

**FUNCTIONAL CONSEQUENCES OF SOUTH AFRICAN
MUTATIONS OF THE HIV-1 CO-RECEPTOR, CCR5**

by

Asongna Theresia Forkem Folefoc

University of Cape Town

Thesis presented for the Degree of
Doctor of Philosophy
In the Division of Medical Biochemistry
University of Cape Town
August 2007

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

This work is dedicated to my beloved, **Daniel** and our girls,
Anu and Nkolaka

Acknowledgements

I would like to thank the Third World Organization for Women in Science (TWOWS) and the International Academic Programmes Office (IAPO) of the University of Cape Town for financial support.

A grateful thank you to my supervisors: Dr. Colleen Flanagan for her relentless effort in teaching and guiding me and for being there whenever I needed her. She was an inspiration to me. Prof Arie Katz for his support and kind attention. Thank you for your encouragement and for all the inputs in making this thesis complete.

A special thank you to my husband who was always there to cheer me up with loving words of encouragement and support throughout this journey. Thank you for being there and for believing in me. To my girls, Anu and Nkolaka, I would like to thank them for their patience and understanding. Their beautiful smiles and happy faces kept me going. I would also like to say thank you to my uncle, Prof A. A. Amin and his wife, their every support and words of encouragement meant a lot to me.

A word of thank you to everyone I worked with in the Receptor Biology Research Group. They made my time in the laboratory a memorable one. A special thanks to Dr. Bernhard Fromme for his assistance in some experiments and his encouraging words.

I want to acknowledge the support and encouragement of my brother and friend, Mr. Asonganyi Thaddeus for his help and support during the course of this work. I would also like to thank his wife and kid for the friendship and love they provided. Many thank to Mr. Levai Matthew, Mr. Ajong Cletus and the Lebialem family members for being there for me throughout my stay in Cape Town.

I am thankful to Pastor Israel of the Light of the World Ministries for spiritual knowledge. To God Almighty, thank you for Health, Strength and Wisdom. Every good thing comes from you.

Declaration

This study was carried out at the Division of Medical Biochemistry, Faculty of Health Sciences, University of Cape Town, South Africa under the supervision of Dr. Colleen A. Flanagan and Prof. Arieh A. Katz.

These studies represent the unaided, original work of the author and have not been submitted in any form to another University. Where use has been made of the work of others, it has been appropriately acknowledged in the text.

Asongna Theresia Forkem Folefoc

August 2007

List of Conference presentations

[25th-31st March 2007]: Asongna T. Folefoc, Bernhard J. Fromme, Arieh A. Katz and Colleen A. Flanagan. *South African mutations of the CCR5 co-receptor for HIV modify function*. Keystone Symposium on HIV Pathogenesis Whistler, British Columbia, Canada.

[24th - 27th October 2006]: Asongna T. Folefoc, Bernhard J. Fromme, Marla Coetsee, Arieh A. Katz and Colleen A. Flanagan. *South African mutations of the CCR5 co-receptor for HIV modify function*. The First African Structural Biology Conference FASB 2006, The Wilderness, South Africa.

[8th - 11th November 2005]: Asongna T. Folefoc, Bernhard J. Fromme, Marla Coetsee, Arieh A. Katz and Colleen A. Flanagan. *South African mutations of the CCR5 co-receptor for HIV modify function*. Virology Africa-2005, held at Breakwater Lodge V&A Waterfront, Cape Town-South Africa

[22nd - 24th March 2005]: Asongna T. Folefoc, Bernhard J. Fromme, Marla Coetsee, Arieh A. Katz and Colleen A. Flanagan. *Functional consequences of South African mutations of the HIV co-receptor, CCR5*. Opening Conference of the Institute of Infectious Disease and Molecular Medicine, Faculty of Health Sciences at the University of Cape Town.

[12th - 14th January 2005]: Asongna T. Folefoc, Bernhard J. Fromme, Marla Coetsee, Arieh A. Katz and Colleen A. Flanagan. *Functional consequences of South African mutations of the HIV co-receptor, CCR5*. First international conference and trade fair on natural products and molecular therapy, Cape Town, South Africa.

List of Abbreviations

μCi	microcurie
A	Adenine
AGM	African green monkey
AIDS	Acquired immunodeficiency syndrome
AMPS	ammonium persulfate
bp	base pair
BSA	Bovine serum albumin
BV	Baculovirus
C	Cytidine
cAMP	cyclic Adenosine Monophosphate
CCR	CC chemokine receptor
CCR5	CC chemokine receptor 5
cDNA	complementary DNA
C-terminal	Carboxyl terminal
CXCR	CXC chemokine receptor
CXCR4	CXC chemokine receptor 4
D/ERY	Aspartate/Glutamate-Arginine-Tyrosine
DMEM	Dulbecco Modified Eagle's Medium
DNA	Deoxyribonucleic acid
ECL	Extracellular loop
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Env	HIV envelope protein
FACS	Fluorescent activated cell sorting
FCS	fetal calf serum
FS	frame shift
G protein	guanyl nucleotide binding protein
G	Guanine
GDP	Guanosine diphosphate
Gi protein	inhibitory G protein
GnRH	Gonadotropin releasing hormone
GnRHR	Gonadotropin releasing hormone receptor
gp120	glycoprotein 120
gp160	glycoprotein 160
gp41	glycoprotein 41
GPCR	G protein coupled receptor
GRK	G protein coupled receptor kinase
GTP	Guanosine triphosphate
G α	alpha subunit of G protein
G $\beta\gamma$	Beta-gamma subunit of G protein
H ₂ O ₂	hydrogen peroxide
H ₂ SO ₄	sulfuric acid
HAART	Highly active antiretroviral therapy
hCCR5	human chemokine receptor 5
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV-1	Human immunodeficiency virus type 1
HOS	Human osteosarcoma

HRP	Horseradish peroxidase
ICL	Intracellular loop
KDa	kilo Dalton
L	Litre
LTR	Long terminal repeat
Luc	luciferase
M	Molar
mCCR5	murine chemokine receptor 5
mCi	millicurie
MCP	Monocyte chemotatic protein
MIP-1 α	Macrophage inflammatory protein-1 alpha
MIP-1 β	Macrophage inflammatory protein-1 beta
mM	millimolar
M-tropic	Macrophage tropic
NaCl	sodium chloride
NNRTI	non-nucleoside reverse transcriptase inhibitor
N-terminal	Amino terminal
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrine
PI	protease inhibitor
PMSF	phenylmethylsulphonyl fluoride
PT	Petasis toxin
R5 virus	CCR5 using virus
RANTES	Regulated on activation normal T cell expressed and secreted
rpm	rotations per minute
SDF-1	stromal derived factor 1
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SIV	Simian immunodeficiency virus
T	thymidine
TBS	Tris buffered saline
TEMED	N,N,N',N'-tetramethylethylenediamine
TM	Transmembrane
TMB	3,3',5,5'-Tetramethyl benzidine
TRH	Tyrotropin releasing hormone
T-tropic	T-cell tropic
TX	triton X
TXP	Threonine-X-Proline (X is any amino acid)
Tyr (Y)	Tyrosine
U/ml	units per milliliter
V1, V2 or V3 loop	Variable loops 1, 2 or 3
Val (V)	Valine
X4 virus	CXCR4 using virus

List of figures

Figure	Title	Page No
Figure 1.1	Primary structure of a representative member of the rhodopsin family of G protein coupled receptors (β -adrenergic receptor)	10
Figure 1.2	Proposed models of receptor activation	15
Figure 1.3	HIV-1 entry into host cell and the molecular targets for anti-HIV therapy	20
Figure 1.4	Typical chemokine fold of the viral macrophage inflammatory protein-1 (vMIP-I)	26
Figure 1.5	A two-dimensional model of CCR5	35
Figure 1.6	CCR5 activation and signalling via Gi protein	42
Figure 1.7	CCR5 mutants and variants	50
Figure 2.1	Positions of the different primers used for amplification	56
Figure 2.2	Primers used for amplification	56
Figure 3.1	Production of mutant receptors	67
Figure 3.2	Restriction enzyme analysis of mutant receptors	67
Figure 3.3	Stimulation of inositol phosphate production by MIP-1 α and RANTES	69
Figure 3.4	Enhanced basal activity of Arg225Gln CCR5	70
Figure 3.5	Profile of radio-iodinated MIP-1 β	71
Figure 3.6	Fractions of label tested for binding to CCR5	72
Figure 3.7	Competition binding of MIP-1 β	73
Figure 3.8	FACS	75
Figure 3.9	Cell surface expression of wild type and mutant CCR5	76
Figure 4.1	Luciferase activity of cells stably transfected with LTR-Luciferase	81
Figure 4.2	Expression of mutant CCR5 in stably transfected cells	82
Figure 4.3	Effect of hygromycin B treatment on CCR5 expression	83
Figure 4.4	HIV-1 fusion efficiencies and coreceptor function of CCR5 mutants	85
Figure 4.5	Expression of purified gp120	87
Figure 4.6	gp120-CD4 binding	87
Figure 4.7	Typical spline curve for determination of gp120 concentration	88
Figure 4.8	gp120 competition binding	89

Lists of tables

Table	Title	Page No
Table 3.1	Summary of chemokine binding and signalling of wild type and mutant CCR5	74
Table 3.2	Effects of mutations on cell surface expression of the CCR5 receptor	77
Table 4.1	Expression and fusion efficiencies of wildtype and mutant CCR5 in HOS cells after two days selection with hygromycin B	85

TABLE OF CONTENTS

ABSTRACT	4
CHAPTER ONE: INTRODUCTION.....	6
Summary	7
1. INTRODUCTION TO G PROTEIN COUPLED CHEMOKINE RECEPTOR 5 (CCR5) AS A RECEPTOR FOR THE BETA CHEMOKINES AND FOR HIV ...	8
1.1 G PROTEIN COUPLED RECEPTORS.....	8
1.1.1 GPCR numbering scheme.....	10
1.1.2 Ligand binding to and activation of GPCRs.....	11
1.1.3 Models of receptor activation	14
1.1.4 GPCRs as drug targets.....	16
1.2 HIV-1 INFECTION.....	17
1.2.1 Mechanism of host cell infection	18
1.2.2 Chemotherapy of HIV-1 infection.....	19
1.2.3 HIV-1 Vaccine development	24
1.3 CHEMOKINES AND CHEMOKINE RECEPTORS	25
1.3.1 Ligand binding and activation of chemokine receptors.....	27
1.3.2 Chemokines, Chemokine receptors and HIV.....	28
1.4 THE CC CHEMOKINE RECEPTOR 5 (CCR5).....	30
1.4.1 Structure of CCR5.....	32
1.4.1.1 The extracellular domain.....	33
1.4.1.2 Transmembrane domain.....	35
1.4.1.3 The intracellular domain.....	36
1.4.2 CCR5 as a chemokine receptor	37
1.4.3 Ligand binding to CCR5.....	38
1.4.3.1 Structural features of CCR5 important for chemokine binding.....	40

1.4.4 CCR5 activation and signalling.....	41
1.4.5 CCR5 phosphorylation and arrestin interaction.....	43
1.4.6 HIV-1 coreceptor function of CCR5.....	44
1.4.7 Naturally occurring mutations of CCR5.....	47
1.5 CONCLUDING REMARKS.....	51
CHAPTER TWO: MATERIALS AND METHODS.....	54
2.1 Materials.....	55
2.2 Primers	55
2.3 Mutant receptor construction.....	57
2.4 Cell Culture	58
2.5 Transfection	58
2.6 Inositol Phosphate (IP) assay.....	58
2.7 Chemokine competition binding assay	59
2.7.1 Iodination of MIP-1 β	59
2.7.2 Competition binding assay.....	60
2.8 FACS Analysis	60
2.9 DU151 Baculovirus (BV) amplification	61
2.10 Expression and purification of gp120	61
2.11 SDS polyacrylamide gel electrophoresis and western blotting.....	62
2.12 gp120 – CD4 binding assay	63
2.13 gp120 ELISA	63
2.14 HIV Fusion assay	64
CHAPTER THREE: EFFECTS OF CCR5 MUTATIONS ON CHEMOKINE RECEPTOR FUNCTION AND EXPRESSION	65
3.1 INTRODUCTION	66
3.2 Construction of CCR5 mutants.....	66
3.3 Effects of mutations on CCR5 signalling (IP production).....	68
3.4 Ligand binding of CCR5 mutant receptors	70
3.5 Expression of Wild type and mutant receptors	74
3.6 Summary.....	77

CHAPTER FOUR: EFFECTS OF CCR5 MUTATIONS ON HIV-1 CO-RECEPTOR FUNCTION.....	78
4.1 Introduction	79
4.2 Cell-cell fusion.....	79
4.2.1 Generation of cells for fusion assay.....	80
4.2.2 Expression of CCR5 mutants in HOS-CD4-luciferase (CD4-10) cells.....	81
4.2.3 Effects of mutations on cell-cell fusion	84
4.3 CCR5 –gp120 interaction.....	86
4.3.1 Expression and functionality of purified gp120.....	86
4.3.2 Concentration of purified gp120.....	88
4.3.3 CCR5 binding.....	88
4.4 Summary.....	89
CHAPTER FIVE: DISCUSSION AND CONCLUSION.....	91
5.1 DISCUSSION.....	92
5.2 CONCLUSION.....	98
REFERENCES	100

ABSTRACT

The chemokine receptors CCR5 and CXCR4 are the major co-receptors for human immunodeficiency virus type-1 (HIV-1). CCR5 is also a receptor for the CC-chemokines, MIP (macrophage inflammatory protein) 1 α /CCL3 and 1 β /CCL4 and RANTES/CCL5 (regulated on activation normal T cell expressed and secreted) amongst others. Individuals, who are homozygous for the non-functional CCR5 Δ 32 allele, are largely resistant to HIV-1 infection and heterozygous carriers show partial protection. Four mutations of the CCR5 receptor have been identified in South African populations, but the effects of these mutations on CCR5 function and HIV infection are unknown. We have used *in vitro* methods to assess the effect of the mutations, Asp2Val, Leu107Phe, Arg225Gln and Arg225stop, on CCR5 interactions with chemokine ligands and HIV.

The conservative Leu107Phe mutation had no measurable effect on any CCR5 function. The Asp2Val mutation, at the amino-terminal of CCR5, decreased binding affinity for MIP-1 β , decreased chemokine-stimulated intracellular signalling and decreased expression of the receptor. It also decreased HIV-1 envelope-mediated fusion in a cell-cell fusion assay and we showed that this mutation had a higher binding affinity for the gp120 HIV envelope glycoprotein than the wild type receptor. The Arg225Gln mutation had no effect on CCR5 expression or binding affinity for MIP-1 β , but the mutant receptor stimulated intracellular signalling in the absence of added ligand, suggesting that the mutant receptor is constitutively active and stabilised in an activated conformation. Cell-cell fusion experiments suggest that constitutive activity of Arg225Gln mutant does not affect HIV fusion efficiency. The Arg225stop mutant, which truncates the receptor in the third intracellular loop, yielded no measurable chemokine binding or signalling. Fluorescence Activated Cell Sorting (FACS) analysis showed no measurable expression of Arg225Stop mutant at the cell surface or within the cell and consistent with this, it did not support HIV envelope-mediated cell-cell fusion. This phenotype is similar to the CCR5 Δ 32 phenotype and might predict that homozygous individuals are resistant to HIV infection.

These results show that South African variants of the CCR5 chemokine receptor have a range of phenotypes *in vitro* that may reflect altered chemokine responses and susceptibility to HIV infection of individuals who carry these alleles.

University of Cape Town

CHAPTER ONE: INTRODUCTION

University of Cape Town

SUMMARY

Human immunodeficiency virus type 1 (HIV-1) infection of permissive cells requires binding of the virus envelope protein to CD4, a cell surface protein expressed on macrophages and T helper cells and a coreceptor belonging to the G protein coupled receptor (GPCR) family. GPCRs are surface proteins that mediate intracellular actions by activating one or more heterotrimeric G proteins. The involvement of a GPCR in HIV-1 infection provides a target for treatment intervention. Several studies have demonstrated coreceptor activity for many different chemokine receptors or orphan, chemokine receptor-like molecules but CXCR4 and CCR5 molecules remain the principal co-receptors for X4 (T cell line-tropic or syncytium inducing) or R5 (macrophage (M)-tropic or non-syncytium inducing) isolates respectively. CCR5 is of particular interest since it is the coreceptor used by the viruses that are transmitted.

Topologically, CCR5 is arranged with seven membrane-spanning domains, an extracellular N-terminus, an intracellular C-terminus and alternating extracellular and intracellular loops. High affinity ligands and high potency agonists of CCR5 that have been identified include MIP (macrophage inflammatory protein)-1 α /CCL3 and 1 β /CCL4, RANTES/CCL5 (regulated on activation normal T cell expressed and secreted), LD78 β /CCL3L1 and macrophage chemoattractant protein-2 (MCP-2). Binding of ligand to CCR5 leads to activation of the Gi family of G proteins and inhibition of cyclic AMP production and the resultant effect is chemotaxis. Domains of CCR5 involved in both chemokine and Env interaction have been widely studied by analyzing CCR5 chimeras and site-directed mutants, comparing chemokine receptor homologues from different species and using anti-CCR5 antibodies. Even though the results of these studies are complex and in some instances, contradictory, most evidence points to an important role played by the N-terminal segment in Env interaction while chemokine binding to the N-terminus of the receptor seems to facilitate interaction with the extracellular loops, in particular, the second extracellular loop.

1. INTRODUCTION TO G PROTEIN COUPLED CHEMOKINE RECEPTOR 5 (CCR5) AS A RECEPTOR FOR THE CC CHEMOKINES AND FOR HIV

This review will focus on the structure and function of the chemokine receptors as G protein coupled receptors and HIV-1 coreceptors. In the first part, G protein coupled receptors will be briefly reviewed in terms of types, ligand diversity, structure, ligand binding and activation. GPCRs in disease pathogenesis and in particular, the chemokine receptors in inflammation and in HIV-1 infection will be overviewed. Particular focus will be on the interaction between the CC chemokine receptor 5 (CCR5) and its ligands and HIV-1.

1.1 G PROTEIN COUPLED RECEPTORS

G protein coupled receptors (GPCRs) are glycoproteins which mediate their intracellular actions by a pathway involving the activation of one or more heterotrimeric G proteins. These receptors are activated by several different types of chemical messengers in the body including hormones, chemo-attractants, neurotransmitters and paracrine agents. GPCRs are the largest group of cell surface receptor molecules comprising more than 1000 representatives that have been identified and classified into more than 100 subfamilies based on genetic sequence homology, ligand structure and receptor function (Ji *et al.*, 1998). Seven different families have been recognized based on their native ligands, phylogenetic analysis of clustering of genes in the human genome and by certain key sequences: rhodopsin/ β adrenergic related receptors (type A), calcitonin receptor related receptors (type B), large N-terminal family B-7 transmembrane helix, receptors related to the metabotropic glutamate receptors (type C), Frizzled/Smoothed, taste 2 and vomeronasal 1 receptors. (Gether, 2000; Kristiansen, 2004). The rhodopsin-related receptors are the most widely studied. Despite the differences that exist between subfamilies, rhodopsin-like receptors share a considerable structural homology reflecting their common mechanism of action. Studies on more than 100 members of the rhodopsin

GPCR family have shown that they are characterized by an N-terminal segment, seven hydrophobic stretches of 20-25 amino acids which form transmembrane (TM) alpha helices and a C-terminal segment (Strader *et al.*, 1994). The α helices traverse the membranes in an anti parallel manner and are linked by alternating extracellular and intracellular loops thus forming three extracellular and three intracellular loops (Milligan and White, 2001; Mirzadegan *et al.*, 2003); (Strader *et al.*, 1994; Ji *et al.*, 1998). A fourth intracellular loop is formed when the C-terminal segment is palmitoylated at cysteine (Ji *et al.*, 1998). The N-terminal sequence is extracellular and the loops on the extracellular surface are short whereas the C-terminal is intracellular (Milligan and White, 2001). It has been demonstrated that the extracellular domain of type A GPCRs is the least conserved, while considerable conservation is displayed towards the cytoplasmic side (Mirzadegan *et al.*, 2003). GPCRs have been shown to have a number of conserved cysteine residues and at least one concensus sequence for N-linked glycosylation (Strader *et al.*, 1994).

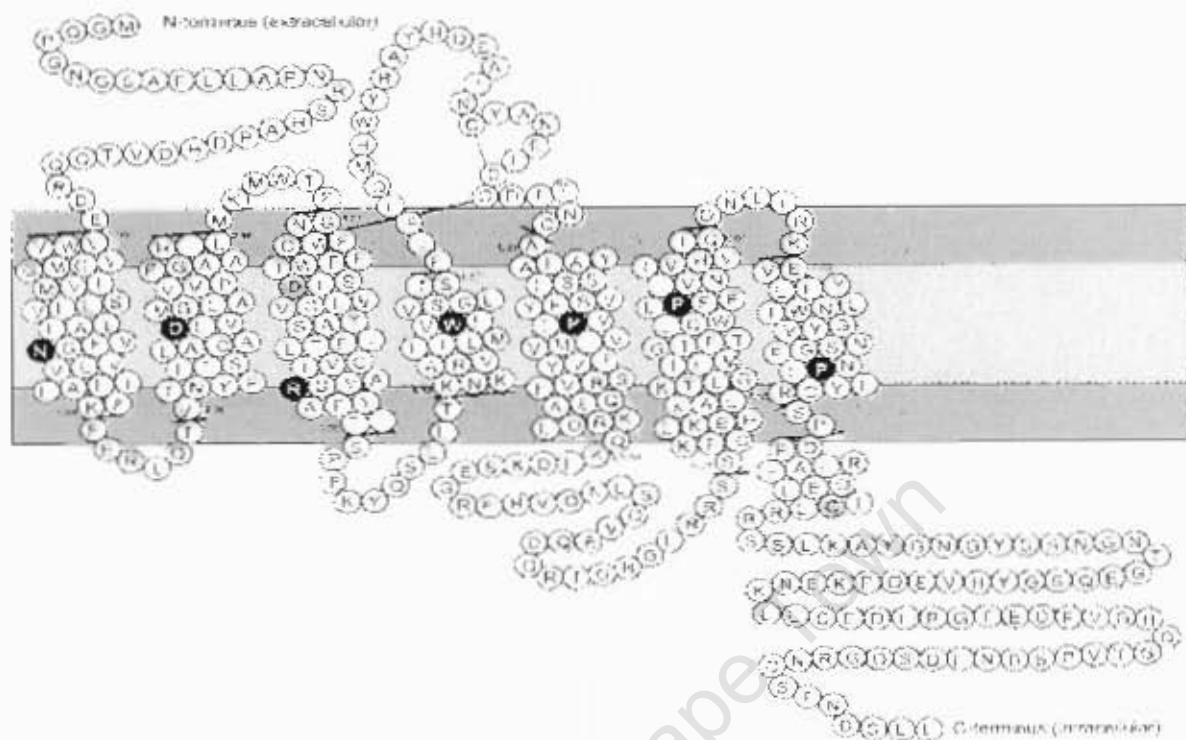


Figure 1.1: Primary structure of a representative member of the rhodopsin family of G protein coupled receptors (β_2 -adrenergic receptor) showing its transmembrane arrangement with the N-terminus on the extracellular and the C-terminus on the intracellular side of the membrane. The most conserved residues of each transmembrane domain among most of family A receptors is indicated (black circles). Disulfide bridge formed between cysteines at extracellular loops 1 and 2 is also depicted (Kristiansen, 2004). The equivalent residues for the CCR5 chemokine receptor is depicted on Figure 1.5.

1.1.1 GPCR numbering scheme

Due to the differences that exist among the lengths of the N-terminal regions and the loops connecting the helices of different members of the GPCR family, there is the necessity for a numbering scheme to allow comparison of equivalent residues in different receptors. Different methods of numbering have been proposed (Baldwin, 1993; Oliveira *et al.*, 1994) but these have a drawback in that they depend on the assignment of the apparent/estimated start of each transmembrane helix. A consensus numbering system has been proposed by Ballesteros and Weinstein (1994) in which positions are allocated independent of the perceived start of the transmembrane (TM). In this general numbering

scheme, amino acid residues are identified relative to a reference residue within the TM which is the most conserved residue (Figure 1.1) within the particular TM and is assigned the number 50. The number of the helix which features the particular residue is allocated first followed by the position relative to position 50 in the helix. Any residue before the conserved residue takes a number below 50 and those that come after the conserved residue take a number above 50 in the right order (Ballesteros, 1995). An extended notation for this numbering system gives a number in parenthesis which identifies the specific sequence position of the amino acid residue in a particular receptor. Thus in the CCR5 molecule, Asp3.49 (125) represents an Aspartic acid residue at position 125 in the amino acid sequence of the receptor and is positioned in helix 3, 1 residue before the most conserved residue (Arg 3.50) in that helix (Kristiansen, 2004). This nomenclature is used in this thesis.

1.1.2 Ligand binding to and activation of GPCRs

GPCRs are the cell surface receptors with the most diverse array of ligands. The family is known to bind and respond to agonists ranging from photons, amino acids, ions, inorganic odorants, nucleosides, nucleotides, peptides and bioactive lipids to large glycoproteins (Strader *et al.*, 1994). GPCRs are involved in ligand binding, signal generation, transmembrane signal transduction and signal transfer to cytoplasmic effector molecules (Strader *et al.*, 1994; Ji *et al.*, 1998; Milligan and White, 2001).

There is a distinction between ligand binding and receptor activation which is supported by the existence of antagonists that bind, but do not activate the receptor and inhibit agonist binding. Interactions of ligands and receptors are thought to involve hydrogen bonds, ion pairs and hydrophobic contacts (Ji *et al.*, 1998). The wide structural difference among the extracellular ligands of rhodopsin-like GPCRs means that each receptor must have evolved specific structural characteristics to link the specific recognition of its cognate ligand to what is believed to constitute a common activation process (Gether, 2000). Several distinct models have been observed for high affinity ligand-binding to GPCR; small molecular weight ligands interact preferentially with the

transmembrane domain, while the relatively high molecular weight ligands tend to bind to the extracellular loops and N-terminus (Youn *et al.*, 2001). The β_2 -adrenergic receptor, which is a prototypical member of family A GPCR, is one of the most extensively studied receptors, thus can provide an excellent model for studying ligand interaction and mechanism of receptor activation. Studies using site directed mutagenesis, molecular modelling techniques and spectroscopic analysis demonstrated that the binding sites for the small molecule agonists of the β_2 -adrenergic receptor are situated in a crevice between TM3, TM4, TM5, TM6 and TM7 (Dixon *et al.*, 1987; Strader, 1987; Tota and Strader, 1990). The positively charged amine group of the ligand was shown to form a salt bridge with the carboxyl side chain of Asp^{3.32}(113) in TM3. This residue is conserved among the biogenic amine receptors and is also bound by the β_2 -adrenergic receptor antagonists for the salt bridge formation (Gether, 2000). Other important interactions of the β_2 -adrenergic receptor include hydrogen bonds between the *para* and *meta* hydroxyl groups of the catechol ring and Ser^{5.43}(204) and Ser^{5.46}(207), both in TM5 (Strader *et al.*, 1989). Ser^{5.42}(203) has also been shown to be accessible on the surface of the binding-site crevice and was suggested to form a hydrogen bond with the heterocyclic ring of the partial agonist, pindolol (Liapakis *et al.*, 2000). A hydrogen bond between Asn^{6.56}(293) in TM6 and the β -hydroxyl of epinephrine has been suggested while Phe^{6.50}(290) also in TM6 is thought to stabilize the catechol ring (Wieland *et al.*, 1996).

In contrast to small molecule ligands, the binding sites of most peptide receptors have been mapped to the extracellular domains but some evidence of the involvement of transmembrane residues have been reported (Gether, 2000; Kristiansen, 2004). For the tripeptide, N-formyl-Met-Leu-Phe, studies have shown that the N-formyl moiety binds to the TM core while the C-terminal region of the ligand interacts with the N-terminal and extracellular loops 1 and 2 (Ji *et al.*, 1998). Mutational analyses of the human neurokinin receptors have identified residues in the N-terminus that are important for ligand interaction even though some residues in TM3, TM5 and TM7 were found to affect binding of the ligands to the receptors suggesting the presence of a binding pocket similar to that found in biogenic amine receptors (Gether, 2000). Other family A peptide and glycoprotein hormone receptors that have been studied including receptors for neuropeptide Y, gonadotropin releasing hormone (GnRH), chemokines, tyrotropin

releasing hormone (TRH) and angiotensin, their ligands were shown to interact directly with residues at the N-terminus and extracellular loops (Gether, 2000; Kristiansen, 2004).

An agonist ligand was first thought to be the regulator which selects an active conformation of the receptor (De Lean *et al.*, 1980) but modern theories have now proposed active receptor states that can exist even in the absence of an agonist. This current model was based on the findings of Samama *et al.* (1993) who showed that mutations in the third intracellular loop of the β_2 -adrenergic receptor resulted in its activation in the absence of a ligand (constitutive activation). These mutants were also demonstrated to have high affinity for agonists in the absence of G proteins but not for antagonists and exhibited increased potency of agonist stimulation of second messengers and increased intrinsic activity for partial agonists (Samama *et al.*, 1993).

A growing amount of biochemical and biophysical data are now available that can help identify the key aspects of receptor activation. The transition from an inactive to an active state of a receptor requires the reorganization of the transmembrane helix bundle. It has been suggested that the cytoplasmic parts of the TM helices move apart to disclose epitopes of the receptor to intracellular signalling molecules and in the rhodopsin-like family of GPCRs, motions of transmembrane helix 3 (TM3) and TM6 during the process of activation have been identified. These TM movements were thought to be effected by disruption of ionic interactions between the highly conserved Arg³⁵⁰(131) at the cytoplasmic end of TM3 with adjacent Asp³⁴⁹(130) and with Glu⁶³⁰(268) at the cytoplasmic end of TM6 of the β_2 -adrenergic receptor. Charge neutralizing mutations of the Glu⁶³⁰(268) and Asp³⁴⁹(130) in the β_2 -adrenergic receptor either alone or in combination were shown to cause an increase in basal and pindolol-stimulated cAMP accumulation in transfected COS-7 cells (Ballesteros *et al.*, 2001). Additional evidence of TM6 movement came from the cysteine accessibility studies of Javitch *et al.* (1997). These authors demonstrated, using a constitutively active β_2 -adrenergic receptor, that a charged, hydrophilic, sulfhydryl-specific reagent could significantly inhibit antagonist binding to the receptor. They suggested that Cys⁶⁴⁷(285) in TM6 may be responsible for the susceptibility of the constitutively active receptor to the sulfhydryl-specific reagent. The accessibility of Cys⁶⁴⁷(285) resulted from rotation or tilting of TM6 as a result of activation

of the receptor, a rearrangement which could bring Cys^{6.47}(285) to the margin of the binding site crevice where it becomes accessible to the sulfhydryl-specific reagent (Javitch *et al.*, 1997). Rigid-body movements of TM5 and TM7 have been proposed for activated receptors (Gether, 2000; Govaerts *et al.*, 2003).

1.1.3 Models of receptor activation

Several theoretical models have been put forward to elucidate the process of activation of GPCRs. One of the first models that was proposed was the ternary complex model (De Lean *et al.*, 1980) which suggests that upon receptor activation, following ligand binding, the receptor adopts a conformation that facilitates its interaction with the G protein. This interaction between the agonist, receptor and the G protein leads to the formation of a "ternary complex" (De Lean *et al.*, 1980). This model did not accommodate subsequent observation by Samama *et al.* (1993) that some receptors couple to and activate the G protein in the absence of ligand. These authors demonstrated that a replacement of the C-terminal region of the third intracellular loop of the β_2 -adrenergic receptor with that of the α_{1B} -adrenergic receptor leads to agonist independent activation of the receptor. This mutant receptor demonstrated an increased affinity for agonist even without G protein and an increased intrinsic activity for partial agonists. No preference was observed for antagonists (Samama *et al.*, 1993).

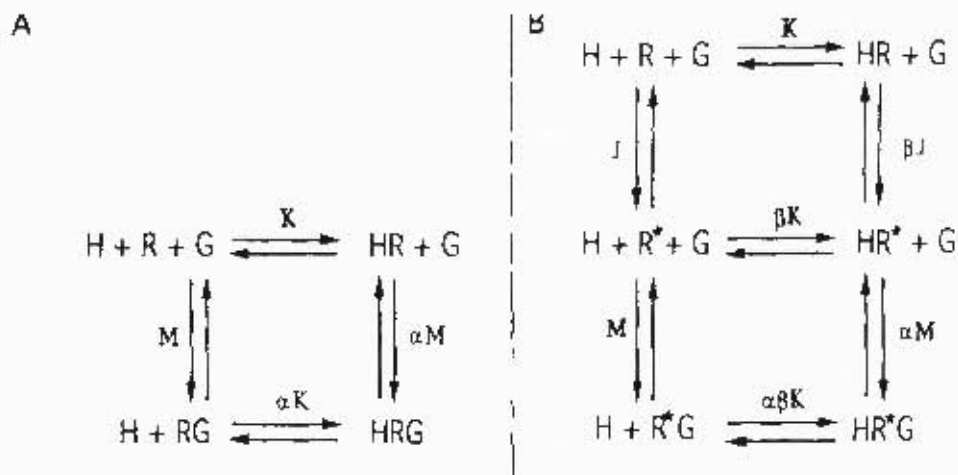


Figure 1.2: Proposed models of receptor activation. (A). The ternary complex model of De Lean *et al.* (1980) depicting the interaction of the hormone, H, the receptor R and the G protein, G. **(B).** The extended ternary complex model in which R undergoes an allosteric transition to an intermediate, R*. R* then interacts with H and G (Reproduced from Samama *et al.*, 1993)

In light of this, an extended ternary complex model was proposed which both accommodates the phenomenon of agonist-independent receptor activity and the complex behavior of different classes of ligands (agonists, partial agonists, inverse agonists, neutral antagonists) (Gether and Kobilka, 1998; Gether, 2000). This two-state model suggests that the receptor exists in an equilibrium between an inactive conformation (R) and an active conformation (R*) and in the absence of bound ligand, the inactive state prevails resulting in low basal levels of signalling in the absence of ligand. Agonist independent (constitutive) activity has been described for several GPCRs and in most cases, mutations of the Glutamic/Aspartic acid residue in the Glu/Asp/Arg/Tyr motif has been implicated. In one study, replacement of the Asp³⁴⁹(142) of the Asp/Arg/Tyr motif of the α_{1B} -adrenergic receptor with alanine conferred high constitutive activity of the receptor (Scheer *et al.*, 1996). Another study with opsin showed that replacement of Glu³⁴⁹(134) of the Glu/Arg/Tyr motif with Gln leads to increased activity in the absence of ligand while aspartic acid at the same position inhibited this activity (Cohen *et al.*, 1993). Rasmussen and collaborators also mutated the Asp residue of this motif in the β_2 -adrenergic receptor, Asp³⁴⁹(130), to Asn and Ala and found that both mutant receptors showed constitutive activity (Rasmussen *et al.*, 1999). These results suggest the importance of the negative charge of Asp/Glu in stabilizing the inactive state of GPCRs.

Agonists are predicted to bind with high affinity to and stabilize the R* conformation thereby shifting the equilibrium and the proportion of receptors in the R* conformation. Inverse agonists (negative antagonists) are thought to move the equilibrium away from the R* state by stabilizing the inactive R state (Gether and Kobilka, 1998; Gether, 2000) while neutral antagonists are compounds that bind with the same affinity to both the R and the R* and have been suggested not to have any effect on the equilibrium (Gether, 2000). The extended ternary complex model is currently the most widely accepted model for GPCR activation but a model which represents only two conformational states of the receptor alone is not sufficient to explain the complex behaviour of many GPCRs.

A multistate model has been proposed in which the receptor is suggested to alternate between multiple active and inactive conformations (Kenakin, 2004). In this model, the conformation to which a ligand binds with highest affinity determines the biological response and pharmacological properties of the ligand (G protein activation, internalization, dimerization etc.), thus reflecting the different kinds of efficacy that different ligands can have (Kenakin, 2002). There is no requirement for a common binding mode for agonist to trigger receptor activation (Gether, 2000).

1.1.4 GPCRs as drug targets

Seven transmembrane receptors that couple to G proteins have proved to be extremely important protein targets for therapeutic intervention and many drugs that target these proteins have passed clinical trials and are now available on the market (Onuffer and Horuk, 2002). Currently, most of the drugs that are in the market target the biogenic amines receptors, such as the 5HT₁, histamine, muscarinic acetylcholine and dopamine receptors and adrenoceptors. Knowledge drawn from the development of these categories of drugs will be essential for the development of small molecule antagonists of other receptors of the same family especially those of peptide and protein receptors. Chemokine receptors have attracted intense interest as targets for drug development since they have been shown to play important roles in many disease processes such as in chronic inflammation, angiogenesis and

angiostasis (Oruffier and Horuk, 2002). These proteins have also been exploited by pathogens, the most important pathogen being the human immunodeficiency virus which uses the chemokine receptors CXCR4 and CCR5 to gain entry into cells. Thus small molecule chemokine receptor inhibitors that target these receptors could be used as anti-HIV-1 drugs to fight the disease. Many chemokine receptor antagonists that target CCR5 are now at an advanced stage of development (Ribeiro and Horuk, 2005; Liste and Telenti, 2007). The structure-function relationship of these receptors and their ligands and the conformational changes that occur after ligand binding and HIV-1-gp120 interaction need to be considered when designing these small molecule inhibitors.

1.2 HIV-1 INFECTION

The human immunodeficiency virus (HIV), the etiologic agent of the acquired immunodeficiency syndrome (AIDS), unknown to medical science before 1982, has now infected millions of individuals worldwide (Farzan *et al.*, 1997b; McCutchan, 2000). Current data show that 39.5 million people are living with HIV/AIDS with 4.3 million new infections in 2006 (UNAIDS, 2006). There are two types of HIVs classified according to genetic and serological differences and also in their geographic distribution. HIV-1 is the most common type found worldwide and HIV-2 is found mostly in West Africa (McCutchan, 2000). HIV-1 is divided into three groups, M, N and O with group M being responsible for most of the disease worldwide. Nine subtypes and fourteen circulating recombinant forms of group M viruses have been characterized. The different subtypes are not evenly distributed in Africa but in Central Equatorial Africa where HIV has been evolving the longest almost all subtypes have been identified (Burgers and Williamson, 2005). In South Africa, most infections are predominantly with subtype C. The high degree of viral diversity is associated with high mutation and recombination rates coupled with rapid turnover of the virus (McCutchan, 2000; Burgers and Williamson, 2005). Since the discovery of this highly infective and deadly virus, research has been focused on understanding its biology and its interaction with host cells leading to possible ways to fight the virus through the use of drugs and vaccines.

1.2.1 Mechanism of host cell infection

HIV enters CD4⁺ T-cells by a cascade of molecular interactions between the virion envelope glycoprotein and two specific receptors on the host cell surface. All well characterized HIV-1 isolates have been shown to be dependent on CD4 as the primary viral receptor, but expression of CD4 alone is not enough to explain why different HIV-1 viruses can infect different subsets of CD4 expressing cells in vitro (Broder and Berger, 1995; Weismann et al, 1995; Berger *et al.*, 1999). It was later found that the receptor (CD4) could render cells permissive for Env-mediated fusion, entry and infection only when expressed on a human cell type, a finding that was supported by cell hybrid experiments in which a conclusion was drawn on the need for a cofactor (co-receptor) that is specific to human cells (Berger *et al.*, 1999). Thus infection of host cells involves the complex interaction between the viral envelop glycoproteins (gp120 and gp41), CD4 and a coreceptor. A straight forward model suggests that gp120 binds CD4, on the target cell surface and this initiates conformational changes in the gp120 glycoprotein and possibly CD4 exposing a previously hidden coreceptor binding site. The CD4-gp120 complex then binds to the coreceptor and stabilizes virus binding triggering a further conformational change, which leads to the apposition of the viral and host cell membranes and exposure of hydrophobic gp41 fusion peptide. Insertion of this fusion peptide into the host cell membrane then induces membrane destabilization, mixing and hence fusion of the virus with the target cell (Broder and Collman, 1997; Howard *et al.*, 1999; Weiss, 2001; Farber and Berger, 2002). Once the virus enters the cell, it releases its RNA and the enzyme reverse transcriptase. This enzyme is known to catalyse the production of viral DNA from viral RNA. The viral DNA then enters the nucleus of the host cell and is incorporated into cellular DNA by the integrase enzyme. The protein synthesis machinery of the cell then produces the protein Gag-Pol which together with the gag and the env proteins is cleaved by the virus encoded aspartyl protease (Kazmierski *et al.*, 2006). Further enzymatic cleavage gives rise to functional proteins that are necessary for the structure of HIV. The fusion/entry process thus presents multiple protein targets for chemotherapeutic attack both on the virus (gp120 and gp41) and the host target cell (CD4 and coreceptors) (Farber and Berger, 2002).

1.2.2 Chemotherapy of HIV-1 infection

In the absence of potent anti-HIV vaccines, other preventive methods have to be employed to fight the ongoing AIDS pandemic (Neurath *et al.*, 2001). This will include the development and application of safer and effective chemotherapeutic agents against the virus or against key processes that lead to infection by the virus.

Great progress in this line was achieved by the discovery of potent inhibitors of the viral reverse transcriptase and protease enzymes. Subsequently, studies proved that triple combination therapy or highly active antiretroviral therapy (HAART) and other triple combinations that included the non-nucleoside reverse transcriptase inhibitors (NNRTIs) were efficient in reducing mortality and suppressing viral load (Pomerantz and Horn, 2003). These combination drugs markedly suppressed viral replication in most treated individuals thus restoring their immune system function (Farber and Berger, 2002; Pomerantz and Horn, 2003).

Despite the use of these inhibitors for HIV therapy, there is still a growing need for improved therapies (Strizki *et al.*, 2001; Takashima *et al.*, 2001; Farber and Berger, 2002) due to problems that limit the current regimen. There is the current problem of resistance to the inhibitors which is mainly due to the low fidelity of the HIV reverse transcriptase coupled with the lack of associated proofreading function. This creates the possibility for a number of mutations in the genes of HIV to emerge and be selected during drug treatment as the predominant strain (Wang *et al.*, 1999; Kim *et al.*, 2001). Apart from the emergence of resistance to most available antiretroviral drugs, there is also the difficulty of adhering to certain treatments that require high pill burdens, multiple daily administration and sometimes disabling side effects (Strizki *et al.*, 2001; Farber and Berger, 2002). In some countries, particularly in Africa where the pandemic is spreading at "an alarming rate" the cost of these drugs are still not affordable (Farber and Berger, 2002; Treurnicht *et al.*, 2002).

Due to drug resistance of HIV that are now associated with the current antiretroviral drugs, most researchers suggest the development of anti-HIV drugs that will target

components of the host cell machinery essential for viral replication. In this case, such molecules will not mutate in the presence of drug pressure (Farber and Berger, 2002). The mechanism by which HIV enters the host cell is proving promising and is receiving greater attention in the current search for new anti-viral agents (Farber and Berger, 2002; Seibert and Sakmar, 2004).

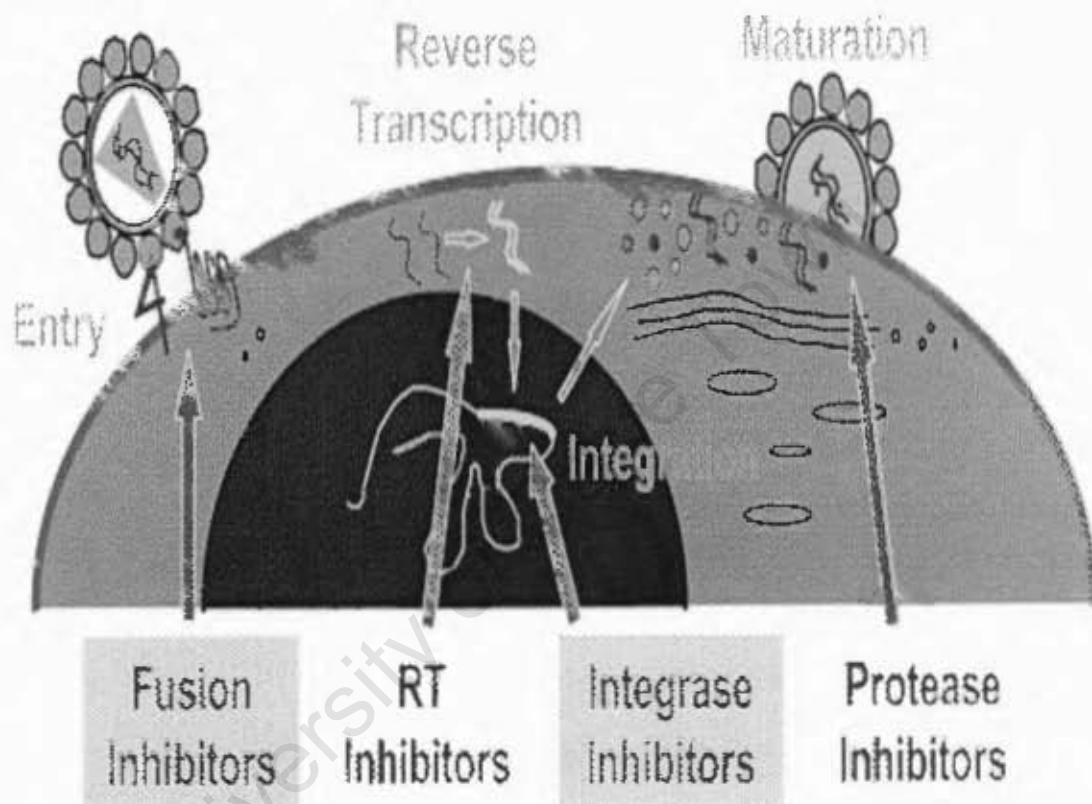


Figure 1.3: HIV-1 entry into host cell and the molecular targets for anti-HIV therapy. Integrase inhibitors are at an advanced stage of development, but not currently available for clinical use while fusion inhibitors are currently in clinical use (Lusso, 2002)

Several entry or fusion inhibitors that are known to block different steps of the fusion/entry process by interacting with the envelope protein gp120 or gp41 or with the target cell proteins, CD4 or the co-receptor are now either in clinical use or are undergoing pre-clinical or clinical development (De Clercq, 2002; Seibert and Sakmar, 2004; Liste and Telenti, 2007). Examples of these fusion inhibitors include T-20 (enfuvirtide) which is in clinical use, binds to gp41 to prevent fusion, T-1249 which has a similar mode of action as T-20 but with enhanced potency, PRO542 which interferes with the gp120-CD4 interaction

(Trkola *et al.*, 1995) and BMS806 and BMS 378806 which bind to gp120 to inhibit the interaction between gp120 and CD4 (Lin *et al.*, 2003).

Two major chemokine receptors have been implicated as co-receptors in this HIV fusion and entry process (Cocchi *et al.*, 1995). The virus strains that are transmitted and which predominate during the asymptomatic phase of the disease (Phase where there are no AIDS symptoms and at times there is normal CD4⁺ T cell count) were shown to be unable to infect CD4⁺ T cell lines, but could infect primary macrophages. These are known as macrophage (M) tropic HIV isolates and have now been shown to use the CCR5 chemokine receptor as a coreceptor (Deng *et al.*, 1996). The essential role of CCR5 in its function as a coreceptor for HIV entry into the host cell has been supported by the findings that human CD4 alone was insufficient to render cells permissive for infection. It was also discovered that the β chemokines, IJ78 α , IJ78 β (both isoforms of macrophage inflammatory protein, MIP-1 α [CCL3, CCL3L1]), MIP-1 β /CCL4 (macrophage inflammatory proteins 1 β) and RANTES/CCL5 (regulated on activation normal T cell expressed and secreted) could inhibit infection of macrophage tropic strains and not T cell tropic HIV-1 isolates (Alkhatib *et al.*, 1996; Deng *et al.*, 1996; Dragic *et al.*, 1996).

Chemokine-mediated inhibition of HIV entry is thought to result from steric blocking (by the chemokine ligand) of interaction between the viral protein, gp120 and the co-receptor or from ligand-induced internalization of receptor (Arenzana *et al.*, 1996; Simmons *et al.*, 1997). There is also evidence of ligand interference with receptor recycling. As levels of chemokines increase, more CCR5 are filled preventing HIV from gaining access to the receptor (Amara *et al.*, 1997; Alkhatib *et al.*, 1997; Brandt *et al.*, 2002). Brandt and collaborators showed that the efficiency of internalization can cause difference in susceptibility to chemokine-induced blocking of HIV entry. They showed that a CCR5 defective of phosphorylation sites did not internalize in response to chemokine and showed resistance to the blocking effects of chemokine to HIV-1 entry (Brandt *et al.*, 2002). After phosphorylation and internalization, receptors may traffic to endosomes resulting in dissociation of ligand and recycling of the receptor back to the cell surface. A defect in the

process of recycling leads to decreased amount of receptors on the surface of the cell causing a decrease in the amount of receptors available for use by HIV.

Chemokine mediation of HIV inhibition and chemokine link to susceptibility to infection by HIV has been associated with copy number of chemokine genes. A direct correlation between the chemokine gene copy number and HIV-1 susceptibility was established by Gonzalez and collaborators (Gonzalez *et al.*, 2005). Their study involved the high affinity ligand of CCR5, CCL3L1 which has been shown to be a more potent inhibitor of HIV-1 infection than RANTES (Nibbs *et al.*, 1999). Gonzalez and collaborators analyzed segmental duplications of the CCL3L1 gene in people from different ethnic groups and found that the copy number of this chemokine varies from one person to another. Their study revealed that this difference in copy number on its own did not determine susceptibility but the gene number relative to the average number for the ethnic group was the determining factor (Gonzalez *et al.*, 2005). Thus individuals with low copy number of CCL3L1 compared to the average for their ethnic population will be more susceptible to HIV-1 than individuals with high copy number from the same racial or ethnic population. Africans have a significantly high number of CCL3L1 gene copies compared to non Africans (Gonzalez *et al.*, 2005).

Further evidence of CCR5 involvement in HIV infection was based on population genetic studies which showed that genetic variants of the CCR5 genes reduce HIV susceptibility and/or progression to AIDS (Howard *et al.*, 1999). The most widely studied variant of CCR5 (CCR5 Δ 32) has a 32 base pair deletion within the coding region of the coreceptor gene which leads to the production of a truncated receptor (Liu *et al.*, 1996; Samson *et al.*, 1996). Individuals who are homozygous for this deletion mutation were shown to be highly resistant to HIV infection. In the heterozygous state, the mutant allele is associated with partial protection against HIV-1 infection and delayed progression to AIDS in seropositive individuals (Liu *et al.*, 1996; Paxton *et al.*, 1996; Samson *et al.*, 1996). Geographic analysis of this mutation shows that the mutation is present almost exclusively in Europe with up to 14% in some northern populations of Eurasia. A North to South cline distribution of this allele has been reported with Sardinia having the lowest frequency (4%) and Finland, the highest (15.8%) (Libert *et al.*, 1998; Zawicki and Witas, 2007). Due to

migration and settlement of Europeans and their descendants in other parts of the world, there are evidences of this mutation out of Europe. The mutation is absent in indigenous Mexicans but present at a frequency of about 4.4% in Hispanic Mexicans (A frequency of 8% is observed in Spain). Some non Caucasians such as Indians and Chinese have been reported to carry this mutation and this may be as a result of gene inflow to ancestral populations (Zawicki and Witas, 2007). The mutation is absent in Africa, South America, Asian the Pacific Islands.

The age of this mutation was estimated to be approximately 700 years and it was suggested that a strong selective pressure imposed by a factor in Europe which gave its carriers some kind of advantage must have pushed the frequency of this allele to its present day distribution (Stephens *et al.*, 1998). The Black Death of 1348 which killed millions in the European continent was an obvious candidate. The bubonic plague was also a suitable candidate. The plague agent was known to infect CCR5 carrying macrophages and thus there was selection of the CCR5 Δ 32 to prevent infection.

As HIV-1 infection progresses, viruses that emerge proved unable to infect macrophages but acquire the ability to infect lymphocytes and CD4⁺ T cells. These are T cell (T) tropic isolates, which use the CXCR4 chemokine receptor either in place of, or in addition to, CCR5 for cellular entry. CXCR4 was the first HIV-1 coreceptor that was identified by Feng and collaborators who used a cloning method that was based on the ability of a cDNA library to allow fusion of cells expressing Env from laboratory adapted strains with a CD4-expressing murine cell. Isolation and sequence analysis of a single cDNA showed that the protein belonged to the GPCR family of transmembrane receptors (Feng *et al.*, 1996; Berger *et al.*, 1999). This protein was named "fusin" and was later shown to be a chemokine receptor that binds and responds to the CXC chemokine, SDF-1(CXCL12) (Bleul *et al.*, 1996).

Structurally modified agonists or agonist derivatives of CCR5 and CXCR4 have been shown to compete with HIV for binding to the receptor and thus contribute to inhibition of infection by the virus (Amara *et al.*, 1997; Arenzana-Seisdedos and Parmentier, 2006). In addition, binding of the natural ligands and these derivatives cause internalization of the

receptors thereby reducing the amount of receptor present on the surface of the cell for interaction with the HIV envelope. These chemokine derivatives such as aminooxypentane (AOP)-RANTES and AOP-LD78 β (AOP-CCL3L1) have been exploited as HIV blocking agents due to their collective capacity to bind the receptor and down regulate receptor expression (Arenzana-Seisdedos and Parmentier, 2006). The major setback for the use of these modified ligands as anti-HIV drugs is their instability, poor pharmacokinetics and high cost which outweigh the benefit. Thus chemokines and chemokine receptors, in particular CCR5 and CXCR4 need to be exploited further for their roles in HIV entry, inhibition of entry and drug design.

1.2.3 HIV-1 Vaccine development

The most powerful and to a great extent the most cost effective measure to combat HIV will be the development and use of an effective prophylactic and protective vaccine against the virus. Taking advantage of the fact that HIV-1 transmission is primarily through mucosal-associated tissues, and the knowledge of the mucosal immune system, development of a mucosal vaccine is now in progress (Yuki *et al.*, 2007). The principle of vaccine development holds that a vaccine should contain immunogenic characteristics of the prevalent HIV subtype(s) circulating in a geographic area (Heyndrickx *et al.*, 2000). A vaccine which blocks replication of the virus has been proposed since infection by HIV cannot be reversed once it has been established. In this case, blocking replication will prevent HIV from entering and infecting fresh target cells. This approach requires neutralizing antibodies directed against the viral envelope protein.

To date, the development of an efficacious vaccine against HIV-1 still poses great problems in the worldwide fight against HIV and AIDS. The relatively high level of genetic divergence and the fact that the correlates of a vaccinated individual are unknown (Anderson *et al.*, 2000; Heyndrickx *et al.*, 2000) remain the major obstacles to vaccine development. Thus, it will be necessary to have a cocktail of different immunogens that are representative of each genotype and phenotype or that are adapted to different geographic regions or over time (Heyndrickx *et al.*, 2000).

1.3 CHEMOKINES AND CHEMOKINE RECEPTORS

In the process of inflammation resulting from injury or infection, there is selective recruitment of leucocytes including T-lymphocytes which traverse the vascular endothelium and migrate into the peripheral tissues in response to a gradient of chemotactic factors such as chemokines (Menten *et al.*, 2002). Chemokines are a group of small, mostly basic protein molecules of 8-14KDa and approximately 70-80 amino acids in length which have been shown to adopt a similar fold even when the overall sequence identity is low. Fractalkine is an unusual membrane-bound member of the family (Stantchev and Broder, 2001; Onuffer and Horuk, 2002). The chemokine family is characterized by the conservation of four cysteines and has been classified into four subfamilies depending on the motif exhibited by the first two cysteines. These are the CXC or alpha chemokines which have an amino acid residue between the first two cysteines, the CC or beta chemokines with the first two cysteines next to each other, the C or gamma (represented by lymphotactin) which has lost the first and the third cysteines and the CX3C or delta chemokine which exhibits three amino acids between the first two cysteines and fractalkine or neurotactin is an unusual membrane-bound member of the family (Rossi and Zlotnik, 2000; Mellado *et al.*, 2001; Menten *et al.*, 2002).

A different chemokine nomenclature presented at the Keystone Chemokine Symposium. This nomenclature is based on the chemokine receptor nomenclature which is being used at present. The chemokine receptor nomenclature uses CC, CXC XC and CX3C with an R (for receptor) at the end followed by a number. Thus the new nomenclature proposed the use of L (for ligand) to replace R when naming a chemokine. In this respect, a CC chemokine receptor 3 will be designated CCR3 while a chemokine ligand will be designated CCL and a number which represents the gene number encoding the chemokine in question (Zlotnik and Yoshie., 2000)

Chemokines share a conserved monomeric fold characterized by a disordered short amino terminus before the first two cysteines, a conserved core region which is made up of the so called 'N-loop', three anti-parallel β -strands separated by short loops and a carboxyl terminal α -helix (Stantchev and Broder, 2001; Bonduc *et al.*, 2002; Blanpain *et al.*, 2003).

Chemokines are constrained by disulfide bridges between cysteine residues (Stantchev and Bröder, 2001).

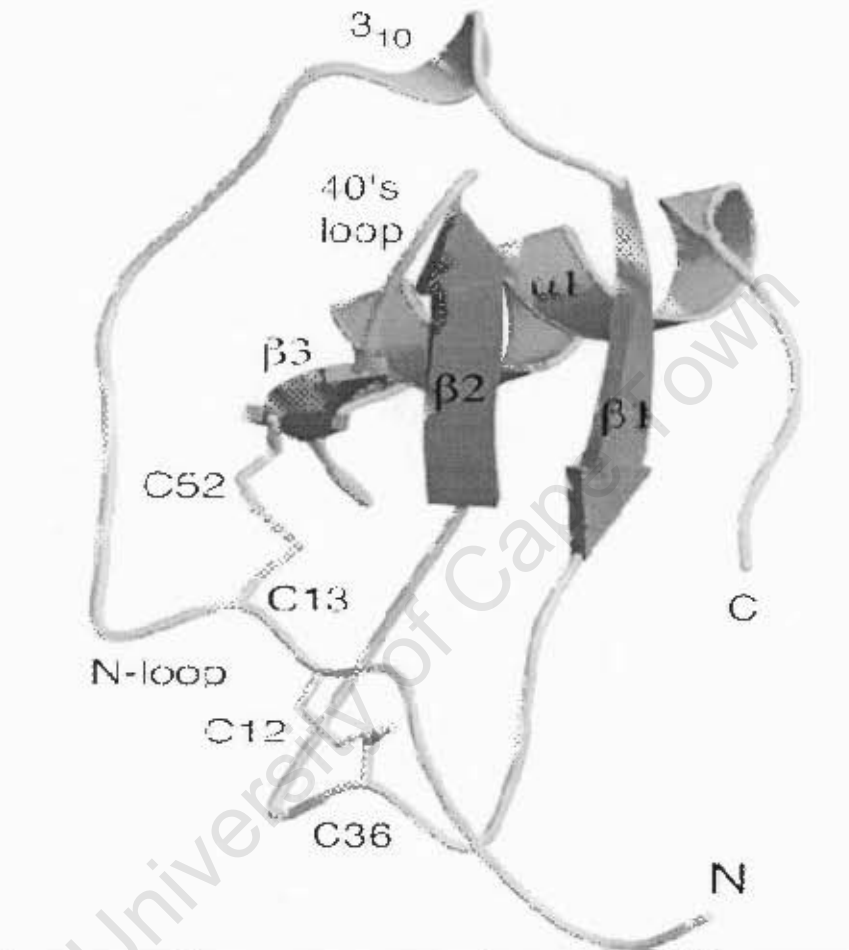


Figure 1.4: Typical chemokine fold of the viral macrophage inflammatory protein-1 (vMIP-1) showing an extended N-terminal segment that contains the 3_{10} helix, three anti-parallel β -sheets, the α -helix structure just before the C-terminal and the cysteine residues forming disulfide linkages (from Luz *et al.*, 2005)

Chemokines exert their effects by interaction with specific G protein coupled receptors present in the membranes of target cells. The chemokine receptor family is the largest subfamily of peptide binding GPCRs that have been described to date. They belong to the class A GPCRs which show high level of homology with rhodopsin (Omiffier and Horuk, 2002). Sequence homology among chemokine receptors may vary from 25% to

80%. Some characteristic features common among chemokine receptors include an acidic N-terminal domain, the DRYLAIVHA sequence or a variation of it in the second intracellular loop, proposed to be involved in G protein interaction, a short basic third intracellular loop and the presence of a cysteine in each of the four extracellular domains (Murphy, 1996; Stantchev and Broder, 2001). The conserved cysteine residue in the first extracellular loop is known to form a disulfide bond with the cysteine in the second extracellular loop. This disulfide linkage is a common feature in the GPCR super-family. Only chemokine receptors share the second disulfide bond which is formed between the N-terminal and the third extracellular loop (Oppermann, 2004). Chemokine receptors can also be grouped into four families depending on the chemokines with which they interact, i.e. CR, CCR, CXCR and CX3CR which interact with the C, CC, CXC, and CX3C chemokines respectively (Murphy *et al.*, 2000). At present, more than 45 chemokines and almost 20 chemokine receptors have been described (Onuffer and Horuk, 2002).

1.3.1 Ligand binding and activation of chemokine receptors

Structure-function studies show that chemokines have two major sites of interaction with their cognate receptors, the flexible N-terminal portion that precedes the first cysteine and the rigid loop that follows the second cysteine (Onuffer and Horuk, 2002). Functional analysis of different chemokines has led to the general conclusion that whereas the N-terminal domain of these proteins is necessary for triggering receptor signaling, the core domain of the molecules contains motifs that are essential for their tight binding to the receptors (Laurence *et al.*, 2000). Even though variations exist among the different chemokine and chemokine receptors in terms of domains involved in binding and activation of intracellular signal, a general model suggests that chemokines along with other cytokines and small peptides bind to the receptor's N-terminus and one or more of its extracellular loops. There is also a possibility for interactions between ligand's C-terminus and the transmembrane core of the receptor (Ahuja *et al.*, 1996; Ji *et al.*, 1998; Stantchev and Broder, 2001). The role of chemokine binding to the N-terminus of the receptor seems to facilitate the subsequent interactions of the ligand with the extracellular loops (Stantchev and Broder, 2001).

Considerable evidence suggests that chemokines can activate nearly all of the downstream signalling pathways associated with GPCRs but factors that determine the pathway that will be preferentially or predominantly activated are not known (reviewed by Stantchev and Broder, 2001). Chemokine receptors are often given as examples of GPCRs that associate with the pertussis toxin (PT) sensitive G_i proteins, the α subunits of which are best known for their ability to suppress adenylylate cyclase (AC) activity (Baggiolini *et al.*, 1997; Beckner, 1997; Neptune *et al.*, 1999; Stantchev and Broder, 2001). Thus it is well established in cells expressing chemokine receptors that chemokines cause a reduction in cyclic AMP (cAMP) (reviewed by Murphy, 1996; Baggiolini *et al.*, 1997).

1.3.2 Chemokines, Chemokine receptors and HIV

A number of other human chemokine receptors and related orphan receptors have been demonstrated to support infection by one or more HIV-1 strains *in vitro* and these include the chemokine receptors CCR2b (Doranz *et al.*, 1996), CCR3 (Choe *et al.*, 1996; Doranz *et al.*, 1996; Alkhatib *et al.*, 1997), CCR8 (Horuk *et al.*, 1998; Jinno *et al.*, 1998), CCR9 (Choe *et al.*, 1998a), CXCR1 (Rucker *et al.*, 1997; Combadiere *et al.*, 1998) and the receptors APJ (Choe *et al.*, 1998a; Edinger *et al.*, 1998). HIV-2 and SIV envelopes may use other coreceptors in addition to CCR5 including STRL33/CXCR6 (Liao *et al.*, 1997), GPR15/BOB (Farzan *et al.*, 1997a), APJ and GPR1.

A valine to isoleucine mutation within the first transmembrane domain of CCR2 (CCR2-V64I) has been described. This mutation occurred at an allele frequency of 10 to 15% among Caucasians and African Americans and is associated with a 2- to 4-year delay in progression to AIDS (Smith *et al.*, 1997; Lee *et al.*, 1998). Lee and collaborators investigated the mechanism of the protective effect of this polymorphism by comparing expression and coreceptor activities of the wildtype and V64I mutant form of the CCR2 isoform, CCR2b. They also studied the effects of expression and agonist binding to the wildtype and mutant CCR2b receptors on the functioning of the two major HIV-1 coreceptors, CCR5 and CXCR4. Their results indicate that the protective effect may be due to an indirect reduction of signalling by the two major coreceptors induced by addition of their ligands (Lee *et al.*, 1998). Subsequent study on the V64I by Mellado and collaborators

proposed heterodimerization of the CCR2bV64I with CCR5 and CXCR4 (Mellado *et al.*, 1999) which may cause a reduction in surface expression of the two major coreceptors. HIV env-induced signalling of CCR5 and CXCR4 have been reported (Davis *et al.*, 1997; Weissman *et al.*, 1997) and since receptor signalling has been suggested to play a role in surface expression of receptor and in post-entry events in viral replication (Chackerian *et al.*, 1997; Edinger *et al.*, 1997), the link between CCR2 and the major HIV-1 receptors, CXCR4 and CCR5 may provide a possible explanation for CCR2 coreceptor activity (Lee *et al.*, 1998). It has also been shown that in the presence of human CD4, Q16 cells expressing CCR2b and CCR3 both support syncytium formation by the dual tropic HIV-1 envelope, 89.6 suggesting the use of a variety of related entry cofactors by some primary virus isolates (Doranz *et al.*, 1996).

The use of CCR3 as an HIV-1 coreceptor was initially confined to microglial infection but it was later reported that CCR3 may commonly be used in chronic infection. CCR3 use was investigated by Choe and co-workers who showed that HeLa cells that were cotransfected with plasmids expressing human CD4 and CCR3 could be infected by the viruses ADA, YU2 and HXBc2 and binding of the CCR3 ligand, cotaxin inhibited infection by these isolates (Choe *et al.*, 1996). Consistent with the result of Choe *et al.*, Alkhatib *et al.* (1997) also showed using an HIV-1 envelope dependent fusion assay that CCR3 can interact with Envs from some M-tropic strains (Ba-I, JR-FL, SF-162 and ADA), T-tropic laboratory adapted strains (HIB and LAV) and the dual tropic primary isolate, 89.6. CCR3 usage by HIV envelopes was shown to be strongly dependent on its surface expression levels with a large number of viral Env proteins being able to use CCR3 as a coreceptor only at higher levels of surface expression (Alkhatib *et al.*, 1997; Rucker *et al.*, 1997). Recent studies have demonstrated efficient use of CCR3 by HIV-1 envelopes from viruses at acute infection (Aasa-Chapman *et al.*, 2006)

CCR8, which is a receptor that binds the CC chemokine I309, has also been implicated in HIV infection. It has been shown that CCR8 can serve as a coreceptor for diverse T-cell tropic, dual tropic and macrophage tropic HIV-1 strains and that its ligand I309 was able to inhibit HIV-1 envelope-mediated fusion and virus infection (Horuk *et al.*, 1998). There has also been a suggestion of CCR8 function as a coreceptor for HIV-1

infection in brain-derived cells as well as T cells (Jinno *et al.*, 1998). Many other chemokine receptors and orphan receptors of the chemokine family have demonstrated coreceptor activity for different HIV-1, HIV-2 and SIV strains (Edinger *et al.*, 1998; Liao *et al.*, 1997; Choe *et al.*, 1998a).

Despite the use of this diverse repertoire of receptors by HIV as co-receptors, CCR5 remains the most important chemokine receptor involved in HIV-1 infection. The almost complete protection against HIV-1 infection of individuals who are homozygous for the A32 CCR5 mutation (Paxton *et al.*, 1996; Samson *et al.*, 1996) and the fact that individuals who lack CCR5 but are infected with HIV-1, carry only the strains that use CXCR4 (Michael *et al.*, 1998; Naif *et al.*, 2002) is evidence for the importance of CCR5 in HIV-1 infection.

1.4 THE CC CHEMOKINE RECEPTOR 5 (CCR5)

The CC chemokine receptor 5 (CCR5) is a member of the chemokine receptor subclass of G protein coupled receptor super-family. It belongs to the CC family of chemokine receptors which interacts with the proinflammatory chemokines, MIP-1 α , MIP-1 β , LD78 β , RANTES and MCP-2 (monocyte chemoattractant protein-2). CCR5 is encoded by a gene located on chromosome 3p21 and shares 71% identity with CCR2B (Raport *et al.*, 1996). It is expressed in lymphoid organs such as the thymus and the spleen as well as peripheral blood leucocytes such as macrophages and T cells. CCR5 was the first example of a human chemokine receptor that signals in response to MIP-1 β and its functions include activation and chemotaxis of various leucocyte populations to site of inflammation (Raport *et al.*, 1996). CCR5 is the major co-receptor for the macrophage tropic strains of the human immunodeficiency virus type 1 (HIV-1).

The involvement of CCR5 in chemotaxis has been demonstrated in inflammatory diseases such as multiple sclerosis, rheumatoid arthritis and diabetes where T cells expressing CCR5 and other chemokine receptors were found to be increased. Variations in CCR5 and its ligands have been shown in these diseases. CCR5 was detected on

lymphocytic cells, macrophages and microglia in actively demyelinating multiple sclerotic brain lesions. Cells expressing CCR5 were high in cerebrospinal fluid. It has been suggested that the CCR5Δ32 mutation does not confer protection from multiple sclerosis, however, there is an association between this mutation and low risk of recurrent clinical disease activity. A late onset of disease was shown for individuals with the CCR5Δ32 allele (Ribeiro and Horuk, 2005). CCL5 expression has been implicated in rheumatoid arthritis.

The ligand binds both CCR1 and CCR5 and thus attracting T cells and monocytes into joints during the onset of disease. This makes CCR1 and CCR5 potential candidates involved in the processes that lead to rheumatoid arthritis. It has been suggested that the CCR5Δ32 mutation is a genetic marker related to severity of the disease with individuals carrying this allele being at high frequency of non severe compared with severe patients in a study involving rheumatoid arthritis patients (Quinones *et al.*, 2004; Ribeiro and Horuk, 2005).

Studies on mouse models have demonstrated an increase in the CCR5 ligands, CCL3 and CCL4 in diabetes, suggesting a role of CCR5 in the development of type I diabetes. Differing results have been obtained for an association between diabetes and the CCR5Δ32 mutation. While partial protection from type I diabetes has been suggested for homozygous individuals, other studies failed to show any association in patients with type I and II diabetes (Ribeiro and Horuk, 2005).

There have been studies which show an influence of CCR5 alleles and the high affinity ligand CCL3L1 with susceptibility to Kawasaki disease, a systemic vasculitis of childhood, common in Asian population with potential infectious etiology. Using CCR5 haplotypes, it was shown that children with the CCR5Δ32 allele were associated with reduced Kawasaki disease susceptibility. Increased production of CCL3L1 as a result of an increase in the copy number of the CCL3L1 gene was also associated with protection against Kawasaki disease (Arenza-Seisdedos and Parmentier, 2006)

In chronic inflammation such as endometriosis, no association was found for the

CCR5 Δ 32 polymorphism. A crucial role of CCR5 has been shown in the regulation of immune response against West Nile Virus (WNV) in mice. Infection by this virus causes encephalitis in several organisms including man and was shown to induce an increase in the expression of CCR5 and its ligand RANTES (CCL5) (Lim *et al.*, 2006; Glass *et al.*, 2005). Glass and Co-workers showed that genetic disruption of CCR5 greatly reduced accumulation of CCR5-expressing NK cells, macrophages and CD4⁺ and CD8⁺ T lymphocytes in WNV infection in mice. Their study suggests that CCR5 may be protective against WNV infection and raised the possibility that individuals with the CCR5 Δ 32 mutation may be at high risk of the disease. Consistent with this suggestion, a follow up study by these authors analysed samples from WNV infected and control patients from two different US states (Colorado and Arizona). Even though the CCR5 Δ 32 homozygosity was 1% of a control group of healthy US Caucasians, 4-8% of symptomatic WNV infection in the study were homozygous for this allele. There was indication of increase death in the CCR5 Δ 32 homozygotes particularly in the Arizona group. Their result confirm that wild type CCR5 mediates resistance to symptomatic infection with WNV.

Apart from its crucial role in WNV infection, no known medical condition has been associated with lack of CCR5 or reduction of the receptor (Zimmerman *et al.*, 1997; Liu *et al.*, 1996). This may be due to the fact that many other chemokine receptors are present in the body that can perform the same function as CCR5 (Carrington *et al.*, 1997; Premack and Schall, 1996). Studies on the CCR5 Δ 32 mutation underscore the need for CCR5 antagonists which could be therapeutically useful in many clinical situations, at the same time pharmaceutical companies should assess the potential side effects of these antagonists with respect to WNV infection.

1.4.1 Structure of CCR5

CCR5 has the same primary structure as other GPCRs, in the rhodopsin family. Models based on the crystal structure of rhodopsin have provided an insight into the structure of other receptors in its family however, local structural motifs like those seen in rhodopsin helices that contain glycine and proline need to be re-examined in the context of

CCR5. Theoretical GPCR models, site directed mutagenesis or molecular modelling techniques have been successfully used for structure validation, determination of ligand binding sites and formulation of hypothesis on the mechanism of receptor activation (Paterlini, 2002; Fano *et al.*, 2006). A model of human CCR5 was built by Fano and collaborators using the crystal structure of bovine rhodopsin. This homology-built CCR5 model was validated through exhaustive conformational sampling and its quality and reliability assessed by analyzing four low-energy conformations relating to available mutagenesis data (Fano *et al.*, 2006).

In addition to the seven helices that traverse the membrane, CCR5 has an eighth helix formed at the C-terminal of the receptor. A fourth intracellular loop is also formed when the C-terminal is palmitoylated at cystein residues (Ji *et al.*, 1998). Even though the sequence identity between CCR5 and bovine rhodopsin is low (<20%), when considering only the transmembrane helices, sequence identity increases to approximately 30% and CCR5 architecture and function are maintained by residues that are conserved among the rhodopsin family (Fanelli and De Benedetti, 2005)

1.4.1.1 The Extracellular Domain

The extracellular domain consists of the N-terminus and the three extracellular loops. There is close proximity among the extracellular domains which may be due to the presence of cysteines. The role of cysteines in GPCRs has been studied and shown to be involved in disulfide bond formation necessary for maintaining the integrity of the receptor which gives a structural conformation that allows for ligand binding (Blanpain *et al.*, 1999b). Two conserved cysteines at the first and second extracellular loops (ECL1 and ECL2) of GPCRs form a disulfide bridge that appears to be important for packaging and stabilization of the seven transmembrane core (Stanchev and Broder, 2001). Two additional cysteines are present in chemokine receptors, located in the N-terminal domain (N₁) and the third extracellular loop (ECL3) and have been shown to form disulfide bonds which provide additional structural constraint contributing to receptor stability and conformation. Alanine substitution of any cysteine on the extracellular domain of CCR5 resulted in decreased cell surface expression and complete loss of chemokine binding

(Blanpain *et al.*, 1999b). In addition to these disulfide bonds, Lee *et al.* showed, using monoclonal antibodies that Lys171 and Glu172 form part of a bridge between extracellular loop 2 (ECL2) and another extracellular loop, with ECL1 being the most likely candidate (Lee *et al.*, 1999). Consistent with this, the model of Fano *et al.* (2006) showed a salt bridge between Lys171 and Asp95 and hydrogen bonds between Glu172, Tyr184 and Asn273 as well as between His181, Lys191 and Gln186 (Fano *et al.*, 2006). The charged or polar side groups of Asp11, Tyr 15, Glu18, Lys22 and Lys26 were proposed to be involved in hydrogen-bonding (side chains of Asp11 and Lys26, Glu18 and Lys22, phenoxy group of Tyr15 and Lys26) amongst themselves or π -cation interactions, the former suggested to be necessary for maintaining the N-terminus of the receptor in a stable motif for substrate recognition (Fano *et al.*, 2006). CCR5 is posttranslationally modified at the extracellular domain by glycosylation and sulfation. Ser6 and Ser7 at the N-terminus were shown to be O-glycosylated in cell lines and in primary macrophages. The O-glycans were shown to be important for MIP-1 α and MIP-1 β binding to CCR5 but their absence had minimal effect on HIV-1 entry (Bannert *et al.*, 2001). A Tyrosine rich region is present at the N-terminus of the receptor proximal to the first cysteine which are critical for HIV co-receptor activity and chemokine interaction (Farzan *et al.*, 1998). These tyrosines have been shown to be enzymatically sulphated in a stepwise manner with Tyr14 and Tyr15 being sulphated first followed by Tyr10 and finally Tyr3 (Seibert *et al.*, 2002). The sulphate moieties were demonstrated to contribute to the ability of CCR5 to function as an HIV-1 coreceptor since tyrosine sulphated peptides based on this CCR5 domain could reduce HIV-1 entry and also less efficiently block MIP-1 α association with the receptor (Farzan *et al.*, 2001; Farzan *et al.*, 2002; Seibert *et al.*, 2002).

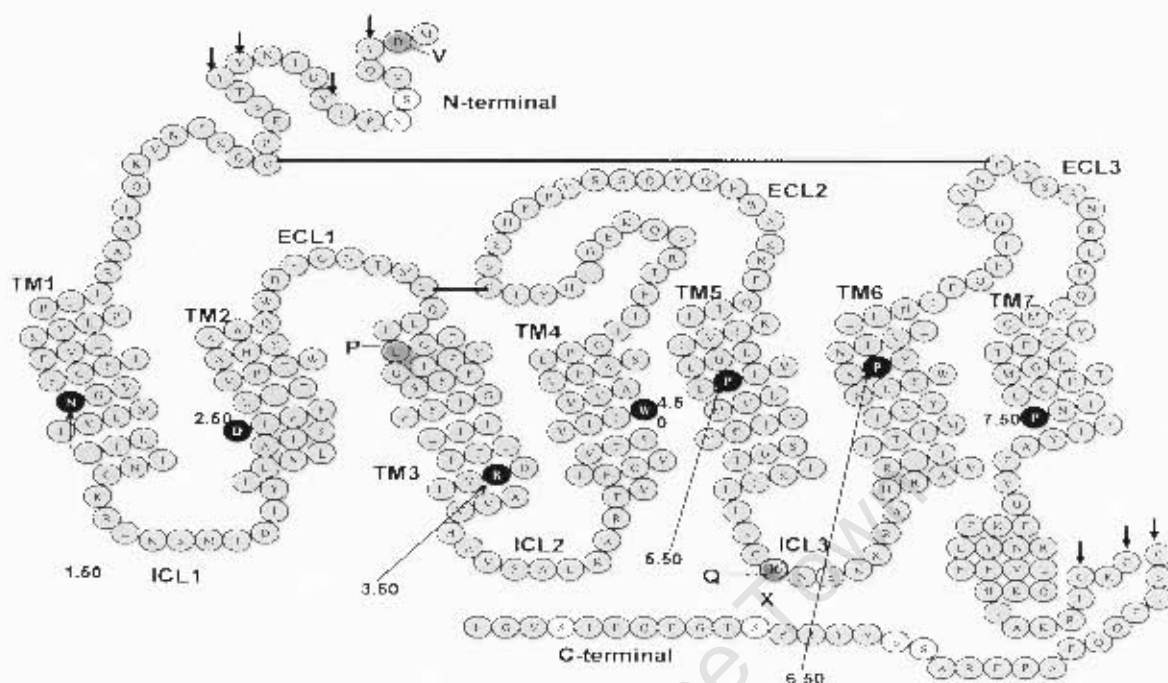


Figure 1.5: A two-dimensional model of CCR5. The structure depicts the N-terminal, the extracellular loops (ECL), the seven transmembrane helices (TM), the intracellular loops (ICL) and the intracellular C-terminal of the co-receptor. The most conserved residue for each TM domain [used for consensus numbering by the Ballesteros scheme (page10)] is indicated in black with its consensus number indicated and the residues studied in this thesis are indicated in dark grey. Arrows pointing to residues at the N-terminal depict tyrosine residues that are involved in sulphation while arrows pointing to residues at the C-terminal are cysteine residues involved in palmitoylation. The four serine residues at the C-terminal (white) indicate phosphorylation sites while those at the N-terminal are glycosylation sites.

1.4.1.2 Transmembrane domain

The transmembrane domain of CCR has been demonstrated to be important for the binding of the small molecule antagonist, TAK779 (Dragic *et al.*, 2000). It was also shown that the CCR5 transmembrane helix bundle interacts with the N-terminus of its chemokine ligand to mediate activation. Hydrogen bond formation, hydrophobic interaction and π -stacking arrangements have been shown among residues in the transmembrane domain of CCR5. In the low-energy conformation study by Fano and collaborators, the phenoxy group of Tyr 108 was shown to be hydrogen-bonded to the carboxyl group of Glu283 which also interacts in a face-to-face π -stacking arrangement with the aromatic ring of Tyr

251 (Fano *et al.*, 2006). These three residues were previously shown to be vital in conferring CCR5 its biological role as an HIV coreceptor (Dragic *et al.*, 2000). The carboxyl group of Glu283 also concurrently hydrogen-bonds to the side chains of Thr177 and Ser179 in ECL2 with the latter observed to be facing a negative charge. The hydrogen bond interaction between Glu283 and the ECL2 residues Thr177 and Ser179 were shown to be retained after a side-chain flip while its interaction with Tyr108 was easily broken suggesting an ECL2 conformation that is consistent with an early activated state of the receptor during ligand binding. Thus, Glu283 is suggested to be important in stabilizing the receptor by tightly packing TM3, TM6, TM7 and ECL2.

Other structural features that are commonly found in CCR5 include a TXP motif in the second transmembrane helix in which a significant correspondence was shown between the modulating effect on the Pro kink angle and helix conformational flexibility by threonine versus other residues in the TXP motif (Govaerts *et al.*, 2001). In the model of Fano *et al.* (2006), this motif is situated in a cavity lined with the polar and hydrophobic residues Tyr37, Trp86, Tyr89, Leu104, Thr284 and Met287. An aromatic pocket consisting of highly conserved residues Phe109, Phe112, Phe113 and Trp248 is formed just below the cavity containing the TXP motif (Fano *et al.*, 2006). Govaerts and co-workers previously showed that these aromatic residues form part of an aromatic cluster that govern the mechanism of action of CCR5 (Govaerts *et al.*, 2003). CCR5 also feature the NPXXY motif in TM7 which has been postulated to be the major determinant for deviation of helix 7 from ideal helicity (Fanelli and De Benedetti, 2005).

1.4.1.3 The Intracellular Domain

The DRY (Asp-Arg-Tyr) motif located at the interface between TM3 and intracellular loop 2 is highly conserved among the rhodopsin family of GPCRs with Tyr being the least conserved among these three amino acids (Mirzadegan *et al.*, 2003). In CCR5, it was shown that mutation of the Arg to a neutral Asn residue abrogates activation of G proteins and β -arrestin-mediated chemotaxis (Lagane *et al.*, 2005). Venkatesan and collaborators working on the C-terminal tail of CCR5 defined three structural requirements in this region

which are necessary for cell surface expression – the length of the C-tail, a membrane-proximal basic amino acid rich domain and cysteine cluster (Venkatesan *et al.*, 2001). The C-terminus contains further structural motifs which are either palmitoylated or phosphorylated. Palmitoylation of the C-terminus of many GPCRs has been suggested to provide a membrane anchor to the C-terminal domain and creates a fourth intracellular loop (Ji *et al.*, 1998; Blanpain *et al.*, 2001). In CCR5, palmitoylation of the C-terminus occurs at cysteine residues and was shown to be important for intracellular trafficking of the receptor and for its coupling to some of its signalling cascade (Blanpain *et al.*, 2001). In addition, alanine scanning mutagenesis studies on C-terminal serines, threonines and tyrosines have identified serine residues at positions 336, 337, 342 and 349 as the exclusive phosphorylation sites (Oppermann *et al.*, 1999).

1.4.2 CCR5 as a chemokine receptor

Like other G protein coupled receptors, CCR5 is involved in signal transduction via G proteins. Originally, CCR5 was characterized as a receptor that responds to and functions physiologically as a receptor for the β -chemokines, LD78 α (MIP-1 α /CCL3), MIP-1 β (CCL4) and RANTES(CCL5) (Guignard *et al.*, 1998; Wang *et al.*, 1999; Kraft *et al.*, 2001). Many more CC chemokines (LD78 β /CCL3L1, MCP-1/CCL2, MCP-2/CCL8, MCP-3/CCL7, MCP-4/CCL13 and eotaxin) have been shown to interact with CCR5 with different affinities and abilities to activate the receptor (Mueller *et al.*, 2002; Blanpain *et al.*, 1999; Ruffing *et al.*, 1998). LD78 β , MCP-2, and MCP-4 are full agonists since they were shown to cause an increase in intracellular calcium in cells transfected with CCR5. MCP-3 is an antagonist since it was able to bind CCR5 with high affinity but could not induce any significant response and was also able to inhibit activation of CCR5 by MIP-1 β (Menten *et al.*, 2002; Mueller *et al.*, 2002; Blanpain *et al.*, 1999; Ruffing *et al.*, 1998). The CC chemokines that have been shown to interact with CCR5 can be divided into two subgroups based on amino acid identity. MIP-1 α , MIP-1 β , LD78 β and RANTES which are full agonists form one subgroup while MCP-1, MCP-2, MCP-3 and MCP-4 which share 60% amino acid identity within the group and approximately 30% identity with MIP-1 α , MIP-1 β and RANTES form another subgroup (Mueller *et al.*, 2002).

Binding of CCR5 to a chemokine ligand initiates a number of events: firstly the association of the receptor with heterotrimeric G proteins leading to activation of the G proteins which in turn leads to activation of signaling such as calcium flux; secondly, phosphorylation of the receptor by a family of G protein coupled receptor kinases (GPKs) leading to association of the receptor with arrestins and desensitization through uncoupling of receptor and G protein (Kraft *et al.*, 2001). Functioning as adaptor proteins, β -arrestins may also lead to receptor internalisation by facilitating clathrin-mediated endocytosis.

1.4.3 Ligand binding to CCR5

The structure function relationships of CC chemokine receptors and their ligands in general have not been well studied. The multiple ligand specificity property of some chemokine receptors is indicative of the existence of a common structural motif with which chemokines fit. There have been little evidence to support the concept that despite obvious similarities in primary and three dimensional structure of the chemokine ligands, they interact with their respective receptors in different ways (Samson *et al.*, 1997). Even though different structural determinants on CCR5 appear to be required by the three major CCR5 chemokine ligands (MIP-1 α , MIP-1 β and RANTES), all three ligands interact apparently in a similar way with the receptor (Samson *et al.*, 1997). A model for chemokine binding to CCR5 proposes a two-step mechanism which involves an initial interaction between the core of the chemokine and the exposed amino terminal receptor domain and the association with regions belonging to the second extracellular loop of CCR5 (Samson *et al.*, 1997; Oppermann, 2004). The free N-terminal domain of the chemokine then interacts with the transmembrane helix bundle which then triggers activation of the receptor (Blanpain *et al.*, 2003; Govaerts *et al.*, 2003; Oppermann, 2004).

Of all the CCR5-binding proteins, MIP-1 β is unique since it has little or no function on other receptors making it possible to understand the structural features responsible for CCR5 binding. In trying to determine the CCR5 binding determinants of MIP-1 β , Bondue and collaborators generated variants of MIP-1 β with mutations that changed the charge,

size and H-bonding properties of the side chains within the N-loop and the 3₁₀ turn and they found that three basic residues (Arg18, Lys19 and Lys22) contributed to the CCR5 binding of MIP-1 β by interacting through their positive charge. Pro21 located within the 3₁₃ turn was also shown to contribute to binding of MIP-1 β to CCR5 (Bondue et al, 2002). A major role of the aromatic side chain of Phe13 in the N-loop of MIP-1 β and basic residues (Arg46 and Lys48) in the so-called "40's loop" in CCR5 binding has been demonstrated (Laurence et al, 2000; Bondue et al, 2002).

Unlike MIP-1 β , MIP-1 α (LD78 α) has been shown to bind other chemokine receptors in addition to CCR5. Evidence of the existence of an isoform of MIP-1 α has been obtained (Nakao *et al*, 1990). This isoform which has a 94% sequence similarity with LD78 α , differing with only three amino acids has been designated LD78 β and has been demonstrated to be the most potent natural CCR5 agonist that has been described (Menten *et al*, 1999). A proline residue at position 2 of the mature LD78 β protein was shown to be responsible for the enhanced potency of LD78 β (Nibbs *et al*, 1999). Additional studies of the N-terminus of this protein using alanine substitutions revealed that Asp6, Pro8 and Thr9 in addition to Pro2 are critical for LD78 β binding to CCR5 (Miyakawa *et al*, 2002).

RANTES is also a ligand for the CC chemokine receptors CCR1, CCR3 and CCR5. Several modifications of the amino terminal of this protein have been shown to produce proteins of different affinities for CCR5, from full agonists through to full antagonists. A truncation of this protein by deletion of the first eight amino acids was shown to produce a protein that could neither mobilize calcium nor induce chemotaxis via CCR5 (Zhang and Rollins, 1995). Coupling of a five-carbon alkyl chain to the amino terminus of RANTES via an oxidized serine residue has been shown to confer this protein with a full agonist property on CCR5 (Proudfoot *et al*, 1996; Simmons *et al*, 1997).

1.4.3.1 Structural features of CCR5 important for chemokine binding

Several different approaches have been used to define the parts of CCR5 that are important for ligand binding. These approaches include site-directed mutagenesis, antibody studies, chimeric receptor studies, molecular dynamic simulations and agonist and antagonist docking. Mutagenesis studies have identified several residues on the extracellular surface of CCR5 that contribute to high affinity binding. N-terminal truncation and alanine substitution studies have identified several charged and aromatic residues that play important roles in chemokine high affinity binding. Alanine substitutions of Asp2, Tyr3, Tyr10, Asp11 and Glu18 and truncations involving the loss of one or more of these residues led to partial or total loss of binding and functional response to chemokines. No aromatic residue is directly adjacent to Glu18 which is a negatively charged residue at the N-terminus, but it was shown that mutation of this residue and Asp11 to alanine completely blocked RANTES binding while alanine substitution of Tyr15 was shown to decrease MIP-1 α binding (Dragic *et al.*, 1998; Farzan *et al.*, 1998; Blanpain *et al.*, 1999a). This result indicates the importance of the negative charges of the Glu and Asp and the polar group of the Tyr in chemokine binding.

Evidence from antibody studies implicates the second extracellular loop of CCR5 as a principal binding element for RANTES, MIP-1 α and MIP-1 β . An antibody to the second extracellular loop (2D7) was shown to inhibit binding of these chemokines whereas monoclonal antibodies recognizing the N-terminus were ineffective (Wu *et al.*, 1997). Consistent with these findings, Lee *et al.* (1999) in mapping the epitope specificities of 18 CCR5 monoclonal antibodies identified three different groups of antibodies: antibodies that bound to the N-terminal epitope, those that bound to extracellular loop 2 (ECL2) epitopes and those that bound to multiple domains on the extracellular surface (multi domain epitopes). They showed that mAbs to ECL2 were more effective at blocking chemokine binding than Env binding whereas mAbs to the N-terminal domain were more effective at blocking gp120 binding with little effect on CCR5-chemokine interactions (Lee *et al.*, 1999).

Youn and collaborators working with CCR5/CCR1 chimeras showed that TM7 plays a critical role in MIP-1 β binding and highlighted the role of Met187 in the binding affinity for MIP-1 β . Using CCR5/CCR1 chimeras (these receptors both bind MIP-1 α and RANTES with high affinity but only CCR5 has high affinity for MIP-1 β and CCR1 has high affinity for leukotactin-1. They demonstrated that the receptor with a CCR5 backbone having its seventh transmembrane domain replaced with that of CCR1 showed impaired signaling ability and binding affinity for MIP-1 β . They also showed that mutant chimeras with CCR5 Met187 residue restored in TM7 showed partial restoration of function (Youn *et al.*, 2001).

1.4.4 CCR5 activation and signalling

Ligand induced activation of receptors lead to receptor-G protein association resulting in activation of signaling, phosphorylation of receptor which subsequently lead to association of arrestin with the receptor and desensitization (Mueller *et al.*, 2002). The molecular mechanisms that underly the transitions between the inactive and the active conformation of GPCRs highlight an important role played by the highly conserved E/DRY motif located between TM3 and the second intracellular loop. This highly conserved DRY sequence motif in the second intracellular loop of CCR5 has been implicated in G protein interaction. Mutation of the first residue of this motif gave agonist-independent activation in numerous receptors while mutation of the Arg residue generally impairs G protein dependent signaling (Lagane *et al.*, 2005). Mutagenesis studies on the Arg residue show that some of the structural determinants needed for CCR5-mediated activation of G proteins are also required for β -arrestin mediated chemotaxis but not for receptor desensitization and internalization. Mutation of the Arg residue was shown to block activation of G protein and chemokine dependent activation of classic second messengers, intracellular calcium flux and cellular response of chemotaxis but preserved phosphorylation and interactions with β -arrestins for endocytosis. β -arrestins are involved in CCR5-mediated chemotaxis but this process is abolished when Arg-126 is mutated (Gosling *et al.*, 1997). Activation of CCR5 causes activation of the pertussis toxin sensitive Gi family of G protein, a process that involves exchange of bound GDP for GTP.

This leads to the dissociation of the $G\alpha$ subunit of the G protein from the $G\beta\gamma$ subunits (Farzan *et al.*, 1997b). Activation of the Gi protein by CCR5 causes inhibition of the adenylyate cyclase pathway. In this pathway, the GTP-bound $G\alpha_i$ inhibits the activity of adenylyate cyclase thereby inhibiting the production of cyclic AMP (cAMP) resulting in intracellular calcium mobilization. Production of inositol-1,4,5- triphosphate (IP_3) and diacylglycerol (DAG) has been associated with activation of phospholipase C (PLC) activation by the $G\beta\gamma$ subunit. IP_3 binds to its specific receptor in the endoplasmic reticulum to mobilize calcium (Mellado *et al.*, 2001).

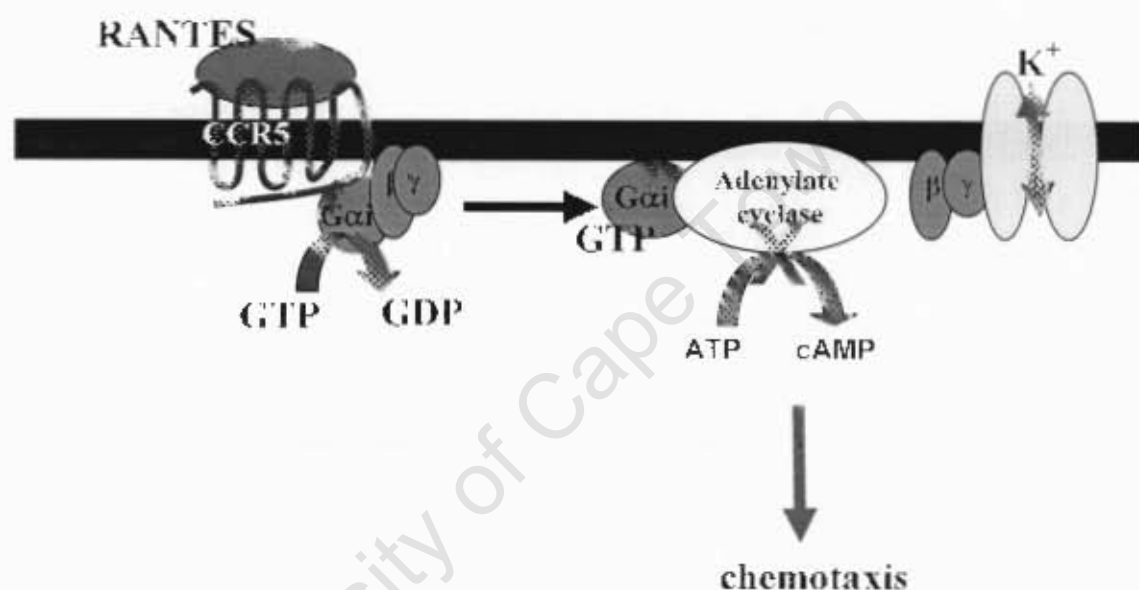


Figure 1.6: CCR5 activation and signalling via Gi protein: Binding of RANTES to CCR5 leads to activation of the receptor causing an exchange of GDP for GTP by the α subunit ($G\alpha_i$) of the Gi protein and dissociation of $G\alpha_i$ from the $G\beta\gamma$ subunit. The GTP bound $G\alpha_i$ then bind adenylyate cyclase inhibiting the formation of cAMP from ATP leading to chemotaxis

A hypothesis has been put forward on the important role of the conserved TXP motif in TM2 for chemokine receptor function. Using mutagenesis studies of the TXP sequence motif Govaerts *et al* showed that an alanine substitution of the proline causes a decrease in binding affinity for chemokines and almost no functional response of the mutated receptor. On the other hand, a change from threonine to Val, Ala, Cys or Ser had no effect in binding

affinity but functional response did depend on the type of side chain substitution (Govaerts *et al.*, 2001).

It has been proposed that conserved aromatic residues do play a role in the activation of GPCRs (Javitch *et al.*, 1998). This suggested a possible interaction between the second and third transmembrane domains of chemokine receptors that have high density of aromatic residues at the top of these two helices. Govaerts and co-workers investigated the effects of these aromatic clusters on activation of CCR5 by mutating them to their CCR2 counterparts either individually or in combination. Their experimental and molecular modelling results demonstrate a crucial role played by these aromatic residues in causing conformational changes of CCR5 leading from ligand recognition to receptor activation (Govaerts *et al.*, 2003). These different effects of CCR5 mutations on binding affinities and functional responses to chemokines highlight the importance of affected residues and domains in the functioning of the CCR5 receptor.

1.4.5 CCR5 phosphorylation and arrestin interaction

Studies on the prototypic β_2 -adrenergic receptor reveal that, agonist activation of receptors lead to their phosphorylation which is an essential step in receptor internalization. Phosphorylation in turn promotes binding of members of the arrestin family, thereby preventing further binding of G proteins. Like other GPCRs, CCR5 is phosphorylated by two major classes of protein kinases. G protein coupled receptor kinase (GRK) and protein kinase C (PKC). Seven mammalian GRKs have been identified (GRK1 to 7) but only GRK2 and GRK3 have been shown to play a prominent role in chemokine induced CCR5 phosphorylation (Oppermann *et al.*, 1999). Four distinct serine residues (Ser 336, 337, 342 and 349) that are located in the C-terminus of CCR5 have been shown to be phosphorylated upon agonist binding to the receptor (Oppermann *et al.*, 1999). The significance of these phosphorylation sites for ability of receptor to interact with β -arrestins and undergo desensitization and internalization has been studied. Results show that β -arrestin binding to CCR5 and receptor internalization are related mechanisms and both require the presence of two C-terminal serine phosphorylation sites (Kraft *et al.*, 2001; Huttenrauch *et al.*,

2002). PKC has been shown to exclusively phosphorylate Ser-337 while GRKs phosphorylate Ser-349 (Pollok-Kopp *et al.*, 2003).

Post-translational modification of G protein coupled receptors by palmitoylation at carboxyl terminal cysteines modify signaling in ways that vary among receptors (Blanpain *et al.*, 2001). CCR5 contains three carboxyl terminal cysteines that conform to predicted palmitoylation sites and were shown to be involved in transport of the receptor to the plasma membrane and also stimulation of endocytosis (Blanpain *et al.*, 2001; Kraft *et al.*, 2001). Carboxyl terminal serine residues have also been demonstrated to be critically involved in CCR5 endocytosis and a tyrosine residue (Tyr-297) in TM7 has been shown to affect CCR5 signaling and agonist induced sequestration (Kraft *et al.*, 2001).

1.4.6 HIV-1 co-receptor function of CCR5

HIV-1 enters host cells by direct fusion of the viral and target cell membranes. This fusion process is mediated by the viral envelope glycoprotein (gp160), CD4 and a co-receptor. HIV-1 envelope glycoprotein is made up of two subunits, an exterior gp120 and a transmembrane gp41. The crystal structure of the core of gp120 complexed with a two domain CD4 and the Fab fragment of the gp120 monoclonal antibody that blocks chemokine receptor binding, 17b has provided insight into the processes of HIV entry (Kwong *et al.*, 1998). The gp120 core is made up of folded inner and outer domains that are connected by a bridging sheet with discontinuous residues from these domains forming the CD4 binding site (Guo *et al.*, 2003; Platt *et al.*, 2005). The conserved residues within the CD4 binding site of gp120 form a deep hydrophobic pocket and interact with phenylalanine 43 residue of CD4 (Kwong *et al.*, 1998; Guo *et al.*, 2003). CCR5 is the major co-receptor involved in the HIV-1 fusion process. Residues in the gp120 bridging sheet which is composed of conserved region 4 (C4), the V1/V2 base and the V3 loop comprise the co-receptor binding site (Kwong *et al.*, 1998; Platt *et al.*, 2005). Efficient binding of CCR5 to gp120 is highly dependent on the presence of the V3 loop. Soluble subtype B gp120 binding to CCR5 has been shown to require two functionally distinct regions of the V3 loop, the stem and the crown, with the crown alone determining co-receptor specificity of the virus (Cormier and Dragic, 2002). Thus the current model of

HIV-1 co-receptor usage suggests that the V3 loop of gp120 dictates the choice of the co-receptor while the conserved bridging sheet residues contribute to binding contacts with the co-receptor (Suphaphiphat *et al.*, 2003).

Studies on structure-function relationships have not been able to clearly map out the distinct domains of CCR5 which are essential for fusion of HIV with host cells. Several different studies (including mutagenesis, chimeric and antibody studies) have been carried out to elucidate the region of the co-receptor involved in envelope interaction. Despite the complex and contradictory results, there is greater evidence which suggests that the envelope probably makes initial contacts with the extracellular regions (Berger *et al.*, 1999).

Mutagenesis studies on the N-terminal domain of CCR5 have identified several charged and aromatic residues which were found to play important roles in both chemokine and Env high affinity binding. These residues include Asp2, Tyr3, Tyr10, Asp11 and Glu18 (Blanpain *et al.*, 1999a; Lee *et al.*, 1999). In one study using monoclonal antibodies directed against the extracellular regions of CCR5, the authors found that the N-terminal mAbs recognized specific residues that span the first 13 amino acids of CCR5 while almost all ECL2 mAbs recognized Tyr-184 to Phe-189. Residue Lys-171 and Glu-172 in ECL2 were recognized by all multi domain antibodies. These results suggest the importance of the N-terminal in Env binding and the extracellular loops in inducing conformational changes in Env that lead to membrane fusion and viral infection (Lee *et al.*, 1999). Dragic and collaborators also working on charged residues in the N-terminal of CCR5 found that alanine substitutions of Asp2, Asp11 and Glu18 either individually or in combination, reduced or completely blocked entry of the M-tropic HIV-1 strains, ADA and JRFL and the dual tropic strain, DH123. They also demonstrated impaired gp120-CCR5 interaction for these mutants (Dragic *et al.*, 1998).

It was shown that a tyrosine- rich region in the N-terminus of CCR5 is important for HIV-1 entry to host cells and mediates association between gp120 and CCR5 (Farzan *et al.*, 1998). It was later shown that sulfation of these tyrosine residues could facilitate electrostatic interactions with positively charged residues in the bridging sheet and the base

of the V3 loop, thus tyrosine sulfation is an optimal requirement for HIV-1 coreceptor function (Farzan *et al.*, 1999; Seibert *et al.*, 2002; Lusso, 2006).

Chimeric study of extracellular loop two indicates that residues between Gly173 and Lys⁵⁴¹(197) which contain six residues that are divergent between the human and the mouse CCR5 play a critical role in the interaction with envelope glycoprotein (Navenot *et al.*, 2001). Interaction between HIV-1 envelope and CCR5 is not only complex but is also subject to marked HIV-1 isolate dependent variation. Bieniasz and collaborators generated humanCCR5/mouseCCR5 chimeric co-receptors by individually substituting the first three extracellular domains of humanCCR5 (H) in a mouseCCR5 (M) context (HMMM, MHMM and MMHM). They co-cultured these constructs with cells expressing the M-tropic HIV-1 isolates (YU2, ADA and Bal), the dual tropic isolate, 89.6 or the T-tropic isolate IIIIB. They found that whereas some HIV-1 strains could effectively utilize chimeras containing any single human extracellular domain, others require any two and still others could only interact in the presence of three human extracellular domains (Bieniasz *et al.*, 1997). In another study by Rucker and co-workers, it was demonstrated using CCR5/CCR2b chimeras and chimeric mutants that the first 20 amino acids of CCR5 N-terminus in a CCR2b receptor could support envelope fusion. Further analysis of this chimera revealed that residues Asp2 to Val5 were important for M-tropic viruses while Ser6 - Ile9 were important for the dual tropic 89.6 virus (Rucker *et al.*, 1996). Antibody studies on CCR5/CCR2b chimeras have also demonstrated the importance of the N-terminus of CCR5 in HIV-1 infection (Wu *et al.*, 1997). Other chimeric studies involved the introduction of the first twenty amino acid residues of CCR5 in other chemokine receptors, in particular, CCR1, CXCR2 and CXCR4. The chimeras were tested for their ability to mediate fusion of the M-tropic isolate JR-FL and the dual tropic viral isolate, 89.6. Results show that the N-terminal domain of CCR5 in the context of these other chemokine receptors results in their ability to mediate fusion by both isolates (Doranz *et al.*, 1997).

Comparing gp120 binding affinities and infectivity of R5 viruses using human and African green monkey (AGM) CCR5, Siciliano and collaborators showed that Glycine 163 is essential for the binding of R5 gp120 and mediation of infection by R5 isolates. These

two receptors differ at position 163 with an Arg for AGM and a Gly for the human CCR5 receptor. AGM CCR5 lacked co-receptor activity but mutation of R163 to G163 confers co-receptor activity while mutation of human CCR5 from G163 to R163 aborts activity (Siciliano *et al.*, 1999). Other single mutations across the CCR5 protein have shown varying effects on HIV-co-receptor function. Blanpain and co-workers showed lower binding of gp120 for R60S, A73V and R223Q mutant CCR5 due to low expression. They also showed partial co-receptor activity by C178R (Blanpain *et al.*, 2000). Loss of co-receptor function was demonstrated for I12T and C20S (Howard *et al.*, 1999).

Together, mutagenesis, chimeric and epitope mapping analyses have revealed the important role of the extracellular domains of CCR5 in its co-receptor function but the role of the transmembrane and/or cytoplasmic regions cannot be completely ignored. These regions of the co-receptor also critically influence activity, probably by affecting display of the extracellular regions (Berger *et al.*, 1999). Though each extracellular region has been implicated in co-receptor function, several studies have suggested a particular important role of the N-terminal segment (Blanpain *et al.*, 1999b; Lee *et al.*, 1999; Picard *et al.*, 1997). Therefore the N-terminal domain maybe more important for gp120 binding whereas the extracellular loops are important for inducing conformational changes in envelope that lead to membrane fusion and viral infection.

1.4.7 Naturally occurring mutations of CCR5

Several naturally occurring mutations across the CCR5 receptor have been studied for their effects on receptor and co-receptor function. The most widely studied CCR5 mutation is the CCR5Δ32 mutation discussed on page 20-21. Carrington and collaborators identified sixteen CCR5 mutations three of which were silent mutations populations of different ethnic origin (Carrington *et al.*, 1997). In the search for functional consequences of these mutations, Blanpain and co-workers showed that mutations involving cysteine residues at the extracellular surface and a frame shift which resulted in a modified end of TM7 and the absence of the C-terminal resulted in poorly expressed receptors (C20S, FS299) or no expression at all (C101X, C178R) as a result of intracellular trapping (Blanpain *et al.*, 2000).

C20S which disrupts the disulfide bond linking the N-terminal to extracellular loop 3 (E-CL3) did not bind nor respond functionally to chemokines (Howard *et al.*, 1999; Blanpain *et al.*, 2000). This mutation was observed in less than one percent in Caucasians and absent in African Americans (Carrington *et al.*, 1997). C101X which truncates the receptor to just two transmembranes behaved like the A32 mutant with only four transmembranes. They both showed neither chemokine receptor nor HIV-1 co-receptor function. A less than one percent frequency of this mutation was observed in African Americans. An alanine to serine mutation at the N-terminal (A29S) showed similar expression as the wildtype receptor but lower binding and impaired functional response to MIP-1 β , no functional response to MIP-1 α but could bind and respond to another CCR5 ligand, MCP-2 suggesting that different chemokines can interact with different residues of the same receptor (Howard *et al.*, 1999; Blanpain *et al.*, 2000). This mutation had a 1.5% frequency in African Americans (Carrington *et al.*, 1997). A moderate decrease in functional response to MIP-1 β with no change in binding affinity was observed for a TM mutation, L55Q while I42F and A73V both showed similar responses and affinities to MIP-1 β as the wild type receptor (Blanpain *et al.*, 2000). Both the I42F and the A73V mutations were at less than 1% in Caucasians but absent in the African American population. The frequency of the L55Q mutation in Caucasians was about 4% while it occurred in less than 1% in the African American population (Carrington *et al.*, 1997).

A naturally occurring mutation in the first intracellular loop of CCR5 (R60S) has been proposed to have an analogous effect on receptor expression as the R89C mutation of the Duffy receptor which confers protection against malaria (Carrington *et al.*, 1997; Tamasauskas *et al.*, 2001). The R89C mutation was shown to cause a 5-fold decrease in expression of the Duffy receptor in transiently transfected cells. Replacement of the arginine residue with a number of different amino acids showed that only a lysine substitution was fully expressed as the wild type receptor. This implies that the positive charge at this position is more important for expression than disulfide bonding. Studies on this mutation (R60S) show that the receptor is less well expressed than wild type CCR5, an expression that was associated with reduced CCR5 receptor and co-receptor function (Blanpain *et al.*, 2000; Tamasauskas *et al.*, 2001). Similar to the result of the Duffy receptor, only a substitution with a positively charged lysine was able to rescue surface

expression to wild type (R60) levels (Tamasauskas *et al.*, 2001). A 1% frequency of the R60S mutation was observed in African American population (Carrington *et al.*, 1997). Other naturally occurring mutations that have been studied include I121F (Howard *et al.*, 1999), I121L, R223Q, S215L, G301V, R334Q, R335V, Y339F and a K228 deletion (Blanpain *et al.*, 2000; Dong *et al.*, 2005) with varying effects on expression, chemokine receptor and HIV co-receptor functions. The R223Q mutation was observed in 1.6% of Caucasians and as high as 3-5% in Asian populations. It occurred in less than 1% in African Americans. More than 2% of the R335V and Y339F mutations occurred in the African American population. (Carrington *et al.*, 1997) A CCR5-893(-) mutation involving a single nucleotide deletion which produces a CCR5 with no C-terminal tail was observed in Asians (Ansari-Lari *et al.*, 1997). Analysis of this C-terminal defective CCR5 showed that expression on cell surface and HIV-1 co-receptor activity were impaired but intracellular expression was comparable to that of wild type CCR5. These results suggested a possible defect in intracellular trafficking of this mutant receptor and possibly affects HIV-1 transmission (Shioda *et al.*, 2001).

It has been hypothesised that variations in the promoter region of CCR5 may have an effect on AIDS progression. This may be due to the fact that this region controls the level of CCR5 expression at any given time. A number of CCR5 promoter mutations and their correlation with HIV infection and AIDS progression have been described. An alanine to glycine mutation (59029 base pair, Genbank U95626) found in the first intron of CCR5 has been associated with disease progression. Homozygous wildtype (A/A) individuals were reported to progress rapidly to AIDS in the absence of the CCR5 Δ 32 or the CCR2-64I mutations (McDermott *et al.*, 1998). It was shown that there was a reduced transcriptional activity for the promoter region encoding the 59029G variant which was associated with lower CCR5 expression in PBMCs (Arenzana-Seisdedos and Parmentier., 2006; McDermott *et al.*, 1998). Mummidi and collaborators have reported a C to T mutation at position 59653 (Genbank U95626) located in intron 2 of CCR5. This mutation was found in 100% linkage disequilibrium with the CCR2-64I. Because the CCR2-64I mutation is protective, it is not certain if protection in individuals with this mutation is coming from the CCR2 mutation or from the CCR5 promoter mutation. Other mutations identified in the promoter region of CCR5 in Caucasians and African Americans include A58755G, G58934T, C59353T, C59356I and A59402G (Mummidi *et al.*, 1997; Mummidi *et al.*, 1998; Carrington *et al.*, 1999). These mutations and the two promoter mutations described above distinguish four common promoter alleles designated CCR5P1, CCR5P2, CCR5P3 and CCR5P4. These alleles were observed at frequencies of 0.56, 0.085, 0.014 and 0.354 respectively in Caucasians (Carrington *et al.*, 1999).

1.6 CONCLUDING REMARKS

The recognition that CCR5 is the molecular factor that mediates entry of the transmitted macrophage tropic strain of HIV-1 came from the discovery of a mutant CCR5 allele that has a 32 base pair deletion in region of the open reading frame encoding the second extracellular loop (CCR5 Δ 32). This deletion mutation causes a frame shift and a premature stop codon in the fifth transmembrane domain of CCR5 giving rise to a truncated protein which is not expressed on the cell surface (Berger *et al.*, 1999) and thus

protects against HIV infection. Population studies of the $\Delta 32$ CCR5 mutation reveal that it is most common in caucasian individuals of Europe and the United States and absent or very rare in black Africans, Japanese and Venezuelan populations (Broder and Collman, 1997; Petersen *et al.*, 2001). Several other mutations of the CCR5 gene do exist, but are less common than the $\Delta 32$. Some of these mutations have been studied *in vitro* and their effects on co-receptor function determined (Blanpain *et al.*, 2000).

Analysis of mutant receptors in recombinant systems can lead to better understanding of CCR5 receptor function and indicate the susceptibility of carriers to HIV infection. Despite the apparent absence of the $\Delta 32$ mutation in Africans, there are other mutations on the CCR5 gene, which potentially protect against or enhance HIV entry in the African population. A single nucleotide polymorphism in the coding region of CCR5 has been associated with decreased disease progression from HIV-1 infection to AIDS in an African population (Hayes *et al.*, 2002). These authors showed that a conservative amino acid change from alanine to valine at position 335 of CCR5 had an allelic frequency of 4/30 in slow progressors as opposed to 1/16 in normal and 0/32 in fast progressors within the African and coloured population. Other mutations of CCR5 have also been identified in the diverse South African population (Petersen *et al.*, 2001). In this study, 206 HIV seropositive patients (140 Africans, 52 Coloureds and 14 Caucasians) and 176 HIV-seronegative controls (128 Africans, 144 Coloureds and 4 Caucasians) were analyzed for CCR5 mutations. Six of these mutations (L55Q, S75, $\Delta 32$, R223Q, A335V and Y339F) have been previously reported (Carrington *et al.*, 1997; Howard *et al.*, 1999; Blanpain *et al.*, 2000; Dong *et al.*, 2005). The L55Q mutation was seen in one HIV-1 positive Caucasian while the S75 occurred in one HIV-1 positive African. The $\Delta 32$ mutation occurred at an allele frequency of 0.035 in HIV-1 negative Coloureds but was seen in one HIV positive Coloured and one HIV positive Caucasian. The R223Q mutant was had an allele frequency of 0.007 in negative Coloureds while the Y339F occurred at a frequency of 0.008 in HIV negative Africans (Petersen *et al.*, 2001). As with the study of Hayes and collaborators, a high frequency of the A335V mutation was seen in the African population (0.029 HIV positive and 0.016 HIV negative). It also occurred in one HIV positive and two HIV negative Coloureds.

Seven (D2V, P35, Y89, L107F, P162, R225Q and R225X) were novel mutations. The D2V mutation was seen in one HIV-1 positive African and one HIV-1 negative Coloured. The silent mutation P35 occurred at high frequencies in both Africans and Coloureds (0.043 HIV-1 positive and 0.07 HIV-1 negative Africans and 0.173 HIV-1 positive and 0.069 HIV-1 negative Coloured) but was not seen in Caucasians. Y89 and P162 which are also silent mutations were seen only in one HIV positive African and one HIV negative Coloured respectively. The L107F and the R225X mutations occurred together at a frequency of 0.007 and 0.016 in HIV positive and HIV negative Africans respectively. It was also seen in one HIV positive Coloured. R225Q mutation occurred in one HIV negative Coloured. Little is known about the consequences of these mutations for interaction of the CCR5 receptor with chemokines or HIV-1. Some of these mutations may ultimately result in structural changes in the CCR5 protein, which may affect interactions with chemokines or HIV.

Four of the unique South African mutations lead to changes in the amino acid sequence of CCR5 that potentially affect the structure of the protein and will be studied in this project. The first mutation is at codon 2 (GAT to GTT), which results in a non conservative amino acid change from Aspartate to Valine. The second mutation, at codon 107 (CTC to TTC), involves an amino acid change from Leucine to Phenylalanine, thereby including an aromatic side chain, which may have an effect on the structure and function of the transmembrane domain of the CCR5 protein. The next mutation involves a truncation of the co-receptor through a conversion of an Arginine at position 225 (CGA) to a Stop (TGA) and the last, a non conservative amino acid change also at position 225 (CGA) from arginine to glutamine (CAA) (Petersen *et al.*, 2001). Studying these CCR5 mutants *in vitro* will lead to better understanding of molecular function of CCR5 and indicate the susceptibility of carriers of these alleles to HIV infection.

This thesis aims at investigating the effects of these four mutations of the chemokine receptor, CCR5 on both chemokine receptor and HIV-1 co-receptor function using *in vitro* methods.

CHAPTER TWO: MATERIALS AND METHODS

University of Cape Town

2.1 Materials

Analysis of the mutant CCR5 receptor function was focused on the *in vitro* study of the effects of mutations on chemokine binding, G protein activation, signal transduction and HIV-1 infection. Genomic DNA of a patient with homozygous wildtype CCR5 genotype was provided by Dr. V. Hayes of the University of Stellenbosch. HOS-CD4 and HOS-CD4-CCR5 cells were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. Nathaniel Landau (Landau and Littman, 1992; Deng *et al.*, 1996). C12Th/syn CCR5 cells were also obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. Tajib Mirzabekov and Dr. Joseph Sodroski (Mirzabekov *et al.*, 1999). Plasmids encoding rev, tat, and LTR-luciferase genes were provided by Stephen Jenkinson of GlaxoSmithKline Research and Development, Research Triangle Park, North Carolina. HEK 293 cells stably expressing the chimeric G protein, G_αqi (HEK-Gqi) and human CD4 (HEK-CD4) were prepared in the laboratory by Vathiswa Papu. G_αqi contains the G_αq backbone with a modified C-terminal containing the last few amino acids of the G_αi (Knight and Grigliatti, 2004; Kostenis *et al.*, 2005). DU151 subtype C envelope gene was from Dr. C. Williamson of the University of Cape Town and DU151 baculovirus were prepared in the lab by Bernhard Fromme. The subtype B envelope (YU2) was kindly provided by Lishun Su of the University of North Carolina and subtype C, Mole 1 in pSVIII was from Thumbu Ndung'u and Max Essex of Botswana and Harvard School of Public Health. Recombinant HIV-1 HIB gp120 (bac) was from NIBSC, Hertfordshire, UK.

2.2 Primers

Primers containing the mutations for amino acid changes Asp2Val, Leu107Phe, Arg225Gln and Arg225Stop were designed to include a silent restriction endonuclease site and the desired mutations.

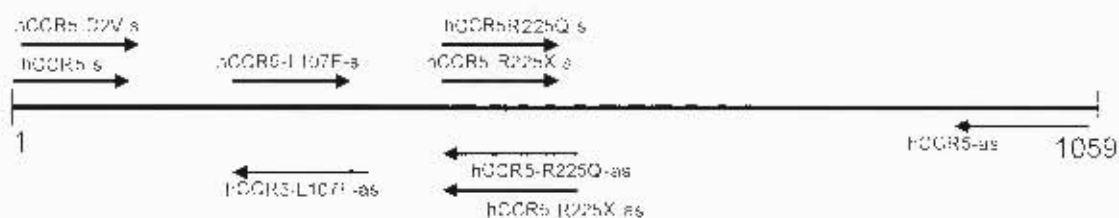


Figure 2.1: Representation of the positions of the different primers used for amplification. The solid horizontal line marked 1-1059 represents the CCR5 gene. The forward arrows represent the different sense primers and the backward arrows indicate the reverse or anti-sense primers.

hCCR5-s: 5'GCGCGCA**AAGCTT**ATGGATTATCAAGTGTCAAGTCC3'

↑ M D Y Q V S S P
HindIII

hCCR5-as: 5'CCGGCC**CTCGAG**TCACAAGCCCACAGATAATTCC3'

↑
XhoI

hCCR5-D2V-s: 5'GCGCGCA**AAGCTT**ATGG**TT**ATCAAGTGTCAAG3'

↑ M V Y Q V S S
HindIII

hCCR5-L107F-s: 5'TGTCAATTG**TTAACAGGGTTC**TATTTTATAGGC3'

C Q L **L** ↑ T G F Y F I G
HpaI

hCCR5-L107F-as: 5'AAAATAGAACCC**TGTTAACAGT**IGACACATTG13'

hCCR5-R225Q-s: 5'CTGCTTCGATGCC**AAAATGAGAAGAAG**3'

L L R C Q N E K K

hCCR5-R225Q-as: 5'CTCAATTT**GGCATCGAAGCAGAG**TTT13'

↑
SfaNI

hCCR5-R225X-s: 5'CTGCTTCGGTGT**TGATCAGAGAAGAAGAGG**3'

↑
BclI

hCCR5-R225X-as: 5'CCCTCTTCTTCT**TGATCAACACCGAAGCAG**3'

Figure 2.2: Primers used for amplification. Desired mutations and the silent restriction sequences are shown in bold. Arrows below sequences depict the restriction site of the enzymes.

2.3 Mutant receptor construction

Wildtype CCR5 was amplified by polymerase chain reaction (PCR) from genomic DNA of a patient with homozygous wildtype CCR5 using a sense primer that contained a HindIII site and an antisense primer containing an XhoI site to facilitate sub cloning. The amplified product was digested with HindIII and XhoI (Amersham, NJ, USA) and cloned into the expression plasmid pcDNA 3.1(+) (Invitrogen, San Diego, USA). Mutant primers were used for PCR based site-directed mutagenesis. Two methods of mutagenesis were used.

In the first method (DpnI method), primers containing the desired mutation and a high fidelity DNA polymerase (DeepVent, New England Biolabs, UK) were used for amplification at 94°C for 5 minutes, sixteen cycles of 94°C for 5 minutes, 55°C for 30 seconds 72°C for 12 minutes, and 72°C for 20 minutes. The product of the PCR reaction was incubated with 2µl of DpnI restriction endonuclease which digests only methylated DNA (New England Biolabs) for one hour at 37°C. Five µl of the digestion reaction was used to transform competent DH10B cells.

The second method (bridge PCR) involved two rounds of PCR. In the first round, two reactions were set up, one using a primer complementary to the 5' end of CCR5 (hCCR5-s) and the mutant antisense primer and the other using the mutant sense primer and the primer complementary to the 3' end of the receptor (hCCR5-as). Amplification was performed at 94°C for 2 minutes, thirty cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 2 minutes and a final extension at 72°C for 7 minutes. The products of this first round of PCR (1µl each) were used as template for the second round using primers that are complementary to both the 5' and the 3' terminal of CCR5 (hCCR5-s and hCCR5-as) and same temperature conditions as for the first round. The primers were added after five cycles. The fragment generated was digested with HindIII and XhoI and subcloned into pcDNA3.1(-) and used to transform competent DH10B cells.

Plasmid DNA was then extracted from resulting colonies using the Nucleobond PC500 kit (Machery-Nagel, Duren, Germany) and analyzed using the restriction endonucleases for the silent sites. Positive samples were sequenced to confirm mutation.

2.4 Cell Culture

HEK 293 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) containing 10% fetal calf serum (FCS) (Highveld Biologicals, Johannesburg, South Africa). HEK-Gqi and HEK-CD4 cells were maintained in DMEM supplemented with 10% FCS and 200µg/mL G418. HOS-CD4 and HOS-CD4-CCR5 were maintained in DMEM supplemented with 10%FCS, 400µg/mL G418 and 1µg/mL puromycin. All mammalian cells were cultured at 37°C and 10% carbon dioxide (CO₂). Insect cells (Sf21) were maintained at 27°C in TC 100 (Highveld Biologicals) containing 10% FCS and later adapted to serum free Sf900II (Gibco).

2.5 Transfection

Transient transfection: Cells were transiently transfected using the FuGENE6 reagent (RocheTM, Indianapolis, USA). For 100mm culture dish (Corning, Cambridge, USA), 6µg of plasmid DNA and 18µl of FuGENE6 reagent were mixed in 600µl of serum free medium (DMEM), incubated for 30-45 minutes and diluted with 10mL of DMEM 10%FCS before adding to the cells. Cells were then cultured for approximately 42 hours and then used for assay.

Stable transfection: Two days after FuGENE6 transfection, selection antibiotics were added to the medium at the concentration that was found to kill cells that were not transfected. Discrete colonies were picked, plated into 24-well culture plates, propagated and then characterized using an appropriate assay.

2.6 Inositol Phosphate (IP) assay

IP assay was performed following the method of (Millar, 1995). HEK 293 cells stably expressing a chimeric G protein, Gqi [which couples CCR5 stimulation to activation of phospholipase C (Knight and Grigliatti, 2004)] were plated (6×10^5 cells/dish) into 100mm tissue culture dishes (Corning, Cambridge, USA). Cells were transiently transfected with wildtype or mutant CCR5 plasmids and plated into 12-well plates one day later. The cells were then cultured overnight at 37°C in DMEM supplemented with 10% FCS before labeling

with 2 μ Ci/ml of ³H-myoinositol (Amersham Life sciences, Buckinghamshire, England) in Medium 199 with 2% FCS. The labeled cells were incubated with 1ml/well buffer 1 (140mM NaCl, 4mM KCl, 20mM HEPES, 8mM glucose, 1mM CaCl₂, 1mM MgCl₂, 0.1% BSA, Phenol red, 10mM LiCl) for 15 minutes. Cells were then stimulated with different concentrations of either MIP-1 α or RANTES (Peprotech, Rocky Hill, NJ) at 37°C for one hour. Cells were lysed with 10mM formic acid (1ml/well) for at least 30 minutes at 4°C to extract inositol phosphate. Separation of inositol phosphate from cell extract was done on columns with 1ml 1X8-200 DOWEX-1 ion exchange resin (Sigma, Bellefonte, USA). The columns were charged with 3ml 3M ammonium formate with 0.1M formic acid and washed with 10ml distilled water. Cell extracts were loaded on to the charged columns, washed with 10ml H₂O, followed by 5ml 5mM myoinositol with 0.1M formic acid. Total inositol phosphates were eluted with 3ml 1M ammonium formate with 0.1M formic acid into scintillation vials containing 16ml of scintillation fluid (Zinsser Analytical, Frankfurt, Germany) and the radioactivity was counted with a scintillation counter.

2.7 Chemokine competition binding assay

2.7.1 Iodination of MIP-1 β

A 7.8 μ g aliquot of MIP-1 β (10 μ L, Cytolab Ltd, Rehovot, Israel) was mixed with 10 μ L of 1mCi Na¹²⁵I and 15 μ L of 0.5M phosphate buffer (pH 7.4). ChloraminT (10 μ L of 1mg/ml, in 0.5M phosphate buffer) was added and the reaction incubated for one minute. The reaction was stopped by adding 50 μ L of sodium metabisulfide (1mg/mL in phosphate buffer). The reaction mixture was applied on to a pre-packed G-25 column and eluted with PBS containing 0.1% BSA. Fractions were collected after every 90sec. Each fraction (10 μ L) was diluted in a 1:100 ratio and counted using a gamma counter. Fractions with high radioactivity (fractions 10, 11, 12, 22, 23 and 24) (figure 3.3) were tested for specific binding to CCR5 using thymocyte cells stably expressing high amounts of CCR5 (C12Th/synCCR5). Fractions that were displaced by unlabelled MIP-1 β were used for subsequent competition binding assays

2.7.2 Competition binding assay

HEK 293 cells or HEK-CD4 cells were transiently transfected with wildtype or mutant CCR5 in 10cm dishes. After two days cells were detached from dishes with a Ca^{2+} and Mg^{2+} free detaching buffer (5mM EDTA, 50mM HEPES, 100mM NaCl), pelleted and resuspended in binding buffer (50mM HEPES, 1mM CaCl_2 , 5mM MgCl_2 , 0.5% BSA). Cells were incubated with labelled chemokine ligand ^{125}I -MIP-1 β (as tracer) (50,000 cpm, iodinated by the chloramine T method) and various concentrations of unlabelled chemokine ligand (MIP-1 β) or gp120 (DU151) in a reaction volume of 0.2ml for one hour at 27°C. Bound tracer was then separated by filtration through glass fibre filters (GF/C, Whatman, Maidstone, England) presoaked in 1% BSA, washed twice with a buffer containing 50mM HEPES, 1mM CaCl_2 , 5mM MgCl_2 and 0.5M NaCl and counted in a gamma counter. Binding parameters were determined with the Prism software (GraphPad) using nonlinear regression for a one-site competition (Howard *et al.*, 1999; Blanpain *et al.*, 1999a).

2.8 FACS Analysis

Cells transiently expressing wild type or mutant receptors were detached from tissue culture dishes using detaching buffer (5mM EDTA, 50mM HEPES pH 7.4, 100mM NaCl) and transferred to 15ml tubes. Cells were centrifuged at 1000 rpm for 5 minutes. Supernatant was removed and the cells were washed with PBS containing 0.5 % BSA (10ml PBS and centrifuging at 1000 rpm for 5 minutes). After washing, cells were resuspended in 500 μl of PBS + 0.5 % BSA. 20 μl of cell suspension was incubated with 1 μl of phycoerythrin (PE)-conjugated anti CCR5 antibody (2D7) (Pharmingen) for 60 minutes in the dark. The cells were then washed twice with PBS-BSA (1.5 ml into each tube and spinning for 10 minutes at 2000rpm) to remove any unbound antibody. Cells were resuspended in 500 μl of PBS-BSA for acquisition and analysis using a FACS calibur (Becton Dickinson). Two parameters were used for acquisition, which are the cell size (forward scatter) and cell granularity (side scatter). A gate between channel 100 and 300 on the forward scatter axis was set to capture a distinct cell population. HEK 293 cells transfected with empty vector, both stained and unstained were used as a negative control and to set the gate. The threshold for cells that

were labeled with PE was set at channel 100 in the FL2 wavelength and cells that were positive for PE label were identified as cells fluorescing above 100 in channel FL2.

2.9 DU151 Baculovirus (BV) amplification

Six million Sf21 cells grown in medium supplemented with 10% fetal calf serum (TC100, Highveld Biologicals, South Africa) were plated into 60mm culture dishes (Corning, Cambridge, USA) and allowed to settle at the bottom of the dish for approximately one hour in a 27°C incubator. Ten µl of first generation DU151-baculovirus was added to the cells and incubated at 27°C for up to five days, when more than half of the cells were dead. The virus was harvested by pipetting the medium off the cells and centrifuging at 1000rpm for 5 minutes. The supernatant was retained as second generation DU151-BV that was used for subsequent amplification.

Sf21 cells in suspension culture (TC100 with 10% FCS, 450ml of 0.5×10^6 cells/ml) were centrifuged (1000 rpm for 5 minutes at room temperature) and re-suspended in 2ml of second generation DU151-BV stock. After incubation with virus (one hour, 27°C on a shaking platform, 50 rpm) cells were diluted and centrifuged at 1000 rpm for 5 minute (to remove excess virus). Pellets were resuspended in 450 ml fresh growth medium (TC100 containing 10% FCS, 100U/ml penicillin and 100µg/ml streptomycin, Highveld Biologicals, South Africa). The cells were allowed to grow for at least three days (shaking at 100 rpm, 27°C) before virus was harvested by centrifuging the cells at 1000 rpm for 5 minutes at room temperature. The supernatant was retained as third generation DU151-BV and stored at 4°C.

2.10 Expression and purification of gp120

Sf21 cells were adapted to serum free medium (Sf 900 II, (Gibco) supplemented with 100U/ml penicillin and 100µg/ml streptomycin) to a density of 2×10^5 cells/ml in 500ml. Cells were transferred from a tissue culture flask to a sterile 500ml centrifuge bottle (Beckman JA- 20), centrifuged for 15 minutes at 1000 rpm at room temperature, resuspended in 50 ml of Baculovirus-gp120 (DU151). The cells were incubated with virus for one hour at 27° C on a shaking platform (50 rpm). 450 ml of Sf 900II + 100 U/ml penicillin and 100

$\mu\text{g/ml}$ of streptomycin was then added to the cells and incubated for three days at 27°C shaking at 130 rpm. On the third day of infection, cells were centrifuged at 2000 rpm for 15 minutes at 4°C . Supernatant was transferred to a sterile bottle on ice and protease inhibitors (PI) added (0.1M PMSF and $10\ \mu\text{g/ml}$ leupeptin). This was immediately loaded onto a lentil lectin column (4°C) that has been equilibrated with 10 volumes of equilibration buffer (20 mM Tris pH 7.5, 0.5 M NaCl, 1mM Mn^{2+} , 1mM Ca^{2+}) containing protease inhibitors (0.1M PMSF + $10\ \mu\text{g/ml}$ leupeptin). The column was washed with the same buffer. The gp120 (DU151) was eluted with 50ml of 1M α -methyl-mannopyranoside in 20mM Tris, 0.5M NaCl, 0.1 % T x 100 pH 5.0 with protease inhibitors. To concentrate the eluted material, the 50 ml eluate was diluted with 50 ml of sterile water containing protease inhibitors and loaded (15 ml at a time) onto an amicon ultra centrifugal filter device (Millipore). This was centrifuged each time at 4500 rpm for 20 minutes. The concentrated material was collected and aliquots were stored (-80°C).

2.11 SDS polyacrylamide gel electrophoresis and western blotting

SDS polyacrylamide gel electrophoreses were performed under reducing conditions. Two layers of analytical gels were prepared. The lower layer (the resolving gel) contained 8% acrylamide, 380mM Tris pH8.8, 0.1% SDS, 0.1% ammonium persulfate (AMPS) and $4\ \mu\text{l}$ of TEMED (N,N,N',N'-Tetramethylethylenediamine). The second layer (stacking gel) had a larger pore size and contained 5% acrylamide, 125mM Tris pH6.8, 0.1% SDS, 0.1% AMPS and TEMED. Protein samples were mixed with an equal volume of 2X Laemli solution (8%SDS, 5%v/v 2-mercaptoethanol, 10% glycerol, 25mM Tris pH 7.0 and bromophenol blue) and separated on the stacking gel at 70 Volts and on the resolving gel at 120 Volts in a running buffer containing 24.8mM Tris, 192mM glycine and 0.1% SDS. After separation by SDS-PAGE, western blotting experiments were performed to confirm the integrity and the size of the protein. The separated protein on the SDS-PAGE gel were electroblotted for one hour at 100 volts onto a PVDF membrane (HybondP, Amersham BioscienceTM, Buckinghamshire, England) in electroblotting buffer (19.84mM Tris, 1.5M glycine and 20% methanol). The membrane was blocked for one hour with 5%(w/v) fat free milk powder in Tris buffered saline (TBS, 20mM Tris pH7.5, 150mM NaCl) and incubated overnight in

2.5% fat free milk powder in TBS containing 1:10,000 dilution of horseradish peroxidase (HRP)-conjugated anti-HIV-1 envelope antibody (BiogenesisTM Poole, England). The membrane was rinsed once with TBS, then once with TBS-0.5% Tween 20 for 5 minutes (shaking). Residual Tween 20 was removed by washing four times (5 minute each) with TBS. Proteins were detected using an ECL⁺ kit (AmershamTM Buckinghamshire, England) which contains an HRP substrate reagent. The light produced when HRP on the membrane comes in contact with its substrate was captured on a light sensitive film (hyperfilm MP, AmershamTM, Buckinghamshire, England). Proteins were detected after developing and fixing of the film.

2.12 gp120 – CD4 binding assay

HEK 293 cells stably expressing CD4 were plated (1.0×10^6 cells/well, 12-well plate, Corning, Cambridge, USA) two days before the assay. As control, HEK 293 cells without CD4 were also plated at the same density. Cells were washed once (1ml/well) with HEPES-DMEM (Gibco). Purified gp120 was diluted in HEPES-DMEM to a final volume of 250 μ l. The diluted gp120 was added to the cells and cells were incubated at 27°C (shaking at 90 rpm) for 60 minutes. Cells were washed twice (HEPES-DMEM) solubilized with 50mM Tris pH7.4, 1% Triton X100, 1mM EDTA containing protease inhibitors (0.1M PMSF and 50mg/ml leupeptin). The solubilized material was centrifuged for 20 minutes at 14,000 rpm and the supernatant was retained for SDS-PAGE and western blot analysis.

2.13 gp120 ELISA

Half area flat bottom high binding 96-well ELISA plates (Costar Life Science) were coated with anti HIV-1 gp120 capture antibody, D7324 (Alto BioReagents, Dublin, Ire) (5 μ g/ml in carbonate buffer, 100mM sodium carbonate pH8.5, overnight at 4°C). This is based on previously described methods by Moore and Jarrett (1988). The plate was washed three times with PBS containing 0.1% TritonX-100 (PBS-TX). After blocking with 3% BSA for two hours (room temperature), serially diluted (in PBS-BSA) standard control gp120 (HIV-1 HIB gp120, EVA607, NIBSC) and test (purified) gp120 were added (50 μ l/well) and

incubated at room temperature for two hours. Plates were washed three times with PBS-TX and HRP-conjugated anti-HIV-1 gp120 (BiogenesisTM Poole, England) was added to each well at 5µg/ml and incubated at room temperature for 1 hour. The plate was washed five times with PBS-TX and TMB substrate containing a 1:1 mixture of TMB and H₂O₂ (Pierce) was added. The reaction was stopped after 30 minutes by addition of 2M H₂SO₄ and the absorbance was read on a plate reader at 450nm.

2.14 HIV Fusion assay

This assay was based on the method developed by Jenkinson *et al* (2003) and it models the interaction and fusion of HIV with the host cell (Jenkinson *et al*, 2003). HEK 293 cells were transfected with the membrane-bound precursor of the viral envelope proteins (gp120 and gp41), gp160 (YU2, Mole1, DU151), the viral protein Rev and the cytoplasmic viral transcription factor, Tat. Cells were cultured for two days to allow for protein expression. Human osteosarcoma (HOS) cells stably expressing CD4 were stably transfected with a long terminal repeat (LTR)-luciferase reporter construct. These cells were then transiently transfected with either wildtype or mutant CCR5 in pcDNA3.1 (hygro+) expression vector. After CCR5 transfection, cells were cultured in DMEM supplemented with 10% FCS and 200µg/mL hygromycin B (to select for cells that were successfully transfected with CCR5), 400µg/mL G418 and 1µg/mL puromycin (to select for LTR-luc expressing cells) for two days. After two days, HOS cells were harvested and plated at a density of 6000 cells per well of a 96-well plate in a volume of 50µL of DMEM containing 2% FCS. HEK 293 cells expressing the envelope, Rev and Tat were also harvested at a density of 12,000 cells per well in 50µL of DMEM-2% FCS and layered on to the HOS cells. Cells were allowed to fuse over night before determining the luciferase activity.

Luciferase activity was determined using the luciferase assay system (Promega). Cells were washed once with cold PBS and lysed with the cell culture lysis buffer. The cells were transferred to 1.5mL tubes and centrifuged at 12,000rpm for 2 minutes. The supernatant was transferred to a white flat bottom 96-well plate (Dynex technology) for luminometer (Veritas, Promega) reading.

**CHAPTER THREE: EFFECTS OF CCR5
MUTATIONS ON CHEMOKINE RECEPTOR
FUNCTION AND EXPRESSION**

3.1 Introduction

The CC chemokine receptor 5 (CCR5) binds and responds to the chemokine ligands, MIP-1 α , MIP-1 β and RANTES, in addition, it interacts with the HIV-1 envelope glycoprotein gp120 required for viral fusion and entry. Site directed mutagenesis, chimeric and antibody studies have shown that the interaction between CCR5 and its chemokine ligands involves the second extracellular loop of the receptor. Residues at the intracellular surface of the receptor are involved in G protein activation, receptor phosphorylation and β -arrestin binding. Four novel CCR5 mutations found in South Africa result in amino acid changes (Asp2Val, Leu107Phe, Arg225Gln and Arg225Stop) and it is not known what effect these changes may have on binding and responding to chemokine ligands.

3.2 Construction of CCR5 mutants

The four CCR5 mutants (1.1kb) were generated by polymerase chain reaction based mutagenesis as described in materials and methods and Figure 3.1. The mutant genes were analysed for the presence of desired mutation by digestion with the restriction enzymes that recognise the created silent restriction sites (Fig 3.2) and cloned into pcDNA 3.1 (+). Mutations were confirmed by sequencing.

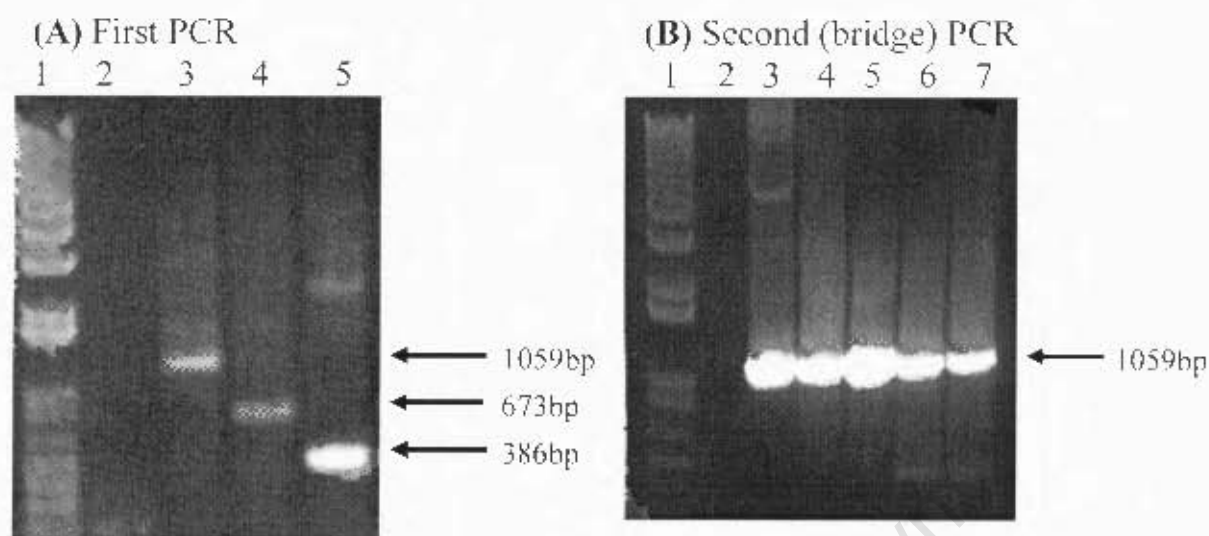


Figure 3.1: Production of mutant receptors. (A) Typical gel picture of first PCR reaction (Gel shows production of the two fragments of the Arg225Stop mutant). Lane 1 is the molecular weight marker, lane 2 is the negative control, lane 3 is full length wild type CCR5, 1059bp (positive control), lanes 4 and 5 are the two fragments of the Arg225Stop mutant (673bp and 386bp) (B) Full length PCR products of mutant receptors from second (bridge) PCR. Lane 1, molecular weight marker, lane 2, negative control, lane 3 wild type CCR5 (positive control), lanes 4, 5, 6 and 7 are full length PCR products of all four mutants (Asp2Val, Leu107Phe, Arg225Gln and Arg225Stop)

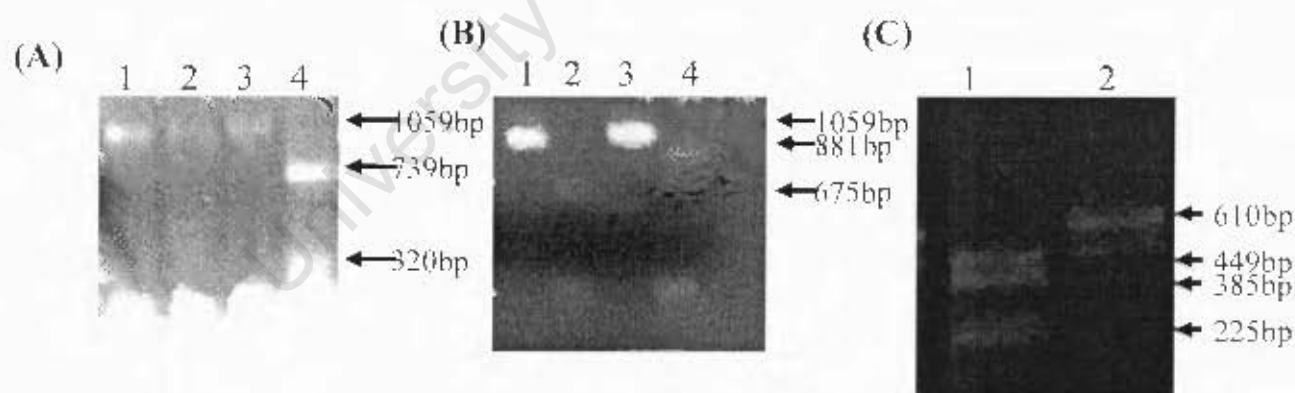


Figure 3.2: Restriction enzyme analysis of mutant receptors: (A) Undigested (lanes 1 and 3) and HpaI digests (lanes 2 and 4) of wild type and Leu107Phe mutant respectively. Digestion of wild type CCR5 with HpaI had no effect on the receptor (lane 2) but when the mutant was digested, two fragments (739bp and 320bp) were obtained (lane 4) (B) Undigested (lanes 1 and 3) and SfaNI digest of wild type and Arg225Gln mutant CCR5. SfaNI cuts the wildtype receptor to give three fragments of sizes 675bp, 206bp and 178bp (lane 2). Presence of the Arg225Gln mutation abolishes one restriction site and two fragments of sizes 881bp and 178bp were obtained. (C) BclI digestion of wild type (lane 1) produced three fragments, 449, 385 and 225bp while digestion of the Arg225Stop mutant (lane 2) gave two fragments of sizes 610 and 449bp).

3.3 Effects of mutations on CCR5 signalling (IP production)

HEK 293 cells stably expressing the chimeric G protein, Gqi (couples CCR5 stimulation to activation of phospholipase C) were transfected with wild type and mutant CCR5 which were then used for inositol phosphate (IP) assays. This assay was used to determine the functional response of the mutant receptors to chemokine stimulation. Two different CCR5 ligands, MIP-1 α (Figure 3.3A) and RANTES (Figure 3.3B) were used to stimulate receptor transfected cells. Addition of increasing concentrations of ligand leads to an increase in inositol phosphate production by the wildtype receptor (EC₅₀ = 8.81 \pm 2.5 nM and 9.95 \pm 1.69 nM for MIP-1 α and RANTES respectively) (Figure 3.3 and Table 3.1). The Asp2Val mutant exhibited a decreased functional response to MIP-1 α and RANTES with an EC₅₀ of 265.3 \pm 178 nM and 261.5 \pm 170 nM respectively compared with the wildtype. This mutant receptor also showed low E_{max} for both ligands at 0.1 μ M stimulation (7506 and 5499 cpm for MIP-1 α and RANTES respectively) compared with the wildtype (14,052 and 13,991 cpm respectively). This suggests that the mutant receptor may be poorly expressed or it may have a low coupling efficiency or decreased affinity for the ligand.

The Leu107Phe mutant had similar EC₅₀ (13.09 \pm 4.9 nM and 8.7 \pm 0.6 nM, Table 3.1) and E_{max} (18799 and 18582 cpm) for both MIP-1 α and RANTES respectively as the wild type CCR5 receptor. Comparable IP results were obtained for the Arg225Gln with EC₅₀ of 7.1 \pm 2.02 nM and 10.05 \pm 0.25 nM for MIP-1 α and RANTES respectively. There was no measurable response for the Arg225Stop mutant. This suggests that this mutant may be poorly expressed or it is unable to bind chemokine ligands or it may not be coupling efficiently.

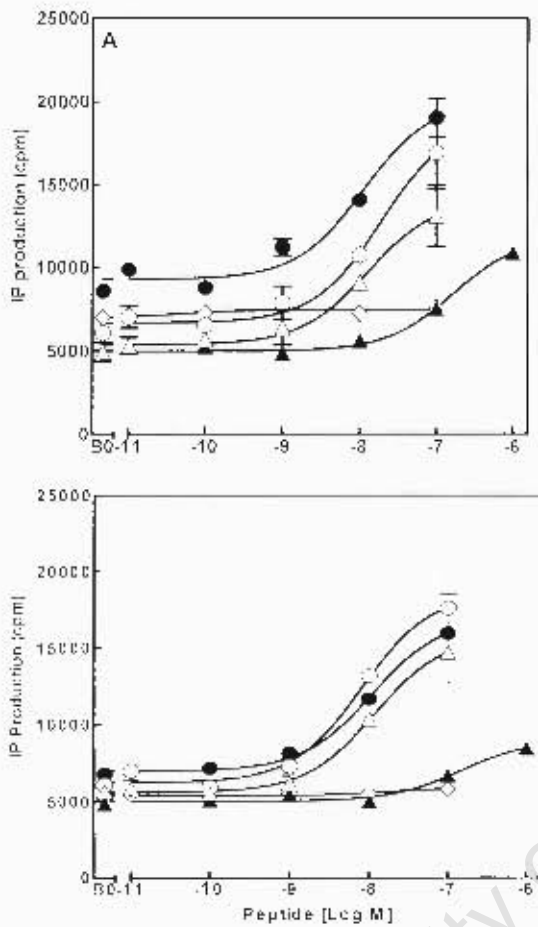


Figure 3.3: Stimulation of inositol phosphate production by MIP-1 α (A) and RANTES (B). HEK cells stably expressing a chimeric G protein, Gqi were transiently transfected with wildtype CCR5 (Δ), Asp2Val (\blacktriangle), Leu107Phe (\circ), Arg225Gln (\bullet) or Arg225Stop (\circ) mutant CCR5 receptors, incubated with 3 H-myoinositol and stimulated with various concentrations of MIP-1 α or RANTES. Data presented are from single experiments that are representative of at least three independent experiments performed in duplicate. Error bars indicate the standard error for each triplicate.

It was noted that even though the Arg225Gln mutant showed the same maximal responses and EC₅₀ values as the wildtype receptor, it exhibited an increase in basal activity (response in the absence of ligand) compared to the wildtype (Figure 3.3). This suggests either increased expression or the receptor may be constitutively active. An IP assay was performed to compare the basal activity of an empty vector, the mutant receptor and that of the wildtype receptor. Analysis of the results showed that there was a slight increase in the basal activity of the wildtype receptor (Figure 3.4). A statistically significant increase (p value less than 0.05) was observed in the signalling activity of the Arg225Gln mutant

compared to the wildtype receptor ($p = 0.0293$) in the absence of ligand. In the presence of ligand, there was no significant difference in the signalling activity of both the wildtype and the Arg225Gln mutant receptors. Cells transfected with empty vector (pcDNA3.1) which was used as a negative control showed no change in the presence of the ligand (Figure 3.4).

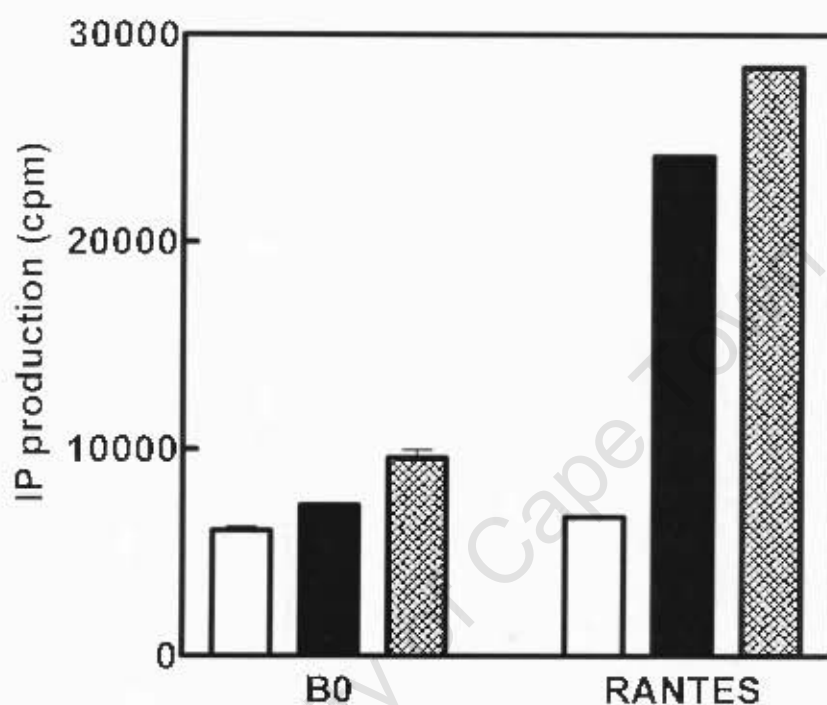


Figure 3.4: Enhanced basal activity of Arg225Gln CCR5. HEK cells stably expressing Gq1 were transiently transfected with empty vector (clear bars), wildtype CCR5 (solid bars) or Arg225Gln CCR5 (hashed bars), incubated in ^3H -myoinositol and stimulated with either buffer or RANTES (10^{-7} M). Each bar represents the mean of duplicate values for each sample. Lack of error bars in some of the bars reflects the closeness of the duplicate values. B0 indicates basal signalling response (signalling in the absence of ligand).

3.4 Ligand binding of CCR5 mutant receptors

A competition binding assay was used to determine the affinities of wild type and mutant receptors for ligand (MIP-1 β). MIP-1 β was radio-iodinated in the laboratory using the chloramine T method. Radio-labelled peptide was purified through a G-25 column and fractions were collected and the amount of radioactivity determined using a gamma counter. The elution profile of a typical MIP-1 β labelling experiment is shown in Figure 3.5.

Fractions 10, 11, 12, 13, 22, 23 and 24 were tested for CCR5 binding using Cf2Th/syn CCR5 cells (thymocytes stably expressing a high amount of CCR5) by competition with 10^{-7} M of unlabelled MIP-1 β . Fractions 10, 11, 12, and 13 showed binding (labelled peptide) (Figure 3.6) and were used for competition binding assay to determine binding of mutant CCR5. Fractions 22, 23 and 24 did not bind CCR5 (free iodine).

Specific binding was measured using a single concentration of tracer (125 I-MIP-1 β) and the concentration of the ligand that showed fifty percent displacement of the tracer (IC₅₀) and the affinity of MIP-1 β for the wildtype and mutant receptors were determined.

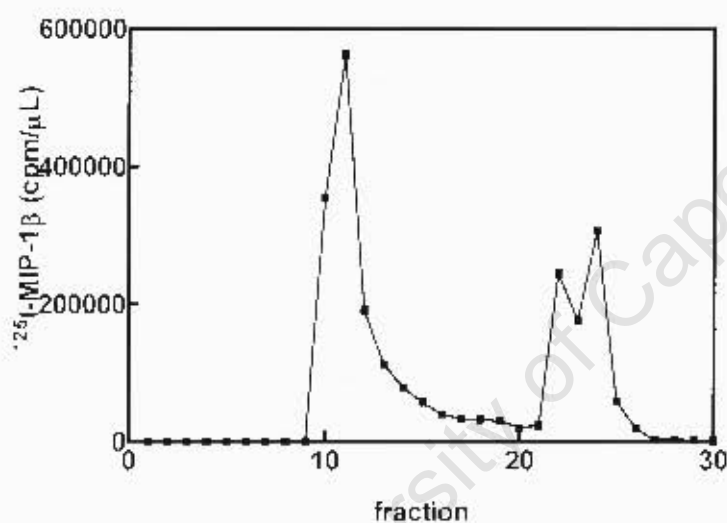


Figure 3.5: Profile of radio-iodinated MIP-1 β . 7.8 μ g of MIP-1 β was mixed with 10 μ l. of 1 mCi Na¹²⁵I and 10 μ l. of 1mg/ml of chloramine T in a reaction volume of 45 μ l. in phosphate buffer. The reaction was stopped with 50 μ L of sodium meta bisulfide and the labelled peptide was purified through a G-25 column. Fractions were collected and counted on a gamma counter.

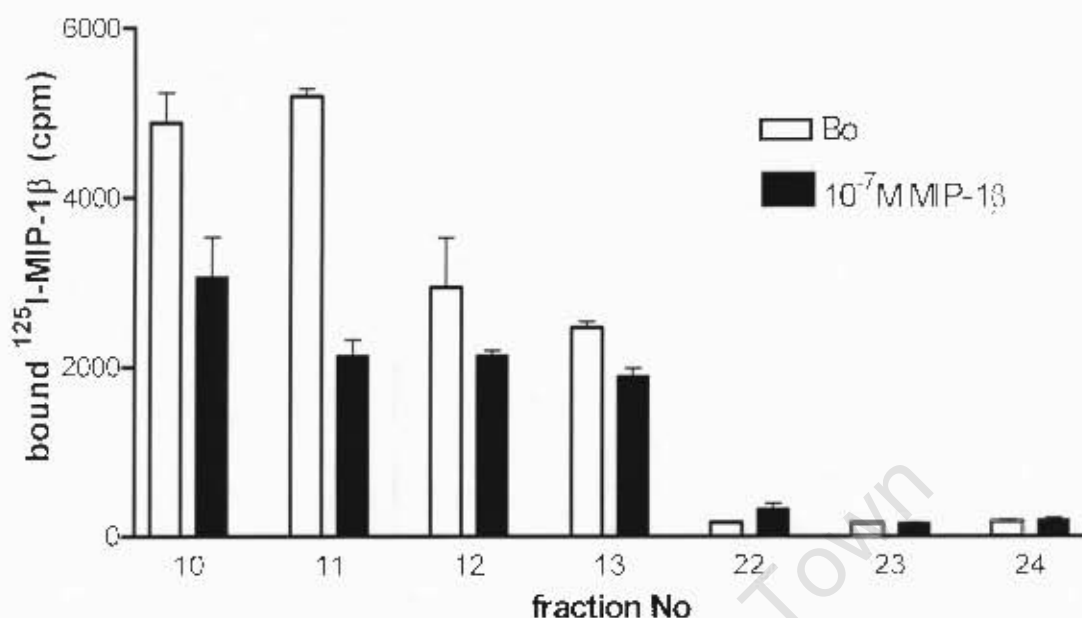


Figure 3.6: Fractions of label tested for binding to CCR5. C12Th/syn CCR5 cells were incubated with labelled MIP-1 β in the absence (clear bars) or presence of 10^{-7} M MIP-1 β (solid bars). B₀ represents no added ligand.

Addition of increasing concentrations of unlabelled MIP-1 β to HEK293 cells expressing the wild type receptor leads to displacement of the labelled MIP-1 β with an IC₅₀ of 15.36 ± 6.87 nM (Figure 3.7 and Table 3.1). The Asp2Val mutant showed both low total binding and decreased affinity for MIP-1 β (IC₅₀ of 63.26 ± 20.48 nM). Both the Leu107Phe and Arg225Gln mutant affinities were comparable to that of the wild type receptor (IC₅₀ 12.38 ± 3.57 nM and 7.22 ± 1.41 nM respectively). No binding was observed for the Arg225Stop mutant (Figure 3.7 and Table 3.1). This suggests that the truncated receptor may not be expressed on the cell surface.

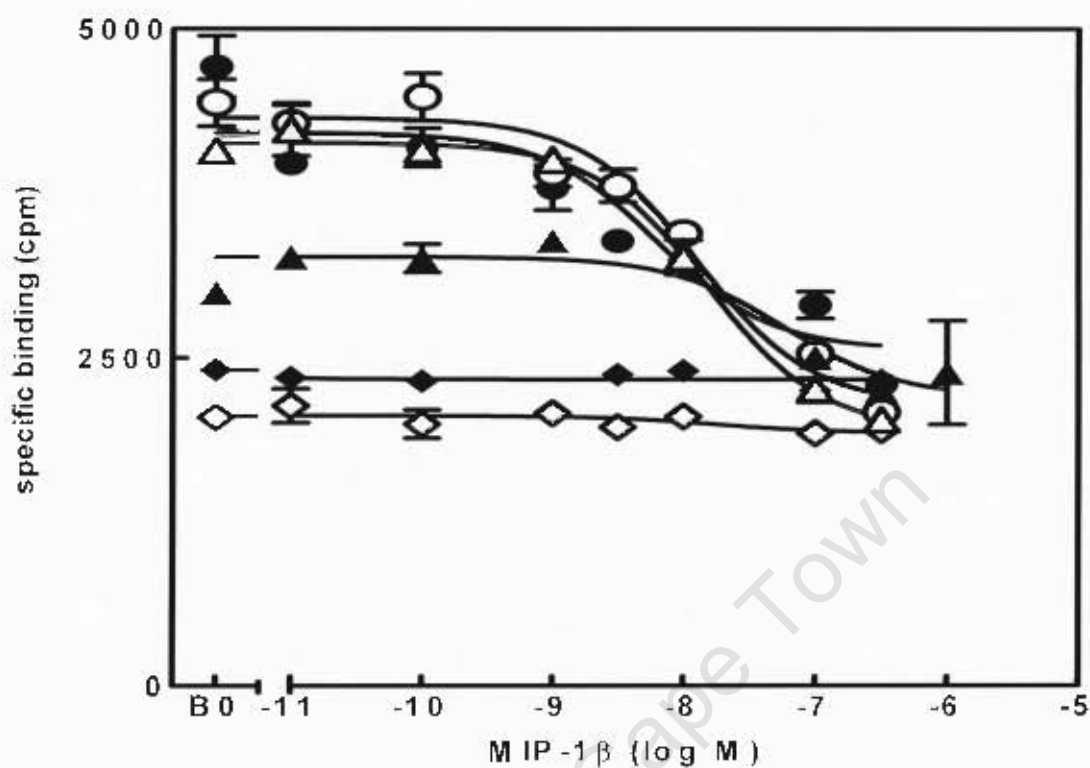


Figure 3.7: Competition binding of MIP-1 β . HEK293 cells were transiently transfected with wildtype receptor (Δ), Asp2Val (\blacktriangle), Leu107Phe (\circ), Arg225Gln (\bullet) or Arg225Stop (\diamond) mutant receptors or untransfected HEK293 cells (\blacklozenge) and incubated with labelled chemokine ligand (^{125}I MIP-1 β as tracer) and various concentrations of unlabelled ligand (MIP-1 β). Data displayed is representative experiment of three independent experiments performed in triplicate. B0 represent buffer with no MIP-1 β . Error bars represent standard errors for each triplicate.

Table 3.1: Summary of chemokine binding and signalling of wild type and mutant CCR5

Results are the means and standard errors calculated from three experiments performed independently. (EC50 is the concentration of ligand that gave half maximal response.). A Mann-Witney two tailed t test was done to compare values obtained for each mutant (except for the Arg225Stop) with the wild type. The P values are indicated in bold in brackets (statistical difference, $p < 0.05$).

Receptor	EC50 (nM)		binding IC50 (nM)
	MIP-1 α	RANTES	MIP1 β
Wild type	8.81 \pm 2.5	9.95 \pm 1.69	15.36 \pm 6.87
Asp2Val	265.3 \pm 178 (0.022)	261 \pm 170.3 (0.028)	63.26 \pm 20.48 (0.042)
Leu107Phe	13.09 \pm 4.9 (0.7)	8.7 \pm 0.6 (0.8)	12.38 \pm 3.57 (1.00)
Arg225Gln	7.1 \pm 2.02 (1.00)	10.05 \pm 0.25 (1.00)	7.22 \pm 1.41 (0.7)
Arg225Stop	Noneasurable response	No measurat response	no binding

3.5 Expression of Wild type and mutant receptors

The lack of binding and signalling by the Arg225Stop mutant suggested a possible lack of receptor expression on the cell surface. To determine expression of wild type and mutant CCR5 receptors FACS analysis was performed. Phycoerythrin-conjugated 2D7, a fluorescent labelled monoclonal antibody against CCR5 which recognizes a conformational epitope located in the second extracellular loop of the receptor was used to detect CCR5. The level of receptor expression was assessed by the intensity of fluorescence of the antibody. The wild type receptor was well expressed at the cell surface with a mean fluorescence of approximately 33 (which gives an indication of the average amount of receptors expressed on the cell surface) and more than 80% of cells expressing the receptor (Figure 3.8). This result indicates greater than 80% transfection efficiency. Both the Leu107Phe and the Arg 225Gln were expressed at similar levels (greater than 90% of wild type expression, table 3.2) to the wildtype receptor, while the Asp2Val mutant was expressed at a lower level (66%, Table 3.2) compared to wild type CCR5. The Arg225Stop mutant was not detected on the cell surface, 7.8 \pm 1.81% of cells expressing receptor and a mean

fluorescence of 23.8:1.45 (Figure 3.8 and Table 3.2) which is comparable to the stained vector transfected cells, 16.02:6.95 % of cells expressing receptor and mean fluorescence of 4.96:2.78 %, respectively.

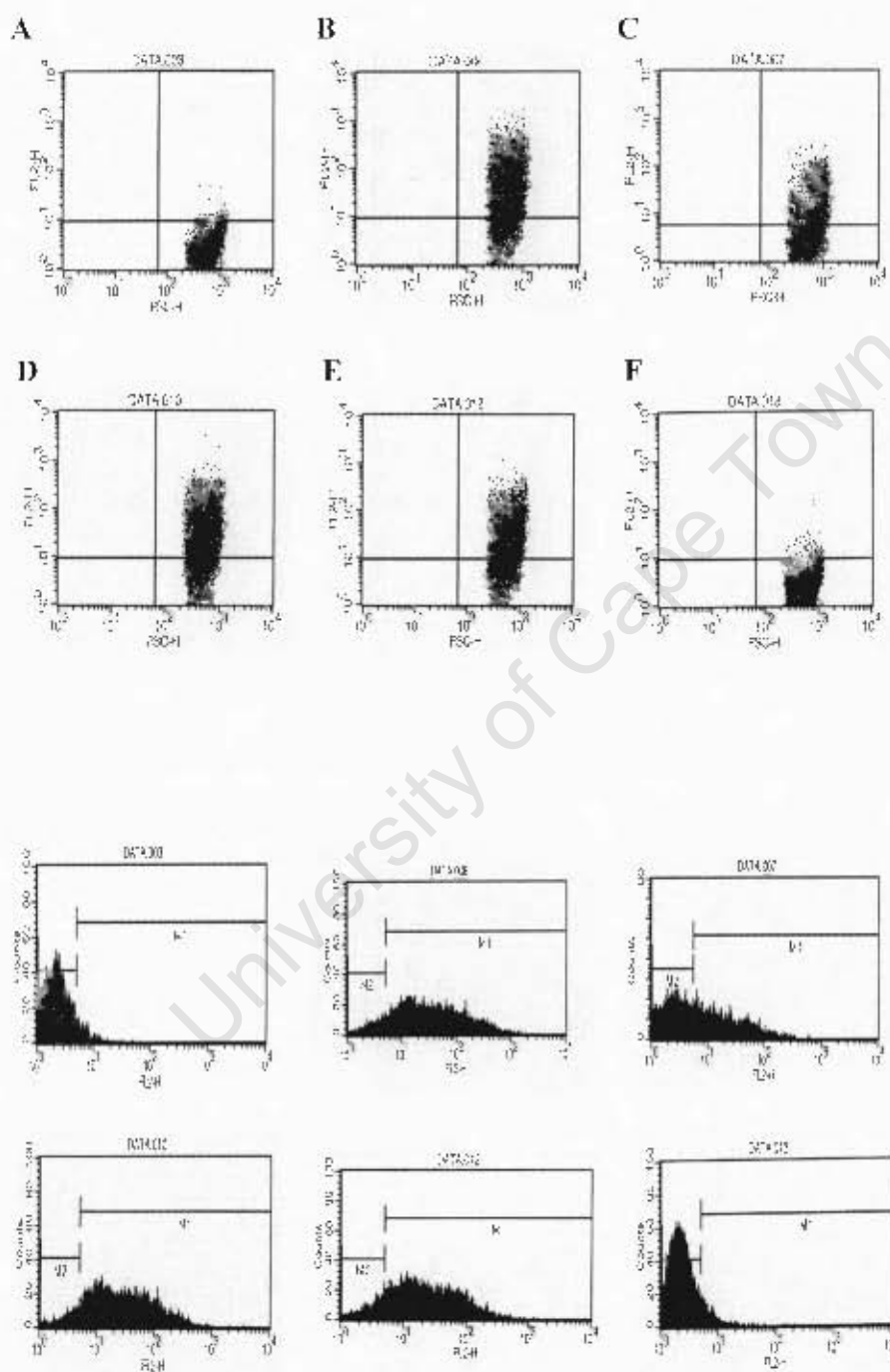


Figure 3.8 FACS: Representative dot plots (top panels) and histograms (bottom panels) of 11Fk cells stained with antibody (A), Wild type CCR5 (B), Asp2Val (C) Leu107Phe (D) Ar225Gln (E) and Arg225Stop (F) mutant CCR5.

In order to measure intracellular expression of receptors, cells were permeabilized with 0.05% saponin, labelled with 2D7 and analyzed using FACS. Results showed increased expression in permeabilized cells (compared to unpermeabilized cells) expressing the wild type receptor and all mutants except for the Arg225Stop mutant (data not shown). This suggests that the Arg225Stop mutant is neither expressed on the cell surface nor on intracellular membranes.

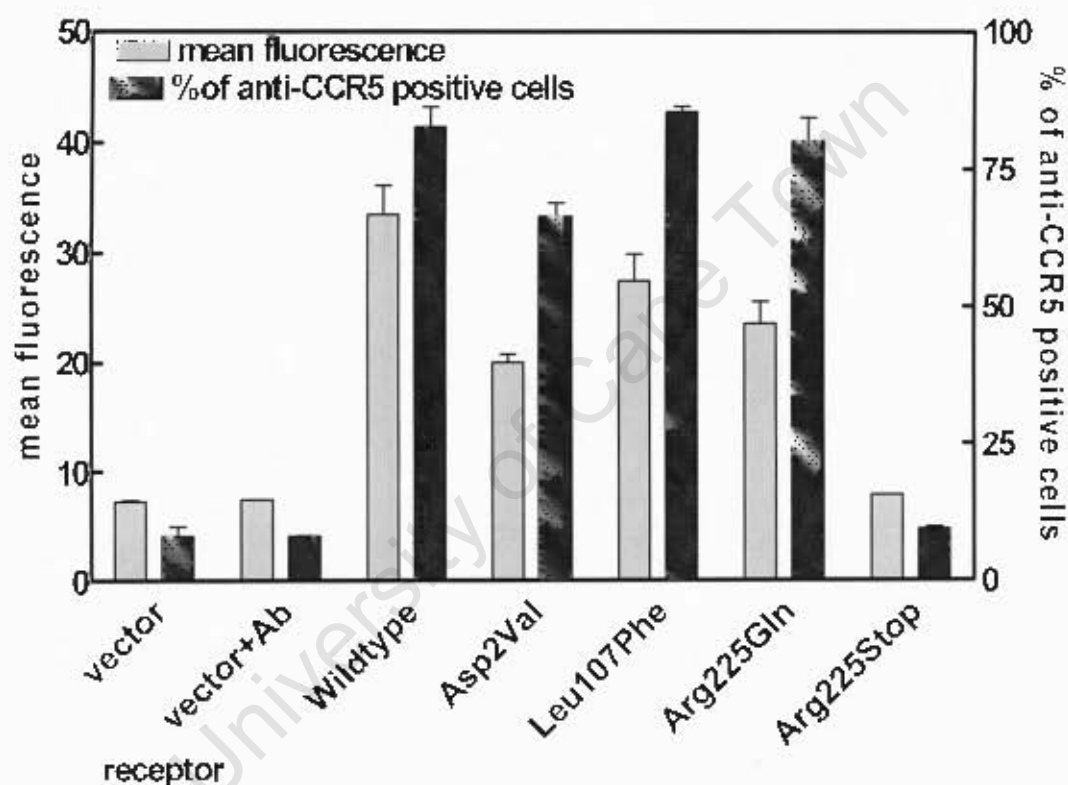


Figure 3.9: Cell surface expression of wildtype and mutant CCR5. HEK 293 cells were transiently transfected with plasmids encoding Wild type, Asp2Val, Leu107Phe, Arg225Gln and Arg225Stop CCR5. Cells were stained with 2D7-PE and analysed by FACS. Unstained and stained vector transfected cells were used as negative controls. The left Y-axis (mean fluorescence) represents the intensity of the fluorescent label recorded for each receptor. The right Y-axis (% of cells expressing receptor) indicates the gated event for each receptor. Representative of three experiments performed independently is presented.

Table 3.2: Effects of mutations on cell surface expression of the CCR5 receptor

Mean fluorescence is the mean intensity of the fluorescent label recorded for each receptor which gives an indication of the average number of receptors expressed. Percentage (%) of cells expressing receptor indicates the gated events and gives an indication of transfection efficiency. Values are expressed as percentage of wild type and results are means and standard errors calculated from three independent experiments. A Mann-Whitney two tailed t test was done to compare values obtained for each mutant (except for the Arg225Stop) with the wild type. The P values are indicated in bold in brackets (statistical difference, $p < 0.05$).

Receptor	Mean fluorescence	% of cells expressing receptor
Wildtype	100	100
Asp2Val	58.6±3.47 (0.0405)	66.1±10.56 (0.004)
Leu107Phe	86.1±4.89 (0.1)	92.6±6.60 (0.405)
Arg225Gln	70.3±0.1 (0.22)	93.6±3.32 (0.179)
Arg225Stop	23.8±1.45 (0.002)	7.87±1.81 (<0.0001)
Vector + Ab	16.02±6.95	4.96±2.78

3.6 Summary

Mutant receptors constructed were analysed by restriction enzyme digestion and confirmed by sequencing. Chemokine receptor function was determined by a signalling assay (IP production) and a competition binding assay. Results show decreased IP response and decreased affinity for the Asp2Val mutant. This mutant also showed low total binding. Both the Leu107Phe and the Arg225Gln showed the same IP response and binding affinities as the wild type receptor. There was a significant increase in basal response for the Arg225Gln mutant compared to the wild type receptor. The Arg225Stop mutant did not bind ligand and did not show any signalling response to either MIP-1 α or RANTES.

Cell surface expression of the different mutant receptors was analysed by FACS. Results showed that both the Leu107Phe and the Arg225Gln were expressed at the same level as the wildtype receptor. There was decreased expression for the Asp2Val mutant while there was no measurable response for the Arg225Stop mutant both on the cell surface and intracellularly.

**CHAPTER FOUR: EFFECTS OF CCR5
MUTATIONS ON HIV-1 CO-RECEPTOR FUNCTION**

4.1 Introduction

Since the discovery that CCR5 acts as a co-receptor for HIV-1 entry into host cells, several studies have been carried out to map out the region on CCR5 and specific residues involved in this entry process. CCR5 is known to interact directly with the envelope glycoprotein, gp120 before fusion is effected. Site directed mutagenesis, chimeric studies, comparison of chemokine receptor homologues from different species and antibody studies have revealed that gp120 interacts mostly with residues at the N-terminal of the receptor. The other three extracellular regions of CCR5 have been shown to be involved in the HIV-1 fusion/entry process but evidence suggests that these regions are important for inducing the conformational changes necessary for membrane fusion (Blanpain *et al.*, 1999a; Lee *et al.*, 1999). In this study, co-receptor function of the four mutant CCR5 were studied using a cell-cell fusion assay to determine their effect on fusion efficiency and a gp120 binding assay to determine the effects of the mutations on interaction with gp120 and their binding affinities.

4.2 Cell-cell fusion

Fusion of HIV with host cells is mediated by the envelope glycoprotein, gp160 which is made up of two subunits (gp120 and gp41). A viral free cell-cell fusion assay system that models the interaction and fusion of HIV with the host cell was used to determine the effect of the four CCR5 mutations on fusion efficiency (Jenkinson *et al.*, 2003). In this assay, the virus was replaced with a cell line (HEK 293) that transiently expresses the HIV envelope glycoprotein, gp160, the viral transcription factor, Tat and the viral protein, Rev. The target cell was a human osteosarcoma (HOS) cell line stably expressing the human CD4 receptor and LTR-luciferase reporter, transiently transfected with wildtype or mutant CCR5. Mixing of these two cell types allows binding of the gp120 to the CD4 and CCR5 resulting in fusion of the cells on which the different proteins are expressed. When fusion occurs, there is mixing of the two cytoplasm and the Tat protein from the HEK 293 cell cytoplasm binds to the LTR on the promoter of the luciferase in the HOS cell and activates transcription of the luciferase gene. The activity of the luciferase is measured as an end point.

4.2.1 Generation of cells for fusion assay

Stable LTR-luciferase (LTR-luc) cell lines were generated in order to perform this assay. HOS cells expressing both human receptors (CD4 and CCR5) and the LTR-luciferase were first prepared. Geneticin (G418) toxicity was first tested on HOS cells stably expressing CD4 (HOS-CD4) and HOS cells stably expressing both CD4 and wildtype CCR5 (HOS-CD4-CCR5) plated in 10cm dishes. The cells were treated with varying concentrations (100µg/ml. - 1000µg/ml.) of the antibiotic and monitored for cell death. Concentrations greater than 700µg/ml. killed all cells after four days and 500µg/ml. after one week. Concentrations less than 300µg/ml. could completely kill cells after ten days. For stable selection, 400µg/mL of G418 was used.

HOS cells stably expressing both CD4 and wildtype CCR5 (HOS-CD4-CCR5) were transfected with the neomycin resistant plasmid (pcDNA3.1+) encoding the LTR-luciferase gene. Transfected cells were selected with 400µg/ml. of G418 and positive clones were identified for luciferase activity by transiently transfecting them with Rev and Tat and assayed using the luciferase assay system (Promega). Four HOS-CD4 clones were positive for the presence of luciferase (CD4-8, CD4-10, CD4-15 and CD4-16). Of the four positive clones, CD4-10 showed highest activity (Figure 4.1) and was later used for subsequent experiments. Only one HOS-CD4-CCR5 clone (CCR5-13) was positive for luciferase and this clone was used as positive control for all fusion experiments (Figure 4.1).

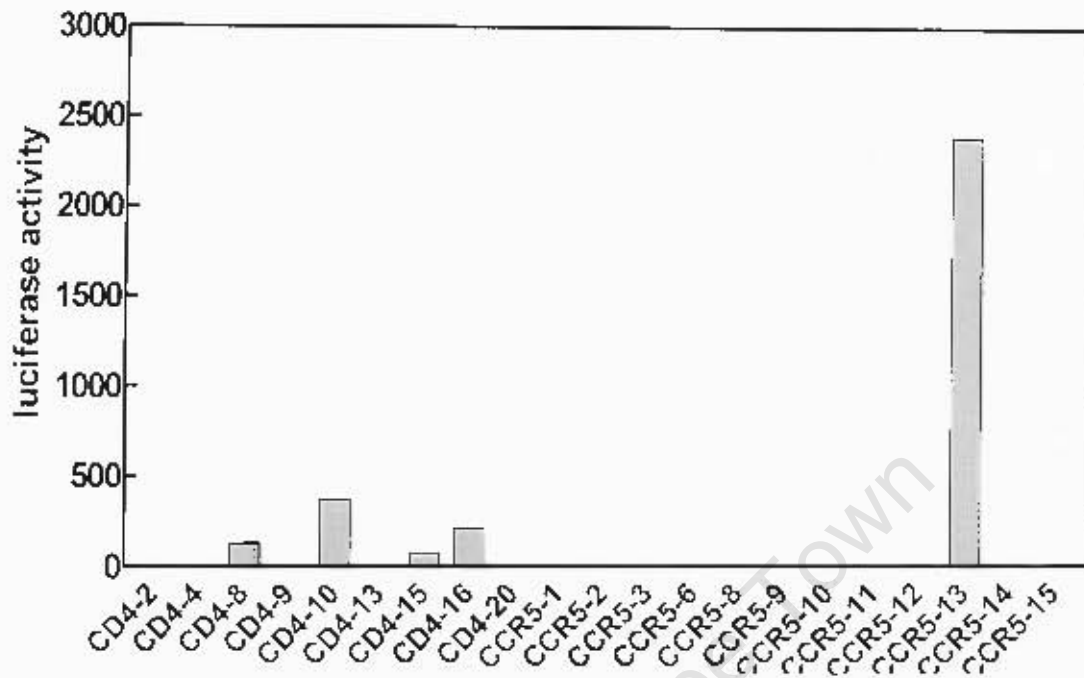


Figure 4.1: Luciferase activity of cells stably transfected with LTR-Luciferase. HOS-CD4 and HOS-CD4-CCR5 were transfected with pcDNA3.1/Neo(-) plasmid encoding the LTR-luciferase gene. Transfected cells were selected and propagated in medium containing 400µg/ml of G418. Positive clones were transiently transfected with rev and tat and after two days the cells were analysed for luciferase activity. Cells were lysed with the luciferase lysis buffer and luciferase activity was measured on a Luminoscan Luminometer using the Ascent software.

4.2.2 Expression of CCR5 mutants in HOS-CD4-luciferase (CD4-10) cells

Wild type and mutant CCR5 genes were subcloned into the hygromycin resistant vector. HOS-CD4-10 cells were transfected with wildtype and mutant CCR5 in pcDNA3.1/Hygro(+) and transfected cells were selected with 200µg/mL hygromycin B (Invitrogen). Stable expression of wildtype and mutant receptors was analysed by FACS analysis. All clones tested were negative for CCR5 expression (Figure 4.2). The lack of expression may have been due to the treatment of the cells with three different antibiotics. These cells were treated with puromycin, G418 and then Hygromycin B. It is possible that the antibiotic pressure could have been too high for them to perform optimally. Since stable transfection was not successful, transient transfection was attempted. HOS-CD4-10 cells were transfected with receptors and FACS analysis done after two days. Expression levels

(both mean fluorescence and % of cells expressing receptor) after transient transfection were very low (Figure 4.3A). Cells were transfected and treated with antibiotic (hygromycin B) for one or two days. Antibiotic treatment for one day improved expression by two fold while two days selection further improved expression by three fold (Figure 4.3B). Three days selection showed further improvement in expression but total cell count after selection was very low. For subsequent fusion assays, HOS-CD4-luciferase cells were transfected with wildtype and mutant CCR5, selected with hygromycin B for two days before use for fusion experiments. Each fusion experiment was performed alongside FACS analysis to ascertain expression of the different mutant receptors

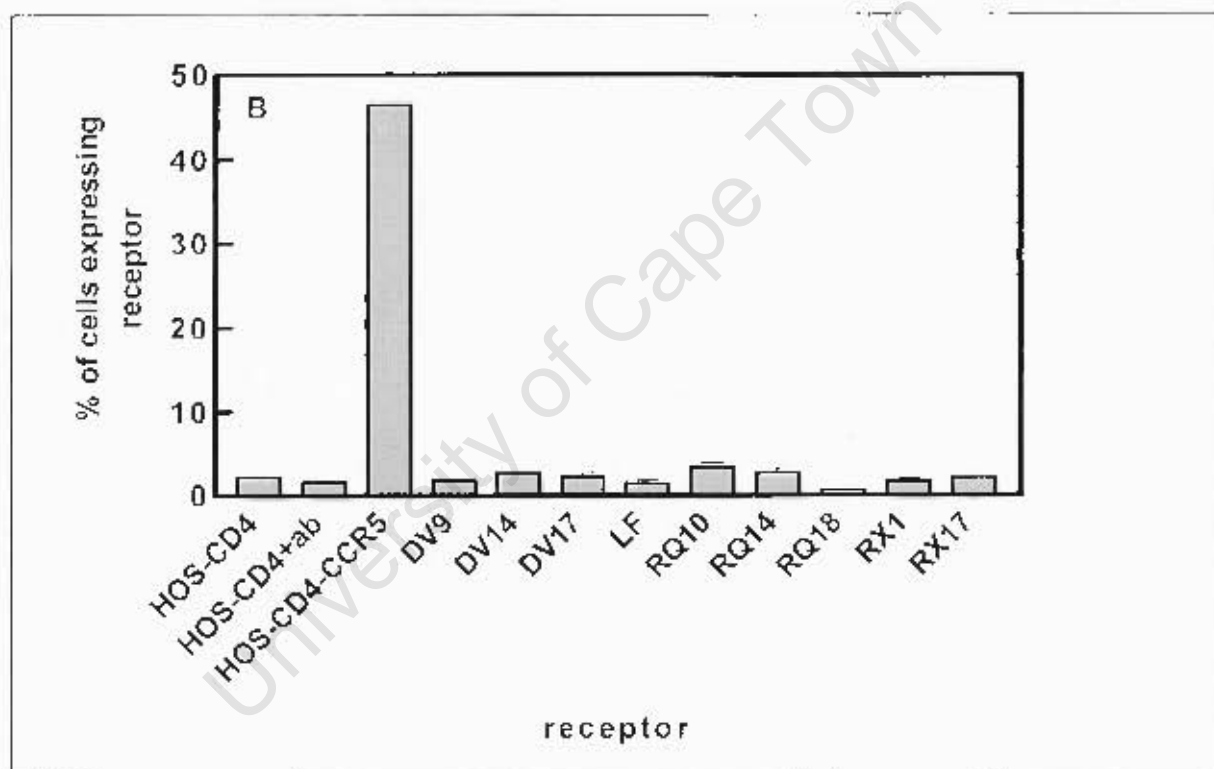


Figure 4.2: Expression of mutant CCR5 in stably transfected cells. HOS cells stably expressing CD4 and the LTR-luciferase (CD4-10, Figure 4.1) were transfected with Asp2Val, Leu107Phe, Arg225Gln and Arg225Stop mutant CCR5 in pcDNA hygro(+) plasmids. Stably transfected cells were selected by propagation in medium containing 200µg/mL of hygromycin B (Invitrogen) and analysed for the presence of CCR5 receptor by FACS analysis. HOS-CD4 and HOS-CD4+ab are negative controls (no receptor present), HOS-CD4-CCR5 is a positive control (cells stably expressing wild type CCR5). DV, LF, RQ and RX are clones from cells transfected with Asp2Val, Leu107Phe, Arg225Gln and Arg225Stop respectively.

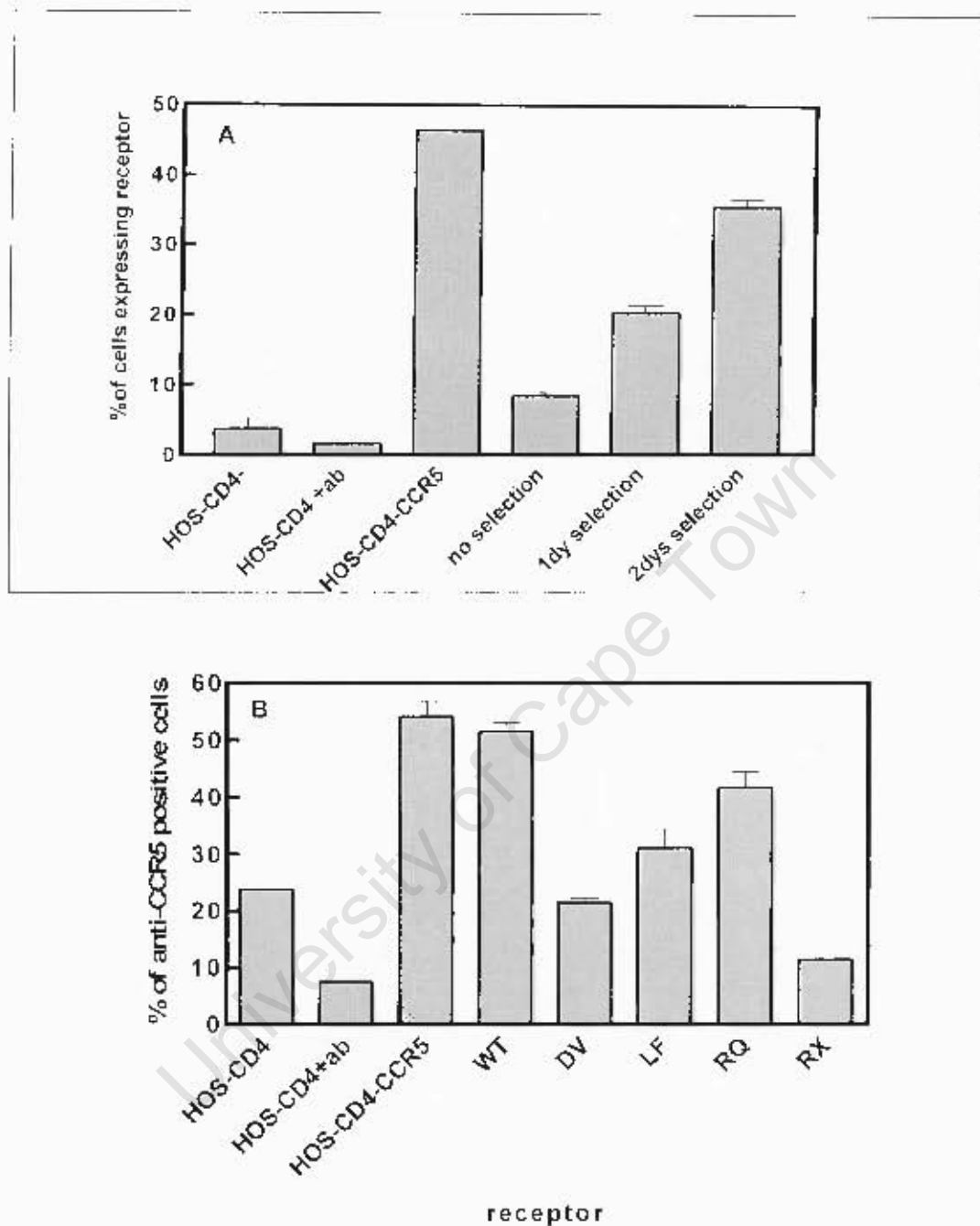


Figure 4.3: Effect of hygromycin B treatment on CCR5 expression. (A) Comparison of length of hygromycin B selection on expression of CCR5 in HOS cells. HOS cells stably expressing CD4 and LTR-luciferase (HOS-CD4) were transfected with plasmids encoding wild type CCR5. Cells were incubated with hygromycin B for one or two days and expression was determined by FACS analysis. (B) Expression of wildtype and mutant CCR5 after two days selection with hygromycin B. Untransfected HOS-CD4 cells (stained and unstained) were used as negative controls while HOS-CD4 cells stably expressing wild type CCR5 (HOS-CD4-CCR5) were used as positive controls.

4.2.3 Effects of mutations on cell-cell fusion

A cell-cell fusion assay was used to assess the ability of CCR5 mutants to mediate fusion of host cells with membranes bearing HIV-1 Env protein. To assess the effect of CCR5 mutations on HIV-1 fusion, three different HIV-1 envelopes together with the viral proteins, Tat and Rev were expressed in HEK 293 cells and incubated with reporter HOS cells expressing the LTR-luciferase and CD4 with wildtype or mutant CCR5. The ability of the HEK cells to fuse with the HOS cells was determined by measuring luciferase activity. The HOS transfected cells used for each fusion assay was analysed using FACS to determine expression of the receptors (Table 4.1). The expression and fusion results in the Table represent the average of three independent experiments expressed as a percentage of wild type while the fusion result on the graph is a single experiment from three independent assays performed. The wild type receptor was able to fuse with the three envelopes. Low fusion was observed for the subtype B envelope, YU2 while the two South African subtype C envelopes, MOLE1 and DU151 showed higher fusion (Figure 4.4). In some fusion assays, slightly higher values were obtained for the Leu107Phe and the Arg225Gln mutant compared to the wild type. A 2-tailed student t-test was done using the GraphPad Prism software to compare fusion of wild type with those of the Leu107Phe and the Arg225Gln mutant (if there is significant difference, $p < 0.05$). Results showed that both Leu107Phe and Arg225Gln showed similar fusion activity (YU2, $p=0.3865$ and 0.3865 , MOLE1, $p=0.6048$ and 0.6665 and DU151, $p=0.9314$ and 0.6665 for the Leu107Phe Arg225Gln mutants respectively) as the wildtype receptor (Figure 4.4). This suggests that the conformational changes needed for HIV-1 fusion was not affected by either the presence of Phenylalanine at position 107 or Gln at position 225 respectively. This result also suggests that constitutive activity of the Arg225Gln mutant did not affect fusion efficiency. There was decreased fusion for the Asp2Val mutant compared to the wildtype and no measurable fusion for the Arg225Stop mutant. Decreased fusion exhibited by the Asp2Val mutant may have been due to decreased expression or low affinity of this mutant receptor for HIV-gp120. The Arg225Stop mutant, since it was not expressed on the cell surface, it was not expected to fuse with any envelope.

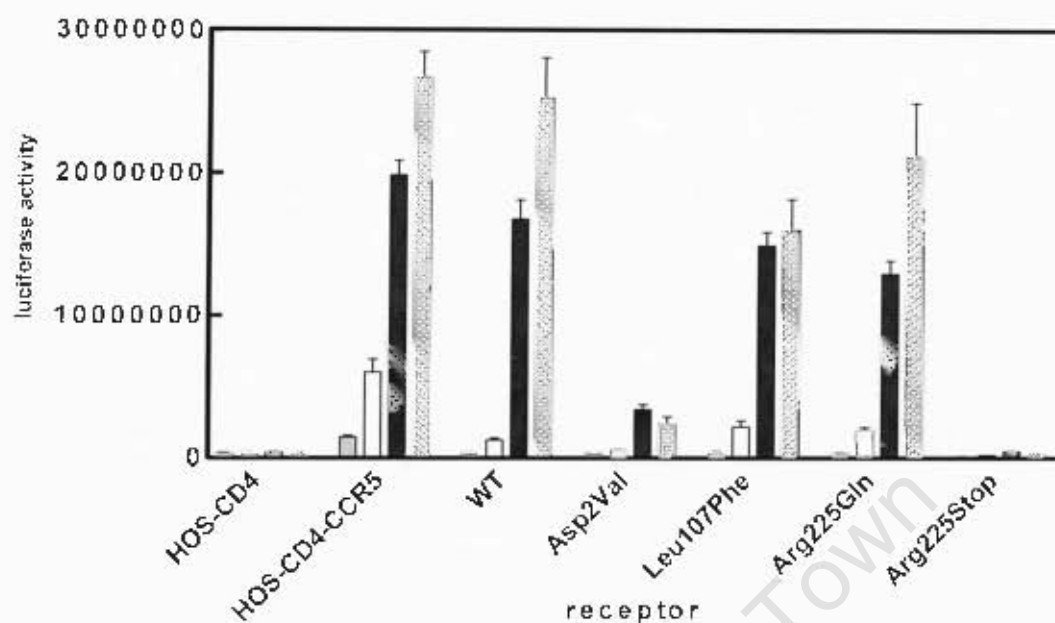


Figure 4.4: HIV-1 fusion efficiencies and coreceptor function of CCR5 mutants. Untransfected HEK 293 cells (grey bars) or HEK 293 cells transiently transfected with rev, tat and either the subtype B HIV-1 envelope, YU2 (open bars) or the subtype C envelopes, Mole1 (solid bars) or DU151 (hashed bars) were mixed with HOS cells expressing CD4, I.TR-luciferase and either wildtype or mutant CCR5. One day after mixing, cells were assayed for luciferase activity using the luciferase assay system (Promega). HOS-CD4 (no receptor) and HOS-CD4-CCR5 (cells stably expressing wild type CCR5) were used as negative and positive controls respectively.

Table 4.1: Expression and fusion efficiencies of wildtype and mutant CCR5 in HOS cells after two days selection with hygromycin B. Values are expressed as percentage of wild type and results are means and standard errors calculated from three independent experiments

Receptor	Expression			Fusion	
	mean fluorescence	% of ce expressing	YU2	Mole1	DU151
Wildtype	100	100	100	100	100
Asp2Val	57.09±3.7	53.67±0.5	56.80±15.3	53.94±32.8	15.79±3.8
Leu107Phe	101.67±5.3	122.25±8.9	143.77±23.3	138.57±25.1	105.38±23.8
Arg225Gln	92.19±11.8	118.17±21.1	148.08±8.3	131.77±47.9	116.35±35.2
Arg225Stop	42.02±2.8	17.53±0.8	32.24±7.6	7.81±2.9	2.83±1.1

4.3 CCR5 –gp120 interaction

To determine the binding affinities of the wild type and the different mutant receptors for gp120, a competition binding assay was performed using purified subtype C gp120 (DU151). HEK 293 cells stably expressing CD4 were transiently transfected with wild type or mutant CCR5 and incubated with a fixed concentration of labelled MIP-1 β (as tracer) and various concentration of purified gp120.

4.3.1 Expression and functionality of purified gp120

The subtype C gp120, DU151 was purified from insect cells (Sf21) infected with baculovirus through a lentil lectin column. The purified gp120 was concentrated with an amicon ultra centrifugal filter device (Millipore). Western blot analysis was used to determine expression and integrity of the purified gp120 and a CD4 binding assay was used to assess the functionality of the protein. Western blot analysis showed that the purified gp120 was expressed and was still intact after the purification process (Figure 4.5). Since gp120 was purified from insect cells, there is less glycosylation which reflects the decrease in the size of the protein obtained (97KD). The intensity of the band was low suggesting that the concentration of the protein was low. This was confirmed by ELISA. CD4 binding assay revealed that the purified gp120 was functional since it could bind CD4 expressed on the surface of HEK 293 cells (Figure 4.6). gp120 from four different purifications were pooled, and re-concentrated before use for binding assays.

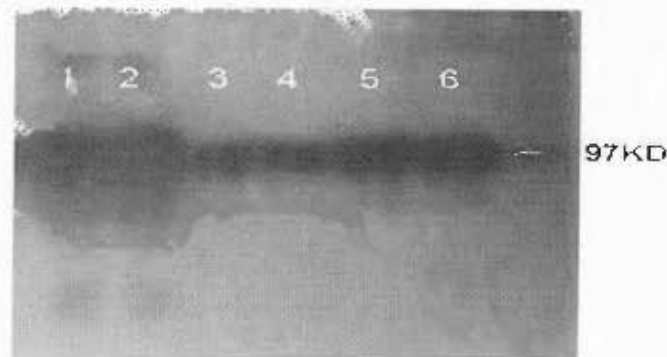


Figure 4.5: Expression of purified gp120. Sf21 cells were infected with DU151 baculovirus for three days. Supernatant was collected and applied on to a lentil lectin column. After washing, gp120 was eluted with α -D methylmannopyranoside and concentrated with an amicon ultra centrifugal filter device. Concentrated protein was separated on an SDS-PAGE gel, transferred to a nitrocellulose membrane and incubated with an HRP-conjugated anti HIV envelope antibody (BiogenesisTM Poole, England). Protein was detected using an ECL+ kit (Amersham, Buckinghamshire, England). Lane 1 and 2, positive control (previously purified functional gp120), Lanes 3, 4, 5 and 6 are purified gp120 (DU151).



Figure 4.6: gp120-CD4 binding. HEK 293 cells stably expressing CD4 were incubated with purified gp120. Cells were solubilised, centrifuged and supernatant used for western blot analysis using an anti HIV envelope antibody. Lane 1 is the protein marker, lanes 2 and 3, CD4 cells with no gp120 (negative control), lanes 4 and 5, HEK 293 cells with gp120 (no CD4) (negative control), lanes 6 and 7, previously purified functional gp120 (positive control), lanes 8 and 9 and lanes 10 and 11 are tested gp120 from two different purifications.

4.3.2 Concentration of purified gp120

Concentration of purified gp120 was determined by ELISA. Both standard gp120 and purified test gp120 were assayed on the same plate and the concentration of the test sample was extrapolated from a standard curve of absorbance versus concentration (figure 4.7) of standard gp120.

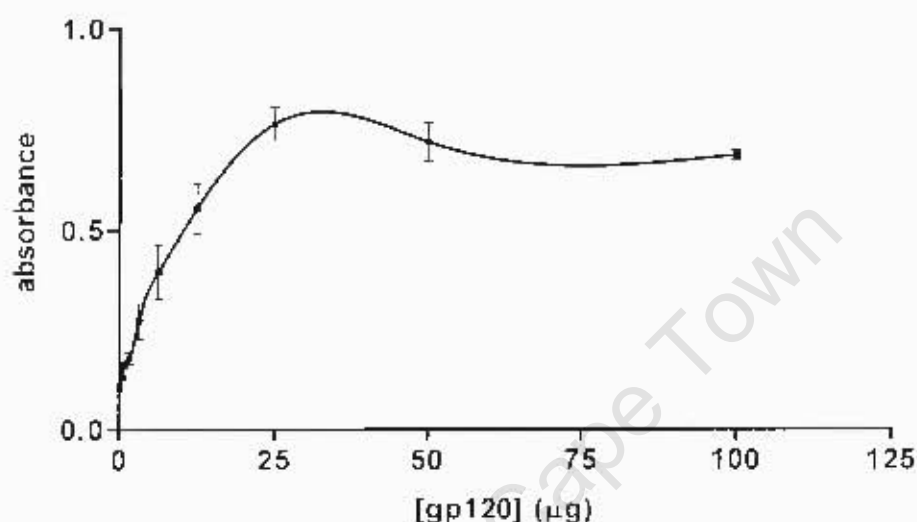


Figure 4.7: Typical spline curve for determination of gp120 concentration. Different concentrations of standard HIV-1 IIIB gp120 and purified samples were captured on an ELISA plate by an HIV-1 IIIB antibody (see materials and methods). After blocking and incubation with an HRP-conjugated anti-gp120 antibody, an HRP substrate was added (blue colour was observed) and the reaction stopped after 15 minutes (reaction appears yellow). Absorbance was read at 450nm. A spline curve of absorbance versus concentration of standard IIIBgp120 was used to determine concentration of sample gp120.

4.3.3 CCR5 binding

MIP-1 β binds CCR5 with high affinity and addition of a competitor such as gp120 will displace MIP-1 β in a concentration dependent manner. gp120 competition binding results showed that addition of increasing concentrations of gp120 leads to 125 I- MIP-1 β displacement from the Leu107Phe and Arg225Gln mutant receptors with IC₅₀s of 0.42 and 0.24 μ M respectively (Figure 4.8). Cell surface expression results (Figure 3.8 and Table 3.2) showed that these two mutants are expressed at similar levels as the wild type receptor and they both fuse with HIV-1 envelopes with the same efficiency as the wild type receptor. However, our gp120 competition binding result showed that the wild type receptor had a higher IC₅₀ (2.57 μ M) than these mutants. These differences in IC₅₀s may

be attributed to the difference in affinities for gp120 with the two mutants showing higher affinities than the wild type receptor. Constitutive activity of Arg225Gln did not seem to play a role in its binding affinity since it showed similar affinity as the Leu107Phe mutant which was not constitutively active. The Asp2Val mutant did not show any binding (Figure 4.8). This may be due to its low surface expression or lack of affinity for gp120. Since the Arg225Stop mutant was not expressed and was non functional, no gp120 binding was done for this mutant. It should be however indicated here that even though we did more than three independent experiments, the results were not reproducible and as such, a representative result of the two most convincing is presented.

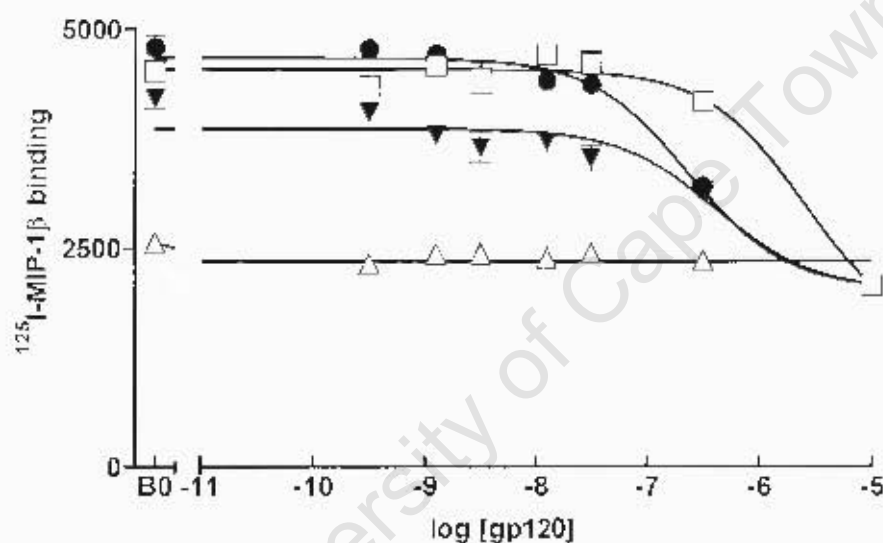


Figure 4.8: gp120 competition binding: HEK-CD4 cells were transiently transfected with wildtype receptor (□), Asp2Val (Δ), Leu107Phe (▼) or Arg225Gln (●) mutant receptors and incubated with labelled chemokine ligand (125 I MIP-1 β as tracer) and various concentrations of gp120.

4.4 Summary

HIV-1 co-receptor activity was analyzed using a fusion assay and a gp120-MIP-1 β competition binding assay. For fusion assay, HOS cells expressing CD4 were successfully transfected stably with LTR-luc. Stable wild type and mutant CCR5 transfection was not successful. Transient transfection of wild type and mutant CCR5 with two days selection with hygromycin B was used to generate acceptor (HOS-CD4-CCR5-Luc) cells for fusion assay. Fusion results showed that the Asp2Val had decreased fusion activity for all three

envelopes tested. Both the Leu107Phe and the Arg225Gln showed comparable fusion with the wild type receptor and no fusion was observed for the Arg225Stop mutant. The subtype C envelopes (DU151 and MOLE 1) showed higher fusion efficiency than the subtype B envelope (YU2).

For gp120 competition binding, gp120 was purified through a lentil lectin column. Western blotting results showed that gp120 was expressed and could bind CXCR4 (Figure 4.5 and 4.6). Concentration of purified gp120 was determined by ELISA. Competition binding assay showed that gp120 could displace labelled MIP-1 β at the Leu107Phe and the Arg225Gln mutant receptors with similar IC₅₀s. The Asp2Val did not show any binding in this assay. Since the Arg225Stop mutant was not expressed and did not bind chemokine ligand, no gp120 binding was done for this mutant.

**CHAPTER FIVE: DISCUSSION AND
CONCLUSION**

5.1 DISCUSSION

CCR5 has in recent years attracted attention since it was discovered that it plays a major role in HIV-1 transmission and pathogenesis. This critical role of CCR5 was demonstrated by the finding that individuals homozygous for the $\Delta 32$ gene variant showed strong resistance to HIV-1 infection and heterozygotes show partial protection and slower progression to AIDS (Smith *et al.*, 1997). It was also shown that the natural ligands of CCR5, some chemokine analogs and monoclonal antibodies directed against CCR5 can inhibit HIV-1 infection (Alkhatib *et al.*, 1996; Choe *et al.*, 1996; Deng *et al.*, 1996). Population genetic studies show that the $\Delta 32$ mutation is common in the Caucasians and absent or rare in black Africans and Japanese population (Broder and Collman, 1997). Studies have also demonstrated less disease associated with inflammation and rheumatoid arthritis for individuals who are heterozygous for the $\Delta 32$ mutation (Zapico *et al.*, 2000). Therefore it is of interest to know the effects of CCR5 mutations on chemokine and HIV function. Blanpain and co-workers identified 16 natural CCR5 variants in various human populations and analysed their functions in terms of expression, chemokine receptor and HIV co-receptor function (Blanpain *et al.*, 2000). Their results revealed varying effects of the different mutations on the function of CCR5, with some mutations having no measurable effects. Other studies on allelic variants of CCR5 that alter function were done by Carrington and collaborators but the sample size and the abundance of variants make it difficult for population studies to be carried out (Carrington *et al.*, 1997). In the absence of large population samples it is therefore important to identify allelic variants of CCR5 and perform *in vitro* studies to characterize them in terms of chemokine receptor and HIV-1 co-receptor function and thus be able to predict their effects *in vivo*.

Presently, subtype C HIV-1 is the most predominant subtype and it is the most common subtype in South Africa (McCutchan, 2000; Williamson *et al.*, 2003). Subtype C is also the most rapidly spreading subtype worldwide (Osmanov *et al.*, 2002). Studies have shown that infection of women with HIV-1C cause a significant reduction in CD4 levels compared with those infected with subtypes A or D, and subtype C can easily establish itself as the major subtype in a region that harbours other subtypes (Renjifo *et al.*, 1998; Neilson *et al.*, 1999). It has been shown that HIV-1C viruses mainly use CCR5 for infection of host

cells and maintain their CCR5 usage throughout infection compared to other subtypes that are able to switch to CXCR4 usage as disease progresses (Tscherning *et al.*, 1998; Morris *et al.*, 2001). Thus, studying the function of CCR5 and the effects of CCR5 variants in a region where HIV-1C is prevalent may contribute to the understanding of the pathogenesis and spread of subtype C HIV-1.

Petersen and collaborators identified seven novel CCR5 mutations in a population of predominantly South African descent (Petersen *et al.*, 2001). Four of these mutations were non-conservative and we therefore predicted that they may have a significant effect on the normal functioning of CCR5. Altered CCR5 function is expected to contribute to host variability and susceptibility to HIV-1 infection and/or progression to AIDS within this population. This thesis was aimed at examining the consequences of the four non-conservative South African mutations of CCR5 on chemokine receptor function, expression and HIV-1 coreceptor function using *in vitro* methods.

Wild type CCR5 and mutant CCR5 were expressed in a range of recombinant systems. Chemokine receptor functions of these CCR5 mutants were determined using both signaling and binding assays. Analysis of IP results showed that the conservative amino acid change from leucine to phenylalanine at the third transmembrane domain (Leu107Phe) did not affect functional response to chemokine. It had EC₅₀s of 13.09±4.9nM and 8.7±0.6nM for MIP-1 α and RANTES respectively which were comparable to those of the wild type receptor (8.8±2.5nM and 9.95±1.69 for MIP-1 α and RANTES respectively) (Figure 3.3 and Table 3.1). This mutant receptor showed similar total binding and affinity (IC₅₀ of 12.38±3.57nM) as the wildtype receptor (15.36±6.87) (Figure 3.7 and Table 3.1). These findings suggest that this residue does not contribute to or is not involved in the conformational changes that lead to activation of the receptor. Thus the aliphatic nature of the hydrophobic side chain of leucine at position 107 does not seem to be important for ligand binding and signaling of the receptor. The role of aromatic residues at the extracellular border of helices 2 and 3 in receptor activation has been demonstrated by Govaerts and collaborators. They showed that aromatic clusters at the TM2-TM3 interface of CCR5 are involved in chemokine-induced activation and interhelical interactions

necessary for conformational changes in the helices that may govern ligand specificity (Govaerts *et al.*, 2003). In this context, it is expected that a change from an aliphatic to an aromatic residue at position 107 in TM3 would enhance or have little or no effect on CCR5 function. Consistent with this, a similar mutation at position 104, also in TM3 (Leu104Phe) showed binding properties similar to the wild type receptor (Govaerts *et al.*, 2003).

The Arg225Gln had EC₅₀s for both MIP-1 α and RANTES as the wild type receptor (Table 3.1 and Figure 3.3). This mutant receptor also showed a significant increase in basal signaling compared to the wild type receptor (signal in the absence of added ligand $p=0.0293$) (Figure 3.4). This slightly higher basal response was suggestive of constitutive activity since expression and maximum binding were both similar to those of the wild type receptor. Activation and signal transduction through a GPCR involves rearrangement and orientation of residues, helices and receptor domains to allow interaction of the receptor with the G protein. This is usually achieved when the receptor binds to its ligand. However, there is evidence to support the fact that activation of intracellular messengers in many members of this family of receptors can occur in the absence of agonist. Though some receptors naturally exhibit agonist independent activation, constitutive activity of many receptors result from mutations of certain residues in different regions of the receptor (Cotecchia *et al.*, 1990; Kjelsberg *et al.*, 1992; Cohen *et al.*, 1993; Ren *et al.*, 1993; Samama *et al.*, 1993; Hogger *et al.*, 1995; Arias *et al.*, 2003). Typically, mutations involving the Aspartic/Glutamic acid of the DRY motif have been shown to confer constitutive activity in many receptors (Cohen *et al.*, 1993; Scheer *et al.*, 1996; Rasmussen *et al.*, 1999). In the α_1B -Adrenergic receptor, systematic substitution of Ala293 in the C terminal part of the receptor to the other 19 amino acids conferred variable levels of constitutive activity (Kjelsberg *et al.*, 1992). Similar mutations induced constitutive activities in the α_{2A} - and β_2 - Adrenergic receptors (Ren *et al.*, 1993; Samama *et al.*, 1993)

Constitutive activity has been demonstrated to have varying effects on ligand binding affinities. In some receptors, it was shown to enhance binding affinity for agonist whereas others cause a decrease in affinity which may be an indication of a conformational shift of the extracellular domains involved in the binding site (Gether, 2000; Arias *et al.*, 2003). In

CCR5, a mutation from threonine in the highly conserved TXP motif (Thr-82) to proline in TM2 conferred constitutive activity to the receptor with varying affinities for the different CCR5 ligands. Mutation of this threonine residue to lysine also showed a strong constitutive activity but was impaired in its responsiveness and showed decreased affinities for the natural ligands of CCR5 (Govaerts *et al.*, 2001; Arias *et al.*, 2003). In the case of the Arg225Gln mutant in this study, the increased basal activity did not affect its binding affinity for MIP-1 β (figure 3.7).

In terms of expression, the presence of the bulkier aromatic Phenylalanine did not have any effect on folding and trafficking of the receptor to the surface of the cell. This mutant showed similar expression as the wild type receptor (Figure 3.8 and Table 3.2). Thus if the Leucine at this position is involved in any interaction that affects receptor folding and trafficking, it is likely to be hydrophobic interactions regardless of the size of the hydrophobic group. The mutation (Arg225Gln) at the third intracellular loop also showed a similar expression pattern as the wild type receptor (Figure 3.8). A similar expression result was obtained for an arginine to glutamine mutation at position 223 (Arg223Gln) in the same region of the receptor. This suggests that the positive charges of the arginines at these positions may not be important for receptor folding and trafficking to the cell surface.

Both the Leu107Phe and the Arg225Gln showed similar fusion results as the wild type receptor (Figure 4.4). This suggests that the amino acid changes at these positions did not alter the conformation of the receptor necessary for interaction with the HIV-1 envelope protein. Therefore, the fusion efficiency of the CCR5 receptor was not affected by either the presence of a large hydrophobic side chain at position 107 at the third transmembrane domain or a glutamine at position 225 at the third intracellular loop.

The mutation involving a change from a charged aspartic acid residue at the extreme N-terminus (position 2) to a hydrophobic valine residue (Asp2Val) caused decrease in functional response to chemokine (Figure 3.3). This mutant receptor showed a decrease in both E_{max} s (7506 and 5499) and EC_{50} s (265 ± 178 nM and 261 ± 170.3) for MIP-1 α and RANTES respectively compared to the wildtype receptor (E_{max} , 14,052 and 13,991 and

In agreement with its decreased ability to bind and functionally respond to chemokines, the Asp2Val mutant also lost some of its co-receptor activity as shown in fusion assay. In cell-cell fusion assay, this mutant showed a reduced ability to mediate fusion (figure 4.4). Studies on the amino terminus of CCR5 and tyrosine-sulfated peptides based on CCR5 amino terminus have shown that the polar aspartic acid residue at this position (Asp2) of CCR5 is adjacent to one of the tyrosine residues (Tyr3) which is most efficiently sulfated. However, this sulfation motif is not required for efficient HIV-1 entry into target cells (Farzan *et al.*, 1999; Farzan *et al.*, 2000). Alanine substitution of this aspartic acid residue either alone or in combination with alanine substitution of Asp11 and/or Glu18 has been shown to reduce or completely block entry of AIDA, JRFL, and DHH23 HIV-1 strains (Dragic *et al.*,

The 122V mutant also showed a reduction in total binding which was shown to be as a result of a decrease in its affinity for agonist. The expression levels of the receptor on the cell surface were also low. This expression result contrasts those of Blampain and collaborators and Dragic and collaborators who previously showed that an alanine substitution of this aspartic acid residue or amino terminal truncations that involved this residue did not affect surface expression of the CCR5 (Dragic *et al.*, 1998; Blampain *et al.*, 1999a). This contrasting result suggests that the substitution of the negative charge of the aspartic acid with a larger hydrophobic side group might have caused a disruption of a conformation that is well suited for the efficient trafficking of the receptor to the cell surface.

The 122A mutant also showed a reduction in total binding which was shown to be as a result of a decrease in its affinity for agonist. The expression levels of the receptor on the cell surface were also low. This expression result contrasts those of Blampain and collaborators and Dragic and collaborators who previously showed that an alanine substitution of this aspartic acid residue or amino terminal truncations that involved this residue did not affect surface expression of the CCR5 (Dragic *et al.*, 1998; Blampain *et al.*, 1999a). This contrasting result suggests that the substitution of the negative charge of the aspartic acid with a larger hydrophobic side group might have caused a disruption of a conformation that is well suited for the efficient trafficking of the receptor to the cell surface.

LC50, 8.81±2.5 and 9.95±1.69 for MIP-1 α and KANTLES respectively) (Figure 3.3 and Table 3.1) This decrease in functional response was a result of a 3-4 fold decreased affinity of this mutant receptor for its chemokine ligand (figure 3.7). Previous studies involving this aspartic acid residue at position 2 showed that chemokine receptor function was impaired when this residue was deleted (A2) or substituted with a small hydrophobic alanine residue (122A) (Blampain *et al.*, 1999a). This negatively charged residue together with other charged and aromatic residues in the N-terminus of CCR5 contribute to high affinity binding of chemokines. Valine which has a larger hydrophobic side chain than alanine at this position of the receptor also showed a decrease in binding affinity and functional response to chemokines thereby confirming the importance of its negative charge in the normal functioning of CCR5.

1998). This is consistent with our fusion result for a valine substitution at this position which reduced the fusion ability of the subtype B (YU2) and subtype C (MOL.1 and DU.151) envelopes. Thus the negative charge of Asp2 is important both for chemokine receptor and HIV-1 coreceptor function.

The mutation at the third intracellular loop at position 225 from arginine to a stop codon leads to truncation of the receptor leaving just five transmembrane domains. We could not demonstrate any expression of this receptor on the cell surface. Even though the wild type and the other three mutants showed increased expression on intracellular membranes, FACS analyses carried out on permeabilized cells transfected with Arg225Stop mutant showed that there was no measurable intracellular expression of this mutant receptor. The fluorescent antibody used to detect expression of CCR5 (2D7) in this study is a conformational antibody which binds CCR5 only when it is folded in a particular conformation. There is therefore a possibility that this receptor (Arg225Stop) may be present on the cell surface or on intracellular membranes but not folded in the conformation that is recognised by 2D7. In terms of function, this mutant did not bind chemokine and did not show any signalling response to chemokine. These results are comparable with the results obtained for the CCR5 Δ 32 mutant which has just four transmembrane domains. This mutant which has been associated with strong resistance to HIV has of recent emerged to be a host genetic risk factor for fatal West Nile Virus (WNV) infection in humans (Diamond and Klein, 2006; Glass *et al.*, 2006; Lim *et al.*, 2006). Thus lack of CCR5 expression, while protecting against HIV-1 infection may pose a high risk in individuals in WNV endemic areas.

Binding of soluble HIV-1 envelope glycoprotein to CCR5 receptors are generally thought to correlate with the fusion potential of the viruses. It is however important to note that binding experiments are usually carried out using monomeric gp120s which may not represent the actual role of the native trimeric Env as well as the potential contribution of the gp41 ectodomain (Choe *et al.*, 1998b). We therefore compared the fusion efficiencies (using full envelope gp160) of wild type and mutant receptors with binding affinities for gp120. Our results showed that the affinity for the wild type receptor was lower than those of the Leu107Phe and Arg225Gln mutants which showed similar fusion as the wildtype receptor.

These two mutants were expressed at similar levels as the wildtype receptor thus expression was not a determining factor of the differences in affinity. No binding was observed for the Asp2Val mutant probably due to low expression or low concentration of the gp120. Even though we experienced similar fusion efficiencies for the wild type and the Leu107Phe and Arg225Gln mutants, we also experienced a trend in all mutants (except the Arg225Stop mutant) that indicated that the subtype B envelope fused less efficiently than the subtype C envelopes (Figure 4.4). These results support the fact that subtype C viruses have higher affinities for CCR5 than subtype B viruses subtypes (Renjifo *et al.*, 1998; Neilson *et al.*, 1999).

5.2 Conclusion

Four natural mutations of the CCR5 receptor were analysed in this study. Two of the mutations (Leu107Phe and Arg225Gln) produced receptors that were expressed at similar levels as the wild type receptor. Functional responses to chemokine ligands and binding affinities for these two mutant receptors were comparable to that of the wildtype. They also showed comparable HIV-1 fusion efficiency as the wild type receptor (Figure 4.4) but their affinities for gp120 were higher than that of the wild type (Figure 4.8). It is noteworthy that binding of soluble HIV-1 envelope glycoprotein to CCR5 receptors are generally thought to be comparable with the fusion potential of the viruses. It is however important to note that binding experiments are usually performed using monomeric gp120 which may not reflect the actual role of the native trimeric Env as well as the potential contributory role of the gp41 ectodomain (Choe *et al.*, 1998b).

One mutation (Asp2Val) produced a receptor that showed low expression, reduction in chemokine induced signalling, decreased chemokine binding affinity and low fusion efficiency for both the subtype B and subtype C envelopes. The last mutation (Arg225Stop) which truncates the receptor to just five transmembrane domains was not expressed on the cell surface, did not bind nor respond functionally to chemokine ligands. No fusion activity was observed for this mutant receptor.

Together, these results suggest different effects of mutations on the functioning of CCR5 both as a chemokine receptor and as an HIV-1 co-receptor. Even though some mutations did not have any effect on function, it may be possible that some slight modifications of the properties of the receptor may have occurred that could not be detected by the assays performed. It is also of interest to note that experiments performed *in vitro* may give an indication of the functioning of these receptors but may not necessarily reflect the actual effects in individuals carrying these mutations. However, our *in vitro* studies suggests that individuals who are homozygous for the Asp2Val mutation might show reduced ability for their CCR5 to bind chemokine which also leads to decrease in signalling function. They will also show decreased ability of their CCR5 to bind gp120 and decreased ability of their CCR5 to mediate HIV fusion. Individuals homozygous for the Arg225Stop mutant, as with the CCR5 Δ 32 mutation will be resistant to HIV infection. Their CCR5 will be non-functional.

It will be of interest to do a wider population analysis of these mutations that showed alteration of CCR5 functions to determine the effect of homozygosity and heterozygosity of these mutations on HIV-1 infection and progression to AIDS.

REFERENCES

- Aasa-Chapman, M. M., Aubin, K., Williams, J. and McKnight, A. (2006). "Primary CCR5 only using HIV-1 isolates does not accurately represent the in vivo replicating quasi-species." *Virology* **351**(2): 489-496.
- Ahuja, S. K., Lee, J. C. and Murphy, P. M. (1996). "CXC chemokines bind to unique sets of selectivity determinants that can function independently and are broadly distributed on multiple domains of human interleukin-8 receptor B. Determinants of high affinity binding and receptor activation are distinct." *J Biol Chem* **271**(1): 225-232.
- Alkhatib, G., Berger, E. A., Murphy, P. M. and Pease, J. E. (1997). "Determinants of HIV-1 coreceptor function on CC chemokine receptor 3. Importance of both extracellular and transmembrane/cytoplasmic regions." *J Biol Chem* **272**(33): 20420-20426.
- Alkhatib, G., Combadiere, C., Broder, C. C., Feng, Y., Kennedy, P. E., Murphy, P. M. and Berger, E. A. (1996). "CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1." *Science* **272**(5270): 1955-1958.
- Amara, A., Gall, S. L., Schwartz, O., Salamero, J., Montes, M., Loetscher, P., Baggolini, M., Virelizier, J. L. and Arenzana-Seisdedos, F. (1997). "HIV coreceptor downregulation as antiviral principle: SDF-1alpha-dependent internalization of the chemokine receptor CXCR4 contributes to inhibition of HIV replication." *J Exp Med* **186**(1): 139-146.
- Anderson, J. P., Rodrigo, A. G., Learn, G. H., Madan, A., Delahunty, C., Coon, M., Girard, M., Osmanov, S., Hood, L. and Mullins, J. I. (2000). "Testing the hypothesis of a recombinant origin of human immunodeficiency virus type 1 subtype E." *J Virol* **74**(22): 10752-10765.
- Ansari-Lari, M. A., Liu, X. M., Metzker, M. L., Rut, A. R. and Gibbs, R. A. (1997). "The extent of genetic variation in the CCR5 gene." *Nat Genet* **16**(3): 221-222.
- Arenzana-Seisdedos, F. and Parmentier, M. (2006). "Genetics of resistance to HIV infection: Role of co-receptors and co-receptor ligands." *Semin Immunol* **18**(6): 387-403.
- Arenzana-Seisdedos, F., Virelizier, J. L., Rousset, D., Clark-Lewis, I., Loetscher, P., Moser, B. and Baggolini, M. (1996). "HIV block by chemokine antagonist." *Nature* **383**(6599): 400.
- Arias, D. A., Navenot, J. M., Zhang, W. B., Broach, J. and Peiper, S. C. (2003). "Constitutive activation of CCR5 and CCR2 induced by conformational changes in the conserved TXP motif in transmembrane helix 2." *J Biol Chem* **278**(38): 36513-36521.

- Baggiolini, M., Dewald, B. and Moser, B. (1997). "Human chemokines: an update." *Annu Rev Immunol* **15**: 675-705.
- Baldwin, J. M. (1993). "The probable arrangement of the helices in G protein-coupled receptors." *Embo J* **12**(4): 1693-1703.
- Ballesteros, J. A., Jensen, A. D., Liapakis, G., Rasmussen, S. G., Shi, L., Gelber, U. and Javitch, J. A. (2001). "Activation of the beta 2-adrenergic receptor involves disruption of an ionic lock between the cytoplasmic ends of transmembrane segments 3 and 6." *J Biol Chem* **276**(31): 29171-29177.
- Ballesteros, J. A. and Weinstein, H (1995). Integrated methods for the construction of three dimensional models and computational probing of structure-function relations in G-protein coupled receptors. *Methods in Neuroscience, Receptor Molecular Biology*. S. C. Sealton. San Diego, Academic Press. **25**: 366-428.
- Bannert, N., Craig, S., Farzan, M., Sogah, D., Santo, N. V., Choe, H. and Sodroski, J. (2001). "Sialylated O-glycans and sulfated tyrosines in the N112-terminal domain of CC chemokine receptor 5 contribute to high affinity binding of chemokines." *J Exp Med* **194**(11): 1661-1673.
- Beckner, S. K. (1997). "G-protein activation by chemokines." *Methods Enzymol* **288**: 309-326.
- Berger, E. A., Murphy, P. M. and Farber, J. M. (1999). "Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease." *Annu Rev Immunol* **17**: 657-700.
- Bieniasz, P. D., Fridell, R. A., Aramori, L., Ferguson, S. S., Caron, M. G. and Cullen, B. R. (1997). "HIV-1-induced cell fusion is mediated by multiple regions within both the viral envelope and the CCR-5 co-receptor." *Embo J* **16**(10): 2599-2609.
- Blanpain, C., Doranz, B. J., Bonduc, A., Govaerts, C., De Leener, A., Vassart, G., Doms, R. W., Proudfoot, A. and Parmentier, M. (2003). "The core domain of chemokines binds CCR5 extracellular domains while their amino terminus interacts with the transmembrane helix bundle." *J Biol Chem* **278**(7): 5179-5187.
- Blanpain, C., Doranz, B. J., Vakili, J., Rucker, J., Govaerts, C., Baik, S. S., Lorthioir, O., Migeotte, I., Libert, F., Baleux, F., Vassart, G., Doms, R. W. and Parmentier, M. (1999a). "Multiple charged and aromatic residues in CCR5 amino-terminal domain are involved in high affinity binding of both chemokines and HIV-1 Env protein." *J Biol Chem* **274**(49): 34719-34727.

- Blanpain, C., Lee, B., Tackoen, M., Puffer, B., Boom, A., Libert, F., Sharron, M., Wittamer, V., Vassart, G., Doms, R. W. and Parmentier, M. (2000). "Multiple nonfunctional alleles of CCR5 are frequent in various human populations." *Blood* **96**(5): 1638-1645.
- Blanpain, C., Lee, B., Vakili, J., Doranz, B. J., Govaerts, C., Migeotte, J., Sharron, M., Dupriez, V., Vassart, G., Doms, R. W. and Parmentier, M. (1999b). "Extracellular cysteines of CCR5 are required for chemokine binding, but dispensable for HIV-1 coreceptor activity." *J Biol Chem* **274**(27): 18902-18908.
- Blanpain, C., Wittamer, V., Vanderwinden, J. M., Boom, A., Remmeboog, B., Lee, B., Le Poul, E., El Asmar, L., Govaerts, C., Vassart, G., Doms, R. W. and Parmentier, M. (2001). "Palmitoylation of CCR5 is critical for receptor trafficking and efficient activation of intracellular signaling pathways." *J Biol Chem* **276**(26): 23795-23804.
- Bleul, C. C., Farzan, M., Choe, H., Parolin, C., Clark-Lewis, I., Sodroski, J. and Springer, T. A. (1996). "The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry." *Nature* **382**(6594): 829-833.
- Bondue, A., Jao, S. C., Blanpain, C., Parmentier, M. and LiWang, P. J. (2002). "Characterization of the role of the N-loop of MIP-1 beta in CCR5 binding." *Biochemistry* **41**(46): 13548-13555.
- Brandt, S. M., Mariani, R., Holland, A. U., Hope, T. J. and Landau, N. R. (2002). "Association of chemokine-mediated block to HIV with co-receptor internalization." *J Biol Chem* **277**(19): 17291-17299.
- Broder, C. C. and Berger, E. A. (1995). "Fusogenic selectivity of the envelope glycoprotein is a major determinant of human immunodeficiency virus type 1 tropism for CD4+ T-cell lines vs. primary macrophages." *Proc Natl Acad Sci U S A* **92**(19): 9004-9008.
- Broder, C. C. and Collman, R. G. (1997). "Chemokine receptors and HIV." *J Leukoc Biol* **62**(1): 20-29.
- Burgers, W. A. and Williamson, C. (2005). "The challenges of HIV vaccine development and testing." *Best Pract Res Clin Obstet Gynaecol* **19**(2): 277-291.
- Carrington, M., Kissner, T., Gerrard, B., Ivanov, S., O'Brien, S. J. and Dean, M. (1997). "Novel alleles of the chemokine-receptor gene CCR5." *Am J Hum Genet* **61**(6): 1261-1267.
- Carrington, M., Dean, M., Martin, M. P. and O'Brien, S. J. (1999). "Genetics of HIV-1 infection: chemokine receptor CCR5 polymorphism and its consequences." *Hum Mol Genet* **8**(10): 1939-1945.

- Chackerian, B., Long, E. M., Luciw, P. A. and Overbaugh, J. (1997). "Human immunodeficiency virus type 1 coreceptors participate in postentry stages in the virus replication cycle and function in simian immunodeficiency virus infection." *J Virol* **71**(5): 3932-3939.
- Choe, H., Farzan, M., Konkel, M., Martin, K., Sun, Y., Marcon, L., Cayabyab, M., Berman, M., Dorfl, M. E., Gerard, N., Gerard, C. and Sodroski, J. (1998a). "The orphan seven-transmembrane receptor apj supports the entry of primary T-cell-line-tropic and dualtropic human immunodeficiency virus type 1." *J Virol* **72**(7): 6113-6118.
- Choe, H., Farzan, M., Sun, Y., Sullivan, N., Rollins, B., Ponath, P. D., Wu, L., Mackay, C. R., LaRosa, G., Newman, W., Gerard, N., Gerard, C. and Sodroski, J. (1996). "The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates." *Cell* **85**(7): 1135-1148.
- Choe, H., Martin, K. A., Farzan, M., Sodroski, J., Gerard, N. P. and Gerard, C. (1998b). "Structural interactions between chemokine receptors, gp120 Env and CD4." *Semin Immunol* **10**(3): 249-257.
- Cohen, G. B., Yang, T., Robinson, P. R. and Oprian, D. D. (1993). "Constitutive activation of opsin: influence of charge at position 134 and size at position 296." *Biochemistry* **32**(23): 6111-6115.
- Combadiere, C., Salzwedel, K., Smith, F. D., Tiffany, H. L., Berger, E. A. and Murphy, P. M. (1998). "Identification of CX3CR1, A chemotactic receptor for the human CX3C chemokine fractalkine and a fusion coreceptor for HIV-1." *J Biol Chem* **273**(37): 23799-23804.
- Cormier, E. G. and Dragic, T. (2002). "The crown and stem of the V3 loop play distinct roles in human immunodeficiency virus type 1 envelope glycoprotein interactions with the CCR5 coreceptor." *J Virol* **76**(17): 8953-8957.
- Cotecchia, S., Exum, S., Caron, M. G. and Lefkowitz, R. J. (1990). "Regions of the alpha 1-adrenergic receptor involved in coupling to phosphatidylinositol hydrolysis and enhanced sensitivity of biological function." *Proc Natl Acad Sci U S A* **87**(8): 2896-2900.
- Davis, C. B., Dikic, I., Unutmaz, D., Hill, C. M., Arthos, J., Siani, M. A., Thompson, D. A., Schlessinger, J. and Littman, D. R. (1997). "Signal transduction due to HIV-1 envelope interactions with chemokine receptors CXCR4 or CCR5." *J Exp Med* **186**(10): 1793-1798.
- De Clercq, E. (2002). "New developments in anti-HIV chemotherapy." *Biochim Biophys Acta* **1587**(2-3): 258-275.

- De Lean, A., Stadel, J. M. and Lefkowitz, R. J. (1980). "A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled beta-adrenergic receptor." *J Biol Chem* **255**(15): 7108-7117.
- Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., Di Marzio, P., Marmon, S., Sutton, R. E., Hill, C. M., Davis, C. B., Peiper, S. C., Schall, T. J., Littman, D. R. and Landau, N. R. (1996). "Identification of a major co-receptor for primary isolates of HIV-1." *Nature* **381**(6584): 661-666.
- Diamond, M. S. and Klein, R. S. (2006). "A genetic basis for human susceptibility to West Nile virus." *Trends Microbiol* **14**(7): 287-289.
- Dixon, R. A., Sigal, J. S., Candelore, M. R., Register, R. B., Scattergood, W., Rands, E. and Strader, C. D. (1987). "Structural features required for ligand binding to the beta-adrenergic receptor." *Embo J* **6**(11): 3269-3275.
- Dong, H. F., Wignmore, K., Carrington, M. N., Dean, M., Turpin, J. A. and Howard, O. M. (2005). "Variants of CCR5, which are permissive for HIV-1 infection, show distinct functional responses to CCL3, CCL4 and CCL5." *Genes Immun* **6**(7): 609-619.
- Doranz, B. J., Lu, Z. H., Rucker, J., Zhang, T. Y., Sharron, M., Cen, Y. H., Wang, Z. X., Guo, H. H., Du, J. G., Accavitti, M. A., Doms, R. W. and Peiper, S. C. (1997). "Two distinct CCR5 domains can mediate coreceptor usage by human immunodeficiency virus type 1." *J Virol* **71**(9): 6305-6314.
- Doranz, B. J., Rucker, J., Yi, Y., Smyth, R. J., Samson, M., Peiper, S. C., Parmentier, M., Collman, R. G. and Doms, R. W. (1996). "A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors." *Cell* **85**(7): 1149-1158.
- Dragic, T., Litwin, V., Allaway, G. P., Martin, S. R., Huang, Y., Nagashima, K. A., Cayanan, C., Maddon, P. J., Koup, R. A., Moore, J. P. and Paxton, W. A. (1996). "HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5." *Nature* **381**(6584): 667-673.
- Dragic, T., Trkola, A., Lin, S. W., Nagashima, K. A., Kajumo, F., Zhao, L., Olson, W. C., Wu, L., Mackay, C. R., Allaway, G. P., Sakmar, T. P., Moore, J. P. and Maddon, P. J. (1998). "Amino-terminal substitutions in the CCR5 coreceptor impair gp120 binding and human immunodeficiency virus type 1 entry." *J Virol* **72**(1): 279-285.
- Dragic, T., Trkola, A., Thompson, D. A., Cormier, E. G., Kajumo, F. A., Maxwell, E., Lin, S. W., Ying, W., Smith, S. O., Sakmar, T. P. and Moore, J. P. (2000). "A binding pocket for a

- small molecule inhibitor of HIV-1 entry within the transmembrane helices of CCR5." Proc Natl Acad Sci U S A **97**(10): 5639-5644.
- Edinger, A. L., Amodee, A., Miller, K., Doranz, B. J., Endres, M., Sharron, M., Samson, M., Lu, Z. H., Clements, J. E., Murphey-Corb, M., Peiper, S. C., Parmentier, M., Broder, C. C. and Doms, R. W. (1997). "Differential utilization of CCR5 by macrophage and T cell tropic simian immunodeficiency virus strains." Proc Natl Acad Sci U S A **94**(8): 4005-4010.
- Edinger, A. L., Hoffman, T. L., Sharron, M., Lee, B., Yi, Y., Choe, W., Kolson, D. L., Mitrovic, B., Zhou, Y., Faulds, D., Collman, R. G., Hesselgesser, J., Horuk, R. and Doms, R. W. (1998). "An orphan seven-transmembrane domain receptor expressed widely in the brain functions as a coreceptor for human immunodeficiency virus type 1 and simian immunodeficiency virus." J Virol **72**(10): 7934-7940.
- Este, J. A. and Telenti, A. (2007). "HIV entry inhibitors." Lancet **370**(9581): 81-88.
- Fanelli, F. and De Benedetti, P. G. (2005). "Computational modeling approaches to structure-function analysis of G protein-coupled receptors." Chem Rev **105**(9): 3297-3351.
- Fano, A., Ritchie, D. W. and Carrieri, A. (2006). "Modeling the structural basis of human CCR5 chemokine receptor function: from homology model building and molecular dynamics validation to agonist and antagonist docking." J Chem Inf Model **46**(3): 1223-1235.
- Farber, J. M. and Berger, E. A. (2002). "HIV's response to a CCR5 inhibitor: I'd rather tighten than switch!" Proc Natl Acad Sci U S A **99**(4): 1749-1751.
- Farzan, M., Choe, H., Martin, K., Marcon, L., Hofmann, W., Karlsson, G., Sun, Y., Barrett, P., Marchand, N., Sullivan, N., Gerard, N., Gerard, C. and Sodroski, J. (1997a). "Two orphan seven-transmembrane segment receptors which are expressed in CD4-positive cells support simian immunodeficiency virus infection." J Exp Med **186**(3): 405-411.
- Farzan, M., Choe, H., Martin, K. A., Sun, Y., Sidelko, M., Mackay, C. R., Gerard, N. P., Sodroski, J. and Gerard, C. (1997b). "HIV-1 entry and macrophage inflammatory protein-1beta-mediated signaling are independent functions of the chemokine receptor CCR5." J Biol Chem **272**(11): 6854-6857.
- Farzan, M., Choe, H., Vaca, L., Martin, K., Sun, Y., Desjardins, E., Ruffing, N., Wu, L., Wyatt, R., Gerard, N., Gerard, C. and Sodroski, J. (1998). "A tyrosine-rich region in the N terminus of CCR5 is important for human immunodeficiency virus type 1 entry and mediates an association between gp120 and CCR5." J Virol **72**(2): 1160-1164.

- Farzan, M., Chung, S., Li, W., Vasilieva, N., Wright, P. L., Schnitzler, C. E., Marchione, R. J., Gerard, C., Gerard, N. P., Sodroski, J. and Choe, H. (2002). "Tyrosine-sulfated peptides functionally reconstitute a CCR5 variant lacking a critical amino-terminal region." *J Biol Chem* **277**(43): 40397-40402.
- Farzan, M., Mirzabekov, T., Kolehinsky, P., Wyatt, R., Cayabyab, M., Gerard, N. P., Gerard, C., Sodroski, J. and Choe, H. (1999). "Tyrosine sulfation of the amino terminus of CCR5 facilitates HIV-1 entry." *Cell* **96**(5): 667-676.
- Farzan, M., Schnitzler, C. E., Vasilieva, N., Leung, D., Kuhn, J., Gerard, C., Gerard, N. P. and Choe, H. (2001). "Sulfated tyrosines contribute to the formation of the C5a docking site of the human C5a anaphylatoxin receptor." *J Exp Med* **193**(9): 1059-1066.
- Farzan, M., Vasilieva, N., Schnitzler, C. E., Chung, S., Robinson, J., Gerard, N. P., Gerard, C., Choe, H. and Sodroski, J. (2000). "A tyrosine-sulfated peptide based on the N terminus of CCR5 interacts with a CD4-enhanced epitope of the HIV-1 gp120 envelope glycoprotein and inhibits HIV-1 entry." *J Biol Chem* **275**(43): 33516-33521.
- Feng, Y., Broder, C. C., Kennedy, P. E. and Berger, E. A. (1996). "HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor." *Science* **272**(5263): 872-877.
- Gether, U. (2000). "Uncovering molecular mechanisms involved in activation of G protein-coupled receptors." *Endocr Rev* **21**(1): 90-113.
- Gether, U. and Kobilka, B. K. (1998). "G protein-coupled receptors. II. Mechanism of agonist activation." *J Biol Chem* **273**(29): 17979-17982.
- Glass, W. G., McDermott, D. H., Lim, J. K., Lekhong, S., Yu, S. F., Frank, W. A., Pape, J., Cheshier, R. C. and Murphy, P. M. (2006). "CCR5 deficiency increases risk of symptomatic West Nile virus infection." *J Exp Med* **203**(1): 35-40.
- Gonzalez, E., Kulkarni, H., Bolivar, H., Mangano, A., Sanchez, R., Catano, G., Nibbs, R. J., Freedman, B. L., Quinones, M. P., Bamshad, M. J., Murthy, K., Rovin, B. H., Bradley, W., Clark, R. A., Anderson, S. A., O'Connell, R. J., Agan, B. K., Ahuja, S. S., Bologna, R., Sen, L., Dolan, M. J. and Ahuja, S. S. (2005). "The influence of CCR5 gene-containing segmental duplications on HIV-1/AIDS susceptibility." *Science* **307**: 1434-1440.
- Gosling, J., Montecarlo, F. S., Atchison, R. E., Arai, H., Tsou, C. L., Goldsmith, M. A. and Charo, I. F. (1997). "Molecular uncoupling of C-C chemokine receptor 5-induced chemotaxis

- and signal transduction from HIV-1 coreceptor activity." *Proc Natl Acad Sci U S A* **94**(10): 5061-5066.
- Govaerts, C., Blanpain, C., Deupi, X., Ballet, S., Ballesteros, J. A., Wodak, S. J., Vassart, G., Pardo, L. and Parmentier, M. (2001). "The TXP motif in the second transmembrane helix of CCR5. A structural determinant of chemokine-induced activation." *J Biol Chem* **276**(16): 13217-13225.
- Govaerts, C., Bondue, A., Springael, J. Y., Olivella, M., Deupi, X., Le Poul, E., Wodak, S. J., Parmentier, M., Pardo, L. and Blanpain, C. (2003). "Activation of CCR5 by chemokines involves an aromatic cluster between transmembrane helices 2 and 3." *J Biol Chem* **278**(3): 1892-1903.
- Guignard, F., Combadiere, C., Tiffany, H. L. and Murphy, P. M. (1998). "Gene organization and promoter function for CC chemokine receptor 5 (CCR5)." *J Immunol* **160**(2): 985-992.
- Guo, Q., Ho, H. T., Dicker, L., Fan, L., Zhou, N., Friberg, J., Wang, T., McAuliffe, B. V., Wang, H. G., Rose, R. E., Fang, H., Searnati, H. T., Langley, D. R., Meanwell, N. A., Abraham, R., Colonna, R. J. and Lin, P. F. (2003). "Biochemical and genetic characterizations of a novel human immunodeficiency virus type 1 inhibitor that blocks gp120-CD4 interactions." *J Virol* **77**(19): 10528-10536.
- Hayes, V. M., Petersen, D. C., Scriba, T. J., Zeier, M., Grimwood, A. and Janse Van Rensburg, E. (2002). "African-based CCR5 single-nucleotide polymorphism associated with HIV-1 disease progression." *Aids* **16**(16): 2229-2231.
- Heyndrickx, L., Janssens, W., Zekeng, L., Musonda, R., Anagonou, S., Van der Auwera, G., Coppens, S., Vereecken, K., De Witte, K., Van Rempelbergh, R., Kahindo, M., Morison, L., McCutchan, F. E., Carr, J. K., Albert, J., Essex, M., Goudsmit, J., Asjo, B., Salminen, M., Buve, A. and van Der Groen, G. (2000). "Simplified strategy for detection of recombinant human immunodeficiency virus type 1 group M isolates by gag/env heteroduplex mobility assay. Study Group on Heterogeneity of HIV Epidemics in African Cities." *J Virol* **74**(1): 363-370.
- Hogger, P., Shockley, M. S., Lameh, J. and Sadec, W. (1995). "Activating and inactivating mutations in N- and C-terminal i3 loop junctions of muscarinic acetylcholine 1m1 receptors." *J Biol Chem* **270**(13): 7405-7410.
- Horuk, R., Hesselgesser, J., Zhou, Y., Faulds, D., Halks-Miller, M., Harvey, S., Taub, D., Samson, M., Parmentier, M., Rucker, J., Doranz, B. J. and Doms, R. W. (1998). "The CC

- chemokine I-309 inhibits CCR8-dependent infection by diverse HIV-1 strains." *J Biol Chem* **273**(1): 386-391.
- Howard, O. M., Shirakawa, A. K., Turpin, J. A., Maynard, A., Tobin, G. J., Carrington, M., Oppenheim, J. J. and Dean, M. (1999). "Naturally occurring CCR5 extracellular and transmembrane domain variants affect HIV-1 Co-receptor and ligand binding function." *J Biol Chem* **274**(23): 16228-16234.
- Huttenrauch, F., Nitzki, A., Lin, F. T., Honing, S. and Oppermann, M. (2002). "Beta-arrestin binding to CC chemokine receptor 5 requires multiple C-terminal receptor phosphorylation sites and involves a conserved Asp-Arg-Tyr sequence motif." *J Biol Chem* **277**(34): 30769-30777.
- Javitch, J. A., Ballesteros, J. A., Weinstein, H. and Chen, J. (1998). "A cluster of aromatic residues in the sixth membrane-spanning segment of the dopamine D2 receptor is accessible in the binding-site crevice." *Biochemistry* **37**(4): 998-1006.
- Javitch, J. A., Fu, D., Liapakis, G. and Chen, J. (1997). "Constitutive activation of the beta2 adrenergic receptor alters the orientation of its sixth membrane-spanning segment." *J Biol Chem* **272**(30): 18546-18549.
- Ji, T. H., Grossmann, M. and Ji, J. (1998). "G protein-coupled receptors. I. Diversity of receptor-ligand interactions." *J Biol Chem* **273**(28): 17299-17302.
- Jinno, A., Shimizu, N., Soda, Y., Haraguchi, Y., Kitanura, T. and Hoshino, H. (1998). "Identification of the chemokine receptor TER1/CCR8 expressed in brain-derived cells and T cells as a new coreceptor for HIV-1 infection." *Biochem Biophys Res Commun* **243**(2): 497-502.
- Kazmierski, W. M., Kenakin, T. P. and Gudmundsson, K. S. (2006). "Peptide, peptidomimetic and small-molecule drug discovery targeting HIV-1 host-cell attachment and entry through gp120, gp41, CCR5 and CXCR4." *Chem Biol Drug Des* **67**(1): 13-26.
- Kenakin, T. (2002). "Drug efficacy at G protein-coupled receptors." *Annu Rev Pharmacol Toxicol* **42**: 349-379.
- Kenakin, T. (2004). "Principles: receptor theory in pharmacology." *Trends Pharmacol Sci* **25**(4): 186-192.
- Kim, E. Y., Winters, M. A., Kagan, R. M. and Merigan, T. C. (2001). "Functional correlates of insertion mutations in the protease gene of human immunodeficiency virus type 1 isolates from patients." *J Virol* **75**(22): 11227-11233.

- Kjelsberg, M. A., Cotecchia, S., Ostrowski, J., Caron, M. G. and Lefkowitz, R. J. (1992). "Constitutive activation of the alpha 1B-adrenergic receptor by all amino acid substitutions at a single site. Evidence for a region which constrains receptor activation." *J Biol Chem* **267**(3): 1430-1433.
- Knight, P. J. and Grigliatti, T. A. (2004). "Chimeric G proteins extend the range of insect cell-based functional assays for human G protein-coupled receptors." *J Recept Signal Transduct Res* **24**(4): 241-256.
- Kostenis, E., Waelbroeck, M. and Milligan, G. (2005). "Techniques: promiscuous Galpha proteins in basic research and drug discovery." *Trends Pharmacol Sci* **26**(11): 595-602.
- Kraft, K., Olbrich, H., Majoul, I., Maek, M., Proudfoot, A. and Oppermann, M. (2001). "Characterization of sequence determinants within the carboxyl-terminal domain of chemokine receptor CCR5 that regulate signaling and receptor internalization." *J Biol Chem* **276**(37): 34408-34418.
- Kristiansen, K. (2004). "Molecular mechanisms of ligand binding, signaling, and regulation within the superfamily of G-protein-coupled receptors: molecular modeling and mutagenesis approaches to receptor structure and function." *Pharmacol Ther* **103**(1): 21-80.
- Kwong, P. D., Wyatt, R., Robinson, J., Sweet, R. W., Sodroski, J. and Hendrickson, W. A. (1998). "Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody." *Nature* **393**(6686): 648-659.
- Lagane, B., Ballet, S., Planchenault, T., Balabanian, K., Le Poul, E., Blanpain, C., Percherancier, Y., Staropoli, I., Vassart, G., Oppermann, M., Parmentier, M. and Bachelier, F. (2005). "Mutation of the DRY motif reveals different structural requirements for the CC chemokine receptor 5-mediated signaling and receptor endocytosis." *Mol Pharmacol* **67**(6): 1966-1976.
- Landau, N. R. and Littman, D. R. (1992). "Packaging system for rapid production of murine leukemia virus vectors with variable tropism." *J Virol* **66**(8): 5110-5113.
- Laurence, J. S., Blanpain, C., Burgner, J. W., Parmentier, M. and LiWang, P. J. (2000). "CC chemokine MIP-1 beta can function as a monomer and depends on Phe13 for receptor binding." *Biochemistry* **39**(12): 3401-3409.
- Lee, B., Doranz, B. J., Rana, S., Yi, Y., Mellado, M., Frade, J. M., Martinez, A. C., O'Brien, S. J., Dean, M., Collman, R. G. and Doms, R. W. (1998). "Influence of the CCR2-V64I polymorphism on human immunodeficiency virus type 1 coreceptor activity and on

- chemokine receptor function of CCR2b, CCR3, CCR5, and CXCR4." *J Virol* **72**(9): 7450-7458.
- Lee, B., Sharron, M., Blanpain, C., Doranz, B. J., Vakili, J., Setoh, P., Berg, E., Liu, G., Guy, H. R., Durell, S. R., Parmentier, M., Chang, C. N., Price, K., Tsang, M. and Doms, R. W. (1999). "Epitope mapping of CCR5 reveals multiple conformational states and distinct but overlapping structures involved in chemokine and coreceptor function." *J Biol Chem* **274**(14): 9617-9626.
- Liao, F., Alkhatib, G., Peden, K. W., Sharma, G., Berger, E. A. and Farber, J. M. (1997). "STRL33, A novel chemokine receptor-like protein, functions as a fusion cofactor for both macrophage-tropic and T cell line-tropic HIV-1." *J Exp Med* **185**(11): 2015-2023.
- Liapakis, G., Ballesteros, J. A., Papachristou, S., Chan, W. C., Chen, X. and Javitch, J. A. (2000). "The forgotten serine. A critical role for Ser-2035.42 in ligand binding to and activation of the beta 2-adrenergic receptor." *J Biol Chem* **275**(48): 37779-37788.
- Liberti, F., Cochaux, P., Beckman, G., Samson, M., Aksenova, M., Cao, A., Czeizel, A., Claustres, M., de la Rua, C., Ferrari, M., Ferrec, C., Glover, G., Grinde, B., Guran, S., Kucinskas, V., Lavinha, J., Mercier, B., Ogur, G., Peltonen, L., Rosatelli, C., Schwartz, M., Spitsyn, V., Timar, M., Beckman, L., Parmentier, M. and Vassart, G. (1998). "The deltaacer5 mutation conferring protection against HIV-1 in caucasian populations has a single and recent origin in North Eastern Europe." *Hum Mol Genet* **7**: 399-406
- Lim, J. K., Glass, W. G., McDermott, D. H. and Murphy, P. M. (2006). "CCR5: no longer a "good for nothing" gene—chemokine control of West Nile virus infection." *Trends Immunol* **27**(7): 308-312.
- Lin, P. F., Blair, W., Wang, T., Spicer, T., Guo, Q., Zhou, N., Gong, Y. F., Wang, H. G., Rose, R., Yamanaka, G., Robinson, B., Li, C. B., Fridell, R., Deminie, C., Demers, G., Yang, Z., Zadjura, L., Meanwell, N. and Colonna, R. (2003). "A small molecule HIV-1 inhibitor that targets the HIV-1 envelope and inhibits CD4 receptor binding." *Proc Natl Acad Sci U S A* **100**(19): 11013-11018.
- Liu, R., Paxton, W. A., Choe, S., Ceradini, D., Martin, S. R., Horuk, R., MacDonald, M. E., Stuhlmann, H., Kouy, R. A. and Landau, N. R. (1996). "Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection." *Cell* **86**(3): 367-377.

- Lusso, P. (2002). "HIV and chemokines: implications for therapy and vaccine." *Vaccine* **20**(15): 1964-1967.
- Lusso, P. (2006). "HIV and the chemokine system: 10 years later." *Embo J* **25**(3): 447-456.
- Luz, J. G., Yu, M., Su, Y., Wu, Z., Zhou, Z., Sun, R. and Wilson, J. A. (2005). "Crystal structure of viral macrophage inflammatory protein 1 encoded by Kaposi's sarcoma-associated herpesvirus at 1.7Å." *J Mol Biol* **352**(5): 1019-1028.
- McCutchan, F. E. (2000). "Understanding the genetic diversity of HIV-1." *Aids* **14**(Suppl 3): S31-44.
- McDermott, D. H., Zimmerman, P. A., Guignard, F., Kleeberger, C. A., Leitman, S. F. and Murphy, P. M. (1998). "CCR5 promoter polymorphism and HIV-1 disease progression. Multicentre AIDS cohort study (MACS)." *Lancet* **352**(9131): 866-870.
- Mellado, M., Rodriguez-Frade, J. M., Manes, S. and Martinez, A. C. (2001). "Chemokine signaling and functional responses: the role of receptor dimerization and TK pathway activation." *Annu Rev Immunol* **19**: 397-421.
- Mellado, M., Rodriguez-Frade, J. M., Vila-Coro, A. J., de Ana, A. M. and Martinez, A. C. (1999). "Chemokine control of HIV-1 infection." *Nature* **400**(6746): 723-724.
- Menten, P., Struyf, S., Schutysse, E., Wuyts, A., De Clercq, E., Schols D., Proost, P. and Van Damme, J. (1999). "The ED78 β isoform of MIP-1 α is the most potent CCR5 agonist and HIV-1-inhibiting chemokine." *J Clin Invest* **104**(4): 1-5.
- Menten, P., Wuyts, A. and Van Damme, J. (2002). "Macrophage inflammatory protein-1." *Cytokine Growth Factor Rev* **13**(6): 455-481.
- Michael, N. L., Nelson, J. A., KewalRamani, V. N., Chang, G., O'Brien, S. J., Mascola, J. R., Volzky, B., Louder, M., White, G. C., 2nd, Littman, D. R., Swanstrom, R. and O'Brien, T. R. (1998). "Exclusive and persistent use of the entry coreceptor CXCR4 by human immunodeficiency virus type 1 from a subject homozygous for CCR5 delta32." *J Virol* **72**(7): 6040-6047.
- Millar, R. P., Davidson J., Flanagan C.A and Wakefield I. (1995). Ligand binding and second messenger assays for cloned Gq/G11-coupled neuropeptide receptors: the GnRH receptor. *Methods in Neurosciences. Receptor Molecular Biology*. S. C. Sealfon. San Diego, Academic press: 145-162.
- Milligan, G. and White, J. H. (2001). "Protein-protein interactions at G-protein-coupled receptors." *Trends Pharmacol Sci* **22**(10): 513-518.

- Mirzabekov, T., Bannert, N., Farzan, M., Hofmann, W., Kolebinsky, P., Wu, L., Wyatt, R. and Sodroski, J. (1999). "Enhanced expression, native purification, and characterization of CCR5, a principal HIV-1 coreceptor." *J Biol Chem* **274**(40): 28745-28750.
- Mirzadegan, T., Benko, G., Filipek, S. and Paleczewski, K. (2003). "Sequence analyses of G-protein-coupled receptors: similarities to rhodopsin." *Biochemistry* **42**(10): 2759-2767.
- Miyakawa, T., Obaru, K., Maeda, K., Harada, S. and Mitsuya, H. (2002). "Identification of amino acid residues critical for LD78 β , a variant of human macrophage inflammatory protein-1 α , binding to CCR5 and inhibition of R5 human immunodeficiency virus type 1 replication." *J Biol Chem* **277**(7): 4649-4655.
- Moore, J. P. and Jarrette, R. F. (1988). "Sensitive ELISA for the gp120 and gp160 surface glycoprotein of HIV-1." *AIDS Res Hum Retroviruses* **4**(5): 369-379.
- Morris, L., Cilliers, T., Bredell, H., Phoswa, M. and Martin, D. J. (2001). "CCR5 is the major coreceptor used by HIV-1 subtype C isolates from patients with active tuberculosis." *AIDS Res Hum Retroviruses* **17**(8): 697-701.
- Mueller, A., Mahmoud, N. G., Goedecke, M. C., McKeating, J. A. and Strange, P. G. (2002). "Pharmacological characterization of the chemokine receptor, CCR5." *Br J Pharmacol* **135**(4): 1033-1043.
- Mummidi, S., Ahuja, S. S., McDaniel, B. L. and Ahuja, S. K. (1997). "The human CC chemokine receptor 5 (CCR5) gene. Multiple transcripts with 5'-end heterogeneity, dual promoter usage, and evidence for polymorphisms within the regulatory regions and noncoding exons." *J Biol Chem* **272**(49): 30662-30671.
- Mummidi, S., Ahuja, S. S., Gonzalez, E., Anderson, S. A., Santiago, S. N., Stephan, K. T., Craig, F. E., O'Connell, P., Tyrone, V., Clark, R. A., Dolan, M. J. and Ahuja, S. K. (1998). "Genealogy of the CCR5 locus and chemokine system gene variants associated with altered rates of HIV-1 disease progression." *Nat Med* **4**(7): 786-793.
- Murphy, P. M. (1996). "Chemokine receptors: structure, function and role in microbial pathogenesis." *Cytokine Growth Factor Rev* **7**(1): 47-64.
- Murphy, P. M., Baggolini, M., Charo, I. F., Hebert, C. A., Horuk, R., Matsushima, K., Miller, J. H., Oppenheim, J. J. and Power, C. A. (2000). "International union of pharmacology. XXII. Nomenclature for chemokine receptors." *Pharmacol Rev* **52**(1): 145-176.

- Naif, H. M., Cunningham, A. L., Alali, M., Li, S., Nasr, N., Buhler, M. M., Schols, D., de Clercq, E. and Stewart, G. (2002). "A human immunodeficiency virus type 1 isolate from an infected person homozygous for CCR5Delta32 exhibits dual tropism by infecting macrophages and MT2 cells via CXCR4." *J Virol* **76**(7): 3114-3124.
- Nakao, M., Nomiya, H. and Shimada, K. (1990). "Structures of human genes coding for cytokine LD78 and their expression" *Mol Cell Biol* **10**(7) 3646-3658.
- Navenot, J. M., Wang, Z. X., Trent, J. O., Murray, J. L., Hu, Q. X., DeLeeuw, J., Moore, P. S., Chang, Y. and Peiper, S. C. (2001). "Molecular anatomy of CCR5 engagement by physiologic and viral chemokines and HIV-1 envelope glycoproteins: differences in primary structural requirements for RANTES, MIP-1 alpha, and vMIP-II Binding." *J Mol Biol* **313**(5): 1181-1193.
- Neilson, J. R., John, G. C., Carr, J. K., Lewis, P., Kreiss, J. K., Jackson, S., Nduati, R. W., Mbori-Ngacha, D., Panteleeff, D. D., Bodrug, S., Giachetti, C., Boff, M. A., Richardson, B. A., Bwayo, J., Ndinya-Achola, J. and Overbaugh, J. (1999). "Subtypes of human immunodeficiency virus type 1 and disease stage among women in Nairobi, Kenya." *J Virol* **73**(5): 4393-4403.
- Neptune, E. R., Iiri, T. and Bourne, H. R. (1999). "Galphai is not required for chemotaxis mediated by Gi-coupled receptors." *J Biol Chem* **274**(5): 2824-2828.
- Neurath, A. R., Strick, N., Li, Y. Y. and Debnath, A. K. (2001). "Cellulose acetate phthalate, a common pharmaceutical excipient, inactivates HIV-1 and blocks the coreceptor binding site on the virus envelope glycoprotein gp120." *BMC Infect Dis* **1**(1): 17.
- Nibbs, R. J., Yang, J., Landau, N. R., Mao, J. H. and Graham, G. J. (1999). "LD78 β , a non-allelic variant of human MIP-1 α (LD78 α), has enhanced receptor interactions and potent HIV suppressive activity." *J Biol Chem* **274**(25): 17478-17483.
- Oliveira, L., Paiva, A. C., Sander, C. and Vriend, G. (1994). "A common step for signal transduction in G protein-coupled receptors." *Trends Pharmacol Sci* **15**(6): 170-172.
- Onuffer, J. J. and Horuk, R. (2002). "Chemokines, chemokine receptors and small-molecule antagonists: recent developments." *Trends Pharmacol Sci* **23**(10): 459-467.
- Oppermann, M. (2004). "Chemokine receptor CCR5: insights into structure, function, and regulation." *Cell Signal* **16**(11): 1201-1210.

- Oppermann, M., Mack, M., Proudfoot, A. E. and Olbrich, H. (1999). "Differential effects of CC chemokines on CC chemokine receptor 5 (CCR5) phosphorylation and identification of phosphorylation sites on the CCR5 carboxyl terminus." *J Biol Chem* **274**(13): 8875-8885.
- Osmanov, S., Pattou, C., Walker, N., Schwarlander, B. and Esparza, J. (2002). "Estimated global distribution and regional spread of HIV-1 genetic subtypes in the year 2000." *J Acquir Immune Defic Syndr* **29**(2): 184-190.
- Paterlini, M. G. (2002). "Structure modeling of the chemokine receptor CCR5: implications for ligand binding and selectivity." *Biophys J* **83**(6): 3012-3031.
- Paxton, W. A., Martin, S. R., Tse, D., O'Brien, T. R., Skurnick, J., VanDevanter, N. L., Padian, N., Braun, J. F., Kotler, D. P., Wolinsky, S. M. and Koup, R. A. (1996). "Relative resistance to HIV-1 infection of CD4 lymphocytes from persons who remain uninfected despite multiple high-risk sexual exposure." *Nat Med* **2**(4): 412-417.
- Petersen, D. C., Kotze, M. J., Zeier, M. D., Grimwood, A., Pretorius, D., Vardas, E., van Rensburg, E. J. and Hayes, V. M. (2001). "Novel mutations identified using a comprehensive CCR5-denaturing gradient gel electrophoresis assay." *Aids* **15**(2): 171-177.
- Picard, L., Simmons, G., Power, C. A., Meyer, A., Weiss, R. A. and Clapham, P. R. (1997). "Multiple extracellular domains of CCR-5 contribute to human immunodeficiency virus type 1 entry and fusion." *J Virol* **71**(7): 5003-5011.
- Platt, E. J., Shea, D. M., Rose, P. P. and Kabat, D. (2005). "Variants of human immunodeficiency virus type 1 that efficiently use CCR5 lacking the tyrosine-sulfated amino terminus have adaptive mutations in gp120, including loss of a functional N-glycan." *J Virol* **79**(7): 4357-4368.
- Pollok-Kopp, B., Schwarze, K., Baradari, V. K. and Oppermann, M. (2003). "Analysis of ligand-stimulated CC chemokine receptor 5 (CCR5) phosphorylation in intact cells using phosphosite-specific antibodies." *J Biol Chem* **278**(4): 2190-2198.
- Pomerantz, R. J. and Horn, D. L. (2003). "Twenty years of therapy for HIV-1 infection." *Nat Med* **9**(7): 867-873.
- Proudfoot, A. E., Power, C. A., Hoogewerf, A. J., Montjovent, M. O., Borlat, F., Offord, R. E. and Wells, T. N. (1996). "Extension of recombinant human RANTES by the retention of the initiating methionine produces a potent antagonist." *J Biol Chem* **271**(5): 2599-2603.
- Quinones, M. P., Ahuja, S. K., Jimenez, F., Schaefer, J., Garavito, E., Rao, A., Chenux, G., Reddick, R. L., Kuziel, W.A. and Ahuja, S. S. (2004). "Experimental arthritis in CC

- chemokine receptor 2-null mice closely mimics severe human rheumatoid arthritis. *J Clin Invest* **113**(6): 856-866.
- Raport, C. J., Gosling, J., Schweickart, V. L., Gray, P. W. and Charo, I. F. (1996). "Molecular cloning and functional characterization of a novel human CC chemokine receptor (CCR5) for RANTES, MIP-1beta, and MIP-1alpha." *J Biol Chem* **271**(29): 17161-17166.
- Rasmussen, S. G., Jensen, A. D., Liapakis, G., Ghanouni, P., Javitch, J. A. and Gether, U. (1999). "Mutation of a highly conserved aspartic acid in the beta2 adrenergic receptor: constitutive activation, structural instability, and conformational rearrangement of transmembrane segment 6." *Mol Pharmacol* **56**(1): 175-184.
- Ren, Q., Kurose, H., Lefkowitz, R. J. and Cotecchia, S. (1993). "Constitutively active mutants of the alpha 2-adrenergic receptor." *J Biol Chem* **268**(22): 16483-16487.
- Renjifo, B., Chaplin, B., Mwakagile, D., Shah, P., Vannberg, F., Msamanga, G., Hunter, D., Fawzi, W. and Essex, M. (1998). "Epidemic expansion of HIV type 1 subtype C and recombinant genotypes in Tanzania." *AIDS Res Hum Retroviruses* **14**(7): 635-638.
- Ribeiro, S. and Floruk, R. (2005). "The clinical potential of chemokine receptor antagonists." *Pharmacol Ther* **107**(1): 44-58.
- Rossi, D. and Zlotnik, A. (2000). "The biology of chemokines and their receptors." *Annu Rev Immunol* **18**: 217-242.
- Rucker, J., Edinger, A. L., Sharron, M., Samson, M., Lee, B., Berson, J. F., Yi, Y., Margulies, B., Collman, R. G., Doranz, B. J., Parmentier, M. and Doms, R. W. (1997). "Utilization of chemokine receptors, orphan receptors, and herpesvirus-encoded receptors by diverse human and simian immunodeficiency viruses." *J Virol* **71**(12): 8999-9007.
- Rucker, J., Samson, M., Doranz, B. J., Libert, F., Berson, J. F., Yi, Y., Smyth, R. J., Collman, R. G., Broder, C. C., Vassart, G., Doms, R. W. and Parmentier, M. (1996). "Regions in beta-chemokine receptors CCR5 and CCR2b that determine HIV-1 cofactor specificity." *Cell* **87**(3): 437-446.
- Samama, P., Cotecchia, S., Costa, T. and Lefkowitz, R. J. (1993). "A mutation-induced activated state of the beta 2-adrenergic receptor. Extending the ternary complex model." *J Biol Chem* **268**(7): 4625-4636.
- Samson, M., LaRosa, G., Libert, F., Painsavoine, P., Detheux, M., Vassart, G. and Parmentier, M. (1997). "The second extracellular loop of CCR5 is the major determinant of ligand specificity." *J Biol Chem* **272**(40): 24934-24941.

- Samson, M., Libert, F., Doranz, B. J., Rucker, J., Liesnard, C., Farber, C. M., Saragosti, S., Lapoumeroulie, C., Cognaux, J., Forceille, C., Muyldermans, G., Verhofstede, C., Burtonboy, G., Georges, M., Imai, T., Rana, S., Yi, Y., Smyth, R. J., Collman, R. G., Doms, R. W., Vassart, G. and Parmentier, M. (1996). "Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene." *Nature* **382**(6593): 722-725.
- Scheer, A., Fanelli, F., Costa, T., De Benedetti, P. G. and Cotecchia, S. (1996). "Constitutively active mutants of the alpha 1B-adrenergic receptor: role of highly conserved polar amino acids in receptor activation." *Embo J* **15**(14): 3566-3578.
- Seibert, C., Cadene, M., Sanliz, A., Chait, B. T. and Sakmar, T. P. (2002). "Tyrosine sulfation of CCR5 N-terminal peptide by tyrosylprotein sulfotransferases 1 and 2 follows a discrete pattern and temporal sequence." *Proc Natl Acad Sci U S A* **99**(17): 11031-11036.
- Seibert, C. and Sakmar, T. P. (2004). "Small-molecule antagonists of CCR5 and CXCR4: a promising new class of anti-HIV-1 drugs." *Curr Pharm Des* **10**(17): 2041-2062.
- Shioda, T., Nakayama, E. E., Tanaka, Y., Xin, X., Liu, H., Kawana-Tachikawa, A., Kato, A., Sakai, Y., Nagai, Y. and Iwamoto, A. (2001). "Naturally occurring deletional mutation in the C-terminal cytoplasmic tail of CCR5 affects surface trafficking of CCR5." *J Virol* **75**(7): 3462-3468.
- Siciliano, S. J., Kubmann, S. E., Weng, Y., Madani, N., Springer, M. S., Lineberger, J. E., Danzeisen, R., Miller, M. D., Kavanaugh, M. P., DeMartino, J. A. and Kabat, D. (1999). "A critical site in the core of the CCR5 chemokine receptor required for binding and infectivity of human immunodeficiency virus type 1." *J Biol Chem* **274**(4): 1905-1913.
- Simmons, G., Clapham, P. R., Picard, L., Offord, R. E., Rosenkilde, M. M., Scharz, T. W., Buser, R., Wells, T. N. and Proudfoot, A. E. (1997). "Potent inhibition with HIV-1 infectivity in macrophages and lymphocytes by a novel CCR5 antagonist." *Science* **276** (5310): 276-279.
- Smith, M. W., Dean, M., Carrington, M., Winkler, C., Huttley, G. A., Lomb, D. A., Goedert, J. J., O'Brien, T. R., Jacobson, L. P., Kaslow, R., Buchbinder, S., Vittinghoff, E., Vlahov, D., Hoots, K., Hilgartner, M. W. and O'Brien, S. J. (1997). "Contrasting genetic influence of CCR2 and CCR5 variants on HIV-1 infection and disease progression. Hemophilia Growth and Development Study (HGDS), Multicenter AIDS Cohort Study (MACS), Multicenter

- Hemophilia Cohort Study (MHCS), San Francisco City Cohort (SFCC), ALIVE Study," *Science* **277**(5328): 959-965.
- Stanchev, T. S. and Broder, C. C. (2001). "Human immunodeficiency virus type-1 and chemokines: beyond competition for common cellular receptors." *Cytokine Growth Factor Rev* **12**(2-3): 219-243.
- Strader, C. D., Candelore, M. R., Hill, W. S., Sigal, I. S. and Dixon, R. A. (1989). "Identification of two serine residues involved in agonist activation of the beta-adrenergic receptor." *J Biol Chem* **264**(23): 13572-13578.
- Strader, C. D., Fong, T. M., Tota, M. R., Underwood, D. and Dixon, R. A. (1994). "Structure and function of G protein-coupled receptors." *Annu Rev Biochem* **63**: 101-132.
- Strader, C. D., Sigal, I. S., Register, R. B., Candelore, M. R., Rands, E. and Dixon, R. A. F. (1987). "Identification of residues required for ligand binding to the β -adrenergic receptor." *Proc Natl Acad Sci U S A* **84**(13): 4384-4388.
- Strizki, J. M., Xu, S., Wagner, N. E., Wojcik, L., Liu, J., Hou, Y., Endres, M., Palani, A., Shapiro, S., Clader, J. W., Greenlee, W. J., Tagat, J. R., McCombie, S., Cox, K., Fawzi, A. B., Chou, C. C., Pugliese-Sivo, C., Davies, L., Moreno, M. E., Ho, D. D., Trkola, A., Stoddart, C. A., Moore, J. P., Reyes, G. R. and Baroudy, B. M. (2001). "SCH-C (SCH 351125), an orally bioavailable, small molecule antagonist of the chemokine receptor CCR5, is a potent inhibitor of HIV-1 infection in vitro and in vivo." *Proc Natl Acad Sci U S A* **98**(22): 12718-12723.
- Suphaphiphat, P., Thitithanyanont, A., Paea-Uccaralertkun, S., Essex, M. and Lee, T. H. (2003). "Effect of amino acid substitution of the V3 and bridging sheet residues in human immunodeficiency virus type 1 subtype C gp120 on CCR5 utilization." *J Virol* **77**(6): 3832-3837.
- Takashima, K., Miyake, H., Furuta, R. A., Fujisawa, J. I., Iizawa, Y., Kanzaki, N., Shiraishi, M., Okonogi, K. and Baba, M. (2001). "Inhibitory effects of small-molecule CCR5 antagonists on human immunodeficiency virus type 1 envelope-mediated membrane fusion and viral replication." *Antimicrob Agents Chemother* **45**(12): 3538-3543.
- Tamasauskas, D., Powell, V., Saksela, K. and Yazdanbakhsh, K. (2001). "A homologous naturally occurring mutation in Duffy and CCR5 leading to reduced receptor expression." *Blood* **97**(11): 3651-3654.

- Tota, M. R. and Strader, C. D. (1990). "Characterization of the binding domain of the beta-adrenergic receptor with the fluorescent antagonist carazolol. Evidence for a buried ligand binding site." *J Biol Chem* **265**(28): 16891-16897.
- Treurnicht, F. K., Smith, T. L., Engelbrecht, S., Claassen, M., Robson, B. A., Zeier, M. and van Rensburg, E. J. (2002). "Genotypic and phenotypic analysis of the env gene from South African HIV-1 subtype B and C isolates." *J Med Virol* **68**(2): 141-146.
- Trkola, A., Pomales, A. B., Yuan, H., Korber, B., Maddon, P. J., Allaway, G. P., Katinger, H., Barbas, C. F., 3rd, Burton, D. R., Ho, D. D. and et al. (1995). "Cross-clade neutralization of primary isolates of human immunodeficiency virus type 1 by human monoclonal antibodies and tetrameric CD4-IgG." *J Virol* **69**(11): 6609-6617.
- Tscherning, C., Alaeus, A., Fredriksson, R., Bjornstal, A., Deng, H., Littman, D. R., Fenyo, E. M. and Albert, J. (1998). "Differences in chemokine coreceptor usage between genetic subtypes of HIV-1." *Virology* **241**(2): 181-188.
- UNAIDS (2006). AIDS Epidemic Updated. **2006**.
- Venkatesan, S., Petrovic, A., Locati, M., Kim, Y. O., Weissman, D. and Murphy, P. M. (2001). "A membrane-proximal basic domain and cysteine cluster in the C-terminal tail of CCR5 constitute a bipartite motif critical for cell surface expression." *J Biol Chem* **276**(43): 40133-40145.
- Wang, J., Guan, E., Roderiquez, G. and Norcross, M. A. (1999). "Inhibition of CCR5 expression by IL-12 through induction of beta-chemokines in human T lymphocytes." *J Immunol* **163**(11): 5763-5769.
- Weiss, R. A. (2001). Gulliver's travels in HIVland. Macmillan Magazines Ltd: 963-967.
- Weissmann, D., Li, Y., Ananworanich, J., Zhou, L.-L., Adelsberger, J., Tedder, T. F., Baseler, M. and Fauci, A. S. (1995). "Three populations of cells with dendritic morphology exists in peripheral blood, only one of which is infectable with human immunodeficiency virus type 1." *Proc Natl Acad Sci USA* **92**: 826-830
- Weissman, D., Rabin, R. L., Arthos, J., Rubbert, A., Dybul, M., Swofford, R., Venkatesan, S., Farber, J. M. and Fauci, A. S. (1997). "Macrophage-tropic HIV and SIV envelope proteins induce a signal through the CCR5 chemokine receptor." *Nature* **389**(6654): 981-985.
- Wieland, K., Zuurmond, H. M., Krasel, C., Ijzerman, A. P. and Lohse, M. J. (1996). "Involvement of Asn-293 in stereospecific agonist recognition and in activation of the beta 2-adrenergic receptor." *Proc Natl Acad Sci U S A* **93**(17): 9276-9281.

- Williamson, C., Morris, L., Maughan, M. F., Ping, L. H., Dryga, S. A., Thomas, R., Reap, E. A., Cilliers, T., van Harmelen, J., Pascual, A., Ramjee, G., Gray, G., Johnston, R., Karim, S. A. and Swanstrom, R. (2003). "Characterization and selection of HIV-1 subtype C isolates for use in vaccine development." *AIDS Res Hum Retroviruses* **19**(2): 133-144.
- Wu, L., LaRosa, G., Kassam, N., Gordon, C. J., Heath, H., Ruffing, N., Chen, H., Humblas, J., Samson, M., Parmentier, M., Moore, J. P. and Mackay, C. R. (1997). "Interaction of chemokine receptor CCR5 with its ligands: multiple domains for HIV-1 gp120 binding and a single domain for chemokine binding." *J Exp Med* **186**(8): 1373-1381.
- Youn, B. S., Yu, K. Y., Alkhatib, G. and Kwon, B. S. (2001). "The seventh transmembrane domain of cc chemokine receptor 5 is critical for MIP-1beta binding and receptor activation: role of MET 287." *Biochem Biophys Res Commun* **281**(3): 627-633.
- Yuki, Y., Nochi, T. and Kiyono, H. (2007). "Progress towards an AIDS mucosal vaccine: An overview." *Tuberculosis (Edinb)*.
- Zapico, I., Coto, E., Rodriguez, A., Alvarez, C., Torre, J. C. and Alvarez, V. (2000). "CCR5 (chemokine receptor-5) DNA-polymorphism influences the severity of rheumatoid arthritis." *Genes Immun* **1**(4): 288-289.
- Zawicki, P. and Witas, H. W. (2008). "HIV-1 protecting CCR5-A32 allele in medieval Poland." *Infect Genet Evol* **8**(2): 146-151.
- Zhang, Y. and Rollins, B. J. (1995). "A dominant negative inhibitor indicates that monocyte chemoattractant protein 1 functions as a dimer." *Mol Cell Biol* **15**(9): 4851-4855.
- Zimmerman, P. A., Buckler-White, A., Alkhatib, G., Spalding, T., Kubofcik, J., Combadiere, C., Weissman, D., Cohen, O., Rubbert, A., Lam, G., Vaccarezza, M., Kennedy, P. E., Kumaraswami, V., Giorgi, J. V., Detels, R., Hunter, J., Chopek, M., Berger, E. A., Fauci, A. S., Ntman, T. B. and Murphy, P. M. (1997). "Inherited resistance to HIV-1 conferred by an inactivating mutation in CC chemokine receptor 5: Studies in populations with contrasting clinical phenotypes, defined racial background and quantified risk." *Mol Med* **3**: 23-36.