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STRUCTURAL AND FUNCTIONAL STUDIES OF MEMBRANE-SPANNING DOMAIN OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 ENVELOPE GLYCOPROTEIN IN VIRAL REPLICATION

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By

LIANG SHANG
M.Sc., Fudan University, 2003

Advisor: **ERIC HUNTER**, Ph.D.

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ABSTRACT

STRUCTURAL AND FUNCTIONAL STUDIES OF THE MEMBRANE-SPANNING DOMAIN OF HUMAN IMMUNODEFICIENCY VIRUS TYPE I ENVELOPE GLYCOPROTEIN IN VIRAL REPLICATION

LIANG SHANG

The membrane-spanning domain (MSD) of the human immunodeficiency viruses type I (HIV-1) envelope glycoprotein (Env) is critical for its biological activities. In this dissertation, genetic approaches are used to study the structural and functional roles of the MSD in viral replication. By examining a series of C-terminal truncation mutants of HIV-1 gp41, we show that the entire MSD is required for normal Env incorporation and viral infection. In contrast, a region of 17 residues (K681 to A698) is sufficient to stably anchor the protein in the membrane, allow efficient transport to the plasma membrane, and mediate WT levels of cell-cell fusion. Truncation to the residues that are N-terminal to F697 resulted in an Env complex that was secreted from the cells. Based on the analysis of these mutants, a “snorkeling” model is proposed for the structure of HIV-1 MSD. In this model, the 12 residues (L682-L693) form an intramembrane helical “core” and the flanking residues K681 and R694 are buried in the lipid while their side chains interact with polar head groups. In order to understand roles of the highly conserved hydrophobic residues and glycine motifs in the MSD “core”, we constructed recovery-of-function mutants by initially replacing the “core” with 12 leucine residues and then reintroducing the specific residues. We show here that conservation of the MSD core sequence is not required for normal expression, processing, intracellular transport, and viral incorporation of HIV-1 Env. However, specific residues in the MSD do play a critical role in the Env-mediated virus-cell fusion and subsequent viral infection by modulating conformational integrity of the Env ectodomain. Substitutions in the MSD “core” result in several minor and localized conformational changes in gp120 and the gp41 ectodomain. These conformational changes impair initiation of the early events that are required for formation of the 6-helix bundle and subsequent fusion pores; while they does not influence the specific virus-cell attachment, nor the recognition of HIV-1 Env to the CD4 receptor and CXCR4 coreceptor.

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DEDICATION

I dedicate this thesis to my mom, Yunxia Yang, my dad, Zhangtao Shang, and my sister, Yang Shang, for their love and support of so many years.

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INTRODUCTION

Enveloped Viruses and Envelope Glycoproteins (Env)

Enveloped Viruses. Enveloped viruses are viruses that have viral envelopes covering their core structures that are mainly composed of nucleocapsids. These viruses include both the DNA and RNA virus, including flaviviruses, togaviruses, retroviruses, coronaviruses, filoviruses, rhabdoviruses, bunyaviruses, orthomyxoviruses, paramyxoviruses, arenaviruses, hepadnaviruses, herpesviruses, baculoviruses, and poxviruses. Viral envelopes are made up of lipid bilayers and are typically derived from host cellular membrane, either plasma membrane or intracellular membranes.

Envelope Glycoproteins. Virus-specific transmembrane glycoproteins, which are encoded by viral *env* genes, are incorporated in the envelopes during viral assembly. Structurally, most viral envelope glycoproteins are the type I integral membrane protein, with a single transmembrane α-helix linking the ectodomain and the cytoplasmic domain tail. In some positive-strand RNA viruses, such as the flaviviruses, where structural proteins derive from a polyprotein precursor, the transmembrane region may be a α-helix hairpin that traverses the membrane twice. Viral envelope glycoproteins (Envs) must carry out at least two functions: binding to receptors/coreceptors on the surface of target cells and mediating the fusion between the viral envelope and the cellular membrane. The extracellular domains of viral Env, which also tends to be the most glycosylated region and major antigenic domain of the Env, typically performs both functions. The membrane-spanning domain (MSD) of viral Env is usually responsible for anchoring the entire glycoprotein in the viral envelope and may be involved in the oligomerization of Env monomers. Most viral envelope glycoproteins have a small cytoplasmic domain; however, some retroviruses, such as Human and Simian Immunodeficiency Virus (HIV and SIV), have a long cytoplasmic domain of approximate

150 amino acid residues. The long cytoplasmic domain interacts with capsid proteins during viral assembly and usually contains signals for the intracellular trafficking of the Env. The enveloped viruses and their envelope glycoproteins are summarized in Table 1. In addition to the virus-specific envelope glycoproteins, viral envelopes also contain some nonspecific host membrane proteins, such as the MHC molecules and integrins (238).

Types of Envelope Glycoproteins. The viral envelope glycoproteins can be categorized based on either **(I)** the number of membrane-spanning domains and their orientations (Fig. 1) (138), or **(II)** the mechanisms by which viral Envs mediate membrane fusion.

(I) Translation of the Env mRNA takes place on the surface of the ER membrane. The N-terminal signal peptides of envelope glycoproteins introduce nascent peptides to a ribonucleoprotein complex known as the signal recognition particle (SRP), where the translation continues and is coupled with the translocation of polypeptides across the membrane into the ER lumen. The number of membrane-spanning domains in an Env protein is determined by the number of stop-transfer, membrane-anchor regions. Most viral envelope glycoproteins contain one stop-transfer, membrane-anchor region, which is generally located towards the C-terminus of the protein. The translocation process of such proteins results in Envs of a Type I orientation with a relatively long N-terminal region in the ER lumen, one transmembrane domain, and a relatively short C-terminal tail in the cytosol. In some viral envelope proteins, such as the influenza virus neuraminidase, a signal peptide at the N-terminus, which is not cleaved, also functions as the membrane anchor with the sequences C-terminal to it are translocated into the ER lumen. The product of this process is an Env of a Type II orientation,

with a long C-terminal region in ER lumen, a transmembrane domain, and a relatively short N-terminal region in the cytosol. The envelope glycoprotein of a Type III orientation possesses multiple hydrophobic sequences that are recognized as stop-transfer signals, which result in a multiple membrane-spanning domain.

(II) One of the basic functions of envelope glycoproteins is to mediate the virus-cell membrane fusion. As to date, three different classes of viral fusion proteins have been revealed (Fig. 2), based on their structures and mechanisms of mediating membrane fusion (see the section " Viral Membrane Fusion" for more discussion).

Table 1. The Enveloped Viruses and Their Envelope Glycoproteins

Family/Virus	Env	Receptor/coreceptor	Orientation	Fusion
Flaviviridae				
TBEV	E	Laminin receptor	I	II
DENV	E	DC-SIGN, Bip (144, 294, 308)	I	II
JEV	E	HSP70 (72)	I	II
YFV	E	Lectin-independent (13)	I	II
WNV	E	DC-SIGNR, integrin (61, 73)	I	II
HCV	E1, E2	CD81, SR-B1, LDLr (4, 16, 187, 254)	I	II
Togaviridae				
Alphavirus				
Semliki Forest Virus	E1, E2	MHC (5, 128)	I	II
Sindbis	E1, E2	Laminin receptor(325)	I	II
Coronaviridae				
SARS-CoV	M, E, S, HE	ACE2 (185)	III I	 I
Rhabdoviridae				
Rabies Virus	G	NACR, CD56, $p75^{NTR}$ (108, 150, 311, 315)	I	-
VSV	G	Negative charged lipid	I	III
Filoviridae				
Marburg Virus	GP	AsialoGP receptor (18)	I	I
Ebola Virus	GP	Integrin (304)	I	I
Paramyxoviridae				
Mumps Virus	F, HN, SH	Sialic acid	I, II II	I
SV5	F, HN, SH	Sialic acid	I, II II	I
NDV	F, HN	Sialic acid	I, II	I
Parainfluenza Virus	F, HN	Sialic acid	I, II	I
Sendai Virus	F, HN	Sialic acid	I, II	I
Measles Virus	F, H	Sialic acid, CD150 (232)	I, II	I
HRSV	F, G, SH	Heparan sulfate	I, II II	I I
Orthomyxoviridae				
Influenza Virus	HA NA	Sialic acid	I II	I
Bunyaviridae				
Hantavirus	Gn Gc	Integrin β1, β3 (109)	I I	II
Arenaviridae				
LCMV	GPC	αDG (43)	III	I
Lassa Virus	GPC	αDG (168)	III	I
Retroviridae				
ALSV	Env	LDLR-like (17)	I	I
MPMV	Env	B^0 (123, 266)	I	I
MuLV	Env	mCAT-1 (156)	I	I
FeLV	Env	D-glucarate transport like (262)	I	I
HTLV	Env	GLUT-1 (199)	I	I
HIV-1	Env	CD4, CXCR4, CCR5	I	I
SIV	Env	CD4, CXCR4, CCR5	I	I

Table 1. The Enveloped Viruses and Their Envelope Glycoproteins (Continued)

Family/Virus	Env	Receptor/coreceptor	Orientation	Fusion
Herpesviridae				
Herpes Simplex Virus	gC, gB, gD, gH, gL	GAG, Nectin, HVEM, 3-OS HS (65, 218, 294)	-	III
Varicella-Zoster Virus	gB, gC, gE, gD, gH, gL	Heparan Sulfate, IDE, MPR^{ci} (54, 140, 184)	-	-
HCMV	gB, gH:gL, gM:gN	Heparan Sulfate	-	-
EBV	gp350/220	CD21, HLA-II (306)	-	III
KSHV	K8.1, gB, gH, gL	Heparan Sulfate, Integrin $\alpha 1\beta 3$, xCT (5, 6, 150)	-	-
Hepadnaviridae				
HBV	L, M, S	-	III	-

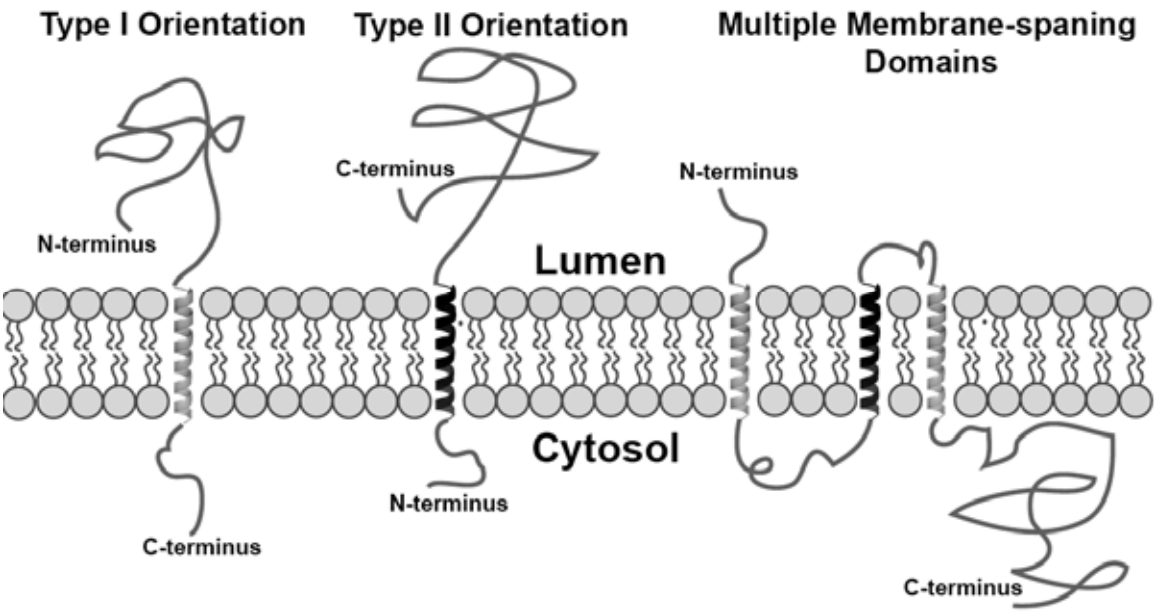

Fig. 1 The orientation of viral envelope glycoproteins in lipid bilayer.

Membrane Fusion and Fusion Mediated by Viral Envelope Glycoproteins.

Membrane fusion is a process of merging two separate lipid bilayers to become one, in which the original asymmetry of lipid components is maintained. It is essential for the communication between membrane-delineated compartments in all eukaryotic cells (201). Many cellular processes require membrane fusion events including: the transport of intracellular vesicles to target membranes in the secretory pathway, merge between late endosomes and lysosomes, fusion of mitochondria during its development, cell-cell fusion events which are critical for fertilization, development and immune responses, and entry of enveloped viruses into target cells (201, 341). Our current knowledge of the molecular mechanism involved in membrane fusion derives from studies in three areas. First, the protein-free physical models of lipid bilayers described, in detail, the process at a molecular level. Then the structural and mechanistic insights obtained from the investigation of viral fusion proteins revealed the critical functions of proteins during lipid rearrangement. Moreover, the study of fusion events in eukaryotic cells, mainly the SNARE proteins system, uncovered the regulatory mechanisms of cellular membrane merging.

Rearrangement of Lipid Bilayers. Our current understanding of merging of two lipid bilayers has been modeled in a fusion-through-hemifusion pathway summarized in Fig. 3A. Membrane fusion is a spontaneous process; however, it has to overcome a high energy barrier without certain artificial conditions (123). For the initiation of membrane fusion, two lipid bilayers must be in close contact to each other. But even a long-term contact is not sufficient for initiation of membrane fusion. In order for lipid mixing to occur, membranes need some distortions in their local structures, for example point-like protrusions. In a protein-free lipid system, such a structure forms through spontaneous curvature of the membrane monolayer and is regulated by its lipid composition (58). Lipids such as lysophosphatidylcholine (LPC) and polyphosphoinositides tend to self-

assemble into curved monolayers bulging to polar heads, while the monolayers formed by unsaturated lipids such as phosphatidylethanolamine and diacylglycerol tend to bulge in the opposite direction. Following the lipid mixing of the outer monolayers of the membrane, the fusion process presents a hemifusion intermediate, in which two inner monolayers are combined into a lipid bilayer structure. Further membrane-disruptive forces break this temporary structure and result in a small fusion pore between the two lipid bilayers. The fusion of lipid compartments continues as the fusion pore expands until the stress forces that drive the expansion disappear.

Hemifusion. The hemifusion step allows membrane fusion to proceed without disrupting the barrier function of the membrane and also prevents contents from leaking (Fig. 3A). The hemifusion intermediate in eukaryotic cells was initially identified in the study of influenza virus HA-mediated cell-cell fusion. Recent reports suggest that SNARE-mediated membrane fusion also occurs via a hemifusion stage following the two-membrane-spanning protein intermediate (194). The hemifusion intermediate to full membrane fusion is detected as lipid mixing in the absence of content mixing. Hemifusion connections are reversible structures and are probably stabilized by protein scaffolds.

Fusion Proteins. Although membrane fusion can occur as a spontaneous process, all biological fusion events are mediated by fusion proteins, such as viral envelope glycoproteins and vesicular SNARE proteins. The main role of fusion proteins is presumably to generate and control the membrane elastic stresses, helping overcome the high energy barrier between the prefusion stage and the postfusion stage of membrane fusion (57). When activated, fusion proteins undergo structural rearrangement, which brings adjacent membranes together to form a much closer contact and also generates stresses by curving membrane monolayers. Fusion proteins may drive membrane merging by influencing the local lipid composition that may results

in different curvatures of membrane monolayers (41, 159, 270). Moreover, some amino acid sequences in fusion proteins contribute to decrease membrane stability, such as fusion peptides and HIV-1 C-terminal lipid lytic peptides (LLPs). Fusion proteins and their associated proteins may drive hemifusion and fusion pore expansion towards an irreversible direction (166).

Viral Membrane Fusion. Virus-cell membrane fusion is an essential process in the life cycle of enveloped viruses. This process is mediated by virus-specific surface glycoproteins. The viral fusogenic glycoproteins when expressed on the plasma membrane of a host cell are capable of mediating cell-cell fusion that contributes to viral spread and pathogenesis. The study of crystal structures of pre-fusion and post-fusion forms of these viral glycoproteins has unraveled some of the molecular mechanisms leading to the merge of lipid bilayers. Viral fusion proteins have a rich diversity of their structural properties that determine the molecular details of protein rearrangement during membrane fusion (340). Currently, all viral fusion proteins are categorized into three classes (Table. 1 and Fig. 2).

Class I: The structure of class I viral fusion proteins have been extensively studied in the Influenza HA, Paramyxovirus F, and HIV-1 gp41 (87, 340). Fusion proteins in this group are trimers in prefusion forms with a linear fusion peptide at the N-terminus of each monomer. In post-fusion forms, a central N-terminal trimeric α-helical coiled coil is surrounded and packed tightly by three anti-parallel C-terminal helices, forming a 6-helix bundle structure. This structural rearrangement brings the N-terminal fusion peptides of fusion protein, which are released and inserted into target membranes after being activated by receptor-binding or low pH, close to its C-terminal membrane-spanning domains in the viral envelope; thereby, forming a trimer of hairpins and initiating virus-cell membrane fusion.

Class II. Alphaviruses and flaviviruses have class II fusion proteins (155, 300). The pre-fusion form of this class is an anti-parallel homodimer lying along the viral surface. They are associated with a viral chaperon protein prM, which is cleaved during or soon after viral assembly. Each monomer contains three domains that all mainly consist of β-sheet structures. Fusion proteins in this class contain an internal fusion loop at the N-terminal of domain III. During fusion, three homodimers realign into two homotrimers that project from the viral membrane in a threefold axe. This presents the fusion loop in domain III to the target membrane. Subsequently, the linker between domain I and III allows domain III to fold back to the side of domain I; thereby, clustering three fusion loops and the three C-terminal transmembrane anchors at one end and resulting in a similar hairpin structure as that of the class I fusion proteins.

Class III: This class of fusion proteins has recently been identified in rhabdoviruses and herpesviruses (12, 127, 271). They are homotrimers of the subunits composed of five distinct domains. The domain I harbors bipartite fusion loops in two independent regions of the β-sheet (303). The domain III is a long central α-helix, which forms a trimeric coiled coil with the helices provided by the other two monomers. During fusion, only domain III undergoes significant conformational changes and results in the transformation of three coiled-coil structures in a dimer into a 6-helix bundle. This process results in a complete flip-over of fusogenic domain I from viral membrane towards to target membrane, allowing the interaction of the bipartite fusion loops with target lipid monolayers. In the postfusion form, the fusion loops and viral transmembrane regions are close together, forming a trimer-of-hairpin structure. Another distinguishing property of class III fusion proteins is that the conformational changes are reversible.

In general, in spite of the difference in the molecular details of membrane fusion mediated by above three classes of viral fusion proteins, the common theme among them is that, after being activated, all fusion proteins convert from a fusion-competent state to a trimeric pre-hairpin, and then to a trimer-of-hairpins, which brings the target membrane-attached fusion peptide and viral membrane-anchored transmembrane domain into close proximity; and thereby, facilitating the merge of viral and cellular membrane (Fig. 3B).

Mechanisms of Triggering Membrane Fusion. Although viral fusion proteins have converged on the same general strategy to mediate fusion, they are not of fusion-competent at native states on the viral surface. The fusogenic states of these proteins must be activated by an environmental fusion trigger. A number of different stimuli can induce the change to a fusion-competent state (340).

(I) Low pH. Low pH is the only known fusion-triggering factor for orthomyxoviruses, rhabdoviruses, alphaviruses, flaviviruses, bunyaviruses, and arenaviruses. Attachment receptors are a prerequisite for virus-cell entry but do not appear to play an active role in mediating membrane fusion. These viruses enter cells through endocytosis and then are subject to low pH value in early or late endosomes where the fusion-inducing conformational changes take place.

(II) Cellular Receptor Binding. The fusion proteins of most paramyxoviruses, retroviruses, herpesviruses, and some coronaviruses are activated at neutral pH by binding to host cellular receptors. For some viruses, like MoMLV, the interaction of fusion protein with a single receptor is enough to trigger the fusion-competent state (340). On the other hand, the activation process of paramyxoviruses and herpesviruses involves an independent viral surface glycoprotein, which binds to cellular receptor and subsequently triggers the

fusion-competent state of the viral fusion protein (127, 174, 224). For lenti-retroviruses (*e.g.* HIV and SIV), multiple cellular receptors sequentially interact with the viral fusion protein, and the accumulated changes in the conformation of the glycoproteins release the previously restricted fusion state.

(III) Receptor Binding Followed by Low pH. Avian retroviruses (ASLV) require a two-step fusion activation process (340). Binding of the surface glycoprotein of ASLV to the cellular receptor causes limited conformational changes that result in a pre-hairpin structure. Subsequent exposure to low pH in endosomes after endocytosis converts the pre-hairpin to a 6-helix bundle structure and then mediate membrane fusion.

(IV) The Ebola virus glycoprotein GP uses an apparently novel fusion-triggering mechanism (340). It seems to require both low pH and endosomal cathepsins (B and L), implicating a proteolytic processing of the glycoprotein during the activation reaction.

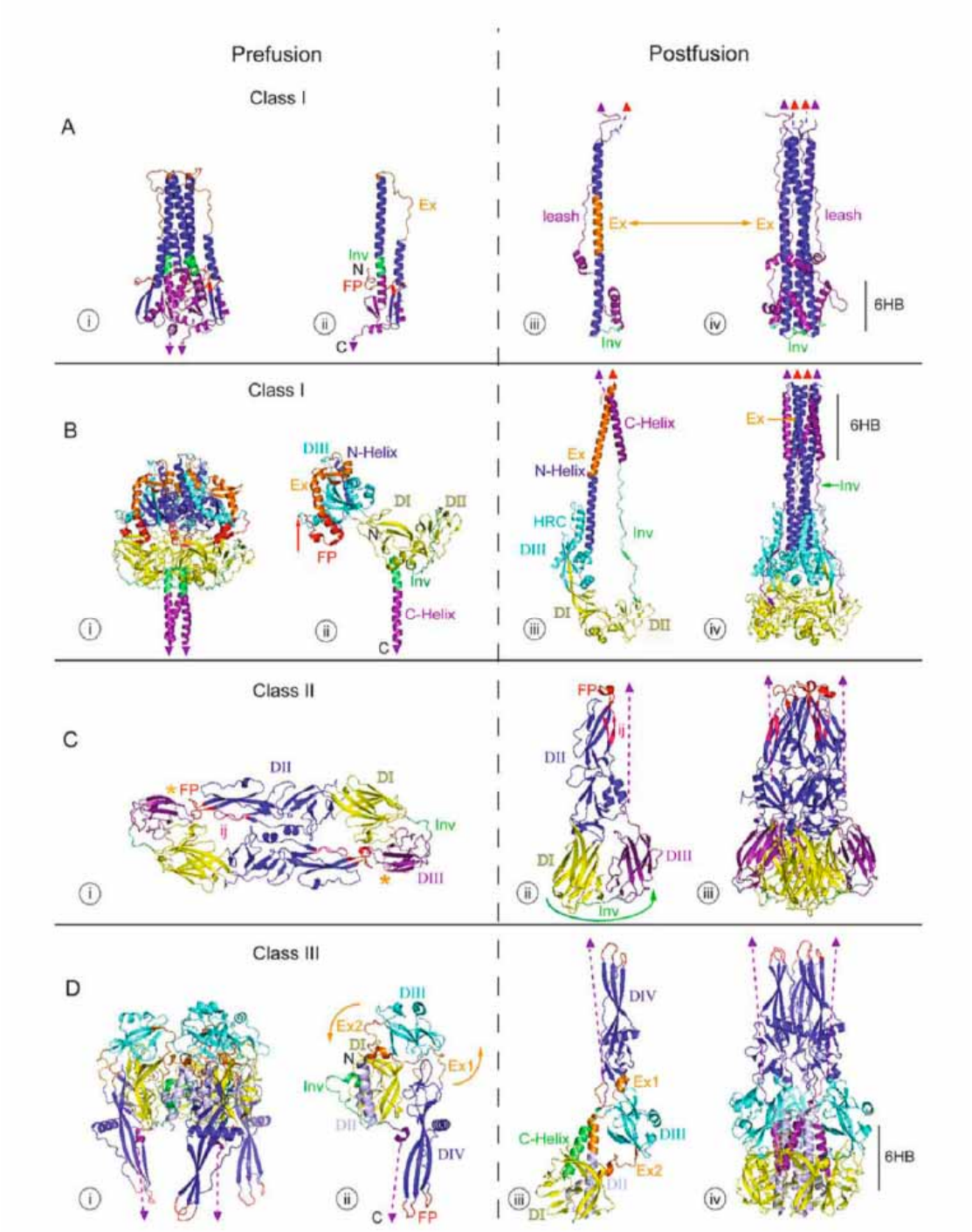

Fig. 2 Viral fusion proteins in the pre- and post-fusion forms: (A) Influenza virus HA, (B) Paramyxovirus F, (C) TBEV E, and (D) VSV G. The domains in dark blue contain the fusion peptides in red. The membrane-connecting C-terminal domains are in purple. Dashed purple lines, purple triangles, and red triangles represent the C-terminal linkers, transmembrane domains and fusion peptides not visible in the structures. (White, JM, Delos,SE, Brecher M, and Schornberg, K, *Crit Rev Biochem Mol Biol.* 2008, 43(3):189-219. Copyright © 2008 Taylor & Francis)

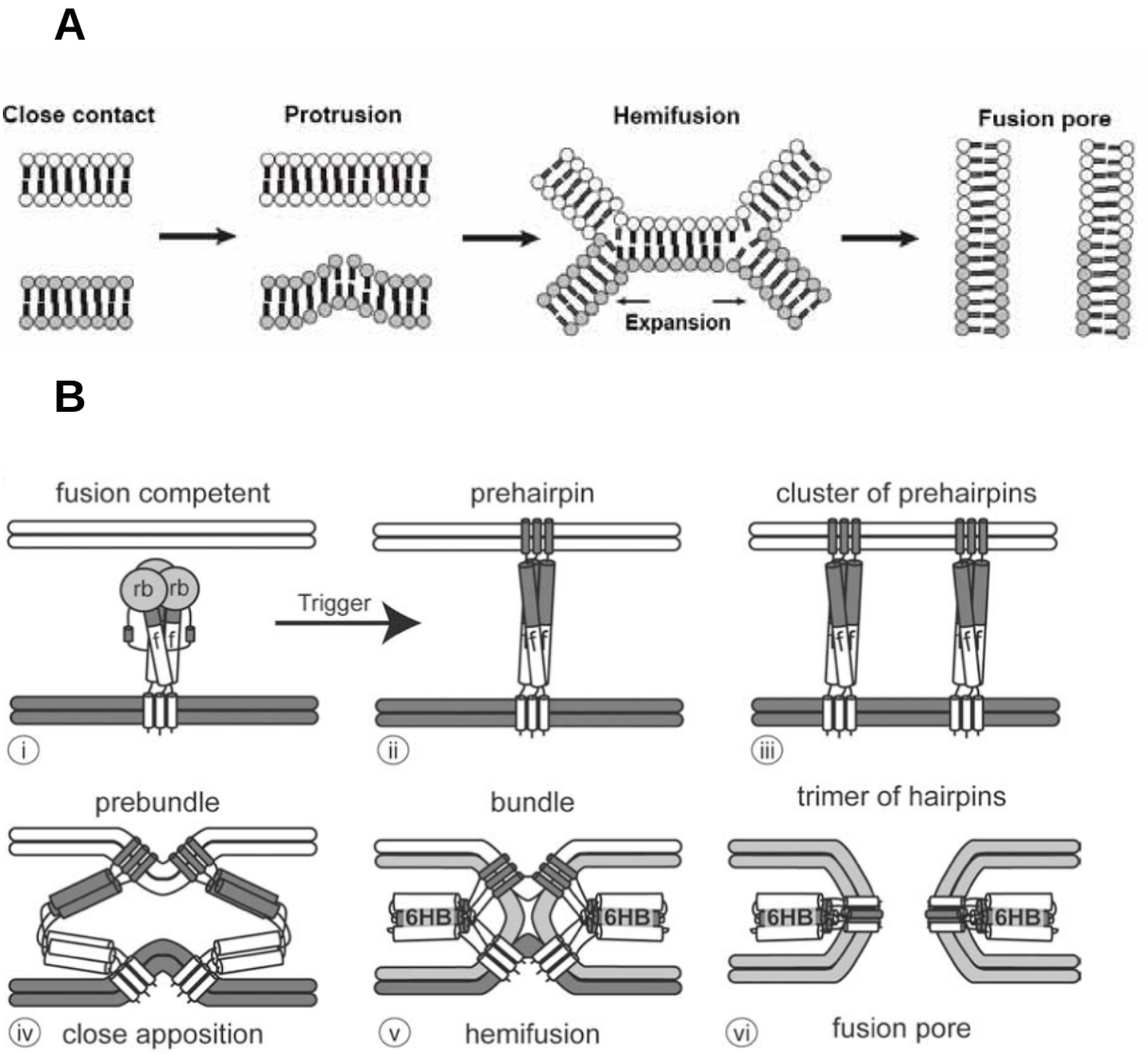

Fig. 3 Membrane fusion. (A) The fusion-through-hemifusion model of lipid bilayer fusion. (B) The trimer-of-pairpins model of membrane fusion (White, JM, Delos,SE, Brecher M, and Schornberg, K, *Crit Rev Biochem Mol Biol.* 2008, 43(3):189-219. Copyright © 2008 Taylor & Francis)

Human Immunodeficiency Virus Type I (HIV-1)

HIV-1 is the viral etiology of the Acquired Immunodeficiency Syndrome (AIDS) and is currently a global epidemic in human population. As of 2007, an estimated 33 million people were living with HIV and 2.7 million became HIV infected (UNAIDS, 2008), mainly through sexual, maternal and parenteral transmission. HIV directly infects and kills cells that are critical for effective immune responses. This, combined with a chronic activation of the immune system, results in a progressive and ultimately profound defect in the cell-mediate immune protection against a variety of normally innocuous agents. This immune disregulation is eventually the major reason for the morbidity and mortality in the AIDS population.

Classification. HIV-1 is a lentiviruse in the retroviridae family and is phylogenetically closed to Simian Immunodeficiency Virus (SIV) from chimpanzees (SIV_{cpz}). It is now generally accepted that the origin of HIV-1 derived from the cross-species transmission events from SIV_{cpz} in central Africa to human beings, and HIV-2 from SIV_{sm} in west Africa (103, 104). Based on the genetic distance, the HIV-1 primary isolates are classified into three groups, M, N, and O. Group M, accounting for the 95% HIV-1 global isolates, consists of at least nine discrete clades or subtypes (A, B, C, D, F, G, H, J, and K) and 15 circulating recombinant forms (Fields Virology, 5^{th} p2110). In group M, the interclade genetic distances are 15%-20% in *gag* gene and 20%-30% in *env* gene. The intraclade distances are 3%-10% for *gag* and 5%-12% for *env*. Coinfection of a single cell with two genetically distinct viruses gives rise to a high rate of recombinant strains as a result of template switching between two different viral RNAs during reverse transcription.

Viral Structures. HIV-1 is an enveloped positive single-strand RNA virus with a cone-shaped, cylindrical core in the mature form (Fig. 4A). As shown in Fig. 4B, the RNA genome is about 10 kb in length and encodes 3 structural (Gag, Pol, and Env) and 6 nonstructural proteins (Vif, Tat, Rev, Vpu, Vpr, and Nef) (Table 2). The long terminal

repeats (LTRs) sequences at the 5'- and 3'- end of a HIV-1 genome contain multiple cis-acting RNA elements that direct the balanced and coordinated production of progeny virions. These elements include the Tat-responsive region (TAR) for mediating transcriptional elongation of viral RNA transcripts, major splice donor for viral RNA splicing, and polyadenylation factor-binding site. A single HIV-1 virion contains two copies of the viral genome that are associated with each other through the RNA packaging site ψ. The virus is initially assembled and released from infected cells as immature particles containing the genomic RNAs and unprocessed Gag/Gag-pol precursors. The immature viral particles are spherical with 100nm in diameter with a characteristic electron-lucent center. During maturation, Gag precursors are cleaved, which leads to drastic changes in the structure and morphology of HIV-1 virions and results in particles exhibiting a buoyant density in sucrose in the range of 1.16 to 1.18 g/ml and a sedimentation rate of about 600S. The viral proteins of HIV-1 and their main functions are summarized in Table 2.

HIV-1 Replication. The Figure 5 schematically presents the life cycle of HIV-1 viruses. HIV-1 viruses use CD4 as the receptor and a chemokine coreceptor (CCR5 or CXCR4) during entry into susceptible cells. The main target cells of the HIV-1 virus are the CD4+ T-helper subset of lymphocytes, CD4+ cells of the macrophage lineage, and some subsets of dendritic cells (DC). Most human CD4 T-cell lines are susceptible to HIV-1 infection and able to release large quantities of progeny virions. The X4-tropic (CXCR4) virus strains only replicate in T-cell lines (TCL-tropic), not in macrophage cells. These viruses usually grow rapidly with high titers of infectivity and often induce the formation of syncytia. In contrast, the R5-tropic (CCR5) viruses grow relatively slow and yield lower viral titers. These viruses are infectious to macrophage cells (M-tropic) and do not form syncytia. Some viral isolates showed a dual-tropism and are able to infect both macrophages and T cell lines. Like X4-tropic viruses, the dual-tropic viruses grow

rapidly and induce the formation of syncytia. Viruses isolated from individuals shortly after they have been infected with HIV-1 are frequently R5-tropic (272). This is consistent with the fact that the CCR5-expressing memory CD4+ T lymphocytes, are depleted during the acute phase of HIV-1 infection in vivo (209). The X4-tropic virus strains appear during the later symptomatic stage of infection in approximately 50% infected persons (163, 290). The emergence of X4 viruses is associated with the accelerated CD4+ T-cell depletion and more rapid progression to AIDS (67, 163). Dendritic cells express relatively low levels of CD4, CCR5, and CXCR4, but higher levels of DC-SIGN that captures HIV by binding to the virion-associated gp120 (110). DC-SIGN expressing cells have the capacity to retain viruses for several days and to efficiently transfer virions to CD4+ T-cells.

Virus Binding and Entry. The HIV-1 infection begins with an interaction between viral envelope glycoproteins (Envs) and cell surface CD4 molecules (31). This interaction results in conformational changes in gp120 and initiates its recognition of the chemokine coreceptors CCR5 or CXCR4. Additional conformational changes following coreceptor binding activate the gp41 fusion protein that mediates the fusion between viral envelope and cell membrane. While this is the primary means of HIV-1 entry, some studies reported that certain viral isolates of HIV-1, HIV-2, and SIV enter target cells through a CD4-independent pathway (91, 202, 258, 268). Furthermore, in addition to CCR5 and CXCR4, many other chemokine receptors have been found to serve as alternate coreceptors during HIV/SIV infection, including CCR3, CCR2b, CCR8, APJ, STRL33, GPR15, and US28 (21). The gp41 fusion protein mediates membrane fusion by a spring-loaded mechanism, in which the N-terminal fusion peptide is released and then binds to the target membrane when gp41 is activated. The trimeric heptad repeats 1 and 2 (HR1 and HR2) generate a 6-helix bundle with a central coiled-coil structure formed by HR1 and tightly packed by HR2. The conformational changes in gp41 result

in a trimer-of-hairpin structure that brings the fusion peptide and the transmembrane domain close together, thereby, decreasing the distance between viral envelope and target membrane.

Reverse Transcription. After cellular entry, the viral nucleocapsid core is partially uncoated in the cytoplasm, and the reverse transcription of the viral RNA genome into a double-stranded DNA sequence initiates (2, 95). First, the $tRNA^{lys3}$ binds to the primer-binding site (pbs) in the 5'-end of the RNA genome and the synthesis of minus-strand DNA is initiated from the 3'-OH of the tRNA. Second, RNaseH digests the RNA portion of newly formed RNA-DNA hybrid. Third, the strong-stop DNA is transferred to the 3'-end of the genome where it binds to a homologous sequence in the 3'-R region. Fourth, the synthesis of minus-strand DNA continues and is coupled with the degradation of RNA templates by RNaseH except for the polypurine tract (PPT) region. Fifth, a fragment of RNA at the PPT region is used as the primer to initiate the plus-strand DNA synthesis. Sixth, the synthesis continues until a portion of tRNA has been copied, which allows the hybridization between 5'- end of plus-strand DNA and 3'- end of minus-strand DNA. Seventh, the plus-strand DNA synthesis continues until it reaches the central termination signal. The newly synthesized dsDNA is transported through cytoplasm and into the nucleus in the form of a preintegration complex (PIC) that, in addition, contains the reverse transcriptase (RT), integrase (IN), MA, NC, and Vpr. The MA, IN, and Vpr have been shown to regulate nucleus import of the PIC (36, 141). A cis-acting, centrally located, and triple-stranded DNA structure known as the central DNA flap has also been proposed to facilitate nucleus import of the PIC (295, 360).

Integration. The integration of viral DNA is catalyzed by HIV-1 integrase. The IN generates a preintegration substrate with 3'-recessed ends by removing two or three nucleotides from the 3' ends of each DNA strand. In cellular target DNA, the IN produces two 5'-overhanging termini that join the 3'-recessed ends of viral DNA. The

gaps are filled by cellular DNA repair machinery. This process results in two 5 bp direct repeats that flank the integrated viral genome. In addition to the linear and integrated viral DNA, 1-LTR circles and 2-LTR circles are also generated in the nucleus as the result of a homogenous recombination and a nonhomogenous end joining (182), respectively. Several cellular factors have been shown to facilitate the integration. The high-mobility group A1 (HMGA1) protein stimulates strand transfer activity in vitro (93, 183). The IN interactor 1 (IN1) protein binds HIV-1 IN and target the viral DNA to transcriptionally active regions in the host genome (151). Another cellular factor BAF (barrier-to-autointegration factor) prevents the viral DNA termini from integrating into internal sites in the same viral genome (53). In addition, a number of functions have been proposed for the lens epithelium-derived growth factor (LEDGF/p75) in HIV integration. This protein facilitates the strand transfer in integration, enhances the binding of IN to DNA, and protects IN from degradation (39, 56, 188).

Gene Expression. The transcription of the integrated HIV-1 genome is carried out by the host cell RNA pol II holoenzyme. The 5'-LTR plays an important role in the regulation of viral gene expression by providing cis-acting elements for both cellular and viral transcriptional factors. The recognition of the HIV-1 promoter, which is mainly composed of the Sp1 elements (121) and the TATA box, requires several general transcriptional factors from host cells, including the TFIID that binds TATA box, TFIIB that recruits RNA pol II, and TFIIH that releases the transcriptional complex from the promoter. After binding to the promoter, the elongation activity of RNA pol II is triggered by phosphorylation of its C-terminal domain (CTD). The cellular kinases which induce the hyperphosphorylation states of the CTD include the CDK7/cyclin H subunits of the CDK-activating kinase (CAK) complex associated with TFIIH, CDK8/cyclin C component of the RNA pol II, and CDK9/cyclin T subunits of the positive transcription elongation factor b (p-TEF-b). In T lymphocytes, three transcriptional factors, NF-κB, NFAT, and

AP-1, which are activated by the interaction between the TCR/CD3 complex and peptide-loaded MHC molecules, are the main regulators of cellular and viral RNA synthesis. The HIV-1 5'- LTR usually contains two NF-κB-binding elements upstream the Sp1 element. The cooperation of NF-κB and Sp1 enhances the binding of both factors to their cis-acting elements in HIV-1 5'-LTR, and then dramatically upregulates viral transcription (248). NFAT binds to sequences at the 3'-terminal portion of the NF-κB elements in the LTR (157), and then acts in synergy with NF-κB to regulate HIV-1 gene transcription. When activated by JNK and ERK, AP-1 binds to DNA sequences upstream of NF-κB elements (152) and cooperates with NF-κB to stimulates transcription from the viral promoter (356). In addition to cellular regulators, HIV-1 gene expression is also regulated by virus-encoded regulatory proteins, Tat and Rev. The Tat protein enhances the steady-state levels of viral RNA by increasing the processivity of the RNA pol II transcription complex. Initially, Tat interacts with the cyclin T1/CDK9 complex by binding its activation domain to cyclin T1. Then the binding of human P-TEFb to Tat induces a conformational change in Tat, which trigger the binding of Tat to TAR, a stem-loop structure in viral RNA. Subsequently, the P-TEFb kinase phosphorylates the RNA pol II CTD and then stimulates its transcriptional processivity (249, 327). The newly synthesized viral RNAs are exported from the nucleus to the cytoplasm, where they function as either the viral genomic RNA for packaging during viral assembly or the mRNA template for translation. Generally, only mRNA that does not contain intronic sequences is transported out of nucleus; however, HIV-1 RNAs contain many intronic sequences due to the multiple splice sites in the genome. This inhibition of incompletely spliced RNA from nuclear export is resolved by the HIV-1 encoded Rev protein (302). The Rev protein recognizes and binds to the RRE element (Rev Responsive Element) in viral mRNA, which spans the junction between the gp120

and gp41 region. After translation, the Rev is imported into nucleus by binding to the importin-β and then dissociate with it via interacting with the RanGTP in nucleus. The free Rev protein binds to RRE-containing viral mRNA. Subsequently, the additional binding of the CRM1 and RanGTP converts the Rev-RRE RNA complex into an active nuclear export structure, which mediates the transport of intronic viral pre-mRNA out of the nucleus.

Virus Assembly. After its synthesis, HIV-1 Gag Pr55 precursor is rapidly targeted to the plasma membrane, where it assembles into infectious virions with the viral genomic RNA and other viral proteins. In certain cell types, particularly MDM, the virus assembly and budding takes place in an intracellular compartment, the multivesicular body (237). The Gag-Gag interaction is sufficient for the assembly of a noninfectious virus-like particle (VLP) without the genomic RNA or other viral proteins. Shortly after budding, the viral protease cleaves the Pr55 precursor into the MA, CA, NC and p6 plus two spacer peptides SP1 (CA-NC) and SP2 (NC-p6). The membrane-binding region of Gag has been mapped to the MA domain, in which both the myristylation at the N-terminus and the downstream sequence that is rich in basic residues contribute to the membrane association of the MA. NMR and X-ray crystallographic analyses suggest a patch of basic residues on the membrane-contacting face of MA (204). Moreover, MA is also shown to interact with host factors such as the phosphatidylinositol (4,5) bisphosphate and AP-3, which, thereby, regulate the intracellular localization and trafficking of the Gag (81, 234). The CA domain of Gag is composed of a N-terminal domain (NTD) and a C-terminal domain (CTD), both of which are highly helical. The NTD contains a cyclophilin A (CypA) loop that incorporates CypA into virions (101). The CypA is important for enhancing viral infectivity (125, 298). The CTD of CA mainly mediates the CA interaction, which results in a hexametric lattice of CA in the shape of spheres, cones, or cylinders (34, 102). The main function of NC domain of HIV-1Gag is to encapsulate the

viral genomic RNAs. This is carried out by binding the two zinc-finger motifs of CA to the RNA packaging signal ψ. It has also been shown that the NC-RNA interaction is critical for stabilizing Gag multimerization. In addition, the NC functions as a nuclear acid chaperon that facilitates the reverse transcription (181). The C-terminal domain of Gag is p6, previously known as the late domain, which interacts with the host cellular ubiquitination pathway and endosomal-sorting pathway (77, 222). The p6 binds to the ESCRT-I component Tsg101 via its PTAP motif (107). It has also been shown to interact with the cellular factor Alix (301). In addition to associating with the cellular endosomal sorting machinery and promoting virus budding, p6 mediates the incorporation of Vpr and Vpx into virions (246, 349). The incorporation of viral Env gp120/gp41 into virions mainly attributes to the interaction between the MA and the cytoplasmic domain of HIV-1 Env (82, 98, 225, 226, 348).

Virus Maturation. Soon after virus budding, the PR catalyzes an intramolecular cleavage of Gag Pr55 precursors to release the viral protease (250). Subsequently, the Gag cleavage takes place as an ordered, step-wise cascade. This results in a dramatic change in virion morphology. The immature HIV-1 particles appear doughnut-shaped vs. cone-shaped as in mature virions.

Viral Pathogenesis. Although the course of disease caused by HIV infection varies dramatically among infected persons. The time from the acute infection to the development of AIDS can be as rapid as 6 months or more than 25 years. It is now generally accepted that early events at the time of acute infection such as viral load and CD4 counts, together with viral and host genetics, play a critical role in and can be used to predict, the ultimate clinical outcome of the disease (196). The median plasma viral load at the time of peak viremia in the acute infection is approximately 10^6 to 10^7 RNA copies/ml, which drops to a mean of 30,000 copies/ml within the first 6 to 12 months (196, 273). Infection of a large number of the memory CD4+ T-cells (both the resting

and activated) in the gut during the acute infection may be the source of initial viremia peaks, which also stresses the following adaptive immune responses (205, 209). Multiple mechanisms of cell death appear to account for the CD4+ T-cell depletion including the accumulation of unintegrated viral DNA (291), interference with cellular RNA processing (160, 299), loss of plasma membrane due to vial budding (179), elimination of HIV-1 infected cells by immune responses, syncytia formation (94, 263), autoimmunity, innocent bystander killing (227, 330), inhibition of hemapoiesis, and apoptosis. During acute infection, roughly $1x10^6$ HIV-1 infected resting memory CD4 cells harbor the integrated latent proviruses, which establish a latent viral reservoir (62, 361).

Immune Response. HIV-1 infection induces a broad host immune response in an infected individual. Viral RNA activates TLR-7 on plasmacytoid dendritic cells and stimulates the production of IFN-α and IL-6, which induce a profound activation of the immune system (19, 126). The number of NK cells increases in the acute infection, which is associated with the decline in viremia; while in chronic infection these cells seem anergic (10). Moreover, the expression of KIR3DS1, a NK cell inhibitory receptor, is associated with slower disease progression (203). Most of the anti-HIV antibodies generated by humoral immune responses are not neutralizing because of the viral escaping mutations. Those who have the capacity to neutralize virions usually target to the CD4-gp120 binding events or membrane fusion. Because of the high mutation rate of HIV-1, the immune pressure from neutralizing antibodies contributes mainly to the evolution of viruses in infected individuals, rather than to clear virus particles (269, 329). The appearance of CD8+ T-cells is associated with the decline in viremia after the primary peak of viral loads (30, 164). Although all the viral proteins of HIV-1 contain stimulating epitopes, the Gag, Nef, and Env seem to be the main targets. However, the breath and magnitude of CD8 T-cell response varies widely among infected individuals

and are not correlated with the control of viremia (26) except for some HLA B57 positive individuals. In contrast to the wide spectrum of CD8 T-cell responses, the CD4 T-cell response mainly targets to the Gag. At the early stage of infection, a large number of the virus-specific CD4 T-cells appear, which is correlated with the induction of CD8 T-cells, suggesting that its function is to help the CD8 T-cell response. Although HIV-1 infection induces a wide spectrum of innate and adaptive immune responses in infected persons, they fail to control viral replication and disease progression. This is partially due to the functional defects in these immune cells. CD8+ T-cells may become exhausted and are prevented from progressing to memory cells in the presence of chronic antigenic stimulations (338, 339). The overexpression of PD-1 can also render these cells ineffective (14). The high mutation rates in HIV-1 replication contribute to the failure of immune control as well. The HIV-1 viruses can rapidly escape from the epitopes targeted by current circulating neutralizing antibodies and CD8 T-cells when they are under immune pressure.

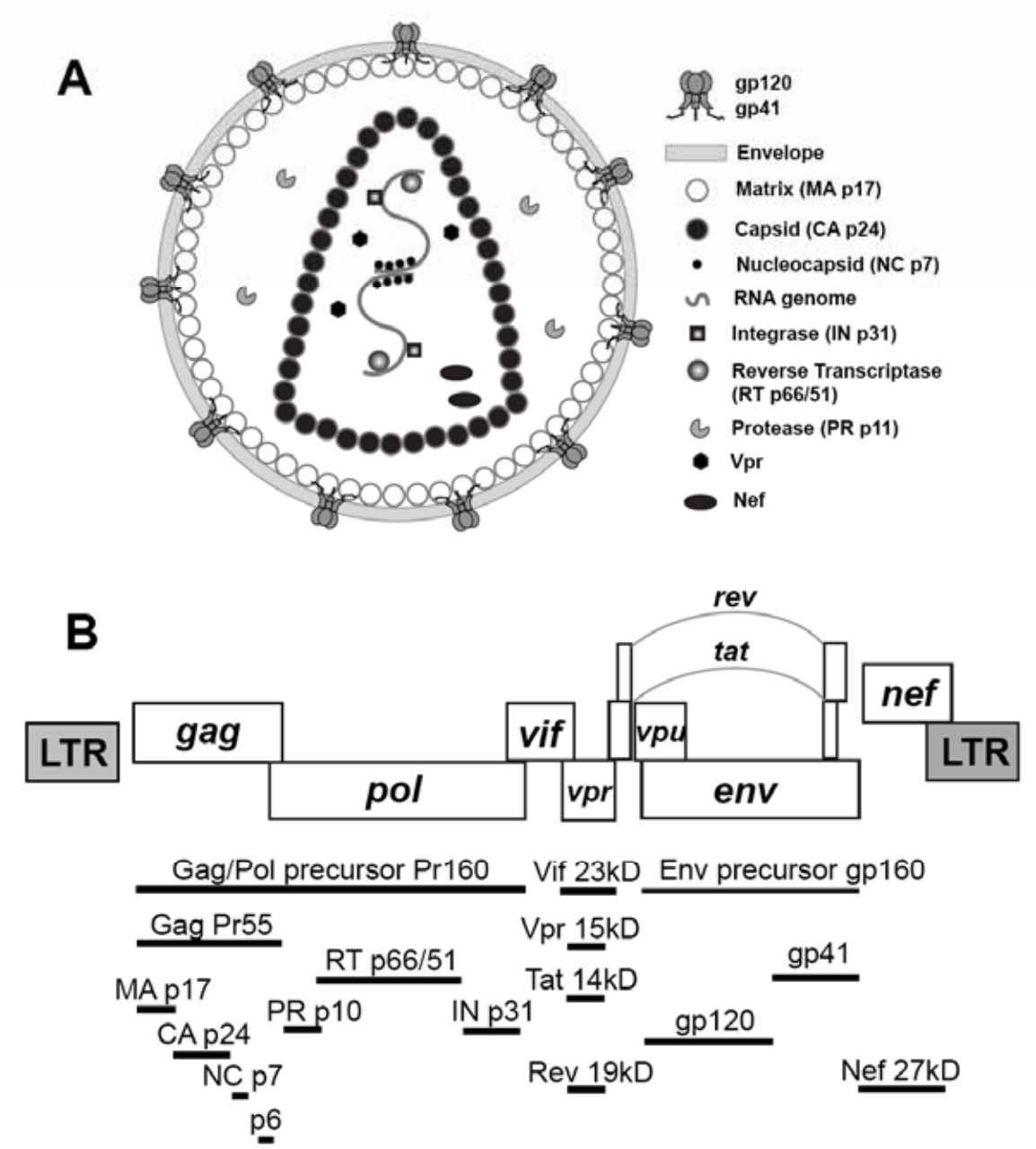

Fig. 4 The structure of a mature HIV-1 virion (A) and its RNA genome (B).

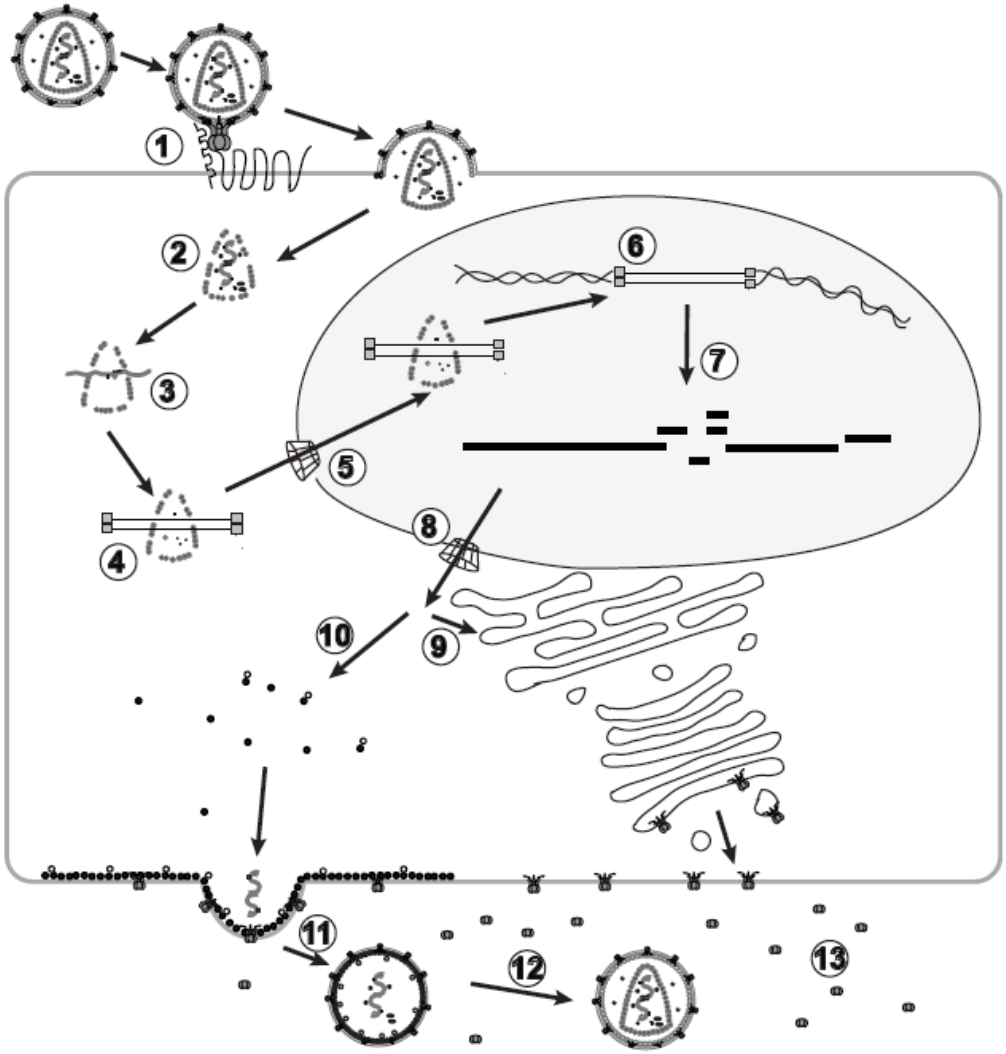

Fig. 5 HIV-1 life cycle. (1) HIV-1 infections begin with the attachment of cell-free virions to the cell surface and then the interaction of viral envelope glycoproteins with the CD4 receptor and chemokine coreceptors. (2) HIV-1 virus enters target cells through virus-cell membrane fusion meditated by the viral gp41 glycoprotein. (3) Following the cellular entry, the nucleocapsid core undergoes partially uncoating. (4) Reverse transcription occurs within the subviral particles in the cytoplasm of the infected cells. (5) The preintegration complex (PIC) containing the double-stranded DNA is transported into the nucleus through the nucleus pore. (6) The viral DNA genome is integrated into the host chromosomal DNA by viral integrase (IN). (7) The integrated viral DNA serves as a template for the host RNA pol II and leads to the production of viral genomic RNA and mRNA. (8) The viral mRNAs are transported into cytoplasm and undergo different splicing processes. (9) The envelope protein is translated and transported to the plasma membrane through the host vesicle transport system. (10) The Gag protein is transported to plasma membrane. (11) The viral assembly and budding takes place at the plasma membrane. (12) The viral protease cleaves the Gag/Gag-pol precursors leading to a mature particle of virus. (13) The gp120 Env glycoprotein sheds off the cell surface.

Table 2. The Virus-encoded Proteins of HIV-1/SIV

Protein	Major Proposed Functions
Structural Protein	
Gag	Capsid protein, including matrix (MA), capsid (CA) and Nucleocapsid (NC) in mature viral particles
Pol	RNA-dependent DNA polymerase, Integrase, Viral Protease,
Env	Surface glycoprotein, containing surface unit gp120 (gp140 in SIV) and transmembrane unit gp41, Receptor/coreceptor-binding & Virus-cell fusion
Nonstructural Protein	
Vif	Deactivation of APOBEC family of cytidine deaminase
Vpr	Cell cycle arrest, Import of viral preintegration complexes (PIC) into nucleus, viral and cellular Transcriptional activator, apoptosis inducer
Vpx	Nuclear import
Vpu	Enhancement of particles release via interacting with tetherin, CD4 degradation
Nef	Downregulation of cellular surface protein such as CD4, MHC-I, MHC-II, CD3, and CD28; enhancement of viral infectivity
Tat	Activation of viral transcription via binding to TAR
Rev	Binding to RRE (Rev-responsive element) in vial genomic RNA and mediating its nuclear export

HIV-1 Envelope Glycoprotein (Env)

HIV-1 Env (gp120/gp41) is located on the surface of virions and plays a critical role in viral replication. The Env interacts with the cellular CD4 receptor and chemokine coreceptors, and mediates virus-cell membrane fusion. Additionally, it induces both the humoral and cellular immune responses in infected persons.

Env Structure. The HIV-1 envelope glycoprotein is encoded by the viral *env* gene (HXB2 K03455 6225-8795 2571bp) (Fig. 4). The *env* gene contains overlapping coding sequences with the *vpu* gene (6062-6310), and the second exons of the *tat* gene (8379-8469) and the *rev* gene (8379-8605 or 8653). The RRE sequence is also mapped in the *env* coding region (7710-8061). HIV-1 Env glycoprotein is synthesized from a 4.3kb, single-spliced bicistronic mRNA, which is also used as translation templates for Vif, Vpr, Vpu and Tat. This pre-mRNA is transported into the cytoplasm via the Rev-RRE nuclear export pathway that requires the cellular importin-β nuclear transport machinery. The product of the *env* gene expression is a 160 kD glycoprotein precursor that is cleaved into a surface unit (SU) gp120 and a transmembrane unit (TM) gp41 by a protease of the furin family in the trans-Golgi network. The HIV-1 Env complex on viral surface is a trimer of gp120/gp41 heterodimers. The ectodomain of the Env contains the SU gp120 and the N-terminal portion of the TM gp41. The gp41 glycoprotein has a single membrane-spanning domain (MSD) followed by a long cytoplasmic domain (about 150 aa). The examination of the Env complexes on viral surface by cryoelectron tomography (cryoET) has demonstrated that the viral Env trimer displays a tripod-like configuration with a gp120 head and gp41 stalk regions (274, 362, 363). The sequence structure of HIV-1 Env is shown in Fig. 6. The biological functions of the HIV-1 Env is summarized in Fig. 7 and Fig. 8.

(I) Surface unit gp120. The HIV-1 gp120 consists of 5 conserved domains (C1-C5) and 5 variable domains (V1-V5). **(i)** Constant Regions. The C1 region is predicted to be an amphipathic helical structure and the C5 region is shown to have a turn-helix configuration in solution (117). Several studies suggest that the C1 and C5 regions directly interact with the immunodominant loop in gp41 (28, 129, 139). The C1 region has the capacity to directly interact with complement factor H (255, 256), which may contribute to the early steps of viral infection. Antibodies against the C5 region showed cross reactivity to the HLA class I on activated cells, which is due to the factor that epitopes in C5 region mimic the HLA class I alpha 1 peptide-binding domain (75, 190). Monoclonal antibodies against the C2 region are not able to bind the gp120 on viral surface, even in the presence of sCD4, indicating that this region is sequestered in the oligomeric structure (177). Further studies demonstrate that the C2 region is involved in the oligomerization of gp120 (286). The binding of CD4 to HIV-1 Env requires multiple conserved regions in the gp120, including C1 (167, 239), C4 (223), and C3 (133, 134). The V3 region may interact with the C4 region, which is also important for the CD4-gp120 binding (220). The comparison of sequence profile of HIV-1 and SIV Env shows that the C2-V3-C3 region is involved in the contact with chemokine receptors (292). The C4 region is accessible to antibodies on the surface of gp120 (207). The fact that mutations in the C1 and C4 regions were reversed by those in the V1V2 region indicated a possible interaction between these regions (326). Additional studies also showed that the V1V2 region binds o the C4 region (97). Some studies have shown that the HIV-1 gp120 is able to crosslink membrane IgM on normal human B cells and to induce their activation. This superantigen (Sag) property is attributed to the C2 and C4

regions that may be involved in the contacts (154). **(ii)** Variable Regions. The V3 loop is a hyper variable disulfide-bonded structure that determines the tropism of HIV-1 virions (124), but is not involved in CD4-binding (353). The crown portion of the V3 loop has been shown to carry a homologous structure to the β2-β3 loops in the ligands of CXCR4 and CCR5 (44). The structural modeling of the V3 loop shows a charged patch with positively charged amino acid residues at positions 11, 24, or 25 for X4-tropic viruses and neutral ones for R5-tropic viruses (44, 124). The V3 region is accessible on the surface of most viruses for neutralizing antibodies (114); however, its tremendous variability protects it from being targeted by either generating escaping mutations in epitopes or introducing glycan shields (124). The V1V2 region also influences the HIV-1 cellular tropism. A single amino acid substitution in the V1 region of HIV-1 JRCSF alters its cellular tropism and enables it to infect X4 T cell line (33). In the macrophage-tropic strain, HIV-1 SF162mc, the V1 and V2 regions, in addition to the V3 loop, are required to maintain the full macrophage tropism, suggesting an interaction between the V1V2 and V3 regions (161). Consistent with these finding, the V1V2 and V3 regions contain the major determinants of the SI capacity (116). In addition, the V1V2 and V3 regions have been demonstrated to play a more important role in using other chemokine coreceptors such as CCR2b, CCR3, STRL33, and APJ, in addition to CCR5 or CXCR4 (132). Moreover, the V1V2 and V3 regions are not involved in the interaction with gp41 or CD4; however, they do demonstrate the capacity to mask conserved neutralization epitopes of the HIV-1 gp120 glycoprotein from antibodies (208, 353). Some studies showed that the V1V2 could influence the replication in macrophages by affecting viral spread (313). Other studies suggest that the elongation of the V2 loop is

a good predictor for slower disease progression, while rapid progression is associated with basic residues in the V3 loop and a shorter V2 region (293). The V4 and V5 regions of gp120 are necessary for efficient utilization of CXCR4 (60, 172). Substitutions in the V4 region dissociate gp120 and gp41. Three N-linked glycans in the V4 and V5 region are dispensable for CD4-binding and fusion activity of the gp120 (115). In the dual tropic strain 89.6, the V3, V4, and V5 regions are involved in the CCR5, CXCR4, and CCR3 utilization (297). Comparisons of the population changes in the V4/V5 and V1/V2 suggest that the V1/V2 and V4/V5 regions often evolve independently during chronic infection (122). **(iii)** Glycosylation. HIV-1 Env ectodomain is extensively glycosylated with approximately 25 potential N-linked glycosylation sites and 8 O-linked glycosylation sites in the gp120, and 3-4 sites in the gp41 (25, 111, 145). Some of these glycosylation sites are critical to maintain the biological functions of HIV-1 Env such as the viral infectivity, receptor binding, oligomerization, and gp120-gp41 interaction (230, 326). However, many N-linked glycans on the HIV-1 Env ectodomain are not necessary for viral replication (76, 88). It is generally accepted that the heavy glycosylation plays an important role in protecting HIV Env from neutralizing antibodies by forming a glycan shield covering the epitopes of neutralizing antibodies (253). In the V1V2 region, some glycans may interact with the C1 and C4 regions, which could be critical for WT viral infectivity (326). The loss of some other glycosylation sites in the V1V2 regions results in an increased sensitivity to a number of neutralizing antibodies and a loss of dependence on CD4, which is probably due to a global change in the gp120 structures (162). These changes also depend on the sequence context of glycosylation

sites. Consistently, the glycans in the V3 and V4 regions were shown to mask neutralization epitopes in the gp120 (89, 284).

(II) Transmembrane unit gp41: The HIV-1 gp41 glycoprotein is the fusion machinery that directly mediates virus-cell membrane fusion. When activated, the ectodomain of gp41 carries out the fusion function, while the membrane-spanning domain and cytoplasmic domain are both important for its fusogenecity. The ectodomain consists of a fusion peptide, two heptad repeats (HR1 and HR2), and a membrane proximate tryptophan rich domain. The cytoplasmic domain contains signals for intracellular trafficking of the Env, and three lipid lytic peptides (LLP1, LLP2, and LLP3) that are suggested to play important roles in membrane fusion. The details of the structures of the gp41 and their functions will be discussed below.

Env Biosynthesis and Transport. The translation of the Env mRNA occurs on the rough endoplasmic reticulum (ER) associated with ribosomes. The nascent gp160 peptide is immediately glycosylated and forms disulfide bonds that link the V1 and V4 regions with the help of the host chaperon BiP/GRP78 (86, 178, 344). The gp160 undergoes trimerization in the ER, which is necessary for transport from the ER to the Golgi apparatus (240). The determinants of Env trimerization have been mapped in the N-terminus of gp41 (85 aa) (259), while the V1V2 region in the gp120 appears to be necessary for a correct oligomerization (49, 78). Studies, in which the gp160 remained in the ER after translation, showed that the events during the expression of gp160 do not influence the later viral assembly events (251, 279). The gp160 trimers are transported to the Golgi, where (in trans Golgi network) they are proteolyctically cleaved into gp120 and gp41 subunits by the protease of cellular furin or furin-like protease family that cleave a conserved sequence motif at Lys/Arg-X-Lys/Arg-Arg (118). The gp120 and gp41 are noncovalently associated by weak interactions and a substantial amount of

gp120 is shed from the surface of Env-expressing cells. The gp160 undergoes additional glycosylation processing in the Golgi apparatus, which results in the factor that approximately half of its molecular mass is composed of oligosaccharide side chains. Some studies also observed a sulfation modification of the gp160 (24). During the synthesis of the Env, the majority of gp160 is retained in ER and subsequently degraded via the proteasome pathway. Only a small portion is correctly folded, cleaved, modified, and then transported to the plasma membrane (PM). Two segments of peptide (750-763, 764-785) have been shown to be critical for transporting Env from the Golgi to the PM (37). Following its arrival at the plasma membrane, the HIV-1 Env is rapidly internalized through the clathrin-dependent endocytosis pathway (171, 275). This has been attributed to the interaction between the membrane-proximal tyrosine based signal Y(712)SPL in the cytoplasmic domain of the gp41 and the μ2 chain of the clathrin-associated AP-2 complexes (37, 69, 231). Recently, the C-terminal dileucine motif (LL) has also been demonstrated to mediate Env endocytosis via the clathrin and AP-2 pathway (40). Truncation of the cytoplasmic domain of gp160 results in an increased level of Env on cell surface. Some studies suggested that another membrane-proximal tyrosine based signal Y(802)W(803) is also a major determinant of the intracellular transport of the HIV-1 Env (175). This motif interacts with TIP47, a protein required for the transport of mannose-6-receptors from the endosomes to the trans-Golgi network (TGN), and facilitates the retro-transport of the Env to the TGN after endocytosis (29). Overexpression of the Env decreased the cell surface level of CD4 by forming a gp160-CD4 complex that is retained in the ER (70). This can be reversed by additional coexpression of the HIV-1 Vpu, which is probably due to the fact that the Vpu can induce the degradation of CD4 via the cellular proteasome pathway (71, 345, 346). The HIV-1 Nef downregulates the surface levels of the Env in a CD4-dependent manner (285). The interactions between the Nef, Vpu, Env, and CD4 can be reconciled as that the Nef and

Vpu induce the degradation of CD4 via lysosome and proteasome, respectively, and the Env is bystandly degraded by forming gp160-CD4 complexes (173, 252, 342).

Env Incorporation. A single HIV-1 virion contains approximately 14 Env complexes on its surface (362). While the assembly of viral genomes and Gag do not require the Env, the Env directs viral budding at the basolateral side of polarized cells, probably through the interaction between the cytoplasmic domain of the Env and the matrix domain of the Gag (189, 242). The mechanism by which the Env glycoprotein is incorporated into budding virions remains ambiguous. Several studies showed evidences suggesting the direct interaction between MA and the Env cytoplasmic domain. Mutations in the matrix domain block the Env incorporation, which can be reversed by removing the cytoplasmic domain or pseudotyping virions with a heterologous Env (82, 96, 98, 198, 358). A small deletion of the α-helix 2 motif in the HIV-1 Env cytoplasmic domain impaired its incorporation, while it was reversed by an additional mutation in the matrix protein at V34I (225). The C-terminal truncation of Env at Y(712) blocks its viral incorporation in PBMC and macrophages, even though the mutant can be incorporated in certain cell lines such as Hela and MT4 (226). Shorter C-terminal truncations are deleterious to Env incorporation (84). Therefore, the cytoplasmic domain of the Env plays a critical role in Env incorporation. Presumably, the long cytoplasmic tails take a tripod-like configuration and fit into the 3-axes symmetric gaps between the HIV-1 Gag that is organized as hexamers array on membrane (7, 8). Additionally, some other indirect interactions are probably also involved in Env incorporation. HIV-1 Env and Gag may be associated to each other by both binding to TIP47 (191). HIV-1 virions can incorporate heterologous Env glycoproteins of other viruses such as MuLV, HTLV, VSV, and HCV. The HIV-1 Gag is associated with lipid rafts through its M domain, I domain and myristate group (80, 228, 235, 260). However, the association between HIV-1 Env and lipid rafts appears to depend on the Env-Gag interaction (27). Nevertheless, some studies show

that the coexpression of the HIV Env and Ebola GP in the presence of HIV-1 Gag results in two distinct viral population carrying different surface glycoproteins because of the segregation of them in lipid rafts (180). These suggest that the association with lipid rafts may contribute to Env incorporation in an unknown way. In infected CD4 T-cells, Both actin and tubulin are required to direct the clustering of Env and Gag at a polarized, capped, raft-like assembly domain (148).

Virus Release. In T-cells and DC cells, the HIV-1 Env and Gag are accumulated in a tetraspanin-enriched microdomain (CD9, CD63, CD81, and CD82) for replication, assembly, and budding (106, 149, 229). This domain is associated with the Tsg101, VPS28, and AP-3 (105, 229). Tetraspanin has been reported to function as a costimulatory signal to increase the expression of HIV-1 genes through the NF-κB, NFAT, and AP-1 pathway in the primary CD4+ T-cells (307). Moreover, overexpression of tetraspanins appears to negatively modulate the Env-mediated cell-cell fusion (113, 335).

Binding and Entry. The first step of HIV infection is the attachment of virions to cell surface. Both specific and nonspecific interactions contribute to the virus-cell attachment. Although the interaction between CD4 and gp120 provides a viral specific binding to target cells, the binding of CD4 to virus-associated gp120 is weak, and infection can take place in a CD4-independent pathway (316). Studies have shown that the cyclophilin A that is incorporated into virions is translocated to the surface during maturation, and then plays an important role in the virus-cell attachment by binding to heparan (280, 281). However, nonspecific interactions have been demonstrated to be a dominant force to mediate virus-cell attachment as well. These include the binding of gp120 to the HSPG, mannose receptor, and Galcer, and the interaction of adhesion molecules on virus and cell surface such as Integrin and ICAMs (314, 316). However, for most of HIV-1 strains, the binding of gp120 to CD4 is critical for a successful entry

into target cells. CD4 is a 55kD member of the Ig family. It is composed of four extracellular domains, one transmembrane domain, and a highly charged cytoplasmic domain. The gp120-binding site on CD4 has been mapped to a small fragment in the N-terminal extracellular domain. The CD4-binding sites in gp120 are located in the C3 and C4 regions (Fig. 5), which is conformation-dependent. In the crystallographic structure of the gp120-CD4 complex (170, 350), the CD4-binding site has been demonstrated to span parts of the outer domain and portions of the bridging sheet that is composed of the V1V2 regions. These regions are predicted to form an open cavity, which is occupied by the Phe43 in CD4 molecules and probably is shielded by glycans. The Env-CD4 interaction is not sufficient to induce virus-cell membrane fusion. Some chemokine receptors, which are members of the G protein-coupled receptor superfamily with seven transmembrane domains, have been identified to be required for the activation of fusogenecity of the gp41 subunit. The two major one are CXCR4 and CCR5. The viral determinants of coreceptor usage are located mainly in the V3 loop of gp120, while the V1V2 regions are also involved in the selection. The binding of coreceptors to gp120 is highly stimulated by CD4 (282, 310). This suggests that HIV-1 binding and entry consists of sequential events: first, gp120 interacts with CD4; conformational changes in the gp120-CD4 complex expose the coreceptor-binding sites; further binding to the coreceptor triggers the activity of gp41 to mediate membrane fusion. However, the appearance of CD4-independent virus isolates and the discovery of a number of other chemokine coreceptors such as the CCR2b, CCR3, CCR8, APJ, Bonzo (STRL33), BOB (GPR15), and US28 indicated that the coreceptor-binding event is probably the key step to activate the gp41 fusion machinery (21, 131, 162). The binding of gp120 to CD4 induces the reorganization of the actin skeleton and the clustering of CD4 and chemokine coreceptors particular in the viral synapses (147). The Env-coreceptor interaction activates Rac-1 GTPase (257). Studies have shown that moesin, a

membrane-actin linker, and filamin-A, an actin-binding protein, are involved in the actin reorganization process (15, 143). The primary function of the Env glycoprotein is to promote the virus-cell entry by mediating the fusion between the viral envelope and the cellular membrane. After the sequential binding of gp120 to CD4 and chemokine coreceptors, the conformation of gp120 undergoes dramatic changes that result in the exposure of gp41, which is previously shielded by gp120, to target membrane. The gp41 glycoprotein is the fusion competent part of HIV-1 Env. When activated, its N-terminal 30-aa hydrophobic peptide, known as the fusion peptide, is released from a compact structure of the gp41 and becomes associated with the outer monolayer of the target membrane. Following the fusion peptide are two conserved and amphipathic heptad repeat sequences, HR1 and HR2. The structure of the gp41 ectodomain has been resolved by crystallography and nuclear magnetic resonance spectroscopy (42, 50, 305, 332). These studies demonstrated that the HR1 and HR2 are both α-helices, and packed antiparallelly with a loop connecting the C-terminus of HR1 and the N-terminus of HR2. In the postfusion form of the gp41 trimer, the three HR1 segments form a coil-coil structure in the center, and the three HR2 segments are tightly packed into the hydrophobic grooves of the coiled-coil structure on the outside. This results in a six-helix bundle, which is similar to the fusion-competent form of influenza HA and SIV gp41. The anti-parallelly packaged heptad repeat sequences in the postfusion form of the gp41 trimer suggests a model of membrane fusion, in which the formation of a 6-helix bundle brings the viral membrane and cell membrane close together to facilitate lipid mixing. However, little has been known about the details of how two lipid bilayers merge into one. Several questions still remained to be answered: what is the factor that triggers lipid mixing; does the HIV-1 Env-mediated membrane fusion has a detectable hemifusion stage; what is the viral and/or cellular factors that contribute to the expansion of fusion pore? Nevertheless, the sequences C-terminal to the HRs are also important

to the fusogenecity of gp41. The membrane proximal tryptophan-rich domain which is C-terminal to HR2 has been shown to be important to the Env-mediated cell-cell fusion and viral infectivity (278). This domain forms a well-defined amphipathic α-helix that is predicted to locate at membrane-water interface (283) and to contribute the disruption of viral membrane during fusion (319). The cytoplasmic domain of gp41 also plays a role in modulating the fusogenecity of HIV-1 Env by regulating the surface level of the Env via its Y(712)SPL, Y(802)W, and the C-terminal dileucine motif, and by influencing the conformational integrity of the Env ectodomains (32, 354). Moreover, this region contains three lipid lytic peptide segments (LLP1-3) that are all amphipathic and suggested to be associated with the inner monolayer of viral membrane (55, 158). The synthetic peptides of these LLPs have been shown to induce the leaking and fusion of artificial lipid vesicles; however, their roles in the context of the gp41 sequence are not known yet (66, 212, 309). Additionally, the lipid composition appears to influence the virus-cell membrane fusion as well (236). HIV viral envelope is rich in lipid rafts components (9) and the membrane fusion may take place at lipid raft microdomains. Studies have demonstrated that cholesterol and glycosphingolipids are critical for virus-cell membrane fusion (35, 137, 321).

Env Immune Response. The HIV-1 Env is a strong immunogen and elicits a number of antibodies. However, only a small number of them are able to neutralize virions. There are two groups of anti-Env neutralizing antibodies that have been studied (153). The first class is anti-V3 loop antibodies, which are usually narrow in their range of neutralization due to the hypervariability of the V3 loop. These neutralizing antibodies can be rapidly escaped by viral mutations in the V3 loop. The other class consists of some rare antibodies with broadly neutralizing activity against functionally conserved surface epitopes in the context of functional gp120 or gp41. Only 4 broadly neutralizing antibodies have been well defined, b12, 2G12, 4E10, and 2F5. Based on the epitopes

of these Nabs, several strategies have been explored to design novel immunogens to elicit Env neutralizing antibodies. The CD4-binding site seems to be prospective for immunogen design because b12 binds to this region and results in a broadly neutralizing activity (38). Studies have shown that the CD4-stabilized CD4-binding site approach increases its ability to elicit neutralizing antibodies (79, 355). The 2G12 binds to glycans on Env surface, suggesting that the alteration of surface oligosaccharides based on the 2G12-glycan structures could be a very promising idea. Other targets in the gp120 for immunogen design include the V3 loop and the regions involved in CCR5 binding. In the gp41 glycoprotein, the membrane proximal external region, where the epitopes of 2F5 and 4E10 are located, may present another interesting target for the immunogen design. In general, the current immunogen design to elicit neutralizing humoral immune response against HIV-1 Env is focused on either the concensus sequences that are functionally conserved, or the surface glycan structure of gp120/gp41. HIV-1 Env also elicits virus-specific CD4 and CD8 T-cell immune responses. Both the MHC class I and II pathway are involved in the epitope-presenting process (176). However, viruses can escape the T-cell mediated immune attack by generating mutations that influence the processing of epitopes and their binding to the MHC molecules. These epitope-escaping events may be associated with certain HLA genotypes.

Env Signaling and Pathogenesis. It has been reported that HIV-1 gp120 induces apoptosis in CD4+ T-cells, CD8+ T-cells, and neurons (20, 23, 130, 211). The Env related cell death can be induced by several distinct mechanisms (247). The soluble gp120 can kill cells through signals that are transmitted by CXCR4 chemokine receptors. The Env-CD4 complexes on the surface of HIV-1 infected cells can kill uninfected bystander cells that express CD4 and CXCR4 or other chemokine receptors. The Env or Env-CD4 complexes can be transferred to CD4-expressing target cells by transient contacts with lipid exchanges, and then induce cell death in the target cells. Moreover,

Env-mediated fusion between infected cells and bystander cells forms syncytia that then yield to apoptosis. This occurs through a complex signaling pathway that involves the activation of several kinases such as Cdk1, Chk2, mTOR, p38 MAPK, and IKK, as well as the activation of several transcription factors including NF-kappaB and p53. However, if the Env-expressing cell is at an early stage of the imminent apoptosis, the apoptosis in syncytia occurs via a mitochondria-dependent pathway but does not involve Cdk1, mTOR, p38 or p53. Additionally, HIV-1 Env contains two calmodulin-binding domains in its cytoplasmic domains, which are involved in the Fas-mediated cell death (213, 243, 264). Binding of HIV-1 Env to the chemokine receptors CCR5 or CXCR4 activates calcium flux and chemotaxis in CD4+ T-cells (333). This calcium flux is both CD4- and coreceptor-dependent; however, the physiological threshold of signal activation is decreased to as low as two virions per cell compared to the calcium flux induced by coreceptors and their natural ligands (210). The consequence of this calcium signal has not been understood yet. Recently, studies have demonstrated that the entry of virions requires actin reorganization in target cells. The binding of Env to CD4 and coreceptors activates a signaling cascade resulting in activation of Rac-1 GTPase and subsequent actin cytoskeletal reorganization that is critical for virus-cell membrane fusion. It has been suggested that the Galpha(q) signaling pathway may be involved in the signal transductions (120). The cell surface level of CD4 is regulated by the HIV-1 Env via two mechanisms. First, the Env-CD4 complex on a cell surface triggers the internalization of CD4 molecules (31). Second, the Env-CD4 complex formed intracellularly can retain CD4 in intracellular membrane compartments for degradation mediated by Vpu and Nef. Some studies showed that dendritic cells secret IFN-γ in the presence of viral particles probably through the interaction of gp120 and mannose receptors (214). The cross linking of CD4 with viral particles induces the signaling pathways that activate cellular transcriptional machinery (20).

Entry Inhibitors. The virus-cell entry step is one the potential target for anti-HIV drug development. So far several HIV entry inhibitors have been found and some of them are under clinical trials. These inhibitors include small molecules, antibodies and covalently modified natural ligands, and can be grouped into three classes: attachment inhibitors, chemokine receptor antagonists, and fusion inhibitors (92, 169, 261). Initially, recombinant soluble CD4 was used as the molecular decoy; however, it is not able to neutralize primary HIV isolates. An exotoxin (PE40) conjugated rsCD4 only showed the modest reduction of viral RNA levels. Several small molecules, BMS-378806 and BMS-488043, which block the gp120-CD4 interaction, have potential anti-HIV activities, but their neutralization activities are either not broad or high dose-dependent. A monoclonal antibody, ibalizumab, has the ability to lock CD4 conformation after attachment and inhibit following events. Initial clinical trial study has shown its potency to decrease the viral RNA level in plasma. A number of the small molecule CCR5 antagonists have been tested for their anti-HIV activities. Four of them, aplaviroc, maraviroc, vicriviroc, and INCB009471 showed potent inhibition of both the lab-adapted and primary isolates, and are in the process of advanced clinical trials. A few CXCR4 antagonists, AMD11070, AMD3100, and plerixafor (AMD conjugated with G-CSF) have shown potent inhibition activity as well. Several broad anti-CCR5 monoclonal antibodies including HGS004 and PRO140 have been tested in HIV-1 infected individuals and proved to efficiently decrease viral RNA level and increase CD4 T-cell level (233). Several synthetic peptides overlapping the HR2 and MPER regions of gp41 have been shown to inhibit the membrane fusion mediated by HIV-1 Env. Among these peptides, T20 (enfuvirtide) was demonstrated to be the most beneficial to inhibit viral replication and valuable to be added into the salvage treatment armamentarium (197).

Env Vaccine. The purpose of Env-based HIV vaccine is mainly to induce an anti-Env humoral immune response containing broadly neutralizing antibodies. So far, HIV

vaccine design has still been unsuccessful. However, studies have suggested several strategies for Env vaccine design. First, the polyvalent Env glycoprotein composed of sequence from different genotypes and a cocktail of Env glycoproteins in vaccine could be a valuable method to elicit broadly neutralizing antibodies. Second, based on currently available broadly neutralizing anti-Env monoclonal antibodies, modifications of the Env sequence at functionally conserved regions such as the CD4-binding site, coreceptor-binding site, and MPER, provide novel strategies for immunogen designs. Finally, since the Env glycoprotein is heavily glycosylated and the 2G12 (against mannose group on Env glycans) antibody shows broadly neutralizing activity, anti-glycan antibodies could be a new source of HIV-1 neutralizing agents. These issues have been discussed in several reviews (135, 206, 244, 245, 253, 296, 312).

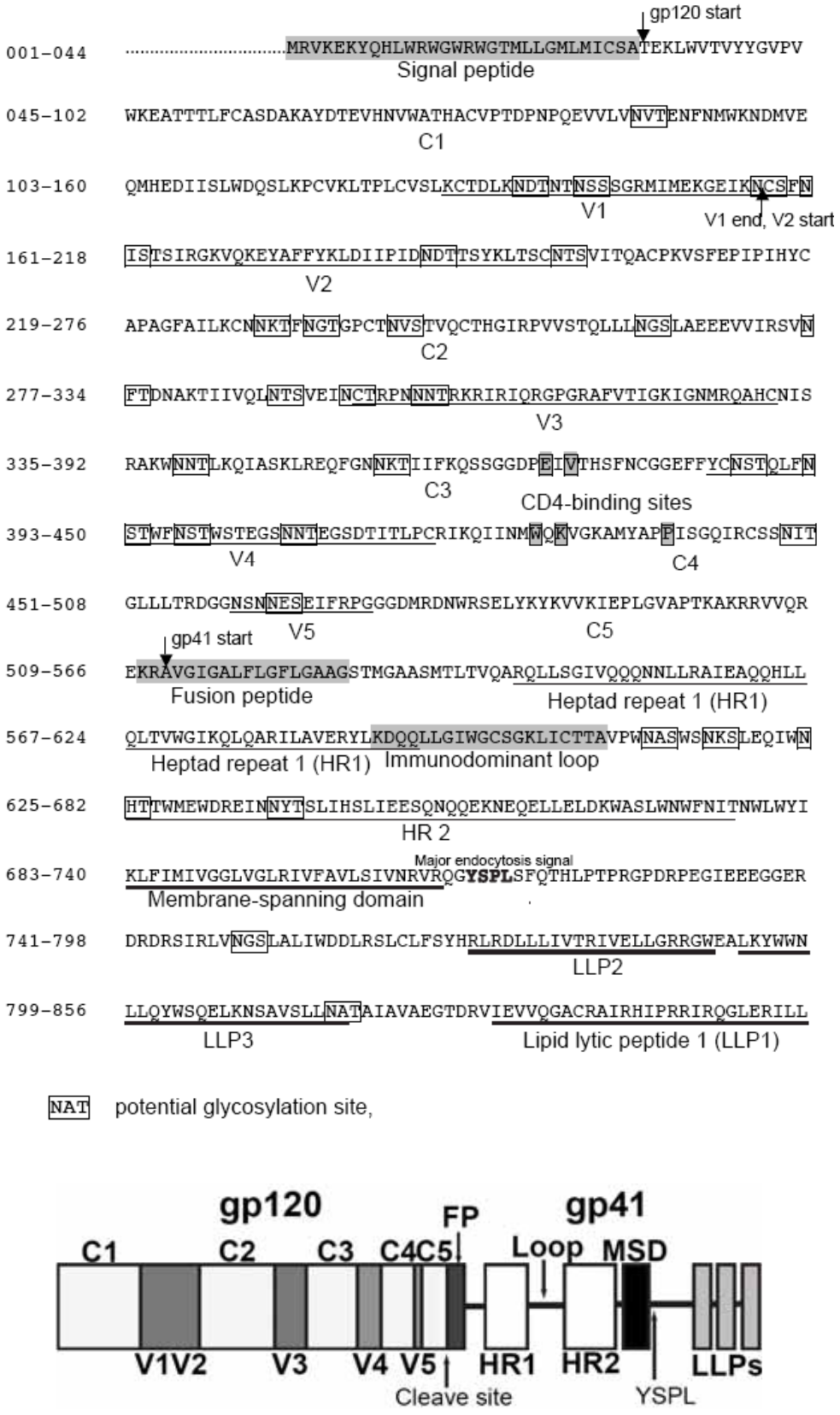

Fig. 6 The Env sequence structure (NL4-3).

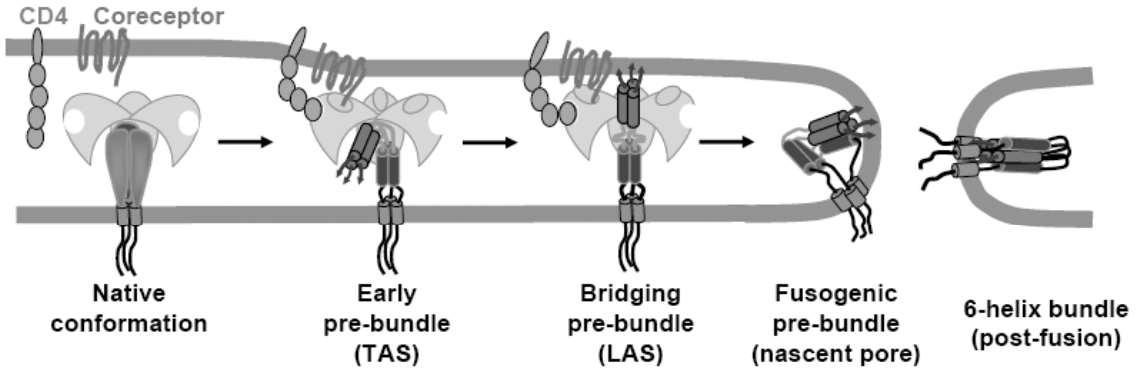

Fig. 7 Membrane fusion mediated by the HIV-1 Env. (Melikyan, G.B. *Retrovirology*, 2008, 5:111. ©Biomed Central)

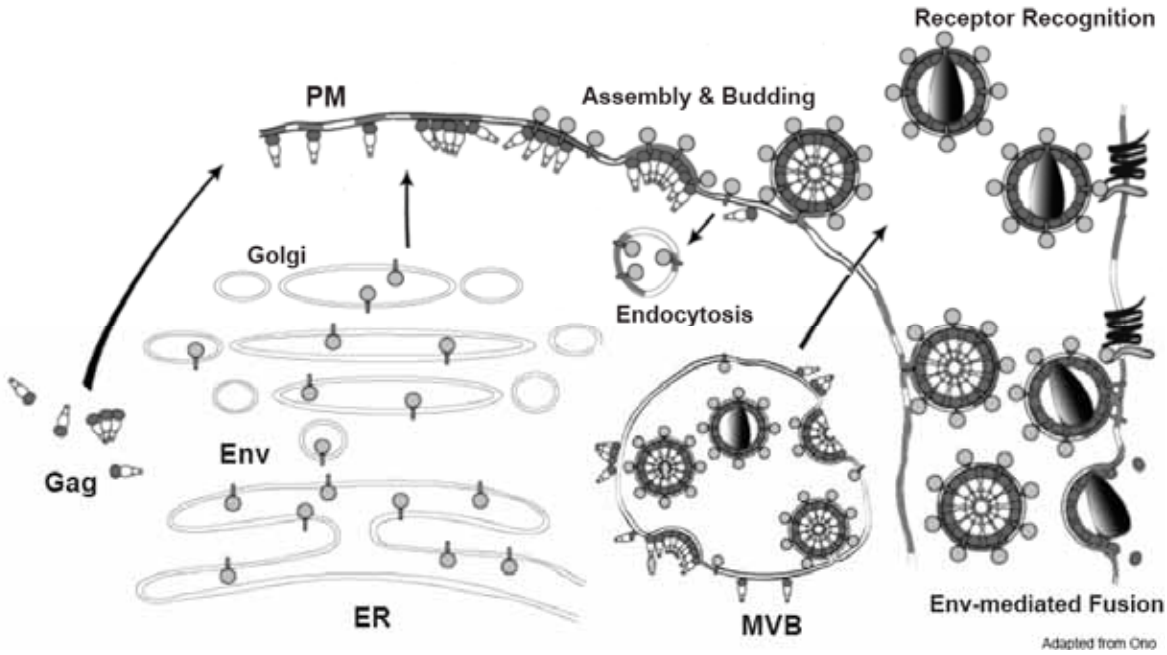

Fig. 8 The biological activities of the HIV-1 Env. (Adapted from figures on the website of Ono Lab at University of Michigan)

The Membrane-spanning Domain of HIV-1 Env

HIV-1 MSD Structure. An important function of gp41 is to anchor the glycoprotein complex within the host-derived viral membrane (165). The precise boundaries of the HIV-1 MSD have not been clearly defined; however, the MSD is one of the most conserved regions in the gp41 sequence. Based on the initial functional studies of HIV-1, the MSD of Env was defined as a stretch of 25 predominantly hydrophobic amino acids that spanned residues K681 to R705 in the NL4-3 sequence (100, 129, 165). These residues were suggested to cross the viral membrane in the form of a α-helix, the length of which is approximately equal to the theoretical depth of a membrane bilayer. A major caveat of this model is that it places a basic amino acid residue (R694) into the hydrophobic center of the lipid bilayer. While some transmembrane proteins do contain charged amino acid residues in their membrane-spanning domains, it is normally considered to be energetically unfavorable without some mechanism to neutralize the charge (68, 99). Point mutation studies have yielded variable results but in general substitution of K681 is detrimental to fusion and infectivity while mutation of R694 or R705 has only a limited effect on these activities (129, 242). On the other hand, accumulating data argues for a different intramembrane structure of the HIV-1 MSD. Serial small deletions (3 amino acid residues) in the region between R694 and R705 showed normal cell-cell fusion, although larger deletions were detrimental (242), suggesting that, with respect to the biological functions of the Env glycoprotein, the length of this region is more important than its amino acid conservation.

A "Snorkeling" Model of the MSD. Previous C-terminal truncation studies of SIV Env (186, 336) suggested that the entire 27 amino acid region is not required for the biological function of this protein. In the case of SIV only the 15 apolar amino acids flanked by K689 and R705 (equivalent to K681 and R694 in HIV) and 6 additional amino

acids (for a total of 23 amino acids) were required for near WT fusion (186, 336). Two more subsequent residues were required (25 amino acids totally) for virus-cell entry and infectivity; while a length of 21 amino acid residues was sufficient for SIV Env to be incorporated into viral particles. These results led to a basic amino acid "snorkeling" model for the SIV MSD (336). In this model, the lysine and arginine (NL4–3 equivalents of K681 and R694) are buried in the lipid bilayer, while their long side chains are proposed to extend outward to the membrane surface and present the positively charged amino groups to the negatively charged head groups of the lipid bilayers. Applied to HIV-1 MSD, this model would predict a hydrophobic intramembrane core of only 12 amino acid residues (compared to 15 amino acid residues in the SIV MSD) between K681 and R694. The hydrophobic region C-terminal to K681 is not sufficient to effectively anchor the protein, since mutation of R694 to a stop codon yielded a non-functional protein that appeared to be retained in the ER (84). This contrasts truncation experiments with the VSV-G glycoprotein, which have shown that a region of 12 hydrophobic amino acids flanked by basic residues is sufficient to anchor the protein in the membrane (3).

The MSD "Core" Domain. The high degree of conservation of the HIV-1 Env MSD, particularly within the "core" region, argues for a functional role in the biological activity of the HIV-1 Env complexes. Substitution of the HIV-1 MSD by equivalent regions from other transmembrane proteins has yielded variable results. Substitution of the HIV-1 MSD with a glycophospholipid anchor abrogated both cell-cell fusion and virus-cell fusion though the chimeric protein could be normally expressed, processed and incorporated into virions (277). Moreover, the replacement of HIV-1 MSD with the MSD of other transmembrane proteins, such as glycophorin A, Vesicular Stomatitis Virus G protein (VSV-G) and influenza hemagglutinin (HA), resulted in an impaired ability of Env complexes to mediate membrane fusion (216, 334). On the other hand, the replacement

of the MSD of Env with that of CD22 (343) did not appear to block the replication of HIV in MT4 cells, and an Env chimera containing the transmembrane region of CD4 (265) has also been shown to induce normal membrane fusion. According to the consensus sequence of the HIV-1 MSD, the 12 amino acid residues in the core region are generally more conserved than those C-terminal to this region (from R694 to R705). Recent studies of the MSD core region have focused on the GXXXG motif in which the glycine residues are the most conserved among all MSD residues. Statistical studies and bacterial models suggested that the GXXXG motif probably constructs a framework for the association of transmembrane helixes (276, 287, 288). It has been demonstrated that the GXXXG motif in the E1 glycoprotein of hepatitis C virus is important for the heterodimerization of its E1 and E2 envelope glycoproteins (63). The membrane-spanning domain of VSV-G also contains a GXXXG motif. Alanine and leucine scanning experiments showed that single mutation of the two glycines reduced the fusion activity of VSV-G to half of WT, but simultaneous mutation of both residues almost completely blocked membrane fusion (64). Similar results were reported for the GXXXG motif in HIV-1 MSD core, in which the fusogenicity of Env started decreasing when more than one glycine residue was substituted for alanine or leucine (215, 216).

In This Dissertation. In order to further understand the structural and functional roles of the HIV-1 MSD in viral replication, we initially substituted all of the residues for leucine within the MSD core, and then sequentially reintroduced the GGXXG motif, and then F683 with V687. We show here that the specific amino acid residues in the helical core of HIV-1 MSD are critical for the fusogenicity of Env complexes and infectivity of HIV-1. The loss of infectivity correlated with impaired virus-cell fusion. However, these mutations in the MSD core did not influence the biogenesis, intracellular transport and incorporation of Env complexes. In further study, we examined the effects of the MSD core “recovery-of-function” mutants on Env-mediated membrane fusion in a molecular

level. We showed that the amino acid composition of MSD core does not influence viral attachment to cell surface, nor CD4 and CXCR4 recognition of HIV-1 Env. However, the cell-cell fusion kinetics of the recovery-of-function mutants was delayed compared to WT. The impaired fusogenecity was not due to arresting of cell-cell fusion at hemifusion stage. Amino acid substitutions within the MSD core can result in limited and localized conformational changes in the gp120 and gp41 ectodomains. In order to understand if the “snorkeling” model is applicable to the HIV-1 MSD, we have constructed a series of nonsense mutants that truncate the HIV-1 gp41 in single amino acid steps at the C-terminus from residue R707 to residue R694. For each mutant Env we have determined membrane stability, fusogenicity and the ability to mediate infectivity. The results of these studies suggest that the 14-residue “core” plus three subsequent hydrophobic amino acids is the minimal anchor domain for HIV-1 Env and as well as the minimal sequence to mediate cell-cell fusion. In contrast to SIV Env, HIV-1 Env requires the entire 25 amino acid region from R707 to K681 to mediate near WT incorporation and infectivity.

TRUNCATION OF THE MEMBRANE-SPANNING DOMAIN OF HUMAN IMMUNODEFICIENCY VIRUS TYPE I ENVELOPE GLYCOPROTEIN DEFINES ELEMENTS REQUIRED FOR FUSION, INCORPORATION AND INFECTIVITY

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ABSTRACT

The membrane-spanning domain (MSD) of the envelope (Env) glycoprotein from human and simian immunodeficiency viruses (HIV and SIV) plays a key role in anchoring the Env complex into the viral membrane, but also contributes to its biological function in fusion and virus entry. In HIV-1, it has been predicted to span 27 amino acids from lysine residue 681 to arginine 707, and encompasses an internal arginine at residue 694. By examining a series of C-terminal truncation mutants of the HIV-1 gp41 glycoprotein that substituted termination codons for amino acids 682 to 708, we show that this entire region is required for efficient viral infection of target cells. Truncation to the arginine at residue 694 resulted in an Env complex that was secreted from the cells. In contrast, a region from residue 681 to 698, which contains highly conserved hydrophobic residues and glycine motifs and extends four amino acids beyond 694R, can effectively anchor the protein in the membrane, allow efficient transport to the plasma membrane, and mediate WT levels of cell-cell fusion. However, these fusogenic truncated Env mutants are inefficiently incorporated into budding virions. Based on the analysis of these mutants, a “snorkeling” model, in which the flanking charged amino acid residues at 681 and 694 are buried in the lipid while their side chains interact with polar head groups, is proposed for the HIV-1 MSD.

INTRODUCTION

Human immunodeficiency virus Type I (HIV-1) infection is initiated by fusion of the viral membrane with that of the target cell and is mediated by the viral envelope glycoprotein (Env). HIV-1 Env, a type-I membrane-spanning glycoprotein, is a trimeric complex composed of three non-covalently linked heterodimers of gp120, the receptor-binding surface (SU) component, and gp41, the membrane-spanning, trans-membrane (TM) component (87, 219, 347, 352). The gp120 and gp41 glycoproteins are synthesized as a precursor gp160 glycoprotein, which is encoded by the *env* gene. The gp160 precursor is co-translationally glycosylated and, following transport to the trans-Golgi network, is cleaved into the mature products by a member of the furin family of endoproteases (352). Mature Env proteins are transported to the plasma membrane, where they are rapidly endocytosed or incorporated into virions (40, 275, 343). Recent evidence suggests that endocytosis and intracellular trafficking of Env is required for its interaction with Gag precursors and for efficient assembly into virions (191).

HIV-1 Env molecules function as quasi-stable “spring-loaded” fusion machines. Recent studies have suggested that several regions of gp120 are reoriented following CD4 binding so that a planar “bridging sheet”, which forms the binding site for the co-receptor (CCR5 or CXCR4) can form (51, 52). Coreceptor binding is necessary for additional conformational changes in gp41 and for complete fusion (21). The gp41 monomer has three subdomains, an ectodomain, a membrane-spanning domain (MSD) and a cytoplasmic domain (331). The ectodomain of gp41, which mediates membrane fusion, is composed of a fusion peptide, two heptad repeats and a tryptophan-rich membrane proximal external region (MPER). Following the binding of gp120 to the CD4 receptor and CCR5/CXCR4 coreceptor, conformational changes are induced in Env and result in the exposure of the gp41 fusion peptide (267). This peptide inserts into the target cell

membrane, allowing gp41 to form a bridge between the viral and cellular membranes. Interaction of the heptad repeats to form a six-helix bundle then brings the target and viral membranes together, allowing membrane fusion to occur (200).

While heptad repeat regions (HR1 and HR2) in the N-terminal ectodomain play key roles in Env-mediated fusion by bringing the viral and cell membranes in close proximity, an important function of gp41 is to anchor the glycoprotein complex within the host-derived viral membrane (165). The precise boundaries of the HIV-1 MSD have not been clearly defined; however, the MSD is one of the most conserved regions in the gp41 sequence. Based on the initial functional studies of HIV-1, the MSD of Env was defined as a stretch of 25 predominantly hydrophobic amino acids that spanned residues K681 to R705 in the NL4-3 sequence (100, 129, 165). These residues were suggested to cross the viral membrane in the form of an alpha-helix, the length of which is approximately equal to the theoretical depth of a membrane bilayer. A major caveat of this model is that it places a basic amino acid residue (R694) into the hydrophobic center of the lipid bilayer. While some transmembrane proteins do contain charged amino acid residues in their membrane-spanning domains, it is normally considered to be energetically unfavorable without some mechanism to neutralize the charge (68, 99). Point mutation studies have yielded variable results but in general substitution of K681 is detrimental to fusion and infectivity while mutation of R694 or R705 has only a limited effect on these activities (129, 241). On the other hand, accumulating data argues for a different intramembrane structure of the HIV-1 MSD. Serial small deletions (3 amino acid residues) in the region between R694 and R705 showed normal cell-cell fusion, although larger deletions were detrimental (241), suggesting that, with respect to the biological functions of the Env glycoprotein, the length of this region is more important than its amino acid conservation.

Previous C-terminal truncation studies of SIV Env (186, 336) suggested that the entire 27 amino acid region is not required for the biological function of this protein. In the case of SIV only the 15 apolar amino acids flanked by K689 and R705 (equivalent to K681 and R694 in HIV) and 6 additional amino acids (for a total of 23 amino acids) were required for near WT fusion (186, 336). Two more subsequent residues were required (25 amino acids totally) for virus-cell entry and infectivity; while a length of 21 amino acid residues was sufficient for SIV Env to be incorporated into viral particles. These results led to a basic amino acid "snorkeling" model for the SIV MSD (336). In this model, the lysine and arginine (NL4–3 equivalents of K681 and R694) are buried in the lipid bilayer, while their long side chains are proposed to extend outward to the membrane surface and present the positively charged amino groups to the negatively charged head groups of the lipid bilayers. Applied to HIV-1 MSD, this model would predict a hydrophobic intramembrane core of only 12 amino acid residues (compared to 15 amino acid residues in the SIV MSD) between K681 and R694. The hydrophobic region C-terminal to K681 is not sufficient to effectively anchor the protein, since mutation of R694 to a stop codon yielded a non-functional protein that appeared to be retained in the ER (84). This contrasts truncation experiments with the VSV-G glycoprotein, which have shown that a region of 12 hydrophobic amino acids flanked by basic residues is sufficient to anchor the protein in the membrane (3).

In order to understand if the "snorkeling" model is applicable to the HIV-1 MSD, we have constructed a series of nonsense mutants that truncate the HIV-1 gp41 in single amino acid steps at the C-terminus from residue R707 to residue R694. For each mutant Env we have determined membrane stability, fusogenicity and the ability to mediate infectivity. The results of these studies suggest that the 12-residue "core" (289) plus three subsequent hydrophobic amino acids is the minimal anchor domain for HIV-1 Env

and as well as the minimal sequence to mediate cell-cell fusion. In contrast to SIV Env, HIV-1 Env requires the entire 25 amino acid region from K681 to R707 to mediate near WT incorporation and infectivity.

MATERIALS AND METHODS

Cells and antibodies. COS-1, 293T (American Type Culture Collection, Rockville, MD) and JC53BL (available from the NIH AIDS Research and Reference Program as TZM-BL) cells were maintained in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and penicillin-streptomycin (all from Gibco-BRL, Rockville, MD). Cells were passaged 3 times per week under conditions of 37°C and 5% CO_2 in humidified incubators and were transfected at 50-70% confluency. The anti-gp120 902 monoclonal antibodies, and anti-gp41 monoclonal antibodies T32, D50 and 2F5 along with HIV-1 patient Ig were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health. The pooled HIV-1 patient sera were provided through the Emory CFAR Clinical Core. The sheep anti-HIV-1 gp120 polyclonal Ab and goat anti-human IgG (H+L) HRP were purchased from Cliniqa Corp (San Marcos, CA) and Pierce (Rockford, IL), respectively.

Glycoprotein and proviral expression constructs. Construction of the HIV-1 Env expression vector pSRHS, which encodes full-length *env, tat*, and *rev* genes from NL4-3, has been described previously (277). This simian virus (SV40 late promoter) based vector contains a Mason Pfizer Monkey Virus (MPMV) derived long terminal repeat providing a polyadenylation signal. A unique *Xba*I site (nucleotide 8213, NL4-3) was previously introduced into the pNL4-3 *env* gene in both pSRHS and the proviral vector pNL4-3. In this study, we employed a two-step overlapping PCR approach to generate DNA fragments containing mutated membrane-spanning domains. Individual stop codons were inserted during the1st round PCR by using forward oligonucleotide primers that contained the required mutations and a reverse oligonucleotide primer *env*M2 (NL4-

3 nucleotides 8500-8534). The 1st round PCR fragment was then used as a reverse mega primer, together with the *env*B7 forward primer (NL4-3 nucleotides 7786-7809) to generate 2nd round PCR fragments, which includes the unique XbaI and BamHI sites. Following digestion with XbaI and BamHI, fragments containing the truncation mutations were subcloned into pSRHS and pNL4-3 to create Env expression vectors and genomic vectors that encode mutants in which the gp41 is truncated in single amino acid steps from residue 707 to residue 694 (Fig.1). All mutations were confirmed by DNA sequencing using a primer approximately 100bp upstream from the MSD sequence. In this study, nucleotides and amino acid residues were labeled according to the NL4-3 sequence (GeneBank accession: AF324493) if not being specifically notified.

Glycoprotein expression and immunoprecipitation. The pSRHS Env expression vectors or proviral DNA were transfected into COS-1 cells using Fugene6 (Roche, Indianapolis, IL) in 6-well plates. At 36-48 h posttransfection, cells were starved for 15 min in methionine-free and cysteine-free DMEM and then labeled for 30 min in methionine-free and cysteine-free DMEM supplemented with ^{35}S-methionine and ^{35}S-cysteine (125µCi/well). The labeled cells were then chased in complete DMEM for 5 h prior to harvest of the supernatant and lysis of the cells. All medium was filtered through a 0.45µm membrane to remove cellular debris. Cells were lysed by a 10 min incubation on ice in lysis buffer (1% Triton X-100, 50 mM NaCl and 0.1% sodium dodecyl sulfate [SDS] in 25 mM Tris-HCl [pH8.0]). Cellular debris was removed by microcentrifugation at 13,200 rpm for 1 min at 4℃. HIV-1 viral proteins were immunoprecipitated from cell lysates and supernatants, by incubating overnight at 4℃ with pooled HIV-1 patient sera plus anti-gp41 monoclonal antibodies T32 and D50. Immune complexes were incubated overnight at 4℃ with fixed *Staphylococcus aureus* cells (or protein-G agarose beads) and pelleted in a microcentrifuge. The pellets were

then washed three times in lysis buffer, and labeled proteins resolved by 10% SDS-PAGE were visualized by autoradiography. The glycoprotein bands were quantified using a Cyclone phosphorimaging system (Packard, Meriden, CT) as previously described (336).

Cell-cell fusion assay. COS-1 cells were transfected with pSRHS expression vectors and 293T cells were transfected with proviral vectors pNL4-3 by using Fugene6. At 36-48h after transfection, cells resuspended by trypsinization were combined with JC53BL indicator cells at a 1:5 ratio. Cell mixtures were incubated for 6 h, 12 h, and 24 h (In the cell-cell fusion assay in which proviral vector pNL4-3 was used, 10uM AZT was added into the cell mixture in order to avoid spread of infectious virions), and then were lysed in luciferase reporter lysis buffer (Promega, Madison WI) using two freeze-thaw cycles. Cellular debris was removed by centrifugation at 13,200 rpm for 5 min at 4℃ in a microcentrifuge (Beckman, Palo Alto, CA). Luciferase substrate at a volume of 100µl (Promega, Madison, WI) was added to 10µl of each cell lysate and light emission was quantified using a Synergy multi-detector microplate reader (Biotek, Vinooski VT).

Cell surface expression of Env glycoprotein. COS-1 cells were transfected with expression vectors, cultured for 36-48 h, and then after removal from the plate, fixed for 20 min at 4℃ in 4% paraformaldehyde (in PBS [pH7.2]). Fixed cells were stained for 1 h at room temperature with 5µg/ml of an Alexa488-conjugated anti-gp120 mAb 902. Stained cells were subjected to flow cytometry analysis using the FACS Calibur system. For some experiments a relative mean fluorescence index (RMFI) was calculated from the product of (% transfected cells) X (mean fluorescence intensity). The Alexa488 conjugation kits were obtained from Invitrogen (Carlsbad, CA).

Infectivity of HIV-1 viruses in JC53BL indicator cells. Medium from 293T cells transfected with proviral vectors was harvested 72 h posttransfection, filtered through a 0.45μm membrane to remove cellular debris, and total virions quantified using a p24 ELISA assay. The p24-normalized virus-containing supernatant (5ng/well) was added to $1X10^5$ JC53BL indicator cells cultured in DMEM containing 1% fetal bovine serum and 80μg/ml DEAE-Dextran. Complete DMEM was added following a 2h incubation and cells were analyzed for luciferase activity 48 h post infection.

Single-round infection. 293T cells were cotransfected with pSRHS expression vectors and the pSG3Δ*env* proviral vector. Medium was collected 72 h posttransfection and then was subjected to p24 ELISA assays. The p24-normalized virus-containing supernatants (5ng/well) were used to infected JC53BL indicator cells. Luciferase activity was measured 48 h after infection.

Incorporation of Env into viral particles. Medium collected from 293T cells 72 h after transfection with proviral vectors was filtered through 0.45um membrane and viral particles were pelleted by ultracentrifugation (100,000×g, 2.5 h) through a 25% sucrose cushion. The viral pellets were resuspended in PBS (pH7.2), and the amount of p24 and gp120 was measured using p24 and gp120 ELISA assays (119). The amount of gp41 in virions was quantitated using chemiluminescence in a Western blot assay with anti-gp41 monoclonal antibody 2F5. Incorporation efficiencies in the viral pellets were compared using the ratio of the amount of gp120 or gp41 to that of p24 for each mutant.

RESULTS

In this study, we constructed a series of C-terminal truncation mutants to define the minimal requirement of the MSD for the structural and functional roles of HIV-1 Env in viral assembly and entry. The amino acid sequences and nomenclature of the MSD truncation mutants are shown in Figure 1. The more conserved HIV-1 MSD "core" region remained intact in all of the mutants.

MSD truncation mutants exhibit normal Env biosynthesis and processing but show differential membrane-anchoring. WT and mutant *env* sequences, in addition to full-length *tat* and *rev* genes, were cloned into the pSRHS vector, in which expression of Env is under the control of the late promoter from simian virus 40 (SV40) and a polyadenylation signal from Mason Pfizer Money Virus (MPMV) (84, 146). Mutant Env glycoproteins expressed in COS-1 cells were metabolically labeled and chased over the course of 5 h. Viral glycoproteins were subsequently immunoprecipitated with pooled HIV-1 patient sera and analyzed by SDS-PAGE followed by autoradiography. Quantification of protein bands was carried out using a phosphorimager (Packard, Meriden, CT). As shown in Figure 2A, with the exception of mutants Env694R-697F, we observed similar ratios of the cell-associated gp160 and gp120 in COS-1 cells expressing either WT or mutant Envs after a 5 h chase. For mutants Env694R-697F the gp120 band was poorly defined and this may reflect increased secretion of these proteins (see below). A majority of the MSD mutants exhibited levels of truncated gp41 that were similar to WT with mutant gp41 bands migrating faster than that of the WT. These data suggested that the introduction of stop codons in the MSD region C-terminal to the "core" residues did not alter the biosynthesis of the glycoprotein precursor or its transport to the Golgi network for completion of carbohydrate processing and cleavage to the SU and TM subunits.

An examination of labeled Env glycoprotein in the culture supernatant showed that levels of gp120 secretion were similar to WT for most of the truncation mutants (Fig. 2B and 2C). However, the gp120 subunit of the shortest Env construct Env694R was secreted 3.2 times more efficiently than that of the WT. Moreover, for this mutant, bands corresponding to both the gp160 precursor and gp41 were also clearly secreted into the culture supernatant, consistent with inefficient anchoring of the truncated Env. Less intense but still elevated levels of gp41 secretion were also observed for the mutants Env695I-700L (Fig. 2B and 2C), while their shedding of gp120 was similar to WT. The remainder of the mutants, Env701S-707R, exhibited slightly lower levels of gp120 shedding to that observed with the wild-type Env and no secreted gp41. These results demonstrate that truncation of gp41 to residue 694, the internal arginine, resulted in an Env protein that is unstable in the plasma membrane and is partially secreted. The mutant, ERRS, in which 11 amino acids, containing a K(X)KXX Endoplasmic Reticulum retention signal, were inserted at the C-terminus of gp41 and which results in the retention of the gp160 precursor in the ER (279) was used as a negative control.

Truncated Env glycoproteins exhibit differential cell surface expression of the Env glycoprotein. Since synthesis and processing of the truncated MSD mutant HIV-1 Env glycoproteins appeared normal, we next examined the surface expression of these mutant proteins to determine whether they were transported to, and retained normally in, the plasma membrane. Transfected COS-1 cells were fixed 48h post-transfection in 4% paraformaldehyde and stained with Alexa488-conjugated anti-gp120 mAb 902. The cells were then subjected to flow cytometry analysis. As shown in Figure 3A, with the exception of mutant Env706V, mutants Env698A-707R exhibited increasing amounts of Env surface expression (105% to 225%) compared to WT (Fig. 3A). Consistent with the

observed secretion of gp41 into the supernatant for mutants Env694R-697F, low levels of Env surface expression (14-35% of WT) was observed (Fig. 3A). Although mutants Env698A-700L also exhibited low levels of truncated gp41 secretion (Fig. 2C), Env was stably presented on cell surface at relatively high levels (120-150% of WT) for these mutants. The enhanced levels of surface expression observed for a majority of the truncation mutants may reflect the absence of endocytosis signals located in the cytoplasmic domain of the WT gp41 (22, 74, 231, 337).

Analysis of truncated Envs reveals a minimal MSD sequence to mediate cell-cell fusion. Since a majority of the HIV-1 Env truncation mutants were expressed stably on the cell surface, we investigated whether a specific number of amino acid residues were necessary in this region to confer on Env the capacity to mediate cell-cell fusion. Transiently transfected COS-1 cells were co-cultured with JC53BL indicator cells, which contain a luciferase reporter gene under the control of the HIV-1 LTR promoter and which can be activated by the Tat protein (328). Cell-cell fusion was measured by calculating the relative luciferase enzyme activity in cells expressing the mutant Envs compared to those expressing WT Env at 6 h, 12 h, and 24 h after co-culture. The results of a representative experiment are shown in Figure 3B, and demonstrate that the three shortest Env proteins, Env694R-696V, were biologically inactive. The inability of these mutants to mediate cell-cell fusion is consistent with their lower levels of surface expression and reduced anchorage. At 24 h, mutant Env697F exhibited 20% WT fusion, but the remaining mutants induced near WT levels (60-93%) of cell-cell fusion. Thus an Env protein with just 4 amino acids extending beyond 694R exhibited effective WT fusion as shown by this assay.

In order to examine whether the impaired cell-cell fusion of the Env truncation mutants is also because of fusion arrest at a hemifusion stage, we performed a three-color cell-cell fusion experiment. The Env-expressing 293T cells were stained with a cytoplasmic dye, Cell Tracker Green (Invitrogen), and the JC53BL target cells were double stained with a cytoplasmic dye, Cell Tracker Blue (Invitrogen), and a membrane dye, DiI (Invitrogen). The cells were subjected to microscope observation after a co-culture of 2 h. We did not find any evidence of hemifusion intermediates, in which DiI was transferred from the plasma membrane of target cells to that of donor cells, but the intracellular dyes Cell Tracker Blue and Cell Tracker Green did not (data not shown).

MSD truncation mutants show different patterns of cell-cell fusion in the context of virus. It has been known that the intracellular behavior of HIV-1 Env can change in the presence of other viral proteins (242). Therefore, we examined the biosynthesis, surface expression and induction of cell-cell fusion by these mutants in the context of virus by substituting the mutant *env* genes into the proviral vector pNL4-3. In pulse-chase experiments similar to those shown for the pSRHS expression vector (Fig. 2), we observed that in the context of the pNL4-3 genome all but one of the truncated Env precursor gp160s were normally expressed and proteolytically processed to gp120 and gp41. The exception was Env706V, which exhibited a drastic reduction in *env* gene products (Fig. 4A). This unexpected effect of a single point mutation within this codon was confirmed through mutagenesis of the 707R codon (AGG) to TAG and TAA, in addition to the initial TGA – in all cases synthesis of Env was reduced. Additional PCR studies have shown that, for this mutant, Env mRNA levels are reduced (Data not shown), and it is likely that bases in this codon are involved in facilitating Env mRNA splicing. Flow cytometric analyses of the provirus-expressing cells showed a similar

pattern of cell surface staining to that in Figure 4, again with the exception of Env706V which exhibited background staining (data not shown).

In order to determine the ability of virus producing cells to mediate cell-cell fusion, 293T cells transfected with proviral DNAs were co-cultured with JC53BL indicator cells for 6 h, 12 h, and 24 h in the presence of 10μM AZT to prevent additional rounds of virus replication and then assayed for luciferase activity. In the context of virus, we again observed that MSD mutants Env694R-696I were unable to mediate cell-cell fusion (Fig. 4B). Surprisingly, mutant Env697F in this context induced WT cell-cell fusion rather than the reduced level observed in the context of pSRHS-based expression in COS-1 cells.

MSD truncation mutant viruses show defects in infectivity: Virions, produced by transfection of 293T cells and normalized based on p24 content, were used to infect JC53BL indicator cells. At 48h post-infection luciferase activity in the indicator cells was quantitated. As shown in Figure 5A, only mutant Env707R exhibited any significant evidence of infection (30% that of WT), the remaining mutants induced luciferase levels close to background. These data suggest that the region C-terminal of the MSD core (residues 695-707) is critical for Env-mediated infectivity in the context of the virions. In order to rule out possible problems with the proviral constructs, these results were confirmed in a single-round infection pseudotyped virus assay (Fig. 5B). In this assay, pSG3Δ*env* was pseudotyped with WT and mutant Envs expressed in COS cells from the pSRHS vector, and then the pseudotyped virions were used to infect JC53BL indicator cells. A similar pattern of infectivity to that observed in the NL4-3 infectivity assay (Fig. 5A) was seen in this assay, with near background levels of infectivity for mutants 694-703, confirming the importance of the MSD core proximal region for infectivity. In this pseudotype virus system a small increase in virus infectivity was observed for mutants

Env704N-706V, while a lower level of infection was seen for Env707R (14.6%) relative to WT.

MSD truncation mutant Envs are incorporated into viral particles at decreased levels. In order to determine whether a defect in Env incorporation might contribute to the impaired infectivity of the MSD mutants, we measured the incorporation efficiency of a subset of the mutated Envs into viral particles. The amount of gp120 and p24 in viral pellets, after ultracentrifugation through a 25% sucrose cushion, was analyzed using gp120 and p24 ELISAs. We observed that the shortest Env mutant 694R incorporated 83.8% less gp120 into virus particles relative to WT (Fig. 6A). This increased to approximately 25% of WT incorporation for mutants 697A and 702I, and to a maximum of almost 50% that of WT for mutant 707R.

Because we were concerned that the C-terminal truncation of HIV-1 Env may cause shedding of gp120 from the viral surface, we also examined the amount of gp41 associated with viral pellets using Western blot with anti-gp41 mAb 2F5. As shown in Fig. 6B and 6C, similar to its incorporation of gp120, the Env mutant 694R incorporated 79.2% less gp41 into viral particles than WT. However, the addition of only two amino acid residues following arginine 694 significantly enhanced the gp41 incorporation of Env mutant 696V to 40% of WT. This increased to approximately 80% of WT for mutant 697F and 702I. The Env mutant 707R has approximately equivalent amount of gp41 incorporated into viral particles to WT. These results indicated that the MSD “core” region plus three following amino acid residues is sufficient for efficient incorporation of Env glycoprotein into viral particles; however, the lack of residues C-terminal to this region decreased the stability of gp120 on the viral surface. Consistent with its

expression profile in the context of a provirus, Env mutant 706V showed undetectable levels of either gp120 or gp41 in viral pellets.

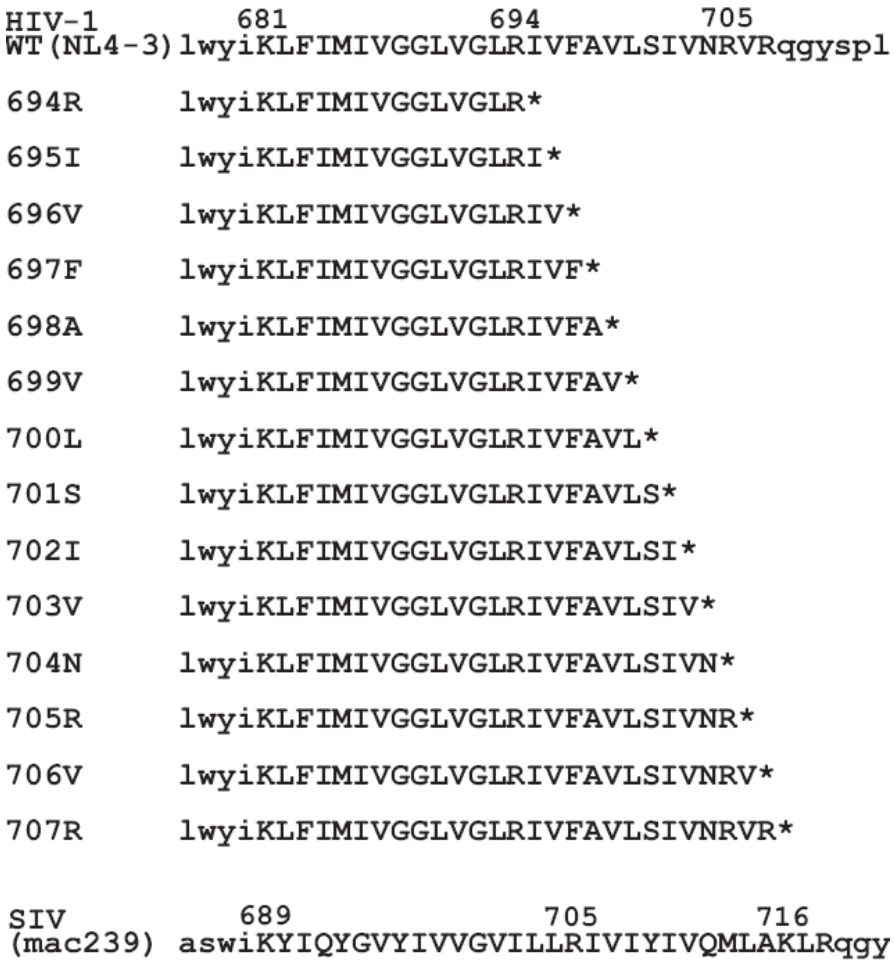
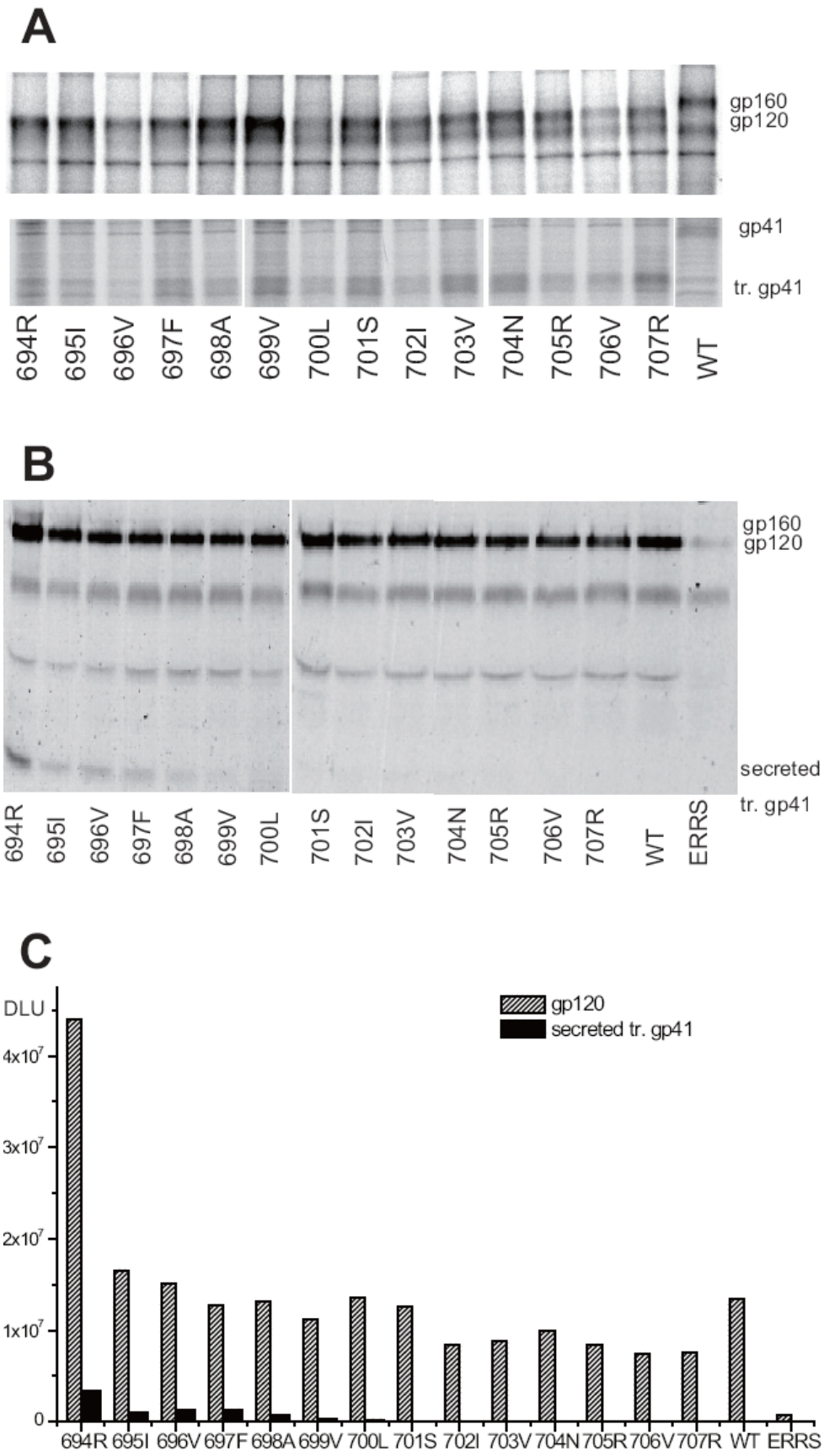


Fig.1 Amino acid sequence of HIV-1 MSDs. The amino acid residues of the putative MSD region are shown as uppercase letters and the flanking sequences as lowercase ones. Truncation mutations were generated by PCR mutagenesis to insert a stop codon (*) subsequent to amino acid number (i.e., 694R has a stop codon in place of amino acid 695). The position of the MSD in the sequence of HIV-1 NL4-3 is marked above the positively charged amino acid residues. The MSD of SIVmac239 is shown as a reference.

Fig. 2 Expression profiles of HIV-1 Env glycoprotein. COS-1 cells transiently transfected with SV40-based Env expression vectors were metabolically labeled and chased over 5 h. Both cellular Env glycoprotein (A) and those in supernatant (B) were immunoprecipitated, followed by 10% SDS-PAGE analysis and autoradiography. (C) The bands of gp120 and truncated gp41 in supernatant were quantified by phosphorimager calculation, and the intensity of bands is shown as digital light units (DLU).


A
gp160
gp120
gp41
tr. gp41
694R
695I
696V
697F
698A
699V
700L
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705R
706V
707R
WT
B
gp160
gp120
secreted
tr. gp41
694R
695I
696V
697F
698A
699V
700L
701S
702I
703V
704N
705R
706V
707R
WT
ERRS
C
DLU
4x10^7
3x10^7
2x10^7
1x10^7
0
gp120
secreted tr. gp41
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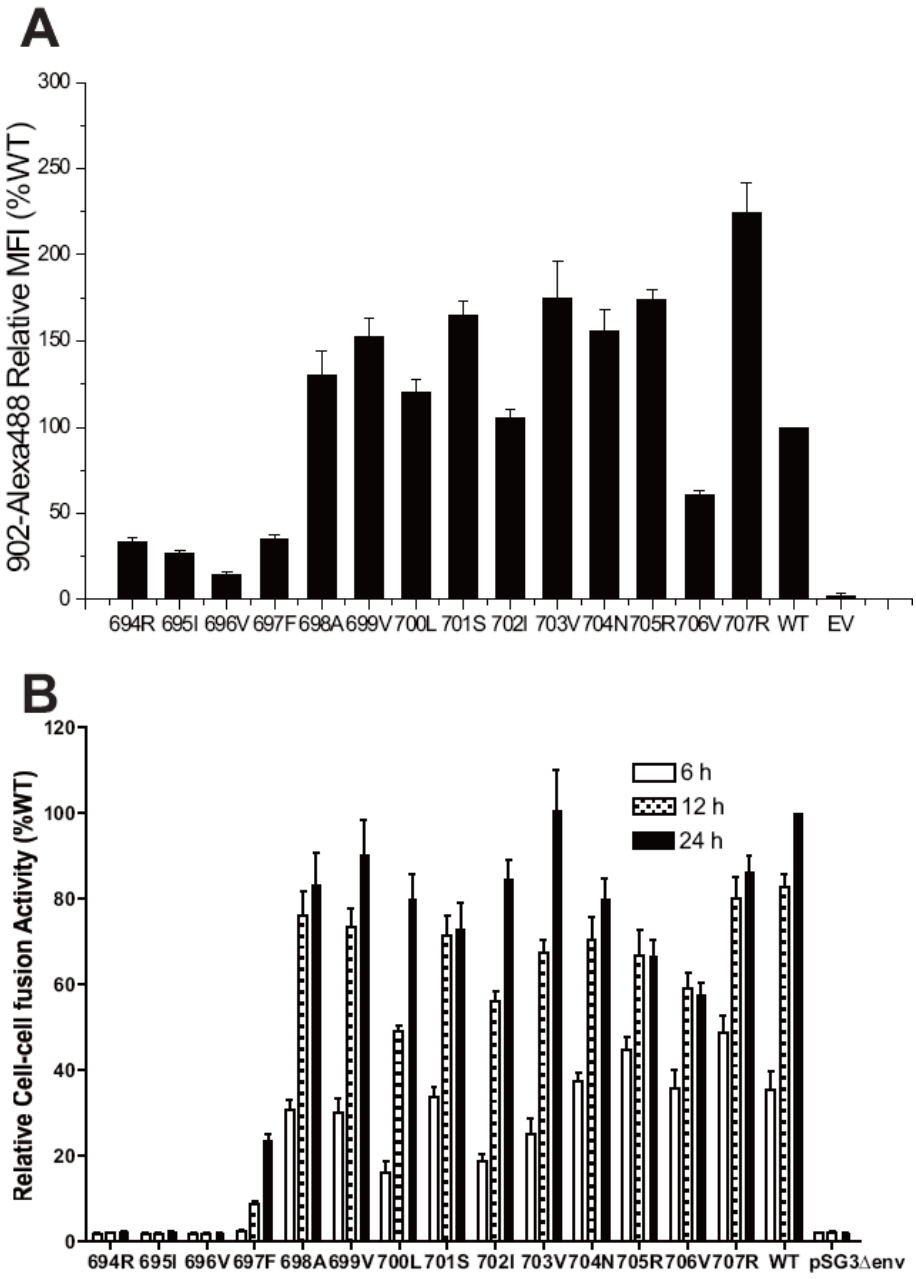

Fig. 3 Cell surface level of Env glycoprotein and cell-cell fusion examined using SV40-based Env expression vectors. (A) COS-1 cells transfected with pSRHS expression vectors were fixed and labeled with Alexa 488-conjugated anit-gp120 mAb 902, then analyzed by flow cytometry. The surface levels of Env glycoprotein are shown as relative mean fluorescence index (MFI) compared to that of WT. (B) Cell-cell fusion assay. COS-1 cells transfected with pSRHS expression vectors were cocultured with JC53BL indicator cells. Cell mixtures were lysed after the incubation of 6 h, 12 h and 24 h, and then luciferase activity was measured. Results are from at least three independent experiments; error bars represents standard deviations from the means. EV: empty vector.

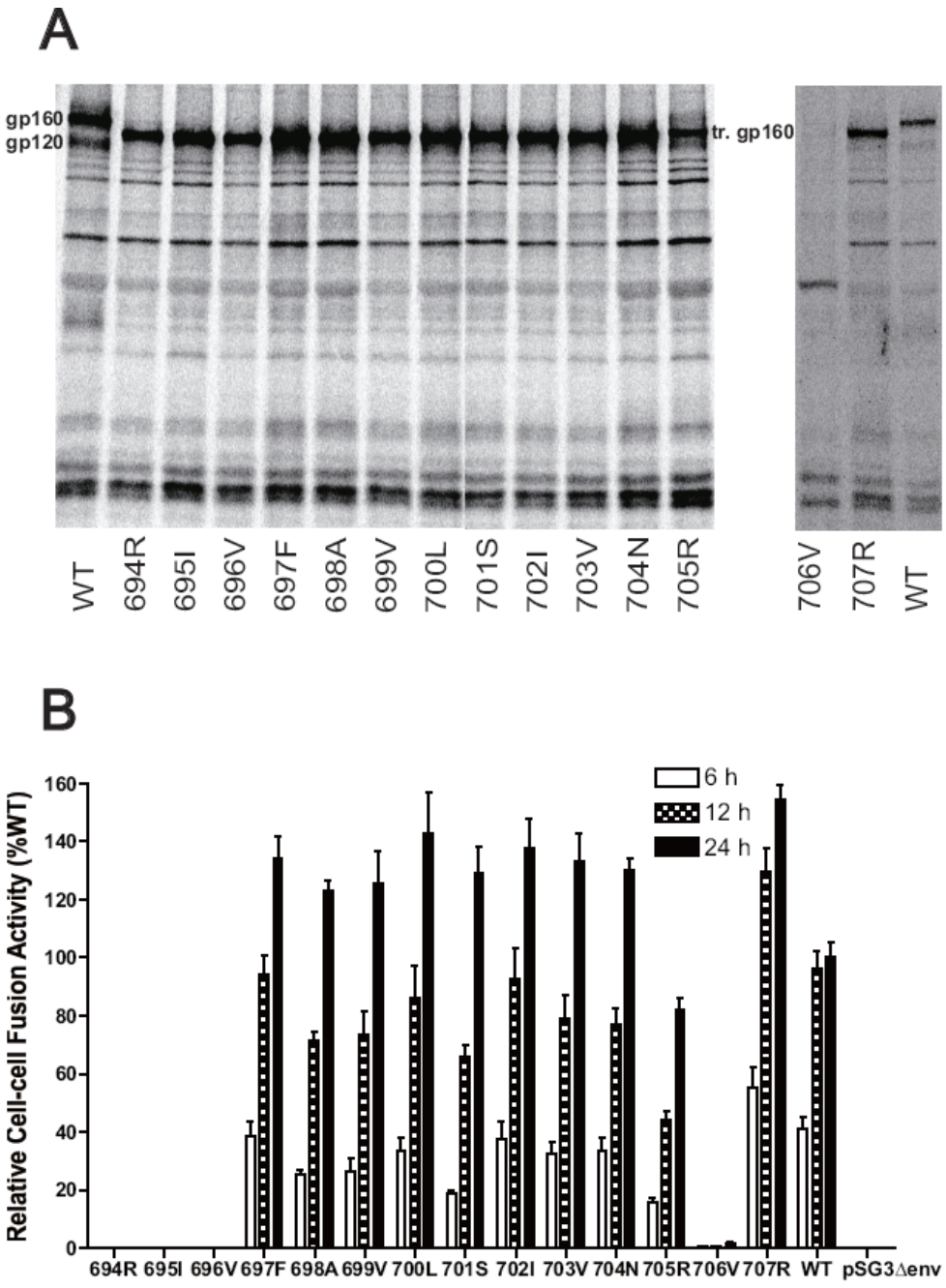

Fig. 4 Cell-cell fusion examined in the context of provirus expression. (A) 293T cells transiently transfected with proviral vectors were metabolically labeled and chased over 5 h. Cellular Env glycoprotein was immunoprecipitated, followed by 10% SDS-PAGE analysis and autoradiography. (B) 293T cells transfected with proviral DNA were mixed with JC53BL indicator cells and cocultured for 6 h, 12h and 24 h with 10um AZT before the measurement of luciferase activity. Results are from at least three independent experiments; error bars represent standard deviations from the means.

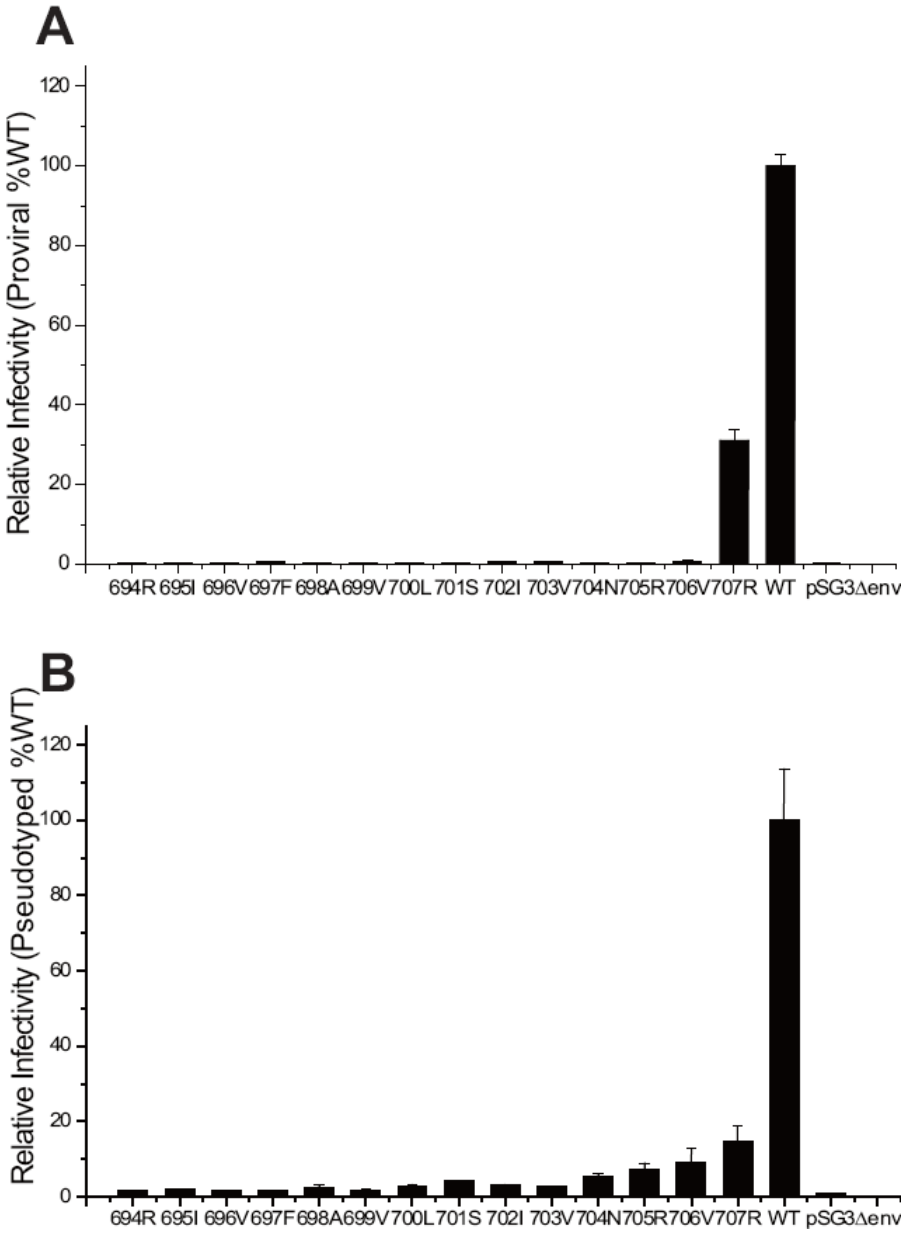

Fig. 5 Infectivity of HIV-1 Env truncation mutants. (A) Mutant viruses containing truncated Env glycoprotein were produced by transfecting proviral DNA into 293T cells. The p24 normalized cell culture supernatant was used to infect JC53BL indicator cells. Cells were lysed after 48 h of incubation and luciferase enzyme activity was assayed. (B) Single-round infectivity assay. Env-defective proviral construct pSG3Δ*env* was cotransfected into 293T cells with WT and mutant Env expression vectors. Pseudotyped viruses in the cell culture supernatant were quantified using a p24 ELISA assay. The p24-normalized supernatant was then used to infect JC53BL indicator cells. Infectivity of MSD truncation mutants is shown as relative luciferase activity compared to that of the WT. Results are from at least three independent experiments; error bars represent standard deviation from the means.

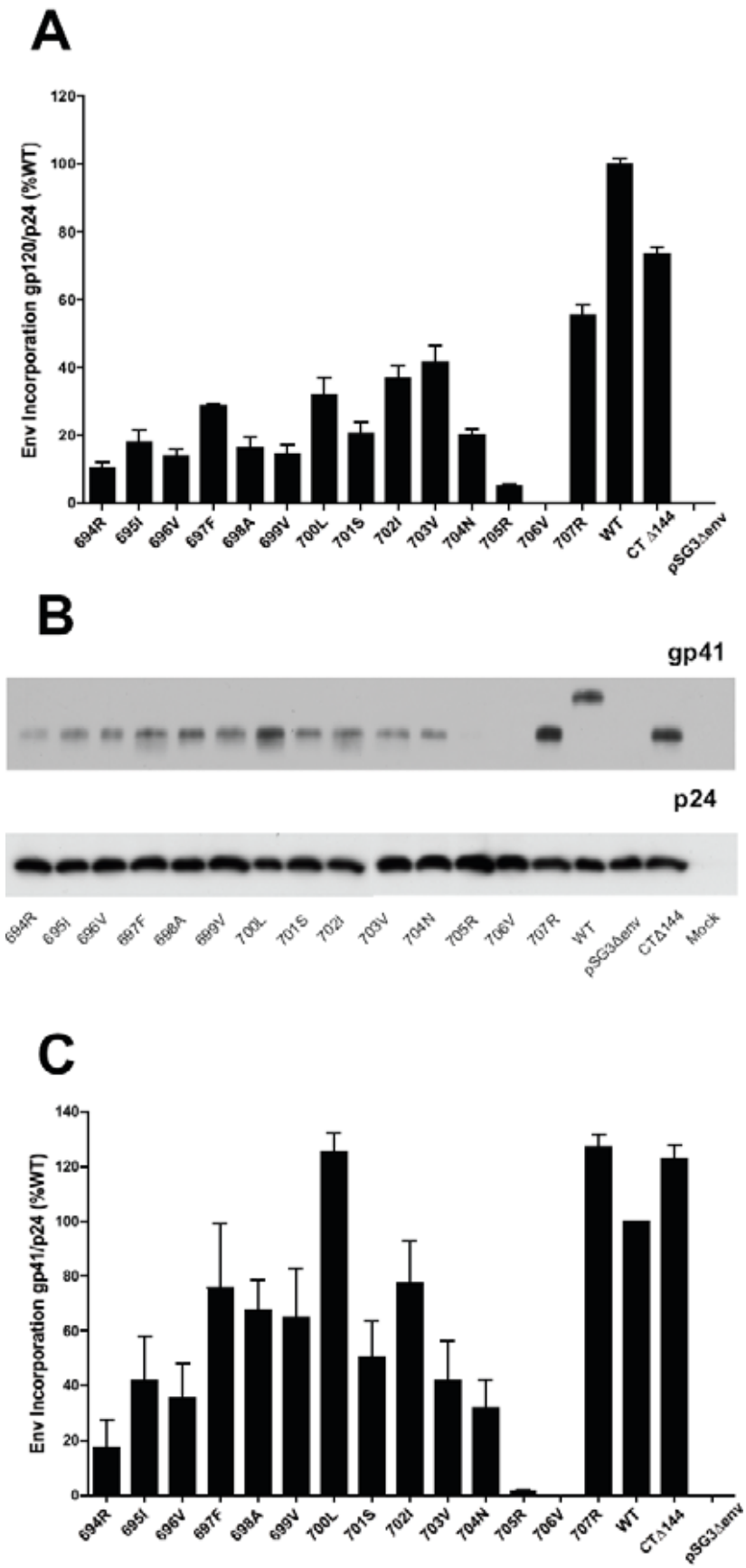
A
Env Incorporation gp120/p24 (%WT)
120
100
80
60
40
20
0
694R
695I
696V
697F
698A
699V
700L
701S
702I
703V
704N
705R
706V
707R
WT
CT Δ144
pSG3Δenv
B
gp41
p24
694R
695I
696V
697F
698A
699V
700L
701S
702I
703V
704N
705R
706V
707R
WT
pSG3Δenv
CTΔ144
Mock
C
Env Incorporation gp41/p24 (%WT)
140
120
100
80
60
40
20
0
694R
695I
696V
697F
698A
699V
700L
701S
702I
703V
704N
705R
706V
707R
WT
CTΔ144
pSG3Δenv

Fig. 6 Incorporation of Env into virions. 293T cells were transfected with proviral DNA and cultured for 72 h. Viral particles in the medium were pelleted through a 25% sucrose cushion by ultracentrifugation. (A) Viral pellets were subjected to gp120 ELISA and p24 ELISA. The incorporation efficiency is calculated as the ratio of the concentration of gp120 to that of p24 in virions. The standard deviations of three repeats for each experiment are shown as error bars. (B and C) The viral pellets were subjected to western blot with anti-gp41 monoclonal antibody 2F5 and anti-p24 monoclonal antibody 183-12H-5C. The gp41 and p24 bands were quantified using a OptiQuant software (Packard, Meriden, CT).

DISCUSSION

The membrane-spanning domain (MSD) of the HIV-1 gp41 glycoprotein – the transmembrane subunit of Env – is essential for anchoring the Env complex in the viral membrane. However, the region likely plays additional roles during virus entry, since substitutions of the MSD with those of other transmembrane proteins were not able to fully restore the biological activities of HIV-1 Env glycoprotein (216, 265, 334, 343). Although the hydrophobicity of amino acid residues predicts an MSD region containing 25 amino acid residues, spanning from 681K to 707R, the precise boundaries of the gp41 MSD have not been clearly defined. Previously, we have reported that, within this predicted MSD, the N-terminal "core" region (12 highly conserved hydrophobic amino acid residues) is necessary for the biological functions of Env (289). In contrast, other groups have shown that the C-terminal region (13 amino acid residues, less conserved than the "core" region) of this 25 amino acid MSD of HIV-1 Env was more sensitive to changes in length than to changes in sequence (241). We, and others, have shown that expression of Env truncation mutants, which have stop codons just upstream of the MSD, results in secretion of gp120 and gp41 (84, 100, 318). In the current study, we used serial C-terminal MSD truncation mutants to understand the structural requirements of the gp41 membrane-spanning domain and its relationship to the biological activities of HIV-1 Env. The properties of these truncated Envs are summarized in Table 1.

The primary function of the HIV-1 MSD is to serve as a membrane anchor of the Env glycoprotein in both viral and cellular membranes. Our results demonstrate that not all 25 amino acid residues are required for this function (Table 1). While the 12 amino acid MSD "core" region alone (following insertion of a stop codon at 694R) is not enough to

stably anchor the Env glycoprotein in the membrane, one additional subsequent amino acid residue can significantly reduce the amount of truncated gp41 and gp160 that is

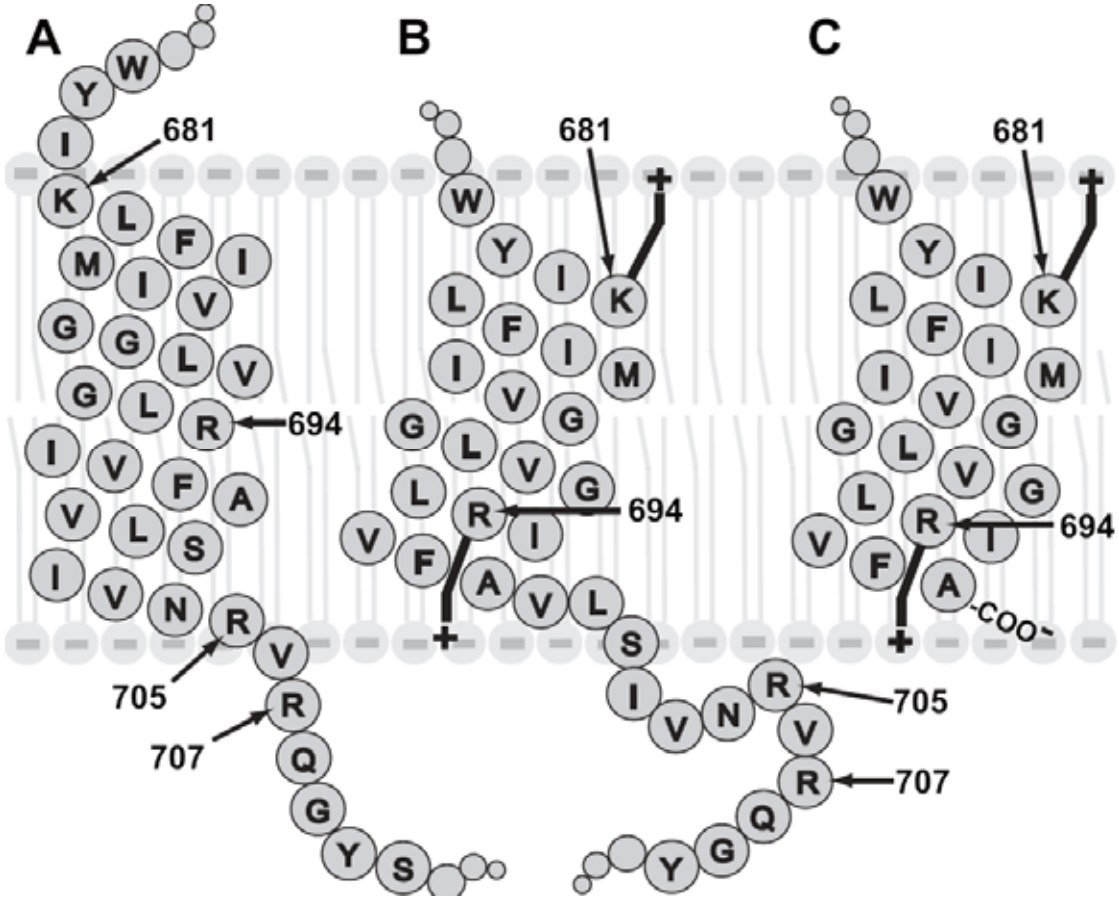

Fig. 7 Two alternative structures for the HIV-1 MSD. (A) The original HIV-1 MSD model predicts a structure of 25 amino acid residues from K681 to R705 in the form of a α -helix. The charged amino acid R694 is buried in the membrane. (B) An alternative "snorkeling" model of HIV-1 MSD is supported by the data presented in this study. This model predicts a α-helical "core" region (12 hydrophobic residues between K681 and R694) and provides a potential mechanism for reduction in the size of the membrane anchor and neutralization of the intramembrane lysine/arginine side chains. Interactions of the positively charged (+) side chains of K681 and R694 with the negatively charged phospholipid head groups (-) are shown. (C) In the "snorkeling" model of truncation mutant 698A, the carboxyl group of the C-terminal alanine 698 is excluded from the hydrophobic environment by interacting with the aqueous environment at the membrane interface.

secreted. Moreover, the addition of 3 more amino acids (mutant 698A) further stabilizes the protein in the membrane and restores fusion capacity to wild-type levels - even though extending Env beyond residue 701 was required to completely prevent secretion. Therefore, a 17 amino acid MSD is the minimal requirement to anchor Env on the cellular surface. These results are consistent with our previous analysis of the SIV MSD (336), and of a model in which Lysine 681 and Arginine 694 "snorkel" within the hydrophobic region of the lipid bilayer (Fig. 7B). Theoretically, the alpha-helix formed by the 14 amino acid residues (K681-R694) would span 21Å. The length of the side chain of lysine is approximately 6.4Å, and arginine 7.0Å. Thus the addition of the length of the 14-aa alpha-helix and those of the two side chains of K681 and R694 reaches 34.4Å. Therefore, the "snorkeling" structure is long enough to span a biological lipid bilayer, which is usually 30-40Å in depth.

In the context of this model, the C-terminal carboxyl group of the arginine 694 in the shortest truncation mutant, Env694R, would be positioned within the hydrophobic core of the lipid bilayer, in an energetically unfavorable position. By contrast, addition of four amino acids (mutant Env698A) could provide the required peptide length for this carboxyl group to reach the polar head groups of the membrane and stabilize the protein-membrane interaction (Fig. 7C).

The length of a glycoprotein's MSD is critical not only for membrane anchoring, but also for normal intracellular trafficking and cell surface transport. Internal deletion studies of VSV-G MSD have shown that at least a 14 amino acid MSD (flanked by a lysine and an arginine) was necessary to transport VSV-G to cell surface (3). Shorter ones (8 to 12 amino acid residues) still appeared to be able to span intracellular membranes, but were retained in the Golgi region of the cell. In contrast, all of the mutants of HIV-1 described

here, including Env694R, undergo intracellular trafficking from the endoplasmic reticulum to the plasma membrane. High levels of surface expression do however require at least 4 additional residues subsequent to the MSD “core” region (mutant Env698A).

The amino acid length of an MSD is also critical for the fusogenicity of glycoproteins. For influenza HA, results from analogous C-terminal truncation mutants have shown that MSDs shorter than 15 amino acids could not be stably presented on the cell surface and that at least a 17 amino acid MSD is required to mediate full cell-cell fusion (11). The 2 amino acid shorter form of the HA MSD resulted in hemifusion intermediates. The minimal MSD requirement for HIV-1 Env to mediate WT cell-cell fusion is the “core” region plus 4 additional amino acids (mutant 698A with an 18 amino acid MSD); although, mutant Env697F exhibited approximately 20% the fusion activity of WT when expressed from the pSRHS expression vector in Cos-1 cells. Interestingly, when Env697F was expressed in the context of the pNL4-3 provirus in 293T cells WT cell-cell fusion was observed after mixing with JC53BL cells. It is not clear at the present time whether this reflects an enhancing interaction of the mutant Env with another viral encoded protein or a difference in the relative fusogenicity of the producer cells (293T cells vs Cos-1 cells).

Truncation mutagenesis of the SIV Env showed that the entire cytoplasmic domain as well as 4 amino acids of the C-terminal domain of the MSD is dispensable for fusogenicity and the production of infectious virions (336). In contrast, the majority of the truncated HIV-1 Env MSD mutants were completely defective for infectivity, with the exception of mutant 707R which was only 30% as infectious as WT. Although the MSD truncation mutants also impaired the incorporation efficiency of Env mutants into viral particles, the defects in infectivity can only partially be explained by reduced

incorporation. The cytoplasmic domain of HIV-1 gp41 also plays critical roles in viral infection. Studies have shown that the Env truncation mutant with a stop codon in place of that for Y710 (based on NL4-3 sequence) is incorporated into viral particles at near WT levels when produced in 293T cells, but is not able to mediate multiple-round infection in PBMC due to a lack of Env incorporation (226). Therefore, an intact gp41 MSD is necessary but not sufficient for HIV-1 Env to initiate normal viral infection. It is worthy to note that for both Y710stop and mutant Env707R, gp41 was incorporated with WT efficiency into viral particles but gp120 incorporation was reduced by 25% and 50% respectively. This, coupled with the reductions in both gp41 and gp120 observed with further truncation of the MSD, demonstrates that Env incorporation and stability are very sensitive to the number of amino acid residues at the C-terminal boundary of the gp41 MSD, and that the entire MSD region of HIV-1 Env and the cytoplasmic domain is required for its stable assembly into virions.

Based on the original assignment of residues 681-705 as the MSD of HIV-1 Env, the most perplexing aspect is that this region contains an arginine residue at position 694 (Fig. 7A). Several mechanisms have been proposed to account for the compatibility of charged amino acid residues with the hydrophobic environment of membrane. One hypothesizes that a protonated arginine or lysine residue could remain in an energetically favorable state within membrane, but only in the very middle of the lipid bilayers (357). This argument, however, was based solely on chemical modeling and mathematical calculations and lacks empirical evidence. An alternative argument, given that charged amino acid residues are not energetically favorable in lipid bilayers, is that these residues have to be either restrained in certain structures (e.g. ion channels) or electronically neutralized by counter ions from interacting proteins (e.g. the MHC-II complex) to avoid contact with the hydrophobic environment of the membrane (68, 195).

Neither of the latter two mechanisms appears to be applicable to either HIV-1 or SIV Env, since there is no evidence for additional proteins associated with the MSD of the Env trimer in the virion and the results of the mutagenesis experiments described previously for SIV and here for HIV-1 would result in the asymmetric positioning of R694 in the membrane. A third suggestion is that the positively charged group of R694 could form a cation-π interaction with the benzyl circles of aromatic amino acid residues, thereby neutralizing its charge (83). However, at the amino terminal end of the region, the vertical distance (18 Å) between F683 and R694 on the surface of alpha-helix is more than the length to which the side chains of the two residues could reach (phenylalanine 5.17 Å, arginine 7.0 Å). Moreover, at the carboxy end of the region, the residue F697 is unlikely to contribute to the intramembrane stability of R694 because a deletion of three amino acid including F697 had no effect on the fusogenicity of HIV-1 Env (241). Therefore, the cation-π interaction is probably not applicable to the HIV-1 MSD. The snorkeling model we have proposed previously (336) would account for both the high level of conservation observed for the N-terminal "core" region of the MSD and would provide a mechanism through which this relatively short (12 and 15 amino acids in HIV-1 and SIV respectively) region could be stably accommodated within the membrane (Fig 7B).

Nevertheless, it is clear that simply anchoring the HIV-1 Env protein in the membrane is insufficient for a stable gp41-gp120 trimer to be incorporated into virions or to function effectively to mediate viral entry. At this time the role played by additional residues C-terminal to 698A is not clear, but it is possible that in the absence of this region, the short MSD is unable to provide stability to the trimer structure needed to maintain a biologically functional "unsprung" structure (192, 193).

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ROLE OF THE MEMBRANE-SPANNING DOMAIN OF HUMAN IMMUNODEFICIENCY VIRUS TYPE I ENVELOPE GLYCOPROTEIN IN CELL-CELL FUSION AND VIRUS INFECTION

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ABSTRACT

The membrane-spanning domain (MSD) of the Human Immunodeficiency Virus Type I (HIV-1) gp41 glycoprotein is critical for its biological activity. Previous C-terminal truncation studies have predicted an almost invariant core structure of 12 amino acid residues flanked by basic amino acids in the HIV-1 MSD that function to anchor the glycoprotein in the lipid bilayer. To further understand the role of specific amino acids within the MSD core, we initially replaced the core region with 12 leucine residues and then constructed recovery-of-function mutants in which specific amino acid residues (including a GGXXG motif) were reintroduced. We show here that conservation of the MSD core sequence is not required for normal expression, processing, intracellular transport, and incorporation into virions of the envelope glycoprotein (Env). However, the amino acid composition of the MSD core does influence the ability of Env to mediate cell-cell fusion and plays a critical role in the infectivity of HIV-1. Substitution of conserved amino acid residues with leucine blocked virus-to-cell fusion and subsequent viral entry into target cells. This restriction could not be released by C-terminal truncation of the gp41 glycoprotein. These studies imply that the highly conserved core residues of the HIV Env MSD, in addition to serving as a membrane anchor, play an important role in mediating membrane fusion during viral entry.

INTRODUCTION

The envelope glycoprotein (Env) of Human Immunodeficiency Viruses Type I (HIV-1) is a trimeric complex composed of three non-covalently linked dimers of gp120, the receptor-binding surface (SU) component, and gp41, the membrane-spanning, trans-membrane (TM) component (87, 219, 347, 352). The gp120 and gp41 glycoproteins are synthesized as a precursor gp160 glycoprotein, which is encoded by the *env* gene. The gp160 precursor is co-translationally glycosylated and, following transport to the trans-Golgi network, is cleaved into the mature products by a member of the furin family of endoproteases (352). Mature Env proteins are transported to the plasma membrane, where they are rapidly endocytosed or incorporated into virions (40, 275, 343). Recent evidence suggests that endocytosis and intracellular trafficking of Env is required for its interaction with Gag precursors and for efficient assembly into virions (191).

The gp41 monomer has three subdomains, an ectodomain, a membrane-spanning domain (MSD) and a cytoplasmic domain (331). The ectodomain of gp41, which mediates membrane fusion, is composed of a fusion peptide, two heptad repeats and a tryptophan-rich membrane proximal external region (MPER). Following the binding of gp120 to the CD4 receptor and CCR5/CXCR4 coreceptor, conformational changes are induced in Env and result in the exposure of the gp41 fusion peptide (267). This peptide inserts into the target cell membrane, allowing gp41 to form a bridge between the viral and cellular membranes. Interaction of the heptad repeats to form a six-helix bundle then brings the target and viral membranes together, allowing membrane fusion to occur (200).

The membrane-spanning domain is one of the most conserved regions of the gp41 sequence. Currently, two models have been proposed to explain the intramembrane

topology of MSD from several genetic studies. In the initial model, 25 amino acid residues (from K681 to R705 in the NL4-3 sequence) were suggested to cross the viral membrane in the form of alpha-helical structure (100, 129, 165), the length of which is approximately equal to the theoretical depth of a membrane bilayer. A major caveat of this model is that it places a basic amino acid residue (R694) into the hydrophobic center of the lipid bilayer without any known mechanism to neutralize the basic amino group. Point mutation studies have yielded variable results but in general substitution of K681 is detrimental to fusion and infectivity while mutation of R694 or R705 has only a limited effect on these activities (129, 241). On the other hand, accumulating data argues for a different intramembrane structure of the HIV-1 MSD. Serial small deletions (3 amino acid residues) in the region between R694 and R705 showed normal cell-cell fusion, although larger deletions were detrimental (241), suggesting that, with respect to the biological functions of the Env glycoprotein, the length of this region is more important than its amino acid conservation. Moreover, previous C-terminal truncation studies of SIV Env (186, 336) and more recently of HIV-1 Env (Yue *et al*., in preparation) have demonstrated that the entire 25 amino acid region is not required for the biological function of Env. In the case of SIV only the 15 apolar amino acids flanked by K689 and R705 (equivalent to K681 and R694 in HIV) and 6 additional amino acids (for a total of 23 amino acids) were required for near WT fusion (186, 336), and in HIV we have shown that 18 amino acid residues (from K681 to A698) are enough to anchor the truncated Env glycoprotein in the membrane and allow it to mediate cell-cell fusion. From these data, we have proposed a topology where the flanking K681 and R694 residues “snorkel” in the membrane (336). In this model for HIV-1 Env, 12 amino acid residues (from L682 to L693) form a hydrophobic core buried within the membrane, while the backbones of K681 and R694 residues, which are flanking the hydrophobic core region, are also buried within the membrane. However, their long side chains are proposed to

extend outward to the surface and present the positively charged amino groups to the negatively charged head groups of the lipid bilayers – hence the term “snorkel”. Arginines 705 and 707 are located at the interface of membrane and cytoplasm in this model.

The high degree of conservation of the HIV-1 Env MSD, particularly within the “core” region, argues for a functional role in the biological activity of the HIV-1 Env complexes. Substitution of the HIV-1 MSD by equivalent regions from other transmembrane proteins has yielded variable results. Replacement of the HIV-1 MSD with a glycophospholipid anchor abrogated both cell-cell fusion and virus-cell fusion though the chimeric protein could be normally expressed, processed and incorporated into virions (277). Moreover, the replacement of HIV-1 MSD with the MSD of other transmembrane proteins, such as glycophorin A (216), Vesicular Stomatitis Virus G protein (VSV-G) (216) and influenza hemagglutinin (HA) (334), resulted in an impaired ability of Env complexes to mediate membrane fusion. On the other hand, the replacement of the MSD of Env with that of CD22 (343) did not appear to block the replication of HIV in MT4 cells, and an Env chimera containing the transmembrane region of CD4 (265) has also been shown to induce normal membrane fusion.

According to the consensus sequence of the HIV-1 MSD, the 12 amino acid residues in the core region are generally more conserved than those C-terminal to this region (from R694 to R705) (Fig. 1). Recent studies of the MSD core region have focused on the GXXXG motif in which the glycine residues are the most conserved among all MSD residues. Statistical studies and bacterial models suggested that the GXXXG motif probably constructs a framework for the association of transmembrane helixes (276, 287, 288). It has been demonstrated that the GXXXG motif in the E1 glycoprotein of

hepatitis C virus is important for the heterodimerization of its E1 and E2 envelope glycoproteins (63). The membrane-spanning domain of VSV-G also contains a GXXXG motif. Alanine and leucine scanning experiments showed that single mutation of the two glycines reduced the fusion activity of VSV-G to half of WT, but simultaneous mutation of both residues almost completely blocked membrane fusion (64). Similar results were reported for the GXXXG motif in HIV-1 MSD core, in which the fusogenicity of Env started decreasing when more than one glycine residue was substituted for alanine or leucine (215, 216).

In order to further understand the biological functions of the more conserved HIV-1 MSD core structure, we initially substituted all of the residues for leucine within the core, and then sequentially reintroduced the GGXXG motif, and then F683 with V687. We show here that the specific amino acid residues in the helical core of HIV-1 MSD are critical for the fusogenicity of Env complexes and infectivity of HIV-1. The loss of infectivity correlated with impaired virus-cell fusion. However, these mutations in the MSD core did not influence the biogenesis, intracellular transport and incorporation of Env complexes. Truncation of the cytoplasmic domain of HIV-1 Env was not able to compensate for the fusion defects caused by MSD core mutations.

MATERIALS AND METHODS

Cells and antibodies. COS-1, 293T and JC53BL cells were maintained in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and penicillin-streptomycin (all from Gibco-BRL, Rockville, MD). Cells were grown under conditions of 37°C and 5% CO_2 in humidified incubators. The anti-gp120 b12, anti-gp160 Chessie 13-39.1, and anti-p24 183-H12-5C monoclonal antibodies along with HIV-1 patient Ig were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health. The pooled HIV-1 patient sera were provided through the Emory CFAR Clinical Core. The sheep anti-HIV-1 gp120 polyclonal Ab and goat anti-human IgG (H+L) HRP were purchased from Cliniqa Corp (San Marcos, CA) and Pierce (Rockford, IL), respectively.

Glycoprotein and proviral expression constructs. Construction of the HIV-1 Env expression vector pSRHS, which encodes full-length *env, tat*, and *rev* genes from NL4-3, has been described previously (278). This simian virus (SV40 late promoter) based vector contains a Mason Pfizer Monkey Virus (MPMV) derived long terminal repeat providing a polyadenylation signal. A unique *Xba*I site (nucleotide 8213, NL4-3) was previously introduced into the pNL4-3 *env* gene in both pSRHS and the proviral vector pNL4-3. In this study, we employed a two-step overlapping PCR approach to generate DNA fragments containing mutated membrane-spanning domains. Briefly, forward and reverse oligonucleotides were designed to span the mutagenesis region between lysine681 and arginine694. The oligonucleotide sequences were: L12FOR, 5'-TTACTACTGTTATTACTGCTCTTGTTACTGTTAAGAATAGTTTTTGCTG-3'; L12REV, 5'-CAGTAACAAGAGCAGTAATAACAGTAGTAATAATTTTATATACCACAGCC-3';

L12GFOR, 5'-TTACTACTGTTATTAGGAGGCTTGTTAGGTTTAAGAATAGTTTTTGCTG-3';
L12GREV, 5'-ACCTAACAAGCCTCCTAATAACAGTAGTAATAATTTTATATACCACAGCC-3';
L12G2FOR, 5'-TTCCTACTGTTAGTAGGAGGCTTGTTAGGTTTAAGAATAGTTTTTGCTG-3'; and
L12G2REV, 5'-ACCTAACAAGCCTCCTAGTAACAGTAGGAATAATTTTATATACCACAGCC-3'. We also designed a forward primer (Primer$_{XbaI}$) overlapping the unique *Xba*I site and a reverse primer (Primer$_{BamHI}$) overlapping a unique *Bam*HI site downstream of the MSD sequence. In the first step of PCR, Primer$_{XbaI}$ and reverse primers amplified sequence A of each MSD mutant, while Primer$_{BamHI}$ and forward primers amplified sequence B of each MSD mutant, using wild type NL4-3 *env* as templates in both reactions. The mixed A and B amplicons were then used as templates to amplify target sequences primed by Primer$_{XbaI}$ and Primer$_{BamHI}$. The wild type NL4-3 *env* region was replaced with mutated sequences by insertion into the *Xba*I and *Bam*HI sites in both SV40-based expression vector pSRHS and proviral vector pNL4-3. We constructed at least three independent clones for the WT and each mutant. All mutations were confirmed by DNA sequencing using a primer approximately 100bp upstream from the MSD sequence. In this study, nucleotides and amino acid residues were labeled according to the NL4-3 sequence (GeneBank accession: AF324493) if not being specifically notified.

Glycoprotein expression and immunoprecipitation. The pSRHS Env expression vectors were transfected into COS-1 cells and CMV-based Env-expression vectors as well as proviral vectors were transfected into 293T cells, using Fugene6 (Roche, Indianapolis, IL) in 6-well plates. The pSRHS vectors do not express in 293T cells

because transcription from the SV40 late promoter is inhibited in these cells. At 36-48 h posttransfection, cells were starved for 15 min in methionine-free and cysteine-free DMEM and then labeled for 30 min in methionine-free and cysteine-free DMEM supplemented with ^{35}S-methionine and ^{35}S-cysteine (125µCi/well). The labeled cells were then chased in complete DMEM for 1h, 2h, 3h, and 5h. All medium was filtered through a 0.45µm membrane to remove cellular debris. For 293T cells transfected with proviral vectors, viral particles were pelleted through a 25% sucrose cushion by ultracentrifugation (100,000×g, 2.5 h). Cells and viral pellets were lysed by a 10 min incubation on ice in lysis buffer (1% Triton X-100, 50 mM NaCl and 0.1% sodium dodecyl sulfate [SDS] in 25 mM Tris-HCl [pH8.0]). Cellular debris was removed by microcentrifugation at 13,200 rpm for 1 min at 4°C. HIV-1 viral proteins were immunoprecipitated from cell lysates, supernatants, and viral pellets by incubating overnight at 4°C with pooled HIV-1 patient sera. Immune complexes were incubated overnight at 4°C with fixed *Staphylococcus aureus* cells (Staph A) and pelleted in a microcentrifuge. The pellets were then washed three times in lysis buffer, and labeled proteins resolved by 8% SDS-PAGE were visualized by autoradiography. The glycoprotein bands were quantified using a Cyclone phosphorimaging system (Packard, Meriden, CT) as previously described (336).

Cell-cell fusion assay. COS-1 cells were transfected with pSRHS expression vectors and 293T cells were transfected with proviral vectors pNL4-3 by using Fugene6. At 36-48h after transfection, cells resuspended by trypsinization were combined with JC53BL indicator cells at a 5:1 ratio. Cell mixtures were incubated for 6 h, 12 h, and 24 h, and then were lysed in luciferase reporter lysis buffer (Promega, Madison WI) using two freeze-thaw cycles. Cellular debris was removed by centrifugation at 13,200 rpm for 5

min at 4℃ in a microcentrifuge (Beckman, Palo Alto, CA). Luciferase substrate 100µl (Promega, Madison, WI) was added to 10µl of each cell lysate and light emission was quantified using a Synergy multi-detector microplate reader (Biotek, Vinooski VT).

Cell surface expression of Env glycoprotein. COS-1 cells and 293T cells were transfected with expression vectors and proviral vectors, respectively, cultured for 36-48 h, and then after removal from the plate fixed for 20 min at 4℃ in 4% paraformaldehyde (in PBS [pH7.2]). Fixed cells were stained for 1 h at room temperature with 5µg/ml of an Alexa647-conjugated anti-gp120 mAb b12. Then cell membranes were permeabilized using the Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences, San Diego, CA). Permeabilized COS-1 and 293T cells were further stained with Alexa488-conjugated anti-gp160 Chessie13-39.1 or Alexa488-conjugated anti-p24 183-H12-5C monoclonal antibodies, respectively, in order to normalize transfection efficiency. For some experiments a relative mean fluorescence index (RMFI) was calculated from the product of (% transfected cells) X (mean fluorescence intensity). Both Alexa647 and Alexa488 conjugation kits were obtained from Invitrogen (Carlsbad, CA). Double stained cells were subjected to flow cytometry analysis using the FACS Calibur system.

Infectivity of HIV-1 viruses in JC53BL indicator cells. Medium from 293T cells transfected with proviral vectors was harvested 72 h posttransfection, filtered through a 0.45µm membrane to remove cellular debris, and total virions quantified using a p24 ELISA assay. The p24-normalized virus-containing supernatant was added to JC53BL indicator cells cultured in DMEM containing 1% fetal bovine serum and 80µg/ml DEAE-Dextran. Complete DMEM was added following a 2 h incubation and cells were analyzed for luciferase activity 48 h post infection.

Single-round infection. 293T cells were cotransfected with pSRHS expression vectors and the pSG3Δ*env* proviral vector. Medium was collected 72 h posttransfection and then was subjected to p24 ELISA assays. The p24-normalized virus-containing supernatants were used to infected JC53BL indicator cells. Luciferase activity was measured 48 h after infection.

Incorporation of Env into viral particles. Two methods were used to measure the incorporation efficiency of Env glycoprotein into viral particles. (a) Medium collected from 293T cells 72 h after transfection with proviral vectors was filtered and viral particles were pelleted through a 25% sucrose cushion ultracentrifugation (100,000×g, 2.5 h). The supernatant including the sucrose solution was collected and viral pellets were resuspended in PBS (pH7.2). The amount of p24 and gp120 in both supernatants and viral pellets was measured using a p24 ELISA assay and a gp120 ELISA assay (119). Incorporation efficiencies in the viral pellets were compared using the ratio of gp120 to that of p24 for each mutant. The gp120/p24 ratio in the supernatant reflected the relative amount of shed gp120. (b) Transfected 293T cells were starved for 15 min in methionine-free and cysteine-free DMEM and then labeled for 30 min in methionine-free and cysteine-free DMEM supplemented with ^{35}S-methionine and ^{35}S-cysteine (125μCi/well). The labeled cells were then chased for 24 h. Viral particles in supernatant were pelleted by ultracentrifugation (100,000×g, 2.5 h) through a 25% sucrose cushion followed by lysis in lysis buffer. HIV-1 viral proteins were immunoprecipitated by incubating overnight at 4℃ with pooled HIV-1 patient sera. Immune complexes were incubated overnight at 4℃ with fixed *Staphylococcus aureus* cells (Staph A) and pelleted in a microcentrifuge (13,200rpm, 1 min). Labeled proteins resolved by 8% SDS-PAGE were visualized by autoradiography and quantified using a

phosphorimager, as described above. Incorporation efficiencies were calculated as the ratio of the digital light units (DLU) of gp120 bands to those of p24 bands.

Virus-cell fusion. A virion-based virus-cell fusion assay was performed as described previously (46-48). Briefly, 293T cells were cotransfected with proviral vector pNL4-3, pCMV-BlaM-Vpr construct (kindly provided by W. Greene, UCSF), and pAdvantage construct (Promega, Madison, WI) using calcium phosphate precipitation. The pCMV-BlaM-Vpr vector contains a CMV promoter-driven β-lactamase coding region fused to the N-terminus of the HIV-1 *Vpr* gene. At 48 h posttransfection, medium filtered through a 0.45μm membrane was loaded onto a 25% sucrose cushion (in PBS [pH7.2]) and centrifuged at 100,000×g for 2.5 h at 4℃. Pelleted virus was resuspended in serum-free DMEM, titered using a p24 ELISA assay, and then 200 ng p24 equivalents were added to $3x10^5$ JC53BL cells cultured in CO_2-independent medium (Gibco-BRL, Rockville, MD) supplemented with 1% fetal bovine serum. After a 6 h incubation at 37℃, free viruses were washed away in serum-free CO_2-independent medium. The fluorescent dye, CCF2-AM, was then loaded into these cells during a 2 h incubation at room temperature according to the Beta-lactamase Loading Kit (Invitrogen, Carlsbad, CA). The extracellular dye was then washed away in serum-free CO_2-independent medium and cells were resuspended in CO_2-independent medium supplemented with 10% fetal bovine serum and 2.5 mM probenecid. The cells were then incubated for 16 h at room temperature in the dark, fixed with 4% paraformaldehyde for 20 min at 4 ℃, and analyze by flow cytometry in a Beckman Dickson LSR2 cytometer.

RESULTS

In this study, we used a recovery-of-function mutagenesis strategy to define the role of specific amino acid residues within the 12 amino acid MSD core in HIV-1 virus assembly and entry. The amino acid sequences and nomenclature of the MSD core mutants are shown in Fig. 1. In the L12 mutant, the 12 amino acid residues between K681 and R694 were replaced by a polyleucine peptide of identical length. Leucine is the most hydrophobic amino acid and a polyleucine peptide would be expected to form a homogenous hydrophobic, helical structure in the lipid bilayer. Subsequently, in the L12G mutant we reintroduced the conserved GGXXG motif into the polyleucine backbone. Finally, V687, which is immediately upstream of the GGXXG motif and has been postulated to contribute to the helix-helix interactions mediated by the GXXXG motif, as well as the highly conserved bulky amino acid, F683, were reintroduced into the MSD core of L12G mutant.

MSD core mutants exhibit normal Env biosynthesis and processing. WT and mutant *env* sequences encoding, in addition, full-length *tat* and *rev* genes were cloned into the vector, pSRHS, in which expression of Env is under the control of the late promoter from simian virus 40 (SV40) and a polyadenylation signal from Mason Pfizer Money Virus (MPMV) (84, 146). Mutant Env glycoproteins expressed in COS-1 cells were metabolically labeled in a 30 min pulse and chased in complete medium over the course of 5 h. Viral glycoproteins were subsequently immunoprecipitated with pooled HIV-1 patient sera and analyzed by SDS-PAGE followed by autoradiography. Quantification of protein bands was carried out using a phosphorimager (Packard, Meriden, CT). As shown in Fig. 2A, we observed similar profiles for the cell-associated Env glycoproteins in COS-1 cells expressing either WT or mutant Envs after a 5 h chase. In a time course experiment (Fig. 2B), the MSD core mutants had similar

processing kinetics to WT Env, with the gp160 precursors decreasing and gp120 and gp41 subunits increasing at similar rates. These data suggested that the replacement of specific amino acid residues in MSD core did not alter the biosynthesis of the glycoprotein precursor or its transport to the Golgi network for completion of carbohydrate processing and cleavage to the SU and TM subunits.

MSD core mutants exhibit normal cell surface expression of Env glycoprotein. Since synthesis and processing of MSD-mutated HIV-1 Env glycoprotein appeared normal, we examined the surface expression of these mutant proteins to determine whether they were transported to, and retained normally in, the plasma membrane. Transfected COS-1 cells were fixed in 4% paraformaldehyde and stained with Alexa647-conjugated anti-gp120 mAb b12 then cells were subjected to flow cytometry analysis. Transfection efficiency was monitored by intracellular staining following permeabilization using Alexa488-conjugated anti-gp160 mAb Chessie13-39.1. As shown in Fig. 3A, the MSD-mutated Env glycoproteins were presented on the cell surface at levels equivalent to that of the WT glycoprotein with mean fluorescent intensity (MFI) values varying from 54.8 for WT to 63.5 for L12G, indicating that the GGXXG motif and specific amino acid residues in the MSD core region are not required for stable surface expression of the Env glycoprotein.

MSD core mutants exhibit minor defects in cell-cell fusion when expressed at high levels. Because all of the MSD core mutants and WT were expressed at similar levels on the surface of COS-1 cells following transfection with the pSRHS vectors, we determined whether these mutant Env glycoproteins had an equivalent capacity to mediate cell-cell fusion. Transiently transfected COS-1 cells were co-cultured with JC53BL indicator cells, which are susceptible to infection by both R5 and X4 HIV-1

isolates and contain a luciferase reporter gene under the control of the HIV-1 LTR promoter and which can be activated by the Tat protein (328). Cell-cell fusion was measured by calculating the relative luciferase enzyme activity in cells expressing the mutant Envs compared to those expressing WT Env at 6 h, 12 h and 24 h after co-culture. Each of the mutants exhibited reduced fusion relative to WT at 6 h, with L12 inducing 70% the fusion observed with WT. This effect was reduced at 12 h and lost at 24 h (Fig. 3B). Similar results were observed when the same *env* sequences were cloned into the pCDNA3.1 vector (CMV promoter) and transfected 293T cells were usedas the effector cells (Fig 3C). In this case fusion induced by the L12 mutant was only 44% that of WT at 6 h (L12G - 83%, L12G2 - 86%), but increased to 56% at 12 h and was equivalent to WT at 24 h after mixing. The small differences in fusion defects observed in the two expression vectors could reflect differences in surface expression levels since in general the MFIs observed with the pSRHS vectors were double that of the pCDNA3.1 vector. Moreover, transfection with 300ng (10-fold reduction) of the pCDNA3.1 vector yielded surface expression levels approximately 5-fold less than that observed with the pSRHS vector and under these conditions we observed a 50% decrease in fusion by the L12 mutant relative to WT at 24h post-co-culture (data not shown).

MSD core mutant viruses show defects in infectivity: In order to analyze whether the MSD core mutations impacted virus infectivity, each mutated *env* gene was substituted into the proviral vector pNL4-3, and virions were produced by transfection of 293T cells. Following normalization based on p24 content, these virions were used to infect JC53BL indicator cells. The substitution of a poly-leucine peptide for the MSD core in the L12 mutant reduced infectivity on JC53 cells more than 100-fold (Fig. 4A). The single reintroduction of the GGXXG motif in L12G mutant increased infectivity to

8.4% that of WT, and the further addition of conserved amino acid residues, F683 and V687, into the L12G2 mutants resulted in another 2-fold increase in infectivity to 17.6% that of WT. An *env*-deletion mutant of HIV-1 (pSG3Δ*env*) exhibited only background infectivity (0.75% that of WT) in this assay. These data suggest that the specific amino acid residues and conserved motifs in the MSD core region are critical for Env-mediated infectivity in the context of the virion.

MSD core mutant Envs expressed in the context of virus are processed normally but show defects in cell-cell fusion: In order to understand the mechanism of the defect in infectivity caused by the MSD core mutations, we examined the biosynthesis, surface expression and induction of cell-cell fusion by these mutants in the context of virus. In pulse-chase experiments similar to those shown for the pSRHS expression vector (Figure 2), we observed that in the context of the pNL4-3 genome the MSD core mutant Env precursor gp160 was expressed and proteolytically processed to gp120 and gp41 with similar kinetics to the wild type gp160 (data not shown). Flow cytometric analyses of cells stained with Alexa647-conjugated anti-gp120 mAb b12 showed that, while the WT and MSD mutants had equivalent levels of surface Env (Fig. 5A and 5B), the mean fluorescent index for the viral-based vector was 4-fold lower than for the pSRHS vector.

In order to determine the ability of virus producing cells to mediate cell-cell fusion, 293T cells transfected with proviral DNA were cocultured with JC53BL indicator cells for 6 h, 12 h and 24 h in the presence of 10μM AZT to prevent virus replication and then assayed for luciferase activity. In the context of virus, we found significant differences ($p<0.01$) in the abilities of the MSD mutants to mediate cell-cell fusion (Fig. 5C). Mutant NL4-3 L12 induced only 35% of the cell-cell fusion observed with WT NL4-3. When the

GGXXG motif was reintroduced into the mutated MSD core, fusion increased to 56% WT, while the further addition of V687 and F683 increased fusion to 72% that of WT. The difference in cell fusion observed in the context of Env expression vectors and the NL4-3 provirus are most likely explained by differences in the amount of Env expressed at the cell surface. Therefore, under conditions where cell surface expression levels were reduced to that characteristic of virus infected cells, the mutants did exhibit a reduced ability to mediate cell-cell fusion, although the extent of this defect was less than that observed in infectivity assays.

MSD core mutant Envs are incorporated efficiently into viral particles: In order to determine whether a defect in Env incorporation might also contribute to the MSD core mutant impaired infectivity, we utilized two different approaches to measure the incorporation efficiency of mutated Envs into viral particles. In the first, virions released from provirus-transfected 293T cells that were pulse-labeled and chased for 24h were pelleted through a sucrose cushion and analyzed by SDS-PAGE (Figure 6A). The ratio of gp120 to p24 for WT and mutant proviruses was calculated using a phosphoimager, and, as can be observed in Figure 6B, similar levels of glycoprotein incorporation were observed for each construct. To confirm this result, the amount of gp120 and p24 in viral pellets and supernatants was analyzed using gp120 and p24 ELISAs after centrifugation of unlabeled transfected 293T cell culture fluids. We observed that the WT and mutant virions had similar gp120/p24 ratios (Figure 6D), confirming that the MSD core mutations did not alter the incorporation of Env complexes into viruses. Moreover, the gp120 glycoprotein of the MSD mutants appeared to be as stable as the WT, because equivalent amount of gp120 were found in medium after centrifugation (Figure 6C and 6E).

Pseudotyped virions containing MSD core mutant Envs are defective for entry and virus-cell fusion: In order to determine if the MSD core mutants were defective at the stage of entry, and specifically at the stage of virus-cell fusion, we utilized a single-round infection pseudotyped virus assay. In order to compare infectivity of these viruses with the NL4-3 constructs, pSG3Δ*env* was pseudotyped with WT and mutant Envs expressed from the pSRHS vector, and then the pseudotyped virions were used to infect JC53BL indicator cells. A similar pattern of defective infectivity to that observed in the NL4-3 infectivity assay (Fig. 4A) was seen in this assay although the extent of the defect was reduced (Fig. 4B). The L12 mutant induced only 17% of the luciferase activity observed for WT Env pseudotyped virus, but reintroduction of conserved amino acid residues increased it to 39% in mutant L12G and 54% in mutant L12G2.

Because the expression of luciferase in JC53BL cells depends on the expression of the *tat* gene, a late event after viral entry, it was possible that the defect of the MSD core mutants in single-round infection could be at a post-entry step. Therefore, we utilized WT and mutant NL4-3 viruses that contained a β-lactamase-Vpr fusion protein (BlaM-Vpr) to pinpoint the stage at which virus infection was blocked. JC53BL target cells infected with the BlaM-Vpr viruses and then loaded with the fluorescent dye CCF2-AM (green), which can be cleaved by β-lactamase into CCF2 (Blue) and AM immediately after viral entry. This method is a sensitive indicator of the membrane fusion between the viral envelope and cell plasma membrane. As shown in Fig. 7A and 7B, the L12 mutation decreased virus-cell fusion by approximately 92% compared to WT, but reintroduction of GGXXG motif increased fusion almost three-fold to 21% that of WT. Further addition of F683 and V687 increased virus-cell fusion to 41% that of WT. Virus attachment assays carried out at 4°C, 10°C and 25°C showed that WT and mutant viruses bound to the target cells with equivalent efficiency (data not shown). These data confirm that the MSD core mutants are blocked at the stage of virus-cell fusion during

the entry process and that conserved amino acid residues play a key role in this process.

C-terminal truncated MSD core mutants. SIV Env and HIV Env have a remarkably long cytoplasmic domain. It has been recently reported that the second site reversion mutants of SIV and HIV-1 that were resistant to amphotericin B methyl ester (AME), a cholesterol-binding compound, had a truncated Env cytoplasmic domain (321). It was suggested that the truncated protein had a higher potential for movement in the lipid bilayer, which resulted in the escape from inhibition by AME. Since it was possible that the loss of conserved amino acid residues in the MSD "core" region altered the mobility of the Env glycoprotein in the membrane at the time of fusion pore formation, we determined whether truncation of the cytoplasmic domain of HIV-1 Env could relieve the defect observed in the absence of the glycine motif. Each mutant and WT was therefore reconstructed into the pNL4-3 Δ144 proviral vector, in which a stop codon was engineered into the gp41 cytoplasmic domain of the WT and MSD core mutants at Y710 (Y712 in HXB2). Incorporation of truncated Env complexes was reduced for both the MSD WT and "core" mutants (Fig. 6D). In addition, the amount of gp120 shed into the supernatant was also decreased (Figure 6E). Moreover, we did not observe any reversal of the infectivity defect in either NL4-3-based provirus assays (Figure 4C) or in pseudotyped virus single cycle assays (Figure 4D). While a pNL4-3 provirus encoding a truncated Env was approximately twice as infectious as wild-type pNL4-3, this was not the case for virus pseudotyped with the truncated Env. Indeed in general viruses encoding the truncated mutant Envs were less infectious than their non-truncated counterparts (compare Figs 4A and 4B with 4C and 4D), with viruses pseudotyped with the truncated WT Env exhibiting a 50% decrease relative to its non-truncated counterpart. Even taking into account this reduced WT infectivity, the L12 and L12G

mutants showed equivalent defects in infectivity to that observed with their non-truncated versions. Similar results were also observed in virus-cell fusion assays based on viruses containing the BlaM-Vpr fusion protein (Fig. 7A and 7C). Thus truncation of the gp41 cytoplasmic domain is not able to complement the fusion and infectivity defects observed with the MSD core mutants.

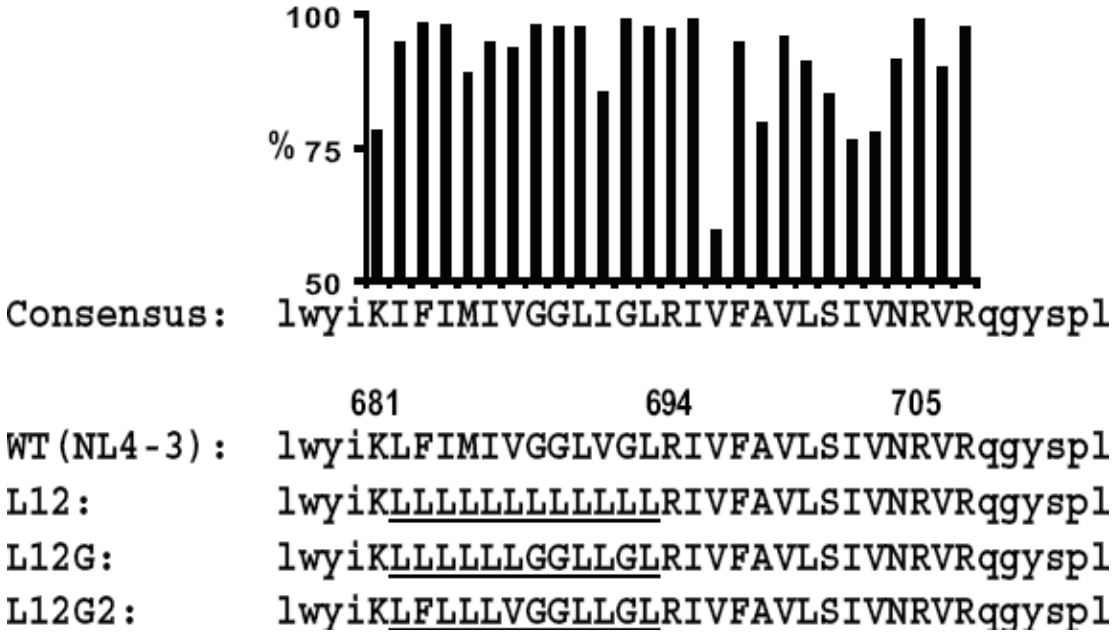
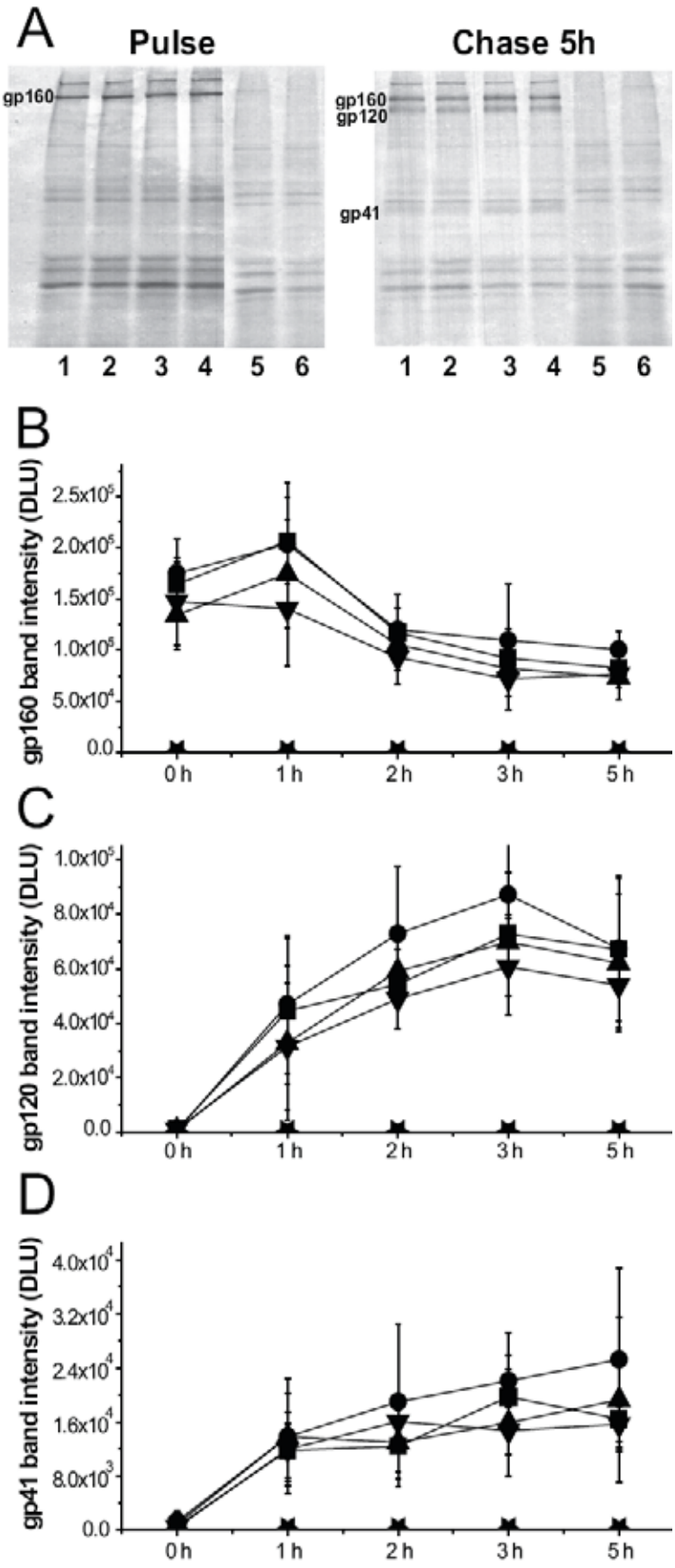


FIG. 1. Amino acid sequences of HIV-1 MSD. The consensus sequence of HIV-1 MSD was generated by the alignment of the Env MSD sequences of all M and N group HIV-1 isolates from the Los Alamos HIV Sequences Database (updated to 06/07). The percentages of isolates with the conserved amino acid residue are shown above the consensus sequence as a bar chart. The amino acid residues of the putative MSD region are shown as upper case letters. The flanking sequences of HIV-1 MSD are shown as lower case letters. The position of the MSD in the sequence of HIV-1 NL4-3 is marked above the positively charged amino acid residues. The mutated portions of the HIV-1 MSD are underlined.

FIG. 2. Expression profiles of HIV-1 Env glycoprotein. (A) COS-1 cells transiently transfected with SV40-based Env expression vectors were metabolically labeled and chased over 5 h. Env glycoprotein was immunoprecipitated followed by 8% SDS-PAGE analysis and autoradiography (Lane 1, pSRHS WT; Lane 2, pSRHS L12; Lane 3, pSRHS L12G; Lane 4, pSRHS L12G2; Lane 5, empty vector; and Lane 6, mock). (B) The bands of gp160 precursors, gp120 and gp41 in cell lysates at each time point were quantified by phosphorimager calculation and the intensity of bands is shown as digital light units (DLU) (square: pSRHS WT; circle: pSRHS L12; upward triangle: pSRHS L12G; downward triangle: pSRHS L12G2; leftward triangle: empty vector; and rightward triangle: mock). The figure shows the results from four independent experiments; error bars represent standard deviation from the mean.


A
Pulse
Chase 5h
gp160
gp160
gp120
gp41
1 2 3 4 5 6
1 2 3 4 5 6
B
gp160 band intensity (DLU)
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2.0x10^5
1.5x10^5
1.0x10^5
5.0x10^4
0.0
0 h
1 h
2 h
3 h
5 h
C
gp120 band intensity (DLU)
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8.0x10^4
6.0x10^4
4.0x10^4
2.0x10^4
0.0
0 h
1 h
2 h
3 h
5 h
D
gp41 band intensity (DLU)
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3.2x10^4
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8.0x10^3
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0 h
1 h
2 h
3 h
5 h

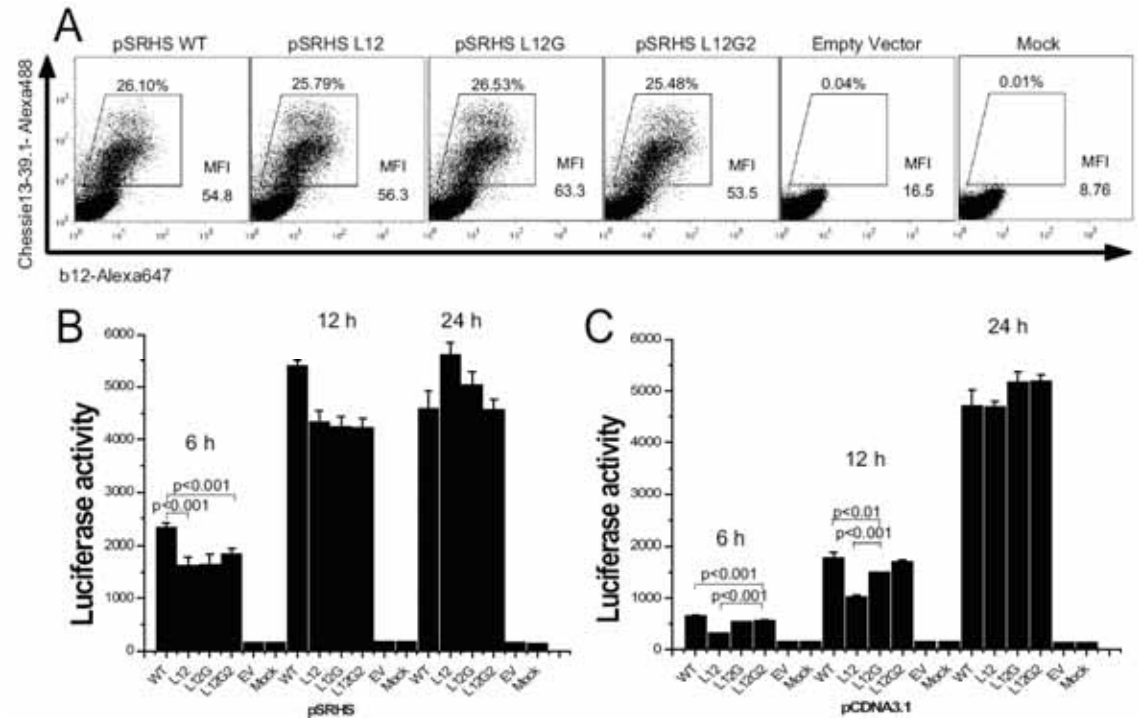


FIG. 3. Cell surface level of Env glycoprotein and cell-cell fusion examined using SV40-based Env expression vectors. (A) COS-1 cells transfected with pSRHS expression vectors were fixed and were labeled with Alexa647-conjugated anti-gp120 mAb b12. Cells were then permeabilized and stained with Alexa488-conjugated anti-gp160 mAb Chessie 13-39.1 as a transfection efficiency control. (B) Cell-cell fusion assay. COS-1 cells transfected with the pSRHS Env expression vectors were cocultured with JC53BL indicator cells. Cell mixtures were lysed and luciferase activity was measured after 6 h, 12 h and 24 h incubation. (C) Cell-cell fusion assay. 293T cells transfected with pCDNA3.1 Env expression vectors were co-cultured with JC53BL indicator cells. Cell mixtures were lysed and luciferase activity was measured after 6 h, 12 h, and 24 h incubation. The figures show the results from three independent experiments; error bars represent standard deviation from the mean. (EV: Empty vector).

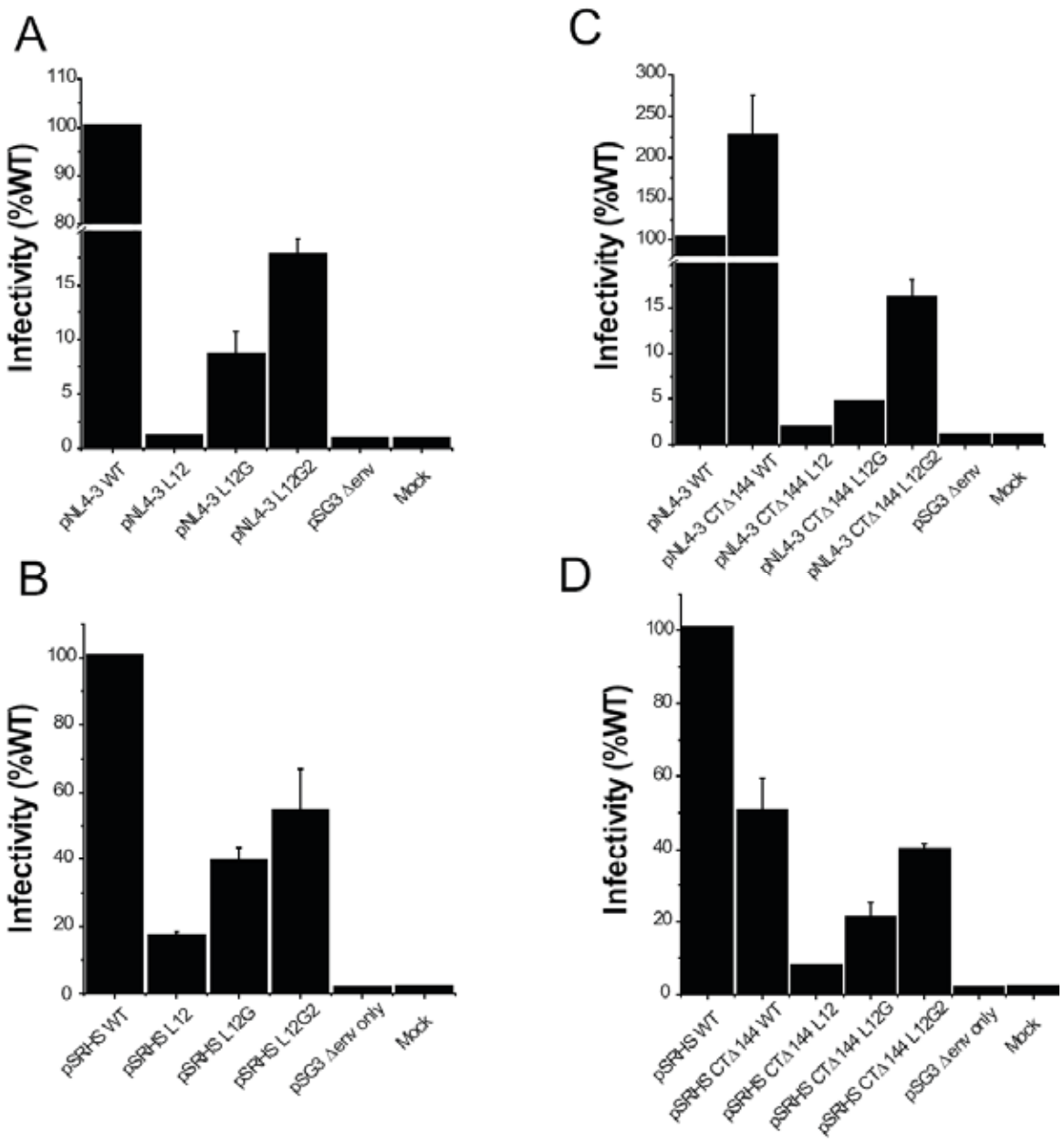


FIG. 4. Infectivity of HIV-1 Env mutants. HIV-1 viruses containing the Env MSD "core" mutations (A) and viruses containing truncated Env plus the MSD "core" mutations (C) were produced by transfecting proviral DNA into 293T cells. The p24-normalized cell culture supernatant was used to infect JC53BL indicator cells. Cells were lysed and luciferase enzyme activity was assayed after 48 h incubation. (B and D) Single-round infectivity assay. Env-defective proviral construct pSG3Δ*env* was cotransfected into 293T cells with wild type and mutant Env expression vectors (B) and by cytoplasmic domain truncated Envs (D). Pseudotyped viruses in the cell culture supernatant were quantitated using a p24 ELISA assay. The p24-normalized supernatant was then used to infect JC53BL indicator cells. Infectivity of MSD mutants is shown as relative luciferase activity compared to that of wild type. The figures show the results from three independent experiments; error bars represent standard deviation from the mean.

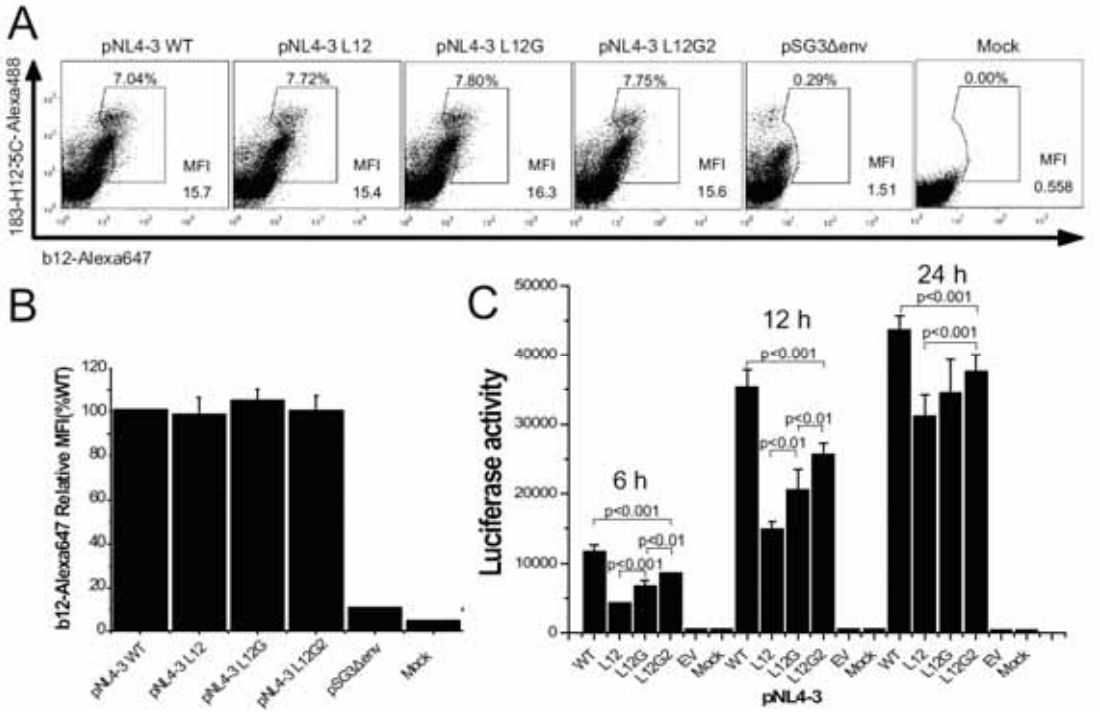
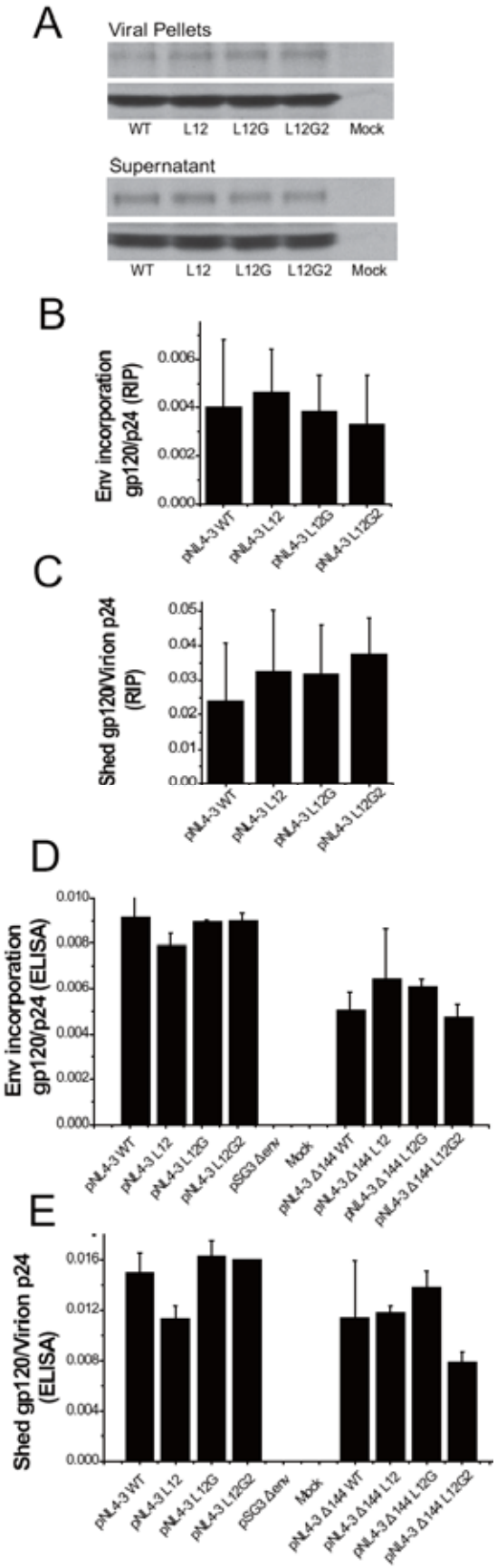


FIG. 5. Cell surface level of Env glycoprotein and cell-cell fusion examined in the context of provirus expression. (A) 293T cells transfected with pNL4-3 proviral vectors were fixed and were labeled with Alexa647-conjugated anti-gp120 mAb b12. Cells were then permeabilized and stained with Alexa488-conjugated anit-p24 mAb 183-H12-5C as a transfection efficiency control. (B) The surface levels of Env glycoprotein are shown as relative mean fluorescent index (RMFI) compared to that of wild type. (C) Cell-cell fusion in the context of viruses. 293T cells transfected with proviral DNA were mixed with JC53BL indicator cells and cocultured for 6 h, 12 h and 24 h before the measurement of luciferase activity. P values were calculated using the Student's t-test. The figure shows the results from three independent experiments; error bars represent standard deviation from the mean.

FIG. 6. Incorporation of Env into virions (A) 293T cells transfected with proviral DNA were pulse labeled with ^{35}S-methionine and ^{35}S -cysteine and chased over 24 h. Viral particles in medium were pelleted through a 25% sucrose cushion by ultracentrifugation. Viral proteins were immunoprecipitated with pooled AIDS patient sera and analyzed on 8% SDS-PAGE followed by autoradiography. (B) The viral gp120 and p24 bands in viral pellets and supernatants after ultracentrifugation were quantified by phosphorimaging analysis. The incorporation efficiency of Env into viral particles is shown as the ratio of the band intensity of gp120 bands in virions to those of p24 in virions. (C) The relative amount of gp120 shed into medium is shown as the ratio of the band intensity of gp120 bands in supernatant after ultracentrifugation to those of p24 in virions. (D) Unlabeled viral pellets and supernatants were subjected to gp120 ELISA assay and p24 ELISA assay. The incorporation efficiency is shown as the ratio of the concentration of gp120 to that of p24 in virions for both full-length (left) and truncated Envs (right). (E) The degree of shedding of gp120 is shown as the ratio of the amount of gp120 in supernatant after centrifugation to that of p24 in virions for both full-length (left) and truncated Envs (right). The standard deviations of three repeats for each experiment are shown as error bars.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Viral Pellets
WT
L12
L12G
L12G2
Mock
Supernatant
WT
L12
L12G
L12G2
Mock
B
Env incorporation gp120/p24 (RIP)
0.006
0.004
0.002
0.000
pNL4-3 WT
pNL4-3 L12
pNL4-3 L12G
pNL4-3 L12G2
C
Shed gp120/Virion p24 (RIP)
0.05
0.04
0.03
0.02
0.01
0.00
pNL4-3 WT
pNL4-3 L12
pNL4-3 L12G
pNL4-3 L12G2
D
Env incorporation gp120/p24 (ELISA)
0.010
0.008
0.006
0.004
0.002
0.000
pNL4-3 WT
pNL4-3 L12
pNL4-3 L12G
pNL4-3 L12G2
pSG3 Δenv
Mock
pNL4-3 Δ144 WT
pNL4-3 Δ144 L12
pNL4-3 Δ144 L12G
pNL4-3 Δ144 L12G2
E
Shed gp120/Virion p24 (ELISA)
0.016
0.012
0.008
0.004
0.000
pNL4-3 WT
pNL4-3 L12
pNL4-3 L12G
pNL4-3 L12G2
pSG3 Δenv
Mock
pNL4-3 Δ144 WT
pNL4-3 Δ144 L12
pNL4-3 Δ144 L12G
pNL4-3 Δ144 L12G2

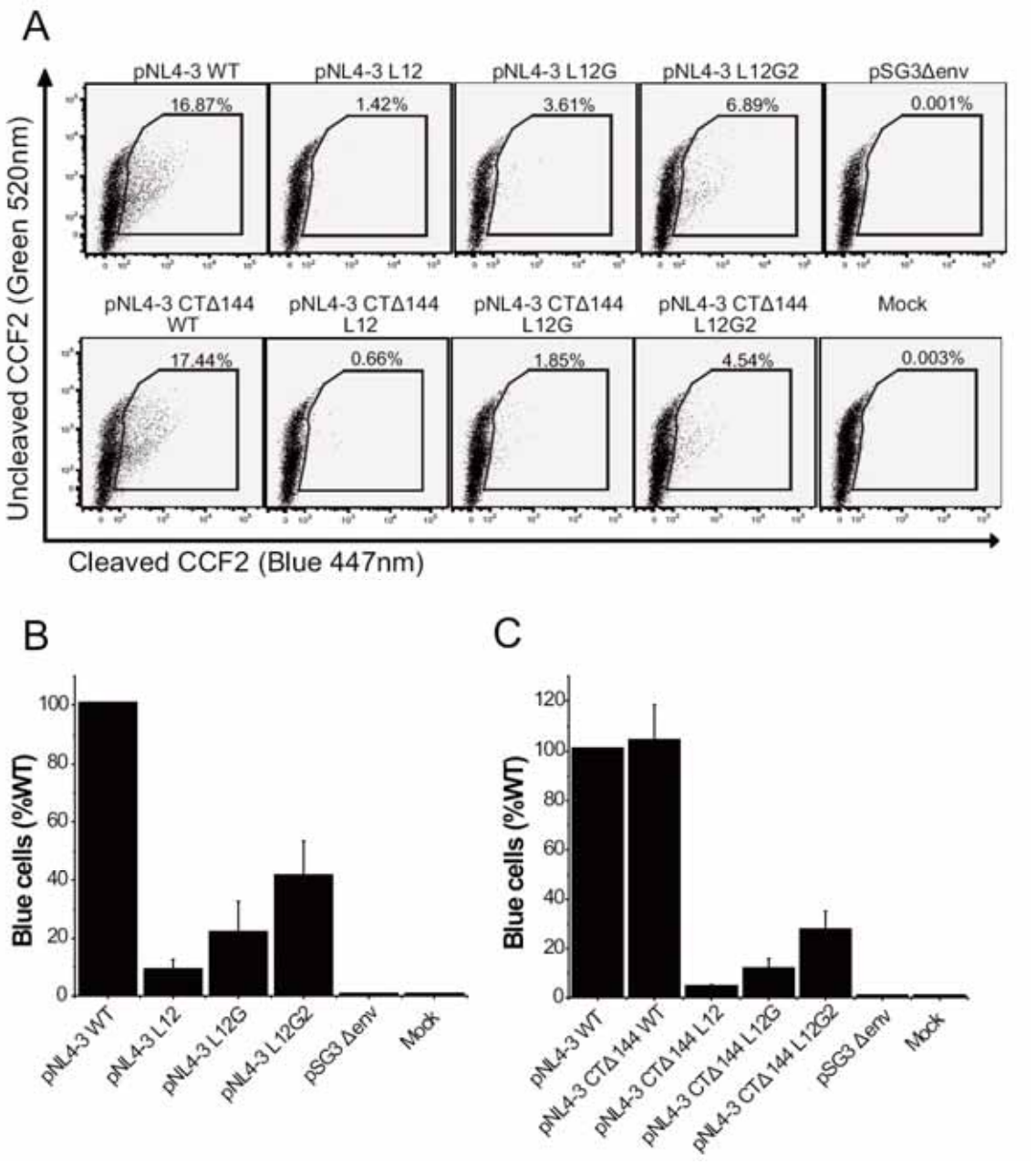

FIG. 7. Virus-cell fusion assay. (A) Proviral constructs were cotransfected into 293T cells with pCMV-BlaM-Vpr vectors. Viruses containing the BlaM-Vpr fusion protein were pelleted by ultracentrifugation through 25% sucrose. Viral pellets normalized by p24 ELISA assays were used to infect JC53BL indicator cells. Then cells were loaded with fluorescent dye CCF2-AM. Blue cells were counted by flow cytometry analysis after 16 h incubation. The ability of the Env MSD mutants (B) and the truncation mutants (C) to mediate virus-cell fusion is shown as relative numbers of blue cells compared to those of wild type. The experiment was repeated at least three times; standard deviations were shown in the error bars.

DISCUSSION

The primary function of the HIV-1 MSD is as a membrane anchor of the Env glycoprotein in both viral and cellular membranes - deletion of this region results in secretion of soluble gp160 molecules (100, 318). However, studies have suggested that this domain is involved in other important biological functions during the viral life cycle: (i) sequence alignments of the MSD from more than nine hundred HIV isolates (in Los Alamos HIV Database) showed that the amino acid sequence of this domain is more highly conserved than might be expected to merely anchor the protein in the membrane, (ii) replacement of the HIV-1 MSD with the equivalent regions of glycophorin A, the VSV-G protein or the influenza hemagglutinin (HA) leads to significant defects in cell-cell fusion mediated by Env glycoprotein (216, 334), and (iii) synthetic peptides corresponding to the HIV-1 MSD region are able to mediate phospholipid-mixing and then fusion between two lipid vesicles (221).

In the absence of any crystallographic or NMR-derived structural information on the HIV-1 MSD the exact length of this region remains unresolved. Two topological models have been proposed to explain the intramembrane structure of the HIV-1 MSD, and they differ in the location of the three basic residues (K681, R694, and R705) with respect to the lipid bilayer (217, 336). In order to better understand the biological functions of this highly conserved MSD domain and the roles played by conserved motifs in this region, we defined an MSD “core” region (K681 to R694), which is more conserved than the rest of the HIV-1 MSD and is buried within the lipid bilayer in both models. Employing a recovery-of-function approach by first replacing the entire MSD “core” with leucine residues, we then determined the effect of reintroducing conserved residues and motifs into this leucine backbone.

A highly conserved GGXXG motif is present in the HIV-1 MSD core region, and it has been postulated that this motif on an intra-membrane helix forms a framework for transmembrane helix-helix association. The absence of long side chains on the two glycine residues that are present on the same face of the helix is postulated to provide a flat platform to mediate helix-helix interactions (33). For HCV Env, substitution of the glycine residues in the GXXXG motif of the E1 protein by tryptophan residues impaired the heterodimerization of E1E2 protein and the following fusion steps (63). The results of the studies presented here, however, show that specific amino acid residues between K681 to R694 are not required for the normal expression, carbohydrate modification and proteolysis of Env glycoprotein precursor. There was no evidence that loss of the glycine-rich GGXXG motif or aromatic residues in the MSD interfered with the intracellular trafficking of Env from the endoplasmic reticulum to the trans-Golgi network, where the gp160 precursor is cleaved into gp120 and gp41 subunits. This argues that trimerization of Env monomers was unaffected by loss of these residues since the transport of Env glycoprotein from the ER to Golgi apparatus has been shown to require the oligomerization of gp160 monomers (240). This conclusion is consistent with the results observed when the membrane-spanning regions of other transmembrane proteins were used to substitute the MSD of the HIV-1 Env (216, 334, 343). In all these cases, the chimeric Env glycoproteins were synthesized and cleaved as efficiently as WT Env, arguing that the HIV-1 MSD domain does not play a critical role in intracellular trafficking of Env. We also observed wild-type surface levels of the mutated Env glycoproteins on cells transfected with either an SV40 promoter-based expression vector in the absence of other viral proteins or in the context of full-length proviral vectors, suggesting that specific amino acid residues within the HIV-1 MSD “core” are also dispensable for the intracellular transport of Env to the plasma membrane. Moreover, the fact that mutant Envs were incorporated into viral particles as efficiently as WT

argues that specific residues in the MSD are not required for this process either. The levels of shed gp120 were similar between the MSD mutants and WT, indicating that the MSD mutant Envs have the same stability as WT after reaching the plasma membrane.

In contrast, the amino acid composition of the MSD “core” clearly influences the fusogenicity of Env glycoprotein. Mutant L12 Env, in which the entire 12 amino acid residues of the MSD “core” were composed of leucine, was defective in mediating virus infectivity and cell-cell fusion. The inability of mutant viruses to infect target cells was correlated with defective fusion between the viral envelope and the cellular membrane, which could be rescued at least in part when conserved amino acid motifs (GGXXG, F683 and V687) were reintroduced into the MSD “core”. These results argue that in addition to its membrane anchoring function the HIV-1 MSD also plays an active role in gp41 mediated fusion during viral entry. Although its structural role is still unclear, the GGXXG motif is critical to the membrane fusion mediated by the HIV-1 Env, since single reintroduction of this motif into the leucine “core” of L12 mutant significantly increased the efficiency of cell-cell fusion and virus-cell fusion. Further improvement of Env fusogenicity was achieved by reintroducing additional F683 and V687, indicating that these two amino acid residues also contribute to membrane fusion. It is worth mentioning that the fusogenicity and infectivity of mutant L12G2 are still less than those of WT, so the remaining mutated amino acid residues (I684, M685, I686, and V692) appear to also facilitate the fusion process. These results argue that all of the 12 amino acid residues in the HIV-1 MSD “core” region are involved in the efficiency of Env-mediated membrane fusion – again consistent with the high conservation of this region. In initial mutagenesis studies, single mutations within the GGXXG motif failed to show a significant role for this motif in membrane fusion (216), perhaps because the remaining

unmodified residues were able to compensate for the single change. In this respect the "recovery-of-function" approach described here provides added power to the analysis.

According to the current model of HIV Env-mediated fusion, merging of the two membranes after the formation of the gp41 six-helix bundles consists of two major events, membrane disruption and fusion pore formation/expansion. It has been reported that the expression of peptides corresponding to the HIV-1 MSD plus four adjacent amino acid residues significantly increased membrane permeability in bacteria (22). Moreover, the replacement of the HIV-1 MSD with those of other transmembrane protein impaired the efficiency of fusion pore formation/expansion (216). Therefore, the highly conserved MSD "core" region may contribute to both processes in membrane fusion, particularly when one considers the fact that the specific amino acid residues in the rest of the HIV-1 MSD (from I694 to N704) are not required to mediate efficient cell-cell fusion (241).

Interestingly, the defect in cell-cell fusion appeared to be modulated by the level of Env expressed on the cell surface. Although each of the mutants Envs (L12, L12G and L12G2) exhibited reduced levels of fusion 6 h after co-culture when expressed from the SV40-based or CMV-based vectors, greater differences were observed when Env was expressed in the context of the proviral genome. While we cannot rule out a modifying effect of Env:Gag interactions (242), this difference most likely reflects the higher surface expression levels of the Env glycoprotein by the SV40- and CMV-promoter based expression vectors. Proviral expression resulted in cell surface levels of Env approximately 4-fold lower than that observed with the pSRHS Env expression vector and analogous reductions in cell fusion were observed with the pCDNA3.1 vector when reduced input DNA resulted in equivalently low surface expression levels. A similar

dependency on surface expression levels has been reported for C-terminally truncated Env mutants, where cell-cell fusion ability also increased with Env expression levels (186), and it may reflect the increased chance of fusion when higher numbers of molecules are present at the cell surface.

Because it was possible that modifications of the MSD “core” sequence could alter its interaction with and mobility in the lipid bilayer, we hypothesized that this might be the basis for decreased fusion. Previous work by Freed and colleagues (322), had shown that a defect in virus replication imposed by the cholesterol-binding compound AME could be relieved by truncating the cytoplasmic domains of Env; presumably because this increased its mobility in the membrane. In contrast, we observed that C-terminal truncations were not able to rescue the defects imposed by the loss of conserved residues in the MSD “core”. C-terminal truncation of gp41 has also been shown to induce conformational changes in the ectodomains of gp120 and gp41 (90, 320, 354), and in the case of the truncated WT Env we did observe an approximately two-fold enhancement of infectivity in the pNL4-3 construct. Viruses encoding mutant Envs on the other hand exhibited, for the most part, lower infectivity and lower virus-cell fusion than their full-length counterparts, suggesting that a combination of the two mutations was detrimental to biological activity rather than compensatory. Indeed, such mutants appear to be incorporated less efficiently into virions.

In summary, the experimental approaches described here have demonstrated that despite its high sequence conservation, the native MSD “core” amino acid sequence of HIV Env is not required for normal biosynthesis and intracellular transport. It does play a critical role in mediating efficient membrane fusion during viral infection, and at this stage the almost invariant GGXXG motif appears to most important. Since the GXXXG motif

has been postulated to provide a potential flat interface through which intra-membrane helix-helix associations can occur (32, 33) it is tempting to speculate that it may facilitate MSD-MSD interactions during formation of the higher order fusion pore rather than function during trimer assembly in the ER. Nevertheless, each amino acid residue within this “core” region appears to contribute to optimal fusogenicity of the HIV-1 Env. Further clarification of the mechanisms by which this region facilitates membrane fusion may provide insights into the intramembrane topology of the HIV-1 MSD and protein-lipid interactions of the region during the process of membrane fusion.

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Residues in the Membrane-spanning Domain core contribute to conformational integrity and fusogenicity of the Human Immunodeficiency Virus Type I Envelope Glycoprotein

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ABSTRACT

The membrane-spanning domain (MSD) of Human Immunodeficiency Virus Type I (HIV-1) envelope glycoprotein (Env) is critical for its biological activity. Initial studies have defined an almost invariant “core” structure in the MSD and demonstrated that it is crucial for anchoring Env in the membrane and for virus entry. We show here that amino acid substitutions in the MSD core do not influence specific virus-cell attachment, nor CD4 receptor and CXCR4 coreceptor recognition by Env. However, substitutions within the MSD “core” delayed the kinetics and reduced the efficiency of cell-cell fusion mediated by Env. Although we observed no evidence that membrane fusion mediated by the MSD core mutants was arrested at a hemifusion stage, impaired Env fusogenicity was correlated with minor conformational changes in the V2, C1, and C5 regions in gp120 and the HR1 and immunodominant loop in gp41. These changes could delay initiation of the conformational changes required in the fusion process.

INTRODUCTION

Human immunodeficiency virus Type I (HIV-1) infection is initiated by fusion of the viral membrane with that of the target cell and is mediated by the viral envelope glycoprotein (Env). On the virus surface, a trimer, composed of three non-covalently linked dimers of gp120, the receptor-binding surface (SU) component, and gp41, the membrane-spanning, trans-membrane (TM) component, forms the functional Env complex (87, 219, 347, 352). The gp120 and gp41 glycoproteins are synthesized as a precursor gp160 glycoprotein, which is encoded by the *env* gene. The gp160 precursor is co-translationally glycosylated and, following transport to the trans-Golgi network, is cleaved into the mature products by a member of the furin family of endoproteases (352). Mature Env proteins are transported to the plasma membrane, where they are rapidly endocytosed or incorporated into virions (40, 275, 343).

The HIV-1 gp120 glycoprotein binds to the CD4 receptor and chemokine coreceptors and consists of 5 conserved domains (C1-C5) and 5 variable domains (V1-V5) (Fig. 1A). Several studies suggest that the C1 and C5 regions directly interact with the immunodominant loop in gp41 (28, 129, 139). The C2 region is sequestered in the oligomeric structure and involved in the oligomerization of the gp120 (177). The binding of CD4 to HIV-1 Env requires multiple conserved regions in the gp120, including C1 (167, 239), C3 (133, 134), and C4 (223). A comparison of the sequence and structural profiles of HIV-1 and SIV Env shows that the C2-V3-C3 region is involved in the contact with chemokine receptors (51, 292). The V3 loop is a hyper variable disulfide-bonded structure and is the major determinant of the tropism of HIV-1 virions (124). The V1V2 region also influences HIV-1 cellular tropism, probably via an interaction with regions of the V3 loop (33, 161). The V4 and V5 regions of gp120 may also be necessary for efficient utilization of CXCR4 (60, 172). Moreover, the V1V2 and V3 regions have been

demonstrated to play a more important role when HIV-1 uses, in addition to CCR5 or CXCR4, other chemokine coreceptors such as CCR2b, CCR3, STRL33, and APJ (132). In the dual tropic strain 89.6, the V3, V4, and V5 regions are involved in CCR5, CXCR4, and CCR3 utilization (297).

The HIV-1 gp41 glycoprotein is the fusion machinery that mediates virus-cell membrane fusion. When activated, the ectodomain of gp41 carries out the fusion function, while the membrane-spanning domain and cytoplasmic domain are both important for its fusogenecity. The ectodomain consists of a fusion peptide, two heptad repeats (HR1 and HR2) with the immunodominant loop in between, and a membrane proximal tryptophan-rich domain (Fig. 1A). The cytoplasmic domain contains signals for intracellular trafficking of the Env, and three lipid lytic peptides (LLP1, LLP2, and LLP3) that play important roles in membrane fusion (66, 212, 309).

After the sequential binding of gp120 to the CD4 receptor and chemokine coreceptors, changes in the conformation of gp120 activate the fusion competency of gp41. The N-terminal hydrophobic fusion peptide is released from a compact structure of gp41 and becomes associated with the outer monolayer of the target membrane. Meanwhile, the ectodomains of the gp41 trimer are rearranged into a 6-helix bundle structure with the three HR1 segments in the center, forming a coiled-coil structure, and the three HR2 segments on the outside, being tightly packed into the hydrophobic grooves of the coiled-coil. This process brings together the cell membrane associated fusion peptides with the viral membrane-binding MSDs, and results in a close proximity between the viral envelope and the cellular membrane, which is necessary for virus-cell membrane fusion (87, 340).

The membrane-spanning domain of Env is defined as a stretch of 25 predominantly hydrophobic amino acids that spans residues K681 to R705 (NL4-3). In the previous C-terminal truncation studies of HIV-1 Env, we have demonstrated that the entire 25 amino acid region is not required for the biological function of Env (359). The 17 amino acid residues (from K681 to A697) are sufficient for stably anchoring the truncated gp41 in the membrane and mediating cell-cell fusion at a WT level. Serial small deletions (3 amino acid residues) in the region between R694 and R705 showed normal cell-cell fusion, although larger deletions were detrimental, suggesting that, with respect to the biological functions of the Env glycoprotein, the length of this region is more important than its amino acid conservation (242). From these data, we have proposed a topology where the flanking K681 and R694 residues “snorkel” in the membrane, and 12 amino acid residues (from L682 to L693) form a hydrophobic core buried within the membrane. Arginines 705 and 707 are located at the interface of membrane and cytoplasm in this model (359).

The membrane-spanning domain, particularly within the “core” region, is one of the most conserved sequences in Env. This argues for a functional role of the MSD “core” in the biological activity of the HIV-1 Env complexes. Recent studies of the MSD core region have focused on the GXXXG motif in which the glycine residues are the most conserved among all MSD residues. Statistical studies and bacterial models suggested that the GXXXG motif probably constructs a framework for the association of transmembrane helixes (276, 287, 288). Previously, we substituted all of the residues for leucine within the HIV-1 MSD core, and then sequentially reintroduced the GGXXG motif, and then two other highly conserved residues, F683 with V687 (289). These studies showed that the specific amino acid residues in the helical core of HIV-1 MSD are critical for the fusogenicity of Env complexes and infectivity of HIV-1 virions. The loss of infectivity

correlated with impaired virus-cell entry. However, these mutations in the MSD core did not influence the biogenesis, intracellular transport and incorporation of Env complexes.

In order to further understand the roles played by the more conserved HIV-1 MSD core residues in virus-cell entry, we attempted in the current study to define the mechanism by which the MSD core mutants impact Env-mediated membrane fusion. We show here that the amino acid composition of the MSD core does not influence viral attachment to the cell surface or CD4 and CXCR4 recognition by HIV-1 Env. However, the cell-cell fusion kinetics of the recovery-of-function mutants was delayed compared to WT, and minor conformational changes in localized regions of gp120 and gp41 were observed, suggesting that these changes might delay the triggering of the fusion process after receptor binding.

MATERIALS AND METHODS

Cells and antibodies. COS-1, 293T, JC53BL cells and KABAT cell lines were maintained in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and penicillin-streptomycin (all from Gibco-BRL, Rockville, MD). Cells were grown under conditions of 37°C and 5% CO_2 in humidified incubators. The anti-gp120 monoclonal antibodies b12 (38), 697-30D (364), 902 (59), F425b4a1, ID6 (112), Chessie6 (1), Chessie13-39.1 (1), 670-30D (364), F425a1g8, 17b (170), E51 (136), A32 (351), and anti-gp41 monoclonal antibodies NC-1 (142), T32 (85), and D50 (85) along with HIV-1 patient Ig, fusion inhibitors (T20 and C34) were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health. The pooled HIV-1 patient sera were provided through the Emory CFAR Clinical Core.

Cell-cell fusion kinetics. MSD mutants were cloned into a proviral vector pTN6-GFP, in which the *nef* gene has been replaced by a GFP reporter gene. 293T cells, which were transfected with these proviral vectors, were resuspended 48 h posttransfection and were mixed with JC53BL indicator cells, which had been loaded with fluorescent dye CMAC-blue (Invitrogen, Carlsbad, CA). The cell mixtures were incubated at 37°C and 5% CO_2 for 0.5 h, 1 h, 2 h, 4 h, 6 h, 12 h, and 24 h. Cell-cell fusion was observed and quantitated by fluorescent microscopy.

CPZ treatment of cell-cell and virus-cell fusion. 293T cells were transfected with pCDNA3.1 Env expression vectors and resuspended by trypsinization 36-48h posttransfection. For all transfections, Fugene6 (Roche, Nutley, NJ) was used according to the manufacturer's directions. The transfected 293T cells were cocultured with

JC53BL indicator cells at a 5:1 ratio. Cell mixtures were incubated for 2 h, and then were treated with 0.5mM chlorpromazine (CPZ, in complete DMEM) for 30 seconds. Control cells were treated in parallel with complete DMEM alone. After the CPZ treatment, cells were washed three times in PBS and were incubated in complete DMEM for 24 h. Then cells were lysed in the luciferase reporter lysis buffer (Promega, Madison WI) using two freeze-thaw cycles. Cellular debris was removed by centrifugation at 13,200 rpm for 5 min at 4℃ in a microcentrifuge (Beckman, Palo Alto, CA). Luciferase substrate 100µl (Promega, Madison, WI) was added to 10µl of each cell lysate and light emission was quantified using a Synergy multi-detector microplate reader (Biotek, Vinooski VT).

A modified virion-based virus-cell fusion assay was performed based on the method described previously (46-48). Briefly, 293T cells were cotransfected with the proviral vector pNL4-3, pCMV-BlaM-Vpr construct (kindly provided by W. Greene, UCSF), and pAdvantage construct (Promega, Madison, WI) using calcium phosphate precipitation. The pCMV-BlaM-Vpr vector contains a CMV promoter-driven β-lactamase coding region fused to the N-terminus of the HIV-1 *Vpr* gene. At 48 h posttransfection, medium filtered through a 0.45µm membrane was loaded onto a 25% sucrose cushion (in PBS [pH7.2]) and centrifuged at 100,000×g for 2.5 h at 4℃. Pelleted virus was resuspended in serum-free DMEM, titered using a p24 ELISA assay, and then 200 ng p24 equivalents were added to $3x10^5$ JC53BL cells cultured in CO_2-independent medium (Gibco-BRL, Rockville, MD) supplemented with 1% fetal bovine serum. After a 2 h incubation at 37℃, cells were treated in serum-free CO_2-independent medium containing 0.5mM CPZ for 30 seconds or with medium alone. Free viruses and drug were washed away in serum-free CO_2-independent medium. Then the fluorescent dye, CCF2-AM, was loaded

into the cells during a 2 h incubation at room temperature according to the Beta-lactamase Loading Kit (Invitrogen, Carlsbad, CA). The extracellular dye was then washed away in serum-free CO_2-independent medium and cells were resuspended in CO_2-independent medium supplemented with 10% fetal bovine serum and 2.5 mM probenecid. The cells were incubated for 16 h at room temperature in the dark, fixed with 4% paraformaldehyde for 20 min at 4 ℃, and analyzed by flow cytometry in a Beckman Dickson LSR2 cytometer.

Three color microscopy of cell-cell fusion. 293T cells were transfected with pTN6-GFP proviral vectors. At 48 h after transfection, cells were resuspended by trypsinization. JC53BL indicator cells were resuspeded by trypsinization and washed once in serum-free DMEM. 5 ul DiI loading solution (Invitrogen, Carlsbad, CA) was added into 1ml of JC53BL cells (10^6 cells per ml) in serum-free DMEM. Cells were washed three times in serum-free DMEM after 30 min incubation at 37 ℃ and were resuspended in serum-free DMEM to 10^6 cells per ml. Then cells were incubated with 10ug/ml CMAC-blue (Invitrogen, Carlsbad, CA) for 30 min followed by three washes in serum-free DMEM. The transfected 293T cells were mixed with the double-stained JC53BL cells (1:1) in complete DMEM and the cell mixtures were cocultured at 37 ℃ for 2 h. Cell-cell fusion events were observed by fluorescent microscopy.

Virus capture assay. Viruses were harvested from the supernatant of proviral vector transfected 293T cells by ultracentrifugation through 25% sucrose cushion. Pelleted virus (in PBS) was added into 96-well plates (10ng p24 per well), which had been coated with anti-gp120 and anti-gp41 monoclonal antibodies in 50mM carbonate/bicarbonate buffer pH9.6, and then incubated at 37 ℃ for 2 h. For studies involving CD4-induced

exposure of epitopes, 1ug/ml sCD4 was mixed with the virus immediately prior to addition to the wells. Unbound viral particles were removed by extensive washes in PBS. Bound viruses were lysed in PBS containing 0.5% Triton X-100 and then subjected to a p24 ELISA assay.

C34 and T20 inhibition of cell-cell fusion and viral infection. 293T cells were transfected with pCDNA3.1 expression vectors. At 36-48h after transfection, cells resuspended by trypsinization were combined with JC53BL indicator cells at a 5:1 ratio. Cell mixtures were incubated for 24 h in the presence of T20 or C34 in specific concentrations (0, 0.008, 0.04, 0.2, 1, and 5 ug/ml). Luciferase activity was quantitated as described above.

Medium from 293T cells transfected with proviral vectors was harvested 72 h posttransfection, filtered through a 0.45μm membrane to remove cellular debris, and total virions were quantified using a p24 ELISA assay. The p24-normalized virus-containing supernatant was added to JC53BL indicator cells cultured in DMEM containing 1% fetal bovine serum, 80μg/ml DEAE-Dextran and T20 or C34 in specific concentrations. Complete DMEM was added following 2 h incubation and cells were analyzed for luciferase activity at 48 h postinfection.

RESULTS

Previously, we used a recovery-of-function mutagenesis strategy to define the roles of the specific amino acid residues within the 12-amino acid MSD "core" in HIV-1 assembly and entry. The amino acid sequences and nomenclature of the MSD "core" mutants are shown in Fig. 1B. We have shown that the MSD "core" region of HIV-1 is critical for its viral infectivity. Replacement of the conserved amino acid residues with leucine residues blocked virus entry and subsequent viral infection. Truncation of the cytoplasmic domain of the Env was not able to rescue the impaired virus entry. In this study, we examined the competence of these MSD "core" mutants at each step of virus entry, in order to further understand the mechanisms of the defects observed in this process. Initially, we carried out a virus-cell attachment assay at low temperatures (4°C, 10°C, and 25°C for 2 h). Virions of the MSD mutants adsorbed to the cell surface with the same efficiency as WT, indicating that the amino acid composition of the MSD "core" does not affect the ability of HIV-1 Env to mediate a virus-specific binding to the cell surface (data not shown). Additionally, using an Env-free virus, we demonstrated that nonspecific binding accounts for approximately 59% of virus-cell attachment (data not shown).

MSD mutants showed delayed cell-cell fusion kinetics. In order to examine whether the MSD "core" mutants influence the fusogenicity of HIV-1 Env, we measured the ability of Env to mediate cell-cell fusion, using a fluorescent marker transfer system. The WT Env and MSD mutants were cloned into the pTN6-GFP provrial vector, in which the *nef* gene is replaced by a GFP reporter gene, and then were transfected into 293T cells. The transfected cells were mixed with JC53BL cells, which were pre-loaded with the fluorescent dye CMAC-blue, and cell mixtures were cocultured for 0.5 h, 1 h, 2 h, 4 h, 6 h, 12 h, and 24 h. Fused cells fluorescing with both colors were then quantitated as a

percent of total cells. As shown in the Fig. 1C, the cell-cell fusion kinetics mediated by the Env MSD mutants was slower than that of WT. After a lag period of approximately 1 h, WT Env showed the highest initial rate of mediating cell-cell fusion (28.87% cells per hour) between 1 h and 2 h and a decreased rate (35.3% of the initial rate) in the following 4 h. Cell-cell fusion rates of the mutants L12G and L12G2 were relatively constant during the first 12 h of incubation and were nearly 25% of the WT initial rate. The L12 mutant exhibited a prolonged lag period of between 1 h and 4 h prior to a linear increase in cell-cell fusion rate, which is equivalent to those of the other two mutants. The WT Env reached 50% fusion at 4 h after cell mixing, while the mutants L12G and L12G2 were at 6 h, and the L12 mutant was at 10 h. Moreover, at 12 h after cell mixing, cell-cell fusion mediated by the Env mutants L12G and L12G2 was equivalent to that of WT, while even at 24 h mutant L12 showed only 84% of WT cell-cell fusogenicity. These data indicated that the MSD “core” mutants impaired the rate of cell-cell fusion by Env.

CPZ treatment did not increase cell-cell fusion or virus-cell fusion. The delayed cell-cell fusion kinetics suggested that the MSD “core” mutants might have to overcome a higher energy barrier to mediate membrane fusion than WT. In order to analyze whether the higher energy barrier was created by a hemifusion intermediate, we treated cells with 0.5 mM CPZ (chlorpromazine) for 30 seconds during the cell-cell fusion mediated by the Env. CPZ specifically disrupts the lipid bilayer structure in a hemifusion intermediate, which is formed by the two inner layers of the membranes involved in the fusion, thereby enhancing membrane fusion. As shown in Fig. 2A, CPZ treatments did not enhance the relative cell-cell fusogenicity of the Env mutants compared to WT, nor change the ratios of fusogenicity among these mutants. Overall, the CPZ-treated set showed slightly lower cell-cell fusion than the non-treated control set, likely due to the

toxicity of CPZ to cells even in a 30 second treatment. Moreover, in the BlaM-Vpr virus-cell fusion assay (46), CPZ-treatment was unable to promote fusion for any of the mutants (Fig. 2B). These results argue that the reduction in fusion observed with the MSD "core" mutants is not due to fusion-arrest at a hemifusion intermediate.

MSD "core" mutants can mediate full cell-cell fusion. In order to confirm the absence of hemifusion intermediates during membrane fusion mediated by the MSD mutants, we used three-color microscopy to examine cell-cell fusion mediated by the mutant L12, which is the least fusogenic. 293T cells transfected with pTN6-GFP proviral DNA were mixed with JC53BL indicator cells, which were double stained with the lipid dye DiI (red) and cytoplasmic dye CMAC-blue. Even though cell-cell fusion induced by mutant L12 was only 1.8% of WT after 2h of coincubation, only complete cell-cell fusion events, in which both the lipid dye and cytoplasmic dyes are mixed, were observed in greater than 50 cells (Fig. 2C). Thus in this assay, we did not observe any hemifusion intermediates, in which the lipid dye but not the cytoplasmic dyes are mixed. These results also indicated that those fusion pores that did form, during fusion by the MSD mutant L12, were expanded to a size that allows at least 30 KD molecules (GFP) to pass through.

High levels of CD4 receptors or CXCR4 coreceptors on the surface of target cells can not rescue the defects of the MSD "core" mutants in viral infectivity. In order to determine whether the MSD core mutants exhibited differential utilization of the CD4 receptor and CXCR4 co-receptor, cell lines expressing different levels of these molecules were employed. In the first series of experiments, the HI-J, and RC-25 cell lines (Kabat), which express similar levels of CXCR4 but differ in their CD4 expression by 15-fold (HI-J>>RC-25) were utilized. The WT and Env mutants were cloned into the

pTN7 proviral vector, in which the *nef* coding sequence is replaced by a renilla luciferase reporter gene. Viruses produced by transfecting 293T cells with these proviral DNAs were used to infect the two cell lines. At 48 h postinfection, cells were subjected to a luciferase activity assay. For each mutant, the viral infectivity in the HI-J cell lines was measured as 100% and compared with that in the RC-25 cell line. As shown in Fig. 3A, the higher level of surface CD4 enhanced virus infectivity of both the WT and MSD mutants, with both the MSD mutants and the WT exhibiting an approximately 2-fold increase in infectivity in the HI-J cells.

To investigate sensitivity to changes in CXCR4, the HI-R and RC-49 cells were utilized. These cells have equivalent levels of surface CD4, but a 6-fold difference in the surface level of CXCR4 (HI-R>>RC-49). In contrast to changes in CD4, changes in the surface level of CXCR4 had no effect on the infectivity of either the WT or MSD "core" mutants (Fig. 3B). These results suggest that the amino acid substitution in the MSD "core" does not affect CD4 or CXCR4 recognition by Env during viral entry.

MSD core mutants change the accessibility of epitopes to monoclonal antibodies in the gp120 glycoprotein. In order to determine whether the MSD mutants alter the conformational integrity of gp120, we measured the binding of anti-gp120 monoclonal antibodies to the Env glycoprotein on the viral surface by a virus capture assay. Viruses purified by ultracentrifugation through 25% sucrose were incubated in 96-well plates precoated with the specific anti-gp120 mAbs. The bound viruses were lysed after extensive washing and then subjected to a p24 ELISA assay. Two groups of mAbs were used to capture viruses, including (i) the CD4-independent mAbs: b12 (Fig. 4A), which binds to the CD4-binding site; 697-30D (Fig. 4B) - the V2 region; F425-B4a1 and 902 (Fig. 4C) - the V3 loop; ID6 and Chessie6 (Fig. 4D) - the C1 region; Chessie13-39.1 (Fig.

4E) - the C2 region; 670-30D (Fig. 4F) - the C5 region, and (ii) the CD4-induced mAbs: F425 A1g8 (Fig. 4G), 17b (Fig. 4H) and E51 (Fig. 4I) which bind the coreceptor-binding region; and A32 (Fig. 4J) - the C1/C4 region. The MSD mutants L12 and L12G showed an approximately 2-fold decrease in the binding of antibodies to epitopes in the V2 and C5 region (mAbs 697-30D and 670-30D), while the mutant L12G2 showed a WT binding ability to the mAbs in these regions. Moreover, the MSD "core" mutants resulted in a 2- to 4-fold increases in the binding of mAbs (ID6 and Chessie6) to epitopes in the C1 region. Consistent with the conformational changes in the C1 region, the binding of A32 to its conformational epitope in the C1 and C4 regions also increased approximately 2-fold. In spite of the conformational changes observed in the V2, C1, and C5 region, binding of antibodies to the V3 and C2 regions was not affected by the MSD "core" mutants. In addition, the accessibility of the mAb b12 to the CD4-binding region and CD4i mAbs F425a1g8, 17b and E51 to coreceptor-binding regions were not affected either, which is consistent with the infectivity data based on the Kabat cell lines, indicating an intact CD4 binding and co-receptor recognition of the Env mutants.

MSD "core" mutants change the conformation of the gp41 ectodomain. We used the same virus capture assay to examine potential conformational changes in the gp41 ectodomain. Three anti-gp41 mAbs were used: NC-1 (Fig. 5A) specifically binds to the 6-helix bundle, T32 (Fig. 5B) to the immunodominant loop in between the HR1 and HR2 regions, and D50 (Fig. 5C) to the HR2 region. Binding of antibody to the 6-helix bundle in the mutant L12 decreased to 50% of WT following induction by sCD4, while the mutants L12G and L12G2 were not significantly affected. Moreover, the exposure of the immunodominant loop was also reduced in the MSD mutants: L12 43% of WT, L12G 57%, and L12G2 81%, which suggested a stoicmetric relationship between the conformational changes in the gp41 ectodomain and the number of substitutions in the

MSD “core” region. However, the HR2 regions of the MSD “core” mutants were fully accessible to the mAb D50 compared to WT, indicating an intact conformation in this region. These results were confirmed by immunoprecipitating viral gp41 with the same mAbs in the presence or absence of sCD4, followed by a western blotting using the anti-gp41 mAb Chessie8 (data not shown).

MSD “core” mutants exhibit the same sensitivity to the fusion inhibitors, T20 and C34, as WT. In order to understand the degree to which the conformation of the gp41 ectodomain has been changed by the MSD “core” mutants, we compared sensitivity of the Env mutants to the fusion inhibitors, C34 and T20, to that of WT. The inhibition kinetics of cell-cell fusion mediated by the Env was measured by treating cells with the fusion inhibitors in a gradient of concentrations (Fig. 6A and 6B). The MSD mutants were as sensitive to either T20 or C34 as WT. Similar results were observed in the experiment measuring the T20 and C34 inhibition kinetics of viral infectivity (Fig. 6C and 6D). These data indicated that alterations in the gp41 ectodomain induced by the MSD “core” mutants were unlikely to slow formation of the 6-helix bundle once conformational changes were initiated and that access to the HR1 coiled-coil was retained in the MSD mutants, allowing the binding of the inhibitor peptides.

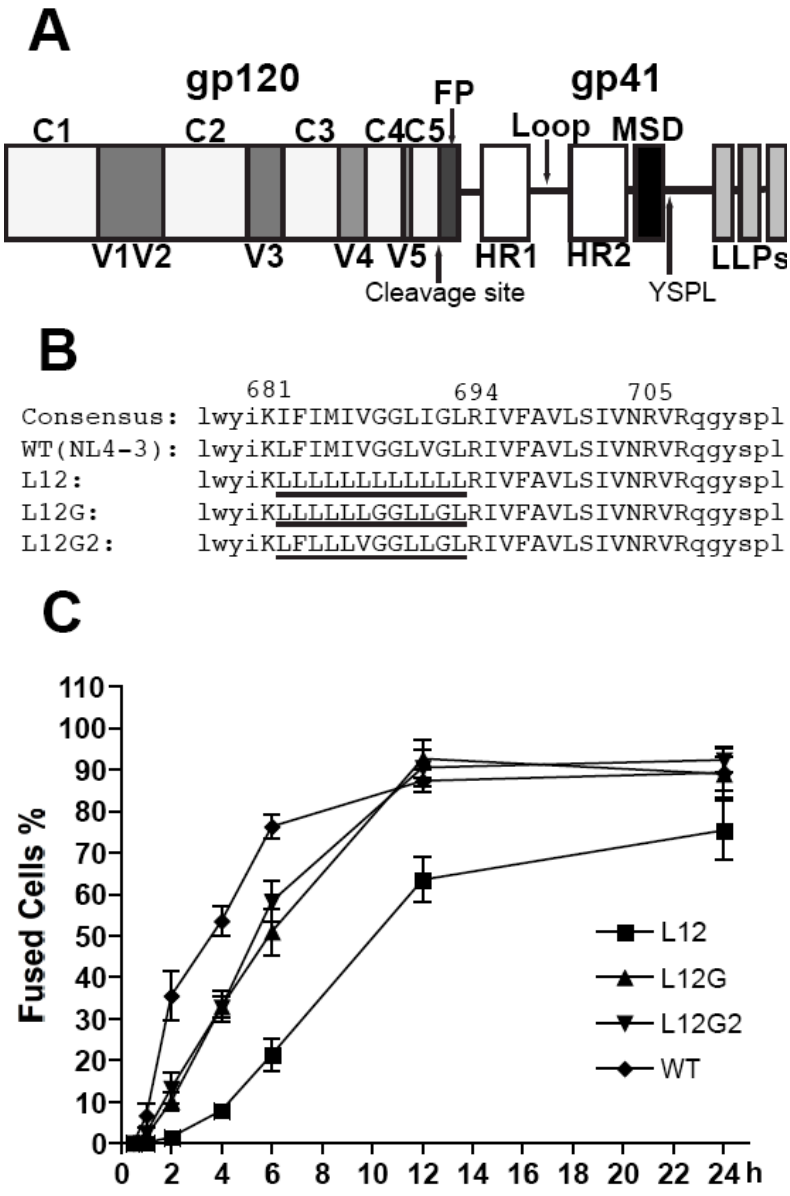

FIG. 1. Kinetics of cell-cell fusion mediated by the Env**.** (A) A schematic structure of the HIV-1 Env sequence (FP: fusion peptide; HR: heptad repeat; Loop: immunodominant loop; MSD: membrane-spanning domain; LLP: Lentivirus lytic peptide). (B) Amino acid sequences of HIV-1 MSDs. The consensus sequence of the HIV-1 MSD was generated by the alignment of the Env MSD sequences of all M and N group HIV-1 isolates from the Los Alamos HIV Sequences Database. The amino acid residues of the putative MSD region are shown as upper case letters. The position of the MSD in the sequence of HIV-1 NL4-3 is marked above the positively charged amino acid residues. The mutated portions of the HIV-1 MSD are underlined. (C) Cell-cell fusion kinetics. 293T cells were transfected with the proviral vector, pTN6-GFP, which carries the MSD mutant and a GFP reporter gene. At 48 h posttransfection, the transfected 293T cells were cocultured with the CMAC-blue-loaded JC53BL indicator cells for specific times. Fused cells were observed and quantitated by fluorescent microscopy.

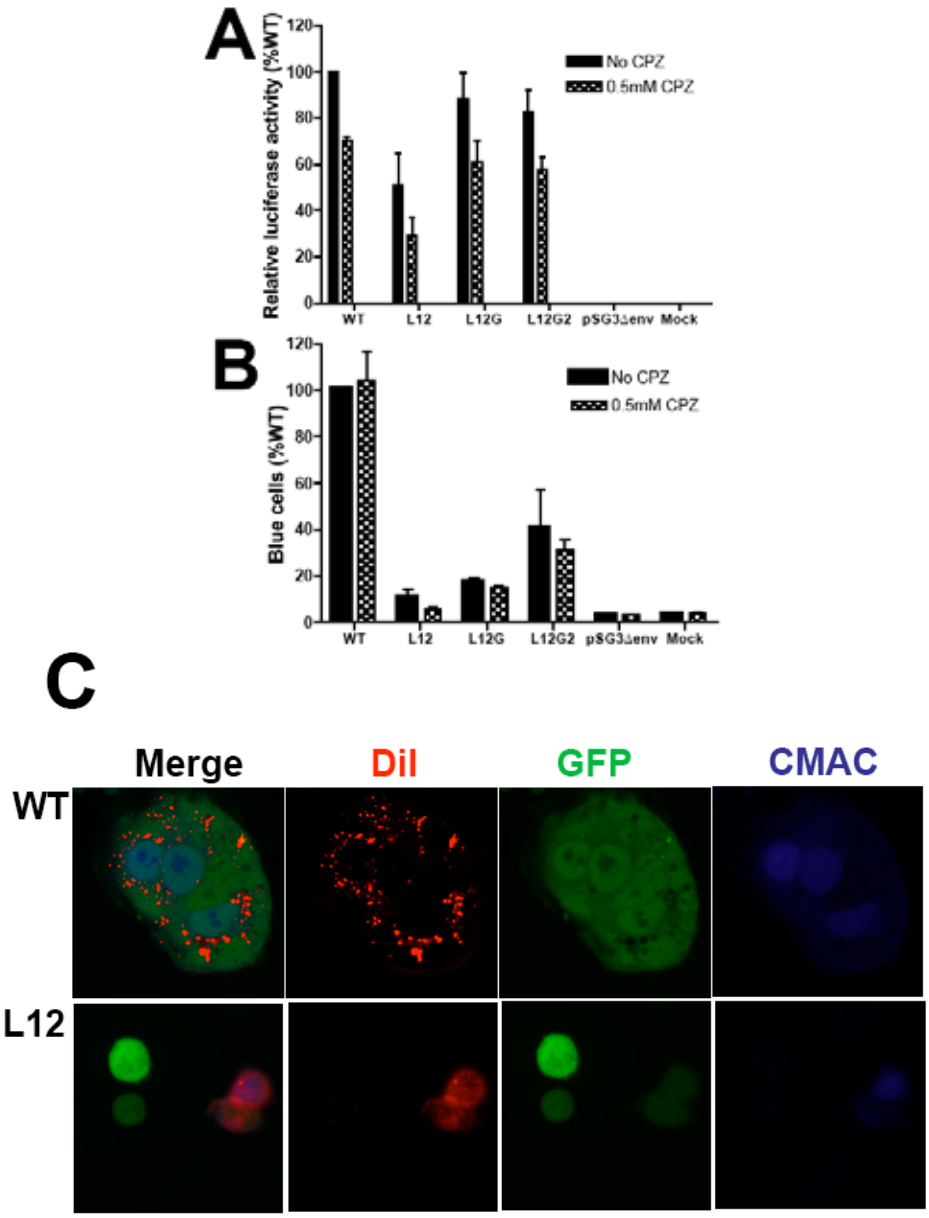

FIG. 2. Examination of hemifusion intermediates**.** (A) Cell-cell fusion mediated by Env. 293 T cells, which were transfected with the Env expression vector, pCDNA3.1, and JC53BL indicator cells were cocultured for 2 h and then were treated with 0.5mM CPZ for 30 seconds. After extensive wash, cells were incubated in complete DMEM for 24 h and then were subjected to a luciferase activity assay. (B) Virus-cell entry assay. Proviral constructs were cotransfected into 293T cells with pCMV-BlaM-Vpr vectors. Viruses containing the BlaM-Vpr fusion protein were pelleted by ultracentrifugation through 25% sucrose. Viral pellets normalized by p24 ELISA were used to infect JC53BL indicator cells for 2 h. The cells were then treated with 0.5mM CPZ for 30 seconds. After extensive wash, cells were loaded with fluorescent dye CCF2-AM. Blue cells were counted by flow cytometry analysis after 16 h of incubation. (C) Three color microscopy of cell-cell fusion mediated by the Env. 293T cells, which were transfected with pTN6-GFP proviral vectors, were cocultured with JC53BL indicator cells, which were double stained with DiI (red) and CMAC-blue, for 2 h. Fused cells were observed by fluorescent microscopy.

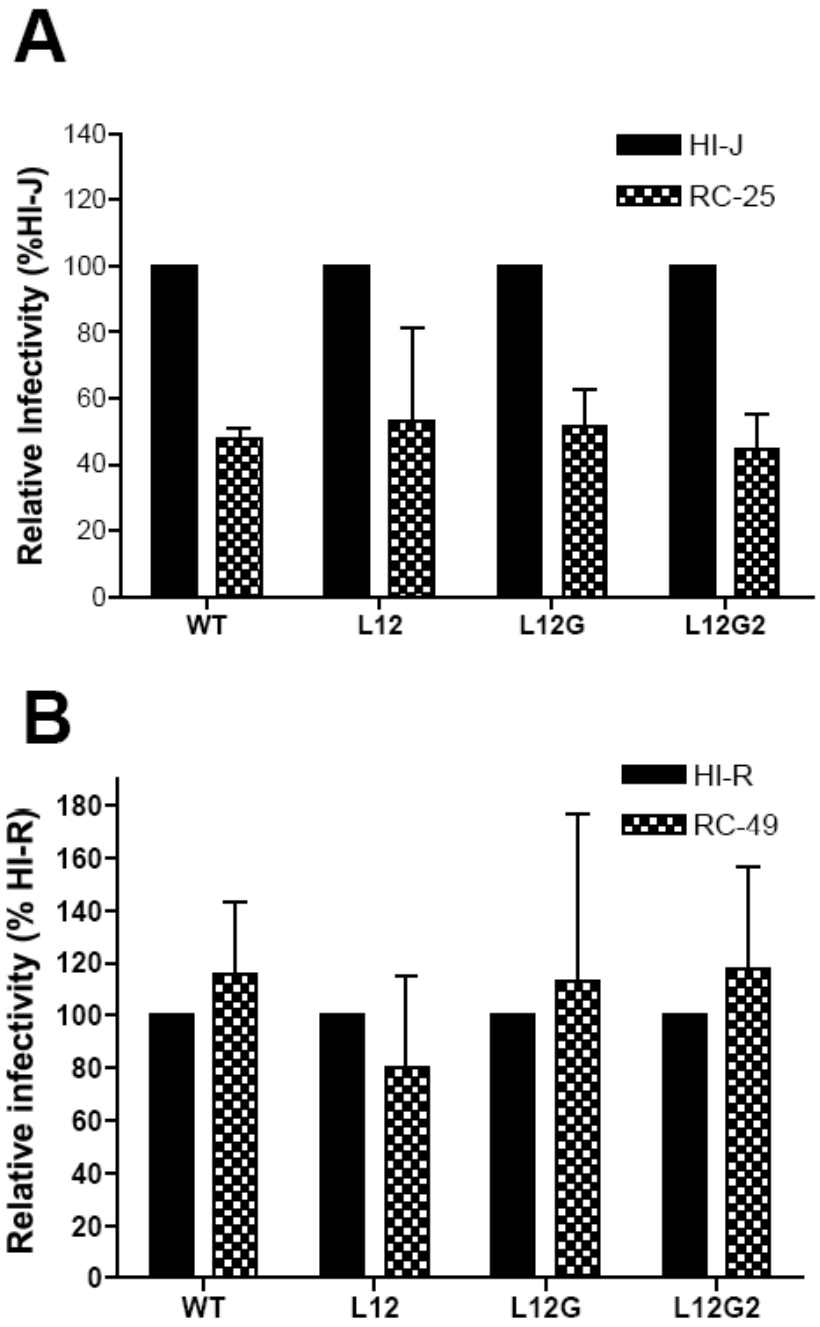

FIG. 3. Sensitivity of MSD core mutants to the levels of CD4 and CXCR4 on cell surface. The Env mutants were cloned into a proviral vector, pTN7, in which the *nef* coding sequence was replaced by a renilla luciferase reporter gene. Viruses produced by transfecting 293T cells with pTN7 constructs were used to infect HI- and RC- cell lines (Kabat). Luciferase activities were measured 48 h postinfection. (A) Cell lines HI-J and RC-25 have the same surface level of CXCR4, but the CD4 level of HI-J is 15-fold higher than that of RC-25. (B) The cell surface levels of CD4 are similar between HI-R and RC-49 cells. But the surface level of CXCR4 on RC-49 is 6-fold lower than that of HI-R cells.

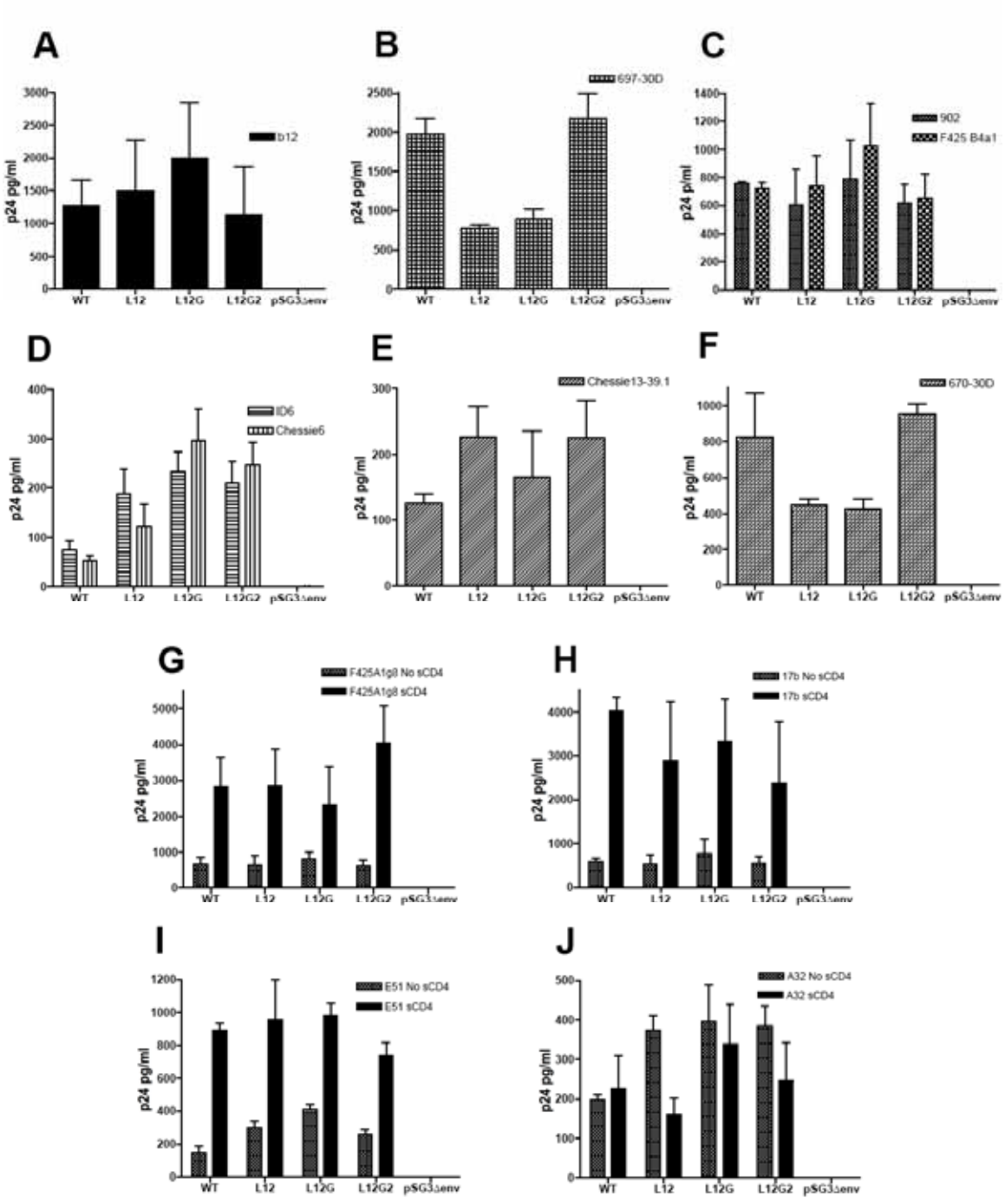

FIG. 4. gp120 virus capture assay**.** Purified viruses were incubated at 37°C in 96-well plates which have been pre-coated with anti-gp120 monoclonal antibodies. The coating antibodies target to different epitopes of the gp120 glycoprotein: b12 (A) binds to the CD4-binding site, 697-30D (B) to the V2 region, F425 B4a1 and 902 (C) to the V3 loop, ID6 and Chessie6 (D) to the C1 region, Chessie13-39.1 (E) to the C2 region, 670-30D (F) to the C5 region, F425 A1g8 (G), 17b (H) and E51 (I) to the coreceptor-binding region, and A32 (J) to the C1/C4 region. The bound viruses were lysed in PBS containing 0.5% Triton X-100 and then subjected to a p24 ELISA assay.

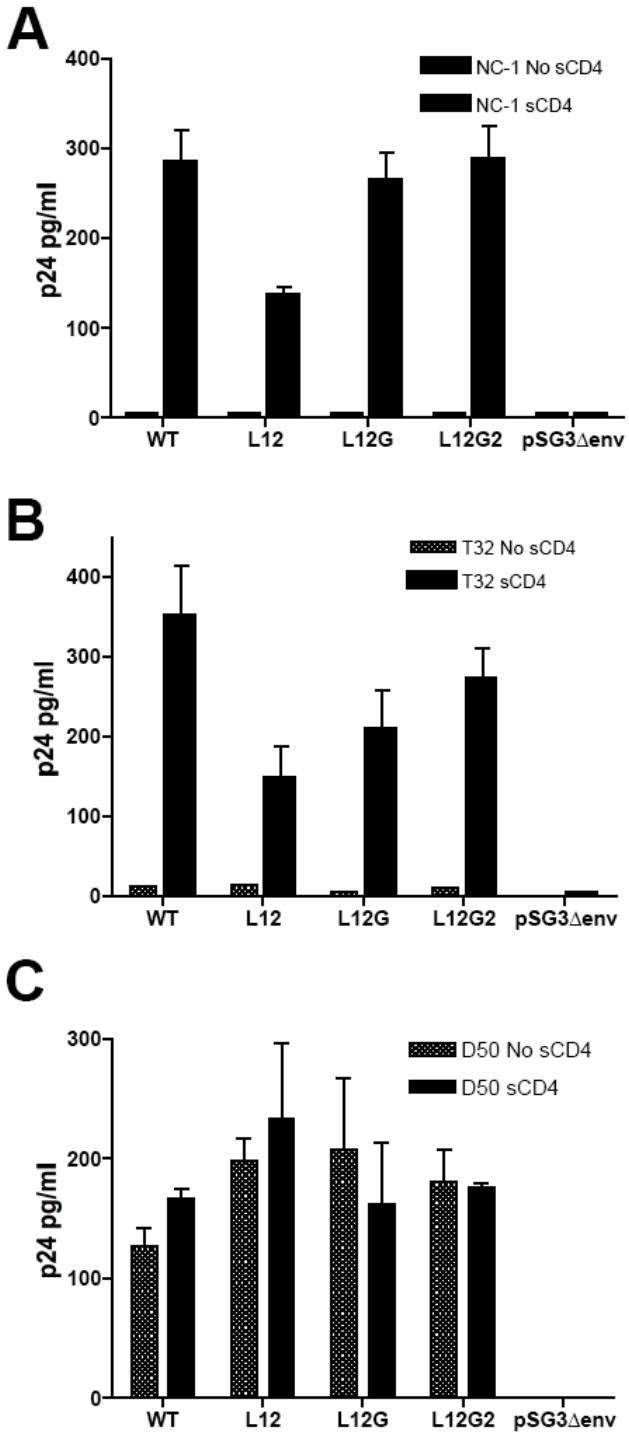

FIG. 5. gp41 virus capture assay**.** Purified viruses were incubated at 37°C in 96-well plates which have been pre-coated with anti-g41 monoclonal antibodies. The coating antibodies target to different epitopes of the gp41 glycoprotein: NC-1 (A) to the 6-helix bundles, T32 (B) to the immunodominant loop of gp41 ectodomain, and D50 (C) to the HR2 region. Bound viruses were lysed in PBS containing 0.5% Triton X-100 and then subjected to a p24 ELISA assay.

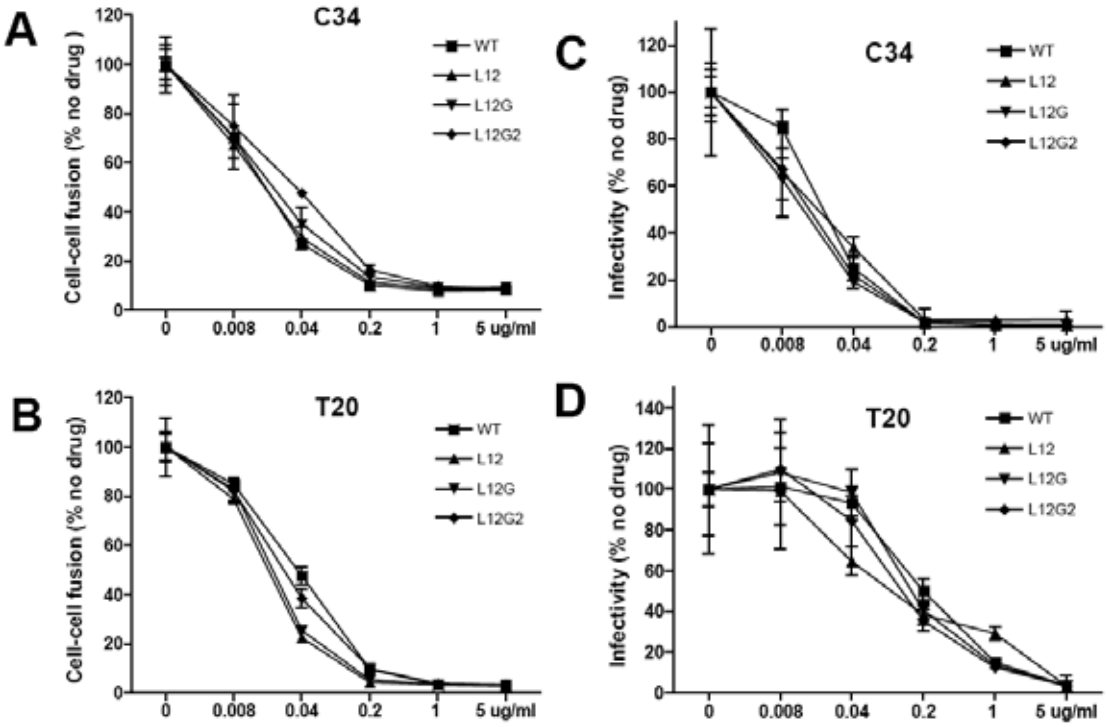

FIG. 6. C34 and T20 inhibition of cell-cell fusion mediated by the Env and viral infection**.** 293T cells transfected with the Env expression vector, pCDNA3.1, were cocultured with JC53BL indicator cells. Cell mixtures were incubated for 24 h in the presence of fusion inhibitors, C34 (A) and T20 (B), at specific concentrations, and then were subjected to a luciferase activity assay. JC53BL indicator cells were infected with the p24-normalized viruses in DMEM containing 1% fetal bovine serum, 80μg/ml DEAE-Dextran and fusion inhibitors, C34 (C) and T20 (D), at specific concentrations. Complete DMEM was added following 2 h incubation and cells were analyzed for luciferase activity 48 h post infection.

DISCUSSION

Previous studies have demonstrated that, in addition to anchoring the Env in membrane, the membrane-spanning domain of HIV-1 gp41 plays a role in Env incorporation and virus-cell entry during the viral life cycle (289, 359). The MSD, and in particular its N-terminal "core" region, is a very highly conserved region in the Env sequence. We have demonstrated that the most conserved residue F683 and GGXXG motif in the MSD "core" are critical for the virus-cell entry and subsequent viral infectivity by using a "recovery-of-function" mutagenesis approach (289). Truncation of the cytoplasmic domains of the MSD "core" mutants was not able to rescue the defect in virus-cell entry. In the current study, we investigated the molecular basis for the defects in viral entry observed with the MSD "core" mutants through an examination of conformational changes in the extracellular domains of Env, as well as changes in the efficiency of virus-cell attachment, receptor/co-receptor recognition, and membrane fusion.

Virions carrying the MSD "core" mutants all exhibited WT binding to the surface of target cells at different temperatures, indicating that substitutions in the MSD "core" regions did not influence the specific virus-cell binding mediated by the interaction of HIV-1 Env with CD4 receptors and chemokine coreceptors on cell surface. Consistent with this result, we demonstrated that changes in the surface levels of CD4 receptors and CXCR4 coreceptors on target cells gave rise to similar variations in viral infectivity for the WT and for the MSD mutants. This result suggested that substitutions in the MSD "core" regions did not affect the utilization of CD4 receptors and CXCR4 coreceptors on cell surface by HIV-1 Env. Thus, in spite of the altered MSD "core" region in the gp41 glycoprotein, the general structure of the Env glycoprotein of the MSD mutants was retained, and the conformation of the CD4-binding site and chemokine receptor-binding site remained intact. However, the MSD "core" mutants showed significant differences

in the kinetics of cell-cell fusion mediated by HIV-1 Env. This was most pronounced for L12, which exhibited a clear lag phase prior to initiation of fusion and a slower rate of increase in the extent of fusion. The mutants L12G and L12G2 demonstrated intermediate kinetics between L12 and WT in both the initiation and expansion period of cell-cell fusion. Therefore, the defect in virus-cell entry of the MSD mutants is most likely due to the impaired membrane fusion mediated by the Env mutants, in particular at the initiation stage.

The utilization of a panel of MAb in a solid state virus binding assay allowed us to indirectly probe for changes in the conformation of gp120 and gp41 by determining whether exposure of a variety of epitopes had been altered by the MSD mutants. Consistent with the equivalent entry of all three mutants into cells expressing different levels of CD4, each mutant bound to the CD4-binding site antibody b12 similarly to WT. In addition, the regions involved in coreceptor recognition bound to the CD4 inducible MAbs (F425A1g8, 17b and E51) at WT levels. However, minor conformational changes, which were restricted to specific regions of gp120, were observed in the MSD mutants. Among all the constant and variable regions of gp120, only the V2, C1, and C5 regions exhibited altered binding to their cognate MAbs (697-30D, ID6, Chessie 6, 670-30D), while the remaining regions in gp120 showed no differences in antibody binding. Since C1 and C5 have been implicated in the non-covalent interactions between gp120 and gp41, the altered accessibility of these regions to antibodies could reflect a perturbation in the gp120-gp41 interface.

While CD4 induced epitopes in the bridging sheet were presented equally by the mutants and WT, minor localized conformational changes were also observed in the gp41 ectodomain of the MSD mutants by the reductions in the formation of the 6-helix

bundle (MAb NC-1) and exposure of the immunodominant loop to MAb T32. This suggested that even though the open configuration of gp120 with an exposed bridging sheet could be stabilized by the binding of CD4 in the MSD mutants, it was less efficient at inducing downstream conformational changes in gp41. Moreover, this could result in the lag phase and slower kinetics of fusion observed for the MSD "core" mutants. It is difficult to define quantitatively how the conformational changes observed above might correlate with the decrease in fusogenicity. However, one possible interpretation of these results is that changes in the MSD core alter in a subtle manner the interaction of gp120 and gp41 in such a way that there is a greater energy barrier to conformational changes in gp41 induced by CD4 and CXCR4. This would result in a reduction in overall efficiency of the fusion process. It seems likely, however, that once the conformation change is initiated it progresses with equivalent kinetics to WT, since all of the mutants showed similar sensitivity to the fusion inhibitors T20 and C34, which bind to the trimeric coiled-coil of HR1 and prevent 6-helix bundle formation. Previous studies have shown that reductions in the kinetics of gp41 conformation changes result in enhanced inhibition by these fusion inhibitors, and this was not observed with the MSD "core" mutants. The lack of any evidence for hemifusion intermediates or defects in fusion pore expansion in these studies, suggests that the defect in the MSD core mutants is in the initiation of conformational changes required for fusion rather than at these later stages of the fusion process.

How might changes in the amino acid composition of the MSD "core" induce conformational changes and inhibit processes within the ectodomain of Env? The highly conserved GGXXG motif in the HIV-1 MSD "core" region has been postulated to facilitate the intramembrane helix-helix association by providing a relatively flat platform for the contact of the three helixes (276, 288). Moreover, the F683 and V687 are also

suggested to contribute to such an interaction via a (FXX)(V)GGXXG motif (317). It is possible that these interactions are optimized to allow some flexibility within the trimer in order to facilitate conformational changes in gp41. By substituting a stretch of hydrophobic leucine residues into this region, it is likely that affinity of both protein-protein and protein-lipid interactions will be increased, or that the orientation of helixes in the membrane would be altered. If this resulted in a more rigid trimeric interaction, it could explain altered interactions with gp120 and the imposition of a greater energy barrier to gp41 conformational changes, with subsequent impact on the formation of 6-helix bundles and membrane fusion.

In summary, the experimental approaches described here have demonstrated that consistent with its high sequence conservation, the native MSD “core” amino acid sequence of HIV Env is critical for efficient membrane fusion during viral infection. Substitutions in the MSD “core” region reduced the fusogenicity of the Env mainly by inducing structural changes in the gp41 ectodomain, influencing gp120-gp41 interactions and consequently impairing initiation of the fusion process.

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DISCUSSION

The membrane-spanning domain (MSD) of the human immunodeficiency viruses type I (HIV-1) envelope glycoprotein (Env) is critical for its biological activities. In this dissertation, we studied the intramembrane topology of the MSD and its biological functions during viral replication through genetic approaches. By examining a series of C-terminal-truncation mutants of the HIV-1 gp41 glycoprotein that substituted termination codons for amino acids 682 to 708, we showed that this entire region is required for efficient incorporation of HIV-1 Env into budding virions and WT levels of viral infectivity. In contrast, a region from residues K681 to A698 (18 aa) can effectively anchor the protein in the membrane, allow efficient transport to the plasma membrane, and mediate WT levels of cell-cell fusion. Based on the analysis of these mutants, a "snorkeling" model is proposed for the HIV-1 MSD. In this we hypothesize that the 12 hydrophobic residues (L682 to L693) form an intramembrane α-helix – the "core" region – and the flanking charged amino acid residues at 681 and 694 are buried in the lipid while their charged amino groups, via their long side chains, are presented to and neutralized by polar head groups of membrane. The MSD "core" region defined in the "snorkeling" model is composed of highly conserved hydrophobic residues and glycine motifs. By examining the "recovery-of-function" mutants, in which the MSD "core" region was initially replaced by 12 leucine residues and then specific residues and glycine motifs were reintroduced, we demonstrated that the highly conserved "core" region is not necessary for the normal expression, processing, intracellular transport, and viral incorporation of HIV-1 Env. It is, however, critical for the cell-cell and virus-cell membrane fusion mediated by Env complexes and consequently for viral infectivity. Further studies showed that defects of the MSD "core" mutants in membrane fusion are correlated with minor conformational changes in the V2, C1, and C5 regions in gp120

and the HR1 and immunodominant loop in gp41, while these changes do not influence specific virus-cell attachment, nor CD4 receptor and CXCR4 coreceptor recognition by the Env. Moreover, we presented evidence that membrane fusion mediated by the MSD "core" mutants was unlikely to be arrested at the hemifusion stage, and that alterations in the gp41 ectodomain induced by the MSD "core" mutants were unlikely to slow the formation of the 6-helix bundle once conformational changes were initiated. These results argued that the highly conserved residues in the MSD "core" region modulate certain early events of HIV-1 Env, which are required to initiate the formation of the 6-helix bundle and the subsequent process of membrane fusion.

MSD Structure

In order to understand the intramembrane structure of a MSD, we have to answer several basic questions including: where is the MSD located; how many amino acid residues are involved; and what structure it is? Based on reported studies, it has been generally accepted that the HIV-1 Env contains a single membrane-spanning domain that is located in the most hydrophobic region (K681 to R707) in the gp41 glycoprotein. However, the exact length, N- and C- termini, and intramembrane topology of the MSD still remained unclear. Theoretically, a single MSD forms an intramembrane α-helix and requires 24-27 amino acids to span a typical biological membrane (35-40Å in depth). This leads to the original model of the HIV-1 MSD, in which the 27 residues span the membrane in the form of a α-helix with the residues K681 and R707 located at membrane surface. However, this would result in a highly charge residue, R694, being positioned in the center of the lipid bilayer, which is predicted to be energetically unfavorable. In this study, we demonstrated that 18 amino acid residues are sufficient to constitute a biologically functional transmembrane region, which is consistent with the report that 17 residues in the MSD of influenza HA are the minimal requirement for a

stable membrane anchor and cell-cell fusogenecity. Our results argue that, for transmembrane proteins, the length of their MSDs could be much shorter than the theoretical model and that the intramembrane structure should not be simply explained as a α-helical region.

Therefore, we proposed the “snorkeling” model to explain the intramembrane topology of HIV-1 MSD with 18 residues. Several things are notable in this model. First, the “snorkeling” model is a semi-stable structure. The vertical length of the “snorkeling” structure, including the α-helical “core” region (21 Å) and side chains of lysine 681 (6.4 Å) and arginine 694 (7.0 Å), reaches 34.4 Å, which is equivalent to the depth of biological membrane. Therefore, in order to span the entire membrane, the “core” region and side chains have to be maximally extended in the vertical direction, resulting in a less flexible intramembrane structure. When the stress, which is generated by the conformational changes in the gp41 ectodomain, forces the MSD to move relatively to membrane, the charged groups of K681 and R694 could be easily pulled into hydrophobic environment and then disrupt the local structure of a lipid monolayer. In this way the semi-stable “snorkeling” structure could contribute to initiating lipid mixing during membrane fusion mediated by HIV-1 Env.

Second, the “snorkeling” model provides a novel strategy to harbor charged amino acid residues within the membrane without generating energy barriers. As pointed out above, in the original model, residue R694 was positioned in the middle of membrane, which is energetically unfavorable. Several strategies have been reported to neutralize or accommodate intramembrane charged residues: (1) An intramembrane channel (similar to a potassium channel) could be formed by multiple α-helixes or β-sheets with their hydrophobic residues in contact with the lipid and the charged residues pointing

into the channel (195); (2) A binding partner containing oppositely charged groups could neutralize the charges in the membrane (such as with the MHC-II) (68), or (3) A cation-π interaction could form between positively charged groups and benzene rings from aromatic residues (83). However, as we describe in Paper 1, none of these alternates are applicable to HIV-1 MSD. The "snorkeling" model not only solves the caveats of the original model, but also is consistent with the report that the R694 is critical for the biological activities of HIV-1 Env (241).

Moreover, in the "snorkeling" model, the sequence C-terminal to the 18 residues in HIV-1 MSD is located in cytoplasm and presumably associated with membrane at the cytoplasmic interface. It has been reported that the amino acid composition of this region is less important than its length for stabilizing HIV-1 Env in membrane and for its fusogenecity (241). In addition, this region is less conserved than the MSD "core" domain. Therefore, although the role played by this C-terminal region in the MSD is still unclear, it is likely to fill a certain membrane proximal space, between the "snorkeling" structure and cytoplasmic domain of Env, which is critical for the biological activities of HIV-1 Env. One of the possibilities is that the C-terminal region of the HIV-1 MSD is used to keep the cytoplasmic domains in the gp41 trimer at a defined distance and thus prevent steric hindrance. The arginine residues at 705 and 707 are likely to stably associate with the membrane through the interaction between their positively charged amino groups and the negatively charged phosphate groups at the membrane surface. This structure could enhance the anchor function of the HIV-1 Env in membrane. On the other hand, it could also contribute to the structural and functional separation of the MSD from the cytoplasmic domain of HIV-1 Env, thereby, prevent the changes in the MSD during fusion from affecting functions of the downstream cytoplasmic domain.

As to the N- and C- termini of HIV-1 MSD, although the relatively rigid “snorkeling” structure narrows down the flanking residues of the “core” region, it is still difficult to identify the real time termini since the MSD is a dynamic region and keeps moving relative to membrane under the force generated during the Env activities.

MSD Function

The primary function of the HIV-1 MSD is as a membrane anchor of the Env glycoprotein in both viral and cellular membrane. According to the “snorkeling” model, the “core” structure is the major determinant of the minimal requirement in length for a stable anchor of the Env in membrane. Moreover, evidence presented in this work also suggests that the specific amino acid composition in the MSD is not required for stably anchoring HIV-1 Env in membrane when the hydrophobicity of the MSD residues is retained. It is likely that during the process of expression, proteolytic processing, and intracellular transport of HIV-1 Env, the MSD only functions as a membrane anchor; therefore, both the C-terminal truncation mutants and MSD “core” mutants demonstrated similar intracellular trafficking behaviors to WT. However, in other biological activities of HIV-1 Env, such as viral incorporation, membrane fusion, and subsequent viral infection, the native MSD is required and plays a key role in these processes.

The mechanism of HIV-1 Env incorporation is not completely understood. Although the interaction between the cytoplasmic domain and matrix protein (MA) has been suggested to be important for Env incorporation, the truncation mutant CTΔ144 is incorporated into viruses at a WT level. The association of Env:Gag with lipid rafts also has been shown to play a role in Env incorporation. In this study, we demonstrated that although specific residues in the N-terminal “core” region are not critical for Env incorporation, the C-terminal portion of the MSD plays a key role in the incorporation of

HIV-1 Env (as assessed through gp41 incorporation) into budding virions and maintaining the stability of Env (gp120 stability) on the viral surface. So far the molecular mechanism of these observed facts still remains unclear. However, it is possible that the C-terminal portion of the MSD is associated with lipid rafts and contributes to viral incorporation of gp41. Moreover, this region could also be important to maintain conformational integrity of the gp41 ectodomain. Therefore, C-terminal truncations into this region impaired gp41 incorporation and gp120 stability.

In contrast, the MSD "core" region is critical for Env-mediated membrane fusion, while the C-terminal portion of the MSD is dispensable. The highly conserved residues in the MSD "core" are important to maintain the normal conformation of the gp120 glycoprotein and the gp41 ectodomain. Although the alteration of these residues only resulted in minor and localized conformational changes in Env, these structural changes modulated the gp120:gp41 interaction and impaired initiation of conformation changes that are required for formation of the 6-helix bundle and subsequent fusion pore. Therefore, the amino acid composition of the MSD "core" region influences some early events that initiate the gp41 fusion machine into the process of membrane fusion. However, so far, we are not able to quantitatively correlate these conformational changes to the defects in fusogenicity and viral infectivity, due to lack of an approach to consolidate conformational changes in different regions. Thus the possibility remains that the mutations in the "core" region may generate other structural or functional changes in Env that have not been detected yet in this work.

It is notable that the defects of the MSD "core" mutants in cell-cell fusion can be rescued by higher levels of HIV-1 Env on cell surface. This suggests that the "core" region regulates the Env fusogenicity in a stoicmetric manner. It is likely that the number of Env

incorporated into HIV-1 virions is far less than that required to reach the threshold of rescue. Nevertheless, the concentration-dependent membrane fusion mediated by the Env mutants also argues that a single HIV-1 Env complex is not sufficient to mediate membrane fusion. The minimal number of Env to mediate membrane fusion remains unclear, but is likely to be influenced by multiple factors, such as the Env sequence, lipid composition, number of receptors and coreceptors, and environmental parameters (*e. g*. temperature).

The current model of membrane fusion predicts a hemifusion intermediate. In the process of membrane fusion, the outer leaflets of two membranes merge first, whereas the distal membrane leaflets remain separate, forming a lipid bilayer structure – the hemifusion intermediate. In the MSD "core" mutants and C-terminal truncation mutants, we did not observe any evidence of hemifusion intermediates during cell-cell fusion mediated by the HIV-1 Env. This is probably because that these Env mutants influence membrane fusion before the hemifusion stage and therefore do not arrest at a stable hemifusion intermediate. On the other hand, it is also possible that the cell-cell fusion mediated by HIV-1 Env does not go through a hemifusion stage at all. The outer and inner leaflets of two cellular membranes could break at the same time and then merge, thereby, skipping the hemifusion stage.

It has been reported that the HIV-1 Env glycoprotein is associated with lipid rafts and depletion of cholesterol in membrane impairs Env incorporation into virions and viral infectivity (45, 323, 324). Although, in this work, we did not present direct evidences showing the MSD:lipid raft interaction, our results leave open the possibility that the C-terminal portion of the MSD could be associated with lipid rafts and contribute to Env incorporation. Moreover, it is possible that the specific amino acid composition of the

MSD “core” region also plays a role in the association of HIV-1 Env with lipid rafts; however, multiple regions (e.g. the MPER region and the LLPs) in the Env could also be involved in such an interaction, thereby, rescuing Env incorporation of the MSD “core” mutants. The role of the Env:raft association in viral replication is still not completely understood. As to the HIV-1 MSD, lipid rafts comprise a thinner membrane and are therefore more suitable for the “snorkeling” structure. Moreover, lipid rafts tend to be more rigid and may have lower energy barriers to initiate lipid mixing during membrane fusion.

The MSD “core” region sequence is highly conserved within gp41, this is particularly true of the GGXXG motif and the phenylalanine 683. The GGXXG motif has been proposed to generate a relatively flat platform on surface of the intramembrane α-helix, which facilitates a close contact between helices in the membrane and then contributes to oligomerization of transmembrane proteins (287, 288). Although it is unlikely that the GGXXG motif in the MSD plays a major role in driving trimerization of HIV-1 Env, oligomerization of the MSD mediated by this motif could be critical for the biological activities of gp41. We have shown that the GGXXG motif is important to maintain the fusion-competent conformation of the gp41 ectodomain. Trimerization of the MSD could arrange and lock the gp41 ectodomain and cytoplasmic domain into the orientations that are optimal for the gp120:gp41 interaction and gp41:Gag hexamers interaction, thereby, contributing to the activation of gp41 fusion competency and Env incorporation into virions. Moreover, oligomerization of the MSD would form a “snorkeling” MSD trimer, which would be more stable than a single MSD “snorkeling” structure in membrane.

The phenylalannine 683 in the MSD “core” region is also important for the Env fusogenicity and viral infectivity. It has been reported that the phenylalanine residue is

able to promote self-interaction of transmembrane domains by stabilizing the GXXXG motif, which is C-terminally close to the phenylalanine residue (317). On the other hand, although the intrahelical interaction of F683 and R694 through a cation-π bond has been ruled out for HIV-1 MSD, it would still be possible for this phenylalanine residue to interact with K681 on the same MSD and the positively charged residues on other helixes by forming cation-π bonds. Further studies in the structural and functional roles of the F683 could help reveal the N-terminal topology of the MSD "core".

In this dissertation, we have examined the structural and functional roles of HIV-1 MSD in viral replication. The MSD is critical for Env fusogenecity by modulating its conformational integrity. Some questions still remain to be answered in order to fully understand, at a molecular level, the role of the MSD "core" in the Env mediated membrane fusion: e.g. Does the MSD structurally and functionally modulate the fusion peptide in gp41? Are the MSD "core" mutants able to completely expand fusion pores? Does the MSD region influence Env flexibility or mobility in membrane? What is the dynamic topology of the MPER-MSD junction? What is the structural and functional role of the C-terminal portion of HIV-1 MSD? And is the MSD associated with lipid rafts? Future studies will undoubtedly provide new insights into these issues.

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LIST OF ABBREVIATIONS

293T	human kidney epithelial cell line
AIDS	acquired immunodeficiency syndrome
AP-2	clathrin-associated protein 2
AP-3	clathrin-associated protein 3
ATP	adenosine triphosphate
bp	base pair
CA	capsid domain or capsid protein
capsid	protein shell of a virion
CD4	complement determinant
cDNA	complementary DNA
COS-1	African green monkey kidney fibroblast-like cells
CT	cytoplasmic tail
Cys	cysteine
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
env	envelope gene
Env	Envelope glycoprotein
F-actin	filamentous actin
FITC	fluorescein isothiocyanate
gag	group specific antigen gene
Gag	group specific antigen polyprotein
GFP	green fluorescent protein
GTPase	guanine nucleotide phosphatase
HA	hemagglutinin
HIV	human immunodeficiency virus

HIV-1	HIV type I
HTLV	Human T-cell leukemia virus
I domain	interaction domain
IN	integrase protein
L domain	late domain
LTR	long terminal repeat
MA	matrix domain or matrix protein
μm	micrometer
MMTV	mouse mammary tumor virus
M-PMV	Mason-Pfizer monkey virus
MSD	membrane-spanning domain
mRNA	messenger RNA
MuLV	Moloney murine leukemia virus
MVB	multi-vesicular body
NC	nucleocapsid domain or nucleocapsid protein
nm	nanometer
NMR	nuclear magnetic resonance
nt	nucleotide
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pen	penicillin
pH	potential of hydrogen
Phe	phenylalanine
PIC	preintegration complex
pol	polymerase gene
Pol	polymerase protein domain

poly(A)tail	adenine nucleodies attached to messenger RNA
PPT	polypurine tract
PR	protease protein
pro	protease gene
retrovirus	RNA tumor virus
Rev	regulator of expression viral proteins
RNA	ribonucleic acid
RRE	Rev response element
RT	reverse transcriptase
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SIV	simian immunodeficiency virus
SRP	signal recognition particle
strep	streptomycin
SU	surface unit
TBS	Tris-buffered saline
TGN	trans-Golgi network
Thr	threonine
TM	transmembrane domain or protein
tRNA	transfer RNA
Tsg101	tumor susceptibility gene 101
Tyr	tyrosine
VSV	vesicular stomatitis virus
VSV-G	glycoprotein of VSV
WT	wild type