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ABSTRACT

Title of Thesis:
Characterization and Enhanced Processing of Soluble, Oligomeric gp140 Envelope Glycoproteins Derived from Human Immunodeficiency Virus Type-1 Primary Isolates

Agnes Y. Jones-Trower, Ph.D., 2001

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HIV-1 first interacts with host cells through its envelope glycoprotein (Env), the major target of neutralizing antibodies. The biologically relevant form of Env is an oligomer, both in its presentation to the immune system and in virus entry. Also, native Env is cleaved into its gp120 and gp41 non-covalently associated subunits. Env-based vaccines have thus far failed to efficiently generate broadly cross-reactive, neutralizing antibodies towards primary isolates. Possible explanations for their failure are either they were derived from laboratory-adapted strains or they have not preserved the conformational structures necessary to elicit broadly reactive responses. Producing a broadly effective Env-based vaccine is also potentially complicated by the existence of multiple HIV-1 genotypes. Here, I have constructed a panel of truncated *env* genes from primary isolates of several different HIV-1 clades. Recombinant vaccinia viruses expressing these genes produce a secreted Env known as gp140. These gp140s were characterized by sucrose density gradient centrifugation and size exclusion chromatography analyses to determine oligomeric status and degree of processing. While most processed gp140s dissociated to monomeric forms, there was evidence that certain

isolates could retain gp120 in an oligomer. A large scale purification scheme was developed using lentil lectin affinity and size exclusion chromatographies to prepare gp140 oligomers. Pre- and post-production processing enhancers (furin and plasmin) were examined as means to bolster the amount of processed gp140, and reducible crosslinkers were employed to analyze the ability of processed gp140 to maintain oligomeric forms. The antigenic properties of the gp140s, both cleaved vs. uncleaved, and crosslinked vs. non-crosslinked, were analyzed by immunoprecipitation with a panel of well-characterized human and mouse antibodies. The purified, crosslinked, cleaved oligomers retained important epitopes and the ability to undergo receptor-induced conformational change as could uncleaved gp140. Finally, a novel antigen delivery system (Matrix III) was investigated where oligomeric gp140 was encapsulated and administered to animals. Analysis of these sera has indicated that Matrix III does not seem to alter the quantity or quality of elicited antibody. Taken together, these experiments have provided critical information and material towards testing a variety of oligomeric Env-based vaccine strategies for the prevention of HIV-1 infection.

CHARACTERIZATION AND ENHANCED PROCESSING OF SOLUBLE, OLIGOMERIC GP140 ENVELOPE GLYCOPROTEINS DERIVED FROM HUMAN IMMUNODEFICIENCY VIRUS TYPE-1 PRIMARY ISOLATES

by

Agnes Yuko Jones-Trower

Dissertation submitted to the Faculty of the Department of Molecular and Cell Biology of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy 2001

DEDICATION

To my patient and encouraging family

Ron - the 'never give up' guy

Sara and Kevin – the best children in the world

To my supportive mom

Hideko Jones

And

In loving memory of my dad

Charles T. Jones

who taught me learning is a lifelong experience

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INTRODUCTION

HIV-1 OVERVIEW

Pathogenesis

Human immunodeficiency virus type 1 (HIV-1) is the virus that causes acquired immunodeficiency syndrome (AIDS), a combination of diseases that has decimated the population of entire countries. Since the beginning of the AIDS epidemic, 21.8 million people have died, 3 million of which succumbed in 2000 alone. Current statistics from the United Nations AIDS Organization indicate that as of December 2000, 5.3 million people worldwide were newly infected with HIV-1, bringing the total number of people living with HIV/AIDS to a staggering 36.1 million. This is more than 50% higher than what World Health Organization's Global Programme on AIDS projected in 1991 on the basis of the data then available (1). Dissemination of knowledge and drug therapy has not stemmed the pandemic, but has only slowed the rate of increase in infection and death, and only in selective regions of the world.

For progression to disease, HIV-1 has to accomplish the following: enter the host, replicate in cells of the immune system, elicit acute symptoms, establish latency and a reservoir of the virus, replicate again and disseminate new virions, and finally, produce end-organ damage (159). Development of disease may take up to 10 years (38, 159, 163, 246). During this period of clinical latency, an immunopathic process that occurs in the lymph nodes of the infected individuals is the primary factor in the eventual destruction of the immune system (94, 214).

Infection with HIV-1 is characterized by an initial phase of high-level viremia followed by a long latency period during which persistent virus replication occurs at a low level (57, 65, 68, 101, 133). The primary indicator of AIDS is a progressive decline in the number of $CD4^+$ lymphocytes (215) with an accelerated rate of decline associated with the appearance of syncytium-inducing variants of the virus (148). Widespread infection of non-lymphoid tissues such as brain, lung, liver, spinal cord, kidney, and heart can be detected during the symptomatic stages of HIV-1 infection. Gastrointestinal symptoms such as chronic intestinal malabsorption, diarrhea and wasting are commonly observed during the later stages of the disease. These symptoms are sometimes due to opportunistic infections associated with immune deficiency, but may also be the direct result of HIV-1 infection of the bowel mucosa. However, it is usually the acquisition of one or more opportunistic infections or malignancies that eventually leads to the death of the infected individual.

Extensive research has been conducted to determine why some HIV-1 infected individuals do not develop AIDS although they have been infected with the virus well beyond the ten year period of latency. Some host-related factors have been identified that account for long-term nonprogression. These include genetic polymorphisms in the cellular coreceptor CCR5 and a strong cellular immune response (43, 170, 216). The polymorphisms in CCR5 are most often represented as a thirty-two base pair deletion within the CCR5 gene (70, 167, 249, 302), or a mutation in the promoter of the CCR5 gene (178). Long-term nonprogressors may also be attributed to infection with an attenuated strain of HIV-1. Nef and Gag deletions, amino acid insertions in Vpu and a single amino acid deletion in gp41 of Env have all been found in slow progressors and nonprogressors

(2, 8, 146). Continued studies of these individuals may provide insights to combat the HIV-1 pandemic.

Retroviruses: Virus Structure

Human immunodeficiency viruses (HIV-1, HIV-2) are classified in the family of Retroviridae, genus Lentivirinae (lentiviruses) (117, 160). The virion measures 80-130 nm in diameter and has the typical cone-shaped viral core seen in other lentiviruses. The viral core is comprised of the p24 capsid protein and a ribonucleoprotein complex which contains the following: viral reverse transcriptase (p55/66); integrase (p32); nucleocapsid proteins (p6/7); two identical copies of plus-stranded viral RNA genome. Just below the lipid bilayer envelope lies the myristylated matrix protein (p17). The envelope, derived from the host cell membrane, contains both viral and cellular proteins, of which seventy-two "knobs" of viral envelope glycoprotein oligomers are an integral part (213).

Genome

HIV-1 is a complex retrovirus. Its genome is about 9.8 kilobases in size and encodes structural, regulatory and accessory proteins. These genes are flanked by long terminal repeats (LTRs). The HIV-1 genes are transcribed from the integrated DNA provirus which results in the production of several subclasses of unspliced, singly spliced or multiply spliced viral mRNAs. The group-specific antigen (*gag*) gene product consists of matrix protein, the capsid structural protein and two RNA binding proteins. The *pol* gene encodes the viral protease, reverse transcriptase and integrase. The *env* gene encodes the envelope glycoprotein (gp160) precursor. *Tat* and *rev* genes, which flank the *env* coding sequence, encode positive regulators of HIV-1 replication.

The accessory proteins of HIV are less well understood but are important during *in vivo* virus growth and pathogenesis. The *nef* gene product may be required for maintenance of high virus load during in vivo infection (145). The myristylated N-terminal region of Nef has severe membrane disordering properties and, when present in the extracellular medium, causes rapid lysis *in vitro* of a wide range of $CD4^+$ and non-$CD4^+$ cells. This reaction suggests a role for extracellular Nef in the depletion of bystander cells (10). Since Nef does not appear to alter virion binding or entry but does enhance proviral DNA synthesis, an early post-entry action of Nef at the level of viral uncoating or reverse transcription has been proposed (259).

The *vif* gene product increases the stability of virion cores. This may permit efficient viral DNA synthesis by preventing premature degradation or disassembly of viral nucleoprotein complexes during early events after virus entry (209). Vif associates with RNA in vitro as well as with viral genomic RNA in virus-infected cells, suggesting a novel role for Vif in the regulation of efficient reverse transcription through modulation of the virion nucleic acid components (75).

Vpr is homologous to Vpx, however HIV-1 expresses only the former, while HIV-2 contains both. Extracellular Vpr and C-terminal fragments of Vpr cause membrane permeabilization and apoptosis of T-lymphocytes and a wide range of non-$CD4^+$ cells, a possible mechanism in the $CD4^+$ cell depletion during AIDS progression (10, 179). Vpr may also increase apoptosis in human neuronal cells, therefor causing injury to the central nervous system (220). This effect may be a byproduct of its other functional properties of induction of G2/M cell cycle arrest and positive effects on virion production and replication (122, 139).

Vpu, an 81 residue membrane protein, facilitates the budding of new virus particles from infected cells due to ion channel activity of its transmembrane helical domain (172). The second function indirectly regulates processing of gp160 in the trans-Golgi compartment by targeting CD4 for proteosome-mediated degradation to decrease the formation of stable complexes with CD4 in the endoplasmic reticulum (ER) (173).

HIV-1 ENVELOPE GLYCOPROTEIN

HIV-1 first interacts with host cells through its envelope (Env) glycoprotein (gp). The HIV-1 gp is a complex composed of two components, the gp120 and gp41 subunits. The gp120 subunit binds the virus to receptors on the cell surface and contains the major neutralization epitopes of the virus. Gp120 interaction first occurs with the CD4 glycoprotein on the host cell, and is followed by engagement with a coreceptor, a member of the chemokine receptor family. The CD4 glycoprotein can be found on the surface of T lymphocytes, monocytes, macrophages, dendritic cells, and brain microglia. The requirement for CD4 is not universal, since CD4-independent laboratory strains of HIV-1 and primary isolate simian immunodeficiency virus (SIV) strains have been reported (56, 83, 93, 95, 96, 174, 231). Binding occurs at the most amino-terminal of the four immunoglobulin-like domains of CD4 (126, 181). The binding of CD4 to the Env of some HIV-1 isolates, mostly T-cell line adapted (TCLA) strains, induces the shedding of gp120 into the surrounding medium (193).

The most widely accepted model for the Env-mediated fusion process is that conformational changes within gp120 occur after initial CD4 binding, which exposes the binding site for coreceptor (reviewed in (22, 78)). CXCR4 and CCR5, the chemokine

receptors most commonly used by HIV-1 as coreceptors, are G-protein coupled receptors. The third variable (V3) loop of gp120 is the principal determinant of chemokine receptor specificity, with more conserved gp120 structures exposed after CD4 binding also involved (262). Most primary, clinical isolates use CCR5 – termed (R5) isolates - for entry into target cells (3, 54, 73, 80, 102), while isolates that arise later in the course of infection tend to use CXCR4. The latter are called (X4) isolates (19). The R5 HIV-1 strains are also often referred to as non-syncytium–inducing strains that exhibit "slow/low" replication kinetics in contrast to the X4 strains that have "rapid/high" kinetics (9, 51, 104, 271).

Following the binding of an appropriate coreceptor, additional conformational changes occur to expose the fusion domain of gp41. The gp41 subunit is comprised of an ectodomain, a transmembrane domain and a cytoplasmic tail. Besides serving as a membrane anchor, gp41 also facilitates fusion of the viral and cellular membranes, is responsible for Env gp oligomerization (87), and also possesses neutralizing domains (49, 69).

Processing and Oligomerization

The Env glycoprotein is synthesized as a monomeric, cotranslationally glycosylated, polyprotein precursor of 845-870 amino acids (gp160). Analysis of the gp120 sequences reveals an N-terminal signal peptide, and five variable regions (V1 through V5) interspersed with five conserved regions (C1-C5) (266). The gp120 variable regions, V1 through V4, form large loop-like structures due to intramolecular disulfide bonds in gp 120 (157). These are well exposed on the surface of the glycoprotein (197, 225, 291). The

Env precursor attaches to the ER via its signal peptide (77), which is removed after the protein has entered the lumen of the ER. The stop transfer sequence is located in the middle of the transmembrane domain of gp160. After asparagine-linked, high mannose sugars are added (glycosylation), the individual precursor molecules fold and assemble to form an oligomeric structure, most likely a trimer (48, 87, 88, 157, 169, 224, 282). There are some twenty-four glycosylation sites in gp120 and four sites in gp41, most of which are conserved among the different isolates. The fully modified envelope is 45% - 50% carbohydrate in mass, which accounts for a 160 - 200 kDa range of molecular mass in contrast to the predicted 88 kDa. The stable oligomer is transported from the ER to the Golgi complex where the bulk of the mannose-rich oligosaccharide side chains are processed to hybrid/terminally glycosylated forms (136). In the trans-Golgi network, cellular proteases, most likely furin and/or PC7, cleave the gp160 into subunits gp120 and gp41 (4, 71, 125, 241, 279). These subtilisin-like proteases recognize and cleave at a highly conserved basic sequence (Arg- X-X-Arg), cleaving immediately after the Arg511 of the Arg-Glu-Lys-Arg ↓ sequence in the C5 domain of HIV-1 (151, 176). A second putative cleavage site is located eight amino acid residues upstream of the physiological cleavage site and plays an important role in the accessibility of the physiological cleavage site to the proteases (106, 123). Cleavage at the secondary site leads to the formation of gp41 presenting a polar amino acid sequence to the fusion peptide that can impair the fusion activity of the Env (198). The gp120 and gp41 subunits remain non-covalently associated, most likely due to residues within the conserved regions C1 and C5 in gp120 (129) and residues 528-562 within gp41 (42). Conformational changes likely occur after cleavage, consistent with studies on the influenza virus (285). Assembled Env oligomers

are incorporated into budding virus particles. **Figure 1** is a representation of the oligomeric structure of HIV-1 envelope glycoprotein in the context of virus entry into a cell.

Tertiary and Quaternary Structure

Though it has not been possible to crystalize the native oligomeric envelope, Wyatt and colleagues have succeeding in crystallizing gp120 by complexing the core of gp120 with two- domain soluble CD4 and the Fab fragment of a neutralizing antibody (290). The core gp120 was chemically deglycosylated, contained only one of the four variable loops, and had N- and C-terminus sequences deleted. Though greatly modified, the gp120 used in this study maintained some degree of structural integrity as evidenced by its ability to bind CD4 and antibodies to the CD4 binding site (CD4bs) as well as the ability to retain some CD4 inducible (CD4i) epitopes. The X-ray crystal structural analysis revealed the core of gp120 to be composed of an inner and an outer domain, and a bridging sheet that does not belong to either domain. The inner domain faces the trimer axis, whereas the outer domain is mostly exposed on the surface of the trimer. The V1/V2 stem projects from the distal end of the inner domain. The stacked double-barreled outer domain lies alongside the inner domain and contains the V4/V5 loops at its proximal end. The inner domain is appreciably more conserved than the outer domain when sequence of HIV-1 strains is compared. The large electroneutral surface on the inner domain is the probable site of trimer packing based on its lack of glycosylation and other factors. Glycosylation sites are all surface exposed. The seven disulfide bridges

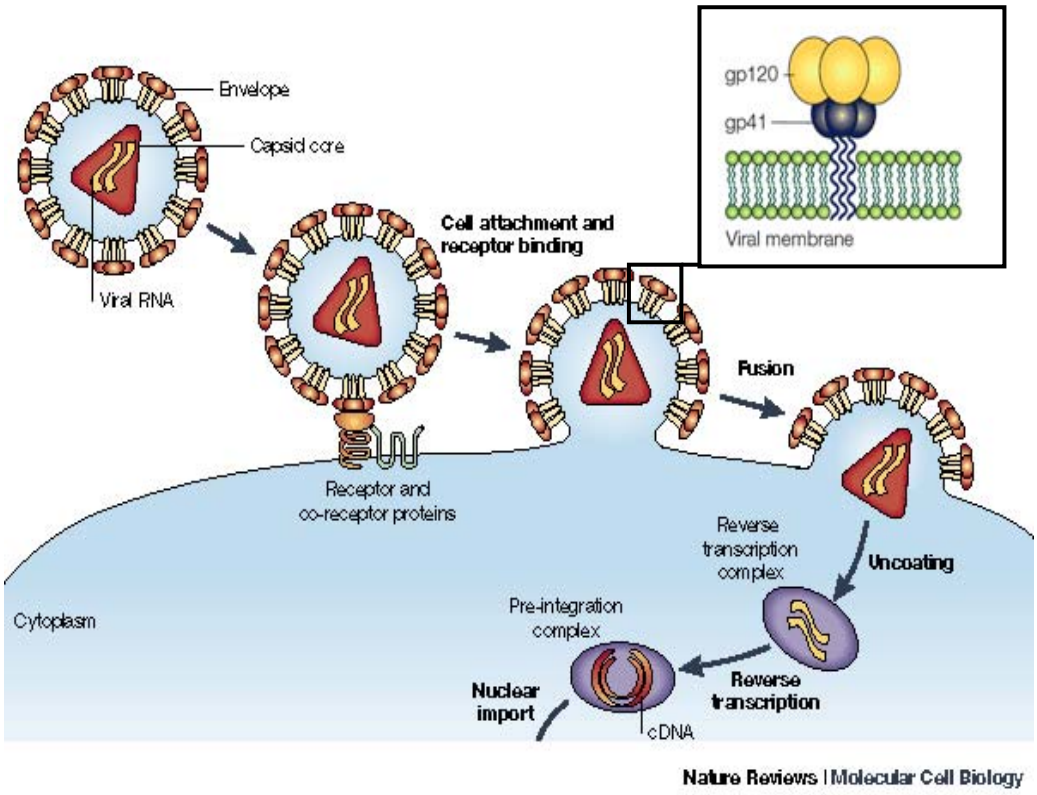


Figure 1. Illustration of HIV-1 binding and entry into a cell. The enlarged view illustrates the trimeric structure of HIV-1 Env as it exists in the cell membrane (modified from Nature Reviews, 2000).

retained in the core gp120 are highly conserved among the virus isolates and are mostly buried within the protein.

Based on the crystal structure of gp120, it is expected that the gp41 interactive regions extend away from the gp120 core towards the viral membrane (288). The crystal structure of peptide fractions of the gp41 ectodomain has revealed a bundle of six α-helices: three α-helices pack in an anti-parallel manner against a central, three stranded coiled coil (48, 283). The glycine-rich fusion peptide at the extreme N-terminus is hydrophobic and inserts into the membrane of the target cell during the Env-mediated fusion process. A 4-3 hydrophobic repeat (leucine zipper-like) region, capable of forming a coiled-coil structure, follows the fusion peptide domain (110). The carboxy terminus is adjacent to the transmembrane helix anchored in the viral membrane. The thermostable coiled-coil gp41 ectodomain is believed to represent the post-fusion form of the gp41 ectodomain, in contrast to the native Env complex, which is thermolabile. The native or non-fusogenic state has been shown in the native HA1/HA2 influenza complex to have the heptad-repeat region fold as a nonhelical loop (285) and converts to its fusogenic coiled coil form upon exposure to low pH. In contrast, the conversion to the fusogenic form of HIV-1 Env evolves from the extensive conformational changes which occur following receptor binding, which have yet to be fully understood, since the resting non-fusogenic structure of Env is unknown.

Genetic Variation

The high levels of genetic variability associated with HIV-1, noted when the virus was first sequenced (250, 281), may likely have a major impact on the efficacy of any Env-

based vaccine that would be designed. Sequencing of the *gag* and *env* genes has led to the identification of two major groups of HIV-1 viruses, M (major) and O (outliers) (202). O subtypes are found in Cameroon and Gabon and in individuals with connections to those countries, but contribute to only a small percentage of the total number of HIV-1 infections (124, 137, 297). The HIV-1 strains responsible for the global pandemic are in the M group. These subtypes have been labeled alphabetically: A, B, C, D, F1, F2, G, H, J, K. Former subtypes E and I are both now defined as circulating recombinant forms (CRF) (240).

There is a rapid evolution of HIV-1 within an infected individual, due in part to the rapid virus replication rate of, on average, 150 replication cycles a year (61). This, combined with the error-prone reverse transcriptase enzyme, leads to approximately one base misincorporation per genome per replication cycle (221, 227, 232, 238). During the first 7 to 10 weeks of infection, there are low levels of sequence variability, especially in the envelope gp120 (299, 301). During late term HIV-1 infection, however, Env gp becomes the HIV-1 protein with the greatest number of mutations, leading to the generation of quasispecies within any one infected individual (55).

HIV-1 Envelope's Significance in Pathogenesis

Understanding the influences of HIV-1 Env gp on cells of the immune and nervous systems provides insights into HIV-1 disease pathogenesis and carries implications for the trials of HIV-1 Env vaccines and immunotherapeutics (52). The HIV-1 Env has been implicated as a factor in the progression of immune system destruction. Noteworthy biological effects on the immune system include: effects on cellular differentiation,

activation and cytokine secretion patterns of T- cells; induction of cellular apoptosis; and modulation on macrophage function (14, 52, 261). These effects have been observed in the absence of infectivity, with direct interaction of HIV-1 Env with CD4 and chemokine receptors. This interaction triggers cellular signaling events. For example, calcium mobilization and chemotaxis of dendritic cells indicate that activation of T-cells has occurred. Envelopes derived from HIV-1 strains that are able to replicate in macrophages mobilized large stores of calcium, contrary to X4 and R5 strains that do not replicate in macrophages (6, 265).

The interaction of R5 HIV-1 Env with CD4 leads to phosphorylation of the tyrosine kinases Lck, ZAP70 and Pyk2. Tyrosine phosphorylation of focal adhesion kinase (FAK) and CCR5, followed by redistribution of focal adhesion complexes and the association of FAK with CCR5, has been shown to follow T-cell activation and also as a response to chemotactic stimuli. This, in turn, leads to alterations in cytoskeletal structure, which is important in facilitating HIV-entry.

Interestingly, activation of caspases can also occur subsequent to interaction of R5 and X4 Env with primary T-cells. Apoptosis is usually the result of the cascade of events that follows activation of these enzymes by HIV-1 Env. However, though dependent on CD4 interaction, Env-induced caspase activation is Fas and Fas ligand independent. Fas and Fas ligand are usually involved in the execution of apoptotic programs. Apoptosis of CD4+ T lymphocytes could contribute to the cell depletion seen with AIDS. Virion and cell-free gp120 may cause the negative effects of attracting cells to sites of viral replication and inducing apoptosis in bystander cells, but may also concurrently cause down-regulation of chemokine receptors, thus suppressing monocyte activation by

chemoattractants. This chemoattractant response induced by CCR5 signaling might be a contributing factor to the pathogenesis of HIV-1 *in vivo* by attracting activated CD4+ cells to sites of viral replication. Viral replication is then enhanced by increasing the activation state of target cells (284).

The effects of HIV-1 Env on the nervous system manifest as AIDS dementia complex (ADC) and painful sensory neuropathy. HIV-1 gp120, in the absence of infectivity, has also been shown to bind various sensory neurons, sensory nerve glycolipids and myelin-associated glycoproteins and has produced neuronal injury by macrophage and possible astrocyte activation (165). The development of ADC has been associated with the loss of neurons from the frontal cortex, most likely through apoptosis, though neurons are not directly infected with HIV-1 (248). The HIV-1 Env gp may also stimulate the release of toxic pro-inflammatory cytokines from brain macrophages, inducing apoptosis (97). Herzberg and colleague have been able to induce long-lasting central neuropathology in the rat by directly applying gp120 to the sciatic nerve (131), but others have not found chronic, systemic administration of gp120 to have any effect on the rodent brain or blood-brain barrier (223).

HIV-1 IMMUNOGENICITY AND ANTIGENICITY

HIV-1 immunogenicity, or its ability to elicit an immune response is well known; infection with HIV-1 elicits potent humoral and cellular responses. The ability to elicit an immune response is not affected by replication phenotype, i.e., classification as X4 vs. R5 (200). Detectable antibody titers two to three weeks after infection demonstrates that the humoral arm of the immune system is responsive to viral infection. Analyzing the

antibody isotypes of individuals with AIDS, it was determined that IgG1 is the predominant subtype, followed by IgG3, IgG4 and IgG2 (168). During natural infections, disassembled envelope glycoproteins are presented to the immune system and may act as decoys to push the immune system towards production of antibody to non-neutralizing determinants. Non-neutralizing antibodies are made to occluded regions of gp120 that are exposed only when gp120 is shed in a monomeric form (195, 287), whereas neutralizing antibodies are not found until after the initial phase of high level viremia and entrenchment of HIV-1 within host cells has occurred (162, 203, 204). Neutralizing antibodies that can inhibit a wider range of isolates are generally not found until late in the course of infection (25, 141, 189, 228).

There are neutralizing determinants in both the gp120 and gp41 subunits. Most of these conserved or variable epitopes lie near the receptor binding regions (132, 226, 273, 286). Both the V2 and V3 loops contain the variable epitopes for strain-restricted neutralizing antibodies (109, 230, 245). What has been known as the principal neutralizing domain (PND) is found within the V3 loop, which lies at the crown of the loop and contains the GPGRAF sequence shared by many HIV-1 isolates (36). Though neutralizing, anti-V3 loop antibodies are mostly restricted to the recognition of the V3 loop of the particular strain against which they are raised and are thus strain specific, with minimal cross-reactivity to other viral isolates. These hypervariable epitopes tend to dominate the humoral immune response, which causes failure of induction of broadly protective humoral immunity.

Neutralizing antibodies to conserved regions are more broadly neutralizing. These are often exclusively reactive to discontinuous conserved epitopes found within three

regions of the Env. The greatest number of neutralizing antibodies is made to the CD4 binding site (164, 226, 272), where both linear and conformational determinants are important to this region that lies between V2 and V3 (268) and contains part of the C4 region (152). Elements of both domains and the bridging sheet contribute to CD4 binding, which binds in a recessed pocket on gp120. Of the two cavities evident at the gp120-CD4 interface, the deep cavity is the most interesting. Here the residues previously identified as important for CD4 binding (66, 151, 156, 211) surround the cavity opening and interact with the critical binding amino acid for CD4, namely phenylalanine-43 (7, 222, 247). The neutralizing antibodies are effective because they block the gp120-CD4 interaction.

Antibodies to the CD4 inducible (CD4i) epitope interfere with binding of the gp120-CD4 complexes to chemokine receptors (276, 286). The amino acids that interact with this set of antibodies probably consists of elements near or within the bridging sheet and V3 loop; mutagenic analysis supports this hypothesis (235). These residues are highly conserved with the exception of Thr 202 and Met 434 (288). Crystal structure analysis supports the suggestion that CD4 binding repositions the V1/V2 loops, and, to a lesser extent, the V3 loop, thus exposing the CD4i epitiopes (288, 289).

The last region of conserved amino acids recognized by neutralizing antibodies is defined by the monoclonal 2G12. This antibody does not efficiently block receptor binding of either CD4 or coreceptor, and so its mechanism for neutralization remains unknown (276). Only six known amino acids in gp120 are involved with this interaction and are located on the outer domain, opposite to and approximately 25 Angstroms away from the CD4 binding site (CD4bs) (288). Glycosylation plays heavily into the

neutralizing activity of this antibody, which may also explain the rarity of generation of this type of antibody. The extensive glycosylation of gp120 protects the virus from humoral immunity, not just by blocking antibody attachment, but by inhibiting the process of production of antibodies by reducing the binding of gp120 to the B-cell receptor (186).

Several regions of gp41 are recognized by sera from individuals infected with HIV-1. The extracellular domain of gp41 contains six regions of seroreactivity. The best characterized antigenic determinant is the immunodominant epitope that lies within the central region of the ectodomain of gp41 and is termed Cluster I (292). This epitope is centered between residues 598 and 613, and contains a short disulfide-bonded loop (116). However, antibodies reactive to this epitope are non-neutralizing and may actually enhance infectivity (242). This antigenic structure is conserved across clades and is probably important in the oligomeric structure (85). The second seroreactive ectodomain region, known as Cluster II, is located proximal to the membrane-spanning region of gp41 (292). This region spanning amino acids 644 to 667, does not usually elicit antibodies that are neutralizing. One exception is the human monoclonal antibody 2F5 that neutralizes a variety of primary isolates of HIV-1 (63, 201). The amino acid sequence ELDKWA (residues 671-676 by HXB2 numbering) has been defined as the epitope recognized by this antibody (201), but the 2F5 epitope may also involve residues flanking this sequence (304). Additionally, MAbs Z13 and 4E10, generated from HIV-1 seropositive donors, have recently been shown to neutralize primary isolates from clades B, C and E (304). The apparently linear epitope for these two antibodies lies proximal to

the 2F5 epitope in gp41, though direct Env conformation changes or epitope masking may limit MAb accessibility to the epitope.

Other distinct conformational antigenic determinants have been mapped to regions of gp41 where structure is influenced by quaternary interactions (85). One determinant lies near the C terminus of the gp41 ectodomain and overlaps Cluster II. Antibodies to this region were only moderately prevalent in HIV-positive human sera. Another determinant in the C terminus mapped to the region defined by amino acids 641-683, a region to which other conformation dependent antibodies have previously been ascribed (135, 270). Two additional antigenic determinants - not very likely to be immunogenic - were defined by two linear MAbs. These regions mapped to the amino terminal 68 amino acids of gp41 and amino acids 611 through 640. The latter domain is highly glycosylated, which may account for its poor immunogenicity (85).

Even the cytoplasmic tail of gp41 has been implicated in Env immunogenicity. The controversial "Kennedy peptide" contains residues 731-752, which are in the cytoplasmic region of gp41. This peptide does contain a conformation-dependent, neutralizing epitope (ERDRD) and an immunodominant, linear, non-neutralizing epitope (IEEE) (60). Cleveland, et. al. has proposed this region lies on the exterior of free infectious virus particles. Antibodies raised against this hydrophilic peptide neutralize a broad range of HIV-1 isolates (69, 98). It is possible that neutralization by antibodies is mediated through cell surface proteins incorporated in the HIV-1 lipid envelope, and not the Env itself (136).

Envelope-Based Therapeutics

Drug development for HIV-1 therapeutics has been successful in lowering viral load, but not in achieving total elimination of the virus from the body. The promising protease inhibitors and reverse transcriptase inhibitors work at the level of inhibiting HIV-1 replication after the virus has already infected the host cell. Though effective, they are costly and not well tolerated. The first step of the HIV-1 life cycle, however, is attachment to and entry into that host cell. To stop the virus at this critical step, the design and development of HIV-1 entry inhibitors has become a major focus in drug development. Lalezari and colleagues (155) have presented the first controlled clinical trial data on such a drug (T-20), a peptide derived from one of the three helical coil domains of gp41. The domain that T-20 binds to becomes exposed following binding of gp120 to CD4 and the chemokine coreceptor. The preliminary results of this safety study are encouraging in that reductions in plasma HIV-1 RNA levels were seen.

Along the same lines, the fusion inhibitor 5-Helix shows promise in *in vitro* studies. This large polypeptide inhibits HIV-1 replication at nanomolar concentrations and is active against both laboratory-adapted and clinical isolates from several different HIV-1 subtypes (243). This fusion protein consists of 5 of 6 helical coils expected to be present in the trimeric fusion domain (244), and its large size predisposes it to be produced as a recombinant peptide in bacteria, in contrast to the chemical synthesis required for T-20. It remains to been seen whether these antiviral therapeutics will be better tolerated than the currently available drugs.

VACCINES

Animal Models

There are many examples throughout history when vaccine development has been successful without the use of animal models. Non-human primate models in HIV research have so far resulted in neither a clear definition of what immune responses will be required for vaccine-induced protective immunity, nor given consistent results on the potential efficacy of various vaccine approaches (138). The role of non-human primate models in HIV-1 vaccine research is continually debated. However, useful data has been generated and further development and improved uniformity in the use of animal models may facilitate the development of an HIV vaccine.

Since the only species other than human that are susceptible to infection by HIV-1 are nonhuman primates, only two models have emerged, each with its own inherent pros and cons. The chimpanzee (*Pan troglodytes*) has been the most carefully studied as the only available HIV challenge model. All known strains of HIV-1 capable of infecting humans are very closely related to SIVcpz, a simian immunodeficiency virus (SIV) lineage found in chimpanzees. The natural home range for *P.t. troglodytes* coincides with the areas of emergence of HIV-1 groups M, N and O, and this subspecies may in fact be a primary reservoir for HIV-1 (205, 251). Though this model has similar infection patterns to humans, the responses to the virus differ. Rarely does the chimpanzee develop full-blown AIDS, and there is no observable decline in CD4+ cells and only low CTL activity to HIV-1 antigens. A strain of HIV-1 that is pathogenic in chimpanzees has emerged, but a continuing debate over the ethics, lack of characterized similarity to human isolates and uncertainty about how it causes disease, is enough to prevent

advancement in its use. Prohibitive costs, attention required, availability and the status of the chimpanzee as an endangered species further limit its usefulness as an animal model.

The Asian rhesus and pig-tailed macaques, though more genetically distant from humans than chimpanzees, are other frequently used animal models for HIV-1. However, these animals can only be infected with simian versions of HIV, namely SIV or the chimeric HIV-SIV virus (SHIV). Most primate lentiviruses that infect African nonhuman primate species are not pathogenic to their natural hosts, but a subset has been found that does act differently in the Asian macaque monkeys. These pathogenic strains are not naturally present in this species. SIV can establish infection by various routes as HIV does, and the similarity of SIV infection in the macaque monkeys - increased viral load, a decrease in CD4+ lymphocytes and the eventual development of AIDS-like symptoms - warrants its use in HIV-1 therapeutic and challenge studies (74). However, the divergent SIV envelopes present antigenic and structural differences from HIV-1 that are expressed as differences in coreceptor usage (158, 205). Most SIV strains require the CCR5 coreceptor. However, many SIV strains fail to use CXCR4, except for a few exceptions. The chimeric SHIV viruses have been developed that provide an SIV pathogenic backbone and gain entry to the cell via an HIV-1 envelope. Interestingly, all pathogenic SHIVs are isolates, or virus quasispecies, isolated from an infected animal in contrast to the nonpathogenic SHIV construct clones (138). The outbred nature of macaques results in variability of response to SIV infection, which may be advantageous in predicting the variety of responses expected in a human population.

The decision to evaluate a candidate vaccine in certain populations may depend upon protection demonstrated in an animal model. To date, HIV-1 vaccine candidates

that are being used in clinical trials have proven to be at least partially efficacious in a non-human primate animal model. Correlates of immune protection have not been established, however. Both antibody-mediated immune protection and cellular immunity have been observed in animal models. Standardization of protocols with regard to challenge virus strain, route of challenge and doses needs to occur for data comparison to be valid and to maximize the potential of animal model use.

Vaccine Strategies

Successful containment of this worldwide pandemic will eventually require an effective vaccine. Most successful vaccines against viral diseases have been killed or attenuated viruses, but this has not been the case for HIV-1 and the closely related SIV. The correlates of a protective immune response have been determined in part by a potential vaccine's ability to elicit both cell-mediated and humoral responses (41, 128). Killed HIV-1 induces a poor neutralizing antibody response and virtually no cytotoxic T-lymphocyte (CTL) response, while attenuated SIV has caused an AIDS-like disease in monkeys (11, 12). Although alternative live virus vaccines in the form of recombinant viral vectors have been able to produce a CTL and humoral response (134, 260), they have been unable to induce high-titer neutralizing antibodies and failed to protect non-human primates against HIV-1 challenge.

Virus neutralization epitopes have been exposed when complexes of Env and its cellular binding partners have been used as immunogens. Using a complex of envelope and CD4 as an immunogen results in generation of a polyclonal antibody response that neutralizes laboratory adapted strains and primary isolates (76, 140). Expanding this

further, a "fusion competent" complex, which contained the former with the addition of the CCR5 receptor, was used as an immunogen in a transgenic mouse model (154). These formaldehyde-fixed, whole-cell vaccines elicited antibodies that were capable of neutralizing infectivity of 23 of 24 HIV-1 isolates from diverse geographic locations and genetic clades A to E. These results suggest the process was successful in capturing transient envelope-CD4-coreceptor structures that arise during HIV binding and fusion. However, there have been no published reports reproducing these findings.

Even gene therapy applied in the context of HIV-1 infection shows promise. Successful transduction and engraftment of hematopoietic stem cells (CD34+) in humans has led to continued expression of genes encoding anti-HIV-1 antisense RNA in HIV-1 infected subjects nine months post infusion (166). Long term follow up and *ex vivo* challenge will determine if this will be ultimately be successful.

Live recombinant viral vectors (eg. vaccina virus, rabies virus) and DNA vaccines engineered to express HIV-1 proteins are known to elicit CTL response (18, 256, 269). Though DNA has been shown to elicit excellent CTL response, multiple DNA immunizations are typically required to elicit even transient, modest titers of HIV-1 neutralizing antibodies (233). Protein boosting can increase titer, persistence, homologous neutralizing activity and avidity of anti-Env responses, and studies to date indicate that the induction of a potent humoral response to HIV-1 Env is best achieved through the administration of recombinant Env protein (59, 67, 118, 119, 233). A combination regimen, consisting of a recombinant virus or DNA as a prime injection followed by protein boosts, is capable of inducing a superior cell-mediated and humoral response, especially if the DNA is accompanied by co-delivery of genes coding for

cytokines (27, 53, 90, 115, 280). Most studies have focused on using clade B, TCLA HIV-1 Env as protein boost components (119, 233, 303).

Augmentation of the immune response by cytokines has also been seen when cytokines have been used as an adjunct to HIV-1 drug therapy. The cytokine interleukin-2 (IL-2) has been shown to increase CD4+ cell counts and stimulate immune function in conjuction with highly active antiretroviral therapy (HAART), but does not having any direct virologic effect in inhibiting HIV-1 replication (84, 161, 180). Second generation DNA/protein vaccines may also benefit from the use of polylactide co-glycolide (PLG) microparticles as DNA carriers and electric current mediated electroporation to increase transfection efficiency *in vivo* (16, 212) . Both have been shown to be potent inducers of cytotoxic T-lymphocyte and CD4+ T-helper cell responses.

There is evidence that in a natural HIV-1 infection, the humoral response is mainly directed to viral debris and not against the intact virus (218). Of the neutralizing antibodies made, the majority present in HIV-1-positive human sera are to determinants in the V3 loop of gp120 and those that block gp120-CD4 interactions. Antibodies to the V3 loop are usually independent of the oligomeric status of gp120. However, those antibodies that block gp120-CD4 binding recognize conserved, discontinuous conformational epitopes in gp120 and can be more broadly neutralizing (34). Constructing a truncated Env to retain gp120 and the ectodomain of gp41 results in the expression of a secreted protein of 140 kDa (gp140). As an oligomeric (trimer) complex, this would most closely mimic the antigenic structure of the virion-associated Env complex. Studies have shown that this soluble, oligomeric form of HIV-1 Env gp140 (IIIB/BH8), expressed by recombinant vaccinia virus can elicit a high proportion of

antibodies to conserved conformation-dependent epitopes (86). In contrast, antibodies elicited by monomeric gp120 are often to linear epitopes like the V3 loop and many do not recognize native oligomeric Env on the surface of virus or HIV-infected cells. Monomeric gp120 from various clades has been used unsuccessfully as a vaccine prototype in clinical trials. Though safety issues have been addressed by these trials, the ineffectiveness of monomeric, non-native gp120 has been determined by the inability of this Env form to elicit potent, neutralizing antibodies to primary isolates (62, 64, 120, 175, 278). Though oligomeric, clade B TCLA gp140 immunogens have been shown to produce a more diverse antibody response in animals than gp120, these uncleaved oligomers did not induce generation of broadly cross-reactive neutralizing antibodies to primary isolate HIV-1 (90, 183) . Uncleaved Env also has exposed epitopes not found on virions (85, 86), and this form also interacts inefficiently with coreceptor (92). Novel strategies via structural alterations have been implemented in an attempt to increase immunogenicity of Env subunits from various HIV-1 clades. These alterations have focused on increasing gp160 processing and increasing oligomeric Env stability. These strategies and recent data are addressed in the discussion.

SPECIFIC AIMS

An effective vaccine should elicit potent, cross-clade neutralizing antibodies to a number of epitopes. Most current vaccine strategies rely on the recombinant, monomeric form of HIV-1 Env gp120. Studies to date have shown this approach has failed to generate broadly cross-reactive neutralizing antibodies. Our long-term goal is to determine if using soluble, oligomeric envelope glycoproteins from primary HIV-1

isolates as antigens will elicit more broadly cross-reactive neutralizing antibodies than has been observed with monomeric Env immunogens to date. This dissertation will focus on the following specific aims.

Specific Aim # 1: Generation and characterization of primary isolate gp140s.

The hypothesis is that truncated HIV-1 primary Envs are processed differently than truncated laboratory strains of HIV-1 Env, and structurally, the non-covalent association is less labile than laboratory strains after secretion. A panel of genetic constructs that encode the gp140 Env glycoprotein will be made from a variety of HIV-1 primary isolates from several HIV-1 clades. These new constructs will be used to generate recombinant vaccinia viruses to express the Env gp140. The characterization of these gp140s will be performed by sucrose density centrifugation and profiles of each Env will determine their oligomeric nature and whether oligomeric Env possessing cleaved gp120/41 subunits are present. These experiments will determine: 1) whether any primary isolate Env gp140 is cleaved to its gp120/41 subunits as compared to laboratory strain IIIB/BH8 gp140, and 2) whether primary isolate Env gp140s are more likely than IIIB/BH8 gp140 to be secreted as cleaved oligomers. A subaim will be to produce preparative amounts of gp140 from each of clades A, B, C, D and E. These gps will be analyzed for oligomeric status and antigenicity. Results of this subaim will be important in future studies to assess these gp140s as polyvalent immunogens.

Specific Aim #2: Generation of soluble, cleaved oligomeric gp140.

The results of these experiments will address the hypotheses that: i) a primary isolate derived, soluble Env remains stable as a processed oligomer; ii) processing of primary soluble Envs can be enhanced and these processed Env can be held together biochemically; and iii) a biochemically crosslinked, enhanced-processed, soluble Env oligomer retains important structural features. Experiments with plasmin, as well as with recombinant vaccinia virus expressing the human cell protease furin, will be used to determine if there are co- or post-expression options for enhancing the cleaving of gp140 into its gp120/41 subunits. Crosslinking experiments with thiol-cleavable crosslinkers will be performed to examine if the process of ultracentrifugation causes dissociation of the cleaved oligomeric Env into monomeric forms. These experiments will determine whether a stable, soluble, cleaved oligomeric form of primary isolate Env gp140 can be produced. This will provide very valuable information for future experiments in comparisons of cleaved vs. uncleaved soluble oligomeric gp140.

Specific Aim #3: Characterize the novel antigen delivery system Matrix III.

The hypothesis is that encapsulation of soluble, uncleaved oligomeric and/or monomeric Env with Matrix III will provide slow release of antigen, thus allowing for continuous stimulation of the humoral arm of the immune system leading to qualitative and quantitative improvement of antibody production. The product Matrix III is a proprietary inorganic biopolymer composite prepared from a slurry of calcium sulfate hemihydrate, other inorganics and water soluble polymers. Solidification and entrapment of antigen occurs at room temperature and neutral pH. A key feature of this technology

is the method of pore-size control in manufacturing; the antigen encapsulated in this product maintains its native conformation. The initial data of this system will be the determining factor of whether it will be incorporated into the major project of a polyvalent vaccine. The primary objective will be to evaluate the quantitative (ELISA titer) and qualitative (neutralization assay) immune responses elicited by both oligomeric gp140 and monomeric gp120 from the IIIB isolate - an Env of a T-cell laboratory adapted strain - in encapsulated versus non-encapsulated form in rabbits. The issues addressed by this specific aim are: 1) whether the encapsulated form of Env will be further investigated in rabbits; 2) whether the oligomeric gp140 induces a more potent neutralizing response than gp120; and 3) whether oligomeric gp140 is a better choice than monomeric gp120 as an immunogen.

MATERIALS AND METHODS

Cells and culture conditions. Human HeLa (CCL-2), HeLaS3 (CCL-2.2) and HuTk$^-$ 143B (CRL 8303) cells, simian BSC-1 (CCL-26) and CV-1 (CCL-70) cells were obtained from the American Type Culture Collection, Rockville, Md. Cell cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. HeLa cell monolayers were maintained in Dulbecco's modified Eagle's medium (Quality Biological, Inc., Gaithersburg, MD) supplemented with 10% fortified bovine calf serum (Hyclone, Logan, Utah) and 2 mM L-glutamine (DMEM-10). HuTk$^-$, BSC-1 and CV-1 cells where maintained in Eagle's minimum essential medium (Quality Biological, Inc., Gaithersburg, MD) supplemented with 10% fortified bovine calf serum and 2 mM L-glutamine (EMEM-10). HeLa S3 cells were maintained in vented Spinner bottles at 37°C in Eagle's minimum essential spinner medium (Quality Biological, Inc., Gaithersburg, MD), 5% horse serum (Sigma, St. Louis, MO) and 2mM glutamine. Roller bottle cultures of BSC-1 cells were maintained in EMEM-10 at 37°C at 0.5 revolutions/minute.

Plasmids. For production of soluble secreted envelope glycoproteins (gp140s), a battery of plasmid constructs encoding a truncated version of an Env gene from several HIV-1 primary isolates was generated. Plasmids containing functional gp160 *env* genes from eight HIV-1 clades under the control of the T7 promoter were obtained from the NIH AIDS Research and Reagent Program (Rockville, MD). These are (HIV-1 Env genetic subtype and geographical location are given in parentheses): 92UG037.8 (A, Uganda);

92HT593.1 (B, Haiti); 93MW965.26 (C, Malawi); 92UG024.2 (D, Uganda); 93ZR001.3 (D, Zaire); 92TH022.4 (E, Thailand); 93BR019.10 (FB, Brazil); 92UG975.10 (G, Uganda). The gp140 construct for CM243 (E, Thailand) was made from pCB53 which contains the full-length *env* in pSC59 (85). The gp140 construct for JRFL (B, United States) was made from pCB28, which contains the full-length *env* in pSC59 (32). Additionally, two plasmid constructs in pSC59 were made that were not made into recombinant vaccinia viruses: a gp140 *env* of 93BR029.2 (F, Brazil), and a full length CM243 *env* with coding for an additional four amino acids at the C-terminus of gp41. A three-step process was used to construct the gp140's. First, a PCR product corresponding to the Env C-terminus was generated that included two translation termination codons inserted after the lysine residue at amino acid 683 (HIV_{HXB2} numbering (149) is used), just prior to the transmembrane domain of gp41. Instead of lysine, arginine is at that position for isolates 92UG024.2 and 92UG975.10. The following primer sets were used to construct the Env truncations unless otherwise noted: 5'-GGAAGCACAATGGGC-GCGGCGTCAAT-3' (sense) and 5'-GCCTCCTACTATCATTATTTATTATTTTAT-ATACCACAGCCA-3'(antisense). For 92UG024.2 and JRFL, the following sense primer was used: 5'-CAACAGCGTATGTTGCAACTCACAGTCTG-3'. The antisense primer for 92UG024.2 was: 5'- GCTTCCTACTATTATTATTTATTATCTTATATAC-CACAGCCA. For the 93ZR001.3 plasmid, the following sense primer was used: 5'-AGGAAGCACGATGGGCGCAGCGTCAGTG-3'. For 93BR029.2 the following sense primer was used: 5'-CGTCGATAACGCTGACGGCACAGGCCAGACAATTAT. For 92UG975.10, the following primers were used: 5'-GGGCATTAAACAGCTCCAGGCA-AGAGTCCTGGCTCTA-3' (sense) and 5'-ACCTCCTACTATCATTATTTATTATCT-

TATATACCATAG-3' (antisense). TOPO cloning (Invitrogen, San Diego, CA) was used to generate a plasmid construct (pCR2.1) containing the PCR product. Restriction digests of each plasmid with *BlnI* and *HindIII* (clade A), *BlnI* and *BamHI* (clade B (593.1), C, D, F (19.10), G), *BlnI* and *SpeI* (clade E (CM243), B (JRFL), F (29.2)), or *BamHI* and *NdeI* (clade E (22.4)) were used to subclone a small *env* fragment containing the stop codons into the original full length *env* in pCRII. Recombinant vaccinia virus (rvv) expressing bacteriophage T7 polymerase (vTF7.3) (108) was used to infect BSC-1 cells transfected with the gp140 *env* constructs to test for expression since the T7 promoter is present in the cloning vector. For CM243 and JRFL, the PCR fragment was subcloned directly into a pSC59 construct containing the full-length *env*, bypassing the next steps. When expression was confirmed, the entire *env* gp140 cassettes were then subcloned into a transfer vector which linked the truncated *env* gene to a strong synthetic vaccinia virus early-late promoter (pSC59) (47)). The restriction digests used for this procedure were: *EcoRI* (clade A, D (24.2), E (22.4)); *BamHI* (Klenow)/*XhoI* into *StuI*/*XhoI* pSC59 (clade B (593.1); *SpeI/XhoI* (clade C, D (1.3), F).

Plasmids for use in transfections were purified using a standard plasmid polyethylene glycol (PEG) purification procedure. Other plasmid preparations were made using the Plasmid Midi or Mini Kits (Qiagen, Valencia, CA). Each recombinant clone was sequenced to ensure that there were no PCR or subcloning errors.

Recombinant vaccinia viruses. A detailed protocol for making recombinant vaccinia viruses (rvv) (33) is referenced for the following procedures, and modified where noted. CV-1 monolayers were infected with wild-type vaccinia virus strain WR

(ATCC#VR1354) and then transfected with the gp140 plasmid constructs to generate rvv. Briefly, a 25-cm^2 flask of CV-1 cells approximately 95% confluent (seeded with 10^6 cells 18 h prior) is infected with 1 ml of sonicated, diluted WR at 3.0 x 10^4 pfu/ml (MOI of 0.05 pfu/cell). After a 2 hour incubation, the transfection mixture is prepared: 5 μg of gp140 plasmid construct is mixed with 2X HBS (280mM NaCl, 10mM KCl, 1.5mM Na2HPO4, 12mM dextrose, 50mM HEPES) in a 1 ml final volume in a 12 x 75 mm polystyrene tube; 50 μl of 2.5 M CaCl is added dropwise and tapped gently. The mixture is incubated at room temperature for 20 minutes until a fine, milky precipitate forms. The virus inoculum is aspirated, and the cells are overlaid with the transfection mixture. After the flask undergoes a 30 minute incubation at room temperature, 9 ml of MEM-10 is added to the flask. 10 ml of fresh medium is added after a 3.5 h incubation at 37°C in a 5% CO_2 atmosphere. After 100 percent cytopathic effect (CPE) is evident (usually 2-3 days), the cells are scraped off the flask, pelleted and resuspended in 1 ml of EMEM-2.5. Three freeze/thaw cycles on dry ice, followed by 30 sec. sonication in a cup sonicator in ice-cold water frees the virus from the cells and the CV-1 crude lysate is stored frozen at -70°C until selection.

The rvv was selected by three rounds of virus plaque assays using a modified immunostaining procedure for MVA (personal communication Dr. Gerd Sutter). Briefly, the CV-1 crude lysate being screened for rvv was diluted out three to five logs by serial 10-fold dilution, and deposited as an overlay onto HuTk$^-$ monolayers in 6-well plates. The recombinant virus was allowed to grow for two days. An initial incubation with a 1:800 dilution of polyclonal anti-gp140 rabbit sera R2143 (see Antibodies section) in Dulbecco's PBS (Sigma, St. Louis, MO) containing 3% F-BCS was followed by

incubation with a goat anti-rabbit IgG conjugated to horseradish peroxidase (Pierce, Rockford, IL). Positive orange/brown-stained foci were identified 15 minutes post incubation with the substrate O-dianisidine (Sigma, St. Louis, MO) in PBS and 0.03% hydrogen peroxide. The third and final round of purification was a standard agarose-overlay plaque assay. Briefly, second round picks undergo freeze/thawing and sonication as described above. 30 μl of each lysate is used to initiate the first in a series of 10-fold dilutions. One milliliter of each 10^{-2} and 10^{-3} dilution is placed on HuTk$^-$ monolayers in 6-well plates and overlaid with 4 ml/well of a 1:1 mix of 2% low melting point (LMP) agarose (Life Technologies, Gaithersburg, MD) and 2X MEM-10% FBS, with a final concentration of 0.025 mg/ml BrdU, solidified at room temperature and incubated at 37°C for 2-3 days until plaques are seen. The plaques are picked after staining with a LMP agarose overlay containing a final concentration of 100 μg/ml neutral red. Agarose plugs containing infected cells are each resuspended in 0.5 ml of MEM-2.5% FBS. Half the volume of the freeze/thawed, sonicated final recombinant virus pick is amplified in HuTk$^-$ monolayers in a 24-well plate, and expression verified by Western analysis of the infected cell lysate and supernatant.

Amplification of the recombinant virus is used to generate virus stock. The clonally pure third round plaque pick was expanded first in a 25-cm^2 flask of HuTk$^-$ monolayers, using 220 μl of the plaque pick. The infected cells demonstrating 100% CPE are harvested, subjected to freeze/thaw cycles and sonication. Half of the 0.5 ml of crude lysate made from this infection is used to amplify the virus in one 150-cm^2 flask of HuTk$^-$ monolayer to generate 2 ml of crude lysate. The same freeze/thaw cycle as described above is used to generate a crude lysate, stored at –70°C and sonicated before each use.

Amplification then takes place in five 150-cm^2 flasks of HeLa monolayers. For the final amplification to make a crude stock, twenty 150-cm^2 flasks of HeLa monolayers are infected with trypsinized, sonicated lysate. An equal volume of crude lysate and 0.25 mg/ml trypsin is mixed together, incubated 30 min. in a 37oC water bath, and sonicated in a cup sonicator in ice-water for 30 sec. The trypsinized virus is diluted in MEM-2.5% FBS to optimally infect the cells at an MOI of 1-3 pfu/ml. The 50 ml of crude virus stock produced is then titered as described below.

Purified recombinant virus stock is made by infection of HeLaS spinner culture cell suspensions plated in twenty 150-cm^2 flasks with trypsinized crude stock at a MOI of 5-8 pfu/cell. The infected cells are harvested, centrifuged and resuspended in 10 mM Tris-HCl, pH 9.0 and stored at –70°C until purification. The virus is sonicated with a probe sonicator for 4 x 15 sec. intervals, and purified on a 36% sucrose cushion, which is centrifuged 80 min. at 32,900 x g at 4°C. The pellet is resuspended in 1 ml of 1 mM Tris-HCl, pH 9.0, sonicated and titrated.

Titration of the vaccinia virus stock is performed as follows. Crude stock is first trypsinized as described previously. However, the purified stock is not trypsinized but only sonicated in a cup sonicator prior to titration. Ten-fold serial dilutions are made in MEM-2.5% FBS, beginning with a 10^{-2} dilution (30 μl of virus stock in 3 ml of diluent), ending with a 10^{-9} dilution for crude stock, and 10^{-10} for purified virus stock. The last 3 dilutions are used to infect BSC-1 cell monolayers in 6-well plates in duplicate. Plates are stained with a 0.1% crystal violet solution 2 days after infection. The virus titer is determined by counting plaques in both wells, dividing by 2 and multiplying by the

dilution factor of those wells, taking into account the 1:1 dilution for the trypsinized virus.

Recombinant vaccinia virus expressing furin was a gift from Gary Thomas (Oregon Health Sciences University, Portland, OR). Additionally, the previously described rvvs vCB14 (IIIB gp140, cleavage site intact), vPE12B (IIIB gp140, cleavage site removed) (86), vCB5 (sCD4) (31), vPE50 (IIIB gp120) (44) were used. Purified vaccinia virus stocks were used at a multiplicity of infection of 10 PFU/cell.

Antibodies and reagents. Polyclonal anti-gp140 rabbit antiserum (R2143) was prepared against purified reduced and denatured IIIB gp140. The monoclonal antibodies 17b, 48d, and 23e were a gift from James E. Robinson, Tulane University Health Science Center, New Orleans, LA. Four domain soluble CD4 (sCD4) and monoclonal antibody 2G12 (Hermann Katinger (37, 276)) were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. Monclonal antibody 2F5 was a gift from Hermann Katinger, Polymun Scientific Inc., Vienna, Austria or Viral Testing System Corp., Houston, TX. IgGb12 was a gift from Dennis Burton, The Scripps Research Institute, LaJolla, CA. CD4-IgG was a gift from James Binley, Aaron Diamond AIDS Research Center, New York, NY. Monoclonal antibodies T3, T4, T6, T8, T9, T10, T30, D11, D12, D20, and D61 from a panel of 138 murine MAbs raised by immunization with soluble monomeric or oligomeric Env was described previously (86). Crosslinking reagents used were: Dithiobis (succinimidylpropionate) (DSP); dithiobis-(sulfosuccinimidylpropionate) (DTSSP); and ethylene glycolbis (succinimidylsuccinate) (EGS), all obtained from Pierce, Rockford, IL.

Western blot analysis. Supernatants from 6-well preparations were centrifuged to remove cell debris and used without concentration. Cell lysates were made by incubating cell pellets with 0.5% Triton X-100 in 20 mM Tris-HCL, pH 8.0, 100 mM NaCl on ice for 30 min.; the cell nuclei were removed by centrifugation. Purified Env (50 ng), cell supernatants or lysates were mixed with 2X SDS-PAGE sample buffer, boiled at 100° C for 5 minutes, and run on SDS-PAGE gels. Separated proteins were transferred to nitrocellulose membranes at 40 mA overnight at 4° C or 400 mA for 1 hr at 4° C in transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3). Membranes were individually blocked in 50 ml of 5% non-fat milk, PBS, and 0.1 % Tween 20 (PBS/T) for 1 hr at room temperature or overnight at 4° C. Primary anti-Env antibody was diluted 1:1000 in PBS/T and incubated with the membrane for 1 hr. room temperature. The membrane was washed three times with PBS/T, then incubated with a 1:40,000 dilution of secondary antibody, either goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (IgG-HRP) or goat anti-mouse IgG- HRP (Pierce, Rockford, IL) in blocking buffer for 1 hr at room temperature. Protein bands were visualized with SuperSignal chemiluminescent substrates (Pierce, Rockford, IL) using the manufacturer's protocol.

Large scale protein expression and purification. HIV-1 Env proteins were produced by infection of monolayers of BS-C-1 cells in roller bottles with recombinant vaccinia viruses at a multiplicity of infection (MOI) of 10 pfu/cell. The secreted Env was obtained by harvesting the medium (OPTI-MEM, Life Technologies, Inc., Rockville, MD) of infected cells after a 30 hour incubation. Envelope proteins were first affinity purified

over lentil lectin Sepharose 4B (Pierce, Rockford, IL) columns. Briefly, serum-free medium containing the glycoproteins was pumped over the lentil lectin columns at a rate of 4ml/min using a peristaltic pump (ISCO, Lincoln, NE). The column was washed with two column volumes of a salt buffer (20 mM Tris pH 7.5, 0.3 M NaCl, 0.5% Triton X-100), followed by one column volume wash with the same buffer without Triton X-100. The proteins were eluted with 0.5 M methyl mannopyranoside (Sigma, St. Louis, MO) in PBS pH 7.4, concentrated on Amincon Centriprep columns (MWCO 50) (Millipore, Bedford, MA) to 1-2 ml, filter sterilized through a 0.22 μm PVDF low protein binding syringe filter (Millipore, Bedford, MA) and stored at 4°C. The Env was further separated into oligomeric and monomeric fractions by size exclusion chromatography with sterile, degassed PBS pH 7.4 (Quality Biologic, Inc., Gaithersburg, MD) over a HiLoad 16/60 Superdex 200 prep grade column (Amersham Pharmacia Biotech, Piscataway, NJ) using an Amersham Pharmacia Biotech P-500 pump to achieve a constant flow rate of 90 ml/hr, with a 1.5 MPa limit. Protein elution was followed by UV absorption at 280 nm concurrently plotted on a chart. Protein was visualized on a colloidal Coomassie blue stained (Novex, Carlsbad, CA) SDS-PAGE gel and was quantitated by image analysis with NIH Image v.1.62 by comparison to a previously prepared reference standard of IIIB gp120 purified under identical conditions and quantitated by amino acid analysis (P. Earl, personal communication). For the encapsulation project, IIIB Env oligomeric gp140 (uncleaved) and IIIB Env monomeric gp120 were produced and purified by lentil lectin chromatography as described above. The Envs were quantitated and sent to Buford Biomedical, Inc., Frederick, MD for encapsulation in Matrix III.

Metabolic labeling, size fractionation and immunoprecipitation. BS-C-1 cell monolayers in 6-well plates or T-75 flasks were infected with recombinant vaccinia viruses at a MOI of 10 pfu/cell. Coexpression of furin required a coinfection of the rVV expressing Env with rVV-furin at an MOI of 10. At 6 hr post infection, the virus inoculum was replaced with L-methionine-free or L-methionine and L-cysteine-free high glucose Dulbecco's minimal essential medium (Life Technologies, Inc., Rockville, MD), 2 mM glutamine and 2.5% dialyzed fetal bovine serum (Life Technologies, Inc., Rockville, MD) containing 200 μCi/ml of stabilized [^{35}S]- methionine (NEN) or Promix [^{35}S]cysteine/methionine (Amersham Pharmacia Biotech, Piscataway, NJ) and incubated overnight.

Density gradient centrifugation is most often used for separation and purification of a variety of biological materials. The density gradient method involves a column of preformed sucrose gradient whose density increases toward the bottom of the tube. Under centrifugal force, the sample solution particles will begin sedimentation through the gradient in separate zones, each zone consisting of particles characterized by sedimentation rate. For sucrose density gradient centrifugation analysis, supernatants were concentrated ten-fold in an Amicon Centricon spin column (MWCO 50) (Millipore, Bedford, MA) and loaded onto a 5 to 20% (wt/vol) continuous sucrose gradient. For crosslinked Env, microgram amounts of room temperature crosslinker were weighed out to make a 25 mg/ml stock solution. Water-insoluble crosslinkers (DSP, EGS) were dissolved in DMSO such that the final concentration of DMSO was less than 10% of the final reaction volume. Water-soluble DTSSP was mixed with sterile double distilled water at the same molar concentrations. All crosslinkers were prepared just prior to use.

Stock crosslinker was added to the concentrated Env supernatants for final concentrations of 0.25 – 5mM; the concentration chosen for all future crosslinker experiments is 5mM. Samples were centrifuged in an SW40 rotor at 40,000 rpm for 20h at $4^{o}C$. Fractions were collected from the bottom of the centrifuge tube using a fraction collector and peristaltic pump; 30 aliquots were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Separation into oligomeric and monomeric fractions was done by first concentrating the supernatants as above and then pumping the concentrate over a Superdex 200 HR 10/30 size exclusion column (Amersham Pharmacia Biotech, Piscataway, NJ). Fractions were collected as above, except the flow rate was 60 ml/hr of PBS, 1.5 Mpa limit. Immunoprecipitations were performed by incubating Env in 200 μl immunoprecipitation binding buffer (100mM Tris, 140 mM NaCl, 0.1% Triton X-100) with 2-10 μg of MAb, 100 μl of hybridoma supernatant, or 1 μl of polyclonal serum for 1 h at room temperature. 50 μl of 20% Protein G Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ) were then added followed by rotation for 1 h to overnight at $4^{o}C$. The Sepharose beads were washed three times with 1 ml of Triton buffer (50mM Tris-HCl, pH 8.0, 300mM NaCl, 0.5 % Triton X-100). Proteins were eluted in sample buffer at $100^{o}C$ for 5 minutes and analyzed by SDS-8 % PAGE.

Amino acid sequence analyses. The ClustalW alignment algorithm and dendrogram analysis features in MacVector v.6.5.3 software were used to compare the amino acid sequences from various HIV-1 isolates.

Plasmin Experiments. 100 μg of human plasminogen (Sigma) was activated with 92.5 Plough units of urokinase (Sigma) in PBS and 0.02M lysine at 37°C for 30 minutes. Activation was confirmed by incubation of 22 μg of plaminogen/urokinase reaction mix with 0.3M D-val-leu-lys p-Nitroanilide (Sigma) in PBS at 37°C for 5 minutes and looking for a colorimetric change (clear to yellow). Various concentrations (0 to 4.4 μg) of the activated plasminogen were added to 1 μg of IIIB gp140 in PBS. The reaction was allowed to proceed at 37°C overnight. Cleaved Env was analyzed by Western as described previously.

Animal Studies. For the mouse study, BALB/C mice were immunized and maintained at the USUHS animal facility in accordance with guidelines described in the Guide for the Care and Use of Laboratory Animals. Calculated 30 μg of lentil lectin purified HIV-1 89.6 gp120 (Group I), 30 μg 89.6 gp120 encapsulated in Matrix III (Groups II and III), and 30 mg of Matrix III alone (Group IV) were resuspended in 650 μl of PBS-emulsified RIBI MPL-SE adjuvant (Corixa/RIBI ImmunoChem Research, Inc., Hamilton, MT) or 650 μl of PBS alone (Group III only). The adjuvant is a 1.0 % (vol/vol) squaline oil-in-water emulsion containing 250 μg of monophosphoryl lipid A per ml. The injection material was divided equally among the three mice in each group. Mice were injected with 100 μl subcutaneously and 100 μl intraperitoneally (IP) on Day 0, Day 30 and Day 80. Tail bleeds to collect 200-500 μl of blood were done at day 0 before the first injection, 8 days after the second injection and 8 days after the third injection. The blood was allowed to clot at room temperature for 4 hours, and then centrifuged at 2,700 X g for 10 minutes. The serum from around the pelleted clot was collected, recentrifuged

under the same conditions and transferred to a new screw top tube. Serum samples were stored at -80°C until ELISAs were performed.

For the rabbit study, New Zealand white rabbits were immunized and maintained at Spring Valley Laboratories, Sykesville, MD. Calculated 5 μg (Group A) or 30 μg (Group B) amounts of encapsulated Env gp140, 30 μg of encapsulated Env gp120 (Group C) and only 30 μg amounts of non-encapsulated Env (Group D/E) were resuspended in 1 ml of PBS-emulsified RIBI MPL-SE adjuvant per rabbit, two rabbits per group. One control of beads-plus adjuvant was included (Group F). Rabbits were injected intramuscularly (0.4 ml each hind leg) and subcutaneously (0.1 ml in each of two sites in the neck). Prebleeds were done prior to first injection at day 0 and test bleeds done every 7 days until day 98 for only those rabbits injected with 30 μg of encapsulated gp140 (Group B) and encapsulated gp120 (Group C). A test bleed for all animals was done on day 56 prior to a second injection boost. Additional boosts followed on day 128 (followed by a test bleed on day 138) and day 170 for a total of four injections. Multiple crop bleeds for all animals were done on days 63, 112, 140, 180. The terminal bleed was performed on day 209. Serum was prepared from each bleed, aliquoted and frozen at $^{-}80^{o}$ C.

ELISA. Dynatech Immulon III 96-well plates (Dynatech Laboratories, Inc., Chantilly, VA) were coated with purified 89.6 gp120 (mouse studies) or purified IIIB gp120 (rabbit studies) at 50 ng per well in 50 μl of coating buffer (18 mM Na_2CO_3, 45 mM $NaHCO_3$, pH 9.6) overnight at 4°C. Plates were washed three times with PBS-T (PBS containing 0.05% Tween 20). 200 μl of blocking buffer (1% BSA in PBS-T) was added and incubated for 1 hour at room temperature. The plates were washed three times with PBS-

T. 50 µl of antiserum was added to the first well, serially diluted in the 96-well plate using a multichannel pipettor, and incubated for 1 hour at room temperature. The plates were washed three times with PBS-T, 50 µl of goat anti-rabbit IgG conjugated to peroxidase (IgG-POD) (0.8mg/ml, from Accurate Chemical & Scientific Corporation, 1:10,000 dilution) or goat anti-mouse IgG-POD (40U, from Boehringer Mannheim, 1:600 dilution) was added and incubated for 1 hour at room temperature. The plates were washed three times with PBS-T, and 50 µl of 2,2'-Azino-di{3-ethylbenzthiazoline sulfonate(6)}diammonium salt (ABTS) substrate (Roche Molecular Biochemicals, Indianapolis, IN) was added. The absorbency was measured at 405 nm after 30 minutes using a 96-well plate spectrophotometer (Dynatech Laboratories, Inc.).

Endpoint titer. ELISA readings were determined in duplicate for each rabbit. The endpoint titer is defined as the reciprocal of the highest analyte dilution that gives a reading above the cutoff. The cutoff values were calculated by a statistically defined method (107). The duplicate titers were translated to logarithm base 2 values, and the geometric mean with standard deviations was plotted for each group.

Neutralization Assays. Dr. David Montefiori's group (Department of Surgery, Duke University Medical Center, Durham, NC) performed all neutralization assays in human T-cell lymphotropic virus type 1 (HTLV-1) transformed $CD4^+$ T-lymphoblastoid cell line MT-2, using SHIV-HXBc2 as the target virus. This assay has been previously described (185, 234). Briefly, cell-free virus (100 $TCID_{50}$ of virus) was added to multiple two-fold serial dilutions (range 1:4 to 1:512) of serum samples in growth medium in triplicate

wells of 96-well microtiter plates coated with poly-L-lysine. After a one hour incubation in an atmosphere of 5% CO_2 at 36°C, dispersed MT-2 cells are added, and the incubation continued for four days. Neutralization was measured by staining viable cells with Finter's neutral red when cytopathic effects in control wells were >70% but less than 100%. This was performed by transferring an aliquot of cells to a poly-L-lysine coated 96-well plate containing 0.014% Finter neutral red dye and staining for one hour at 37°C. The dye is extracted from the washed cells with acidified alcohol (50%ethanol in 1% acetic acid), and the extracted dye solution is quantitated colorimetrically at 540 nm. Percent protection was determined by calculating the difference in absorption (A_{540}) between test wells (cells + serum sample + virus) and virus control wells (cells + virus) and dividing this result by the difference in absorption between cell control wells (cells only) and virus control wells (virus only). Neutralizing titers are reported as the reciprocal of the serum dilution that protected 50% of cells from virus-induced cell killing as measured by neutral red dye uptake. Fifty percent protection corresponds to approximately 90% reduction in $p27^{gag}$ antigen synthesis in this assay (39).

RESULTS

Generation and characterization of primary isolate gp140s.

Historically, phylogenetic analysis of HIV-1 Env sequences has been used to group viral strains into multiple clades or genetic subtypes. Recently, the nomenclature for HIV-1 strains has been reassessed, such that the designation of subtypes A-D, F-H, J and K will be retained, but subtype E will now be referred to as CRF01_AE (circulating recombinant form) to reflect its distinctive phylogenetic clustering in Env relative to the rest of the genome, and to recognize the predominant view that it is an A/E recombinant (239). To avoid confusion, we use the clade E nomenclature since we are primarily using originally designated clade E isolates. HIV-1 strains are also categorized according to their chemokine coreceptor usage: T-cell-tropic HIV-1 strains use the chemokine receptor CXCR4 (X4); macrophage-tropic strains use the chemokine receptor CCR5 (R5). Many HIV-1 primary isolates are dual-tropic (X4R5) (reviewed in (24, 35)). The ten HIV-1 primary isolate plasmid *env* clones used to make recombinant vaccinia viruses expressing HIV-1 Env (**Table 1**) were chosen using the following criteria: genetic subtype, coreceptor usage, and functionality in membrane fusion (45, 111).

In designing the gp140 constructs, we included the entire ectodomain of gp41, which should keep the protein folded in a more natural configuration. More importantly, the inclusion of the ectodomain of gp41 should maintain the Env glycoprotein as an oligomer because the amino acids responsible for oligomerization are within this region. Excluding the transmembrane and cytoplasmic tail of the glycoprotein should allow the recombinant protein to be secreted into the media and subsequently simplify purification

Clade	Isolate	Coreceptor	Lab Designation
A	92UG037.8	R5	vAT1; vAT1F
B	JRFL	R5	vAT13
B	92HT593.1	X4/R5	vAT2
C	93MW965.26	R5	vAT3
D	92UG024.2	X4	vAT10; vAT10F
D	93ZR001.3	X4	vAT11
E	92TH022-4	R5	vAT5
E	CM243	R5	vGK4; vAT7.120
F	93BR019.10	X4	vAT6; vAT6F
F	93BR029.2	R5	pAT9[a]
G	92UG975.10	R5	vAT12

Table 1. Primary isolate env gp120, gp140 and gp160 (F) constructs. [a]One construct was not made into a recombinant virus.

of the antigen using conditions not expected to perturb or denature the glycoprotein. A linear representation of the genetic constructs designed and generated here is illustrated in **Figure 2**.

It should be noted that classification of some primary isolates is variable because of their recombinant status. Isolate 93BR019, listed as clade FB by the NIH repository when this project was first begun, is a recombinant that has approximately 200 bp of sequence from the 3' region of gp41 clustered in subtype B, but the remainder of *env* is subtype F. Since the gp140 constructs contain only the ectodomain of gp41, this Env is effectively clade F. This same reasoning also applies to primary isolate 92UG975, classified as subtype G, though the 3' end (cytoplasmic tail) of gp41 is subtype A.

The alignment of the HIV-1 primary isolate Env gp140 amino acid sequences in **Figure 3** highlights the homologous and heterogenous regions and defines several important regions in each subunit. The Env sequence for HXB2 (GenBank accession number K03455) is included in the comparison because this virus is the most commonly used reference strain for many different kinds of functional studies, and is used by the HIV Sequence Database to facilitate a common numbering system among strains. Other than the noted variable regions (V-1 through V-5), the amino acid sequences appear to be mostly conserved. Each sequence contains the primary cleavage site (REKR↓)

Another way to look at the similarities and differences among the Envs chosen for this study can be represented in a dendrogram. A dendrogram consists of many upside-down, U-shaped lines connecting objects in a hierarchical tree showing relationships between members of a group. The dendrogram in **Figure 4** illustrates the genetic relatedness of HXB2 with other HIV-1 strains. These relationships that the ClustalW

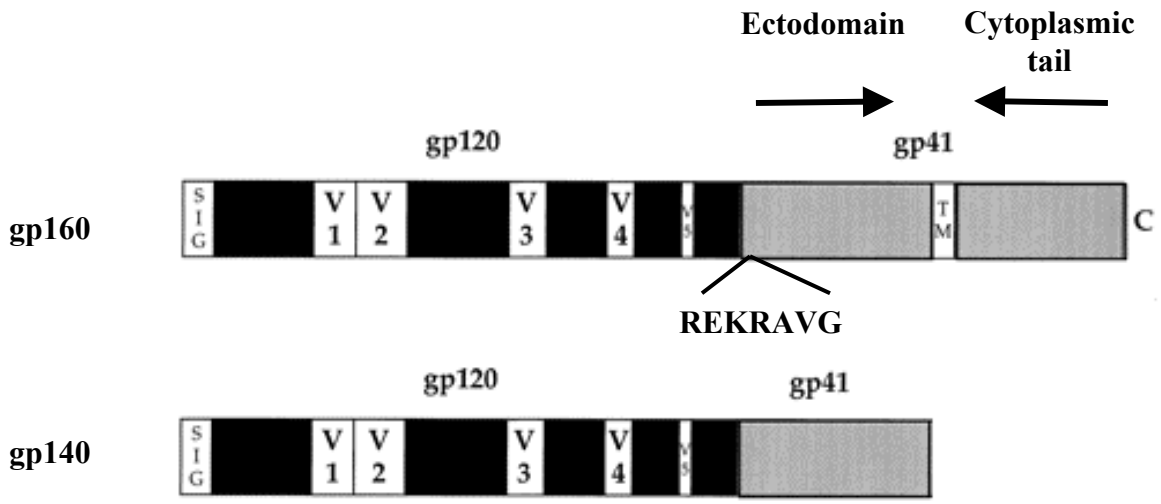
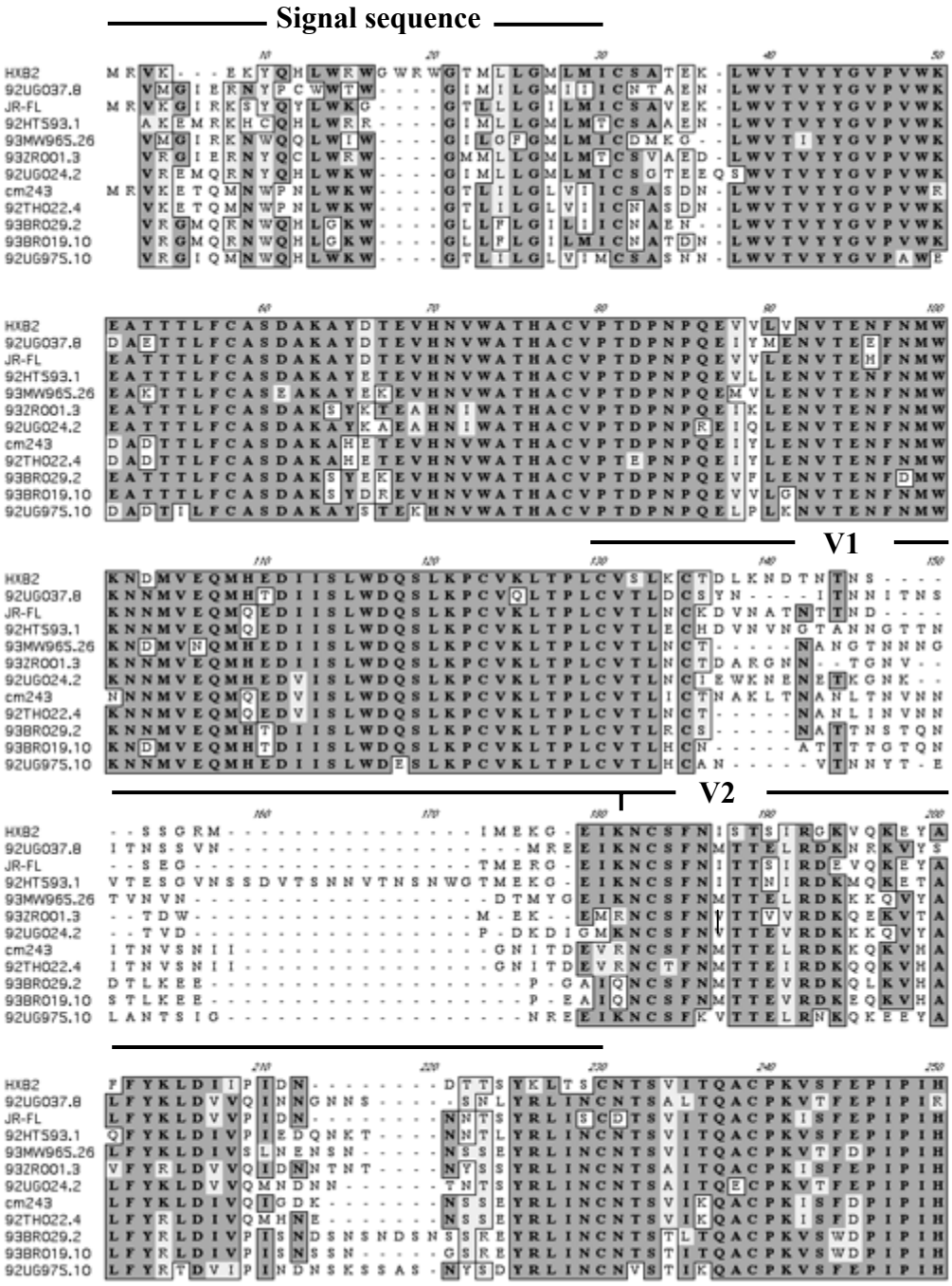
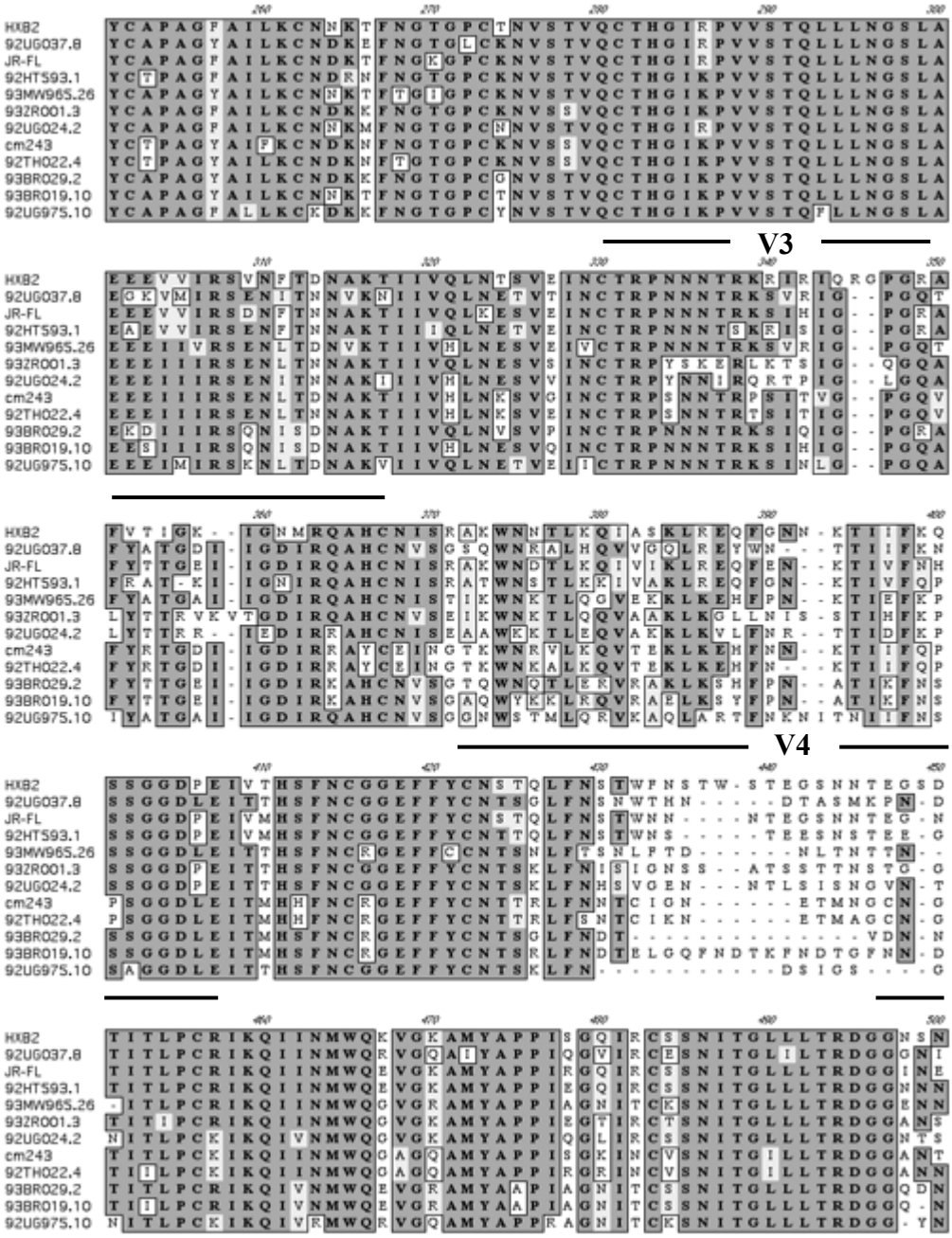
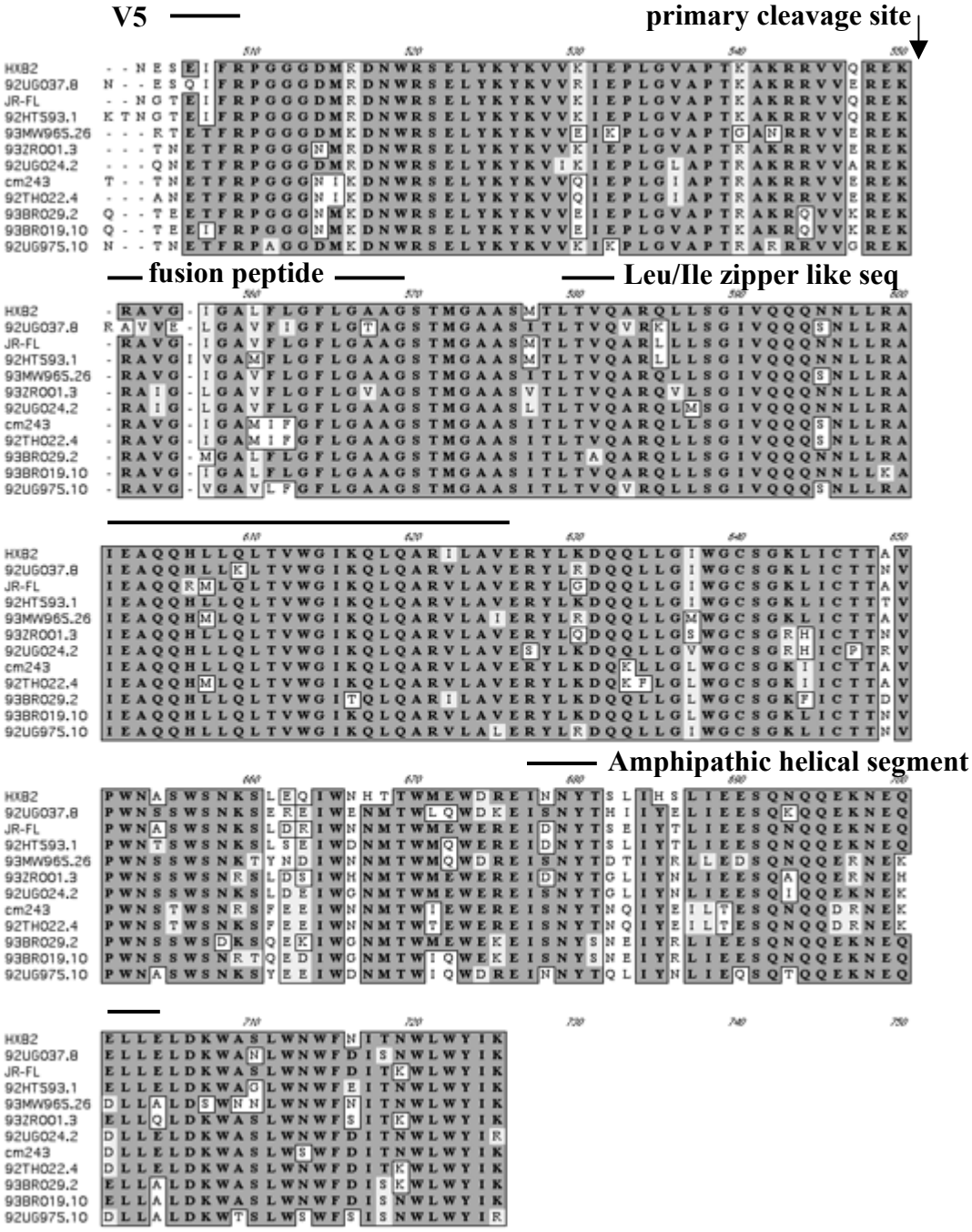


Figure 2. Illustration of the Env construct as native full length (gp160) and soluble truncated version (gp140). The schematic structure of HIV-1 Env gp160 precursor protein is shown at the top, including its signal sequence (SIG), gp120 with defined variable regions (V1 – V5), and gp41 containing ectodomain, transmembrane segment (TM) and cytoplasmic tail. The amino acid sequences represent the junction residues of gp120 and gp41, with the REKR sequence defining the primary cleavage site.

Figure 3. Amino acid alignment of HIV-1 primary isolate Env gp140 with HIV-1 HXB2. V# designates variable loops within gp120. The areas of interaction within the gp41 ectodomain are named.


Signal sequence
V1
V2
HXB2
92UG037.8
JR-FL
92HT593.1
93MW965.26
93ZR001.3
92UG024.2
cm243
92TH022.4
93BR029.2
93BR019.10
92UG975.10


V3
V4


V5
primary cleavage site
fusion peptide
Leu/Ile zipper like seq
Amphipathic helical segment
HXB2
92UG037.8
JR-FL
92HT593.1
93MW965.26
93ZR001.3
92UG024.2
cm243
92TH022.4
93BR029.2
93BR019.10
92UG975.10

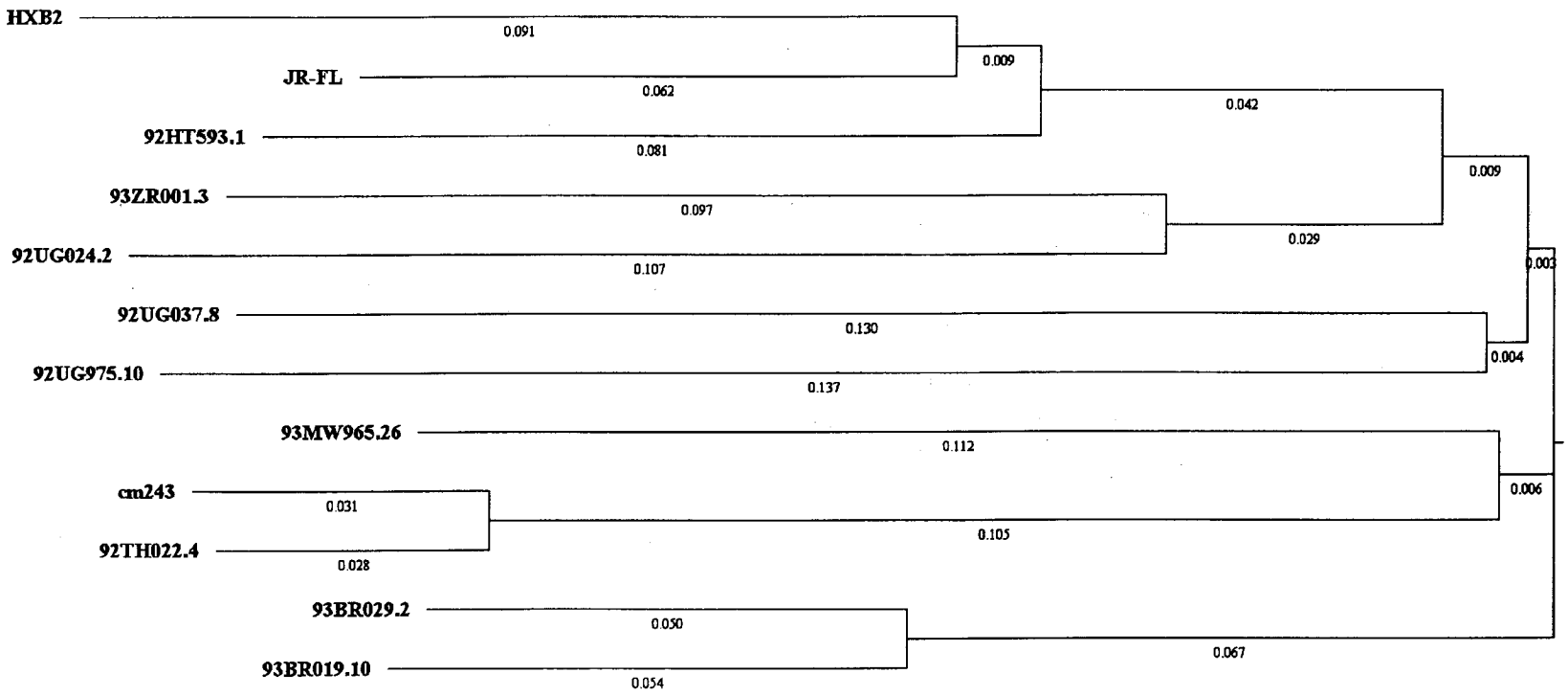


Figure 4. Dendrogram of pairwise comparison of HIV-1 primary isolate Env amino acid sequences. Numbers designate distance between isolates and are equivalent to changes per residue. A value of 0.1 corresponds to approximately a 10% difference between sequences.

algorithm (used to generate the alignment in Fig. 3) generates when doing pairwise alignments is shown in **Figure 4** as a 'phenogram', with the distances between nodes shown in phylogenetic units on the phenogram. As an approximate guide, a value of 0.1 corresponds to a difference of 10% between two sequences. However, this phenogram does not represent a true phylogenetic tree in that it does not give any indication of temporal change. A phenogram type of dendrogram only shows the similarity of characters without regard to distinctions recognized in phylogeny, which shows the unique historical relationship resulting from evolution. Therefore, we can only say that the Envs chosen for this study do not differ from each other in amino acid sequence by much more than 13%.

Proteolytic processing, or cleavage of Env is necessary for the glycoprotein to assume a mature state capable of mediating membrane fusion activity. Though much of the native Env on the surface of a cell is cleaved, many reports using tissue culture laboratory-adapted (TCLA) strains of HIV-1 have demonstrated a high degree of instability where gp120 can readily dissociate from the oligomeric complex (218). This dissociation could also be accelerated by the binding of the CD4 receptor (23, 126, 193). However, in retrospect the gp120 dissociation phenomena was much less prevalent in primary isolate strains of HIV-1 (23, 127, 147, 191-193). Previous investigations have shown that wild type Env, as well as Env with a mutated cleavage site, of a TCLA strain of HIV-1 IIIB (LAI/IIIB clone BH8) synthesized in cells by recombinant vaccinia virus as a gp140 truncated construct is synthesized as a monomer, forms stable homodimers and a higher order oligomer of presumably trimer or tetramer, but these are less stable to detergent solubilization and centrifugation after cleavage (87). In this study, our focus

was to use recombinant vaccinia viruses that express secreted wild type gp140 from primary isolate HIV-1 strains. We also chose to use Envs with the cleavage sites intact to determine the amount of processing that each primary isolate undergoes, and if the instability observed with the Envs of TCLA HIV-1 strains is also applicable to the HIV-1 primary isolate Envs.

For the initial analysis, density gradient centrifugation was used to analyze supernatants containing ^{35}S-methionine metabolically labeled, secreted HIV-1 Env gp140s. Because of the power of the recombinant vaccinia virus expression system- where host protein synthesis is inactivated in infected cells, coupled with a strong promoter - a recombinant protein secreted from infected cells represents the primary product in the "conditioned" medium. Density gradient centrifugation is most often used for separation and purification of a variety of biological materials. The density gradient method involves a column of preformed sucrose gradient whose density increases toward the bottom of the tube. Under centrifugal force, the sample solution particles will begin sedimentation through the gradient in separate zones, each zone consisting of particles characterized by sedimentation rate. Earl and colleagues (87) had previously demonstrated that full-length HIV-1 IIIB Env is recovered from two peak fractions from a 5 – 20% sucrose gradient, with corresponding calculated sedimentation coefficients of approximately 10.8S and 7.2S. The sedimentation coefficient gives a relative value that can be used in a qualitative way to characterize a protein's molecular weight (MW). Calculation of the MW for each form of the recovered full length HIV- 1 IIIB Env protein analyzed by SDS-PAGE suggested dimer (310 kDa) and monomer (167 kDa)

forms were present. The truncated, secreted form of IIIB gp140 formed oligomers as well.

The truncated, secreted HIV-1 primary isolate HIV-1 gp140 Envs were analyzed by density gradient centrifugation using the same parameters as those used in the experiments described above. First, sucrose density centrifugation was used to analyze both metabolically labeled HIV-1 IIIB cleavable gp140 Env (vCB14 product) and primary isolate HIV-1 clade D gp140. The HIV-1 laboratory strain IIIB was included to determine if the results obtained by Earl and colleagues (87) were reproducible and to have a cleavage profile with which to compare and contrast the HIV-1 primary isolate gp140 profiles. BSC-1 cells were infected with purified recombinant vaccinia virus, metabolically labeled with ^{35}S-methionine and the concentrated supernatant was centrifuged on a 5-20% sucrose gradient. After fractionation, the distribution of the Env across the gradient was determined by autoradiography of the dried SDS-PAGE gel. Representative fractions from each gradient (fractions marked with an *) were also immunoprecipitated with polyclonal rabbit serum made to IIIB gp140 to differentiate between the reduced forms of Env and other labeled proteins. **Figure 5** shows a comparison of IIIB gp140 and clade D primary isolate gp140 sucrose gradient profiles and the results of the immunoprecipitation. Though many viral proteins are co-labeled with the Env proteins, the uncleaved gp140 and the gp120 cleavage product are distinguishable from the other proteins within the proposed tetramer or trimer (T), dimer (D) and monomer (M) regions. To verify the T, D, or M forms and to estimate their size, non-reduced aliquots of the EGS crosslinked fractions were analyzed by SDS-PAGE and autoradiography (**Figure 6**). Env MW was determined by comparison to molecular

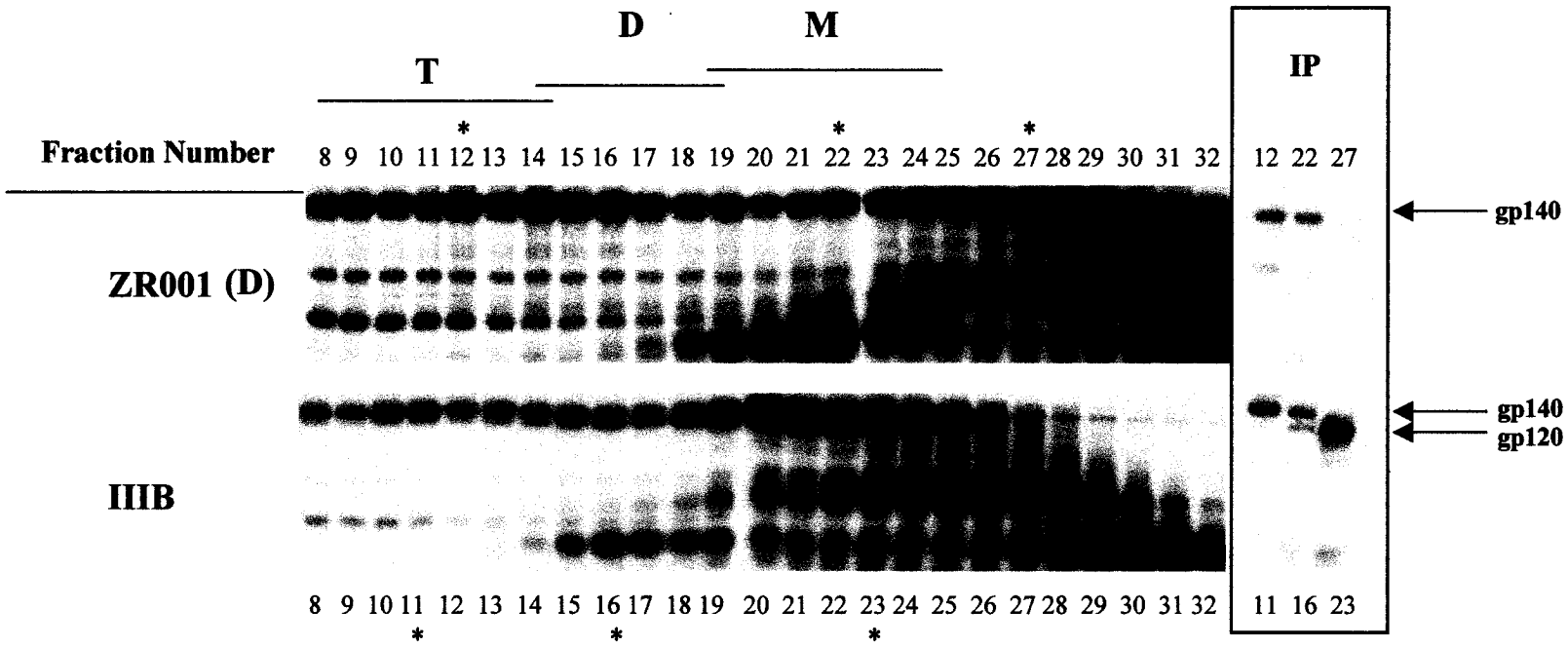


Figure 5. Comparison of sucrose gradient profiles of a primary isolate and TCLA strain gp140s. Supernatants containing ^{35}S-methionine labeled Env were produced by BSC-1 cells infected with rvv expressing a clade D (ZR001) primary isolate gp140 and the clade B TCLA IIIB gp140. Each supernatant was concentrated and separated on a 5-20% sucrose gradient. The distribution of Env across the gradient was determined by running fraction aliquots (8-32) on 10% SDS-PAGE gels. Fractions from each of the proposed oligomeric (T,D) and monomeric (M) regions (marked with *) were immunoprecipitated with polyclonal anti-gp140 rabbit serum and run on 10% SDS-PAGE gels (results are seen in the IP panel).

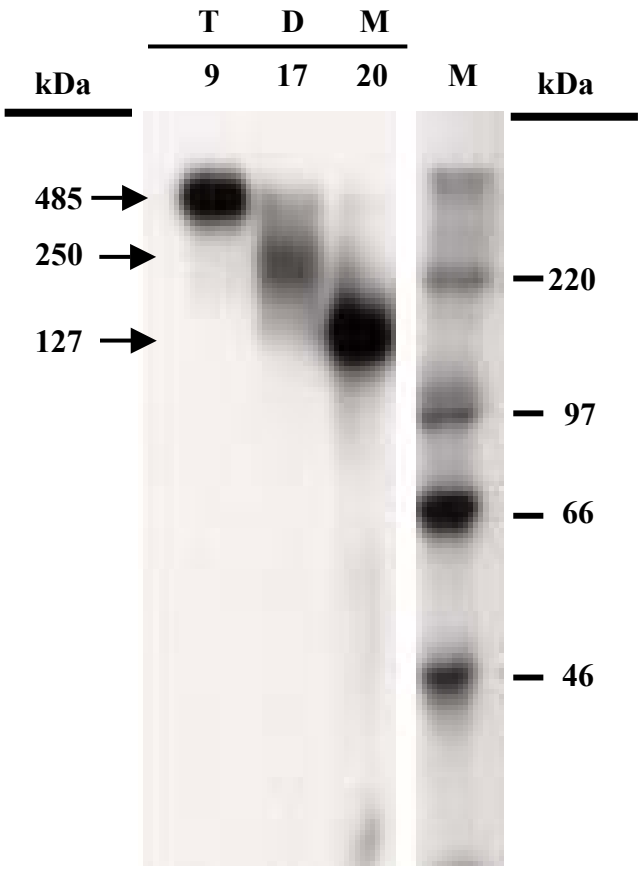


Figure 6. Molecular weight determination of metabolically labeled clade D primary isolate Env sucrose density centrifugation fractions. Metabolically labeled supernatant containing a clade D gp140 is concentrated, crosslinked and separated over a 5-20% sucrose gradient. Representative samples from regions of trimer (T), dimer (D) and monomer (M) corresponding to fractions 9, 17 and 20 respectively, are analyzed by SDS-PAGE without reducing agent (βME). The molecular weights were determined by Eagle-Eye densitometry and integration against marker (M).

weight standards. Though the sedimentation coefficients were not determined, the approximate molecular mass for the T fractions is calculated to be between what would be expected for trimeric (420 kDa) or tetrameric (560 kDa) gp140. These sizes correlate to those determined by Earl and colleagues and are consistent with the proposed oligomeric nature of the Env. The more exacting method of determining molecular mass is sedimentation equilibrium centrifugation, though this was not available to us at the beginning of this thesis work. This is a method for measuring protein molecular masses in solution and for studying protein-protein interactions. It is particularly valuable for establishing whether the native state of a protein is a monomer, dimer, trimer, etc. In sedimentation equilibrium the sample is spun in an analytical ultracentrifuge at a speed high enough to force the protein toward the outside of the rotor, but not high enough to cause the sample to form a pellet. As the centrifugal force produces a gradient in protein concentration across the centrifuge cell, diffusion acts to oppose this concentration gradient. Eventually an exact balance is reached between sedimentation and diffusion, and the concentration distribution reaches an equilibrium. This equilibrium concentration distribution across the cell is then measured while the sample is spinning, using absorbance detection. Most importantly, the concentration distribution at equilibrium depends only on molecular mass, and is entirely independent of the shape of the molecule. The precision of the molecular masses determined by this technique is usually 1-2%.

Figure 7 shows sucrose density gradient profiles for most of the rvv primary isolate gp140s produced for this study. The clade C (MW965) and other clade D (UG024) Env secreted is mostly unprocessed, and much oligomeric uncleaved gp140 is observed. The

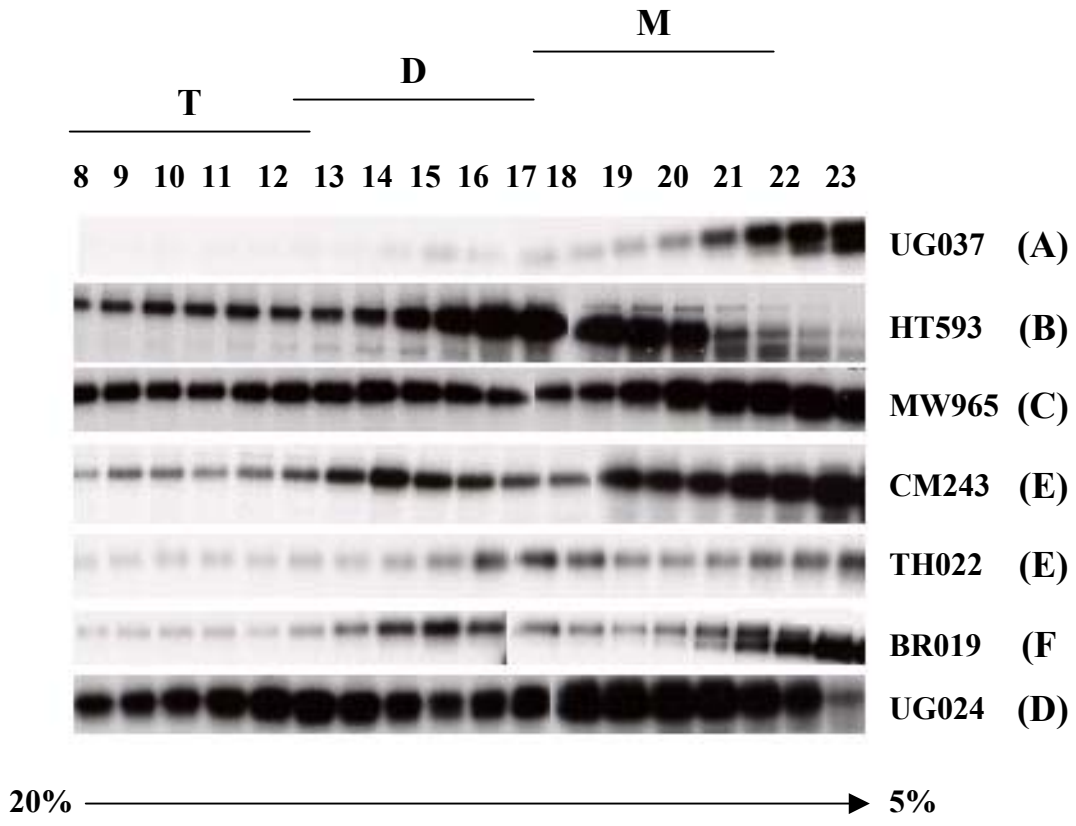


Figure 7. Sucrose gradient profiles of HIV-1 primary isolate gp140s from various clades. Supernatants containing ^{35}S-methionine labeled Env were produced by BSC-1 cells infected with rvv expressing gp140. Each supernatant was concentrated and separated on a 5-20% sucrose gradient. The distribution of Env across the gradient was determined by running fraction aliquots (8- 26) on 10% SDS-PAGE gels. Each profile is labeled with partial isolate name and (clade).

greatest degree of cleavage to monomeric gp120 appears to occur with the clade B primary isolate HT593. The JRFL R5 clade B strain was also cleaved almost entirely to gp120 (not shown). Overall, results from sucrose gradient centrifugation suggest that most of the oligomeric forms of Env dissociate into monomers. However, these results are inconclusive because neither crosslinking nor immunoprecipitation was carried out on any of these samples to verify oligomeric status or HIV-1 Env identity.

To determine whether a chemical crosslinking reagent could be used to enhance the recovery of stable, soluble, cleaved oligomeric gp140 Env, a variety of experiments using reducible crosslinkers was performed to control for possible dissociation of oligomers during centrifugation. EGS, a hydroxylamine cleavable crosslinker, and DSP and its water-soluble analog DTSSP, which are thiol-cleavable crosslinkers, were each tested on ^{35}S-methionine labeled IIIB Env supernatants. Each of these crosslinkers are homobifunctional (intramolecular crosslinkers) N-hydroxysuccimide esters (NHS-ester) and react significantly with the primary amine groups present on the N-termini of peptides and proteins and the ε-amine of lysines. A range of the EGS crosslinker (0.25 to 5mM) was used to determine the least amount that could be used to link the oligomeric molecules. Cells were infected with purified rvv expressing IIIB gp140 and metabolically labeled with ^{35}S-methionine. The crosslinker was added to the concentrated supernatant before separation over a sucrose gradient. After centrifugation, individual fractions were collected across the gradient. Fractions were then analyzed under non-denaturing conditions on SDS-PAGE. Based on these results, a final concentration of 5mM was chosen to test on the thiol-reducible crosslinkers. Since similar results were obtained with each of the crosslinkers, 5mM DTSSP was chosen for

use in all subsequent experiments because it is soluble in aqueous solutions, whereas the others require a solvent.

Furin enhances processing of primary isolate HIV-1 Env

In prior studies using vaccinia virus to produce gp140 (87), the removal of the cleavage site allowed for a higher yield of oligomeric material, but its removal is not necessary for retention of oligomeric forms. However, a non-cleaved Env may not necessarily be presented in its exact native conformation. To determine if proteolytic processing of gp140 into gp120/41 subunits can be enhanced, treatment with the serine protease plasmin *in vitro*, as well as coexpression with recombinant vaccinia virus producing the human protease furin, was investigated.

Recently, it was reported that plasmin (PLA) could affect gp160 processing (210). To investigate whether PLA is effective at cleaving our soluble preparations of HIV-1 Env gp, purified IIIB gp140 with the cleavage site intact was incubated with the activated plasminogen. Plasmin is the active form of a cell surface serine protease. The plasminogen precursor is activated by urokinase, another serine protease which is involved in matrix degradation. After conditions for activating plasminogen were optimized, studies on PLA effectiveness were initiated by mixing 10 μg of purified IIIB gp140 and 0.012 to 1.2 μg of PLA and incubating for 30 minute and 1 hour. Aliquots were run on SDS-PAGE gels, western blotted and detected. No observable differences in Env were seen after Western analysis. An overnight incubation with only 1 μg of purified IIIB Env and a range of PLA yielded the results shown in **Figure 8**.

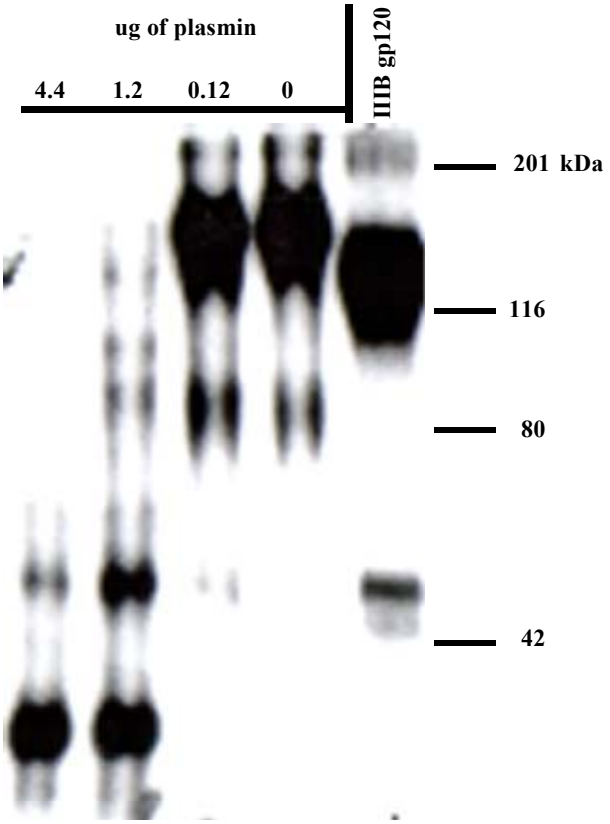


Figure 8. Western analysis of purified IIIB after incubation with activated plasminogen. Samples were incubated overnight at 37° C, and 50 ng of each sample was resolved on a 10% SDS-PAGE gel. After transfer, the Western blot was probed with polyclonal rabbit serum made to IIIB gp140. 50 ng of purified IIIB gp120 is included as a control for MW comparison.

Overdigestion of Env resulted from the high concentration of PLA (4.4 μg), while 0.12 μg of PLA did not cleave at all. Only 1.2 μg of PLA generated the gp120 band, but this concentration also yielded more secondary cleavage product than gp120. The results obtained here with purified soluble IIIB gp140 indicate this approach to enhancing cleavage post Env production is possible, but it appeared that large quantities of the purified protease would be required. Therefore, the additional activation step of PLA coupled with the challenge of repurification of gp140 warranted no further investigation of this procedure.

Furin is a member of a cellular subtilisin-like protease family that recognizes the amino acid sequence RXXR. Previous work has shown that furin is most likely to be the cellular protease responsible for HIV-1 envelope processing (71, 72, 199) as well as other viral membrane glycoproteins (112). To determine whether coexpression of furin can enhance cleavage of HIV-1 primary isolate Envs, we used a recombinant vaccinia virus that expresses furin. BSC-1 cells were coinfected with rvv expressing cleavable IIIB gp140 and rvv-furin and metabolically labeled. HIV-1 Env from supernatants and cell lysates were immunoprecipitated with a polyclonal serum and Protein G-Sepharose beads. Precipitated Env was eluted from the beads with SDS-PAGE sample buffer and the material was separated by 12% SDS-PAGE. The gel was then dried and Env was visualized by autoradiography. The densities of the gp140, gp120 and gp41 bands were determined by scanning and densitometry analysis (area of band translated to a numerical value) with NIH Image software. To determine percent cleaved material, the following calculation was performed: the area of the gp120 band (number of pixels) divided by the total area (area of gp120 plus area for gp140). The results are shown in **Table 2**.

Virus MOI vCB14/furin	Area		% Cleaved
	gp140	gp120	
10/0	1180	190	14
10/2	910	610	40
10/5	990	860	46
10/10	790	770	49

Table 2. Effect of furin coexpression on IIIB gp140 processing. An MOI of 10 for vCB14 (rvv expressing IIIB gp140) was used to coinfect BSC-1 cells with varying MOIs of rvv expressing furin. The ^{35}S-methionine labeled supernatants were immuno-precipitated with rabbit polyclonal serum raised to IIIB gp140 and analyzed by SDS-PAGE. Densitometry analysis of the gp140 and gp120 bands was used to determine percent of total Env cleaved.

Preliminary coinfection experiments with rvvs expressing furin and IIIB Env gp140 were promising; cleavage was enhanced by approximately 50%. However, secondary cleavage of IIIB was evident for dimer and monomer fractions (**Figure 9**). This cleavage appears similar to that which occurs at the tip of the V3 loop at the trypsin /chymotrypsin sensitive site, GPGR/GPGRAF, respectively, which has been previously reported (58, 121).

Viral multiplicity of infection (MOI) combinations were examined to determine the vaccina virus infection ratio that produced the greatest degree of cleaved Env. When the most effective combination of rvv-Env and rvv-furin was determined, experiments were performed to investigate whether cleavage of a primary isolate Env could be enhanced, and whether using crosslinker would increase the amount of recoverable cleaved, oligomeric material from sucrose gradient preparations. Rvv-furin co-infection experiments with clade D (vAT11) envelope-expressing virus and a rvv expressing clade E Env have resulted in 100% processing to gp120 and gp41 subunits, with no secondary cleavage at the V3 loop as found with IIIB (**Figure 10**). Based on the results shown in Figure 10, we determined that the centrifugal forces appear great enough to increase the loss of oligomeric fractions through the dissociation of processed gp120 from the oligomeric complex.

Separation of subsequent Env-containing supernatants was performed by size exclusion chromatography to expedite the procedure and to allow for a greater yield of oligomeric material. To demonstrate that equivalent results can be obtained by purifying crosslinked, furin processed Env by column chromatography methodology, the following experiment was performed. A metabolically labeled clade E primary isolate Env was

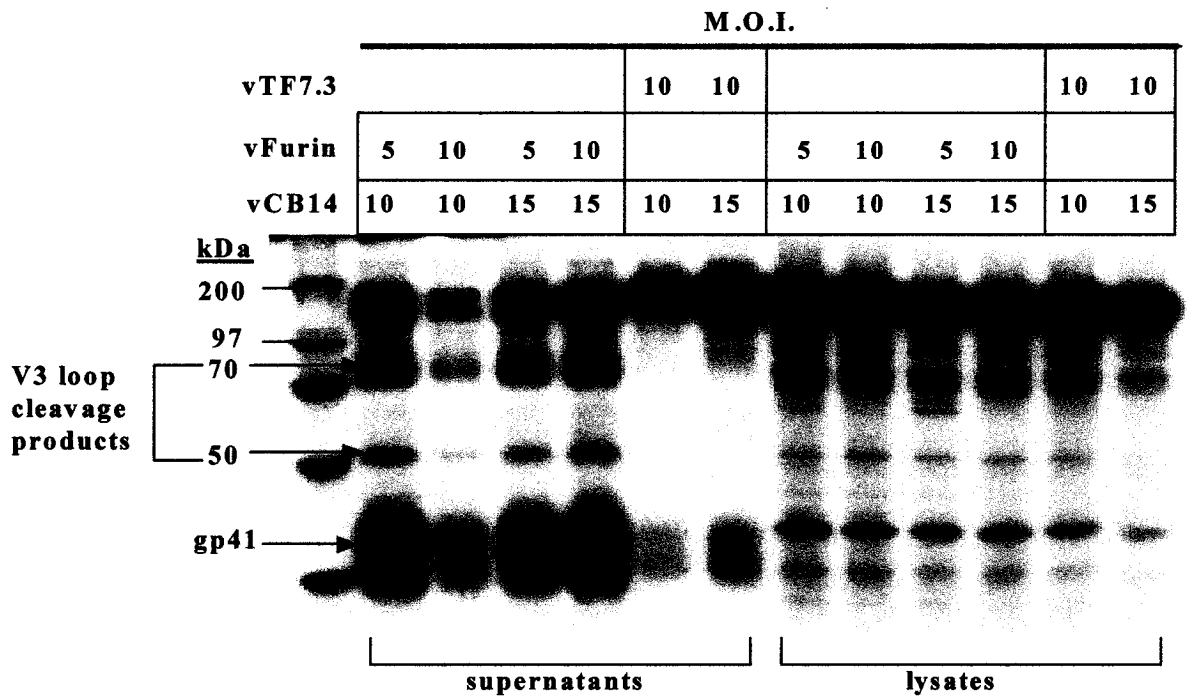


Figure 9. Comparison of metabolically labeled supernatants resulting from infection with various MOI combinations of vfurin and rvv expressing IIIB gp140. Various MOI combinations of vCB14 (expressing IIIB gp140) and vfurin were used to determine the most effective combination for the greatest yield of processed gp140 without decrease in total yield of Env. The secondary cleavage that occurs at the tip of the V3 loop is demonstrated by the 70 kDa and 50 kDa bands.

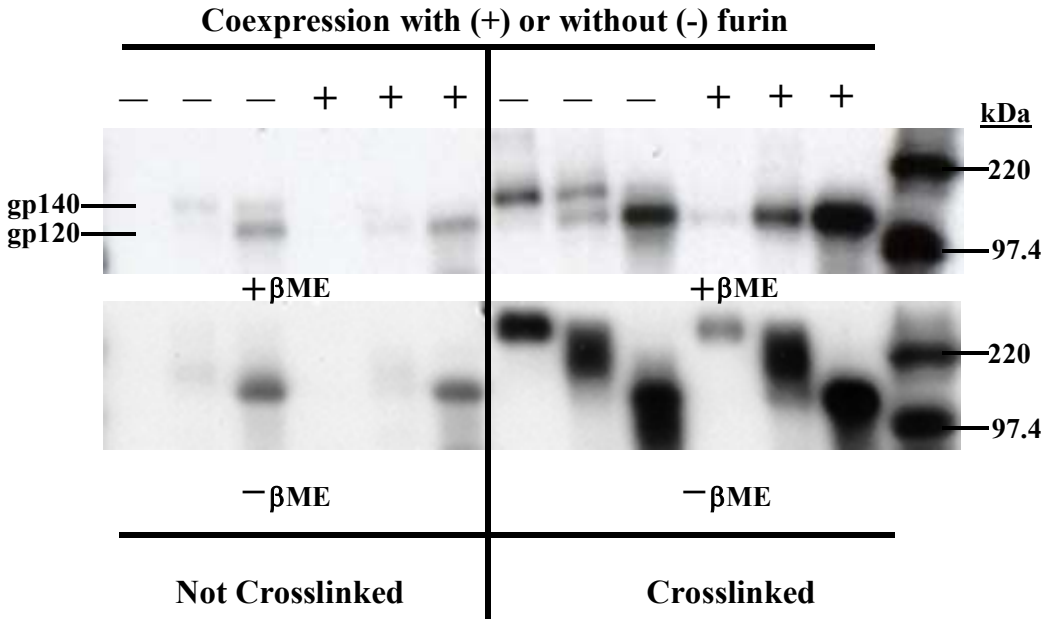


Figure 10. Analysis of a clade D primary isolate gp140 produced with and without coexpression with furin and crosslinking. Half of each concentrated supernatant containing metabolically labeled clade D gp140 coexpressed with and without furin was crosslinked with 5mM DTSSP. All supernatants were individually fractionated over a sucrose gradient and representative T, D and M fractions were analyzed by 10% SDS-PAGE under reducing (+ βME) and non-reducing conditions.

coexpressed with furin, and the concentrated supernatant was crosslinked and subjected to size exclusion chromatography using Superdex 200, a unique composite matrix of dextran and agarose with a separation range (M_r) of 10,000 to 600,000 globular protein. Aliquots of all the collected fractions were analyzed by autoradiography of dried SDS-PAGE gels. Representative samples from each of the regions defined as trimer (T), dimer (D) and monomer (M) were run under reducing and non-reducing conditions on a 8% SDS-PAGE gel along with an aliquot of Env not expressed in conjunction with furin. These results (**Figure 11**) exemplify that Env of another clade can be 100% processed by furin, and oligomeric fractions can be separated from monomers by size exclusion chromatography.

Large Scale Antigen Preparation and Analysis

In preparation for future polyvalent immunization studies, as well as structural and functional studies, we had to produce and analyze preparative amounts of gp140 from a representative of each of clades A, B, C, D and E. The gp140 Envs were produced, initially purified by lentil lectin chromatography and subsequently by size exclusion chromatography. The protein complexes with the largest molecular weights elute off the size exclusion column first. From previous studies it was determined that the first peak consisted of trimers or tetramers, the second peak dimers and the third monomers. To determine the extent of processing of the Env gp140, representative samples from each peak (trimer, dimer and monomer) were analyzed by chemiluminescent Western blot using a rabbit polyclonal raised to denatured IIIB gp140. **Figure 12** shows the chromatograms from each size exclusion run, and the coordinate peak samples as

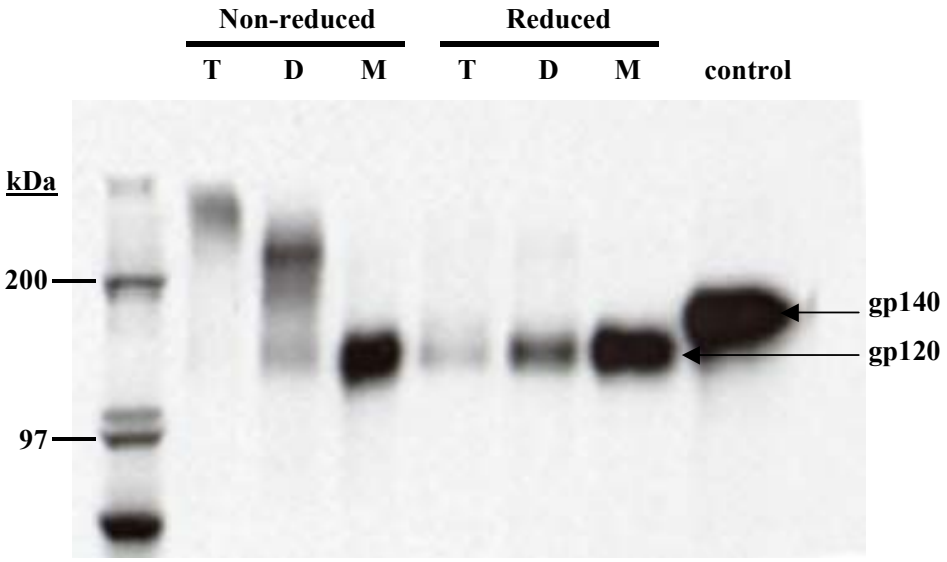


Figure 11. Analysis of oligomeric fractions from size exclusion chromatography of supernatant containing metabolically labeled clade E gp140. Supernatant containing ^{35}S-methionine labeled clade E primary isolate gp140 coexpressed with furin is concentrated, crosslinked and purified by size exclusion chromatography. Fractions from T (trimer), D (dimer) and M (monomer) regions are run in SDS-PAGE sample buffer without and with βME on an 8% SDS-PAGE gel. An aliquot of the clade E gp140 not coexpressed with furin is included for MW comparison.

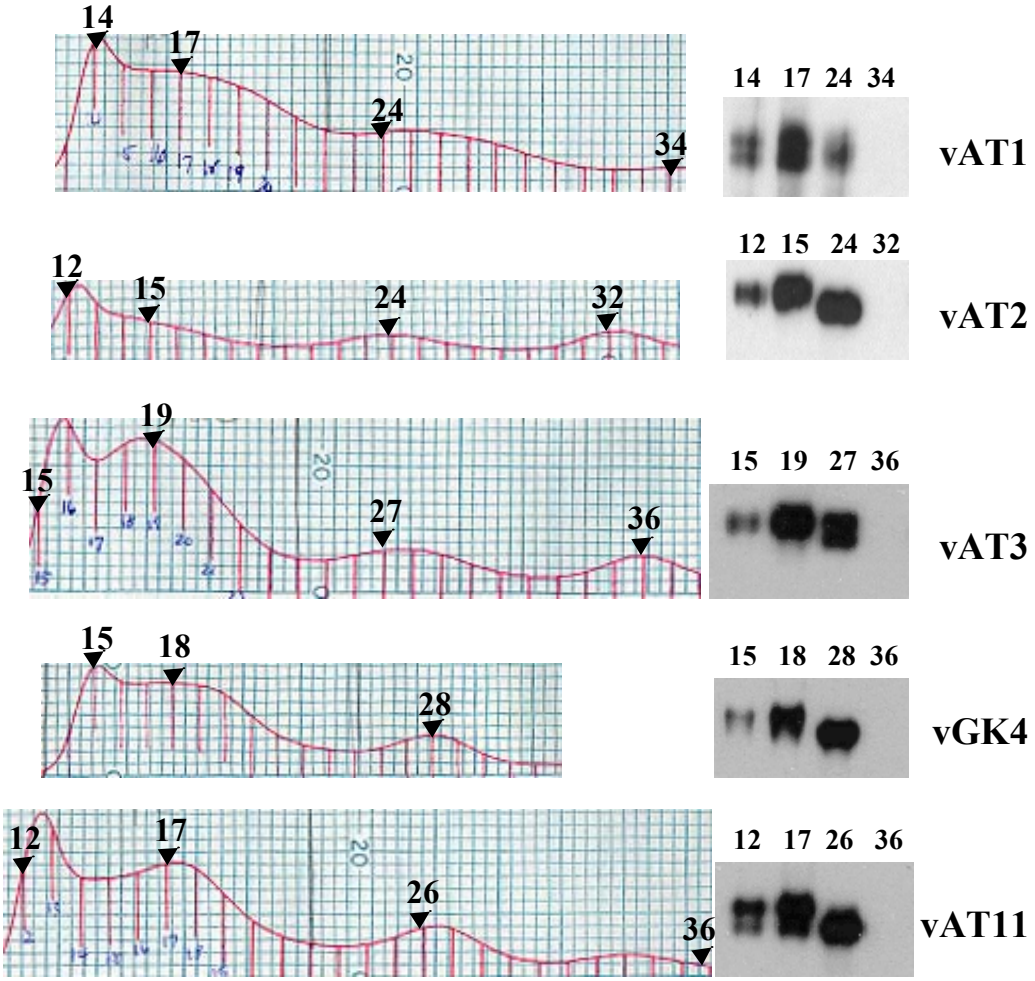


Figure 12. Chromatograms and western analysis of lentil lectin purified HIV-1 primary isolate gp140s. Total protein as measured by absorbance at 280 nm during size exclusion chromatography is translated to a chromatogram for each of the large preparations of previously lentil lectin-purified HIV-1 primary isolate gp140 as they are eluted from the column. Adjacent to each printout is the Western analysis of fraction representatives of each peak, probed with a rabbit polyclonal serum raised to IIIB gp140.

resolved on a Western blot. The cleaved and uncleaved nature of each Env oligomeric fraction can be determined. It is interesting to note that for most isolates, the proposed dimer peak contains mostly uncleaved gp140, yet some (clade C, clade D) contain some cleaved material as well. The panel showing the aliquots from the clade D Env fractions is most interesting because there appears to be cleaved oligomeric gp120 as well as uncleaved gp140 present in the oligomeric fractions- which has never before been observed- though much of the cleaved material has dissociated to monomeric gp120. Center and colleagues have recently shown that some percentage of IIIB gp120 is able to dimerize independent of gp41 (44), though this has not been shown for any other HIV-1 isolate. The gp120 band seen in the dimer peak may be this dimer of monomers. Based on this analysis, the first two peaks were pooled. The pooled trimer and dimer fractions were concentrated and quantitated by densitometry analysis of colloidal Coomassie stained SDS-PAGE gels, which includes a IIIB gp140 Env concentration standard. The quantity recovered for each of the oligomeric Env gp140s to be used in the polyvalent immunization study are listed in **Table 3**. Storage of these glycoproteins has been at 4° C for over a year with no visible deterioration in quality as determined by recent Western analysis, demonstrating the high degree of stability of oligomeric gp140.

To further verify the oligomeric nature of each fraction, representative samples of each region (T, D, M) of the clade D purified Env were crosslinked and resolved on a 6% SDS-PAGE gel under non-reducing conditions. Western blot analysis was performed using the polyclonal rabbit serum raised to denatured IIIB Env gp140. These results (**Figure 13**) show the molecular weights as determined by extrapolation from standard protein markers resolved on the same SDS-PAGE gel. The molecular weight

Env Clade (isolate)	Size Exclusion Oligomer Total µg
B (IIIB-unc)	345
A (UG037)	2000[a]
B (HT593)	240
C (MW965)	506
D (ZR001)	330
E (CM243)	154

Table 3. Quantification of column chromatography purified oligomeric HIV-1 gp140s for use in the polyvalent vaccine experiment. These Envs were purified from supernatant from four roller bottles of BSC-1 monolayers infected with MOI of 10 for each rvv. Supernatants were first purified by lentil lectin chromatography followed by size exclusion chromatography to separate oligomeric fractions from monomeric fractions. The oligomeric fractions were then pooled and quantitated by Coomassie staining of SDS-PAGE gel with HIV-1 IIIB gp140 standard of known concentration. IIIB-unc is the TCLA HIV-1 strain with the cleavage site removed. [a] Original amount recovered after lentil lectin chromatography. Degradation of protein occurred after size exclusion chromatography.

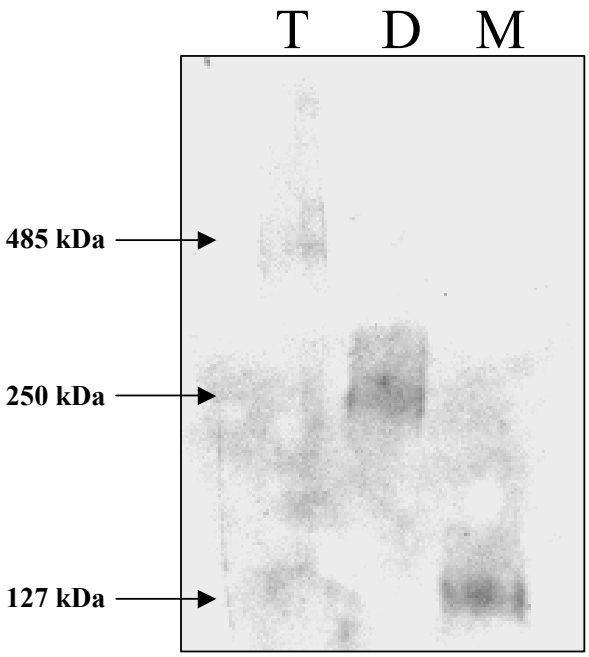


Figure 13. Western analysis of fractions of size exclusion purified clade D gp140, cross-linked post purification. T,D and M represent the proposed trimer, dimer and monomer fractions. Cross-linked frations were not reduced prior to resolution by 6% SDS-PAGE. Western blot was probed with a rabbit polyclonal serum raised to IIIB gp140

determinations are consistent with trimer, dimer and monomer forms of gp140 Env.

Cleaved, Soluble Crosslinked Primary Isolate Env Binds Potent HIV-1 Human Neutralizing Antibodies

The human monoclonal antibodies IgG1b12, 2G12 and 2F5 are presently the three MAbs generally accepted as having significant neutralizing ability against primary isolates from a variety of HIV-1 clades, despite the fact that these were isolated from individuals infected with clade B strains of HIV-1 (81, 82, 144, 188, 275). IgG1b12, which consists of recombinant human Fab fragments, recognizes a discontinuous epitope in the CD4 binding domain (237). It recognizes monomeric gp120, but has preferential reactivity with the oligomeric form (237, 254). IgG1b12 is cross-reactive with many primary isolate gp120s from clades A-D, but it is not reactive to gp120 from clades E or F (190). 2G12, a recombinant human monoclonal antibody IgG1, recognizes a conformationally sensitive and carbohydrate-dependant epitope that is distinct from the CD4bs MAb epitope but lies within gp120 (37, 276).

Unlike the other two human monoclonals, 2F5, an IgG3 kappa isotype, recognizes an epitope in gp41. The core epitope is LDKW (275) within the general epitope of ELDKWA in the C-terminus of the ectodomain of gp41 (37, 201). Considering its neutralizing titer, this MAb is paradoxically weak in binding to virus (40). When compared to the other potent, neutralizing MAbs, 2F5 has been shown to be more potent and have greater breadth against primary isolates (258, 274, 275).

The CD4-IgG_2 molecule was included in the panel of monoclonal antibodies used to assess the antigenic properties of the soluble Env from primary isolates. This molecule is

a novel fusion protein comprising human IgG_2 in which the Fv portions of both heavy and light chains have been replaced by the V1 and V2 domains of human CD4 (5). CD4-IgG_2 can also neutralize a broad panel of primary isolates, as would be expected for those isolates dependent upon CD4 for initial binding to the cell (275).

To determine whether soluble, gp140 oligomers with or without enhanced processing and crosslinking retain both CD4 binding activity and important conformational epitopes, the following studies were performed. First, concentrated, labeled supernatants were evaluated for the ability of the Env to be recognized by the potent neutralizing human monoclonals. As seen in **Figure 14**, clades A and B react with the gp120 antibodies IgG1b12, 2G12, CD4IgG as well as with anti-gp41 2F5. Any substitutions at the D or K position of the LDKW core epitope leads to neutralization resistance (229). The clade C isolate we used has an S substitution for the K, which explains the inability of this Env to be precipitated by 2F5.

When the concentrated supernatants are crosslinked, separated into oligomeric and monomeric fractions and the trimer form is immunoprecipitated with saturating quantities of the human monoclonals, both gp140 and gp120 are still recognized by these antibodies (**Figure 15**). Though the clade B gp140 is not processed 100%, gp41 can still be observed for each immunoprecipitation. Most exciting are the clade D and E Envs processed to gp120 and gp41 by furin. Not only is the processed gp120 recognized by the anti-gp120 MAbs, but gp41 is coprecipitated with these antibodies also, suggesting the crosslinker is able to stabilize the oligomer. The clade E Env, which doesn't react with IgG1b12 (noted previously), is reactive to 2F5, so the gp120 observed here must be associated with the gp41. Notably, the clade D Env is not recognized by the 2F5

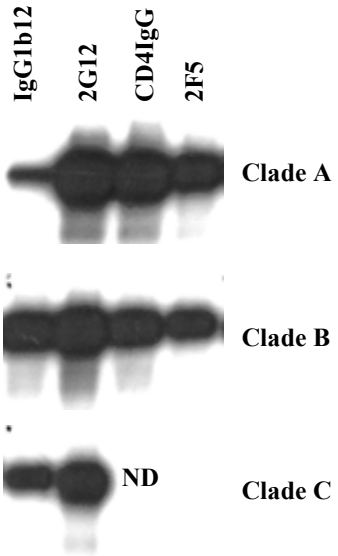


Figure 14. Immunoprecipitations with human neutralizing MAbs performed on metabolically labeled HIV-1 primary isolate gp140 supernatants for various clades. ND indicates no data for this MAB on the clade C gp140.

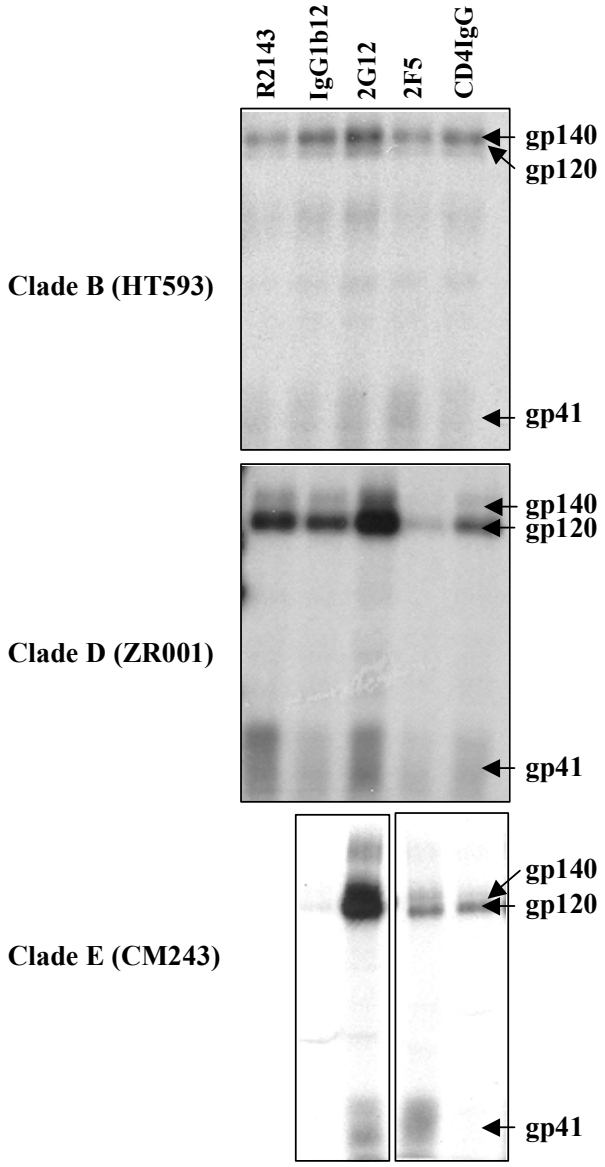


Figure 15. Immunoprecipitations with human neutralizing MAbs performed on metabolically labeled crosslinked purified trimers of HIV-1 primary isolate gp140 from various clades, coexpressed with furin.

antibody. Within the 2F5 epitope, ELDKWA (aa662-667 HXB2 numbering), there is an E662Q substitution for the clade D (ZR001) gp140. Glutamine at this position seems conserved within clade D envs. Of 41 clade D HIV-1 primary isolates recently sequenced, 65% have the E662Q substitution and 23% have other substitutions; only 12% have E662 (79). This change also does not fall under the known D or K substitution effect on neutralization resistance, previously mentioned for the clade C isolate (229). Also, a multi-laboratory study found 2F5, which is neutralizing to many HIV-1 primary isolates, unable to neutralize a D subtype primary isolate (82). A similar lack of reactivity to this MAb was found with HIV-1 pediatric isolates with alanine and lysine substitutions of the E662 amino acid (113). Overall, however, the results of MAb immunoprecipitation of purified, crosslinked oligomer signifies several important epitopes in Env are seemingly unperturbed by the addition of crosslinker. Comparison to the amount of Env immunoprecipitated by R2143 - the polyclonal serum that should immunoprecipitate all forms of Env- indicates equivalent amounts immunoprecipitated. The percent of Env immunoprecipitated by the various human neutralizing MAbs calculated as a percentage of the amount precipitated by R2143 is summarized in **Table 4**.

Cleaved, Soluble, Crosslinked Primary Isolate Env is Recognized by Conformationally-Dependent Monoclonal Antibodies

A panel of 138 monoclonal antibodies had been made from mice that had been immunized with non-denatured tetramer/trimer, dimer or monomers of IIIB secreted Env gp140 that lacked the cleavage site (86). The nomenclature used to identify the monoclonals is based on what form of Env was used to generate the antibodies: T for

		CLADE					
		A	**B***	**C**	**D**	**D-furin/***	**E-furin/***
gp120	**IgG1b12**	10%	100%	50%	90%	80%	0%
	2G12	100%	100%	100%	100%	125%	100%
	CD4IgG	100%	80%	(ND)	50%	75%	10%
gp41	**2F5**	50%	50%	0%	0%	0%	10%

Table 4. Summary of neutralizing antibody reactivities to HIV-1 Envs from various clades. The percent reactivity is calculated based on 100% reactivity with polyclonal sera R2143 or with 2G12 if R2143 was not used, based on the results in Figures 14 and 15. The asterisk (*) indicates crosslinked Env.

tetramer/trimer, D for dimer and M for monomer. Previous studies had determined the reactivities of these MAbs as related to the Env quaternary structure (34): T4, T6, T9 and T10 recognize conformation dependent, oligomeric specific gp41 epitopes; D11 and D12 recognize conformation dependent gp41 epitopes; T8 recognizes a linear gp120 epitope; D61 recognizes a linear gp41 epitope in Cluster I. Cross reactivities to some primary isolate Envs from clades A, B, C and E have also been determined (85). To assess the oligomeric nature of the secreted primary isolate Envs we have selected, these monoclonals were used in immunoprecipitation assays on concentrated ^{35}S labeled supernatants of a representative of each of clades A through E. Wild type IIIB gp140, with cleavage site intact, and IIIB gp140, with the cleavage site removed (IIIB-unc) were included as controls. As seen in **Figure 16**, all expressed gp140s of R5 HIV-1 isolates were immunoprecipitated by all the conformation dependent as well as conformation independent antibodies, with a variable degree of binding. Only the 140 kDa band is seen for the conformation dependent antibodies. This is expected as the Envs naturally processed to gp120 and gp41 ectodomain may dissociate under the immunoprecipitating conditions. Immunoprecipitation of clade A gp120 by T8 supports the findings that clade A naturally processes to its gp120-gp41 subunits, as seen with IIIB. The linear gp120 T8 antibody does not recognize the clade C gp140. Immunoprecipitation of the clade C Env with T8 was repeated and the results of this immunoprecipitation confirmed this initial finding. Of note are the results for the X4 clade D soluble gp140 Env, which is only recognized weakly by T9 and T10, and more strongly by D61 (linear epitope), though overall not very reactive.

To further characterize the oligomeric nature of the naturally processed, purified,

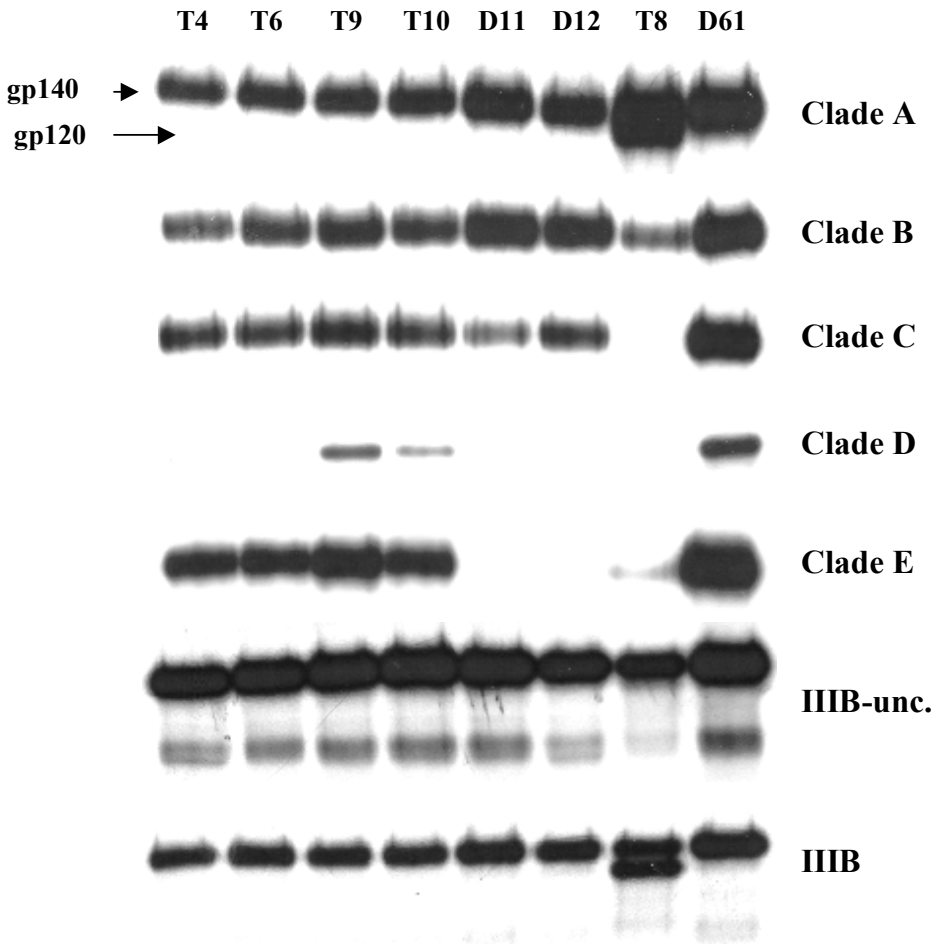


Figure 16. Immunoprecipitations with murine MAbs specific for gp120 or gp41 performed on metabolically labeled supernatants. Metabolically labeled supernatants of HIV-1 primary isolate gp140 immunoprecipitated with Mabs to gp41 and gp120 as described in the text. IIIB gp140 wild type and with cleavage site removed (IIIB-unc) shown for comparison.

soluble Envs, immunoprecipitations with MAbs that recognize linear and conformational epitopes were performed. The oligomeric (pooled trimer and dimer fractions) and monomeric fractions from the clade D and clade E Envs purified by molecular sieve gel filtration chromatography were immunoprecipitated in independent reactions with: i) a conformation-dependent, oligomer specific anti-gp41 MAb (T9); ii) a conformation-independent anti-gp41 MAb (T3) and; iii) as a control for total Env recovered, the anti-gp140 polyclonal serum. The polyclonal serum was also used as the probe for the Western analysis. **Figure 17** demonstrates that for both clades E and D, only Env oligomeric fractions can be immunoprecipitated by anti-gp41 antibodies, indicating that the monomeric fractions almost exclusively contain soluble gp120. However, the majority of secreted uncleaved gp140 is oligomeric. In addition, for both primary isolate clade E and D oligomeric gp140, some amount of processed oligomer is observed. That is gp120, along with gp140, can be precipitated with MAbs reactive to gp41 epitopes which is seen with both the oligomer specific T9 and linear T3 MAbs. Specifically, some processed oligomers exist for the clade E gp140 that maintain native conformation as evidenced by the precipitation of the gp120 subunit with the oligomeric specific, gp41 reactive, T9 antibody.

To determine if these same antibodies recognize cleaved, crosslinked forms of the primary isolate Envs, we performed immunoprecipitations on the purified oligomeric (trimer) fractions of metabolically labeled primary isolate secreted Env gp140 that was coexpressed with rvv furin. D20 is a conformation-dependent anti-gp120 antibody that maps to the CD4 binding site (85), in contrast to the conformation dependent, oligomeric specific T4 and T9 anti-gp41 MAbs. D20 will precipitate gp120 and gp140 that is

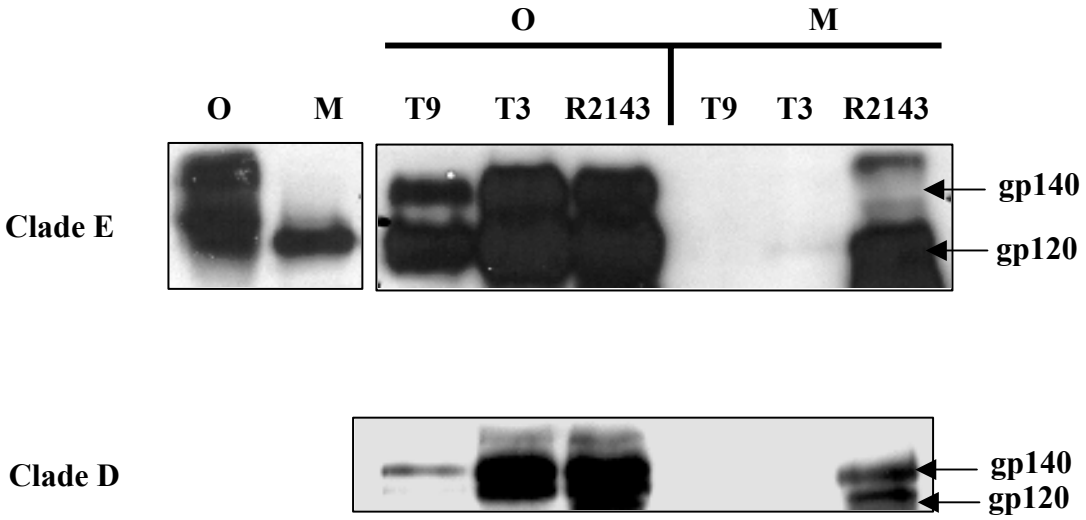


Figure 17. Western analysis of size exclusion chromatography purified, pooled oligomer (O) and monomeric (M) fractions immunoprecipitated with 1) a Mab that recognizes a gp41 oligomer specific epitope (T9); 2) a Mab that recognizes a linear gp41epitope (T3); 3) a rabbit polyclonal serum to IIIB gp140 (R2143) used as a control to immunoprecipitate all Env. The left panel for clade E Env includes non-immunoprecipitated aliquots of O and M. The Western blot was probed with R2143.

properly folded. T4 and T9 will precipitate the gp120/gp41 cleaved complex or the uncleaved gp140 and would indicate whether the bond between the gp120 and gp41 was intact as evidenced by a band at gp120 for both MAbs. Results for the concentrated metabolically labeled supernatants displayed in **Figure 18** indicate all clades have recognizable gp140 oligomers, but after processing, the gp120-gp41 association is not stable for the clade C and clade D Envs, which were processed 100%. Clade A and clade B gp140 did not process to a great extent, though there appears to be some gp120 precipitated with both D20 and T4, evident for both Envs coexpressed with furin. **Figure 19** results are immunoprecipitations performed on metabolically labeled concentrated supernatants containing clade E Env expressed with and without furin, as well as immunoprecipitations performed on crosslinked, size exclusion chromatography purified trimer pools of clade E Env either coexpressed with furin or not. Looking at the results for immunoprecipitation of the concentrated supernatants, D20 does not appear to have a particularly high affinity for the clade E Env; T9 has a higher affinity. 100% processing is evident by the gp120 bands present for the Envs coexpressed with furin, and some oligomeric processed Env may be present as evidenced by the gp120 band for the T9 precipitation. The low affinity of D20 for this clade E Env may explain the lack of signal for this lane. However, immunoprecipitation results for the crosslinked oligomer fractions display gp120 and gp41 bands for each MAb, suggesting the crosslinker was able to stabilize the oligomers and did not interfere with antibody recognition. **Table 5** is a summary of the reactivities of these monoclonals with Env.

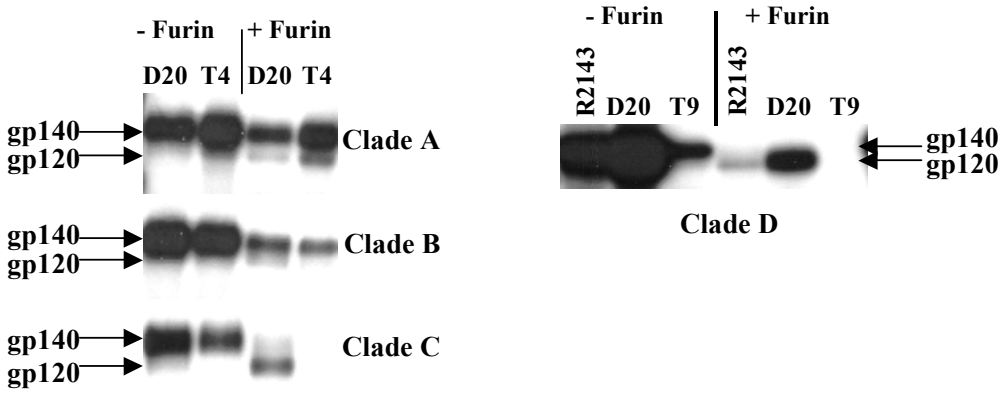


Figure 18. Immunoprecipitation of metabolically labeled supernatants containing HIV-1 primary isolate gp140 from various clades either coexpressed with furin or not. Conformation dependent anti-gp120 (D20) and either anti-gp41T4 or T9 Mabs were used for immunoprecipitation; rabbit polyclonal raised to IIIB gp140 (R2143) only used on clade D gp140.

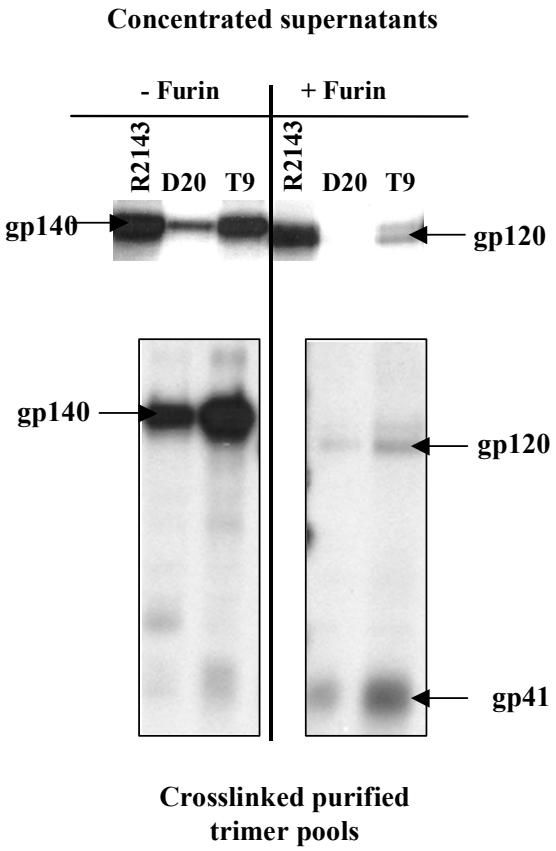


Figure 19. Comparison of immunoprecipitations of metabolically labeled gp140s in concentrated supernatants and crosslinked, purified, trimer pools. Immuno-precipitation was performed on metabolically labeled supernatants and crosslinked, purified trimer pools containing clade E gp140 coexpressed with or without furin, using conformation dependent anti-gp120 (D20) and anti-gp41 (T9) Mabs and analyzed on 10% SDS-PAGE gels. Top panel shows results for concentrated supernatants; bottom panels shows results for crosslinked purified oligomeric pools.

		A	A-furin	B	B-furin	C	C-furin	D	D-furin	E	E-furin
linear gp120	T8	+		+		–		–		–	
	D19							–	–	+	+
	D47							–	–	–	–
	T12									+	
	T18							+		+	
	T36							(+)		+	
	T50							+		+	
conformation dependent gp120	D25							+	+	–	–
	M12							–	–	–	–
	T22							+	+	+	+
	D20	+	+	+	+	+	+	+	+	+	–
linear gp41	D50	–		+		–		+	+	–	–
	D61	+		+		+		+	+	+	
	T3	–		+		+		+		+	
conformation dependent, oligomeric sensitive gp41	D10	+		+		+					
	D11	+		+		+		–			
	D12	+		+		+		–		–	–
conformation dependent, oligomeric specific gp41	T4	+	+	+	+	+	+	–		+	+
	T6	+		+		+		–		+	
	T9	+		+		+		(+)	–	+	+
	T10	+		+		+		(+)		+	

Table 5. Cross reactivities of MAbs with Env from primary HIV-1 isolates. Metabolically labeled gp140s - coexpressed with furin and crosslinked where noted - were immunoprecipitated with MAbs and analyzed by SDS-PAGE as shown in Figures 16, 17 and 18. Env proteins were derived from primary isolates as indicated in parentheses: clade A (UG0337.8); clade B (HT593.1); clade C (MW965.26); clade D (ZR001.3); clade E (CM243). +, strong reactivity; (+), decreased reactivity; –, lack of reactivity. Shaded cells represent novel findings; the unshaded cells are reproduced findings of PL Earl and colleagues (see text).

Cleaved, Soluble, Crosslinked Primary Isolate Env is Conformationally Altered After CD4 Binding

Previous studies have shown that upon binding of CD4, conformational changes occur and exposure of certain epitopes is enhanced; namely CD4 inducible (CD4i) epitopes. These structural changes also result in the exposure of the binding site for the coreceptor (152, 236). The ability to bind CD4 suggests Env folded as is found on the native virion. An increased ability of Env to bind the MAbs known as CD4i – 17b, 48d, 23e, A32 – indicates a change in epitope exposure. Deletion mutant experiments have demonstrated a significant involvement of the V2 loop for binding by these antibodies (289). The 48d epitope lies within a region between the 17b epitope and the V3 loop, partially overlapping both. Another antibody that recognizes a CD4-induced gp120 epitope overlapping the 48d epitope- 23e - competes with a sulfated N-terminal CCR5 peptide on gp120, but the epitope as yet has not been mapped (100). Studies have shown that neutralization is not predicted by MAb (17b) binding to monomer, but is associated with oligomeric Env binding; without CD4, 17b only bound monomer (105). A32 was included in this study because this MAb recognizes discontinuous epitopes that are sensitive to amino acid substitutions in several regions of gp120, but particularly the C1 and C4 domains (196) and the epitope might lie in proximity to the C1-C5 structure involved with gp41 association (195). The site is clearly distinct from the epitopes recognized by 48d and 17b. Though it was considered a CD4-induced epitope (195), this notion has changed (James Robinson, personal communication) and A32 actually serves as a conformation inducing MAb that mimics sCD4, and allows for enhanced binding of 17b, 48d and 23e.

CG10 is a unique MAb that reacts exclusively with the sCD4-gp120 complex, and not with sCD4 or gp120 alone (114). CG10 also competes with MAb 17b, binding near the conserved bridging sheet of gp120 (236). To determine whether soluble primary isolate oligomeric gp140s are able to bind CD4 and are able to undergo a conformational change when crosslinked, metabolically labeled oligomeric clade B Env coexpressed with furin was incubated with soluble CD4 (sCD4) and those MAbs that recognize epitopes available after Env binding to CD4. After immunoprecipitation with Sepharose G beads and elution, the Envs were resolved on a 10% SDS-PAGE gel. The results of these experiments for the clade B gp140 are displayed in **Table 6**, along with a representative autoradiograph of the clade B gp140 under these conditions (**Figure 20**). The percent increase immunoprecipitated Env in **Table 6** was determined by comparing the density of the band resulting from sCD4 and MAb incubation to the band resulting from MAb alone, coordinate to the results for the clade B results in Figure 20. There was a greater effect seen for MAb 48d, and the detectable increase for CG10 implies the CD4-gp120 complex was present. The decrease in precipitated Env for the polyclonal serum is consistent with competition for binding sites on gp120 between sCD4 and antibodies present in the polyclonal serum. The results indicate that there was a detectable increase in MAb binding with the addition of sCD4 for all MAbs, supporting the hypothesis that soluble, crosslinked gp140 Env is able to undergo conformational change after CD4 binding.

Additional experiments using preparatory amounts of Env were conducted to determine CD4 binding activity and reactivity with CD4i antibodies. To evaluate the reactivity of purified, soluble, oligomeric HIV-1 primary isolate Env proteins with

	-CD4	+CD4	
MAb	**Area of gp140/120**	**Area of gp140/120**	**% Increase**
CG10	336	565	68
17b	1067	1573	47
48d	496	768	55
R2143	3110	2821	(-9)

Table 6. Percent increase in immunoprecipitated Env after incubation with sCD4. Metabolically labeled, crosslinked clade B (HT593) gp140 coexpressed with furin, was purified by size exclusion chromatography, and the purified trimer fractions were immunoprecipitated with respective antibodies either with or without prior incubation with sCD4.

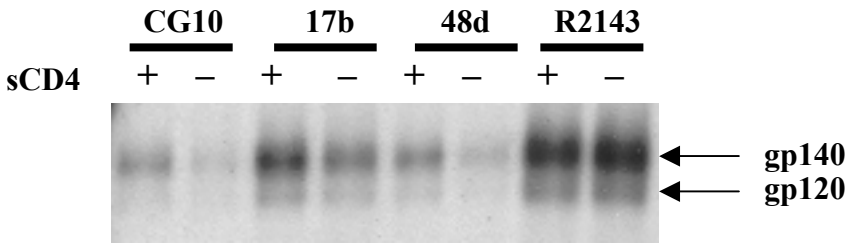


Figure 20. sCD4 binding induces conformational change of a purified, cross-linked oligomeric primary isolate Env. Metabolically labeled, crosslinked clade B (HT593) HIV-1 primary isolate gp140 coexpressed with furin was purified by size exclusion chromatography. The identified trimer fractions were pooled, incubated with or without soluble CD4, and immunoprecipitated with MAbs that recognize CD4 inducible epitopes.

different MAbs in the presence and absence of soluble CD4 (sCD4), size exclusion purified oligomeric gp140 Env was incubated with sCD4 overnight at 4°C, followed by incubation with MAb 17b, 48d, 23e or A32. Immunoprecipitated Env was resolved on 10% SDS-PAGE gels, Western blotted and probed with rabbit polyclonal serum raised to IIIB gp140 (R2143). In **Figure 21**, the results of the chemiluminescent visualization of Envs are shown. Interestingly, 17b and 48d bound three different gp140s (clades B, C and D) in the absence of sCD4, while at the same time the CD4i feature of 23e binding was retained. Also, in all cases, MAb A32 could substitute for sCD4 for 17b, 48d, and 23e binding. These results suggest that soluble gp140 can bind CD4 and is able to undergo conformational changes that expose previously obscured or partially hidden epitopes.

In summary, these results indicate primary isolate gp140s can be: readily produced; receptive to enhanced processing such that some retention of gp120 in oligomeric form is possible; processed and crosslinked and retain important structural features; uncleaved and retain the ability to bind CD4 and coreceptor.

Matrix III Encapsulation Studies

The purpose of these studies was to explore whether Matrix III may be suitable for the delivery of HIV-1 Env for the purposes of immunization. Matrix III may offer Env stability and release of antigen over a long period of time. The initial studies were performed using mice and lentil lectin purified HIV-1 89.6 gp120 Env to compare the effect of encapsulation on antibody elicitation, the contribution of RIBI adjuvant, and the immunogenicity of the beads alone. Three injections at 0, 31 and 51 days were done

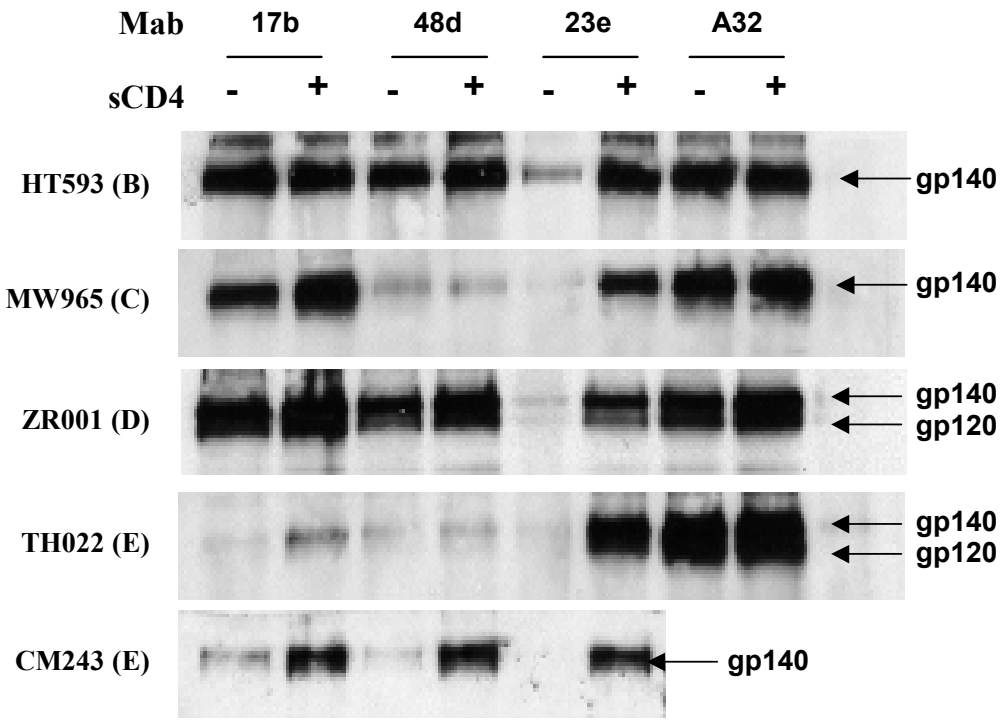


Figure 21. Immunoprecipitations of lentil lectin, size exclusion chromatography purified oligomeric Env with MAbs that recognize CD-4 inducible epitopes. Lentil lectin, size exclusion chromatography purified, oligomeric Env for each clade was incubated with or without sCD4 before immunoprecipitation with the respective antibody. The Env eluted from the Sepharose G beads was resolved on SDS-PAGE, Western blotted and probed with rabbit polyclonal serum raised to IIIB gp140.

intraperitoneally and subcutaneously. Sera from tail bleeds at 8 and 35 days after the second injection, and 8 days after the third injection were analyzed by ELISA. The ELISA is a standard antigen on plate followed by primary antibody absorption, with 25 ng of gp120 coated per well. Sera were diluted 1:5000, tested in duplicate for each of the mice and the absorbance values at 405 nm were averaged. Average value and standard deviation of each group were calculated using the results of the three mice from each group. The highest titers were generated by immunization with encapsulated gp120 (**Figure 22**), with the mice administered encapsulated antigen with RIBI adjuvant (Group II) producing sera that has a titer twice that of sera from mice that received encapsulated gp120 in PBS (Group III). Significantly, the ELISA results show that immunization of mice with the encapsulated gp120 in RIBI adjuvant (Group II) have yielded sera which is approximately 8 fold the titer compared to gp120 in RIBI adjuvant alone (Group I). Qualitative antibody analysis was attempted, but because of the high level of non-specific neutralization activity seen in these mice, rabbits were chosen for a follow-up experiment.

Experience has shown that the rabbit antibody response better predicts the antibody response in humans and non-human primates (26, 182) and evidence that the rabbit may serve as a useful model for studying HIV-1 infection and pathogenesis has also been demonstrated (255). In addition, the recommendation we have received from Dr. Montefiori (Duke University) has been to specifically avoid the use of mice in our immunization studies, primarily because of the variable and sometimes potent non-specific neutralizing activity seen even in the sera of control or unimmunized animals. An additional benefit is the increased volume of sera available, affording multiple

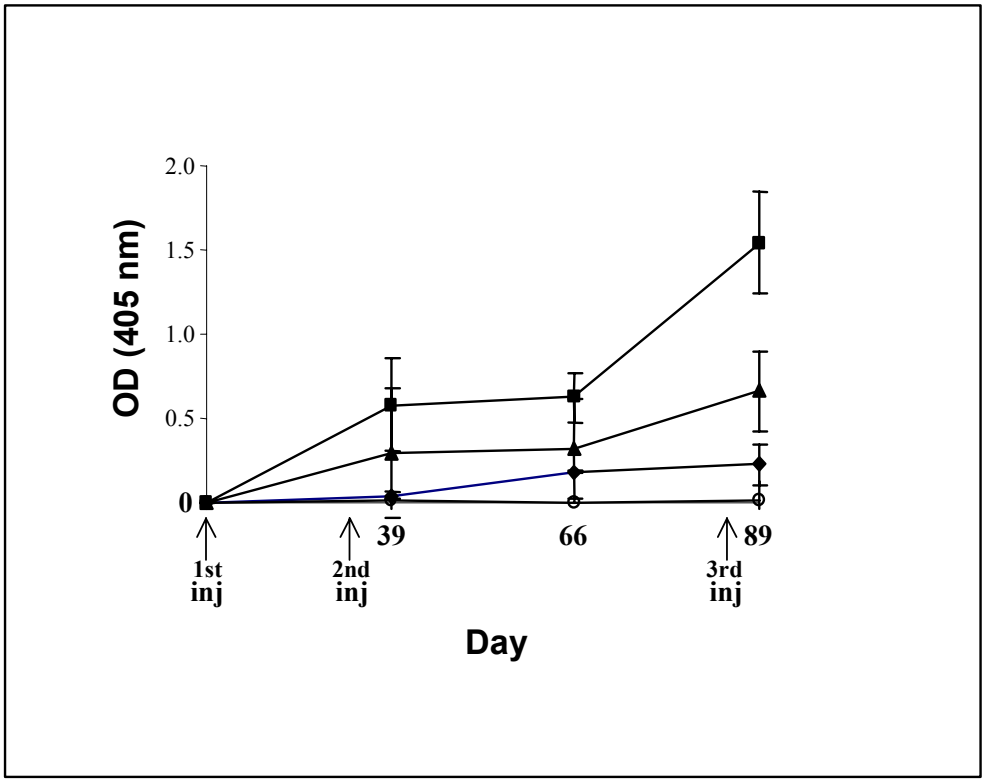


Figure 22. Generation of anti-HIV Env antibodies during immunization with encapsulated or non-encapsulated 89.6 gp120. Mouse sera was diluted 1:5000 and analyzed by ELISA against HIV-1 89.6 gp120. Average OD readings with standard deviation at each point are for three mice. Group I (♦) is 89.6 gp120 without encapsulation with RIBI adjuvant; Group II (■) is Matrix III/89.6 gp120 in RIBI adjuvant; Group III (▲) is Matrix III/89.6 gp120 in PBS; GroupIV (○) is Matrix III only in RIBI.

titrations and the ability to perform repetitive assays on multiple HIV-1 isolates for neutralization tests and ELISAs.

Briefly, the rabbit study entailed administering lentil lectin purified IIIB gp120 and gp140 in the context of Matrix III encapsulation. Two rabbits defined each group, and the groups received the following: 5 μg encapsulated IIIB gp140 (Group A); 30 μg of IIIB gp140, encapsulated (Group B) and not (Group D); 30 μg IIIB gp120, encapsulated (Group C) and not (Group E); or encapsulation material (beads) only (Group F). To compare the relative immunogenicity of gp120 and gp140, the rabbits were immunized four times with the respective Envs in RIBI adjuvant. To determine if there was a difference quantitatively between encapsulated vs. non-encapsulated antigen (HIV-1 IIIB gp120 and gp140) stimulation of antibody production in rabbits, ELISAs were performed on the sera obtained from rabbit crop bleeds taken over time. Endpoint ELISA titers using IIIB gp120 were determined in duplicate for each rabbit at each time point, translated to the logarithm in base 2, and the geometric mean with standard deviation was calculated per group. These results were plotted at various timepoints. Injections occurred on days 0, 56, 128 and 170. Results show that initially, 56 days after the first injection, the response was greater overall for the non-encapsulated forms of Env (**Figure 23**), implying a slower release of antigen for the encapsulated forms. Since gp120 was the capturing antigen in the ELISA, differences between the responses to the gp120 and gp140 immunogens were not due to the presence of gp41 in the gp140 immunogen. However, by the third injection, there were no significant differences in the antibody titers. Interestingly, the antibody titer elicited by the 5 μg of encapsulated gp140 was

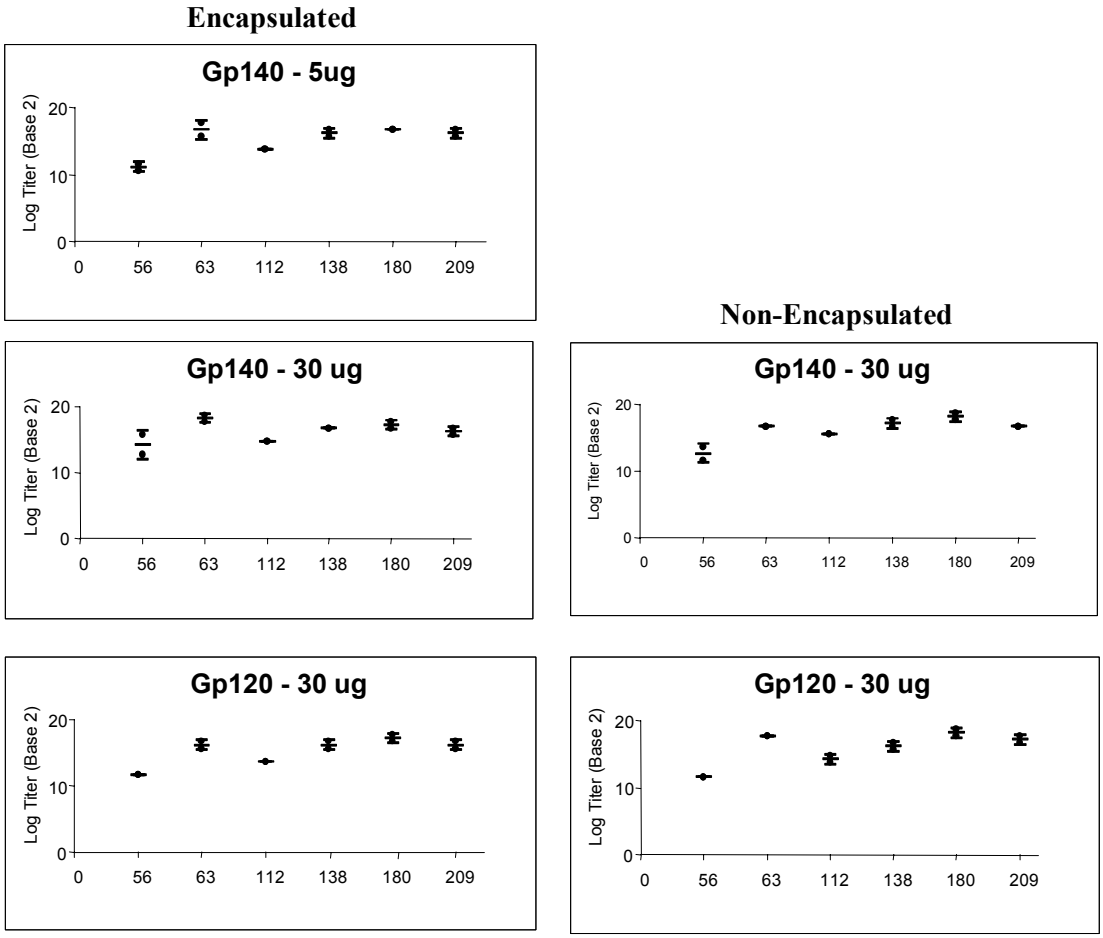


Figure 23. Comparative rabbit sera endpoint titers over time after immunization with encapsulated or non-encapsulated IIIB gp140 or 120. The geometric mean and standard deviation of the ELISA-derived log endpoint titers were calculated for each rabbit group of three and plotted for various timepoints numbered as days along the X-axis. Injections occurred at day 0, 56, 128 and 170.

equivalent to that generated by the 30 μg encapsulated and nonencapsulated forms of Env.

To determine if a qualitative difference existed between encapsulated vs. non-encapsulated antigen stimulation of antibody production, neutralization assays were performed. The initial neutralization assay was performed using rabbit sera from three time points (Days 0, 56, and 138) with SHIV-HXBc2 (IIIB) as the challenge virus in the MT-2 neutralization assay. Titers are the serum dilution at which 50% of cells were protected from virus-induced killing as measured by neutral red uptake. Fifty percent protection corresponds to approximately 90% reduction in $p27^{gag}$ antigen synthesis in this assay (39). Results of the initial neutralization assays (**Table 7**) performed by Dr. Montefiori's group demonstrated that, with the exception of the control animals injected with beads, all other animals developed low to moderate neutralization titer. The neutralizations assay was also attempted with HIV-1 MN, but the background activity of the prebleeds was so high that the results were non-interpretable. Because of nonspecific effects of whole sera, however, a repeat of the neutralization assays was performed using purified, concentrated IgG from the pre-bleed and terminal bleeds for only two rabbits was used to compare the neutralization response of rabbits immunized with encapsulated gp140 to non-encapsulated gp140. These neutralization titers displayed in **Table 8** as μg/ml of IgG needed for 50% neutralization of SHIV-HXBc2 indicate a better neutralization response using the non-encapsulated IIIB gp140 compared to encapsulated IIIB gp140. While this suggests Matrix III does not enhance the humoral response, it also indicates that Matrix III does not make an aberrant neutralization response.

		Titer at study day		
Animal no.	**Immunogen**	**0**	**56**	**138**
A1	5 μg enc-gp140	78	90	130
A2		42	44	64
B1	30 μg enc-gp140	<20	132	39
B2		119	68	92
C1	30 μg enc-gp120	<20	<20	184
C2		<20	<20	44
D1	30 μg gp140	<20	<20	226
D2		<20	<20	37
E1	30 μg gp120	<20	<20	82
E2		<20	<20	35

Table 7. Neutralizing antibody titers for rabbit sera from Matrix III study. Neutralizing antibodies generated by encapsulated or non-encapsulated IIIB gp140 or gp120 immunization of rabbits were titered by the MT-2 assay. Neutralizing antibody titers to SHIV-HXBc2 are given as the reciprocal serum dilution at which 50% of cells were protected from virus-induced killing. Immunizations were performed on days 0, 56, 128 and 170.

		Titer at study day	
Animal no.	**Immunogen**	**0**	**209**
B1	30 μg enc-gp140	594	280
D1	30 μg gp140	210	13

Table 8. Comparison of neutralizing antibody titers of purified, concentrated rabbit IgG from Matrix III study. The MT-2 assay was used to evaluate neutralizing antibody titer of purified, concentrated IgG from rabbits injected with encapsulated and non-encapsulated IIIB gp140. Neutralizing antibody titers are given as μg/ml of IgG needed for 50% neutralization of SHIV-HXBc2.

DISCUSSION

This thesis has focused on the development and characterization of soluble and secreted oligomeric forms of several envelope glycoproteins derived from primary isolates of HIV-1. My approach has been to express these genetic constructs using recombinant vaccinia virus technology, which has allowed for large-scale recombinant protein production. The results of this dissertation research are significant. First, the Env glycoprotein from several HIV-1 primary isolates of several different genetic clades have been identified that are proteolitically processed to varying degrees when expressed as soluble and secreted forms in the recombinant vaccinia virus system using a particular cell line. Further, we have been able to demonstrate for the first time that certain soluble primary isolate gp140 Envs can maintain their association as an oligomeric complex following cleavage. Second, we have shown that some HIV-1 primary isolate-derived gp140s can be 100% cleaved (processed) to its gp120/gp41 subunits by coexpression of recombinant furin. This secreted, cleaved Env can be stabilized with a chemical cross-linker in an apparent trimeric configuration as it exists in the membrane of the virus or infected cells, mimicking the native Env configuration. Though the methods used do not unequivocally prove that the secreted, processed, crosslinked Env is a trimer, we have shown that it is oligomeric, unlike the proposed oligomeric disulfide-lined SOS gp140 recently produced by others and described in more detail below (28). These chemically-crosslinked, oligomeric forms of HIV-1 primary isolate Envs can be purified from the monomeric subunits by size exclusion chromatography without aggregation. The chemical crosslinking process did not seem to have significantly interfered with or altered

Env conformation, as these oligomeric gp140s were recognized by potent neutralizing and conformation-dependent monoclonal antibodies to both gp120 and gp41. Furthermore, these purified Env oligomers were also recognized by CD4, the native receptor to which HIV-1 Env initially binds. More importantly, though crosslinked, these purified Env oligomers remain capable of conformational alterations upon CD4 binding. Third, these soluble gp140s can be produced in milligram amounts and the oligomeric forms can be separated from the monomeric forms, a necessity to test the HIV-1 primary isolate gp140 as an immunogen in future experiments. These facts are critical for investigating whether soluble HIV-1 primary isolate gp140 is capable of eliciting broadly cross-reactive, cross-neutralizing antibodies. Furthermore, these new materials will also make it possible to examine whether significant antigenic differences exist between the uncleaved and cleaved forms of oligomeric HIV-1 Env, which can now be addressed in future experiments. Finally, the results of the animal study have addressed the issue of using a novel adjuvant-like material (Matrix III) in conjunction with the HIV-1 Env immunogen. Though the immune sera elicited by the non-encapsulated gp140 had a greater neutralizing titer to the homologous isolate (i.e. IIIB) than the encapsulated form, there were no significant differences in anti-Env titer as a result of using Matrix III. However, the finding that equivalent titers are elicited by reduced amounts of encapsulated antigen would be important for cost effective and time saving measures. These findings are individually addressed below.

An effective vaccine should elicit potent, cross-clade neutralizing antibodies to a number of epitopes. Extensive variability of the HIV-1 Env protein, evidenced by the growing number of subtypes and recombinant forms, presents a major obstacle in

designing an appropriate immunogen. Current subunit vaccine strategies remain focused on recombinant, monomeric forms of the HIV-1 envelope glycoprotein subunit 120 (gp120) (**Figure 24)**.

Clinical trials to date indicate this approach has failed to generate broadly cross-reactive neutralizing antibodies (20, 64, 277). Sera from gp120 vaccinees react preferentially with denatured gp120 and primarily contain a predominance of antibody reactive to the V3 loop, antibodies ineffective in neutralizing diverse isolates (184). The elicitation of such restricted neutralizing antibodies may be explained by the differences in epitope exposure between monomeric and oligomeric Env. Many MAbs that recognize exposed epitopes on monomeric gp120 bind poorly to native oligomeric Env (195). Conversely, binding of MAbs to oligomeric but not monomeric Env has been correlated to neutralization (219, 253). Indeed, sera from HIV-1 infected individuals do contain cross-reactive antibodies that are directed against discontinuous epitopes that would only be found on native oligomeric Env (189). Oligomeric gp140 Envs also contain the gp41 ectodomain, which contains at least two important epitopes recognized by potent neutralizing antibodies, 2F5 and MAb Z13 (304); monomeric Env is comprised of only gp120. Therefore, soluble and secreted primary isolate-derived Envs may display a variety of epitopes that could prove critical in eliciting antibodies capable of neutralizing primary HIV-1 isolates.

Though it has been shown that all the Envs chosen for this study were functionally competent, potentially important amino acid variations exist in some of the clones which may have an impact upon the Env structure. 92HT593.1(B) contains an unusually long V1 loop (111). The clade E HIV-1 isolates contain an extra cysteine pair in V4, which

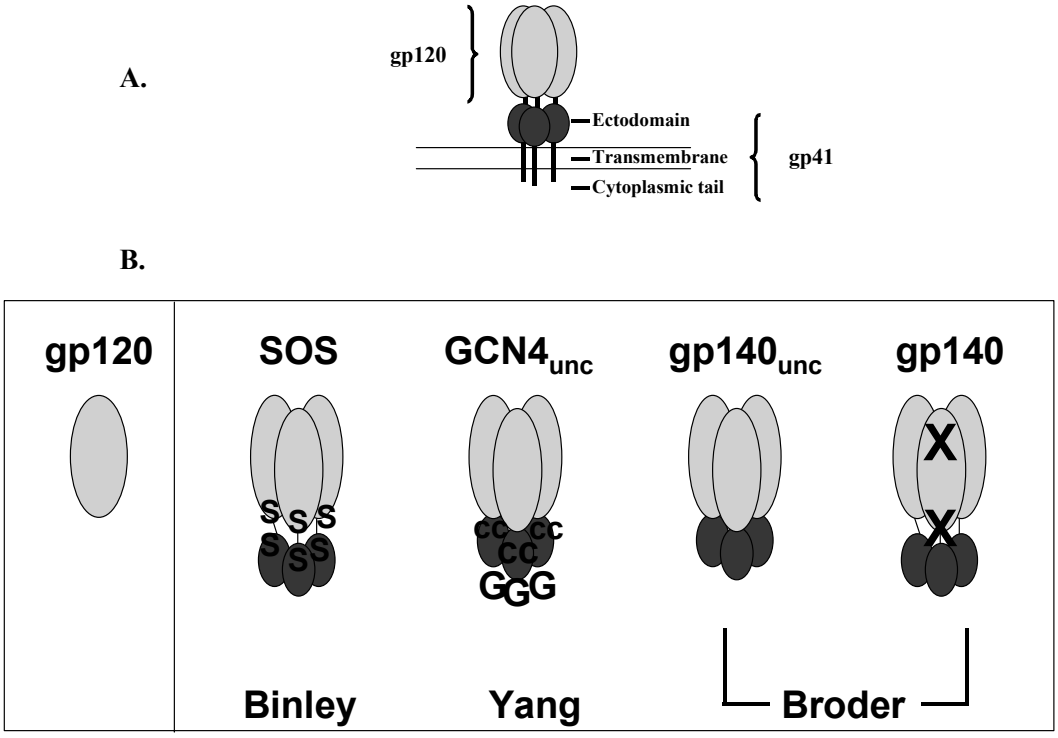


Figure 24. Illustrations of HIV-1 Env forms. A) HIV-1 full length Env in the context of expression in the cell membrane. Env is cleaved and oligomeric, with gp120 and the ectodomain of gp41 extending outside the cell membrane. B) Illustrations of Env in experimental and clinical evaluation. Monomeric gp120 has been the only Env form evaluated in clinical vaccine trials. Other Env forms containing gp120 and the ectodomain of gp41- cleaved and uncleaved, with and without mutations or crosslinking (X) - are in the early stages of experimental evaluation as vaccine candidates.

creates a unique structure of the V4 loop not found in any of the six African isolates studied in 1996 (177). 93MW965.26 (C) contains unpaired cysteine residues that are expected to give rise to an inappropriately folded glycoprotein. However, infectivity assays and fusion assays done by our group and others demonstrate levels comparable to positive controls (46, 111). Therefore, it is not surprising to find variability in results for natural processing and responsiveness to enhanced processing among the various clades.

More recent evidence in support of a native trimeric configuration comes from analyses of an HIV-1 primary isolate gp140 and an HIV-1/SIV gp 140 chimera (298) as well as from studies of SIV Env oligomers derived from both virions and recombinant SIV gp140 constructs (44, 50). Zhang and colleagues produced soluble, trimeric HIV Env gp140 and a chimeric HIV gp120/SIV gp41 ectodomain using expression plasmids in mammalian cells (298). These recombinant proteins were found to maintain structural fidelity equivalent to that of the virion Env as suggested by gel filtration chromatography, analytical ultracentrifugation, sCD4 binding and MAb reactivity analysis. SIV is a member of the same family of retroviruses (Lentiviruses) as HIV and causes AIDS-like symptoms in rhesus macaques. The SIV Env is synthesized, glycosylated, undergoes oligomerization, and is cleaved into gp120 and gp41 subunits that remain non-covalently associated analogous to the HIV-1 Env. The SIV Env shares an approximate 25% sequence homology with gp160 from HIV-1, as well as a gp41 ectodomain with the trimeric structure analogous to that of HIV-1 (171). Purified, soluble SIV envelope glycoprotein has been shown to be trimeric by chemical cross-linking and gel filtration chromatography analysis and forms tight complexes with CD4 and a number of neutralizing antibodies (50). Analysis of gel filtration fractions by chemical crosslinking,

sedimentation equilibrium, and scanning transmission electron microscopy have established that both soluble recombinant vaccinia virus expressed gp140 and virion-associated forms of SIV Env are trimeric (44). The gp140 Env molecules were visualized as triangular or tri-lobed structures, indicating that the determinants of trimeric structure reside within the ectodomain - as they do for HIV-1 Env - and are not negated by loss of membrane anchoring during folding and oligomerization within the ER. This evidence supports our findings that a portion of soluble, oligomeric HIV-1 gp140 produced by the rvv system is likely trimeric as well.

The fusion-competent form of native HIV-1 Env expressed on a virion is cleaved. Creating an immunogenic mimic of this form requires the production of a stable, trimeric, cleaved glycoprotein. Recently conducted experiments have attempted to stabilize soluble, cleaved gp140 Envs to demonstrate that the lability of the non-covalent bonds between gp120 and gp41 can be overcome (**Figure 24**). Binley and associates have addressed this issue by introducing a disulfide bond pair between gp120 and the gp41 ectodomain while maintaining the natural cleavage site of clade B HIV-1 truncated Envs (28). This Env, known as SOS gp140, was expressed as a A501C/T605C double cysteine mutant. Only partial disulfide bond formation was observed in these SOS constructs, though antigenic recognition was maintained. However, recent findings presented at the Keystone Symposium (March, 2001) have cast some doubt on this approach of using disulfide-linked HIV-1 gp140 as an antigen, as the disulfide-linked oligomer has failed to elicit neutralizing antibodies in rabbits. In addition, upon further analysis of the SOS gp140, the purified protein used for the immunogen study was found to actually be monomeric, in contrast to non-purified Env preparations, which presented as an oligomer.

Here we have been successful in producing soluble, cleaved, stable oligomeric gp140 from a variety of HIV-1 clades. This Env is recognized by important neutralizing human MAbs, as well as MAbs that recognize conformation dependent epitopes in both gp120 and gp41. We have also demonstrated CD4 binding to these Env forms, and induced conformational changes of crosslinked material. Although we have been successful in enhancing cleavage of gp140 by coexpression with furin, and recovering more cleaved oligomeric material with the use of a chemical crosslinker, this has only been done using small amounts of metabolically labeled Env. This method requires testing of preparatory amounts of Env. This issue of enhanced cleavage and chemical crosslinking for large preparations is addressed in Future Directions.

Immunization with oligomeric HIV-1 Env preparations has been shown to generate antibodies that preferentially recognize oligomeric Env (34, 86) and similar findings have been reported for immunizations with oligomeric SIV (91). Recently, experiments with soluble, uncleaved gp140 trimers of a TCLA strain of HIV-1 have demonstrated these oligomers to be more effective than gp120 at eliciting neutralizing antibodies (NAbs) in rhesus macaques. The NAbs elicited by the soluble, uncleaved oligomers are qualitatively as well as quantitatively superior to the NAbs elicited by gp120 when tested against the homologous strain of virus. However, neither neutralization of primary isolates nor robust neutralization against heterologous strains was seen when the sera of animals injected with the soluble, uncleaved TCLA gp140 oligomers was tested, although significant protection against a homologous SHIV challenge was seen (90). Similar results in humoral response were evident when Yang and colleagues tested soluble, uncleaved, stabilized oligomers of an HIV-1 primary isolate (**Figure 24**). They chose to

disrupt the cleavage sites in the truncated *env*s of HIV-1 TCLA strain HXBc2 and a clade B HIV-1 R5 primary isolate, YU2, extend the heptad repeat region of gp41 with the addition of GCN4 sequence, and insert an additional cysteine pair in the gp41 coiled-coil, in an attempt to stabilize the oligomers. They found it necessary to prevent cleavage because, even with these additional mutations to enhance monomer interaction, they also observed cleaved proteins to be monomeric (293, 294). Mouse studies were performed using this form of gp140 as the immunogen, but results indicate minimal neutralizing activity against another clade B HIV-1 primary isolate with antibodies elicited by the YU2 Env (295).

Taken together, these studies have indicated the importance of continued investigation of the oligomeric, antigenic structure of Env from primary isolates from a variety of clades, with the overall goal of developing an effective Env-based HIV-1 subunit vaccine. Our studies have addressed this issue by characterizing the processing, oligomeric nature and antigenicity of a variety of HIV-1 gp140 Envs from several alternative clades. A similar approach was developed by others using the Semliki Forest Virus expression system to express gp140, but only three clade B HIV-1 primary isolates of unknown coreceptor usage were examined by one group (30) and primary isolate representatives from each of HIV-1 clades A, B and E were investigated by the other group (267). We have characterized representatives from clades A, B, C, D, and E and have made preparative amounts of soluble oligomeric gp140 from each clade suitable for large animal studies.

Future Directions

Testing the immunogenicity of the oligomeric soluble Envs from different clades in a polyvalent vaccine is beyond the scope of this dissertation project, but the work described here has allowed this concept to be tested; these studies are now in progress in our laboratory. The Envs will be tested individually and in two groups - clades A, C, D and B, C, E – to mimic the distribution of clades prominent in HIV-1 infections in Africa and Asia, respectively. These Envs are concurrently being tested in rabbits with an adjuvant that is in clinical trials, namely QS-21. QS-21 (supplied in collaboration with Antigenics, Inc., New York) is a natural product, a triterpene glycoside (or "saponin") purified from the bark of a South American tree called Quillaja saponaria. QS-21 has been tested in over 2,500 patients in 50 clinical trials and has proven to be far more effective in stimulating antibody responses than aluminum hydroxide or aluminum phosphate, the only adjuvants in use in approved vaccines in the U.S. today (143). Up to 10% of the bark from Quillaja is composed of saponins, of which QS-21 is typically one of the more predominant (142). QS-21 is well characterized with a known molecular structure, thus distinguishing it from other adjuvant candidates, which are typically emulsion, polymers or biologicals (143). This adjuvant has also been used successfully in HIV-1 DNA and subunit vaccine studies, enhancing humoral and cell-mediated response (99, 252). Results from the polyvalent experiment using this adjuvant will determine the ability of multiple HIV-1 primary isolate gp140 Envs to induce cross-reactive, neutralizing antibodies to HIV-1 primary isolates.

There are other possible improvements to the gp140 immunogen that can be addressed to achieve broader and more potent neutralization, particularly with respect to

divergent primary isolates. These improvements include: processed or cleaved oligomers; elimination of potential N-linked glycosylation sites; deletion of variable loops; gp140s from more clinically relevant, primary isolates or immunotypes; and Env-CD4-coreceptor complexes. Though this dissertation research has demonstrated that soluble HIV-1 Env that is coexpressed with a recombinant vaccinia virus expressing furin can be processed 100% to subunits gp120 and gp41 ectodomain, this was only done with small amounts of metabolically labeled Env. Size exclusion chromatography of a large-scale preparation of crosslinked and non-crosslinked Env coexpressed with furin had been attempted once using the clade D HIV-1 gp140. This experiment was unsuccessful in recovering cleaved, crosslinked material as verified by Western analysis. However, more experiments need to be done to ensure the validity of these results.

Evaluation of glycosylation and variable loop contribution to epitope masking on oligomeric Env will be important in determining if simple changes can be made to enhance cross-reactive neutralizing antibody production. The study of HIV-1 Env gp140 in our laboratory has been extended to include the generation and characterization of a specific set of N-linked glycosylation site mutants in the context of oligomeric primary isolate clade E gp140 Env. Though HIV-1 gp120 has approximately 24 to 30 N-linked glycosylation sites, the gp41 glycoprotein has four highly conserved potential N-linked glycosylation sites (89). This glycosylation of Env has been postulated to play a role in shielding the virus from antibody responses (13, 21, 29, 103, 130). Because there have been many studies evaluating the effects of glycosylation site removal in gp120, our laboratory has focused on four mutations within the gp41 ectodomain. These mutants have been made in the clade E primary isolate (CM243) gp140 generated as part of this thesis

dissertation. Preliminary studies indicate that oligomeric specific MAbs that map to cluster I have better reactivity with two of the glycosylation mutants, suggesting these mutations expose a previously hidden domain. Preliminary ELISA data with rabbit sera raised to these mutants exhibit reactivity with several HIV-1 primary isolates across clades. However, neutralization data not yet generated will specifically address the immunogenicity of these mutants.

Another approach involves deletions of variable loops V1 and V2 in conjunction with the removal of a relevant glycosylation site in gp120 of a clade E HIV-1 primary isolate gp140 that was constructed as part of this thesis dissertation. This was done to determine whether the variable loop structures play a role in masking any antigenic or immunogenic epitopes in native gp140 oligomer. The glycosylation sites deleted within the combination mutant, whether due to removal of a variable loop or site directed mutagenesis, were those found to be deleted in the novel CD4-independent mutant of HIV-1 IIIB (8x) (153). These mutant Envs will be characterized for their antigenic and oligomeric nature, as well as immunogenic potential in rabbits.

A series of studies using the SF162 HIV-1 R5 isolate has been performed with V1 and/or V2 loop deletions (264). The V2 loop delete version of SF162 has also been prepared as a soluble gp140 construct (263). However, most of the later studies with these constructs have been performed only via DNA immunizations (17). Thus far, data from these studies indicate that V2-loop deleted gp140 constructs do show improvement in elicitation of neturalizing antibodies. But again, boosting of animals (monkeys) with purified proteins was needed to raise serum antibody levels. It should be noted however,

that there have been no published data on the biochemical analysis of these V-loop deleted gp140s following purification.

Most of the antigenic and structural information available on HIV-1 Envs has been obtained by immunochemical studies using soluble subunit proteins (188, 194). The study of the antigenic conservation of epitopes shared among intact virions of different HIV-1 clades has been used to identify regions that are well exposed on the surfaces of these viruses. Similarities exist among isolates of different clades of HIV-1 conferred by both sequence and conformational homologies, irrespective of their genetic subtypes or phenotypes (206). Studies using soluble gp120 monomers have led to the conclusion that the conserved C5 region of HIV-1 is buried (194, 195). However, data from virus binding ELISAs, in which the native gp120 structure is presented, yielded results suggesting the C5 region is well exposed on the surfaces of viruses from clades A, B and D (206). In addition, binding studies using anti-V3 MAbs resulted in cross-reactive virion binding across several clades, suggesting antigenic similarity (206). There is also growing evidence that the role of conformational dependent anti-V3 antibodies in protective immunity may be more important than previously thought (217, 257, 300). As the next step in developing a global HIV-1 vaccine, defining immunologic subgroups of HIV-1, in contrast to the genetic classification currently available, will be relevant. Analysis of the nucleotide sequences has not been useful in the development of serotype vaccines for other microorganisms, such as *S. pneumoniae* and the polio virus. Likewise, this approach has not defined any immunologically related groups among HIV-1 (150, 187, 208). Mathematical analysis of serologic ELISA data from HIV-1 infected persons have identified 5 to 9 serologic groups, suggesting clades A and C present conserved antigenic

properties, while clade D is quite divergent (15). Another group, led by Dr. G. van der Groen, identified immunologically related groups using spectral map analysis of neutralization titers (208). Spectral map analysis is defined as principal-component analysis of a logarithmic and double-centered data table. This multivariate statistical method of data analysis had been used previously to study the interaction between drugs and receptors and between viruses and antivial compounds. The goal was to identify sera and HIV-1 isolate neutralization serotypes and to study how they were correlated to genetic subtypes. This study strongly suggests that genetic subtypes do not correlate with neutralization clusters. However, since these cluster analyses were based on data generated with polyclonal sera, the conserved epitopes characterizing each serotype could not be interpreted. Recently, the same method of multivariate analysis of binding data generated by ELISA with anti-HIV-1 human MAbs and intact native HIV-1 primary isolates of Group M, revealed three immunotypes of HIV-1 and five MAb groups useful for immunotyping HIV-1 (207). This study revealed that there are fewer immunotypes than genotypes of HIV-1, and that clustering of the isolates did not correlate with genotypes, coreceptor usage, or geographic origin of the isolates.

I have described the construction and evaluation of several primary isolate gp140s that may potentially serve as a kind of immunotype, since two to three of these gp140 Envs appear to have pre-exposure of multiple CD4i epitopes. Making constructs expressing soluble Env gp140s from the primary isolates defined by the immunotype study that have the common antigenic epitopes could be useful for selecting the minimum number of HIV-1 strains to be combined into a polyvalent vaccine. However, developing Envs with features such as pre-exposed epitopes (eg. CD4i epitopes) with the belief that

these will be beneficial remains to be formerly tested. Recently such assumptions have been called into question from studies of matched molecular clones where multiple methods were used to demonstrate that there is equal binding of MAbs to neutralization-resistant primary isolate viruses and their neutralization-sensitive TCLA virus derivatives (296). These findings stand in apparent contradiction to the current model that suggests that differences in neutralization sensitivity of primary isolate and TCLA viruses reflect differences in the ability of antibodies to bind to the respective Env complexes. Although it is true that among TCLA viruses and their neutralizing antibodies, occupancy of sites on the virion determines neutralization, an extrapolation of these findings to primary isolates is questionable (219, 296). These recent findings offer structural and mechanistic differences between the functioning of TCLA and primary isolate Env binding, fusion and entry as speculative support, with proof as yet to be demonstrated by future studies.

Our laboratory has recently provided evidence that supports the hypothesis that the first step in the virus entry process is the formation of a trimolecular complex composed of the HIV-1 Env, CD4 and a coreceptor molecule, and that membrane fusion could be correlated to the formation of such a complex. We have also demonstrated a constitutive cell surface association between CD4 and CCR5. Upon interaction of these molecules, conformation changes that take place may expose new epitopes not previously exposed in the trimolecular complex, and neutralizing antibodies specific for this 'fusion-competent' intermediate could potentially be elicited. The "fusion competent vaccine" proof-of-principle was suggested by LaCasse and colleagues (154) using mixtures of chemically fixed HIV-1 Env-expressing and receptor-expressing cells undergoing membrane fusion in CD4-CCR5 transgenic mice. The polyclonal antibody responses generated from these

mixtures were broadly cross-reactive to a variety of HIV-1 primary isolates. Presently the development and evaluation of soluble HIV-1 Env-CD4-coreceptor complexes as immunogens in CD4-CCR5 transgenic mice is underway in our laboratory as the next step in potentially uncovering hidden immunogenic epitopes in soluble, oligomeric Env in the context of a 'fusion competent' complex.

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