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Human T-Cell Leukemia Virus Type I Envelope Protein Maturation Process: Requirements for Syncytium Formation

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The human T-cell leukemia virus type I (HTLV-I) envelope protein is synthesized as a gp61 precursor product cleaved into two mature proteins, a gp45 exterior protein and a gp20 anchoring the envelope at the cell membrane. Using N-glycosylation inhibitors and site-directed mutagenesis of the potential glycosylation sites, we have studied the HTLV-I envelope intracellular maturation requirements for syncytium formation. We show here that experimental conditions resulting in the absence of precursor cleavage (tunicamycin, monensin treatments, and use of inhibitors of the reticulum steps of the N glycosylations) also result in no cell surface expression of envelope protein. The lack of syncytium formation observed in these cases is thus explained by incorrect intracellular transport. When the precursor is cleaved in the Golgi stack (no treatment or treatment with inhibitors of the Golgi steps of the N glycosylations), it is transported to the cell surface in all the cases examined. Syncytium formation is markedly reduced, however, when Golgi glycosylations are incorrect, which shows that the sugar moieties are involved in the envelope functions. Site-directed mutagenesis demonstrates that each of the five potential glycosylation sites is actually glycosylated. Glycosylation of sites 1 and 5 is required for normal maturation, whereas that of sites 2, 3, and 4 is dispensable. Glycosylation of each site, however, is required for normal syncytium formation. Altogether, the restraints exerted by the cell for the HTLV-I envelope to be transported and functional are very high, which might play a role in the observed conservation of the envelope amino acid sequence between various strains.

The human T-cell leukemia virus type I (HTLV-I) is the causative agent of adult T-cell leukemia (31, 47) and is associated with a neurological disease, tropical spastic paraparesis, also called HTLV-I-associated myelopathy (12, 26). The HTLV-I virally encoded envelope, as with other retroviruses (6), is synthesized as a precursor gp61 protein, which is cleaved into two products (16, 21): a small gp20 product, responsible for anchoring the protein at the cell membrane, and a larger gp45 product, probably responsible for the interaction of the envelope with a yet undefined target receptor, encoded by a gene assigned to the human chromosome 17 (37).

Prior to the virus budding from the cell, the viral envelope has to be expressed at the cell membrane and thus has to follow the intracellular transport leading to cell surface expression. As for most surface glycoproteins, this transport depends upon a correct folding of the protein in the endoplasmic reticulum and intrachain disulfide bonding, as well as normal oligomerization of the protein (for a review, see reference 17). Cell surface proteins are N glycosylated, and the contribution of the glycosylations to the efficiency of assembly and transport varies between proteins: in some cases, blocking of the glycosylation does not affect the efficiency of assembly or transport, while other proteins are retained in the endoplasmic reticulum when glycosylations are affected (17). In addition to their role in the intracellular transport, the sugar moieties can play a direct role in the protein function itself.

The posttranslational modifications involved in the viral envelope protein's maturation and function have been widely studied with viruses (1, 45) as well as with retroviruses (5, 8, 24, 29). In the human immunodeficiency virus type 1 (HIV-1), it has been shown that N-glycosylation inhibitors abolish both the syncytium-forming ability of infected cells and the infectivity of viral particles (5, 15, 28, 43). Moreover, in vitro deglycosylation decreases the interaction between CD4 and purified (23) or virus-associated (10) gp120 envelope protein. The sugar moieties might also play a role in postbinding events, since castanospermine, an inhibitor of the early steps of the glycosylation process, does not affect the binding capacity of the HIV envelope but does affect its ability to form syncytia (43).

The HTLV-I envelope maturation process has not been fully analyzed so far. A complete understanding of the HTLV-I envelope maturation process should be useful as a basis for future mutational analysis designed to define functional regions of this protein. Since this envelope bears only five potential glycosylation sites (Asn-X-Ser/Thr) (36), the contribution of each of these sites to the envelope functions can be directly studied. The HTLV-I envelope is a very conserved protein among different isolates (3, 4, 14, 18, 27, 35); it is also a very small protein in size compared with other retroviral envelopes. We have previously shown that introduction of random mutations along the HTLV-I envelope gene results, in most cases, in a nonfunctional envelope (30). The limiting step is the intracellular maturation which is usually affected. To further analyze whether the level of stringency in the intracellular quality control is high for the HTLV-I envelope, we have studied the HTLV-I envelope maturation process and analyzed the glycosylation contribution to the envelope function using N-glycosylation inhibitors and site-directed mutagenesis.

MATERIALS AND METHODS

Cell lines. The COS-1 cells, simian virus 40-transformed African green monkey kidney cells (13), were obtained from the American Type Culture Collection and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal

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calf serum. The sarcoma virus-transformed XC cells (38) used as indicator cells in the syncytium formation assay were a generous gift from S. Gisselbrecht (Hôpital Cochin, Paris, France). They were grown in minimum essential medium containing 10% fetal calf serum. The C91/PL cell line is an HTLV-I-infected T-cell line (32) and was grown in RPMI 1640 medium supplemented with 10% fetal calf serum. All cell lines were maintained at 37°C in a 5% CO_2 atmosphere.

HTLV-I envelope expressor plasmid. The HTLV-I envelope expressor plasmid is the HTE-1 expressor previously described (7). Briefly, HTE-1 contains an HTLV-I promoter and all the viral sequences corresponding to the *env*, *tax*, and *rex* genes. A negative-control plasmid (HTE-24stop) was obtained by the insertion of a stop codon at position 5251 (36) in the *env* gene at the *Pvu*II restriction site. In the envelope protein sequence, this is located just after the sequence coding for the signal peptide.

Transfection procedure. Plasmid (3 μg) was transfected into 0.5×10^6 COS-1 cells according to the procedure described by Cullen (2) with DEAE dextran and chloroquine (Sigma, La Verpillière, France). At 48 h posttransfection, cells were labeled for immunoprecipitation or used for syncytium formation assay.

Glycosylation inhibitors used in this study. The glycosylation inhibitors were obtained from Sigma and were added into the labeling medium for 16 h at final concentrations of 2.4 μM tunicamycin, 3 mM deoxynojirimycin (DNM), 5 mM 1-deoxymannojirimycin (MNM), 60 μM swainsonine, and 1 μM monensin.

Metabolic labeling of transfected or infected cells. C91/PL infected cells or COS-1 transfected cells were labeled for 16 h with 3 ml of cysteine-free RPMI medium containing 100 μCi of [^{35}S]cysteine per ml (Amersham, Les Ulis, France) and 10% dialyzed fetal calf serum. Cells were lysed as described previously (30), and the lysates were used for immunoprecipitation.

Cell surface labeling. Radioiodination of surface proteins was performed by using the lactoperoxidase method (22). The cells (2×10^6) were washed twice in phosphate-buffered saline (PBS) containing 1 mM of $CaCl_2$ and $MgCl_2$ to prevent cellular aggregation and resuspended in 200 μl of the same medium. Then, 0.2 mCi of ^{125}I (Amersham) was added, and supplementation with 10 μl of lactoperoxidase (200 IU/ml; Calbiochem Corp., La Jolla, Calif.) and 10 μl of H_2O_2 (0.1%) was done every 5 min. After 20 min, the labeling reaction was stopped by the addition of 10 μl of sodium bisulfite (10 mg/ml). The labeled cells were washed twice in PBS and lysed in 500 μl of buffer containing 50 mM Tris (pH 8.0), 0.15 M NaCl, 20 mM iodoacetamide, 5 mM $MgCl_2$, 5 mM KCl, 1% Nonidet P-40, and 50 mM phenylmethylsulfonyl fluoride. Lysates were then directly used for immunoprecipitation.

Immunoprecipitation of the envelope products. The envelope products were immunoprecipitated as previously described (30) using a tropical spastic paraparesis patient's serum (a gift from A. Lever, London, United Kingdom). For endoglycosidase H (endoH) digestion, immunoprecipitates were washed twice in 50 mM sodium acetate (pH 5.5) containing 0.025% sodium dodecyl sulfate (SDS) and then digested with 8 mU of endoH (Sigma) for 6 h at 37°C. The envelope proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions, unless otherwise stated.

Syncytium formation assay. The assay was previously described (30) and involved the coculture of envelope bearing cells with indicator XC cells. After 24 h of coculture, Giemsa coloration was performed, and syncytia containing more than 20 nuclei were then counted with an inverted microscope.

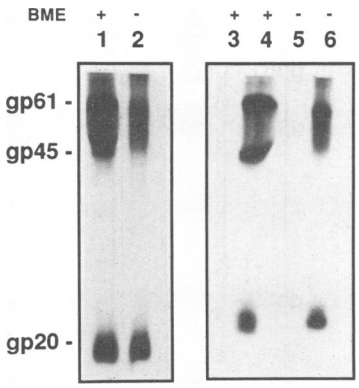

FIG. 1. Electrophoretic analysis of the HTLV-I envelope proteins under reducing and nonreducing conditions. Lanes: 1 and 2, immunoprecipitates from C91/PL-infected cells; 3 to 6, immunoprecipitates from COS-1 cells transfected with the HTE-24stop negative-control plasmid (lanes 3 and 5) or with the HTE-1 envelope expressor (lanes 4 and 6). The addition of beta-mercaptoethanol (BME) is indicated.

Site-directed mutagenesis of the glycosylation sites. For each glycosylation site (Asn-X-Ser/Thr), the codon corresponding to the asparagine was changed to a glutamine. The site-directed mutagenesis was performed by using the Kunkel method (20). A 448-bp *Sph*I-*Sal*I fragment and a 778-bp *Xho*I-*Xho*I envelope fragment were subcloned into the pGEM-7ZF(+) plasmid (Promega). Single-stranded DNA template containing uracil was prepared into the *ung* mutant *dut* mutant *Escherichia coli* bacterial strain RZ1032 (a gift from P. Legrain, Institut Pasteur, Paris, France) after superinfection with the M13K07 helper phage. The mutagenic oligonucleotide (20-mer) was hybridized to the single-stranded DNA, and the double-stranded DNA was then synthesized with T4 DNA polymerase and T4 DNA ligase (Boehringer). The reaction mixture was used to transform *ung*$^+$ *dut*$^+$ *E. coli* HB101. After plasmid DNA preparation, the mutations were confirmed by direct DNA sequencing with a Sequenase kit version 2.0 as recommended by the manufacturer (United States Biochemical Corp., Cleveland, Ohio). The envelope fragments bearing the desired mutation were then subcloned into the HTE-1 plasmid, and the mutated envelope expressor was resequenced in the envelope region before being used for transfection.

RESULTS

The HTLV-I envelope mature products are not covalently associated. To analyze whether the gp45 and the gp20 mature envelope products are covalently associated, the immunoprecipitated envelope products obtained from infected cells (Fig. 1, lanes 1 and 2) or transfected cells (lanes 3 to 6) were run onto acrylamide gels, under reducing or nonreducing conditions. It can be seen in Fig. 1 that under nonreducing conditions (lanes 2 and 6), the mobility of the gp20 is the same as under reducing conditions (lanes 1 and 4). This glycoprotein is thus not covalently linked to the gp45 protein. Under reducing conditions, the migration of the larger envelope mature product is slightly affected (lanes 2 and 6);

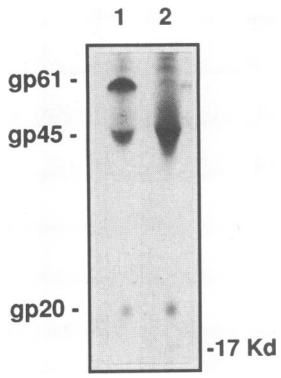

FIG. 2. EndoH digestion of the HTLV-I envelope products. Lentil-lectin-absorbed glycoproteins from HTE-1-transfected COS-1 lysates were immunoprecipitated and untreated (lane 1) or treated with endoH (lane 2) before electrophoretic separation.

this fact may indicate a high amount of intrachain disulfide bonding.

N-glycosylation type of the HTLV-I envelope products. Whether the gp61, gp45, and gp20 envelope proteins belong to the mannose-rich, hybrid, or complex-type glycoprotein was determined with endoH. Once the glycosylation process has been achieved, this enzyme is able to eliminate the sugar residues of mannose-rich or hybrid glycoproteins but not those of complex glycoproteins (41). As shown in Fig. 2 (lane 2), the gp61 precursor is totally sensitive to endoH digestion, which brings its molecular size down to 43 kDa. The gp61 thus belongs to the mannose-rich or hybrid-type glycoproteins. In contrast, the gp45 outer membrane protein is totally resistant to endoH digestion since no smaller product can be detected. This result identifies the gp45 as a complex-type glycoprotein. The anchored-membrane protein gp20 is partially digested by endoH, leading to a 17-kDa product in addition to residual gp20. An incomplete digestion of gp20 by endoH seems unlikely, since with the same conditions, the gp61 was totally digested. The gp20 is thus probably a mixture of both mannose-rich or hybrid glycoproteins and complex glycoproteins.

Effect of glycosylation inhibitors on the intracellular envelope maturation process. Inhibitors affecting different steps of the glycosylation process were used, and their effect on the maturation of envelope products was analyzed by SDS-PAGE. Tunicamycin is an antibiotic which prevents any N glycosylation (9). DNM, a glucosidase inhibitor (34), affects early steps of the glycosylation process occurring in the endoplasmic reticulum. The intracellular transport from the endoplasmic reticulum to the Golgi complex can be blocked by the addition of the carboxylic ionophore monensin (39). MNM (11) and swainsonine (42) are inhibitors of mannosidase I and II, respectively, which are enzymes working in the late stages of the glycosylation process in the Golgi stack.

The effects of the inhibitors on HTLV-I-infected cells or HTE-1-transfected cells were analyzed and are shown in Fig. 3. Tunicamycin treatment leads to the accumulation of a 42-kDa product (Fig. 3A, lanes 3 [negative control] and 4) in the absence of any detectable gp45 and gp20. This corresponds to the envelope peptidic core in the absence of any glycosylation. In the presence of DNM, a glucosidase I inhibitor, the majority of the envelope proteins are in the uncleaved form, since only small amounts of gp45 and gp20

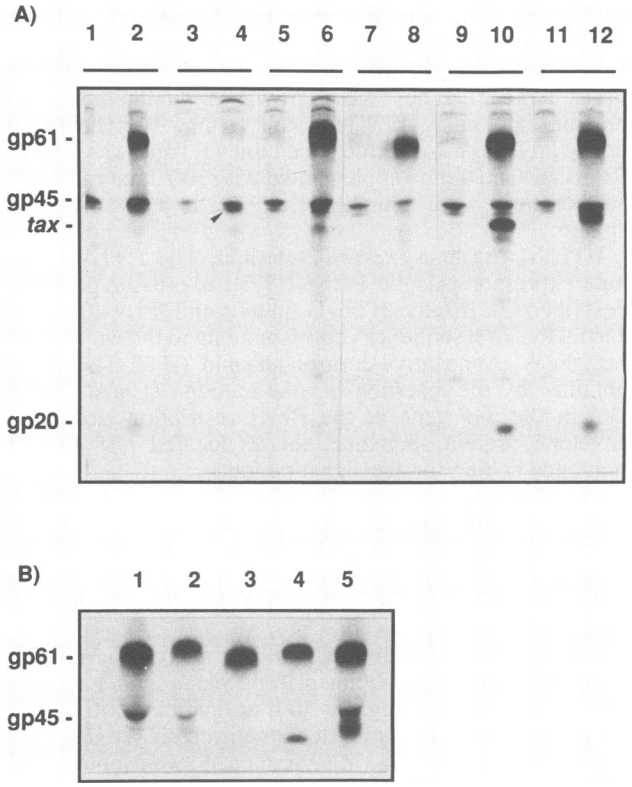

FIG. 3. Immunoprecipitation of the envelope proteins after treatment with glycosylation inhibitors. (A) Proteins obtained from COS-1 cells transfected with a negative-control plasmid (lanes 1, 3, 5, 7, 9, and 11) or with the HTE-1 envelope expressor (lanes 2, 4, 6, 8, 10 and 12). Two days after transfection, COS-1 cells were incubated for 16 h with medium alone (lanes 1 and 2) or with medium containing tunicamycin (lanes 3 and 4; the arrow indicates the 42-kDa protein described in Results), DNM (lanes 5 and 6), monensin (lanes 7 and 8), MNM (lanes 9 and 10), or swainsonine (lanes 11 and 12). (B) Immunoprecipitates from HTLV-I-infected C91/PL cells, untreated (lane 1) or treated with DNM (lane 2), monensin (lane 3), MNM (lane 4), or swainsonine (lane 5). Inhibitor concentrations are described in Materials and Methods.

are visible (Fig. 3A, lanes 5 [negative control] and 6), whereas more slowly migrating products (61 to 65 kDa) are detected. The presence of a small quantity of gp45 and gp20 mature products might be due to incomplete inhibition of the glycoprotein processing by the inhibitor or may represent proteins synthesized before the addition of the inhibitors and stable long enough to be detected.

Addition of monensin also results in the accumulation of an uncleaved product (Fig. 3A, lanes 7 [negative control] and 8) with an electrophoretic mobility identical to that of the precursor protein seen with untreated cells. No mature product can be detected.

The blocking of mannosidases I and II was done with MNM or swainsonine treatment, respectively. These inhibitors have no effect on the gp61 cleavage since a normal level of gp20 is observed (Fig. 3A, lanes 10 and 12 and controls lanes 9 and 11). The molecular size of the outer envelope protein is affected by these inhibitors since proteins of 39 kDa (MNM, lane 10) and 41 kDa (swainsonine, lane 12) are detected. The electrophoretic mobilities of the gp61 and gp20 do not vary under these conditions.

The results obtained with HTE-1-transfected cells or

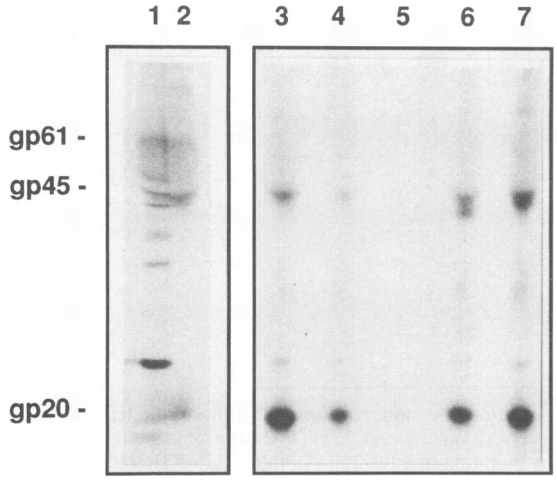

FIG. 4. Radioiodination of surface proteins after glycosylation inhibitor treatment. C91/PL cells were incubated with medium alone (lane 3) or with medium containing DNM (lane 4), monensin (lane 5), MNM (lane 6), or swainsonine (lane 7). Cells were labeled with ^{125}I and lysed, and the envelope proteins were immunoprecipitated as described in Materials and Methods. Crude immunoprecipitate (lane 1) or lentil-lectin-retained glycoproteins (lane 2) from [^{35}S]cysteine labeled C91/PL-infected cell lysates were used as migration controls.

C91/PL HTLV-I-infected cells are in every case similar (compare panels A and B in Fig. 3) for all the inhibitors (data not shown for tunicamycin).

Effect of the glycosylation inhibitors on the cell surface expression of the envelope products. Cell surface expression of the different envelope products in the C91/PL cells was monitored by labeling the cell surface proteins with iodine. The results obtained after treatment with the glycosylation inhibitors are shown in Fig. 4. In each case, the same amount of labeled lysate was loaded onto the gel (125 μg of proteins per lane). In untreated cells (Fig. 4, lane 3), only gp45 and gp20 can be detected, showing that the gp61 envelope precursor is not transported to the cell surface. In the presence of DNM (Fig. 4, lane 4) partial inhibition of surface expression is observed, whereas in the presence of the mannosidase inhibitors (MNM and swainsonine, lanes 6 and 7, respectively), the amount of gp45 and gp20 detected is comparable to that of untreated cells. This shows that the intracellular transport of the envelope from the Golgi complex to the cell surface is not affected by these inhibitors. Faster migration products can be also detected in addition to gp45. These proteins are efficiently transported to the cell membrane. In contrast, monensin (Fig. 4, lane 5) completely abrogates the transport of the envelope proteins at the cell surface.

Effects of the glycosylation inhibitors on syncytium formation. HTE-1-transfected cells were treated for 16 h with the various inhibitors and then cocultivated for an additional 16 h with XC cells before syncytium formation scoring. The results in Table 1 show that there was no syncytium formation after treatment with tunicamycin or monensin. This correlates with the absence of envelope mature products observed in the cells treated with these inhibitors (Fig. 3). In the presence of DNM, a highly reduced amount of syncytia was observed. The residual syncytia may be due to the low amounts of gp45 and gp20 which were detectable in the immunoprecipitation (Fig. 3, lane 6). Treatment with a mannosidase I inhibitor, MNM, resulted in a low level of syncytium formation, whereas treatment with the mannosidase II inhibitor, swainsonine, allowed 50% of syncytium formation relative to that with the untreated cells. As the reduction of syncytium formation is not a consequence of defective transport of the molecule to the surface (Fig. 4), these results show that the glycosylations play a role in the envelope functions.

TABLE 1. Effects of glycosylation inhibitors on envelope cleavage, surface expression, and syncytium formation

Cell treatment	Precursor cleavage[a]	Transport to the membrane[b]	Syncytium formation[c]
None	++++	++++	++++
Tunicamycin	−	NT[d]	−
Monensin	−	−	−
DNM	+	+	+/−
MNM	++++	+++	+
Swainsonine	++++	++++	++

[a] Data from metabolic labeling (Fig. 3).
[b] Data from surface labeling (Fig. 4).
[c] Number of syncytia per well: ++++, about 50 syncytia; ++, about 25 syncytia; +, about 10 syncytia; +/−, about 5 syncytia; −, less than 3 syncytia.
[d] NT, Not tested.

Effect of site-directed mutagenesis of the glycosylation sites on envelope maturation and syncytium formation. Each of the five potential glycosylation sites (Asn-X-Ser/Thr) present on the envelope sequence was mutated individually. To do so, the asparagine was changed to a glutamine residue. Sites were numbered 1 to 5, 1 being the most N-terminal one and 5 being the most C-terminal one. The different mutants are described in Table 2; they were named dGX, with X referring to the position (1 to 5) of the mutated glycosylation sites. The maturation process and the syncytium-forming capacity of the different mutants were analyzed after transfection of the constructions into COS-1 cells, and the results appear in Fig. 5. For each of the single-site mutants, a 59-kDa precursor protein can be immunoprecipitated from the transfected

TABLE 2. Effects of site-directed mutagenesis of glycosylation sites on envelope cleavage and syncytium formation

Plasmid	Position of mutations in amino acid sequence[a]	Apparent mol size of precursor (kDa)	Precursor cleavage	Syncytium formation[b]
HTE-24stop[c]	24	None	−	−
HTE-1	none	61	++++	++++
dG1[d]	140	59	−	−
dG2	222	59	+	+
dG3	244	59	++	−
dG4	272	59	++	+
dG5	404	59	−	−
dG3/4	244/272	54	+	−
dG1/3/4	140/244/272	49	−	−
dG3/4/5	244/272/404	48	−	−
dG1/2/3/5	140/222/244/404	47	−	−
dG1/3/4/5	140/244/272/404	47	−	−
dG2/3/4/5	222/244/272/404	47	−	−

[a] Counting from the Met corresponding to the initiation codon of the envelope.
[b] Four independent experiments were performed. ++++, about 50 syncytia per well; +, about 10 syncytia per well; −, less than 3 syncytia per well.
[c] Introduction of a nonsense codon.
[d] In each potential N-glycosylation site, the asparagine codon was changed to a glutamine codon.

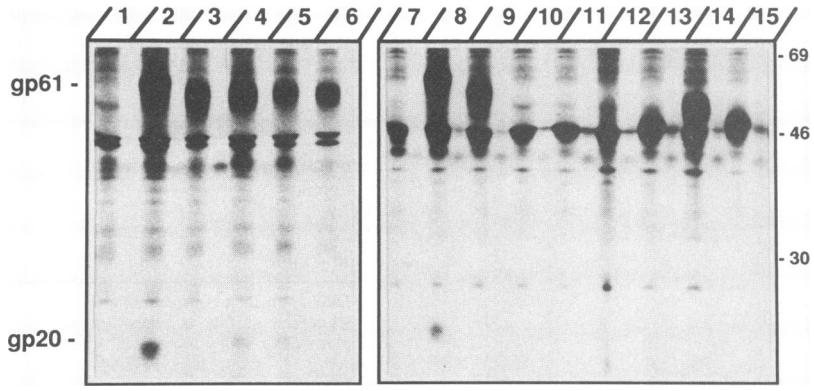

FIG. 5. Immunoprecipitation of the envelope proteins expressed by the glycosylation site mutants. For each mutant, the left and right lanes correspond to immunoprecipitation of cell lysate and cell supernatant, respectively. Lanes: 1 and 7, HTE-24stop negative control; 2 and 8, HTE-1; 3, dG1; 4, dG3; 5, dG4; 6, dG5; 9, dG2; 10, dG2/3/4/5, i.e., simultaneous mutation of sites 2, 3, 4, and 5; 11, dG1/3/4/5; 12, dG1/2/3/5; 13, dG3/4/5; 14, dG3/4; 15, dG1/3/4. The descriptions of the mutations are given in Table 2. On the right, molecular weight markers are indicated.

cells. This suggests that each site bears an equivalent amount of oligosaccharides. Effects of the mutations on gp61 cleavage are also shown in Fig. 5. It can be seen that mutation of site 1 (dG1) or of site 5 (dG5) results in the absence of precursor cleavage since neither gp45 nor gp20 is detected (Fig. 5, lanes 3 and 6, respectively). When sites 3 or 4 are mutated (dG3 and dG4 mutants), a partial cleavage can be observed (lanes 4 and 5, respectively). Likewise, partial cleavage products were detectable with cells transfected with dG2 and dG3/4 mutants, albeit to a very low level (only visible on the original film). For mutants giving a partial cleavage, the migration of the anchored envelope protein is slightly increased (giving an apparent molecular size of 22 kDa). This could be a consequence of a difference in protein folding. As the gp45 protein is masked by the background of proteins migrating around 45 kDa (Fig. 5, lanes 1 and 7, negative control), the sizes of the mutated outer envelope proteins in dG2, dG3, and dG4 could not be determined. In the dG4 mutant, the amount of the gp59 precursor is decreased relative to the other mutants, perhaps indicating decreased stability of the protein when site 4 is mutated (lane 5). Mutants with several deleted glycosylation sites were also constructed, resulting in mutated envelopes bearing two, three, or four mutated glycosylation sites. The results shown in Fig. 5 show that maturation of the envelope protein could not be observed for any of these mutants except for the dG3/4 mutant in which a low level of gp20 is detected (lane 14).

The effects of the glycosylation site mutations on syncytium formation were also analyzed. Results are shown in Table 2. It can be seen that in all cases, syncytium formation was greatly diminished (mutation at glycosylation sites 2 and 4) or abolished (all other mutants).

DISCUSSION

We have analyzed the HTLV-I envelope maturation process as well as the glycosylation requirements for functionality of this protein. Our results first show that the envelope mature products gp45 and gp20 are not covalently associated, as they migrate as distinct proteins under nonreducing conditions (Fig. 1). In this regard, the HTLV-I envelope is different from the avian retroviral envelope mature products, which are linked by disulfide bonds (6). In contrast, there is no covalent association between the mature HIV-1 envelope products (19), and shedding of gp120 from the cell surface is often observed with this virus. With HTLV-I, however, we have never been able to immunoprecipitate envelope external protein from infected or transfected cell supernatants (30). This means that either the association between gp45 and gp20 is very tight or the gp45 when released from the cell is very unstable. The larger envelope mature protein migration is slightly different in reducing and nonreducing conditions, an observation made with other systems when a large amount of intrachain disulfide bonding has been demonstrated. The exterior envelope protein sequence indeed bears 14 cysteine residues (36), which could account for such bond formation. We also showed that the HTLV-I envelope precursor protein is a mannose-rich glycoprotein, as shown by its complete sensitivity to endoH digestion (Fig. 2). This is also the case with the HIV-1 envelope precursor protein (28) and corroborates prior work by Lee et al. (21). The glycosylations of the mature HTLV-I envelope proteins are of the complex type for the gp45 and consist of both mannose-rich or hybrid and complex glycosylations for the gp20 protein. With the HUT-102-infected human cells (21), it was previously suggested that the gp45 is sensitive to endoH digestion. This discrepancy might be due to variation of glycosylations in monkey COS cells versus human cells, although we have never observed any difference between the human C91/PL-infected cells and the COS-1 transfected cells when glycosylation inhibitors were used.

We have observed a lack of envelope precursor cleavage with several experimental situations. These included treatment blocking the entire glycosylation process (tunicamycin) or treatment preventing vesicular transport (monensin) (39), as well as inhibition of the early steps of the glycosylation process (DNM). The HTLV-I envelope cleavage takes place after transport in the Golgi, as shown by the absence of precursor cleavage observed after treatment with monensin (Fig. 3). In the absence of cleavage of the precursor, transport to the cell surface does not seem to occur (Fig. 4), probably because the envelope precursor is retained in the endoplasmic reticulum. It is well-known that the cell imposes conformational transport restraints on unassembled as well as misfolded proteins. Misfolding or incorrect oligomerization usually results in retention in the endoplasmic reticulum (17). Whether the HTLV-I envelope protein forms oligomers is not known yet, but this is very probable since membrane glycoproteins in general and retroviral envelopes

in particular (8, 40, 44) tend to be oligomeric. Our results suggest that glycosylation of the HTLV-I envelope precursor is implicated in oligomer formation and/or in correct folding of the envelope protein. Proteins retained in the endoplasmic reticulum are degraded at variable rates. The nonglycosylated HTLV-I envelope precursor appears rather stable as it accumulates in amounts comparable to that of the normally glycosylated precursor.

Our results show that correct cleavage correlates with cell surface expression of the envelope, as observed when the cells are untreated or treated with inhibitors effective at late stages of the glycosylation process (MNM and swainsonine) (Fig. 3 and 4). With these conditions, the intracellular transport of the envelope protein being unaffected, the role of the glycosylations in the envelope functions can be examined. Our results show that the glycan moieties are implicated in posttransport events: they play a role in the envelope functions since the syncytium formation capability is greatly affected when cells are treated with MNM and swainsonine (Table 1). This could mean either that sugar chains participate directly in envelope functions (by binding to the receptor) or influence the local conformation of functional regions such that their absence gives rise to dysfunction. With HIV-1, the glycosylations have been shown to play a role both in the binding of the envelope to the CD4 receptor (23) and in postbinding events (43) or in infectivity (5, 15, 28). It has also been suggested that abnormal glycosylation of the envelope protein accounts for the lack of infectivity of a feline retrovirus (33). For the HTLV-I envelope, the exact stage at which glycosylations are implicated remains to be established, but this is rendered difficult by the lack of an available binding assay.

The individual contributions of the five glycosylation sites of the HTLV-I envelope protein were analyzed. The asparagine required for N glycosylation was changed to a glutamine in each of the five positions individually, a conservative change in terms of amino acids. Our results show that each of the five potential sites is indeed glycosylated, as opposed to what was previously proposed from careful sequence analysis of the HTLV-I envelope gene sequence (25). A normal glycosylation of sites 1 and 5 is essential for normal intracellular maturation, since no cleavage is observed when these sites are mutated (Fig. 5). As discussed above, the absence of cleavage likely means retention in the endoplasmic reticulum. In this regard, the observed inability of these mutants to form syncytia is interpreted as a consequence of the absence of cell surface expression. Sites 2, 3, and 4 do not need be glycosylated for a correct envelope intracellular transport, since a partial cleavage was observed in the corresponding mutants. Glycosylation of these sites is, however, required for functionality of the envelope as measured by syncytium formation (Table 2), again showing the role of the sugar moieties in the envelope functions. Any combination of two or more glycosylation site mutations results in a lack of syncytium formation. Taken together, these results demonstrate that each of the glycosylation sites of the HTLV-I envelope needs be glycosylated for the envelope to be fully functional, either because it is important for intracellular transport or because it is involved in the functions.

From all the results discussed above, several rules emerge, which should be useful for designing or interpreting mutagenesis of the HTLV-I envelope protein. (i) The absence of precursor cleavage probably means retention in the endoplasmic reticulum, i.e., defect in transport. A mutant with such a phenotype is useless for functional studies. (ii) Cleavage occurs in the Golgi stack, and once the protein has reached the Golgi, it goes to the cell surface in all the cases examined to date. Observation of cleaved products is thus probably indicative of cell surface expression. (iii) Most of the nonfunctional mutants are likely to be defective in the maturation process. Indeed, any mutation affecting the glycosylation process (this study) and most of the mutations affecting the peptidic backbone (our previous study [30]) result in retention of the precursor in the endoplasmic reticulum. These rules should be kept in mind if one wants to define an HTLV-I envelope receptor-binding region.

Finally, our results show that the intracellular maturation process of the HTLV-I envelope is a very tightly controlled process. Indeed, affecting the glycosylations, either by preventing terminal branching of the sugar moieties on all the sites or by removing an individual site, results in a nonfunctional protein. In a previous work, we have shown that alteration of the peptidic backbone also leads, in most cases, to a nonfunctional protein (30). The HTLV-I envelope protein is a very conserved envelope compared with the HIV-1 one, for instance (3, 4, 14, 18, 27, 35). This conservation probably results from the convergence of several features, including (i) host parameters, like immune surveillance; (ii) the virus biology, as the replication rate of HTLV-I is probably low and the virus might be essentially transmitted from cell to cell as a gene included in the cellular genome (46); and (iii) the intrinsic characteristics of the viral proteins, which have to be functional for the virus to survive. In this regard, the HTLV-I envelope is a very "demanding" protein, since slightly affecting any of its components results in a lack of functionality. This is probably one of the features contributing to its conservation.

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