Evidence That the Transmembrane Domain Proximal Region of the Human T-cell Leukemia Virus Type 1 Fusion Glycoprotein gp21 Has Distinct Roles in the Prefusion and Fusion-activated States‡

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To investigate the structural context of the fusion peptide region in human T-cell leukemia virus type 1 gp21, maltose-binding protein (MBP) was used as an N-terminal solubilization partner for the entire gp21 ectodomain (residues 313–445) and C-terminally truncated ectodomain fragments. The bacterial expression of the MBP/gp21 chimeras resulted in soluble trimers containing intramonomer disulfide bonds. Detergents blocked the proteolytic cleavage of fusion peptide residues in the MBP/gp21-(313–425) chimera, indicating that the fusion peptide is available for interaction with detergent despite the presence of an N-terminal MBP domain. Limited proteolysis experiments indicated that the transmembrane domain proximal sequence Thr425–Ala439 protects fusion peptide residues from chymotrypsin. MBP/gp21 chimera stability therefore depends on a functional interaction between N-terminal and transmembrane domain proximal regions in a gp21 helical hairpin structure. In addition, thermal aggregation experiments indicated that the Thr425–Ser436 sequence confers stability to the fusion peptide-containing MBP/gp21 chimeras. The functional role of the transmembrane domain proximal sequence was assessed by alanine-scanning mutagenesis of the full-length envelope glycoprotein, with 11 of 12 single alanine substitutions yielding oligomerization domain that forms the core of the gp21 trimer. The gp21 ectodomain is anchored to the viral envelope by a central coiled-coil, a disulfide-bonded loop that stabilizes a chain reversal, and an antiparallel C-terminal segment packed on the outside of the coiled-coil (12). gp21-(338–425) is similar in overall architecture to TM protein core fragments derived from other retroviruses (13–17), a filovirus (18, 19), paramyxoviruses (20), and the low pH fusogenic conformation of influenza virus (orthomyxovirus) HA2 (21, 22). The three-dimensional structures of influenza virus HA2 in the prefusion (23, 24) and low pH fusogenic states (21, 22) have illustrated the conformational changes associated with fusion activity and provide a paradigm for studying retroviral fusion.

Retroviral envelope glycoproteins (Env) are synthesized as inactive precursors that are processed in the Golgi apparatus to yield a functional hetero-oligomeric complex. The Env complex is composed of a surface-exposed subunit (SU) that mediates attachment to cellular receptors, triggering the membrane fusion activity of the noncovalently associated transmembrane (TM) protein, leading to viral entry. Human T-cell leukemia virus type 1 (HTLV-1) is a retrovirus that is associated with various diseases including adult T-cell leukemia/lymphoma, HTLV-1-associated myelopathy/tropical spastic paraparesis, uveitis, and infectious dermatitis of children (1). HTLV-1 transmission occurs mainly via fusion between infected Env-expressing cells and receptor-bearing cells, because infection by cell-free HTLV-1 is inefficient in vitro and in vivo (2–5). The receptor(s) recognized by the HTLV-1 SU-TM protein (gp46-gp21) complex are yet to be identified; however, the results of monoclonal antibody blocking studies and protein expression studies suggest that HTLV-1 fusion depends on multiple cellular factors (6–11). The ectodomain of HTLV-1 TM protein (gp21) contains an N-terminally located fusion peptide, an ~15-residue hydrophobic sequence that inserts into target cellular membranes and is critical for membrane fusion activity. The fusion peptide is connected through a glycine-rich segment to a coiled-coil forming oligomerization domain that forms the core of the gp21 trimer. The gp21 ectodomain is anchored to the viral envelope via an ~20-residue transmembrane domain (TMD) (Fig. 1). Our previous crystallographic study of a gp21 core fragment, gp21-(338–425), revealed a trimeric helical hairpin structure comprising a central coiled-coil, a disulfide-bonded loop that stabilizes a chain reversal, and an antiparallel C-terminal segment packed on the outside of the coiled-coil (12). gp21-(338–425) is similar in overall architecture to TM protein core fragments derived from other retroviruses (13–17), a filovirus (18, 19), paramyxoviruses (20), and the low pH fusogenic conformation of influenza virus (orthomyxovirus) HA2 (21, 22). The three-dimensional structures of influenza virus HA2 in the prefusion (23, 24) and low pH fusogenic states (21, 22) have illustrated the conformational changes associated with fusion activity and provide a paradigm for studying retroviral fusion.

The structural changes accompanying HA2 fusion-activation include helical extension at the N terminus of the central coiled-coil and refolding of the C-terminal portion of the ectodo-

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main such that TMD proximal sequences pack on the outside of the coiled-coil in a hairpin conformation. This helical hairpin architecture implies that the TMD is close to the N terminus of fusion-activated TM proteins. The currently available three-dimensional structures of viral TM proteins lack the N-terminal fusion peptide region.

A striking feature of the low pH-induced HA2 conformational change is a loop-to-helix transition that extends the N terminus of the central coiled-coil by ~100 Å (21, 25). This helical extension would translocate the fusion peptide from the HA2 core to the tip of the helical hairpin rod for membrane insertion. The location of fusion peptides in prefusogenic retroviral Env is not known; however, in the helical hairpin they are also likely to be displayed at the coiled-coil N terminus. An early stage of membrane fusion is thought to involve the insertion of fusion peptides into the outer leaflet of the target membrane (26, 27). Spectroscopic studies of the interactions between water-soluble model HA2 fusion peptides and hydrated lipid bilayers indicated that membrane insertion correlates with a change in fusion peptide structure from random coil to cated that membrane insertion correlates with a change in energy to help destabilize the closely apposed viral and target membranes for fusion (22, 39).

The oblique insertion of fusion peptides into membranes is due to extend away from the 3-fold symmetry axis (22, 36). The monomeric insertion of fusion peptides into membranes and the oblique mode of insertion, and insertion depths have also been observed for a trimeric N-terminal HA2 fragment, where 10 of the HA2 fusion peptide insert at an oblique angle (32). Similar a-helical content, oblique mode of insertion, and insertion depths have been observed with retroviral fusion peptides suggesting a conserved mechanism of membrane insertion (28, 29, 33–35). The monomeric insertion of fusion peptides into membranes may be facilitated by N-capping structures that terminate the HA2 and gp21 central coiled-coils, directing N-terminal residues to extend away from the 3-fold symmetry axis (22, 36). The oblique insertion of fusion peptides into membranes is considered to expand the center of the target bilayer, introducing negative curvature strain and instability to the site of insertion (37, 38). Refolding of the ectodomain into a hairpin then presumably draws the TMDs and associated viral envelope toward the site of fusion peptide insertion in the target membrane. The refolding process is considered to provide free energy to help destabilize the closely apposed viral and target membranes for fusion (22, 39–42).

To further understand the structural context of the HTLV-1 gp21 fusion peptide region, we biochemically characterized MBP/gp21 ectodomain chimeras containing the fusion peptide. The bacterially expressed chimeras form soluble trimers with intact intramonomer disulfide bonds. Limited proteolysis and thermal aggregation experiments indicated that the gp21 TMD proximal sequence (Thr425–Ala439) protects fusion peptide residues from proteolysis and confers stability to the fusion peptide-containing MBP/gp21 chimeras. Alanine-scanning mutagenesis of the Thr425–Ser436 sequence in HTLV-1 Env resulted in enhanced fusion activity in 11 of 12 alanine mutants. The enhanced fusogenicity of Env mutants is likely due to effects on prefusion Env because alanine substitutions did not significantly affect MBP/gp21 chimera stability. The function of the TMD proximal sequence in the prefusion Env complex may be distinct from its role in stabilizing the fusion peptide and adjacent glycine-rich region in the fusion-activated gp21 helical hairpin.

**Experimental Procedures**

MBP/gp21 Escherichia coli Expression Vectors—A modified version of the MBP/gp21 expression vector pMBPL−gp21(338–425) was used for expression of fusion peptide-containing MBP/gp21 chimeras. The PvuII site that links MBP and gp21 moieties was replaced with a unique Ndel site. PCR was used to generate a series of gp21 ectodomain fragments encoding the gp21 amino acids Ala313 to Asn329, Thr425, Asn330, Leu333, Ser336, Ala339, or Thr445 using pCEL1.3 as the template (36). The forward primer, 5′-CCCGCGCGCCGCCCGGTTGCGGTC, incorporates the N-terminal gp21 amino acid, Ala313 (underscored and labeled with an asterisk). Residues that were substituted with alanine in HTLV-1 Env are indicated by underlined residue numbers. The three major chymotrypsin C-tail sites are indicated by an arrow and labeled Chy. Residues that were substituted with alanine in HTLV-1 Env are indicated by asterisks.

Expression and Purification of MBP/gp21 Chimeras—MBP/gp21 chimera expression was induced in E. coli strain BL21(DE3) cells by the addition of 0.2 mM isopropyl-β-D-thiogalactopyranoside and purified as described previously (43). MBP/gp21 trimers were isolated by Superdex 200 (HiLoad 26/60) gel filtration chromatography (Amersham Biosciences) in S-buffer (43). Gel filtration experiments were calibrated with thyroglobulin (669 kDa), ferritin (440 kDa), ovalbumin (43 kDa) (Amersham Biosciences), and MBP/gp21-(338–425) trimer (151 kDa) (12, 43); blue dextran was used to determine the void volume (V0). The purity and stability of MBP/gp21 trimers were monitored by reducing SDS-PAGE and analytical Superdex 200 (PC3.2/30) gel filtration chromatography.

Limited Proteolysis of MBP/gp21 Chimeras—MBP/gp21 trimers at 0.5 mg/ml in 10 mM Tris (pH 8.5), 30 mM sodium chloride, and 0.1 mM EDTA (250 μg of protein) were proteolyzed with sequencing grade chymotrypsin (Roche Molecular Biochemicals) using a 1:150 ratio of protease to protein (w/w). Proteolysis was performed for 1, 5, 10, 30, and 60 min at 37 °C. Aliquots were taken at each time point and quenched by the addition of 0.1% trifluoroacetic acid (v/v) for mass spectrometry (25 μg of protein) or quenched by boiling for 5 min in SDS-PAGE sample buffer containing 1% β-mercaptoethanol for SDS-PAGE in 10–17% gradient gels (5 μg of protein). For detergent binding assays, MBP/gp21-(313–425) trimers (0.5 mg/ml) were preincubated for 16 h at room temperature.
temperature with high purity detergents at critical micellar concentrations, prior to digestion with chymotrypsin (1:100 protease/protein, w/w, 5 min at room temperature). The detergents tested included non-ethanlyl glycerol monoo-n-dodecyl ether (C12E8), octaethylene glycol monoo-n-dodecyl ether (C12E10), octoethylene glycol monoo-n-dodecyl ether (C12E10), N,N,N′-tri(o-chlorophenyl) decyl ether (C12E10), n-decyl maltoside (CYMAL-5), n-nonyl o-glucoside, n-octanoylsucrose, and cyclohexylpropyl β-o-maltoside (CYMAL-3) (Hampton Research, Laguna Niguel, CA). Chymotrypsin activity in the presence of detergents was monitored with the chymotrypsin substrate Suc-Ala-Ala-Pro- Phe-pNA (BACHEM, Bubendorf, Switzerland) by photochemical detection of pTMB at release at 405 nm.

Assessment of Thermostability of MBP/gp21 Chimeras—The purified MBP/gp21 trimers or chymotrypsin-treated MBP/gp21 samples were desalted and concentrated by precipitation in methanol/chloroform and infused directly into a PE Scie PI + mass spectrometer in 15% (v/v) acetic acid, 50% (v/v) acetonitrile using a nanoelectrospray ion source (44). The ion spectrum was visualized with Tune 2.5-FPU software and deconvoluted using the Hypermass facility in MacSpec 3.3 (PE Sciei). The redox state of cysteine residues was identified by mass spectrometry analysis of chimeras treated with the alkylating agent 4-vinylpyridine (4-VP, Sigma) as described previously (43).

Cell Lines and Viruses—293T and HeLa cells were maintained in Dulbecco’s modification of minimal essential medium with 10% fetal calf serum and transfected using Fugene-6 according to the manufacturer’s specifications (Roche Molecular Biochemicals). The recombinant vaccinia virus vTF7.3, which drives expression of bacteriophage T7 polymerase, was obtained from T. M. Fuerst and B. Moss (45). vaccinia virus vTF7.3, which drives expression of bacteriophage T7 polymerase, was obtained from T. M. Fuerst and B. Moss (45).

Mammalian Expression Vectors and pMBP/gp21—(313–439) Alanine Substitution Mutants—The vector pCEL7.1 directs cytomegalovirus promoter-driven expression of HTLV-1 Env, C-terminally tagged with the monoclonal antibody (mAb) C8 epitope (36, 43, 46), and is a modification of pCMV-ENV (47). The alanine substitution mutants T425A, G426A, W427A, G428A, L429A, N430A, W431A, D432A, L433A, G434A, L435A, and S436A were introduced into a Kpn1-Nost pCEL7.1 fragment (env nucleotides 939–1383) by PCR mutagenesis. The sequences of pCEL7.1 mutants were confirmed by the ABI Prism BigDye terminator system (Applied Biosystems, Foster City, CA). The pTM luc effector was cotransfected with pTM luc and wild-type or mutated pCEL7.1 vectors. In parallel, HeLa target cells were infected with vTF7.3 at a multiplicity of infection of 1 plaque-forming unit per cell. At 16-h postinfection, HeLa cells were resuspended in phosphate-buffered saline containing 50 μM EDTA, washed twice in complete medium, and cocultured with transfected 293T cells for a further 12 h in the presence of 1 μg/ml of actinomycin D and 40 μg/ml of cytosine arabinoside at 37°C. Cells were then lysed and assayed for luciferase activity using the Promega (Madison, WI) luciferase assay system.

RESULTS

E. coli Expression and Purification of Trimeric MBP/gp21 Chimeras Containing the Fusion Peptide—Previously we found that expression in E. coli of a chimera comprising MBP linked to the gp21(338–425) ectodomain fragment lacking the fusion peptide resulted in high yields of soluble trimer suitable for x-ray crystallographic studies (12, 43). We therefore examined the utility of the MBP expression system for production of gp21 ectodomain fragments containing the fusion peptide and the adjacent glycine-rich segment (Fig. 1). A series of mbp/gp21 chimeras were generated comprising MBP linked via NAA to the gp21 N-terminal residue, Ala127. The chimeras were terminated at Thr445, Asn446, Leu448, Ser449, Ala450, or Thr445; Thr445 is predicted to be the last residue of the gp21 ectodomain (Fig. 1). The chimeras were expressed in E. coli and MBP/gp21 trimers purified by amylose-agarose affinity chromatography and Superdex 200 gel filtration. The purified chimeras coelute with trimeric MBP/gp21(338–425) in analytical Superdex 200 gel filtration experiments (Fig. 2A). Therefore, the tendency of hydroporphic fusion peptides to cause precipitation or aggregation of the TM protein ectodomain, as has been observed for other viral TM proteins (49), is mitigated by the N-terminal MBP moiety.

The gp21 ectodomain contains a conserved disulfide-bonded loop formed by Cys439–Cys468, whereas Cys403 is unpaired (Fig. 1) (12). The disulfide-bonded loop is associated with a chain-reversing structure at the base of the helical hairpin and is a key determinant of Env fusogenicity (36). We therefore assessed the redox state of MBP/gp21 chimeras by treatment with the alkylating agent 4-VP followed by electrospray mass spectrometry to detect the covalent modification of free sulfhydryls (43). The molecular mass of 4-VP-treated chimeras increased by −105 Da (4-VP, M, 105) and is consistent with the presence of a single free cysteine (Fig. 2B). The molecular mass of chimeras increased by −315 Da when pretreated with the reducing agent dithiothreitol prior to alkylation, confirming the presence of three cysteine residues per monomer (data not shown).

The gp21 TMD Proximal Residues, Gly426 to Ala439, Protect the Fusion Peptide Region of MBP/gp21 Chimeras against Limited Proteolysis with Chymotrypsin—The purified chimeras, with the exception of MBP/gp21(313–425), were stable following storage for 4 weeks at 4°C. SDS-PAGE revealed proteolysis of the shortest chimera, MBP/gp21(313–425), suggesting that the TMD proximal residues Gly426-Thr445 are required for chimera stability. We therefore assessed the role of

Mass Spectrometry—Purified MBP/gp21 trimers or chymotrypsin-treated MBP/gp21 samples were desalted and concentrated by precipitation in methanol/chloroform and infused directly into a PE Scie PI + mass spectrometer in 15% (v/v) acetic acid, 50% (v/v) acetonitrile using a nanoelectrospray ion source (44). The ion spectrum was visualized with Tune 2.5-FPU software and deconvoluted using the Hypermass facility in MacSpec 3.3 (PE Sciei). The redox state of cysteine residues was identified by mass spectrometry analysis of chimeras treated with the alkylating agent 4-vinylpyridine (4-VP, Sigma) as described previously (43).

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Antibodies—mAb C8, directed to the HIV-1 gp41 cytoplasmic domain, was obtained from G. Lewis (46), and mAb 46, directed against HTLV-1 gp46, was a gift from David Tribe (The University of Melbourne, Victoria, Australia). Immunoglobulin G was purified from the plasma of an HTLV-1-infected individual (anti-HTLV-1) using protein A-Sepharose (Amersham Biosciences).

Western Blotting—At 24 h posttransfection, Env-expressing 293T cells were lysed for 10 min on ice in phosphate-buffered saline containing 1% Triton X-100, 0.02% sodium azide, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 mM dithiothreitol. Lysates were clarified by centrifugation at 10,000 × g at 4°C prior to SDS-PAGE in 12% polyacrylamide gels under reducing conditions. Proteins were transferred to nitrocellulose prior to Western blotting with mAb C8. Immunoblotting was carried out using the chemiluminescence blotting substrate procedure (Roche Molecular Biochemicals).

Bio synthetic Labeling and Immunoprecipitation—At 16 h posttransfection, 293T cells were incubated for 30 min at 37°C in cysteine- and methionine-deficient medium (ICN, Costa Mesa, CA) and then labeled with 120 μCi of [35S]methionine (ICN) per well for 24 h. Immunoprecipi-
Structure-Function Analysis of the HTLV-1 gp21 Ectodomain

**Fig. 2.** MBP/gp21 chimeras containing the fusion peptide form stable trimers containing intramonomer disulfide bonds. A, analytical Superdex 200 gel filtration shows that fusion peptide-containing MBP/gp21 chimeras coelute with the 151-kDa MBP/gp21-(338–425) trimer (12, 43). The peak elution times of gel filtration calibration markers, blue dextran (V), thyroglobulin (669 kDa), ferritin (440 kDa), ovalbumin (43 kDa), and MBP/gp21-(338–425) trimer (151 kDa) (12, 43) are marked. B, treatment of MBP/gp21 chimeras with 50 mM 4-VP results in the addition of ~105 mass units, consistent with the alklylation of one Cys residue. Listed are the relative molecular weights (Mₐ) of each MBP/gp21 chimera and the molecular masses of untreated (mᵤᵤ) and 4-VP alkylated (mᵥᵥ) MBP/gp21 chimeras as determined by electrospray mass spectrometry.

The gp21 TMD proximal residues in chimera stability by subjecting freshly purified MBP/gp21 trimers to limited proteolysis with chymotrypsin. Chymotrypsin cleaves on the C-terminal side of the amino acids Tyr, Phe, and Trp and to a lesser extent Leu, Met, Ala, Asp, and Glu. The gp21 ectodomain contains 55 chymotrypsin targets with 22 target residues in the N- and C-terminal regions for which there is no available three-dimensional structure (Fig. 1). MBP/gp21 trimers (250 μg, 0.5 mg/ml) were digested at 37 °C with limiting amounts of chymotrypsin (1:150 ratio of protease/protein (w/w)) for 1 to 60 min, and the digestion patterns were analyzed by SDS-PAGE. Fig. 3A reveals that protease resistance correlated with chimera length. The shortest construct, MBP/gp21-(313–425), was the most sensitive to chymotrypsin with >50% cleavage occurring after 1 min and almost 100% cleavage after 5 min. Incremental increases in chimera stability were seen with gp21 C-terminal extensions. Approximately 50% of MBP/gp21-(313–430) was cleaved after 10 min and almost 100% cleaved after 60 min. MBP/gp21-(313–433) and MBP/gp21-(313–436) required 60 min for ~50% cleavage, whereas only ~10% of MBP/gp21-(313–439) was cleaved after 60 min.

Electrospray mass spectrometry of the proteolyzed chimeras identified three major sites of cysteine cleavage within the gp21 moiety. The mass spectrometry profile of MBP/gp21-(313–430) after digestion with chymotrypsin for 30 min illustrates the pattern of proteolysis (Fig. 3B). The 53,130-Da species corresponds to undigested MBP/gp21-(313–430) (Mₐ 53,126), whereas the 41,160-Da species corresponds to a protease-resistant fragment, MBP/gp21-(313–319) (MBP/gp21(313–319), Mₐ 41,160), indicating a cleavage event at gp21 residue Trp³¹⁹. The 11,218-Da species corresponds to the protease-resistant gp21 fragment, gp21-[325–427] (gp21(325–427), Mₐ 11,216), consistent with secondary cleavage at Leu⁴²⁴ and Trp⁴³⁹, a transient 11,979-Da fragment corresponding to gp21-[320–430] (Mₐ 11,983) was detected at earlier time points. The C-terminally truncated chimera MBP/gp21-(313–427) was not detected, therefore it follows that cleavage at Trp³¹⁹ (Fig. 1, Chy1) is required for subsequent cleavage at Leu³²⁴ and Trp⁴³⁹ (Fig. 1, Chy2). Mass spectrometry analysis of the other chimeras at various time points revealed the same sites of chymotrypsin cleavage, giving rise to the protease-resistant gp21 core, gp21-[325–427] or gp21-[325–425] from the shortest chimera, MBP/gp21-(313–425) (see supplementary Table I). In the context of a MBP/gp21 chimera containing the fusion peptide, the gp21 TMD proximal residues 431–439 are required for protection of the gp21 fusion peptide region from proteolysis. Simultaneously, fusion peptide residues 313–319 are required for the protection of the TMD proximal residues 427–439 against proteolysis.

The gp21 TMD Proximal Residues, Gly⁴²⁶ to Ser⁴³⁶, Confer Thermostability to MBP/gp21 Chimeras Containing the Fusion Peptide—To confirm the role of the TMD proximal sequence in stabilization of fusion peptide-containing MBP/gp21 chimeras, we devised a thermostability assay based on the temperature-dependent conversion of MBP/gp21 trimers to high molecular weight aggregates as monitored by Superdex 200 gel filtration. The thermal aggregation assay distinguishes gp21 unfolding from MBP domain unfolding; the monomeric structure of MBP is not affected by treatment at 60 °C, 5 min, and limited proteolysis of heat-induced (50 °C, 5 min) MBP/gp21-(313–436) aggregate releases intact monomeric MBP, whereas the gp21 fragment is degraded (data not shown). MBP/gp21 trimers (~100 μg, 2 mg/ml in 50 mM sodium chloride, 50 mM glycine, pH 8.3) were subjected to heat treatment over a temperature range of 37–50 °C for 5 min. The Superdex 200 profiles of the MBP/gp21 chimeras, before and after heat treatment at 46 °C (Fig. 4A), illustrate incremental increases in resistance to thermal aggregation with extension of the gp21 chimeras from Thr³⁴⁵ to Asn³⁴⁰, Leu³⁴³, and Ser³⁴⁶. Chimeras terminated at
Ser^{436}, Ala^{439} (Fig. 4A), and Thr^{445} (data not shown) were the most stable with comparable thermostability values. This trend in chimera thermostability was consistent over the temperature range 37–52 °C (data not shown) and is reflected in the chimera $T_{\text{MAX,TRI}}$ (maximum temperature at which >95% trimeric structure was maintained) and $T_{\text{MIN,AGG}}$ (the minimum temperature required to convert >95% of trimer to soluble aggregate) (Fig. 4B). These results indicate that the presence of the fusion peptide and glycine-rich segment (residues 313–337) has a destabilizing influence on the shorter TMD proximal residues protect N-terminal gp21 residues against limited proteolysis with chymotrypsin.

The Detergents n-Octanoyl Sucrose and CYMAL-3 Protect the Fusion Peptide Region against Limited Proteolysis with Chymotrypsin—Previous studies have shown that synthetic peptide analogs of the HIV-1 gp41 and influenza virus HA fusion peptides can insert into detergent micelles (28, 31, 50). We therefore tested the ability of the fusion peptide in MBP/gp21-(313–425) to interact with detergents by determining whether various detergents can protect the fusion peptide residues Trp^{319} and Leu^{324} from chymotrypsin proteolysis. SDS-PAGE indicated that chymotrypsin treatment for 5 min at room temperature of MBP/gp21-(313–425) led to substantial amounts of MBP/gp21_{FRAG} and gp21_{CORE} (Fig. 5, Chy). A 16-h preincubation of MBP/gp21-(313–425) with the detergents C_{12}E_{8}, C_{12}E_{9}, and Decoy BigChap at critical micromolar concentrations, prior to chymotrypsin treatment, had no effect on the digestion profile (Fig. 5, D1, D2, and D3). In contrast, preincubation with n-octanoylsucrose and CYMAL-3 led to the protection of MBP/gp21-(313–425) from chymotrypsin cleavage (Fig. 5, D7 and D8), the protected MBP/gp21 protein migrating as a single ~53-kDa band corresponding to untreated MBP/gp21-(313–425) (Fig. 5, Unt). Other detergents, n-decyl-β-D-maltoside, CYMAL-5, and n-nonyl-β-D-glucoside were partially protective (Fig. 5, D4, D5, and D6), although n-nonyl-β-D-glucoside promoted the degradation of MBP/gp21_{FRAG} and gp21_{CORE}. The detergents did not affect chymotrypsin activity as assessed using the chromogenic substrate Suc-Ala-Ala-Pro-Phe-pNA under identical digestion conditions (data not shown). We also tested the effects of detergents on the proteolysis of more stable MBP/gp21 chimeras, which require an incubation temperature of 37 °C for significant cleavage. However, digestion at 37 °C in the presence of detergents promoted the degradation of MBP and gp21 domains, probably caused by the denaturing effects of heat plus detergent. These results indicate that the fusion peptide in MBP/gp21-(313–425) can interact with n-octanoylsucrose and CYMAL-3 preventing proteolysis at fusion peptide residues Trp^{319} and Leu^{324}.

Alanine Substitutions in the gp21 TMD Proximal Ectodomain Sequence Thr^{425}-Ser^{436} Result in Enhanced Cell-Cell Fusion Activity of HTLV-1 Env—The biochemical characterization of fusion peptide-containing MBP/gp21 chimeras indicated that residues Thr^{425}-Ser^{436} were important for stability. To determine whether these residues were also important for Env function, we performed alanine-scanning mutagenesis of the Thr^{425}-Ser^{436} sequence in full-length HTLV-1 Env and tested the effects of the mutations on gp62 precursor processing, gp46-gp21 association, cell surface expression, and cell-cell fusion activity.

The HTLV-1 Env precursor (gp62) is cleaved in the Golgi apparatus to yield gp46 and gp21, which remain noncovalently

**Fig. 3. TMD proximal residues protect N-terminal gp21 residues against limited proteolysis with chymotrypsin.** A, SDS-PAGE of purified trimeric MBP/gp21-(313–425) (a), MBP/gp21-(313–430) (b), MBP/gp21-(313–433) (c), MBP/gp21-(313–436) (d), and MBP/gp21-(313–439) (e) after limited proteolysis with chymotrypsin (1:150 ratio of protease/protein (w/w)) for 0, 1, 5, 10, 30, or 60 min at 37 °C. The digestion products were visualized following staining of gels with Coomassie Brilliant Blue. Full-length MBP/gp21 and the proteolytic products, MBP/gp21_{FRAG} and gp21_{CORE}, are labeled. Asterisks indicate chymotrypsin (25 kDa) and contaminating *E. coli* histone-like protein-1 (15.6 kDa). This figure was prepared using Adobe Photoshop 6.0 software. B, electrospray mass spectrometry of MBP/gp21-(313–430) digested with chymotrypsin for 30 min at 37 °C. The left panel illustrates an 11,218-Da species corresponding to the protease-resistant core, gp21-(325–427). The right panel illustrates a 53,130-Da species corresponding to MBP/gp21-(313–390).
The effects of heat treatment on the trimeric structures of the core domain of HTLV-1 Env proteins were determined (46). Freshly purified MBP/gp21-(313–425) trimer was preincubated for 16 h at room temperature with various detergents at critical micellar concentrations prior to digestion with chymotrypsin. Digestion in the absence of detergent (Chy); digestion in the presence of detergents: C₁₀E₅ (D₁), C₁₀E₆ (D₂), Deoxy BigChap (D₃), n-decyl-β-D-maltoside (D₄), CYMAL-5 (D₅), n-nonyl-β-D-glucoside (D₆), n-octanoylsucrose (D₇), CYMAL-3 (D₈); or undigested control (Unt). Full-length MBP/gp21-(313–425), MBP/gp21FRAG (MBP/gp21-(313–319)), and gp21CORE (gp21-(325–425)) are labeled. The sizes of protein molecular mass markers (kDa) are indicated at the left. The asterisks indicate chymotrypsin (25 kDa) and contaminating E. coli histone-like protein-1 (15.6 kDa). The figure was prepared from a single gel using Adobe Photoshop 6.0.

**FIG. 5.** Detergents protect the fusion peptide region in MBP/gp21-(313–425) from chymotrypsin proteolysis. Freshly purified MBP/gp21-(313–425) trimer was preincubated for 16 h at room temperature with various detergents at critical micellar concentrations prior to digestion with chymotrypsin (1:100 protease/protein (w/w), 5 min at room temperature). Digestion was monitored by SDS-PAGE in 10–17% gradient gels and Coomassie Brilliant Blue staining. Digestion in the absence of detergent (Chy); digestion in the presence of detergents: C₁₀E₅ (D₁), C₁₀E₆ (D₂), Deoxy BigChap (D₃), n-decyl-β-D-maltoside (D₄), CYMAL-5 (D₅), n-nonyl-β-D-glucoside (D₆), n-octanoylsucrose (D₇), CYMAL-3 (D₈); or undigested control (Unt). Full-length MBP/gp21-(313–425), MBP/gp21FRAG (MBP/gp21-(313–319)), and gp21CORE (gp21-(325–425)) are labeled. The sizes of protein molecular mass markers (kDa) are indicated at the left. The asterisks indicate chymotrypsin (25 kDa) and contaminating E. coli histone-like protein-1 (15.6 kDa). The figure was prepared from a single gel using Adobe Photoshop 6.0.

**FIG. 4.** Thermal aggregation-gel filtration analysis of fusion peptide-containing MBP/gp21 chimeras. The elution profiles of untreated (dashed line), and heat-treated (solid line) MBP/gp21 chimeras are shown. The elution times of gel filtration calibration markers (669 kDa), ferritin (440 kDa), MBP/gp21-(338–425) trimer (151 kDa) (12, 43), and ovalbumin (45 kDa) are marked. B, summary of thermal aggregation-gel filtration data. The maximum temperature at which >95% trimeric structure was maintained (TMAX.TRI) and the minimum temperature required for conversion of trimers to >95% soluble aggregates (TMIN.AGG) are listed for each chimera.

associated (51). The effect of the Ala substitutions on gp62 synthesis and processing to gp21 was assessed by Western blotting with mAb C8, which is directed to an epitope tag joined to the gp21 C terminus. The 12 point mutants were expressed and processed to yield gp21 at levels comparable with wild type (Fig. 6A) indicating intracellular translocation of cleavage-competent Env structures. We next determined whether the gp21 mutants had retained the ability to anchor the SU (gp46). Transfected 293T cells were metabolically labeled with [35S]Cys, and HTLV-1 Env proteins were immunoprecipitated from cell lysates and clarified culture supernatants with mAb 46, which is directed to gp46. We also used the control mAb C8 to help distinguish gp46 in cell lysate immunoprecipitations. Both gp62 and gp46 were immunoprecipitated by mAb 46 from lysates of wild-type and mutant Env-expressing cells, whereas mAb C8 immunoprecipitated gp62 but not gp46 (Fig. 6B). Only gp46 was obtained from corresponding clarified culture supernatants (Fig. 6C). Similar levels of cell-associated and shed gp46 were observed for wild-type and mutant Env indicating that the mutations had not significantly affected the gp46-anchoring ability of gp21. We verified that the Env mutants were expressed at the cell surface using a surface binding assay employing 125I-labeled anti-HTLV. The levels of cell surface expression of five mutant Env glycoproteins (T425A, G426A, G428A, L429A, and S436A) were comparable with wild-type surface expression (Fig. 7). Seven of the mutants (W427A, N430A, W431A, D432A, L433A, G434A, and S436A) exhibited slightly elevated cell surface expression at less than 1.4 times the wild-type level. These results are consistent with the normal maturation of Env mutants.

Finally, the cell-cell fusion activity of wild-type and mutated HTLV-1 Env glycoproteins was determined using a luciferase reporter assay employing HeLa cells as fusion targets. Fig. 7 shows that following a 12-h coculture between Env-expressing 293T cells and vTF7.3-infected HeLa targets 11 of the 12 mutants exhibited enhanced fusion activity. The increases in fusion activity ranged from 150–200% for T425A, G426A, G428A, L429A, and S436A; 300–350% for T425A, G428A, L429A, N430A, and L433A and 400–450% for W431A and L435A. Only one mutant, D432A, exhibited an ~25% reduction in fusion activity. These results indicate that Ala substitutions in the
TMD proximal sequence did not affect Env maturation but led to significant enhancement of fusion activity for 11 of 12 mutants.

Alanine Substitutions Do Not Affect MBP/gp21-(313–439) Chimera Stability—To determine whether the enhanced fusion activities associated with Ala substitutions were related to changes in gp21 helical hairpin stability, we introduced T425A, G428A, W431A, D432A, and L433A substitutions into the MBP/gp21-(313–439) chimera. As observed for the wild-type MBP/gp21-(313–439) chimera, the alanine mutants acquired trimeric structures (Fig. 8A) with an intact disulfide bond in each monomer (data not shown). Thermal aggregation analysis indicated that single alanine mutations had no significant effect on chimera stability. The gel filtration profiles of MBP/gp21-(313–439) mutants treated at 48 °C were comparable with 48 °C-treated wild-type chimera (Fig. 8A). The stability of wild-type and mutant chimeras was also comparable over the temperature range 37–51 °C (data not shown). All MBP/gp21-(313–439) chimeras had a T MAX.TRI of 45 °C, and 4 of 5 mutated chimeras showed a wild-type T MIN.AGG of 50 °C;
T425A exhibited a TMIN.AGG of 51 °C (Fig. 8B). The comparable stability of wild-type and mutated chimeras was also reflected in limited proteolysis experiments using chymotrypsin (data not shown). These results indicate that multiple residues within the TMD proximal and fusion peptide/glycine-rich regions contribute to MBP/gp21-(313–439) stability, thereby overriding the potential effects of single residue substitutions. The enhanced fusion activities of HTLV-1 Env mutants are likely to be a result of subtle alterations to the prefusion Env complex.

**DISCUSSION**

Previously, we used MBP as an N-terminal solubilization partner to aid the bacterial expression, crystallization, and structure determination of a trimeric HTLV-1 gp21-(338–425) ectodomain fragment lacking the fusion peptide region (residues 313–337) and TMD proximal sequence (residues 422–445) (12, 43). These studies indicated that the gp21 fragment had acquired a helical hairpin conformation resembling the low pH-induced fusogenic form of influenza virus HA2 (12, 21, 22). We now show that the MBP expression system also confers solubility to fusion peptide-containing gp21 ectodomain constructs, enabling purification as soluble trimeric MBP/gp21 chimeras. Therefore, the MBP expression system provides a new means of expressing an entire viral TM protein ectodomain in addition to a previously described method employing the highly polar FLAG peptide as an N-terminal solubilization partner for influenza virus HA2 (49).

Thermal aggregation experiments illustrated the destabilizing influence of the fusion peptide region on MBP/gp21, because the MBP/gp21-(313–425) chimera was significantly less stable than MBP/gp21-(338–425). The results of studies with hydrated model fusion peptides indicate that they are in a disordered, high entropy state, but adopt ordered a-helical structures when inserted in detergent micelles or lipid bilayers (29–31, 50). The instability of the fusion peptide-containing chimera, MBP/gp21-(313–425), may be due in part to exposure of fusion peptide residues to aqueous solvent leading to disorder in this region. This idea is supported by the observation that fusion peptide residues Trp319 and Leu324 in MBP/gp21-(313–425) were rapidly hydrolyzed by chymotrypsin. However, the Trp319 and Leu324 sites were not hydrolyzed in the presence of the detergents n-octanoyl sucrose and CYMAL-3, indicating that the fusion peptide within MBP/gp21-(313–425) becomes inaccessible to chymotrypsin when it is in a detergent-bound state.

Inclusion of the TMD proximal sequence Gly426–Ala439 also confers stability to fusion peptide-containing MBP/gp21 chimeras. Incremental increases in chimera thermostability and resistance to chymotrypsin cleavage were observed with extension of the gp21 ectodomain C terminus from Thr425 to Asn430, Leu433, Ser436, or Ala439. Furthermore, initial cleavage at Trp319 within the fusion peptide is required for cleavage at the TMD proximal residue Trp427, indicating that an intact gp21 N-terminal region confers stability to the TMD proximal region. Inclusion of TMD proximal residues beyond Thr425 may enable contacts to form with the exterior of the gp21 N-cap that terminates the coiled-coil and with residues of the glycine-rich segment thereby imposing structural order to this region and improving the overall stability of chimeras. Consistent with this idea was the finding that the protease-resistant core, gp21-(325–427), retained an intact glycine-rich segment (325–337) and a portion of the TMD proximal sequence (419–427) despite
the presence of potential internal chymotrypsin sites (Ala325, Met326, Ala328, Ala331, Leu341, Glu342, and Leu343). In functional terms, interactions between N and C termini in the gp21 helical hairpin may result in a stable structure at the membrane-inserted end of the hairpin, its formation contributing free energy to help drive membrane fusion (22). Alternatively, the TMD proximal sequence may bind to another region of gp21 contributing to helical hairpin stability via an allosteric mechanism.

In contrast to the glycine-rich segment, residues Trp319 and Leu324 of the fusion peptide were targets for chymotrypsin even in the most stable chimera, MBP/gp21(313–439), after extended incubations with protease. This observation is consistent with a theoretical requirement that the fusion peptide in the fusion-activated gp21 helical hairpin does not mediate contacts in order that it is free to insert into a target membrane. Our results indicate that the glycine-rich segment is stabilized by contacts with TMD proximal residues in the helical hairpin; however, transient flexibility in the glycine-rich segment may be favorable at the early pre-hairpin stages of gp21 refolding induced by SU receptor binding. A flexible glycine-rich linker may decouple unstable transiently hydrated fusion peptides from the N-cap of the coiled-coil, thereby maintaining a stable core while allowing the fusion peptide to attain a favorable membrane-inserted conformation. This scenario is supported by the NMR structure of the detergent-associated HIV-1 gp41 N-terminal region, where fusion peptide residues 8–14 adopt α-helical structure and are linked through a flexible segment (residues 15–23) to the coiled-coil (29).

Alanine-scanning mutagenesis of the Thr345–Ser346 sequence in full-length HTLV-1 Env led unexpectedly to an α-helical hairpin, its formation contributing free energy to help drive membrane fusion (22). Alternatively, the TMD proximal sequence may bind to another region of gp21 contributing to helical hairpin stability via an allosteric mechanism.

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Evidence That the Transmembrane Domain Proximal Region of the Human T-cell Leukemia Virus Type 1 Fusion Glycoprotein gp21 Has Distinct Roles in the Prefusion and Fusion-activated States

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