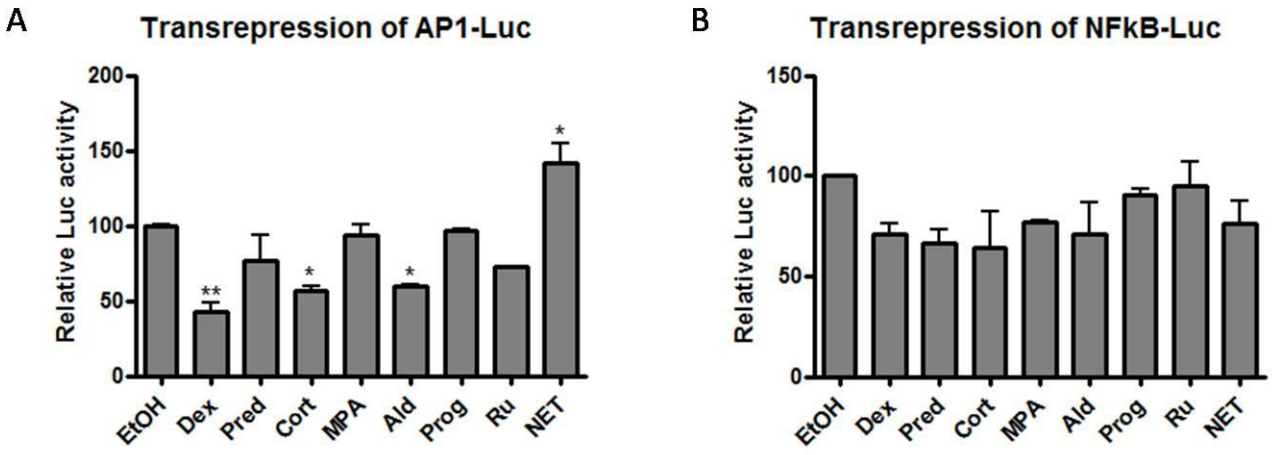


Figure 4.5. Endogenous GR can weakly transrepress in a ligand-selective, promoter-specific fashion. U2OS cells were co-transfected with a β-gal reporter and either (A) an AP-1-Luc or (B) and NFκB-Luc reporter plasmid, and treated with saturating concentrations of different ligands (100 nM dex, 1 µM pred, 1 µM cort, 100 nM MPA, 10 µM ald, 1 µM prog, 100 nM RU486, and 10 µM NET), in the presence of PMA, as described in materials and methods. Thereafter luciferase activity was measured and normalised to β-gal to control for transfection efficiency. Pooled results of two independent experiments, each performed in triplicate, are shown. One-way Anova with Dunnett's post-test relative to EtOH treated cells was performed.

Transrepression of endogenous genes is ligand-selective and promoter specific

In order to investigate whether the transrepression results observed on synthetic promoter reporters provide an accurate reflection of the transrepression of endogenous genes, with a complex chromatin structure, transrepression of endogenous genes was measured by quantitative real time PCR. Several endogenous genes, with different promoter architecture were selected to represent different modes of GR-mediated transrepression.

TNFα is a pro-inflammatory cytokine, the expression of which is auto-induced and transrepressed by GCs (Beutler *et al.,* 1986). TNFα is involved in the pathogenesis

of osteoporosis (Pacifici, 1996). Expression of the TNFα gene was strongly induced by incubation of cells in the presence of TNFα. However, of all the ligands, only dex was capable of eliciting significant transrepression on this promoter via endogenous GR in U2OS cells (figure 4.6A).

Interleukin 8 (IL8) is a proinflammatory chemokine released from a variety of cells in response to inflammatory stimuli (reviewed in Atta-ur-Rahman *et al.,* 1999). The IL8 promoter is repressed by GR binding to an NFκB response element (Nissen & Yamamoto, 2000; Garside *et al.,* 2004; Rogatsky *et al.,* 2002). Although strong induction of this gene was observed upon PMA treatment (figure 4.6B), the partial agonist prog caused a further significant increase in IL8 expression. No significant transrepression of IL8 was observed with any ligand. This is in stark contrast to results observed by Rogatsky and co-workers (Rogatsky *et al.,* 2002) who report a 56-fold repression of IL8 mRNA in U2OS.rGR cells upon 2 h simultaneous treatment with 25 ng/ml PMA and 100 nM dex. These are the same treatment conditions as used in the present study, except for the fact that Rogatsky and co-workers (2002) performed experiments in U2OS cells stably transfected with GR. One must therefore conclude that GR expression levels play a critical role in determining the extent of transrepression elicited by the GR. Indeed, this was confirmed using reporter assays on synthetic AP-1 and NFκB promoter-reporter constructs in the absence and presence of overexpressed hGR (Appendix D, figure D.2). However, as this study aimed to examine the effects of endogenous GR, further experimentation in the presence of overexpressed GR was not conducted.

Collagenase 3 (COL3) is a matrix metalloprotease that has been implicated in tumour migration and metastasis (reviewed in Leeman *et al.,* 2002). GR represses COL3 expression by binding to an AP-1 response site in the promoter of the COL3 gene (Tuckermann *et al.,* 1999). In stably transfected U2OS.GR cells, COL3 is maximally induced by PMA at 2 h, and induction by PMA has been shown to be stronger than that induced by TNFα or LPS. Reporter assays showed that 25 and 50 ng/ml PMA resulted in a 15- or 20-fold increase in promoter activity respectively, which was completely lost upon addition of 100 nM dex in U2OS.rGR cells

(Rogatsky *et al.,* 2001). This is in contrast to the results observed in WT U2OS cells (figure 4.6C), where an induction of only 4-fold was measured upon treatment with 25 ng/ml PMA for 2 h. On this promoter dex, pred, cort and ald resulted in significant repression. Similarities were noted between the results observed on reporter and endogenous genes. For instance, dex, cort and ald gave significant transrepression on an AP-1 reporter (figure 4.5A), and on COL3, an AP-1-regulated endogenous gene (figure 4.6C). Similarly, significant transrepression was not observed on the NFκB-regulated promoter in a reporter assay (figure 4.5B) nor on an endogenous gene (figure 4.6C).

The last model gene examined was osteocalcin (OCN). The OCN promoter is negatively regulated by glucocorticoids because the GR response element, which resembles the consensus GRE, except for three non-conserved bases, overlaps with the TATA box, thereby forming a competitive nGRE (Strömstedt *et al.,* 1991). U2OS cells constitutively express OCN, detectable by reverse transcription PCR (Benayahu *et al.,* 2001), and it has been shown that treatment with 1 µM dex for 42 h completely depletes OCN mRNA in an osteoblast cell line (Leclerc *et al.,* 2005). In a promoter reporter study, U2OS.rGR cells were treated overnight with 25 ng/ml PMA in the presence or absence of 100 nM dex, resulting in about 10-fold repression of the reporter gene by dex (Rogatsky *et al.,* 2002). Thus, in previous reports of dex-mediated transrepression of the OCN promoter treatment times of at least 16 h were used. However, for the real-time PCR experiment in the present study, it was desirable to avoid indirect effects of GCs (i.e. GC alters transcription of an intermediate transcription factor which subsequently regulates OCN). For this reason, incubation times were kept as short as possible in the present study. In the case of OCN, induction with 50 ng/ml PMA was performed for 5 h. Unfortunately, no transrepression was observed for any ligand under these conditions (figure 4.6D). This is unlikely to be due to excessive gene induction, such that the limited endogenous GR was not able to transrepress the gene adequately, since the induction observed upon PMA treatment is only approximately 20% (figure 4.6D).

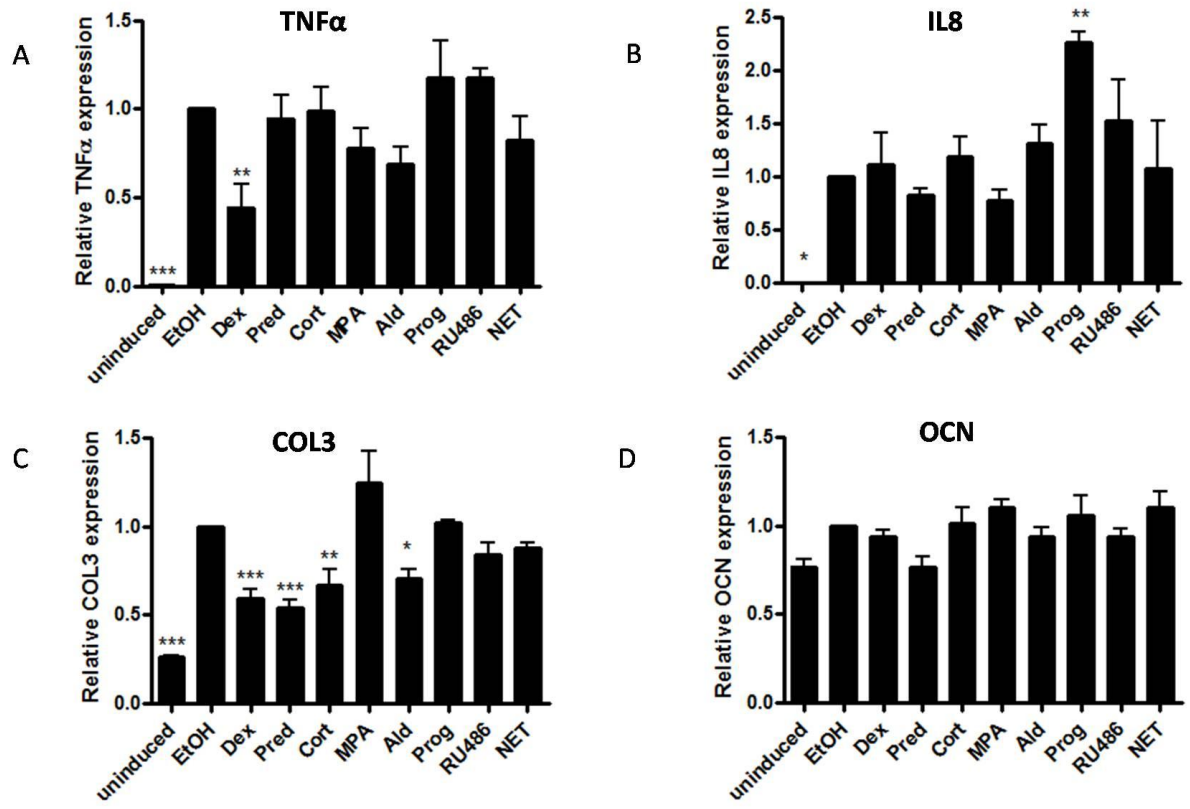
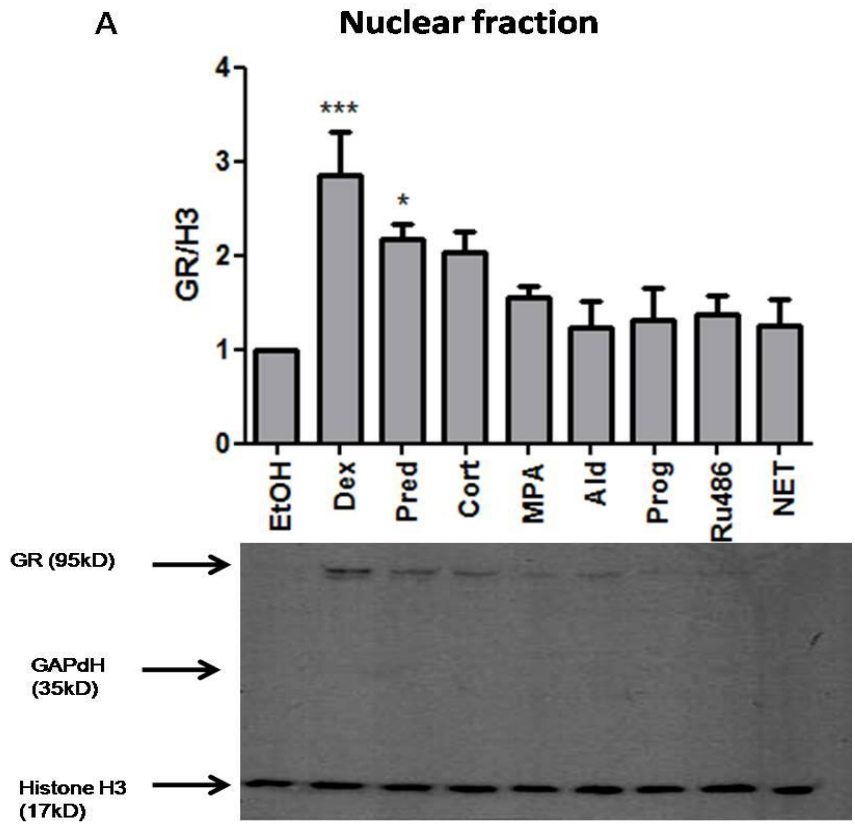


Figure 4.6. GR ligands elicit weak transrepression of endogenous genes in a ligand-selective fashion. U2OS cells were either uninduced or treated with TNFα or PMA to induce gene expression. Treatment with saturating concentrations of different GR ligands (100 nM dex, 1 µM pred, 1 µM cort, 100 nM MPA, 10 µM ald, 1 µM prog, 100 nM RU486, and 10 µM NET) was performed as described in materials and methods. RNA was harvested, reverse transcribed and expression of (A) tumour necrosis factor α (TNFα), (B) interleukin 8 (IL8), (C) collagenase 3 (COL3), and (D) osteocalcin (OCN) were analysed by real time PCR, normalising to GAPDH for each sample. Uninduced treatment refers to expression in the absence of stimulation by TNFα (A) or PMA (B-D). Pooled results of three independent experiments are presented as mean +/- SEM for each gene, and analysed by one-way Anova with Dunnet's post-test relative to EtOH treated cells.

Different ligands induce different extents of GR nuclear localisation

Having shown that different ligands result in ligand-specific differences in maximal transactivation and transrepression in a promoter-specific fashion in U2OS cells (figures 3.2- 3.7), the mechanism by which these differences arise was investigated. In order to examine whether differential GR nuclear localisation could be the cause, nuclear localisation of the GR was investigated by both biochemical fractionation and immunofluorescence microscopy.

It was decided to examine nuclear localisation of GR under the same conditions under which GILZ transactivation was examined (i.e. 2 h serum starvation followed by 2 h GC treatment at saturating concentrations). This treatment time was also used for analysis of IL8 and COL3 expression, in the presence of PMA. When comparing the subcellular localisation of GR after a 2 h GC treatment, ligand-specific differences were observed. Dexamethasone caused a significant three-fold increase in GR nuclear localisation (figure 4.7A), while the GR partial agonists and antagonists caused no increase in GR nuclear localisation compared to untreated cells. Although the quantification of GR in the cytoplasmic fraction did not yield statistically significant differences between different ligands, a decrease in cytoplasmic GR was observed after dex treatment (figure 4.7B). The co-incubation of blots with a mix of antibodies for GR and cytoplasmic and nuclear marker proteins (GAPDH and histone H3, respectively), shows that the total protein loaded per lane is equal, and indicates successful separation of nuclear and cytoplasmic fractions.


A
Nuclear fraction
GR/H3
4
3
2
1
0
***
*
EtOH
Dex
Pred
Cort
MPA
Ald
Prog
Ru486
NET
GR (95kD)
GAPdH
(35kD)
Histone H3
(17kD)

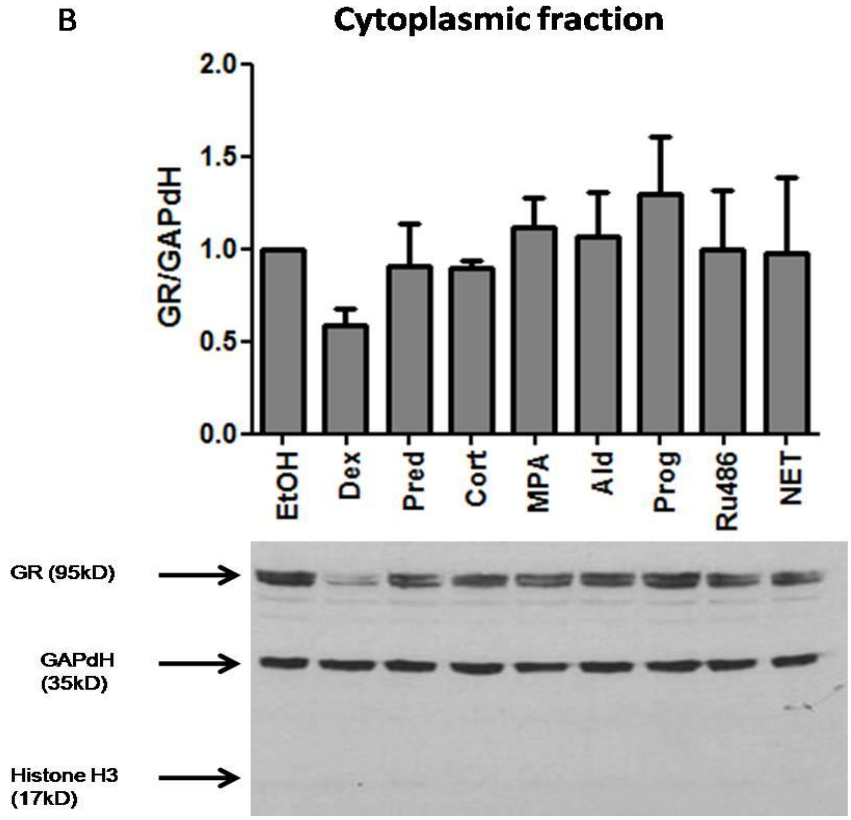
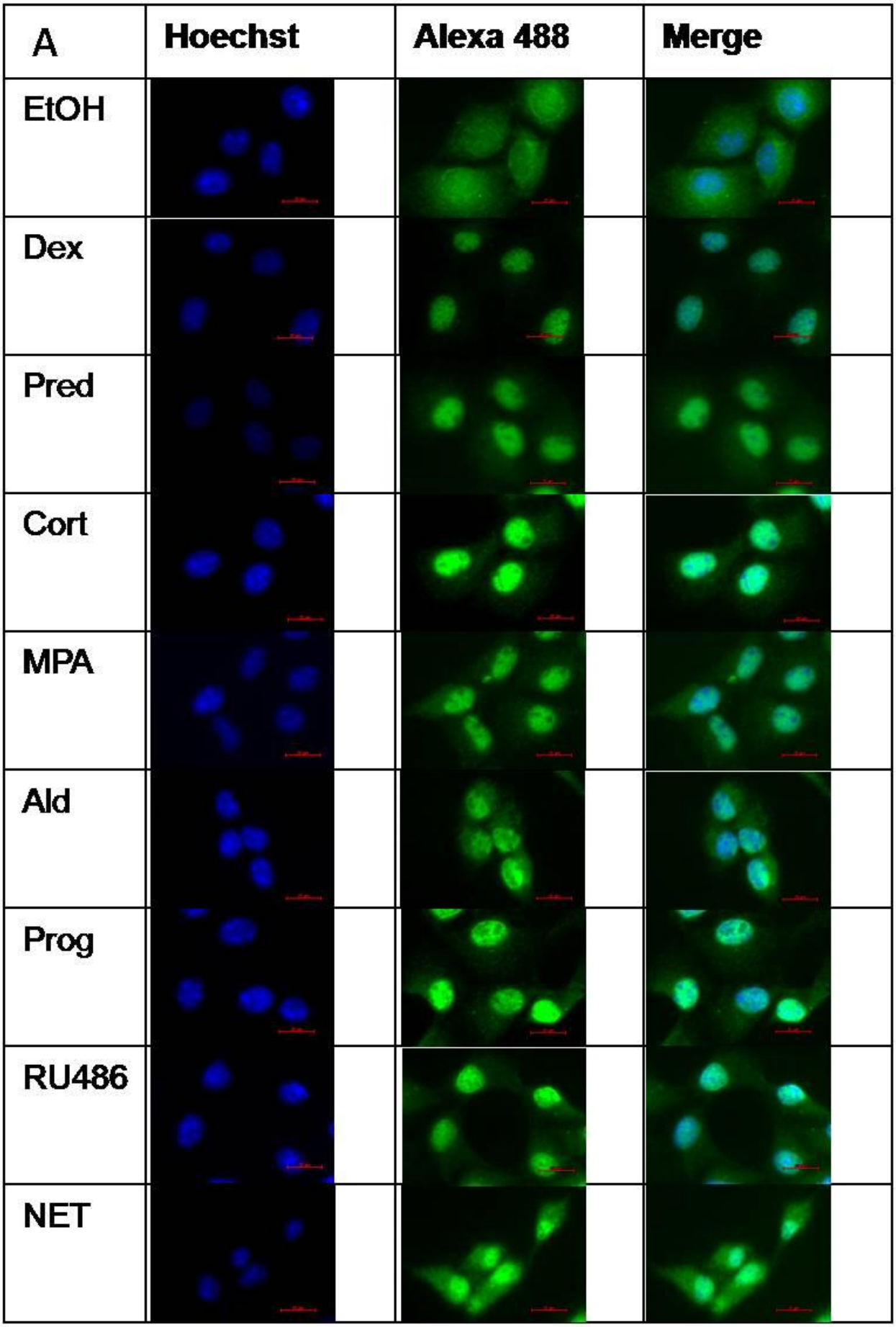

B
Cytoplasmic fraction
GR/GAPdH
2.0
1.5
1.0
0.5
0.0
EtOH
Dex
Pred
Cort
MPA
Ald
Prog
Ru486
NET
GR (95kD)
GAPdH
(35kD)
Histone H3
(17kD)

Figure 4.7. Ligand-specific differences in nuclear import. U2OS cells were treated with saturating concentrations of different steroidal ligands (100 nM dex, 1 µM pred, 1 µM cort, 100 nM MPA, 10 µM ald, 1 µM prog, 100 nM RU486, and 10 µM NET) for 2 h, before being fractionated into (A) nuclear and (B) cytoplasmic fractions. The fractions were analysed by Western blot, with GAPDH and histone H3 serving as markers of efficient separation of nuclear and cytoplasmic fractions, and as controls for equal protein loading. Bands on Western blots were quantified using Alphaease software, and the intensity of the GR signal was normalised to Histone H3 (nuclear fractions) or GAPDH (cytoplasmic fractions) to control for differences in protein loading. Histograms show pooled results from 3 independent experiments, analysed by One-way Anova with Dunnet's post-test relative to EtOH treated cells. Western blots are representative.

In order to confirm the ligand-selective nuclear localisation of endogenous GR observed upon subcellular fractionation, an immunofluorescence approach was employed. GR subcellular localisation was examined by indirect immunofluorescence, under exactly the same treatment conditions (i.e. 2 h serum starvation followed by 2 h GC treatment at saturating concentration) (figure 4.3). Images were selected randomly by selecting fields of view based on Hoechst stain. There was quite a high degree of variability in the intensity of the Alexa 488 immunostain across each coverslip, however randomly selected images (figure 4.3A) show a high ratio of nuclear to cytoplasmic stain for dex-treated cells, and a much lower ratio for NET, following the same trend as observed upon biochemical fractionation. Despite the large error, quantification of the relative intensity of Alexa 488 stain for GR within the nucleus was possible by measuring 40 nuclei for each treatment, and pooling data from four independent experiments (figure 4.3B). Interestingly, GR distribution appeared to be predominantly nuclear for all the ligands.


A
Hoechst
Alexa 488
Merge
EtOH
Dex
Pred
Cort
MPA
Ald
Prog
RU486
NET

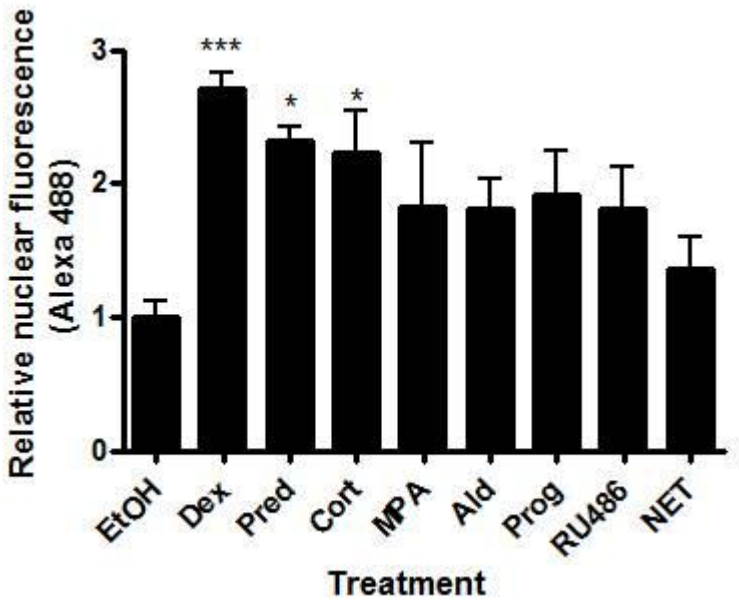


Figure 4.8. Differential nuclear localisation of endogenous GR detected by immunofluorescence (A) U2OS cells were treated with saturating concentrations of different steroidal ligands (100 nM dex, 1 µM pred, 1 µM cort, 100 nM MPA, 10 µM ald, 1 µM prog, 100 nM RU486, and 10 µM NET) for 2 h and GR localisation was examined by indirect immunofluorescence. Scale bars (red) represent 10 µm. (B) Nuclear fluorescence was quantified in 40 randomly selected cells from 4 fields of view using Axiovision Rel. 4 software. Background fluorescence was subtracted from these values, and EtOH was set as 1. Results of 4 independent experiments were pooled and one way Anova with Dunnet's post-test was performed to determine statistical significance relative to EtOH.

While only dex and pred showed significantly more nuclear GR by biochemical fractionation, cortisol was also significantly different from vehicle control (EtOH) in this experiment. However, on the whole, the same pattern is observed as for biochemical fractionation, with full agonists exhibiting the greatest extent of nuclear localisation.

Different ligands cause differential phosphorylation of S211 of hGRα

GR phosphorylation at Serines 211 (S211) and 226 (S226) is a well-known marker of GR activation (Ismaili & Garabedian, 2004). In order to determine whether S211 phosphorylation would correlate with transactivation or nuclear translocation for any of the ligands investigated, S211 phosphorylation was examined by Western blot, using an antibody that specifically recognises GR phosphorylated at S211 (GR-P211). Blots were then stripped and reprobed for total GR levels as a loading control.

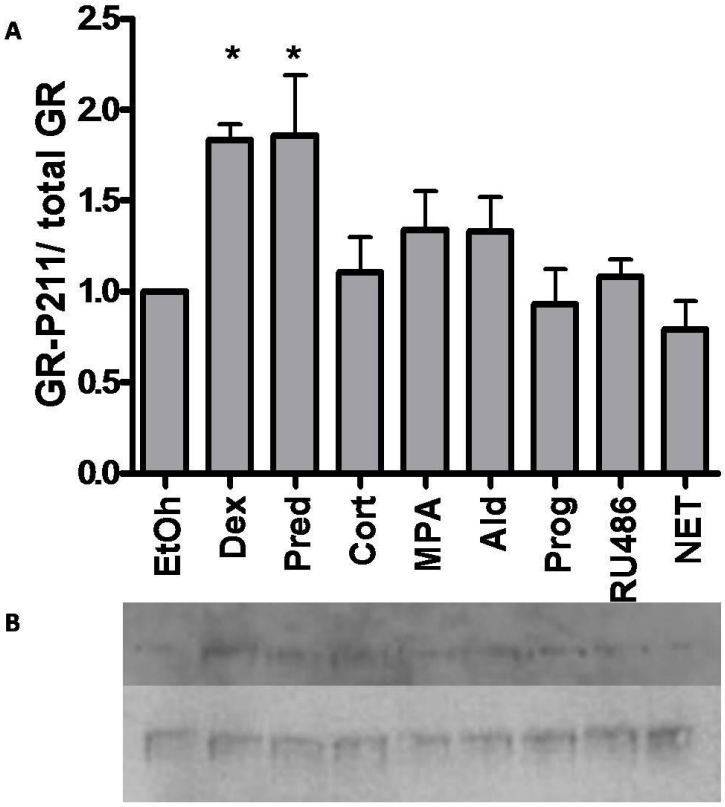


Figure 4.9. Differential phosphorylation of S211 of endogenous GR in U2OS cells. U2OS cells were plated in 12 well plates and treated with vehicle (EtOH) or different ligands at saturating concentrations (100 nM dex, 1 µM pred, 1 µM cort, 100 nM MPA, 10 µM ald, 1 µM prog, 100 nM RU486, and 10 µM NET) for 2 h before being lysed and analysed by Western blot, probing for GR phosphorylated at S211, and total GR. (A) Bands on phospho-Western blots were quantified using Alphaease software, normalised to total GR levels, and plotted relative to EtOH, which was set as 1. Results from four independent experiments were pooled and analysed by one way Anova, with Dunnett's post-test, relative to EtOH. (B) A representative Western blot is shown, with the top panel showing GR phosphorylated at S211and the bottom panel showing total GR levels.

As shown in figure 4.9, different ligands resulted in different extents of phosphorylation of GR at S211, under the same conditions at which nuclear localisation was measured. Wang et al (2002) have shown differential phosphorylation at GR S211 using a small panel of ligands in U2OS overexpressing GR. Similar to the results shown here, agonists dex and pred caused phosphorylation of GR at S211, while the antagonist RU486 did not. Thus there did appear to be a correlation with transactivation, but this was not quantified. Furthermore, the concentration selected for treatment of different ligands were not saturating for all ligands, and the issue of differential GR binding by different ligands was not corrected for. To the best of the present author's knowledge, the current study is the first time differential phosphorylation of endogenous GR with different ligands has been reported, although similar studies have been performed using overexpressed GR (E. Stubsrud, 2005, M.Sc. thesis; Chen *et al.*, 2008).

Correlation between nuclear import, S211 phosphorylation and transcriptional response on a GRE-reporter and the endogenous GILZ gene

Correlation analysis of transactivation, S211 phosphorylation, and nuclear localisation was performed using a Pearson two-tailed correlation analysis on GraphPad Prism software, and the results (showing R^2 and p values) are summarised in Table 4.1. Graphs of these correlations plotted in GraphPad Prism are shown in Appendix D, figure D.3, showing a good distribution of points along the curve.

Table 4.1. Summary of correlation analysis

	Reporter transactivation	Fractionation	Immunofluorescence	GILZ transactivation	GR-P211	
Reporter transactivation		0.8133	0.775	0.7728	0.7024	
Fractionation	0.0009 ***		0.8168	0.8124	0.8124	
Immunofluorescence	0.0017**	0.0008***		0.7122	0.7392	R^2
GILZ transactivation	0.0040**	0.0024**	0.0084**		0.5705	
GR-P211	0.0048**	0.0009***	0.0030**	0.0302*		
	P value					

Values obtained for GR-mediated reporter transactivation (figure 4.2), GR nuclear localisation as measured by biochemical fractionation (figure 4.7A), GR nuclear localisation as measured by immunofluorescent microscopy (figure 4.8B), transactivation of the endogenous GILZ gene by GR (figure 4.3) and GR phosphorylation at S211 (GR-P211) (figure 4.9) for saturating concentrations of different ligands, were analysed by two-tailed Pearson correlation. The R^2 values (coefficient of determination) give an indication of the fraction of the variance shared by the two factors being compared. The p values give an indication of whether the correlation is statistically significant.

Good, and significant correlations were obtained between measurements of transactivation, nuclear localisation and phosphorylation. There was no correlation between transrepression and nuclear import, except for transrepression on the AP-1 reporter construct, which showed a weak (R^2= 0.5415) but significant (p= 0.0238) correlation with the nuclear immunofluorescence measurements. Transrepression on the AP-1 reporter also correlated weakly with phosphorylation at S211 (R^2= 0.5586, p=0.0206). The fact that correlations between transrepression and nuclear import were weaker than those between transactivation and nuclear localisation of the GR, could indicate that some additional factors are involved in transrepression compared to transactivation.

DISCUSSION

This report has shown that different GR ligands elicit differences in maximal transcriptional effect for both transactivation and transrepression on both endogenous and synthetic promoters. To further investigate the mechanism underlying these differences, GR nuclear localisation was examined by biochemical fractionation and immunofluorescence microscopy, and was found to correlate with the transactivation efficacy for a panel of eight different ligands. Furthermore, a correlation was identified between nuclear translocation and GR phosphorylation at Serine-211.

The U2OS osteosarcoma cell line was used in this study, as a model to investigate the effects of GR on transactivation and transrepression. This model system was selected after stably transfected U2OS cells exhibited reduced GR expression over time (Appendix E, figure E.5.1), which highlights the importance of regular monitoring of the expression from exogenous constructs in stably transfected cell lines. It was interesting that WT U2OS, which are widely believed not to express GR (Rogatsky *et al.,* 1997), gave significant transactivation in response to dex on both synthetic and endogenous promoters. Although this finding in this system may cast doubt on the authenticity and purity of the cell line used, it is supported by Rogatsky *et al.,* (1997), who detected a band corresponding to GR when WT U2OS cell lysates were immunoprecipitated with an antibody against GR. GR expression levels have been shown to affect both the potency and efficacy of GR-regulated transactivation (Vanderbilt *et al.,* 1987) and transrepression (Zhao *et al.,* 2003). This implies that results obtained in model cell systems with high GR levels may not represent the effects occurring in cells/tissues with low GR levels. This makes the results reported here more significant since this study examines the effects of different GR ligands in a model cell system expressing relatively low levels of endogenous GR. Since the panel of ligands selected included some compounds with high binding affinity for other steroid receptors, endogenous expression levels of other closely related steroid receptors of the nuclear receptor superfamily (i.e. AR, MR, PR) (Lu *et al.,* 2006) were examined by reporter assay and Western blot. No steroid receptor-

specific agonists besides dex gave a response on a synthetic HRE-containing reporter in U2OS, and it was thus inferred that all transcriptional responses observed were mediated by the GR. Furthermore, involvement of the GR in observed transactivation was confirmed by specific protein knockdown, which validated the selected model system.

In order to assess the validity of using promoter-reporter assays as models of endogenous genes, correlation analyses were performed between the efficacies of different ligands on synthetic and endogenous promoters. There was a significant correlation between transactivation of the synthetic GRE promoter and the endogenous GRE-containing GILZ promoter (R^2= 0.7728, p=0.004). This supports the physiological relevance of the results on synthetic promoters. However, no correlation was observed between activity on synthetic and endogenous promoters containing either AP-1 or NFκB response elements (COL3 and IL8 respectively). This suggests that other factors (besides AP-1 and NFκB) or cis-elements may play a role in transrepression of these endogenous genes, and highlights a fundamental mechanistic difference between transactivation and transrepression downstream of ligand binding and nuclear translocation.

Another surprising difference identified between transactivation and transrepression, was that transrepression was generally much weaker than transactivation measured in WT U2OS. Maximum transrepression observed was about 50% with dex on the AP-1-Luc reporter (figure 4.5) and TNFα and COL3 promoters (figure 4.6), while an approximately 6-fold increase in transactivation was observed on the endogenous GILZ promoter (figure 4.3), and a 50-fold increase in reporter activity was measured (figure 4.2). Reporter assays conducted in the absence and presence of exogenous GR showed that higher levels of GR caused a greater extent of transrepression on AP-1 and NFκB promoters (Appendix D, figure D.2.). This suggests that low levels of GR present in U2OS cells were responsible for the weak transrepression. This finding may thus indicate that relative efficacy for transrepression is dependent on receptor levels, and caution should be taken when comparing results in different cell lines, or in cells expressing different levels of GR. Indeed, the efficacy of GR for

transrepression has previously been reported to increase with increasing receptor levels (Zhao *et al.,* 2003). Furthermore, some ligands (MPA and RU486) have been identified which switch biocharacter from full agonists for transrepression at high receptor concentration to antagonists for transrepression at low receptor concentration (Zhao *et al.*, 2003). Another possibility for the weak transrepression may be the treatment times used in this study. These were kept short in order to avoid indirect effects. Although the treatment conditions used here for IL8 (Rogatsky *et al.,* 2002) and COL3 (Rogatsky *et al.,* 2001) expression had previously been shown to yield close to 100% transrepression in U2OS cells stably transfected with GR in the presence of dex, it is possible that at low concentrations of GR the kinetics of transrepression may be slower, and thus a longer incubation with ligand may be necessary for complete transrepression.

Despite the very weak levels of GR-mediated transrepression observed in this study, interesting ligand- and promoter specific differences in GR mediated transcription were identified. When investigating differential transrepression of the IL8 gene, it was found that Prog caused a significant 2-fold increase in IL8 expression. This was unexpected, but similar upregulation of IL8 by progesterone has been observed in endocervical cells (N. Verhoog, Hapgood laboratory, unpublished observation). The effect of NET on transactivation appears to be promoter-specific, since no transactivation of the GILZ gene was induced by NET in this model cell system, after a 2 h treatment (figure 4.3), although an increase in transcription of a GRE-Luc reporter plasmid was observed upon treatment with NET (figure 4.2). Alternatively, the effect on the reporter construct could be indirect. In the reporter assay cells are incubated for 24 h in the presence of ligand, which could allow time for upregulation of another transcription factor in response to NET, which may subsequently increase transcription from the TAT-GRE promoter. This would not be observed in the real time PCR assay, as the incubation time of 2 h would be too short to allow for translation of this hypothetical intermediary transcription factor. Similarly, the partial agonist MPA elicited a response on the synthetic reporter promoter (figure 4.2), but failed to elicit a significant response on the endogenous GILZ promoter (figure 4.3).

Having identified ligand-specific differences in transactivation and transrepression efficacy, the mechanism by which these arise was investigated, and ligand-specific differences in the extent of GR nuclear localisation were found (figure 4.7 and 4.8). Nuclear localisation of GR measured by both biochemical fractionation and immunofluorescence microscopy was found to correlate with efficacy for transactivation. It was also shown that differential nuclear import of the GR correlates well with its ligand-dependent phosphorylation at S211, which has not previously been shown for endogenous GR. This could be explained by one of three possibilities: either (1) differential phosphorylation caused by binding of different ligands causes differential nuclear import, or (2) differential nuclear import causes differential phosphorylation at S211 of the GR, or (3) a common upstream event determines ligand-selectivity of both nuclear import and phosphorylation at S211.

Wang *et al.* (2002) observed that GR P211 was localised to the nucleus, and speculated that GR is phosphorylated at Serine-211 before entering the nucleus, which is in agreement with possibility 1 above. However, a closer look at their results reveals a subtle difference between the subcellular localisation observed by biochemical fractionation and immunofluorescence microscopy, similar to what was observed in this study when comparing total GR localisation by the two different techniques. Upon dex treatment, it appears that the majority of GR is nuclear by immunofluorescent microscopic analysis, while biochemical fractionation indicates that a large amount of GR remained in the cytoplasm, notwithstanding differences in protein loading. Similarly Wang *et al.* (2002) observed that upon dex treatment, the majority of GR-P211 was nuclear when examined by immunofluorescence microscopy, but biochemical fractionation indicated that the majority of the GR-P211 remained cytoplasmic. One possible explanation for this apparent discrepancy is that the GR becomes phosphorylated at S211 on interaction with the nuclear envelope, and harsh biochemical fractionation conditions result in the nuclear envelope-associated GR remaining in the cytoplasmic fraction, as in hypothetical possibility (2) above, that differential nuclear import leads to differential phosphorylation of GR. Studies with mutant GR constructs in which phosphorylation at S211 is abolished are also consistent with this hypothesis, since such mutations had no effect on GR nuclear localisation (Webster *et al.,* 1997; Avenant *et al.,* 2010). Thus it seems likely

that either GR is phosphorylated on or after nuclear import, or a common upstream event determines ligand-selectivity of both nuclear import and phosphorylation at S211. Differences in the conformation of GR when bound to different ligands could represent a likely possibility for this common upstream event.

This report has investigated the extent of GR nuclear localisation at a fixed time point (2 h), at saturating concentrations of GR ligand. Thus the question of nuclear import rate has not been examined, and it would be interesting to find out whether the observed differences are a result of different kinetics of nuclear import and export, or lower maximal nuclear import. The former seems a more likely explanation in light of published observations that different ligands caused different rates of nuclear import (Vicent *et al.,* 2002). However Robertson *et al.,* (2009) performed a time course analysis of nuclear import of GR when liganded to dex versus the dissociated GR ligand Compound A, and found that dex caused higher maximal GR nuclear localisation than Compound A. Initial investigations into the kinetics of GR nuclear localisation by the present author (data not shown) were confounded by the effect of ligand-dependent GR turnover, which has been shown to be more rapid when GR is liganded to more potent ligands (Dr C. Avenant, PhD thesis). Differential GR turnover caused different amounts of total GR to be present after treatment with different ligands, thus it was not possible to quantify the percentage of total GR in the nucleus after long treatments. Since it takes approximately 8 h for 50% reduction of GR levels in the presence of dex (Dr C. Avenant, PhD thesis), the effect of turnover would be negligible after 2 h treatment, and would only became problematic after longer incubation times.

In conclusion, nuclear import of the GR is not simply a result of GR ligand binding, but the nature of the ligand which binds can determine the extent of nuclear localisation of the GR. Nuclear localisation correlates well with GR transactivation, probably as a result of the nuclear fraction of total GR binding DNA, and recruiting cofactors to increase gene transcription from a GRE. This suggests that nuclear translocation may be a rate-limiting step for transactivation. GR nuclear localisation with different ligands also correlates with GR phosphorylation at S211, which could

suggest GR becomes phosphorylated on interaction with the nuclear envelope or nuclear pore complex. The importance of differential GR nuclear localisation induced by different ligands should not be overlooked when studying steps downstream of nuclear import.

Chapter 5

Future perspectives and conclusions

This research work has further raised many interesting possibilities for future research, which could not be addressed in this project due to time-constraints. These will be discussed below, under the subheadings **Acetylation** and **Nuclear localisation**, as they pertain to Chapter 3 and Chapter 4, respectively.

4.1 Acetylation

The present studies have provided valuable insight into the molecular mechanism of action of the GR. It has been shown that lysines 494 and 495 play a critical role in GR transactivation, probably through acetylation, which modulates ligand binding, and thus downstream steps including nuclear translocation and phosphorylation. ChIP assays have implicated the immunophilin FKBP52 as a GR cofactor on the GILZ gene, and suggest the interaction may be modulated by GR acetylation at K494/495.

The extent of ligand-dependent acetylation of GR in intact cells is an important issue that was not conclusively determined in the present study. Since immunoprecipitation results presented here, showing no increase in overall GR acetylation in response to dex or PMA (figure 3.1), contradict previously published results stating that “GR becomes acetylated after ligand binding” (Ito *et al.,* 2006), this is clearly an area which warrants further research. As mentioned in Chapter 3, this poses some technical difficulties. To the best of the author’s knowledge, all the studies showing ligand-dependent SR acetylation *in vivo* employ a pre-treatment with HDAC inhibitors, before examining the acetylation state of the SR (Ito *et al.,* 2006; Fu *et al.,* 2002; Kim *et al.,* 2006), presumably since acetyl groups are vulnerable to cleavage by HDACs during the immunoprecipitation step, and HDACs must therefore be inhibited prior to cell lysis. This is in contrast to studies in which

HDAC pre-treatment of cells was not performed, which largely show ligand-independent acetylation of SRs as reported here (Wang *et al.,* 2001; Gaughan *et al.,* 2002). In addition, some studies are inconclusive due to the lack of loading controls (Fu *et al.,* 2003; Fu *et al.,* 2006). Furthermore, ligand-dependent acetylation of GR was only shown upon co-incubation with IL1β and dex (Ito *et al.,* 2006), or overexpression of the HAT proteins CLOCK and BMAL1 (Nader *et al.,* 2009) which further complicates interpretation of the results. These discrepancies should be addressed by a comprehensive analysis of GR acetylation in the absence and presence of IL1β and alternative pro-inflammatory stimuli such as PMA, to determine whether the effect of IL1β is specific, or a general feature of the GR response to pro-inflammatory stimuli. Comparison should also be made between GR acetylation in the absence or presence of pre-treatment with HADC inhibitors, and in the absence and presence of CLOCK. Furthermore, WT hGR and K494/495 mutants need to be compared in parallel to conclusively ascertain whether the specific residues K494 and K495 are dex-dependently acetylated in addition to changes in overall GR acetylation status. These studies could be done by an immunoprecipitation approach, or by incubation with tritiated acetyl-CoA, to examine incorporation of the radioactive acetyl group.

Another approach to answer the question of *in vivo* GR acetylation involves development of an antibody specific towards acetyl-GR K494/495. This has already been developed by the Ito laboratory (Prof K. Ito, personal communication), but unfortunately could not be supplied on request. This will be useful in determining the acetylation status of the GR in liganded and unliganded states, and should ideally be used in experiments with minimal post-treatment processing steps, in order to minimise the chance of HDAC removal of acetyl groups, for example immunofluorescence microscopy and ChIP assays.

It has been assumed, that since the K494/495A hGR mutant construct used in this study, and a similar K494/495Q hGR mutant construct to the one used in this study, have previously been shown to have lower levels of acetylation than WT hGR (Ito *et al.,* 2006), that the same would apply to the model system used here. Ideally, this

should have been tested by the same technique used by Ito and co-workers. This approach involved immunoprecipitation of WT and mutant GR after dex treatment and analysis of total acetylation levels by Western blotting. This would lend strong support to the argument that changes in acetylation status under the conditions used in the current study are responsible for the functional consequences observed with the mutant GR constructs, rather than some other non-specific effect of the mutations. To further strengthen the argument that acetylation at K494/495 influences the ligand binding capacity of the GR, competitive binding curves could be performed, to establish the K_d of WT versus mutant GR.

Although the acetylation deficient mutant caused significantly decreased transactivation of a synthetic promoter-reporter construct, and decreased nuclear translocation of GR, it may be argued that this should be confirmed on an endogenous gene, such as GILZ, by quantitative real-time PCR. Similarly, performing a ChIP assay to show a lack of recruitment of K494/495A hGR to the GILZ promoter would strongly support other findings presented here, such as a lack of nuclear localisation and ligand-binding displayed by this mutant. As mentioned in Chapter 3, the lack of *in vitro* DNA binding by the K494/495A hGR mutant may reflect a resistance to heat activation of the mutant rather than a decrease in DNA binding activity *per se*. To clarify this issue, WT hGR and K494/495A hGR could be separated by centrifugation through a sucrose gradient after a heat transformation treatment, or corresponding control treatment. Untransformed GR would be expected to be found in a 9S complex, while after heat transformation, a 4S complex would be expected (reviewed in Pratt, 1987). If K494/495A hGR did not form a 4S complex after heat transformation, this would indicate that the difference in *in vitro* DNA binding capacity between WT and mutant is most probably due to difference in response to heat activation.

In this study, mutation of K494/495 of GR was shown to affect GR transactivational ability. As mentioned in chapter 3, it would be very interesting to investigate the role of these residues in GR-mediated transrepression, especially since acetylation of these residues has been implicated in modulating the interaction between GR and

the NFκB subunit p65 (Ito *et al.,* 2006). Ito and co-workers (2006) used co-immunoprecipitation assays to show an interaction between p65 and K494/495A hGR, but did not include the K494/495Q mutant as a control, which would be useful. Further investigation of the functional effect of K494/495 acetylation on transrepression could be done quite simply by promoter-reporter assays with WT and mutant GR constructs, using reporter constructs containing NFκB-sites. Results obtained in promoter-reporter assays could further be confirmed on endogenous genes using quantitative real-time PCR, and compared to transrepression via AP-1 sites, to investigate whether the effect is specific to NFκB, or a general feature of GR transrepression. If the effects of acetylation on transactivation and transrepression are found to be different, then modulation of GR acetylation status could play an important role in the development of selective GR agonists with greater potency for transrepression than transactivation.

The present study showed, for the first time, that mutation of lysine residues 494/495 of human GR to abrogate or mimic acetylation at these sites modulated the phosphorylation status of the GR at S211 and S226. It would be very interesting to examine the effect of these mutations on other known phosphorylation sites such as S203 and S404. GR phosphorylated at S203 has been found to localise mainly to the cytoplasm, to be relatively high in the absence of ligand (Wang *et al.,* 2002), and to be greater when S226 is not phosphorylated (Wang *et al.,* 2007a). These factors indicate this is a potential phosphorylation site in K494/495A hGR, which does not bind ligand, translocate to the nucleus, or display phosphorylation at S226. If the K494/495A mutant were found to be a target of phosphorylation at S203, it would strengthen the argument that this mutant is correctly folded and active in some respect, rather than simply being a null-mutant. Furthermore, GR phosphorylation at S404 has been implicated in the interaction between GR and p65 (Galliher-Beckley, Williams, Collins, & Cidlowski, 2008). Since acetylation of GR at K494/495 has also been implicated in this interaction (Ito *et al.,* 2006), it is probable that there may be an interplay between these two post-translational modifications, which would be interesting to investigate.

In addition to examining the role of K494/495 in GR transactivation, this study also implicated FKBP52 as a GR interacting partner via acetylated K494/495 residues, and as a GR cofactor on the GILZ promoter. However, many elements of this work are speculative, and further experimentation is needed to make the speculations presented more conclusive. For instance, ChIP analysis of GR occupancy at the GILZ promoter using an anti-HA antibody did not show increased levels of K494/495Q HAhGR on this promoter in response to dex, although quantitative real-time PCR indicated that the mutant GR must have been recruited in order to cause increased GILZ transcription in response to dex. This argument would have been strengthened by immunoprecipitation with antibodies recognising other epitopes of the GR, which may have shown an increase in total mutant GR recruitment to the promoter. Furthermore, ChIP assays with an FKBP52 antibody failed to show significant recruitment of FKBP52 to the GILZ promoter by WT HAhGR. This was perplexing given that FKBP52 has been shown to augment the GR-mediated transcriptional response at the GILZ gene in the presence of WT GR (Wolf *et al.,* 2009). As speculated in chapter 3, this could be due to formation of a multi-protein complex at the GILZ promoter, which prevented access of the polyclonal anti-FKBP52 antibody to the FKBP52 present on the promoter. Low levels of endogenous FKBP52 may also contribute to this problem, therefore a possible solution may include stably transfecting a tagged FKBP52 which could be immunoprecipitated by its tag. As mentioned in chapter 4, transient transfection is unlikely to be useful for ChIP analysis because only a small percentage of cells express the exogenous protein, and the promoter of interest becomes a limiting factor. It would also be interesting to examine the differences in recruitment of other cofactors between WT HAhGR and K494/495Q HAhGR, for instance members of the p160 family such as GRIP1 and SRC1. This may give a better picture of the members of the complex recruited to the GILZ promoter by each GR construct. Furthermore it would be intriguing to investigate whether FKBP52 is accompanied by Hsp90 to the GILZ promoter by performing a ChIP assay using an anti-Hsp90 antibody. Hsp90 has been shown to be recruited to other endogenous promoters (Freeman & Yamamoto, 2002), where it is implicated in rapid cycling on the chromatin. The presence of Hsp90 on the GILZ promoter in the presence of WT HAhGR would support the notion that FKBP52 may also be recruited but its presence is masked by the multitude of other cofactors recruited. The line of

investigation could also be pursued in a cell line with high levels of endogenous GR, such as A549.

The results presented in chapter 3 support a model in which GR interaction with FKBP52, via acetylated lysine residues in its DBD/hinge region, modulates its ligand-binding ability, and hence its nuclear translocation, phosphorylation and transactivation. Furthermore, FKBP52 is implicated as a gene-specific coactivator of the GILZ promoter. This last supposition would be strengthened by analysis of FKBP52 recruitment to another gene that has previously been shown to be unaffected by FKBP52 knockdown, such as SGK or p21 genes (Wolf *et al.,* 2009). FKBP52 levels would be expected to show no increase on this promoter, although WT HAhGR recruitment would be expected to increase in a dex-dependent manner. Quantitative real-time PCR should first be used to compare the transactivational ability of WT HAhGR and K494/494Q HAhGR on the SGK promoter, as this may differ significantly from the GILZ promoter. It would then be interesting to examine recruitment of K494/495Q HAhGR to the SGK promoter, as well as other cofactors such as GRIP-1 and SRC1.

The argument that GR acetylation at K494/495 modulates its interaction with FKBP52, as speculated here, could certainly be strengthened by further experimentation. Although this hypothesis is well-supported by the data presented in chapter 3, the precise role of acetylation at these sites has not been conclusively shown. This could be done by immunoprecipitation of WT and mutant GR protein, followed by analysis of co-immunoprecipitated immunophilins by Western blotting. Since FKBP51 has been shown to have an opposite effect to that of FKBP52 on GR ligand binding and transactivation (Denny *et al.,* 2000), it would be interesting to use this technique to examine the hypothesis that FKBP51 is the predominant immunophilin present in the Hsp90 heterocomplex in the case of K494/495A hGR, in contrast to K494/495Q HAhGR which would be expected to be found in a complex comprising Hsp90, and FKBP52 as the predominant immunophilin. Another TPR protein found in Hsp90 heterocomplexes with GR is PP5. This would be an interesting candidate to investigate, since it has been shown to modulate the

phosphorylation status of the GR (Wang *et al.,* 2007a; Zhang *et al.,* 2009), and the present study has shown that alteration of GR acetylation status differentially affects phosphorylation at S211 and S226. It is possible that this could also be a result of differences within the Hsp90 heterocomplex brought about by GR acetylation status. Co-immunoprecipitation experiments conducted by the present author and other members of the Hapgood laboratory proved inconclusive due to technical difficulties. The conditions required to maintain the interaction between GR and immunophilin were insufficiently stringent to allow separation of exogenous GR from background levels of GR expressed in COS-7 cells. However, optimisation of this immunoprecipitation technique is a subject of ongoing research in the Hapgood laboratory. The temperature of incubation with ligand is a factor which must be optimised, as GR-immunophilin interaction in the cytoplasm is observed on incubation at 4˚C (Davies *et al.,* 2002), while ChIP assays suggest an interaction at 37˚C after nuclear localisation of the GR. It may be necessary to perform the treatments at both 4˚C and 37˚C to fully elucidate the role of GR-immunophilin interactions.

If an immunoprecipitation approach should prove inconclusive, an alternative approach would be the use of fluorescence resonance energy transfer (FRET). In this technique labelled fluorescent proteins are expressed in combination so that a close association between proteins is indicated by excitement of one protein being induced by the fluorescence emission of the other. FRET may thus allow detection of differences in GR- immunophilin interactions between WT and mutant GR in COS-7 cells.

4.2 Nuclear localisation

The present study show for the first time, using a large panel of GR ligands, and an endogenous GR model system, that the extent of nuclear localisation of the GR is selectively affected by the nature of the activating ligand, which correlates with the ligand-selective transactivation efficacy of the GR on the GILZ gene, and

phosphorylation at S211, but not transrepression on a number of endogenous genes.

As highlighted in Chapter 1, the question of ligand-selective differences in nuclear translocation is often overlooked when investigating ligand-selective SR transcriptional responses (Wang *et al.,* 2006; Garside *et al.,* 2004; Kang *et al.,* 2004). The results shown in Chapter 4 show that different ligands at saturating concentrations cause different extents of GR localisation, and this correlates well with ligand efficacy for transactivation of a synthetic reporter construct and an endogenous gene. Interestingly, no correlation was observed between nuclear localisation of the GR and efficacy for transrepression on promoter-reporter constructs or several endogenous genes. It is plausible that the use of PMA in transrepression assays may modulate nuclear translocation of the GR, and this could be investigated by repeating fractionation and immunofluorescence microscopy experiments in the absence and presence of PMA. However the weak transrepression observed in U2OS, as a result of the low endogenous GR levels (Appendix D figure D.2) makes it difficult to draw a robust conclusion. It would be interesting to repeat the transrepression experiments in a cell line with higher levels of endogenous GR, such as A549, which would be likely to yield higher levels of GR-mediated transrepression. Use of a cell line with endogenous GR would be preferable to transfected GR, as transiently transfected constructs do no distribute evenly between the entire cell population, while stably transfected constructs have to be carefully monitored to ensure their expression levels do not decrease over time as shown in Appendix E, figure E.5.1.

Another disappointing finding probably attributable to the low levels of GR in the selected U2OS model system was that the ChIP assay on the GILZ promoter did not yield reproducible results in the U2OS cell line, as this would have allowed further dissection of the relative roles of ligand-selective nuclear translocation and ligand-selective promoter occupancy of GR in the transcriptional response. Examination of ligand-selective promoter occupancy by GR would very likely be possible using the A549 cell line, which has higher GR levels and gave a higher fold recruitment of GR

to the GILZ promoter in the ChIP assay. However, since it is established that varying GR levels may impact the transcriptional response (Vanderbilt *et al.,* 1987; Zhao *et al.,* 2003), it would be necessary to repeat experiments to investigate differential nuclear translocation and biological activity in the new model cell line. In itself this would be interesting, as the effect of GR levels on ligand-selective nuclear localisation is not known, however this could not be further investigated in this project due to constraints on time and budget. Furthermore, differential cofactor recruitment has been demonstrated for a wide panel of ligands *in vitro* and in mammalian two-hybrid assays (Ronacher *et al.,* 2009), and it would be very interesting to examine recruitment of cofactors to endogenous promoters using the ChIP assay, to see whether observations from *in vitro* assays are reflected *in vivo*.

One of the most interesting directions for follow-up from this research would be to uncover the mechanism of differential nuclear localisation of GR when bound to different ligands. The basis for these differences is likely to be different conformations of the GR when bound to structurally different ligands. As it has been shown that GR ligand-binding elicits a swop of FKBP51 for FKBP52 on the Hsp90 heterocomplex (Davies *et al.,* 2002), this was initially suspected to play a role. However, despite following two different published protocols, the present author was unable to detect a dex-mediated immunophilin swop on endogenous GR in U2OS (data not shown) or A549 cells (Appendix E., figure E.3.2). Thus, the possibility of differential immunophilin swop as a mechanism for differential GR nuclear translocation has not been excluded, but requires further optimisation. Alternative explanations which could also be explored include differential interaction of liganded GR with proteins of the nuclear pore complex, or importins.

This report has investigated the extent of GR nuclear localisation at a fixed time point (2 h), at saturating concentration of GR ligand. Thus the question of nuclear import rate has not been examined, and it would be interesting to find out whether the observed differences are a result of different kinetics of nuclear import and export, or lower maximal nuclear import. Initial investigations into the kinetics of GR nuclear localisation by the present author (data not shown) were confounded by the effect of

ligand-dependent GR turnover, which has been shown to be more rapid when GR is liganded to more potent ligands (Dr C. Avenant, PhD thesis), thus it was not possible to quantify the percentage of total GR in the nucleus after long treatments. A possible strategy to avoid this complication, and investigate the kinetics of GR nuclear localisation with different ligands, would be to use live cell imaging of fluorescently labelled GR, which would allow constant monitoring of nuclear GR levels, by measuring nuclear fluorescence intensity from the moment of treatment to the maximal accumulation within the nucleus, and subsequent decline in GR levels due to turnover. This could give some basis for comparison of the rate of GR nuclear localisation elicited by different ligands, while taking into account the different rates of ligand-mediated GR turnover.

In chapter 4 it was proposed that GR phosphorylation at S211 may occur in the nucleus or on interaction with the nuclear membrane, due to differences in subcellular localisation of GR-phospho-S211 observed with biochemical and microscopic techniques (Wang *et al.,* 2002). This is an interesting hypothesis which warrants further investigation. Results from the Hapgood laboratory indicate that phosphorylation at S211 is not required for nuclear import (Avenant *et al.,* 2010), which may indicate that the strong correlation observed between nuclear localisation and S211 phosphorylation reported in chapter 4 is a result of a causative effect of nuclear translocation on S211 phosphorylation. For further study in this area on endogenously expressed GR, it would be advantageous to use a cell line expressing higher levels of endogenous GR than U2OS (e.g. A549), since detection of phosphorylated GR was very difficult in this cell line. Detection of the S211 phosphoisoform of GR in biochemically fractionated nuclear and cytoplasmic fractions of A549 cells, would allow confirmation of the results observed for overexpressed GR by Wang and co-workers (2002), with endogenous GR. Furthermore, use of a nuclear-translocation deficient mutant may yield interesting results, as phosphorylation of all known phosphorylation sites could be examined in the absence and presence of ligand, using Western blotting and phospho-specific antibodies. Lack of phosphorylation in the nuclear translocation deficient mutant GR would confirm that hypothesis that nuclear import is required for phosphorylation. However, if this mutant were phosphorylated, this could indicate that either GR

phosphorylation occurs in the cytoplasm, or on interaction with the nuclear envelope. One could further speculate that the subcellular location at which GR phosphorylation takes place may be ligand-selective, as comparison of the compartmentalisation of GR phospho-isoforms has only been performed in the presence of dex (Wang *et al.,* 2002). This could be investigated by comparing the extent of phosphorylation of a nuclear translocation deficient GR mutant in the presence of different ligands.

The finding that GR-mediated transactivation efficacy by a panel of ligands correlates with nuclear localisation, and phosphorylation at S211, as well as recruitment of the coregulators GRIP-1, SRC1, SMRT (Ronacher *et al.,* 2009) is interesting. One might be tempted to speculate that ligand-selective differences based on differences in GR conformation at an upstream step cause the difference in downstream steps. If this is the case, nuclear import is unlikely to represent the key upstream step, since ligand-selective differences in cofactor recruitment (correlating with transactivation in COS-1 cells) are observed *in vitro* in whole cell lysates (Ronacher *et al.,* 2009). GR phosphorylation is another unlikely possibility, since phosphorylation deficient mutants have been shown to exhibit unaltered nuclear localisation (Avenant *et al.,* 2010). The key "first step" which defines these correlations thus remains to be established, but it seems unlikely that the GR conformation *per se* could affect interactions of the GR-ligand complex with several different interacting proteins to the same relative extent. It may be possible that an as-yet unidentified protein interacts with GR to variable degrees depending on the conformation induced by the particular ligand. As discussed in Chapter 3, a multifunctional protein like FKBP52 can modulate GR function at a number of steps, for example ligand binding, nuclear translocation, and by acting as a coregulator, making it a possible candidate. Thus the notion of an interacting partner as the reason for the strong correlation between several different steps of the GR transcriptional response is plausible. However work needs to be done to establish whether this is the case.

4.3 Conclusions

Transcriptional regulation by the GR is a complex, multistep process, which is not fully understood. The present research has made an important contribution to our understanding of two aspects of ligand-selective GR activity. Firstly, it has investigated the functional effects of GR acetylation at lysines 494 and 495. Results strongly suggest that acetylation at K494/495 is essential for ligand binding and transactivation by the GR, possibly through a mechanism involving FKBP52 interaction. Secondly, it has highlighted the importance of differential nuclear import as a key step in regulating ligand-specific transcriptional efficacy of the GR. This should not be overlooked when investigating the effects of different GR ligands at steps downstream of GR nuclear import, such as DNA binding, protein tethering or cofactor recruitment. Although many unanswered questions remain regarding the molecular mechanism of action of the GR, any advances in the field of GR function could one day have important implications for treatment of a multitude of diseases for which the GR is a drug target.

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Appendix A : GR ligands

Chemical structures of the GR ligands used in this study are shown in figure A.1.

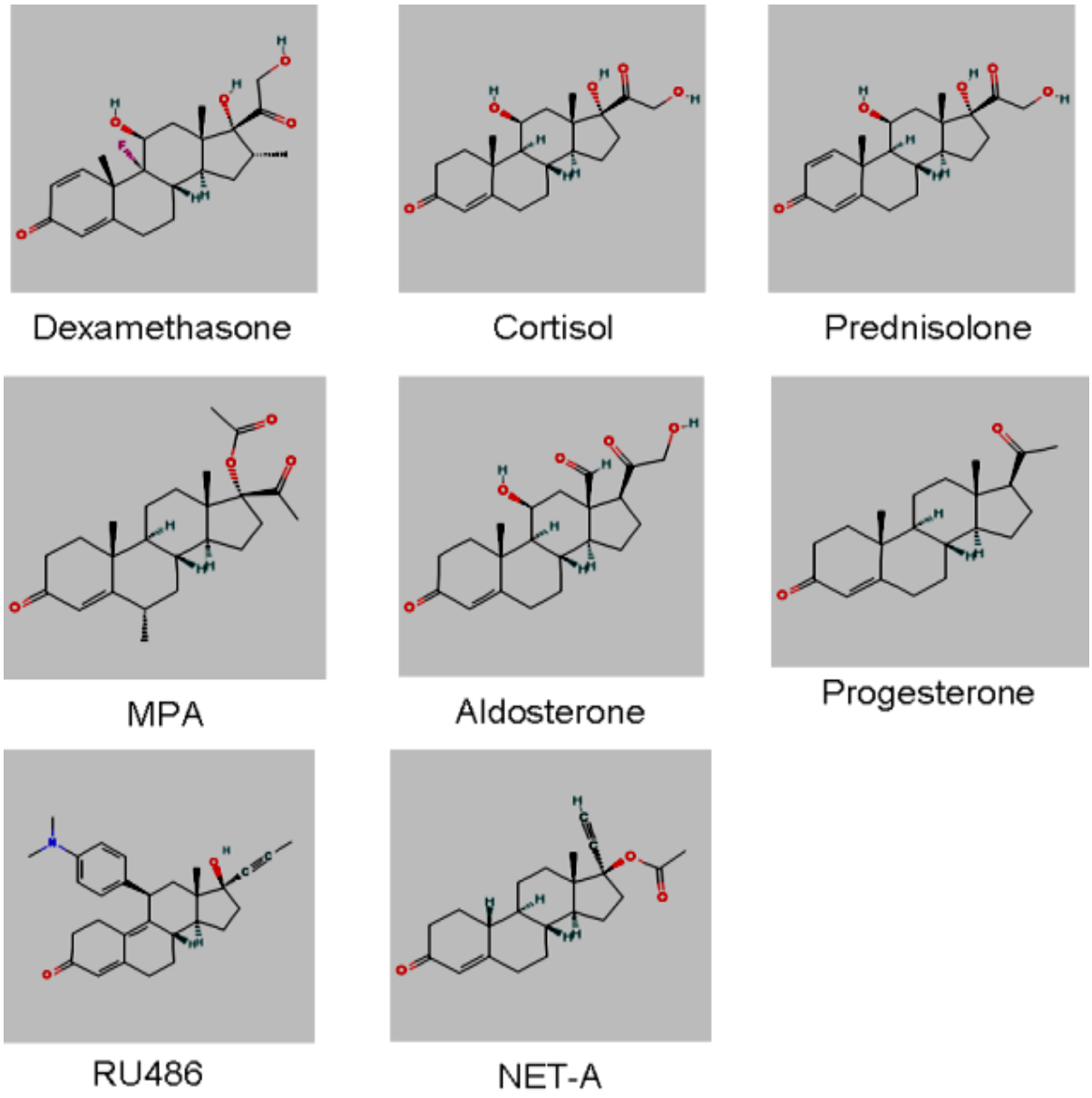


Fig. A1. Chemical structures of GR ligands used in this thesis

Receptor theory dictates that ligand binding to GR must obey the basic laws of thermodynamics. In a given pool of receptor and ligand, a certain proportion of receptor will be occupied by ligand. This proportion depends on the rate of association (K_{on}) and dissociation (K_{off}) of the ligand-receptor complex. At

equilibrium, the rate of complex formation and dissociation are equal, and the ratio of K_{off}/K_{on} is the equilibrium dissociation constant (K_D) of a given ligand, which gives an indication of the concentration of ligand which will saturate 50% of the available receptors.

The fractional occupancy of receptors refers to the proportion of total receptors which are occupied at a given concentration of ligand.

$$F.O. = \frac{(liganded\ receptor)}{(liganded\ receptor\ +\ free\ receptor)}$$

By substituting in the equation for K_D,

$$\mathbf{Kd} = \frac{\mathbf{Koff}}{\mathbf{Kon}} = \frac{[\mathbf{ligand}].[\mathbf{free\ receptor}]}{[\mathbf{ligand\ receptor\ complex}]}$$

We find that

$$\mathbf{F.O.} = \frac{[\mathbf{ligand}]}{[\mathbf{ligand}] + \mathbf{Ki}}$$

Thus, the fractional occupancy of the GR by a given ligand can be calculated if the K_D is known. K_D values may be found in the literature, or determined experimentally using competitive binding assays, in which cells are incubated with a single concentration of a radiolabelled ligand, and increasing concentrations of the unlabelled ligand of interest. The concentration of ligand which displaces 50% of the radiolabelled ligand is known as the IC_{50} for that ligand. At equilibrium, the rate of and the ratio of K_{off}/K_{on} for the unlabelled competing ligand is the equilibrium inhibitor dissociation constant (K_i) of that ligand, which gives an indication of the concentration of ligand which would saturate 50% of the available receptors in the absence of radioligand (Cheng & Prusoff, 1973).

$$Ki = \frac{IC50}{1 + \frac{[radioligand]}{Kd(radioligand)}}$$

The relative fractional occupancy of different ligands can then be determined using

$$F.O. = \frac{[ligand]}{[ligand] + Ki} \times 100\%$$

Table A1. IC_{50} concentrations, Ki values and relative fractional occupancies of GR ligands resulting in GR occupancy of at least 96.5% (Ronacher *et al.*, 2009)

Ligand	IC_{50} (nm) as determined by competitive binding curves	K_d or K_i (nM) $\frac{IC50}{1+\frac{[radioligand]}{Kd(radioligand)}}$	relative fractional occupancy at 100nM ligand	relative fractional occupancy at 1µM ligand	relative fractional occupancy at 10µM ligand
Dex	3	0.39	99.610		
Pred	68.3	8.91	91.820	99.117	
Cort	152.75	19.92	83.386	98.047	
MPA	18.8	2.45	97.607		
Ald	1130	147.39	40.422	87.154	98.547
Prog	274	35.74	73.671	96.549	
RU486	1.2	0.16	99.844		
NET	1688	220.17	31.233	81.956	97.846

Cells with grey fill indicate the fractional occupancy of GR at the final concentration used in experiments.

Calculation of the relative fractional occupancies of the different ligands as shown in table A1, is based on binding and dose response analysis performed by previous members of the Hapgood lab. It was important to control for the different GR binding affinities of the ligands in the panel, rather than using a single concentration for all ligands. This is because, if the concentration selected was too low, differential saturation of GR would occur, and prevent analysis of ligand-specific effects. Similarly, if the selected concentration were too high, spurious non-specific effects could be elicited, as have been observed in the Hapgood lab upon treatment of COS-1 cells with 10 µM RU486 (data not shown).

For use as a MR-specific agonist (figure 4.1A) ald was used at 10nM, at which concentration, it would saturate 100% of MR present, but only about 6% of GR present, since the K_d of Ald for the MR is 0.15 nM (Dr D. Africander, PhD thesis), compared to the K_i of Ald for the GR, which is 147.39 nM.

Appendix B: Dilutions and diluents of primary and secondary antibodies used in Western blot

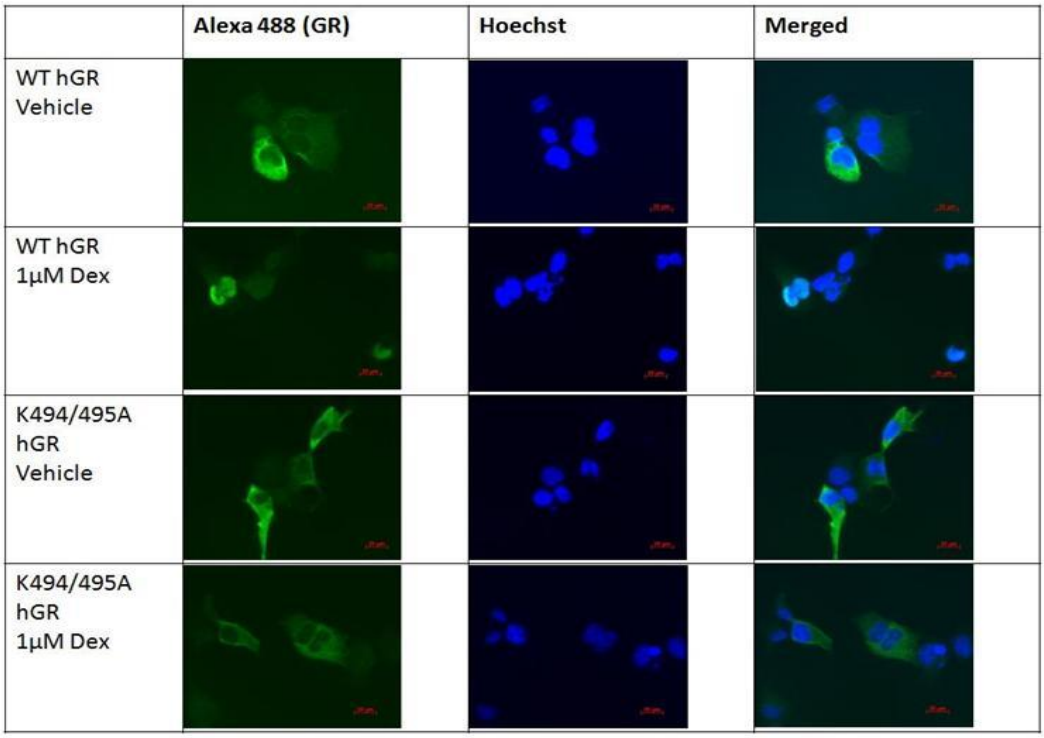
Table B1. Conditions of Western blot incubations with primary and secondary antibody

Protein of interest	Primary antibody dilution	Primary antibody diluent	Secondary Antibody	Secondary antibody dilution	Secondary antibody diluent
Acetyl lysine	1:1000	4% ECL-TBST	Anti-rabbit HRP	1:5000	5% non-fat milk powder in TBST
AR	1:10000	TBST	Anti-mouse HRP	1:10000	10% non-fat milk powder in TBST
FKBP52	1:1000	TBST	Anti-goat HRP	1:5000	5% non-fat milk powder in TBST
GAPDH	1:1000	4% ECL-TBST	Anti-rabbit HRP	1:5000	5% non-fat milk powder in TBST
GR (endogenous GR in U2OS)	1:1000	TBST	Anti-rabbit HRP	1:5000	5% non-fat milk powder in TBST
GR (overexpressed in COS-7 cells)	1:5000	TBST	Anti-rabbit HRP	1:5000	5% non-fat milk powder in TBST
GR-Phospho S211	1:10000	4% ECL-TBST	Anti-rabbit HRP	1:5000	5% non-fat milk powder in TBST
GR-Phospho S226	1:10000	4% ECL-TBST	Anti-rabbit HRP	1:5000	5% non-fat milk powder in TBST
GR-β	1:5000	4% ECL-TBST	Anti-rabbit HRP	1:10000	5% non-fat milk powder in TBST
Histone H3	1:4000	4% ECL-TBST	Anti-rabbit HRP	1:5000	5% non-fat milk powder in TBST
MR	1:10000	4% ECL-TBST	Anti-goat HRP	1:5000	5% non-fat milk powder in TBST
PR A/B	1:10000	4% ECL-TBST	Anti-rabbit HRP	1:5000	5% non-fat milk powder in TBST
β-actin	1:2000	TBST	Anti-rabbit HRP	1:10000	5% non-fat milk powder in TBST
β-tubulin	1:5000	TBST	Anti-mouse HRP	1:20000	10% non-fat milk powder in TBST

TBST: 50 mM Tris, 150 mM NaCl, 0.1% (v/v) Tween.

Appendix C: Supplementary results pertaining to Chapter 3

A



B

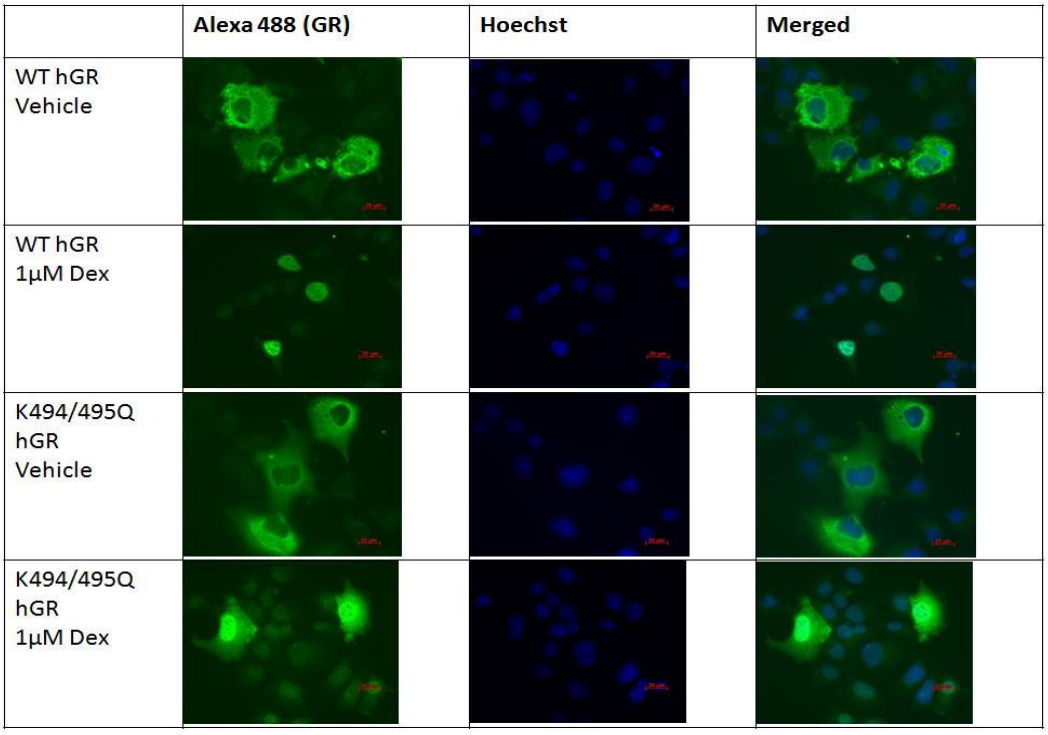


Figure C.1. K494/495A hGR and K494/495Q HAhGR exhibit less nuclear translocation that WT GR after 16 h of dex treatment. COS-7 cells were plated and transfected with WT hGR and K494/495A hGR (A) or WT HAhGR and K494/495Q HAhGR (B) as described for figure 3.3, but

treated with vehicle (EtOH) or 1 µM dex for 16 h before being fixed and immunostained as described in materials and methods, chapter 3. Results are representative of at least 100 cells from a single experiment.

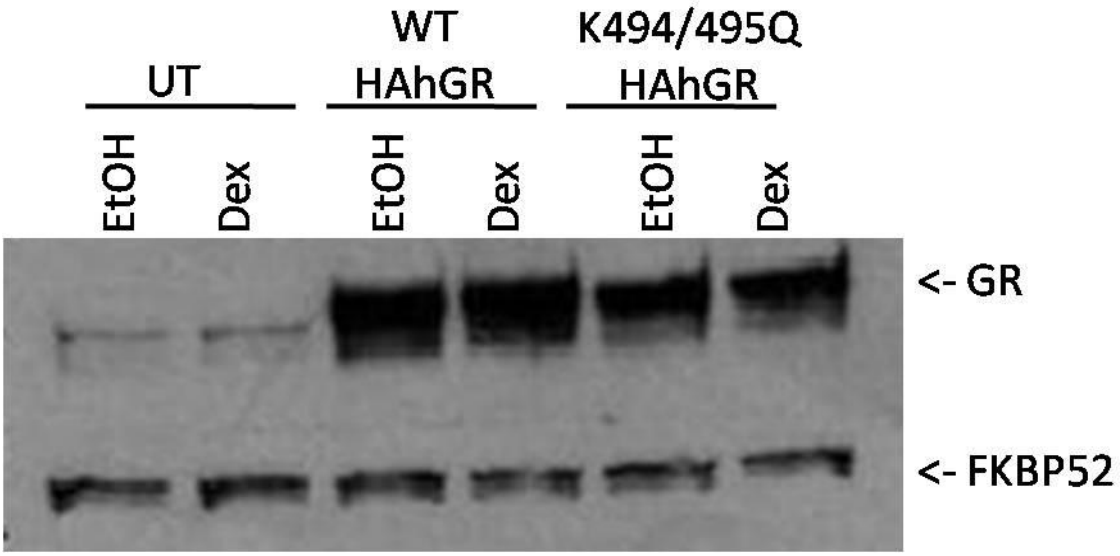


Figure C.3. FKBP52 levels do not change following 1 h treatment with 1 μM dex. COS-7 cells were plated and transfected with WT HAhGR or K494/495Q HAhGR as described in materials and methods for ChIP. Cells were treated for 1 h with 1 μM dex before whole cell lysates were harvested and analysed for GR and FKBP52 levels by Western blotting.

Appendix D: Supplementary results pertaining to Chapter 4

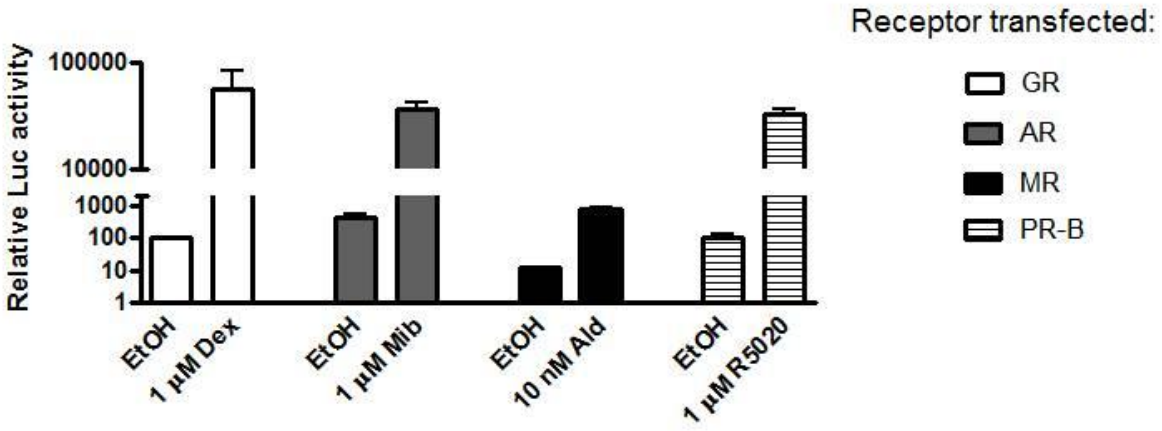


Figure D.1. Steroid receptor-specific agonists give a transcriptional response on pTAT-GRE-E1b-luc in the presence of the receptor of interest. U2OS cells were transfected with pCMV β-galactosidase reporter and the GR responsive luciferase reporter construct pTAT-GRE-E1b-luc as well as plasmids encoding either the GR, AR, MR or PR-B. Cells were treated with vehicle (EtOH) or 1 µM dex, 1 µM mib, 10 nM Ald or 1 µM R5020, respectively for 24 h. Luciferase activity of cell lysates was monitored and normalised to β-galactosidase activity for each well to control for transfection efficiency. Histogram shows results of a single experiment performed in triplicate.

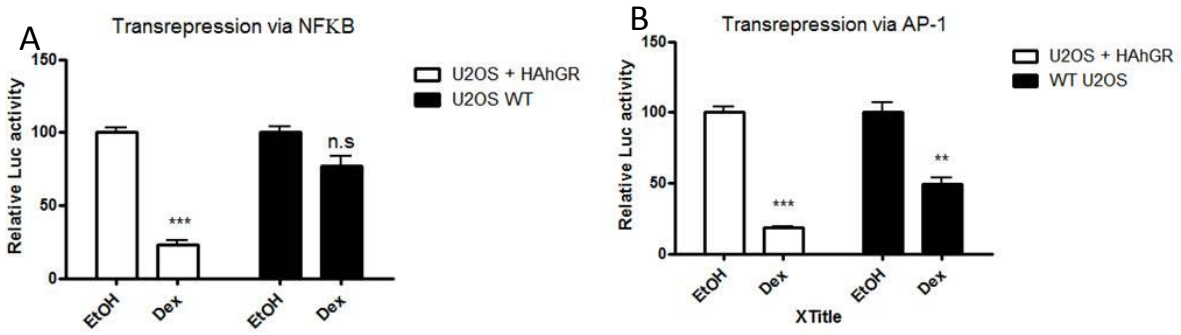


Figure D.2. Transrepression of NFκB and AP-1 promoters in U2OS cells is greater in the presence of exogenous HAhGR. U2OS cells were transfected with pCMV β-galactosidase reporter and (A) NFκB-Luc or (B) AP-1-Luc reporter constructs. Cells were treated with vehicle (EtOH) or 1 μM dex in the presence of 20 ng/ml PMA for 24 h. Luciferase activity of cell lysates was monitored and normalised to β-galactosidase activity for each well to control for transfection efficiency. Histogram shows pooled results of two independent experiments each performed in triplicate. Statistical significance was determined by a paired t-test.

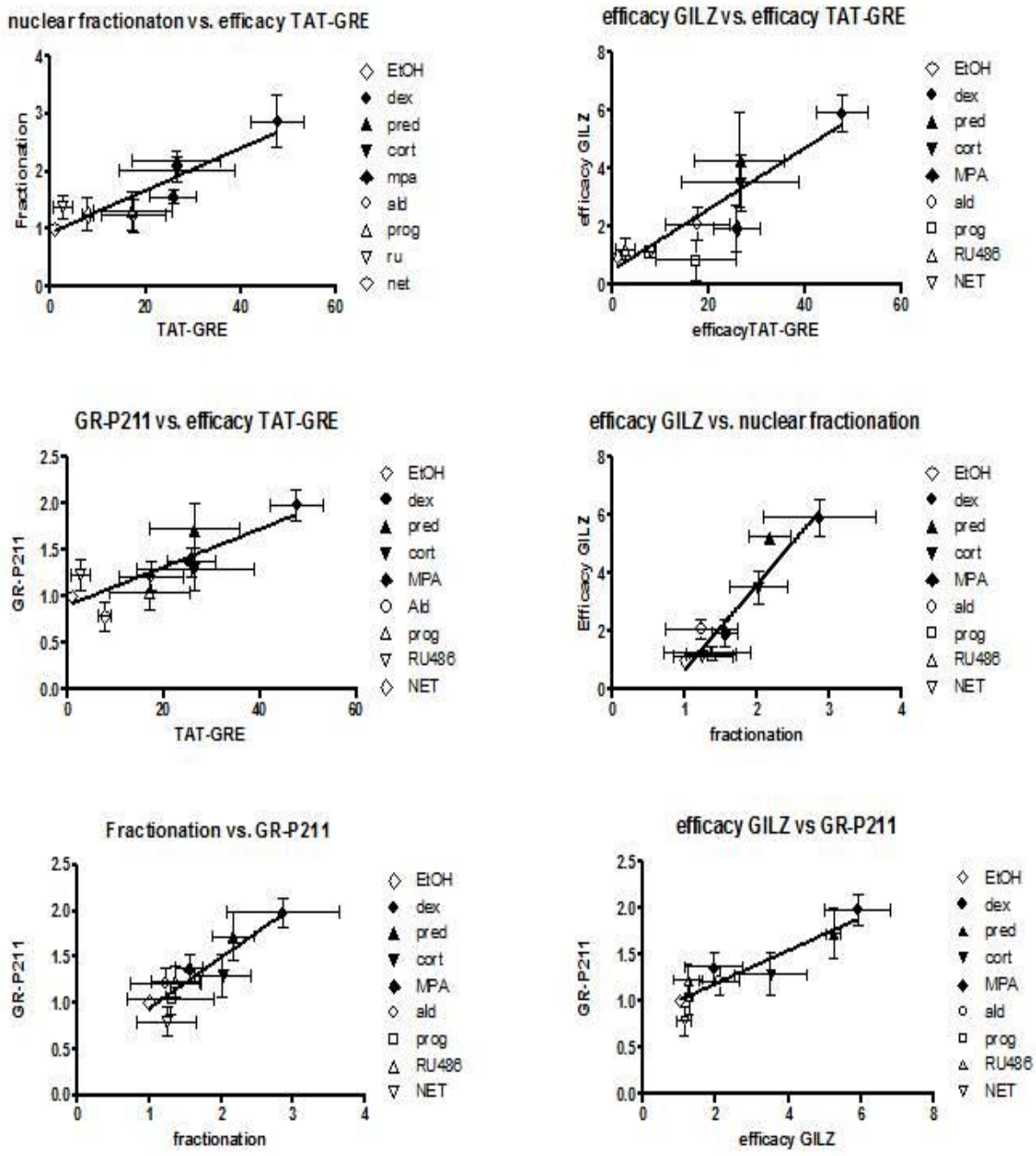


Figure D.3 (continued on next page)

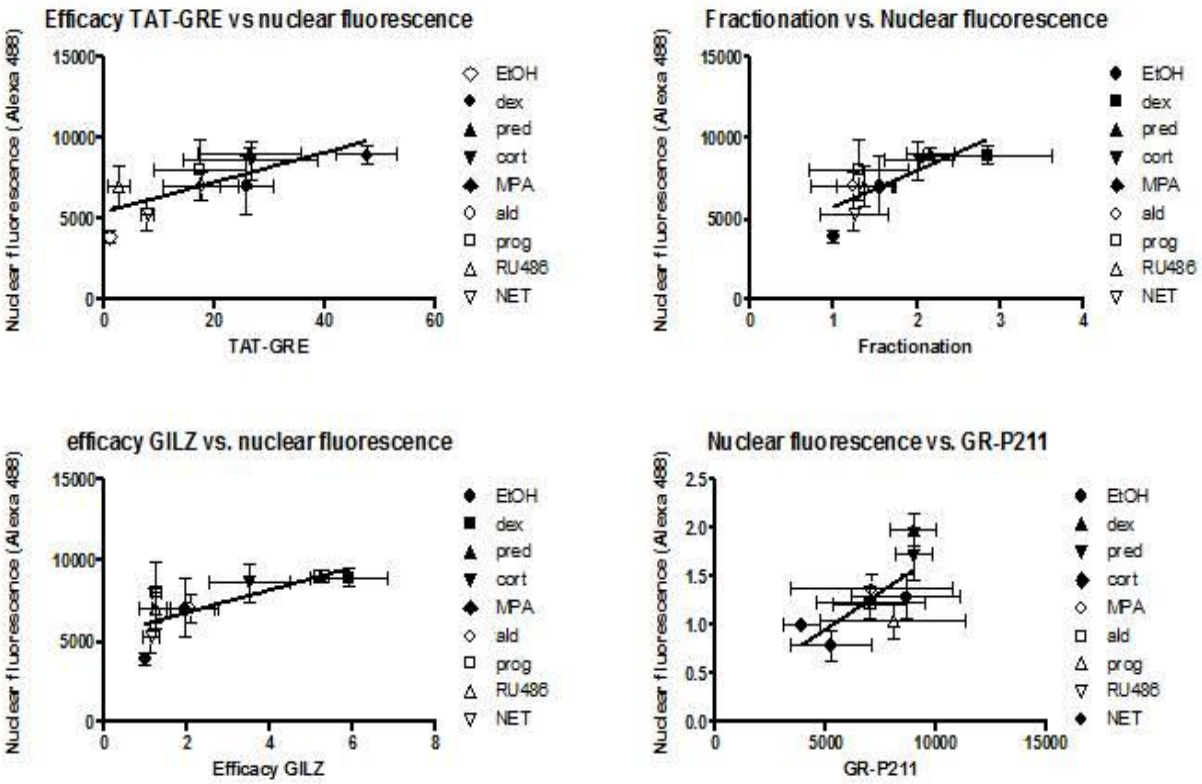


Figure D.3. Graphs of correlation analyses. Correlation analyses of transactivation, S211 phosphorylation, and nuclear localisation were performed in GraphPad Prism using two-tailed Pearson correlations, as described in materials and methods and shown in table 4.1.

Appendix E: Optimisation of experimental techniques

E.1. Quantitative Real Time PCR

Analysis of the relative expression level of a gene was performed using two-step quantitative real-time PCR in a Corbett Rotorgene thermal cycler. Following isolation of RNA, the two-step protocol entails reverse transcription of the RNA to cDNA, and subsequent analysis by PCR. Performing reverse transcription and PCR in separate reactions (rather than in the PCR reaction, as in a one-step protocol) facilitates trouble-shooting of these steps independently.

Since RNA is a relatively unstable molecule, every sample was analysed by agarose gel electrophoresis prior to the reverse transcription. Agarose gel electrophoresis gave an indication of the integrity of the RNA sample, which was evaluated on the extent of smearing, and the ratio of the intensity of 18S:28S bands which should ideally be 1:2. A representative RNA gel is shown in figure E.1.1. Furthermore, absorbance analysis was used to indicate the purity of the RNA sample. Pure RNA has a 260:280 ratio greater than or equal to 2.0 (Sambrook *et al.*, 1989), therefore samples with a 260:280 ratio below 1.9 were not used due to the presence of contaminating molecules, such as DNA.

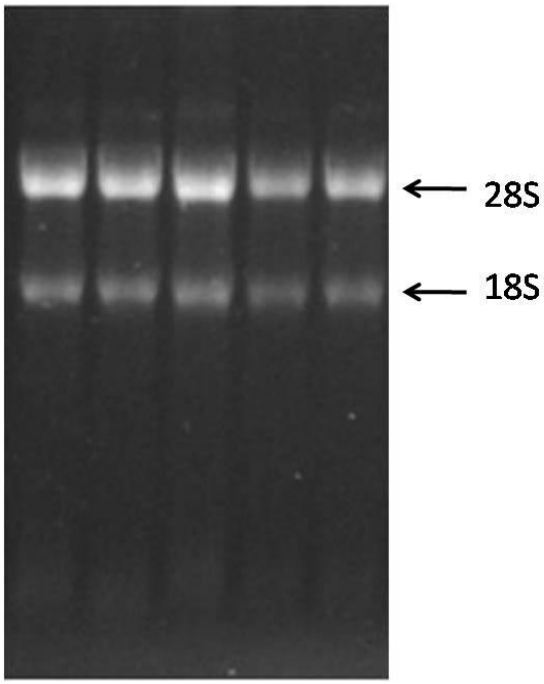


Figure E.1.1. Representative RNA agarose gel. 500 ng of RNA isolated from U2OS cells was analysed on a formaldehyde agarose gel. The 18S and 28S RNA bands are indicated by arrows.

Initial real time PCR experiments were found to be unreproducible, and in order to troubleshoot the cause of the variability, a single RNA sample was used for several parallel cDNA synthesis experiments, and each cDNA sample was analysed in duplicate PCR reactions. As shown in figure E.1.2, there was little variation between duplicate PCR samples, however variability was observed between the amount of PCR product detected from amplification of the cDNA samples reverse transcribed in parallel. This indicated that the likely cause of the variability was introduced during the cDNA synthesis step, and not the PCR step.

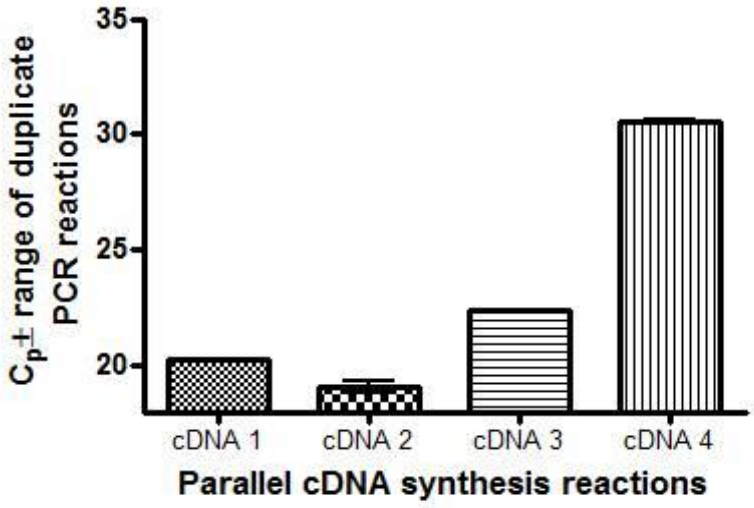


Figure E.1.2. Evaluation of parallel cDNA syntheses and PCR reactions. A single RNA sample was reverse transcribed in four parallel reactions (cDNA 1- 4). Each cDNA sample was subsequently amplified in parallel, in duplicate PCR reactions. The crossing point (C_p) of each reaction above threshold is plotted ± the range of the duplicate PCR reactions. The histogram shows results of a single experiment.

In order to eliminate this problem, several different cDNA synthesis kits from three different manufacturers, were compared in three parallel reactions on a single RNA sample. Each cDNA sample was subsequently amplified by PCR using primer sets for GILZ and GAPDH. The relative abundance of the GILZ transcript was calculated for each sample, as shown in figure E.1.3. A high degree of variability was detected for kits P and C. It was thus decided to use kit R, which showed the highest degree of reproducibility.

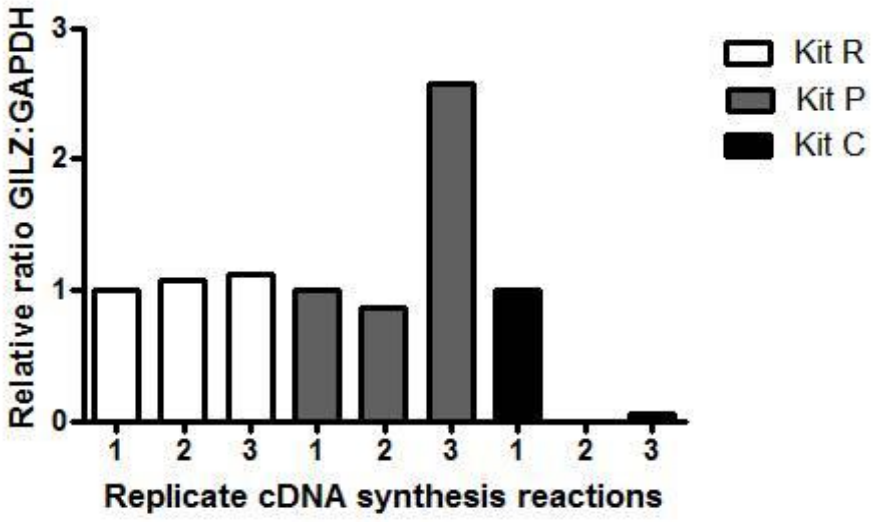


Figure E.1.3. Comparison of different cDNA synthesis kits. Parallel cDNA synthesis reactions were performed on a single RNA sample in triplicate, using three different kits designated R, P and C. The relative abundance of the GILZ transcript was subsequently analysed in each sample by duplicate quantitative real time PCR reactions in parallel. The average C_p of duplicate PCR samples amplified with GILZ primers was normalised to the average C_p of duplicate PCR samples analysed with GAPDH primers to give the relative expression of GILZ in each cDNA sample, which was plotted.

In order to compare the extent of transrepression elicited by different ligands via the GR, the extent of transrepression elicited by dex was compared under different conditions, in order to determine which condition would yield optimal transrepression. Important parameters include the type and concentration of pro-inflammatory compound used for induction and the time of treatment with GC and pro-inflammatory compound. Preliminary time course experiments performed in the stably transfected U2OS.HAhGR cell line indicated that maximal transrepression of the IL8 gene occurred at 2 h of treatment, as shown in figure E.1.4.

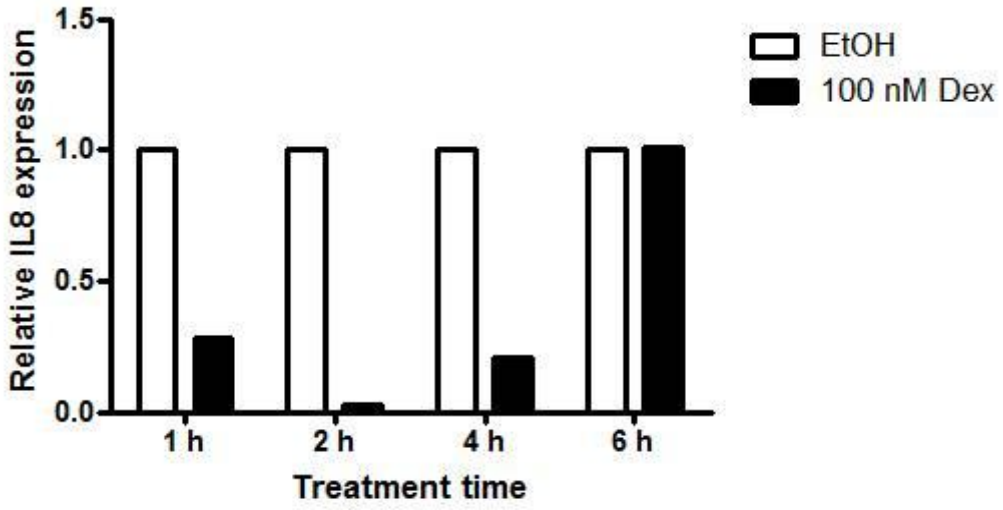


Figure E.1.4. Time course of transrepression of IL8 gene in U2OS.HAhGR cells. U2OS.HAhGR cells were plated in 12 well plates at a density of 1 x 10^5 cells per well. Cells were treated with 50ng/ml PMA and 100 nM dex simultaneously for 2 h, after which the relative abundance of the IL8 transcript was quantified, and plotted relative to vehicle treated cells.

Having determined that a 2 h treatment resulted in a high level of transrepression, this was further investigated in WT U2OS cells, and a comparison between simultaneous and staggered PMA and dex treatment was conducted. On both the IL8 and COL3 genes, it appeared that simultaneous treatment gave a higher degree of transrepression than when PMA treatment was delayed by 30 min, as shown in figure E.1.5. Simultaneous treatment with PMA and GC was thus used in subsequent experiments. Although the extent of transrepression observed in this experiment is greater than that shown in figure 4.6, for both IL8 and COL3, this graph shows the result of a single experiment. Variability between experiments was such that the mean transrepression from three experiments was less than shown here.

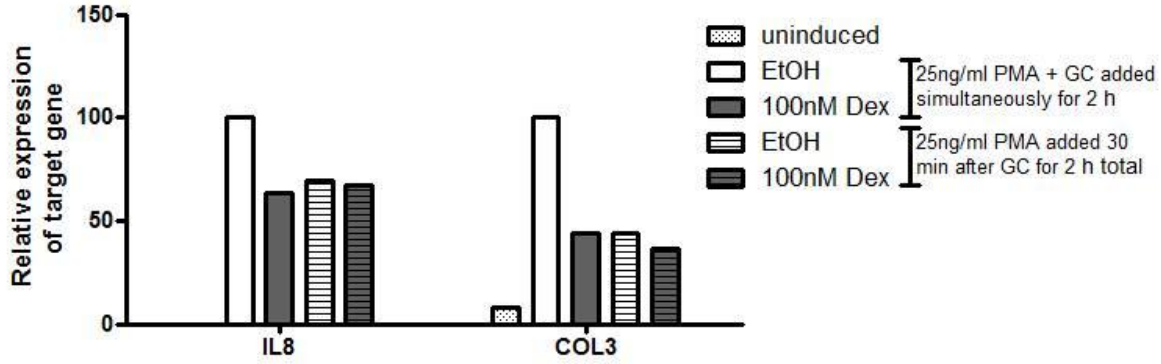


Figure E.1.5. Comparison of treatment conditions on IL8 and COL3 transrepression in U2OS cells. U2OS cells were treated with PMA and 100 nM dex as indicated, and the relative abundance of IL8 and COL3 transcripts was measured by realtime PCR as indicated in materials and methods.

Transrepression of the TNFα gene was subsequently investigated, and a comparison was made between induction by PMA and TNFα, and simultaneous versus staggered treatment. In this instance, slightly better transrepression was observed upon TNFα stimulation 30 min after GC treatment of cells, as shown in figure E.1.6. This condition was therefore used for subsequent analysis of TNFα transrepression by different ligands in U2OS cells. Although the extent of transrepression observed in this experiment is greater than that shown in figure 4.6 for TNFα, this graph shows the result of a single experiment in duplicate for addition of TNFα 30 min after dex treatment. Variability between experiments was such that the mean transrepression from three experiments was only approximately 50% (figure 4.6).

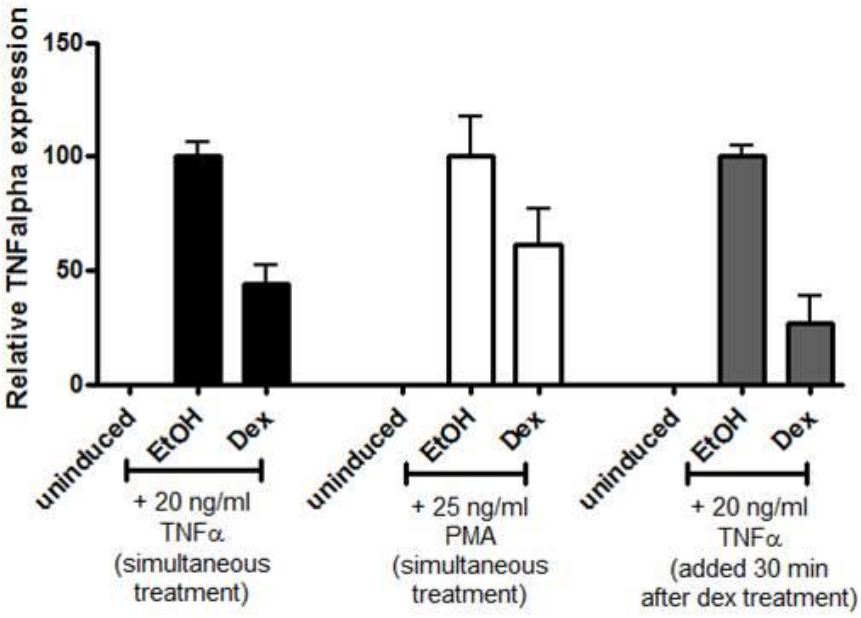


Figure E.1.6. Comparison of treatment conditions for transrepression of TNFα. U2OS cells were treated with PMA or TNFα and vehicle or 100 nM dex as indicated, and the relative abundance of TNFα transcripts was measured by realtime PCR as indicated in materials and methods. Pooled results of one to three experiments, each performed in duplicate, are shown.

E.2. ChIP Assay

The ChIP assay is a multistep assay in which proteins are cross-linked to DNA (Ma, *et al.,* 2003). The DNA is subsequently fragmented by sonication, prior to immunoprecipitation. After reversal of the crosslinks, the immunoprecipitated DNA is purified, and analysed by PCR. Sonication is a crucial step in this assay, as it not only facilitates the rupture of the crosslinked cells, but also determines the average size of the DNA fragments. This can determine the success of the assay, as fragments that are too large will be retarded in the immunoprecipitation step, and fragments that are too small may be lost during DNA cleanup, or not successfully amplified during the PCR step. The recommended average size of DNA fragments is between 300 bp and 800 bp (Ma *et al.,* 2003). To this end, the sonication step was optimised to yield DNA fragments of an appropriate size. Since the crosslinked chromatin also contains heat-labile proteins, the sonication must be performed in

short bursts, with an intermittent rest period on ice, to prevent heating. The time required for “on” and “off” periods, as well as the number of cycles, and the power setting of the sonicator must all be optimised for the particular sonicator to be used. A representative gel of different sonication conditions is shown in figure E.2.1. The selected sonication parameters for initial ChIP optimisation experiments in U2OS cells were those in the third lane, which gave slightly larger fragments (average size approximately 250 bp) than the other conditions (average size approximately 150 bp).

On	10s	10s	10s	20s
Off	20s	20s	20s	40s
Cycles	20	10	20	2
Power	4	4	3	5

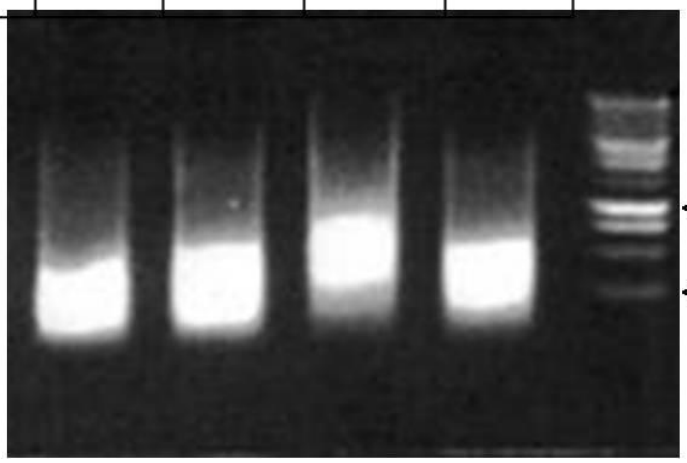


Figure E.2.1. Optimisation of sonication conditions using probe. U2OS cells were plated, crosslinked, and scraped as indicated in materials and methods for a ChIP assay. Cells were sonicated under different conditions as indicated using a Misonix Sonicator 3000 with microtip, before crosslinks were reversed overnight. DNA was purified using QiaQuick columns, and approximately 1 µg was analysed by agarose gel electrophoresis.

For subsequent ChIP experiments in COS-7 cells, a Bioruptor (Diagneode, Belgium) was available. This is a water-bath based sonicator which allows simultaneous sonication of up to six samples. This is advantageous as it increases reproducibility of sonication between samples. Furthermore, sonication occurs in sealed tubes, which prevents cross-contamination of samples. The manufacturer's recommended sonication parameters for mammalian cells including COS cells was 10 cycles of 30s on, and 30s rest on ice. When these parameters were used, they were found to yield fragments between 300 bp and 500 bp, as shown in figure E.2.2.

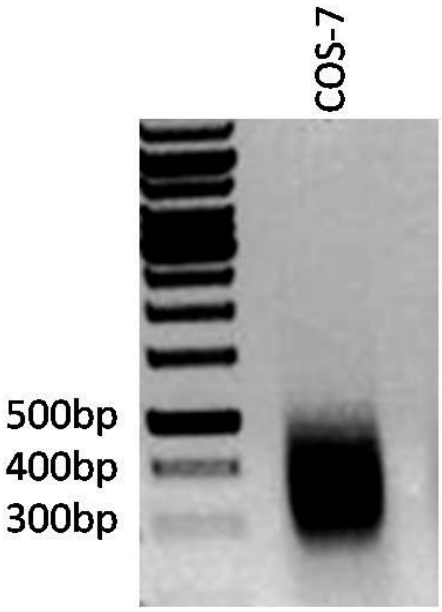


Figure E.2.2. Sonication in Bioruptor. COS-7 cells were plated, crosslinked, and scraped as indicated in materials and methods for a ChIP assay. Cells were sonicated in a Bioruptor before crosslinks were reversed overnight. DNA was purified using QiaQuick columns, and approximately 1µg was analysed by agarose gel electrophoresis.

ChIP is a powerful tool for elucidating interactions between transcription factors and DNA in intact cells. Cellular proteins are crosslinked by incubation with formaldehyde, which reduces the occurrence of false-positive results caused by interaction of proteins following lysis. ChIP analysis was performed to investigate whether promoter occupancy at the endogenous GILZ promoter by activated GR correlated with transactivational efficacy and nuclear localisation. Accordingly, ChIP

technique was extensively optimised, but failed to yield reproducible GR recruitment to the GILZ promoter in U2OS cells. Since the ChIP assay is a complicated multi-step assay, there are numerous steps at which the assay can be compromised, and trouble-shooting is an arduous and time-consuming, not to mention expensive exercise. After several attempts proved fruitless, it was decided to try the ChIP assay in another cell line, to check if the experimental procedure was working. A549 cells were selected, since recruitment of endogenous GR to the GILZ promoter has been reported in these cells (Wang *et al.,* 2004).

As shown in figure E.2.3A, ChIP in A549 cells yielded an approximately 7-fold increase in GR recruitment to the GILZ promoter upon dex treatment. This is in line with reported values of 8-10 fold recruitment reported in these cells by Wang *et al.* (2004), using different primers that fall within approximately the same region of the GILZ promoter. Pooled results of three independent ChIP experiments in U2OS show a large amount of error, and fail to show a significant difference in GR recruitment between EtOH and dex.

Endogenously expressed levels of GR in A549, COS-1 and U2OS cells (figure E.2.3B) were subsequently examined. There is a clear difference in the amount of GR expressed by the three cell lines, with A549 levels being the highest. This may indicate that the relatively low GR levels in U2OS account for the weak recruitment of GR to the GILZ promoter observed in the ChIP assay, and that the ChIP assay was not sufficiently sensitive to detect low levels of GR on an endogenous promoter.

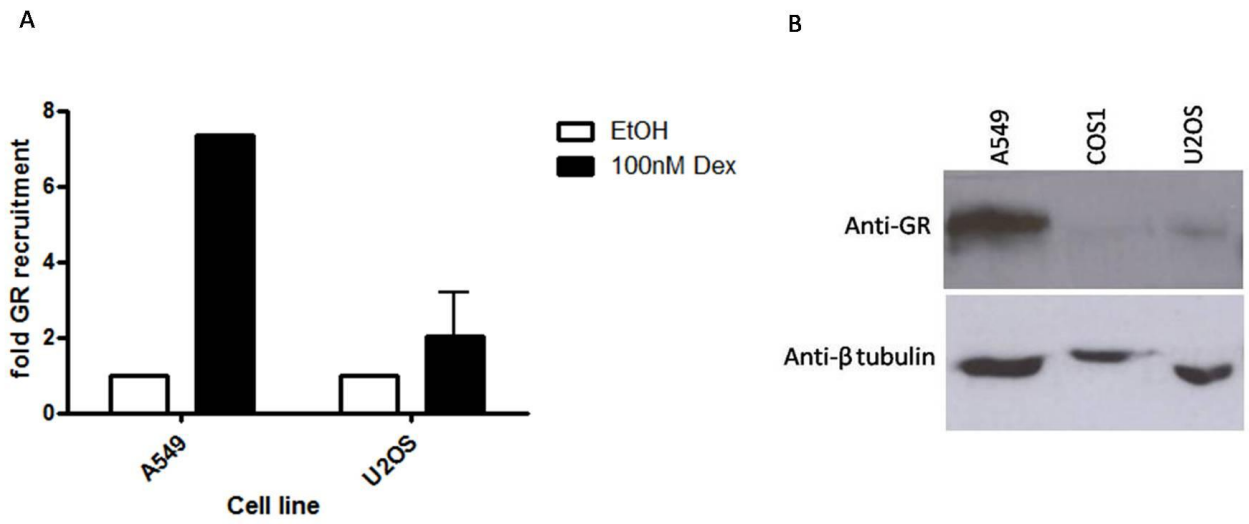


Figure E.2.3. GR ChIP assay is not reproducible in U2OS cells (A) ChIP assay was performed in parallel in A549 and U2OS cells, to measure recruitment of endogenous GR to the GILZ promoter. One experiment is shown for A549, while pooled results of three independent experiments are presented for U2OS, presented as mean +/- SEM (B) Relative levels of GR are shown in A549, COS-1 and U2OS cells, as analysed by Western blot.

E.3. Co-immunoprecipitation

Co-immunoprecipitation is a useful technique for evaluation of protein-protein interactions. Cells are lysed, and the protein of interest is immunoprecipitated, followed by elution from beads, and analysis of the eluate by Western blot to detect the presence of a specific interacting protein. As a positive control for the experimental technique, co-immunoprecipitation was used to detect ligand-specific interactions between overexpressed GR and GRIP-1 in COS-1 cells, as shown in figure E.3.1. However, co-immunoprecipitation of endogenous proteins in U2OS was not successful in the present author's hands.

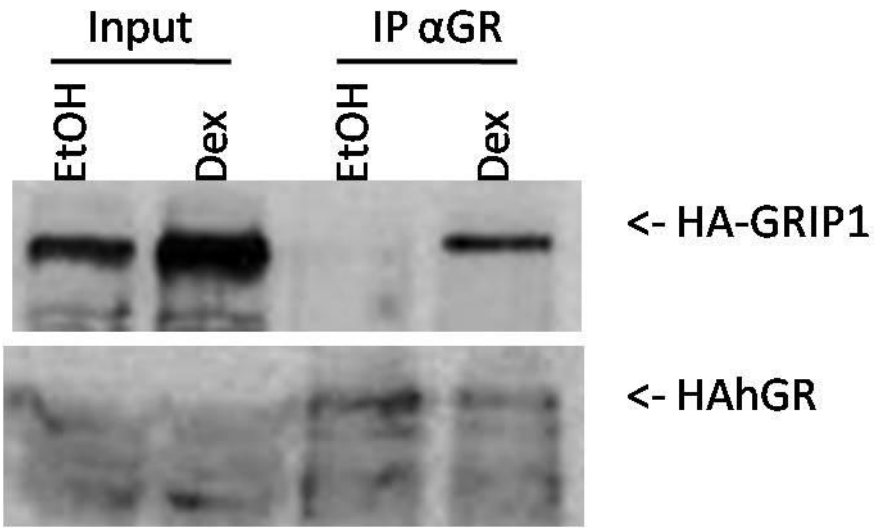


Figure E.3.1. Co-immunoprecipitation of overexpressed HAhGR and HA-GRIP1 for COS-1 cells. COS-1 cells were transfected with 3 µg each of HAhGR and HA-GRIP1 in 10cm plates, and treated for 1 h with 100 nM dex or vehicle (EtOH). Cells were then lysed and incubated with an anti-GR antibody and protein A/G agarose beads overnight. Beads were then washed and immunoprecipitated proteins were analysed for total GR and HA-GRIP1 by Western blotting.

The interaction between GR and immunophilins was subsequently investigated by co-immunoprecipitation. The interaction between GR and immunophilins in the Hsp90 heterocomplex is quite unstable, and particular conditions are required to maintain this association during immunoprecipitation (Davies *et al.,* 2002; Echeverría *et al.,* 2009). Several reports have been published demonstrating a ligand-selective switch in immunophilin association of the GR, using different techniques (Davies *et al.,* 2002; Echeverría *et al.,* 2009) and this has been shown to occur in A549 cells (Croxtall *et al.,* 2003). However, experiments in the present authors hands failed to show a ligand-dependent change in the levels of GR:FKBP52 association in A549 cells, as shown in figure E.3.2. The two protocols used (Davies *et al.,* 2002; Croxtall *et al.,* 2003) differ considerably in treatment time and temperature of the cells, the lysis procedure, and the quantity of input material. It is possible that some unpredictable difference in conditions or experimental protocol prevented successful immunoprecipitation of the complex.

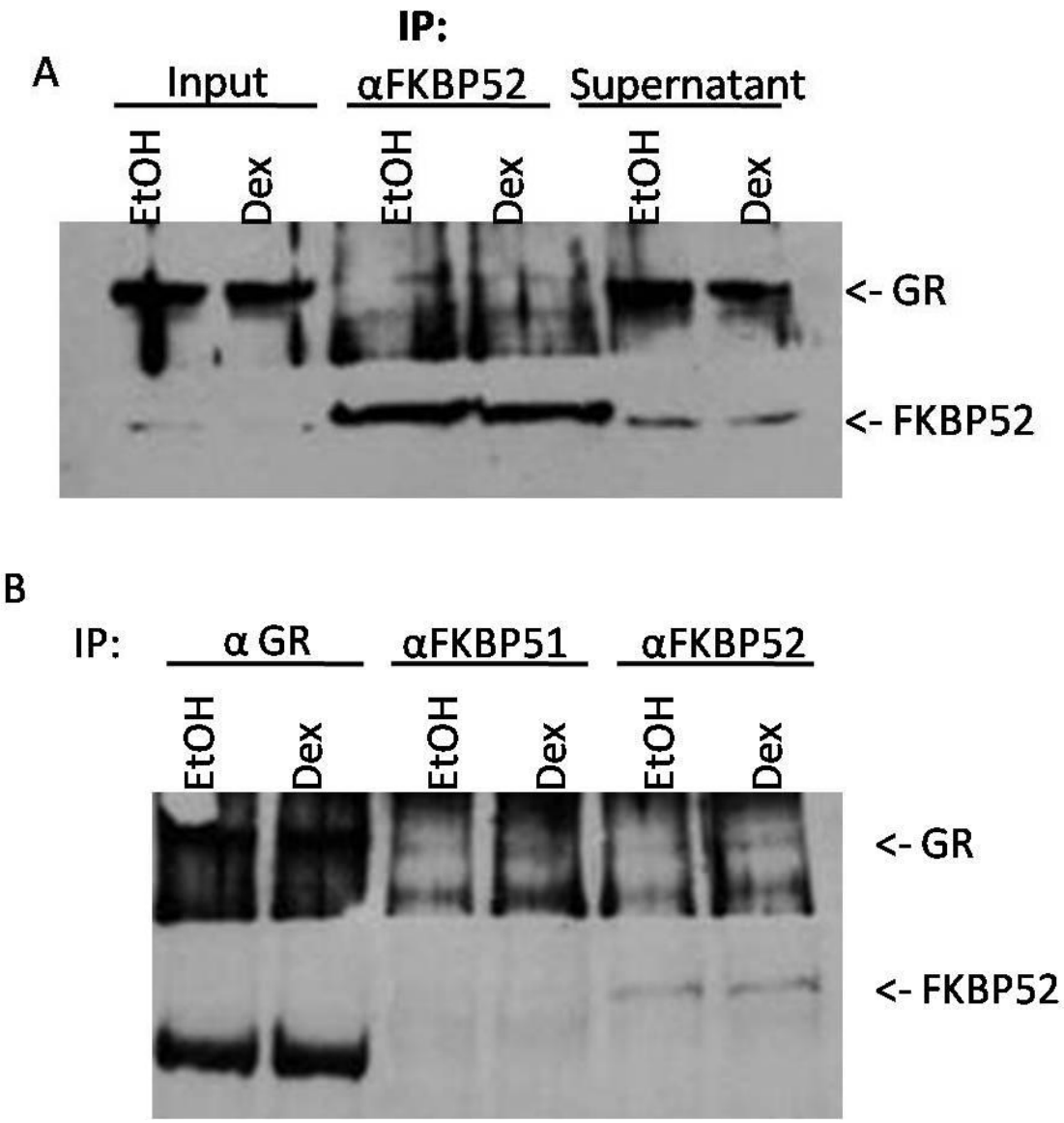


Figure E.3.2. Co-immunoprecipitation of GR and immunophilins. (A) A549 cells were plated, treated with EtOH or dex and subjected to immunoprecipitation with an anti-FKBP52 antibody, according to a published protocol (Davies *et al.,* 2002). After immunoprecipitation, the supernatant was harvested, and analysed by Western blot, alongside the eluate from the beads, and input samples harvested prior to immunoprecipitation. The Western blot was probed for FKBP52 and GR. (B) A549 were plated, treated with EtOH or dex, and immunoprecipitated according to a published protocol (Croxtall *et al.,* 2003). Immunoprecipitation was performed using antibodies towards GR, FKBP51, or FKBP52, and protein A/G agarose beads. Eluted proteins were analysed by Western blotting, probing for GR, FKBP51 and FKBP52.

E.4. RNA interference (RNAi)

Transfection of short interfering RNA (siRNA) causes knockdown of specific mRNA transcripts, allowing analysis of the role of a particular protein by examining the effect of reduced protein levels. siRNA strands cause activation of the RNA-induced silencing complex (RISC), which allows sequence recognition and mRNA degradation. The experimental protocol requires careful optimisation to allow maximal knockdown, with minimal off-target effects, and cell death. Where available, pre-validated siRNA sequences from commercial vendors can save a lot of time. These are often supplied with a transfection reagent. In this case HiPerfect transfection reagent and pre-validated siRNA sequences (GR5, GR6, and non-silencing control, NSC) were purchased from Qiagen. The optimal concentration of HiPerfect was determined by comparing the success of knockdown at high and low concentrations within the recommended range, as shown in figure E.4.1. GR5, GR6 and a combination of 5 and 6 all gave efficient knockdown of GR at both low and high concentrations of HiPerfect transfection reagent. However, at high concentrations of transfection reagent, a high level of cell death was apparent on microscopic examination (data not shown). This is also evident from the reduced levels of β-tubulin observed upon Western blotting (figure E.4.1, right hand panel). Based on this experiment, it was decided to continue optimisation using a low concentration of transfection reagent, and the siRNA sequence GR6.

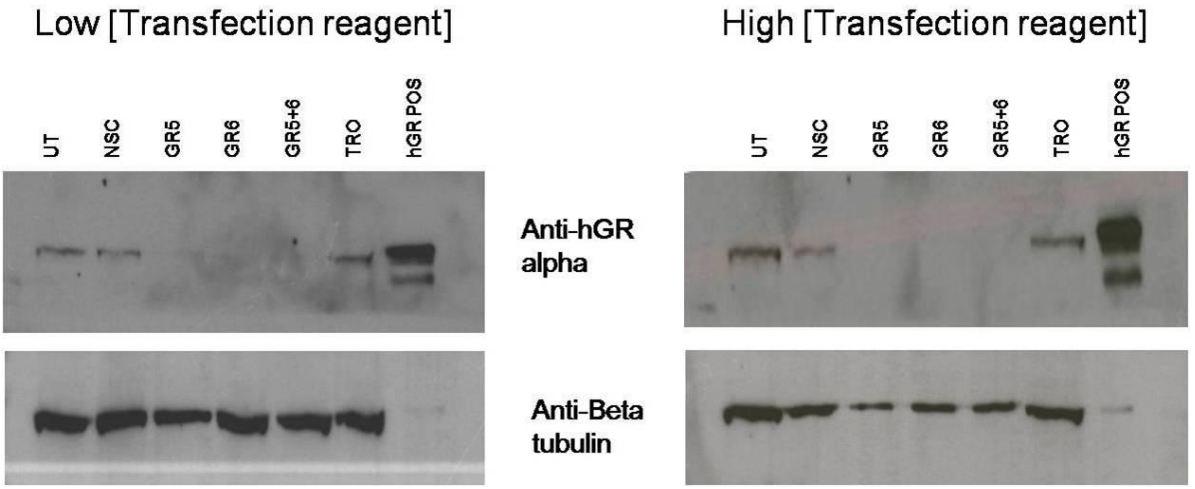


Figure E.4.1. Comparison of different siRNA sequences and different concentrations of transfection reagent. U2OS cells were plated in 6 well plates and left untreated (UT) or transfected with 100 nM GR siRNA or NSC, using 7 µl (low) or 21 µl (high) of HiPerfect transfection reagent per well, or transfection reagent only (TRO). Cells were incubated for 48 h before harvesting and analysis of GR and β-tubulin protein expression levels by Western blotting using GR and β-tubulin specific antibodies, as listed in materials and methods.

Having determined a suitable concentration of transfection reagent, it was necessary to determine the lowest effective concentration of siRNA. This was established by transfecting different concentrations of GR siRNA and monitoring GR expression levels relative to β-tubulin, as shown in figure E.4.2. GR levels were reduced at all concentrations of GR6 siRNA transfected. In this Western blot the GR antibody detected two bands, with the lower band most likely representing degradation products of GR. Although GR levels appeared similar in cells transfected with 10 nM and 1 nM GR6 siRNA, the lower GR band was slightly stronger in the cell transfected with 1 nM GR6 than 10 nM, indicating possible degradation of GR in this sample. Therefore, it was decided that 10 nM of GR6 siRNA would be suitable for subsequent knockdown experiments.

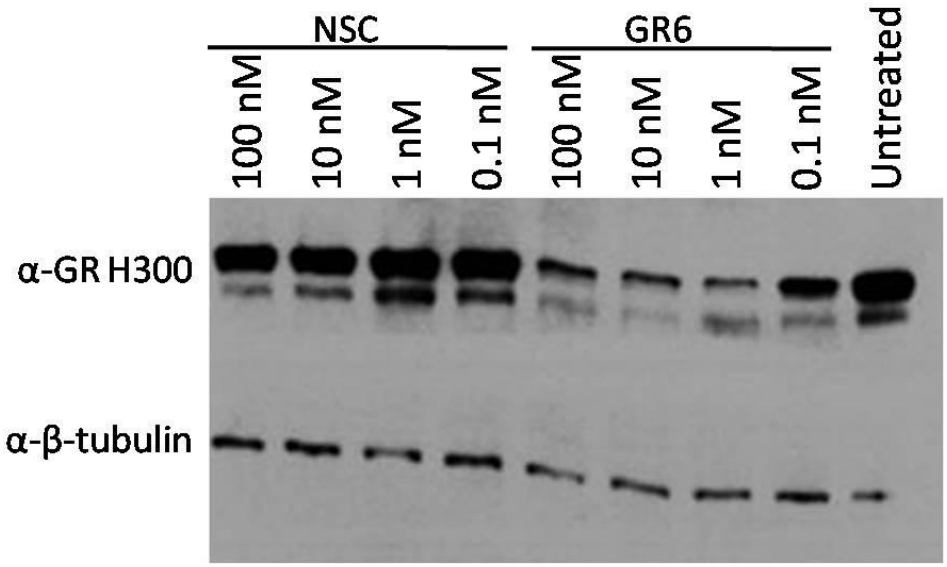


Figure E.4.2. Dose response of siRNA transfection in U2OS cells. U2OS cells were transfected with serial dilutions of siRNA GR6, as

indicated, using 7 µl of transfection reagent per well. Cells were incubated for 48 h before analysis of GR and β-tubulin levels by Western blotting.

In order to determine the optimal time for knockdown of GR expression, a time course analysis was performed, with transfected cells being harvested over a period of five days. As shown in figure E.4.3., successful knockdown was observed at every time point examined. A time point of 48 h post-transfection was selected as a suitable time for analysis of knockdown effects in subsequent experiments.

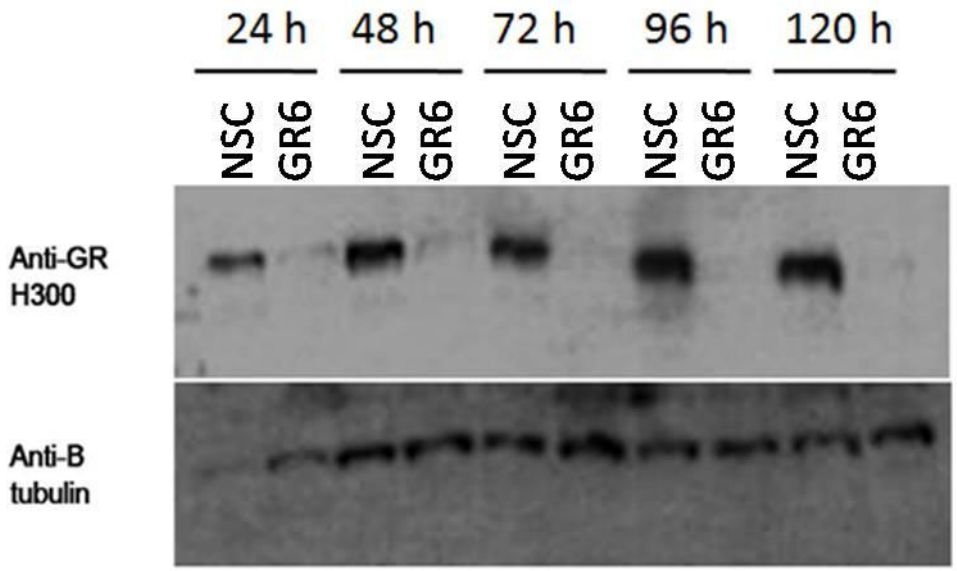


Figure E.4.3. Time course analysis of GR expression knockdown by NSC and GR6 transfection in U2OS cells. U2OS cells were plated and transfected with 10 nM of GR6 siRNA or NSC siRNA, and incubated for the indicated times before proteins were harvested. Expression levels of GR relative to β-tubulin were analysed by Western blot.

As shown in figure 4.1A, only GR, and not AR, MR or PR gave a ligand-dependant transcriptional response in a reporter assay. Since SRs have previously been reported to regulate each others' expression (reviewed in Schmidt & Meyer, 1994; Slayden & Brenner, 2004) the effect of GR knockdown on the activity of AR, MR and PR was examined using reporter assays and receptor-specific ligands. Expression of the luciferase reporter gene was normalised to expression of a co-transfected β-

galactosidase plasmid to normalise for transfection efficiency. As shown in figure E.4.4, knockdown of GR reduced the dex-mediated response as expected. However, GR knockdown did not affect the response to mibolerone (mib), aldosterone (ald) or R5020, indicating that knockdown of GR did not increase the activity of AR, MR and PR respectively.

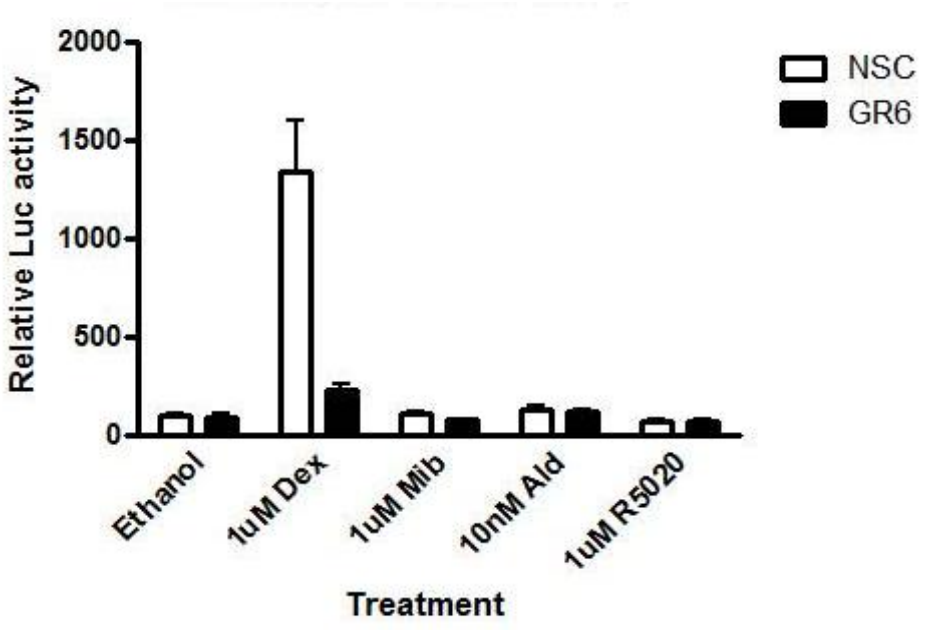


Figure E.4.4. Effect of transfection of NSC or GR6 on response to receptor-specific ligands in U2OS cells. U2OS cells were transfected with NSC or GR6 siRNA, the HRE-luciferase reporter plasmid pTAT-GRE-E1b-Luc and pCMV-β galactosidase as a transfection control. After 24 h cells were treated as indicated for a further 24 h, before cells were lysed and luciferase activity was measured, and normalised to β-galactosidase activity. The graph shows pooled results of two independent experiments performed in triplicate.

E.5. Effect of stable and transient overexpression of GR in U2OS cells

Stable transformation of U2OS cells with HAhGR to create the U2OS.HAhGR cell line was performed by Dr Ronacher, a previous member of the Hapgood lab. Stably transfected colonies were selected by G418 screening. After selection of several

colonies, these clones were propagated in the presence of G418, and lysates were harvested over several weeks to analyse GR expression levels over time. This was observed to decrease over time, as shown by Western blotting (figure E.5.1). Although it has previously been shown that U2OS cells express endogenous GR which is detectable by Western blotting (figure 4.1B) levels of GR in U2OS cells appear low here, probably due to variation in Western blotting conditions used, including primary antibody concentration and exposure time.

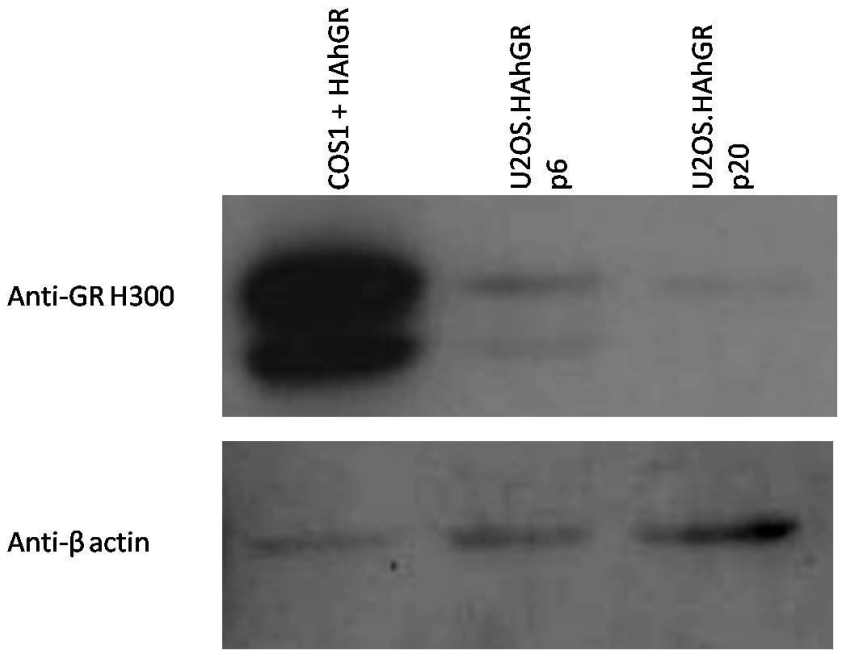


Figure E.5.1. Changes in stably transfected GR expression levels over time. Lysates of transiently transfected COS-1 cells were analysed by Western blot in parallel with lysates of stable transfected U2OS.hGR cells harvested at passage 6 and passage 20. Western blots were probed with antibodies towards GR and β-actin.

The decrease in GR expression over time may have been due to hypermethylation of the exogenous promoter, with no apparent effect on the expression of genes required for G418 resistance. Several attempts were made to prevent loss of HAhGR expression, including varying antibiotic concentration, testing different clones, and

carefully monitoring levels at every passage to discern whether certain clones may exhibit reliably stable expression for at least a few passages. However, it was ultimately decided that HAhGR expression of the stably transfected cells was too unpredictable for a study in which GR expression levels could have such a significant impact.

Having ascertained that stably transfected U2OS-HAhGR cells would not be a suitable model system for the present study, the effect of transient GR overexpression was examined in U2OS cells. As shown in Appendix D, figure D2, significant dex-mediated transrepression was observed on AP-1 and NFκB reporters in the presence of overexpressed GR. In contrast WT U2OS cells, expressing endogenous GR exhibited significant dex-mediated transrepression on the AP-1 promoter, but not the NFκB promoter. Since endogenous GR in U2OS cells caused significant repression in a promoter-specific fashion, it was decided to use this model system for further investigation. Use of untransfected U2OS cells presented the additional advantage of even GR distribution in all cells, as opposed to transiently transfected cells, in which only a small percentage of cells would express high levels of the transfected construct. Unfortunately, the lack of reproducibility of the ChIP assay in U2OS, which is likely attributable to low endogenous GR levels, was only identified after the initial experiments on transactivation and transrepression were completed.