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A Novel Human T-Leukemia Virus Type 1 Cell-to-Cell Transmission Assay Permits Definition of SU Glycoprotein Amino Acids Important for Infectivity

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Human T-leukemia virus type 1 (HTLV-1) envelope glycoproteins play a major role in viral transmission, which in the case of this virus occurs almost exclusively via cell-to-cell contact. Until very recently, the lack of an HTLV-1 infectivity assay precluded the determination of the HTLV-1 protein domains required for infectivity. Here, we describe an assay which allows the quantitative evaluation of HTLV-1 cell-to-cell transmission in a single round of infection. Using this assay, we demonstrate that in this system, cell-to-cell transmission is at least 100 times more efficient than transmission with free viral particles. We have examined 46 surface (SU) glycoprotein mutants in order to define the amino acids of the HTLV-1 SU glycoprotein required for full infectivity. We demonstrate that these amino acids are distributed along the entire length of the SU glycoprotein, including the N-terminus and C-terminus regions, which have not been previously defined as being important for HTLV-1 glycoprotein function. For most of the mutated glycoproteins, the capacity to mediate cell-to-cell transmission is correlated with the ability to induce formation of syncytia. This result indicates that the fusion capacity is the main factor responsible for infectivity mediated by the HTLV-1 SU envelope glycoprotein, as is the case for other retroviral glycoproteins. However, other factors must also intervene, since two of the mutated glycoproteins were correctly fusogenic but could not mediate cell-to-cell transmission. Existence of this phenotype shows that capacity for fusion is not sufficient to confer infectivity, even in cell-to-cell transmission, and could suggest that postfusion events involve the SU.

The retroviral envelope glycoproteins all share a common organization, with an external surface glycoprotein (SU) and a transmembrane (TM) glycoprotein. These two mature glycoproteins derive from a precursor, cleaved in the Golgi compartment by a cellular protease. In the viral entry process, the SU glycoprotein is responsible for binding to the cell receptor; this binding is followed by postbinding events required for fusion (20, 21). Such events probably include conformational changes in the SU, since receptor binding induces exposure of epitopes of the SU which were masked prior to the binding event (50). The fusion process per se is mediated by the TM glycoprotein, which bears an N-terminal fusion peptide. That fusion competence requires conformational rearrangements of the viral glycoproteins is probably a general rule in enveloped viruses and is reminiscent of the viral spike glycoprotein changes undergone in alphaviruses and flaviviruses before fusion (2, 18).

The involvement of the human T-leukemia virus type 1 (HTLV-1) glycoproteins in the infectivity process has not been directly studied due to the lack of an infectious provirus clone until recently (10, 23). As a consequence, glycoprotein functions could be studied only by analysis of syncytium formation (8). HTLV-1 can be distinguished from all other retroviruses on the basis of the infection process: it is transmitted almost exclusively via cell-to-cell contact, and the free viral particle is not, or is very poorly, infectious. Indeed, seroconversion is observed in transfusion recipients of HTLV-1-positive blood cellular components but not in recipients of positive noncellular units (24, 27, 30). In vitro, cell-free viral infection is extremely difficult to achieve (29, 35, 51), although a low level of successful infection could be obtained for both primary cells (10) and cell lines (6, 13). The envelope glycoproteins are believed to be responsible for the lack of infectivity of the free viral particle, since pseudotypes between HTLV-1 envelope glycoproteins and murine retroviral cores either cannot mediate cell-free transmission or do so extremely inefficiently (46, 49), whereas the murine retroviral envelope particle can (26). To better understand the requirements for this cell-to-cell transmission of HTLV-1, we have developed a quantitative infectivity assay which measures HTLV-1 cell-to-cell transmission in a single round of infection. Mutated SU envelope glycoproteins were examined in this assay. We show that in most instances, the fusion capacity of the envelope glycoprotein parallels its capacity for infection. Two glycoprotein mutants, however, were not infectious even though they displayed a substantial fusion capacity. This demonstrates that in cell-to-cell transmission, SU determinants of infectivity differ from those of fusion competence.

MATERIALS AND METHODS

Construction of the indicator provirus and of mutated *env* genes. The indicator provirus (pCS-HTLV-neo) was constructed from the pCS-HTLV-1 proviral clone (3) (a kind gift from D. Derse, National Cancer Institute, Frederick, Md.), where the *env* gene was replaced by the neomycin resistance gene under the control of the simian virus 40 promoter. To do so, a provirus fragment extending from a *Sal*I site at position 5695 in the sequence determined by Seiki et al. (38) (hereafter referred to as Seiki's sequence) to an *Eco*RI site in the 3′ long terminal repeat (LTR) flanking region was subcloned into the PGEM-11Zf(+) vector (Promega), giving the pGEM-SE construct. In this construct, the *Sal*I-to-*Pst*I fragment corresponding to most of the *env* gene (positions 5695 to 6752 in Seiki's sequence) was replaced by an *Acc*I-to-*Sma*I fragment from the pSV2*neo* construct (Clontech) in which the *Acc*I site was changed to a *Sal*I site; this resulted in the pGEM-SE*neo* construct. The *Sal*I-to-*Cla*I fragment of pGEM-SE*neo* was then inserted back into the sequence of pCS-HTLV-1, in place of the

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proviral *Sal*I-to-*Cla*I fragment (positions 5695 to 7495 in Seiki's sequence), resulting in the pCS-HTLV-neo clone. To permit evaluation of reverse transcription, a new *Xho*I site was introduced into the 3′ LTR of pCS-HTLV-neo, by changing one base (G to A) at position 8447. Two steps were required to obtain this pCS-HTLV-neo-Xho construct; the first involved site-directed mutagenesis in pGEM-11-SE*neo*, and the second involved subcloning of the mutation in the pCS-HTLV-neo construct by using a *Cla*I-to-*Eco*RI fragment (positions 7495 to the 3′ LTR flanking region).

The HTLV-1 envelope expressor plasmid used in this study is HTE-1, constructed as previously described (12) except that the HTLV-1 promoter was replaced by the simian cytomegalovirus (CMV) promoter, resulting in the CMV-ENV construct. Site-directed mutagenesis of the *env* gene was performed as previously described (8, 32). The mutants were named *X*amino acid position-*Z*, where *X* and *Z* are the wild-type and mutant amino acids, respectively, and amino acid position 1 corresponds to the initiator methionine. All experiments included a negative control, the CMV-ENVΔPvuII construct in which a stop codon was inserted at a *Pvu*II site (position 5274 in Seiki's sequence), in the *env* open reading frame of the CMV-ENV construct, using an *Nhe*I 12-bp linker (New England Biolabs, Ozyme, France).

Cell lines. The cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (FCS) and gentamicin. COS-1 (16) and HOS (28) cells were obtained from the American Type Culture Collection. 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.). CosLTRLacZ and HeLa-Tat cells are stable transfectants described elsewhere (9) and were a kind gift from M. Alizon (ICGM, Hôpital Cochin, Paris, France). 293-TSA cells (17) were obtained from J. Neyton (Ecole Normale Supérieure, Paris, France).

Expression of mutated envelope glycoproteins. COS-1 cells were transfected with the envelope expressor plasmid DNA as described previously (7). At 48 h posttransfection, the transfected cells were labeled with [^{35}S]cysteine (Amersham) for 16 h as previously described (8). The cells were lysed, and the glycoproteins were immunoprecipitated with sera from HTLV-1-infected individuals (8). Immunoprecipitates were analyzed on sodium dodecyl sulfate-polyacrylamide gels; the gels were dried and exposed with a Fuji Imaging plate (type BAS-IIIS), allowing quantitative measurement of the bands' radioactivity with a phospho imager (Fuji type BAS 1000 Fujix). The amount of cleaved envelope, or cleavage index, was estimated as follows: {[radioactivity in the TM band (mutated protein)/radioactivity in the precursor band (mutated protein)]/[radioactivity in the TM band (wild-type protein)/radioactivity in the precursor band (wild-type protein)]} × 100.

Cell surface expression of mutated envelope glycoproteins. The cell surface expression of the glycoproteins was monitored by using an indirect immunofluorescence assay. Twenty-four hours after transfection, transfected 293-TSA cells were collected and washed three times in phosphate-buffered saline (PBS) containing 2% FCS. Saturating amounts of the anti-HTLV-1 envelope MET-3 mouse monoclonal antibody (42) (50 μl of a 1/35 dilution; a kind gift from Y. Tanaka, Kitasato University, Kitasato, Japan) were then added to 2×10^5 cells for 45 min at 4°C. The cells were washed three times in PBS–2% FCS and then incubated with phycoerythrin-conjugated rabbit anti-mouse immunoglobulin G (50 μl of a 1/100 dilution; Caltag Laboratories) for 45 min at 4°C. The cells were washed twice in PBS–2% FCS and then once in PBS, resuspended in 400 μl of PBS, and analyzed by flow cytometry with a FACScan (Becton Dickinson).

Syncytium formation of mutated envelope glycoproteins. To evaluate the abilities of the envelope glycoproteins to induce the formation of syncytia, we used a system, described by others (9), which allows quantitative evaluation of syncytium formation via a β-galactosidase assay. Briefly, the envelope constructs were transfected into CosLTRLacZ cells, which are COS-1 cells stably expressing a β-galactosidase gene under the control of the human immunodeficiency virus type 1 (HIV-1) LTR; 48 h later, the transfected cells were cocultivated with HeLa cells stably transfected with a *tat* gene expressor (HeLa-Tat indicator cells). Upon envelope-induced fusion of the transfected CosLTRLacZ cells with the HeLa-Tat cells, the HIV-1 LTR is transactivated by the Tat protein, resulting in β-galactosidase expression.

After a 24-h coculture of the transfected cells with the indicator cells, the amount of β-galactosidase was evaluated by using a chemiluminescence assay for this enzyme (Tropix) and a chemiluminometer (Lumat LB9501; Berthold). For each envelope glycoprotein mutant, the relative amount of syncytium formation was calculated as {[β-galactosidase (mutated protein) − β-galactosidase (negative control)]/[β-galactosidase (wild-type protein) − β-galactosidase (negative control)]} × 100. For all mutated glycoproteins, syncytia were also stained by using a previously described procedure (9) and counted under a microscope. The amount of β-galactosidase measured in the chemiluminescence was always proportional to the number of syncytia directly counted under the microscope (data not shown).

Virion incorporation of mutated envelope glycoproteins. The pCS-HTLV-neo construct was cotransfected with the envelope expressor plasmids into 1.2×10^6 293-TSA cells, employing a procedure using polyethyleneimine (1). Twenty-four hours later, the cells were metabolically labeled with 700 μCi of [^{35}S]cysteine-methionine promix (Amersham) per ml. The cell supernatants were collected, centrifuged for 10 min at 1,200 rpm to remove cell debris, and filtered through a 0.45-μm-pore-size filter. The supernatants were then layered onto discontinuous sucrose gradients (3 ml of 20% sucrose on top of 3 ml of 60% sucrose) and centrifuged for 2 h at 4°C at 25,000 rpm, using an SW41 rotor. The virus sedimented at the interface between the two layers and was collected in a volume of 1 ml, to which 200 μl of 5× lysis buffer (8) was added. The viral proteins were then immunoprecipitated with sera from HTLV-1-infected patients.

TABLE 1. Cell-to-cell transmission of HTLV-1

COS-1 cells transfected with[b]:	No. of neomycin-resistant clones after COS-1 cell coculture with[a]:	
	HOS cells	B5 cells
pCS-HTLV-neo + CMV-ENVΔPvuII[c]	0	10
pCS-HTLV-neo + CMV-ENV[d]	50	>200

[a] The indicator cells were placed into selection medium 48 h after the beginning of coculture with the transfected COS-1 cells. The neomycin-resistant colonies were counted 3 weeks later. No neomycin-resistant colonies were found in the absence of coculture.

[b] COS-1 cells were transfected as indicated in Materials and Methods and treated with mitomycin before coculture with the indicator cells.

[c] Cotransfection of the pCS-HTLV-neo indicator provirus with a control plasmid which has a nonsense codon in the *env* gene.

[d] Cotransfection of the pCS-HTLV-neo indicator provirus with an envelope expressor plasmid.

Infectivity assay. pCS-HTLV-neo (0.75 μg) and the envelope expressor (0.75 μg) were cotransfected into COS-1 cells seeded at 3×10^5 cells per 60-mm-diameter dish the day before, as described previously (7). Forty-eight hours later, the cells were treated with 10 μg of mitomycin per ml for 3 h at 37°C. After treatment, the cells were washed five times with PBS, trypsinized, and seeded with 5×10^5 B5 cells in a 60-mm-diameter cell culture dish. After 48 h of coculture, the cells were trypsinized and half of the cells were transferred to medium containing geneticin (G-418 sulfate [125 μg/ml]; Gibco). Colonies began to appear after 2 weeks. A few days later, colonies were counted after fixation with methanol and staining with a Giemsa blue solution.

For each mutated glycoprotein, the relative infectivity was evaluated by counting the number of neomycin-resistant colonies, and the infectivity index was determined as follows: {[number of colonies (mutated protein) − number of colonies (negative control)]/[number of colonies (wild-type protein) − number of colonies (negative control)]} × 100.

PCR amplification of the LTRs in neomycin-resistant clones. Neomycin-resistant colonies were picked by using cloning cylinders and grown individually in cell culture plates. After several days, 10^6 cells were lysed, the DNA was extracted, and the 5′ LTR sequences were amplified by using LTR forward primer (5′AGCCTCCCAGTGAAAAACATT3′) and Gag reverse primer (5′CGGAC GAGCCCCCAACTGTG3′). Primers were added at 20 μM to 100 ng of template genomic DNA. PCR amplification was performed for 35 cycles of 20-s denaturation at 94°C, 30-s annealing at 55°C, and 60-s extension at 72°C.

RESULTS

HTLV-1 cell-to-cell transmission in a single round of infection. We have developed a quantitative infectivity assay which permits evaluation of HTLV-1 transmission in a single round of infection by cell-to-cell contact. For this purpose, two complementary constructs were used. The first was an HTLV-1 indicator provirus with the *env* gene replaced by a selectable marker, and the second construct allowed the expression of the *env* gene. The two constructs were cotransfected into COS-1 cells, which were treated with mitomycin to prevent growth and cocultivated with target cells. After growth in selection medium, the neomycin resistant colonies were stained and counted.

As target cells (Table 1), we tested two cell lines, HOS and the B5, which had been previously infected by others (4, 48) with HTLV-1. The B5 cells allowed the outgrowth of neomycin-resistant colonies, in the range of 200 to 400 colonies depending on the experiment (Table 1).

The mitomycin treatment could prevent the growth of transfected cells, since no neomycin-resistant colony was obtained from transfected COS-1 cells in the absence of coculture. Also, a very low background was obtained in the absence of the HTLV-1 envelope, as shown by cotransfection of the indicator provirus with a negative control containing a nonsense codon

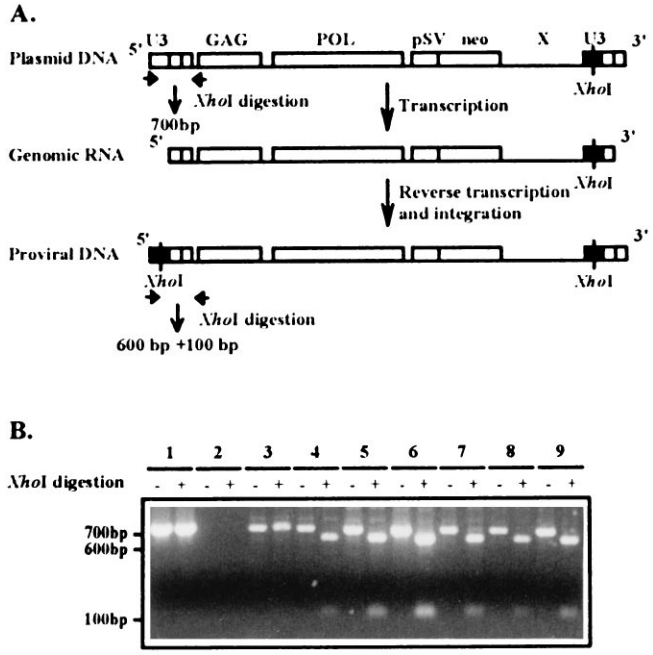

FIG. 1. Provirus integration into the B5 target cells requires a reverse transcription step. (A) Schematic representation of the different steps of viral replication, of the new *Xho*I restriction site introduced into the proviral 3′ LTR, and of the primers (arrows) used to amplify the 5′ LTR from the B5 cell DNA. (B) 5′ LTR fragments amplified from the DNA of infected B5 cells. Lane 1, DNA amplified from an HTLV-1-infected cell line (positive control); lane 2, DNA amplified from B5 cells in the absence of coculture (negative control); lanes 3 to 9, DNA from independent neomycin-resistant clones without digestion (−) and after digestion with *Xho*I (+).

at the beginning of the *env* gene, CMV-ENVΔPvuII. Thus, the glycoproteins are required for infectivity in this assay. The addition of transfected cell supernatant to the B5 target cells did not result in any neomycin-resistant colony (data not shown). Taken together, our results indicate that cell-to-cell transmission is the essential mode of transmission in this system and that it is at least 100 times more efficient than transmission through free viral particles.

Cell-to-cell transmission in the infectivity assay requires reverse transcription. Neomycin-resistant cells selected in the infectivity assay could theoretically originate from two processes: either viral transmission, which requires a reverse transcription step, or direct transfer of plasmid from the transfected to the target cells. Proviral DNA integrated after a reverse transcription step should contain two identical U3 regions in the LTRs, one of which, that of the 5′ LTR, is copied from the other during the reverse transcription process (5). In contrast, integration of the plasmid does not result in any remodeling of the LTR sequences. To distinguish between the two processes, an *Xho*I restriction enzyme site was introduced into the U3 sequence of the 3′ LTR of pCS-HTLVI-neo, resulting in the pCS-HTLV-neo-Xho construct (Fig. 1A). After reverse transcription, B5 cells infected with a virus derived from this construct should contain a proviral DNA with an *Xho*I restriction site, also in the 5′ LTR sequence. By contrast, an integrated provirus of plasmid origin should contain only a single *Xho*I restriction site, in the 3′ LTR sequence.

The 5′ LTR was amplified by PCR using appropriate primers after extraction of the genomic DNA from seven independent neomycin-resistant clones. The amplified LTR DNAs were digested with *Xho*I (Fig. 1). Six of seven of the colonies tested contained an *Xho*I site in the 5′ LTR (Fig. 1B, lanes 4 to 9). This result shows that proviral integration involves a reverse transcription step, indicating that a large majority of the neomycin-resistant clones obtained in our assay essentially result from cell-to-cell transmission of HTLV-1 virus rather than from direct transmission of the plasmid.

Construction of the HTLV-1 SU glycoprotein mutants. We examined the contribution of single amino acids of the SU glycoprotein in the cell-to-cell transmission process. To do so, we used 46 SU glycoprotein mutants with a single amino acid substitution, 23 of which have been previously described (8). This first set of mutated glycoproteins had nonconservative substitutions at positions conserved in HTLV-1; most of them were also conserved in HTLV-2 (39, 40) and simian T-leukemia virus type 1 (47), which share a common cell surface receptor, but not in bovine leukemia virus (36, 37), which uses a different cell surface receptor on human cells (41). The second set of mutations included 23 nonconservative substitutions randomly introduced along the entire length of the SU, or else targeted to a GYDPI motif which is conserved in oncoretroviruses (15), and is located at amino acid positions 169 to 173 in the HTLV-1 SU glycoprotein. The 46 mutants are listed in Table 2.

The effects of mutations on envelope glycoprotein precursor cleavage, on SU-to-TM association, on syncytium formation, and on cell-to-cell transmission of the virus were analyzed by using quantitative assays in each case (see Materials and Methods). The results appear in Table 2, Fig. 2, and Fig. 3. Figures 2 and 3 show immunoprecipitations only for the mutated SU glycoproteins which were not published previously by Delamarre et al. (8).

Precursor cleavage-defective SU glycoprotein mutants are distributed along the entire length of the SU glycoprotein. For each mutated protein, the relative amount of precursor cleavage was evaluated by comparing the TM/precursor ratio obtained with the mutated protein to that of the wild-type glycoprotein in the same experiment.

As shown in Table 2 and Fig. 2A and B, 21 of the 46 changes assayed greatly affected the precursor cleavage (compared to the wild-type glycoprotein). These are the proteins with less than 30% cleavage, which we estimate as the lower limit for cleavage detection. As previously observed, some of these mutations are clustered in the central part of the SU (amino acids 109 to 178), which confirms the critical role of this domain in the envelope maturation process. However, the N- and C-terminus parts of the SU glycoprotein are also important for correct intracellular maturation, since some mutations in these regions prevent precursor cleavage (Ser25-Arg, Gln45-Leu, Leu52-Arg, Asp53-Val, Ser58-Leu in the N-terminus part and Ser292-Tyr and Leu295-Arg in the C-terminus part of the SU [Table 2]). In most mutants, the observed lack of cleavage is probably a consequence of retention of the misfolded proteins in the endoplasmic reticulum, which prevents the mutants from being transported to the Golgi compartment, where cleavage normally occurs. Also, most of the mutations (Gly169-Ala, Asp171-Tyr, and Ile173-Ser) introduced into the GYDPI motif, which is conserved among oncoretroviruses, result in glycoproteins which are not processed. This finding shows the importance of this motif in glycoprotein conformation.

Syncytium formation and infectivity require precursor cleavage. Figure 2C plots the syncytium formation and infectivity indices of the 21 precursor cleavage-defective glycoprotein mutants. For 15 of them, the lack of precursor cleavage results in absence of syncytium formation, and the mutated glycoproteins are not able to mediate cell-to-cell transmission of the virus.

TABLE 2. Effects of HTLV-1 glycoprotein mutations on precursor cleavage, SU-to-TM association, syncytium formation, and infectivity[a]

Protein	Precursor cleavage (%)[b]	SU in the supernatant[c]	Syncytium formation (%)[d]	Infectivity (%)[e]
Wild type	100	−	100	100
Mutants				
Ser25-Arg	22	−	6	2
Val33-Asp	37	−	30	35
Ser35-Leu	41	−	29	89
Gln45-Leu	21	−	29	102
Leu52-Arg	<20	−	0	0
Asp53-Val	<20	−	0	0
Ser58-Leu[f]	<20	−	0	0
Ser75-Phe[f]	67	−	60	89
Ser81-Phe[f]	47	−	60	81
Lys90-Ile	30	−	12	71
Asn93-Ile	87	−	103	71
Arg94-Glu	45	−	35	9
Asn95-Ile[f]	100	−	60	86
Ser101-Leu[f]	73	−	50	6
Ser105-Leu[f]	74	−	60	77
Ser109-Phe[f]	<20	−	60	45
Ser119-Leu[f]	<20	−	0	0
Tyr124-Asp	<20	−	0	0
Ser130-Ile[f]	<20	−	0	0
Lys134-Thr	48	−	54	101
Asn149-Ile	60	−	60	87
Lys156-Ile[f]	<20	−	10	68
Ser162-Phe[f]	<20	−	0	0
Gly169-Ala	25	−	11	37
Tyr170-Ser[f]	51	+++	30	65
Asp171-Tyr	<20	−	0	0
Ile173-Ser	<20	−	0	0
Asn177-Tyr	45	−	64	81
Thr178-Ala[f]	<20	−	0	0
Ser181-Ile[f]	43	−	20	74
Ser194-Phe[f]	34	−	110	88
Asn195-Ile[f]	65	−	50	77
Asp197-Val[f]	49	−	10	62
Ser203-Phe[f]	86	−	70	84
Ser208-Leu[f]	56	−	20	58
Thr212-Ile[f]	<20	−	30	71
Ser220-Ile[f]	100	−	80	76
Ser233-Ile[f]	39	−	60	87
His238-Leu	21	−	0	0
Ser252-Phe	57	−	58	78
Leu262-His	29	−	10	6
Gln282-Leu	45	−	41	64
Ser286-Phe[f]	44	−	40	82
Ser292-Tyr	<20	−	0	0
Leu295-Arg	20	−	0	0
Ser299-Tyr	62	−	36	42

[a] Data represent the means of at least two or three independent experiments.
[b] Percentage of cleavage of the mutated protein relative to the wild-type glycoprotein, calculated as described in Materials and Methods.
[c] Results of immunoprecipitation of the transfected cell supernatant.
[d] Percentage of β-galactosidase activity for each mutated protein relative to a value of 100% for the wild-type envelope glycoprotein (see Materials and Methods for description of the calculation).
[e] Infectivity of mutated glycoproteins relative to that of wild-type glycoprotein was measured by counting neomycin-resistant colonies appearing after growth in selection medium as described in Materials and Methods.
[f] Previously described (8).

Six processing-defective mutants (Gln45-Leu, Lys90-Ile, Ser109-Phe, Lys156-Ile, Gly169-Ala, and Thr212-Ile) are, however, still capable of mediating cell-to-cell transmission despite a very low cleavage index. Three of them (Gln45-Leu, Ser109-Phe, and Thr212-Ile) also have significant fusion capacity, as indicated by the syncytium formation assay. This finding suggested that although we cannot detect any cleavage of the precursor, functional glycoproteins that do mediate fusion are produced. The two functional assays thus seem to be more sensitive than cleavage measurement. The other three processing-defective glycoproteins (Lys90-Ile, Lys156-Ile, and Gly169-Ala) have a very low capacity to form syncytia and still can mediate cell-to-cell transmission. The amounts of processed protein required for infectivity are thus lower than those required for fusion. An alternative explanation for the surprising phenotype of these mutants (cleavage defective but inducing cell fusion and/or infectivity) is that cell fusion and/or infectivity in some instance can be achieved with uncleaved glycoproteins.

Fusion capacity requires amino acids spanning the entire SU, including the C terminus. We evaluated the syncytium formation obtained with the 25 glycoproteins displaying more than 30% precursor cleavage; the results are presented in Table 2 and Fig. 3. All of these glycoproteins were expressed at the cell surface, as measured by indirect immunofluorescence (data not shown).

The fusion capacities of these mutants, as measured using the chemiluminescence assay (see Materials and Methods), are plotted in Fig. 3B together with the precursor cleavage indices. Syncytia were also stained in situ, and their size and number were determined under a microscope. The amount of β-galactosidase measured in the chemiluminescence assay was proportional to the number of syncytia directly counted under the microscope. Also, the number of syncytia was correlated to their size (number of nuclei), with one exception (see below).

For 16 of the 25 mutated glycoproteins, the amount of syncytium formation is comparable to the extent of precursor cleavage (less than 20% difference between the two indices). Such a phenotype is a characteristic of all of the mutated positions in the N-terminal region of the SU. In the case of these mutants, fusion competence is clearly correlated to the amount of mature glycoproteins.

The remaining nine mutated glycoproteins, despite substantial precursor cleavage, have a low syncytium formation capacity. This finding indicates the involvement of the mutated amino acids in the glycoprotein conformation required for the fusion process. Some of the mutated amino acids are located in regions previously described as being important for fusion (Asn95-Ile, Ser101-Leu, Ser181-Ile, and Asp197-Val). We noted that among these mutated glycoproteins, Ser101-Leu induced syncytia with fewer than 10 nuclei. Additional amino acids, located in the C-terminal part of the SU, are also very important for the fusion capacity of the glycoprotein, as shown by the results obtained with the Ser208-Leu and Ser299-Tyr mutated glycoproteins.

One mutated glycoprotein, Ser194-Phe, repeatedly exhibited a very high fusion capacity despite low amounts of precursor cleavage. This mutant protein likely has a conformation more favorable for receptor recognition or fusion, resembling the phenotype of the Ser109-Phe mutant (Fig. 2A and B).

Infectivity in cleavage-competent glycoprotein mutants is distinct from their fusion capacity. We analyzed the cell-to-cell transmission of the 25 cleavage-competent mutants. Cell-to-cell transmission capacity is plotted together with syncytium formation capacity in Fig. 3C.

Of the 25 precursor-cleavage-competent proteins, 21 mediate cell-to-cell transmission, and this correlates with fusion capacity. As a rule, in these mutants, infectivity is more efficient than fusion. This is the case in particular for the Tyr170-Ser mutation, which results in secretion of the SU into the supernatant. In one instance (Asp197-Val), substantial viral transmission was observed although this mutant exhibited very little syncytium formation. This finding again suggests that infectivity requires less glycoprotein than does cell fusion, as

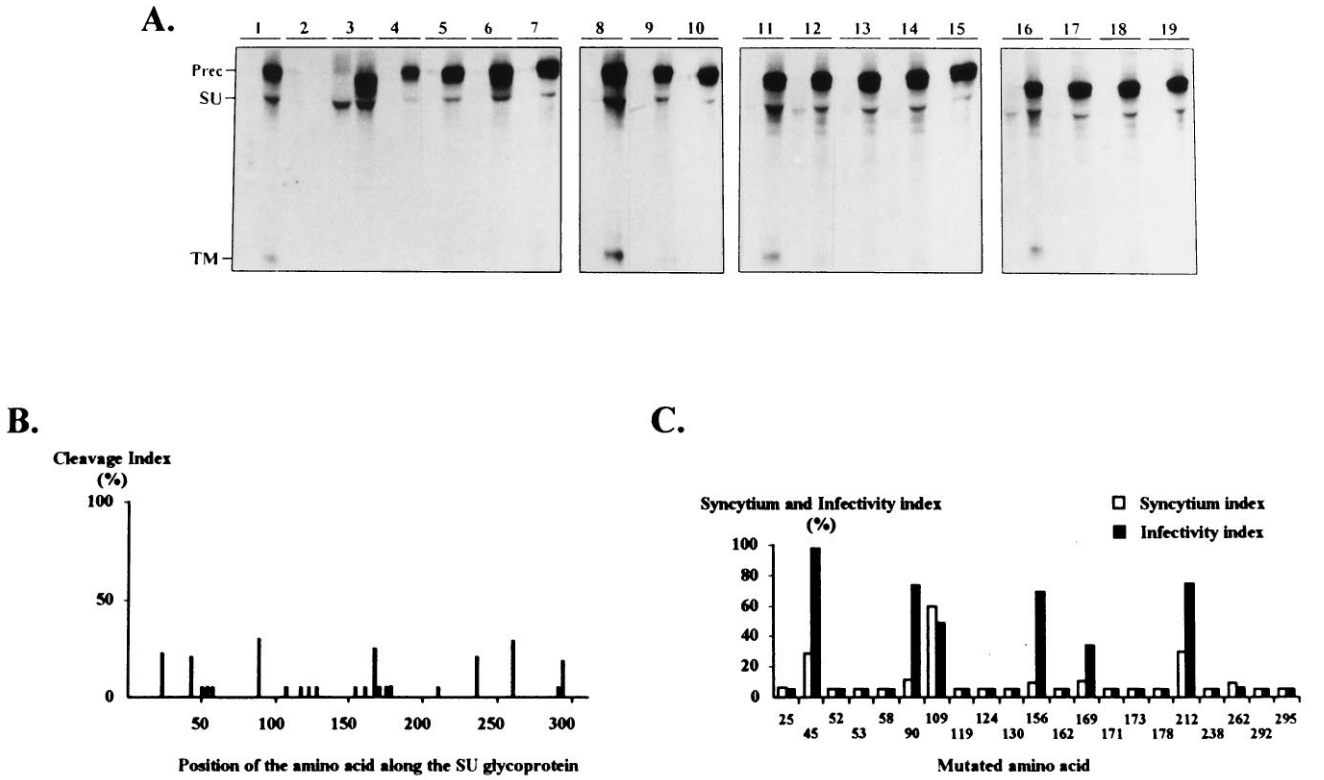

FIG. 2. Mutated glycoproteins with low precursor cleavage. (A) Immunoprecipitation of the envelope glycoproteins from transfected COS-1 cells. In each case, the left and right lanes correspond to immunoprecipitation of cell supernatant and cell lysate, respectively. Lanes 1, 8, 11, and 16, wild-type envelope positive control (cells transfected with the CMV-ENV construct); lane 2, negative control (cells transfected with the CMV-ENVΔPvuII construct); lane 3, soluble glycoprotein construct (cells transfected with the HTE-438 construct [33]); lane 4, Ser25-Arg; lane 5, Gln45-Leu; lane 6, Leu52-Arg; lane 7, Asp53-Val; lane 9, Lys90-Ile; lane 10, Tyr124-Asp; lane 12, Gly169-Ala; lane 13, Asp171-Tyr; lane 14, Ile173-Ser; lane 15, His238-Leu, lane 17, Leu262-His; lane 18, Ser292-Tyr; lane 19, Leu295-Arg. Prec, precursor. (B) Schematic representation of the cleavage indices of the mutated glycoproteins plotted against their positions along the SU glycoprotein. (C) Schematic representation of the syncytium formation and infectivity indices of the mutated glycoproteins.

observed (see above) with three of the cleavage-defective mutated glycoproteins.

However, four mutated glycoproteins (Asn93-Ile, Arg94-Glu, Ser101-Leu, and Ser194-Phe) do not fulfill the above rule, since their fusion capacity is higher than their cell-to-cell transmission capacity. Two of them (Arg94-Glu and Ser101-Leu) have a significantly reduced infectivity despite substantial fusion capacity, as measured by syncytium formation. This finding shows that fusion capacity is not sufficient to confer infectivity.

Cell surface expression and virion incorporation of the two SU glycoprotein mutants with fusion capacity but no infectivity. To determine the phenotypes of the two mutated glycoproteins with no infectivity despite substantial fusion capacity, it was important to quantitate the glycoprotein levels at the cell surface and in the virions. Immunofluorescence experiments were performed to examine cell surface expression of the Arg94-Glu and Ser101-Leu mutated glycoproteins. The results appear in Fig. 4A, which shows that the two proteins are expressed at the cell surface.

We also monitored incorporation of the two glycoproteins into virions (Fig. 4B). It should be first noted that the variation range that we observe between negative (Fig. 4B, lane 1) and positive (Fig. 4B, lane 2) incorporation is quite narrow. The negative control consists of the glycoproteins detected in the gradient, in the absence of pCS-HTLV-neo coexpression with the envelope expressor. In this case, no viral particles should form. However, after the required 2-month exposure of the gels, we consistently detect glycoproteins in this control, despite filtration and sucrose sedimentation. This is probably due to cell membrane debris contamination of the gradient (Fig. 4B, lane 1). The level of wild-type glycoprotein incorporation detected is quite low (Fig. 4B, lane 2), despite the very long exposure of the gels. This means either that incorporation is very low in HTLV-1 or, more likely, that the glycoproteins are very unstable once at the virion surface. The two mutated glycoproteins with discordance between fusion capacity and infectivity were tested in this assay. Whereas the Arg94-Glu mutated glycoprotein seems to be normally incorporated into the viral particle (compare lanes 2 and 3 in Fig. 4B), the amount of incorporation of the Ser101-Leu mutant appears to be lower (Fig. 4B; compare lanes 1, 2, and 4). These results show that the infectivity defect has different causes for the two mutated glycoproteins. In the case of Ser101-Leu, the mutation results in several differences with respect to the wild-type glycoprotein which could all contribute to the infectivity defect: low surface expression, reduced fusion capacity, and low or no incorporation of the glycoprotein into virions, which is indicative of a modified glycoprotein presentation at the cell surface. In Arg94-Glu, the absence of infectivity is not due to such deficiencies.

DISCUSSION

The HTLV-1 infectivity assay developed in this study is the first to permit the evaluation of cell-to-cell transmission of HTLV-1 in a single round of infection. Since this assay requires the coculture of cells transfected with a provirus together with indicator cells, it was important to rule out the possibility that plasmid transmission had occurred between

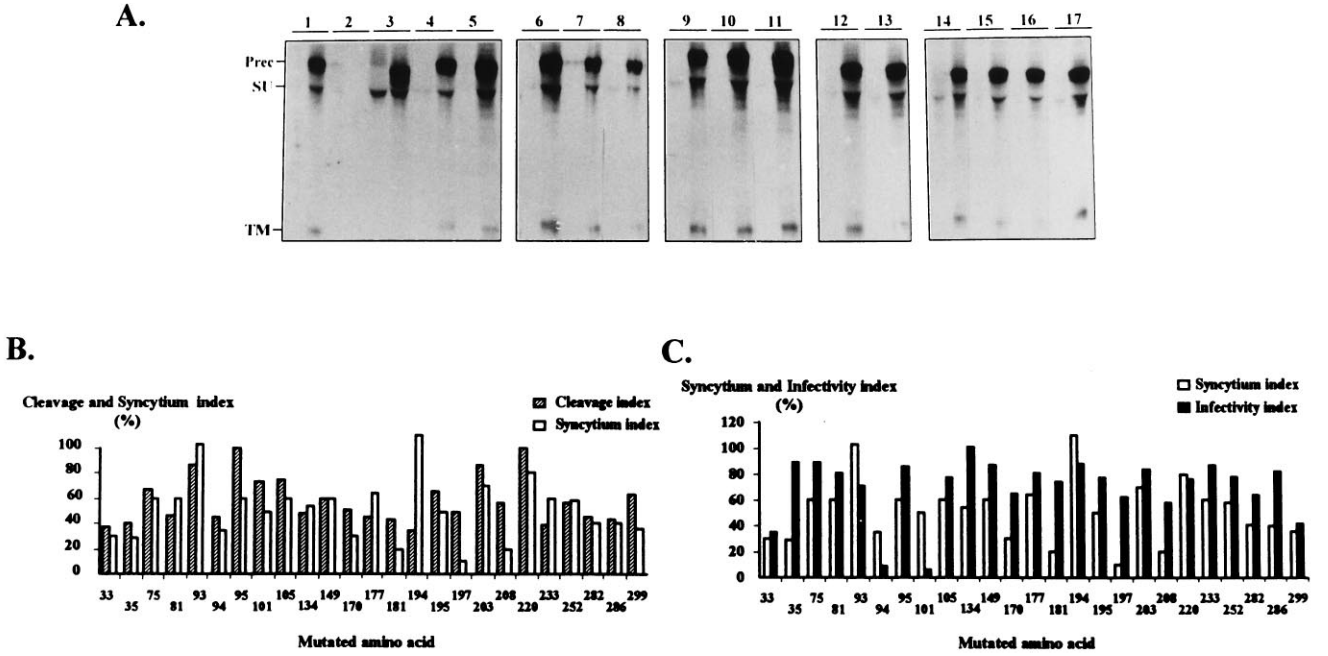

FIG. 3. Mutated glycoproteins with substantial precursor cleavage. (A) Immunoprecipitation of the envelope glycoproteins from transfected COS-1 cells. In each case, the left and right lanes correspond to immunoprecipitation of cell supernatant and cell lysate, respectively. Lanes 1, 6, 9, 12, and 14, wild-type envelope positive control (cells transfected with the CMV-ENV construct); lane 2, negative control (cells transfected with the CMV-ENVΔPvuII construct); lane 3, soluble glycoprotein construct (cells transfected with the HTE-438 construct, [33]); lane 4, Val33-Asp; lane 5, Ser35-Leu; lane 7, Asn93-Ile; lane 8, Arg94-Glu; lane 10, Lys134-Thr; lane 11, Asn149-Ile; lane 13, Asn177-Tyr; lane 15, Ser252-Phe; lane 16, Gln282-Leu; lane 17, Ser299-Tyr. Prec, precursor. (B) Schematic representation of the cleavage and syncytium indices of the mutated glycoproteins, plotted against their positions along the SU. (C) Schematic representation of the syncytium formation and infectivity indices of the mutated glycoproteins.

cells. We have demonstrated that transmission depends on glycoprotein expression. Furthermore, transmission requires a reverse transcription step. Cell-to-cell transmission was compared to transmission with free viral particles. When filtered particles were used instead of transfected cells to infect the B5 cells, no resistant colony indicative of infection could be obtained. This shows that in this assay, HTLV-1 transmission with free virus particles is at least 100 times less efficient than cell-to-cell transmission. It would be interesting to quantify cell-free and cell-to-cell transmission for other retroviruses and to determine whether the difference in efficiency of the two modes of transmission lies within the same range in the other retroviruses. This comparison may give a clue to explain why HTLV-1 is almost exclusively transmitted via cells rather than via free particles in vivo and whether this is an intrinsic property of HTLV-1 or just the reflection of a lower threshold of infectivity compared to other retroviruses.

The infectivity assay allowed us to examine the involvement of HTLV-1 SU glycoprotein in the transmission process, using a collection of 46 mutated SU glycoproteins, in order to determine whether this glycoprotein behaves like the other retroviral SU glycoproteins. As expected, cell-to-cell transmission is not observed in the absence of glycoproteins or when the mutated glycoprotein precursor is not cleaved, as indicated by the results obtained with the vast majority of the mutated glycoproteins displaying a very low amount of precursor cleavage. However, three mutated glycoproteins which are cleavage defective (Ser109-Phe, Lys156-Ile, and Thr212-Ile) can efficiently induce cell fusion or infectivity. This odd phenotype could indicate a very low sensitivity of the cleavage detection compared to that of the functional assays. However, these three mutants are as infectious as some of the cleavage-positive viruses. An alternative explanation to the cleavage-negative infectivity-positive phenotype would be that in some instance, a transported uncleaved glycoprotein is biologically active.

The conformation of the SU which allows precursor cleavage depends on amino acids which span the entire SU glycoprotein, although as we previously noted (8), some of them cluster in the central part of the molecule. In most mutants, this defect in cleavage is probably due to a retention of the misfolded proteins in the endoplasmic reticulum, which prevents their transport to the Golgi compartment, where cleavage normally occurs. How do these mutations affect the conformation of the HTLV-1 SU glycoprotein? It is extremely difficult to speculate on the three-dimensional conformation of the molecule in the absence of a crystal structure for any retroviral glycoprotein and in the absence of data concerning intrachain disulfide bondings for the HTLV-1 glycoprotein. A model of the retroviral SU glycoproteins has been proposed recently (15). According to this model, all of the SU would have three structural and functional domains interspersed by conserved hinges. In the present work, we have introduced mutations into the GYDPI motif, a motif conserved among oncoretroviruses which was proposed as one of the hinges. This motif is located at positions 169 to 173 in the HTLV-1 glycoprotein. Most mutations in this motif result in the absence of precursor cleavage. This shows the importance of this motif for glycoprotein conformation, since the absence of cleavage very likely results from retention in the endoplasmic reticulum due to misfolding of the protein (22). Mutation of the tyrosine at position 170 in the motif results in secretion of the SU into the supernatant (8). This finding indicates that this tyrosine residue in the hinge is fundamental for protein-protein interactions, probably involved in SU-to-TM association. Confirmation that such a phenotype exists for other oncoretroviral SU glycoproteins would be an interesting finding.

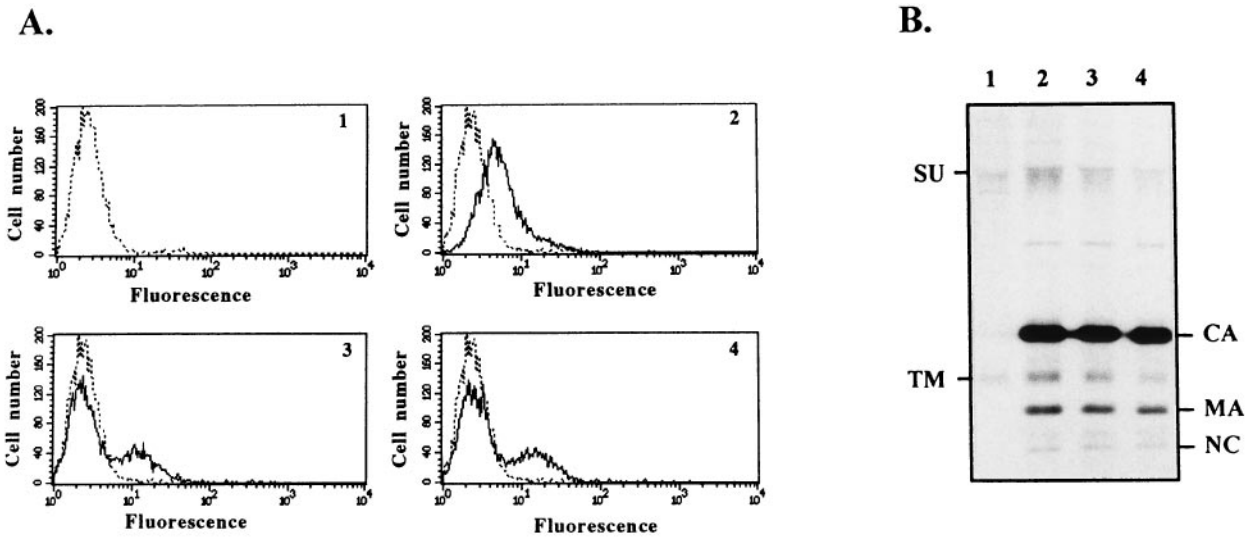

FIG. 4. Cell surface expression and virion incorporation of the mutated glycoproteins with fusion capacity but no infectivity. (A) Cell surface expression, as measured by indirect immunofluorescence. 1, negative control (cells transfected with the CMV-ENVΔPvuII construct); 2, wild-type envelope positive control (cells transfected with the CMV-ENV construct); 3, cells transfected with the Arg94-Glu construct; 4, cells transfected with the Ser101-Leu construct. (B) Incorporation of glycoproteins into virions. Lane 1, negative control (cells transfected with the CMV-ENV construct alone); lane 2, wild-type envelope positive control (cells cotransfected with the CMV-ENV and pCS-HTLV-neo constructs); lane 3, cells cotransfected with the Arg94-Glu construct; lane 4, cells cotransfected with the Ser101-Leu construct.

In this study, we found that the fusion competence of the HTLV-1 glycoprotein involves amino acids along the entire length of the SU. This includes two regions previously defined by us and others (8, 11, 25, 31, 34, 43), around amino acids 100 and 194 of the SU. These regions are known to be targets for neutralizing antibodies. Amino acids located in the C terminus of the SU glycoprotein, at positions 208 and 299, are shown here to be also important for fusion. The exact role of each of these domains in the fusion process cannot be determined, since no existing assay allows discrimination between binding and fusion per se by the HTLV-1 glycoprotein. Also, since no antienvelope conformational monoclonal antibody is currently available, it is not possible to determine whether the various amino acids important for fusion are all part of the same epitope, as has been demonstrated for the HIV-1 glycoprotein (44, 45).

The mutated proteins with correct precursor cleavage permitted a comparison of the amino acid requirements for fusion, as indicated by syncytium formation, and for infectivity. We observed that the cell-to-cell transmission is in general (21 of 25 mutated glycoproteins) less affected by mutations of the SU glycoprotein than is the fusion capacity and that a glycoprotein with almost no fusion capacity in the syncytium assay could still mediate cell-to-cell transmission. This finding could indicate that the amount of envelope required at the cell surface for full membrane fusion between cells is higher than that required for viral transmission, which might imply that limited (in area) local fusions are sufficient to ensure viral transmission. Transmission competence in fusion-defective mutants was previously observed with HIV-1 glycoproteins (19). That cell-to-cell fusion requires less glycoprotein than does viral transmission might thus be a general rule for retroviruses rather than a special feature of HTLV-1.

Finally, we also obtained two mutated glycoproteins with the reciprocal phenotype, i.e., a substantial fusion capacity but very low cell-to-cell transmission capacity. One mutation (Ser101-Leu) results in several differences with respect to the wild-type glycoprotein which could all contribute to the infectivity defect: low surface expression, reduced fusion capacity, and low or no incorporation of the glycoprotein into virions, indicative of defects in protein presentation at the cell surface. The other mutated glycoprotein (Arg94-Glu) seems normally incorporated into the virion. Existence of this phenotype suggests that the HTLV-1 SU glycoprotein could be implicated in postfusion events required for cell-to-cell transmission of the virus. In other retroviruses, and in HIV-1 in particular, the SU glycoprotein is believed to play a role in receptor binding as well as in postbinding events required for the fusion process itself. These postbinding events include conformational changes of the SU required for the exposure of the TM N-terminal fusion peptide (50). Mutations in the HIV-2 SU (14) and in the HIV-1 TM (19) glycoproteins have also revealed the existence of additional events required, after fusion, for full infectivity. These postfusion events are poorly defined but could include glycoprotein conformational changes required for entry of the viral core into the target cell. Their exact nature remains to be defined.

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