The Role of the CCR5 Co-receptor Conformation in HIV Fusion

By

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Declaration

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Abstract

The CCR5 chemokine receptor is the major co-receptor mediating fusion of human immunodeficiency virus type-1 (HIV-1). The role of this host protein in fusion is well defined. CCR5 also binds chemokines, RANTES, MIP-1α (LD78α and -β) and MIP-1β. When HIV binds to CCR5 it can initiate G protein-mediated signal transduction. However mutants of the CCR5 receptor that are unable to signal are nevertheless able to mediate fusion suggesting that an inactive conformation of the CCR5 co-receptor mediates fusion. This thesis aimed to better define the co-receptor conformation by determining whether mutants of the CCR5 receptor stabilized in the active conformation were less able to mediate HIV fusion

Eight mutant receptors were generated by site-directed mutagenesis and screened for constitutive activity using intracellular signaling assays. Expression of the mutant receptors was assessed by FACS analysis, while the binding affinity of constitutively active mutants was evaluated with homologous competition binding assays. The fusion efficiency of constitutively active mutants was evaluated using Env-directed cell fusion assays.

Receptors with mutations in the conserved DRY motif, Asp$^{3.49(125)}$Ala and Asp$^{3.49(125)}$Asn, or at position Arg$^{5.69(225)}$ were not constitutively active. Receptors with mutations in the conserved TXP motif, Thr$^{2.56(82)}$Lys and Thr$^{2.56(82)}$Pro, were identified as constitutively active. The Thr$^{2.56(82)}$Pro mutant showed expression similar to the wild type receptor, while the Thr$^{2.56(82)}$Lys mutant was poorly expressed. When these mutations were combined with the Arg$^{5.69(225)}$Gln mutation the expression of the Thr$^{2.56(82)}$Lys/Arg$^{5.69(225)}$Gln mutant was enhanced. The Thr$^{2.56(82)}$Lys mutant showed low total binding due to low expression and therefore affinity could not be measured. The remaining constitutively active mutants displayed binding affinity not significantly different to the wild type receptor. Mutants with Thr$^{2.56(82)}$ mutated to Lys showed poor fusion efficiency, while mutants with Pro in position 82 showed fusion efficiency comparable to the wild type receptor.

These results suggest that mutants with Lys and Pro at position 82 are stabilized in distinct active conformations of CCR5 that have differential abilities to mediate Env-directed fusion.

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List of Abbreviations

BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CAM receptor	constitutively active mutant receptor
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle medium
EC_{50}	peptide concentration required to half-maximally stimulate production of inositol phosphates
EDTA	ethylenediamine tetraacetic acid (disodium salt)
G protein	guanine nucleotide binding protein
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IC_{50}	peptide concentration required to half-maximally inhibit binding of labeled MIP-1ß.
IP_3	inositol 1,4,5 – triphosphate
IP	inositol phosphates
mRNA	messenger ribonucleic acid
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKC	protein kinase C
PLC	phosphatidyl inositol-specific phospholipase C
PIP_2	phosphatidyl 4,5-biphosphate
R	inactive state of the receptor
R*	active state of the receptor

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1. Introduction

1.1 The Human Immunodeficiency Virus

The Human Immunodeficiency Virus (HIV) is an enveloped retrovirus which belongs to the group of Lentiviruses (Janeway, 2005a). Two copies of the viral RNA genome are contained within a viral capsid, which surrounds each virion. The viral enzymes, reverse transcriptase, integrase and protease, are also contained within the viral capsid. These enzymes facilitate infection of host cells as well as integration of the viral RNA into the host genome. The viral envelope protein (Env) is expressed on the surface of the viral capsid. Env consists of two non-covalently bound subunits, gp120 and gp41, expressed on the cell surface as a heterotrimer (Berger et al., 1999).

HIV enters the host cell via a two-step process where the Env protein sequentially interacts with two host cell receptors. The first step in the process of HIV fusion is the binding of the gp120 subunit to the CD4 receptor expressed on the host cell surface. This binding step leads to a conformational change in Env, transiently exposing a binding site for the chemokine co-receptor which is usually either the CCR5 or CXCR4 chemokine receptor (Berger et al., 1999). The gp120 subunit binds to the chemokine co-receptor, causing a second conformational change in Env, leading to penetration of the gp41 subunit into the host cell membrane. Penetration of gp41 in the host cell membrane leads to the fusion of the viral and host cell membrane and transfer of the viral core into the host cell cytoplasm (Berger et al., 1999). The advantage of employing a two-step process of binding to host cell receptors is that the binding surface of gp120 that contacts the co-receptor is only transiently exposed once binding to CD4 has occurred, thereby keeping the conformation hidden from neutralizing antibodies (Lusso, 2006).

1.2 Chemokines and chemokine receptors:

During the onset of inflammation usually in response to injury or early stages of infection the affected tissue releases cytokines which belong to the family of chemoattractant cytokines known as chemokines (Janeway, 2005b). These chemokines bind to their cognate receptors and induce a chemotactic response in nearby cells (Janeway, 2005b). Chemokines are small proteins approximately 70-80 amino acids in length and 8-14kDa in size (Stantchev and Broder, 2001). Chemokines have been categorized according to the cysteine residue sequence near the amino terminus (Janeway, 2005b; Lagerstrom, 2008; Murphy et al., 2000). These chemokines can be divided into four families, CXC, CC, C and CX3C based on the number and position of conserved cysteine residues. The chemokines denoted CXC, CC and CX3C have four conserved cysteines, while the C family of chemokines only has two conserved cysteines. CC chemokine receptors have two adjacent conserved cysteines, while in the case of CXC and CX3C receptors, these cysteines are separated by either one or three amino acid residues respectively (Murphy et al., 2000). Chemokines have a homologous structure with a short N-terminus preceding the first two conserved cysteine residues. Following the first two conserved cysteines is a loop region, three anti-parallel β-strands and an α-helix at the C-terminal end (Stantchev and Broder, 2001). There are disulfide bridges between the first and third and the second and fourth conserved cysteines which are thought to constrain the molecule (Stantchev and Broder, 2001).

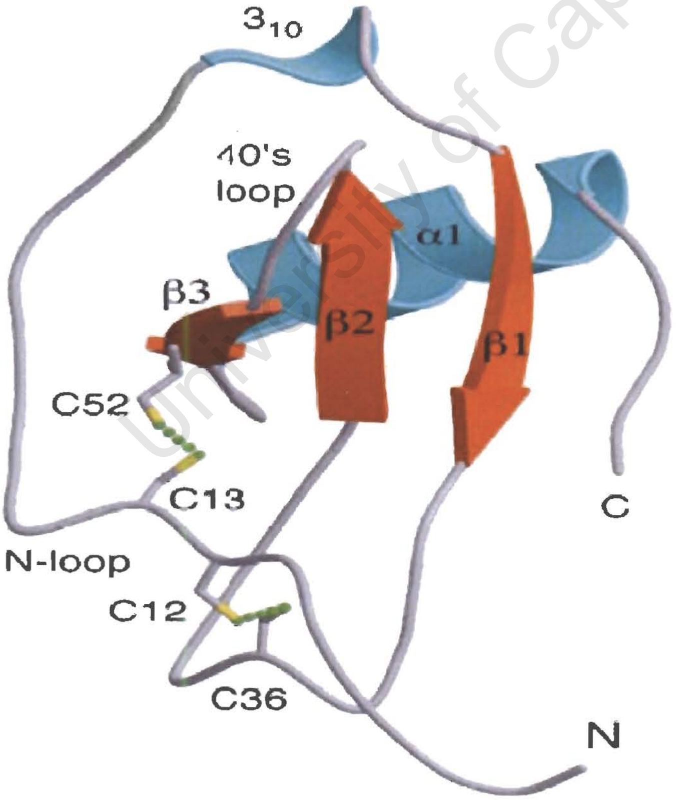


Figure 1.1: **Typical chemokine fold of vMIP-I.** Viral macrophage inflammatory protein I is a CC chemokine and homolog of human MIP-1α. The structure of vMIP-I depicts the typical chemokine fold, which consists of an extended N-terminal region containing a short 3_{10} helix. Following the N-terminal region are three anti-parallel β-strands, an α-helix and C-terminal domain. The chemokine is stabilized by two disulfide bridges between cysteine residues 12 and 36 and between cysteine residues 13 and 52. *Taken from Luz. et al (2005)*

Chemokine names were usually derived from their function or the cell type from which the chemokine originates, which resulted in some chemokines having several names. A unifying nomenclature was proposed at the Keystone symposium in 1999, where it was decided that the chemokine designation would parallel that of chemokine receptors (Murphy et al., 2000). According to this nomenclature chemokines are now designated CCL1 to 27, CXCL1 to 14, XCL1, XCL2 and CXC3CL1 (Murphy et al., 2000). CC-chemokine receptors e.g. CCR5 bind to CC-chemokines and CXC-chemokine receptors e.g. CXCR4 bind to CXC-chemokines. Chemokine receptors are able to recognize more than one chemokine, but the repertoire of chemokines recognized by a particular receptor is usually restricted to a subclass of chemokines (Stantchev and Broder, 2001).

Thus far 18 human proteins have been classified as chemokine receptors. Chemokine receptors have a specific nomenclature which reflects the subclass of chemokines to which the receptor binds (Murphy et al., 2000). Generally chemokine receptors have a fairly conserved structure, with amino acid identity ranging from 25-80%. Despite the big range in amino acid identity between chemokine receptors, there are a few structural features that these receptors have in common. These structural features include the presence of a cysteine residue in each of the four extracellular domains, the presence of the conserved sequence, DRYLAVHA, in the second intracellular loop, a third intracellular loop which is short and basic and an acidic N-terminal segment (Murphy et al., 2000). Chemokine receptors also range in length from 340 to 370 amino acids and contain a motif located in the N terminus which promotes tyrosine sulfation. This motif has been shown to be vital for the co-receptor activity of CCR5 (Farzan et al., 1999; Murphy et al., 2000). Disulfide bonding occurs between the first and the third cysteine residues and between the fourth and the second cysteine residues, which serves to constrain the structure of the receptor (Murphy et al., 2000). Chemokine receptors also couple to the Gi class of G proteins, which facilitates chemotactic signaling (Murphy et al., 2000). Chemokine receptors play a biological role in the trafficking of leukocytes and therefore influence related processes which include immune surveillance, immune responses and inflammation (Foxman et al., 1997; Murphy et al., 2000; Springer, 1994). The specificity of the biological role played by each chemokine receptor is determined by the subset of leukocytes on which the receptor is expressed as well as the regulation of the expression of the cognate ligand (Murphy et al., 2000). Besides its important role in the immune system, chemokine receptors have also been shown to be exploited as portals of entry into host cells by pathogens. These include the use of CCR5 and CXCR4 by HIV-1 and the use of the Duffy receptor by the pathogen *Plasmodium Vivax* in the transmission of malaria (Murphy et al., 2000).

1.2.2 Discovery of chemokine receptors as HIV co-receptors:

The discovery of the role of chemokine receptors as HIV co-receptors stemmed from two observations that were initially quite difficult to explain. Firstly, it was noted that HIV-1 entry was specific to certain cell types and that the expression of the primary receptor CD4 was not sufficient to render nonhuman cells susceptible to infection by HIV-1 (Berger, 1997). In addition HIV-1 displayed a distinct tropism for certain human target cell types that were nevertheless $CD4^+$ (Berger et al., 1999). Many HIV-1 isolates were able to replicate in primary $CD4^+$ T-lymphocytes, but the distinct tropism arose in the case of continuous $CD4^+$ T-cell lines and primary macrophages. Certain viral isolates were able to infect and replicate efficiently in continuous $CD4^+$ T-cell lines, but had poor infectivity and replicative abilities in primary macrophages; while other viral isolates displayed the opposite phenotype. Viral isolates able to infect and replicate in continuous $CD4^+$ T-cell lines were designated T-cell line tropic (TCL-tropic), while isolates able to infect and replicate in primary macrophages were designated macrophage-tropic (M-tropic). There also existed viral isolates able to infect and replicate efficiently in both cell lines and these isolates were designated dual-tropic (Berger et al., 1999). These observations suggested that the expression of CD4 was not sufficient to explain the distinct tropism of HIV-1 in certain human target cells and this suggested the presence of a cofactor or co-receptor unique to human cells.

Attention was then focused on the identification of this co-receptor. Initial studies used the method of a functional cDNA cloning strategy, which relied on a cDNA library rendering murine cells, expressing the CD4 receptor, susceptible to HIV-1 infection (Feng et al., 1996). Cells expressing the envelope protein from HIV-1 isolates which were TCL- tropic were used. As a result a single cDNA was isolated and subsequently sequenced. Sequencing results identified the protein as a member of the superfamily of G protein-coupled receptors and the protein shared the highest sequence homology with a group of receptors known as peptidergic receptors, which included chemokine receptors (Berger et al., 1999). However no cognate ligands for the receptor were identified and it was therefore categorized as an orphan receptor. The orphan receptor was termed "Fusin" to reflect its role in fusion with the envelope protein of HIV-1 (Feng et al., 1996). Its role as a HIV-1 co-receptor was further confirmed by demonstrating that the expression of Fusin, along with the CD4 receptor, rendered nonhuman cells susceptible to HIV-1 infection and that with the addition of Fusin-specific antibodies this newly acquired function was subsequently lost (Feng et al., 1996). To further support the specificity of the role of Fusin, it was also shown that these effects were only seen with TCL-tropic HIV-1 isolates, initially used to render murine cells permissive to infection, and not with M-tropic HIV-1 isolates.

The next step was the discovery of the co-receptor for M-tropic HIV-1 isolates and this search was boosted by a second seemingly unrelated observation. This was the discovery of certain soluble suppressor factors capable of controlling HIV-1 infection. These soluble suppressor factors were identified as the CC chemokines RANTES, MIP-1α and MIP-1ß released by CD8$^+$ T-lymphocytes (Cocchi et al., 1995). In addition to their identification as CC chemokines, it was further observed that the suppressive factors were able to control HIV-1 infection by isolates that were M-tropic, but had little effect on TCL-tropic isolates. Since the sequencing of Fusin had shown high sequence homology with chemokine receptors, it was suggested that the suppressive chemokines bind to a chemokine receptor, which acts a co-receptor for M-tropic viral isolates, blocking the co-receptor and thereby inhibiting the binding of Env. The identification of the M-tropic chemokine co-receptor was made possible after the identification of the chemokine receptor for the soluble suppressor chemokines RANTES, MIP-1α and MIP-1β (Raport et al., 1996; Samson et al., 1996a). The receptor was termed CCR5, because it was the fifth CC chemokine receptor to have been cloned. The role of CCR5 as the M-tropic co-receptor was confirmed by experiments showing that the expression of CCR5 along with CD4 rendered nonhuman cells permissive to entry of M-tropic HIV-1 isolates and that this gain of function was subsequently lost with the addition of CCR5 specific ligands (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996). The identification of the M-tropic co-receptor as a chemokine receptor quickly led to the confirmation that Fusin was a chemokine receptor specific for the CXC chemokines stromal derived factor-1α (SDF-1α) and -1β (SDF-1β) (Bleul et al., 1996; Oberlin et al., 1996). Fusin was therefore renamed CXCR4, since it was the fourth CXC chemokine receptor to have been cloned.

A simple model was therefore proposed to explain the tropism of different HIV-1 isolates. HIV-1 strains expressing Env that preferentially bind to the CXCR4 chemokine co-receptor, infect and replicate efficiently in continuous T-cell lines which express high levels of CXCR4; while HIV-1 strains which express Env that preferentially bind to CCR5, infect and replicate efficiently in primary macrophages which express high levels of CCR5 (Berger et al., 1999). Both TCL-tropic and M-tropic strains can infect and replicate efficiently in primary $CD4^+$ T-cells, since these cells express both CXCR4 and CCR5.

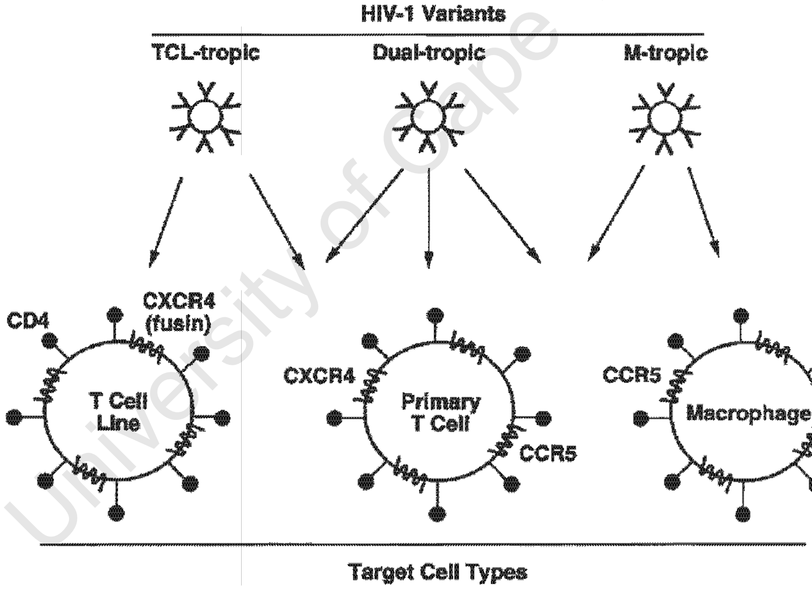


Figure 1.2 Model of HIV-1 viral tropism. TCL tropic or X4-tropic viral isolates use the CXCR4 co-receptor for entry into host cells and are able to infect primary $CD4^+$ T-cells and continuous $CD4^+$ T-cell lines. M-tropic or R5 tropic isolates use the CCR5 co-receptor for entry into host cells and are able to infect primary $CD4^+$ T-cells and primary macrophages. Dual-tropic or R5X4 tropic isolates can use either the CXCR4 or CCR5 co-receptor for entry into host cells and can infect primary $CD4^+$ T-cells, continuous T-cell lines and primary macrophages. *Figure taken from Berger et al. 1999*

As a result of this model the tropism of the HIV-1 strains were renamed to reflect the co-receptor usage as opposed to the preferred cell type. M-tropic HIV-1 strains were therefore designated R5-tropic, while TCL-tropic HIV-1 strains were designated X4-tropic and dual-tropic HIV-1 strains were designated R5X4-tropic (Berger et al., 1998). Since the discovery of the role of CCR5 and CXCR4 in HIV infection a number of other chemokine receptors have been shown to display co-receptor activity through recombinant studies. These chemokine receptors include CCR2b (Doranz et al., 1996), CCR3 (Choe et al., 1996; Doranz et al., 1996), CCR8 (Horuk et al., 1998; Jinno et al., 1998), CCR9 (Choe et al., 1998), and CXCR1 (Combadiere et al., 1998; Rucker et al., 1997). Despite this diverse repertoire of chemokine receptors with co-receptor activity *in vitro*, CCR5 remains the most important co-receptor *in vivo* and has now been established as the principal co-receptor for sexually transmitted or R5-tropic HIV-1 isolates (Berger et al., 1999).

1.3 G protein-coupled receptors:

G protein-coupled receptors (GPCRs) make up the largest single family of membrane proteins and mediate a large number of physiologically important processes. GPCRs mediate responses to a large and diverse array of ligands, including peptides, glycoproteins, lipids, nucleotides and ions by facilitating the transduction of extracellular signals to the interior of the cell (Flanagan, 2005; Gether, 2000; Karnik, 2003). Mutational abnormalities of GPCRs cause a number of disorders ranging from colour blindness to precocious puberty (Karnik, 2003). Approximately half of the current prescription drugs available today are targeted at GPCRs (Flanagan, 2005).

1.3.1 GPCR structure

GPCRs consist of a single polypeptide that characteristically traverses the cell membrane seven times with three extracellular loops and three intracellular loops joining the transmembrane helices. The receptor also contains an intracellular carboxy-tail terminal domain (COOH-terminal domain) and an extracellular amino-terminal domain (NH_2-terminal domain) (Gether, 2000; Karnik, 2003). There are five major families of human GPCRs which are categorized according to the degree of sequence homology between the receptors (Karnik, 2003; Lagerstrom, 2008). These five families are the rhodopsin-like family, the glutamate family, the adhesion receptor family, the secretin family and the frizzled/taste 2 receptor family. The chemokine receptor, CCR5, belongs to the rhodopsin-like family. This family forms the largest group of GPCRs with 670 members of the 802 known and predicted GPCRs (Lagerstrom, 2008) and is also the most studied group of G protein-coupled receptors (Gether, 2000). The overall sequence homology between the receptors in the family is low, but there are several key conserved features in the secondary structure shared between the members of this family (Figure 1.2). The presence of highly conserved residues amongst GPCRs has allowed the development of a consensus numbering scheme in order to facilitate the comparison of corresponding residues in different rhodopsin-like GPCRs (Ballesteros, 1995). In this numbering scheme the most conserved residue within the transmembrane helix is arbitrarily assigned the number 50. The remaining residues in each helix are numbered relative to the reference residue and three generalized numbers are associated with each amino acid. The first number indicates the transmembrane helix where the amino acid is located. The second number is its position relative to the reference residue, while the third number identifies the sequence position of the amino acid in the particular receptor (Ballesteros, 1995). As an example, the arginine residue in the highly conserved DRY-motif is the reference residue in helix 3 and is therefore assigned the position number 50. In the CCR5 receptor the arginine residue is located at position 126, therefore the amino acid identifier for this arginine residue within the CCR5 receptor would be $R^{3.50(126)}$. The consensus numbering scheme accommodates mutations by indicating the mutant amino acid after the wild type amino acid sequence. In the case of the arginine residue being mutated to alanine (A), the mutation would be designated $R^{3.50(126)}A$.

Conserved features amongst rhodopsin-like GPCRs include the disulfide bridge between transmembrane helix 3 and extracellular loop 2, the NPxxY-motif in transmembrane helix 7, which is thought to play an important role in both structure and function (Karnik, 2003) and the highly conserved "DRY"-motif (aspartate (D)-arginine (R)-tyrosine (Y)) in transmembrane 3. The high degree of conservation of these residues and motifs amongst receptors in this family suggests that they have an important role in the structure and therefore function of these GPCRs. They are thought to provide a structural basis for a common mechanism of activation (Gether, 2000; Karnik, 2003).

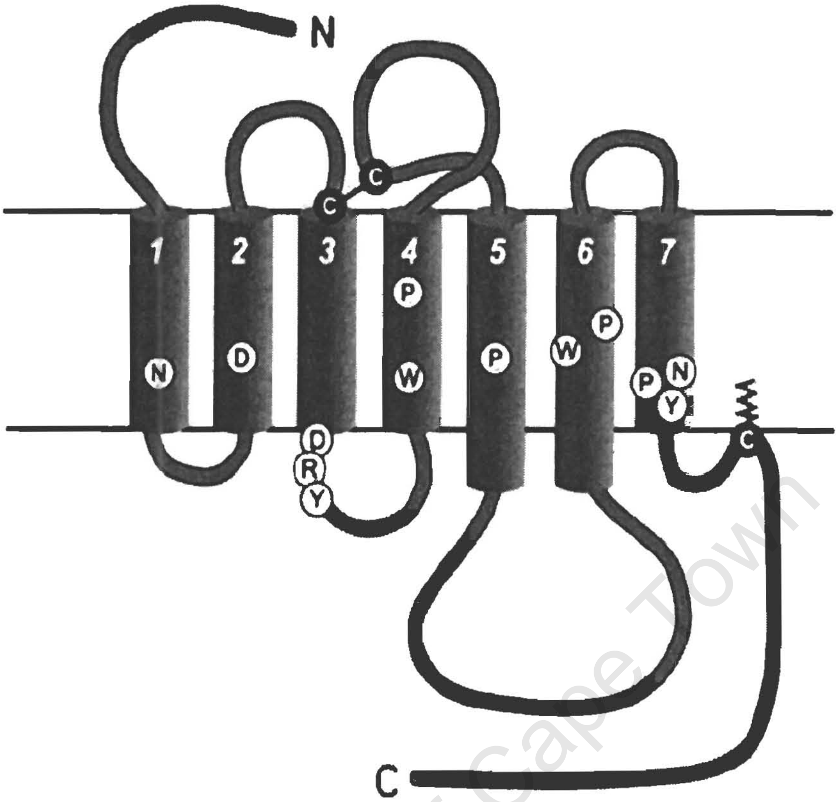


Figure 1.3: General structure of rhodopsin-like G protein-coupled receptors. The receptors consist of a single polypeptide that traverses the cell membrane seven times with three extracellular loops joining the transmembrane helices. The receptor has an intracellular carboxy-tail terminal domain and an extracellular amino-terminal domain. There are several conserved residues shared between the members of the rhodopsin-like receptor family. These residues are illustrated in white circles with black letters. In most rhodopsin-like receptors there is a disulfide bridge between extracellular loops 2 and 3, illustrated by black circles with white letters. *Taken from Gether et al.2000*

1.3.2 GPCR activation and regulation:

There is a high degree of diversity amongst the endogenous ligands that bind to GPCRs. Despite this high degree of diversity in ligands, GPCRs share a common activation process (Gether, 2000). Activation of GPCRs leads to initiation of signaling and transduction of the signal across the membrane which is mediated via interaction with a heterotrimeric G protein (Gether, 2000). The downstream effects of GPCR activation can be mediated by a number of different G proteins as well as non-G protein signaling molecules such as β-arrestin (Gether, 2000). Once the receptor is activated, it needs to be to be regulated and this is achieved by an interplay of receptor phosphorylation, internalization, desensitization and recycling.

1.3.2.1 GPCR activation

When an agonist binds to the GPCR, the agonist stabilizes a specific receptor conformation. The stabilization of this conformation can result in an activated receptor. The activation of the receptor in many cases involves the movement and rearrangement of transmembrane helices relative to one another as a result of constraining intramolecular interactions being relaxed (Karnik, 2003). These conformational changes allow the receptor to interact with a cytosolic G protein leading to a conformational change in the α-subunit of the G protein and the generation of a downstream signal transduction (Karnik, 2003). The process of agonist activation of GPCRs and subsequent signal transduction is thought to involve four main components, the agonist or ligand, the receptor, an intermediate guanyl nucleotide binding protein (G protein) and an effector protein (Gether, 2000; Karnik, 2003). The effector protein is the component responsible for generating a secondary messenger. The cytosolic G protein has a trimeric structure consisting of α, ß, and γ-subunits. The α-subunit is able to convert between an inactive GDP- bound and an active GTP-bound form. The GDP-bound form of the G protein is the inactive form and in this conformation all three subunits remain associated. Once the G protein is activated the bound GDP is released and it binds GTP. The GTP-bound α-subunit disassociates from the ßγ-dimer and is now free to interact with and activate an effector protein, which in turn catalyzes the formation of a secondary messenger (Karnik, 2003; Stryer, 2000). The ßγ-dimer is also able to modulate several signaling pathways (Karnik, 2003). The generation of the secondary messenger is terminated by the hydrolysis of GTP to GDP, the consequent inactivation of the G protein and the reassociation of the α-subunit with the βγ-dimer.

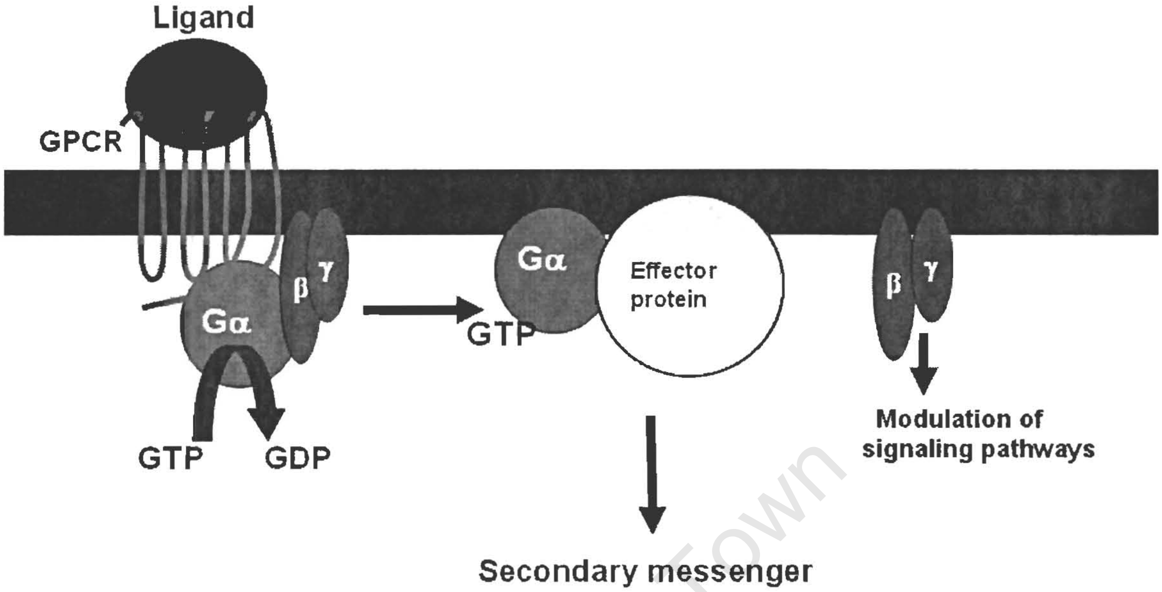


Figure 1.4: Signal transduction of GPCRs. Agonist binding selects for and stabilizes a receptor conformation able to interact with the cytosolic G protein. The inactive heterotrimeric G protein exchanges bound GDP for GTP. Once GTP is bound the α-subunit of the G protein disassociates from the βγ-subunit and activates an effector protein, which in turn catalyzes the formation of a secondary messenger. The βγ-subunit it also able to modulate several signaling pathways, including activation of phospholipases and ion channels.

Many GPCRs display a certain level of basal activity and are able to interact with and activate the G protein in the absence of bound agonist (Gether, 2000). It has been shown that conservative mutations can result in a big increase in the level of this basal constitutive activity, thereby increasing agonist-independent receptor activity (Gether, 2000). Lefkowitz and co-workers demonstrated that simply by replacing the alanine residue, $A^{6.34(293)}$, in the α_{Ib}-adrenergic receptor with any other amino acid residue resulted in an increase in agonist-independent receptor activation (Gether, 2000; Kjelsberg, 1992). These results suggested that receptors are constrained in an inactive state by a few intramolecular interactions which, when disrupted, results in a constitutively active receptor (Gether, 2000). In many GPCRs the separation of transmembrane helices 3 and 6 is common during the process of receptor activation and it has been shown that inhibiting the movement of these helices with the use of Zinc(II) bridges results in the inhibition of receptor activation in members of the rhodopsin-like and secretin families (Karnik, 2003; Sheikh, 1999).

1.3.2.2 GPCR desensitization, internalization and down-regulation:

In addition to the activation of G proteins, agonist activation of GPCRs also leads to the initiation of three important processes which include feedback regulation of the receptor through desensitization, internalization of the receptor as well the coupling of the receptor to signaling pathways independent of G protein activation (Ferguson, 2001). Desensitization is the process whereby the receptor becomes less responsive to continuous agonist stimulation and this process protects against acute and chronic overstimulation of the receptor (Carman, 1998; Ferguson, 2001). The mechanism of desensitization amongst GPCRs is fairly universal and requires the coordinated actions of three families of regulatory proteins, 2nd messenger dependant protein kinases, protein kinase A (PKA) and protein kinase C (PKC), GRKs and the arrestin proteins (Kohout, 2003). Once the receptor binds agonist there is a rapid decrease in the receptor responsiveness (Ferguson, 2001) which occurs as a result of these regulatory proteins. Firstly, the receptor is phosphorylated, on serine and threonine residues located in intracellular loops and the carboxy tail by GRKs, PKA or PKC (Carman, 1998; Ferguson, 2001). The GRK-mediated receptor phosphorylation allows translocation and binding of the adaptor protein, ß-arrestin, to the receptor. This physically occludes the interaction between the receptor and the G protein, uncoupling the receptor from the G protein and attenuating G protein-mediated signaling (Carman, 1998; Kohout, 2003). In addition to uncoupling from G proteins, ß-arrestin targets the receptor for internalization by recruiting clathrin-coated pits. As a consequence the number of receptors expressed on the cell surface is downregulated. Receptor downregulation also occurs by decreasing receptor mRNA and protein synthesis and by the lysosomal degradation of existing receptors (Ferguson, 2001). In most cases GPCR signaling is terminated at the level of the receptor, but this can also be done at the level of the G protein. Proteins known as regulators of G protein signaling (RGS) terminate signaling at the level of the G protein by increasing the rate at which GTP is hydrolyzed to GDP (Ferguson, 2001).

Receptor resensitization is also an important regulatory process, which ensures that the receptor does not remain desensitized to agonist stimulation for prolonged periods of time. It is thought that, in response to agonist stimulation, receptors are translocated to endosomes with GPCR-specific phosphatase activity (Ferguson, 2001). The receptors are therefore dephosphorylated and recycled back to the cell membrane.

1.3.3 Theoretical models of GPCR activation:

Several theoretical models have been developed describing the process of GPCR activation. These models have developed over time as more experimentally-based information has become available regarding the process of activation.

1.3.3.1 The Ternary Complex Model:

The Ternary Complex Model (TCM) is a theoretical model of GPCR activation developed in 1980 by De Lean and colleagues (DeLean et al., 1980). The TCM proposes that upon receptor activation in response to agonist binding, it is able to interact with a cytosolic membrane protein, the G protein. In addition the model states that the intrinsic activity of the agonist is highly correlated with the affinity of the G protein to the agonist-bound receptor. The interaction between the agonist, receptor and G protein results in the formation of a "high affinity ternary complex" which leads to the initiation of a signaling cascade (DeLean et al., 1980). The Ternary Complex model therefore implies that the unliganded form of the receptor cannot interact with the G protein. The limitations of this model were revealed by the discovery of constitutively active receptors, which were able to activate G protein-mediated signaling in the absence of bound ligand (Samama et al., 1993). As a result of these limitations the model was extended to the Extended Ternary Complex model proposed by Samama and colleagues (Samama et al., 1993).

1.3.3.2 The Extended Ternary Complex Model:

The Extended Ternary Complex model (ETCM) also referred to as the two-state model proposes that the receptor exists in an equilibrium of two functionally distinct states i.e. the inactive (R) and the active (R^*) state (Samama et al., 1993). In the absence of agonist the equilibrium is shifted towards R, but there still remains a fraction of receptors within the R^* state. R* has a higher affinity for agonist and binding of the agonist stabilizes and consequently shifts the equilibrium to R*. The R* state of the receptor is thought to be the only conformation of the receptor capable of interacting with the cytosolic G protein. Studies with the β_2-adrenergic receptor where a portion of the third intracellular loop was replaced with the corresponding region in the α_{1B}-adrenergic receptor, provided evidence of receptors able to exist in the R* state in the absence of agonist. The mutant receptor displayed activation of the effector protein in the absence of any agonist and was therefore able to interact with the G protein in the absence of bound agonist (Samama et al., 1993).

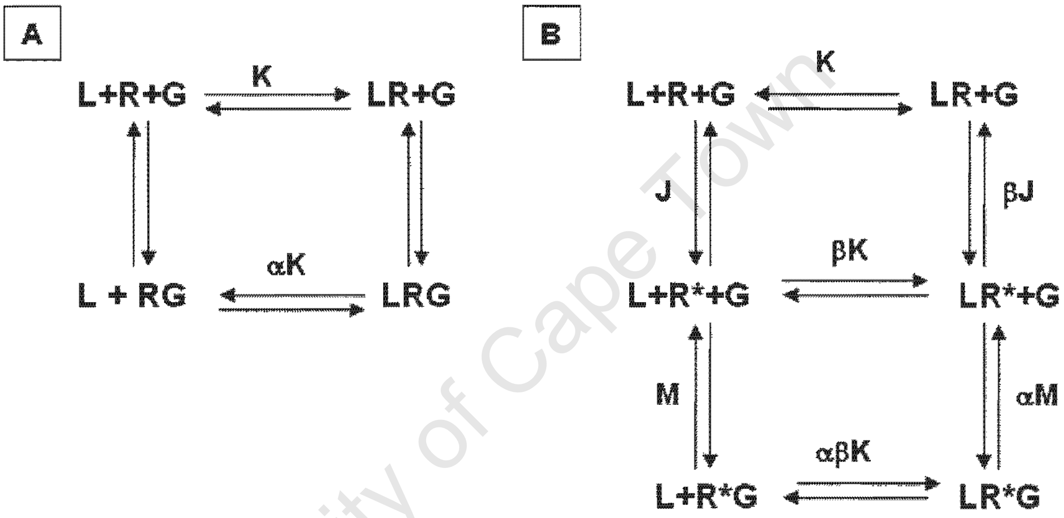


Figure 1.5 Models of receptor activation: A. The Ternary Complex model. L, Ligand; R, receptor; G, G protein. LRG represents the agonist promoted high affinity ternary complex which is a prerequisite for the activation of the effector protein. LR represents the low affinity complex and RX represents a complex that is spontaneously formed and which may be able to activate the effector protein. **B. The Extended Ternary Complex.** In this model there is an explicit isomerization of R to an R* intermediate. The R* intermediate is able to interact with the L and G in the same way as R in the TCM (Lefkowitz et al., 1993; Samama et al., 1993).

Further structural and functional studies on GPCRs have highlighted certain limitations of the ETCM. One such study looked at the effect of mutating certain serine residues in transmembrane helix 5 of the Dopamine D2 receptor, on receptor activation. The mutations resulted in the loss of functional coupling in response to certain agonists, but not to others (Wiens, 1998). These results suggest that there actually exist multiple active conformations or R* states of the receptor and that each of these conformations is stabilized by a different agonist (Wiens, 1998). In addition to this study it was also shown that synthetic agonists to the D1-like dopamine receptor selectively couple to distinct second messenger pathways, again suggesting the existence of multiple receptor conformations stabilized by different agonists and mediating distinct signaling pathways (Gether, 2000).

1.3.3.3 Multiple conformational state model of GPCRs

It is now generally accepted that GPCRs exist in an ensemble of conformations and different ligands selectively bind to and stabilize an ensemble of receptor conformations for which it has the highest affinity (Kenakin, 2002; Kenakin and Onaran, 2002; Weiss et al., 1996). The binding of the ligand will elicit the biological response corresponding to that particular ensemble of stabilized conformations and will therefore bias receptor activity (Kenakin, 2002). Each ensemble of receptor conformation represents proteins constantly folding and unfolding local regions and these proteins exists in microstates and each ensemble can be represented by a normal distribution of these microstates (Kenakin, 2002). Each biological response, such as G protein activation, internalization or phosphorylation, can be represented by an ensemble (Kenakin, 2002). There exists a degree of overlap between these ensembles and therefore ligand binding can elicit more than one biological response or efficacy. This model therefore eliminates the necessity for a common binding mode for different agonists to result in receptor activation (Gether, 2000). In addition to ligands binding and stabilizing certain ensembles of receptor conformation, point mutations of the receptor can have a similar effect. Mutational studies done by Lefkowitz and co-workers also support the view that receptors actually co-exist between multiple active conformational states (Kjelsberg, 1992). The group found that when substituting A293 in the α_{1B}- adrenoreceptor with any one of the twenty amino acids, each substitution resulted in an active mutant receptor as indicated by the levels of constitutive activity (Kenakin, 2003; Kjelsberg, 1992). Unless one assumes that the resultant mutant receptors have identical tertiary conformations, the work done provides evidence of different conformations of the receptor capable of exposing the regions necessary to facilitate interaction with the G protein (Kenakin, 2003).

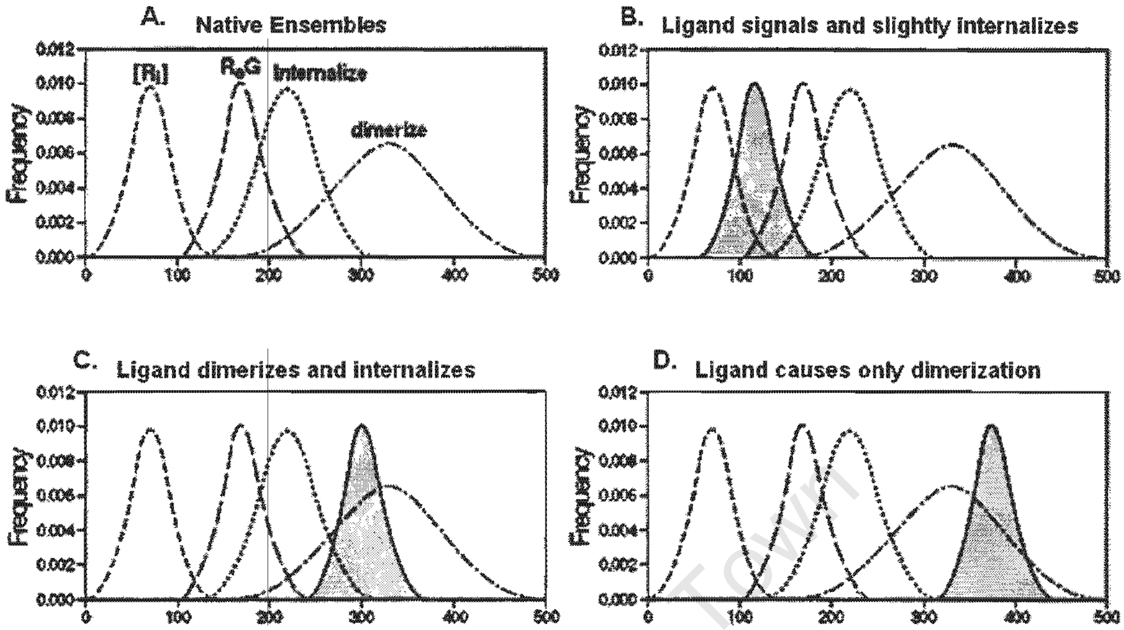


Figure 1.6 Ensembles of receptor conformations. A. Four ensembles of receptor conformations each representing a receptor state. $[R_i]$ represents the inactive state, R_aG represents the receptor state able to bind to the G protein and initiate a response followed by an ensemble able to internalize and an ensemble able to dimerize. **B.** The hypothetical ligand (grey curve) stabilizes an ensemble of conformations which overlaps with the signaling and internalization ensemble. **C.** This hypothetical ligand (grey curve) stabilizes an ensemble of receptor conformations overlapping with the internalization and dimerization ensembles. **D.** This hypothetical ligand (grey curve) stabilizes an ensemble of receptor conformations only able to dimerize. *Taken from (Kenakin, 2002).*

One of the earlier studies to provide us with evidence for agonist selective receptor conformation ensembles was that of transfection studies (Perez, 2005). These studies were carried out with the type-1 pituitary adenylyl cyclase-activating polypeptide (PACAP) receptor, using the cognate ligands PACAP-27 and PACAP-38 (Spengler et al., 1993). Both of these ligands were able to stimulate the effector protein adenylate cyclase with equal potencies, but only PACAP-38 could activate phospholipase C and induce the inositol phosphate (IP) response (Spengler et al., 1993). These results demonstrate two agonists stabilizing different ensembles of receptor conformations as indicated by the divergent physiological responses elicited. Another related example is demonstrated by the serotonin receptor, 5-HT$_{2c}$, which is able to couple to two divergent signaling pathways in Chinese Hamster Ovary (CHO) cells. The receptor is able to interact with the secondary messenger, phospholipase C, and result in the production of IP as well as phospholipase A2 which results in the release of arachidonic acid (AA) (Berg et al., 1998). The relative efficacy of 5-HT$_{2c}$ agonists, DOI (1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane) and quipazine is reversed depending on the signaling pathway surveyed. In the case of AA release, DOI produces a higher maximal stimulation and therefore has a higher efficacy than quizapine; while when measuring IP production the maximal efficacies of the agonists are reversed (Berg et al., 1998). This suggests that the receptor is not interacting with the agonists in an identical manner and that the active conformation stabilized by DOI is distinct from that stabilized by quipazine (Kenakin, 2001).

Multiple G protein couplings in the same receptor are another indication of multiple conformational receptor states. One such example is that of human dopamine 2 long (D2L) receptor which has been shown to interact with the pertussis toxin sensitive G proteins, G$_{i/o}$. The D2L receptor is able to selectively couple to G$_{i/o}$ proteins including G$\alpha_{1\text{-}3}$ and Gα_o and the G protein specific signaling cascade, depending on the agonist used (Gazi et al., 2003). Fluorescent studies have been a very useful tool in visualizing conformational changes of the receptor as seen by spectral changes resulting from shifts in protein helices or changes in protein-protein contacts (Perez, 2005). Studies were carried out with the purified preparations of the ß$_2$- adrenoreceptor where a fluorophore was attached to the cysteine 265 residue, located in the third intracellular loop (Ghanouni, 2001). Distinct conformations were visible when the receptor was bound to a full agonist and this conformation was visibly different from one where the receptor was bound to a partial agonist (Ghanouni, 2001).

Protean agonism provides additional evidence for the existence of multiple activated states. Protean agonists can result in positive or negative agonism depending on the system in which it is observed. (Kenakin, 2001; Kenakin, 2002). A single agonist may therefore result in either positive or negative agonism, depending on the signaling pathway assessed and it is this reversal of agonism that resulted in the term "protean agonism" after the Greek God "Proteus" who could change his shape and appearance at will (Kenakin, 2001). If the GPCR system displays no constitutive activity i.e. most of the receptors are in the inactive conformation, then the ligand will result in an increase of activity by shifting the equilibrium of receptors from the inactive (R) state to the active

(R*) state and this is observed as positive agonism. If the agonist is applied to a system where the majority of the receptors are already in a spontaneously adopted active conformation i.e. constitutively active, the agonist will result in the decrease of activity by shifting the equilibrium from the highly efficacious constitutively active state to the agonist-induced active conformation of lower efficacy and this is observed as negative agonism (Kenakin, 2001). This negative or inverse agonism suggests that the receptor is capable of forming at least two active receptor conformations i.e. the spontaneously formed constitutively active conformation and agonist-induced active conformation of lower efficacy (Kenakin, 2001; Kenakin, 2002).

The idea of multiple conformational states of the receptor which is ligand-selective opens up the possibility of designing drugs to block certain conformations of the receptor and as result also selectively inhibit a particular pathway or aspect of the receptor behavior (Kenakin, 2003). This idea becomes particularly relevant in the case of the chemokine receptor CCR5. CCR5 is involved in a number of natural physiological and immunological processes and also serves as the point of entry into host cells for R5-tropic and dual-tropic HIV-1 strains. Therefore it would be ideal to block the CCR5 receptor conformation mediating HIV-1 entry without affecting the conformation mediating effects as a result of the binding of chemokines to the receptor (Kenakin, 2003).

1.4 CCR5 GPCR and HIV-1 Co-receptor Function:

1.4.1 CCR5 structure:

CCR5 has a molecular mass of 40.6 kilodaltons (kDa) and contains a total of 352 amino acids (Oppermann, 2004). A high-resolution crystal structure is not currently available for any GPCR besides Rhodopsin (Oppermann, 2004; Palczewski et al., 2000) and the β2-adrenergic receptor (Cherezov et al., 2007; Rasmussen et al., 2007). Therefore to infer information about the 3D structure of CCR5, the 3D structure of the Rhodopsin receptor was used as a template (Oppermann, 2004). CCR5 has a typical conserved GPCR structure, consisting of seven transmembrane helices, an extracellular amino-terminal domain and a cytosolic carboxy-terminal domain. There are seven conserved residues in each transmembrane helix of GPCRs and these residues are used as reference residues in the GPCR numbering scheme (Ballesteros, 1995). CCR5 contains all seven of these reference residues (Fanelli and De Benedetti, 2005). A common GPCR structural motif is the presence of conserved cysteine residues, particularly in the extracellular loop regions. These cysteine residues are thought to play an important role in maintaining the structural integrity of the receptor to facilitate ligand binding (Blanpain et al., 1999b). CCR5 has two conserved cysteines in the first and second extracellular loops, which form a disulfide bridge, as well as two additional cysteine residues which are thought to form a disulfide bridge between the N-terminus and the third extracellular loop. Both of these disulfide bridges are thought to facilitate packaging, thereby stabilizing the receptor in a conformation able to bind ligand (Oppermann, 2004; Stantchev and Broder, 2001). All four of the cysteine residues were mutated to alanine in the CCR5 receptor and the mutant receptors were unable to bind chemokine, had decreased cell surface expression and impaired HIV co-receptor function (Blanpain et al., 1999b; Stantchev and Broder, 2001). CCR5 contains the GPCR conserved NPxxY motif in transmembrane helix 7, which is thought to play a role in receptor activation as well as the highly conserved DRY-motif which is a conserved motif amongst rhodopsin-like receptors, located at the border between the third transmembrane helix and the second intracellular loop. The arginine residue is the only residue conserved amongst all the rhodopsin-like receptors (Gether, 2000). The DRY-motif has been implicated in stabilizing the inactive conformation of the receptor (Ballesteros et al., 1998). It was shown that a CCR5 mutant with the arginine residue of the DRY-motif mutated to asparagine, $R^{3.50(126)}N$, failed to activate the G protein and activate β-arrestin mediated chemotaxis (Lagane et al., 2005).

In addition to the broadly conserved GPCR structure, chemokine receptors share the conserved amino acid sequence, DRYLAVHA, in the second intracellular loop, which is thought to play a role in G protein interaction and a short third intracellular loop enriched in basic residues (Murphy et al., 2000; Oppermann, 2004) CCR5 also contains another conserved motif amongst chemokine receptors, located within the second transmembrane helix of chemokine receptors, the Threonine – X – Proline (TXP) motif, thought to be an important structural feature in receptor activation (Govaerts et al., 2001; Oppermann, 2004). Chemokine receptors have a number of serine and threonine residues in the carboxy-terminal tail, that are most likely phosphorylated after receptor activation by protein kinases, resulting in receptor desensitization (Murphy, 1996). In addition, to facilitate cell surface expression, three structural requirements in the C-terminal tail have been described which include the length of the C-terminal tail, the presence of a cysteine cluster and a amino acid domain rich in basic residues (Venkatesan et al., 2001).

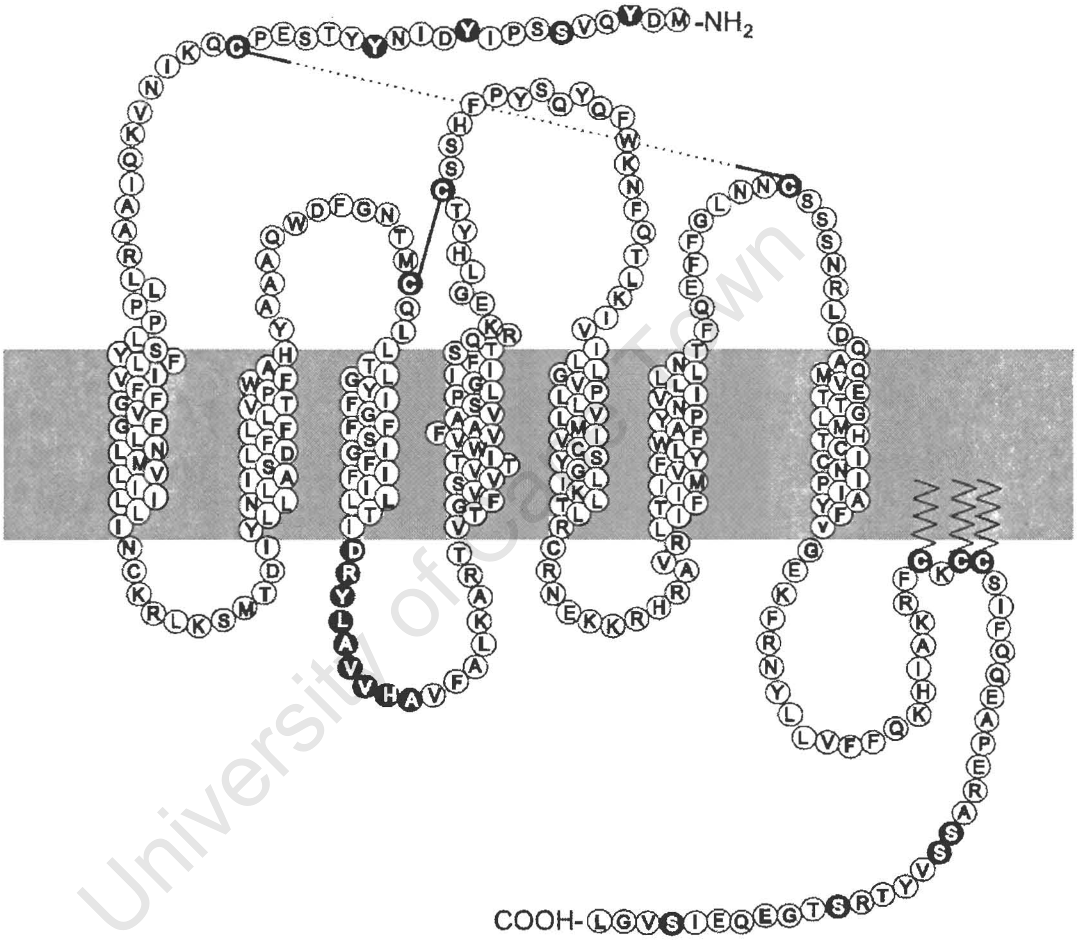


Figure 1.7: A two dimensional model of the CCR5 receptor. CCR5 has a typical GPCR structure with the N-terminal domain, three extracellular loops, seven transmembrane helices, three intracellular loops and a C-terminal domain. Conserved residues are depicted in black circles with white letters. *Figure taken from Opperman, 2004.*

1.4.2 CCR5 signaling pathways:

1.4.2.1 Chemokine activation

The natural ligands of CCR5 include the chemokines, macrophage inflammatory protein 1-α (MIP-1α, CCL3) and –ß (MIP-1ß, CCL4), monocyte chemoattractant protein-2 (MCP-2, CCL8) and Regulated upon activation normal T-cell expressed (RANTES, CCL5). LD78α (CCL3) and LD78ß (CCL3-L1) are two nonallelic isoforms of the MIP-1α protein, the sequences of which differ by only three amino acids (Aquaro et al., 2001). The LD78ß isoform was shown to be the most potent chemokine in the induction of intracellular calcium signaling and chemotaxis as well as in the inhibition of CCR5-dependant HIV-1 replication (Aquaro et al., 2001; Menten et al., 1999; Nibbs et al., 1999). The anti-HIV activity was shown to be achieved by the downregulation of the surface expression of CCR5 (Aquaro et al., 2001). LD78ß showed similar levels of viral inhibition to RANTES, previously reported to have the highest anti-HIV activity against R5-tropic primary isolates (Trkola et al., 1998), at a concentration five times lower than RANTES and exhibited a five-fold lower EC_{50} value than the LD78α isoform of MIP-1α (Aquaro et al., 2001). Further characterization of LD78β showed that a naturally occurring variant with the first two amino terminal residues truncated by the protease dipeptidyl peptidase IV (DPP IV), LD78β(3-70), exhibited enhanced anti-HIV activity when compared to the full length LD78β(1-70) (Struyf et al., 2001). It was reported that LD78β(3-70) achieved 50% inhibition of viral replication at a concentration less than half that required for the LD78β(1-70) isoform to achieve the same level of inhibition (Struyf et al., 2001). Competition binding assays demonstrated that LD78β(3-70) had a 10-fold increase in affinity for CCR5 when compared to LD78β(1-70) and this was the most likely reason for the increased anti-viral potency of the isoform (Struyf et al., 2001).

It is proposed that during the process of chemokine activation of the receptor, the N-loop region makes initial contact with the receptor and this is then followed by the interaction of the chemokines N-terminus with the receptor (Blanpain et al., 2003; Govaerts et al., 2003). Chemokines bind to the N-terminus of the receptor and also make contact with one or more of the extracellular loops of the receptor (Stantchev and Broder, 2001). In the case of CCR5, the second extracellular loop of the receptor has been shown to play an important role in determining ligand specificity (Samson et al., 1997). Studies with chimeras of the CCR5 receptor with the CCR2b receptor have implicated a vital role for the second extracellular loop of CCR5 in specific agonist recognition (Samson et al., 1997). Chimeras were created with CCR5 and CCR2b, two chemokine receptors which share a high degree of homology, but share no overlap in agonist repertoire (Samson et al., 1997). Chimeras were created where the NH_2-terminus and extracellular loops were individually replaced with the CCR2b corresponding region. When the first extracellular loop of the CCR5 receptor was replaced with than of CCR2b (5255), the receptor was still able to bind MIP-1α. However when the second extracellular loop was replaced with that of CCR2b (5525), MIP-1α binding was completely inhibited, suggesting an important role of this region in the recognition of the chemokine. This role was further supported with the observation that when the second extracellular loop of CCR5 was placed within an entirely CCR2b context (2252) the receptor was able to bind MIP-1α with the same affinity as the wild type CCR5 receptor (Samson et al., 1997). In addition the biological response of the mutant receptors correlated with the binding data. Metabolic activity in response to chemokines MIP-1α, -ß and RANTES, as measured with a microphysiometer, was compared to baseline activity in the absence of added chemokines. Mutant receptors 5255 and 2252 showed a percentage increase in activity when compared to baseline activity, while mutant receptor 5525 showed no increase at all (Samson et al., 1997). Therefore the second extracellular loop is not only important for the recognition and binding of CCR5 chemokines, but is also important in mediating the resultant cellular responses (Samson et al., 1997).

Following chemokine activation of CCR5, there are proposed conformational changes in the transmembrane regions of the receptor and subsequent exposure of residues in the intracellular loops of the receptor that mediate interaction with a cytosolic G protein which is sensitive to inhibition by the pertussis toxin i.e. the Gi protein (Oppermann, 2004). The Gi protein can be linked to two effector proteins, adenylate cyclase and phospholipase C (PLC), and these effector proteins can be regulated by either the αi-subunit or the ßγ-dimer of the Gi protein (Oppermann, 2004). Once the Gi protein binds the receptor it exchanges the bound GDP nucleotide for GTP. This exchange leads to the release of the GTP-bound αi- subunit of the receptor. The αi- subunit then binds to and inhibits the protein adenylate cyclase, which leads to an inhibition of cyclic AMP (cAMP) production and the mobilization of intracellular calcium.

1.4.2.2 CCR5 desensitization, internalization and resensitization:

In addition to intracellular signaling, chemokine activation of CCR5 leads to the subsequent regulation of receptor activity by the process of receptor desensitization. Both GRKs and PKC mediate receptor regulation via the process of receptor phosphorylation. In addition to PKC, CCR5 is also phosphorylated by the serine threonine protein kinases i.e. G protein-coupled receptor kinases (GRKs) (Oppermann, 2004). These carboxy-terminal serine residues need to be phosphorylated in order to facilitate binding of β-arrestin (Huttenrauch et al., 2002; Kraft et al., 2001). Interaction with β-arrestin leads to receptor desensitization, by the inhibition of receptor-G protein interactions, and to clathrin-mediated endocytosis (Oppermann, 2004). Studies have shown that internalization of CCR5 is dependant on the expression of both GRK as well as ß-arrestins i.e. ß-arrestin-1 and ß-arrestin-2 (Aramori et al., 1997). Co-expression of CCR5, GRK-2 and dominant negative mutants of ß-arrestin i.e. ß-arrestin-1-V53D and ß-arrestin-2-V54D, which are unable to promote receptor internalization, inhibited receptor internalization (Aramori et al., 1997). After receptor internalization, CCR5 is recycled back to the cell surface in a dephosphorylated form. Desensitization and internalization of CCR5 in response to chemokine activation has been well established and receptor internalization leading to decreased cell surface expression of CCR5 is one of the mechanisms of chemokine inhibition of HIV infection (Alkhatib et al., 1997; Brandt et al., 2002). However studies have indicated that the role of the CCR5 as a HIV-1 co-receptor is not dependant on its ability to be desensitized and internalized (Aramori et al., 1997). To assess the need for desensitization and internalization of CCR5 for its function as a co-receptor, a CCR5 mutant receptor was constructed, $Y^{7.53(297)}A$, which displayed impaired receptor signaling and internalization, even when co-expressed with GRK2 (Aramori et al., 1997). Despite impaired signaling and internalization, the mutant receptor was able to support HIV-1 fusion and internalization (Aramori et al., 1997). Although internalization is not essential to CCR5 co-receptor function, the amount of receptors expressed on the cell surface does have an effect on the efficiency of HIV-1 fusion and infection (Lin et al., 2002; Reynes et al., 2000). This is clearly illustrated by the high resistance to HIV-1 infection by individuals expressing the CCR5 mutant with a 32-base pair deletion (Δ-32) which results in mutants not expressed on the cell surface (Dean et al., 1996; Samson et al., 1996b).

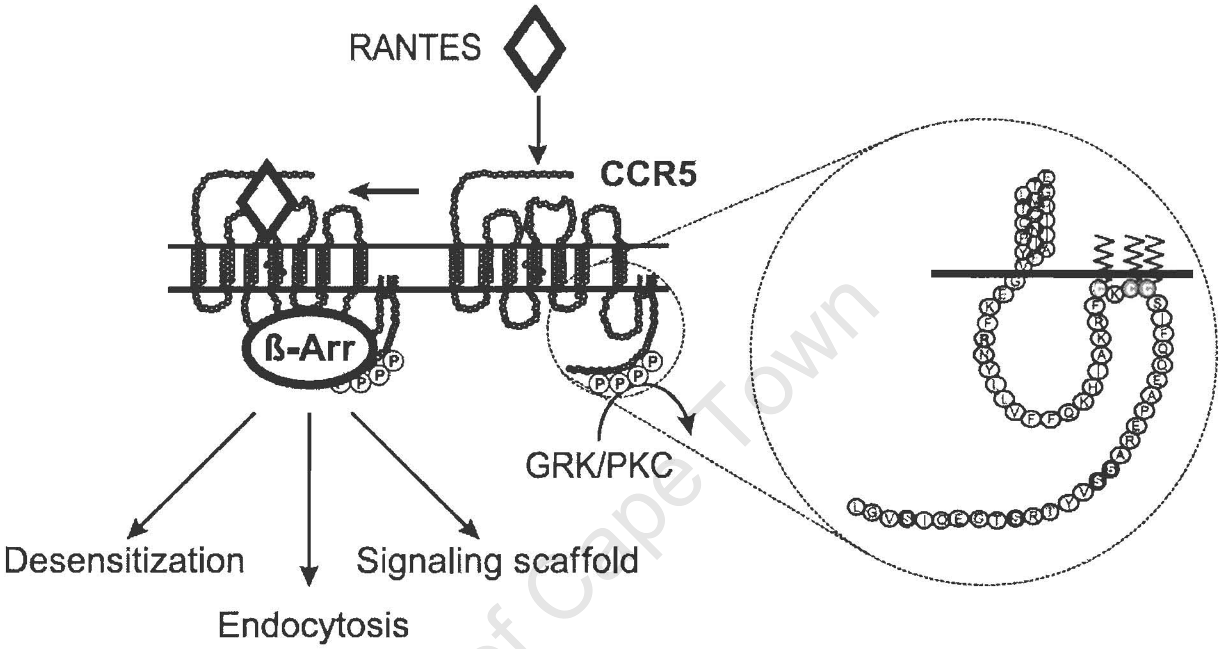


Figure 1.8: Desensitization and endocytosis of the CCR5 receptor. This diagram illustrates the sequence of events leading to receptor desensitization and endocytosis of CCR5. RANTES binds to CCR5 and leads to phosphorylation of serine residues (black circles) by receptor kinases, GRK and PKC. ß-arrestin binds to the phosphorylated receptor and occludes the binding of the G protein thereby uncoupling the receptor from G protein activation. B-arrestin also acts an adaptor protein recruiting clathrin and initiating clathrin-mediated endocytosis. *Figure taken from Opperman, 2004*

1.4.2.3 CCR5 dimerization

There have been a number of functional and biochemical studies that have suggested that CCR5 can form both homo- and heterodimeric structures (Blanpain et al., 2002; Kenakin, 2002; Kuhmann et al., 2000; Rodriguez-Frade et al., 2001; Vila-Coro et al., 2000). The concept of CCR5 oligomerization has become particularly relevant since the suggestion that CCR5 dimerization may prevent HIV infection (Vila-Coro et al., 2000). This suggestion was based on a study where an anti-CCR5 mAb was found to potently inhibit HIV-1 infection. The mAb was generated by immunizing mice with synthetic peptides which corresponded to various extracellular domains of hCCR5. The antibody, CCR5-02, which blocked HIV-1 infection was specific to the N-terminal domain of CCR5. CCR5-02 did not affect RANTES binding or RANTES-induced responses, therefore it did not compete with chemokine binding to the receptor. CCR5-02 did not trigger a Ca^{2+} flux or affect binding of the M-tropic JFRL gp120 envelope protein (Vila-Coro et al., 2000). It was therefore proposed that the antibody inhibited HIV-1 infection by inducing receptor dimerization (Vila-Coro et al., 2000). CCR5-transfected Human Embryonic Kidney 293 (HEK 293) cells were treated with RANTES or CCR5-02 and then crosslinked with 2mM disuccinimidyl suberate (DSS). Immunoprecipitation of the cell lysates with a different mAb, CCR5-03, detected a high molecular mass receptor species of approximately 75kDa, corresponding to the molecular mass of two CCR5 molecules (Vila-Coro et al., 2000).

1.4.2.4 The HIV-1 envelope protein as a ligand:

As in the case of chemokine binding to the CCR5, the envelope protein of HIV-1 is also able to activate the Gi protein mediated signaling cascade (Davis et al., 1997). In order to assess the ability of Env to stimulate this signaling cascade, the phosphorylation status of a protein tyrosine kinase, Pyk2, in response to chemokine CCR5 activation as well as envelope binding to CCR5 was evaluated. Pyk2 is downstream of the CCR5 receptor and it is phosphorylated after the ligand activation of GPCRs (Davis et al., 1997). Human Leukemia cells (HL60) expressing CCR5 were treated with RANTES and MIP-1ß and it was observed that RANTES rapidly induced Pyk2 phosphorylation. In addition the phosphorylation of Pyk2 in response to RANTES was abrogated by the pre-treatment of cells with the pertussis toxin, indicating that the phosphorylation of Pyk2 was mediated by the Gi protein (Davis et al., 1997). In addition, 293T cells were transiently transfected with envelope proteins from both the R5-tropic isolate, JRFL, and the X4 viral isolate, HXB2. The Env-expressing 293T cells were mixed with HL60 cells, which are able to fuse with X4-tropic envelopes, or with DU6 cells, which allow fusion of both X4- and R5-tropic envelope proteins (Davis et al., 1997). When 293T cells expressing JRFL were mixed with HL60 cells, Pyk2 was not phosphorylated, but was phosphorylated when JRFL expressing 293T cells were mixed with DU6 cells. In the case of HXB2 expressing 293T cells, phosphorylation of Pyk2 occurred both when mixed with HL60 and DU6 cells (Davis et al., 1997). These results indicate that fusion of the envelope protein to the chemokine co-receptor does result in the activation of signaling cascades typically induced by chemokine activation of CCR5.

Mutants of the CCR5 receptor impaired in their function to initiate Gi mediated signaling, nevertheless were still able to support HIV-1 internalization (Gosling et al., 1997). The cytoplasmic tail of the receptor was truncated to 8 amino acids by the introduction of a stop codon after the Leu amino acid in position 398 (CCR5Δ2) (Gosling et al., 1997). This mutation was proposed to impair the interaction of the receptor via critical cytoplasmic regions with the Gi protein. As a result the mutant receptor, when co-transfected with chimeric Gqi5, failed to produce inositol phosphate, failed to mobilize intracellular stores of calcium in response to agonist binding and failed to inhibit the effector protein adenylate cyclase. These processes are typical of chemokine mediated responses of CCR5 and therefore indicate impaired chemokine receptor function. However, when the mutant CCR5 receptor was incubated with the HIV-1 strain, BaL, it was still able to support internalization of the virus at levels comparable to the wild type receptor, thereby indicating that the co-receptor function of the mutant was not in any way affected (Gosling et al., 1997). Similarly a CCR5 mutant with amino acid changes in the DRY-motif ($D^{3.49(125)}$G; $R^{3.50(126)}$G; $Y^{3.51(127)}$A; $F^{3.59(135)}$A), proposed to play a critical role in GPCR activation, bound chemokines MIP-1α and MIP-1ß with lower affinity than wild type CCR5 and when co-transfected with Gqi5, failed to produce measurable levels of inositol phosphate and initiate mobilization of intracellular calcium, but was unaffected in its ability to support the internalization of HIV-1 (Gosling et al., 1997).

Mutational studies by Farzan et al. (1997) support the work done by Gosling et al. Mutations in CCR5 where the arginine residue in the DRY-motif was mutated to asparagine ($R^{3.50(126)}$N) and the aspartate residue was mutated to asparagine ($D^{3.49(125)}$N), resulted in receptors that were unable to mobilize internal stores of calcium after binding of MIP-1ß. Despite the inability to activate the G protein, the mutants were capable of supporting HIV-1 entry at levels comparable to the wild ~~type receptor (Farzan et al.,~~ 1997). Similar results were reported when CCR5 receptors with the $R^{3.50(126)}$N mutation were expressed in T-lymphocytes and macrophages derived from individuals with the homozygous 32-base pair deletion in their CCR5 gene (Δ-32) (Amara et al., 2003). These studies lead to the conclusion that receptor signaling is not required for HIV-1 fusion and entry into host cells and that the chemokine-activated CCR5 conformation and the CCR5 HIV-1 co-receptor conformation are distinct conformations.

1.4.2.5 Determinants of CCR5 co-receptor function:

It is not very well established how CCR5 facilitates Env-mediated fusion of R5-tropic isolates, however important structural determinants both in the co-receptor and gp120 subunit of the envelope protein have been described. Attempts to identify determinants of the hCCR5 co-receptor include studies using chimeric CCR5 co-receptors which comprise of segments of murine CCR5 (mCCR5) or divergent chemokine receptors such as CCR2b, which are unable to mediate HIV infection (Dragic, 2001). mCCR5 and hCCR5 have an 82% amino acid homology and have identical third extracellular loops (Doranz et al., 1997). The two receptors differ by 20 extracellular amino acid residues and this difference renders mCCR5 incapable of supporting fusion and infection of host cells by any HIV-1 isolate tested (Ross et al., 1998). Chimeric hCCR5/mCCR5 co-receptors have therefore been a useful tool in trying to identify which amino acid residues or domains of hCCR5 are able to confer co-receptor activity onto mCCR5. Chimeric hCCR5/mCCR5 receptors were generated by individually substituting the first, second and third extracellular domain of hCCR5 in an entirely mCCR5 context (HMMM, MHMM and MMHM respectively) (Ross et al., 1998). The chimeric constructs were co-cultivated with cells expressing R5-tropic envelope proteins (ADA, BaL and YU2), a dual tropic envelope (89.6) and a X4-tropic envelope (IIIB) (Ross et al., 1998). R5-tropic viruses varied in their ability to use the different hCCR5/mCCR5 chimeras, while the dual tropic isolate 89.6 and the R5-tropic isolate, BaL, were unable to use any of the chimeras (Ross et al., 1998). This finding indicated that although viral isolates make use of the hCCR5 co-receptor to fuse and infect host cells, they still differ in terms of the specific domains recognized by the envelope protein. There was a degree of functional redundancy between the three extracellular domains of hCCR5, as illustrated by the ability of ADA and Yu2 to effectively use all three chimeras to mediate host cell fusion and infection (Ross et al., 1998). Since the mCCR5 and hCCR5 have identical third extracellular loops, it was suggested that determinants of co-receptor activity were located in the first or second extracellular loops of hCCR5 (Doranz et al., 1997).

In an attempt to identify residues important to co-receptor activity, all of the fifteen charged amino acids located in extracellular regions of hCCR5 were individually mutated to uncharged Ala. No single mutation completely inhibited co-receptor activity and the most notable reduction in co-receptor activity occurred with the mutation of Asp at position 11 to Ala. This residue is located within the amino-terminal of CCR5 and resulted in the reduction of co-receptor activity by 50% when compared to the wild type receptor (Doranz et al., 1997). When the Ala mutations were combined in a single receptor, the most significant reductions occurred when Asp11Ala was combined with Lys197Ala, located in the second extracellular loop, and with Asp276Ala, located in the third extracellular loop. These two double mutants D11A/K197A and D11A/D276A showed reductions of 20-40% in co-receptor activity when compared to the wild type receptor, and when all three mutations were combined in a single receptor complete inhibition of co-receptor activity occurred (Doranz et al., 1997).

In other studies the first 20 amino acid residues of hCCR5 were introduced in more divergent chemokine receptors i.e. CCR1, CXCR2 and CXCR4, which were shown not to support Env fusion, and the transfer of these amino acids was enough to confer co-receptor activity to these receptors (Doranz et al., 1997). Truncation mutants of the amino-terminal domain exhibited decreased binding affinity for R5-tropic envelope proteins (Blanpain et al., 1999a). Env-binding affinity decreased by greater than 80% when amino-terminal residues 2-5 were truncated (Blanpain et al., 1999a). In addition the sequential truncation of amino-terminal residues also impaired the ability of the co-receptor to mediate HIV-1 fusion and infection of host cells (Blanpain et al., 1999a). Site-directed mutagenesis of charged amino acid residues located in the N-terminal domain to Ala, identified several residues that played an important role in Env-binding affinity. These charged aromatic residues included Asp-2, Asp-11 and Glu-18 (Blanpain et al., 1999a). The important role played by these residues was confirmed by another study which made use of alanine-scanning mutagenesis (Dragic et al., 1998). These three residues were mutated to alanine individually or in combination and in each case Env-mediated fusion was abrogated. In addition only Asp-11 was found to be important for CC-chemokine mediated inhibition of HIV-1 entry (Dragic et al., 1998). These findings suggest that the N-terminal domain of hCCR5 does indeed play a role a vital role in facilitating Env-mediated fusion. Chimeric studies, on the other hand, have shown that when replacing the N-terminal domain of CCR5 with divergent chemokine receptors or with that of the murine chemokine receptor, there was no significant decrease in co-receptor function (Bieniasz et al., 1997; Doranz et al., 1997; Picard et al., 1997). This suggests that the amino-terminal domain may function in concert with other extracellular domains, such as extracellular loops one and two, in mediating co-receptor activity or that the key residues in these divergent co-receptors are probably conserved to facilitate chemokine binding.

The results from these studies are difficult to interpret and do not define distinct amino acid residues or domains in hCCR5 that are essential for co-receptor function. Part of the difficulty in the interpretation of the results is the inherent differences in the methodology employed, such as the chimeras generated, different viral isolates and the variety in receptor systems. Another shortcoming with the use of receptor chimeras is that conserved regions between the receptors can be easily overlooked and therefore not be taken into consideration when investigating important structural determinants of co-receptor function (Blanpain et al., 1999a). However, despite the discrepancies, preliminary conclusions about the determinants of CCR5 co-receptor function can be drawn. Firstly, the important amino acid residues seem to be located on the three of the four extracellular domains; however these residues are not localized at one specific point. Secondly, it is clear that different HIV-1 envelope proteins recognize different hCCR5 epitopes. Lastly, it is also evident that some hCCR5 residues are also functionally redundant and can independently mediate HIV-1 fusion when expressed within a divergent chemokine receptor or murine chemokine receptor context (Ross et al., 1998).

1.4.3 Constitutively active mutants of GPCRs and CCR5

Constitutively active mutant (CAM) receptors are proposed to be impaired in their ability to constrain the cytosolic peptide regions from interaction with the G protein and as a consequence CAMs characteristically have a higher basal activity, in the absence of agonist, when compared to the wild type receptor. It is thought that CAMs display a shift in the equilibrium from R to R^*, thereby increasing the number of receptors capable of interacting with the G protein and activating a signal transduction cascade, in the absence of bound agonist (Tiberi and Caron, 1994). In addition these CAMs also have a higher affinity for agonist and an increased coupling efficiency to signaling molecules (Samama et al., 1993; Tiberi and Caron, 1994) . According to the ensemble model of protein conformations constitutive activity results from proteins stabilized in microconformations which overlap between the inactive state and the active state of the receptor which is able to signal to G proteins (Kenakin, 2002).

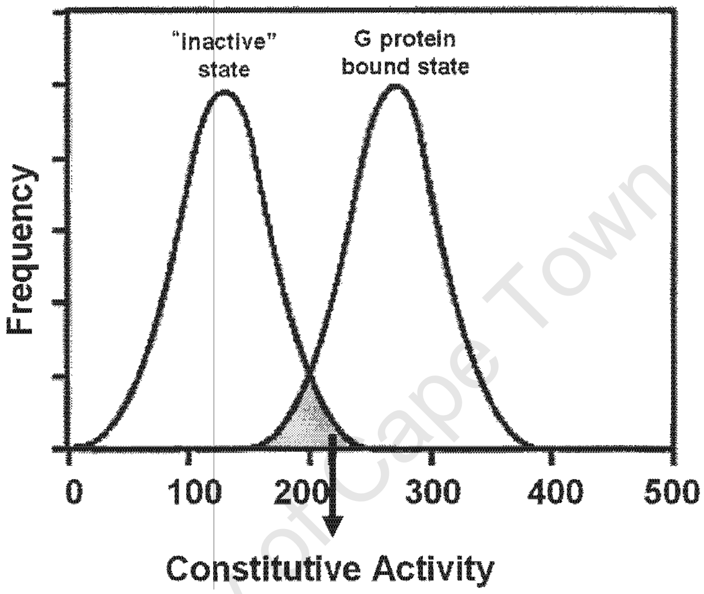


Figure 1.9: The inactive and G protein bound ensembles. The two curves represent two receptor ensembles; the inactive and G protein bound state. The shaded region represents receptor microstates common to both states which are able to spontaneously activate the G protein i.e. constitutive activity. *Taken from (Kenakin, 2002).*

CAMs may be particularly relevant in the CCR5 co-receptor context, since the report that CCR5 dimers may be impaired in their ability to support HIV-1 fusion and infection of host cells (Vila-Coro et al., 2000). The formation of CCR5 homodimers have been reported in response to chemokine activation (Blanpain et al., 1999a; Kenakin, 2002; Kuhmann et al., 2000; Rodriguez-Frade et al., 2001), therefore implying that the chemokine-activated CCR5 conformation may be less able to mediate HIV-1 fusion. CAMs of CCR5 are essentially "stuck" in the activated conformation and therefore may also be constitutively dimerized. Constitutively activated and dimerized receptors could therefore be proposed to be less efficient at mediating HIV-1 fusion.

Structural studies of a β_2-adrenergic CAM receptor (Lefkowitz et al., 1993) have suggested that CAMs are characteristically unstable proteins (Gether et al., 1997). Conformational changes of the CAM, both in the absence and presence of agonist, were visualized by labeling the receptor with the cysteine-reactive fluorophore, IANBD (Gether et al., 1997). When the β_2-adrenergic receptor is activated in response to agonist binding there is a consequent decrease in fluorescent activity and the degree of change in activity is positively correlated with the intrinsic efficacy of the agonist, suggesting that the decrease in fluorescence is indicative of a conformational change. The decrease in fluorescence also suggests that upon activation one or more labeled cysteine residues are exposed to a more polar environment (Gether et al., 1995). The β_2-adrenergic CAM displayed much greater changes in fluorescence in response to activation with the full agonist, isoproterenol or partial agonist, salbutamol, when compared to the wild type receptor, suggesting that the CAM was a lot more conformationally flexible (Gether et al., 1997). In addition the CAM was poorly expressed when compared to the wild type receptor and treatment with agonists, inverse agonists or neutral agonists enhanced expression equally well. The similar effect of these pharmacologically different ligands suggested that the decreased expression of the CAM was not as a result of constitutive activation and therefore constitutive downregulation, but rather as a result of downregulation and enhanced degradation of a structurally unstable protein (Gether et al., 1997). This hypothesis was further supported by the 4-fold increase in the rate of denaturation of the CAM at 37°C when compared to the wild type receptor (Gether et al., 1997). Many congenital as well as acquired conformational diseases have been caused by the intracellular retention of structurally unstable CAM receptors by the ER, these include diabetes insipidus, retinitis pigmentosa and familial obesity (Leskela et al., 2007; Morello et al., 2000). The relevance of structurally unstable receptors in pathological diseases has led to work being done to try and stabilize the folding of these receptors in an attempt to enhance cell surface trafficking and expression of the receptor.

1.4.3.2 Enhancing cell surface expression of GPCRs in the presence of molecular chaperones

The endoplasmic reticulum serves as a quality control check before transporting receptors to the cell surface and this quality control check is dependant on conformational criteria (Ellgaard et al., 1999). CAMs result from changes in the primary structure of the wild type receptor and are therefore conformationally different to the wild type receptor. The CAM receptor may still be fully or partially functional but the changes in conformation can result in the receptor not being transported to the cell surface and instead retained in the ER. These receptors are retained in the ER and prematurely degraded in proteasomes. Work has been done to try and stabilize the folding of the receptors and as a result increase the trafficking of the receptor to the cell surface. The V_2 vasopressin receptor, mutants of which are linked to insipidus diabetes, was the first GPCR to be targeted with ligands that were membrane permeable (Leskela et al., 2007; Morello et al., 2000). It was shown that a mutant V2 vasopressin receptor was retained in the ER by calnexin. The misfolded mutant receptor was stabilized by a small lipophilic receptor antagonist, able to cross the membrane, and once expressed on the cell surface most of the receptor was functional and able to produce cAMP in response to vasopressin (Leskela et al., 2007; Morello et al., 2000). Since the work done with the vasopressin receptor, the model has been applied to a number of other mutant receptors including rhodopsin, GnRHR and melanin-concentrating hormone receptor (Fan et al., 2005; Janovick et al., 2003; Janovick et al., 2002; Noorwez et al., 2003; Saliba et al., 2002). These membrane permeable molecules have been termed "pharmacological chaperones" and are proposed to increase cell surface expression of GPCRs by binding to immature receptors in the ER and stabilizing the protein during folding (Leskela et al., 2007).

The human δ-opioid receptor (hδOR) is a receptor, which in its wild type state is poorly processed due to the inherent complications with the correct folding of this protein. It was shown that only 40% of newly synthesized proteins were actually processed to the cell surface (Petaja-Repo et al., 2000). When cells expressing the hδOR were treated with opioid antagonist naltrexone (NTX) there was a 2-fold increase in the number of mature receptors when compared to untreated cells and a significant decrease in the half-life of the immature receptor species. The half-life of the mature receptor species was unaffected in the presence of NTX suggesting that the antagonist did not enhance receptor folding at the cell surface but rather intracellularly (Petaja-Repo et al., 2002). To test this hypothesis the effects of a membrane impermeable ligand, Leu-enkephaline (LE), was assessed. LE treatment alone did not lead to enhancement of receptor maturation, but when used in conjunction with NTX, the results mimicked those obtained with NTX treatment alone, suggesting that NTX action was not blocked by the presence of LE (Petaja-Repo et al., 2002). Later studies with the hδOR provided direct evidence for the site of action of these pharmacological chaperones (Leskela et al., 2007). In order to separate immature receptors localized in the ER from mature receptors expressed on the cell surface, a HEK293 cell line which expressed the hδOR under tetracycline induction was prepared (Leskela et al., 2007). Newly synthesized receptors were retained in the ER using Brefeldin A (BFA) treatment and it was shown that these immature receptors were still able to bind opioid antagonist, [^{3}H]diprenorphine, with high affinity (Leskela et al., 2007). When receptors were treated with both BFA and NTX the binding capacity of receptors, tested by saturation binding assays, increased 2-fold. Furthermore it was shown that treatment of the Asp95Ala hδOR mutant, which has reduced receptor maturation, with opioid antagonists decreased the amount of calnexin, an ER chaperone, that co-immunoprecipitated with the receptor (Leskela et al., 2007). These studies support the potential role to be played by pharmacological chaperones in enhancing expression of poorly processed wild type and mutant receptors as a result of conformational instability.

1.4.3.3 Mutations of the highly conserved "DRY"-motif

There are a number of intramolecular interactions within various domains of the receptor that serve to constrain the tertiary conformation and thereby stabilize the inactive conformation. Mutations that result in CAMs disrupt these intramolecular constraints leading to the destabilization of the inactive conformation and activation of the receptor.

Mutations of the highly conserved DRY-motif, proposed to stabilize the inactive receptor conformation (Ballesteros et al., 1998), have resulted in CAMs of some GPCRs. The side chain of the Arg residue, $R^{3.50}$, is proposed to ionically interact with the adjacent Asp/Glu residue, $D/E^{3.49}$, and the Asp/Glu residue ,$D/E^{6.30}$, at the cytosolic end of transmembrane helix 6, thereby constraining the $R^{3.50}$ residue within a conserved "polar pocket" (Scheer et al., 1997). The ionic interaction between these residues is facilitated by the negative side chain of residues $D/E^{3.49}$ and $D/E^{6.30}$ and the positive side chain of the $R^{3.50}$ residue. It is proposed that the protonation and therefore neutralization of these side chains would disrupt this ionic interaction and result in the reorientation of the $R^{3.50}$

residue, allowing it to shift out of the polar pocket and as a result destabilize the inactive conformation and result in receptor activation. A number of studies support the theoretical role of the DRY-motif. In the rhodopsin-like GPCR, gonadotrophin releasing hormone (GnRH) receptor, the $R^{3.50}$ residue was mutated to the uncharged Asn ($R^{3.50}N$) residue and the mutant receptor displayed slightly enhanced receptor coupling and signaling (Ballesteros et al., 1998). In another study where the role of the $D^{3.49(142)}$ residue in the α_{1B}-adrenoreceptor was investigated, the residue was mutated to every possible amino acid (Scheer et al., 1997). All the resulting mutant receptors exhibited constitutive activity to varying degrees (Scheer et al., 1997). Mutation of $D^{3.49(142)}$ to neutral Ala ($D^{3.49(142)}A$), resulted in high levels of constitutive activity. However when $D^{3.49(142)}$ was mutated to neutral residue Asn ($D^{3.49(142)}N$), it resulted in a mutant receptor with only modest levels of constitutive activity. This suggests that the neutralization of the charge on the $D^{3.49}$ side chain is not the only factor to consider (Scheer et al., 1997). The study therefore concluded that the equilibrium between inactive R and active R^{*} state of the adrenoreceptor was in part regulated by the deprotonated (negatively charged) or protonated (neutral) status of the $D^{3.49(142)}$ residue (Scheer et al., 1997). In a similar study, the effects of mutating the $D^{3.49(115)}$ residue in the histamine H_2 receptor was investigated (Alewijnse et al., 2000). H_2 is a relatively unique GPCR since it exhibits constitutive activity at low levels of expression. Mutation of the $D^{3.49(115)}$ residue to uncharged Ala ($D^{3.49(115)}A$) or Asp ($D^{3.49(115)}N$) in this receptor resulted in highly constitutively active receptors (Alewijnse et al., 2000). The CAMs exhibited high agonist affinity and increased signaling properties and a characteristically high structural instability. In addition the study also looked at the effects of mutating $Arg^{3.50(116)}$ to Ala, $R^{3.50(116)}A$, and Asp, $R^{3.50(116)}N$. These mutations led to a decrease in signal transduction and a significant decrease in the levels of mutant receptor expressed on the cell surface when compared to the wild type receptor (Alewijnse et al., 2000).

The results obtained from mutational studies of the "DRY"-motif in different GPCRs, suggest an important role for this conserved amino acid triplet in the process of receptor activation. Neutralization of the $D^{3.49}$ residue through mutagenesis has resulted in constitutive activation of GPCRs (Alewijnse et al., 2000; Ballesteros et al., 1998; Scheer et al., 1997) and therefore suggests that the unprotonated $D^{3.49}$ residue plays a role in maintaining the receptor in an inactive conformation. These conclusions are consistent with microdomain modeling of the GnRH receptor (Ballesteros et al., 1998) corroborated by the high resolution structure of the inactive state of the Rhodopsin receptor (Ballesteros et al., 2001).

The DRY-motif has been investigated in the CCR5 receptor in studies to elucidate the molecular mechanisms underlying CCR5 activation and coupling. The CCR5 receptor has the conserved $D^{3.49(125)}$ and $R^{3.50(126)}$ residues, but instead of the negatively charged $D^{6.30}$ it has a positively charged Arg residue in this position, which is more likely to sterically repel $R^{3.50(126)}$ (Springael et al., 2007). Consistent with results in other rhodopsin-like GPCRs, mutation of the $R^{3.50(126)}$ residue to neutral Asp ($R^{3.50(126)}N$) impaired receptor signaling (Farzan et al., 1997; Lagane et al., 2005). The mutant receptor, $R^{3.50(126)}N$, displayed agonist affinity comparable to the wild type receptor, therefore the impaired signaling must have been as a result of impaired receptor activation of the G protein (Lagane et al., 2005). However, in contrast to other GPCRs, when $D^{3.49(125)}$ was mutated to neutral Asn, $D^{3.49(125)}N$, the mutant receptor was uncoupled from the G protein and showed decreased receptor activity, both basal and in response to agonist, suggesting an alternative role for the DRY motif in CCR5 (Lagane et al., 2005). To further investigate the alternative role of the DRY motif in CCR5, the $R^{6.30}$ residue was mutated to neutral Ala and negatively charged Glu ($R^{6.30(223)}E$) , Asp ($R^{6.30(223)}D$) and Gln ($R^{6.30(223)}Q$) (Springael et al., 2007). When the charge on $R^{6.30(223)}$ was neutralized by mutating the residue to neutral Ala, the receptor displayed decreased constitutive activity at levels half that of the wild type receptor. When $R^{6.30(223)}$ was mutated to negatively charged amino acids Glu, Gln and Asp, thereby constituting the classical DRY ionic lock present in other GPCRs such as rhodopsin, the receptors did not display any detectable levels of constitutive activity (Springael et al., 2007). These results suggest that the presence of $R^{6.30(223)}$ instead of $D/E^{3.60}$ in the classical DRY lock, may account for the basal levels of constitutive activity of the wild type receptor. However when the DRY lock is introduced into the receptor, CCR5 is able to mimic the structural changes necessary to maintain the DRY ionic lock (Springael et al., 2007).

In an attempt to uncouple CCR5 from Gi protein dependant signaling, both the $D^{3.49(125)}$ and $R^{3.50(126)}$ were mutated to Gly ($D^{2.49(125)}G$ and $R^{3.50(126)}G$) and the Tyr residue was mutated to alanine ($Y^{3.51(127)}A$) in a single receptor construct (Gosling et al., 1997).

The mutant receptor displayed a marked decrease in agonist affinity compared to the wild type receptor, did not display any measurable generation of secondary messenger inositol phosphate (IP) when co-transfected with chimeric Gqi5 and failed to inhibit adenylate cyclase or increase intracellular levels of calcium in response to chemokine stimulation (Gosling et al., 1997). The mutations therefore successfully uncoupled the receptor from the G protein and these results suggested a similar role for the DRY-motif in the CCR5 receptor, however final conclusions are difficult to make since all of these point mutations were combined into a single receptor construct (Gosling et al., 1997).

1.4.3.4 The "TXP"-motif

The amino acid triplet, Threonine-X-Proline, is a highly conserved motif in the second transmembrane domain of chemokine receptors (Govaerts et al., 2001). The conserved nature of the TXP-motif implicates it in chemokine receptor function. In molecular modeling studies, the proline residue, $P^{2.58(84)}$, was proposed to have a kinking effect on the helical structure of the transmembrane domain and this effect was thought to be enhanced by the threonine residue, $T^{2.56(82)}$ (Govaerts et al., 2003). This hypothesis was further investigated using site-directed mutagenesis of the $T^{2.56(82)}$ and $P^{2.58(84)}$ residues within the CCR5 receptor (Govaerts et al., 2003). Mutagenesis of $P^{2.58(84)}$ to Ala resulted in impairment of receptor function, while the mutation of $T^{2.56(82)}$ to amino acids Ala, Cys, Ser and Val had no effect on the ability of the receptor to bind RANTES (Govaerts et al., 2003). In another study, random mutagenesis in the presence of manganese resulted in the constitutively activating mutations of $T^{2.56(82)}$ residue to Lys ($T^{2.56(82)}$K), Pro ($T^{2.56(82)}$P) and Arg ($T^{2.56(82)}$R) (Arias Alvarez et al., 2003). In addition to enhanced basal signaling the $T^{2.56(82)}$P mutant displayed increased sensitivity to chemokine RANTES, when compared to the wild type receptor, but did not display any difference in IC_{50} values, in homologous competition binding assays with MIP-1α. The $T^{2.56(82)}$K mutant showed marked decreases in sensitivity to chemokines, most notably in competition binding assays (Arias Alvarez et al., 2003). It was proposed that the introduction of a second proline residue in transmembrane helix 2 disrupted the helix and most likely induced additional structural constraints, while the mutation from threonine to lysine at the same position resulted in a change in charge and this led to a constitutively active mutant (Arias Alvarez et al., 2003).

1.5 Concluding remarks

The discovery of the major role played by the CCR5 co-receptor in HIV-1 infection has resulted in the convergence of previously unrelated fields of basic research (Berger et al., 1999). Chemokine receptors have extended their role beyond that of leukocyte trafficking and now represent primary targets for the prevention of HIV-1infection. The intersection between basic immunology, virology and GPCR theory has opened up a number of new avenues to explore in terms of HIV prevention. The GPCR identity of CCR5 enables us to look at the receptor within a GPCR theoretical framework which is relevant to its role as HIV-1 co-receptor. CAM receptors have proven to be a useful tool when investigating the mechanisms of activation and regulation of GPCRs as well the pathophysiology of GPCRs (Parnot et al., 2002). The potential of use CAMs in screening for novel drugs has also been highlighted (Chen et al., 2000). This thesis aims to use CAMs of CCR5 as a tool to define the co-receptor conformation of CCR5, by determining whether mutants of CCR5 are less able to support HIV-1 fusion. Once these differences are delineated we can exploit them to stabilize the receptor in a conformation permissive to chemokine binding but not HIV-1 fusion. We have used the abundant GPCR literature to design mutants of CCR5 proposed to be constitutively active and have evaluated the efficiency of these mutant receptors to support HIV-1 fusion.

2. Materials and Methods:

2.1 CCR5 Constructs and Expression

2.1.1 Mutant Receptor Construction:

Polymerase chain reaction-based mutagenesis (based on the Stratagene QuikChange site-directed mutagenesis kit, La Jolla, CA), the *Dpn* I method, was used to generate CCR5 mutants. Synthetic oligonucleotide primers containing the desired mutations, flanked by unmodified nucleotide sequence were designed. To facilitate identification of mutations the mutagenic primers were designed to either introduce or remove a silent restriction endonuclease recognition sequence at the site of the specific mutation (table 2.1). The mutants were further confirmed by sequencing. The wild type CCR5 sequence cloned into the pcDNA3.1(+) expression vector (Invitrogen, Carlsbad, CA) was used as template DNA for the PCR-based mutagenesis. The concentration of the template DNA (1ng/μl) was kept low to maximise mutagenesis. High fidelity thermophilic, Deep Vent DNA Polymerase (New England BioLabs, Ipswich, CA) (2000 U/ml), which has a 3′ to 5 ′ proofreading exonuclease activity was used. All PCR reaction mixtures were performed in a total volume of 50μl and cycling conditions consisted of an initial denaturation step at 95°C for 5 minutes, followed by 16 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 75°C for 12 minutes. The final extension step was carried out at 75°C for 20 minutes. The cycles of denaturation, annealing and extension were restricted to 16 cycles to minimize errors. To allow adequate extension of the insert and vector, the extension cycle time was increased to 12 minutes and in addition the final extension time was extended to 20 minutes. After the PCR cycling was completed, the reaction mixture was cooled to 37°C and 2μl of the restriction endonuclease, *Dpn* I (20 U/μl, New England Biolabs), was added directly to the PCR reaction and incubated (37°C, 60 minutes). *Dpn* I digests methylated DNA template. DNA that is amplified using PCR is not methylated and therefore is not digested by *Dpn* I. The restriction enzyme digestion results in the degradation of the wild type template DNA and increases the proportion of mutant DNA relative to wild type DNA in the subsequent transformation.

Eight mutant receptors were designed targeting three amino acid locations in the wild type CCR5 receptor. Threonine, located at position 82 in the second transmembrane helix was mutated to proline ($T^{2.56(82)}P$), lysine ($T^{2.56(82)}K$) and arginine ($T^{2.56(82)}R$). Aspartate^{125} ($D^{3.49(125)}$), located at the boundary of transmembrane helix 3 and the second intracellular loop was mutated to alanine ($D^{3.49(125)}A$) and asparagine ($D^{3.49(125)}N$). The third position targeted was amino acid arginine 225 ($R^{5.69(225)}$), located in the third intracellular loop, because previous work in the laboratory had shown increased basal signaling of $R^{5.69(225)}Q$ (Folefoc et al., in preparation). $R^{5.69(225)}$ was mutated to amino acids alanine ($R^{5.69(225)}A$), aspartate ($R^{5.69(225)}D$) and glutamate ($R^{5.69(225)}E$). Single mutations were then combined to create double mutants. The $R^{5.69(225)}Q$ was used as a template and the $T^{2.56(82)}K$ and $T^{2.56(82)}P$ mutations were incorporated into the receptor.

Table 2.1: Mutagenic primers used to generate CCR5 mutants. Silent restriction endonuclease sequences were introduced or removed at the site of mutation to facilitate diagnostic restriction digests of CCR5.

Construct	Mutagenic primer sequence 5' to 3'	Restriction endonuclease
$T^{2.56(82)}$ wild type	**CTT - ACT - GTC - CCC - TTC** **L T V P F**	-
$T^{2.56(82)}R$	CTA - CGC - GTT - CCC - TTC L R V P F	*Mlu* I
$T^{2.56(82)}P$	CTA - CCG - GTC - CCC - TTC L P V P F	*Age* I
$T^{2.56(82)}K$	CTT - AAG - GTC - CCC - TTC L K V P F	*Afl* II
$D^{3.49(125)}$ wild type	**ATC - GAT - AGG – TAC - CTG** **I D R Y L**	-
$D^{3.49(125)}A$	ATC - GCG - AGG - TAC - CTG I A R Y L	*Nru* I
$D^{3.49(125)}N$	ATC - AAC - CGG - TAC - CTG I N R Y L	*Age* I
$R^{5.69(225)}$ wild type	**CGG - TGT - CGA - AAT - GAG** **R C R N E**	*Taq* I
$R^{5.69(125)}A$	CGG - TGC - GCA - AAT - GAG R C A N E	*Taq* I
$R^{5.69(225)}D$	CGG - TGT - GAC - AAT - GAG R C D N E	*Taq* I
$R^{5.69(225)}E$	CGG - TGT - GAG - AAT - GAG R C E N E	*Taq* I

2.1.2 Expression of CCR5 constructs

CCR5 wild type and mutant receptor constructs were transiently transfected into Human Embryonic Kidney Cells (HEK 293) or HEK 293 cells stably expressing the chimeric G protein, Gαqi (HEK Gqi). CCR5 couples to the Gαi protein and direct measurement of Gαi activation is complex and expensive. The chimeric G protein, Gαqi, couples to GαI coupled GPCRs, such as CCR5, and leads to activation of phospholipase C (PLC) which produces a robust increase in inositol triphosphate (IP$_3$) (Kostenis, 2001). HEK Gqi cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Invitrogen, Paisley, Scotland) supplemented with 10% foetal calf serum (FCS) (Highveld Biologicals, Johannesburg, South Africa) and the selection antibiotic G418 (200μg/ml, Sigma, St. Louis, Missouri). HEK 293 cells, not expressing Gqi, were used in the chemokine competition binding assays and fluorescent activated cell sorting (FACS) analysis. HEK 293 cells were maintained in DMEM supplemented with 10 % FCS.

One day before transfection cells were plated into 10cm^2 dishes (3 X 10^6 cells, Corning, Cambridge, USA) in a final volume of 10ml DMEM supplemented with 10% FCS. Wild type and mutant DNA constructs (6μg) were incubated with 30μl FuGene HD (Roche Diagnostics Corp., Indianapolis, USA) in serum-free DMEM (final volume of 500μg, room temperature, 30 minutes) and added directly to the 10ml medium in the 10cm^2 dishes. Cells were incubated overnight (37°C; 5% CO$_2$) to express the transiently transfected DNA.

2.2 Inositol Phosphate Assay:

The inositol phosphate (IP) assay, in HEK Gqi cells, is a means of measuring the signaling capacity of wild type or mutant receptors. Basal production of IP (without the addition of any ligand) provides an indication of the constitutive basal activity of the receptor.

The day after transfection with wild type or mutant CCR5 receptors, cells were detached (detaching buffer 5mM EDTA, 50mM HEPES, 100mM NaCl), centrifuged (1 000 rpm, 5 minutes) and re-suspended in 24ml DMEM supplemented with 10% FCS. One milliliter of the cell re-suspension was added to each well in a 12-well plate (Corning) and left overnight to incubate (37°C, 5% CO$_2$) to allow cells to attach. Cells were radio-labeled with 1µCi/ml ^{3}[H]myoinositol (Amersham Life Sciences, Buckinghamshire, England) and incubated overnight (16-18 hours at 37°C, 5% CO_2) to allow metabolism of the [^{3}H]myoinositol by the cells to [^{3}H]phosphatidyl inositol- 4, 5-biphosphate (PIP_2). The hydrolysis of PIP_2 by phospholipase C (PLC) generates two second messengers, diacylglycerol (DAG) and [^{3}H]inositol phosphate. Medium was aspirated and cells were pre-incubated with Buffer I (NaCl; 40 mM, KCl; 4 mM, HEPES; 20 mM, glucose; 8.3 mM, $CaCl_2$; 1 mM, $MgCl_2$; 1 mM, LiCl; 10 mM, 0.1 % BSA, 0.4 % phenol red; for 15 minutes at 37°C). Cells were incubated with CCR5 chemokine ligand MIP-1β (60 minutes, 37°C, Peprotec, Rocky Hill, NJ) at various concentrations (0 M to 10^{-7}M) in duplicate after which the medium was replaced with 1ml of pre-cooled formic acid (10 mM, 4°C, 30 minutes). The formic acid lyses the cell membrane resulting in the transfer of soluble intracellular content into the surrounding medium, allowing determination of [^{3}H]IP, extracted on DOWEX-1 ion exchange columns (Sigma, Bellefonte, USA). Columns were washed with 3 ml of 3 M ammonium formate/ 0.1 M formic acid to negatively charge the resin and thereafter with 10 ml of distilled water to wash off the excess ammonium formate. Samples were applied to the columns and bind to the positively charged resin. The columns were washed with 5 ml of 0.1 M formic acid/ 5 mM inositol to wash off unbound [^{3}H]myoinositol. Bound [^{3}H]IP was eluted with 3 ml of 1 M ammonium formate/ 0.1 M formic acid into scintillation vials containing 16 ml of scintillation fluid (Quicksafe; Zinnser Analytical, Frankfurt, Germany)and counted by a beta counter (Packard, Palo Alto, CA). Columns were reconstituted with 3 ml of 3 M ammonium formate/ 0.1 M formic acid, followed by 10 ml of dH_2O. EC_{50} values for full curve assays were calculated using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA).

2.3 Chemokine Competition Binding Assay:

HEK 293 cells were transiently transfected with wild type or mutant DNA in 10cm^2 dishes (3X10^6 cells /dish). Cells were incubated (48 hours, 37°C, 5% CO_2), detached, pelleted and re-suspended in binding buffer (HEPES, 50mM, pH 7.4; $CaCl_2$, 1mM; $MgCl_2$, 5mM; BSA, 0.5%). Radio-labeled ligand MIP-1β (50 000 cpm), which serves as a tracer, was added to the cells in the presence of increasing concentrations of unlabelled MIP-1ß (0M to 10^{-7}M) in a total volume of 0.2ml and incubated (60 minutes at 27°C) to achieve equilibrium. MIP-1β was radio-iodinated in our laboratory using the chloramine T method.

Radio-labeled peptide was purified through a G-25 column (Sephadex, Sigma, Bellefonte, USA). Fractions of 1ml were collected and 1μl of label was counted using a gamma counter. The raw counts of each fraction were recorded and an elution profile was generated. Based on the elution profile fractions were selected to be tested using Cf2Th/syn CCR5 cells, which stably express a codon-optimized CCR5 receptor (Mirzabekov, 1999). The labeled ligand binds to the receptor and addition of the unlabelled ligand competes for binding of the receptor, displacing the labeled ligand. Total binding (B0) of the receptor is determined in the presence of radio-labeled ligand only. Non-specific binding (NSB) was determined as the amount of radio-labeled ligand bound in the presence of 10^{-7}M unlabelled MIP-1β. Specific binding of the ^{125}I-MIP-1β is calculated as the difference between B0 and NSB. Bound tracer was separated by filtration with glass-fiber filters (GF/C, Whatman, Maidstone, England) presoaked in 1% BSA. The filters were washed twice with washing buffer (HEPES, 50 mM, pH 7.4; $CaCl_2$, 1 mM; $MgCl_2$, 5 mM; NaCl, 0.5 M) and the radioactivity was counted in a gamma counter. IC_{50} values were calculated for binding curves using Prism software and nonlinear regression for one-site competition (GraphPad Software Inc., La Jolla, CA).

Cf2Th/syn CCR5 cells (Mirzabekov, 1999), were used in MIP-1ß competition binding assays in the development of a protocol to wash off residual TAK-779 (obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: TAK-779 (Baba et al., 1999)).

2.4 Fluorescence Activated Cell Sorting Analysis:

Fourty-eight hours after transfection of HEK 293 or HOS-CD4-luc cells with wild type or mutant DNA, cells were detached from the dishes ($10cm^2$) and washed with 10 ml of phosphate buffered saline (PBS) (NaCl, 137 mM; KCl, 2.7 mM; KH_2PO_4, 1.4 mM; $Na_2HPO_4.7H_2O$, 4.3 mM, pH 7.3) with 0.5 % BSA and were centrifuged (1000 r.p.m, 10 minutes). The cell pellet was re-suspended in 0.5 ml of PBS-0.5% BSA and 20 μl of re-suspended cells was incubated with 2 μl of phycoerythrin labeled 2D7 mouse anti-hCCR5 antibody (PE-2D7) (25 μg/ml, BD BioSciences Pharmingen, Franklin Lakes, NJ) in the dark (room temperature, 60 minutes). Samples were centrifuged (2000 r.p.m, 10 minutes), washed in 1.5 ml of PBS-0.5 % BSA, centrifuged (2000 r.p.m, 10 minutes) and re-suspended in 500 μl of PBS-0.5 % BSA for analysis. FACS analysis of labeled cells was performed using a FACScalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ). HEK 293 cells were acquired using the two parameters of cell size (forward scatter) and cell integrity (side scatter). Forward scatter was set on a logarithmic scale, due to the large size of the cells and side scatter was set on a linear-scale side scatter to eliminate the large amount of cell debris particles which accumulated during the labeling of the cells. A distinct cell population was gated between channel 100 and 300 on the forward scatter axis. Untransfected, labeled HEK 293 cells were used as a negative control to gate for unlabelled cells. The threshold for cells positive for PE labeling was set at channel 100 in the FL-2 wavelength channel. Positive cells labeled with PE-2D7 were therefore identified as cells fluorescing above channel 100 in FL-2.

2.5 Env-directed cell fusion assay

The fusion assay was developed by Stephen Jenkinson (Jenkinson, 2003) and models the interaction of the host cell receptors with the envelope protein expressed by the HI virion. In this assay two sets of cells are used i.e. HEK 293 cells transiently transfected with the envelope protein, gp160, which is the membrane-bound precursor of gp120 and gp41, the viral protein tat and the viral transcription factor rev. The second set of cells used is human osteosarcoma cells stably expressing hCD4 (HOS-CD4.pBABE-puro, NIH NAID Research and Reference Reagent Program, Division of AIDS USA) and a luciferase reporter construct adjacent to the long terminal repeat promoter (pHIV-1LTR-Luc, gift from Stephen Jenkinson, GlaxoSmithKline). HOS cells stably expressing both CD4 and CCR5 (HOS-CD4-CCR5, NIH NIAID Research and Reference Reagent Program, Division of AIDS, (Deng et al., 1996) and stably transfected with pHIV-1LTR-Luc were used a positive control in the fusion assay. The HOS-CD4-Luc cells were transiently transfected with wild type or mutant CCR5 receptor DNA cloned in the hygromycin resistant vector (hygro+) (Invitrogen). After transfection the HOS cells were cultured in DMEM medium supplemented with 10% FCS, 400μg/ml G418 and 200μg/ml of hygromycin (Sigma, St. Louis, Missouri) to allow for a 48-hour long selection of cells resistant to hygromycin to enrich for cells expressing CCR5. HEK 293 cells were transfected with the gp160 (Du151, gift from Carolyn Williamson, University of Cape Town , (Burgers et al., 2006), rev and tat (both gifts from Stephen Jenkinson, GlaxoSmithKline) 24 hours after transfection of HOS-CD4 cells. HOS cells were harvested and seeded in triplicate at a density of 6 000 cells per well in a 96-well plate (Corning). HEK 293 cells expressing gp160, tat and rev were harvested and layered onto HOS-CD4 cells at increasing densities (30 cells/well – 48 000 cells/well). The cells were co-cultured overnight to allow fusion to occur. It is thought that binding of Env on the HEK 293 cells to CD4 and CCR5 on the HOS cells allows fusion of the cells and tat expressed in HEK 293 cells is able to activate Luc expression via binding to the LTR promoter in the HOS-CD4-luc cells. Luciferase activity was determined using the Luciferase assay system (Promega, Madison, WI). Cells were washed with 100µl PBS containing 0.5% BSA (100µl) and incubated with lysis buffer (100µl, 15 minutes) and left to shake gently to allow lysis of cells. Cell debris was pelleted by centrifugation (14 000 rpm, 5 minutes) and 40µl of supernatant was transferred to each well in a 96-well white flat bottom plate (Dynex Technologies, Chantilly, VA). Luciferase substrate (75µl) was automatically dispensed into each well and luciferase activity was measured using a luminometer (Veritas, Promega).

3. Results

3.1 Effects of single amino acid mutations on CCR5 signalling

3.1.1 Screening for constitutively active mutants using the inositol phosphate assay:

Eight mutant CCR5 receptors with a single amino acid changes were generated using site-directed mutagenesis. These mutants were proposed to be constitutively active i.e. able to produce increased levels of second messenger in the absence of chemokine ligand. The IP assay was used to identify constitutively active mutants by comparing the basal IP production of mutant receptors to that of the wild type receptor in HEK Gqi cells.

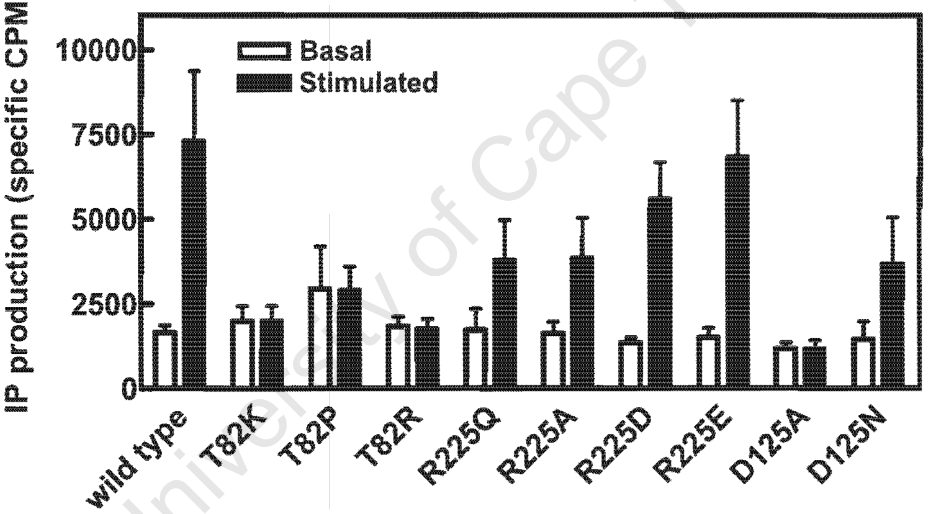


Figure 3.1: IP production by wild type and mutant receptors in the absence and presence of MIP-1β. HEK Gqi cells were transiently transfected with wild type or mutant CCR5 receptors, labelled with [^{3}H]myoinositol and incubated without (basal) or with chemokine agonist, MIP-1β (10^{-7}M, stimulated). Basal signalling (basal) of the receptors was assessed in the absence of MIP-1β. Results are from a representative experiment performed at least three times in duplicate.

Cells expressing the wild type CCR5 receptor displayed increased basal IP production when compared to mock-transfected cells and showed increased IP production in response to 10^{-7}M MIP-1β (figure and table 3.1). Mutants with substitutions of the $T^{2.56(82)}$ residue all displayed enhanced basal IP production when compared to the wild type receptor. The $T^{2.56(82)}$K mutant displayed basal counts 2.6 fold higher than the wild type receptor, while the $T^{2.56(82)}P$ mutant displayed basal signalling 6.9 fold higher than the wild type receptor (figure and table 3.1). The $T^{2.56(82)}R$ mutant displayed basal IP production 1.4 fold higher than the wild type receptor (figure and table 3.1). All three mutants showed no further increase in IP production in response to 10^{-7}M MIP-1β. These results are consistent with previous reports where mutants with lysine ($T^{2.56(82)}K$), proline ($T^{2.56(82)}P$) or arginine ($T^{2.56(82)}R$) in position 82 identified as constitutively active mutants using PCR-based mutagenesis in the presence of manganese (Arias Alvarez et al., 2003).

Table 3.1: Summary of basal and stimulated IP production. Cells were transfected with wild type or mutant CCR5 receptor. Mean basal counts were determined in the absence of MIP-1β, while mean stimulated counts were determined in the presence of 10^{-7}M MIP-1β. Results are mean values ± SEM calculated from at least three independent experiments performed in duplicate.

CCR5 Receptor Construct	Basal (cpm)	Basal % WT	Stimulated (cpm)	Stimulated % WT
Wild type	1 736 ± 294	-	15 684 ± 1198	-
$T^{2.56(82)}K$	4 500 ± 1110*	307 ± 67	4 516 ± 915	30 ± 8
$T^{2.56(82)}R$	2 385 ± 373	154 ± 25	2827 ± 802	49 ± 5
$T^{2.56(82)}P$	11 940 ± 2 791*	761 ± 122	12 382 ± 3 161	78 ± 15
$R^{5.69(225)}Q$	1 014 ± 148	83 ± 20	4 127 ± 132	84 ± 36
$R^{5.69(225)}A$	1 438 ± 360	87 ± 13	6 197 ± 2 550	73 ± 10
$R^{5.69(225)}D$	1 664 ± 259	78 ± 12	6 446 ± 1 556	94 ± 14
$R^{5.69(225)}E$	1 808 ± 418	82 ± 19	6 697 ± 2 022	114 ± 31
$D^{3.49(125)}A$	1 811 ± 368	79 ± 11	1 799 ± 680	28 ± 5
$D^{3.49(125)}N$	1 378 ± 338	71 ± 12	2 827 ± 802	53 ± 8

(* : value is significantly different to wild type basal; $p\text{-value} < 0.05$)

The $D^{3.49(125)}$ mutants were proposed to disrupt the ionic lock within the conserved DRY motif thereby releasing the $R^{3.50(126)}$ amino acid sidechain to stabilize the active conformation. The $D^{3.49(125)}A$ mutant displayed similar levels of basal IP production with 79 ±11 % of wild type levels, but displayed no further increase in IP production in response to 10^{-7}M MIP-1ß (table and figure 3.1), suggesting that it was uncoupled from G protein activation. The $D^{3.49(125)}N$ mutant displayed basal IP production 71 ±12 % of wild type basal IP production and this increased to 2 827 ± 802 cpm in the presence of 10^{-7}M MIP-1ß (table and figure 3.1).

The naturally occurring CCR5 mutant, $R^{5.69(225)}Q$, was previously found to be slightly constitutively active, when compared to the wild type receptor, in our laboratory (Folefoc et al., in preparation). In this study basal IP levels as well as IP production in the presence of 10^{-7}M MIP-1β of the $R^{5.69(225)}Q$ mutant was not significantly different from the wild type receptor. (table and figure 3.1). The $R^{5.69(225)}A$ mutant displayed basal IP production 87 ± 13 % of wild type production, while IP production increased to approximately 6 197 ± 2 550 cpm in the presence of 10^{-7}M MIP-1ß (table and figure 3.1). The $R^{5.69(225)}D$ and $R^{5.69(225)}E$ mutants displayed similar levels of basal IP production (78 ± 12 and 82 ±19 % of wild type levels respectively, table and figure 3.1).

3.1.2 Expression of mutant CCR5 receptors using fluorescent activated cell sorting analysis

The levels of basal and stimulated IP production mediated by each mutant receptor may be affected by the numbers of mutant receptors expressed on the cell surface. In order to distinguish effects of different receptor expression levels from effects of constitutive activity, expression of mutant and wild type CCR5 receptors was determined using FACS analysis. Transiently transfected HEK cells were stained with a phycoerythrin labeled anti-CCR5 antibody, 2D7, and the mean fluorescence of cells expressing CCR5 was measured (figure and table 3.2). The mean fluorescence per cell is used as an indication of the average receptor number per cell, while the percentage gated gives an indication of transfection efficiency.

The $T^{2.56(82)}P$ mutant showed expression levels comparable to the wild type receptor (mean fluorescence levels 92 ± 15 % of the wild type receptor, figure and table 3.2). The $T^{2.56(82)}K$ receptor however was poorly expressed when compared to the wild type receptor (mean fluorescence levels only 6 ± 1.5 % of wild type levels), while the $T^{2.56(82)}R$ receptor also had lower levels of surface expression (mean fluorescence 19 ± 3 % of wild type levels, figure and table 3.2).

The $R^{5.69(225)}Q$ mutant showed expression levels slightly but not significantly higher than the wild type receptor. Similarly the $R^{5.69(225)}D$ mutant had expression levels comparable to the wild type receptor (mean fluorescence 72 ± 24 % of wild type levels).

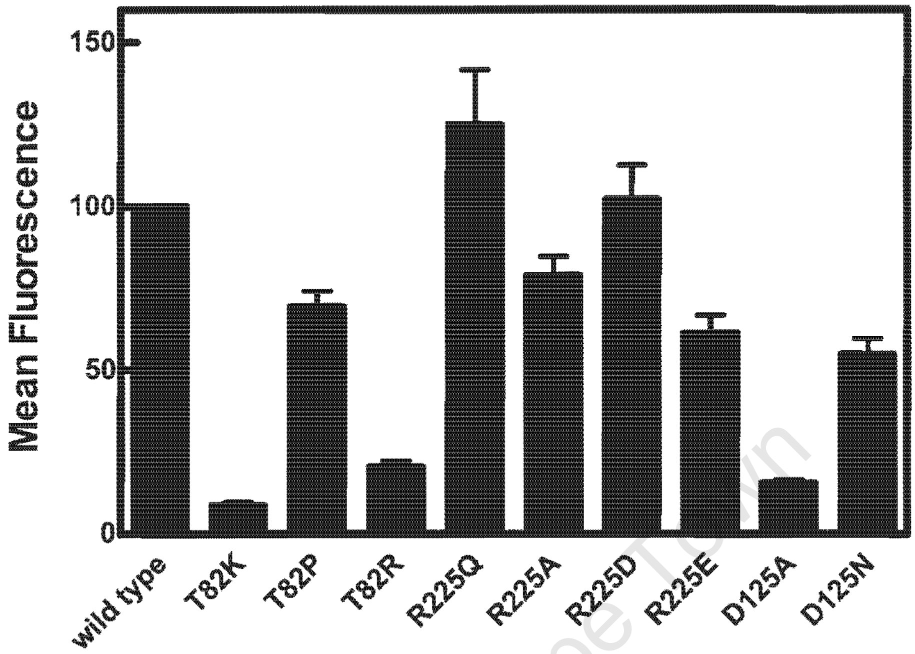


Figure 3.2: Cell surface expression of wild type and mutant CCR5 receptors. HEK cells were transiently transfected with either wild type or mutant CCR5 receptors. Cells were then stained with PE-labelled anti-CCR5 mAb, 2D7, and analysed by FACS. Data are representative of at least three independent experiments performed in duplicate.

The $R^{5.69(225)}$A and $R^{5.69(225)}$E mutants had expression levels lower than the wild type receptor (mean fluorescence levels 63 ± 10.4 % and 43 ± 11.6 % of wild type receptor levels respectively, table and figure 3.2).

Both $D^{3.49(125)}$A and $D^{3.49(125)}$N mutants had decreased expression levels when compared to the wild type receptor (mean fluorescence 47 ± 8.5 % and 19 ± 3 % of the wild type receptor respectively, table and figure 3.2).

Table 3.2: Summary of expression levels of wild type and mutant CCR5 receptors. HEK 293 cells were transiently transfected with wild type or mutant CCR5 receptors. Mean fluorescence of mutant receptors is expressed as a percentage of wild type expression levels. Results represented are mean values ± SEM calculated from at least three independent experiments performed in duplicate.

CCR5 Construct	**Mean Fluorescence (% of WT)**	**Percentage of cells expressing receptor**
Wild type	100	86 ± 0.5
$T^{2.56(82)}K$	6 ± 1.5	8 ± 0.5
$T^{2.56(82)}P$	92 ± 15	47 ± 6.7
$T^{2.56(82)}R$	19 ± 3	51 ± 0.8
$R^{5.69(225)}Q$	125 ± 16	50 ± 9
$R^{5.69(225)}A$	63 ± 10.4	57 ± 11.3
$R^{5.69(225)}D$	72 ± 24	61 ± 19.3
$R^{5.69(225)}E$	43 ± 11.6	69 ± 5.0
$D^{3.49(125)}A$	11 ± 1.7	46 ± 1.8
$D^{3.49(125)}N$	47 ± 8.5	74 ± 0.3

3.1.3 Env-directed cell fusion

A viral-free cell-cell fusion assay was used to assess the fusion efficiency of the mutant receptors. This fusion assay models the interaction and fusion of HIV with the host cell (Jenkinson, 2003). HOS cells stably expressing the CD4 receptor and a luciferase reporter gene and transiently transfected with wild type or mutant CCR5 were mixed with HEK cells expressing the envelope protein, MOLE-1, and HIV proteins tat and rev. HOS cells stably expressing both the CD4 and CCR5 co-receptor and a LTR-luciferase reporter gene (HOS-CD4-CCR5-luc), that is activated by tat, were used as a positive control. Fusion efficiency was assessed by measuring the luciferase activity resulting from the transfer of tat into HOS cells expressing the luciferase reporter. Initially only preliminary experiments were carried out using a subset of mutant receptors. HOS cells stably expressing wild type CCR5 showed high fusion efficiency with HEK cells expressing MOLE-1 (figure 3.3, top panel). HOS cells transiently transfected with wild type CCR5 showed lower fusion efficiency than those stably expressing CCR5 (figure 3.3, top panel). The $R^{5.69(225)}A$ mutant displayed fusion efficiency slightly higher than the wild type receptor, while the $R^{5.69(225)}E$ and $T^{2.56(82)}P$ mutants displayed fusion efficiency comparable to the wild type receptor (figure 3.3, top panel). The $T^{2.56(82)}K$ and $R^{5.69(225)}D$ mutants displayed poor fusion efficiency lower than the wild type receptor (figure 3.3, top panel).

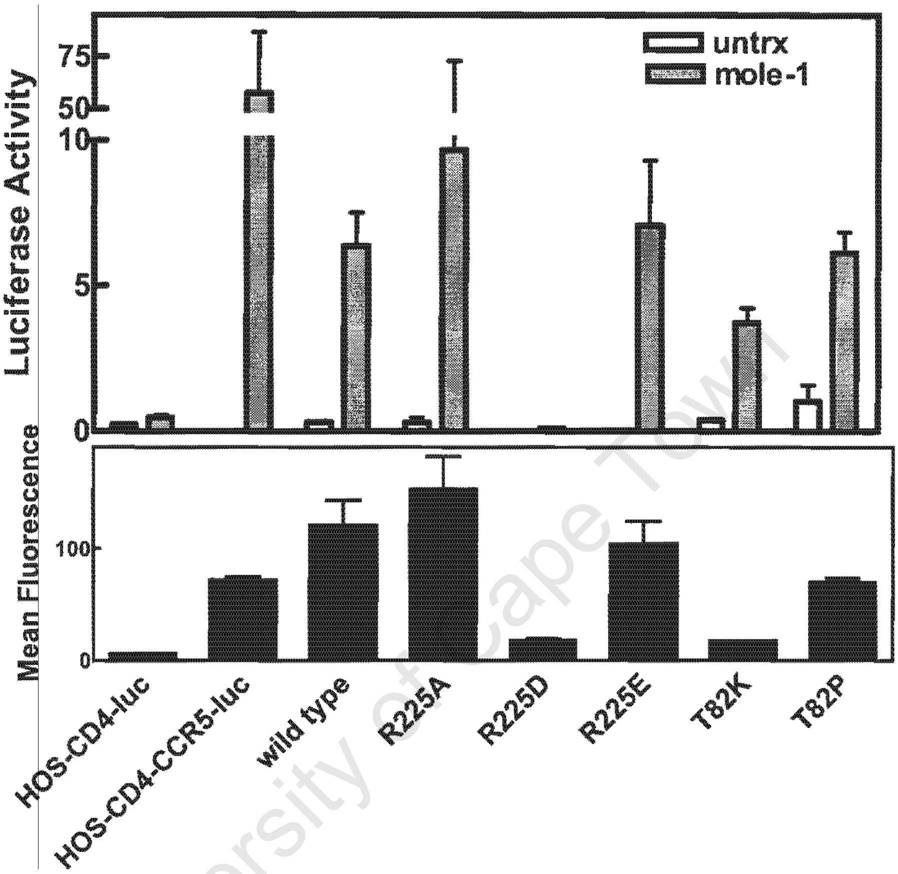


Figure 3.3: Fusion efficiency and mean fluorescence of wild type and mutant CCR5 in HOS cells. Top panel: HOS-CD4-luc cells (6 000) either stably expressing CCR5 (HOS-CD4-CCR5-luc) or transiently transfected with wild type or mutant CCR5 receptors were mixed with HEK cells (12 000) transiently transfected with rev, and tat and envelope gene, Mole-1. Cells were lysed and luciferase activity was measured. Bottom panel: To determine expression levels cells were stained with PE-labelled anti-CCR5 mAb, 2D7, prior to FACS analysis.

FACS analysis performed on the cells used in the cell-cell fusion assay showed highly variable expression of the different mutant receptors in HOS-CD4-luc cells. The $R^{5.69(225)}A$, $R^{5.69(225)}E$ and $T^{2.56(82)}P$ mutants showed expression levels similar to the wild type receptor (figure 3.3., bottom panel). The $T^{2.56(82)}K$ and $R^{5.69(225)}D$ mutants showed low expression levels when compared to the wild type receptor (figure 3.3, top panel). Because of the low expression of the $T^{2.56(82)}K$ and $R^{5.69(225)}D$ mutants, it was not possible to determine whether low fusion activity was due to low expression or due to constitutive activity.

The variation in the expression of the mutant receptors of CCR5 made it difficult to interpret the cell-cell fusion assay. We therefore attempted to enhance the expression of poorly expressed receptors such as the $T^{2.56(82)}K$ receptor using two different approaches, molecular chaperones and further mutagenesis.

3.1.3.1 Effect of TAK-779 on cell surface expression of CCR5

It has been reported that receptor ligands, able to diffuse across biological membranes, enhance cell surface expression of their receptors (Leskela et al., 2007; Petaja-Repo et al., 2002). They are proposed to act as "molecular chaperones" that stabilize folding of nascent receptors (Petaja-Repo et al., 2002; Petaja-Repo et al., 2000). TAK-779 is a well characterized small molecule inverse agonist of CCR5 and we therefore investigated whether it could enhance expression of CCR5 (Dragic, 2000). TAK-779 (1nM) was added to medium 24 hours after transfection of HEK 293 cells with wild type or mutant CCR5 receptors and stained with the PE-labelled anti-CCR5 mAb 2D7 for FACS analysis.

All three receptors tested, wild type, $R^{5.69(225)}A$ and $T^{2.56(82)}P$, showed increased mean fluorescence as well as an increase in the percentage of cells gated after TAK-779 treatment (figure 3.4). The increase in mean fluorescence indicated an increase in the average number of receptors per cell, while the increase in the percentage of cells gated suggested that a higher proportion of cells were expressing detectable amounts of receptor. The wild type receptor, in the absence of TAK-779, showed a low percentage of cells gated. This was in contrast to results previously obtained in the laboratory and suggested low transfection efficiency as a result of poor quality DNA in this experiment. $R^{5.69(225)}A$ showed the highest increase in mean fluorescence with a percentage increase of 528 % in the presence of TAK-779 and a 812 % increase in the proportion of cells gated (figure 3.4). The $T^{2.56(82)}P$ receptor displayed a 325 % increase in the mean fluorescence of cells and a 59 % increase in the proportion of cells gated. These results suggested that TAK-779 treatment enhances the cell surface expression of the wild type and mutant CCR5 receptors.

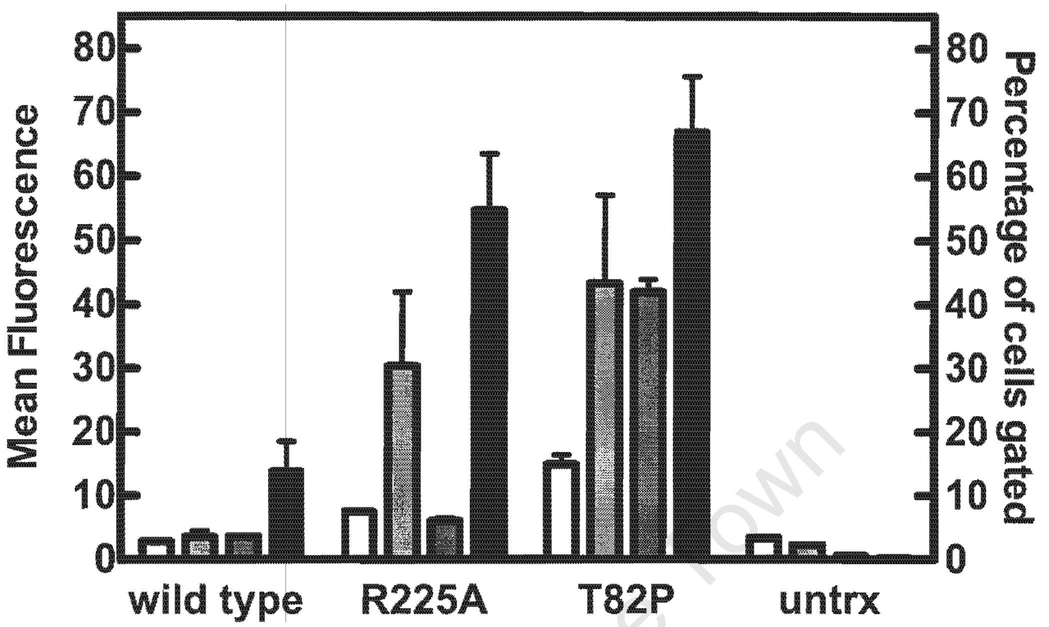


Figure 3.4: Cell surface expression of the wild type and mutant receptors in the absence or presence of inverse agonist TAK-779. HEK cells were transiently transfected with receptor DNA. TAK-779 was added to samples at a concentration of 1 nM 24 hours after transfection and left in the medium overnight. Cells were stained with PE-labelled anti-CCR5 mAb, 2D7 for FACS analysis. The mean fluorescence of cells was determined in the absence (white bars) and presence of 1nM TAK-779 (light grey bars). The percentage of cells gated was determined in the absence (dark grey bars) and presence of TAK-779 (black bars). Results are from a single experiment performed in duplicate.

To optimize the effect of TAK-779 it was added to samples at increasing concentrations to determine whether the increase in mean fluorescence was dose dependant (figure 3.5). The wild type receptor displayed the highest mean fluorescence and the highest proportion of cells gated in the presence of 1nM TAK-779 (figure 3.5). Contrary to the previous experiment (figure 3.4) the $R^{5.69(225)}A$ receptor displayed the highest levels of mean fluorescence in the absence of any added TAK-779 (figure 3.5). The $T^{2.56(82)}P$ mutant did not display any significant increase in mean fluorescence or percentage of cells gated in the presence of TAK-779 (figure 3.5). There was no significant change in mean fluorescence or percentage of cells gated with untransfected cells, eliminating any non-specific effects of TAK-779 (figure 3.5).

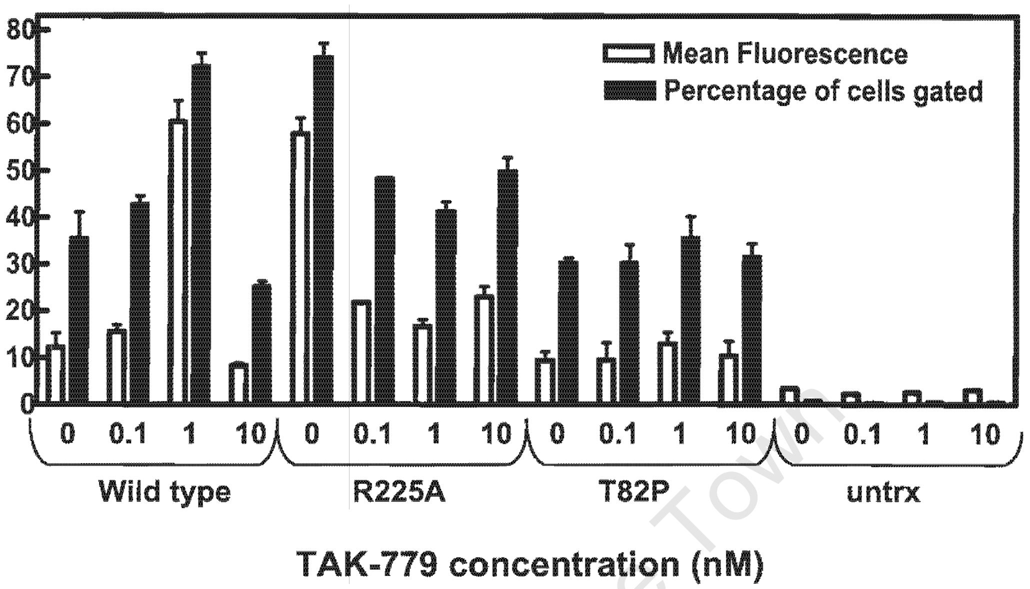


Figure 3.5: Cell surface expression of the wild type and mutant receptors in the absence or presence of increasing concentrations of inverse agonist TAK-779. HEK cells were transiently transfected with receptor DNA. TAK-779 was added to samples at a concentration of 0.1 μM 24 hours after transfection and left in the medium overnight. Cells were stained with anti-CCR5 mAb 2D7 and analysed. Results are representative of a single experiment.

Since TAK-779 inhibits CCR5 binding of agonists and HIV envelope, it must be removed from cells to be used in functional assays such as IP production, ligand binding and fusion assays. Therefore if TAK-779 was to be used to increase expression of receptors it would be necessary to develop a protocol to remove the TAK-779 from cells before functional assays without damaging the integrity of the cells. In order to investigate both the effect of TAK-779 on functional assays and to develop a method of removing the inverse agonist from the medium, Cf2Th/syn CCR5 cells were used (Mirzabekov, 1999). Cf2Th/syn CCR5 cells were used because they express high levels of the codon-optimized wild type CCR5 receptor that facilitated the measurement of the effect of residual TAK-779 on ligand binding. The effect of TAK-779 was evaluated using MIP-1β homologous competition binding assays. Two different wash procedures were employed: a DMSO wash which entailed adding 1% DMSO to the medium (DMEM with 10% FCS) and incubation for 15 minutes. After 15 minutes the medium containing DMSO was removed and replaced with normal DMEM containing 10% FCS. The second wash procedure consisted of HEPES/DMEM supplemented with 10% FCS, which was added to the cells for 15 minutes and then replaced with DMEM supplemented with 10% FCS. The effects of these wash procedures on subsequent ligand binding assays was assessed in cells that had not been treated with TAK-779, while their effectiveness in removing TAK-779 was assessed in cells that had been treated with TAK-779 (1nM).

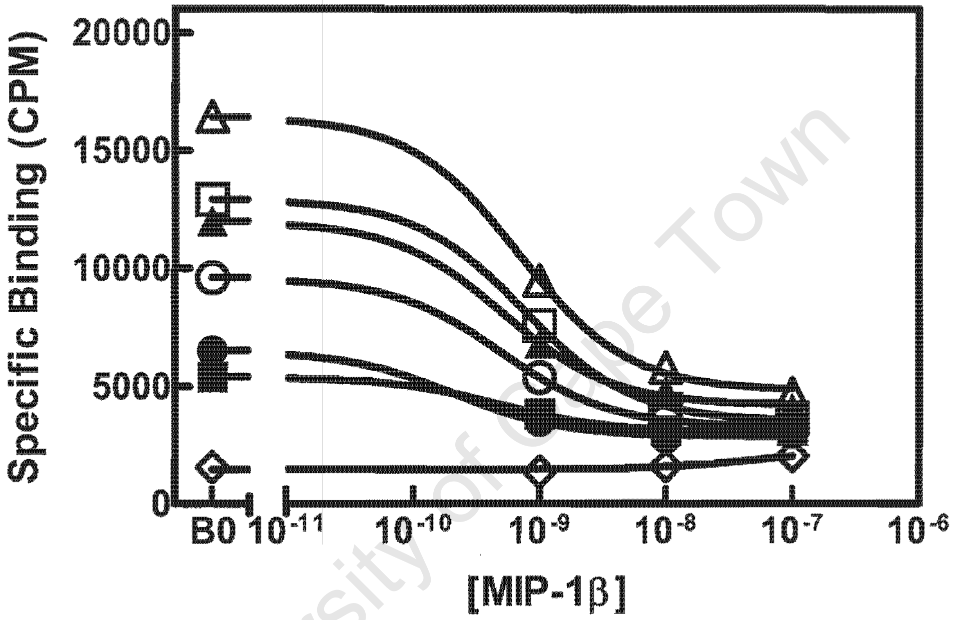


Figure 3.6: Effects of washing procedures on competition binding with MIP-1ß in Cf2Th/syn CCR5 cells. Cf2Th/syn CCR5 cells were incubated with a constant concentration of ^{125}I-MIP-1β and increasing concentrations of MIP-1ß (10^{-11} to 10^{-7} M). Total binding was compared between cells incubated with TAK-779 (1nM, filled symbols) or without TAK-779 (open symbols). Cells underwent no wash protocol (■ ,□), a HEPES/DMEM wash (▲, Δ) or a DMSO wash (•, ○).Untransfected HEK cells were used as a negative control and underwent a DMSO wash (◊).

When looking at Cf2Th/syn CCR5 cells in the absence of TAK-779, cells without any wash protocol showed high total binding and cells having undergone the HEPES/DMEM wash showed comparable total binding to cells without any wash protocol. Cf2Th/syn CCR5 cells subjected to the DMSO wash showed decreased total binding when compared to cells subjected to either no wash or a HEPES/DMEM wash (figure 3.6). When looking at Cf2Th/syn CCR5 cells in the presence of TAK-779 (1nM), cells not having undergone any wash protocol had significantly decreased total binding, confirming that residual TAK-

779 has a suppressive effect on ligand binding, while cells having subjected to the HEPES/DMEM wash showed total binding comparable to cells washed with HEPES/DMEM in the absence of TAK-779 (figure 3.6). This suggests that the HEPES/DMEM wash may be effective in removing residual TAK-779. Cf2Th/syn CCR5 cells which underwent the DMSO wash had significantly decreased total binding comparable to cells that did not undergo any wash protocol in the presence of TAK-779 (figure 3.6), suggesting that the DMSO wash was not effective in removing residual TAK-779 or damaged the cells. Untransfected HEK cells in the absence of TAK-779 and having undergone the DMSO wash showed very low total binding, thereby eliminating non-specific effects of DMSO.

3.2. Double mutants

A second approach to enhancing expression of constitutively active mutants was to use site-directed mutagenesis to combine the mutations that caused the highest constitutive activity with the $R^{5.69(225)}Q$ mutation, which caused increased basal IP production in some experiments, but yielded expression that was equal or higher than the wild type receptor.

3.2.1 Effects of double amino acid mutations on CCR5 signaling

The double mutants, $T^{2.56(82)}K/R^{5.69(225)}Q$ and $T^{2.56(82)}P/R^{5.69(225)}Q$, displayed enhanced basal IP production when compared to the wild type receptor. The $T^{2.56(82)}K/R^{5.69(225)}Q$ receptor produced levels of basal IP 8-fold higher than the wild type receptor, while the $T^{2.56(82)}P/R^{5.69(225)}Q$ receptor displayed basal production 10-fold higher than that of the wild type receptor (figure 3.7, table 3.3). Increasing concentrations of MIP-1ß did not further increase IP production (figure 3.7, table 3.3). Basal IP production of the double mutants was higher than the basal IP production of the single mutants and comparable to the maximum stimulated production of the wild type receptor. These results suggest enhanced expression of the double mutants and that these mutants were fully constitutively active (figure 3.7, table 3.3).

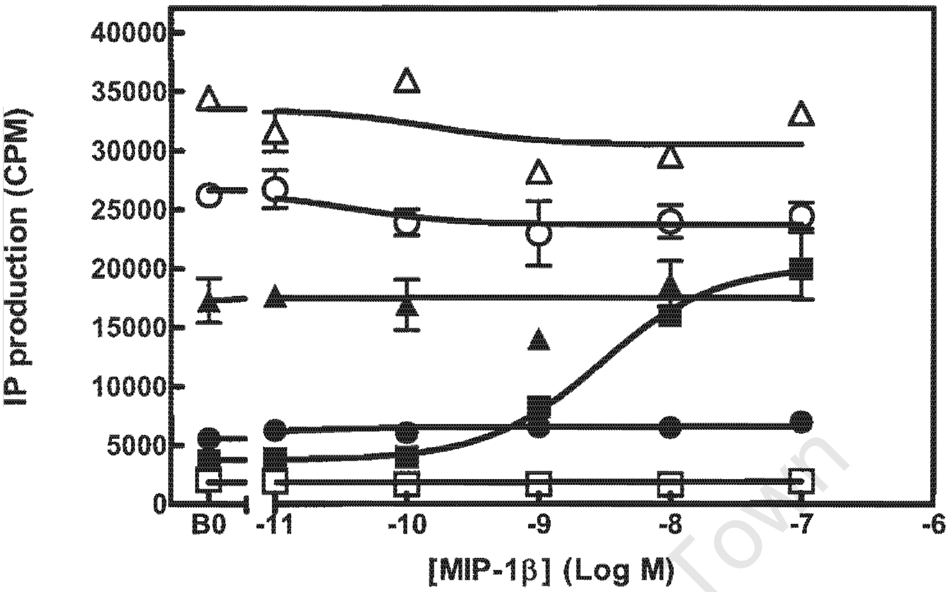


Figure 3.7: IP production in HEK Gqi cells expressing CCR5 with mutations of $T^{2.56(82)}$ and $R^{5.69(225)}$. HEK Gqi cells transfected with wild type (■) or mutant CCR5 receptors $T^{2.56(82)}$K (●), $T^{2.56(82)}$P (▲), $T^{2.56(82)}$K/$R^{5.69(225)}$Q (○) or $T^{2.56(82)}$P/$R^{5.69(225)}$Q (Δ). Untransfected cells (□) were used as a negative control. Cells pre-labelled with [^{3}H]myoinositol were incubated with increasing concentrations of MIP-1β (10^{-11} – 10^{-7}M). Data are from a single experiment that is representative of at least three independent experiments performed in duplicate

Table 3.3: IP production in HEK Gqi cells expressing wild type CCR5 or mutants with substitutions at $T^{2.56(82)}$ and $R^{5.69(225)}$. Results are means ± SEM calculated from at least three independent experiments. Basal refers to IP production in the absence of MIP-1β, while stimulated refers to IP production in the presence of 10^{-7}M MIP-1β, as it was not possible to calculate Emax for constitutively active mutants.

CCR5 Receptor Construct	Basal (cpm)	Basal (cpm) % WT	Stimulated (cpm)	Stimulated (cpm) % WT
Wild type	1 736 ± 294	-	15 684 ± 1198	-
$T^{2.56(82)}$K	4 500 ± 1110*	307 ± 67	4 516 ± 915	30 ± 8
$T^{2.56(82)}$P	11 940 ± 2 791*	761 ± 122	12 382 ± 3 161	78 ± 15
$T^{2.56(82)}$K/$R^{5.69(225)}$Q	13 419 ± 3 259**	811 ± 195	14 187 ± 4 320	88 ± 21
$T^{2.56(82)}$P/$R^{5.69(225)}$Q	16 158 ± 4 484**	1 013 ± 280	18 038 ± 6 700	111 ± 33

(*: value is significantly different to wild type basal; p-value < 0.05)
(**: value is significantly different to wild type basal; p-value < 0.01)

3.2.2 Competition Binding Assays:

Homologous competition binding assays were used to assess expression and affinity of wild type and mutant receptors for the chemokine MIP-1β. In cells expressing the wild type CCR5 receptor, unlabelled MIP-1β displaced the labeled MIP-1ß with an IC_{50} of 32.6nM ± 6.5nM (figure 3.8, table 3.4).

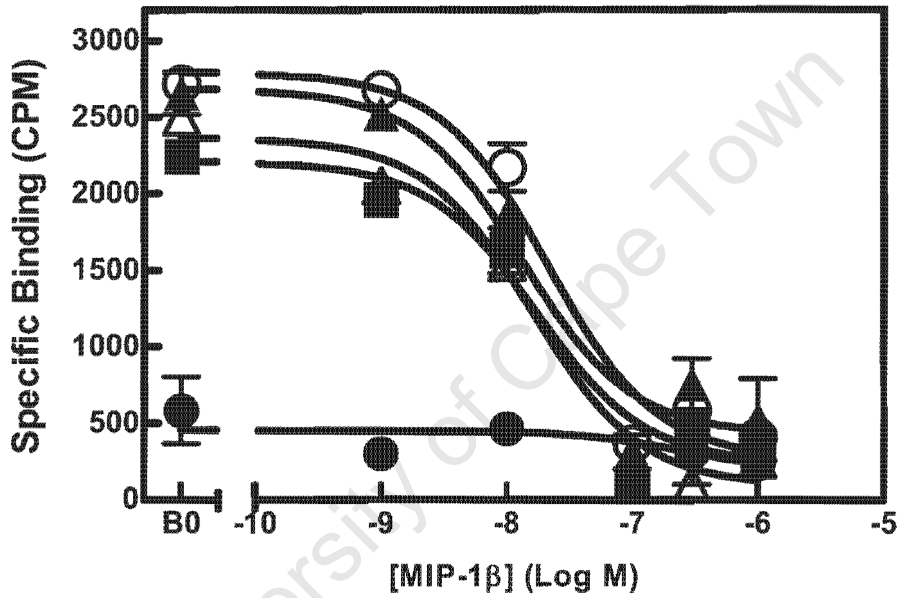


Figure 3.8: Competition binding of MIP-1ß in cells expressing wild type and mutant CCR5 receptors. HEK 293 cells were transiently transfected with wild type (■) or mutant CCR5 receptors, $T^{2.56(82)}$ K (•), $T^{2.56(82)}$P (▲), $T^{2.56(82)}$K/RQ (○) or $T^{2.56(82)}$ P/RQ (Δ). Cells were incubated with a constant concentration of labeled chemokine ligand (^{125}I-MIP-1ß, 50 000 cpm) and various concentrations of unlabelled chemokine ligand (MIP-1ß). Data are from a single experiment which is representative of at least three independent experiments performed in triplicate.

The $T^{2.56(82)}$K receptor showed specific binding that was too low for calculation of an IC_{50} value, suggesting that this receptor is poorly expressed. This result is consistent with the FACS analysis which showed low expression of this mutant The double mutant, $T^{2.56(82)}K/R^{5.69(225)}Q$, displayed total binding comparable to the wild type receptor with an IC_{50} value of 20.67 ± 4 nM (figure 3.8, table 3.4). Similarly both mutants with proline in position 82, $T^{2.56(82)}$P and $T^{2.56(82)}P/R^{5.69(225)}Q$, displayed total binding and affinity comparable to the wild type receptor with IC_{50} values of 31.9 ± 7.4 nM and 30.6 ± 13nM respectively (figure 3.8, table 3.4). IC_{50} values for the mutant receptors were not significantly different from the wild type receptor.

Table 3.4: Competition binding assays with cells expressing wild type or mutant CCR5 receptors. Summary of maximum binding (B0) and IC_{50} values obtained from homologous MIP-1β competition binding assays. Results are mean values ± SEM calculated from at least three independent experiments.

Receptor	Total binding (cpm)	IC_{50} (nM)
Wild type	1 932 ± 123	32.6 ± 6.5
T82K	169 ± 307	B0 too low to low to calculate IC_{50}.
T82P	2 350 ± 223	31.9 ± 7.4
T82K/R225Q	2 300 ± 223	20.67 ± 4
T82P/R225Q	1 800 ± 373	30.6 ± 13

3.2.3 FACS analysis of double mutants in HEK 293 cells

FACS analysis was used to measure the expression levels of receptors with mutations at both $T^{2.56(82)}$ and $R^{5.69(225)}$ and to compare the expression of these mutants to the wild type receptor. HEK 293 cells were transiently transfected with either wild type or mutant receptors and stained with PE-labelled anti-CCR5 mAb, 2D7. The $T^{2.56(82)}P$ and $T^{2.56(82)}P/R^{65.69(225)}Q$ receptors displayed expression levels comparable to the wild type receptor with no significant increase in expression levels when combining the $T^{2.56(82)}P$ mutation with the $R^{5.69(225)}Q$ mutation (figure 3.9, table 3.5). The $T^{2.56(82)}K$ receptor displayed very poor levels of expression when compared to the wild type receptor, with only 6% of the wild type mean fluorescence levels (figure 3.9, table 3.5). The combination of the $T^{2.56(82)}K$ mutation with the $R^{5.69(225)}Q$ resulted in a mutant with higher levels of expression at 51% of wild type expression levels (figure 3.9, table 3.5).

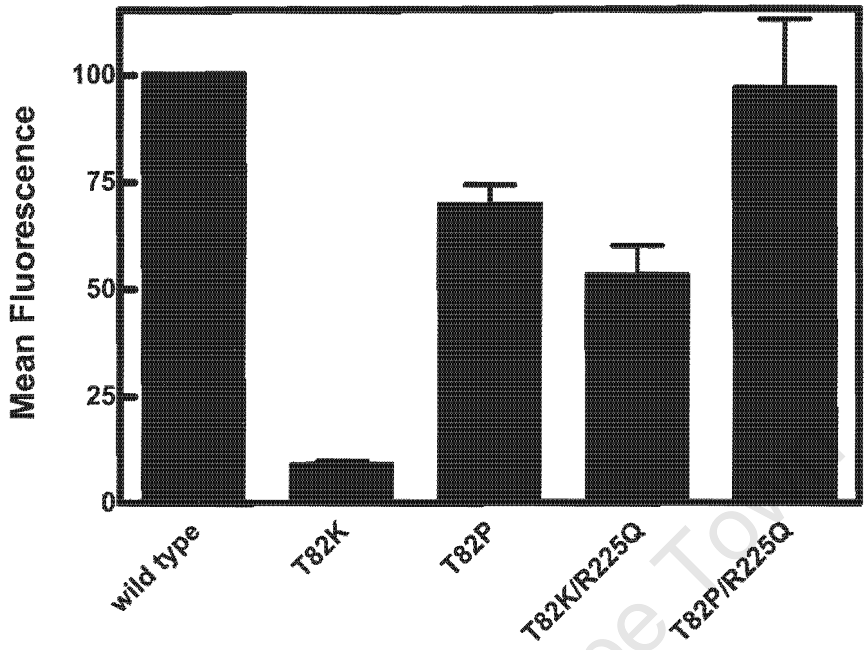


Figure 3.9: Surface expression levels of wild type and mutant receptors in HEK cells. HEK cells were transfected with wild type or mutant CCR5 receptors and stained with PE-labelled anti-CCR5 mAb, 2D7, for FACS analysis. Results are mean values ± SEM calculated from at least three independent experiments performed in duplicate.

Table 3.5: Surface expression levels of wild type and mutant CCR5 receptors. Results are representative of at least three independent experiments performed in duplicate.

CCR5 construct	Mean fluorescence (% of WT)	Percentage of cells expressing receptor
Wild type	100	86 ± 0.5
$T^{2.56(82)}K$	6 ± 1.5	8 ± 0.5
$T^{2.56(82)}P$	92 ± 15	47 ± 6.7
$T^{2.56(82)}K/R^{5.69(225)}Q$	51 ± 12.6	48 ± 7.5
$T^{2.56(82)}P/R^{5.69(225)}Q$	80 ± 21	58 ± 7.9

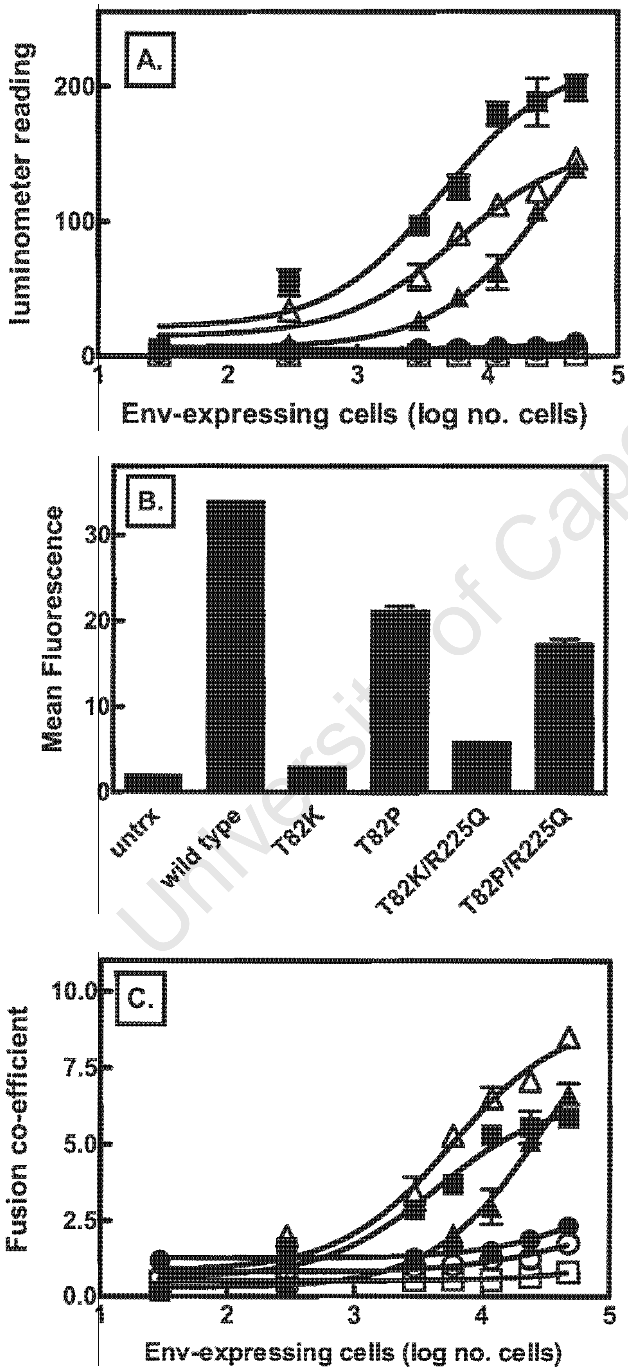
3.2.4 Env-directed cell fusion

To assess the ability of the $T^{2.56(82)}$ mutant receptors to mediate fusion we performed cell-cell fusion assays similar to initial experiments (figure 3.3., top panel) but in this case full dose response curves were carried out. The fusion efficiency coefficient of each receptor construct was determined by dividing the luminometer reading by the average expression, measured by FACS analysis.

The wild type receptor fused well with Env-expressing cells with a maximum fusion coefficient of 11.8 ± 2.2 (figure 3.10, table 3.6). The mutants with Lys in position 82 i.e. $T^{2.56(82)}K$ and $T^{2.56(82)}K/R^{5.69(225)}Q$ both displayed very low fusion efficiency when compared to the wild type receptor, with a maximum fusion coefficient of 0.84 ± 0.2 and 1.9 ± 0.4 respectively (figure 3.10, table 3.6). The mutants with Pro in position 82 i.e. $T^{2.56(82)}P$ and $T^{2.56(82)}P/R^{5.69(225)}Q$ displayed a maximum fusion coefficient comparable to the wild type receptor. The $T^{2.56(82)}P$ had a maximum fusion coefficient of 16.5 ± 4.1, while the $T^{2.56(82)}P/R^{5.69(225)}Q$ had a maximum fusion coefficient of 18.8 ± 5.6 (figure 3.10, table 3.6).

Based on the fusion results the constitutively active mutants fall into two categories, those with Lys in position 82 are poor mediators of fusion, while those with Pro in position 82 are good mediators of fusion. The two classes of constitutively active mutants may define distinct activated receptor conformations.

Figure 3.10: Fusion activity of wild type and mutant CCR5 receptors. HOS cells stably expressing CD4 and the luciferase reporter gene were transiently transfected with wild type (■) or mutant CCR5 receptors, $T^{2.56(82)}K$ (●), $T^{2.56(82)}P$ (▲), $T^{2.56(82)}K/R^{5.69(225)}Q$ (○) or $T^{2.56(82)}P/R^{5.69(225)}Q$ (Δ). Cells were selected for 48 hours in medium containing 200μg/ml hygromycin. After selection, CCR5-expressing HOS-CD4-luc cells were incubated overnight with HEK cells transiently expressing tat, rev and the codon optimized gp150 envelope protein, DU151.



Panel A: Luciferase activity was measured as a measure of fusion efficiency.

Panel B: CCR5-expressing HOS-CD4-luc cells were labeled with PE-labelled anti-CCR5 mAb, 2D7, and analysed by FACS. Mean fluorescence was measured to determine cell surface expression of wild type and mutant receptors.

Panel C: To compare fusion efficiency amongst receptors, the expression was normalized by dividing the luciferase activity by the mean fluorescence of each construct.

Table 3.6: Fusion efficiencies of the wild type and mutant CCR5 receptors in HOS-CD4-luc cells. Values are means ± SEM calculated from at least three independent experiments performed in triplicate.

CCR5 construct	**Maximum fusion efficiency (co-efficient)**[a]	**Maximum fusion (% wild type receptor)**	**EC_{50} (no. Env-expressing cells)**
Wild type	11.8 ± 2.2	100	14 705 ± 4591
$T^{2.69(82)}K$	0.84 ± 0.2	4.6 ± 1.7	fusion activity too low to measure EC_{50}.
$T^{2.69(82)}P$	16.5 ± 4.1	135 ± 10.9	15 384 ± 4818
$T^{2.69(82)}K/R^{5.(225)}Q$	1.9 ± 0.4	16.8 ± 3.1	fusion activity too low to measure EC_{50}.
$T^{2.69(82)}P$ /$R^{5.(225)}Q$	18.8 ± 5.6	134.8 ± 31.1	5545 ± 957

[a] fusion co-efficient: calculated by dividing the luminometer reading by the mean fluorescence of the receptor construct.

In summary, the results show that four constitutively active mutants of CCR5 were generated, $T^{2.56(82)}K$, $T^{2.56(82)}P$, $T^{2.56(82)}K/R^{5.69(225)}Q$ and $T^{2.56(82)}P/R^{5.69(225)}Q$. The $T^{2.56(82)}P$ and $T^{2.56(82)}P/R^{5.69(225)}Q$ mutants were expressed at similar levels to the wild type receptor. The $T^{2.56(82)}K$ mutant had very low levels of expression when compared to the wild type receptor, while the double mutant $T^{2.56(82)}K/R^{5.69(225)}Q$ showed enhanced levels of expression relative to the single mutant $T^{2.56(82)}K$, but was still less well expressed than the wild type receptor. CAMs with Lys in position 82 showed very low fusion efficiency when compared to the wild type receptor, while mutants with Pro in position 82 showed good fusion efficiency comparable to the wild type receptor.

4. Discussion

CCR5 is the major co-receptor in HIV-1 fusion. The central role played by CCR5 and its cognate chemokines in regulating HIV-1 infection has been supported by the discovery of gene variants of both the receptor and chemokine and the effect these variants have on the susceptibility of individuals to infection as well as the time taken to progress to AIDS. Of particular significance was the discovery of the Δ-32 allele that results in a mutant CCR5 receptor that cannot support HIV-1 fusion. Individuals homozygous for this allele exhibit high resistance to HIV-1 infection (Dean et al., 1996; Liu et al., 1996; Samson et al., 1996b). The development of preventative and therapeutic measures against HIV which are directed against viral proteins has been complicated with high rates of viral mutations leading to emerging drug resistance (Hendrickx et al., 2000; Kim et al., 2001). More promising avenues of prevention and treatment have been those targeting host encoded proteins, such as low molecular compounds targeting the chemokine co-receptors (Brass et al., 2008; Seibert and Sakmar, 2004). The role of the host protein CCR5 in the process of HIV-1 fusion is well defined and provides a promising target. An ideal inhibitor of CCR5 would be an allosteric molecule capable of destabilizing the CCR5 conformation able to support fusion, while having no effect on the conformation mediating chemokine receptor function. Development of this allosteric inhibitor would require a better understanding of the functional conformation of the CCR5 co-receptor. It is now well accepted that GPCR proteins, such as CCR5, exist in an ensemble of conformational states (Kenakin and Onaran, 2002). Binding of any particular agonist stabilizes the conformational states that mediate the response appropriate to the ligand e.g. chemokine binding to CCR5 stabilizes receptor conformations that activate the Gi protein and receptor internalization, while HIV binding must stabilize a conformation that facilitates its fusion with the host cell membrane. The CCR5 conformation that mediates HIV fusion is poorly defined. We sought to stabilize the conformations of CCR5 that mediate G protein activation, using site-directed mutagenesis, in order to assess whether these conformations also mediate fusion with membranes expressing HIV Env protein.

4.1 Mutations of $R^{5.69(225)}$ do not result in CAMs of CCR5

The naturally occurring CCR5 $R^{5.69(225)}Q$ mutant was previously found to be partially constitutively active in our laboratory (Folefoc et al., in preparation). In this study we attempted to increase the constitutive activity by mutating the $R^{5.69(225)}$ amino acid in the CCR5 receptor to amino acids Ala ($R^{5.69(225)}A$), Asp ($R^{5.69(225)}D$) and Glu ($R^{5.69(225)}E$). In this study we were not able to replicate the levels of constitutive activity of the $R^{5.69(225)}Q$ mutant. IP production showed that the mutant receptor $R^{5.69(225)}A$ produced basal levels to similar to the wild type receptor and therefore was not constitutively active, indicating that the neutralization of charge in this position did not lead to the stabilization of an active conformation. Similarly mutation of the Arg residue to acidic amino acids Asp and Glu did not result in CAMs of CCR5. These findings suggest that $R^{5.69(225)}$ does not play a role in stabilizing the inactive conformation of the CCR5 receptor. Similar mutations of an Arg residue in CCR5 located two amino acid residues upstream from the $R^{5.69(225)}$ residue, $R^{6.30(223)}$, to Ala, Glu, Gln and Asp also resulted in a decrease of constitutive activity when compared to the wild type receptor (Springael et al., 2007).

4.2 Mutations of $D^{3.49(125)}$ in the conserved DRY motif of CCR5 do not result in CAMs

Mutations of the conserved "DRY" motif, at the border between the third transmembrane helix and the second intracellular loop, have resulted in constitutive activity of some GPCRs (Rasmussen et al., 1999; Scheer et al., 1996). In the CCR5 receptor, mutation of the Arg residue in the DRY motif to Asn ($R^{3.50(126)}N$) resulted in a receptor that was uncoupled from the G protein but constitutively phosphorylated and internalized (Lagane et al., 2005). These findings suggest that the mutation stabilized a conformation that is internalized, but destabilized the conformation that activates Gi. In this study we mutated the aspartate to uncharged Ala ($D^{3.49(125)}A$) and Asn ($D^{3.49(125)}N$) to neutralize the negative charge of $D^{3.49(125)}$ in the DRY motif. This neutralization was proposed to disrupt the ionic interaction with $R^{3.50(126)}$, thereby re-orientating the residue and destabilizing the inactive conformation resulting in constitutive activity. Identical mutations in the β_2-adrenergic receptor ($D^{3.49(130)}A$ and $D^{3.49(130)}N$) resulted in constitutively active receptors displaying elevated levels of basal cAMP when compared to the wild type receptor (Rasmussen et al., 1999). IP results showed that the $D^{3.49(125)}A$ mutation of CCR5 resulted in lower levels of basal IP production when compared to the wild type receptor. The mutant also could not be further stimulated in the presence of MIP-1ß (10^{-7}M). The level of IP production is a factor of the amount of receptor expressed on the cell surface and therefore one possibility for the poor basal IP could be low expression of the receptor. FACS analysis of the $D^{3.49(125)}$A revealed low levels of receptor expression when compared to the wild type receptor. Furthermore IP production could not be further increased in the presence of 10^{-7}M MIP-1ß, suggesting that the mutant receptor may be uncoupled. The mutant may also be constitutively internalized which could also account for low receptor expression on the cell surface resulting in low basal IP and no further increase in IP in the presence of chemokine. The functional profile of this receptor suggests that substitution of the $D^{3.49(125)}$ with the small and neutral residue ,Ala, decreases receptor stability and therefore receptor expression is low due to enhanced receptor downregulation. Previous work with the $D^{3.49(125)}$V CCR5 mutant described a similar phenotype. The $D^{3.49(125)}$V mutant receptor displayed decreased basal signaling as well as decreased coupling to the G protein in the presence of agonist, without affecting affinity for chemokine (Lagane et al., 2005). These results suggest that the Asp residue in the DRY motif of CCR5 may be important for stabilizing the active R* conformation rather than the inactive R conformation, which is in contrast to the β-adrenergic receptor where it stabilizes the inactive R conformation. The $D^{3.49(125)}$N receptor displayed basal IP levels lower than the wild type receptor and showed an increase in IP production in the presence of 10^{-7}M MIP-1ß. The $D^{3.49(125)}$N receptor expression level was lower than that of the wild type receptor but higher than that of the $D^{3.49(125)}$A receptor, perhaps explaining the slight increase in IP production in the presence of 10^{-7}M MIP-1ß. The poor expression of the $D^{3.49(125)}$N receptor may explain the poor basal IP levels when compared to the wild type receptor. In addition, the Asn amino acid is a hydrophilic residue and may therefore be able to partially substitute for the Asp residue in the motif.

4.3 Mutation of the TXP motif stabilizes the activated CCR5 conformation.

The TXP motif is a highly conserved motif in transmembrane helix two of most chemokine receptors (Govaerts et al., 2001). It was shown that the extracellular domain of transmembrane helix 2 played a role in the activation process of CCR5 and it was proposed that the TXP motif may transmit agonist binding signals to the cytosolic surface (Govaerts et al., 2003). The TXP motif has a significant effect on the conformation of this helix, due to the presence of a proline kink enhanced by the threonine residue. The effect of the motif on the conformation of helix 2 as well as the high level of conservation of this motif, led to the hypothesis that the TXP motif played an important role in receptor activation and signaling Previous studies used a PCR-based approach to randomly mutate the CCR5 receptor in the presence of manganese and characterize the resultant mutants (Arias Alvarez et al., 2003). Mutants $T^{2.56(82)}P$, $T^{2.56(82)}K$ and $T^{2.56(82)}R$ displayed levels of constitutive activity when transiently expressed in Chinese Hamster Ovary (CHO) cells (Arias Alvarez et al., 2003). We created these mutants. All three mutants displayed increased basal IP when compared to the wild type receptor and could not be further stimulated in the presence of 10^{-7}M MIP-1ß. The absence of a further increase in IP production in the presence of 10^{-7}M MIP-1β, suggests that the receptors were maximally activated in the absence of chemokine. The $T^{2.56(82)}K$ mutant was poorly expressed when compared to the wild type receptor and this is characteristic of constitutively active mutants (Gether et al., 1997). Low levels of receptor expression could be as a result of constitutive downregulation. Constitutive downregulation results in low levels of cell surface receptor expression and therefore the addition of ligand would not result in a further increase in IP production (Parnot et al., 2002). Constitutive downregulation has been described for many GPCR CAMs (Heinflink et al., 1995; Pei et al., 1994). In certain cases decreased expression of CAMs is a direct result of the instability of the receptor as it has been shown that CAMs often display a high degree of conformational flexibility, producing inherently unstable proteins (Gether et al., 1997). Mutations affecting posttranslational modifications, such as palmitoylation and phosphorylation, of the receptor may also explain the low surface expression. These modifications may lead to enhanced receptor internalization and impaired receptor recycling or enhanced receptor degradation thereby decreasing receptor density on the cell surface (Zaitseva et al., 2003). The $T^{2.56(82)}K$ mutation may result in conformational changes that constitutively expose the serine residues within the cytoplasmic tail to GRKs and PKC leading to constitutive internalization and low cell surface receptor density. The $T^{2.56(82)}P$ mutant produced basal levels of IP that were 6.8 fold that of wild type levels. FACS analysis revealed expression of the $T^{2.56(82)}P$ receptor that was comparable to the wild type receptor. There was no further increase in IP production after adding 10^{-7}M MIP-1ß, suggesting that the receptor is maximally stimulated in the absence of chemokine. These findings support a role for the

TXP motif in receptor activation. The mutations introduced at $T^{2.56(82)}$ stabilize a receptor conformation able to interact with the G protein in the absence of agonist and this may be facilitated by distorting the helical conformation of transmembrane helix two. The differential expression of the $T^{2.56(82)}K$ and $T^{2.56(82)}P$ mutants, despite both showing enhanced basal IP, may suggest that these mutants are stabilized in distinct activated conformations that are differentially sensitive to internalization or misfolding. The $T^{2.56(82)}K$ mutant may be stabilized in a conformation rendering it more susceptible to cellular processes such as phosphorylation and internalization.

4.4 Normalizing receptor expression

The differential expression in mutant receptors was problematic, as the number of CCR5 receptors expressed on the cell surface has been shown to affect HIV infection and fusion efficiency (Lin et al., 2002; Reynes et al., 2000). This was evident in the preliminary fusion assay performed (figure 3.3) where results from poorly expressed $T^{2.56(82)}K$ and $R^{5.69(225)}D$ were difficult to interpret. If fusion efficiency was to be compared amongst mutant receptors, it was necessary to normalize mutant receptor expression and one way of doing this was to try and increase receptor expression of poorly expressed mutants. It has been demonstrated for other GPCRs that the use of membrane-permeable ligands, designated molecular chaperones, can enhance receptor expression by helping to stabilize and correctly fold nascent proteins, increasing protein export to the cell surface (Chen et al., 2006; Leskela et al., 2007; Petaja-Repo et al., 2002). Applying this model to the CCR5 receptor, we used the small molecular inverse agonist, TAK-779, to test whether it would have a similar effect. The effects of TAK-779 were evaluated using FACS analysis and initial results looked promising with an increased mean fluorescence in the wild type and mutant receptors, $R^{5.69(225)}A$ and $T^{2.56(82)}P$, in the presence of TAK-779 (1nM). However, follow up results were not consistent with early observations. The limited role played by TAK-779 as a molecular chaperone in these experiments may be explained by us testing mutant receptors that were fairly well expressed and therefore the effect of TAK-779 may have not been as marked. Follow-up experiments with the poorly expressed mutant receptors, such as $T^{2.56(82)}K$ and $D^{3.49(125)}A$, might have been useful. Another potential complication with using the inverse agonist TAK-779 to enhance receptor expression was removing the molecule from the cells before use in functional assays. Preliminary experiments to develop a wash protocol to ensure that the TAK-779 was effectively removed from the cells without damaging the integrity of the cell, suggested that TAK-779 could be relatively easily removed.

While investigating the potential role of TAK-779, to increase receptor expression of CCR5 mutants, an alternative approach was being carried out. The $R^{5.69(225)}Q$ mutation, which had previously displayed partial constitutive activity and expression levels comparable to the wild type receptor, was combined with either the $T^{2.56(82)}K$ or $T^{2.56(82)}P$ mutations. The intention was to create CAMs of CCR5 with similar expression to the wild type receptor. FACS analysis revealed enhanced expression of mutant $T^{2.56(82)}K/R^{5.69(225)}Q$ compared to the single mutant receptor $T^{2.56(82)}K$, while $T^{2.56(82)}P/R^{5.69(225)}Q$ displayed expression levels comparable to the wild type receptor and to the single mutant receptor $T^{2.56(82)}P$. These findings suggest that the $R^{5.69(225)}Q$ mutation may stabilize folding of the $T^{2.56(82)}K$ mutant or reduce constitutive downregulation of the mutant, thus increasing cell surface expression.

IP production of the double mutants showed the highest basal IP when compared to both the wild type and its single mutation counterparts. Similar to the single mutant phenotypes, the IP production did not increase in the presence of increasing concentrations of MIP-1ß, suggesting that these receptors were fully activated and that most receptor molecules were stabilized in a conformation that activates G protein signaling.

4.5 Ligand affinity of the $T^{2.56(82)}$ mutants

Constitutive activity has been reported to have varying effects on ligand binding affinities. In some receptors constitutive activity increased affinity for agonist ligands (Samama et al., 1993), while in others agonist affinity was decreased (Arias Alvarez et al., 2003). In a previous study, when the $T^{2.56(82)}$ residue was mutated to Pro, the receptor displayed affinity for MIP-1β, comparable to the wild type receptor in heterologous competition assays, while the $T^{2.56(82)}K$ mutant had a 5-fold decrease in affinity for MIP-1β (Arias Alvarez et al., 2003). In this study homologous competition binding assays were carried out with MIP-1ß. The $T^{2.56(82)}K$ mutant showed significantly decreased binding of ligand, due to decreased expression, when compared to the wild type receptor and as a result we could not determine an IC_{50} value. The receptors $T^{2.56(82)}K/R^{5.69(225)}$, $T^{2.56(82)}P$ and $T^{2.56(82)}P/R^{5.69(225)}Q$ displayed IC_{50} values comparable to the wild type receptor.

4.6 Differential fusion efficiency of $T^{2.56(82)}$ mutants reveals distinct activated conformations

Maximum fusion efficiency of mutant receptors was measured and compared to the wild type receptor. The mutants with Lys in position 82, $T^{2.56(82)}K$ and $T^{2.56(82)}K/R^{5.69(225)}Q$, both showed low fusion efficiency when compared to the wild type receptor. Both of these mutant receptors are fully stabilized in an activated conformation but have greatly decreased fusion efficiency. In contrast mutant receptors with Pro in position 82, $T^{2.56(82)}P$ and $T^{2.56(82)}P/R^{5.69(225)}Q$, displayed good fusion efficiency comparable to the wild type receptor. These receptors are also fully stabilized in an activated conformation but have contrasting fusion phenotypes when compared to the mutants with Lys in position 82. These results suggest that the receptors with Lys in position 82 and the receptors with Pro in position 82 represent two distinct activated conformations. CCR5-specific antibody studies have suggested that the co-receptor may exist in multiple conformational states (Lee et al., 1999). These conformational states may differ in their ability to support viral fusion (Zaitseva et al., 2003). Distinct activated conformations of CCR5 might explain why fully constitutively active mutant receptors, defined by G protein signaling, differ in the ability to support HIV fusion in cell-cell fusion assays.

These differences in conformation are not fully evident when comparing receptors using the IP assay. However there is a clear distinction when comparing the fusion efficiencies as well as expression levels of the receptors. In terms of the ensemble model of receptor conformations the mutation of $T^{2.56(82)}$ to Pro may be stabilizing an ensemble of microconformations which overlap between the G protein activated ensemble of conformations and the ensemble of co-receptor conformations of CCR5, however when $T^{2.56(82)}$ is mutated to Lys, only the G protein activated ensemble of conformations is stabilized. These findings highlight the importance of using multiple functional assays to evaluate and compare differences in receptor conformation.

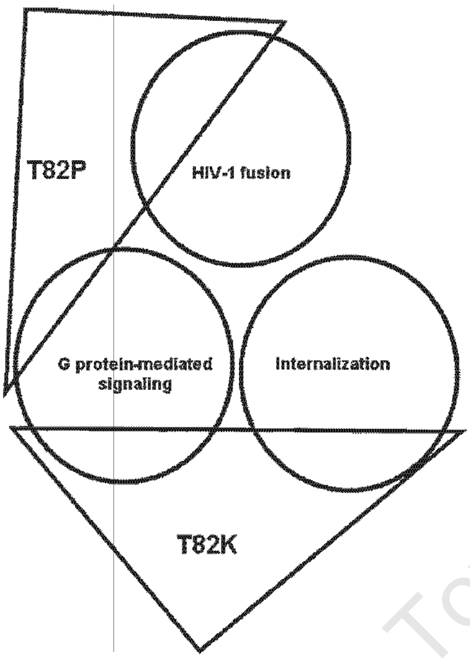


Figure 4.1: Venn diagram depicting ensembles of receptor conformations stabilized by $T^{2.56(82)}$ mutations. Mutation of Thr in position 82 to Lys leads to the stabilization of an ensemble of receptor conformations able to activate G protein-mediated signaling, but unable to support HIV-1 fusion. In addition this mutation may stabilize the receptor in an ensemble of conformations more susceptible to internalization. Mutation of the Thr in position 82 to Pro leads to the stabilization of an ensemble of receptor conformations able to activate G protein-mediated signaling and support HIV-1 fusion. *Adapted from (Kenakin, 2005).*

4.7 Final conclusions:

The different fusion efficiencies of our mutant CCR5 receptors suggest that mutation of the $T^{2.56(82)}$ residue to Lys stabilizes the receptor in an ensemble of activated conformations, which is able to interact with the G protein but unable to support HIV-1 fusion efficiently. Mutation of the $T^{2.56(82)}$ residue to Pro stabilizes the receptor in an ensemble of activated conformations, which is able to interact with the G protein and able to support HIV fusion efficiently. These results suggest that there are subtle structural differences between the chemokine receptor and co-receptor conformation of CCR5.

The existence of distinct activated conformations of CCR5 with differential ability to support HIV-1 fusion opens up the possibility of developing agonists capable of selecting for specific receptor conformations. This ligand bias can be therefore exploited for therapeutic value as in the case of HIV-1 infection.

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