Truncation of the Membrane-Spanning Domain of Human Immunodeficiency Virus Type 1 Envelope Glycoprotein Defines Elements Required for Fusion, Incorporation, and Infectivity[▽]

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The membrane-spanning domain (MSD) of the envelope (Env) glycoprotein from human (HIV) and simian immunodeficiency viruses 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 type 1 (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 residues 681 to 698, which contains highly conserved hydrophobic residues and glycine motifs and extends 4 amino acids beyond 694R, can effectively anchor the protein in the membrane, allow efficient transport to the plasma membrane, and mediate wild-type 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.

Human immunodeficiency virus type 1 (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 1 membrane-spanning glycoprotein, is a trimeric complex composed of three noncovalently linked heterodimers of gp120, the receptor-binding surface (SU) component, and gp41, the membrane-spanning, transmembrane (TM) component (12, 26, 44, 45). The gp120 and gp41 glycoproteins are synthesized as a precursor gp160 glycoprotein, which is encoded by the *env* gene. The gp160 precursor is cotranslationally glycosylated and, following transport to the *trans*-Golgi network, is cleaved into the mature products by a member of the furin family of endoproteases (45). Mature Env proteins are transported to the plasma membrane, where they are rapidly endocytosed or incorporated into virions (5, 33, 43). Recent evidence suggests that endocytosis and intracellular trafficking of Env is required for its interaction with Gag precursors and for efficient assembly into virions (20).

HIV-1 Env molecules function as quasistable “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 coreceptor (CCR5 or CXCR4), can form (6, 7). Coreceptor

binding is necessary for additional conformational changes in gp41 and for complete fusion (3). The gp41 monomer has three subdomains, an ectodomain, a membrane-spanning domain (MSD), and a cytoplasmic domain (39). 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. Following the binding of gp120 to the CD4 receptor and the CCR5/CXCR4 coreceptor, conformational changes are induced in Env that result in the exposure of the gp41 fusion peptide (32). 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 (24).

While heptad repeat regions 1 and 2 in the N-terminal ectodomain play key roles in Env-mediated fusion by bringing the viral and cell membranes into close proximity, an important function of gp41 is to anchor the glycoprotein complex within the host-derived viral membrane (18). 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 span residues K681 to R705 in the NL4-3 sequence (14, 16, 18). 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

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the hydrophobic center of the lipid bilayer. While some trans-membrane proteins do contain charged amino acid residues in their MSDs, it is normally considered to be energetically unfavorable without some mechanism to neutralize the charge (8, 13). Point mutation studies have yielded varying 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 (16, 29). On the other hand, accumulating data argue 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 (29), 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 simian immunodeficiency virus (SIV) Env (19, 41) suggested that the entire 27-amino-acid region is not required for the biological function of the 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-wild-type (WT) fusion (19, 41). Two subsequent residues were required (total, 25 amino acids) 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 (41). 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 predicts 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 nonfunctional protein that appeared to be retained in the endoplasmic reticulum (11). This contrasts with truncation experiments with the vesicular stomatitis virus (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 (1).

In order to understand if the “snorkeling” model is applicable to the HIV-1 MSD, we constructed a series of nonsense mutants with HIV-1 gp41 truncated in single-amino-acid steps at the C terminus from residue R707 to residue R694. For each mutant Env, we determined the membrane stability, fusogenicity, and ability to mediate infectivity. The results of these studies suggest that the 12-residue “core” (36) plus three subsequent hydrophobic amino acids is the minimal anchor domain for HIV-1 Env, 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, Manassas, VA), and JC53BL (available from the NIH AIDS Research and Reference Program as TZM-BL) cells were maintained in complete Dulbecco's mod-

HIV-1	681	694	705
WT (NL4-3)	lwyiKLFIMIVGGLVGLRIVFAVL	SIVNRVR	aggyspl
694R	lwyiKLFIMIVGGLVGLR*		
695I	lwyiKLFIMIVGGLVGLRI*		
696V	lwyiKLFIMIVGGLVGLRIV*		
697F	lwyiKLFIMIVGGLVGLRIVF*		
698A	lwyiKLFIMIVGGLVGLRIVFA*		
699V	lwyiKLFIMIVGGLVGLRIVFAV*		
700L	lwyiKLFIMIVGGLVGLRIVFAVL*		
701S	lwyiKLFIMIVGGLVGLRIVFAVLS*		
702I	lwyiKLFIMIVGGLVGLRIVFAVLSI*		
703V	lwyiKLFIMIVGGLVGLRIVFAVLSIV*		
704N	lwyiKLFIMIVGGLVGLRIVFAVLSIVN*		
705R	lwyiKLFIMIVGGLVGLRIVFAVLSIVNR*		
706V	lwyiKLFIMIVGGLVGLRIVFAVLSIVNRV*		
707R	lwyiKLFIMIVGGLVGLRIVFAVLSIVNRVR*		
SIV	689	705	716
(mac239)	aswiKYYQYGVYIVGVILLRIVYIVQMLAKLR	qggy	

FIG. 1. Amino acid sequences of HIV-1 MSDs. The amino acid residues of the putative MSD region are shown as uppercase letters and the flanking sequences as lowercase letters. Truncation mutations were generated by PCR mutagenesis to insert a stop codon (*) subsequent to the 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 for reference.

ified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and penicillin-streptomycin (all from Gibco-BRL, Rockville, MD). The cells were passaged three times per week under conditions of 37°C and 5% CO₂ in humidified incubators and were transfected at 50 to 70% confluence. The anti-gp120 902 monoclonal antibodies (MAbs) and anti-gp41 MAbs T32, D50, and 2F5, along with HIV-1 patient immunoglobulin, 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. Sheep anti-HIV-1 gp120 polyclonal antibody and goat anti-HIV-1 immunoglobulin G (H+L)-horseradish peroxidase were purchased from Clinia Corp. (San Marcos, CA) and Pierce (Rockford, IL), respectively.

Glycoprotein and proviral expression constructs. Construction of the HIV-1 Env expression vector pSRHS, which carries full-length *env*, *tat*, and *rev* genes from NL4-3, has been described previously (34). This simian virus (simian virus 40 [SV40 late promoter])-based vector contains a Mason-Pfizer monkey virus-derived long terminal repeat providing a polyadenylation signal. A unique XbaI 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 MSDs. Individual stop codons were inserted during the first-round PCR by using forward oligonucleotide primers that contained the required mutations and a reverse oligonucleotide primer *env*M2 (NL4-3 nucleotides 8500 to 8534). The first-round PCR fragment was then used as a reverse megaprimer, together with the *env*B7 forward primer (NL4-3 nucleotides 7786 to 7809), to generate second-round PCR fragments, which included 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 encoded mutants in which gp41 was 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 100 bp upstream from the MSD sequence. In this study, nucleotides and amino acid residues were labeled according to the NL4-3 sequence (GenBank accession no. AF324493) unless otherwise indicated.

Glycoprotein expression and immunoprecipitation. The pSRHS Env expression vectors or proviral DNA were transfected into COS-1 cells using Fugene6 (Roche, Indianapolis, IN) in six-well plates. At 36 to 48 h posttransfection, the

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 (125 μ Ci/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 media were filtered through a 0.45- μ m membrane to remove cellular debris. The 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 [pH 8.0]). Cellular debris was removed by microcentrifugation at 13,200 rpm for 1 min at 4°C. HIV-1 proteins were immunoprecipitated from cell lysates and supernatants by incubating them overnight at 4°C with pooled HIV-1 patient sera plus anti-gp41 MAbs T32 and D50. Immune complexes were incubated overnight at 4°C 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 the labeled proteins resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE) were visualized by autoradiography. The glycoprotein bands were quantified using a Cyclone phosphorimaging system (Packard, Meriden, CT) as previously described (41).

Cell-cell fusion assay. COS-1 cells were transfected with pSRHS expression vectors, and 293T cells were transfected with the proviral vector pNL4-3 by using Eugene6. At 36 to 48 h after transfection, cells resuspended by trypsinization were combined with JC53BL indicator cells at a 1:5 ratio. The cell mixtures were incubated for 6 h, 12 h, and 24 h (in the cell-cell fusion assay in which the proviral vector pNL4-3 was used, 10 μ M zidovudine [AZT] was added to the cell mixture in order to avoid the spread of infectious virions) and then 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°C in a microcentrifuge (Beckman, Palo Alto, CA). Luciferase substrate at a volume of 100 μ l (Promega, Madison, WI) was added to 10 μ l of each cell lysate, and the light emission was quantified using a Synergy multidetector microplate reader (Biotek, Winooski, VT).

Cell surface expression of Env glycoprotein. COS-1 cells were transfected with expression vectors, cultured for 36 to 48 h, and then, after removal from the plate, fixed for 20 min at 4°C in 4% paraformaldehyde (in phosphate-buffered saline [pH 7.2]). The fixed cells were stained for 1 h at room temperature with 5 μ g/ml Alexa 488-conjugated anti-gp120 MAb 902. The stained cells were subjected to flow cytometry analysis using the FACSCalibur system. For some experiments, a relative mean fluorescence index was calculated from the product of the percentage of transfected cells and the mean fluorescence intensity. The Alexa 488 conjugation kits were obtained from Invitrogen (Carlsbad, CA).

Infectivity of HIV-1 in JC53BL indicator cells. Medium from 293T cells transfected with proviral vectors was harvested 72 h posttransfection and filtered through a 0.45- μ m membrane to remove cellular debris, and the total virions were quantified using a p24 enzyme-linked immunosorbent assay (ELISA). The p24-normalized virus-containing supernatant (5 ng/well) was added to 1×10^5 JC53BL indicator cells cultured in DMEM containing 1% fetal bovine serum and 80 μ g/ml DEAE-dextran. Complete DMEM was added following a 2-h incubation, and the cells were analyzed for luciferase activity 48 h postinfection.

Single-round infection. 293T cells were cotransfected with pSRHS expression vectors and the pSG3 Δ env proviral vector. Medium was collected 72 h posttransfection and then subjected to p24 ELISAs. The p24-normalized virus-containing supernatants (5 ng/well) were used to infect 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 a 0.45- μ m membrane, and viral particles were pelleted by ultracentrifugation (100,000 $\times$ g; 2.5 h) through a 25% sucrose cushion. The viral pellets were resuspended in phosphate-buffered saline (pH 7.2), and the amounts of p24 and gp120 were measured using p24 and gp120 ELISAs (15). The amount of gp41 in virions was quantitated using chemiluminescence in a Western blot assay with anti-gp41 MAb 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 for the MSD in 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 Fig. 1. The more conserved HIV-1 MSD "core" region remained intact in all of the mutants.

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 the supernatant (B) were immunoprecipitated, followed by 10% SDS-PAGE analysis and autoradiography. (C) The bands of gp120 and truncated (tr.) gp41 in the supernatant were quantified by phosphorimager calculation, and the intensities of bands are shown in digital light units (DLU).

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 SV40 and a polyadenylation signal from Mason-Pfizer monkey virus (11, 17). 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 Fig. 2A, with the exception of mutants Env694R to Env697F, 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 to Env697F, 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 trun-

cated gp41 that were similar to WT levels, with mutant gp41 bands migrating faster than that of the WT. These data suggested that the introduction of stop codons into 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 into the SU and TM subunits.

An examination of labeled Env glycoprotein in the culture supernatant showed that levels of gp120 secretion were similar to those of the WT for most of the truncation mutants (Fig. 2B and C). 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 to Env700L (Fig. 2B and C), while their shedding of gp120 was similar to that of the WT. The remainder of the mutants, Env701S to Env707R, exhibited slightly lower levels of gp120 shedding than that observed with the WT 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, which resulted in the retention of the gp160 precursor in the endoplasmic reticulum (35), was used as a negative control.

Truncated Env glycoproteins exhibit differential cell surface expression of the Env glycoprotein. Since the 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 48 h posttransfection in 4% paraformaldehyde and stained with Alexa 488-conjugated anti-gp120 MAb 902. The cells were then subjected to flow cytometry analysis. As shown in Fig. 3A, with the exception of mutant Env706V, mutants Env698A to Env707R exhibited increasing amounts of Env surface expression (105% to 225%) compared to the WT (Fig. 3A). Consistent with the observed secretion of gp41 into the supernatant for mutants Env694R to Env697F, low levels of Env surface expression (14 to 35% of WT) was observed (Fig. 3A). Although mutants Env698A to Env700L also exhibited low levels of truncated gp41 secretion (Fig. 2C), Env was stably presented on the cell surface at relatively high levels (120 to 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 (4, 9, 28, 42).

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 was necessary in this region to confer on Env the capacity to mediate cell-cell fusion. Transiently transfected COS-1 cells were cocultured with JC53BL indicator cells, which contain a luciferase reporter gene under the control of the HIV-1 long

terminal repeat promoter and which can be activated by the Tat protein (38). Cell-cell fusion was measured by calculating the relative luciferase enzyme activities in cells expressing the mutant Envs compared to those expressing WT Env at 6 h, 12 h, and 24 h after coculture. The results of a representative experiment are shown in Fig. 3B, and they demonstrate that the three shortest Env proteins, Env694R to Env696V, 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% of WT fusion, but the remaining mutants induced near-WT levels (60 to 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 was also due to 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 coculture of 2 h. We did not find any evidence of hemifusion intermediates, in which DiI transferred from the target cell to that of the Env-expressing donor cells in the absence of intracellular dye (Cell Tracker Blue in target cells, GFP in donor cells) transfer (data not shown).

MSD truncation mutants show different patterns of cell-cell fusion in the context of virus. It is known that the intracellular behavior of HIV-1 Env can change in the presence of other viral proteins (30). 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 into 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 pattern of cell surface staining similar to that in Fig. 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 cocultured with JC53BL indicator cells for 6 h, 12 h, and 24 h in the presence of 10 μ M 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 to Env696I were unable to mediate cell-cell fusion (Fig. 4B). Surprisingly, mutant Env697F in this context induced WT cell-cell fusion rather than the

FIG. 3. Cell surface levels 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 anti-gp120 MAb 902 and then analyzed by flow cytometry. The surface levels of Env glycoprotein are shown as the relative mean fluorescence index (MFI) compared to that of the WT. (B) Cell-cell fusion assay. COS-1 cells transfected with pSRHS expression vectors were cocultured with JC53BL indicator cells. The cell mixtures were lysed after incubation for 6 h, 12 h, and 24 h, and then luciferase activity was measured. The results are from at least three independent experiments; the error bars represent standard deviations from the means. EV, empty vector.

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 48 h postinfection, luciferase activity in the indicator cells was quantitated. As shown in Fig. 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 to 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, pSG3Δenv 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 pattern of infectivity similar 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 to 703, confirming the importance of the MSD core-proximal region for infectivity. In this pseudotyped-virus system, a small increase in virus infectivity was observed for mutants Env704N to Env706V, while a lower level of infection was seen for Env707R (14.6%) relative to the 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 amounts of gp120 and p24 in viral pellets, after

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. tr., truncated. (B) 293T cells transfected with proviral DNA were mixed with JC53BL indicator cells and cocultured for 6 h, 12 h, and 24 h with 10 μ M AZT before the measurement of luciferase activity. The results are from at least three independent experiments; the error bars represent standard deviations from the means.

ultracentrifugation through a 25% sucrose cushion, were analyzed using gp120 and p24 ELISAs. We observed that the shortest Env mutant, 694R, incorporated 83.8% less gp120 into virus particles than the 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 the WT for mutant 707R.

Because we were concerned that the C-terminal truncation of HIV-1 Env might cause shedding of gp120 from the viral surface, we also examined the amount of gp41 associated with viral pellets by using Western blotting with the anti-gp41 Mab 2F5.

As shown in Fig. 6B and C, similar to its incorporation of gp120, the Env mutant 694R incorporated 79.2% less gp41 into viral particles than the WT. However, the addition of only 2 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 mutants 697F and 702I. The Env mutant 707R had an amount of gp41 approximately equivalent to that of the WT incorporated into viral particles. 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.

DISCUSSION

The MSD of the HIV-1 gp41 glycoprotein, the TM 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 replacements of the MSD with those of other transmembrane proteins were not able to fully restore the biological activities of the HIV-1 Env glycoprotein (25, 31, 40, 43). 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 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 (36). 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 (29). 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 (11, 14, 37). In the current study, we used serial C-terminal MSD truncation mutants to understand the structural requirements of the gp41 MSD 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 amounts of truncated gp41 and gp160 that are secreted. Moreover, the addition of three more amino acids (mutant 698A) further stabilizes the protein in the membrane and restores fusion capacity to WT 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 (41) and of a model in which lysine 681 and arginine 694 “snorkel” within the hydrophobic region of the lipid bilayer (Fig. 7B). Theoretically, the α helix formed by the 14 amino acid residues (K681 to R694) would span 21 Å. The length of the side chain of lysine is approximately 6.4 Å, and that of arginine is 7.0 Å. Thus, the addition of the length of the 14-amino-acid α helix and those of the two side chains of K681 and R694 reaches 34.4 Å. Therefore, the “snorkeling” structure is long enough to span a biological lipid bilayer, which is usually 30 to 40 Å in depth.

In the context of this model, the C-terminal carboxyl group of the arginine 694 in the shortest truncation mutant,

FIG. 5. Infectivities of HIV-1 Env truncation mutants. (A) Mutant viruses containing truncated Env glycoproteins were produced by transfecting proviral DNA into 293T cells. The p24-normalized cell culture supernatant was used to infect JC53BL indicator cells. The cells were lysed after 48 h of incubation, and luciferase enzyme activity was assayed. (B) Single-round infectivity assay. The Env-defective proviral construct pSG3Δenv 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. The p24-normalized supernatant was then used to infect JC53BL indicator cells. The infectivities of MSD truncation mutants are shown as relative luciferase activity compared to that of the WT. The results are from at least three independent experiments; the error bars represent standard deviations from the means.

Env694R, would be positioned within the hydrophobic core of the lipid bilayer in an energetically unfavorable position. In contrast, addition of 4 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 the VSV G MSD have shown that at least a 14-amino-acid MSD (flanked by a lysine and an arginine) is necessary to transport VSV G to the cell surface (1). Shorter MSDs (8 to 12 amino acid residues) still appeared to be able to span intracellular membranes, but they were retained in the Golgi region of the cell. In contrast, all of the mutants of HIV-1 described here, including Env694R, undergo intra-

cellular trafficking from the endoplasmic reticulum to the plasma membrane. High levels of surface expression do, however, require at least four 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 virus hemagglutinin, 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 (2). The form of the hemagglutinin MSD that was 2 amino acids shorter 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% of the fusion activity of

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 was 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 blotting with anti-gp41 MAb 2F5 and anti-p24 MAb 183-12H-5C. The gp41 and p24 bands were quantified using OptiQuant software (Packard, Meriden, CT).

TABLE 1. Summary of properties of Env truncation mutants

Env	Expression and processing ^a	gp120 shedding (10 ⁷ DLU ^b)	gp41 secretion (10 ⁷ DLU)	Cell surface expression (%)		Cell-cell fusogenicity (%) ^d		Cell-cell fusogenicity (%) ^e		Infectivity (%)		Single-round infection (%)	
				Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
WT	++	1.34	— ^c	100		100		100		100		100	
694R	++	4.4	0.33	33.4	2.2	3.19	0.7	0.13	0.01	0.13	0.01	1.42	0.2
695I	++	1.66	0.10	26.7	1.8	3.15	0.7	0.13	0.01	0.14	0.02	1.77	0.4
696V	++	1.51	0.13	14.4	1.3	3.09	0.8	0.14	0.01	0.16	0.01	1.51	0.1
697F	++	1.28	0.13	35.2	2.4	20.1	1.1	91.1	2.7	0.55	0.04	1.59	0.3
698A	++	1.32	0.067	130.0	14.0	87.6	11.2	98.8	1.6	0.14	0.01	2.38	0.8
699V	++	1.12	0.031	152.6	10.5	64.7	16.2	84.6	1.1	0.17	0.02	1.57	0.4
700L	++	1.35	0.023	119.8	7.9	71.2	31.9	102.	3.0	0.21	0.03	2.50	0.7
701S	++	1.25	—	164.7	8.9	77.4	19.4	95.3	1.3	0.21	0.03	4.11	0.2
702I	++	0.81	—	105.6	4.8	93.2	23.1	106.	1.2	0.65	0.03	2.91	0.3
703V	++	0.88	—	174.5	21.3	75.5	26.6	63.7	1.4	0.58	0.03	2.66	0.2
704N	++	1.00	—	156.0	12.2	78.1	14.9	66.0	5.5	0.36	0.04	5.22	0.8
705R	+	0.84	—	173.6	6.0	77.6	6.2	37.2	1.0	0.38	0.06	7.18	1.8
706V	—	0.75	—	60.6	2.6	59.5	19.4	1.88	0.08	0.84	0.03	9.03	3.9
707R	++	0.76	—	224.7	17.3	91.5	11.9	89.6	6.7	31.1	2.8	14.6	4.3

^a ++, wild-type level; +, reduced expression; —, the expression level of mutant 706V from the expression vector pSRHS was lower than that from the WT and other mutants but was undetectable when it was expressed from the proviral vector.

^b DLU, digital light units.

^c —, gp41 was undetectable.

^d Cell-cell fusion mediated by Env expressed from the pSRHS vector.

^e Cell-cell fusion in the context of virus in the presence of 10 μ M AZT.

the 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 they were mixed with JC53BL cells.

It is not clear at present whether this reflects an enhancing interaction of the mutant Env with another virus-encoded protein or a difference in the relative fusogenicity of the producer cells (293T cells versus COS-1 cells).

Truncation mutagenesis of 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 (41). 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 the WT. Although the MSD truncation mutants also impaired the efficiency of incorporation of the 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 the 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 peripheral blood mononuclear cells due to a lack of Env incorporation (27). Therefore, an intact gp41 MSD is necessary, but not sufficient, for HIV-1 Env to initiate normal viral infection. It is worth noting 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 are required for its stable assembly into virions.

Based on the original assignment of residues 681 to 705 as the MSD of HIV-1 Env, the most perplexing aspect is that this

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 an alpha helix. The charged amino acid R694 is buried in the membrane. (B) An alternative "snorkeling" model of the HIV-1 MSD is supported by the data presented in this study. This model predicts an alpha-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.

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 the membrane. One hypothesizes that a protonated arginine or lysine residue could remain in an energetically favorable state within the membrane, but only in the very middle of the lipid bilayers (46). 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., major histocompatibility complex class II) to avoid contact with the hydrophobic environment of the membrane (8, 23). Neither of the 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 (10). However, at the amino-terminal end of the region, the vertical distance (18 Å) between F683 and R694 on the surface of the α helix is more than the length to which the side chains of the 2 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 3 amino acids, including F697, had no effect on the fusogenicity of HIV-1 Env (29). Therefore, the cation- π interaction is probably not applicable to the HIV-1 MSD. The snorkeling model we proposed previously (41) would account for the high level of conservation observed for the N-terminal "core" region of the MSD and would provide a mechanism by 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 roles played by additional residues C terminal to 698A are 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 (21, 22).

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REFERENCES

- Adams, G. A., and J. K. Rose. 1985. Structural requirements of a membrane-spanning domain for protein anchoring and cell surface transport. *Cell* 41:1007–1015.
- Armstrong, R. T., A. S. Kushnir, and J. M. White. 2000. The transmembrane domain of influenza hemagglutinin exhibits a stringent length requirement to support the hemifusion to fusion transition. *J. Cell Biol.* 151:425–437.
- Berger, E. A., P. M. Murphy, and J. M. Farber. 1999. Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. *Annu. Rev. Immunol.* 17:657–700.
- Berlitz-Torrent, C., B. L. Shacklett, L. Erdtmann, L. Delamarre, I. Bouchaert, P. Sonigo, M. C. Dokhelar, and R. Benarous. 1999. Interactions of the cytoplasmic domains of human and simian retroviral transmembrane proteins with components of the clathrin adaptor complexes modulate intracellular and cell surface expression of envelope glycoproteins. *J. Virol.* 73:1350–1361.
- Byland, R., P. J. Vance, J. A. Hoxie, and M. Marsh. 2007. A conserved dileucine motif mediates clathrin and AP-2-dependent endocytosis of the HIV-1 envelope protein. *Mol. Biol. Cell* 18:414–425.
- Chen, B., E. M. Vogan, H. Gong, J. J. Skehel, D. C. Wiley, and S. C. Harrison. 2005. Determining the structure of an unliganded and fully glycosylated SIV gp120 envelope glycoprotein. *Structure* 13:197–211.
- Chen, B., E. M. Vogan, H. Gong, J. J. Skehel, D. C. Wiley, and S. C. Harrison. 2005. Structure of an unliganded simian immunodeficiency virus gp120 core. *Nature* 433:834–841.
- Cosson, P., and J. S. Bonifacio. 1992. Role of transmembrane domain interactions in the assembly of class II MHC molecules. *Science* 258:659–662.
- Day, J. R., C. Munk, and J. C. Guatelli. 2004. The membrane-proximal tyrosine-based sorting signal of human immunodeficiency virus type 1 gp41 is required for optimal viral infectivity. *J. Virol.* 78:1069–1079.
- Dougherty, D. A. 1996. Cation- π interactions in chemistry and biology: a new view of benzene, Phe, Tyr, and Trp. *Science* 271:163–168.
- Dubay, J. W., S. J. Roberts, B. H. Hahn, and E. Hunter. 1992. Truncation of the human immunodeficiency virus type 1 transmembrane glycoprotein cytoplasmic domain blocks virus infectivity. *J. Virol.* 66:6616–6625.
- Eckert, D. M., and P. S. Kim. 2001. Mechanisms of viral membrane fusion and its inhibition. *Annu. Rev. Biochem.* 70:777–810.
- Fujiyoshi, Y. 1998. The structural study of membrane proteins by electron crystallography. *Adv. Biophys.* 35:25–80.
- Gabuzda, D., U. Olshevsky, P. Bertani, W. A. Haseltine, and J. Sodroski. 1991. Identification of membrane anchorage domains of the HIV-1 gp160 envelope glycoprotein precursor. *J. Acquir. Immune Defic. Syndr.* 4:34–40.
- Hammonds, J., X. Chen, L. Ding, T. Fouts, A. De Vico, J. zur Megede, S. Barnett, and P. Spearman. 2003. Gp120 stability on HIV-1 virions and Gag-Env pseudovirions is enhanced by an uncleaved Gag core. *Virology* 314:636–649.
- Helseth, E., U. Olshevsky, D. Gabuzda, B. Ardman, W. Haseltine, and J. Sodroski. 1990. Changes in the transmembrane region of the human immunodeficiency virus type 1 gp41 envelope glycoprotein affect membrane fusion. *J. Virol.* 64:6314–6318.
- Johnston, P. B., J. W. Dubay, and E. Hunter. 1993. Truncations of the simian immunodeficiency virus transmembrane protein confer expanded virus host range by removing a block to virus entry into cells. *J. Virol.* 67:3077–3086.
- Kowalski, M., J. Potz, L. Basiripour, T. Dorfman, W. C. Goh, E. Terwilliger, A. Dayton, C. Rosen, W. Haseltine, and J. Sodroski. 1987. Functional regions of the envelope glycoprotein of human immunodeficiency virus type 1. *Science* 237:1351–1355.
- Lin, X., C. A. Derdeyn, R. Blumenthal, J. West, and E. Hunter. 2003. Progressive truncations C terminal to the membrane-spanning domain of simian immunodeficiency virus Env reduce fusogenicity and increase concentration dependence of Env for fusion. *J. Virol.* 77:7067–7077.
- Lopez-Verges, S., G. Camus, G. Blot, R. Beauvoir, R. Benarous, and C. Berlitz-Torrent. 2006. Tail-interacting protein TIP47 is a connector between Gag and Env and is required for Env incorporation into HIV-1 virions. *Proc. Natl. Acad. Sci. USA* 103:14947–14952.
- Lu, M., S. C. Blacklow, and P. S. Kim. 1995. A trimeric structural domain of the HIV-1 transmembrane glycoprotein. *Nat. Struct. Biol.* 2:1075–1082.
- Lu, M., and P. S. Kim. 1997. A trimeric structural subdomain of the HIV-1 transmembrane glycoprotein. *J. Biomol. Struct. Dyn.* 15:465–471.
- Lumms, S. C. 2004. The transmembrane domain of the 5-HT₃ receptor: its role in selectivity and gating. *Biochem. Soc. Trans.* 32:535–539.
- Markovic, I., and K. A. Clouse. 2004. Recent advances in understanding the molecular mechanisms of HIV-1 entry and fusion: revisiting current targets and considering new options for therapeutic intervention. *Curr. HIV Res.* 2:223–234.
- Miyauchi, K., J. Komano, Y. Yokomaku, W. Sugiura, N. Yamamoto, and Z. Matsuda. 2005. Role of the specific amino acid sequence of the membrane-spanning domain of human immunodeficiency virus type 1 in membrane fusion. *J. Virol.* 79:4720–4729.
- Moore, J. P., J. A. McKeating, R. A. Weiss, and Q. J. Sattentau. 1990. Dissociation of gp120 from HIV-1 virions induced by soluble CD4. *Science* 250:1139–1142.
- Murakami, T., and E. O. Freed. 2000. The long cytoplasmic tail of gp41 is required in a cell type-dependent manner for HIV-1 envelope glycoprotein incorporation into virions. *Proc. Natl. Acad. Sci. USA* 97:343–348.
- Ohno, H., R. C. Aguilar, M. C. Fournier, S. Hennecke, P. Cosson, and J. S.

- Bonifacio.** 1997. Interaction of endocytic signals from the HIV-1 envelope glycoprotein complex with members of the adaptor medium chain family. *Virology* **238**:305–315.
29. **Owens, R. J., C. Burke, and J. K. Rose.** 1994. Mutations in the membrane-spanning domain of the human immunodeficiency virus envelope glycoprotein that affect fusion activity. *J. Virol.* **68**:570–574.
 30. **Owens, R. J., J. W. Dubay, E. Hunter, and R. W. Compans.** 1991. Human immunodeficiency virus envelope protein determines the site of virus release in polarized epithelial cells. *Proc. Natl. Acad. Sci. USA* **88**:3987–3991.
 31. **Raja, N. U., M. J. Vincent, and M. Abdul Jabbar.** 1994. Vpu-mediated proteolysis of gp160/CD4 chimeric envelope glycoproteins in the endoplasmic reticulum: requirement of both the anchor and cytoplasmic domains of CD4. *Virology* **204**:357–366.
 32. **Ray, N., and R. W. Doms.** 2006. HIV-1 coreceptors and their inhibitors. *Curr. Top. Microbiol. Immunol.* **303**:97–120.
 33. **Rowell, J. F., P. E. Stanhope, and R. F. Siliciano.** 1995. Endocytosis of endogenously synthesized HIV-1 envelope protein. Mechanism and role in processing for association with class II MHC. *J. Immunol.* **155**:473–488.
 34. **Salzwedel, K., P. B. Johnston, S. J. Roberts, J. W. Dubay, and E. Hunter.** 1993. Expression and characterization of glycopospholipid-anchored human immunodeficiency virus type 1 envelope glycoproteins. *J. Virol.* **67**:5279–5288.
 35. **Salzwedel, K., J. T. West, Jr., M. J. Mulligan, and E. Hunter.** 1998. Retention of the human immunodeficiency virus type 1 envelope glycoprotein in the endoplasmic reticulum does not redirect virus assembly from the plasma membrane. *J. Virol.* **72**:7523–7531.
 36. **Shang, L., L. Yue, and E. Hunter.** 2008. Role of the membrane-spanning domain of human immunodeficiency virus type 1 envelope glycoprotein in cell-cell fusion and virus infection. *J. Virol.* **82**:5417–5428.
 37. **Vincent, N., C. Genin, and E. Malvoisin.** 2002. Identification of a conserved domain of the HIV-1 transmembrane protein gp41 which interacts with cholesterol groups. *Biochim. Biophys. Acta* **1567**:157–164.
 38. **Wei, X., J. M. Decker, H. Liu, Z. Zhang, R. B. Arani, J. M. Kilby, M. S. Saag, X. Wu, G. M. Shaw, and J. C. Kappes.** 2002. Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. *Antimicrob. Agents Chemother.* **46**:1896–1905.
 39. **Weiss, C. D.** 2003. HIV-1 gp41: mediator of fusion and target for inhibition. *AIDS Rev.* **5**:214–221.
 40. **Welman, M., G. Lemay, and E. A. Cohen.** 2007. Role of envelope processing and gp41 membrane spanning domain in the formation of human immunodeficiency virus type 1 (HIV-1) fusion-competent envelope glycoprotein complex. *Virus Res.* **124**:103–112.
 41. **West, J. T., P. B. Johnston, S. R. Dubay, and E. Hunter.** 2001. Mutations within the putative membrane-spanning domain of the simian immunodeficiency virus transmembrane glycoprotein define the minimal requirements for fusion, incorporation, and infectivity. *J. Virol.* **75**:9601–9612.
 42. **West, J. T., S. K. Weldon, S. Wyss, X. Lin, Q. Yu, M. Thali, and E. Hunter.** 2002. Mutation of the dominant endocytosis motif in human immunodeficiency virus type 1 gp41 can complement matrix mutations without increasing Env incorporation. *J. Virol.* **76**:3338–3349.
 43. **Wilk, T., T. Pfeiffer, A. Bukovsky, G. Moldenhauer, and V. Bosch.** 1996. Glycoprotein incorporation and HIV-1 infectivity despite exchange of the gp160 membrane-spanning domain. *Virology* **218**:269–274.
 44. **Willey, R. L., and M. A. Martin.** 1993. Association of human immunodeficiency virus type 1 envelope glycoprotein with particles depends on interactions between the third variable and conserved regions of gp120. *J. Virol.* **67**:3639–3643.
 45. **Wyatt, R., and J. Sodroski.** 1998. The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. *Science* **280**:1884–1888.
 46. **Yoo, J., and Q. Cui.** 2008. Does arginine remain protonated in the lipid membrane? Insights from microscopic pKa calculations. *Biophys. J.* **94**:L61–L63.