Role for the Terminal Clasp of HIV-1 gp41 Glycoprotein in the Initiation of Membrane Fusion*

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The fusion function of gp41 involves the refolding of its core into a 6-helix bundle, which apposes the lipophilic termini of the gp41 hairpin and the associated cell and viral membranes, leading to their fusion. In this study, we examined the functional role of the polar segment and membrane proximal external region (MPER), which link the fusion peptide and transmembrane domain, respectively, to the core domain and interact to form a terminal clasp adjacent to the core. Limited proteolysis indicated that the terminal clasp is destabilized by simultaneous I535A/V539G mutations within the polar segment and mutations within the MPER. The destabilizing effects of I535A/V539G correlated with defective cell-cell fusion, viral entry, and viral replication. By using lipophilic and cytoplasmic fluorescent dye transfer assays, we found that terminal clasp destabilization is linked to a block in the lipid mixing/hemifusion phase of the membrane fusion cascade. Because the biosynthesis of the prefusion gp120-gp41 complex did not appear to be affected by I535A/V539G, we infer that the hemifusion block is due to a specific effect on the trimer of hairpins conformation of gp41. By contrast, the decreased fusion function of the MPER mutants correlated with a decrease in the interfacial hydrophathy of the MPER sequence, suggesting that the prefusion Env complex had been adversely affected in these cases. These findings reveal a novel conserved functional target for the discovery of fusion inhibitors.

The gp120-gp41 envelope glycoprotein (Env) complex of HIV-1 mediates viral entry and is a target of neutralizing antibodies, as well as chemical and protein-based inhibitors of infection (1–3). The gp120–gp41 complex is synthesized as a highly glycosylated precursor (gp160) that is processed in a late Golgi compartment into a trimer of noncovalently associated gp120–gp41 heterodimers (4–7). Binding by gp120 to CD4 and a chemokine receptor (CCR5 or CXCR4) activates the membrane fusion function of the gp41 transmembrane glycoprotein (8). Gp41 is a class I fusion glycoprotein composed of an N-terminal fusion peptide, connected through a polar segment (also referred to as the fusion peptide-proximal region (FPPR) (9)) to a coiled-coil forming amphipathic α-helix (helical region 1 or HR1), a centrally located disulfide-bonded loop region, a C-terminal amphipathic α-helix (helical region 2 or HR2), and a tryptophan-rich membrane-proximal external region (MPER) (10, 11). The gp120–gp41 ectodomain is anchored to the viral envelope by a C-terminal transmembrane domain (TMD), which precedes a cytoplasmic domain.

The binding of gp120 to CD4 causes major structural changes in gp120, which trigger the pre-hairpin intermediate conformation of gp41 that bridges the viral and cellular membranes (5, 12–14). The subsequent engagement of chemokine receptors by gp120–CD4 is believed to enable further structural changes in gp41 including the antiparallel packing of HR2 into hydrophobic grooves on the exterior of the coiled-coil to form the 6-helix bundle core. Six-helix bundle formation brings together the N- and C-terminal membrane-inserted ends (i.e. the fusion peptide and TMD) in a trimer of hairpins structure

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

The abbreviations used are: Env, envelope glycoprotein; MPER, membrane proximal external region; FPPR, fusion peptide-proximal region; HR1, helical region 1; HR2, helical region 2; TMD, transmembrane domain; sCD4, soluble CD4; FBMC, peripheral blood mononuclear cells; MBP, maltose-binding protein; Dil, 1,1’dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate; DiO, 3,3’-dioctadecyloxacarbocyanine perchlorate; ΔG, membrane interface-to-water transfer free energies; LFI, L669G/F673A/I675A mutant; W(1–3)A, W666A/W670A/W672A mutant; W(1–5)A, W666A/W670A/W672A/W680A/W683A mutant.

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that apposes the associated viral and cellular membranes, ready for merger (15–20).

The functional role of the MPER, which links HR2 to the TMD, is of major interest because it encompasses the epitopes of broadly neutralizing mAbs 2F5, 4E10, and Ze13 (10), indicating that it is a conserved antiviral target. Electron density corresponding to the MPER is not observed in a cryotomographic structure of virion gp120-gp41 in which the orientation of the glycoproteins was confirmed using ligands (5), however, biophysical approaches indicate that this sequence is embedded in the viral envelope, most likely at the base of the trimer (21, 22). Spectroscopic studies of synthetic MPER peptides and model membranes have revealed a lipid-immersed α-helix-kink-α-helix conformation. Interestingly, the side chains of the key mAb 2F5- (Trp⁶⁶⁶ to 4E10- (Trp⁶⁷², Phe⁶⁷³, and Ile⁶⁷⁵) binding residues are buried, forming part of the lipid-interactive face of the peptide (22–24). Reinherz et al. (22, 25) proposed an unusual binding mechanism for mAbs 2F5 and 4E10, which can target the MPER in the prefusion gp120-gp41 complex (21, 26), whereby the mAb paratope initially interacts with lipid and then extracts its epitope from the hydrophobic phase. Taken together, these studies indicate that the MPER is immersed in lipid and protein-protein interactions can induce its extraction from this environment.

A number of studies have examined the role of the MPER in the HIV-1 membrane fusion mechanism. Mutational studies have indicated that the conserved Trp⁶⁶⁶- Trp⁶⁷⁰ motif of the MPER functions cooperatively in a late stage of membrane fusion (27, 28). Studies of the interaction between synthetic peptide MPER analogues and liposomes indicate membrane partitioning, lipid mixing, and permeability activities, however, these activities depend on peptide length and lipid composition (see Ref. 10). Whether these observations with peptides reflect the native function of the MPER remains unclear.

We previously reported that an interaction between the MPER and polar segment confers stability to the trimer of the hairpins conformation of gp41 and proposed that a terminal hairpin clamp forms at its membrane-interactive end (29, 30). X-ray crystallography has now revealed that the polar segment and MPER form helical extensions of the central trimeric HR1 coiled-coil and antiparallel HR2 segments, respectively (9) (Fig. 1). The clamp is stabilized by predominantly hydrophobic interactions between residues of the polar segment coiled-coil and adjacent antiparallel MPER helices (Gly⁵³¹-Leu⁵⁷⁹, Ala⁵³³, Trp⁶⁷⁰, Met⁵³⁵-Ile⁶⁷⁵/Asn⁶⁷¹, and Thr⁵³⁶/Leu⁵³⁷-Trp⁶⁶⁶). In this study, we examined the functional role of the terminal clamp by mutagenesis. We found that clamp destabilization in a trimer of hairpins model protein is linked to a block in the lipid mixing/hemifusion phase of the membrane fusion cascade, indicating that terminal clamp formation is important for the initiation of membrane fusion.

**EXPERIMENTAL PROCEDURES**

Env Expression Vectors and Proviral Clones—The preparation of CMV and bacteriophage T7 promoter–driven HIV-1<sub>AD₈</sub> Env expression vectors, pCDNA3.1-AD₈/env and pTM-AD₈/env, respectively, are described elsewhere (29, 31, 32). For Env expression, the cells were cotransfected with pTM-AD₈/env and pCAG-T₇, the latter directing expression of bacteriophage T7 RNA polymerase from a CMV immediate-early enhancer and chicken β-actin promoter (33). The pAD₈ infectious clone was obtained from K. Peden (34). pAD₈ mutants were prepared by transferring the EcoRI-BspMI env-containing fragment from pCDNA3.1-AD₈/env vectors into pAD₈.

**Cell Lines**—293T, BHK21, and JC53 cells were maintained in DMEM, 10% FCS (complete medium) and transfected with expression vectors using FuGENE HD (Roche Applied Science). U87.CD4.CC5R cells were maintained in DMEM, 15% FCS supplemented with puromycin (0.1 mg/ml) and G418 (0.3 mg/ml).

**Antibodies**—The anti-gp41 mAb, C8, and anti-gp120 polyclonal sheep antibody, DV-012, were obtained through the AIDS Research and Reference Reagent Program, National Institute for Allergy and Infectious Diseases, from G. Lewis (35) and Michael Phelan (36, 37), respectively. The human mAb 2G12 (38–40), which is directed to an oligosaccharide cluster of gp120, was purchased from Polymun.

**Western Blotting**—At 24-h post-transfection, 293T cells were lysed for 10 min on ice in PBS containing 1% Triton X-100, 0.02% sodium azide, 1 μl EDTA. Lysates were centrifuged for 10 min at 10,000 X g at 4 °C prior to SDS-PAGE under reducing conditions. Proteins were transferred to nitrocellulose and blotted with mAb C8 and DV-012. The immunoblots were developed with Alexa Fluor 680-conjugated goat anti-mouse or donkey anti-sheep Ig (Invitrogen) and scanned in a LI-COR Odyssey infrared imager.

**Flow Cytometry**—293T cells (600,000 cells/well of a 6-well flat bottomed plate) were cotransfected with pCAG-T₇ and pTM-AD₈/env vectors in triplicate. At 48 h post-transfection, the cells were resuspended in PBS containing 1% FCS and incubated with mAb 2G12, directed to gp120 for 1 h on ice. After 2 washes in PBS, 1% FCS, the cells were incubated with FITC-conjugated goat anti-human Fab for 30 min on ice. After a further 2 washes in PBS, 1% FCS, the cells were fixed with 4% paraformaldehyde/PBS prior to flow cytometry using a FACSCalibur instrument (BD Bioscience) and CellQuest software. The mean fluorescence intensity from the acquisition of 10,000 gated events was used as a measure of binding.

**Soluble CD4 (sCD4)-induced Shedding of gp120**—293T cells were cotransfected with pTM-AD₈/env and pCAG-T₇ plasmids. At 24 h post-transfection, the cells were starved in methionine and cysteine-free medium for 30 min and then pulse labeled with 150 μCi of Tran-35S-label (MP Biomedicals) for 45 min. The labeled cells were washed and then chased for 5 h with complete medium in the presence or absence of sCD4 (15 μg of sCD4/0.6 ml of medium). All incubations were at 37 °C in 5% CO₂. The cells and culture supernatants were processed for immunoprecipitation with mAb 2G12 and protein G-Sepharose and subjected to reducing SDS-PAGE. The labeled proteins were visualized by scanning in a Typhoon TRIO variable mode imager (GE Healthcare).

**Luciferase Reporter Assay of Cell to Cell Fusion**—293T effector cells (250,000 cells well of a 12-well culture plate) were cotransfected with pTM-AD₈/env and pCAG-T₇ plasmids. BHK21 target cells (250,000 cells/well of a 12-well culture plate) were cotransfected with pTM-AD₈/env and pCAG-T₇ plasmids.
CAGTCTCGGACTTTATGGCC (AD8 sequence in bold), and the HIV-2ST (Met565-Ser665)-HIV-1AD8 (Trp666-Asn677) reverse primer, 5’-GGCGGTCAGCTTAATGGTTAGTGC- AACAACTCCAAATTGTCGCTATTTT- TGTTAGTTTCATGATAGCT (Sall site underlined, AD8 sequence in bold). The PCR products were ligated into the MBP expression vector through NotI-Sall. The various substitution mutations were introduced to MBP/gp41(528-L-677)AD-ST by overlap extension PCR. The tr665 construct, which was truncated after residue 665 to remove the MER was produced by PCR using MBP/gp41(528-L-677)AD-ST as the template and 5’-CCGCCGCGCCGCGCAGACATGTCGGCCGGGTTCA- TAACG and 5’-GGCGGTCAGCTTAATGGTTAGTGC-AACAACTCCAAATTGTCGCTATTTTGTTAGTTTCATGATAGCT as forward and reverse primers, respectively. The tr665 PCR product was ligated into the MBP expression vector through NotI-Sall. DNA sequences were confirmed using ABI Prism BigDye terminator (Applied Biosystems). MBP/gp41 chimeras were induced in Escherichia coli strain BL21(DE3) and purified by amylase-agarose affinity chromatography (New England Biolabs) and gel filtration as described (30). MBP/gp41 trimers were proteolyzed with sequencing-grade chymotrypsin (Roche Applied Science and Promega) and analyzed by SDS-PAGE in 12–17% polyacrylamide gradient gels as described (30).

Time of Flight-Mass Spectrometry—Analyses were carried out on an Agilent 6220 Accurate Mass TOF LC/MS Mass Spectrometer coupled to an Agilent 1100 LC system. All data were acquired and reference mass was corrected via a dual-spray electrospray ionization source. Each scan or data point on the total ion chromatogram is an average of 10,000 transients, producing a scan every second. Spectra were created by averaging the scans across each peak. Chromatographic separation was carried out following injection of 10 μl of protein sample (0.5 mg/ml) using a MassPREP On-Line Desalting Cartridge, 2.1 × 10 mm (Waters). Unbound material including chymotrypsin buffer reagents were removed with a one-min 15% “B” (95% acetonitrile in water with 0.1% formic acid) wash that was directed to waste. Eluted molecules were then sent to the mass spectrometer during a 15–80% B gradient at 0.25 ml/min.

Lipid Mixing Assays—293T effector cells (5 × 10^5 cells/well of a 12-well plate) were cotransfected with pCAG-T7 and wild type or mutant Env-expression vectors. At 24 h post-transfection, the cells were stained with the red lipophilic dye Dil (1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate; red fluorescence, excitation at 549 nm, emission at 565 nm (Invitrogen)) for 15 min at room temperature. At the same time, JC53 (46) target cells were labeled with DiO (3,3’-dioctadecyloxycarbocyanine perchlorate; green fluorescence, excitation at 484 nm, emission at 501 nm). After washing 3 times with PBS, the cells were detached with PBS, 1 mM EDTA and resuspended in complete medium. Equivalent numbers of effector and target cells were mixed and added to the wells of fibronectin-coated chamber slides. The cells were allowed to adhere for 2 h at the fusion nonpermissive temperature of 23 °C after which fusion was initiated by raising the temperature to 37 °C.

Following 2 h of coculture at 37 °C, lipophilic dye transfer events were recorded using an API Deltavision RT Deconvolution system and SoftWorx 1.2 software. Images of 10–15 ran-
dom fields (×60 magnification) were collected for each condition. Imaris Colocalization software was used to quantify lipid-mixing events in reconstructed z-stacks by determining the Pearson coefficient of correlation for the location of green and red fluorescing pixels (referred to as voxels).

Cytoplasmic Dye Transfer Assays—Cytoplasmic dye transfer assays were performed essentially as described for lipophilic dyes, above, except that effector cells were labeled for 30 min at 37 °C with CellTracker orange CMTMR (554.04 Da, excitation at 541 nm, emission at 565 nm) and targets were labeled with CellTracker green CMFDA (464.86 Da; excitation at 492 nm, emission at 517 nm).

Molecular Modeling—A homology-based model of HIV-1AD8 gp41 was generated from PDB entry 2X7R (9) using the Modeler algorithm (47) within Discovery Studio, version 3.0 (Accelrys). Five independent models were generated from iterative cycles of conjugate-gradient energy minimization against spatial constraints derived from the template crystal structure. The model with the lowest energy (probability density function) was selected for structural analysis. Point mutations were introduced using the “Build Mutants” routine of Modeler within Discovery Studio and optimized using the same approach as for the wild type AD8 homology model.

RESULTS
Phenotypes of Terminal Clasp Mutants—In this study, we used mutagenesis of HIV-1AD8 Env to determine the role of the terminal clasp in the membrane fusion mechanism. Ile535 and Val539 were initially chosen as mutagenic targets because they were predicted to occupy outward-facing e and b positions, respectively, of the polar segment-HR1 coiled-coil heptad repeat (Fig. 1). Modeling of Ile535 into the terminal clasp of PDB entry 2X7R indicates that it will mediate hydrophobic contacts

![FIGURE 1. Structure of the gp41 trimer of hairpins. The 6-helix bundle core domain comprises an inner trimeric coiled-coil of HR1 helices (magenta) bound to an antiparallel outer layer of HR2 helices (light blue). The polar segment/FPPR (pink) forms a helical extension of the coiled-coil, providing a packing surface for the MPER helix (Trp666, Asn677) (gray). Amino acids targeted with mutations are color-coded: polar segment/FPPR, orange; Trp cluster of the MPER, blue; hydrophobic residues of the MPER, teal. The schematic was drawn using PyMOL and PDB entry 2X7R (9). Inset, alignment of polar segment/FPPR and MPER amino acid sequences. The sequences were selected to reflect the predominant intraclade polymorphisms at amino acid 535.](image-url)
with Asn<sup>671</sup> and Ile<sup>675</sup> of the MPER. On the other hand, Val<sup>539</sup> does not contact other residues.

The individual I535A and V539G mutations led to modest reductions in cell-cell fusion (30 and 25%, respectively; Fig. 2A) and single cycle entry (75 and 60%, respectively; Fig. 2B), whereas both functions were abolished for the combination mutant, I535A/V539G. Consistent with these data, HIV-1<sub>AD8</sub> infectious clones containing I535A or V539G exhibited slightly delayed replication kinetics with respect to wild type in phytohemagglutinin-stimulated PBMCs, whereas the I535A/V539G virus lacked replication competence (Fig. 2C). Importantly, the expression and processing of I535A, V539G, and I535A/V539G to gp120 and gp41 in transfected 293T cells was found to be similar to that of wild type as determined by Western blotting (Fig. 3A) and the mutants were expressed efficiently at the cell surface as determined by flow cytometry using the gp120-specific mAb 2G12 as the primary antibody (Fig. 3B and supplemental Fig. S1). Soluble CD4 induced the shedding of gp120 from cell surface-expressed 35S-labeled glycoprotein complexes into the culture supernatant as determined by immunoprecipitation with mAb 2G12, consistent with a receptor-responsive Env complex (Fig. 3C). Furthermore, Western blotting analysis of Env-pseudotyped luciferase reporter viruses (Fig. 3D) and virions derived from HIV-1<sub>AD8</sub> infectious clones (Fig. 3E) indicated the presence of gp120 and p24 at wild type levels. Taken together, these data indicate that I535A/V539G is specifically blocked in membrane fusion and virus entry function because Env biosynthesis, gp120-gp41 association, and responsiveness to sCD4 are not affected. The previously described L537A mutant, which exhibits a gp120-shedding phenotype in both cellular and viral contexts and causes modest reductions in cell-cell fusion and viral entry (29) was included for comparison with I535A, V539G, and I535A/V539G.

We next targeted the conserved Trp<sup>666</sup>-Trp<sup>670</sup>-Trp<sup>672</sup>-Trp<sup>678</sup>-Trp<sup>680</sup> cluster of the MPER. Trp<sup>666</sup> mediates hydrophobic interactions with Thr<sup>536</sup> and Leu<sup>537</sup>, whereas Trp<sup>672</sup> contacts Ala<sup>533</sup> within the clasp. Tyr<sup>678</sup> and Trp<sup>680</sup> are located outside the clasp in a segment perpendicular to the MPER helix and are proposed to interact with the membrane (9). Because single Trp to Ala substitutions do not affect Env-mediated fusion, we tested the functional effects of the simultaneous W(1–3)A and W(1–5)A mutations, which were shown previously to completely block the cell-cell fusion function of Env derived from the T cell line adapted X4 strain HIV-1<sub>HXB2</sub> (28). The W(1–3)A and W(1–5)A mutations had less severe effects on fusion mediated by the R5 Env of HIV-1<sub>AD8</sub> with ~60 and 80% reductions observed, respectively (Fig. 2A). The mutants appeared to be processed into stable gp120-gp41 complexes in a similar manner to wild type (Fig. 3A, C and D), however, their expression at the cell surface was diminished by ~30% (Fig. 3B). In contrast to the polar segment mutants, pseudovirions produced with W(1–3)A and W(1–5)A lacked gp120 and therefore virus entry function (Figs. 2B and 3D).

The substitution of conserved MPER residues Leu<sup>669</sup>, Phe<sup>673</sup>, or Ile<sup>675</sup> with Ala leads to 1–2 log<sub>10</sub> reductions in virus entry without an effect on cell-cell fusion (29, 48). These data suggest...
that the aromatic (Trp<sup>670</sup>-Phe<sup>673</sup>) and hydrophobic (Ile<sup>535</sup>-Ile<sup>675</sup>, Leu<sup>669</sup>-Trp<sup>666</sup>) interactions mediated by these residues in the terminal clasp are functionally relevant. To further investigate the role of these residues, we produced the multiple mutant L669G/F673A/I675A (LFI). In this case, a small reduction in cell-cell fusion was observed (Fig. 2A) in the absence of any adverse effects on Env biosynthesis (Fig. 3, A-C), whereas the observed ~2 log<sub>10</sub> reduction in virus entry again correlated with low levels of gp120 being incorporated into pseudovirions (Figs. 2B and 3D). Because W(1–3)A, W(1–5)A, and LFI exhibited gp120 virion incorporation defects, we did not further examine these mutations in the context of infectious clones.  

**Effects of Mutations on Terminal Clasp Stability**—We previously used limiting chymotrypsin proteolysis to assess terminal clasp stability in the MBP/gp41(528-L-677) trimer of hairpins model protein, which comprises MBP fused to polar segment-HR1 residues 528–596, a Ser-Gly-Gly-Arg-Gly-Gly linker, and HR2-MPER residues 610–677 of HIV-2ST (29). Although HIV-2<sub>ST</sub> is a useful general model of the gp41 hairpin, it does not allow an examination of HIV-1-specific features of the terminal sequences as HIV-1<sub>ADA</sub> and HIV-2<sub>ST</sub> MPERs exhibit nonconservative amino acid changes: A667D, S668V, and W670G. We therefore swapped the polar segment and MPER of HIV-2<sub>ST</sub> with the corresponding sequences of the HIV-1<sub>ADA</sub> strain that was used in the mammalian cell-based analyses to give MBP/gp41(528-L-677)<sub>ADA-ST</sub> (Fig. 4A). This chimera presented as a trimer with Superdex 200 gel filtration (supplemental Fig. S2). Limiting chymotrypsin treatment of MBP/gp41(528-L-677)<sub>ADA-ST</sub> released ~48, ~41.5, and ~5–7.5-kDa products (Fig. 4B). The identities of the protein bands were inferred by mass spectrometry of the 1:20 protease:protein reaction. Band 1, which is produced first, comprises MBP/gp41(528–593) plus MBP/gp41(528–596) (47,892.91 and 48,280.46 Da, respectively) (Fig. 4C), indicating cleavage events at the C terminus of HR1. The emergence of band 2 is delayed and is concomitant with a decrease in band 1 intensity. Band 2 corresponds to the gp41 helical peptides, gp41(538–596) plus gp41(611–677) (6,866.21 and 7,744.08 Da, respectively). The introduction of L537A inhibited the production of band 2 (Fig. 4, B and D), consistent with the removal of a favored chymotrypsin site from the polar segment. The chymotrypsin sensitivity of the
polar segment was therefore used to monitor clasp stability. The release of band 2 was accelerated by I535A/V539G and the W(1–3)A mutations (Fig. 4, B and D). Note that it was not possible to test W(1–5)A in this context because the chimera is truncated at Asn677 to overcome protein aggregation (30). The LFI mutation was associated with slightly elevated chymotrypsin sensitivity relative to the wild type, however, this difference was not statistically significant. These data indicate that clasp destabilization correlates with loss of fusion function.

We observed that the effects of individual I535A and V539G mutations on cell-cell fusion and virus entry were less severe in comparison to I535A/V539G (Fig. 2, A and B). We therefore asked whether this gradation of functional effects is reflected in decreased clasp stability in the context of MBP/gp41(528-L-677)AD.ST. Pilot studies indicated that the stabilities of I535A and V539G were close to that of wild type therefore harsher proteolysis conditions were employed to reveal differences in chymotrypsin sensitivity. For these experiments, aprotinin was excluded from the quench step. The data indicate stepwise increases in chymotrypsin sensitivity due to mutations in the order: WT/H11021 V539G/H11021 I535A/H11021 I535A/V539G (Fig. 5, A and B). The hierarchy of clasp destabilizing effects due to V539G, I535A, and I535A/V539G, respectively, is consistent with the inhibitory effects of these mutations on cell-cell fusion and virus entry. We conclude that the terminal clasp is an important determinant of membrane fusion and virus entry function.
Evidence That I535A/V539G Exerts Its Functional Effects in the Context of a Terminal Clasp—We next asked whether the effects of I535A/V539G on trimer of hairpins stability is linked to the presence of the MPER by comparing the chymotrypsin sensitivities of MBP/gp41(528-L-677)AD.ST with I535A, V539G, and I535A/V539G mutations. A, chymotrypsin cleavage of wild type and mutated MBP/gp41(528-L-677)AD.ST; and tr665, which was truncated after residue 665 to remove the MPER, in the presence and absence of the polar segment mutation. Analytical Superdex 200 gel filtration was first used to confirm trimeric structure for tr665 and tr665-I535A/V539G, which like MBP/gp41(528-L-677)AD.ST eluted at 27 min (supplemental Fig. S2). Consistent with the data presented in Fig. 4, the release of band 2 from MBP/gp41(528-L-677)AD.ST was significantly enhanced by I535A/V539G (Fig. 6, A and B). The release of band 2 from tr665 by chymotrypsin was even greater than for MBP/gp41(528-L-677)AD.ST-I535A/V539G, suggesting that removal of the outer MPER layer from the trimer of hairpins terminus had exposed the polar segment to the protease. However, in the tr665 context, I535A/V539G was not associated with a further increase in susceptibility to protease, suggesting that a structural defect is associated with the polar segment mutation in the context of the terminal clasp.

Mass spectrometry was used to infer the sites of cleavage within tr665 (Fig. 6C). The spectra indicate that band 2 is likely to comprise MBP/gp41(528–530) and MBP/gp41(528–537) (40,815.85 and 41,429.55 Da, respectively), consistent with cleavage at Met530 and Leu537, respectively, whereas band 3 corresponds to the gp41(538–596) HR1 and gp41(611–665) HR2 peptides (6866.59 and 6210.15 Da, respectively). A similar cleavage pattern was inferred for tr665-I535A/V539G with cleavage at Met530 and Leu537, respectively, whereas band 3 corresponding to MBP/gp41(528–530) and MBP/gp41(528–537) (40,815.82 and 41,387.40 Da, respectively), and band 3 corresponding to gp41(538–596) and gp41(611–665) (6824.37 and 6210.14 Da, respectively). These data suggest that I535A/V539G affects the gp41 structure and function in the context of a terminal clasp.

Effects of Terminal Clasp Mutations on Cytoplasmic Dye Dysfunction—The cell-cell fusion assay measures pore expansion where luciferase induction follows transfer of a large protein probe (T7 RNA polymerase) from gp120-gp41-expressing 293T effectors through an expanded fusion pore to the CD4 + CCR5 + targets harboring the reporter. To determine whether the fusion block occurs at the lipid mixing/hemifusion phase, which precedes pore expansion, we measured the fusion activity of I535A/V539G in a Dil/Dio lipophilic fluorescent dye exchange assay (29). Dil (red)-labeled Env-293T cells were cocultured with Dio (green)-labeled JC53 targets, and fusion events were recorded using an API Deltavision RT system. Fig. 7A shows that Dil and Dio are associated with individual cells for cocultures employing effectors transfected with empty vector (No Env), whereas major dye redistribution with numerous colocalization events was observed for the WT Env coculture, consistent with extensive lipid mixing between effectors and targets. The appearance of cocultures employing I535A/V539G-expressing targets was similar to that of the No Env controls, indicating that lipid mixing is blocked by this mutation. The W(1–5)A mutant, which has been shown to block Dil transfer mediated by HIV-1HXB2 Env between cocultured cells by ~25% (27), was included as a control. In this case (Fig. 7A), small areas of dye colocalization were occasionally observed indicating a more attenuated phenotype for W(1–5)A in the context of the HIV-1AD8 strain.

Imaris Colocalization software was used to calculate the Pearsons correlation coefficient for the locations of green and red fluorescent pixels (voxels) within reconstructed z-stacks of the deconvoluted images. The Pearson coefficient was 0.021 for cocultures with control empty vector-transfected effectors (No Env), whereas 0.33 was obtained for the wild type Env-expressing cells (Fig. 7B, left panel). Pearson coefficients of 0.051 and 0.09 were obtained for I535A/V539G and W(1–5)A, respectively, indicating significant inhibition of lipid mixing activity. The specificity of the lipophilic dye transfer assay was verified using the HR2-based peptidic fusion inhibitor, C34, which blocked lipid mixing to “No Env” levels (Fig. 7B, right panel). These data indicate that the I535A/V539G clasp destabilizing mutation blocks the lipid-mixing phase.

Effect of Terminal Clasp Mutations on Cytoplasmic Dye Transfer between Effectors and Targets—To further investigate the nature of fusion defects associated with I535A/V539G, we monitored the exchange of small cytoplasmic dyes, CellTracker orange CMTMR and CellTracker green CMFDA, between cocultured Env-293T effectors and JC53 target cells, respectively, using the API Deltavision RT system. Both dyes were observed in multinucleated cells in cocultures employing wild type Env (Fig. 8A), consistent with the cytoplasmic contents mixing, whereas the dyes were separately located in distinct cells for cocultures with empty vector-transfected effectors (No
Env). The C34 fusion inhibitor completely blocked the content mixing activity of wild type Env, indicating that cytoplasmic dye transfer requires Env-mediated membrane fusion. In the case of I535A/V539G, the labeling of cocultured cells was for the most part similar to experiments with mock-transfected cells, however, small localized content mixing events were occasionally observed. The extent of dye transfer was larger in area and more frequent in cocultures employing W(1–5)A (Fig. 8A), consistent with the residual cell-cell fusion activity of this mutant (see Fig. 2A). The extent of cytoplasmic content mixing was quantitated by measuring the area occupied by green and red fluorescence within 12–15 fields at 600 magnification per cell.

FIGURE 6. Effects of I535A/V539G on chymotrypsin sensitivity in the presence and absence of the MPER. A, chymotrypsin cleavage of wild type and mutated MBP/gp41(528-L-677)AD.ST. Purified MBP/gp41 trimers were treated with chymotrypsin and analyzed as described in the legend to Fig. 4B. B, quantitation of chymotryptic peptides. The intensities of band 2 released at the various protease:protein ratios were quantified using Odyssey version 1.2 software and expressed as a percentage of the corresponding mock-treated protein. The data are mean ± S.E. from 3 independent experiments. p values: I535A/V539G versus WT, 0.013; tr665 versus WT, 0.002; tr665-I535A/V539G versus WT, 0.009 (1:20 protease:protein ratio, 2-tailed paired t test). C, identification of chymotryptic peptides by mass spectrometry. The 1:20 digests of wild type (upper panels) and I535A/V539G mutated MBP/gp41 proteins were analyzed on an Agilent 6220 Accurate Mass TOF LC/MS mass spectrometer as described for Fig. 4D.
construct. Fig. 8B indicates cytoplasmic dye exchange between wild type Env-transfected 293T cells and target cells, but not for mock, or I535A/V539G, whereas W(1–5)A exhibited an intermediate phenotype, retaining ~20% of wild type content mixing activity.

**DISCUSSION**

We find that a simultaneous I535A/V539G mutation in the polar segment/FPPR led to destabilization of the gp41 terminal clasp, as indicated by an increase in the sensitivity of this region to limited chymotrypsin proteolysis. The clasp destabilizing
Functional Role of the HIV-1 gp41 Terminal Clasp

The structural data of Buzon et al. (9) indicate that the coiled-coil extension formed by the polar segment in the gp41 trimer of hairpins expands outwards from the 3-fold axis and does not exhibit knobs into holes packing of $a$- and $d$-position residues. The terminal clasp is therefore stabilized by hydrophobic interactions between the polar segment helices and adjacent antiparallel MPER helices. Interacting side chains include Gly$^{531}$-Leu$^{679}$, Ala$^{533}$-Trp$^{670}$, Met$^{535}$ (Ile$^{535}$ in AD8)-Ile$^{675}$/Asn$^{671}$, and Thr$^{536}$/Leu$^{537}$-Trp$^{666}$. The small decreases in cell-cell fusion, entry, and clasp stability observed with I535A suggests that the potential consequences of disrupting the Ile$^{535}$-Ile$^{675}$/Asn$^{671}$ cluster, which leads to the potential loss of $\sim 377 \text{ Å}^2$ of buried hydrophobic surface, are mitigated by the remaining interactions. In contrast, Val$^{539}$ does not mediate substantial contacts suggesting that the mild V539G phenotype results from increased conformational flexibility of the backbone at Gly$^{539}$ (49, 50). When combined in I535A/V539G, these mutations may result in a less stable helical structure in the polar segment and therefore more severe disruption of the terminal clasp, resulting in complete ablation of fusion and entry function. The chymotrypsin sensitivity of tr665 that would display a “naked” polar segment due to the absence of MPER was greater than for MBP(528–L–677)AD-ST. However, this sensitivity was not increased further by I535A/V539G, suggesting that Ile$^{535}$/Val$^{539}$ play distinct structural roles before and after clasp formation. Studies with synthetic peptide derivatives of the fusion peptide–polar segment–HR1 region indicate that the helical structure is propagated into the polar segment even when HR2 and the MPER are absent (51, 52). The clasp may therefore assemble in 2 steps whereby the polar segment forms a helical extension of the coiled-coil to provide a docking surface for the MPER. Previously, we found that disruption of the other key Leu$^{537}$-Trp$^{666}$ hydrophobic interaction of the terminal clasp that buries $\sim 636 \text{ Å}^2$ of hydrophobic surface, via L537A/W666A, also led to increased chymotrypsin cleavage of the polar segment (29). However, the functional consequences of the decreased clasp stability were not clear in this case because an earlier stage of the membrane fusion cascade, namely responsiveness to CD4, was compromised by L537A/W666A. With I535A/V539G, we can now assign a function for the terminal clasp in the early lipid mixing/hemifusion phase of the membrane fusion cascade.

In contrast to I535A/V539G, the MPER mutants exhibited complex phenotypes with LFI, W(1–3)A, and W(1–5)A causing $\sim 20–30\%$ reductions in expression of Env at the cell surface and defective incorporation of gp120 into pseudovirions. Because these data are consistent with a compromised fusion glycoprotein complex, the relationship between the mutations’ adverse effects on clasp stability and membrane fusion function is difficult to infer. When immersed in lipid, the MPER forms a kinked helix in the interfacial region that lies roughly parallel to the membrane plane with Trp$^{566}$, Leu$^{669}$, Trp$^{670}$, Trp$^{672}$, Phe$^{673}$, Ile$^{675}$, Leu$^{679}$, Trp$^{678}$, Tyr$^{681}$, and Ile$^{682}$ penetrating into the hydrophobic phase (22). Evidence is accumulating to indicate that this conformation is targeted by MPER-specific mAbs such as 2F5 and 4E10 in the prefusion Env of neutralization-sensitive isolates (21, 22, 25, 26). Consistent with this structural data, a plot of the membrane interface-to-water transfer free energies ($\Delta G$) of the MPER-TMD region revealed 2 positive peaks corresponding approximately to the N- and C-terminal helical segments of the MPER (53) (see also supplemental Fig. S3A). Sáez-Cirión et al. (53) showed that the cell-cell fusion defect associated with W(1–3)A was associated with a decrease in peak 1 and an $\sim 10$-fold reduction in the affinity of an MPER peptide for the interfacial region of a model membrane. By subjecting the MPER mutants to a similar analysis, we have found that peak 1 is partially suppressed by LFI, whereas it is ablated by W(1–3)A. On the other hand, W(1–5)A ablates both peaks (supplemental Fig. S3A). A strong correlation between the average $\Delta G$ for MPER residues 663–683 and the cell-cell fusion activities of the mutants was found (supplemental Fig. 3C), suggesting that the losses in fusion activity in a cellular context were linked to the decreased capacity of the mutated MPER to partition into the membrane interfacial region in prefusion Env. We note that in contrast to our observations that W(1–3)A and W(1–5)A inhibited HIV-1Adap-Env mediated cell-cell fusion by $\sim 65$ and $80\%$, respectively, Salzwedel et al. (28) reported that both mutations completely blocked the fusion activity of Env derived from the T cell line-adapted strain, HIV-1HR2. Another point of difference between the MPERs of HIV-1Adap and HIV-1HXB2 is our finding that W(1–5)A in the former causes a uniform $\sim 80\%$ block in lipid and cytoplasmic content mixing, including reporter gene expression that requires fusion pore expansion, whereas the same mutation in HXB2 Env specifically blocks the pore expansion step (27). These data imply that the fine details of MPER function may vary in an isolate-specific manner, as is observed with other conserved Env determinants, including the receptor/coreceptor binding sites (e.g. 54–56), gp120-gp41 association site (32), and core domain sequences of gp41 that are targeted by fusion inhibitors (e.g. 57, 58). It will be interesting to examine how W(1–5)A affects the fusion cascade in a variety of primary isolates and whether these effects correlate with the mechanism of neutralization by 2F5 and 4E10.

Terminal clasp destabilization by I535A/V539G correlated with the inhibition of lipophilic dye transfer between Env-expressing cells and receptor-expressing cells, indicating a hemifusion block. These data imply that formation of the HR1-HR2 6-helix bundle core, which apposes the virus and cell membranes and in theory can release sufficient free energy to catalyze hemifusion (59), is not sufficient to initiate the fusion cascade. Rather, hemifusion appears to require extension of the HR1-HR2 6-helix bundle core through to the fusion peptide and TMD via assembly of the terminal clasp. This idea is inconsistent with an earlier proposal that the 6-helix bundle folding is completed after fusion pore formation (60). This proposal stems from the finding that cold-arrested fusion pores can be irreversibly closed by peptides targeting the gp41 prehairpin. However, this observation could be due to reversible 6-helix
bundle assembly at low temperatures where hydrophobic interactions tend to be weakened (61) as peptide-mediated pore closure did not occur when growth arrest was induced at 15 °C instead of 4 °C. Furthermore, a recombinant 6-helix bundle is able to inhibit Env-mediated cell-cell fusion and viral entry with a 90% inhibitory concentration of ~3 μM (62), consistent with a reversible 6-helix bundle fold.

A comparison of the detergent- or lipid-immersed structure of the MPER with the polar segment/FPFR-bound state within the trimer of hairpins indicates changes in both conformation and environment during membrane fusion (9, 22). For example, helical extension beyond HR2 by the 666–677 sequence will extract Trp<sup>666</sup>, Trp<sup>670</sup>, and Ile<sup>675</sup> from the bilayer hydrophobic phase enabling contacts with polar segment residues in the terminal clasp. Buzon et al. (9) also observed that the side chains of Trp<sup>678</sup>, Trp<sup>680</sup>, and Tyr<sup>681</sup> do not participate in clasp contacts but are positioned such that they remain associated with the viral envelope. These changes may cause localized alterations in envelope curvature as it becomes apposed with the cell membrane and/or allow appropriate positioning of fusion peptide and TMD sequences to enable fusion to proceed. It is therefore plausible that I535A/V539G renders the transition of the MPER membrane fusion.

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