The Conserved Glycine-Rich Segment Linking the N-Terminal Fusion Peptide to the Coiled Coil of Human T-Cell Leukemia Virus Type 1 Transmembrane Glycoprotein gp21 Is a Determinant of Membrane Fusion Function

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Retroviral transmembrane proteins (TMs) contain an N-terminal fusion peptide that initiates virus-cell membrane fusion. The fusion peptide is linked to the coiled-coil core through a conserved sequence that is often rich in glycines. We investigated the functional role of the glycine-rich segment, Met-326 to Ser-337, of the human T-cell leukemia virus type 1 (HTLV-1) gp21, by alanine and proline scanning mutagenesis. Alanine substitution for the hydrophobic residue Ile-334 caused an ~90% reduction in cell-cell fusion activity without detectable effects on the lipid-mixing and pore formation phases of fusion. Alanine substitutions at other positions had smaller effects (Gly-329, Val-330, and Gly-332) or no effect on fusion function. Proline substitution for glycine residues inhibited cell-cell fusion function with position-dependent effects on the three phases of fusion. Retroviral glycoprotein fusion function thus appears to require flexibility within the glycine-rich segment and hydrophobic contacts mediated by this segment.

The envelope glycoprotein complex (Env) of retroviruses comprises a trimer of surface-exposed glycoproteins (SU), which mediates receptor binding, and a trimer of transmembrane glycoproteins (TMs), which mediates membrane fusion (8). Retroviral TMs are class I fusion proteins (13, 18, 21, 22, 47), and their fusion activity depends on two membrane-interactive sequences: the N-terminal fusion peptide, which inserts into the target cell membrane (6, 17, 53), and the C-terminally located transmembrane domain (TMD) that anchors the glycoprotein complex to the viral envelope and infected cell membrane (2, 30, 37, 42, 50). For retroviruses, the interaction between SU and receptor triggers the refolding of TM into a fusion-active state (14, 20, 27, 36). The N-terminal extension of the central triple-stranded coiled coil of the ectodomain is considered to project the adjacent fusion peptide toward the target membrane for insertion. The C-terminal helical segment then associates with the external grooves of the coiled coil in an antiparallel manner, producing the “trimer of helical hairpins” conformation. The trimer of hairpins formation is considered to draw the TMDs and associated viral envelope toward the site of fusion peptide insertion, resulting in merger between closely apposed and destabilized viral and target membranes (13, 18, 41, 46, 51). Investigations of the class I fusion glycoproteins of influenza virus and human immunodeficiency virus type 1 (HIV-1) have indicated that membrane fusion proceeds through three identifiable phases in the order of lipid mixing, the formation of a small pore through which small solutes can pass, and pore dilation (5, 39). These events precede entry of the viral nucleocapsid into the cytosol.

In retroviruses, the fusion peptide is linked to the central coiled coil through a sequence that is often rich in glycine and serine residues. Such concentrations of glycine and serine residues are often associated with rotational freedom of the polypeptide backbone (48). Amino acid alignments indicate that the overall length and amino acid composition of fusion peptide and glycine-rich segments of TMs is conserved in a number of retroviral genera and in HA2 of influenza virus (Fig. 1) (21, 22). Spectroscopic studies of the gp41 N-terminal region have revealed that residues 8 to 14 of the fusion peptide form an α-helix in the hydrophobic phase, while residues 18 to 27 of the glycine-rich segment form a flexible link to the coiled coil (9-11, 23). The coiled-coil regions of human T-cell leukemia virus type 1 (HTLV-1) gp21 and influenza virus HA2 are terminated by N caps that lock out the glycine-rich segment from the core domain, directing N-terminal sequences away from the threefold symmetry axis for membrane insertion (12, 31, 34). A flexible segment linking the fusion peptide to the coiled coil may have evolved to decouple unstable, transiently hydrated fusion peptides from the core domain in a pre-hairpin intermediate conformation. The subsequent insertion of the fusion peptide into membrane is coupled to α-helix formation and the transfer of free energy from the fusion peptide to membrane, thus leading to membrane destabilization (26, 33).

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correlates with backbone flexibility (48), we also examined the effects of Gly-to-Pro substitutions which will theoretically lock the backbone in a cis or trans conformation at the site of mutation. Our results indicate that the hydrophobicity of Ile-334 is required for the late, pore dilation phase of fusion. Glycine-to-Pro substitutions inhibited distinct phases of fusion in a position-dependent manner indicating that flexibility within the glycine-rich segment is also an important determinant of TM function.

**Biosynthesis of HTLV-1 Env mutants.** We performed an alanine scan across the Met-326–to–Ser-337 glycine-rich segment in pCELT.1, which directs the expression of functional HTLV-1 Env that is C-terminally tagged with the monoclonal antibody (MAb) C8 epitope from the cytomegalovirus immediate-early promoter (1, 7, 34). We confirmed that the presence of the C-terminal epitope tag did not affect the syncytium-forming (cell-cell fusion) function of HTLV-1 Env in transfected HeLa cells. Thus, the mean numbers of syncytia per 12 microscope fields (×40 magnification) in three independent transfections are as follows: wild-type Env, 64; Env-C8, 61; and mock-transfected cells, 4. The mean number of nuclei per syncytium for wild-type and C8-tagged Env was 12; for mock-transfected cells, it was 6. The effect of Ala and Pro substitutions on gp62 precursor synthesis and its processing to gp21 in transfected 293T cells was assessed by Western blotting with MAb C8, as described previously (34). Figure 2A shows that mutant gp62 precursors were expressed and processed to gp21 at levels comparable to those of the wild type, indicating that the mutants are cleaved and that the resulting gp21 is stable. The ability of gp21 mutants to anchor SU was assessed by immunoprecipitation of [35S]Cys-labeled Env proteins from transfected cell lysates and clarified culture supernatants with MAb M46, which is directed to the SU, gp46 (34). The cell association of gp46 has been shown to require membrane-anchored gp21, with removal of the TMD resulting in greatly enhanced gp46 shedding (43). This MAb immunoprecipitated both gp62 and gp46 from lysates of wild-type and mutant Env-expressing cells (Fig. 2B, M46) whereas only gp46 was obtained from corresponding culture supernatants (Fig. 2C). In control experiments, MAb C8 immunoprecipitated gp62 but not gp46 from cell lysates (Fig. 2B, C8). As similar levels of cell-associated and shed gp46 were observed for wild-type and mutant Env, we conclude that the gp46-anchoring ability of gp21 was not significantly affected by the mutations. We also verified that wild-type and mutant Env glycoproteins were similarly expressed at the surface of Env-transfected 293T cells (Fig. 2D) by using a surface-binding assay employing 125I-labeled immunoglobulin G from an HTLV-1-infected patient (34). These results indicate that Ala and Pro substitutions in the glycine-rich segment did not affect Env biosynthesis.

**The glycine-rich segment is a determinant of cell-cell fusion function.** Cell-cell fusion appears to be the major mode of HTLV-1 transmission in vivo, infection by cell-free virions being an inefficient process (15, 16, 19, 28). We therefore assessed the cell-cell fusion activities of wild-type and mutated glycoproteins by using a luciferase reporter assay. 293T cells...
were cotransfected with wild-type or mutated pCELT.1 vectors and pTMlac, which contains the luciferase open reading frame under control of the bacteriophage T7 promoter. Luciferase is induced after fusion with HeLa targets infected with vaccinia virus vTF7.3, which directs expression of T7 RNA polymerase (34, 38, 54). Figure 3 indicates that the I334A mutation caused a >90% decrease in cell-cell fusion activity. Approximately 25 to 40% reductions in fusion were observed for G329A, V330A, and G332A, whereas other Ala substitutions did not significantly decrease cell-cell fusion. In contrast, the five Gly-to-Pro
mutations resulted in the abolition of cell-cell fusion function. These results indicate that the glycine-rich segment of gp21 is a determinant of membrane fusion function.

**Mutants with diminished cell-cell fusion activity vary in their ability to promote the lipid-mixing and pore formation phases of cell-cell fusion.** Cell-cell fusion and virus-cell fusion proceed through the lipid-mixing, pore formation, and pore dilation phases (5, 39). To determine whether the mutants with diminished cell-cell fusion activity retain the ability to promote lipid mixing, we used a flow cytometric assay (3) to reveal lipophilic dye exchange between 293T cells transfected with wild-type or mutant Env vectors and labeled with DiO (green fluorescent probe)-labeled 293T-Env-expressing effector cells. After a 2-h coculture, cells were washed twice in PBS and detached with trypsin, and cells displaying dual fluorescence were detected by flow cytometry analysis. The results are shown as a percentage of the total number of cells. The mean ± standard error (n = 4) is shown. Statistical analysis was performed by using a two-sample t test, assuming unequal variances.

Figure 4A reveals dual fluorescence for 6.2% ± 0.8% (mean ± standard error) of cells for wild-type transfections compared to 0.75% ± 0.15% and 0.51% ± 0.17% for F402A and F402H, respectively. The I334A and V330A mutants, which exhibited...
~90 and 30% reductions in fusogenicity, respectively, retained lipid-mixing ability at levels comparable to those of the wild type. Of the five Gly-to-Pro mutants, all of which lacked cell-cell fusion ability as determined in the luciferase reporter assay (Fig. 3), only G329P exhibited a significant (~75%) reduction in lipid-mixing function (P < 0.01 relative to wild type). Thus, the G329P mutation impairs the early, lipid-mixing stage of fusion.

To determine whether the fusion-defective mutants are able to promote fusion pore formation, we monitored the exchange of small cytoplasmic dyes, CellTracker green (465 Da) and CMTMR (554 Da) (Molecular Probes), between cocultured 293T-Env effectors and HeLa target cells, respectively, using fluorescence microscopy. The use of flow cytometry for these experiments was not feasible, as the available excitation/emission wavelengths could not distinguish CellTracker green fluorescence from that of CMTMR. Briefly, 293T-Env and HeLa cells were labeled with CellTracker green (10 μM) and CMTMR (8 μM), respectively, prior to coculturing for 2 h at 37°C. Adherent cells were fixed with 4% formaldehyde and mounted in Vectorshield (Vector Laboratories), and dually fluorescing cells counted in three random fields (×40 magnification). Figure 4B indicates cytoplasmic dye exchange between wild-type Env-transfected 293T cells and target cells, but not for mock, F402A, and F402H transfections. The G329P mutant was defective in pore-forming ability, consistent with its impaired lipid-mixing function. Despite the retention of wild-type lipid-mixing ability, the G327P, G332P, and G336P mutations were associated with ~50 to 90% decreases in small cytoplasmic dye exchange, consistent with defective pore formation. In contrast, I334A and G333P exhibited substantial pore-forming ability, indicating a block at the pore dilation phase. Thus, individual residues of the glycine-rich linker contribute to the distinct phases of fusion in a position-dependent manner.

We have found that the glycine-rich segment (Met-326 to Ser-337) of HTLV-1 gp21 is a determinant of membrane fusion function. An alanine scan identified Ile-334 as a hydrophobic residue that is important for fusion function with the I334A mutation impairing the late, pore dilation phase of fusion. As the I334A mutation did not appear to adversely affect Env maturation, Ile-334 is likely to play a structural role in a fusion-activated conformation of gp21, perhaps by contributing to hydrophobic contacts outside the Met-338–to–Asn-425 trimer of the hairpins core domain (31). Isoleucine-to-Ala substitutions can destabilize a protein by changing the free energy of unfolding by between ~2.6 and ~4 kcal mol⁻¹ (29, 45, 49, 55). Thus, I334A-mutated gp21 may lack sufficient energy to drive pore dilation. A comparison of glycine-rich sequences from retroviral TMs indicates the presence of hydrophobic residues (Fig. 1), suggesting that hydrophobic contact between the glycine-rich segment and other TM regions is a conserved feature of fusion function. For example, the homologous N-terminal sequence of HIV-2 gp41 cooperates with the membrane-proximal segment in stabilizing the trimer of hairpins (32). The membrane-proximal segment of gp41 contains an aromatic cluster that may interact with hydrophobic residues of the glycine-rich segment in a late fusion intermediate to drive the pore expansion phase of fusion (40, 44). Consistent with this idea, we have found that the I334A mutation destabilizes the gp21 trimer of hairpins in the context of a chimera composed of maltose-binding protein (MBP) linked to the gp21 ectodomain (MBP/gp41[313-436]) (54; K. A. Wilson, A. L. Maerz, S. Bär, M. Alizon, and P. Poubourios, unpublished data).

Fusion function was also blocked by the five Gly-to-Pro substitutions with position-dependent effects on the various phases of membrane fusion. For example, G329P specifically inhibited lipid mixing and G327P and G332P inhibited pore formation, whereas G333P blocked pore dilation. The G336P mutant retained ~50% of pore-forming ability relative to the wild type, suggesting that a defect in pore dilation function also contributes to its lack of cell-cell fusion activity. As for I334A, the Gly-to-Pro substitutions did not discernably affect Env maturation, suggesting that the fusion defects are due to alterations in fusion-activated gp21. Restriction of backbone rotational freedom by Gly-to-Pro mutations can lead to position-dependent changes in protein stability (35). Thus, position-dependent alterations in gp21 structure and/or stability may contribute to the variety of fusion defects observed. Interestingly, G329P blocked fusion at the early, lipid-mixing phase, suggesting that Gly-329 is critical in an early fusion-intermediate form of gp21. For example, restriction of the backbone at position 329 may render the SU/TM complex unable to undergo the correct receptor-induced conformational changes leading to fusion peptide insertion into membrane. Alternatively, decreased flexibility in the glycine-rich segment may result in the transfer of entropy from hydrated fusion peptides to the coiled-coil core domain and the formation of an aberrant trimer of hairpins that cannot effect membrane merger. Flexibility in the glycine-rich segment therefore appears to be important for a functional trimer of hairpins.

In contrast to the Gly-to-Pro mutants, the fusion activity of Gly-to-Ala mutants was either unchanged or was decreased by only ~25 and 40% for G329A and G332A, respectively. Whether a Gly-to-Ala replacement within a turn or random coil will influence a protein’s structure is difficult to predict as stabilizing effects, destabilizing effects, and no effects have been observed in systematic mutagenesis studies of model proteins (4, 24). The retention of function by the Gly-to-Ala mutants therefore suggests that the targeted glycines do not play a direct structural role. Furthermore, the restriction on backbone flexibility imposed by Ala is unlikely to be as severe as Pro (48), consistent with the relative severity of functional defects resulting from Ala and Pro substitutions in gp21. The conserved glycine-rich segment, linking the fusion peptide to the coiled-coil core of gp21, is thus an important determinant of fusion function. Future studies aimed at providing three-dimensional structural data for such functional determinants that are located outside the trimer of hairpins core domain may identify novel targets for antiretroviral agents.

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