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Effects of Stabilization of the gp41 Cytoplasmic Domain on Fusion Activity and Infectivity of SIVmac239

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Abstract

We investigated the effects of introducing specific sequences that are predicted to affect trimer stability into the CT domain of the SIV Env protein. Two constructs, 3HBai and 3HBaa, with additional GCN4-related sequences in the CT domain (45 aa) had enhanced infectivity, and differed in their fusion activity and trimer stability. Another construct, 3HBii, exhibited a very stable trimeric structure. Pseudotyped virions containing 3HBii retained infectivity despite the lack of syncytia formation. In contrast, 3HBai and 3HBaa, which caused extensive syncytia formation, had a less stable trimeric structure. We observed an inverse correlation between trimer stability and fusion activity but no correlation between syncytia formation activity and infectivity. Quantitative cell–cell fusion assays, analysis of Env incorporation, measurement of ectodomain conformation by CD4 binding, and CCR5 blocking assays indicated differential effects on fusion activity and infectivity of the viruses with Env CT modifications. Differences in interaction with CD4 were not affected by trimer stability and were not related to fusion activity or infectivity. The results indicate that changes in the stability of the CT domain can have significant effects on functional activities of the Env external domain and can impact viral biological properties.

Introduction

Enveloped viruses such as HIV and SIV enter cells by fusing the viral envelope with a cellular membrane. The viral Env proteins contain two subunits, a surface subunit gp120 (SU) and a transmembrane subunit gp41 (TM), which are associated by noncovalent interactions and are presented as trimeric complexes on the surfaces of virus particles. The Env protein is responsible for binding to specific cellular receptors and coreceptors. The binding of gp120 to CD4 and a chemokine receptor (usually CCR5) leads to a structural rearrangement in gp41 and insertion of its amino terminus into the host cell membrane.[1–3] Fusion between the viral and cellular membrane progresses through a series of steps. The initial step, binding of the gp120 subunit to CD4, results in the formation of the gp120 bridging sheet that, along with the V3 loop, forms the coreceptor binding site.[4] The recruitment of coreceptors by Env initiates gp41 refolding that progresses through a prebundle intermediate, in which the gp41 N- and C-terminal heptad repeat domains (N-HR and C-HR, respectively) are exposed.[5–8] The heptad repeat domains ultimately coalesce into a stable postfusion conformation referred to as the 6-helix bundle (6HB), which is formed by an antiparallel association of the trimeric N-HR domain (coiled coil) with three peripheral C-HR domains.[9]

The TM gp41 subunits of HIV and SIV Env consist of an ectodomain containing a hydrophobic N-terminal fusion peptide, a membrane-spanning domain, and a carboxy-terminal cytoplasmic tail that in contrast to most retroviral TM proteins is unusually long (about 150–164 amino acids for HIV-1 and SIV, respectively). The TM cytoplasmic tail plays a key role in important viral functions, such as assembly into lipid rafts,[10,11] the regulation of Env expression at the cell surface,[12] basolateral targeting of viral budding, and incorporation of Env into virions.[13] In this regard, the TM cytoplasmic domain contains multiple motifs[12–17] including three highly conserved alpha-helical "lentivirus lytic peptide" domains (LLP-1, LLP-2, LLP-3) implicated in interactions with the plasma membrane, decreasing bilayer stability, altering membrane ion permeability, and mediating cell killing.[18–24]

SIV strains that were passaged on human cell lines frequently acquire a premature stop codon and express a truncated Env protein that lacks all but approximately 20 amino acids of the cytoplasmic domain.[25–27] Such truncations enhance fusion activity of the Env in some cell types.[28–30] However, SIV with a truncated Env exhibits a defect in replication in monkeys[31,32] and in some cell cultures.[11]

To further investigate the effect of the gp41 cytoplasmic domain on fusion activity and infectivity of SIV, we constructed Env proteins with specific modifications in the

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cytoplasmic tail. These Env constructs were designed by the addition of GCN4-related sequences to the C-terminus of SIVmac239 Env with a truncated CT (17 aa). The added sequences were derived from a leucine zipper motif, first described for DNA-binding proteins including c-Myc, c-Jun, and the yeast gene regulatory protein GCN4[33] and have been used for functional study of other transmembrane proteins.[34–36] We examined the effects of these modifications on thermal stability of the trimeric structure of the Env protein, incorporation into pseudotyped virions, fusion activity, and infectivity. We compared the effects of conformational changes and trimer stability with biological characteristics of different forms of Env proteins (fusogenic vs. nonfusogenic, infectious vs. noninfectious). The results are discussed in the context of glycoprotein assembly, trimer stability and conformation, fusion activity, and infectivity.

Materials and Methods

Cells, plasmids, viruses

Hep2, CV-1, 3T3T4R5, NIH3T3, 293T, and JC-53BL cells were maintained as described.[11] The recombinant vaccinia virus vTF7-3 and the wild-type (wt) vaccinia virus strain IHD-J were kindly provided by Bernard Moss (National Institutes of Health, Bethesda, MD). The vaccinia virus stocks were propagated and titrated on CV-1 cells. Plasmid pGINT7 β-Gal was provided by Edward Berger (National Institutes of Health). Plasmids pCMV239Env(FL) and pCMV239Env(T) with tPA signal peptides were described previously.[37]

Env constructions

The gene encoding the Env full-length protein of SIVmac239 virus was amplified by using primers, F with a unique restriction enzyme site *Eco*RI GGCTAAGGCGAATCCA TCTTCTGCATCAAACAAGTAAGTATGGG and R with *Bam*HI TCATATACTGTCCCGGATCCTATTTCTGTCCCTC ACAAG AGAG. For Env-T17 the R primer was designed with a stop codon CAGTGCCGG GTCCTGTTGGATGGATCCC TACTGGAAATAAGAG and for Env-T45, 3HBaa, 3HBai, and 3HBii Envs this R primer was designed without a stop codon. Cytoplasmic three-helix bundle (3HB) domain sequences were designed with unique restriction enzyme sites *Bam*HI and *Sph*I. The amino acid sequence of 3HBii was derived from GCN4-*p*-II.[38] All gene sequences were confirmed by sequencing and cloned into a pSP72 obtained from Promega (Madison, WI) or plasmid vector pCAGGS (kindly provided by Dr. Y. Kawaoka) under the control of a chicken β-actin promoter.

Analysis of protein expression by radioactive labeling, immunoprecipitation, and surface biotinylation

Protein expression was carried out using the recombinant vaccinia virus T7 transient expression system. Briefly, Hep2 cells were seeded in culture dishes and grown to 90% confluence overnight. The cells were then infected with recombinant vaccinia virus VTF7-3 (which expresses the T7 polymerase) for 2 h followed by transfection with indicated DNA constructs using FuGene 6 obtained from Roche (Indianapolis, IN). At 20 h postinfection/transfection, cells were starved in Met, Cys-deficient DMEM for 30 min, and then labeled with [^{35}S]-Met, Cys-labeling mix obtained from Amersham (Piscataway, NJ) for 4 h. Surface expression of the SIV Env protein was detected by a surface biotinylation assay as described in our previous studies.[39] Briefly, after labeling and chase, cells were washed with phosphate-buffered saline (PBS) at 4°C and then incubated with 1 ml NHS-SS-Biotin dissolved in PBS (1 mg/ml) for 30 min at 4°C. After labeling and surface biotinylation, the cells were lysed with lysis buffer and proteins and then precipitated with a polyclonal anti-SIVmac251 antiserum plus protein A–agarose beads obtained from Pierce (Rockford, IL) overnight at 4°C. Proteins bound to protein A agarose beads were washed three times with lysis buffer, solubilized with 10% sodium dodecyl sulfate (SDS), and heated at 95°C for 5 min. Dissociated proteins were precipitated again with streptavidin agarose beads at 4°C for 3 h and then washed three times with lysis buffer. Protein samples were then prepared by the addition of reducing sample buffer, and heated at 95°C for 5 min prior to analysis by SDS–PAGE. The gels were fixed, dried, and exposed to phosphor screens for quantification of the surface and total glycoproteins ratio using a phosphorimager (Molecular Dynamics, Sunnyvale, CA) and ImageQuant software.

Analysis of Env protein incorporation and oligomerization

Pseudotyped virions were obtained by cotransfection of 293T cells with Env CMV plasmid constructs with the pSG3 plasmid expressing env-minus molecular clone SG3 (HIV-1) and after 3 days collected and purified as previously described.[39] To detect protein oligomerization, samples were heated in Laemmli sample buffer at the temperatures indicated and analyzed by SDS–PAGE on 4–15% acrylamide gels and Western blotting by using a polyclonal antibody from human plasma (HIV-Ig) for analysis of Gag proteins or a polyclonal anti-SIVmac251 antiserum for analysis of Env proteins and developed by an ECL kit obtained from Amersham (Piscataway, NJ). We used a negative control (env-minus molecular clone SG3 HIV-1) with WB and did not observe any bands when we used anti-SIVmac251 antiserum. The amounts of proteins were quantitated by densitometer analysis (NIH Image version 1.54).

Cell fusion assays

Colorimetric lysate assay. Dishes (60-mm diameter) of subconfluent NIH3T3 cells were infected for 1 h with the vTF7-3 virus at an MOI of 1–2 and then transfected with 4 μg of Env constructs. A second population of 3T3T4R5 cells was infected with wt vaccinia virus strain IHD-J and transfected with the pGINT7 ß-Gal plasmid, which contains the ß-galactosidase (ß-Gal) gene under the control of the T7 promoter. At 16–20 h posttransfection, the two populations were mixed in a 96-well tissue culture plate and incubated for 2 h 30 min at 37°C, after which cell fusion was quantitated by a colorimetric lysate assay. The data were analyzed with the Delta Soft II Microplate analysis program.

In situ assay. At 16–20 h posttransfection (as described above), the two populations were mixed in a four-well culture slide and incubated for 2 h 30 min at 37°C, after which the cells were fixed and stained, as described by Nussbaum *et al*.[40] Photographs were taken with an inverted

microscope with 10× or 20× phase-contrast and 10× ocular objectives.

gp120-CD4 binding assay

To determine binding of mutant Env proteins to CD4, we used a modified version of a procedure described before.[41] Briefly, 96-well microtiter plates were incubated overnight at 4°C with 125 μl of a solution containing 2 μg/ml of a murine monoclonal antibody against CD4 (OKT4) in 30 mM $NaHCO_3$ (pH 8.5). Plates were washed three times with PBS containing 0.05% Tween 20. To reduce the background, the plates were washed three times with SuperBlock buffer obtained from Pierce (Rockford, IL). The plates were incubated for 2 h at room temperature with SuperBlock buffer containing 1 μg/ml of a soluble CD4. Plates were washed again as described above. A similar amount of pseudotyped virions determined by an ELISA Core Antigen assay obtained from ZeptoMetrix corporation (Buffalo, NY) was diluted in medium with 10% FBS, added to the plate, and incubated overnight at 4°C. Plates were washed five times with PBS containing 0.05% Tween 20. The captured pseudotyped virions were probed with biotin-conjugated polyclonal antibody to SIV. After washing, plates were developed with tetramethylbenzidine (TMB). We then determined absorbance (OD) at 450 nm. For each sample, the OD value was determined after subtracting the value obtained with pseudotyped virions without Env proteins as control. Results represent percentage of gp120 binding of pseudotyped virions compared to that found for wild-type full-length Env-containing samples (100%).

CCR5 blocking assay

JC-53BL cells were preincubated with dilutions of monoclonal antibody (MAb) 2D7 for 1 h at 4°C, as described by Wu *et al*.[42] About 30 infectious particles of pseudotyped virions with each Env protein were added per well. At 3 days postinfection, cells were fixed and stained as described by Chackerian *et al*.[43] The blocking of infection was measured by comparison of the number of infected cells in wells infected with antibody-treated cells to the number in untreated infected cells.

Results

Design of mutant SIVmac239 Env proteins with modifications in the CT domain

To compare biological activity of different (fusogenic or nonfusogenic) forms of Env proteins, we generated mutant Envs based on the hypothesis that the length and sequence of the cytoplasmic tail of Env are involved in regulation of fusion activity and infectivity. Previous studies had indicated that truncation of the CT alters the conformation of the gp41 external domain[26] and affects CD4 dependence and infectivity of SIV.[11,27] To further examine the effects of modifications of the CT structure, we compared constructs having an addition of a three-helix bundle (3HB) domain[38] to the C-terminus of the truncated SIVmac239 Env CT (17 aa). The amino acid sequence of 3HBii forms an isoleucine knob-into-hole helical interaction and two mutants were designed to ablate these interactions (Fig. 1). Three critical isoleucine residues required for 3HB formation were changed to alanine in the 3HBai construct. All hydrophobic isoleucine residues were converted to alanine in the 3HBaa construct. The sequence of the cytoplasmic domain corresponding to the same length of the wild-type SIVmac239 Env (Env-T45) was also used for comparison. The properties of this sequence are completely different, as almost all amino acids are charged or polar residues. The hydropathicity scores of the sequences were determined by Kyte–Doolittle analysis and were in the order Env-T45 < 3HBaa < 3HBai < 3HBii (Fig. 1B).

Mutant Env proteins exhibit high levels of cell surface expression

Wild-type (full-length or truncated) and mutant glycoproteins expressed by T7-expression vector in Hep2 cells were metabolically labeled, and the viral proteins were immunoprecipitated with sera from SIV-infected macaques and analyzed by SDS–PAGE followed by autoradiography. The synthesis and processing of mutant proteins were found to be similar to the wild-type Env (not shown). Cell surface expression levels of Env proteins were determined by surface biotinylation. The ratio of SU to TM subunits on cell surfaces was similar for all constructs including wild-type Env. The

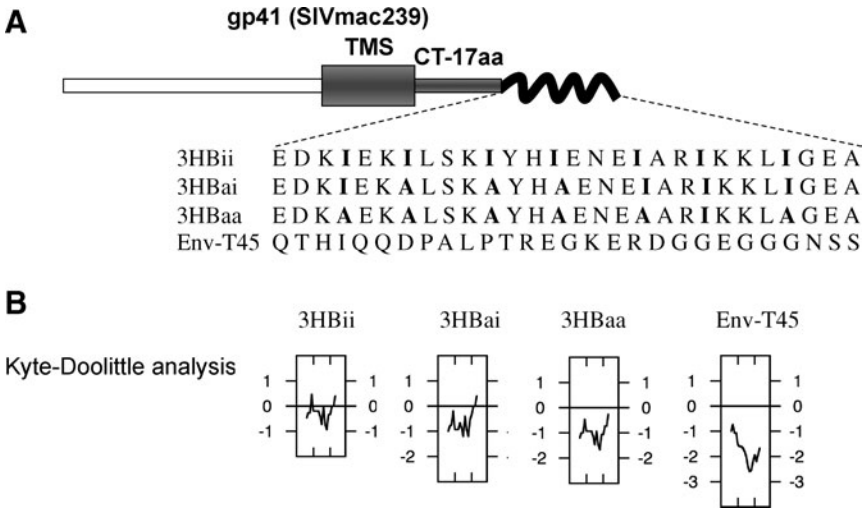


FIG. 1. Schematic representation of mutant (SIVmac239) Env glycoproteins. **(A)** Amino acid sequences of SIVmac239 transmembrane protein gp41 with a truncated cytoplasmic domain (CT-17 aa) and with addition of a cytoplasmic three-helix bundle (3HB) domain. The amino acid sequence of 3HBii is derived from GCN4-*p*-II.[38] Mutants 3HBai and 3HBaa were designed to ablate the isoleucine knob-into-hole interactions. The amino acid sequence of Env-T45 was derived from the original SIVmac239 sequence (accession number M33262). **(B)** Hydropathy plots are based on Kyte–Doolittle analysis.

levels of surface proteins (SU subunit of Env) were quantitated by phosphorimager analysis. The mutant glycoproteins were expressed on the cell surface at levels ranging from 80% to 150% of that of the wild-type full-length Env (Fig. 2). Most of the truncated Env proteins with additional sequences were expressed on the cell surface at levels higher than the full-length wild-type Env. The 3HBai Env was expressed at a lower level, which was 80% of the level of full-length Env. Comparison of cell surface versus total expression levels showed that the 3HBaa was transported to the cell surface most efficiently and wild-type truncated Env-T17 was transported to the cell surface with the lowest efficiency. Previous results demonstrated that the cytoplasmic tail of SIV was able to regulate cell surface expression and endocytosis.[44] The results indicate that both surface expression and transport efficiency of the mutant Env proteins are increased relative to wild-type Envs with full length or truncated CT.

The length and structure of the CT domain affect cell–cell fusion activity of the Env protein

To compare cell–cell fusion activity, we used the T7 vaccinia expression system to express wild-type and mutant Env glycoproteins in NIH3T3 cells and expression of a ß-Gal reporter gene in target cells (3T3T4R5) with SIV-specific receptors and coreceptors (CD4, CCR5). The wild-type Env with a truncated CT (Env-T17) exhibited about 52% of the fusion activity of full-length Env (Fig. 3A). The Env mutant 3HBii with predicted greater helical bundle interactions in the CT exhibited the lowest fusion activity of 45% among the truncated Envs with extended CT sequences. The Env construct 3HBaa in which all of the seven critical isoleucine residues required for 3HB formation were changed to alanine exhibited fusion activity of 405%, which was the highest level observed. Mutant Envs 3HBai in which three of the seven isoleucine residues were changed to alanine, or Env-T45 with an extended SIV sequence (CT of 45 amino acids), also showed high fusion activity of 377 and 330% of wild-type Env, respectively (Fig. 3A). As another approach to confirm the results of quantitation, an *in situ* fusion assay was used (Fig. 3B). We observed large blue-stained syncytia in samples with 3HBai, 3HBaa, and Env-T45, smaller blue-stained syncytia in samples with Env (FL), and no syncytia with truncated Env or 3HBii. Generally, these differences in fusion activity were not correlated with differences in cell-surface density of Env. These data indicate that the length and sequence of the CT domain may regulate fusion activity of SIV Env and that extension of the CT domain can increase or decrease fusion activity depending on the strength of the interactions between monomers in the trimeric protein.

Efficient incorporation of wild-type and mutant Envs into pseudotyped virions

To determine the effect of CT modification on the incorporation of Env proteins into pseudotyped virions, we used a plasmid with an HIV backbone sequence without vpu or env (SG3) and plasmids with CMV promoters for expression of Env proteins (Fig. 4A). To obtain a high level of rev-independent expression of wild-type Env proteins with full-length or truncated CT we inserted these genes into the pJW4303 vector and replaced the SIV Env signal peptide with the tPA signal peptide (Fig. 4B). To analyze the impact the CT modifications on Env incorporation, the wild-type Env protein and each mutant Env were transferred into the pCAGGS vector. We did not observe a significant difference in the levels of incorporation of truncated vs. full-length SIVmac239 Env into virions, as indicated by the Env/Gag ratio (Fig. 4A). All the mutant Env proteins were found to be incorporated into pseudotyped virions at levels ranging from 150% to 393% above that of the wild-type full-length Env (Fig. 4C). The results indicated that mutant proteins with different lengths (17, 45 aa) and sequences of the CT domain can be incorporated into virions with similar efficiency.

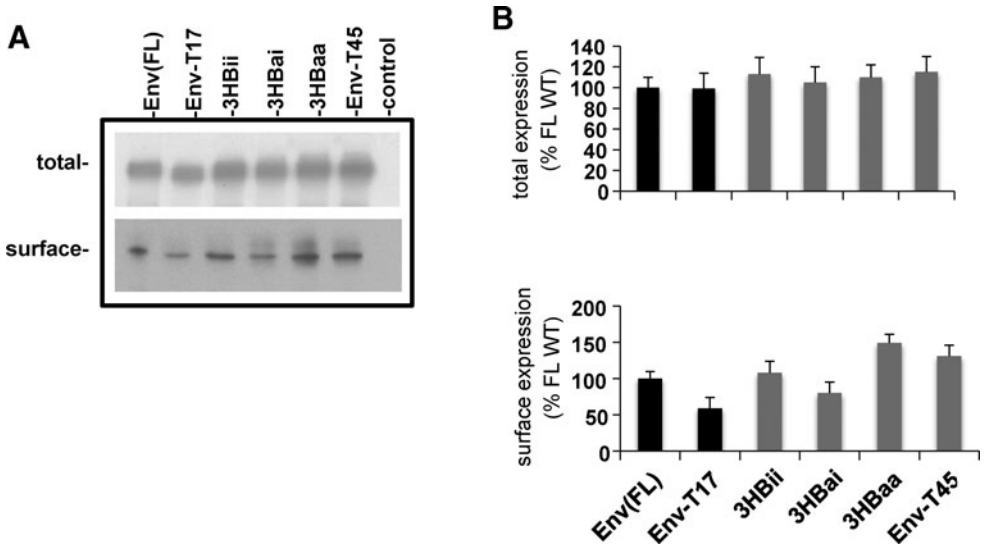


FIG. 2. Analysis of surface expression of mutant and wild-type Env proteins in Hep2 cells. **(A)** Hep2 cells were infected by vaccinia vTF7-3 virus and then transfected by T7 plasmid constructs. After 20 h, infected cells were labeled with [^{35}S]-Met, Cys for 3 h. At the end of the labeling period, the cell surface proteins were biotinylated and lysed, immunoprecipitated, and precipitated with streptavidin-agarose beads (surface) or only lysed (total) as described in Materials and Methods. SIV-specific proteins were immunoprecipitated with a polyclonal anti-SIVmac251 antiserum. **(B)** The levels of total or surface proteins (SU subunit of Env) present in the autoradiograph relative to the FL Env were quantitated by phosphorimager analysis as described in Materials and Methods. Data are plotted as the mean of three experiments. Error bars represent standard deviations.

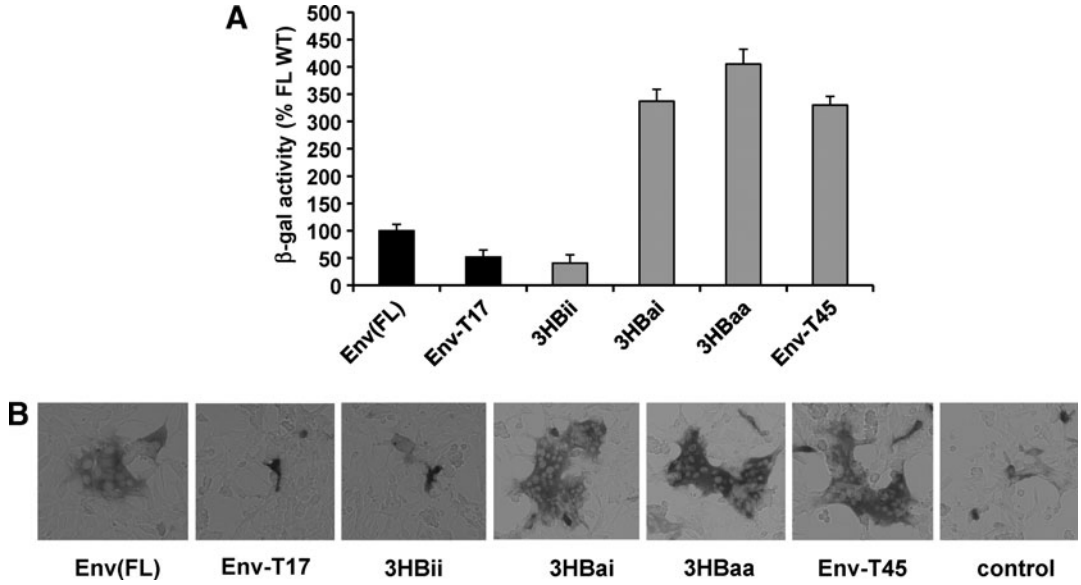


FIG. 3. Effect of modifications of the CT domain of Env on cell–cell fusion activity. **(A)** NIH3T3 cells were infected with a vaccinia vTF7-3 virus and then transfected by T7 plasmid constructs. 3T3T4R5 cells were infected with IHD-J vaccinia virus and transfected with a reporter gene construct. At 18 h posttransfection, the two populations were mixed in a 96-well tissue culture plate and incubated for 2 h 30 min at 37°C, after which cell fusion was quantitated by a colorimetric lysate assay. The data are percentage of the ß-Gal activity observed in WT (full-length Env)-expressing cells. **(B)** *In situ* assay. Cells in a four-well culture slide were fixed and stained as described by Nussbaum *et al.*[40] Micrographs were taken with an inverted microscope with 10×or 20×phase-contrast and 10×ocular objectives.

Infectivity of pseudotyped virions with mutant Env

To compare the infectivity of particles with incorporated full-length wild-type Env, truncated Env, or truncated Env with the addition of 3HB sequences, we used pseudotyped virions prepared by cotransfection of pSG3 with env-expressing plasmids and an indicator cell line JC-53BL (expressing CCR5 and CD4). The pseudotyped virions were obtained in 293T epithelial cells. First, we compared infectivity of pseudotyped virions with identical Env proteins, but with different levels of incorporation by using Env with wild-type or tPA signal peptides. The level of particle production in cells transfected by Env(FL) expressed with tPA signal peptide was 3-fold lower than in cells transfected by Env(FL) (Fig. 5, right). The infectivity titer of pseudotyped virions with tPA Env(FL) was about 21-fold higher than with rev-dependent Env(FL). The level of particle production in cells transfected by Env-T17 expressed with tPA signal peptide was about 2-fold higher than in cells transfected by Env-T17. The titers of Env-T17 with tPA or rev-dependent signal peptides were similar. The results indicate that infectivity titers of Envs with full-length or truncated CT are

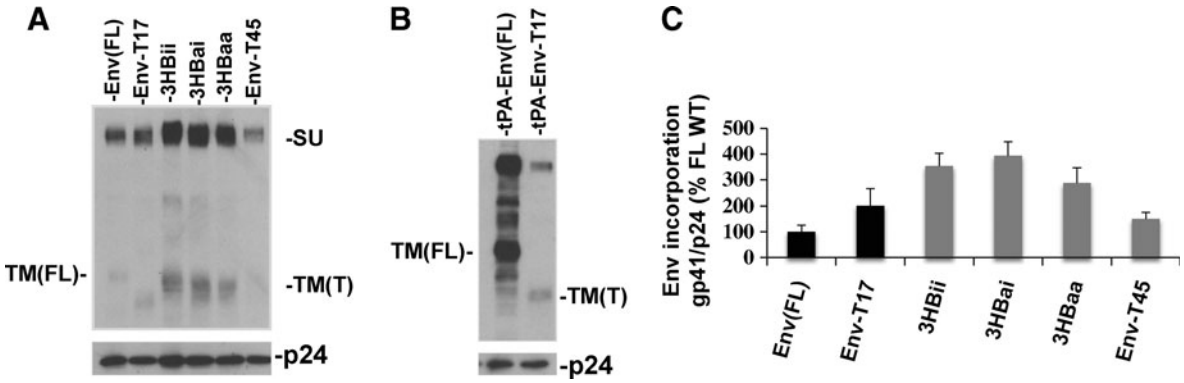
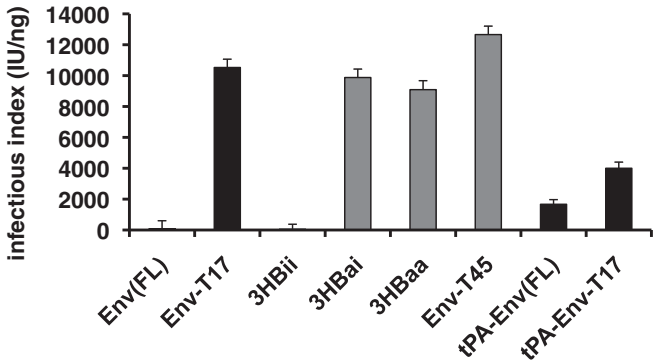


FIG. 4. Analysis of incorporation mutant or wild-type Env proteins into pseudotyped virions. Virions pseudotyped with Env proteins were obtained by cotransfection of 293T cells with Env CMV plasmid constructs pCAGGS **(A)** or pJW4303 (tPA signal peptide) **(B)** with pSG3 plasmid expressing the env-minus molecular clone SG3 (HIV-1) and collected and purified after 3 days. The samples were analyzed by SDS–PAGE and Western blotting by using a polyclonal antibody from human plasma (HIV-Ig) for analysis of Gag proteins or a polyclonal anti-SIVmac251 antiserum for analysis of Env proteins. The amounts of proteins were quantitated by densitometer analysis (NIH Image version 1.54) **(C)**. The levels of Env proteins incorporation were determined by the Env/Gag ratio and indicated as percent of wild-type Env (100%).



Pseudotyped virions obtained after transfection of 293T cells

pst virions	titer (IU/ml)	ELISA (p24) ng/ml	IU/ng*
Env (FL)	$3x10^3$	38	79
Env-T17	$2x10^5$	19	10526
3HBii	$1x10^4$	136	74
3HBai	$8x10^5$	81	9877
3HBaa	$8x10^5$	88	9091
Env-T45	$1x10^6$	79	12658
tPA-Env (FL)	$2x10^4$	12	1667
tPA-Env-T17	$2x10^5$	25	4000

Assays are described in Methods. *IU/ng, infectious units

FIG. 5. Infectivity of virions pseudotyped with mutant and wild-type Env proteins. Infectivity is shown as the ratio between infectious titer, determined by ß-galactosidase assays in JC53-BL, and p24 content of virus stocks determined by the ELISA Core Antigen assay (ZeptoMetrix Corporation).

correlated with levels of Env incorporation into pseudotyped virions.

Second, we compared infectivity titers of virions pseudotyped with different Env structures, with high levels of Env incorporation. The mutant Env 3HBii with the lowest fusion activity exhibited restricted infectivity, which was similar to that observed for Env(FL). Other mutant Env virions demonstrated levels of infectivity substantially higher than virions with wild-type Env (Fig. 5, left). Env-T45, with intermediate fusion activity, exhibited the highest infectivity (Fig. 5, right). The observed infectious indices were 79 IU/ng for Env(FL), 9877 IU/ng for 3HBai, 9091 IU/ng for 3HBaa, and 12658 IU/ng for Env-T45 or differences of 115- to 160-fold, respectively. Taken together, the results indicated that there is no strict correlation between cell–cell fusion activity and infectivity. Multiple factors can exhibit effects on infectivity, including the level of Env incorporation and structure of the CT domain. Some Env proteins containing the addition of α-helical CT structures confer high infectivity despite a lack of detectable fusion activity.

The structure of the Env CT affects the function of CD4 and CCR5-binding sites

To further understand how 3HB sequences and other changes in the CT domain affect the structure or conformation of the Env protein, we determined whether they affected gp120 binding to CD4 by using a CD4-binding ELISA with soluble CD4. We observed that the Env(FL) and Env-T17 exhibited intermediate CD4-binding activity compared with mutant Envs (Fig. 6A). 3HBaa as well as Env-T45 exhibited increased relative CD4-binding activity of about 153%, which is significantly higher than 3HBii ($p = 0.003$). The 3HBii and 3HBai constructs showed reduced binding of soluble CD4 of about 71% and 82%, respectively. We also compared CD4-binding activity of pseudotyped virions with different levels of Env incorporation by comparing tPA vs. rev-dependent Envs, and found that higher levels of Env incorporation increased gp120-CD4 binding activity (not shown). These differences in CD4 interactions were not associated with Env functions (fusion activity and infectivity). However, the sequence and length of the CT domain affected the CD4 binding site exposure.

To measure the effect of CT modification on Env protein binding to CCR5, we used a blocking assay with the indicator cell line JC-53BL and a CCR5-specific (2D7) monoclonal antibody. The binding site for this antibody is located within the extracellular loop 2 of CCR5 and it was shown that 2D7 efficiently inhibits the binding of gp120 to CCR5.[42,45] We observed that 2D7 partially inhibited infection by virus with wild-type or truncated Env in a range from 30 to 60% but was completely ineffective at blocking infection by 3HBii pseudotypes (Fig. 6B). These results indicate that mutant Env proteins modified by the addition of a CT helical structure exhibit decreased exposure of the binding sites recognizing the CD4 receptor and may affect the affinity of binding to the CCR5 coreceptor.

Thermal stability of Env protein trimers depends on the α-helical content of the CT domain

Further evaluation of the functional effect of length and the addition of α-helical structures in the CT domain was addressed by performing an analysis of Env thermostability. Purified pseudotype virions with different Env constructs were incubated in Laemmli sample buffer at a range of temperatures and then analyzed by WB. An increase in temperature to 95°C resulted in the dissolution of the majority of high-molecular-weight oligomeric bands into low-molecular-weight monomeric bands (Fig. 7). We observed that for most Envs, the levels of TM trimers and dimers decrease, whereas bands related to monomers increased (Fig. 7). However, with

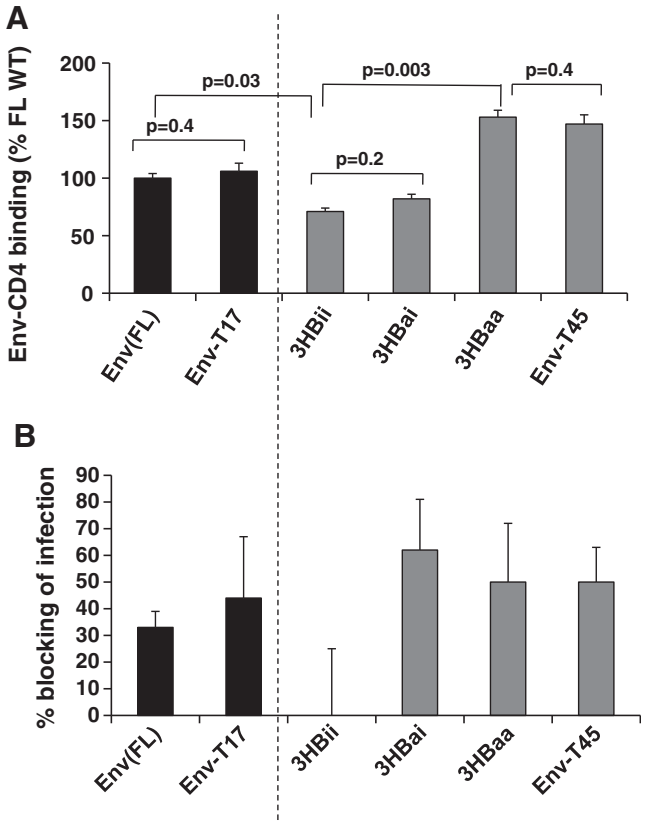


FIG. 6. Reactivity of Env proteins with CD4 and CCR5 receptors. **(A)** Env-CD4 binding. A 96-well plate coated with OKT4 antibody and then with soluble CD4 was incubated with virions pseudotyped with Env constructs. After extensive washes the bound virions were detected by anti-SIV peroxidase-conjugated antibodies. Results represent percentage of bound virions compared to that observed for wild-type full-length Env samples (100%). The results were analyzed by the Microsoft Excel program and the p values obtained for statistical comparison between samples. **(B)** Blocking of infection by SIV Env pseudotyped virions by mAb 2D7. A virus entry assay based on single-cycle infection as described in Materials and Methods was used. The blocking of infection was measured by comparison of the number of blue cells in wells with antibody-treated cells to the number in wells with untreated cells. Results are shown with the standard deviation ($n=3$).

Env-T17, residual trimer and dimers species were still observed at 95°C (Fig. 7D). 3HBii dimer species were observed at 95°C, trimers and dimers were stable up to 85°C, and smaller amounts of monomers were observed at 80–85°C than with other Envs. For Env(FL) and Env-T45 stable trimers and dimers were observed at 85°C, but 3HBaa and 3HBai trimers and dimers dissociated at 85°C. We also observed that the temperature for the dissociation of TM trimers was similar to that of the dissociation of SU trimers. The stability of trimers was approximately in the order Env-T17 > 3HBii > Env(FL) ≈ Env-T45 > 3HBai ≈ 3HBaa.

Discussion

The present results have implications for the mechanism of SIV virus-mediated membrane fusion, and the relationship of structural changes in the CT to exposure of receptor binding sites on fusogenic or nonfusogenic forms of Env proteins. The data indicate that the sequence, the α-helical content, and the length of the CT domain have effects on the stability of the trimeric structure of the Env protein and play different roles in cell–cell fusion and infectivity. Enveloped viruses enter cells by fusion of the viral envelope with a cellular membrane, which is mediated by metastable fusion-mediating glycoproteins that use the energy released from irreversible protein refolding for the work of membrane fusion.[9,35,46] The proteins then undergo a cascade of tightly regulated conformational changes, releasing the fusion proteins to a lower energy state. We found that fusion activation is suppressed by stabilizing the CT structure of Env proteins. An increase in the α-helical content in the CT domain was found to inhibit fusion activity. Previous results demonstrated that an elongated CT of a paramyxovirus F protein interferes with membrane fusion in a sequence-dependent manner.[47] The glycoprotein 3HBaa (CT of 45 aa) with the lowest trimer stability exhibited the highest fusion activity among chimeric mutant Envs, and the glycoprotein 3HBii with the most stable trimeric structure exhibited the lowest level of fusion activity. It was previously shown[29] that both full-length and truncated TM subunits of the SIVmac239 envelope protein form stable oligomers that are expressed on cell surfaces. The Env-T17 protein with a CT domain of 17 aa had optimal fusion activity and was able to form syncytia in an extended range of cells, whereas Envs with shorter (3 aa) or longer CT sequences demonstrated reduced fusion activity.[29] In the present study, we observed that Env-T17 demonstrated stable TM trimer species at 95°C and exhibited similar cell–cell fusion activity compared to 3HBii in mouse fibroblast 3T3T4R5 cells.

We did not observe a correlation between the level of incorporation of Env proteins, fusion activity, and infectivity. Previous studies indicated that the level of incorporation into virions of the Env protein with a full-length cytoplasmic domain was lower than that of Env with truncated CT into SIV or HIV virions.[48] It was also shown that sequence changes in the SU subunit and truncation of gp41 can significantly increase the level of Env incorporation into SIV virions, but SIV with truncated Env exhibits a defect in infectivity.[11,31,32] Despite the apparent importance of fusion activity for early steps of infection, we observed that cytoplasmic domain modifications had differential effects on fusion activity and infectivity, and these effects were dependent on the content and length of the CT. Several mutant Env proteins were incorporated very effectively into pseudotyped virions, which exhibited high specific infectivity. The 3HBii Env with a more stable trimeric structure exhibited low infectivity and fusion activity. Pseudotyped virions with Env-T45 were more infectious than pseudotyped virions with the 3HBai or 3HBaa Env but less fusogenic than 3HBaa. Previously, we observed that replication of SIV with full-length Env was cell type dependent and occurred only in monkey cell lines; in contrast, SIV with truncated Env produced infectious particles in all types of monkey and human cell lines tested.[11] Here, we found that transfected human epithelial 293T cells produced lower levels of infectious virions pseudotyped with full-length or 3HBii Env than with other truncated Env mutants.

Several studies indicate that known coreceptors are not absolutely required for CD4 binding.[49–51] It is possible that

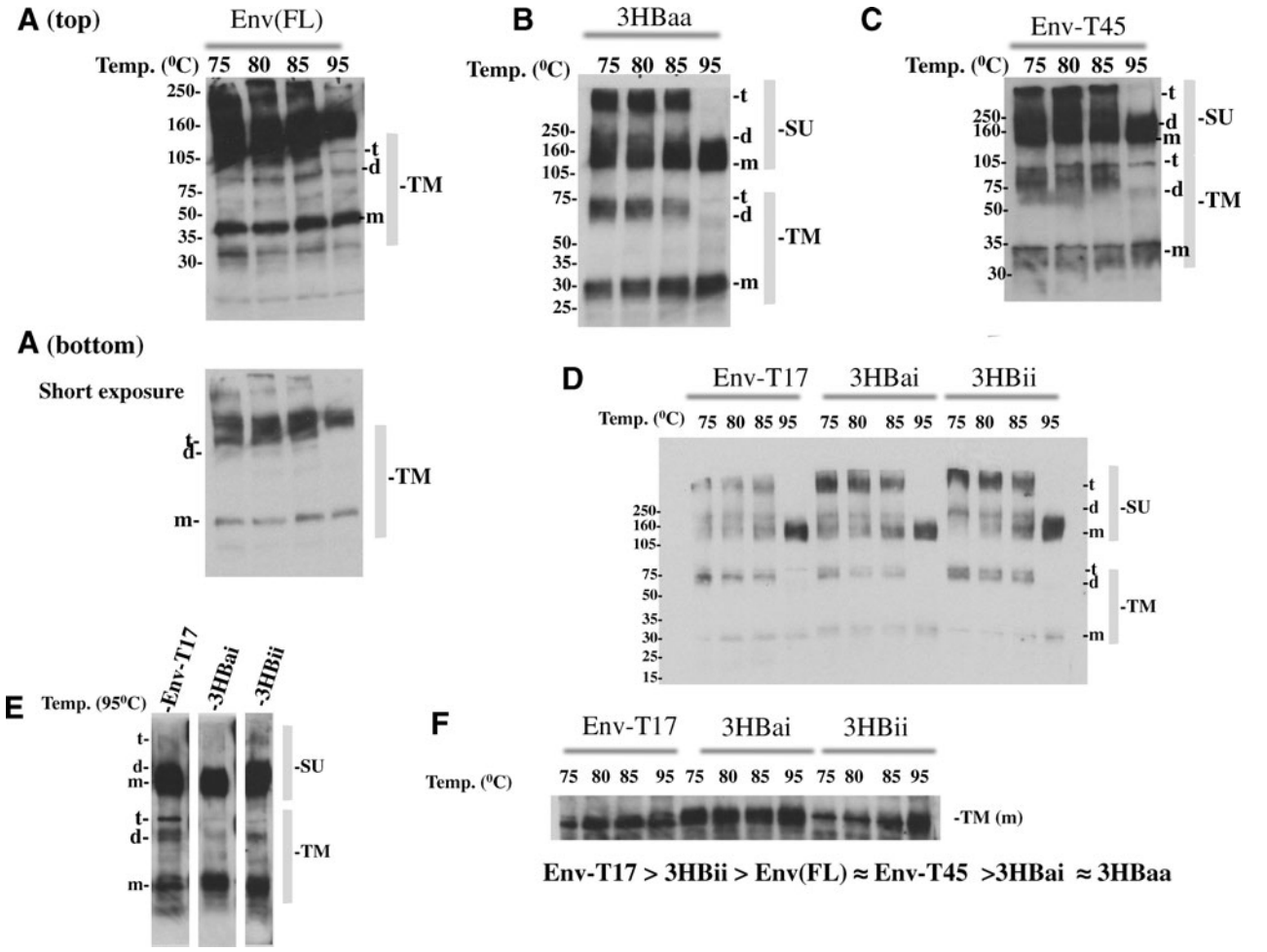


FIG. 7. Env protein trimer stability. Pseudotyped virions with wild-type or mutant Env proteins were obtained as described in the legend to Fig. 4. Samples were heated in Laemmli sample buffer at the temperatures indicated and analyzed by SDS–PAGE on 4–15% acrylamide gels and Western blotting by using a polyclonal anti-SIVmac251 antiserum and developed by ECL kit (Amersham). Env proteins with a chemiluminescent signal were captured on film **(A)** (top), **(B)**, **(C)**, **(E)**, and **(F)** by long exposure (10–15 min) or **(A)** (bottom) and **(D)** short exposure (1 min). Env protein surface subunit, SU, and transmembrane subunit, TM. Env species, m, monomer; d, dimer; t, trimer. The amounts of proteins were quantitated by densitometer analysis (NIH Image version 1.54).

HIV-1 strains become CD4 dependent as a mechanism to shield the conserved coreceptor binding site from neutralizing antibody.[52] We observed that mutant 3HBai Env and 3HBii with three or seven isoleucine residues in GCN4-related sequences added to truncated cytoplasmic tails showed reduced binding activity to CD4 molecules as well as differences in fusion activity and infectivity. 3HBaa with intermediate fusion activity and infectivity, and Env-T45 with the highest infectivity, exhibited the highest CD4 binding activity. We also studied interactions with CCR5 by using a blocking assay with a CCR5-specific (2D7) monoclonal antibody. 2D7 may inhibit HIV-1 binding by steric hindrance rather than by direct interruption of the binding site.[42] These results may indicate that the Env proteins that escape the antibody blocking effect could have higher affinity for CCR5.

Our studies have focused on effects of the cytoplasmic domain on Env structure, trimer stability, and their relation to fusion activity and infectivity. The gp41 ectodomain undergoes a series of conformational changes to form a fusion-active state during virus–cell membrane fusion.[53,54] The differences in reactivity of Envs with CD4 and CCR5 may reflect changes in gp41 ectodomain conformations due to modifications of the CT domain of the prefusogenic forms. We observed that defined modifications of the CT modulate infectivity and cause changes in the stability of the trimeric structure that promote fusion activity. The addition of helical CT structures stabilizes Env oligomers and results in changes in Env incorporation, infectivity, and cell fusion activity. Recent work has shown differences between the mechanisms of cell-to-cell fusion and virus entry.[55] Cell–cell fusion occurs at the cell surface and the role of cortical actin is consistent with this event.[56] Infection, however, occurs after HIV enters cells via endosomes and excludes cell surface proteins from this process.[55] Properties of incoming virus particles including the level of incorporation of Env molecules, their association with lipid rafts, or association with actin filaments may be important for this process.

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Author Disclosure Statement

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References

1. Dimitrov DS: Cell biology of virus entry. Cell 2000;101(7): 697–702.
2. Berger EA, Murphy PM, and Farber JM: Chemokine receptors as HIV-1 coreceptors: Roles in viral entry, tropism, and disease. Annu Rev Immunol 1999;17:657–700.
3. Doms RW: Chemokine receptors and HIV entry. AIDS 2001;15(Suppl 1):S34–35.
4. Wyatt R and Sodroski J: The HIV-1 envelope glycoproteins: Fusogens, antigens, and immunogens. Science 1998;280(5371): 1884–1888.
5. Furuta RA, Wild CT, Weng Y, and Weiss CD: Capture of an early fusion-active conformation of HIV-1 gp41. Nat Struct Biol 1998;5(4):276–279.
6. Gallo SA, Puri A, and Blumenthal R: HIV-1 gp41 six-helix bundle formation occurs rapidly after the engagement of gp120 by CXCR4 in the HIV-1 Env-mediated fusion process. Biochemistry 2001;40(41):12231–12236.
7. Melikyan GB, Markosyan RM, Hemmati H, Delmedico MK, Lambert DM, and Cohen FS: Evidence that the transition of HIV-1 gp41 into a six-helix bundle, not the bundle configuration, induces membrane fusion. J Cell Biol 2000; 151(2):413–423.
8. Sattentau QJ and Moore JP: The role of CD4 in HIV binding and entry. Philos Trans R Soc Lond B Biol Sci 1993;342(1299): 59–66.
9. Eckert DM and Kim PS: Mechanisms of viral membrane fusion and its inhibition. Annu Rev Biochem 2001;70:777–810.
10. Bhattacharya J, Peters PJ, and Clapham PR: Human immunodeficiency virus type 1 envelope glycoproteins that lack cytoplasmic domain cysteines: Impact on association with membrane lipid rafts and incorporation onto budding virus particles. J Virol 2004;78(10):5500–5506.
11. Vzorov AN, Weidmann A, Kozyr NL, Khaoustov V, Yoffe B, and Compans RW: Role of the long cytoplasmic domain of the SIV Env glycoprotein in early and late stages of infection. Retrovirology 2007;4:94.
12. LaBranche CC, Sauter MM, Haggarty BS, *et al.*: A single amino acid change in the cytoplasmic domain of the simian immunodeficiency virus transmembrane molecule increases envelope glycoprotein expression on infected cells. J Virol 1995;69(9):5217–5227.
13. Lodge R, Lalonde JP, Lemay G, and Cohen EA: The membrane-proximal intracytoplasmic tyrosine residue of HIV-1 envelope glycoprotein is critical for basolateral targeting of viral budding in MDCK cells. EMBO J 1997;16(4):695–705.
14. Yang C, Spies CP, and Compans RW: The human and simian immunodeficiency virus envelope glycoprotein transmembrane subunits are palmitoylated. Proc Natl Acad Sci USA 1995;92(21):9871–9875.
15. Berlioz-Torrent C, Shacklett BL, Erdtmann L, *et al.*: 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 1999;73(2):1350–1361.
16. Bowers K, Pelchen-Matthews A, Honing S, *et al.*: The simian immunodeficiency virus envelope glycoprotein contains multiple signals that regulate its cell surface expression and endocytosis. Traffic 2000;1(8):661–674.
17. Sauter MM, Pelchen-Matthews A, Bron R, *et al.*: An internalization signal in the simian immunodeficiency virus transmembrane protein cytoplasmic domain modulates expression of envelope glycoproteins on the cell surface. J Cell Biol 1996;132(5):795–811.
18. Chen SS, Lee SF, and Wang CT: Cellular membrane-binding ability of the C-terminal cytoplasmic domain of human immunodeficiency virus type 1 envelope transmembrane protein gp41. J Virol 2001;75(20):9925–9938.
19. Chernomordik L, Chanturiya AN, Suss-Toby E, Nora E, and Zimmerberg J: An amphipathic peptide from the C-terminal region of the human immunodeficiency virus envelope glycoprotein causes pore formation in membranes. J Virol 1994;68(11):7115–7123.
20. Comardelle AM, Norris CH, Plymale DR, *et al.*: A synthetic peptide corresponding to the carboxy terminus of human immunodeficiency virus type 1 transmembrane glycoprotein induces alterations in the ionic permeability of Xenopus laevis oocytes. AIDS Res Hum Retroviruses 1997;13(17): 1525–1532.
21. Kalia V, Sarkar S, Gupta P, and Montelaro RC: Rational site-directed mutations of the LLP-1 and LLP-2 lentivirus lytic peptide domains in the intracytoplasmic tail of human immunodeficiency virus type 1 gp41 indicate common functions in cell-cell fusion but distinct roles in virion envelope incorporation. J Virol 2003;77(6):3634–3646.
22. Miller MA, Cloyd MW, Liebmann J, *et al.*: Alterations in cell membrane permeability by the lentivirus lytic peptide (LLP-1) of HIV-1 transmembrane protein. Virology 1993;196(1): 89–100.
23. Venable RM, Pastor RW, Brooks BR, and Carson FW: Theoretically determined three-dimensional structures for amphipathic segments of the HIV-1 gp41 envelope protein. AIDS Res Hum Retroviruses 1989;5(1):7–22.
24. Srinivas SK, Srinivas RV, Anantharamaiah GM, Segrest JP, and Compans RW: Membrane interactions of synthetic peptides corresponding to amphipathic helical segments of the human immunodeficiency virus type-1 envelope glycoprotein. J Biol Chem 1992;267(10):7121–7127.
25. Kodama T, Wooley DP, Naidu YM, *et al.*: Significance of premature stop codons in env of simian immunodeficiency virus. J Virol 1989;63(11):4709–4714.
26. Spies CP, Ritter GD Jr, Mulligan MJ, and Compans RW: Truncation of the cytoplasmic domain of the simian immunodeficiency virus envelope glycoprotein alters the conformation of the external domain. J Virol 1994;68(2):585–591.
27. Puffer BA, Pohlmann S, Edinger AL, *et al.*: CD4 independence of simian immunodeficiency virus Envs is associated with macrophage tropism, neutralization sensitivity, and attenuated pathogenicity. J Virol 2002;76(6):2595–2605.
28. Mulligan MJ, Yamshchikov GV, Ritter GD Jr, *et al.*: Cytoplasmic domain truncation enhances fusion activity by the exterior glycoprotein complex of human immunodeficiency virus type 2 in selected cell types. J Virol 1992;66(6):3971–3975.

29. Spies CP and Compans RW: Effects of cytoplasmic domain length on cell surface expression and syncytium-forming capacity of the simian immunodeficiency virus envelope glycoprotein. Virology 1994;203(1):8–19.
30. Ritter GD Jr, Mulligan MJ, Lydy SL, and Compans RW: Cell fusion activity of the simian immunodeficiency virus envelope protein is modulated by the intracytoplasmic domain. Virology 1993;197(1):255–264.
31. Marthas ML, Banapour B, Sutjipto S, *et al.*: Rhesus macaques inoculated with molecularly cloned simian immunodeficiency virus. J Med Primatol 1989;18(3–4):311–319.
32. Luciw PA, Shaw KE, Unger RE, *et al.*: Genetic and biological comparisons of pathogenic and nonpathogenic molecular clones of simian immunodeficiency virus (SIVmac). AIDS Res Hum Retroviruses 1992;8(3):395–402.
33. Landschulz WH, Johnson PF, and McKnight SL: The leucine zipper: A hypothetical structure common to a new class of DNA binding proteins. Science 1988;240(4860):1759–1764.
34. Bernstein HB, Tucker SP, Kar SR, *et al.*: Oligomerization of the hydrophobic heptad repeat of gp41. J Virol 1995;69(5): 2745–2750.
35. Waning DL, Russell CJ, Jardetzky TS, and Lamb RA: Activation of a paramyxovirus fusion protein is modulated by inside-out signaling from the cytoplasmic tail. Proc Natl Acad Sci USA 2004;101(25):9217–9222.
36. Frey G, Peng H, Rits-Volloch S, Morelli M, Cheng Y, and Chen B: A fusion-intermediate state of HIV-1 gp41 targeted by broadly neutralizing antibodies. Proc Natl Acad Sci USA 2008;105(10):3739–3744.
37. Vzorov AN, Lea-Fox D, and Compans RW: Immunogenicity of full length and truncated SIV envelope proteins. Viral Immunol 1999;12(3):205–215.
38. Harbury PB, Zhang T, Kim PS, and Alber T: A switch between two-, three-, and four-stranded coiled coils in GCN4 leucine zipper mutants. Science 1993;262(5138):1401–1407.
39. Vzorov AN, Gernert KM, and Compans RW: Multiple domains of the SIV Env protein determine virus replication efficiency and neutralization sensitivity. Virology 2005; 332(1):89–101.
40. Nussbaum O, Broder CC, and Berger EA: Fusogenic mechanisms of enveloped-virus glycoproteins analyzed by a novel recombinant vaccinia virus-based assay quantitating cell fusion-dependent reporter gene activation. J Virol 1994; 68(9):5411–5422.
41. Moore JP: Simple methods for monitoring HIV-1 and HIV-2 gp120 binding to soluble CD4 by enzyme-linked immunosorbent assay: HIV-2 has a 25-fold lower affinity than HIV-1 for soluble CD4. AIDS 1990;4(4):297–305.
42. Wu L, LaRosa G, Kassam N, *et al.*: Interaction of chemokine receptor CCR5 with its ligands: Multiple domains for HIV-1 gp120 binding and a single domain for chemokine binding. J Exp Med 1997;186(8):1373–1381.
43. Chackerian B, Haigwood NL, and Overbaugh J: Characterization of a CD4-expressing macaque cell line that can detect virus after a single replication cycle and can be infected by diverse simian immunodeficiency virus isolates. Virology 1995;213(2):386–394.
44. Bowers K P-MA, Höning S, Vance PJ, Creary L, Haggarty BS, Romano J, Ballensiefen W, Hoxie JA, and Marsh M: The simian immunodeficiency virus envelope glycoprotein contains multiple signals that regulate its cell surface expression and endocytosis. Traffic 2000;1(8):661–674.
45. Dragic T, Trkola A, Lin SW, *et al.*: Amino-terminal substitutions in the CCR5 coreceptor impair gp120 binding and human immunodeficiency virus type 1 entry. J Virol 1998; 72(1):279–285.
46. Skehel JJ: Receptor binding and membrane fusion in virus entry: The influenza hemagglutinin. Annu Rev Biochem 2000;69:531–569.
47. Seth S, Vincent A, and Compans RW: Mutations in the cytoplasmic domain of a paramyxovirus fusion glycoprotein rescue syncytium formation and eliminate the hemagglutinin-neuraminidase protein requirement for membrane fusion. J Virol 2003;77(1):167–178.
48. Zingler K and Littman DR: Truncation of the cytoplasmic domain of the simian immunodeficiency virus envelope glycoprotein increases env incorporation into particles and fusogenicity and infectivity. J Virol 1993;67(5):2824–2831.
49. Vödrös D, Doms RW, Fenyö EM, and Reeves JD: Evolution of coreceptor use and CD4-independence in envelope clones derived from SIVsm-infected macaques. Virology 2003;316: 17–28.
50. Dehghani H PB, Doms RW, and Hirsch VM: Unique pattern of convergent envelope evolution in simian immunodeficiency virus-infected rapid progressor macaques: Association with CD4-independent usage of CCR5. J Virol 2003; 77(11):6405–6418.
51. Lin G BF, Romano J, Doms RW, and Hoxie JA: Identification of gp120 binding sites on CXCR4 by using CD4-independent human immunodeficiency virus type 2 Env proteins. J Virol 2003;77(2):931–942.
52. Labrijn AF PP, Raja A, Zwick MB, *et al.*: Access of antibody molecules to the conserved coreceptor binding site on glycoprotein gp120 is sterically restricted on primary human immunodeficiency virus type 1. J Virol 2003;77(19):10557–10565.
53. Chan DC and Kim PS: HIV entry and its inhibition. Cell 1998;93(5):681–684.
54. Weissenhorn W, Dessen A, Calder LJ, Harrison SC, Skehel JJ, and Wiley DC: Structural basis for membrane fusion by enveloped viruses. Mol Membr Biol 1999;16(1):3–9.
55. Miyauchi K, Kim Y, Latinovic O, Morozov V, and Melikyan GB: HIV enters cells via endocytosis and dynamin-dependent fusion with endosomes. Cell 2009;137(3):433–444.
56. Yoder A, Yu D, Dong L, *et al.*: HIV envelope-CXCR4 signaling activates cofilin to overcome cortical actin restriction in resting CD4 T cells. Cell 2008;134(5):782–792.

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