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The human and simian immunodeficiency virus envelope glycoprotein transmembrane subunits are palmitoylated

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ABSTRACT **The envelope proteins of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) were found to be modified by fatty acylation of the transmembrane protein subunit gp41. The precursor gp160 was also palmitoylated prior to its cleavage into the gp120 and gp41 subunits. The palmitic acid label was sensitive to treatment with hydroxylamine or 2-mercaptoethanol, indicating that the linkage is through a thioester bond. Treatment with cycloheximide did not prevent the incorporation of [^{3}H]palmitic acid into the HIV envelope protein, indicating that palmitoylation is a posttranslation modification. In contrast to other glycoproteins, which are palmitoylated at cysteine residues within or close to the membrane-spanning hydrophobic domain, the palmitoylation of the HIV-1 envelope proteins occurs on two cysteine residues, Cys-764 and Cys-837, which are 59 and 132 amino acids, respectively, from the proposed membrane-spanning domain of gp41. Sequence comparison revealed that one of these residues (Cys-764) is conserved in the cytoplasmic domains of almost all HIV-1 isolates and is located very close to an amphipathic region which has been postulated to bind to the plasma membrane.**

The envelope proteins of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) are critical for virus infection, as they mediate the binding and fusion to the target cell membranes which are necessary for virus entry into target cells. The envelope protein is synthesized as a gp160 precursor which is cleaved into two subunits, the surface (SU) protein gp120 and the transmembrane (TM) protein gp41, by a cellular protease (1–3). The gp120 subunit mediates binding to the cellular CD4 receptor, while the gp41 subunit contains a hydrophobic domain at its amino terminus which is involved in the viral fusion activity (4, 5). The HIV envelope proteins are extensively modified posttranslationally by both N-linked and O-linked glycosylation (6–8) and sulfation (9). The posttranslational glycosylation of HIV envelope proteins has been shown to be important for the biological functions of the virus (10, 11).

The first proteins that were found to be palmitoylated were the glycoproteins of Sindbis virus and vesicular stomatitis virus (VSV) (12, 13). Subsequently, a number of other viral and cellular proteins have been found to be modified by fatty acylation (14). The function of protein palmitoylation is being elucidated, and it has been reported to affect signal transduction, receptor modulation, protein localization, and lateral diffusion on cell membranes (15–18). The isolation of palmitoyl thioesterases (19) indicates that protein palmitoylation is a dynamic, reversible process, which may play a role in regulation of protein functions (20). Recently the cellular receptor for HIV, CD4, has been reported to be palmitoylated (21). In this study, we have investigated the palmitoylation of the HIV and SIV envelope proteins and have determined that the sites of palmitic acid attachment to the lentivirus envelope proteins exhibit unusual features which may play a role in the interaction of their cytoplasmic domains with cell membranes.

MATERIALS AND METHODS

Cells and Viruses. HeLa and HeLa T4 cells were maintained as described (22). Construction of vaccinia virus recombinants which express full-length and truncated SIVmac239 has been described (22). Recombinant vaccinia virus (vTF7-3) expressing T7 RNA polymerase was provided by B. Moss (National Institutes of Health, Bethesda, MD).

Plasmid Construction and Mutagenesis. The HIV (strain HXB2) envelope protein gene was excised from the plasmid pFN (23) at *Bbs* II and *Xho* I sites and inserted into plasmid pSP72 (Promega) at *Eco*RI and *Xho* I sites under the control of the T7 promoter. The resulting construct has 30 nucleotides between the start codon of the HIV envelope protein and the T7 promoter and is designated as pSP72envEB. HIV envelope protein mutants were generated by oligonucleotide-directed mutagenesis using M13 single-strand DNA mutagenesis (24). The codons for Cys-764 and Cys-837 were changed into codons for serine either individually or together, and the resulting constructs are designated as HC764S, HC837S, or HC764/837S. Primers used for mutagenesis were 5′-CGGAGCCTGTCCCTCTTCAGC-3′ for Cys-764 and 5′-CAAGGAGCTTCTAGAGCTATT-3′ for Cys-837. Mutations were confirmed by DNA sequencing (25).

Protein Expression, Metabolic Labeling, and Immunoprecipitation. Expression with vaccinia virus recombinants was done as described (22). For protein expression using the vaccinia virus/T7 system (26), HeLa cells grown to confluence were infected by vTF7-3, a recombinant vaccinia virus expressing T7 RNA polymerase, at a multiplicity of infection of 10 for 1.5 hr, and then transfected by plasmid DNA constructs by using Lipofectin (GIBCO/BRL) in Dulbecco's modified Eagle's medium (DMEM). Where indicated, cycloheximide was added at a concentration of 50 μg/ml and was present throughout the labeling process. After labeling with either [^{3}H]palmitic acid or [^{3}H]leucine (NEN/DuPont), cells were lysed with lysis buffer (150 mM NaCl/50 mM Tris·HCl, pH 7.5/1 mM ethylenediamine tetraacetate/1% Triton X-100/1% sodium deoxycholate) and immunoprecipitated with appropriate antibodies and staphylococcal protein A-agarose (Pierce) at 4°C overnight. Samples were prepared in either reducing or nonreducing sample buffer [125 mM Tris·HCl, pH 7.5/4% sodium dodecyl sulfate (SDS)/20% (vol/vol) glycerol, plus 10% (vol/vol) 2-mercaptoethanol for 2× reducing sample buffer]. Samples were heated at 95°C for 5 min before SDS/PAGE (27).

Hydroxylamine Treatment. Duplicate samples were subjected to SDS/PAGE, and one of the gels was then treated with either 1 M hydroxylamine, pH 7.5, or 1 M Tris·HCl, pH 7.5, at

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Abbreviations: HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; SU protein, surface protein; TM protein, transmembrane protein.

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room temperature overnight. All protein gels were treated with EnHance (NEN/DuPont) and then fluorographed with Kodak film. Densitometer analysis was performed on a Macintosh (IIci) computer using the public domain National Institutes of Health IMAGE program (written by W. Rasband).

Fusion Assay. The fusion activities of HIV envelope constructs were analyzed by using a quantitative cell fusion assay (28). Confluent HeLa T4 cells in 35-mm dishes were infected with vaccinia virus strain IHD-J at a multiplicity of infection of 10 for 1.5 hr and then transfected with plasmid pG1NT7β-gal (provided by E. Berger, National Institutes of Health, Bethesda) by using Lipofectin. At the same time, HeLa cells grown to confluence were infected with vTF7-3 and transfected with plasmid constructs containing wild-type or mutant HIV envelope genes by using Lipofectin. Twelve hours after infection, infected and transfected HeLa and HeLa T4 cells were detached from plates by treatment with 2 mM EDTA (in Ca^{2+}- and Mg^{2+}-deficient phosphate-buffered saline) and suspended in DMEM with 10% bovine calf serum. They were then mixed and dispensed into individual wells of a 96-well flat-bottom tissue culture plate. In fused cells, plasmid pG1NT7β-gal, which contains the β-galactosidase gene under the phage T7 promoter, will be transcribed by the T7 polymerase produced by vTF7-3, and β-galactosidase will be produced. The extent of cell fusion was quantitated by a colorimetric assay to determine the amount of β-galactosidase (nanograms) produced at different time points.

RESULTS

SIV and HIV Envelope Glycoproteins Are Palmitoylated. To examine whether the envelope proteins of lentiviruses could be labeled with [^{3}H]palmitic acid, we first investigated the HIV-1 envelope protein. As shown in Fig. 1, the HIV-1 envelope protein precursor gp160 and TM protein subunit gp41, but not the SU protein subunit gp120, were labeled by [^{3}H]palmitic acid, whereas all three proteins were labeled with [^{3}H]leucine (Fig. 1, comparing lanes 1 and 3). We obtained similar results for the envelope proteins of SIVmac239 (see below, Fig. 4) and HIV-2/ST (data not shown). These results indicate that the envelope glycoproteins of HIV and SIV are palmitoylated and that this modification occurs on the TM protein subunits.

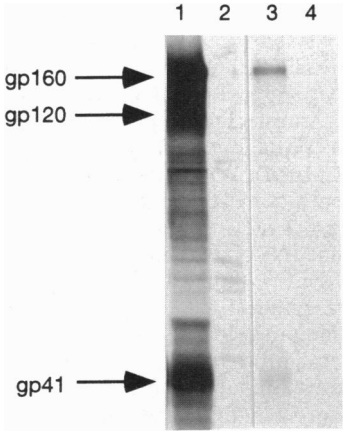

FIG. 1. Labeling of the HIV-1 envelope proteins with [^{3}H]palmitate. HeLa cells were infected with vTF7-3, followed by transfection with pSP72envEB (HIV-1 env) or pSP72 (control). At 12 hr after infection, cells were labeled with either 50 μCi of [^{3}H]leucine (lanes 1 and 2) or 500 μCi of [^{3}H]palmitic acid (lanes 3 and 4) (1 μCi = 37 kBq). Cells were then lysed and immunoprecipitated with polyclonal antibody against HIV-1 and protein A-agarose. Protein samples were prepared using nonreducing sample buffer and analyzed by SDS/PAGE. Lane 1, HIV-1 env labeled with [^{3}H]leucine; lane 2, control cells labeled with [^{3}H]leucine; lane 3, HIV-1 env labeled with [^{3}H]palmitic acid; lane 4, control cells labeled with [^{3}H]palmitic acid.

Previous studies have shown that palmitic acid is usually attached to proteins through a thioester bond to cysteine residues, although some studies indicate a hydroxyester linkage to serine residues (14). The thioester bond can be distinguished from a hydroxyester bond by its sensitivity to treatment by reducing agents (29). We observed that using a reducing sample buffer decreased the amount of [^{3}H]palmitic acid labeling in the HIV envelope protein TM subunit by more than 50% (Fig. 2*A*), whereas the labeling of the HIV envelope proteins with [^{3}H]leucine was not affected. We also observed that after treatment with hydroxylamine (Fig. 2*B*), the [^{3}H]palmitate label of the TM protein was completely removed, while the labeling with [^{3}H]leucine was unaffected. These results indicate that the labeling of HIV envelope proteins with [^{3}H]palmitic acid occurs through a thioester linkage, and they therefore strongly suggest that the HIV envelope glycoproteins are palmitoylated on cysteine residues.

To determine whether treatment with a protein synthesis inhibitor would prevent the palmitoylation of the HIV envelope protein, we treated cells with cycloheximide at 50 μg/ml at various time points before labeling with either [^{3}H]palmitic acid or [^{3}H]leucine. As shown in Fig. 3, cycloheximide treatment reduced the labeling of the HIV envelope protein with [^{3}H]leucine. As determined by densitometer analysis, after 90-min preincubation with cycloheximide at 50 μg/ml, the labeling with [^{3}H]leucine was reduced by about 90%. In contrast, the labeling with [^{3}H]palmitic acid was not affected significantly after 90-min preincubation. These results indicate that the palmitoylation of the HIV envelope proteins is a posttranslational event.

Localization of the Site of Palmitoylation. Earlier studies have suggested that the palmitoylation of most viral glycoproteins occurs on cysteine residues within or proximal to their membrane-spanning domains (14). However, examination of the sequences of the HIV and SIV envelope proteins revealed

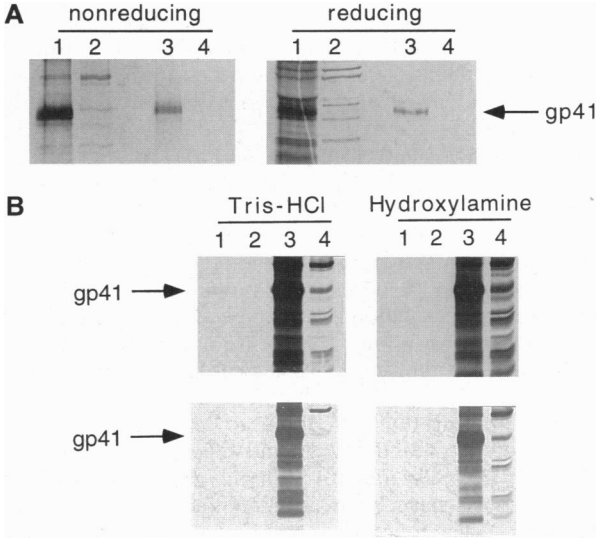

FIG. 2. Effect of treatment with reducing reagents or hydroxylamine. (*A*) Duplicate protein samples labeled with either [^{3}H]palmitic acid or [^{3}H]leucine were prepared by mixing with either reducing or nonreducing sample buffer and were analyzed by SDS/PAGE. Lane 1, HIV-1 envelope protein (env) labeled with [^{3}H]leucine; lane 2, control cells labeled with [^{3}H]leucine; lane 3, HIV-1 env labeled with [^{3}H]palmitic acid; lane 4, control cells labeled with [^{3}H]palmitic acid. (*B*) Protein samples labeled with [^{3}H]palmitic acid (lanes 1 and 2 for HIV-1 env and control, respectively) or [^{3}H]leucine (lanes 3 and 4 for HIV-1 env and control, respectively) were prepared by mixing with nonreducing sample buffer and analyzed by SDS/PAGE. The protein gels were then treated with either hydroxylamine (1 M, pH 7.5) or Tris·HCl (1 M, pH 7.5). The lower panels are shorter exposures of the upper panels.

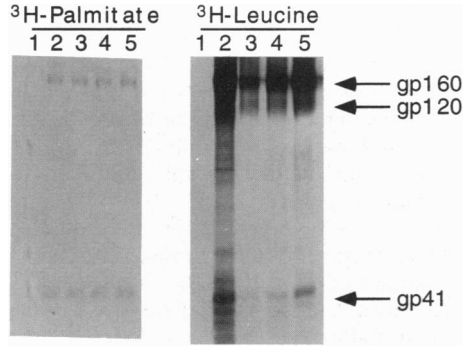

FIG. 3. Resistance of palmitoylation to treatment with cycloheximide. HeLa cells were infected with vTF7-3 and transfected with pSP72envEB (HIV-1 env) as described for Fig. 1. Cycloheximide (final concentration 50 μg/ml) was added to the cells at various times before labeling. Cells were then labeled with either [^{3}H]palmitic acid or [^{3}H]leucine for 2 hr. The cycloheximide was present throughout the labeling period. The cells were then lysed, and proteins were immunoprecipitated and analyzed by SDS/PAGE. Lane 1, control with no cycloheximide added; lane 2, HIV-1 env with no cycloheximide added; lane 3, HIV-1 env with cycloheximide added at 90 min before labeling; lane 4, HIV-1 env with cycloheximide added at 45 min before labeling; and lane 5, HIV-1 env with cycloheximide added at 15 min before labeling.

that there is no cysteine residue within or close to their transmembrane domains. To locate the site where palmitic acid is attached on the envelope proteins, we initially compared the labeling of a series of truncated forms (22) of the SIVmac239 envelope proteins. As shown in Fig. 4, the precursor gp160 and the TM protein subunit gp41 of the full-length SIVmac239 envelope proteins were labeled by [^{3}H]palmitic acid, but the SU protein subunit gp120 was not. A truncated form in which a stop codon was introduced after amino acid 826 was also found to be palmitoylated. However, truncated SIVmac239 envelope proteins containing 781 or 733 amino acids were not labeled by [^{3}H]palmitic acid, while they are readily labeled by [^{3}H]leucine (lanes 3 and 4). These results indicate that palmitic acid is linked to the 45 amino acid region between residues 781 and 826, which encompasses the single cysteine residue, Cys-787, in the cytoplasmic tail of the SIVmac239 envelope protein. Sequence comparison revealed that

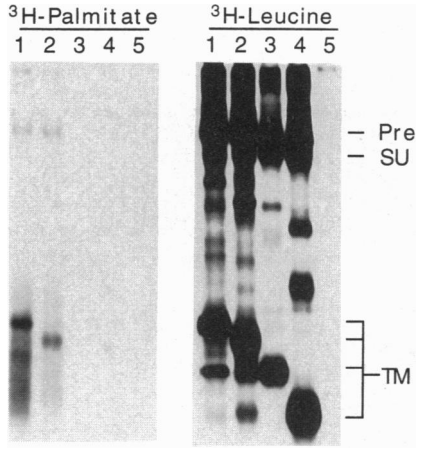

FIG. 4. Labeling by [^{3}H]palmitate of full-length and truncated SIVmac239 envelope proteins. HeLa T4 cells were infected with recombinant vaccinia viruses expressing full-length or truncated SIVmac239 envelope proteins. At 12 hr after infection, cells were labeled with either 50 μCi of [^{3}H]leucine or 500 μCi of [^{3}H]palmitic acid for 4.5 hr. Lane 1, SIVmac239; lane 2, SIVmac239-826T; lane 3, SIVmac239-781T; lane 4, SIVmac239-733T; and lane 5, VVSC11 (control recombinant vaccinia virus). Pre, precursor gp160.

Table 1. Sequence comparison of HIV-1, HIV-2, and SIV subgroups

Virus subgroup	Consensus sequence
HIV-1 group M	
Clade A	AWDDLRSL**C**LFSYHRLRDFILIAA
Clade B	IWDDLRSL**C**LFSYHRLRDLLLIVA
Clade C	AWDDLRSL**C**LFSYHRLRDFILVAA
Clade D	IWDDLRNL**C**LFSYHRLRDLILIAT
Clade E	AWDDLRSL**C**LFSYHRLRDFILIAA
Clade F	VWDDLRNL**C**LFSYHHLRDLILIAA
Clade G	AWDDLRSL**C**LFSYHRLRDFILIVA
HIV-1 group O	LGQKTIEA**C**RLC*AV*QYWLQELQ
HIV-2	LLTWLYSI**C**RDLLSR*FQTLQPIS
SIV	LLTWLFSN**C**RTLLSRAYQILQPIL

The sequences shown above are the consensus sequences of each clade of HIV-1 group M (residues 818–841), HIV-1 group O (807–830), HIV-2 (773–796), and SIV (789–802), numbered as in refs. 30–32. The conserved cysteine residues are shown in boldface. *, Residues not showing consensus within a subgroup..

Cys-787 is conserved among various SIV and HIV-2 isolates (see Table 1).

The envelope protein of HIV-1 strain HXB2 has two cysteine residues in its cytoplasmic tail, Cys-764 and Cys-837. On the basis of their sequence homology, HIV-1 isolates have been grouped into two groups (M and O), and there are several subgroups (clades) within the major group M (30–32). Shown in Table 1 are the consensus sequences of a cytoplasmic tail region in the envelope protein of HIV-1 group M clades A–G, HIV-1 group O, HIV-2, and SIV. As shown by the sequence comparison, one cysteine residue (Cys-764 in HXB2) is conserved among almost all HIV-1 group M isolates. A cysteine residue is also conserved in a region of the envelope protein in HIV-1 group O, HIV-2, and SIV, and some sequence similarity is evident among these viruses.

To determine whether the palmitic acid is linked to cysteine residues in the cytoplasmic tail, we mutated the two cysteine residues in the HXB2 envelope protein cytoplasmic tail individually or together into serine residues by oligonucleotide-directed mutagenesis. These wild-type and mutant HIV-1 envelope proteins were expressed by using the vaccinia virus T7 system and labeled with [^{3}H]palmitic acid (Fig. 5). We observed that the TM proteins of mutants HC764S and HC837S, in which only one of the cysteine residues was mutated, could still be labeled with [^{3}H]palmitic acid (lanes 3 and 4), whereas the TM protein of mutant HC764/837S, in which both cysteine residues were mutated into serines, was not labeled by [^{3}H]palmitic acid (lane 5). A similar level of protein expression was observed with wild-type envelope TM protein, mutant HC764S, and mutant HC764/837S, as shown by labeling with [^{3}H]leucine. The expression level of mutant HC837S as well as its labeling with [^{3}H]leucine was considerably lower than that of the wild-type envelope TM protein

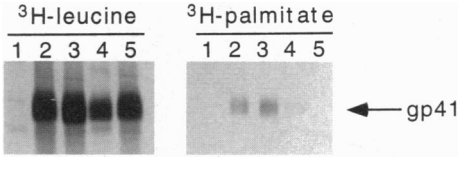

FIG. 5. Effect of cysteine mutations on labeling of the HIV-1 envelope protein with [^{3}H]palmitate. HeLa cells were infected with vTF7-3 followed by transfection with plasmid constructs containing the wild-type or mutant HIV-1 envelope (env) gene. At 12 hr after infection, cells were labeled with either 50 μCi of [^{3}H]leucine or 500 μCi of [^{3}H]palmitic acid for 4.5 hr. Protein samples were prepared using nonreducing sample buffer and analyzed by SDS/PAGE. Lane 1, control; lane 2, HIV-1 env; lane 3, HIV-1 env C764S; lane 4, HIV-1 env C837S; lane 5, HIV-1 env C764/837S.

(comparing lane 4 to lane 2), which possibly resulted from variation in transfection efficiency. We therefore conclude that palmitoylation of the HIV envelope proteins occurs on the two cysteine residues in the cytoplasmic tail of the TM protein.

The Palmitoylation State of the HIV Envelope Protein Does Not Affect Its Cell Fusion Activity. We further investigated whether the palmitoylation state of the envelope protein affects its function in mediating cell fusion, using a quantitative cell fusion assay developed by Nussbaum *et al.* (28). Fig. 6 shows the time course of cell fusion as determined by the accumulation of β-galactosidase. The amounts of β-galactosidase at each time point were similar for the wild-type and the mutant envelope proteins, indicating that palmitoylation is not required for the fusion activity of the HIV envelope protein. Pulse–chase experiments also showed that the transport, processing, and surface expression of the HIV-1 envelope protein, as well as levels of secretion of gp120, were not affected by these cysteine mutations either (data not shown).

DISCUSSION

In this study, we present evidence that the HIV and SIV envelope proteins are palmitoylated. The fact that the label is sensitive to reducing reagents, as shown by treatment with reducing sample buffer and hydroxylamine, indicates that the palmitic acid is attached through a thioester linkage to a cysteine residue. By using site-directed mutations, we determined that palmitic acid is linked to the two cysteine residues of the HIV-1 (strain HXB2) envelope protein in its cytoplasmic tail. In studying the possible functions of palmitoylation, we found that mutations in the HIV-1 envelope protein that change its palmitoylation state do not affect its cell fusion activity, intracellular transport, or surface expression. This is in accordance with results from other studies, which also showed that mutations which alter protein palmitoylation usually do not cause defects in protein transport or surface expression (21, 33).

As shown by sequence comparison, one cysteine residue (Cys-764 in HXB2) in the envelope protein cytoplasmic tail is conserved among almost all HIV-1 isolates (group M). A conserved cysteine residue is also present in the cytoplasmic domain of the envelope protein in a distant group of HIV-1 isolates, group O (30, 31), as well as HIV-2 and SIV isolates. The conservation of cysteine residues implies that they may be important for some aspect of virus infection and replication apart from cell fusion activity. Nevertheless, a few virus isolates have been identified that do not maintain this conserved cysteine residue (34–37). Some of these viruses were isolated from the central nervous system (34–36) and belong to a unique group of virus isolates which are less effective in down-regulating the surface expression of CD4 in infected cells (38). Interestingly, a recent study with α_{2A}-adrenergic receptors reported that palmitoylation of the cytoplasmic tail is important in mediating down-regulation of the receptor by prolonged agonist exposure (18).

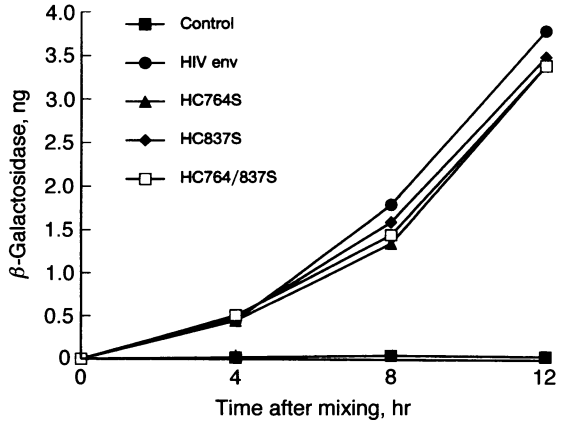

FIG. 6. Fusion activities of the wild-type and mutant HIV-1 envelope proteins. The kinetics of cell fusion induced by HIV-1 envelope protein were determined as described in the text. The amounts of β-galactosidase produced by the wild-type or mutant HIV-1 envelope proteins at intervals after mixing of HeLa and HeLa T4 cells were determined as described by Nussbaum *et al.* (28).

Although the precise functions for the palmitoylation of many cellular and viral proteins are presently unknown, several studies have indicated that the addition of palmitic acid may enhance protein association with cellular membranes (15, 39, 40). In the vast majority of cellular and viral transmembrane proteins which have been reported to be palmitoylated, the site of palmitoylation appears to be within or close to the membrane-spanning domain (14), usually less than six amino acids away from it. In contrast, the palmitoylated cysteine residues in the HIV-1 envelope cytoplasmic tail are about 59 and 132 amino acids away from the proposed membrane-spanning domain (41). However, there are two amphipathic regions in the cytoplasmic domain of the HIV envelope proteins, and it has been proposed that they could associate with the plasma membrane (42, 43). The cysteine residues required for the palmitoylation of the HIV envelope protein are very close to these amphipathic regions. It is interesting to note that the palmitoylated cysteine residue that is conserved in HIV-1 group M viruses is located at the N terminus of the first amphipathic region, whereas it is located at the C terminus of the first amphipathic region in HIV-1 group O, HIV-2, and SIV envelope proteins. Peptides corresponding to these amphipathic regions have been shown to exhibit a cytolytic effect which may play a role in HIV-induced cell lysis (43). Furthermore, Srinivas *et al.* (43) showed that acylation of a peptide mimic of segment 768–788 (the first amphipathic region) of the HIV-1 (strain WMJ-22) envelope protein increased its lipid affinity and cytolytic activity. The site of palmitoylation at the conserved cysteine residue (Cys-764 in strain WMJ-22) is only 4 amino acids from the N terminus of this region. On the basis of these studies and by analogy with other palmitoylated proteins, we propose (Fig. 7) that palmitoylation of the HIV envelope protein is a modification that enables these amphipathic regions in the cytoplasmic domains of the envelope glycoprotein to associate more tightly with the plasma mem-

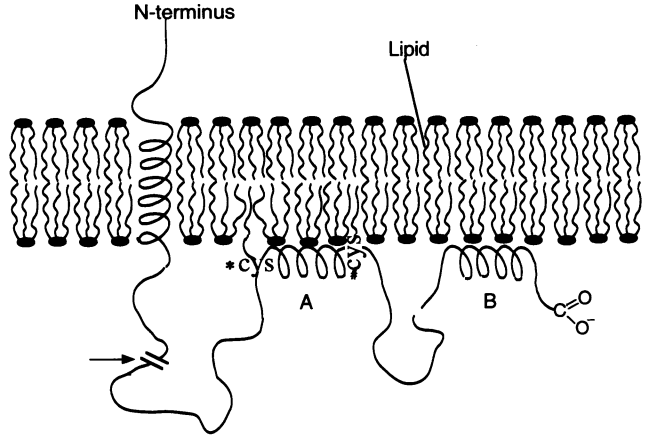

FIG. 7. Schematic diagram showing the proposed conformation of the envelope protein cytoplasmic tail. Interactions between the first amphipathic segment in the cytoplasmic tail of the HIV-1 envelope protein and the plasma membrane are stabilized by insertion of the palmitic acid on the conserved cysteine residue (*) into the plasma membrane. Also indicated is the natural truncation site in HIV-2 and SIV envelope proteins (→) and the conserved cysteine residue (#) in the cytoplasmic tail of HIV-1 group O, HIV-2, and SIV envelope proteins.

brane. The relationship between the structure of the HIV-1 envelope protein cytoplasmic tail and its biological functions is still unknown. Lentivirus envelope proteins have very long cytoplasmic tails (about 150 amino acids) compared with those of other retroviruses (less than 50 amino acids), and for HIV-2 and SIV most of the cytoplasmic tail can be deleted without affecting viability of the virus (44, 45). However, the full-length cytoplasmic tail of SIV appears to be required for induction of disease (45). The cytoplasmic tail of the HIV envelope protein has also been reported to be important for virus infectivity and cytopathicity (46–48), although it is dispensable for its cell fusion activity. It is possible that the palmitoylation of the envelope protein is necessary for proper folding of the cytoplasmic tail, which may be important for its interactions with other viral or cellular proteins. It will therefore be of interest to determine whether the palmitoylation of the HIV and SIV envelope proteins can modulate the biological properties of the viruses.

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