

**EXPRESSION AND CHARACTERIZATION OF FUNCTIONAL HIV-1
SUBTYPE C ENVELOPE GLYCOPROTEINS IN INSECT CELLS**

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ABSTRACT

The full-length envelope glycoproteins from four South African HIV-1 subtype C isolates exhibiting different phenotypic properties were expressed in insect cells, purified, and their biological characteristics investigated. Isolates 98ZA151Du, 99ZACM9, 98ZA179Du and 99ZASW7 that utilize the CCR5, CCR5/CXCR4, CCR5/CXCR4 and CXCR4 coreceptors for cell entry, respectively were selected for this study. All four full-length *env* genes were PCR amplified, cloned into a baculovirus expression system and the recombinant envelope glycoproteins were expressed in *Spodoptera frugiperda* (Sf9) insect cells. The envelope glycoproteins were purified on a large scale using *Galanthus nivalis* lectin and their biological properties were examined by their reactivity with soluble CD4 (sCD4) and a panel of monoclonal antibodies. The conformation of the four purified recombinant HIV-1 subtype C envelope glycoproteins was intact, since they all reacted with the conformational dependent monoclonal antibodies A32, C11, 17b, IgG1b12 and 7b2. However, the IgG1b12 monoclonal antibody was unable to bind to the 98ZA151Du envelope glycoprotein efficiently, suggesting that this CCR5 envelope glycoprotein exhibited lower antigenic properties compared to the CCR5/CXCR4 and CXCR4 envelope glycoproteins. The high levels of reactivity of the recombinant envelope glycoproteins with the A32 and C11 monoclonal antibodies and their lower interaction with IgG1b12 indicated they were purified as monomers. All four recombinant HIV-1 subtype C envelope glycoproteins reacted poorly with sCD4, however their high binding activity with the 17b monoclonal antibody in the presence of sCD4 confirmed that they are functional. Thus the recombinant HIV-1 subtype C envelope glycoproteins purified in this study can be used as reagents to further facilitate functional, structural, antigenicity and immunogenicity studies.

DECLARATION

I declare that this dissertation is my own unaided work. It is being submitted for the degree of Masters of Science in Medicine (Virology) at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any other degree or examination at any other university.



Praise-God Sibusiso Nkosi

20 day of September, 2002

I dedicate this work to my family.

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LIST OF ABBREVIATIONS

ng	nanogram
µg	microgram
µl	microlitre
DMSO	dimethylsulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetate
ELISA	Enzyme linked immunosorbent assay
<i>env</i>	envelope gene
FCS	fetal calf serum
HCl	Hydrochloric acid
HIV-1	Human immunodeficiency virus type 1
KCl	Potassium chloride
MMP	Methyl-α-D-mannopyrannoside
min	minute
MIP-1	Macrophage inflammatory protein 1
MIP-1	Macrophage inflammatory protein 1
ml	millilitre
NaCl	Sodium chloride
NaHPO ₄	Sodium phosphate monobasic
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
RANTES	Regulated on activation normal T-cell expressed and activated
rpm	Revolutions per minute

SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	second
SIV	Simian immunodeficiency virus
TBS	Tris buffered saline

CHAPTER 1

1.1 INTRODUCTION

The human immunodeficiency virus (HIV) is the aetiologic agent of the acquired immunodeficiency syndrome (AIDS) in humans, which is characterized by depletion of CD4-positive lymphocytes (Cao *et al.*, 1993); (Abacioglu *et al.*, 1994). HIV-1 like all other retroviruses is surrounded by an envelope consisting of a host cell derived lipid bilayer and virus encoded envelope glycoproteins (Wyatt and Sodroski, 1998). The envelope glycoprotein of HIV-1 mediates functions that are critical to the viral life cycle, including attachment to target cells and the fusion of viral and cellular membranes. Furthermore, the envelope glycoprotein is the main target of the neutralizing humoral immune response of the host. This protein is thus an ideal target for numerous drug development and vaccine strategies.

The HIV-1 envelope glycoprotein (gp160) is synthesized in the endoplasmic reticulum as an 835 to 870 amino acid precursor depending on the viral strain (Wyatt and Sodroski, 1998); (Yang *et al.*, 2000); (van Harmelen *et al.*, 2001). The gp160 protein precursor results from the addition of N-linked high mannose sugar chains to particular amino acids in the endoplasmic reticulum. Post translational modifications such as folding, disulphide bond formation and oligomerization of gp160 also occur in the endoplasmic reticulum (Poumbourios *et al.*, 1995). Following oligomerization, the gp160 is transported to the Golgi apparatus where it is cleaved by the cellular proteases such as furin into the exterior glycoprotein (gp120) and the transmembrane glycoprotein (gp41), held together by weak noncovalent bonds (Abacioglu *et al.*, 1994); (Farzan *et al.*, 1998). In the Golgi apparatus, carbohydrates are further

modified by the addition of complex sugars to form the mature envelope glycoprotein. The mature envelope glycoprotein complexes are then transported to the surface of the host cells, where they are incorporated into budding virions as an oligomeric complex (Wyatt and Sodroski, 1998); (Yang *et al.*, 2000). The envelope glycoprotein forms 7 to 14 oligomeric spikes on the surface of the virion and/or the infected cells (Chertova *et al.*, 2002). Physical and crystallographic studies have shown that the oligomeric envelope glycoprotein complex is a trimer, consisting of three gp120 subunits noncovalently associated with three gp41 moieties (Center *et al.*, 2001); (Center *et al.*, 2002). The trimeric envelope glycoprotein complex sequentially binds HIV-1 to the host cell surface CD4 receptors and chemokine coreceptors, either CCR5 or CXCR4 and mediates fusion between the viral and cellular membranes.

1.2 Structure of the HIV-1 envelope glycoprotein

1.2.1 Structure of gp120

The gp120 consists of five variable regions (V1-V5) interspersed by five conserved regions namely C1 to C5 (Kwong *et al.*, 1998). The conserved and variable regions of gp120 are heavily glycosylated (Kwong *et al.*, 1998); (Rizzuto *et al.*, 1998). Intramolecular disulphide bonds in the gp120 result in the incorporation of the first four variable regions to form large looplike structures (Figure 1.1) (Kwong *et al.*, 1998); (Wyatt *et al.*, 1998). The conserved regions fold into a gp120 core, composed of an inner domain and an outer domain and a 'bridging sheet' (β sheet) (Rizzuto *et al.*, 1998); (Wyatt and Sodroski, 1998). The domain names are derived from the orientation of gp120 in the trimeric envelope glycoprotein. The inner domain is more conserved than the outer domain. The inner domain interacts with gp41 and the outer domain is exposed on the surface of the trimer (Kwong *et al.*, 1998); (Wyatt and

Sodroski, 1998); (Burton and Parren, 2000). The bridging sheet is a four stranded, antiparallel β sheet that includes the V1 and V2 region stems and strands derived from the fourth conserved gp120 region (Rizzuto *et al.*, 1998). The conserved gp120 regions form discontinuous structures that are important for the interaction with the gp41 ectodomain and with the viral coreceptors on the target cell (Kwong *et al.*, 1998).

The CD4 binding site (CD4bs) on gp120 is situated in a depression at the interface of the inner domain, outer domain and the bridging sheet (Rizzuto *et al.*, 1998); (Burton and Parren, 2000), (Moore *et al.*, 2001). The CD4bs in the gp120 is recessed, flanked by variable regions exhibiting extensive glycosylation (Rizzuto *et al.*, 1998); (Wyatt and Sodroski, 1998). It lacks glycosylation and is relatively conserved among HIV-1 isolates (Rizzuto *et al.*, 1998); (Moore *et al.*, 2001). The gp120-CD4 interface in the gp120 consists of two cavities, a shallow cavity filled with water and a deep cavity extending roughly 10 angstroms ($\text{\AA}$) into the interior of gp120 (Wyatt and Sodroski, 1998). The conserved chemokine receptor binding surface is located at an approximately 90° angle to the CD4bs (Rizzuto *et al.*, 1998). The gp120 residues that form the coreceptor binding site are located near or within the bridging sheet, with additional contributions from residues at the base of the V2 loop (Rizzuto *et al.*, 1998); (Moore *et al.*, 2001). Both the CD4bs and the chemokine receptor binding site are masked by variable regions, mostly the V1/V2 and V3 loops (Moore and Sodroski, 1996); (Kwong *et al.*, 1998); (Moore *et al.*, 2001) (Figure 1.1).

1.2.2 Structure of gp41

The gp41 is a transmembrane glycoprotein that consists of a hydrophobic N-terminus ectodomain which comprises the glycine-rich 'fusion' peptide spanning amino acids 519 to 541, that is essential for membrane fusion (all amino acid numbering is based on the HIV-1 subtype HXB2 isolate, Los Alamos National Laboratory HIV Sequences Database, <http://hiv-web.lanl.gov>) (Poumbourios *et al.*, 1995). The gp41 fusion domain is followed by two regions containing the 4-3 hydrophobic (heptad) repeats, called the N-terminal peptide or N-leucine zipper (amino acids 552 to 597) and C-terminal peptide or C-leucine zipper (amino acids 629 to 674) regions. A loop region containing two cysteines is located between the two heptad repeat regions. This is ensued by an immunodominant epitope (amino acids 620 to 670), a membrane anchoring domain (amino acids 684 to 705) and cytoplasmic region (amino acids 705 to 856) (Figure 1.2) (Poumbourios *et al.*, 1995); (Gorny and Zolla-Pazner, 2000). X-ray crystallographic studies have shown that the gp41 core structure is a trimeric rod composed of an extended amphipathic α -helix (Weissenhorn *et al.*, 1997). This extended α -helical domain contains a heptad repeat of leucine and isoleucine, known as a 'leucine zipper' motif, encompassing approximately residues 552-597 (N-leucine zipper) and 629-674, (C-leucine zipper) and is conserved in the transmembrane proteins of HIV and other enveloped viruses (Chen *et al.*, 1994); (Harbury *et al.*, 1994); (Mobley *et al.*, 2001). Studies have shown that in solution the synthetic peptides based on the N-heptad repeat and C-heptad repeat assume coiled coils (Chan and Kim, 1998); (Gorny *et al.*, 2000); (Mobley *et al.*, 2001). The core structure of gp41 includes a hexameric helix bundle, with the N- and C-leucine zipper domains folded into three hairpins. The N-leucine zipper peptide forms a central three helical core organized as a trimer of coiled coils, while the C-leucine zipper motifs pack antiparallel into hydrophobic grooves on the surface of the N-peptide coiled coil

bundle (Figure 1.3) (Weissenhorn *et al.*, 1996); (Chan *et al.*, 1997); (Weissenhorn *et al.*, 1997); (Chan and Kim, 1998). The C-terminal helices interact with the N-terminal helices through conserved hydrophobic grooves on the surface of the central coiled coil (Weissenhorn *et al.*, 1997).

The trimeric coiled coil interior (N-helix) and three exterior (C-helix) self-assemble to form a thermostable six-helix bundle. This six-helix bundle (hairpin structure) has been shown to be the fusion competent conformation of gp41 (Chan and Kim, 1998); (Weng *et al.*, 2000). The notion that the six-helix bundle represents a fusion active gp41 conformation is supported by the observation that similar structures have been observed in the fusion active envelope glycoproteins of other viruses (Sodroski, 1999). Although the native state of the gp41 (prefusogenic) is undefined, it is thought to be an oligomer of gp41 in which the N-terminal repeat regions of three gp41 molecules self assemble, but are prevented from complexing with the C-terminal peptide region, perhaps by the interaction of gp41 with native gp120 (Gorny and Zolla-Pazner, 2000). Recently, the HIV-1 and SIV envelope glycoproteins in the prefusogenic activated state on the surface of the virion have been shown to be a trimer (Center *et al.*, 2001); (Center *et al.*, 2002). The same group showed that recombinant SIV envelope glycoproteins in the prefusogenic state are trimers (Center *et al.*, 2001). However, the factors that keep the association of the gp41 subunits in the trimer at this state needs to be examined.

“3D” structure of gp160

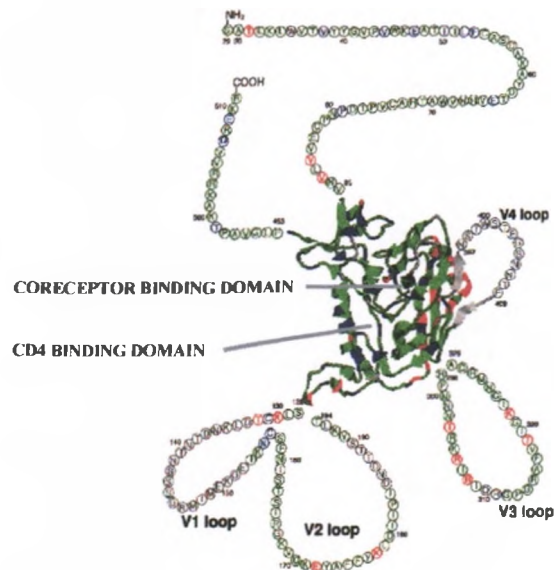
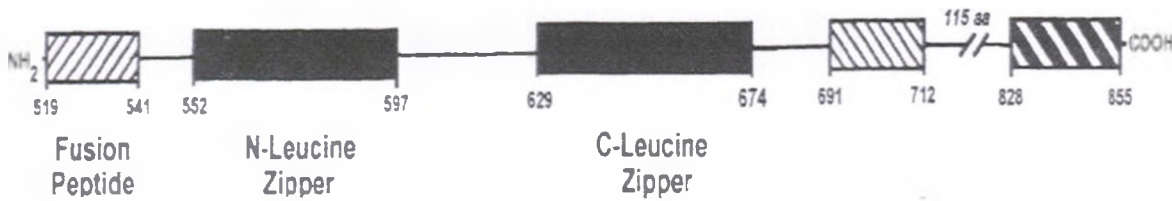
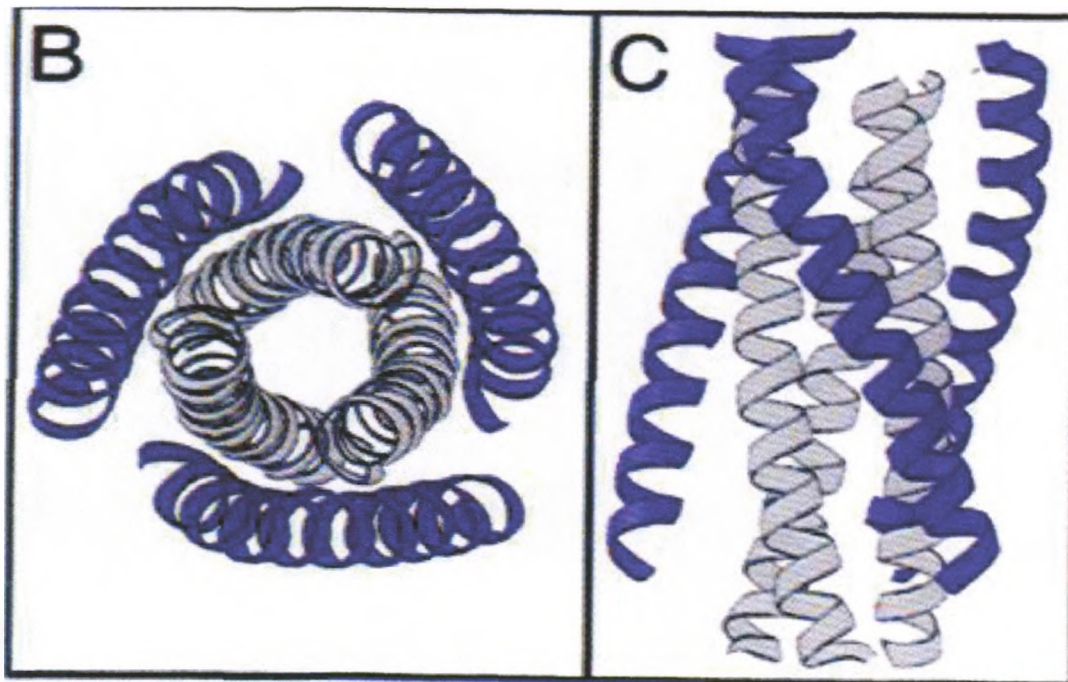


Figure 1.1: Schematic diagram showing the 3D structure of the HIV-1 envelope glycoprotein.



Taken from Mobley *et al.*, 2001

Figure 1.2: Schematic representation of various domains of the HIV-1 gp41 transmembrane protein. Fusion peptide domain (residues 519 to 541), N-leucine zipper region (residues 552 to 597), C-leucine zipper region (residues 629 to 674), Transmembrane region (gp41 residues 691 to 712) and C-terminal peptide (residues 828 to 855).



Taken from Chan and Kim, 1998

Figure 1.3: Core structure of the gp41 ectodomain. B shows the gp41 ectodomain viewed from above, and C from the side. The N-terminal peptides of the gp41 ectodomain (gray) form a trimeric central coiled coil and the three antiparallel C-peptides (blue) are packed in the hydrophobic groove oblique around this coiled coil.

1.2.3 Interaction of HIV-1 gp120 and gp41 subunits.

The association of gp120 with gp41 is unstable, and involves weak noncovalent interactions. The C1 and C5 regions of gp120 are important for its interaction with the gp41 (Wu *et al.*, 1996). Site-directed mutagenesis studies on the envelope glycoprotein of HIV-1 subtype B HXB2 have shown that the highly conserved C1 amino-terminal region (amino acids 36 to 45) consists of hydrophobic amino acids that interact with gp41 (Helseth *et al.*, 1991). The C5 carboxyl terminal region (amino acids 491 to 501) consists of less hydrophobic amino acids that affect the interaction of gp120 with gp41 (Helseth *et al.*, 1991). Amino acid substitutions in the α -helical sequences reduce the gp120 and gp41 subunit associations of HIV-1 subtype B HXB2 and LA1 (Poumbourios *et al.*, 1997). Residues 580, 587, Leu-571, Thr-564, Ile-554 and Leu-550 of the gp41 are required for the gp120 and gp41 subunit interaction (Chen, 1994); (Poumbourios *et al.*, 1997) and mutations in these residues impair the ability of the gp120 to associate with gp41 (Chen, 1994). Sullivan *et al.*, (1993) have

reported that amino acid substitutions in the V2 loop result in the dissociation of gp120 and gp41.

1.3 Oligomerization of HIV-1 envelope glycoproteins

Oligomerization of HIV-1 envelope glycoproteins is essential for the production of trimeric structurally mature and fusion active envelope glycoproteins (Poumbourios *et al.*, 1995); (Richardson *et al.*, 1996). Studies have shown that the amphipathic α -helical N-terminal zipper-like sequence heptad repeats in the gp41 mediate the oligomerization of the envelope glycoproteins in the endoplasmic reticulum (Chen, 1994); (Poumbourios *et al.*, 1995); (Center *et al.*, 1997); (Poumbourios *et al.*, 1997). The amphipathic α -helical hydrophobic repeat residues of the gp41 mediate oligomerization through multiple intermonomer interactions. The intermonomeric interactions result from the formation of the coiled coil structure, where the Leu-550 to Leu-585 hydrophobic repeats form the central coiled coil and hydrophobic grooves that interact with the C-terminal α -helix including residues 631 to 661 (numbering is based on the HIV-1 LA1 envelope sequence) (Poumbourios *et al.*, 1997). Studies have shown that multiple alanine substitutions and deletions of 9 of the 10 residues composing the α -helix 4-3 hydrophobic repeats (Leu-550 to Leu-582) result in the disruption of envelope hetero-oligomerization. (Center *et al.*, 1997); (Poumbourios *et al.*, 1997). These mutations also render the envelope glycoprotein gp160 precursor monomeric (Poumbourios *et al.*, 1997). Interestingly, Poumbourios *et al.*, (1997) showed that a single alanine substitution in the α -helix hydrophobic repeat does not affect gp160 oligomerization but abolished the syncytium formation function. Disruption of the oligomeric structure is accompanied by a change in HIV-1 envelope glycoprotein antigenic structure and function. The formation of the coiled coil

structure is required for the fusion of the viral and host cell membranes (Chan and Kim, 1998). During HIV-1 entry into the host cells the envelope glycoprotein undergoes conformational changes that result in the formation of the coiled coil structure as in Figure 1.3. The factors described above facilitate or stabilise the oligomerisation of the envelope glycoprotein in the fusion active state.

The envelope glycoprotein is very labile and it can easily undergo conformational changes from the prefusogenic state found on the surface of the virion or infected cells to the fusogenic state. This makes it difficult to study the envelope glycoproteins in the prefusogenic state. The oligomeric valency of the virion associated envelope glycoproteins at the prefusogenic state has recently been shown to be a trimer and the factors that mediate oligomerization in this state are not known (Center *et al.*, 2002). The residues that form oligomerisation also play an important role in the trimerization of the envelope glycoprotein in the fusion active state (Chan and Kim, 1998); (Sodroski, 1999). The factors that mediate oligomerization of the envelope glycoproteins in the prefusogenic state might be different from those in the fusogenic state and need to be investigated further. This will enhance our understanding of the factors that keep the association of the gp120 and gp41 subunits in the trimeric structure found on the surface of the virion or the infected cells.

1.4 HIV-1 entry into the host cell

1.4.1 Envelope glycoprotein binding to CD4 and chemokine coreceptors

The entry of HIV-1 into the host cell is initiated by the binding of HIV-1 gp120 with the host CD4 receptors and obligatory chemokine coreceptors, namely CCR5 and CXCR4. CD4 binds to a recessed pocket on gp120, making extensive contacts over

800°Å² of the gp120 surface. The residues of gp120 important for CD4 binding surround the opening of this deep cavity (Wyatt and Sodroski, 1998). The CD4-gp120 complex formation or interaction is associated with an unusually large bonding energy that is used to drive the rearrangement of the gp120 structure (Myszka *et al.*, 2000). Binding of gp120 to CD4 induces conformational changes in the gp120, including the movement of the V1/V2 and V3 loop structures exposing or forming the chemokine coreceptor binding sites (Moore and Sodroski, 1996); (Kwong *et al.*, 1998); (Center *et al.*, 2000); (Myszka *et al.*, 2000). This binding brings the trimeric envelope glycoprotein complex into proximity with the chemokine coreceptors. The formation or the exposure of the chemokine binding site allows the interaction of gp120 with the chemokine coreceptors on the surface of the host cells. The binding of gp120 to the chemokine coreceptors triggers further conformational changes in the envelope glycoprotein that result in the activation of the membrane fusion activity of gp41 (Sullivan *et al.*, 1998); (Center *et al.*, 2000).

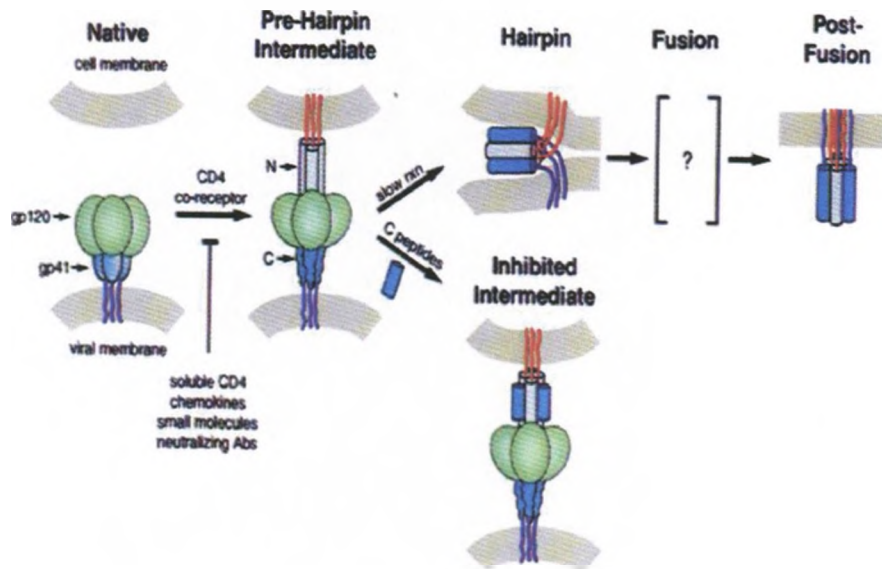
1.4.2 Fusion of the viral and the host cell membranes

The gp41 regions of the envelope glycoprotein mediate the fusion of viral and host cell membranes that leads to viral entry and infection of the host cells. In their native, nonfusogenic state, the gp41 fusion peptides are buried within the envelope glycoprotein complex (Chan and Kim, 1998); (Sodroski, 1999). This native gp41 structure has been suggested to be stabilized by its interaction with gp120 (Chan and Kim, 1998). The binding of the gp120 to the coreceptors induces conformational changes in the gp41 that are thought to be associated with transition from an as yet undefined native (nonfusogenic) to a fusion active (fusogenic) state (Lu *et al.*, 1999); (Suarez *et al.*, 2000). Furthermore, the binding of the gp120 to receptors and

coreceptors is essential for the transformation of a metastable native glycoprotein to an energetically stable fusion competent conformation. Video microscopy and fluorescent dye bisANS studies have shown that conformational changes are initiated within one to four minutes of receptor binding and are completed within 20 minutes (Chan and Kim, 1998).

Figure 1.4 outlines the conformational changes that gp41 undergoes during the fusion process. Firstly, gp41 undergoes its transition to the prehairpin intermediate that allows the exposure of the fusion peptide and its insertion into the target membrane (Chan and Kim, 1998); (Lu *et al.*, 1999); (Munoz-Barroso *et al.*, 1999); (Sodroski, 1999); (Suarez *et al.*, 2000). The fusion peptide which lies on top of the N-peptide coiled coil penetrates the target cell membrane (Chan and Kim, 1998); (Jiang *et al.*, 1998); (Saurez *et al.*, 2000). In the prehairpin intermediate state the C-peptide region of the trimeric coiled coil is not associated with this coiled coil, because it is constrained by an interaction with another region of gp41 and/or with gp120 (Chan and Kim, 1998); (Gorny and Zolla-Pazner, 2000). The conformational change from the intermediate prehairpin to the stable six helical complex may be slow due to constraints on the C-peptide region (Chan and Kim, 1998). Once these constraints are overcome, the interaction of the C-peptide domain with the N-peptide trimeric coiled coil occurs to form the stable fusion active hairpin structure as shown in Figure 1.3 (Chan and Kim, 1998); (Sodroski, 1999). Because the C-peptide of the α -helical domain of the gp41 is located near the viral membrane, the formation of the stable six-helical complex results in the apposition of the viral and the host cell membrane (Chan and Kim, 1998). The mechanism of how membrane apposition leads to fusion

is unclear but probably involves clustering of envelope protein trimers to form fusion pores (Chan and Kim, 1998).



Taken from Chan and Kim, 1998

Figure 1.4: Overview of the conformational changes that gp41 undergoes during the fusion process. In their native conformation the envelope glycoproteins form the trimeric complex with the fusion peptide of the gp41 buried in the complex. Following the interaction of gp120 with CD4 and coreceptors, the envelope glycoprotein complex undergoes conformational changes to form the pre-hairpin intermediate. At this stage the fusion peptide (red lines) is protruded and inserted into the target membrane and the N-terminal central trimeric coiled coil showed by gray is formed. So far the C-peptide is not associated with the N-terminal as shown by blue. The prehairpin intermediate is relatively long lived and is susceptible to C-peptide inhibition. The step at which HIV fusion inhibitors work is shown by blue rods binding to the N-terminal central coiled coil. After the prehairpin intermediate stage the C-peptide regions of the gp41 ectodomain interact with the N-terminal central coiled coils by packaging into the N-terminal hydrophobic grooves, to form six helical bundles that result in viral and cell membrane appositions. The mechanism as to how these membranes fuse is unknown as indicated by (?).

1.5 Coreceptor usage and cell tropism

During entry into the host cell HIV-1 sequentially uses CD4 as well as the seven transmembrane G protein coupled receptors (Chan *et al.*, 1999). CCR5 and CXCR4 are the principal chemokine coreceptors used by HIV-1 for cell entry, however, the use of other chemokine receptors and orphan receptors has been described *in vitro* (Chan *et al.*, 1999). HIV-1 coreceptor usage is the main determinant of cell tropism (Shimizu *et al.*, 1999). Furthermore, the ability of HIV-1 isolates to use different

coreceptors and to utilize different cell types determines AIDS pathogenesis (Shimizu *et al.*, 1999). CCR5 is the major coreceptor used by HIV-1 isolates that infect macrophages hence they are called macrophage tropic (M-tropic) isolates (R5 viruses) and are non-syncytium inducing (NSI) in an MT-2 cell line (Hill *et al.*, 1997). On the other hand, CXCR4 is a coreceptor utilized by T cell line infecting viruses called T-tropic viruses (X4 viruses) and are syncytium inducing (SI) in an MT-2 cell line (Hill *et al.*, 1997); (Wang *et al.*, 2000). Dual tropic viruses use CCR5 and/or CXCR4 for entry into the host cells and are referred to as R5X4 viruses. Furthermore, HIV-1 isolates that use other coreceptors for entry into the host cell such as CCR3, CCR8, BOBGPR 15 (Bob), STRL33 (Bonzo), GPR1, V28/CX₃R1, ChemR23, leukotriene B4 receptor, Apj and human cytomegalovirus (HCMV) encoded US8 have been described (Chan *et al.*, 1999); (Li *et al.*, 1999); (Cecilia *et al.*, 2000). In general, R5 viruses infect macrophages only and cannot infect T cell lines. However, a study has demonstrated that macrophages can express CXCR4 in low quantities (Tokunaga *et al.*, 2001). The same group described two primary X4 viruses that infected macrophages by using the CXCR4 coreceptor only (Tokunaga *et al.*, 2001).

R5 viruses play an important role in sexual, vertical and blood borne HIV-1 transmission and are the predominant viral populations immediately after seroconversion and during asymptomatic infection (Jansson *et al.*, 1999); (Kolchinsky *et al.*, 1999); (Li *et al.*, 1999); (Wang *et al.*, 2000); (Singh and Collman, 2000). A study by Salvatori and Scarlatti, (2001) has suggested that multiple tropic viruses can be transmitted from mother to child. As AIDS develop, 40-50% of individuals infected with HIV subtype B experience a switch in coreceptor use from R5, NSI viruses to X4, SI viruses (Chan *et al.*, 1999); (Li *et al.*, 1999). The emergence of X4

strains that are characterised by high replication kinetics may be responsible for the rapid immune destruction. Although X4 or R5X4 isolates are present at the later stages of the disease, R5 strains are transmitted and/or initiate infection. This reflects the persistence of R5 variants along side with the CXCR4 using SI isolates in the later stage of AIDS (Singh and Collman, 2000). HIV-1 subtype C isolates from India were shown to exclusively use CCR5 coreceptors in advanced AIDS patients (Cecilia *et al.*, 2000). This was confirmed by Morris *et al.*, (2001) who demonstrated that HIV-1 subtype C isolates from South African AIDS patients with tuberculosis (TB) used only the CCR5 coreceptor to enter into the host cells. Studies suggest that the prevalence of TB in HIV/AIDS patients results in the upregulation of the CCR5 coreceptor expression, thus selecting CCR5 using viruses in the late stages of the disease (Fraziano *et al.*, 1999). Furthermore, in Africa, environmental factors have been shown to enhance the expression of the CCR5 coreceptor (Clerici *et al.*, 2000).

1.5.1 Effects of the V1/V2 and V3 loop on coreceptor usage and cell tropism

The V3 loop of gp120 plays a major role in determining HIV-1 coreceptor usage (Shimizu *et al.*, 1999). The V3 domain forms a loop structure and its tip sequence is well conserved among the different HIV-1 subtypes, for example HIV-1 subtype B and subtype C display a characteristic GPGR and GPGQ V3 tip, respectively (Shimizu *et al.*, 1999); (Su *et al.*, 2000). Single amino acid changes in the V3 loop can drastically alter coreceptor specificity (Chan *et al.*, 1999). Several studies have shown that basic amino acid substitutions at one or more positions of the V3 loop, such as 307 and 312 confer the ability of M-tropic viruses to use CXCR4 and induce syncytia (Harrowe and Cheng-Mayer, 1995); (Cheng-Mayer *et al.*, 1997). Furthermore, basic amino acid substitution at positions 306, 321, 322 and 328 result in an increase in the

overall positive charge in the V3 region allowing the HIV-1 strains SF2, SF162, R32, Mu3, Db1 and MuG2 to switch from using CCR5 to CXCR4 (Chesebro *et al.*, 1996); (Speck *et al.*, 1997); (Chan *et al.*, 1999). Interestingly, Su *et al.*, (2000) demonstrated that deletion of the three conserved GPG amino acids in the V3 region did not affect the infectivity of HIV-1 in different cell types.

Conflicting data exists concerning the correlation of the V1/V2 loops of gp120 and the biological phenotype of the virus tropism (Andeweg *et al.*, 1993); (Sullivan *et al.*, 1993); (Koito *et al.*, 1994); (Koito *et al.*, 1995); (Wang *et al.*, 1995), primarily because these studies were undertaken before the CCR5 and CXCR4 coreceptors were identified. Several studies have shown that the V1/V2 sequences in combination with the V3 region influence coreceptor usage (Shimizu *et al.*, 1999); (Su *et al.*, 2000). The role of these hypervariable regions in cell tropism needs to be investigated further, since there is high V1/V2 sequence diversity between HIV-1 isolates.

1.6 Influence of glycosylation on the structure and function of HIV-1 envelope glycoproteins

The HIV-1 envelope glycoprotein is heavily glycosylated with 50% of its molecular mass consisting of glycans (Dirckx *et al.*, 1990); (Fenouillet and Jones, 1995). The gp120 contains approximately 23 to 24 asparagine (N)-linked glycosylation sites, while the gp41 contains three to four N-linked glycosylation sites (Schawaller *et al.*, 1989); (Lee *et al.*, 1992); (Moore *et al.*, 1993); (Fenouillet and Jones, 1995); (Johnson *et al.*, 2001); (Pollakis *et al.*, 2001). The envelope glycoproteins obtained from different HIV-1 viruses were shown to contain mainly, if not exclusively, N-linked

sugars of the high mannose type, the complex type and the hybrid type (Lee *et al.*, 1992); (Fenouillet and Jones, 1995); (Johnson *et al.*, 2001). Carbohydrate moieties on the HIV-1 envelope glycoprotein affect the intracellular transport of these proteins and their structure, and therefore influence the viral phenotype (Ly and Stamatatos, 2000); (Malenbaum *et al.*, 2000). N-linked sugars are essential during biosynthesis of gp160 to sustain the conformation required for envelope function activities (Li *et al.*, 1993); (Fenouillet and Jones, 1995). Several studies have shown that the lack of glycosylation during biosynthesis disrupts gp160 and impairs the envelope mediated fusion capability (Li *et al.*, 1993); (Moore *et al.*, 1993); (Ogert *et al.*, 2001). Furthermore, studies have found that blocking the formation of N-linked sugars or its high mannose type reduces HIV-1 infectivity (Lee *et al.*, 1992); (Fenouillet and Jones, 1995).

N-linked glycosylation in the V2 region was found to affect the binding of the envelope glycoprotein to the CD4 receptor. The removal of N-linked sugars at positions 154 and 195 in the V2 domain of the HIV-1 isolate SF162 viral envelope increased the ability of the glycoprotein to interact with the CD4 receptor but reduced the effective interaction with the CCR5 coreceptor (Ly and Stamatatos, 2000). The removal of the glycosylation site at position 160 in the V2 region of the HIV-1 isolate DH12 completely eliminated the ability of this dual tropic virus to use either the CXCR4 and/or CCR5 coreceptors (Ogert *et al.*, 2001). The absence and presence of glycosylation sites in the V1 region were shown not to influence coreceptor utilization. However, Pollakis *et al.*, (2001) found that removal of glycosylation sites in the V1 region allowed the HIV-1 isolate HXB2 to utilize CCR3 coreceptors. The

mechanism as to how deglycosylation in the V1 region allows the HXB2 virus to use other minor coreceptors need to be investigated further.

N-linked glycosylation within the V3 region of the envelope glycoprotein is significant in determining CXCR4 utilization and in detecting a phenotypic switch from CCR5 to CXCR4 usage (Ogert *et al.*, 2001); (Pollakis *et al.*, 2001). Pollakis *et al.*, (2001) used chimeric viruses possessing different high positive charges in the V3 domain similar to X4 viruses and demonstrated that these chimeras were capable of inducing syncytia. However, this did not alter their R5 phenotype, since they were not able to infect U87.CD4⁺ cells expressing CXCR4. On the other hand, the loss of N-linked glycosylation sites in the V3 region, particularly at position N301 allowed the chimeric viruses with high positive charge to acquire the strong usage of CXCR4, and the ability to use CCR3, and lose the ability to use CCR5 (Pollakis *et al.*, 2001). At the lower V3 loop charges (+3 and +4), no phenotypic changes were observed after the alteration of the N-linked glycosylation site (Pollakis *et al.*, 2001). This is in agreement with observations by Ogert *et al.*, (2001) who showed that the loss of the glycosylation site at position N301 in the V3 region of the dual tropic virus DH12 completely abolished the ability to use CCR5. Furthermore glycosylation site mutations near the V1/V2 loop compromised the use of CCR5 and CXCR4 equally. Upon viral culture, the isolate gained compensatory glycosylation sites in the V1/V2 and V3 regions (but not N301) that subsequently allowed it to use CXCR4, but the ability to use CCR5 was not restored. These findings demonstrate that the increase in positive charge in the V3 loop is not the sole determinant of coreceptor usage. Moreover, these observations indicate that as the positive charge increases in the V3 domain, the affinity of the envelope for CXCR4 is enhanced, but the N-linked glycan

in the V3 region inhibits the virus from being able to utilize CXCR4. Thus the presence of N-linked glycosylation sites in the V3 region particularly N301 allows a virus with a high charge in the V3 loop to maintain CCR5 usage.

A comparison of amino acid sequences from primary HIV-1 isolates from the Los Alamos data base has found a strong correlation between the high V3 positive charge and the loss of N-linked glycosylation sites in the V3 loop for all HIV-1 subtypes except for HIV-1 subtype C (Pollakis *et al.*, 2001). The HIV-1 subtype C isolates from South Africa described by Morris *et al.*, (2001) have a low positive charge in the V3 loop. The same group has recently described HIV-1 subtype C isolates that can use the CXCR4 coreceptor and exhibit a higher positive charge (+6 to +8) in the V3 loop (Cilliers *et al.*, in preparation). Whether these HIV-1 subtype C isolates with a higher positive charge lack glycosylation sites in the V3 loop, particularly at position N301, is under investigation.

Most significantly, the removal of the terminal glycosylation sites in the V3 loop conferred various degrees of enhanced activity to neutralization by monoclonal antibodies directed against the CD4bs (Malenbaum *et al.*, 2000).

1.7 CD4-independent entry of HIV-1 into the host cells

Although the envelope glycoprotein of HIV-1 promotes viral entry by sequentially binding to CD4 and coreceptors, some isolates have been described that productively infect cells in the absence of CD4. An HIV-2 variant (HIV-2/vcp) was shown to enter CD4 negative cells using CXCR4 (Endres *et al.*, 1996). Furthermore, an SIV strain

(SIVmac239) was shown to infect CD4 negative brain capillary endothelial cells and other cell types by using CCR5 as a primary receptor (Edinger *et al.*, 1997).

Primary HIV-1 isolates that directly bind to CCR5 are rare but have been generated in a laboratory, and include the HIV-1 subtype B isolates IIB (La Blanche *et al.*, 1999), HXBc2 (Edwards *et al.*, 2001) and ADA (Kolchinsky *et al.*, 1999). The changes in the envelope glycoprotein of HIV-1 subtype B ADA responsible for the adaptation of CCR5 as a major receptor occurred in the V2 loop and at the V1/V2 stem (Kolchinsky *et al.*, 1999). Furthermore, the V1/V2 changes in the CD4 independent viruses include the removal of an N-linked glycosylation site at position 197 (Kolchinsky *et al.*, 2001). Moreover, deletion of the complete V1/V2 loops of the envelope glycoprotein was sufficient for this primary HIV-1 isolate to acquire CD4 independent entry (Kolchinsky *et al.*, 2001). These observations suggest that the major function of the CD4 binding can be bypassed by changes in the gp120 V1/V2 elements, which allow the envelope glycoprotein to assume a conformation competent for CCR5 binding. In another study, changes in the V1/V2 stem along with the V3 loop were also shown to contribute to the acquisition of CD4 independence by the CXCR4 using HIV-1 isolate (Edinger *et al.*, 1997).

CD4 independent viruses have been shown to be significantly sensitive to neutralization by soluble CD4 and a variety of antibodies (Kolchinsky *et al.*, 2001). The CD4 independent envelope glycoproteins exhibit higher affinity for antibodies against CD4 induced epitopes but not other neutralizing ligands (Kolchinsky *et al.*, 2001).

1.8 Antigenicity and Immunogenicity of HIV-1 envelope glycoproteins

The envelope glycoprotein on the surface of the virion and/or infected cell is the primary target for neutralizing antibodies against HIV-1. Antibodies against the envelope glycoproteins can be detected in the sera of HIV-1 infected individuals within 2 to 3 weeks after infection, however, most of these antibodies are unable to neutralise HIV-1 infection (Moore and Ho, 1993); (Wyatt and Sodroski, 1998); (Sanders *et al.*, 2000). Antibodies that are capable of neutralizing HIV-1 develop later usually after a few months to 1 year (Moore and Ho, 1993); (Burton and Parren, 2000); (Nunberg *et al.*, 2000). By the time antibodies develop HIV-1 infection is already established and they are incapable of neutralizing the virus. Five potent human monoclonal antibodies that are capable of neutralizing HIV-1 viruses have been identified from HIV-1 infected individuals (Posner *et al.*, 1991); (Burton *et al.*, 1994). These include IgG1B12, 2G12, 2F5, 4E10 and Z13 monoclonal antibodies. IgG1B12 is a potent cross clade neutralizing monoclonal antibody that recognizes the epitope spanning the CD4 binding site and blocks the interaction of the gp120 with CD4 receptors on the surface of the host cells (McKeating *et al.*, 1999); (Schutter *et al.*, 1993); (Wyatt *et al.*, 1998). The 2G12 epitope is formed by high mannose N-linked glycans at position 295, 332, 386, 395 and 448 of the gp120 (Wyatt *et al.*, 1998); (Sanders *et al.*, 2002a). 2F5 is the monoclonal antibody that recognises the linear ELDKWA epitope of the gp41 (Wyatt and Sodroski, 1998); (Nunberg *et al.*, 2000). The Z13 and 4E10 monoclonal antibodies recognize the region immediately C-terminal of the 2F5 epitope (Zwick *et al.*, 2001). Interestingly, 2G12 and 2F5 have been shown not to neutralize HIV-1 subtype C viruses (Bures *et al.*, 2002). The IgG1B12, 2G12 and 2F5 monoclonal antibodies have been shown to protect against infection and/or disease in passive immunization studies in animal models (Parren *et*

al., 1995); (Conley *et al.*, 1996); (Mascola *et al.*, 1999); (Mascola *et al.*, 2000); (Armbruster *et al.*, 2002). However, in natural infection few individuals are able to generate these potentially neutralizing antibodies and if generated they are at a very low titres and appear too late to effectively control HIV-1 infection.

The HIV-1 envelope glycoprotein has evolved successful ways to limit the generation of neutralizing antibodies and/or minimize their effects on its life cycle (Moore and Binley, 1998); (Wyatt and Sodroski, 1998); (Burton and Parren, 2000). Lability, variable regions and extensive glycosylation of the envelope glycoprotein are the main factors that contribute to its poor antigenicity and immunogenicity (Kwong *et al.*, 1998); (Wyatt *et al.*, 1998); (Wyatt and Sodroski, 1998). During natural infection the gp120 dissociates from the trimer and the disassembled proteins elicit most of the antibodies. Antibodies directed against this viral debris cannot bind to the functional trimeric envelope glycoprotein complex, therefore they don't exhibit neutralizing activities (Burton, 1997); (Parren *et al.*, 1997); (Moore and Binley, 1998). The disassembled envelope glycoproteins might not only generate an irrelevant immune response, but may even actively decoy antibody production away from functionally important epitopes of the envelope glycoprotein complex (Moore and Binley, 1998).

The V1/V2 and V3 loops reside proximal to the CD4 and chemokine binding site, masking the conserved gp120 elements and present potentially variable epitopes to the immune system (Wyatt *et al.*, 1998); (Wyatt and Sodroski, 1998); (Rizzuto *et al.*, 1998). Furthermore, variable loops minimize the time and the space available for the antibody to interfere with HIV-1 infection (Moore and Binley, 1998); (Rizzuto *et al.*, 1998); (Wyatt and Sodroski, 1998). Variable and conserved regions of the envelope glycoprotein are extensive glycosylated and thus glycosylation shields the

presentation of conserved epitopes to the immune system (Wyatt *et al.*, 1998); (Wyatt and Sodroski, 1998). Most of the carbohydrates may appear as 'self' to the immune system, thus limiting the effect of the neutralizing antibodies (Wyatt and Sodroski, 1998).

Studies have shown that it is possible to raise neutralizing antibodies against HIV-1 using a recombinant envelope glycoprotein as a vaccine immunogen (Moore *et al.*, 1993); (Jones *et al.*, 1995); (LaCasse *et al.*, 1999). However, so far antibodies raised by these immunogens can only efficiently neutralize T-cell line adapted viruses (TCLA) and have little or no activity against circulating primary (clinical) isolates (PI) (Moore *et al.*, 1993). These immunogens probably raise irrelevant antibodies that cannot bind to the native oligomeric envelope glycoproteins on the surface of the virion and therefore are not able to neutralize PI viruses (Binley *et al.*, 2000); (Yang *et al.*, 2000). Studies have shown that there are antigenic differences between the monomeric and oligomeric envelope glycoproteins and between TCLA versus PI viruses (Fouts *et al.*, 1997), (Brand *et al.*, 2000). Most of the epitopes that are present on monomeric envelope glycoproteins are not available or occluded on the oligomeric envelope glycoprotein complexes. Similarly, epitopes that are presented by TCLA viruses are not available or occluded on PI viruses (Trkola *et al.*, 1995); (Fouts *et al.*, 1997). Studies have shown that TCLA viruses assume a more 'open' configuration of the envelope complex compared to PI viruses making them more susceptible to antibody neutralization (Trkola *et al.*, 1995); (Fouts *et al.*, 1997).

Moore and Ho, (1995) and Burton and Parren, (2000) have shown that the binding of antibodies to oligomeric envelope glycoprotein trimers correlates with the

neutralization of PI viruses. Thus the desirable immunogen must mimic the oligomeric envelope glycoprotein trimer found on the surface of the virion or the infected cells. The development of an effective immunogen has been frustrated by the lability of the native envelope glycoprotein, which makes it difficult to obtain soluble trimeric envelope glycoproteins (Binley *et al.*, 2000); (Yang *et al.*, 2000). Various mechanisms have been formulated to stabilize the association between gp120 and gp41 in a trimer in an attempt to produce an immunogen that mimics the trimeric envelope glycoprotein complex (Binley *et al.*, 2000); (Yang *et al.*, 2000); (Zhang *et al.*, 2001); (Grundner *et al.*, 2002); (Sanders *et al.*, 2002b). The modifications include the addition of disulphide bonds, disruption of the cleavage site, deletion of variable loops, manipulation of the amino acids and addition of a GCN4 trimeric helix and T4 bacteriophage fibrin at the C-terminus of the gp41 (Binley *et al.*, 2000); (Sanders *et al.*, 2002b); (Yang *et al.*, 2000); (Zhang *et al.*, 2001); (Grundner *et al.*, 2002); (Sanders *et al.*, 2002b); (Yang *et al.*, 2002). Most of the abovementioned modifications have been performed on the envelope glycoproteins derive from HIV-1 subtype B viruses.

Binley *et al.*, (2000) have modified envelope glycoproteins by adding the disulphide bonds between the C1 and C5 of the gp120 region and an immunodominant region of gp41 to produce an immunogen termed SOS gp140 (gp160 without the transmembrane region of the gp41). The produced SOS gp140 was shown to exhibit antigenic properties similar to the trimeric envelope glycoprotein (Binley *et al.*, 2000). However, following purification this immunogen was shown to fall apart and form monomers (Schulke *et al.*, 2002). Recently the modified version of SOS gp140 has been produced by mutating the isoleucine to proline at position 559 in the N-terminal

repeat region of the gp41 to disrupt the formation of the six helical bundle, thus promoting the stabilization of the trimeric envelope glycoproteins in the prefusogenic state (Sanders *et al.*, 2002b). This immunogen is called SOSIP gp140 and was shown to have antigenic properties similar to those of the trimer, however, it remains empirical whether it will be able to induce potent neutralizing antibodies against PI viruses (Sanders *et al.*, 2002b).

Yang *et al.*, (2000) produced an immunogen that have antigenic properties similar to those of the trimeric envelope glycoprotein by replacing arginine residues at position 508 and 511 with serine to disrupt the proteolytic cleavage site. This immunogen was further manipulated by extending the N-terminal coiled coil with GCN4 sequences at the C terminus of the gp41 and was called gp140 683 (-/GCN4). This immunogen was able to induce neutralizing antibodies against homologous PI viruses but could not neutralize PI viruses from other subtypes (Yang *et al.*, 2001). However, this finding is significant since it indicates for the first time that it is possible to raise neutralizing antibodies against PI using a modified envelope glycoprotein as a vaccine immunogen.

Grunder *et al.*, (2002) recently described the creation of solid-phase gp160 Δ CT proteoliposomes (PLs) as an approach to generate better mimics of the functional HIV-1 envelope glycoprotein trimer. This approach required elimination of the gp120-gp41 cleavage site but no other modifications of the envelope glycoprotein ectodomain. An intact gp41 transmembrane region is maintained, allowing the trimeric envelope glycoprotein complex to be embedded in a reconstituted lipid bilayer in a natural context.

More studies are needed to determine what factors keep the association of the g120 and gp41 subunits in the trimeric envelope glycoproteins in the prefusogenic state (Moore *et al.*, 2001). Identification of such factors is the key in producing a vaccine immunogen that will raise potent broad cross clade neutralizing antibodies against circulating HIV-1 PI.

1.9 Antiviral ligands and agents targeting HIV-1 envelope glycoproteins

The introduction of antiretroviral therapy in 1996 has had a major impact on the AIDS epidemic by changing the morbidity and mortality patterns of AIDS patients. These drugs decrease the viral load and increase CD4 counts, thus improving the well-being of HIV/AIDS patients. The currently available antiretroviral drugs for HIV-1 infection interfere with the function of one of the two viral specific enzymes, namely reverse transcriptase and protease (Kilby *et al.*, 1998); (Derdeyn *et al.*, 2000). Highly active antiretroviral therapy (HAART) regimens targeting these enzymes have a strong antiviral activity. However, many patients are intolerable to the available drugs and develop virological failure because of incomplete viral suppression and emergence of drug resistance (Kilby *et al.*, 1998); (Ruff *et al.*, 2001). The development of a novel class of antiretroviral agents targeting different stages of the viral life cycle with different toxicity profiles is an important challenge.

Entry inhibitors have evolved as potential therapeutic agents that prevent the entry of HIV-1 into the host cells. Several ligands exist naturally and others have been developed that inhibit the interaction of the envelope glycoproteins with host cells at the various stages of cell entry. These ligands block HIV-1 infection by preventing the

interaction of gp120 with the host cell surface CD4 receptors, by blocking the gp120-CD4 complex interaction with the chemokine receptors, or by preventing the fusion of the viral and host cell membrane.

PRO542 is a potent entry inhibitor consisting of four domains of CD4 and prevents HIV-1 infection by blocking the interaction of gp120 with the host surface CD4 receptors (Jacobson *et al.*, 2000). This agent is effective against diverse HIV-1 isolates that are encountered clinically (Jacobson *et al.*, 2000). PRO542 is currently in the second phase of human clinical trials. It was shown to be tolerable in humans and significantly decreased the viral load in phase 1 clinical trials (Jacobson *et al.*, 2000).

The natural ligands, RANTES, MIP-1 α , MIP-1 β and SDF-1 bind to chemokine receptors, such as CCR5 and CXCR4, thus preventing the HIV-1 envelope glycoprotein interaction with the chemokine receptors (Alkhatib *et al.*, 1996); (Doranz *et al.*, 1996). These natural ligands prevent HIV-1 infection by blocking and/or down-regulating chemokine receptors (Jansson *et al.*, 1999). The TCLA viruses are blocked by the SDF-1 α ligand that binds to the CXCR4 coreceptor, whereas M-tropic viruses are inhibited by the β chemokines: RANTES, MIP-1 α and MIP-1 β that bind to CCR5 (Alkhatib *et al.*, 1996). The third variable (V3) loop of the gp120 is important in the gp120-CCR5 interaction and is the main determinant of virus susceptibility to inhibition by β chemokines (Jansson *et al.*, 1999). Single amino acid changes in the V3 region confer resistance to viral sensitivity to β chemokines (Jansson *et al.*, 1999).

CCR5 and CXCR4 antagonists have been developed that block the interaction of the gp120-CD4 complex with the chemokine receptors (Baba *et al.*, 1999). CCR5

antagonists include the nonpeptide compounds TAK799, PRO140, 2D7, anti-CCR5 monoclonal antibodies and derivatives of RANTES (Baba *et al.*, 1999). CXCR4 inhibitors include T22, AMD3100, ALX40c, 12G5 and derivatives of SDF-1. AMD3100 targets the second and the third extracellular loops of CXCR4 and is currently in phase I/II human clinical trials (Baba *et al.*, 1999).

Studies have shown that the heptad repeats (HR) HR1 and HR2 in the gp41 domain are essential for virus cell-to-cell mediated membrane fusion to occur. A synthetic peptide, DP178 containing amino acids 127 to 162 of the HIV-1 gp41 was shown to inhibit HIV-1 infection and virus cell-to-cell mediated fusion (Rimsky *et al.*, 1998). The DP178 synthetic peptide binds to the C-peptide (HR2) preventing the formation of the coiled coil, thus disrupting the gp41 conformational changes needed to mediate viral and cell membrane fusion (Rimsky *et al.*, 1998); (Kilby *et al.*, 1998); (Jin *et al.*, 2000). A 36 amino acid peptide, corresponding to DP178 called T20 was found to be a particularly potent inhibitor of HIV-1 in T cell lines (50% inhibitory concentration $IC_{50} = 1.7\text{ng/ml}$ (Kilby *et al.*, 1998). Currently, T20 is at the third phase of human clinical trial testing and is promising to be a potent novel antiretroviral drug (www.trimerics.com). Mutations in HR1 regions that result in resistance to T20 have already been identified (Rimsky *et al.*, 1998). A derivative of T20, called T1249 has been developed and has a similar mechanism of action as T20. Most importantly, HIV-1 does not develop resistance to T1249 as easily as to T20 and is currently in the first phase of human clinical trials (www.trimerics.com).

Peptide T derived from the V2 region of HIV-1 gp120 has been shown to inhibit the replication of R5 and dual tropic viruses, whereas it has little or no inhibitory effect

on X4 viruses (Ruff *et al.*, 2001). Peptide T suppresses viral replication by blocking the chemokine receptor CCR5 (Ruff *et al.*, 2001). The effectiveness of peptide T blockade is more potent and significant on CCR5 coreceptors compared to CXCR4 coreceptors. The development of nontoxic treatments like peptide T which suppress CCR5 using viruses which work by different mechanisms may be useful to provide novel therapeutic options.

It is thus important to study the structure and function of the HIV-1 envelope glycoproteins and their interaction with the host cells with the possibility of identifying novel target sites for drug development.

1.10 Specific objectives of this study

South Africa has one of the fastest growing HIV-1 epidemics, with an estimated 4.7 million people infected out of a total of 40.6 million (Research, 2001). HIV-1 subtype C is the predominant circulating subtype among heterosexual group in South Africa (van Harmelen *et al.*, 2001). During the early stages of the epidemic, HIV-1 subtype C isolates in South Africa were shown to use the CCR5 coreceptor during all stages of disease. Morris *et al.*, (2001) showed that early HIV-1 subtype C isolates from South African AIDS patients with TB used the CCR5 coreceptor. However, the same group has recently identified HIV-1 subtype C viruses that can use CXCR4 and other coreceptors. This implies that there has been ongoing evolution of HIV-1 subtype C viruses circulating in South Africa. Thus the biological properties of the HIV-1 envelope glycoproteins from subtype C viruses with different phenotypic

characteristics need to be investigated. This has implications for HIV-1 vaccine and drug development studies.

The specific objectives of this study were:

- 1 To construct baculovirus recombinants that express full length, functional envelope glycoproteins (gp160) from selected circulating HIV-1 subtype C isolates with different phenotypic characteristics.
- 2 To obtain purified recombinant envelope glycoproteins in the correct conformation, and in sufficient quantities.
- 3 To examine and compare the biological properties of the purified envelope glycoproteins.

CHAPTER 2

CLONING AND EXPRESSION OF HIV-1 SUBTYPE C ENVELOPE GLYCOPROTEINS IN INSECT CELLS

2.1 INTRODUCTION

The envelope glycoprotein facilitates the entry of HIV-1 into the host cell and plays an important role in AIDS pathogenesis. The HIV-1 envelope glycoprotein (gp160) is synthesized in the endoplasmic reticulum as a precursor polypeptide. It undergoes posttranslational modifications and is cleaved in the Golgi compartments by cellular proteases into the gp120 extracellular subunit and the gp41 transmembrane subunit (Wells and Compans, 1990); (Yeh *et al.*, 1993). In their native conformation, the envelope glycoproteins form trimeric spikes that consist of three gp120 subunits noncovalently associated with three gp41 moieties. These trimeric envelope glycoprotein spikes are exposed on the surface of the virions or infected cells (Binley *et al.*, 2000); (Brand *et al.*, 2000). The gp160 initiates viral entry by binding the gp120 to the host CD4 receptor. This binding induces conformational changes in the gp120 that allow it to bind to the chemokine coreceptors, mainly CCR5 and CXCR4 (Bristow *et al.*, 1994); (Brand *et al.*, 2000). The envelope glycoprotein undergoes further conformational changes, exposing the fusion peptide of the gp41 that brings the viral and host cell membranes together. This is ensued by the formation of the six helical bundle in the gp41 that results in the fusion of the viral and the host cell membrane (Chan and Kim, 1998); (Jiang *et al.*, 1998).

The gp160 is a major target for HIV-1 vaccine based strategies. Several researchers have expressed recombinant gp160, gp140 (gp160 with a truncation in the transmembrane region of the gp41) and gp120 in different expression systems for use as a vaccine immunogen, and for further structure, function, antigenicity and immunogenicity studies (Binley *et al.*, 2000); (Zhang *et al.*, 2001). These include bacterial, baculovirus and mammalian expression systems. The envelope glycoproteins expressed in bacterial systems are nonglycosylated, improperly folded and lack appropriate posttranslation modifications, thus rendering the envelope glycoprotein nonfunctional (Morikawa *et al.*, 1990). The mammalian and baculovirus expression systems are effective in overcoming these problems. The gp160 expressed in mammalian systems undergoes posttranslation modifications in a similar manner to those that occur in the human host cells (Kaufman *et al.*, 2000). The production of the gp160 in mammalian systems is critical because 50% of its molecular mass consists of carbohydrates (Rhodes *et al.*, 1994); (Kaufman *et al.*, 2000). However, the expression of HIV-1 gp160 in mammalian systems is regulated by the viral Rev protein (Ivey-Hoyle and Rosenberg, 1990), and is produced only when coexpressed with Rev. Furthermore, the levels of gp160 produced in mammalian systems are lower compared to those obtained in a baculovirus expression system (Moore *et al.*, 1990).

The baculovirus expression system has been used to express recombinant gp160 under the control of the strong polyhedrin promoter (Rusche *et al.*, 1987); (Wells and Compans, 1990); (Moore *et al.*, 1990); (Yeh *et al.*, 1993); (Bristow *et al.*, 1994). The gp160 expressed in insect cells undergoes posttranslation modifications such as proteolysis, N-and O-linked glycosylation, acylation, amidation, carboxylation,

phosphorylation and prenylation (Yeh *et al.*, 1993). The envelope glycoproteins are produced at high yields in insect cells, and are immunologically, antigenically and biologically active (Wells and Compans, 1990); (Moore *et al.*, 1990). Thus, the baculovirus expression system is suitable for the expression of HIV-1 envelope glycoproteins.

HIV-1 subtype C isolates circulating in South Africa have been shown to predominantly use the CCR5 coreceptors for cell entry both during early and advanced stages of AIDS disease (Morris *et al.*, 2001). This is in contrast to HIV-1 subtype B viruses, where it has been shown that up to 40% of the isolates at the late stages of the disease undergo a coreceptor switch to use CXCR4 for cell entry (Chan *et al.*, 1999); (Li *et al.*, 1999); (Nelson *et al.*, 2000). However, Cilliers *et al.*, (in preparation) have identified HIV-1 subtype C isolates that use the CXCR4 coreceptor, and dual tropic viruses that use both CCR5 and CXCR4 for cell entry. This is the first report of these viruses circulating in South Africa. It is thus important to define the biological characteristics displayed by the envelope glycoproteins from HIV-1 subtype C that use these different coreceptors.

Cloning, expression and characterization of the recombinant gp160 from predominantly subtype B has elucidated the HIV-1 envelope glycoprotein structure, function, antigenicity and immunogenicity. These recombinant proteins have significant potential to facilitate in the development of an effective HIV-1 vaccine. To date, HIV-1 subtype C envelope glycoproteins with different phenotypes have not been purified on a large scale, or characterized. The purpose of this study was to clone HIV-1 subtype C full-length envelope genes from isolates that use CCR5, CXCR4, or

both coreceptors into a baculovirus expression vector, construct recombinant baculoviruses and express the recombinant gp160 in *Spodoptera frugiperda* (Sf9) insect cells.

2.2 MATERIALS AND METHODS

2.2.1 HIV-1 subtype C isolates

Numerous HIV-1 isolates from South African patients have been generated in the last 3 to 4 years at the National Institute for Communicable Diseases. HIV-1 viruses were isolated from patients peripheral blood mononuclear cells (PBMCs) by cocultivation with phytohemagglutinin-stimulated donor PBMCs. Phenotype and coreceptor usage of each strain was established by infecting MT2 cells and U87.CD4 cells expressing CCR5 and CXCR4, respectively (Cilliers *et al.*, in preparation). Four HIV-1 subtype C isolates with different phenotypic characteristics were selected for the purposes of this study (obtained from Prof L. Morris). These included isolates 98ZA151Du (Du151), 99ZASW7 (SW7), 99ZACM9 (CM9) and 98ZA179Du (Du179). The full-length genome sequences of these isolates are available (van Harmelen *et al.*, 2001); (Papathanasopoulous *et al.*, 2002). Clinical information of the patients and the biological phenotype of these isolates is shown in Table 2.1.

2.2.2 Proviral DNA isolation and PCR amplification of the full-length HIV-1 envelope (*env*) gene

Proviral DNA from each strain was extracted from HIV-1 cocultured PBMCs using the High Pure PCR Template Preparation kit (Roche, Germany) as per manufacturers instructions and stored at -20°C . The full-length *env* gene (~2.5 kb) was amplified in a nested PCR reaction. The primers and cycle conditions used were optimised to obtain a single PCR product of the correct molecular size. Primers *env* A and *env* N (Table 2.2); (Gao *et al.*, 1996) were used in the first round PCR amplification using the Expand High Fidelity PCR System (Roche). The optimal first round PCR reaction

contained two master mixes. Master mix 1 contained 10 μ l dNTPs (1mM), 10 μ l proviral DNA and 4 μ l each of *env* A and *env* N (25 pmoles/ μ l) and 22 μ l of water to a final volume of 50 μ l. Master mix 2 contained 10 μ l of 10X buffer, 0.75 μ l enzyme and 39.25 μ l of water to a final volume of 50 μ l. Immediately before cycling, master mix 1 and 2 were combined and PCR amplified in a GeneAmp PCR system 9700 (Perkin Elmer). The optimal cycle conditions included: 1 cycle of denaturation at 94°C for 3 minutes (min), followed by 30 cycles at 94°C for 15 seconds (sec), 61°C for 30 sec and 72°C for 3 min. This was then ensued by a final elongation cycle at 72°C for 10 min and a holding temperature of 4°C.

Primers containing *Hind*III and *Bam*H1 restriction sites namely MAP2 and MAP3 (Table 2.2), respectively were designed and used for the second round PCR amplification. The second round PCR reaction mix contained 5 μ l of 10X buffer, 10 μ l dNTPs (1mM), 2.5 μ l each of MAP2 and MAP3 primers (5 pmoles/ μ l), 2.7 μ l of MgCl₂ (25mM), 2 μ l DNA from the first round PCR product, 0.3 μ l Supertherm Taq (Southern Cross Biotechnologies, South Africa) and 25 μ l of water to a final volume of 50 μ l. PCR conditions included 1 cycle of denaturation at 94°C for 3min. This was ensued by 30 cycles at 94°C for 30 sec, 62°C for 45 sec and 72°C for 3 min. This was followed by a final elongation at 72°C for 10 min, and a holding temperature at 4°C. The amplified PCR products were analyzed by horizontal gel electrophoresis as described by Sambrook *et al.*, (1989), in 1X Tris-Acetate-EDTA (TAE) buffer (pH8.0; Appendix A). Gels contained 1% agarose (Gibco, Scotland) and 10 μ g/ml ethidium bromide (Biorad, USA) (Appendix A) and electrophoresis was performed at a constant voltage (100V) for 2 hours. Gels were viewed with a UV transilluminator

and photographed. The ~2.5kb fragment for each isolate was excised from the gel and purified using the High Pure PCR Product Purification Kit according to manufacturers instructions (Roche).

TABLE 2.1: Clinical information of HIV-1 seropositive patients and biological phenotype of four HIV-1 subtype C isolates from South Africa.

Isolate	Clinical Status	Source	CD4 counts cells/ μ l	Viral load RNA copies/ml	MT-2 assay	Coreceptor	Designation
98ZA151Du	Asymptomatic	PBMC	367	500,00	NSI	CCR5	R5
99ZACM9	Cryptococcal meningitis	PBMC	24	42,545	SI	CCR5/CXCR4	R5X4
98ZA179Du	Asymptomatic	PBMC	394	1,359	SI	CCR5/CXCR4	R5X4
99ZASW7	Pulmonary TB	PBMC	10	n/a	SI	CXCR4	X4

PBMC – Peripheral blood mononuclear cells NSI- Non-syncytium-inducing viruses
SI - Syncytium-inducing viruses R5 - Macrophage-tropic virus X4- T-cell tropic virus
R5X4 - dual-tropic virus n/a-not available

2.2.3 Cloning of HIV-1 subtype C *env* genes into the pFast Bac vector

The clean *env* gene PCR products (section 2.2.2) for each isolate and the pFastBac vector (BAC-TO-BAC Baculovirus Expression System, Gibco) were each restricted with *Bam*HI and *Hind*III restriction enzymes (Roche) at 37°C overnight. The restriction reaction mix consisted of 10 μ l of the clean PCR product, 1 μ l each of *Bam*H1 and *Hind*III enzymes, 2 μ l of the recommended 10X buffer B and 6 μ l of distilled water to a final volume of 20 μ l. The restricted *env* genes and the pFastBac vector were then cleaned using the High Pure PCR Product Purification Kit. The full-

length *env* gene for each of the isolates was ligated into the *Bam*HI and *Hind*III sites of the pFastBac vector. The ligation mix contained 7µl of the restricted PCR product, 1µl of the restricted pFastBac vector, 1µl of 10X ligation buffer and 1µl ligase (Roche) to a final volume of 10µl. The ligation mixture was incubated in a low temperature waterbath at 14°C overnight. Ten microliters of the ligation mix was used to transform 100µl of *Escherichia* (*E.*) *coli* XL-1 blue cells. The transformation mix was plated onto LB agar (Gibco) plates containing 100 µg/ml ampicillin (Roche) and 100 µg/ml tetracycline (Roche) and incubated at 37°C overnight. The observed white colonies were picked and inoculated into LB broth (Gibco) containing 100 µg/ml ampicillin and 100 µg/ml tetracycline and incubated at 37°C overnight with shaking at 200 rpms.

2.2.4 Selection of the recombinant pFastBac-env plasmids

All colonies were screened by PCR amplification to determine the presence of the *env* gene in the pFastBac plasmid. Primers ES7 and ES8 (Table 2.2) were used to amplify an approximately 700bp region encompassing the C2-V5 region of the gp160. The PCR reaction mix contained 5µl of 10X buffer, 10µl dNTPs (1mM), 2µl each of ES7 and ES8 primers (5 pmoles/µl), 3µl of MgCl₂ (25mM), 0.125 Supertherm Taq, 5µl of the overnight LB broth cultures and 22.9µl of water to a final volume of 50µl. PCR conditions included 3 cycles at 94°C for 3 min, 55°C for 45 sec and 72°C for 90 sec. This was ensued by 31 cycles at 94°C for 1 min, 55°C for 45 sec and 72°C 90 sec, followed by a final elongation at 72°C for 10 min and a holding temperature at 4°C. The PCR products were analyzed by 1% agarose gel electrophoresis in 1XTAE buffer at 100V for 2 hours, as described previously. Positive clones were inoculated into 50

ml LB broth containing 100µg/ml ampicillin and 50µg/ml tetracycline and grown overnight at 37°C with shaking at 220 rpms. The recombinant plasmids from each isolate were purified using the HiSpeed Plasmid Midi kit (Qiagen, Germany) as per manufacturers instructions. The recombinant pFastBac plasmids were analysed by restriction enzyme analysis (single and double digests) using *Bam*HI and *Hind*III restriction enzymes to confirm the presence of the *env* genes in the plasmid. The restriction fragments were screened on a 1% agarose gel by electrophoresis at 100V for 2 hours to confirm the recombinant plasmids (single digests) and the vector and *env* genes (double digests) were the correct size.

2.2.5 Transposition of the *env* genes into Bacmid DNA

One hundred microlitres of *E. coli* DH10BAC cells were transformed with 2µl of recombinant pFastBac DNA (2ng) for transposition of the *env* gene into the Bacmid DNA as described in the BAC-TO-BAC Baculovirus Expression System. Transformation mix was plated onto LB agar plates containing 7µg/ml gentamycin (Roche), 50µg/ml kanamycin (Roche) and 10µg/ml tetracycline, 40µg/ml isopropyl β-D-thiogalactopyranoside (IPTG; Roche) and 100µg/ml of 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Roche) and incubated at 37°C overnight. The observed white large colonies were picked and inoculated into 2ml LB broth containing 7µg/ml gentamycin, 50µg/ml kanamycin and 10µg/ml tetracycline and incubated overnight at 37°C with shaking at 200 rpms. PCR amplification using ES7 and ES8 primers (as described in section 2.2.4) was used to detect the presence of the HIV-1 subtype C *env* genes in the Bacmid DNA. Recombinant Bacmid DNA for all isolates was purified as described in the BAC-TO-BAC Baculovirus Expression System manual. Briefly, overnight cultures of positive recombinant *E. coli* DH10BAC

cells were transferred into a 1.5ml microcentrifuge tube and centrifuged at 14000xg for 1 min. The pellet was resuspended in 0.3ml of solution 1 (15mM Tris-HCl (pH 8.0; USB, USA), 10mM EDTA (Merck), and 100µg/ml RNase A (Roche)). Solution 2 (0.3ml) containing 0.2N NaOH (Merck) and 1% SDS (USB) was added and the mixture was incubated at room temperature for 5 min until the solution become translucent. Then 0.3 ml of a 3M potassium acetate solution (Merck) was added and mixed gentle until a thick white precipitate appeared. The mixture was placed on ice for 10 minutes and then centrifuged for 10 minutes at 14000xg. The supernatants were mixed gently with 0.8ml isopropanol (Merck) and kept on ice for a further 10 minutes. This was followed by centrifugation at 14000xg for 15 minutes at room temperature, supernatant was removed and 0.5ml of 70% ethanol was added to wash the pellet by inverting the tube several times. This was centrifuged for 5 minute at 14000xg at room temperature and the supernatant was discarded. The pellet was air dried for 5 to 10 min and the DNA resuspended in 20µl of sterile distilled water and stored at -20°C.

Table 2.2: Primers used to PCR amplify the envelope gene, for screening recombinants and DNA sequencing.

Primer	Sequences of the primers	Position ¹	Source
PCR primers			
<i>envA</i>	5'-GGCTTAGGCATCTCCTATGGCAGGAAGAA-3'	5140-5168	Gao <i>et al.</i> , 1996
<i>env N</i>	5'-CTGCCAATCAGGGAAGTAGCCTTGTGT-3'	8310-8336	Gao <i>et al.</i> , 1996
MAP2	5'-ATGTTTATCTAAGCTTGAGATGCTGCTCC-3'	8049-8078	This study
MAP3	5'-GAAAGAGCAGGGATCCGTGGCAATGAGAG-3'	5401-5429	This study
ES7	5'-CTGTTAAATGGCAGTCTAGC-3'	6191-6210	HMA kit ²
ES8	5'-CACTTCTCCAATTGTCCCTCA-3'	6789-6809	HMA kit
Sequencing primers			
EnvB	5'-AGAAAGAGCAGAAGACAGTGGCAATGA-3'	5400-5426	Gao <i>et al.</i> , 1996
A589	5'-GTGTAAAGTTAACCCCACTCTG-3'	5780-5802	F. McCutchan ³
E16	5'-CCAATTCCCATAACATTATTGTG-3'	6047-6068	F. McCutchan
Envf	5'-CTGTAGAAATTGTGTGTACAAGACCC-3'	6285-6310	F. McCutchan
AES6	5'-GGACAAGCATTCTATGCAACAGGTG-3'	6347-6371	F. McCutchan
E13	5'-ACAAATTATAAACATGTGGCAGG-3'	6631-6653	F. McCutchan
JL109	5'-GTGAATTATATAAATATAAAGTAG-3'	6808-6830	F. McCutchan
Env3f	5'-GGAAGCACTATGGGCGCGGC-3'	6944-6963	F. McCutchan
JL103	5'-TAACAAATTGGCTGTGGTATATAA-3'	7389-7412	F. McCutchan
Env4f	5'-GAGTTAGGCAGGGATACTCACC-3'	7485-7556	F. McCutchan
ZM184D	5'-CCACTCAGCTACTGCTATTGCTATGGT-3'	7835-7872	F. McCutchan
CM3	5'-CCCCCCCCGTCTCAGTCCCTTGAGACTGC-3'	7728-7758	F. McCutchan
TU-H	5'-GCCCCAGACTGTGAGITGCAACAGATG-3'	7055-7092	F. McCutchan
267SEQ2	5'-AGATGCTGTTGCGCCTCTATAGCC-3'	7036-7070	F. McCutchan
A590	5'-AATCGCGAAACCAGCCGGCGCACAAAT-3'	6063-6088	F. McCutchan
793SEQ4	5'-CAGCAGTTGAGTTGATACTACTGG-3'	6170-6204	F. McCutchan

¹Position based on 98ZA151Du sequence (van Harmelen *et al.*, 2001)

²National Institutes of Health; AIDS Research and Reference Reagent Program Catalog

³F McCutchan, pers comm

2.2.6 Growth and maintainance of *Spodoptera frugiperda* (Sf9) insect cells

One vial of frozen Sf9 insect cells (kindly obtained from Prof Huisman; University of Pretoria, South Africa) was thawed rapidly at 27°C and inoculated into 10ml

Grace's medium (Highveld Biologicals, South Africa) in a 25 cm³ tissue culture flask (Corning, USA). The Sf9 insect cells were allowed to attach for 2 to 3 hours at 27°C. Once attached, the Grace's medium was removed and 15ml of fresh Grace's medium containing 10% fetal calf serum (FCS) (Delta Bioproducts, South Africa) and 3% penicillin, streptomycin and fungizone (Highveld Biologicals, South Africa) was added. The Sf9 insect cells were incubated at 27°C for 4 to 7 days until they were confluent. The confluent Sf9 insect cells were scraped, resuspended in 4ml of Grace's medium and transferred into 75cm³ tissue culture flasks. Thirty millilitres of Grace's medium containing 10% FCS, 3% penicillin, streptomycin and fungizone were added into the culture and incubated at 27°C for 4 to 7 days until they were confluent. The confluent insect cells were scraped, suspended in 6ml Grace's medium and transferred into sterile 250 ml flasks. Fifty millilitres of Grace's medium containing 3% penicillin, streptomycin and fungizone, 10% FCS and 400µl Pluronic-F68 (Sigma) were added into the culture. The Sf9 insect cell cultures were incubated at 27°C with shaking at 130rpms until they reach the cell density of 2X10⁶ cells/ml. The Sf9 insect cells were then split back to 0.2X10⁶ cells/ml. Stocks of Sf9 insect cells were prepared by suspending 2X10⁶ cells/ml in 10 ml FCS supplemented with 10% dimethyl sulfoxide (DMSO; Sigma). This was then aliquoted in 1 ml in Nunc cryotube vials (Nunc, Denmark) and frozen down at -70°C for 24 hrs and transferred to liquid nitrogen for long term storage.

2.2.7 Transfection of Sf9 insect cells for production of recombinant baculoviruses

1X10⁶ cells/ml Sf9 insect cells were seeded in six well tissue culture plates (Nunc) and allowed to attach for 1 hour at room temperature. Recombinant Bacmid DNA (section 2.2.6) was used to transfect Sf9 insect cells to generate recombinant

baculoviruses as described in the BAC-TO-BAC Baculovirus Expression System. Briefly, 5 μ l of recombinant Bacmid DNA and 6 μ l of CellFectin (Gibco) were each mixed with 100 μ l of Grace's medium. These solutions were incubated at room temperature for 45 minutes to allow the CellFectin to package the recombinant Bacmid DNA. Eight hundred microlitres of Grace's medium were added into the transfection mix and used to transfect Sf9 insect cells. The transfected cells were incubated at 27°C for 5 hrs, followed by the removal of the transfection mix. Two millilitres of the Grace's medium containing 3% penicillin/streptomycin/fungizone and 10% FCS was added and the transfected Sf9 insect cells were incubated at 27°C for up to 4 days. After each day posttransfection, the supernatant containing recombinant baculoviruses was harvested and stored at 4°C and at -70°C for long-term storage, protected from the light.

2.2.8 Screening of the recombinant baculoviruses for expression of the envelope glycoproteins

Fresh Sf9 insect cells (1X10⁶cell/ml) were seeded in 24 well culture plates (Nunc, Denmark) and allowed to attach for 1 hour at room temperature. Two hundred microlitres of supernatant containing the recombinant baculovirus (obtained in section 2.2.7) from each day were used to transfect the insect cells. The transfection mix was made up to a final volume of 500 μ l by adding Graces medium containing 3% penicillin/streptomycin/fungizone and 10% FCS, and incubated at 27°C for 72 hrs. The whole cell lysate and the supernatant were harvested on day 3 and assayed for the expression of HIV-1 subtype C envelope glycoproteins by 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel and Western blot analysis. The 10% SDS-PAGE gel consisted of a resolving and stacking gel (Appendix A). The whole cell

lysate was prepared by suspending the harvested cells in lysis buffer (Appendix A). The cells were then lysed by freezing and thawing three times. The cell debris was removed by centrifugation and the supernatant was collected. Twenty microlitres of each sample containing 10 μ l sample and 10 μ l reducing loading buffer (Appendix A) were boiled for 5 minutes, allowed to cool and loaded onto duplicate gels and run at a constant current of 30mA for 1.5 hrs in SDS-PAGE running buffer (Appendix A). One of the SDS-PAGE gels was stained with Coomassie blue (Appendix A) for a minimum of 30 min with gentle shaking at room temperature, destained with the destaining buffer (Appendix A) until clear, and then visualised under white light and photographed. The duplicate SDS-PAGE gel was used for Western blot, and first equilibrated with transfer buffer (Appendix A) for 1 hour with gentle shaking. Each of the subsequent steps was performed at room temperature with gentle shaking. The hybridization nitrocellulose membrane (Hybond C; Amersham Pharmacia Biotech, AB, Sweden) was equilibrated with transfer buffer for 15 minutes. The proteins from the SDS-PAGE gel were transferred onto the membrane using a semidry blot apparatus (Bio-Rad) at constant current of 45mA for 45 minutes. The membrane was dried for 1.5 hrs at room temperature and blocked with 5% skimmed milk (Merck) in 50ml 1XTBS (Appendix A) for 1 hour. It was washed 3 times with 1XTBS for 5 minutes. The membrane was then incubated overnight with 50 ml of 0.5% BSA-1XTBS (Bovine serum albumin; Roche) containing 5 μ g/ml of serum from an HIV-1 infected individual as the primary antibody. The membrane was then washed 3 times with 1XTBS for 5 minutes. This was ensued by incubation of the membrane with 1 μ l antihuman IgG (Fc specific) alkaline phosphate conjugate (Sigma) at 1:30000 in 30ml 0.5% BSA-1XTBS for 1 hour. The membrane was washed 3 times with 1XTBS for 5 minutes. The membrane was immersed in a 4 Nitro blue tetrazolium chloride-5-

bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) solution (1 NBT/BCIP tablet (Roche) resuspended in 10ml of distilled water) and incubated in the dark until bands appeared. The reaction was stopped by addition of water.

2.2.9 Optimization of HIV-1 subtype C full-length envelope glycoprotein expression in insect cells

Sf9 insect cells at a density of 1×10^6 cells/ml were seeded into a 75cm^3 tissue culture flask and allowed to attach for 1 hour at room temperature. One and half millilitres of the recombinant baculoviruses were used to transfect each 75cm^3 tissue culture flask. The Grace's medium containing 3% penicillin/streptomycin/fungizone and 10% FCS was added to the flask to a final volume of 20ml. This was then incubated at 27°C for up to five days. The whole cell lysate and the supernatant were harvested from day 1 to day 5 posttransfection and assayed for protein expression using SDS-PAGE and Western blots as described in section 2.2.8.

2.2.10 DNA Sequencing of the positive recombinants

The insert from the recombinant plasmids of each isolate was sequenced using the ABI Prism BigDye Sequencing Ready reaction kit, version 2.0 (PE Applied Biosystems). Sequencing primers are listed in Table 2.2. Reactions were run on an ABI 3100 Automated Sequencer. The entire coding regions were assembled using Sequencer 3.1 (Genecodes Ann Arbor, MI). Predicted amino acids were obtained using the DNAsis package (version 2.5) and compared to the previously published sequences (van Harmelen *et al.*, 2001); (Papathanasopolous *et al.*, 2002).

2.3 RESULTS

2.3.1 PCR amplification of HIV-1 subtype C *env* genes

The *env* gene from each of the four HIV-1 subtype C isolates was successfully amplified in a nested PCR reaction. The clean PCR products were analyzed by horizontal gel electrophoresis and shown to be approximately 2.5kb in size (Figure 2.1).

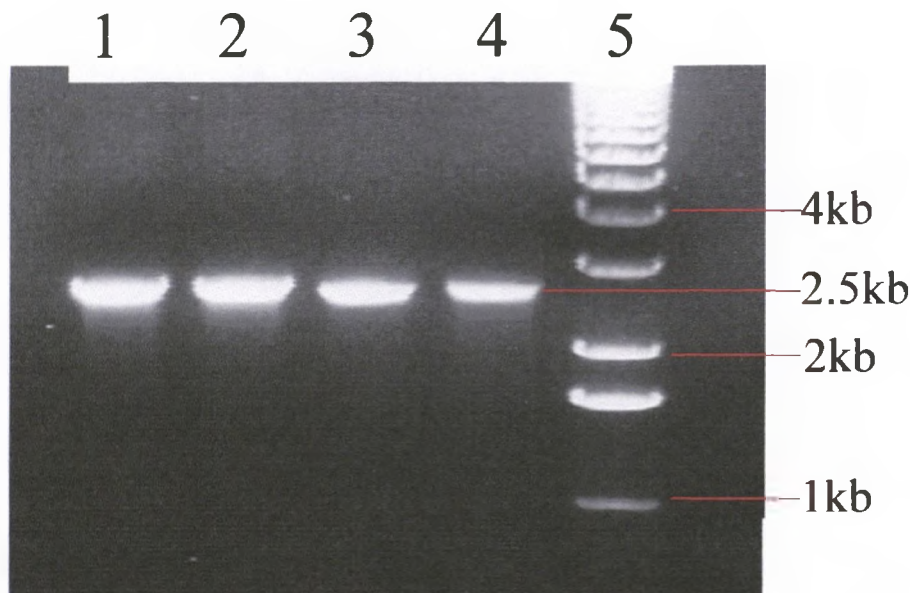


Figure 2.1: PCR amplification of four different HIV-1 subtype C *env* genes (~2.5kb). Lane 1: CM9, Lane 2: Du179, lane 3: Du151, lane 4: SW7, and lane 5: DNA molecular weight marker X (Roche, Germany).

2.3.2 Cloning of the HIV-1 subtype C *env* genes into the pFastBac vector and transposition into the Bacmid DNA

The *env* gene from each of the four HIV-1 subtype C isolates was cloned into the pFastBac vector. The *env* PCR products (~2.5kb) and the pFastBac vector (4.7kb) were each restricted with *Bam*H1 and *Hind*III restriction enzymes, ligated together and transformed into *E.coli* XL-1 blue cells. Recombinant *E.coli* were identified by

PCR amplification using ES7 and ES8 primers. These primers amplify the C2-V5 region (~0.7kb) of the *env* gene (Figure 2.2). A total of five CM9, nine Du151, eight Du179 and seven SW7 colonies were positive. However, only four of the positives for each isolate were randomly chosen for further experiments as shown in Figure 2.2. One clone for each isolate was then screened by restriction enzyme analysis using *Bam*H1 and *Hind*III restriction enzymes to confirm the presence and size of the *env* gene in the recombinant pFastBac plasmid DNA (Figure 2.3). A linear 7.2 kb fragment was observed in single restriction enzyme digests using *Bam*H1. Two fragments of 4.7 kb (pFastBac vector) and ~2.5 kb (*env* gene) were observed in double digests using both *Bam*H1 and *Hind*III restriction enzymes (Figure 2.3). These recombinants were used in subsequent experiments.

The recombinant pFastBac-*env* plasmids for each of the four HIV-1 subtype C strains were successfully transposed into the Bacmid DNA and the presence of the *env* gene in the Bacmid DNA was confirmed by PCR as shown in Figure 2.4. Several clones were positive for each of the four HIV-1 subtype C isolates, however, only three for each isolate were randomly selected for further studies.

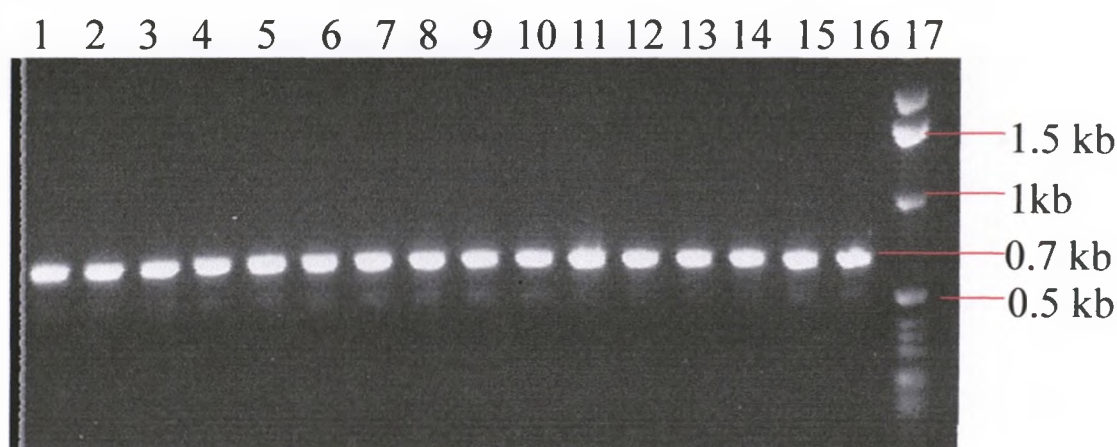


Figure 2.2: PCR amplification of the C2-V5 (~0.7kb) region of gp120 to confirm the presence of the HIV-1 subtype C *env* genes in the pFastBac vector. Lanes 1 to 4: CM9 clones, lanes 5 to 8: Du179 clones, lanes 9 to 12: Du151 clones, lanes 13 to 16: SW7 clones and lane 17: DNA molecular weight marker X (Roche, Germany).

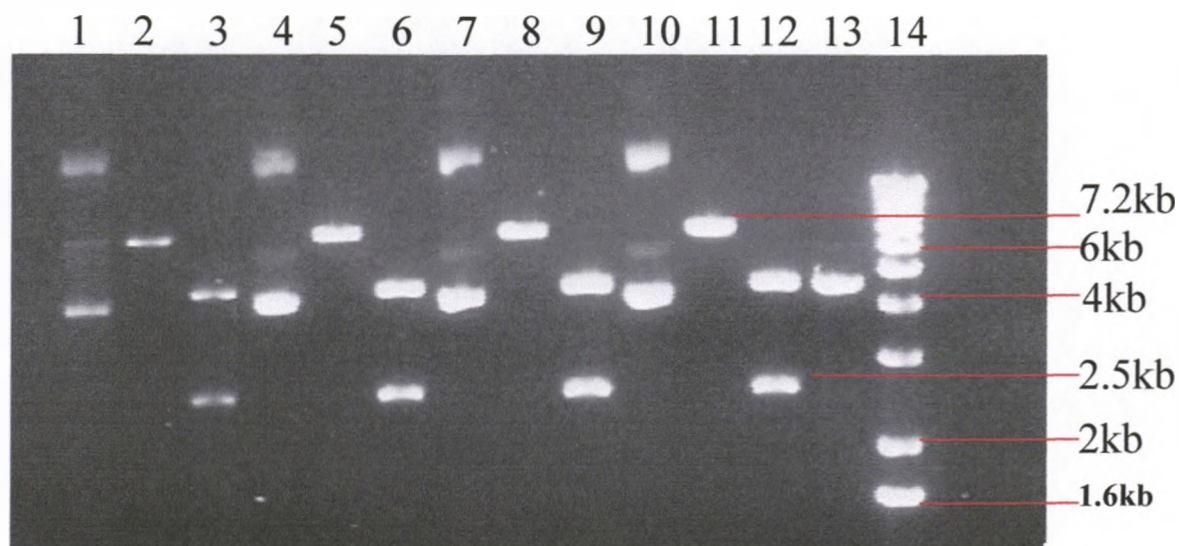


Figure 2.3: Restriction enzyme analysis to confirm the presence of HIV-1 subtype C *env* genes in the pFastBac vector. Lanes 1, 2 and 3: CM9 recombinant pFastBac DNA uncut, single digest and double digests, respectively. Lanes 4, 5 and 6: Du179 recombinant pFastBac DNA uncut, single digest and double digest, respectively. Lanes 7, 8 and 9: Du151 recombinant pFastBac DNA uncut, single digest and double digests, respectively. Lanes 10, 11, and 12: SW7 recombinant pFastBac DNA uncut, single digest and double digests, respectively. Lane 13: pFastBac vector double digest and lane 14: DNA molecular weight marker X (Roche, Germany).

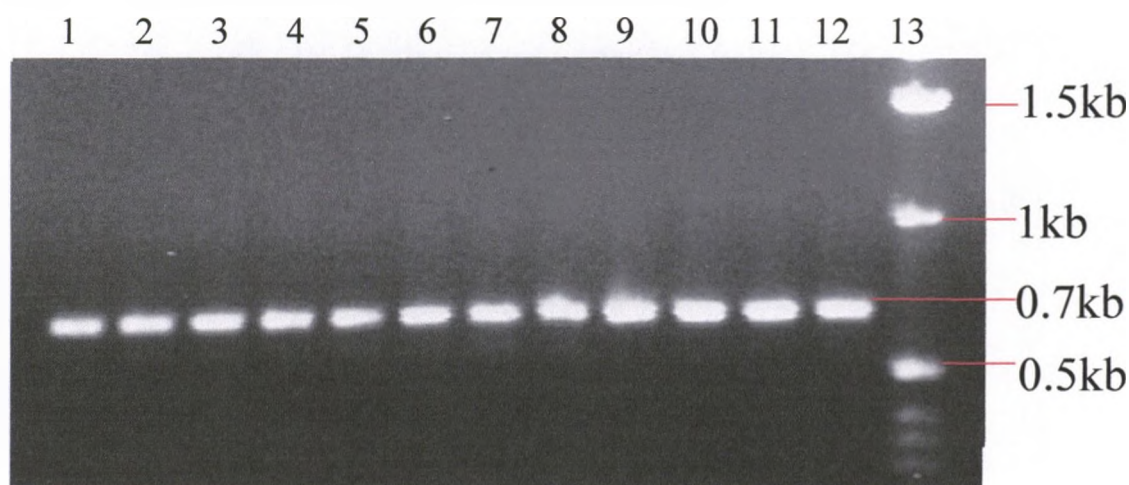


Figure 2.4: PCR amplification of the C2-V5 region (~0.7kb) of gp120 to detect the presence of the HIV-1 subtype C *env* genes in Bacmid DNA. Lanes 1 to 3: CM9 clones, lanes 4 to 6: Du179 clones, lanes 7 to 9: Du151 clones, lanes 10 to 12: SW7 clones, and lane 13: DNA molecular weight marker X (Roche, Germany).

2.3.3 Construction of recombinant baculoviruses expressing HIV-1 subtype C envelope glycoproteins

Purified recombinant Bacmid DNA was used to transfect Sf9 insect cells, to produce recombinant baculoviruses. Two recombinant baculoviruses per isolate were tested for their ability to express the HIV-1 envelope glycoproteins (Figure 2.5). The supernatants containing recombinant baculoviruses were collected from day 1 to day 4 posttransfection. These were analysed for the presence of the recombinant envelope glycoproteins by SDS-PAGE gel and Western blot. The recombinant baculoviruses collected on day 4 were shown to optimally express *env* genes from Du151, CM9, Du179 and SW7 HIV-1 subtype C isolates as determined by the detection of envelope glycoproteins (Figure 2.5). The envelope glycoproteins (gp160) observed have a molecular weight of approximately 116 kDa due to a lack of complete glycosylation in baculovirus expression system (Figure 2.5). The recombinant baculoviruses harvested on day 4 expressed the envelope glycoproteins better compared to those harvested on day 3 where there was little or no protein expression, and none by the recombinant viruses harvested on day 2 and day 1 (data not shown). There was no visible difference in the level of the envelope glycoprotein expression by the two different recombinant baculoviruses per isolate (Figure 2.5). One batch of the recombinant baculoviruses per isolate was selected for further experiments.

2.3.4 Expression of HIV-1 subtype C envelope glycoproteins in insect cells with time

The expression of HIV-1 subtype C envelope glycoproteins was shown to be highest on day 4 for the CM9, Du179 and SW7 isolates (Figures 2.6 and 2.7). The level of HIV-1 subtype C envelope glycoprotein expression in these isolates increased with time, with no envelope glycoproteins detected on day 1 (Figures 2.6 and 2.7). A low

level of envelope glycoprotein expression was observed on day 1 for the Du151 isolate, which peaked at day 2, followed by a constant level of expression on day 3 and slightly diminished on day 4 (Figure 2.6). No envelope glycoprotein was detected for any of the four isolates on day 5. Furthermore, no envelope glycoproteins were detected in the culture supernatant, as determined by SDS-PAGE and Western blot analysis (results not shown). The envelope glycoprotein was not detected in baculovirus wild type and mock infected cell controls (data not shown). The Du151 envelope glycoprotein was partially cleaved into gp120 (Figure 2.6), whereas no cleaved products were visible for CM9, Du179 and SW7 gp160, as shown in Figures 2.6 and 2.7. gp41 was not visible on the western blot. This may be due to western blot conditions that were optimized to detect gp160 and gp120.

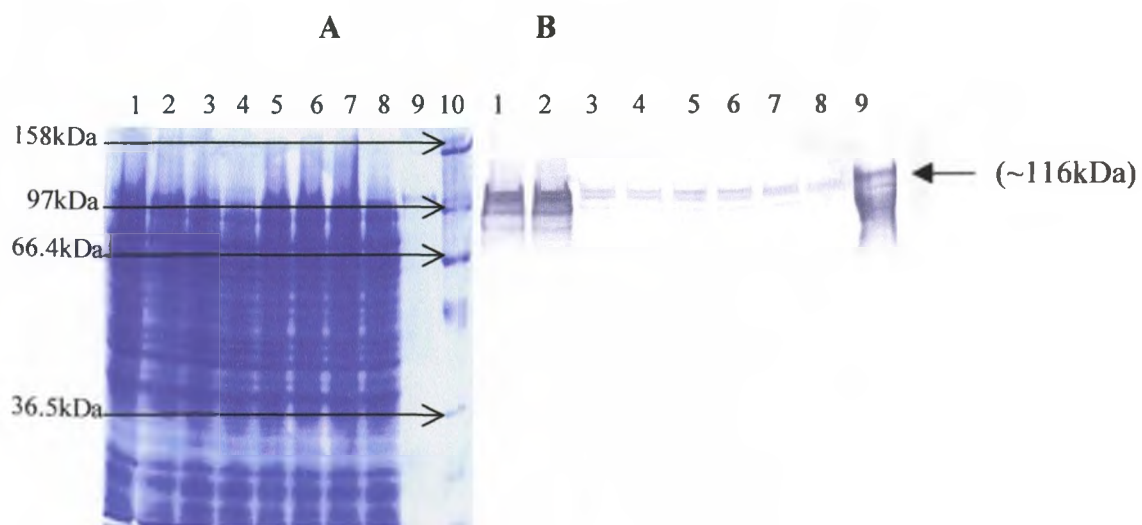


Figure 2.5: Expression of HIV-1 subtype C envelope glycoproteins in Sf9 insect cells transfected with the day 4 recombinant baculoviruses, as determined by SDS-PAGE (A) and Western blot (B) analysis. The cell lysates collected on day 3 are shown in Lanes 1 to 2: Du151-1 and Du151-2; lanes 3 to 4: CM9-1 and CM9-2; lanes 5 to 6: Du179-1 and Du179-2; and lanes 7 to 8: SW7-1 and SW7-2, lane 9: baculovirus gp160 (positive control) and lane 10: protein molecular weight marker (Promega, USA).

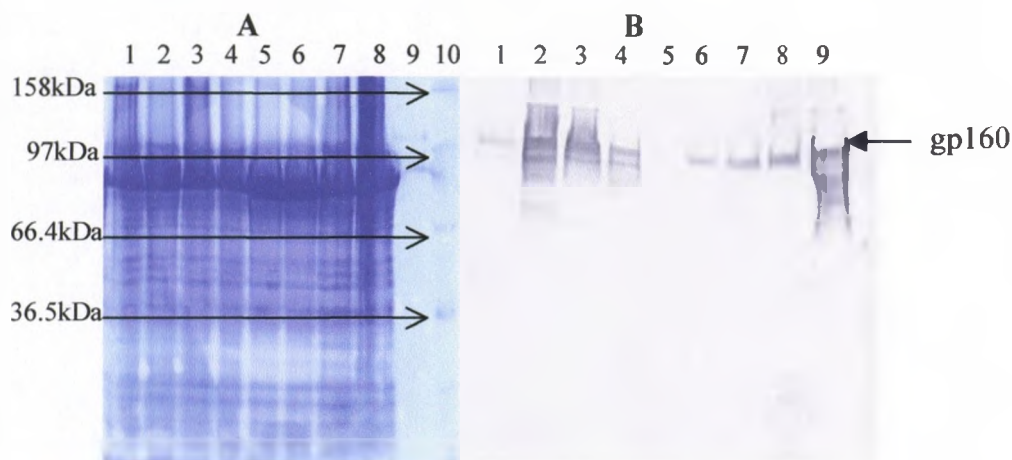


Figure 2.6: Expression of Du151 and SW7 HIV-1 subtype C envelope glycoproteins in Sf9 insect cells with time, as determined by SDS-PAGE (A) and Western blot (B) analysis. Cell lysate for each isolate was harvested from day 1 to day 4 posttransfection, and shown in Lanes 1 to 4: Du151 envelope glycoprotein from day 1 to day 4, respectively; lanes 5 to 8: SW7 envelope glycoprotein from day 1 to day 4, respectively; lane 9: baculovirus gp160 (positive control) and lane 10: protein molecular weight marker (Promega, USA).

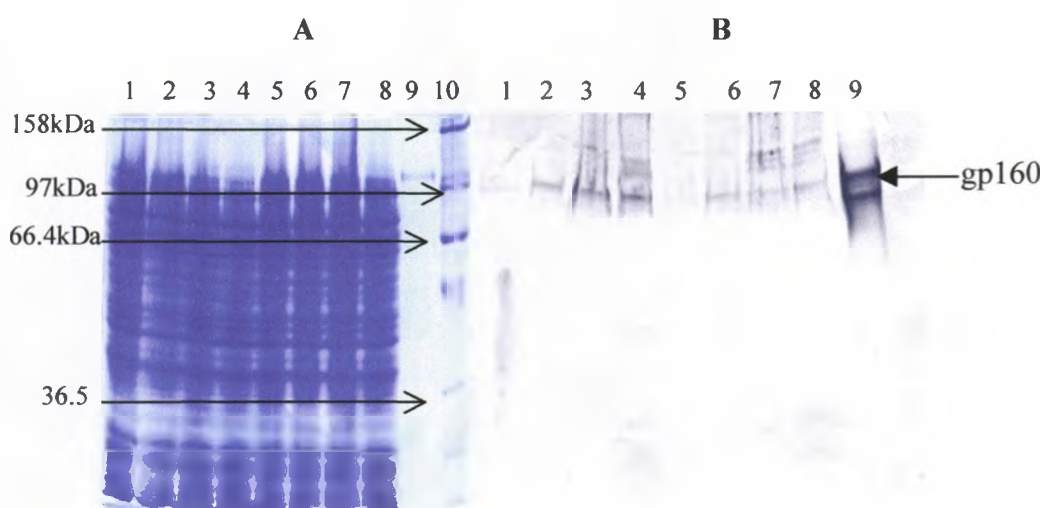


Figure 2.7: Expression of CM9 and Du179 HIV-1 subtype C envelope glycoproteins in Sf9 insect cells with time, as determined by SDS-PAGE (A) and Western blot (B) analysis. Cell lysate for each isolate was harvested from day 1 to day 4 posttransfection, and shown in Lanes 1 to 4: CM9 envelope glycoprotein from day 1 to day 4, respectively; lanes 5 to 8: Du179 envelope glycoprotein from day 1 to day 4, respectively; lane 9: baculovirus gp160 (positive control) and lane 10: protein molecular weight marker (Promega, USA).

2.3.5 DNA sequencing of recombinant pFASTBac-*env*

The recombinant pFASTBac-*env* plasmids that resulted in the recombinant baculoviruses we selected for further protein expression studies were sequenced. The nucleotide sequences of the full-length *env* for each of the four HIV-1 subtype C isolates were analysed, and found to contain no premature stop codons, or gross insertions or deletions. The predicted amino acid sequences showed that the Du151, CM9, Du179 and SW7 recombinant plasmids coded for proteins of 845, 866, 835 and 848 amino acids, respectively (Figure 2.8).

Figure 2.8: Alignment of the full-length predicted amino acid sequences of the four HIV-1 subtype C envelope glycoproteins expressed and purified in this study. The start of the gp120 and the gp41 region are indicated. The constant regions C1 to C5 are interspersed by the five variable regions (V1 to V5). The V1 to V5 regions are highlighted in different colours, and the amino acid residues that form the CD4 binding site are highlighted in pink (see page 52).

gp120 → C1

20 40 60 80 100 120

Du151 : MRVRGIPRNWPQWWIWGILGFWMIIICRVVGNLNLWVTVYGVFVWKEAKTTLFCASDAKAYDKEVHNWATHACVPTDPNPQEIIVLENVTENFNMWKNMDVDMHEDIISLWDQSLKPC : 120
 CM9 : MRVREILRNYQQWWIWSILGFWM--ICNVVG--NLWVTVYGVFVWKEAKTTLFCASDAKAYEKEVHNWATHACVPTDPNPQEMHLENVTENFNMWKNMDVDMHEDIISLWDQSLKPC : 116
 Du179 : MRVRGIQRNWPQWWIWGILGFWMLMICSQGVG--NLWVTVYGVFVWREAKTTLFCASDAKAYDREVHNWATHACVPTDPNPQEIIVMGNVTEFNMWKNMDVDMHEDIINLWDQSLKPC : 118
 SW7 : MRVMGILKNYQQWWIWGILGFWMLIMGSVVG--NLWVTVYGVFVWREAKTTLFCASDAKAYDKEMHNWATHACVPTDPNPQEMVLDNVTEFNMWKNMDVDMHEDIISLWDQSLKPC : 118

V1 V2 C2

140 160 180 200 220 240

Du151 : VKLTPLCVTLNCTNAT-----AYNSSMHGEMKNCENFTTTEIRDRKQKAYALFKKPDVPLRREENNCTG--GYILIN--SSITITQACPKVTFDPIPIHYCAPAGYAILKCNKNTFN : 230
 CM9 : VKLTPLCVTLECTNATFNSTTHNNATYNDTIAGEMKNCENFNTTELDRDKKKEYALFYRLDIVPLGGNSSKGNASKYRLIN--SSITITQACPKVSFDPIPIHYCAPAGYAILKCNKNTFN : 236
 Du179 : VKLTPLCVTLKCS-----TYNGSDTNDMRNCEFTTTEIRDKQOTVYALFYKPDIVPIN---ES---EYILIN--SSITITQACPKVSFDPIPIHYCAPAGYAILKCNKNTFN : 219
 SW7 : VKLTPLCVTLHCADVENSTN-----SMNDSMSREMRNCEFTTTEIRDKQONTVYALFYKPDVPLKSSSNSS---EYILIN--SSITITQACPKVSFEPIPIHYCAPAGFAILKCNKNTFN : 230

V3 C3

260 280 300 320 340 360

Du151 : GTGPCNNVSTVQCTHGKIPVSTQLLNGSLAEEEEIIRSENLTNNIKTIIVHLNKSVEIV--TRPNNN--TRKSIRIGPGQTFYATGEIIGDIRQAH--NISKSKWNETLQMVGKKLKEHF : 348
 CM9 : GTGPCNNVSTVQCTHGKIPVSTQLLNGSLAEEEEIIRSENLTNDVKTIIIVHLNESVPT--CARPGNN--TIKRIRIGPRYAFYAKETIIGDIRQAH--NISEEKWNTLQOVGKKLKEHF : 354
 Du179 : GTGPRQNVSTVQCTHGKIPVSTQLLNGSLAEGEIIIRSENLTNNVKTIIIVHLNESIGIV--TRPGNN--TRKSIRIGPGQAFYTN--HIIGDIRQAH--NISKEWNTLEVRKKLQEHF : 336
 SW7 : GTGPCNNVSTVQCTHGKIPVSTQLLNGSLAEGEIIIRSENLTNNVKTIIIVHLNESVAIV--TRPGSNKQRI--NRITRIGPGRAFTN--GVIGDIRKAY--NISNNHWNKTLEQVKKLGEHF : 349

V4 C4 V5

380 400 420 440 460 480

Du151 : PNKTIIFNPPSGGDLLEVATHSFNCRGEFFY--TEELFS-----NNSDSN-----NETITL--KIKQIINMWQKAGRAMYAPPIEGNITCKSNITGLLLTRDG---CDNTTN--EIFF-- : 452
 CM9 : PNKTIIFAPHSGGDLLEITHSFNCRGEFFY--ISKLFNRPNGTESNSNQTTESNSRTITL--RIQIINMWQEVGQAMYAPPIKGNITCTSNITGLLLTRDG---CDNTTN--EIFF-- : 471
 Du179 : FNKTIKFNSSSGGDLLEITHSFNCRGEFFY--EDTSKLFN-----DSLVIDTE-----SNSTITITL--RIQIINMWQEVGQAMYAPPIAGNITCKSNITGLLLTRDG---CDNTTE--EIFF-- : 442
 SW7 : LNKTIIEFKSPSGGDLLEITHSFNCRGEFFY--EDTSKLFNR-----TNPFNST-----DSNITITL--RIKQIINMWQGVQAMYAPPIEGNITCNSSITGLLLTRDGGISK--TENNTIEFI-- : 460

C5 gp41 →

500 520 540 560 580 600

Du151 : GGNMKDNWRSELYKYKVVEIEPLGVAPTCKSRVVEREKRAGV--LGAVLLGFLGAAGSTMGAASITLTVQARQLLSGIVQQSNLLRAIEAQQHMLQLTVWGIKQLQTRVLAIERYLKD : 571
 CM9 : GGDMDNWRSELYKYKVVEIKPLGIAPTAKRRVVEREKRAGVLLGAVFLGFLGAAGSTMGAASIALTVQTRQLLSGIVQQSNLLRAIEAQQHMLQLTVWGIKQLQTRVLSIERYLKD : 591
 Du179 : GGNMKDNWRSELYKYKVVEIKPLGIAPTEAKRRVVEREKRAGV--IGAVLLGFLGAAGSTMGAASITLTVQARQLLSGIVQQSNLLRAIEAQQHMLQLTVWGIKQLQTRVLAIERYLKD : 561
 SW7 : GGNMKDNWRSELYKYKVIEIRPLGVAPTEAKRRVVEREKRAPV--IGAVFLGFLGAAGSTMGAASITLTVQARQLLSGVVQQSNLLRAIEAQQHMLQLTVWGIKQLQARVLAIERYLQD : 579

620 640 660 680 700 720

Du151 : QQLLGLWGCSGKIICCTTAVPWNSSWSNKSQEDIDWNMTWMQWDREISNYTGTIYRLLEDSONQQEKNKEDLLALDSWKNLWNWFDITNWLWYIKIFIMIVGGLIGLRIIFGVLAIVKVR : 691
 CM9 : QQLLGLWGCSGKRICCTTAVPWNSSWSNKSKEEIDWNMTWMQWDREISNYTDIYRNLEESONQQEENEKELLEDSWKNLWSWFDITQWLWYIKIFIMIIGGLIGLRIIFAVLSMVNRVR : 711
 Du179 : QQLLGLWGCSGKLICTTNVPWNSSWSNKSQQAIDWNMTWMQWDREINNYTNIYQLLEDSDIQEQNEKEDLLALDKWQNLWSWFSITNWLWYIKIFIMIVGGLIGLRIIFAVLSIVNRVR : 681
 SW7 : QQLLGLWGCSGRICCTTNVPWNSSWSNKSKTIDWNMTWMQWDREISNYTDTIYRLLEVSQONQQEKNKEDLLALDKWNSLWSWFSITNWLWYIKIFIMIVGGLIGLRIIFTVLSIVNRVR : 699

740 760 780 800 820 840

Du151 : QGYSPLSFQTLTPNPRGP--DRLGGIEEGGEQDKDRSIRLVSGFLAWDDLRLSLCLFSLHRLADFLIVVTRAVELLGRSSLRGLQRGWEALKYLGLSLVQYWGLELKRSASILLDTIAI : 809
 CM9 : QGYSPLSFQTLTPSRGPP--DRLERIEEGGEQDKDRSVRLVSGFLPLIWDLLRLSLCLFSYHRLRDFILIVVRAVELLGRSSLRGLQRGWEILKYLGLSLVQYWGLELKRSASILLDTIAI : 830
 Du179 : QGYSPLSFQTLTPNPRGP--DRLGEIEEGGEQDRDRSIRLVSGFLPLAWDDLRLSLCLFSYHRLRDFISIAARTVELLGRSSLRGLQRGWEVLKYLGLSLVQYWGLELKRSASILLDTIAI : 799
 SW7 : QGYSPLSFQTLTPNPGGPRPDRLERIEEGGEQDRNRSIRLVNGFLAWDDLRLSLCLFSYHRLRDFILVAARTVEILGR-----RGWEALKYLGLSLVQYWGLELKRSASILLDTIAI : 812

860

Du151 : AVGEGTDRIIEIQRICRAIRNIPTRIQQGFETALL : 845
 CM9 : AVAEGTDRIIEFIQRLGRAIYHIPRRIQQGFEVALQ : 866
 Du179 : AVAEGTDRIIEIQRFCRAICNIPRRIQQGCEAALL : 835
 SW7 : VVAEGTDRIIEVTQRICRAICNIPRRIQQGFEAALL : 848

2.4 DISCUSSION

This study focused on the construction of recombinant baculoviruses expressing HIV-1 subtype C full-length envelope glycoproteins from isolates Du151, SW7, CM9 and Du179, and optimisation of protein expression in baculovirus transfected Sf9 insect cells. Previous studies have only focused on cloning of HIV-1 subtype B envelope glycoproteins (Farmer *et al.*, 1989); (Murphy *et al.*, 1990); (Wells and Compans, 1990). This is the first report of cloned full-length envelope glycoprotein genes from South African HIV-1 subtype C isolates with different phenotypes in a baculovirus system.

The designed MAP2 and MAP3 primers containing *Bam*H1 and *Hind*III restriction sites, respectively were effectively used to PCR amplify full-length HIV-1 subtype C *env* genes from isolates with different phenotypic characteristics as shown in Figure 2.1. PCR and restriction analysis showed that HIV-1 subtype C *env* genes from different isolates were successfully cloned into the pFastBac vector (Figures 2.2 and 2.3). Of note, the recommended *E. coli* DH10BAC cells could not be efficiently transformed with the recombinant pFASTBac vector, and the *E. coli* XL-1 blue cells were shown to be the most efficiently transformed. In a previous study the HIV-1 subtype B *env* gene was first cloned into one vector, excised and then subcloned into the pFastBac vector (Luckow *et.al.*, 1993). The data described in this study showed that PCR amplified HIV-1 subtype C *env* genes from R5, X4 and R5X4 viruses were directly cloned into the pFastBac vector.

All the recombinant baculoviruses tested were shown to express HIV-1 subtype *env* genes as determined by the detection of the envelope glycoproteins by SDS-PAGE and Western blot analysis (Figure 2.5). There were differences in expression levels between the

recombinant baculoviruses expressing the different *env* genes. The Du151, CM9, Du179 and SW7 HIV-1 subtype C envelope glycoproteins were expressed at high levels in insect cells, without the aid of other viral regulatory genes as required in a mammalian expression system (Ivey-Hoyle and Rosenberg, 1990); (Moore *et al.*, 1990). This is the first study showing the expression of HIV-1 subtype C envelope glycoproteins in insect cells. Moreover, for the first time we report the expression of the recombinant envelope glycoproteins from HIV-1 subtype C R5, R5X4 and X4 viruses in insect cells.

The expression of the recombinant HIV-1 subtype C envelope glycoproteins in insect cells was shown to increase with time, with the lowest level detected on day 2 and the highest level observed on day 4 for all the isolates except Du151 (Figures 2.6 and 2.7). This is in contrast with previous findings where HIV-1 subtype B gp160 was detected on day 1 posttransfection in insect cells (Farmer *et al.*, 1989); (Murphy *et al.*, 1990). Low levels of the Du151 envelope glycoprotein were detected on day 1, peaked on day 2, and maintained until day 4. However, a visual inspection of the amount of the Du151 envelope glycoprotein detected on day 1 posttransfection showed that it was at a lower level compared to the Western blots published for HIV-1 subtype B gp160 (Farmer *et al.*, 1989); (Wells and Compans, 1990). Several studies have shown that the level of HIV-1 subtype B gp160 expression in insect cells increased with time, with the lowest level detected on day 1, reaching optimal expression on day 2 and rapidly declining after day 3, with little or no gp160 recovered thereafter (Farmer *et al.*, 1989); (Murphy *et al.*, 1990); (Wells and Compans, 1990). Farmer *et al.*, (1989) showed that the viability of the transfected Sf9 insect cells diminished after three days in culture, resulting in their inability to express the recombinant envelope glycoproteins. By contrast, in our study the

envelope glycoprotein expression was detected until day 4 and was at the highest levels on day 4. Since no protein was detected on day 5, the transfected Sf9 insect cells had probably lost viability at this stage. A recently study has shown that the COOH terminal cytoplasmic domain of the gp160 causes toxicity in mammalian cells, resulting in rounding and detachment of the cells within 48hrs, thus decreasing the amount of the envelope glycoprotein expression (Chakrabarti *et al.*, 2002). Chakrabarti *et al.*, (2002) showed that *env* mutants lacking the cytoplasmic domain do not induce toxicity in mammalian cells, resulting in an increase in the amount of the envelope glycoprotein expressed. The mechanism as to how the cytoplasmic domain of the envelope glycoprotein causes cell cytotoxicity remains to be defined. However Chakrabarti *et al.*, (2000) only used a CXCR4-utilizing virus in their experiments. In our study we observed microscopically that Sf9 insect cells infected with the Du151 recombinant baculoviruses that express the CCR5-utilizing envelope glycoproteins were still intact and healthy after day 3. However, Sf9 insect cells infected with CM9, Du179 and SW7 recombinant baculoviruses that expressed the CCR5/CXCR4 and CXCR4 utilizing envelope glycoproteins, respectively tended to detach by day 3 (data not shown). This observation indicates that the R5 envelope glycoproteins might be less cytotoxic in Sf9 insect cells compared to the R5X4 and X4 envelope glycoproteins. This finding probably accounts for the higher level of the Du151 envelope glycoprotein expression in insect cells. No comparative study has been performed to determine the level of the envelope glycoprotein expression in insect cells with time from viruses that have different phenotypic characteristics. Overall, our data showed that the R5 envelope glycoproteins were expressed earlier and at a higher level in Sf9 insect cells compared to the R5X4 and X4 envelope glycoproteins.

Early detection and high levels of expression of the Du151 envelope glycoprotein could be an intrinsic property of the envelope glycoprotein derived from R5 viruses. The observed differences in the rate and the level of expression of the envelope glycoprotein from the HIV-1 subtype C R5 virus compared to those of R5X4 and X4 viruses may be due to the differences in signal sequences of the gp160 between these viruses. The cleavage of signal sequences has been shown to determine the rate of the envelope glycoprotein folding, intracellular transport and thus the envelope glycoprotein expression in both insect cells and mammalian cells (Li *et al.*, 1994); (Li *et al.*, 1996); (Li *et al.*, 2000). Golden *et al.*, (1998) showed that the replacement of the gp120 signal sequences with tissue plasminogen activator (tpa) or placental alkaline phosphatase signal sequences allowed for a higher level of gp120 expression in a baculovirus expression system. Furthermore, a study by Chen *et al.*, 2000 showed that the fusion of the honeybee mellitin sequence with the N-terminus of SIV gp160 allowed for a higher level of expression and secretion of SIV gp160 in insect cells. However, no study has been performed to determine the effect of signal sequences derived from HIV-1 viruses that use different coreceptors on the level of envelope glycoprotein expression in a baculovirus and/or mammalian expression system. Here we propose that the signal sequence of the Du151 envelope glycoprotein is probably more readily cleaved, accounting for the early and higher level of the envelope glycoprotein expression compared to that of the CM9, Du179 and SW7 envelope glycoproteins.

No gp160 and/or gp120 were recovered in the culture supernatant. Experiments on subcellular location have shown that the recombinant gp160 in insect cells remains cell

associated with little or no gp160/gp120 detected in the supernatant (Farmer *et al.*, 1989). The transmembrane region of the gp160 has been implicated in embedding the recombinant gp160 in the membrane of insect cells (Wells and Compans, 1990). Wells and Compans, (1990) observed that a mutant gp160 with a truncated transmembrane region was more readily released into the medium compared to the wild type.

Previous reports have found conflicting results regarding the cleavage of gp160 into gp120 and gp41 in insect cells. Rusche *et al.*, (1987) and Morikawa *et al.*, (1993) showed that the HIV-1 subtype B gp160 was not cleaved into gp120 and gp41 in insect cells. In contrast, Wells and Compans (1990) showed that the gp160 in insect cells was partially cleaved into gp120. The different results obtained in these studies was probably due to the different envelope glycoproteins used. To date, no comparative study has been performed to look at the cleavage efficiency of the envelope glycoprotein from viruses that use different coreceptors and expressed in insect cells. In our study partial cleavage of the R5 (Du151) recombinant envelope glycoprotein was observed, whereas no cleavage was observed for the X4 (SW7) and R5X4 (CM9 and Du179) envelope glycoproteins. Partial cleavage of the Du151 envelope glycoprotein in insect cells might be another factor that results in the higher level of expression in Sf9 insect cells. Binley *et al.*, (2002) observed that efficient proteolytic cleavage results in a high level of the recombinant envelope glycoprotein in mammalian cells. The cleavage of envelope glycoprotein in insect cells if it happens does not occur at a similar extent as that observed in mammalian system (Wells and Compans, 1990); (Morikawa *et al.*, 1993). This may be due to lower levels of cellular proteases in insect cells. Moreover, insect cells grow at 27°C, thus this temperature may not be suitable for the activity of cellular proteases that cleave HIV-1 envelope

glycoproteins. Furthermore, it is possible that the conformation of the envelope glycoproteins, in particular CM9 and Du179 (R5X4) and SW7 (X4) makes them resistant to cleavage by cellular proteases, as suggested for other HIV-1 subtype B envelope glycoproteins (Morikawa *et al.*, 1993).

Overall, the observed higher level of Du151 envelope glycoprotein expression in insect cells could be the result of several factors. These include, a readily cleaved signal sequence, less cytotoxic effect of its cytoplasmic region, and partial cleavage to gp120 and gp41.

Nucleotide sequence analysis confirmed all four of the HIV-1 subtype C *env* genes had no premature stop codons and were correctly inserted into the pFastBac vector. Analysis of the predicted amino acid sequences revealed that the CM9 clone had 9, SW7 clone had 2, Du179 clone had 5 and the Du151 clone had 41 amino acids residues different from the published sequences (van Harmelen *et al.*, 2001); (Papathanasopoulos *et al.*, 2002). However, the published sequences for all four viruses represents population based sequencing, and thus the predominant quasispecies. The differences noted above for the CM9, SW7 and Du179 clones can be expected, since only one sequence from a pool of quasispecies was cloned into the pFastBac vector. The differences can also be attributed to Taq polymerase error, however, in our experience this is negligible. The differences seen in the Du151 envelope sequences were higher than expected, however we subsequently found that the individual from whom we obtained the viral isolate was dually infected with another HIV-1 subtype C virus, and this clone could thus represent a recombination event between the two viruses in that individual.

HIV-1 subtype C viruses account for more than 50% of HIV-1 infection world-wide, thus it is important to express and characterize recombinant envelope glycoproteins from this subtype. In this study HIV-1 subtype C envelope glycoproteins were successfully expressed in insect cells without the aid of other viral regulatory genes. In previous reports only HIV-1 subtype B envelope glycoproteins have been expressed and characterised in insect cells (Farmer *et al.*, 1989); (Moore *et al.*, 1990); (Wells and Compans, 1990). Furthermore, the recombinant envelope glycoproteins used were derived from only HIV-1 subtype B isolates. Herein the recombinant envelope glycoproteins from South African HIV-1 subtype C viruses that use major coreceptors (CCR5 and/or CXCR4) were expressed in insect cells.

The expression of recombinant HIV-1 envelope glycoproteins in various expression systems has played a crucial role in elucidating their structure and function, as well as their antigenic and immunological properties essential for the design of an effective HIV-1 vaccine. However, an effective vaccine against HIV-1 still remains to be found. There has been a lot of activity in the HIV vaccine field worldwide in the last few years, and an HIV-1 subtype C vaccine is expected to enter Phase I clinical trials in South Africa soon. Thus the expression of HIV-1 subtype C recombinant envelope glycoproteins in large quantities and in the right conformation is important for further structural, functional, antigenic and immunological studies.

CHAPTER 3

PURIFICATION AND BIOLOGICAL CHARACTERIZATION OF HIV-1 SUBTYPE C R5, X4 AND R5X4 ENVELOPE GLYCOPROTEINS

3.1 INTRODUCTION

The envelope glycoprotein (gp160) mediates the entry of HIV-1 into the host cells by first binding the gp120 component of the gp160 with the host cells CD4 receptors and then with chemokine receptors, mainly CCR5 and CXCR4. This binding induces conformational changes in the envelope glycoprotein exposing the fusion peptide of the gp41 component of the gp160, which pierces the host cell membrane thus fusing the viral and the host membrane. Following viral and cell membrane fusion, the viral core enters into the host cell and continues the viral replication. The most recent studies have shown that the envelope glycoprotein forms between 7 to 14 oligomeric spikes that protrude from the mature virions (Chertova *et al.*, 2002). Biophysical and biochemical studies have shown that the oligomeric envelope glycoprotein is a trimer which consists of three gp120 subunits noncovalently associated with three gp41 moieties (Weissenhorn *et al.*, 1996); (Poumbourios *et al.*, 1997); (Lu *et al.*, 1999); (Center *et al.*, 2001); (Center *et al.*, 2002).

The envelope glycoprotein is the main target for vaccine based strategies because it is the only viral protein that can induce neutralizing antibodies against HIV. Thus it is important to express the HIV envelope glycoproteins in various expression systems and study their structure, function, antigenic and immunological properties in order to facilitate the

development of an effective vaccine. The recombinant envelope glycoproteins expressed in mammalian and baculovirus (insect cells) systems have been shown to retain their bioactive conformation and functional activities (Wells and Compans, 1990); (Fenouillet *et al.*, 1996). Recombinant envelope glycoproteins expressed in mammalian cells resemble more closely the gp160 complex on the virion surface than the baculovirus expressed gp160 because they are processed more efficiently and have carbohydrates of mammalian origin (Weiss and White, 1993). The sugar complexes found on the recombinant envelope glycoprotein expressed in insect cells differ from those of mammalian origin, however, they retain similar biological properties (Farmer *et al.*, 1989); (Moore *et al.*, 1990); (Wells and Compans, 1990). Furthermore, the recombinant envelope glycoproteins expressed in insect cells have been shown to induce humoral responses (Rusche *et al.*, 1987). Recombinant envelope glycoproteins expressed in insect cells have been shown to form trimers, bind to CD4 receptors and Fab fragments of neutralizing monoclonal antibodies (Chen *et al.*, 2000). All the abovementioned studies have focused on expression of HIV-1 subtype B envelope glycoproteins. Because the HIV-1 envelope glycoproteins expressed in baculovirus systems are trimeric and functional this system should be suitable and efficient for the expression and biological characterization of the envelope glycoproteins from other HIV-1 subtypes.

In order to determine the biological characteristics of recombinant HIV-1 envelope glycoproteins, they have to be purified in large quantities using methodologies that ensure they will retain their function and conformation integrity. Several recombinant envelope glycoproteins that maintain the right conformation and complete functional activities have been purified in insect cells using immunoaffinity chromatography and lentil lectin

purification methods (Moore *et al.*, 1990); (Gilljam, 1993); (Zhang *et al.*, 2001). In immunoaffinity chromatography, the recombinant expressed protein was attached to monoclonal antibodies and/or the serum from HIV-1 positive individuals and eluted using appropriate buffers (Zhang *et al.*, 2001). Lectin purification methods have been used to successfully purify recombinant gp160 for use in human vaccine trials (Gilljam, 1993). Lentil lectin has been used for purification of recombinant envelope glycoproteins in numerous studies (Moore *et al.*, 1990); (Gilljam, 1993); (Gilljam *et al.*, 1999). This system takes advantage of binding to mannose and glucose sugars of recombinant gp160 (Gilljam, 1993), since the gp160 consists of 24 N-linked and 4 N-linked glycosylation sites in the gp120 subunits and gp41 subunits, respectively (Morikawa *et al.*, 1993); (Johnson, *et al.*, 2001). For example, the *Galanthus nivalis* lentil lectin binds to the 7 to 9 terminal mannose residues of the recombinant envelope glycoproteins (Gilljam, 1993); (Gilljam *et al.*, 1999).

In individuals infected with HIV-1 subtype B, viruses that use the CCR5 coreceptor (R5 viruses) have been shown to dominate early in the infection, and approximately 50% will undergo a coreceptor switch and use CXCR4 (X4 viruses) at the late stages of AIDS (Chan *et al.*, 1999). A study has shown that the recombinant envelope glycoproteins from subtype B R5 viruses expressed on the surface of mammalian cells have low antigenic and immunological properties compared to those derived from X4 viruses (Brand *et al.*, 2000). These findings mark the differences that are displayed by the envelope glycoproteins from HIV isolates that use different coreceptors, even within the same subtype, that are important in vaccine design and diagnostic tests.

South Africa has one of the fastest growing HIV-1 epidemics, with an estimated 4.7 million people infected out of a total population of 40.6 million (Research, 2001). Our current understanding of the pathogenesis and the molecular biology of HIV-1 is based on research done with subtype B viruses and reagents. Only limited reagents are available from other subtypes, particularly subtype C, which constitutes over 56% of all circulating subtypes of HIV-1 group M viruses worldwide (Rodenburg *et al.*, 2001), and is the major subtype responsible for the epidemic among heterosexual groups in this country (van Harmelen *et al.*, 2001). HIV-1 subtype C predominantly use the CCR5 coreceptor for cell entry both during early and advanced stages of disease (Morris *et al.*, 2001). However, Cilliers *et al.*, (in preparation) have identified and characterized HIV-1 subtype C viruses from South African patients that can use other coreceptors. This observation indicates the evolution of HIV-1 subtype C envelope glycoproteins to use other coreceptors, and implies that HIV-1 subtype C envelope glycoproteins that use different coreceptors might have different biological characteristics. Whether differences or similarities exist in these envelope glycoproteins needs to be investigated further. In this study, HIV-1 subtype C envelope glycoproteins that utilize CCR5, CXCR4 or both coreceptors for cell entry were expressed in insect cells, purified using *Galanthus nivalis* lectin, and their biological characteristics were investigated and compared.

3.2 MATERIALS AND METHODS

3.2.1 Recombinant baculoviruses, recombinant proteins and insect cell growth media

Recombinant baculoviruses, recombinant envelope glycoproteins and monoclonal antibodies used in this study are shown in Table 3.1. The insect cells were grown in Grace's medium-complete, comprised of Grace's Insect cell medium (Highveld Biologicals, South Africa) supplemented with 10% Fetal calf serum (FCS, Delta Bioproducts, South Africa) and 3% penicillin/streptomycin/fungizone (Highveld Biologicals). For shaker cultures, 400 μ l of Pluronic-F68 (10% solution; Sigma, Germany) was added to each 50ml of Grace's medium-complete.

3.2.2 Large scale expression of recombinant HIV-1 subtype C envelope glycoproteins and preparation of cell extract

The large scale expression of the four different HIV-1 subtype C envelope glycoproteins in *Spodoptera frugiperda* (Sf9) insect cells was performed in both stationary culture and shaker cultures. In stationary culture, Sf9 insect cells at a density of 1X10⁶ cells/ml as measured by a haemocytometer count, were seeded in 75cm³ tissue culture flasks and infected using 2ml of the recombinant baculoviruses (Table 3.1). In shaker culture, Sf9 cells at a cell density of 2x10⁶cell/ml were added into 250ml EhrlenMeyer flasks and infected with 10ml of the recombinant baculoviruses (Table 3.1) as described in section 2.2.7. The infection mix was made up to 50 ml by adding fresh Grace's medium-complete. The infected stationary and shaker cultures (shaking at 130 rpms) were incubated at 27°C for 3 to 4 days. The supernatant was removed from the stationary cultures and the cells were washed twice with phosphate buffered saline (PBS, pH 7.4; Appendix A). For every

two 75cm³ flasks, infected cells were scraped and resuspended in 1ml lysis buffer (Appendix A). In the shaker culture, the infected cells were pelleted by centrifugation at 2000rpm. The supernatant was removed and the cells were washed twice with PBS and then resuspended in 5ml of lysis buffer. The cell extract was prepared by freezing and thawing the cells in lysis buffer and the cell debris was removed by centrifugation at 2000xg for 10 minutes. The large scale expression of the recombinant HIV-1 subtype C envelope glycoproteins in insect cells was confirmed by SDS-PAGE gel and Western blot analysis as described in section 2.2.8.

3.2.3 Purification of the recombinant HIV-1 subtype C envelope glycoproteins

Attempts to purify the recombinant HIV-1 subtype C envelope glycoproteins were made using the protein A immunoprecipitation kit (Roche, GmbH Germany) and the Seize X mammalian immunoprecipitation kit (Pierce, IL, USA). Finally, the *Galanthus nivalis* lectin method described by Gilljam (1993) was used to purify the recombinant HIV-1 subtype C envelope glycoproteins with a few modifications. All buffers were made in PBS (pH 7.3) and adjusted to a final pH of 7.3. One millilitre of *Galanthus nivalis* attached to agarose (Sigma) was added into a column and allowed to settle. The column was equilibrated with 5 ml of 0.65M NaCl (Merck, Germany) in PBS (pH 7.3). Two millilitres of each of the samples prepared in section 3.2.1 were added into different columns and allowed to pass through by gravity. The sample was recycled for a minimum of five times. Alternatively, the samples were allowed to attach to the lectin by incubating the mixture in the column overnight with gentle shaking at 4°C. The column was then sequentially washed with 15 ml of 0.65M NaCl in PBS, 5ml of PBS, 10ml of 1M NaCl in PBS to remove nonspecific binding and 10 ml PBS to neutralize the column. The samples were

eluted with 2.5ml of 1M methyl- α -D-mannopyranoside (Sigma). The eluates were dialyzed against PBS at 4°C for 20 hours using the slide-A-lyser cassette (Pierce) and concentrated using centricon columns (Amicon Bioseparations, Millipore, USA) as per manufacturers instructions. The purified recombinant envelope glycoproteins were aliquoted into 20 μ l and stored at -70°C.

3.2.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and silver nitrate staining

Purified recombinant HIV-1 subtype C envelope glycoproteins prepared in section 3.2.3 were examined by SDS-PAGE, Western blotting and silver nitrate staining. Ten microlitres of the purified envelope glycoprotein, the cell extract, and the column flow through were each mixed with 10 μ l of the sample buffer (Appendix A). Samples were heated for 5 minutes and loaded into the wells of a 10% SDS-PAGE gel and run in 1X SDS-PAGE running buffer (Appendix A) at 20mA for 1 hour 45 minutes. The gel was stained using the Bio-Rad Silver Staining kit (Bio-Rad, USA) as described below. All the silver staining procedures were performed at room temperature with gentle shaking. The SDS-PAGE gel was incubated in fixative 1 (40% methanol and distilled water) for 1 hour. It was further fixed with fixative 2 (10% ethanol and distilled water) for 30 minutes, followed by fresh fixative 2 for 10 minutes. The gel was then incubated with the oxidizer for 10 minutes, followed by three washes for 15 minutes each using deionized water. Silver nitrate reagent was added onto the gel and incubated for 30 minutes. The gel was then washed with deionized water for 2 minutes and the developer was added until a yellow-brown solution appeared. This solution was discarded and fresh developer was added. The bands were allowed to develop for a further 5 minutes. Fresh developer was

added every 5 minutes until all the bands were clearly visible. The reaction was stopped by adding 5% acetic acid (Saarchem Holpro Analytic, South Africa).

3.2.5 Concentration measurement of the purified recombinant HIV-1 subtype C envelope glycoproteins

The concentrations of the four purified recombinant envelope glycoproteins were measured using their absorbance readings at 280nm in a WPA lightwave spectrophotometer (WPA, Cambridge, UK) and extrapolated from a standard curve based on the known concentrations of bovine serum albumin (BSA; Roche). The BSA was made up in PBS to concentrations ranging from 10µg/ml to 500µg/ml. The purified recombinant envelope glycoproteins were diluted 1:5 in PBS.

3.2.6 Interaction of recombinant HIV-1 subtype C envelope glycoproteins with soluble CD4 (sCD4)

The reactivity of the purified recombinant HIV-1 subtype C envelope glycoproteins from Du151, CM9, Du179 and SW7 with sCD4 (Table 3.1) was determined using an enzyme linked immunosorbent assay (ELISA). The antibodies, sCD4 and envelope glycoproteins were all diluted to the required concentrations using 1XTBS (Appendix A). One hundred microlitres of D7324 polyclonal antibody at 10µg/ml in 0.1M Sodium Bicarbonate (pH8.5) (Sigma) was used to coat each well of the ELISA plate (Corning Incorporated, USA) and incubated at 4°C overnight. The plate was washed twice with 1XTBS (Appendix A) using a PW40 plate washer (Sanoti, Pasteur, France). Each well was then blocked with 200µl of 2% skimmed milk-1XTBS (Merck) at room temperature for an hour. The envelope glycoprotein for all the isolates at different concentrations ranging

from 0.2ng/ml to 100ng/ml was incubated with 1µg/ml of sCD4 for 1 hour at 37°C. The skimmed milk was completely removed from the ELISA plate and 100µl of the gp160-sCD4 complex prepared above was added into the wells and allowed to attach for 1 hour at 37°C. The plate was then washed twice with 1XTBS. The secondary anti-CD4 monoclonal and polyclonal antibodies (Table 3.1) were prepared at a concentration of 5µg/ml in 2% skimmed milk-1XTBS supplemented with 20% sheep serum (Sigma), and 100µl was added into each well and incubated at 37°C for 1 hour. The plate was washed twice with 1XTBS, and then 100µl anti-mouse IgG-Alkaline phosphatase (Sigma) diluted at 1:1000 in 2% skimmed milk-1XTBS was added into each well and incubated at 37°C for one hour. The reactions were developed using an Ampak Kit (Dako, UK). Briefly, the plate was washed a further five times with Ampak buffer. Fifty microlitres of Ampak substrate was added into each well and incubated at room temperature for 1 hour. Following incubation, 50µl of the amplifier was added and the colour was allowed to develop for 3 minutes. The reaction was then stopped by adding 50µl of stop solution into each well. Absorbance readings were taken at 490nm in a VERSAmax™ Tunable Microplate Reader (Molecular Devices Corporation, California, USA).

3.2.7 Reactivity of monoclonal antibodies with recombinant HIV-1 subtype C envelope glycoproteins

The integrity of the recombinant HIV-1 subtype C Du151, CM9, Du179 and SW7 envelope glycoproteins was assessed by their ability to react with monoclonal antibodies using an ELISA. The monoclonal antibodies that were tested are shown in Table 3.1. An ELISA was performed as described in section 3.2.6 with a few exceptions. The

recombinant HIV-1 subtype C envelope glycoprotein for all the isolates at different concentrations was adsorbed onto the plate coated with D7324 polyclonal antibody. Anti-human IgG (Fc specific) Alkaline phosphatase conjugate (Sigma) secondary antibody was used instead of the anti-mouse IgG-Alkaline phosphatase conjugate. In the assay using the 17b monoclonal antibody, the recombinant envelope glycoproteins were preincubated with 1 μ g/ml sCD4 for 1 hour at 37°C, and then added to the wells precoated with the D7324 antibody.

Table 3.1: Recombinant baculoviruses, recombinant proteins and monoclonal/polyclonal antibodies used in this study

REAGENT	SOURCE
Recombinant Baculoviruses	
pBacDu151	Section 2.3.3
pBacCM9	Section 2.3.3
pBacDu179	Section 2.3.3
pBacSW7	Section 2.3.3
Recombinant proteins	
gp160	NIH ¹ Cat no: 1004
gp120	NIH Cat no: 386
soluble CD4	Progenics Pharmaceuticals (NIH)
Monoclonal Antibodies	
A32	James Robinson ²
C11	James Robinson
17b	James Robinson
7b2	James Robinson
IgG1B12	NIH Cat no: 2640
F105	NIH Cat no: 857
2G12	NIH Cat no: 1476
2F5	NIH Cat no: 1475
Polyclonal Antibodies	
D7324	Aalto Bio Reagents Ltd, Ireland. Cat no: D076S
Anti CD4	South African Medical Research Council
Anti CD4	Sigma Cat no: 1805

¹National Institutes of Health, AIDS Research and Reference Reagent Program, Bethesda, MD, USA

²James Robinson, Tulane University, New Orleans, USA

3.3 RESULTS

3.3.1 Detection of the expressed recombinant HIV-1 subtype C envelope glycoproteins in the cell extracts

Expression of the recombinant HIV-1 subtype C envelope glycoproteins as determined by SDS-PAGE and Western blot analysis of the cell extracts is shown in Figure 3.1. The recombinant HIV-1 subtype C envelope glycoproteins were expressed in similar quantities in both stationary and shaker cultures. Western blot analysis of the cell extracts revealed bands for each of the recombinant HIV-1 subtype C envelope glycoprotein in the approximately 116 kDa size range.

3.3.2 Purification of the recombinant HIV-1 subtype C envelope glycoproteins

The recombinant HIV-1 subtype C envelope glycoproteins were purified using immunoprecipitation and immunoaffinity chromatography. However, the purified recombinant envelope glycoproteins were contaminated with antibodies (data not shown). The recombinant HIV-1 subtype C envelope glycoproteins expressed in Sf9 insect cells were successfully purified from cell extract using *Galanthus nivalis* lectin. Analysis of the purified proteins by SDS-PAGE followed by silver nitrate staining revealed bands in the approximately 116kDa size range (Figures 3.2 and 3.3). The molecular weight of the purified recombinant HIV-1 subtype C envelope glycoproteins (gp160) was consistent with that of the control gp160 (Table 3.1). The purity of the recombinant HIV-1 subtype C envelope glycoproteins was approximately 90% and was at a similar purity as the control gp160 (Lane 7 in Figures 3.2 and 3.3). Western blot analysis using serum from an HIV-1

infected individual confirmed that the purified proteins are the recombinant HIV-1 subtype C envelope glycoproteins (data not shown).

3.3.3 Concentrations of the purified HIV-1 subtype C envelope glycoproteins

The absorbance values of different concentrations of BSA at 280nm were measured and used to draw a standard curve. The absorbance values of the purified envelope glycoproteins were measured at 280nm as 0.066 for Du151, 0.017 for CM9, 0.026 for Du179 and 0.027 for SW7. The concentrations of the purified recombinant HIV-1 subtype C envelope glycoproteins were extrapolated from the curve, and multiplied by 5 to correct for the dilution factor. The concentrations of Du151, CM9, Du179 and SW7 were approximately 325 ng/ml, 40 ng/ml, 90 ng/ml and 95 ng/ml, respectively.

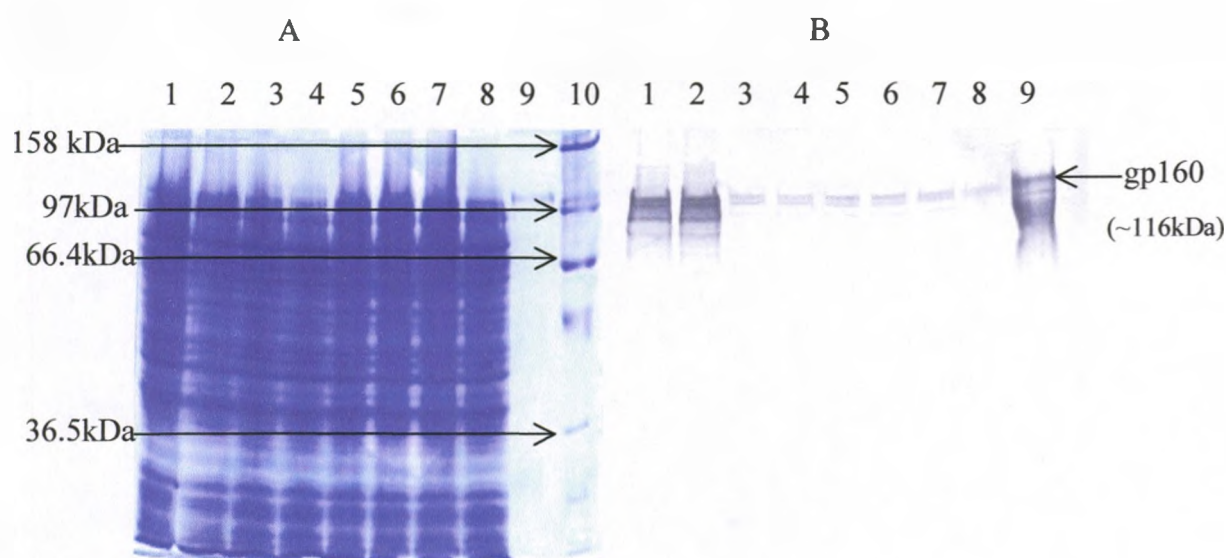


Figure 3.1: SDS-PAGE (A) and western blot analysis (B) of cell extracts showing the envelope glycoproteins expressed in large scale stationary (st) and shaker (sk) insect cell cultures. Lanes 1 to 2: Du151-st and Du151-sk, lanes 3 to 4: CM9-st and CM9-sk, lanes 5 to 6: Du179-st and Du179-sk, lanes 7 to 8: SW7-st and SW7-sk, lane 9: control gp160 (Table 3.1) and lane 10: protein molecular weight marker (Promega, USA). Molecular weights (in kDa) are shown on the left.

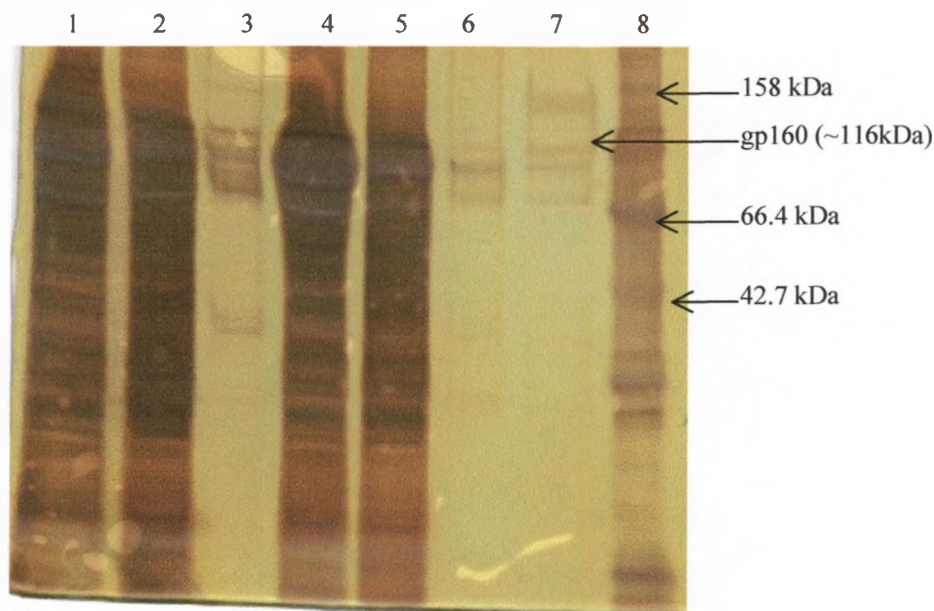


Figure 3.2: Silver nitrate stained SDS-PAGE gel (10%) showing the recombinant envelope glycoproteins (~116 kDa) expressed in insect cells and purified using *Galanthus nivalis* lectin. Lane 1: Du151 cell lysate before purification, lane 2: Du151 flow through, lane 3: purified Du151 gp160, lane 4: SW7 cell lysate before purification, lane 5: SW7 flow through, lane 6: purified SW7 gp160, lane 7: control bacgp160 (Table 3.1) and lane 8: protein molecular weight marker (Promega, USA). Molecular weights (in kDa) are shown on the right.

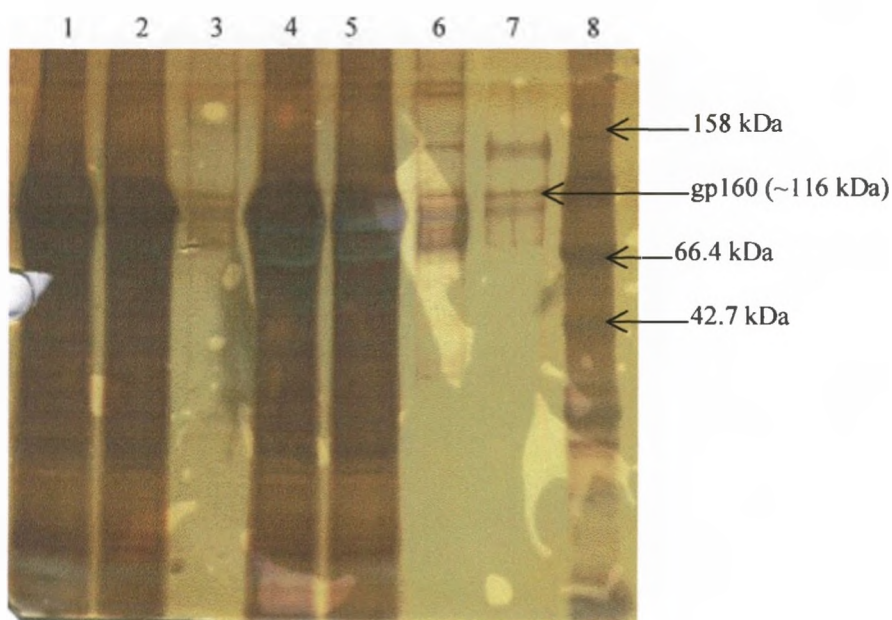


Figure 3.3: Silver nitrate stained SDS-PAGE gel (10%) showing the recombinant envelope glycoproteins (~116 kDa) expressed in insect cells and purified using *Galanthus nivalis* lectin. Lane 1: CM9 cell lysate before purification, lane 2: CM9 flow through, lane 3: purified CM9 gp160, lane 4: Du179 cell lysate before purification, lane 5: Du179 flow through, lane 6: purified Du179 gp160, lane 7: control bacgp160 (Table 3.1) and lane 8: protein molecular weight marker (Promega, USA). Molecular weights (in kDa) are shown on the right.

3.3.4 Biological characterization of purified recombinant HIV-1 subtype C envelope glycoproteins

The conformational stability of the purified recombinant HIV-1 subtype C Du151, CM9, Du179 and SW7 envelope glycoproteins was assessed for the ability to bind to A32, C11, 17b, IgG1b12, F105, 2G12, 2F5 and 7b2 monoclonal antibodies in an ELISA based assay (Figures 3.4 to 3.9).

All the baculovirus expressed HIV-1 subtype C Du151, CM9, Du179 and SW7 envelope glycoproteins reacted with the A32 monoclonal antibody as shown in Figure 3.4. The level of A32 binding to the Du151 envelope glycoprotein and the control gp120 (bacgp120) was highest for all concentrations of protein tested (OD of >1.8), and reached saturation levels at approximately 20ng/ml. Binding of SW7 to A32 reached saturation levels at approximately 40ng/ml, at an OD value of 1.6. Binding of CM9 to A32 reached saturation levels at approximately 20ng/ml, at an OD of 0.9. Binding of Du179 to A32 did not reach saturation levels at the concentrations tested.

The Du151, CM9, Du179 and SW7 recombinant HIV-1 subtype C envelope glycoproteins were able to bind to the C11 monoclonal antibody as shown in Figure 3.5. Again, binding of the Du151 envelope glycoprotein and the bacgp120 to the C11 monoclonal antibody were the highest amongst all proteins tested. The binding of the C11 monoclonal antibody to CM9, Du179, SW7, bacgp120 and bacgp160 envelope glycoproteins did not reach saturation levels at the protein concentrations tested. Binding of bacgp120 to C11 was much higher than that of bacgp160.

The recombinant envelope glycoproteins from CM9, Du179, SW7 and bacgp120 bound to the IgG1b12 monoclonal antibody (Figure 3.6). Binding of Du151 to IgG1B12 was negligible, but slightly increased at the much higher concentrations of protein (>80ng/ml). The binding of IgG1b12 to the bacgp120 envelope glycoprotein was highest while that of CM9, SW7 and Du179 envelope glycoproteins was moderate.

All the recombinant HIV-1 subtype C envelope glycoproteins reacted with the 7b2 monoclonal antibody (Figure 3.7). Du151 and Du179 exhibited the highest level of binding to 7b2, followed by bacgp160, SW7 and lastly CM9.

The functional and conformational stability of the four newly purified recombinant HIV-1 subtype C envelope glycoproteins was assessed by testing their ability to bind to sCD4 in an ELISA. Detection of the rgp160-sCD4 complex using both the anti-CD4 antibodies available (Table3.1) was poor (results not shown). The detection was poor even in the presence of 0.3% to 1% empigen (data not shown).

Binding of the recombinant HIV-1 subtype C envelope glycoproteins to the 17b monoclonal antibody was tested in the presence and absence of sCD4. The Du151, CM9, Du179 and SW7 HIV-1 subtype C envelope glycoproteins and the bacgp120 all reacted with 17b in the absence and presence of sCD4 as shown in Figures 3.8 and 3.9. However, the binding of 17b to the recombinant HIV-1 subtype C envelope glycoproteins and the bacgp120 was augmented in the presence of sCD4. The Du151 recombinant envelope glycoprotein and the bacgp120 had the highest reactivity with 17b at protein

concentrations >80ng/ml, followed by CM9 and Du179 which had intermediate binding, and lastly SW7, which exhibited the lowest binding.

No binding of the F105, 2G12 and 2F5 monoclonal antibodies to the recombinant HIV-1 subtype C envelope glycoproteins was detected (results not shown). However, the binding of these monoclonal antibodies to the bacgp160 from HIV-1 subtype B was observed (data not shown). The level of reactivity of the control bacgp160 with these monoclonal antibodies was very high with an absorbance value of over 1.6.

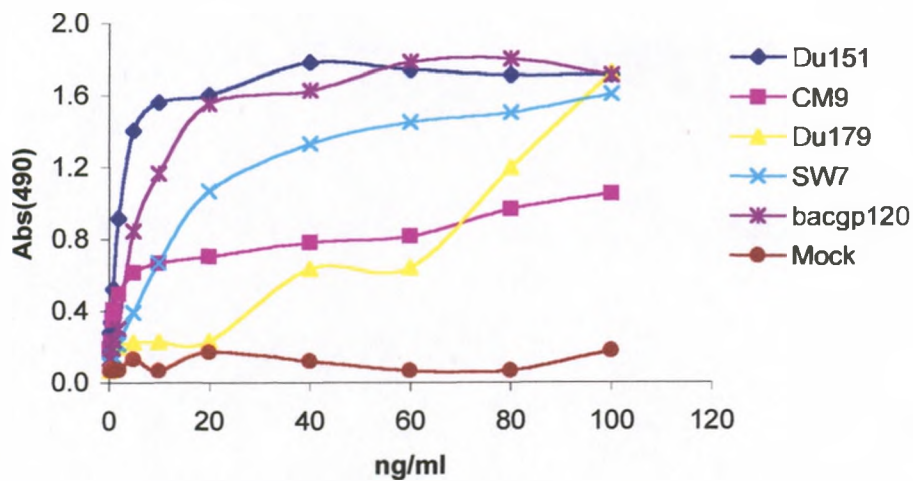


Figure 3.4: Binding of purified Du151, CM9, Du179 and SW7 envelope glycoproteins to the A32 monoclonal antibody using an ELISA. The envelope glycoproteins at different concentrations ranging from 0.4ng/ml to 100ng/ml were bound to immobilized D7324 and reacted with A32 monoclonal antibody at a concentration of 5µg/ml. The envelope glycoproteins from each strain are represented by a different colour line on the right of the graph. Bacgp120 is the control gp120 (Table 3.1) and mock represents purified baculovirus proteins without HIV-1 envelope glycoproteins expressed in insect cells.

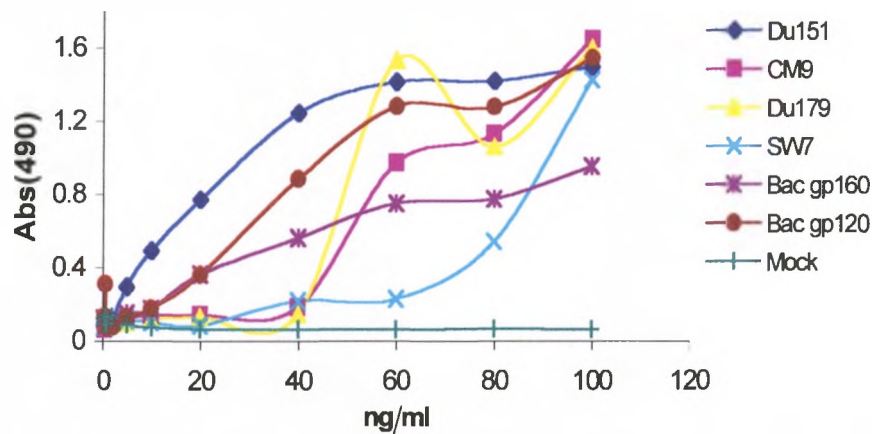


Figure 3.5: Binding of purified Du151, CM9, Du179 and SW7 envelope glycoproteins to the C11 monoclonal antibody using an ELISA. The envelope glycoproteins at different concentrations ranging from 0.4ng/ml to 100ng/ml were bound to immobilized D7324 and reacted with C11 monoclonal antibody at a concentration of 5 μ g/ml. The envelope glycoproteins from each strain are represented by a different colour line on the right of the graph. Bacgp160 is the control full-length envelope glycoprotein, and Bacgp120 is the control gp120 (Table 3.1). Mock represents purified baculovirus proteins without HIV-1 envelope glycoproteins expressed in insect cells.

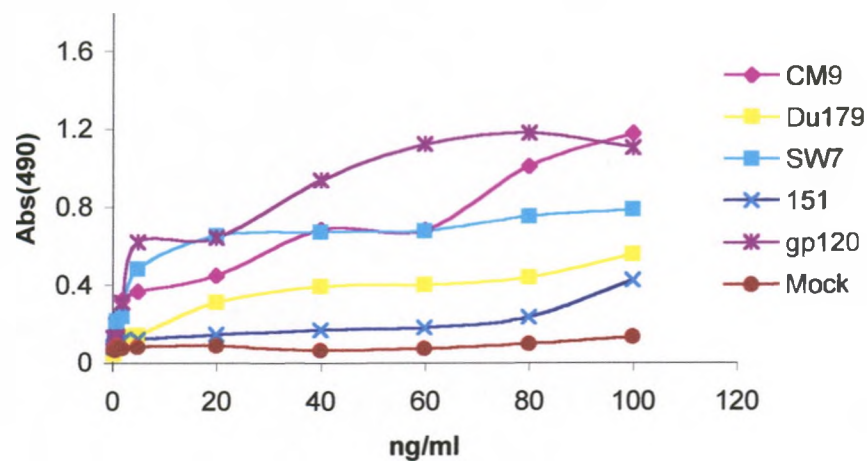


Figure 3.6: Binding of purified Du151, CM9, Du179 and SW7 envelope glycoproteins to the IgG1b12 monoclonal antibody using an ELISA. The envelope glycoproteins at different concentrations ranging from 0.4ng/ml to 100ng/ml were bound to immobilized D7324 and reacted with IgG1B12 monoclonal antibody at a concentration of 5 μ g/ml. The envelope glycoproteins from each strains are represented by a different colour line on the right of the graph. The bacgp120 is the control gp120 (Table 3.1) and mock represents purified baculovirus proteins without HIV-1 envelope glycoproteins expressed in insect cells.

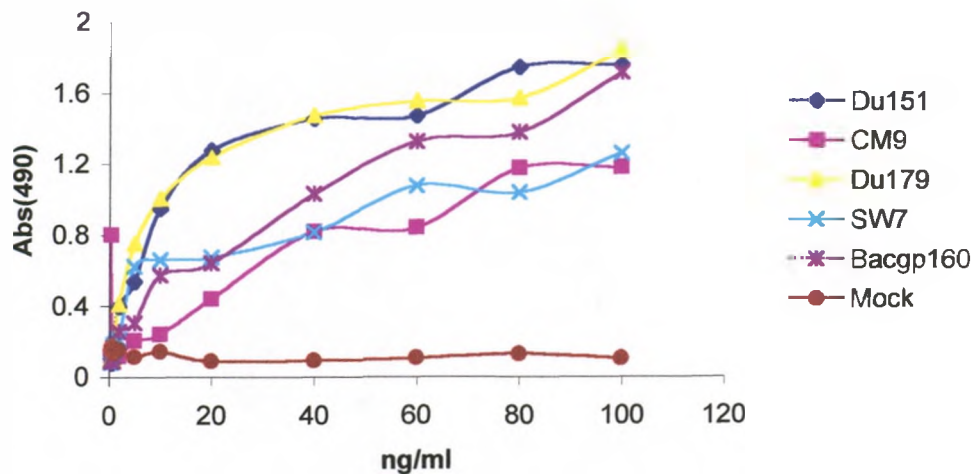


Figure 3.7: Binding of purified Du151, CM9, Du179 and SW7 envelope glycoproteins to the 7b2 monoclonal antibody using an ELISA. The envelope glycoproteins at different concentrations ranging from 0.4ng/ml to 100ng/ml were bound to immobilized D7324 and reacted with 7b2 monoclonal antibody at a concentration of 5µg/ml. The envelope glycoproteins from each strain are represented by a different colour line on the right of the graph. (Table 3.1) Bacgp160 is the control full-length envelope glycoprotein, and mock represents purified baculovirus proteins without HIV-1 envelope glycoproteins expressed in insect cells.

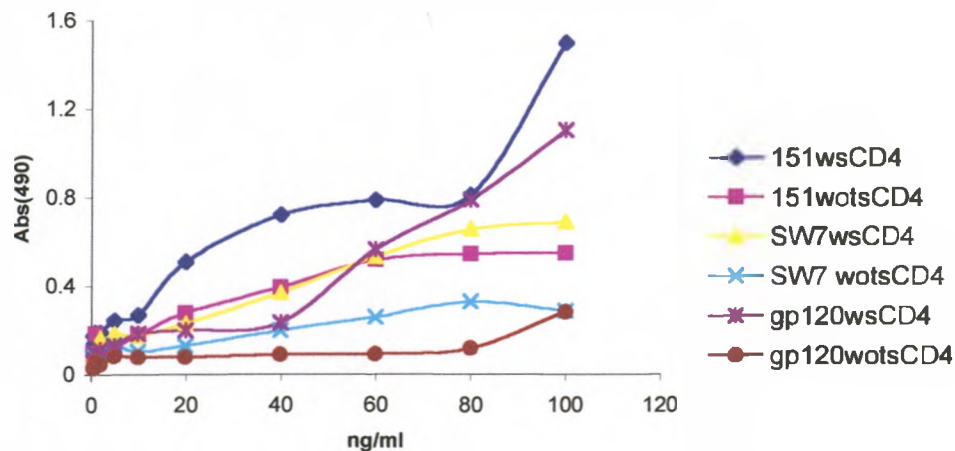


Figure 3.8: Binding of purified Du151, SW7 and the control bacgp120 envelope glycoproteins to the 17b monoclonal antibody in the presence (ws) and absence (wot) of sCD4 using an ELISA. The envelope glycoproteins at different concentrations ranging from 0.4 ng/ml to 100 ng/ml were bound to immobilised D7324 and reacted with 5µg/ml of 17b monoclonal antibody. For the sCD4 binding experiments, the purified recombinant envelope glycoproteins were preincubated with sCD4 before binding to D7324. The envelope glycoproteins from each strain are represented by a different color line as shown on the right of the graph.

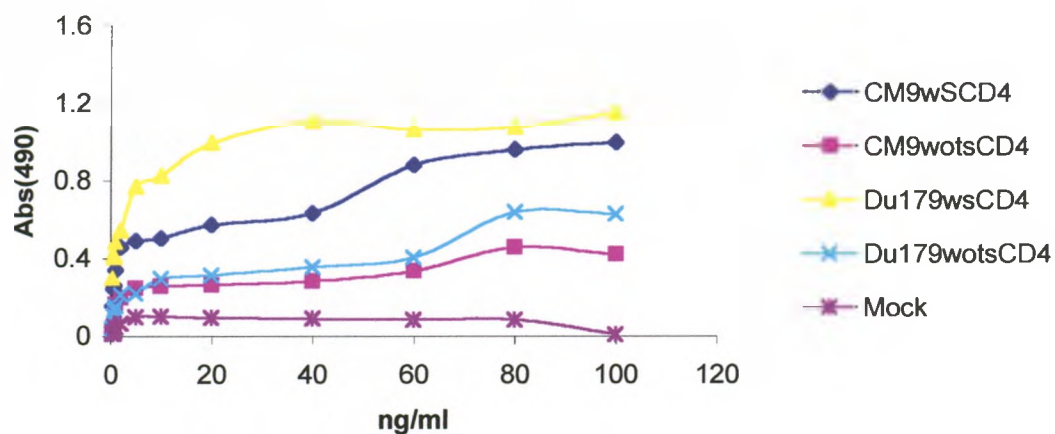


Figure 3.9: Binding of purified CM9 and Du179 envelope glycoproteins to the 17b monoclonal antibody in the presence (ws) and absence (wot) of sCD4 using an ELISA. The envelope glycoproteins at different concentrations ranging from 0.4 ng/ml to 100 ng/ml were bound to immobilised D7324 and reacted with 5µg/ml of 17b monoclonal antibody. For the sCD4 binding experiments, the purified recombinant envelope glycoproteins were preincubated with sCD4 before binding to D7324. The envelope glycoproteins from each strain are represented by a different color line as shown on the right of the graph. Mock represents purified baculovirus proteins without HIV-1 envelope glycoproteins expressed in insect cells.

3.4 DISCUSSION

The envelope glycoproteins are the sole HIV-1 components exposed on the surface of the virion, and therefore represent the only realistic viral target for vaccine induced neutralizing antibody responses. Understanding the biological characteristics exhibited by the envelope glycoproteins derived from HIV-1 subtype C strains that use different coreceptors for cell entry is thus important for rational vaccine design and in the development of reagents for testing immune responses raised by the candidate vaccines. Purification of recombinant HIV-1 subtype C envelope glycoproteins that retain their functional and conformation integrity is essential for further study of their biological properties. In this study the recombinant envelope glycoproteins from HIV-1 subtype C viruses that use different coreceptors were purified and their biological properties evaluated.

HIV-1 subtype C envelope glycoproteins were successfully purified using a modified *Galanthus nivalis* purification method (Gilljam, 1993); (Gilljam *et al.*, 1999). The purity of the recombinant envelope glycoprotein acquired using this method was at least 90% as determined by silver stained SDS-PAGE gel analysis (Figures 3.2 and 3.3). The purity of our recombinant envelope glycoproteins was compared to that of the control bacgp160 (Table 3.1), which was purified to 98% homogeneity using immunoaffinity chromatography. Purification of the HIV-1 subtype C recombinant envelope glycoproteins using the lectin method we established was better than the lentil lectin used in a previous study (Moore *et al.*, 1990). In this study HIV-1 subtype B gp160 expressed in insect cells was only purified to 30% homogeneity (Moore *et al.*, 1990). The gp120 from HIV-1, HIV-

2 and SIV expressed in mammalian cells was purified using *Galanthus nivalis* to 98% purity as determined by silver stained SDS-PAGE gel (Gilljam, 1993); (Gilljam *et al.*, 1999). Gilljam (1993) confirmed that there are no mammalian glycoproteins and/or fetal calf serum that are capable of attaching to *Galanthus nivalis* lectin. However, in both these studies this method was not used to purify the full-length envelope glycoproteins (gp160). Our data showed that the recombinant envelope glycoproteins purified using *Galanthus nivalis* in insect cells contained contaminating proteins. The contaminating proteins detected were similar to those encountered in the control bacgp160 obtained from the NIH (Figures 3.2 and 3.3, lane 7). These contaminating proteins may be due to insect cells glycoproteins and/or baculovirus glycoproteins attaching to the lectin during purification. So far it has not been established whether insect cells and/or baculovirus glycoproteins are capable of binding to *Galanthus nivalis* lectin. Our data suggests that the insect cell glycoproteins may be capable of attaching to *Galanthus nivalis* lectin. Furthermore, the contaminating proteins may be insect cells membrane proteins attached to the hydrophobic transmembrane region of the gp41 subunit. Moore *et al.*, (1990) observed a large amount of contaminating proteins in the purified gp160 using lentil lectin. Because less contaminating proteins were detected in our study compared to the previous study by Moore *et al.*, (1990) and was of similar purity as the immunoaffinity purified control bacgp160, the method we used is suitable for large scale purification of the full-length envelope glycoproteins expressed in insect cells. Although the purified envelope glycoproteins have at least 90% purity, a further purification step will be required to achieve 100% purity. However, the purity of our HIV-1 subtype C envelope glycoproteins is adequate to determine their biological characteristics and for structure and functional studies. Interestingly, modified recombinant envelope glycoproteins (SOS gp140) were

purified using *Galanthus nivalis* lectin, and shown to maintain their oligomeric and conformational properties (Schulke *et al.*, 2002).

Our data showed that the HIV-1 subtype C envelope glycoproteins expressed and purified in insect cells maintained their conformational stability as measured by their ability to react with monoclonal antibodies that recognize conformational dependent epitopes. All the purified recombinant HIV-1 subtype C envelope glycoproteins and the control HIV-1 subtype B gp160 were able to react with A32, C11, IgG1b12, 17b and 7b2 monoclonal antibodies. However, the level of binding of each monoclonal antibody to the purified envelope glycoproteins was different.

All the recombinant HIV-1 subtype C envelope glycoproteins reacted with the A32 monoclonal antibody (Figure 3.4). A32 is the nonneutralizing monoclonal antibody that recognizes the conformational epitopes spanning the C1 to C4 region of the envelope glycoprotein (Los Alamos National Laboratory; <http://hiv-web.lanl.gov/immunology>). The recombinant envelope glycoproteins from Du151 and the control bacgp120 showed the highest affinity for A32. This finding suggests that the A32 conformational epitope might be more readily exposed in the Du151 (R5) and the control bacgp120 (X4) envelope glycoproteins, compared to the SW7 (X4), CM9 (R5X4) and Du179 (R5X4) envelope glycoproteins.

All four recombinant HIV-1 subtype C envelope glycoproteins reacted with the C11 monoclonal antibody (Figure 3.5). C11 is the nonneutralizing monoclonal antibody that recognises conformational epitopes spanning the C1 to C5 regions of the envelope

glycoproteins (Los Alamos National Laboratory; <http://hiv-web.lanl.gov/immunology>). Helseth *et al.*, (1991) and Wu *et al.*, (1996) showed that the C1 to C5 regions of the gp120 interact with the gp41 subunit, thus the C11 epitope is masked in the gp160. This suggests that C11 will not readily bind to gp160 with high affinity. Interestingly, the C11 bound to the HIV-1 subtype C recombinant envelope glycoproteins and the control bacgp120 and bacgp160. However, there was a marked difference between the binding of the bacgp120 relative to the bacgp160, suggesting that the epitope in the bacgp160 was less exposed. Furthermore, the results showed that the recombinant HIV-1 subtype C gp160 have a higher affinity for C11 monoclonal antibody compared to HIV-1 subtype B bacgp160, suggesting that the C11 conformational epitope might be more readily exposed on the recombinant HIV-1 subtype C envelope glycoproteins. The control gp120 had a higher affinity for C11, similar to that of Du151 as a result of the exposure of the C1 to C5 regions in the absence of gp41. The Du151 binding kinetics may be similar to that of the bacgp120 because the purified protein consists of a pool of uncleaved gp160 and cleaved gp120, as discussed in Chapter 2. This result suggests that the C11 conformational epitope is readily exposed on the Du151 (R5) recombinant envelope glycoprotein compared to the CM9 (R5X4), Du179 (R5X4) and SW7 (X4) envelope glycoproteins.

The ability of the recombinant HIV-1 subtype C envelope glycoproteins to react with the conformationally dependent A32 and C11 monoclonal antibodies showed their conformations are intact. Furthermore, the purified recombinant HIV-1 subtype C envelope glycoproteins are monomeric as determined by their high level of binding to A32 and C11 monoclonal antibodies. Studies have shown that monomeric envelope glycoproteins have higher binding activities to A32 and C11 monoclonal antibodies

compared to oligomeric trimeric envelope glycoproteins (Moore *et al.*, 1994); (Yang *et al.*, 2000); (Grundner *et al.*, 2002); (Yang *et al.*, 2002). These findings suggest that the conformational epitopes recognized by A32 and C11 monoclonal antibodies are occluded in the oligomeric trimeric envelope glycoproteins whereas they are readily exposed in the monomeric envelope glycoprotein (Yang *et al.*, 2000).

The IgG1b12 monoclonal antibody bound to CM9, SW7, Du179 and bacgp120 but marginally to Du151. IgG1b12 has been shown to potently neutralize primary and T-cell line adapted isolates and it recognizes a conformational dependent epitope spanning the CD4 binding site of the envelope glycoprotein (Moore *et al.*, 1995); (Trkola *et al.*, 1995); (Fouts *et al.*, 1997). Bures *et al.*, (2002) observed that five of eight South African, and four of eight Malawian HIV-1 subtype C viruses were neutralized by IgG1b12. Of particular interest in this study, the Du151 isolate was neutralized whereas the Du179 isolates was resistant to neutralization by IgGb12. By contrast, our data showed that the recombinant HIV-1 subtype C Du151 envelope glycoprotein did not bind well to IgG1b12, compared to the Du179 envelope glycoprotein. This supports the observation that primary HIV-1 isolates are resistant to neutralization by IgG1b12 compared to T cell-line adapted viruses (Moore *et al.*, 1995); (Fouts *et al.*, 1997). The IgG1b12 monoclonal antibody has been shown to bind to both monomeric and trimeric envelope glycoproteins (Yang *et al.*, 2000). However, trimeric envelope glycoproteins have a higher binding affinity to IgG1b12 compared to monomeric envelope glycoproteins (Yang *et al.*, 2000); (Grundner *et al.*, 2002); (Yang *et al.*, 2002). The recombinant HIV-1 subtype C envelope glycoproteins and the control bacgp120 had a lower affinity for the potently neutralizing IgG1b12 compared to the nonneutralizing antibodies A32 and C11. This observation confirms that the

recombinant HIV-1 subtype C envelope glycoproteins are monomeric. Furthermore, this assay confirmed that the CD4 binding site of the recombinant HIV-1 subtype C envelope glycoproteins is intact.

The Du179 and Du151 recombinant envelope glycoproteins showed a similar and high level of binding to the 7b2 monoclonal antibody compared to CM9 and SW7 (Figure 3.7). This monoclonal antibody is nonneutralizing, and binds to conformational dependent epitopes of the gp41 subunit. This binding assay confirmed that the conformation of the gp41 subunit of the recombinant HIV-1 subtype C envelope glycoproteins is stable.

The detection of the purified recombinant HIV-1 subtype C gp160-sCD4 complexes by anti-CD4 antibodies was poor in an ELISA. This may be due to the disruption of the CD4 binding site epitope during purification of the recombinant envelope glycoproteins. Previous reports have shown that the gp160 binds poorly to sCD4 in an ELISA, because the hydrophobic regions in the gp41 cause the aggregation of the envelope glycoprotein into micelles, reducing the concentration of the free envelope glycoproteins in the solution (Moore and Jarrett, 1988); (Moore *et al.*, 1990); (Moore, 1990). However, the micelle formation was disrupted by adding 1% empigen and the binding of gp160 to sCD4 was enhanced (Moore and Jarrett, 1988), (Moore *et al.*, 1990). In these studies, the OKT4 antibody which binds to the third and fourth domain of sCD4 was used in the ELISA (Moore *et al.*, 1990); (Moore, 1990); (Earl *et al.*, 1992). In our studies, polyclonal anti-CD4 antibody was used to detect the gp160-sCD4 complex in an ELISA. The regions of sCD4 where these polyclonal anti-CD4 antibodies bind are unknown. This antibody might

not be able to bind to the sCD4 in the gp160-sCD4 complex, thus accounting for the poor detection of the gp160-sCD4 complex in the ELISA.

The 17b monoclonal antibody reacted with all the recombinant HIV-1 subtype C envelope glycoproteins (Figures 3.8 and 3.9). 17b is a nonneutralizing monoclonal antibody that recognizes a CD4 induced conformational epitope of the envelope glycoprotein (Los Alamos National Laboratory; <http://hiv-web.lanl.gov/immunology>). The binding of the recombinant HIV-1 subtype C envelope glycoproteins to 17b occurred in both the absence and presence of sCD4. However, the binding of the recombinant HIV-1 subtype C envelope glycoproteins to 17b was increased following their preincubation with sCD4, implying the exposure of the 17b conformationally dependant epitope. Similar trends were seen for all the recombinant HIV-1 subtype C envelope glycoproteins. This binding assay confirmed that the recombinant HIV-1 subtype C envelope glycoproteins are functional since binding to sCD4 exposed the 17b epitope.

No binding of the recombinant HIV-1 subtype C envelope glycoproteins to the F105 monoclonal antibody was observed, however, the bacgp160 bound to F105 (data not shown). This monoclonal antibody recognizes the conformational dependent epitope spanning the CD4 binding site of the envelope glycoprotein (Los Alamos National Laboratory; <http://hiv-web.lanl.gov/immunology>). The inability of the recombinant HIV-1 subtype C envelope glycoproteins to bind to F105 may indicate that the conformational epitope to this antibody was disrupted during purification. Alternatively, HIV-1 subtype C envelope glycoprotein might not harbor the conformational epitope to which the F105 monoclonal antibody binds. However, this needs to be confirmed with the oligomeric

HIV-1 envelope glycoproteins that mimic the envelope glycoproteins found on the surface of the virus. The F105 monoclonal antibody has been shown to recognize both primary and T-cell line adapted HIV-1 subtype B envelope glycoproteins expressed in the Semliki expression system, confirming the integrity of these proteins (Brand *et al.*, 2000).

The 2G12 monoclonal antibody did not bind to the HIV-1 subtype C envelope glycoproteins, however, it reacted with the control bacgp160. Sanders *et al.*, (2002a) have recently showed that high mannose N-linked glycans at position 295, 332, 392, 386 and 448 form the 2G12 epitope on the HIV-1 envelope glycoprotein. Bures *et al.*, (2002) observed that none of the HIV-1 subtype C viruses were neutralized by 2G12, including Du151 and Du179, consistent with our observations. Furthermore, Sanders *et al.*, (2002a) observed that the HIV-1 subtype C Du151, Du179 and Du422 viruses were not neutralized by 2G12. Furthermore, they observed that these HIV-1 subtype C isolates lacked the N-linked glycan at position 295, accounting for the lack of neutralization by 2G12.

The recombinant HIV-1 subtype C envelope glycoproteins were not able to react with the 2F5 monoclonal antibody. However, the HIV-1 subtype B bacgp160 bound to this antibody as assessed by an ELISA and Western blot (data not shown). Bures *et al.*, (2002) showed that 2F5 could not neutralize any South African HIV-1 subtype C isolates. However, they observed that two of eight Malawian HIV-1 subtype C isolates were neutralized by this monoclonal antibody. The 2F5 neutralizing monoclonal antibody recognizes the ELDKWA epitope in the gp41 region of the envelope glycoproteins. HIV-1 subtype C viruses from South African have been shown to have changes in one or more amino acids in this epitope (Papathanasopoulos *et al.*, in preparation), thus accounting for

the inability of 2F5 to recognize HIV-1 subtype C envelope glycoproteins and viruses. Other HIV-1 subtype B viruses have been shown to have amino acids changes in the ELDKWA epitope, rendering the 2F5 monoclonal antibody unable to neutralize them (Bures *et al.*, 2002).

Previous studies have shown that recombinant HIV-1 envelope glycoproteins expressed in insect cells have antigenic properties similar to those of HIV-1 envelope glycoproteins expressed in mammalian systems (Moore *et al.*, 1990); (Morikawa *et al.*, 1990); (Wells and Compans, 1990). However, in these studies only one envelope glycoprotein from a T-cell line adapted isolate (HTLVIIIB) was examined. We describe for the first time the antigenic properties of four envelope glycoproteins from primary HIV-1 subtype C isolates that use different coreceptors for cell entry. In this study, the Du151 (R5) recombinant envelope glycoprotein showed lower antigenic properties compared to CM9 (R5X4), Du179 (R5X4) and SW7 (X4) envelope glycoproteins, as determined by the poor ability or inability of Du151 to bind to the potently neutralizing monoclonal antibody IgG1b12. Interestingly, Brand *et al.*, (2000) showed that an R5 HIV-1 subtype B envelope glycoprotein had lower antigenic properties compared to an X4 envelope glycoprotein expressed in a mammalian system. Thus, the recombinant envelope glycoproteins from the four South African HIV-1 subtype C isolates (that use different coreceptors) expressed in insect cells display antigenic properties similar to recombinant envelope glycoproteins expressed in mammalian systems. Furthermore, for the first time we are showing that recombinant envelope glycoproteins from dual tropic viruses (CM9 and Du179) exhibit antigenic characteristics similar to those observed in X4 viruses (SW7).

Because recombinant HIV-1 envelope glycoproteins expressed in insect cells are not fully glycosylated compared to those expressed in mammalian systems there has been speculation that they are not suitable for developing reagents to test antibody responses and for further structural and functional studies. Li *et al.*, (1993) showed that N-linked glycosylation is essential for correct folding of the envelope glycoprotein so that it is functional but it is not essential for the correctly folded protein to bind to CD4. Furthermore, they also found that glycosylation of the envelope glycoprotein in insect cells is sufficient for its correct folding required for binding to CD4 (Dirckx *et al.*, 1990); (Li *et al.*, 1993). Moreover, expression of recombinant envelope glycoproteins in insect cells is very attractive, because the recombinant envelope glycoproteins are expressed at higher levels, and without the aid of other viral genes compared to mammalian expression systems (Moore *et al.*, 1990); (Wells and Compans, 1990).

Overall, the ability of the recombinant HIV-1 subtype C envelope glycoproteins to react with sCD4 and their high level of reactivity with the 17b monoclonal antibody in the presence of sCD4 showed that they are functional. The recombinant HIV-1 subtype C envelope glycoproteins were able to react with both nonneutralizing and neutralizing conformational dependent monoclonal antibodies and therefore their conformation is intact. The differences seen in the monoclonal antibody binding profiles of the four envelope glycoproteins confirmed there are antigenic differences between the R5, R5X4 and X4 HIV-1 subtype C envelope glycoproteins. Thus, the *Galanthus nivalis* lectin method we developed is ideal for purification of the recombinant HIV-1 subtype C envelope glycoproteins because these proteins retain their function and conformational integrity. Chen *et al.*, (2000) showed that modified recombinant SIV envelope

glycoproteins expressed in insect cells are functional and trimeric. Therefore, the recombinant HIV-1 subtype C envelope glycoproteins expressed and purified in insect cells can be further modified to produce an immunogen that mimics the trimeric envelope glycoproteins found on the surface of the virion or the surface of the infected cells. The purified recombinant HIV-1 subtype C envelope glycoproteins described in this study can be used to develop reagents that will facilitate further studies on structure, function, antigenicity and immunogenicity of the envelope glycoproteins. Such studies are important in the design of a vaccine that will induce broadly cross-clade neutralizing antibodies against HIV-1.

CHAPTER 4

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APPENDIX A

COMPOSITION OF BUFFERS, MEDIA AND GELS

Reagents for agarose gel electrophoresis

Tris Acetate EDTA (TAE) 10X concentration stock in 10 litres (pH7.4)

Tris (MW 121.1)	484g
Glacial acetic acid	114ml
0.5M EDTA pH8.0	200ml

Dissolve Tris in eight litres of distilled water, add glacial acetic acid and EDTA and make up to 10 litres. Filter sterilize and store at 4°C.

For experiments 1X TAE was used.

1% agarose gel

Dissolve 0.5g agarose in 50ml of 1XTAE

Microwave the mixture until the agarose is completely dissolved.

Allow it to cool and add 2µl of 10mg/ml ethidium bromide and pour the gel.

LB broth

Dissolve 20g of in 1 litre of distilled water. Autoclave the broth and allow to cool to 50°C and add appropriate antibiotics.

Reagents for 10% SDS-PAGE gel

40% acrylamide/bis acrylamide electrophoresis reagent

10% SDS

10% Ammonium Persulfate (APS; made fresh)

Baxter water

Temed

0.5M Tris-HCl pH 6.8

1.5M Tris-HCl pH 8.8

Separating SDS-PAGE gel

Baxter water	5.9 ml
40% acrylamide	5.0 ml

1.5M Tris-HCl	3.8 ml
10% SDS	150μl
10% APS	150μl
Temed	15μl

Mix by gentle swirling and pour the gel

Stacking SDS-PAGE gel

Baxter water	2.7 ml
40% acrylamide	670μl
0.5M Tris-HCl	500μl
10% SDS	40μl
APS	40μl
Temed	4μl

Mix by gentle swirling and pour over the separating SDS-PAGE gel

Stock of 10X SDS-PAGE Running buffer (pH7.4)

SDS	10g
Tris	30.3g
Glycine	144.1g

Dissolve these ingredients in 1000ml of distilled water.

For experiments use 1XSDS-PAGE running buffer by diluting 10X buffer 1:10.

2X SDS-PAGE sample buffer

1.5M Tris (pH6.8)	10ml
20% SDS	6ml
glycerol	30ml
β-mercaptoethanol	15ml
Bromophenol blue	1.8mg

Mix these reagents and make it up 100ml by adding distilled water, aliquot to 10ml and store at -20°C.

Reagents for Western blots

Transfer buffer

Tris	5.82g
Glycine	2.93g
10%SDS	3.75ml
Methanol	200ml

Dissolve these reagents in 700ml and adjust the pH 9.2 and add water to make up to 1000ml.

Tris Buffer Saline (1XTBS) (pH7.4)

0.5M Tris-HCl pH 8	20ml
NaCl	8.8g

Dissolve 8.8g NaCl in 800ml and 20ml 0.5M Tris-HCl, adjust to pH8 and make it up 1000ml.

Coomassie blue staining solution

Coomassie Brilliant Blue R250	2.5g
Methanol	450ml
Acetic acid	100ml

Dissolve 2.5g of Coomassie Brilliant Blue in 450ml of distilled water and add 450ml methanol and 100ml acetic acid.

Destaining solution

Methanol	400ml
Acetic acid	70ml

Mix these reagents and add distilled water to make it up to 1000ml.

Lysis Buffer

50mM Tris-HCl, pH 7.5	5ml
150mM NaCl	3.75ml
1% Nonidet and 0.5% sodium deoxycholate	2.5ml
1 Complete tablet (Roche, Germany)	1 tablet

Silver staining reagents

Fixative 1

Add 80ml of methanol in 120ml of deionized water.

Fixative 2

Add 40ml of ethanol in 360ml of deionized water

Oxidizer

Add 20ml of oxidizer in 180ml of deionized water

Silver reagent

Add 20ml of silver reagent in 180ml of deionized water

Developer

Dissolve 1 bottle of the developer in 3.6L of deionized water

Reagents for protein purification

10X PBS

NaCl	80g
KCl	2g
Na ₂ HPO ₄ ·7H ₂ O	26.8g
KH ₂ PO ₄	2.4g

Dissolve all these reagents in 800ml of distilled water and adjust to pH7.3 with HCl and adjust the volume to 1000ml with distilled water.

2M NaCl

Dissolve 116.9g of NaCl in 1000ml of distilled water.

0.65M NaCl in PBS (pH7.3)

Add 165.2ml of 2M NaCl in 334.8ml of PBS, adjust to pH 7.3, filter sterilize and store at 4°C.

1M NaCl in PBS (pH7.3)

Add 250ml of 2M NaCl in 250ml of PBS, adjust to pH 7.3, filter sterilize and store at 4°C.

1M Methyl α -D mannopyranoside (MMP)

Dissolve 9.71g of MMP in 50ml of PBS, adjust to pH 7.3, filter sterilize and store at 4°C.

APPENDIX B

REAGENTS AND SUPPLIERS

β -mercaptoethanol	Sigma, USA
ABI Prism Bigdye Ready Reaction Kit version 2.0	P E Applied Biosystem, USA
Acrylamide	Sigma, USA
Agarose	Gibco, Scotland
Ammonium persulfate	Sigma, USA
Ampak substrate	Dako, UK
Ampicillin	Roche, Germany
Anti-human IgG-alkaline phosphatase	Sigma, USA
Anti-mouse IgG-alkaline phosphatase	Sigma, USA
<i>Bam</i> H1	Roche, Germany
Bovine serum albumin	Roche, Germany
Bromophenol blue	Sigma, USA
CellFectin	Gibco, Scotland
Coomassie Brilliant Blue R250	Sigma, USA
Complete™ Tablets	Roche, Germany
Developer	Bio-Rad, USA
DMSO	Sigma, USA
dNTP's	Roche, Germany
EDTA	Merck, Germany
Ethanol	Associated Chemical Enterprises, SA
Fetal calf serum	Delta Bioproducts, South Africa
<i>Galanthus nivalis</i> lectin	Sigma, USA
Gentamycin	Roche, Germany
Glacial acetic acid	Saarchem, South Africa
Glycerol	NT laboratory suppliers, South Africa
Glycine	Fluka Biochemica, Germany
Grace's medium	Highveld Biologicals, South Africa

High Pure PCR Product Purification kit	Roche, Germany
High Speed Plasmid kit	Qiagen, Germany
<i>Hind</i> III	Roche, Germany
Immunoprecipitation kit (Protein A)	Roche, Germany
Kanamycin	Roche, Germany
LB agar	Gibco, Scotland
LB broth	Gibco, Scotland
Ligase	Roche, Germany
Methanol	Merck, Germany
Methyl α -D mannopyrannoside	Sigma, USA
NBT/BCIP tablets	Roche, Germany
Oxidizer	Bio-Rad, USA
Pluronic-F68	Sigma, USA
Potassium chloride	Merck, Germany
Seize X Mammalian immunoprecipitation kit	Pierce, USA
Silver Reagent	Bio-Rad, USA
Skimmed milk	Merck, Germany
Sodium chloride	Merck, Germany
Sodium dodecyle sulfate	USB, USA
Sodium hydroxide	Merck, Germany
Supertherm Taq	Southern Cross Biotechnology, SA
Temed	Sigma, USA
Tetracycline	Roche, Germany
Tris	USB, USA
Tris-HCl	USB, USA