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# The Ectodomain of the Human T-Cell Leukemia Virus Type 1 TM Glycoprotein Is Involved in Postfusion Events

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**To examine the contribution of the transmembrane envelope glycoprotein (TM) to the infectivity of the human T-cell leukemia virus type 1 (HTLV-1), single amino acid substitutions were introduced throughout its ectodomain. The mutated envelopes were tested for intracellular maturation and for functions, including ability to elicit syncytium formation and ability to mediate cell-to-cell transmission of the virus. Three major phenotypes, defining three functionally distinct regions, were identified. (i) Mutations causing defects in intracellular maturation of the envelope precursor are mostly distributed in the central portion of the TM ectodomain, containing the immunosuppressive peptide. This region, which includes vicinal cysteines thought to form an intramolecular disulfide bridge, is probably essential for correct folding of the protein. (ii) Mutations resulting in reduced syncytium-forming ability despite correct intracellular maturation are clustered in the amino-terminal part of the TM ectodomain, within the leucine zipper-like motif. Similar motifs with a propensity to form coiled-coil structures have been implicated in the fusion process driven by other viral envelope proteins, and HTLV-1 may thus conform to this general rule for viral fusion. (iii) Mutants with increased syncytium-forming ability define a region immediately amino-terminal to the membrane-spanning domain. Surprisingly, these mutants exhibited severe defects in infectivity, despite competence for fusion. Existence of this phenotype indicates that capacity for cell-to-cell fusion is not sufficient to ensure viral entry, even in cell-to-cell transmission. The ectodomain of the TM glycoprotein thus may be involved in postfusion events required for full infectivity of HTLV-1, which perhaps represents a unique feature of this poorly infectious retrovirus.**

Human T-cell leukemia virus type 1 (HTLV-1) is a human retrovirus etiologically associated with a severe T-lymphocyte neoplasia, adult T-cell leukemia (62, 73), and a chronic neuromyelopathy, tropical spastic paraparesis (39), also designated HTLV-1-associated myelopathy (56). HTLV-1 is transmitted through three major routes: from mother to child mainly through breast-feeding, during sexual intercourse mainly from male to female, and by exposure to contaminated blood products through transfusion or needle sharing. Thus, the overall mode of HTLV-1 transmission is reminiscent of that of the human immunodeficiency virus (HIV); unlike HIV, however, HTLV-1 is apparently not transmitted by cell-free body fluids (8, 30, 55). Likewise, in vitro infection by HTLV-1 usually requires cocultivation of target cells with infected cells (24, 72), and cell-free infection, although successfully achieved in some experimental systems, is very inefficient compared with that of other retroviruses (19, 27, 34).

The envelope of a retrovirus is the first element to interact with the target cell and plays a crucial role in the infection process. It is made of a lipid bilayer of cellular origin, which contains the virally encoded glycoproteins. The HTLV-1 glycoproteins, like those of the other retroviruses, are initially synthesized in infected cells as a polyprotein precursor (gp61), which is subsequently cleaved in the Golgi apparatus into two mature products: the entirely extracellular surface glycoprotein (gp45) (SU) and the transmembrane glycoprotein (gp20) (TM), which spans the lipid bilayer (23, 44). These two subunits remain associated with each other through noncovalent interactions (60) and are anchored, via the TM glycoprotein, to the surface of the infected cell or of the virion (59). The envelope glycoproteins govern entry of the virus into the target cell by mediating specific attachment to a cellular receptor followed by fusion between viral and cellular membranes. Schematically, binding of HTLV-1 to its as yet undefined receptor is the property assigned to the SU glycoprotein, although the HTLV-1 SU seems to be also implicated in postbinding events required for viral entry (24); driving of the fusion process per se is attributed to the TM glycoprotein. Fusion is required for penetration of the viral core into the cytoplasm of the target cell. In addition, fusion between envelope-expressing cells and receptor-bearing cells leads to the formation of giant multinucleated cells (syncytia) (48).

Very little information is as yet available on the contribution of the HTLV-1 TM glycoprotein to the processes of membrane fusion and viral entry, but a dynamic model for the structure-function relationships of the HIV TM glycoprotein is emerging with increasing precision (reviewed in reference 54). That several regions of the TM ectodomain are involved in the conformational rearrangements required for the fusion process is likely to be a general feature in retroviruses. Indeed, similarity in the functioning of retroviral TM glycoproteins is strongly suggested by similarity in their sequences and predicted spatial structures (38, 58). Three motifs are highly conserved in the ectodomains of retroviral TM glycoproteins, and HTLV-1 does not depart from this rule (23): (i) an amino-terminal hydrophobic stretch with characteristics of a fusion peptide, which is probably involved directly in the membrane fusion process (61); (ii) a leucine zipper-like motif, which is believed to exhibit an amphipathic α-helical secondary structure capable of self-association as a coiled coil (25); and (iii) a sequence described as an immunosuppressive peptide (ISP) in oncoretro-

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viruses, which contains three cysteine residues thought to form an intramolecular disulfide bridge (17, 64).

To gain insight into the structural features of the HTLV-1 TM glycoprotein that contribute to its functions, we have introduced single amino acid substitutions into its ectodomain. The mutations resulted in three distinct phenotypes, revealing the existence of three functional regions. In addition, evidence is provided here that cell-to-cell fusion and cell-to-cell transmission of the virus are dissociable, albeit related, events, and that the ectodomain of the HTLV-1 TM glycoprotein bears determinants implicated in each of these processes.

## MATERIALS AND METHODS

**Cell lines.** COS-1 cells were obtained from the American Type Culture Collection (ATCC) (40). B5 cells, derived from the DBS-FRhL-2 cells, which are rhesus monkey lung-derived fibroblast-like cells (ATCC CL 160), were a kind gift from D. Waters (Frederick Cancer Research and Development Facility, Frederick, Md.) (68). CosLTRLacZ cells, which are COS cells stably expressing the bacterial β-galactosidase gene under the control of the HIV type 1 (HIV-1) long terminal repeat, and HeLa-Tat cells, which are HeLa cells stably transfected with a *tat* gene expressor, were a kind gift from M. Alizon (Institut Cochin de Génétique Moléculaire, Paris, France) (31). COS-1, B5, and HeLa-Tat cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 50 μg of gentamicin per ml and 5% fetal calf serum (FCS); CosLTRLacZ cells were grown in DMEM supplemented with 50 μg of gentamicin per ml and 10% FCS plus 300 μg of hygromycin B (Calbiochem) per ml. All cell lines were maintained at 37°C in a 5% $CO_2$ atmosphere.

**Plasmids.** The HTLV-1 envelope expression vector used in this study is the previously described CMV-ENV plasmid (24), which contains all the HTLV-1 sequences corresponding to the *env*, *tax*, and *rex* genes, under the control of the simian cytomegalovirus (CMV) promoter. The CMV-ENVΔPvuII plasmid, which was obtained by insertion of a nonsense codon just after the sequence coding for the signal peptide of the envelope protein (24), is the negative control. The CMV-ENV438-stop plasmid, derived from the HTE-438 plasmid (59), encodes a truncated secreted envelope under the control of the CMV promoter. The indicator provirus pCS-HTLV-neo has been described previously (24); it contains the pCS-HTLV-1 provirus (18) with the *env* gene replaced by the neomycin resistance gene under the control of the simian virus 40 promoter.

**Site-directed mutagenesis and cloning.** Oligonucleotide-directed mutagenesis of the sequence encoding the HTLV-1 TM ectodomain was performed by subcloning the 444-bp *Kpn*I-*Nsi*I fragment of the *env* gene (positions 6122 to 6566 in Seiki's sequence [65]) into the pGEM-7Zf(+) vector (Promega) and using the Kunkel method (51). The presence of the desired mutations was checked by DNA sequencing. The mutated *env* fragments were then recloned into the CMV-ENV expressor and resequenced before being used for transfection. The mutants were named "X amino acid position-Y," where X and Y are the wild-type and substituted amino acids, respectively, and amino acid position 1 corresponds to the initiator methionine of the envelope protein.

**Radioimmunoprecipitation of the envelope products.** The envelope constructs were transfected into COS-1 cells according to the procedure described by Cullen, by using DEAE-dextran, chloroquine, and dimethyl sulfoxide (20). Two days posttransfection, the cells were metabolically labeled with [$^{35}$S]cysteine (Amersham) for 16 h, and the envelope products were immunoprecipitated as previously described (61), both from the transfected-cell lysates (to assess envelope precursor expression and cleavage) and from the transfected-cell supernatants (to assess SU-to-TM association), with sera from HTLV-1-infected individuals (a kind gift from Y. Coste, CRTS, Montpellier, France). Immunoprecipitates were electrophoresed in sodium dodecyl sulfate–13% polyacrylamide gels under reducing conditions and visualized by autoradiography. The dried gels were also exposed on an imaging plate (BAS-IIIS; Fuji) allowing quantitative evaluation of the radioactivity of each band with a phosphorimager (FUJIX BAS1000; Fuji). For each envelope glycoprotein mutant, the cleavage index was calculated as follows:

$$\frac{\begin{array}{c}\text{radioactivity of the TM band of the mutated protein/}\\ \text{radioactivity of the precursor band of the mutated protein}\end{array}}{\begin{array}{c}\text{radioactivity of the TM band of the wild-type protein/}\\ \text{radioactivity of the precursor band of the wild-type protein}\end{array}} \times 100$$

The lower limit for cleavage detection is estimated at 20% cleavage.

**Syncytium formation assay.** The syncytium-forming abilities of the envelope glycoproteins were evaluated by use of a quantitative assay designed by others (26) and adapted as previously described (24). Briefly, the envelope constructs (1 μg) were transfected into CosLTRLacZ cells seeded at $3 \times 10^5$ cells per 60-mm-diameter dish the day before (20). Two days posttransfection, $5 \times 10^5$ HeLa-Tat cells were added as indicator cells. After a 24-h coculture, the amount of β-galactosidase was evaluated by a chemiluminescence assay for detection of the activity of this enzyme in cell lysates (Galacto-Light; Tropix) with a luminometer (Lumat LB 9501; Berthold). For each envelope glycoprotein mutant, the syncytium formation index was calculated as follows:

$$\frac{\begin{array}{c}\text{β-galactosidase activity with the mutated protein}\\ -\ \text{β-galactosidase activity in the negative control}\end{array}}{\begin{array}{c}\text{β-galactosidase activity with the wild-type protein}\\ -\ \text{β-galactosidase activity in the negative control}\end{array}} \times 100$$

The validity of this index as a quantitative indicator of syncytium formation was verified as follows: for each of the envelope glycoprotein mutants, syncytia were stained in situ (26), and their number and size were evaluated under the microscope. All the mutants conformed to the rule that the amount of β-galactosidase measured by use of the chemiluminescence assay was proportional to the number of blue foci directly counted under the microscope (24). Also, the number of syncytia was correlated to their size (number of nuclei) in each case (data not shown).

**Infectivity assay.** The abilities of the envelope glycoproteins to mediate HTLV-1 cell-to-cell transmission in a single round of infection were evaluated by use of a previously described quantitative assay (24). Briefly, the envelope constructs (0.75 μg) were cotransfected with the indicator provirus pCS-HTLV-neo (0.75 μg) into COS-1 cells seeded at $3 \times 10^5$ cells per 60-mm-diameter dish the day before (20). Two days posttransfection, the cells were treated with 10 μg of mitomycin per ml (amétycine; Laboratoire Choay) for 3 h at 37°C to prevent growth. After treatment, the cells were washed five times with phosphate-buffered saline, trypsinized, and seeded with $4 \times 10^5$ B5 cells into 60-mm-diameter dishes. After 2 days of coculture, half of the cells were transferred into a 60-mm-diameter dish and subjected to selection for 2 to 3 weeks in complete medium containing 125 μg of G418 sulfate (Geneticin; Gibco) per ml. G418-resistant colonies were counted after fixation with methanol and coloration with a 1:50 dilution of Giemsa stain. For each envelope glycoprotein mutant, the infectivity index was calculated as follows:

$$\frac{\begin{array}{c}\text{number of colonies with the mutated protein}\\ -\ \text{number of negative-control colonies}\end{array}}{\begin{array}{c}\text{number of colonies with the wild-type protein}\\ -\ \text{number of negative-control colonies}\end{array}} \times 100$$

## RESULTS

**Rationale for the site-directed mutagenesis of the HTLV-1 envelope gene.** To define the HTLV-1 TM glycoprotein regions important for envelope-mediated functions, our site-directed mutagenesis was designed on the basis of two main criteria. First, our previous studies demonstrated that most mutations artificially introduced into the HTLV-1 envelope glycoproteins result in retention of the precursor in the endoplasmic reticulum (23, 60, 61). Because our goal was to identify the structural requirements for late functional steps rather than for intracellular maturation, we opted for a fine mutagenesis consisting of single amino acid substitutions. We also excluded changes of cysteine or proline residues, since these amino acids usually play a major role in protein folding. Second, we selected the amino acids to be mutated on the basis of their conservation among both the then-sequenced HTLV-1 isolates and the phylogenetically related retroviruses, including simian T-cell leukemia virus type 1, HTLV-2, and bovine leukemia virus. Indeed, the high degree of conservation of these residues argues strongly for a critical role in envelope functions.

Following this rationale, we selected 20 amino acids along the length of the ectodomain of the HTLV-1 TM glycoprotein, downstream from the fusion peptide. All of them were changed in a nonconservative manner, since this seemed more likely to affect envelope functions. For three positions in the leucine zipper-like heptad repeat, a conservative substitution was also tested. The 23 mutants are listed in Table 1.

The mutated *env* genes were transiently expressed in COS-1 cells, and the resulting phenotypes were compared with that of the wild-type envelope. The envelope precursor processing, SU-to-TM association, syncytium-forming activity, and ability to complement cell-to-cell transmission of the virus were assessed by quantitative assays in each case, as described in Materials and Methods.

The results are presented in Fig. 1 and Table 1. Figure 1

TABLE 1. Phenotypes of HTLV-I TM ectodomain mutants[a]

| Envelope glycoprotein | Precursor cleavage index (%)[b] | Syncytium formation index (%)[c] | Infectivity index (%)[d] |
|---|---|---|---|
| Wild type | 100 | 100 | 100 |
| Ser339-Tyr | 40 | <5 | 7 |
| Lys344-Gln | 106 | 63 | 100 |
| Asp351-His | <20 | <5 | <5 |
| Ser355-Phe | 98 | 55 | 135 |
| Ala360-Glu | 85 | <5 | <5 |
| Ile361-Arg | 81 | <5 | <5 |
| Ile361-Leu | 125 | 15 | <5 |
| Leu368-Arg | 29 | <5 | <5 |
| Leu368-Ile | 130 | 193 | 105 |
| Ala375-Asp | 25 | <5 | 9 |
| Ala375-Val | 51 | 16 | 42 |
| Gln377-Leu | 82 | <5 | 22 |
| Arg379-Gly | <20 | <5 | <5 |
| Asp383-Tyr | 20 | <5 | <5 |
| Glu398-Val | 32 | <5 | <5 |
| Asn407-Tyr | 42 | <5 | <5 |
| Ser408-Phe | 30 | 7 | 5 |
| Glu415-Lys | 28 | 143 | 39 |
| Leu419-Arg | 31 | <5 | <5 |
| Trp431-Gly | 43 | 137 | 5 |
| Asp432-Tyr | 48 | 20 | <5 |
| Ser436-Leu | 41 | 66 | <5 |
| Arg440-Leu | 58 | 207 | <5 |

[a] The cleavage, syncytium formation, and infectivity indices were calculated as described in Materials and Methods. No SU glycoprotein could be immunoprecipitated from the transfected-cell supernatants.

[b] Data represent the means of at least two independent transfections (standard errors were less than 8%).

[c] Data represent the means of at least three independent transfections (standard errors were less than 5%).

[d] Data represent the means of at least two independent transfections (standard errors were less than 8%). All values were obtained from assays in which the number of neomycin-resistant colonies with the wild-type glycoprotein was in the range of 250 to 350.

shows that none of the mutations had a significant effect on envelope product stability, since comparable amounts of the envelope precursor gp61 were detected in all cases. Neither did the mutations appear to disrupt the glycosylation process, since the mobilities of the mutated precursors in sodium dodecyl sulfate-polyacrylamide gels were similar to that of the wild-type gp61. Finally, no envelope protein could be immunoprecipitated from the cell supernatants (Fig. 1, left lane for each mutant), showing that none of the mutations disrupted SU-to-TM association.

From this series of correctly synthesized mutants, four distinct phenotypes emerged.

**Functional TM glycoprotein mutants.** Among the 23 mutations assayed, only 3 spared both intracellular maturation and functions of the envelope glycoproteins (Lys344-Gln, Ser355-Phe, and Leu368-Ile) (Table 1). Careful examination, however, reveals differences in the functioning of these mutated glycoproteins compared to that of the wild-type envelope. Figure 2 plots the cell-to-cell transmission capacity together with the syncytium formation capacity. Two mutants repeatedly exhibited an infectivity index comparable to (Lys344-Gln) or even higher than (Ser355-Phe) that of the wild-type envelope, despite reduced fusogenic properties as evaluated by their syncytium formation index. On the other hand, the conservative substitution at position 368 (Leu368-Ile) enhanced the syncytium-forming activity without affecting infectivity. These observations point to distinct requirements for cell-to-cell fusion and for cell-to-cell transmission of the virus.

**Processing-defective TM glycoprotein mutants.** Of the 23 mutations assayed, 9 greatly affected envelope processing, as evaluated by comparing the TM/precursor ratio obtained with the mutated protein to that of the wild-type envelope. These were Asp351-His, Leu368-Arg, Ala375-Asp, Arg379-Gly, Asp383-Tyr, Glu398-Val, Ser408-Phe, Glu415-Lys, and Leu419-Arg (Table 1).

To examine whether these cleavage-defective mutants could still mediate envelope functions, we analyzed their ability to elicit syncytium formation and to complement cell-to-cell transmission of the virus. As shown in Table 1, the lack of precursor cleavage resulted in a complete loss of function as detected by both assays, with one exception: the Glu415-Lys mutant exhibited a low cleavage index (28% of the wild-type level) yet was highly fusogenic and mediated substantial viral transmission. Thus, barely detectable amounts of mature products can still be sufficient to sustain envelope functions, which is reminiscent of what we already noticed with some mutants of the HTLV-1 SU glycoprotein (22, 24). The two functional assays may indeed be more sensitive than the immunoprecipitation assay for revealing the presence of the mature envelope products.

Although the mutations affecting processing are distributed throughout the TM ectodomain, it is noteworthy that some of them cluster in the central portion, containing the so-called ISP region (amino acids 377 to 402) (Fig. 3).

**Processing-competent fusion-defective TM glycoprotein mutants.** For eight mutants (Ser339-Tyr, Ala360-Glu, Ile361-Arg, Ile361-Leu, Ala375-Val, Gln377-Leu, Asn407-Tyr, and Asp432-Tyr), the syncytium-forming activity was less than might be expected from the extent of precursor cleavage (Table 1). In this respect, the Ala360-Glu, Ile361-Arg, and Gln377-Leu mutations, which completely abolished fusion ca-

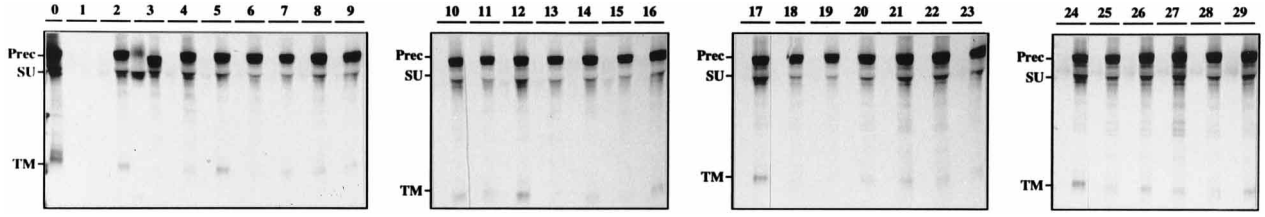


FIG. 1. Immunoprecipitation of the envelope glycoproteins from COS-1 transfected cells. In each case, the left part and the right part of the lane correspond to immunoprecipitation from the cell supernatant and from the cell lysate, respectively. Lane 0, lysate of C91/PL HTLV-1-infected cells (63); lane 1, negative control (cells transfected with the CMV-ENVΔPvuII construct); lanes 2, 10, 17, and 24: wild-type envelope (cells transfected with the CMV-ENV construct); lane 3, soluble envelope (cells transfected with the CMV-ENV438-stop construct); lane 4, Ser339-Tyr; lane 5, Lys344-Gln; lane 6, Asp351-His; lane 7, Ser355-Phe; lane 8, Ala360-Glu; lane 9, Ile361-Leu; lane 11, Ile361-Arg; lane 12, Leu368-Ile; lane 13, Leu368-Arg; lane 14, Ala375-Val; lane 15, Ala375-Asp; lane 16, Gln377-Leu; lane 18, Arg379-Gly; lane 19, Asp383-Tyr; lane 20, Glu398-Val; lane 21, Asn407-Tyr; lane 22, Ser408-Phe; lane 23, Glu415-Lys; lane 25, Leu419-Arg; lane 26, Trp431-Gly; lane 27, Asp432-Tyr; lane 28, Ser436-Leu; lane 29, Arg440-Leu.

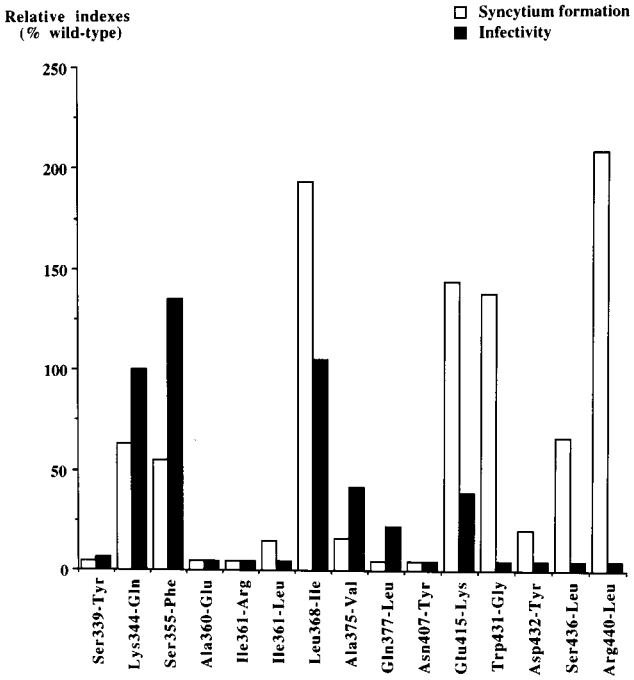


FIG. 2. Schematic representation of the cell-to-cell fusion and cell-to-cell transmission capacities of the processing-competent HTLV-1 TM mutants. For each mutant, the syncytium formation index is plotted together with the infectivity index. The index values are given in Table 1.

pacity despite efficient glycoprotein processing, are particularly outstanding.

Mutations that interfered with cell-to-cell fusion might also prevent cell-to-cell transmission of the virus. Indeed, six of the eight amino acid changes that severely impaired the formation of syncytia also abolished virus infectivity, as expected (Fig. 2). However, the remaining two mutations (Ala375-Val and Gln377-Leu) affected the virus entry function less than they reduced the syncytium-forming activity.

Strikingly, most of the fusion-defective mutants are located in the amino-terminal part of the TM ectodomain, within the leucine zipper-like motif (amino acids 340 to 392) (Fig. 3). The observation that even conservative substitutions at positions 361 and 375 of the heptad repeat greatly impaired the fusogenic properties of the glycoproteins (Ile361-Leu and Ala375-Val) points to a critical role for this region in the membrane fusion process.

**Fusion-competent infectivity-defective TM glycoprotein mutants.** Four mutants (Glu415-Lys, Trp431-Gly, Ser436-Leu, and Arg440-Leu) did not allow cell-to-cell transmission of the virus, despite competence for cell-to-cell fusion (Table 1 and Fig. 2). Strikingly, three of them formed syncytia even more efficiently than the wild-type envelope, and yet their ability to complement virus entry was greatly reduced (Glu415-Lys) or even completely abolished (Trp431-Gly and Arg440-Leu). These functional differences were not simply due to the presence of other HTLV-1 proteins, notably Gag products, in the infectivity assay as opposed to the syncytium formation assay, since the syncytium formation index values were comparable under the experimental conditions of the infectivity assay, i.e., upon cotransfection of the envelope constructs with the provirus pCS-HTLV-neo (data not shown). Therefore, the observed discrepancy between the syncytium-forming activity and the virus transmission ability indicates that fusogenic properties are not sufficient to confer full infectivity.

As shown in Fig. 3, the fusion-competent infectivity-defective mutants clearly define a region immediately amino-terminal to the membrane-spanning domain that is likely to play a crucial role in a postfusion step required for HTLV-1 transmission.

## DISCUSSION

In this study, we have assessed the effects of 23 single amino acid substitutions within the ectodomain of the HTLV-1 TM glycoprotein on envelope maturation and functions. Three major phenotypes were observed, indicating the existence of three functionally distinct regions (Fig. 3).

Our mutagenesis was designed to avoid drastic changes in the structure of the envelope glycoproteins, yet one-third of the mutated proteins did not even complete the maturation process. This corroborates and extends our previous studies, showing that the structural integrity of both the TM segment and the SU segment of the HTLV-1 envelope precursor is required for normal intracellular maturation (22, 23, 60, 61).

The HTLV-1 envelope precursor cleavage takes place after transport into the Golgi apparatus (60). As for most membrane proteins, this transport is thought to depend upon proper disulfide bonding, folding, and oligomerization of the protein in the endoplasmic reticulum (reviewed in reference 29). Interestingly, a cluster of processing-defective mutations can be detected in the ISP region (Fig. 3). This domain, which includes highly conserved cysteines thought to form an intramolecular disulfide bridge (35, 38, 58, 64), is probably critical for correct folding of the protein. Indeed, an extensive deletion of

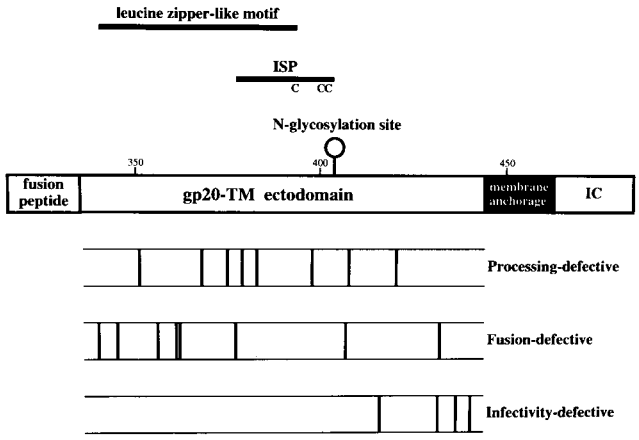


FIG. 3. Summary of HTLV-1 TM mutant phenotypes. The ectodomain, the membrane-spanning domain (membrane anchorage), and the intracytoplasmic domain (IC) of the gp20-TM glycoprotein are depicted. The position of the amino-terminal fusion peptide is indicated, as are those of the leucine zipper-like motif (amino acids 340 to 392) and the ISP (amino acids 377 to 402). This latter region contains the only three cysteine residues (C) of the HTLV-1 TM ectodomain (at positions 393, 400, and 401). The sole N-glycosylation site of the HTLV-1 TM glycoprotein (at position 404) is also represented. Vertical bars mark the positions of the nonconservative amino acid substitutions introduced along the HTLV-1 TM ectodomain. Mutants deficient for precursor processing (processing-defective) have amino acid substitutions distributed throughout the TM ectodomain, but mostly clustered in the central portion, containing the ISP region. Mutants with reduced syncytium-forming activity despite efficient precursor processing (fusion-defective) have amino acid substitutions clustered mainly in the amino-terminal part of the TM ectodomain, within the leucine zipper-like motif. Mutants with impaired infectivity despite fusion competence (infectivity-defective) define a functional region immediately amino-terminal to the membrane-spanning domain.

the ISP region of the Mason-Pfizer monkey virus TM glycoprotein prevented the export of the mutant precursor from the endoplasmic reticulum (3). The substitution of either of the vicinal cysteines in the HIV-1 TM ectodomain also impaired the envelope precursor cleavage (21, 67). Finally, processing defects were elicited by amino acid substitutions within the putative cysteine loop of the feline leukemia virus and the feline immunodeficiency virus TM glycoproteins, indicating that not only the cysteines that form the disulfide bridge but also specific sequences within the loop are required for proper envelope protein maturation (7, 57). Accordingly, at least for the Glu398-Val mutant, the lack of cleavage is likely to result from a folding defect precluding the envelope precursor from being transported to, and consequently processed in, the Golgi complex.

The conserved ISP region has also been postulated to form a contact site for the SU glycoprotein, according to a general model proposing that the mechanism governing the SU-to-TM linkage is conserved among several retroviral families (64). In agreement with this prediction, various changes within the ISP region of the Mason-Pfizer monkey virus TM were found to disrupt the SU-to-TM association (3, 4); yet none of the 23 mutations assayed in this study, whatever their location in the HTLV-1 TM, caused a loss of the SU glycoprotein into the supernatant. It should be kept in mind that cleavage of the envelope precursor into its subunits is a prerequisite for detection of a subunit dissociation phenotype. Since in the case of the HTLV-1 envelope, the completion of the intracellular maturation process is exceptionally sensitive to any change affecting the structural conformation of the protein (23, 60, 61), difficulties in unmasking the amino acids that hold the SU-TM complex together would be expected. Besides, the noncovalent association between the SU and TM glycoproteins probably results from multiple contact areas involving several domains of both subunits, as shown with HIV-1 (9, 15, 36, 43, 45, 50, 53). For the HTLV-1 envelope glycoproteins, which are thought to form a very compact structure (23), this would imply that point mutagenesis could hardly elicit a subunit dissociation phenotype. In this respect, it is noteworthy that among the 76 single amino acid substitutions that we have introduced into the HTLV-1 glycoproteins, only 1 (at position 170 of the SU protein) resulted in shedding of the SU into the cell supernatant (references 22 and 24 and this report).

The present study allowed us to define a region important for the fusion competence of the HTLV-1 envelope in the amino-terminal part of the TM glycoprotein, immediately carboxyl to the fusion peptide (Fig. 3). Further evidence supporting this view is the recent identification of a putative syncytium-neutralizing epitope within the region spanned by amino acids 346 to 368 (28). Structurally, this region contains a leucine zipper-like heptad repeat of hydrophobic residues, which is believed to give rise to an amphipathic α-helix capable of self-association as a coiled-coil structure (25, 38). The corresponding domains in Moloney murine leukemia virus and HIV-1 do indeed form coiled coils in the crystal structure of TM fragments, in both cases as homotrimers (13, 35). Analogous motifs are found not only in all retroviral TM proteins (25) but also in the fusion proteins of other families of enveloped viruses, including orthomyxoviruses, paramyxoviruses, and coronaviruses (2, 5, 12). By analogy with the conformational change undergone by the influenza hemagglutinin upon acidification (6, 11), it has been suggested that binding of the SU glycoprotein to its cellular receptor triggers the heptad repeat region of the TM glycoprotein to adopt an extended coiled-coil structure, which, in turn, propels the fusion peptide into position to interact with the target cell membrane (1, 54, 69). In this model, the propensity of the zipper motif to self-associate is postulated to drive the formation of the fusogenic envelope oligomer rather than the intracellular assembly of the prefusogenic oligomer. A major support for this assumption is that point mutations designed to destabilize the coiled-coil structure of the HIV-1 leucine zipper-like repeat failed to interfere with envelope precursor oligomerization but did impair envelope-mediated fusion and infectivity (16, 32, 69). Likewise, most of the mutations that we have introduced into the leucine zipper-like domain of the HTLV-1 TM affected syncytium formation rather than precursor processing, suggesting that the HTLV-1 envelope is likely to conform to this general model for viral fusion.

In the present work, we have changed some of the core amino acids of the HTLV-1 heptad repeat in a conservative manner. Unlike classical leucine zippers, the hydrophobic heptad repeats of viral fusion proteins, including that of the HTLV-1 TM, contain isoleucine and valine residues in addition to leucine residues (12). When either isoleucines or valines were substituted for leucines in a canonical leucine zipper, a switch among two-, three-, and four-stranded coiled coils was observed (41). This has led to the hypothesis that the presence of isoleucines and valines may be responsible for formation of the higher-order complexes of envelope glycoproteins required for the fusion process (1). In good agreement with this assumption, our results show that the fusion competence of the HTLV-1 envelope is reduced by the change of the isoleucine at position 361 into a leucine, while it is enhanced by the converse mutation in the next heptad repeat unit, i.e., the change of the leucine at position 368 into an isoleucine.

Some of the mutations in the amino-terminal part of the HTLV-1 TM ectodomain affected the virus entry function less than they reduced the syncytium-forming activity (Fig. 2). Transmission competence despite fusion deficiency has previously been observed with mutants of HIV-1 (9, 10, 42, 49) and HIV-2 (66) glycoproteins and has generally been attributed to differences in the requirements for virus-to-cell fusion compared with cell-to-cell fusion. However, this explanation cannot account for our observation with HTLV-1, since this retrovirus can barely be transmitted via cell-free particles. Thus, our results indicate unambiguously that defects in cell-to-cell fusion do not necessarily imply defects in cell-to-cell transmission. Indeed, the syncytium formation assay probably records additional events of fusion subsequent to those required for delivery of the viral core from the cytoplasm of the donor cell to that of the target cell. Our studies indicate that this very final step of the fusion process involves amino acids present in both the TM and the SU glycoproteins of the HTLV-1 envelope (reference 24 and this report).

Finally, most of the mutations that we have introduced into the carboxyl portion of the HTLV-1 TM ectodomain resulted in syncytium-forming activities significantly enhanced over that of the wild-type envelope (Fig. 3). Amino acid changes in the corresponding region of the HIV-1 TM glycoprotein have also been found to increase the capacity for fusion (9). Studies with synthetic peptides modeled after this sequence have led to the proposal that the carboxy-terminal region of the HIV-1 TM ectodomain could interact with the leucine zipper-like region and thereby regulate the availability of this latter domain for the fusion process (13, 14, 54, 70, 71). An alternative hypothesis would implicate the fusion peptide as the target of interaction (46, 47). It would be interesting to determine whether either of these mechanisms is in fact involved in the functioning of the HTLV-1 TM glycoprotein, since that could provide a clue to explaining the observed increase in fusion ability

displayed by the envelopes with mutations in the carboxy-terminal region of the TM ectodomain.

Despite their enhanced competence for fusion, the HTLV-1 mutants with changes in the carboxyl portion of the TM ectodomain exhibited marked defects in infectivity (Fig. 2), in striking opposition to HIV-1 envelopes mutated in the same region (9). It could be argued that envelopes with increased syncytium-forming activities might artificially reduce the number of neomycin-resistant colonies in our cell-to-cell infectivity assay by committing the donor and target cells to form abortive syncytia. However, mutants with equivalent fusogenic activities exhibited widely different capacities to complement cell-to-cell transmission of the virus (compare Trp431-Gly with Glu415-Lys, and Arg440-Leu with Leu368-Ile). Thus, the observed decreases in the infectivity index most likely represent bona fide defects in the virus entry function.

The existence of fusion-competent, infectivity-defective mutants indicates that a capacity for cell-to-cell fusion is not sufficient to ensure cell-to-cell transmission of the virus and suggests that postfusion events mediated by the envelope must also intervene for completion of the virus entry process. For instance, additional conformational changes of the glycoproteins, subsequent to those involved in the fusion process, could be required to propel the viral core through the opening at the fusion site into the cytoplasm of the target cell. The present study allowed us to identify the carboxy-terminal region of the TM ectodomain as a determinant for the postfusion steps required for HTLV-1 transmission. Deletions of the intracytoplasmic domain of the HIV-1 TM glycoprotein have likewise been reported to cause defects in virus entry despite enhanced fusogenic competence (33, 37, 42). To our knowledge, however, defects in infectivity have not previously been observed with mutations of the region located upstream from the membrane-spanning domain in any retroviral envelope studied so far. It would be interesting to determine whether the requirement for the TM region adjacent to the exterior side of the membrane is a feature restricted to HTLV-1 infectivity. By comparison with other retroviruses, infection by HTLV-1 of susceptible cells is very inefficient, as demonstrated by clinical (30) as well as in vitro (34) data, and this property may be attributed, at least in part, to the envelope glycoproteins (52). Further systematic searches for unique features in the functioning of the HTLV-1 envelope may contribute to an understanding of the molecular bases underlying the poor infectivity of this retrovirus.

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