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Wild-Type-Like Viral Replication Potential of Human Immunodeficiency Virus Type 1 Envelope Mutants Lacking Palmitoylation Signals

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Palmitoylation of the cytoplasmic domain of the human immunodeficiency type virus type 1 (HIV-1) envelope (Env) transmembrane protein, gp41, has been implicated in Env targeting to detergent-resistant lipid rafts, Env incorporation into the virus, and viral infectivity. In contrast, we provide evidence here to show that HIV-1 infectivity, Env targeting to lipid rafts, and Env incorporation into the virus are independent of cytoplasmic tail palmitoylation. The T-cell (T)-tropic HXB2-based virus, which utilizes CXCR4 as the entry coreceptor, carrying a Cys-to-Ser mutation at residue 764 or 837 or at both replicated with wild-type (WT) virus replication kinetics in $CD4^+$ T cells. The properties of Env expression, precursor processing, cell surface expression, and Env incorporation of these three mutant viruses were normal compared to those of the WT virus. These three mutant Env proteins all effectively mediated one-cycle virus infection. When the Cys residues were replaced by Ala residues, all single and double mutants still retained the phenotypes of infectivity, Env incorporation, and lipid raft localization of the WT Env. When Cys-to-Ala substitutions were introduced into the macrophage (M)-tropic ConB virus, which utilizes CCR5 as the coreceptor, these mutations did not affect the replication potential, Env phenotypes, lipid raft targeting, or Env assembly into the virus of the WT Env. These T- and M-tropic mutants also productively replicated in human primary $CD4^+$ T cells. Moreover, mutations at both Cys residues significantly reduced the level of palmitoylation of the Env. Our results together support the notion that palmitoylation of the cytoplasmic tail of the HIV-1 Env is not essential for the HIV-1 virus life cycle.

Lipid rafts are highly specialized membrane microdomains present in both the plasma and endosomal membranes of eukaryotic cells (23, 55). These dynamic microdomains, which are organized in the lateral dimension of the plasma membrane, are characterized by detergent insolubility, light density, and enrichment of cholesterol and glycosphingolipids (for reviews, see references 3 and 55). Lipid rafts play important roles in various biological processes such as cell surface signal transduction, T-cell activation, and intracellular trafficking (17, 55, 56, 60). Many membrane-enveloped viruses also assemble and selectively bud from lipid rafts on the surfaces of infected cells; the envelope (Env) glycoproteins of some of these viruses are known to be associated with lipid rafts (2, 34, 62, 69). Acylation of viral Env proteins, particularly palmitoylation, may be important for the targeting of these Env proteins to lipid rafts (for reviews, see references 48 and 53). Lipid rafts also serve as important entry and assembly/budding sites for human immunodeficiency virus type 1 (HIV-1) (30, 33, 41, 46, 64). Removal of cellular cholesterol with β-methyl cyclodextrin was shown to reduce HIV-1 budding and infectivity and Env-mediated syncytium formation (29, 33, 44, 61). Also, depletion of cholesterol in the viral envelope may impair viral internalization (16) and permeabilize the virion envelope (15).

In retroviruses, palmitoylation may play some roles in viral replication. For instance, palmitoylation-deficient Env mutants of murine leukemia virus (MLV) are mostly soluble when extracted using ice-cold Triton X-100, and they stay at the bottom of the sucrose gradient (28). In addition, treatment of cells with β-methyl cyclodextrin abolishes the ability of the MLV Env to associate with lipid rafts (28). Those studies indicated that palmitoylation of the MLV Env is critical for lipid raft association. Nevertheless, mutant Env proteins of MLV are still able to mediate the syncytium-forming ability, suggesting that palmitoylation or raft association is not required for MLV-mediated fusion activity (28). In contrast, palmitoylation of the transmembrane (TM) protein of Rous sarcoma virus was shown to be required for protein stability and virus infectivity (42). Nevertheless, acylated Env glycoproteins of this virus are not sequestered into lipid raft domains (43). The TM proteins of HIV-1 and simian immunodeficiency virus are also posttranslationally modified by the addition of palmitate to the Cys residues located in the cytoplasmic domains of these viruses through a thioester bond (68). Palmitoylation of the T-cell (T)-tropic HXB2 strain of HIV-1 occurs on two Cys residues, Cys-764 and Cys-837, located in the cytoplasmic tail of gp41 (68). Although HIV-1 Env is associated with lipid rafts (41), whether palmitoylation of Env plays a crucial role in Env localization in lipid rafts and viral infectivity has been a longstanding question.

Rousso et al. found that substitutions of the two cytoplasmic Cys residues in the Env of the HXB-2D clone with Ser residues significantly decreased one-cycle viral infectivity by reducing incorporation of the C764S C837S double-mutant Env into the virus (50). Also, most of the double-mutant Envs could be extracted into the soluble fraction with Triton X-100 at 4°C, whereas both the C764S and C837S mutants, just like the wild-type (WT) Env, were still distributed in the membrane

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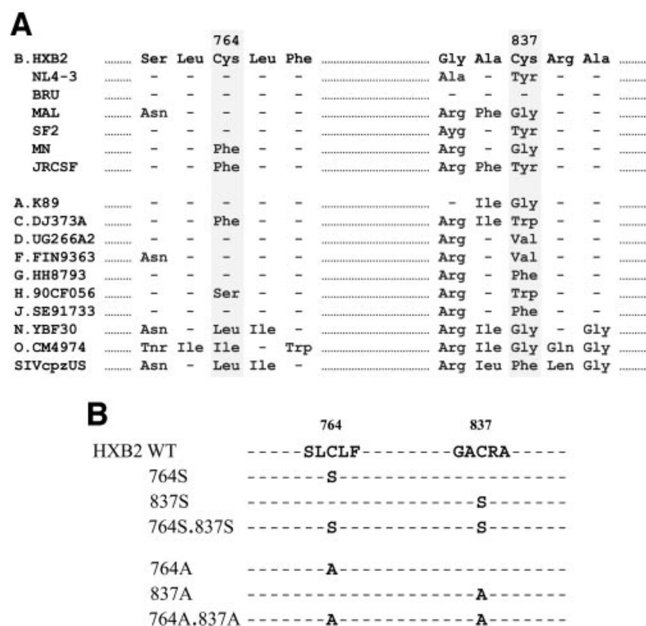


FIG. 1. Amino acid comparison of the palmitoylated Cys residues located in the cytoplasmic tail among different HIV-1 serotypes and construction of HXB2-based mutant proviruses. (A) The Cys-764 and Cys-837 amino acid residues of the putative palmitoylation sites of the HXB2 strain are compared with residues in other representative isolates from clade B and other clades of HIV-1, i.e., clades M, N, and O, and with SIVcpzUS. Dashes indicate that residues are identical to those of the HXB2 strain. Residues corresponding to the two palmitoylated Cys residues are shaded. (B) The Cys residues located at positions 764 and 837 of the Env cytoplasmic domain of the HXB2 strain were individually or together replaced by Ser or Ala residues as described in Materials and Methods.

pellets (50). Their results indicated that palmitoylation of the HIV-1 Env is critical for viral infectivity through the targeting of Env to lipid rafts. Interestingly, the two palmitoylated Cys residues located in the HXB2 strain are not highly conserved among HIV-1 clade B and different HIV-1 clades (Fig. 1A) (22). The NL4-3 virus contains only one Cys residue at position 764, whereas the 837 position in this virus is a Tyr residue (Fig. 1A). Other isolates in the B clade, such as MN and JRCSF, do not contain Cys residues at these two positions (Fig. 1A). Moreover, some representative isolates of other clades neither encode these palmitoylated Cys residues nor contain any other Cys residues in the cytoplasmic domains of their TM proteins (Fig. 1A) (22). This suggests the interesting proposal that palmitoylation of the HIV-1 Env is not required for viral replication.

Bhattacharya et al. recently reported that replacement of both Cys-764 and Tyr-837 of the NL4-3 *env* gene by a Ser or Ala residue still mediated about 10% and 40%, respectively, of the single-cycle infectivity mediated by the WT Env (5). The degree of infectivity of these two mutants paralleled the amount of mutant Env incorporated into the virus. Interestingly, these two mutants were excluded from lipid rafts. These two mutant proteins also mediated cell-cell membrane fusion with an efficiency comparable to that of the WT Env. Bhattacharya et al. also found that substitutions of both Cys-764 and Tyr-837 residues with bulky hydrophobic side chains retained the raft association property of the Env, and these mutant proteins mediated over 80% of the one-cycle infectivity of the WT Env and were effectively incorporated into the virus, implying that these mutations may compensate for the lack of a palmitate group in the cytoplasmic tail of the NL4-3 Env. They therefore concluded that the cytoplasmic cysteines are required for Env targeting to lipid rafts but are not essential for Env incorporation into the virus or for viral infectivity.

Because of the disparate conclusions as to the effects of cytoplasmic tail palmitoylation on viral infection drawn by these two groups, we reexamined the role of cytoplasmic cysteines of the HIV-1 Env in virus replication. To truly monitor replication kinetics of mutant viruses carrying a deacylated Env, we examined the effects of deacylation of both Cys residues of the T-tropic HXB2 strain Env by Ser and Ala substitutions on viral replication, using a continuous cell culture system as well as a single-cycle viral replication assay. The properties of cell surface expression, lipid raft localization, and incorporation into the virus of mutant Env proteins were also studied. We further examined whether the effects of deacylation on viral replication are related to the T or macrophage (M) tropism of the viruses examined, which, respectively, utilize chemokine receptors CXCR4 and CCR5 as the entry coreceptors (and thus are referred to as the X4 and R5 viruses, respectively). In contrast to the earlier results (5, 50), we failed to detect any altered phenotypes of the deacylated Env mutants compared to those of the WT virus in any of the analyses performed. Our results strongly indicate that palmitoylation of Env does not play a critical role in the HIV-1 life cycle.

MATERIALS AND METHODS

Cells, hybridoma, and antibodies. 293T, HeLa, and HeLa-T4 cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum (FBS). CEM-SS, PM1, and H938 cells were cultured in RPMI 1640 supplemented with 10% FBS. Hybridomas 902, Chessie 8, and 183 (clone H12-5C), which produce mouse monoclonal antibodies (MAbs) reactive with HIV-1 gp120, gp41, and p24, respectively, and sheep anti-gp120 were as previously described (9). The SIM2 hybridoma secreted a MAb that specifically recognizes human CD4. A MAb directed against the glycoprotein G of vesicular stomatitis virus (VSV) was purchased from Sigma (St. Louis, MO).

Construction of plasmids. For construction of Cys-to-Ser and Cys-to-Ala mutants of the Env protein of the T-tropic HXB2RU3 provirus (Fig. 1B), oligonucleotide-directed, site-specific mutagenesis using PCR overlap extension was performed as previously described (25). Oligonucleotides 8423f and 8933r were used as the 5′ and 3′ external primers, respectively. The paired internal primers used in the PCR encoding each Cys mutant were as follows: 764S, 5′-CTGCG GAGCCTGAGCCTCTTCAGCTACCACCGC-3′ (sense) and 5′-GTAGCTGA AGAGGCTCAGGCTCCGCAGATCGTC-3′ (antisense); 837S, 5′-GTACAAG GAGCTAGTAGAGCTATTCGCCACATA-3′ (sense) and 5′-GCGAATAGC TCTACTAGCTCCTTGTACTACTTC-3′ (antisense); 764A 5′-CTGCGGAGC CTGGCCCTCTTCAGCTACCACCGC-3′ (sense) and 5-GTAGCTGAAGAG GGCCAGGCTCCGCAGATCGTC-3′ (antisense); and 837A, 5′-GTACAAGG AGCTGCTAGAGCTATTCGCCACATA-3′ (sense) and 5′-GCGAATAGCTC TAGCAGCTCCTTGTACTACTTC-3′ (antisense). To construct the 764S 837S and 764A 837A double mutants, the 764S and 764A PCR products were used as templates, and the paired sense and antisense oligonucleotides for generating the 837S and 837A mutants were used as internal primers, respectively. All PCR amplifications were performed using Vent DNA polymerase (New England BioLabs, Beverly, MA) according to a previously described amplification program (26). The amplified mutated BamHI-XhoI fragments were cloned into the pHXB2RU3 provirus to generate various mutant proviruses. To construct HXB2-based mutant *env* expression plasmids, the BamHI-XhoI fragments isolated from Cys-to-Ser and Cys-to-Ala mutant pHXB2RU3 proviruses were cloned into corresponding sites in pSVE7*puro*, an HIV-1 long terminal repeat (LTR)-directed *env* expression plasmid. To generate ConB provirus-based Cys-to-Ala mutants, BamHI-XhoI fragments isolated from pHXB2RU3 mutant pro-

viruses were used to replace the homologous sequences in the ConB provirus. The KpnI-XhoI fragments isolated from mutant ConB proviruses were cloned in the corresponding sites in pSVE7*puro* to generate ConB-based mutant *env* plasmids. All mutant clones were confirmed by DNA autosequencing.

Plasmid DNA transfection. 293T cells grown in 100-mm petri dishes were transfected with 10 μg each of the WT or mutant proviruses by a standard calcium phosphate coprecipitation method. To prepare VSV glycoprotein G-transcomplemented viral stocks, 293T cells were cotransfected with 7.5 μg of the human cytomegalovirus virus (HCMV) promoter-directed VSV G protein expression plasmid, pHCMV-VSV G, together with 7.5 μg each of the WT or mutant proviruses. To prepare HIV-1 Env pseudotypes, 293T cells were cotransfected with 7.5 μg each of pHXBΔBglCAT with each of the WT or mutant pSVE7*puro* plasmids. Cotransfection with pHXBΔBglCAT and an *env*-defective pSVE7*puro*(ΔKS) was used as the negative control. For protein expression studies, HeLa cells grown in 100-mm petri dishes were transfected with 8 μg each of WT or mutant proviruses by the Lipofectamine transfection method (Life Technologies, Rockville, MD). For the [^{35}S]methionine and [^{3}H]palmitate labeling studies, HeLa cells grown in 6-cm petri dishes were transfected with 4 μg of WT or mutant proviruses or with 3.5 μg of WT or mutant pSVE7*puro* plasmids along with 1.5 μg of the Tat expression plasmid pIIIex*tat*.

Viral infection, RT, and CAT assays. Two days after 293T transfection, cell-free culture supernatants were assayed for reverse transcriptase (RT) activity as previously described (11). Cell-free viruses containing 2×10^4 cpm of RT activity were used to challenge 10^6 CEM-SS or PM1 cells. Postinfection RT activity of viruses obtained from supernatants of infected cultures was then monitored. For VSV G-transcomplemented HIV-1 infection, 10^6 CEM-SS cells were challenged with VSV G-transcomplemented viruses containing 2×10^6 cpm of RT activity. For the *env trans*-complementation assay, Env-pseudotyped HXBΔBglCAT reporter viruses containing 2×10^5 cpm of RT activity were used to challenge subconfluent HeLa-T4 cells grown in 60-mm dishes, or 10^6 CEM-SS or PM1 cells, and chloramphenicol acetyltransferase (CAT) activity was assessed as previously described (10).

Isolation of human PBMCs and viral infection. Peripheral blood mononuclear cells (PBMCs) were isolated from normal donors by using a standard Ficoll/Hypaque separation method. Cells were then stimulated with interleukin-2 and phytohemagglutinin A. After stimulation, 10^6 cells were infected with WT or mutant viruses containing 10^6 cpm of RT activity, and postinfection virus production was then measured.

Western blot analysis. Two days after proviral transfection or 3 days after VSV G-transcomplemented HIV-1 infection, lysates of cells and virions were prepared as previously described (12). Equal volumes of cell and virion lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western immunoblotting analysis using MAbs 902, Chessie 8, and 183 to detect gp120, gp41, and capsid protein p24, respectively. Sheep anti-gp120 was used to detect gp160 and gp120 of the ConB virus. A MAb against VSV G and a MAb secreted from hybridoma SIM2 were used to react with VSV G and CD4, respectively. Horseradish peroxidase-conjugated anti-mouse or anti-sheep immunoglobulin G was used as the second antibody. The immune complexes were visualized with a chemiluminescence detection kit.

Sucrose gradient equilibrium ultracentrifugation and quantification of lipid raft-associated Env proteins. Flotation centrifugation to assess Env localization to lipid rafts was performed according to a previously described procedure (41) with modifications. Phosphate-buffered saline (PBS)-washed transfected 293T cells that expressed Env were extracted on ice with 0.5 ml of 1% Triton X-100 prepared in NTE buffer (25 mM Tris-HCl containing 0.15 M NaCl and 5 mM EDTA; pH 7.5) supplemented with phenylmethylsulfonyl fluoride and the protease inhibitor cocktail for 30 min. Cell lysates were centrifuged at $8,000 \times g$ for 10 min at 4°C. The supernatants were adjusted to a final concentration of 42.5% sucrose prepared in NTE by adding an equal volume of 85% sucrose prepared in NTE. The mixed solutions were then loaded into SW41 tubes, and the extracts were overlaid with 6 ml of 30% sucrose prepared in NTE followed by 5 ml of 5% sucrose prepared in NTE. Tubes were centrifuged at $100,000 \times g$ for 18 h at 4°C. The gradients were fractionated from the bottom of the gradient, and 1 ml was collected per fraction. Aliquots of samples from each fraction were precipitated with trichloroacetic acid and then subjected to SDS-PAGE followed by Western blotting. To quantitate lipid raft-associated Env proteins, soluble fractions 1 to 3 and lipid raft fractions 7 and 8 were individually pooled, and concentrations of total proteins were determined using a Bio-Rad (Hercules, CA) protein assay kit. Aliquots containing equal amounts of proteins from soluble and raft fractions were precipitated with trichloroacetic acid and then subjected to Western blotting using MAb Chessie 8. The ECL blot was scanned using a Microtek (Carson, CA) ScanMarker 8700 and quantitated using the MetaMorph software (Universal Imaging, Downing Town, PA).

Metabolic labeling, cell surface biotinylation, palmitoylation, and immunoprecipitation. Transfected HeLa cells grown in 6-cm petri dishes were metabolically labeled with [^{35}S]methionine overnight, and labeled cells were surface biotinylated with 0.25 mg/ml of membrane-impermeable sulfo-*N*-hydroxysuccinimide-biotin. The levels of total and cell surface Env expressions were then assessed as previously described (9, 25). For palmitoylation studies, a set of transfected dishes was labeled with [^{35}S]methionine for 5 h. Another set of transfected cells was washed twice with PBS and then incubated with 1 ml Dulbecco's modified Eagle's medium containing 5% dialyzed FBS and 5 mM sodium pyruvate at 37°C for 30 min. [^{3}H]palmitic acid (30 to 60 Ci/mmol; New England Nuclear, Boston, MA) was dried to completeness to remove the ethanol and then dissolved in dimethyl sulfoxide before use. One millicurie of [^{3}H]palmitate dissolved in 2 μl of dimethyl sulfoxide was diluted with PBS to 100 μl and added to each dish, and cell cultures were labeled for 5 h. Cell lysates were immunoprecipitated with anti-HIV preadsorbed onto protein A-Sepharose, and the immune complexes were separated by SDS-PAGE under nonreducing conditions.

Flow cytometry analyses. To assess Env expression on the cell surfaces, 293T cells were transfected with WT or mutant pSVE7*puro* plasmids together with pIIIex*tat*. Two days after transfection, cells were divided into two portions. One portion of cells was used to determine total Env expression, and another portion of cells was processed for Env cell surface expression by fluorescence-activated cell sorter (FACS) analysis according to a procedure described previously (9).

RESULTS

Infectivity of gp41 cytoplasmic tail Cys-to-Ser mutants of a T-tropic HXB2 virus. To understand the role of palmitoylation of the Env cytoplasmic domain in viral replication, the two Cys residues located at residues 764 and 837 in the Env of the HIV-1 HXB2 strain were replaced individually or together by a Ser residue by using oligonucleotide-directed, site-specific mutagenesis with PCR overlap extension based on the pHXB2RU3 provirus (59). This molecular clone also carries functional *vpr*, *vpu*, and *nef* genes, which are not present in the parental HXB2 construct. These resultant mutants were referred to as the 764S, 837S, and 764S.837S mutants, respectively (Fig. 1B). Replication of these mutant viruses along with the WT virus was assessed using human CD4^{+} CEM-SS T cells. The 764S.837S double mutant virus, just like the WT and 764S and 837S mutant viruses, was still capable of productively replicating in CEM-SS cells (Fig. 2A). When the replication of these mutant viruses was examined in another CD4^{+} T-cell line, PM1, which is derived from Hut78 and expresses both CXCR4 and CCR5 coreceptors (32), all three of these Cys-to-Ser mutants productively replicated at a level similar to that of the WT virus (Fig. 2B).

Expression of viral proteins encoded by HXB2-based Cys-to-Ser mutants. To examine viral protein expression of these three Cys-to-Ser mutants in CD4^{+} T cells, which are natural target cells for HIV-1 infection, a high-level transient HIV-1 expression system based on pseudotyping with the VSV G protein (4, 31) was employed. This method has been shown to effectively express viral proteins in suspended T cells (9, 25, 40), which are usually transfected less efficiently than is required for biochemical analyses when other commonly used transfection methods are used. Equal amounts of cell-free VSV G-transcomplemented viruses obtained from each cotransfection and after normalization by RT activity were used to challenge CEM-SS cells. Equal portions of cell and virion lysates were analyzed by Western blotting using a cocktail containing the Env- and CA-specific MAbs. The 764S.837S double mutant produced levels of intracellular Gag and Env proteins comparable to those produced by the WT and the

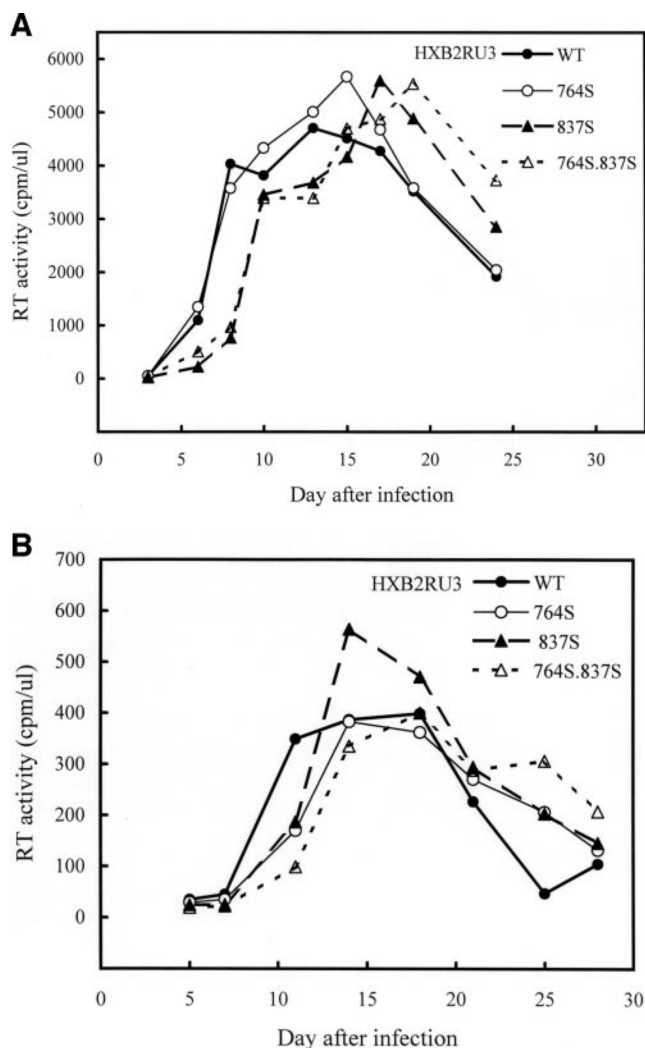


FIG. 2. Effect of Cys-to-Ser mutations on HXB2 viral replication. 293T cells were transfected with the WT or each of the mutant pHXB2RU3 proviruses, and cell-free culture supernatants containing equal amounts of RT activity were used to challenge CEM-SS (A) and PM1 (B) cells. Postinfection virus production, as measured by RT activity, was monitored.

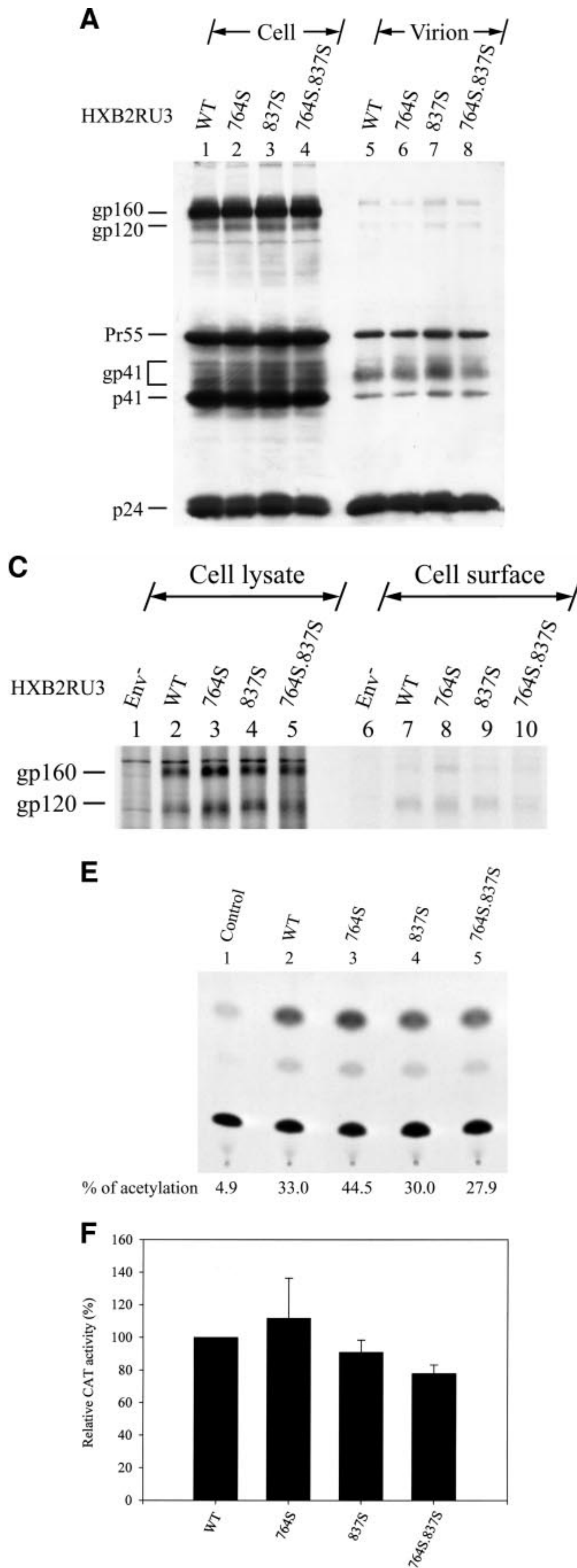
other two Ser mutants (Fig. 3A, lanes 1 to 4). Also, Gag assembly/budding of and Env incorporation into this double mutant virus were normal compared to those observed in the WT and the other two mutant viruses (Fig. 3A, lanes 5 to 8). When 293T cells were directly transfected with each of the WT and Cys-to-Ser mutant proviruses, these mutations did not affect Env synthesis, precursor processing, or Env incorporation into the viruses (Fig. 3B).

To determine whether these mutant Env proteins were expressed on the cell surface, HeLa cells transfected with the WT or each of the mutant proviruses were labeled with [^{35}S]methionine overnight, and cell surface biotinylation using membrane-impermeable sulfo-*N*-hydroxysuccinimide-biotin was performed. This method has been widely used to examine cell surface expression of HIV Env proteins (9, 40, 51). All these mutant proviruses showed levels of the intracellular gp160 precursor and gp120, as well as cell surface gp120, that were comparable to those produced by the WT provirus upon transfection (Fig. 3C). As previously reported (9), no gp120 from WT transfection could be detected on the cell surface when cells were treated with brefeldin A, which prevents transport of glycoproteins out of the ER, prior to biotinylation (data not shown). Also, the cell surface expression of neither HIV-1 Gag Pr55 nor p24 from WT proviral transfection could be detected under this surface biotinylation condition (data not shown).

To confirm that these mutant Env proteins are as efficiently expressed on cell surface as the WT Env, these three mutations were introduced to the HIV-1 LTR promoter-driven *env* expression pSVE7*puro* plasmid. FACS analysis of 293T cells transfected with each of the WT and mutant pSVE7*puro* plasmids along with pIIIex*tat* was performed. The two single mutants as well as the WT constructs produced comparable levels of Env in the cells and on the cell surface upon transfection (Fig. 3D). Although the double mutant produced a higher level of Env on the cell surface (Fig. 3D, bottom panels), this might reflect the fact that this mutant also produced a larger amount of Env in the cells than did the WT construct in this particular transfection study (Fig. 3D, top panels).

Cys-to-Ser mutant Env protein-mediated viral entry into CD4$^+$ host cells. To determine whether these mutant Env proteins expressed on the cell surface are capable of mediating viral entry, an *env trans*-complementation assay which assesses single-cycle viral infectivity was performed. The double mutant, as well as the 764S and 837S mutants, effectively mediated one-cycle viral infectivity in HeLa-T4 cells (Fig. 3E and F).

Lipid raft association of the Cys-to-Ser mutants. To determine whether these Cys-to-Ser mutant Env proteins are localized in lipid rafts, sucrose gradient ultracentrifugation, which was used to separate raft-associated proteins from soluble cytosolic proteins (41), was employed. HeLa cells separately expressing CD4 and VSV G proteins were extracted with 1% Triton X-100 on ice, and extracted cell lysates were analyzed by sucrose gradient ultracentrifugation. VSV G and CD4 were predominantly distributed in the soluble bottom fractions and lipid raft fractions 7 and 8, respectively (Fig. 4A). This result was consistent with the membrane localization properties of these two membrane proteins (8, 45). When HeLa cells transfected with the WT pHXB2RU3 provirus were analyzed, the uncleaved gp160 precursor and gp41 were localized in the lipid raft fractions 7 and 8 (Fig. 4B). p17 was also localized in lipid rafts, whereas CA p24 was localized predominantly in the cytosolic fractions (Fig. 4B). This result was consistent with previous observations that the HIV-1 Env and p17 matrix proteins are located on lipid raft membranes (41, 50). When these HXB2-based Cys-to-Ser mutants were analyzed in parallel, all of the mutant Env proteins were localized in the lipid rafts (Fig. 4C). Next, 293T cells were cotransfected with each of the WT or mutant pSVE7*puro* plasmids and pIIIex*tat*, and cells were extracted with cold Triton X-100 and analyzed by sucrose gradient ultracentrifugation. All three mutant Env proteins were localized in the lipid rafts (Fig. 4D). To quantify the expression of WT and mutant Env proteins in the soluble and raft fractions, the method previously used to quantify lipid raft-associated Nef, CD4, CXCR4, and CCR5 (36, 47, 67, 70) was employed. Soluble fractions 1 to 3 and raft fractions 7 and 8 were separately combined, and equal amounts of proteins from each pool were subjected to Western blotting using MAb


A
Cell
Virion
HXB2RU3
WT
764S
837S
764S.837S
1 2 3 4 5 6 7 8
gp160
gp120
Pr55
gp41
p41
p24
C
Cell lysate
Cell surface
Env⁻
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E
Control
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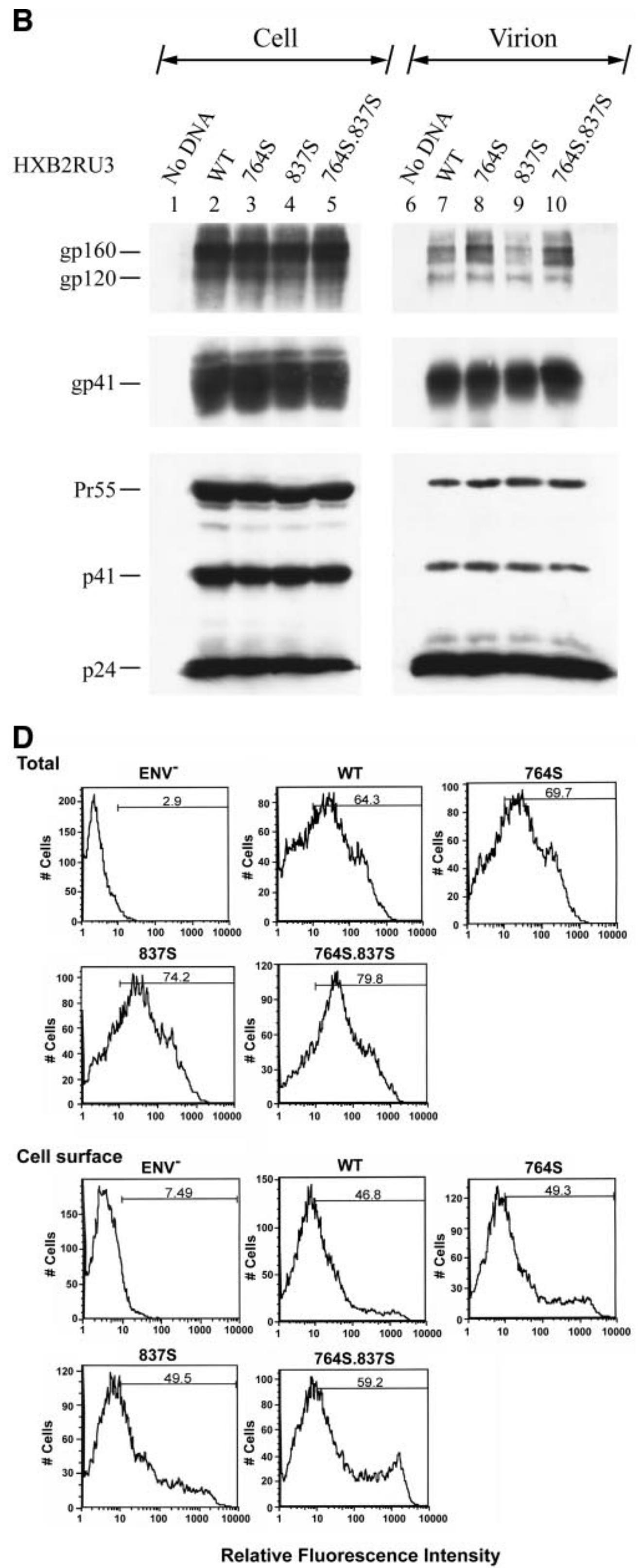
F
Relative CAT activity (%)


B
Cell
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1 2 3 4 5 6 7 8 9 10
gp160
gp120
gp41
Pr55
p41
p24
D
Total
ENV⁻
2.9
64.3
69.7
74.2
79.8
Cell surface
7.49
46.8
49.3
49.5
59.2
Cells
Relative Fluorescence Intensity

Chessie 8. The percentages of mutant proteins distributed in the raft fractions versus the soluble fractions were comparable to those for the WT Env (Fig. 4E).

Characterization of HXB2-based Cys-to-Ala mutant viruses. To confirm that mutations at palmitoylated cysteine do not affect HIV-1 replication, Cys-764, Cys-837, or both residues were replaced by an Ala residue (Fig. 1B). These mutants were termed 764A, 837A, and 764A.837A, respectively. As shown for the Cys-to-Ser mutants, these three Ala-substituted mutants still productively replicated in CEM-SS (Fig. 5A) and PM1 (Fig. 5B) cells. In HeLa cells, mutations at either Cys-764, Cys-837, or both Cys residues did not apparently affect synthesis or precursor processing of Env and Gag (Fig. 5C, lanes 1 to 5), nor did they alter viral Gag budding or Env incorporation into the virus (Fig. 5C, lanes 6 to 10). Also, none of these mutations affected the synthesis or proteolytic cleavage of Gag and Env, Gag assembly/budding, or assembly of Env into the virus in 293T cells (Fig. 5D).

These mutant Env proteins were expressed on the cell surface at levels similar to those observed for the WT Env when cell surface biotinylation of transfected HeLa cells (Fig. 6A) or FACS analyses of transfected 293T cells (Fig. 6B) was performed. These Cys-to-Ala mutant Env proteins also mediated single-cycle viral entry into CEM-SS cells as effectively as did the WT Env (Fig. 6C). Sucrose gradient equilibrium centrifugation of cold Triton X-100-extracted lysates obtained from 293T cells transfected with each of the WT and mutant proviruses showed that these three mutant Env proteins as well as the WT Env were localized in lipid rafts (Fig. 6D). When equal amounts of proteins of the soluble and lipid raft fractions obtained from 293T cells cotransfected with each of the WT or mutant pSVE7*puro* plasmids along with pIIIex*tat* were analyzed, the percentages of WT and mutant proteins localized in the lipid raft fractions versus the soluble fractions were comparable (Fig. 6E). These studies indicated that removal of the cysteine palmitoylation signals located in the cytoplasmic domain of the HXB2 Env did not alter the property of raft membrane localization of the Env.

Characterization of Cys-to-Ala mutants derived from an M-tropic ConB virus. To determine whether Env palmitoylation may function differentially in the life cycles of T- and M-tropic viruses, Cys-to-Ala mutations were introduced into an M-tropic ConB provirus (59), which contains the consensus V3 sequences of HIV-1 subtype B in the backbone of the molecular clone pHXB2RU3 and utilizes CCR5, but not CXCR4, as an entry coreceptor (65). The 764A.837A double mutant as well as the 764A and 837A mutants replicated with kinetics similar to that of the WT virus in PM1 cells (Fig. 7A). When expressed in HeLa cells, synthesis and precursor processing of the Env and Gag proteins of these M-tropic Cys-to-Ala mutants were normal compared to those observed with the WT ConB virus (Fig. 7B, lanes 1 to 5). These mutations did not affect virus assembly/budding into the culture medium or Env incorporation into the virus compared to the WT ConB virus (Fig. 7B, lanes 6 to 10). Furthermore, these mutations did not show an apparent effect on Env incorporation into the virus in 293T cells (data not shown). Similar levels of Env proteins were expressed on the surface of cells expressing either the WT or any of the three Ala substitution mutants when cell surface biotinylation of ConB provirus-transfected HeLa cells was performed (data not shown). Consistent with virus replication in PM1 cells, these Cys-to-Ala mutants also mediated entry of the Env-deficient reporter virus into PM1 cells as effectively as did the WT Env (data not shown). These Cys-to-Ala mutants and the WT ConB Env were all localized in lipid rafts (Fig. 7C).

Replication of mutant viruses in PBMCs. We then determined whether these Cys-to-Ala mutants could replicate in human primary $CD4^+$ T cells. All single and double Cys-to-Ala mutants of the HXB2 and ConB viruses replicated as effectively as the WT virus in human PBMCs (Fig. 8A and B, respectively). These results indicated that no discernible replication potentials could be observed with these mutants even in human primary $CD4^+$ T cells.

Effect of the Env/p24 ratio of the virus on its infectivity. To examine whether the Env/p24 ratios of the "real" viruses used in continuous cell culture assay may differ from those of the Env pseudotypes used in the one-cycle infectivity assay, 293T cells were transfected with WT pHXB2RU3 or with pHXBΔBglCAT and WT pSVE7*puro*, and cell and virion lysates were analyzed by Western blotting. The Env/p24 ratio of the WT Env pseudotype was much higher than that of the WT real virus (Fig. 9A). Since the one-cycle infectivity assay did not differentiate infectivities of WT and mutant Env pseudotypes, we then assayed the one-cycle-like infectivity of WT and mutant viruses produced from full-length proviral transfection. All of the Cys-to-Ala mutant viruses showed levels of infectivity similar to that of the WT virus when determined using H938 cells (Fig. 9B), a *cat* gene-carrying reporter cell line allowing measurement of viral infectivity by the ability of Tat to transactivate HIV-1 LTR-linked *cat* gene expression in this cell line.

FIG. 3. Characterization of Cys-to-Ser mutants. (A) Cell-free VSV G-transcomplemented WT and mutant viruses as indicated, containing 10^6 cpm of RT activity, were used to challenge 2×10^6 CEM-SS cells. Equal portions of cell and virion lysates were analyzed by SDS-10% PAGE followed by Western blotting using MAbs 902, Chessie 8, and 183. (B) Cell and virion lysates obtained from 293T cells transfected with each of the WT or Cys-to-Ser mutant proviruses were subjected to Western blot analysis using the MAbs as indicated in (A). (C) HeLa cells transfected with pHXBΔBglCAT (Env^-), the WT, and each of the mutant proviruses were metabolically labeled with [^{35}S]methionine and cell surface biotinylated with sulfo-*N*-hydroxysuccinimide-biotin. Cell lysates were immunoprecipitated with pooled anti-HIV preadsorbed onto protein A, and the antigens released from the immune complexes were either directly resolved using SDS-PAGE (lanes 1 to 5) or precipitated with neutravidin-agarose prior to SDS-PAGE (lanes 6 to 10). (D) 293T cells were transfected with each of the WT or mutant pSEV7*puro* plasmids together with pIIIex*tat*. Total and cell surface Env expressions were analyzed by FACS as described in Materials and Methods. Transfection with an *env*-defective pSVE7*puro*(ΔKS) (Env^-) was used as a negative control. (E and F) Cell-free, WT, and mutant Env-pseudotyped HXBΔBglCAT viruses containing 2×10^5 cpm of RT activity were used to challenge HeLa-T4 cells, and CAT activity was measured. A representative result is shown in (E). In (F), the degree of WT Env-mediated viral entry into HeLa-T4 was arbitrarily assigned as 100%. Mutant Env-mediated viral entry was expressed as a percentage of that of the WT Env. Results from at least three individual experiments were averaged (mean), and the standard deviation was calculated.

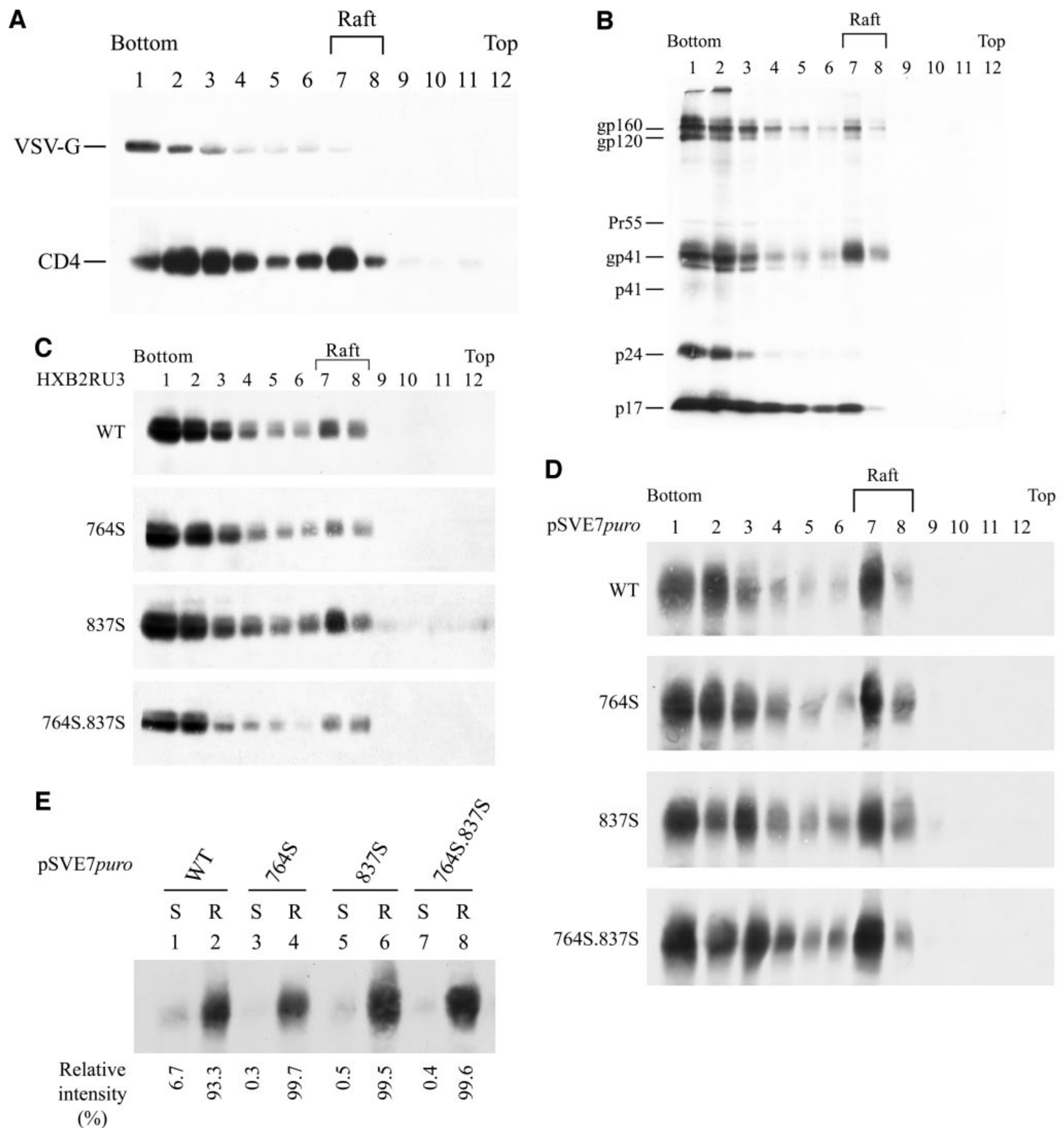

FIG. 4. Effects of Cys-to-Ser mutations on lipid raft localization of the Env protein. (A, B, C) HeLa cells were transfected with either pHCMV-VSV G or pCDNA3-CD4 (A), WT pHXB2RU3 (B), or each of the WT and Cys-to-Ser mutant pHXB2RI3 proviruses (C). Cell lysates prepared by 1% Triton X-100 extraction at 4°C were subjected to sucrose gradient equilibrium ultracentrifugation. After fractionation, proteins in each fraction were analyzed by Western blotting using VSV G or SIM2 MAbs (A); MAbs 902, Chessie 8, and 183 (B); and MAb Chessie 8 (C). The distribution of gp41 in sucrose gradients is shown in panel C. (D and E) 293T cells were cotransfected with each of the WT or mutant pSVE7*puro* plasmids along with pIIIex*tat*. Cell lysates prepared by 1% cold Triton X-100 extraction were subjected to sucrose gradient equilibrium ultracentrifugation and analyzed by Western blotting using Chessie 8 (D). Soluble fractions 1 to 3 and raft-associated fractions 7 and 8 were separately combined and normalized for protein concentrations. Equal amounts of proteins from soluble (S) and raft (R) fractions were subjected to Western blotting using MAb Chessie 8, and the gp41 band is shown (E). The distribution of WT and mutant gp41 in soluble and raft fractions was quantified as described in Materials and Methods.

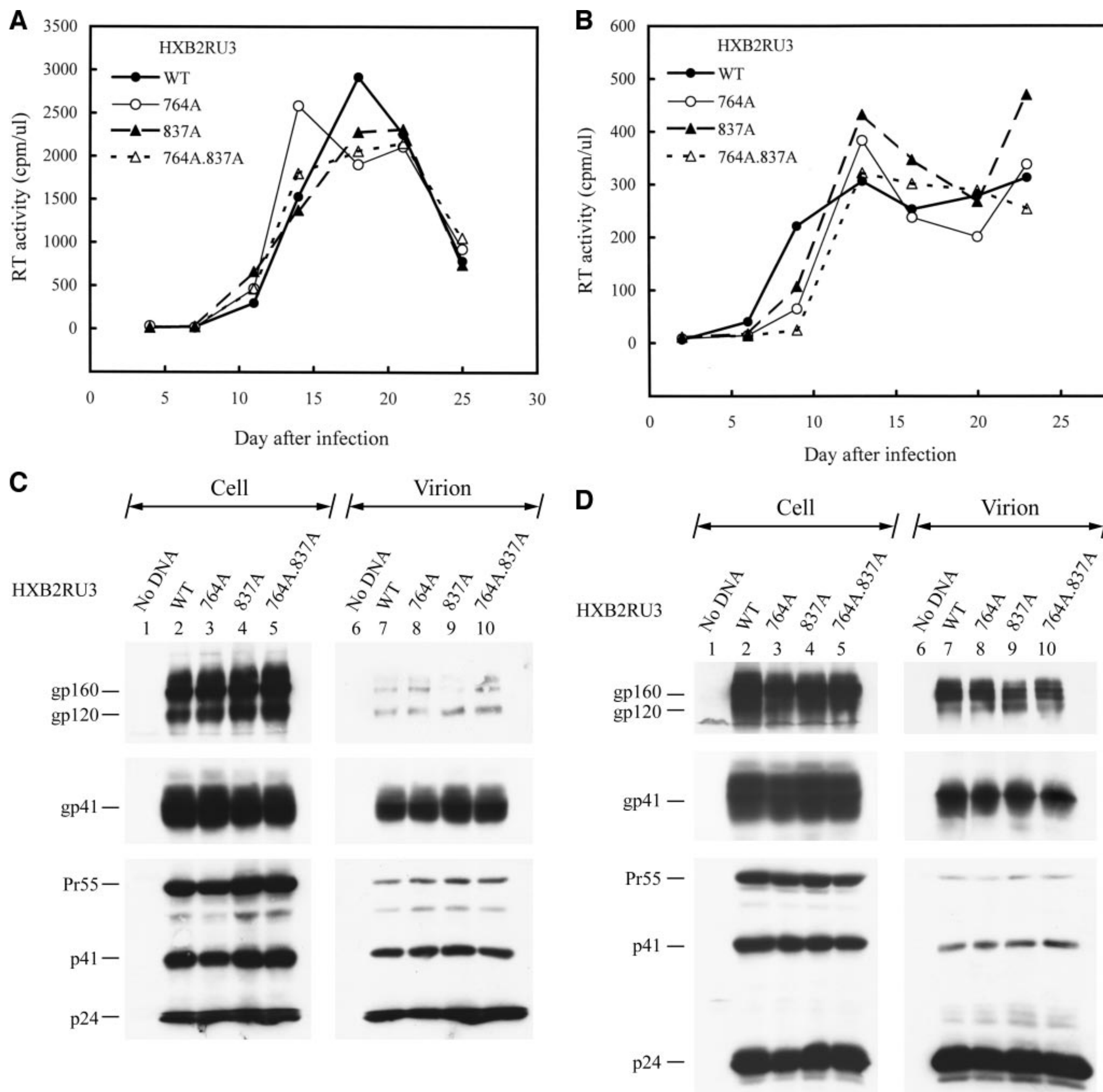

FIG. 5. Viral replication and protein expression of Cys-to-Ala mutants of the HXB2 strain. (A and B) Cell-free, WT, and Cys-to-Ala mutant viruses as indicated were assayed for viral replication kinetics in CEM-SS (A) and PM1 (B) cells. (C and D) HeLa (C) and 293T (D) cells were transfected with the WT or mutant proviruses, and cell and virion lysates were assessed by Western blot analysis.

Palmitoylation state of the Cys-to-Ala mutants. To confirm that the double Cys-to-Ala mutant is indeed deficient in palmitoylation, metabolic labeling with [^{35}S]methionine and [^{3}H]palmitic acid was performed on ConB viruses. A mutation at residue 837, but not at residue 764, significantly reduced incorporation of [^{3}H]palmitate into gp160 and gp41 compared to the WT Env (Fig. 10). Likewise, palmitoylation of the double mutant was also greatly reduced (Fig. 10, lane 10). These results indicated that palmitoylation at residue 837, but not at residue 764, contributed the most to gp41 cytoplasmic tail palmitoylation under the conditions we used.

DISCUSSION

Rousso et al. previously characterized HIV-1 Env mutants in which Cys-764 and Cys-837 located in the cytoplasmic tail of the HXB-2D Env were individually or together replaced by Ser residues, and they concluded that palmitoylation of Env is critical for Env targeting to lipid rafts, Env incorporation into the virus, and viral infectivity (50). However, it was not clear why gp160, but not gp120, was used to assess Env incorporation. The uncleaved gp160 precursor is fusion defective, and gp160 incorporation into virions does not necessarily correlate

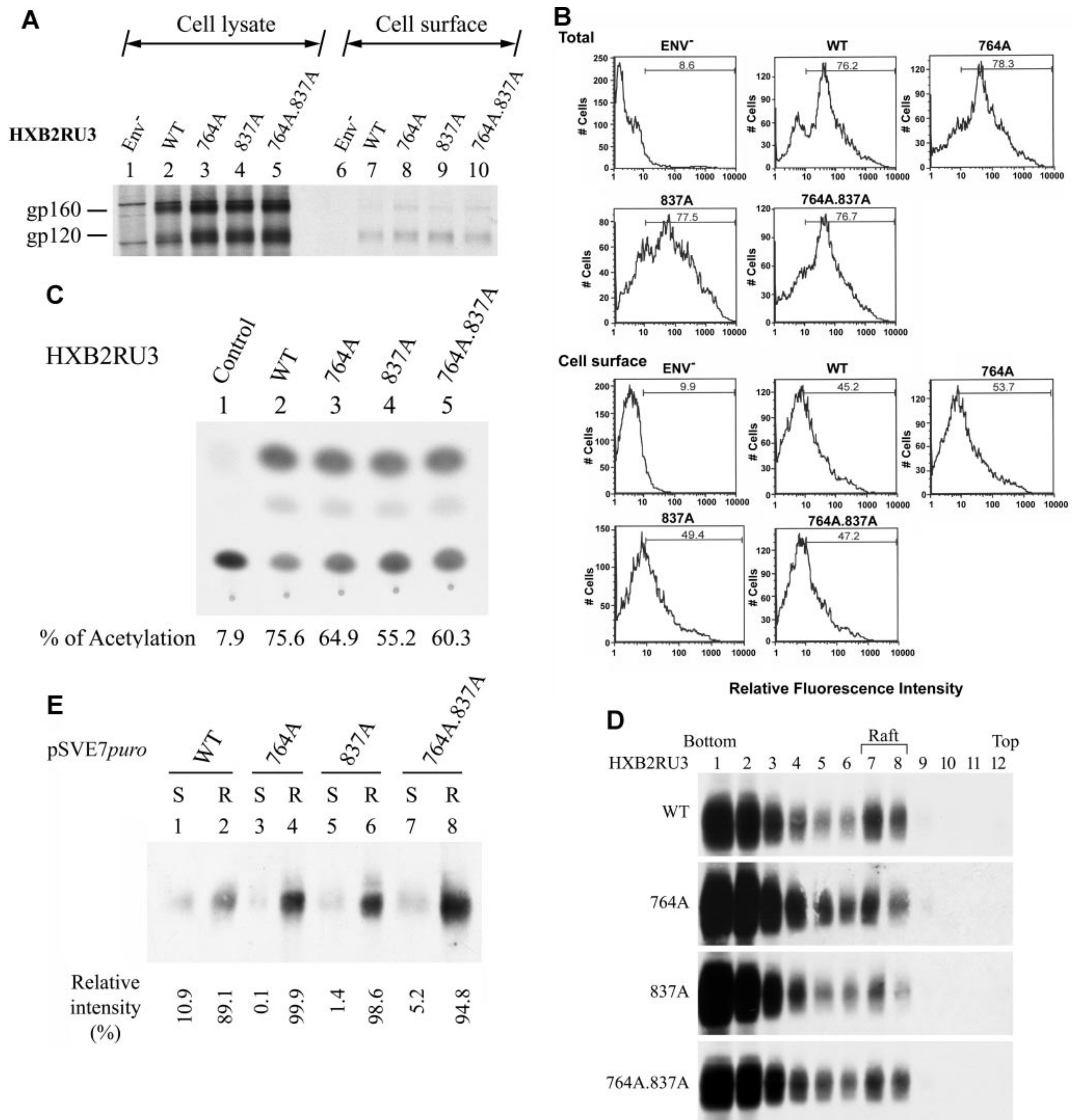

FIG. 6. Characterization of HXB2-based Cys-to-Ala mutants. (A) WT and mutant provirus-transfected HeLa cells were metabolically labeled and surface biotinylated to determine the cell surface expression of Env proteins. (B) 293T cells were cotransfected with pIIIex*tat* and each of the WT or mutant pSVE7*puro* plasmids, and total and cell surface Env expressions were analyzed by FACS. (C) Env-deficient HXBΔBglCAT reporter viruses pseudotyped with the WT or Cys-to-Ala mutant Env proteins were used to challenge CEM-SS cells, and CAT activity was determined to assess the ability of mutants to mediate the single-cycle viral replication potential. (D) 293T cells were transfected with the WT or each of the mutant proviruses as indicated. Cells were then extracted with 1% Triton X-100 on ice for 30 min, and lysates were subjected to sucrose gradient ultracentrifugation followed by Western blotting using Chessie 8 MAb. The distribution of gp41 in gradients is shown. (E) 293T cells were cotransfected with pIIIex*tat* and each of the WT or mutant pSVE7*puro* plasmids. Cold Triton X-100-extracted lysates were subjected to sucrose gradient ultracentrifugation, and equal amounts of proteins from soluble (S) and raft (R) fractions were analyzed by Western blotting using MAb Chessie 8. The relative intensities of soluble and raft fractions of gp41 in total protein were quantitated.

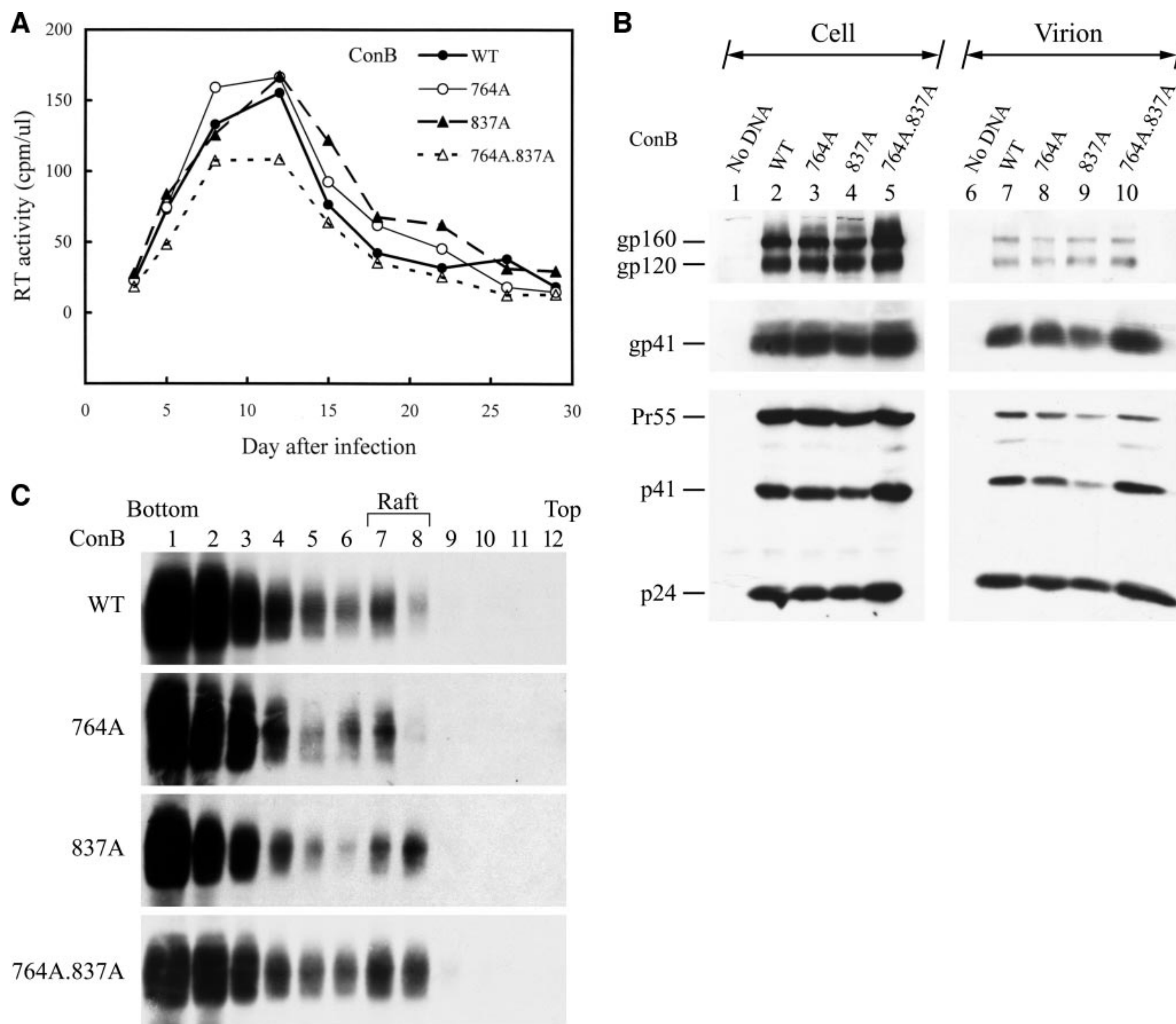

FIG. 7. Analyses of Cys-to-Ala mutants derived from the M-tropic ConB virus. (A) PM1 cells were challenged with WT or each of the Cys-to-Ala mutant ConB viruses, and postinfection RT activities of the culture supernatants were determined. (B) Cell and virion lysates obtained from HeLa cells transfected with the WT or each of the mutant ConB proviruses were analyzed by Western blotting using goat anti-gp120, Chessie 8, and 183 MAbs, respectively. (C) Cold Triton X-100-extracted lysates obtained from WT and mutant provirus-transfected 293T cells were analyzed by the lipid raft flotation assay. After fractionation, proteins in each fraction were analyzed by Western blotting using Chessie 8 MAb.

with viral infectivity. In addition, release of gp160 into the culture medium may occur via its association with microvesicles (63), which are shed from cultured cells. Also, the cell fractionation method they used is not an ideal approach to assessing lipid raft localization, since Triton X-100 insolubility may result from association of target proteins with the cytoskeleton or other insoluble protein complexes. On the other hand, Bhattacharya et al. characterized the effects of replacing Cys-764 and Tyr-837 of the NL4-3 Env with various amino acid residues and concluded that although cytoplasmic cysteines are required for the association of Env with light detergent-resistant membranes, these Cys residues are not essential for Env incorporation or viral infectivity (5). In both of these studies, the single-round infectivity assay was employed to study the functions of mutant Env proteins. However, whether the 764S.837S double mutant replicates at all in $CD4^+$ T cells was not elucidated. The reason for this concern is that the single-cycle infectivity assay measures virus-cell transmission of a defective virus pseudotyped with the Env, whereas replication of HIV-1 in $CD4^+$ T cells also encounters cell-cell transmission. Because of the different results obtained by these two groups and because of the unanswered issues raised above, we reexamined the involvement of cytoplasmic palmitoylated cysteines of HIV-1 in the virus life cycle.

In contrast to the findings of the other two groups (5, 50), we found that in the context of the T-tropic HXB2 virus, substitutions of Ser and Ala for both Cys-764 and Cys-837 did not affect viral infectivity, as judged by continuous cultures and

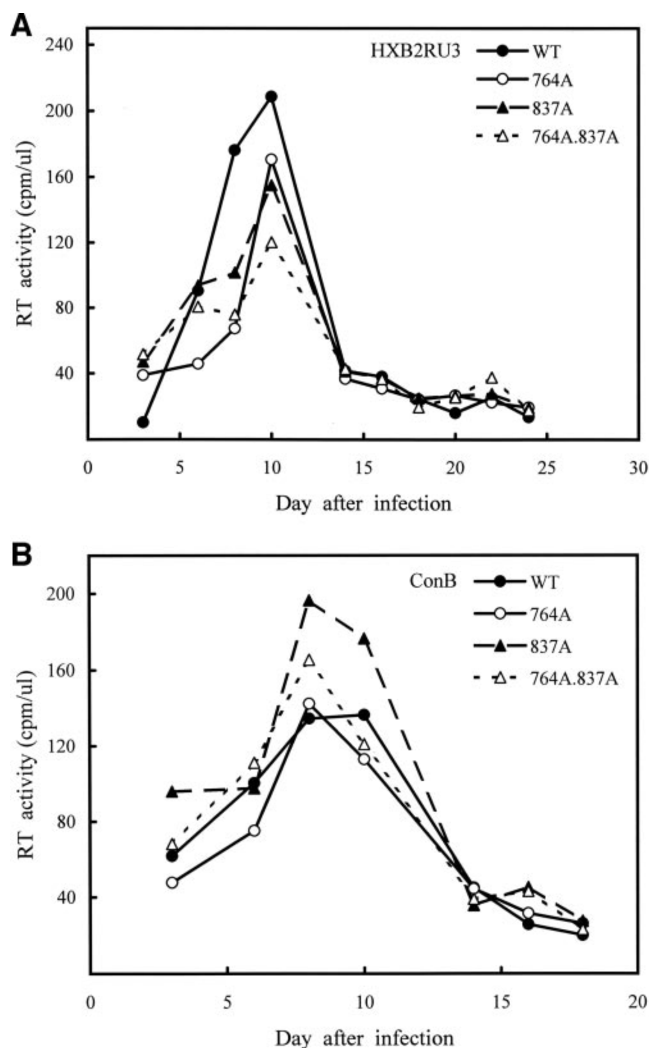

FIG. 8. Replication of Cys-to-Ala mutants in human PBMCs. Cell-free, WT, and mutant HXB2 (A) and ConB (B) viruses containing 10^6 cpm of RT activity were used to challenge activated PBMCs. Infected cells were regularly fed, and postinfection virus production in the culture medium was assessed by RT activity at different times.

single-cycle infectivity assays in various host target cells, including HeLa-T4, CEM-SS, PM1, and human primary $CD4^+$ T cells (Fig. 2, 3E, 3F, 5A, 5B, 6C and 8A). In addition, removal of the putative palmitoylation signals in the cytoplasmic tail of an M-tropic HIV-1 clone, ConB, also exerted no significant effect on viral replication (Fig. 7A and 8B and data not shown). Ser substitutions for either Cys-764 or Cys-837, or both, of the HXB2 strain Env were previously shown not to affect Env transport to the cell surface (68). Although the 764S.837S double mutant showed a higher level of surface expression than did the WT Env (Fig. 3D, bottom panels), this could simply have been due to a higher efficiency of transfection of this mutant in this particular experiment and not necessarily to higher surface expression of this mutant, since this mutant also produced a higher level of total Env in cells than did the WT Env (Fig. 3D, top panels). In support of this notion, surface biotinylation did not differentiate the WT and this double mutant (Fig. 3C), and the T- and M-tropic Cys-to-Ala double mutants showed no increase in surface expression compared to the WT and other single mutants (Fig. 6A and 6B and data not shown). Also, removal of these two cytoplasmic cysteines exhibited no significant effect on Env incorporation into the virus in CEM-SS (Fig. 3A), HeLa (Fig. 5C and 7B), or 293T (Fig. 3B and 5D and data not shown) cells.

On the other hand, abrogation of the palmitoylation signals in the cytoplasmic tail did not affect the raft association property of the T- and M-tropic Env proteins in HeLa (Fig. 4C) or 293T (Fig. 4D, 6D, and 7C) cells. In these lipid raft flotation analyses, only a fraction, ranging between 10% and 25%, of the total WT and mutant proteins was localized in the lipid rafts. When the expression of WT and mutant proteins in the soluble and raft fractions was quantified by normalization of protein amounts used in Western blotting, most of the WT as well as the Cys-to-Ser and Cys-to-Ala mutants was found to be located in the raft fractions, and the percentages of mutant proteins distributed in the raft fractions versus soluble fractions were comparable to those of the WT Env (Fig. 4E and 6E, respectively). These results indicate that the two cytoplasmic Cys residues are not critical for Env association with lipid rafts. Since the majority of proteins in the cells were not associated with lipid rafts, normalization for total proteins in the pooled raft and soluble fractions would result in much lower levels of Env being localized in the soluble fractions than in the raft fractions. Therefore, although this assay provides a means to compare the raft association properties of the WT and mutants, this approach provides no information on the absolute levels of gp41 present in the lipid rafts.

In agreement with previous studies (19, 35), we also noted that gp160 can be detected in viral particles. At a late stage of Env synthesis, the gp160 precursor is proteolytically cleaved by furin-like cellular proteases or other subtilisin-like proteases, most probably in the *trans*-Golgi network, to yield noncovalently associated gp120-gp41 heterodimers. Therefore, gp160 precursor cleavage is dependent on the cell types examined. Also, production of a native gp120-gp41 complex has been hampered by the limited efficiency of Env cleavage (18, 38, 39). These unprocessed gp160 molecules, likely forming homotrimers or heterotrimers with processed gp120-gp41, are also transported to the cell surface (40, 51), where they interact with Gag proteins and are incorporated into the virions.

Rousso et al. (50) employed an *env*-deficient, luciferase-containing NL4-3R^-E^-Luc provirus, which carries the functional *vif* and *vpu* genes but not the *vpr* or *nef* genes, along with the pCDNA3-based *env* gene derived from the HXB-2D strain for Env incorporation and *env trans*-complementation assays. Bhattacharya et al. (5) also used an NL4-3-derived *env*-deficient construct that encodes all viral proteins, except Env, and an HIV-1 LTR-driven NL4-3 *env* plasmid in their studies. Among these four accessory proteins, both Nef and Vpu encode activities that contribute to downregulation of the viral receptor CD4 from the cell surface (for a review, see reference 6). Nef increases viral infectivity (14, 37, 54), and downregulation of CD4 contributes to enhanced viral infectivity (1, 24, 49). Nef-enhanced viral infectivity occurs, at least in part, by an increase in Env incorporation into the virus, likely via Nef-enhanced steady-state levels of cell membrane-associated Env proteins (52). One of the well-characterized functions of Vpu

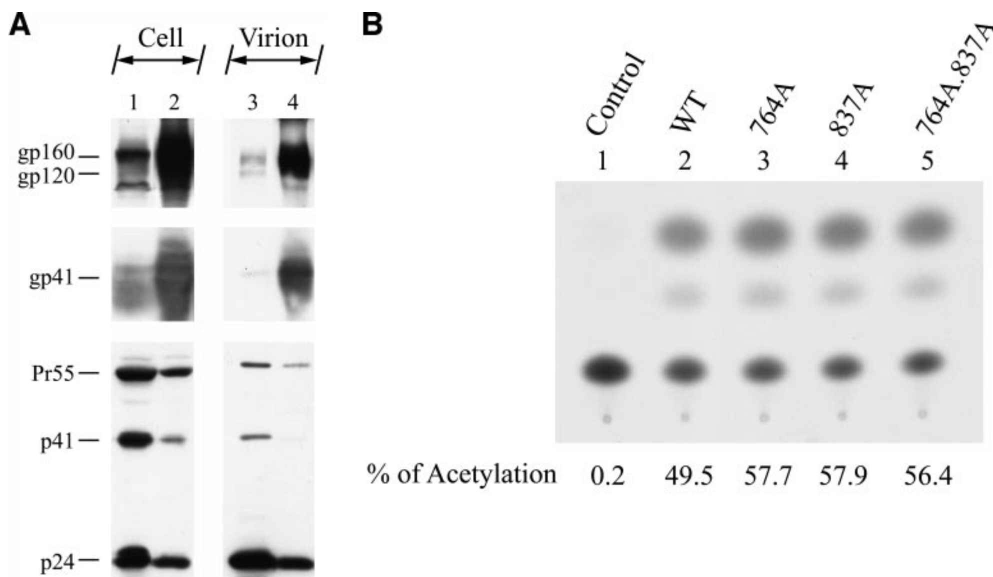

FIG. 9. Assessment of the effect of the Env/p24 ratio of the virus on viral infectivity. (A) 293T cells were transfected with 10 μg of WT pHXB2RU3 (lanes 1 and 3) or with 7.5 μg each of pHXBΔBglCAT and WT pSVE7*puro* (lanes 2 and 4), and cell and virion lysates were analyzed by Western blotting. (B) Viruses produced from transfection with each of the WT and mutant proviruses were normalized for RT and then used to challenge H938 cells. CAT activity was determined 2 days postinfection.

is its ability to induce degradation of CD4 molecules trapped in intracellular complexes with Env, which allows the release of Env for transport to the cell surface (66). Vpu also mediates the efficient release of viral particles from virus-infected cells (21, 57). Vpu positively enhances viral infectivity by down-modulating the cell surface CD4 receptor (27), presumably removing the activity of CD4 that inhibits the virion release-promoting activity of Vpu (7, 58). Although the version of the infectious HXB2 molecular clone used in our study carries functional *vif*, *vpr*, *vpu*, and *nef* genes (59), these accessory genes might not be the primary factor responsible for the differential effects of the cytoplasmic tail palmitoylation observed. This was reasoned out by the observation that the pHXBΔBglCAT provirus used in our single-cycle infectivity assay carries none of the functional *vpr*, *vpu*, and *nef* genes.

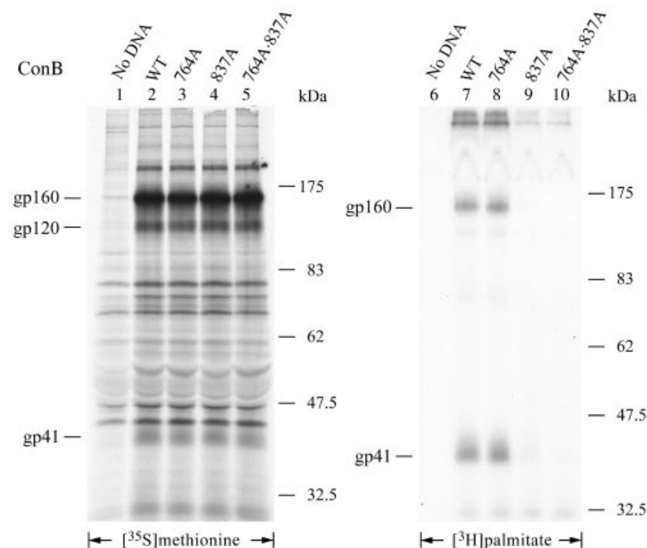

FIG. 10. Palmitoylation of Cys-to-Ala Env mutants. Duplicate sets of HeLa cells cotransfected with WT or mutant pSVE7*puro* plasmids together with pIIIex*tat* were labeled with [^{35}S]methionine (lanes 1 to 5) or [^{3}H]palmitate (lanes 6 to 10). Cell lysates were immunoprecipitated with pooled anti-HIV-1 antisera preadsorbed onto protein A-Sepharose, and the immune complexes were resolved by SDS-PAGE followed by autoradiography.

It was reported that the entire cytoplasmic tail of the Env protein can be deleted without imposing a significant effect on Env incorporation into the virus in 293T cells (40). In the study by Bhattacharya et al. (5), the effect of mutations at positions 764 and 837 in the cytoplasmic tail on Env incorporation depended on the residue to be mutated and on what type of amino acid substitution was used. It is therefore likely that in 293T cells, the cytoplasmic tail per se does not positively contribute to Env incorporation; however, mutations in the cytoplasmic cysteines may induce alterations in Env folding and/or intracellular trafficking, which ultimately affect the effectiveness of Env incorporation into the virus.

Although the Env/p24 ratio of the Env-pseudotyped virus used in our one-cycle infectivity assay was greater than that of the "real" virus prepared from full-length, proviral transfection (Fig. 9A), the possibility that overexpression of mutant Env in pseudotypes may compensate for the impaired infectivity, if any, of the double mutant is unlikely. First, Bhattacharya et al. (5) also used LTR-controlled *env* expression plasmids for pseudotype preparation. Nevertheless, the viral entry of their Ser and Ala substitution double mutants was severely reduced, which is accounted for by insufficient Env incorporation of these two mutants. Second, if the possibility holds, it would be expected that the infectivity of the "real" WT and mutant viruses, which had lower Env/p24 ratios than the Env pseudotypes, would have been differentiated in the one-cycle infectivity

assay. However, the WT and all mutant viruses showed similar levels of one-cycle-like infectivity in the LTR-lined *cat* gene-carrying H938 reporter cell line (Fig. 9B). Therefore, the WT-like infectivity of the mutant Env pseudotypes we examined was clearly due to the WT-like Env incorporation phenotype of the mutants.

A contiguous long sequence located in the cytoplasmic tail, beginning with the first palmitoylation site at Cys-764 and ending at the C terminus, is believed to be embedded in membranes (20). We previously showed that the C-terminal two-thirds of the cytoplasmic domain confers multimerization and plays a role in membrane association (13, 26). It is likely that minor amino acid differences between the *env* clones used in the studies by Rousso et al. (50), Bhattacharya et al. (5), and us may be responsible for the differential effects of the deacylated Env proteins on lipid raft association, Env incorporation, and viral infectivity observed by the three groups. The cytoplasmic tail of Env has specific interactions with cellular membranes, cellular proteins, and the matrix (MA) protein. It is also likely that subtle differences in the modes of interactions between Env and membranes, Env and cellular proteins, and Env and Gag, aroused by amino acid differences in the *env* and *gag* gene products, may contribute to the differential Env phenotypes of the deacylated Env mutants observed among different laboratories.

In the present study, we were unable to ascribe any apparent effects on replication of T- and M-tropic viruses to deacylation of the HIV-1 Env. Consistent with the notion that many isolates representative of different HIV-1 clades and the infectious molecular clones such as BORI, JRCSF, and DH123 do not contain the putative Cys palmitoylation sites in the cytoplasmic domains of their TM proteins, our results strongly support the notion that palmitoylation of the gp41 cytoplasmic tail does not play a critical role in the HIV-1 life cycle.

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