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Human Immunodeficiency Virus Type 1 Envelope Protein Endocytosis Mediated by a Highly Conserved Intrinsic Internalization Signal in the Cytoplasmic Domain of gp41 Is Suppressed in the Presence of the Pr55^{gag} Precursor Protein

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The mechanisms involved in the incorporation of viral glycoproteins into virions are incompletely understood. For retroviruses, incorporation may involve interactions between the Gag proteins of these viruses and the cytoplasmic domains of the relevant envelope (Env) glycoproteins. Recent studies have identified within the cytoplasmic tail of the human immunodeficiency virus type 1 (HIV-1) Env protein a tyrosine-containing internalization motif similar to those found in the cytoplasmic domains of certain cell surface proteins that undergo rapid constitutive endocytosis in clathrin-coated pits. Given that surface expression of the HIV-1 Env protein is essential for the production of infectious virus, the presence of this internalization motif is surprising. We show here that in contrast to the rapid rate of Env protein internalization observed in cells expressing the Env protein in the absence of other HIV-1 proteins, the rate of internalization of Env protein from the surfaces of HIV-1-infected cells is extremely slow. The presence of the Pr55^{gag} precursor protein is necessary and sufficient for inhibition of Env protein internalization, while a mutant Pr55^{gag} that is incapable of mediating Env incorporation into virions is also unable to inhibit endocytosis of the Env protein. The failure of the Env protein to undergo endocytosis from the surface of an HIV-1-infected cell may reflect the fact that the proposed interaction of the matrix domain of the Gag protein with Env during assembly prevents the interaction of Env with host adaptin molecules that recruit plasma membrane molecules such as the transferrin receptor into clathrin-coated pits. When the normal ratio of Gag and Env proteins in the infected cells is altered by overexpression of Env protein, this mechanism allows removal of excess Env protein from the cell surface. Taken together, these results suggest that a highly conserved system to reduce surface levels of the Env protein functions to remove Env protein that is not associated with Gag and that is therefore not destined for incorporation into virions. This mechanism for the regulation of surface levels of Env protein may protect infected cells from Env-dependent cytopathic effects or Env-specific immune responses.

For enveloped viruses, the incorporation of viral glycoproteins into budding virions is essential for the production of infectious virus particles (reviewed in references 16, 17, and 44). The mechanisms involved in the incorporation of viral glycoproteins into virions are incompletely understood. For retroviruses, there is some evidence that incorporation may involve interactions between the Gag proteins of these viruses and the cytoplasmic domains of the relevant envelope (Env) glycoproteins, as was originally suggested by chemical cross-linking studies with Rous sarcoma virus (11). In the case of human immunodeficiency virus type type 1 (HIV-1), several studies suggest that Gag-Env interactions influence incorporation of the Env glycoprotein (3, 8, 28, 31, 35, 49, 50). However, direct biochemical evidence for such interactions is lacking, and incorporation is not strictly dependent on Gag-Env interactions since some truncated Env mutants can be incorporated (8, 9, 31, 48), as can heterologous retroviral Env proteins (24, 30, 43) and certain host membrane proteins (1). Recent work suggests that the incorporation of the full-length Env protein, but not certain C-terminal truncation mutants, is dependent on some form of interaction between the matrix (MA) domain of the Gag precursor protein Pr55^{gag} and the cytoplasmic domain of the gp41 subunit of the Env protein (8, 31).

Regardless of the mechanism of incorporation, it is likely that the level of cell surface expression of viral glycoproteins is a critical factor in the assembly of viruses like HIV-1 that bud from the plasma membrane. In principle, the level of expression of viral glycoproteins on the cell surface can also determine the susceptibility of infected cells to destruction by immunologic effector mechanisms such as antibody-dependent cytolytic effector cells (33). In addition, surface expression of some viral glycoproteins, notably HIV-1 Env, is associated with cytopathic effects such as syncytium formation (26, 40) and Env-dependent single-cell killing (18, 20, 21, 29, 45). It is therefore important to understand the factors that govern cell surface expression of the viral glycoproteins.

The level of cell surface expression of viral glycoproteins is influenced not only by the rate of biosynthesis and the efficiency of transport through the exocytic pathway but also by the rate at which the glycoproteins are removed from the cell surface. Recent studies (37) have identified within the cytoplasmic tail of the HIV-1 Env protein a tyrosine-containing internalization motif similar to those found in the cytoplasmic domains of certain cell surface proteins such as the transferrin

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receptor (TfR) that undergo rapid constitutive endocytosis in clathrin-coated pits (46). When the HIV-1 Env protein is expressed in B-lymphoblastoid cell lines (B-LCL), this tyrosine-containing motif mediates rapid endocytosis of the Env protein at rates comparable to those observed for the TfR ($t_{1/2}$ = 20 min). Mutation of the relevant tyrosine residue in gp41 to alanine results in dramatically reduced rates of endocytosis and, as a consequence, higher steady-state levels of Env expression on the cell surface (37). Interestingly, this tyrosine residue is located in a six-amino-acid region that is absolutely invariant in HIV-1 clade B isolates and highly conserved in other HIV-1 isolates (32). Highly conserved, tyrosine-containing motifs are also found in the corresponding regions of the cytoplasmic domains of the HIV-2 and simian immunodeficiency virus (SIV) Env proteins. LaBranche et al. have recently shown that substitution of the corresponding tyrosine residue of the SIV Env protein results in higher levels of expression of Env protein on the surfaces of chronically infected cells and greater incorporation of Env into SIV virions (22, 23).

The nature of the selective pressure that favors conservation of a functional intrinsic endocytosis signal sequence in the HIV-1 Env protein is unclear. Given that surface expression of the Env protein is essential for the production of infectious virus, the presence of this internalization motif is surprising. To date, quantitative measurements of endocytosis of the HIV-1 Env protein have been carried out in experimental systems in which the Env protein is expressed in the absence of other viral proteins (37). Thus, it has been unclear whether the intrinsic internalization signal in the cytoplasmic domain of HIV-1 gp41 is fully functional in HIV-1-infected cells. This study was undertaken to determine whether the Env protein expressed on the surfaces of HIV-1-infected T cells is subject to rapid endocytosis. We show here that in contrast to the rapid rate of internalization observed in cells expressing the Env protein in the absence of other HIV-1 proteins, the rate of internalization of Env protein from the surfaces of infected cells is extremely slow. The presence of the $Pr55^{gag}$ precursor protein is necessary and sufficient for inhibition of Env protein internalization, while mutant $Pr55^{gag}$ that is incapable of mediating Env incorporation is also unable to inhibit endocytosis of the Env protein. Taken together, these results suggest that a highly conserved system to reduce surface levels of the Env protein functions to remove excess Env protein that is not associated with Gag and that is therefore not destined for incorporation into virions. These results allow for a synthesis of some of the divergent results in the literature on the mechanism of Env incorporation into virions.

MATERIALS AND METHODS

Cell lines. Primary $CD4^+$ lymphoblasts were prepared from peripheral blood mononuclear cells (PBMC) of HIV-1-seronegative donors as follows. $CD3^+$ $CD4^+$ T-cell populations were isolated from PBMC by fluorescence-activated cell sorting using appropriate monoclonal antibodies (MAbs). The cells were then stimulated with phytohemagglutinin and irradiated allogeneic PBMC as previously described (27). The cells were maintained in culture medium (CM) consisting of RPMI (Biofluids, Rockville, Md.) with 10% heat-inactivated fetal calf serum (HyClone, Logan, Utah), penicillin (100 U/ml), streptomycin (100 μg/ml), and glutamine (40 μM). This CM was supplemented with 50 U of recombinant interleukin-2 per ml and 50 U of T-cell growth factor per ml prepared as previously described (27). The cells were restimulated every 2 to 3 weeks by the addition of irradiated PBMC (5,000 rad) and phytohemagglutinin (0.25 μg/ml). The cell lines H9, SupT1, and ACH-2 were obtained from the AIDS Research and Reference Reagent Program, AIDS Program, National Institute of Allergy and Infectious Diseases (contributors, R. Gallo, J. Hoxie, and T. Folks, respectively) and were maintained in CM. In some experiments, ACH-2 cells were activated with phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, Mo.) at a final concentration of 160 nM overnight to upregulate HIV-1 gene expression. Jurkat and H9 cells stably transfected with HXB2 proviral DNA constructs will be described in detail elsewhere (48a).

Infections. Three days after restimulation with phytohemagglutinin and irradiated allogeneic PBMC, $CD4^+$ blasts were incubated with high-titer supernatants from $HIV\text{-}1_{LAI}$-infected SupT1 cells. Following a 2-h incubation at 37°C, the cells were washed and cultured at 10^6/ml for 7 days in supplemented CM.

Vaccinia virus vectors. The vaccinia virus vector vPE16, which expresses the LAI (BH8) *env* gene, was kindly provided by Patricia Earl and Bernard Moss (National Institutes of Health) and has been previously described (6). The vJR3 vector carries a mutant form of the LAI *env* gene with a single tyrosine→alanine substitution at position 707 (Y707A mutation) (37). The vaccinia virus vector vP1287, which contains the entire $Pr55^{gag}$ open reading frame, was a gift of James Tartaglia and Enzo Paoletti (Virogenetics, Troy, N.Y.). The vaccinia virus vector vSC8, which expresses β-galactosidase but no HIV-1 proteins, was used as a negative control. Cells were infected with vaccinia virus vectors for 2 h at 10^7/ml in CM and then incubated overnight at 10^6/ml.

Antibodies. The T32 hybridoma (5) specific for HIV-1 gp41 was obtained from Pat Earl, National Institutes of Health. A MAb to the human TfR (CD71) was obtained from Becton Dickinson, San Jose, Calif.

Flow cytometric analysis of endocytosis. Cells were incubated for 45 min at 0°C with saturating concentrations of the gp41-specific MAb T32, a CD71 (TfR)-specific MAb, or isotype-matched control antibodies. After incubation with the primary antibody, the cells were washed, resuspended in CM at 10^6/ml, and incubated at 37 or 0°C for various periods. The cells were then pelleted and incubated with a 1:40 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (Sigma) for 45 min at 0°C. After incubation with the secondary antibody, the cells were washed and resuspended in 1.5% paraformaldehyde in phosphate-buffered saline at 10^6/ml. Cell surface protein expression was analyzed with a FACScan flow cytometer (Becton Dickinson) running FACScan research software. Dead cells were excluded by appropriate forward and side light scattering gates, and 10^4 events were analyzed for each sample. The forward and side light scatter profiles of cells infected with different vaccinia virus vectors did not differ significantly. For calculation of percent internalization, green fluorescence measurements were made on a linear scale rather than a logarithmic scale, and the mean fluorescence (MF) was determined for each population. The percent internalization was calculated [MF(0°C) − MF(37°C)]/[MF(0°C) − MF(control)] × 100, in which the negative control was either vSC8-infected cells labeled with the relevant MAb or vPE16-infected cells labeled with an isotype-matched irrelevant antibody. Both types of negative control gave the same low level of background staining. All flow cytometric assays shown were repeated a minimum of three times with comparable results.

Western blot (immunoblot) analysis. Jurkat cells or Jurkat cells stably transfected with HIV-1 HXB2 (Jurkat B2) were infected at a multiplicity of infection of 10:1 with a control vaccinia virus vector (vSC8), a vaccinia virus vector expressing wild-type Env (vPE16), or a vaccinia virus vector expressing mutant Env with a Y→A mutation within the Env internalization motif (vJR3). After overnight incubation at 37°C, the cells were lysed in 20 μl of 2× sodium dodecyl sulfate sample buffer for 10 min and boiled for 10 min. The cell debris was pelleted, and the sample was loaded onto an 8% acrylamide separating gel. Proteins were then transferred to nitrocellulose (Bio-Rad, Hercules, Calif.) overnight at 4°C and blocked with powdered milk for 3 h. HIV-1 proteins were visualized by staining with anti-HIV-1 serum from an HIV-1-seropositive patient at a dilution of 1:1,000. The secondary antibody was a goat anti-human immunoglobulin G–horseradish peroxidase conjugate (Bio-Rad) used at a dilution of 1:10,000. Protein antibody complexes were then visualized with the ECL Western Blotting System (Amersham).

RESULTS

Internalization of the HIV-1 Env protein expressed on $CD4^+$ T cells. When expressed in B-LCL by using vaccinia virus vectors, the Env protein of HIV-1 is rapidly internalized from the cell surface (37). To determine whether rapid endocytosis of the Env protein also occurs in cells that are targets for virus replication in vivo, $CD4^+$ T cells were infected with vaccinia virus vectors carrying either the wild-type HIV_{LAI} *env* gene (vPE16) or a mutant form of the *env* gene with a single tyrosine→alanine substitution in the endocytosis motif in gp41 (vJR3). Previously published pulse-chase studies have shown that this mutation at position 707 of the BH8 Env sequence (position 712 of the reference HXB2R isolate) does not affect rates of synthesis or processing of the Env protein (37). Rates of Env internalization in $CD4^+$ T cells were measured by a previously described flow cytometric assay (37). Cells were incubated with an anti-gp41 MAb at 0°C, washed, and then incubated in medium for various times at either 37°C, to allow internalization of surface Env protein, or 0°C, a temperature that is nonpermissive for endocytosis. The cells were then

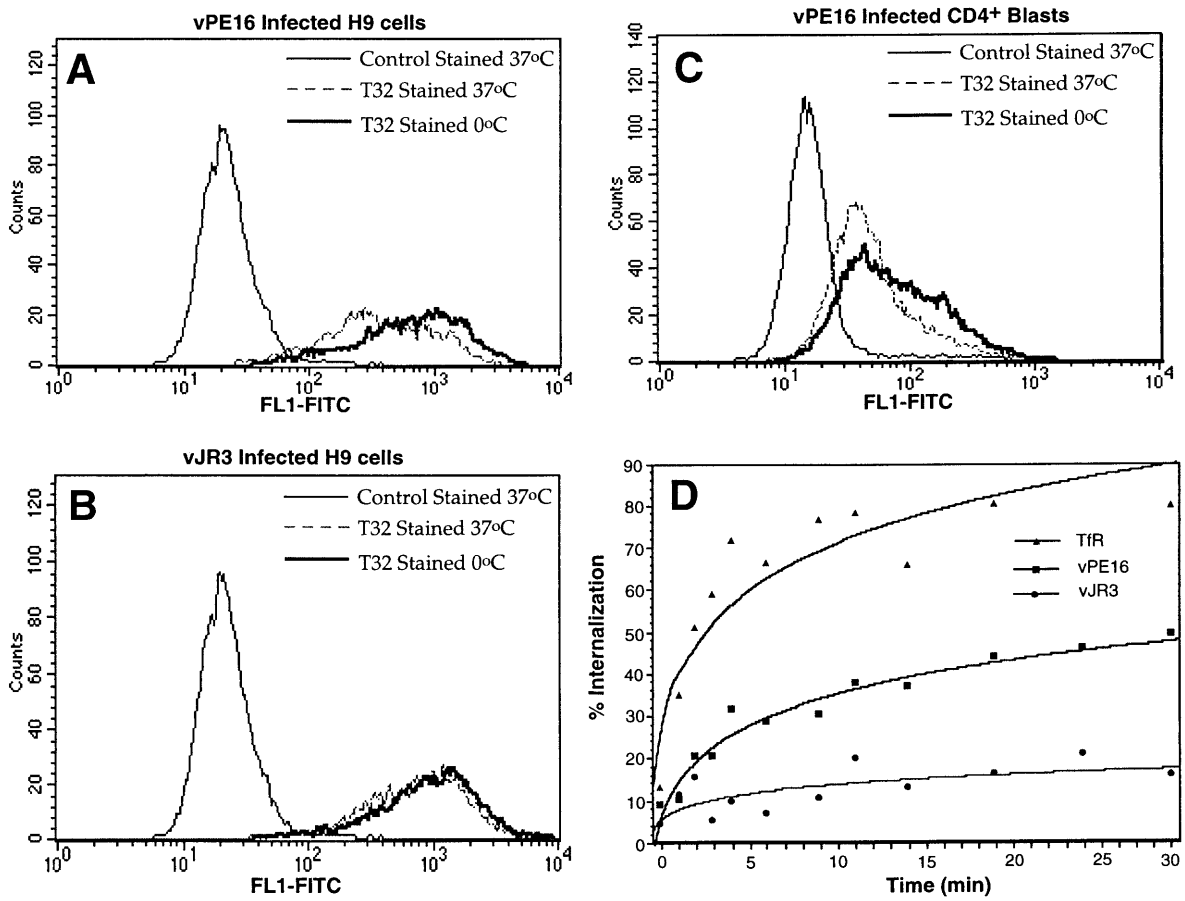

FIG. 1. Internalization of wild-type and mutant forms of the HIV-1 Env protein from the surfaces of $CD4^+$ human T cells. (A) Flow cytometric analysis of internalization of the wild-type Env protein from H9 cells infected with vPE16. H9 cells were infected with vPE16 and incubated overnight at 37°C. Cells were then stained at 4°C with an isotype-matched control antibody or with the anti-gp41 antibody T32. Cells were then incubated at 37 or 0°C for 60 min, at which point all samples were stained with a secondary antibody at 0°C. The degree of internalization of the HIV-1 Env protein in these cells was then measured by flow cytometry. (B) Flow cytometric analysis of internalization of the Y707A mutant Env protein from H9 cells infected with vJR3. (C) Flow cytometric analysis of internalization of the wild-type Env protein from primary $CD4^+$ lymphoblasts cells infected with vPE16. (D) Kinetics of internalization of wild-type and mutant forms of the env protein. Primary $CD4^+$ lymphoblasts were infected with vPE16 or vJR3. The rate of internalization of the Env protein and the TfR (CD71) was measured by the flow cytometric assay. Data are from a single representative experiment.

incubated at 0°C with a FITC-conjugated secondary antibody and analyzed by flow cytometry. An anti-gp41 antibody was used in these experiments because the antibody will not detect shed gp120 bound to CD4 on the surfaces of infected cells. This method is ideal for the quantitative analysis of endocytosis for several reasons. First, because data can be collected on large numbers of individual cells (n = 10,000), even subtle changes in the level of surface expression can be measured with a high degree of statistical accuracy. Second, dead cells can be excluded from the analysis by using appropriate gating parameters. Third, by collection of data using a linear acquisition mode, background fluorescence can be subtracted. In control experiments in which endocytosis was blocked by fixation of cells prior to labeling with primary antibody or by incubation in the presence of hypertonic sucrose, no loss of staining was observed during the 37°C incubation, indicating that the observed decrease in fluorescent signal is due to endocytosis rather than dissociation of the primary antibody during the 37°C incubation (37). In addition, in situations in which endocytosis of the Env protein is blocked, no loss of primary antibody occurs during the 37°C incubation, confirming that the assay measures endocytic processes (see below).

As shown in Fig. 1A, $CD4^+$ H9 cells infected with the vPE16 vaccinia virus vector carrying the wild-type *env* gene showed significant internalization of the Env protein, as indicated by the leftward shift of the fluorescence histogram of cells incubated at 37°C. This shift represents internalization of >50% of surface env protein in 60 min. In contrast, in vJR3-infected H9 cells expressing mutant Env protein with a Y707A mutation, there was a higher steady-state level of Env expression than on vPE16-infected cells and only minimal internalization of Env protein (Fig. 1B). Similar results were obtained in Jurkat cells (data not shown). Most importantly, readily detectable Env internalization also occurred in primary nontransformed $CD4^+$ T lymphoblasts (Fig. 1C). Analysis of the kinetics of internalization in $CD4^+$ lymphoblasts showed that >30% of the surface Env protein was internalized within 5 min of incubation at 37°C (Fig. 1D). A plateau level of 45% internalization was reached within 40 min. Internalization of the TfR on the same cells was also rapid. In contrast, the Y707A Env mutant expressed by the vJR3 vector was internalized to a significantly lower extent. These results demonstrate that the Env protein of HIV-1 can be internalized rapidly from the surface of normal $CD4^+$ lymphoblasts through an endocytic process that depends in part on a tyrosine residue in the cytoplasmic domain of gp41.

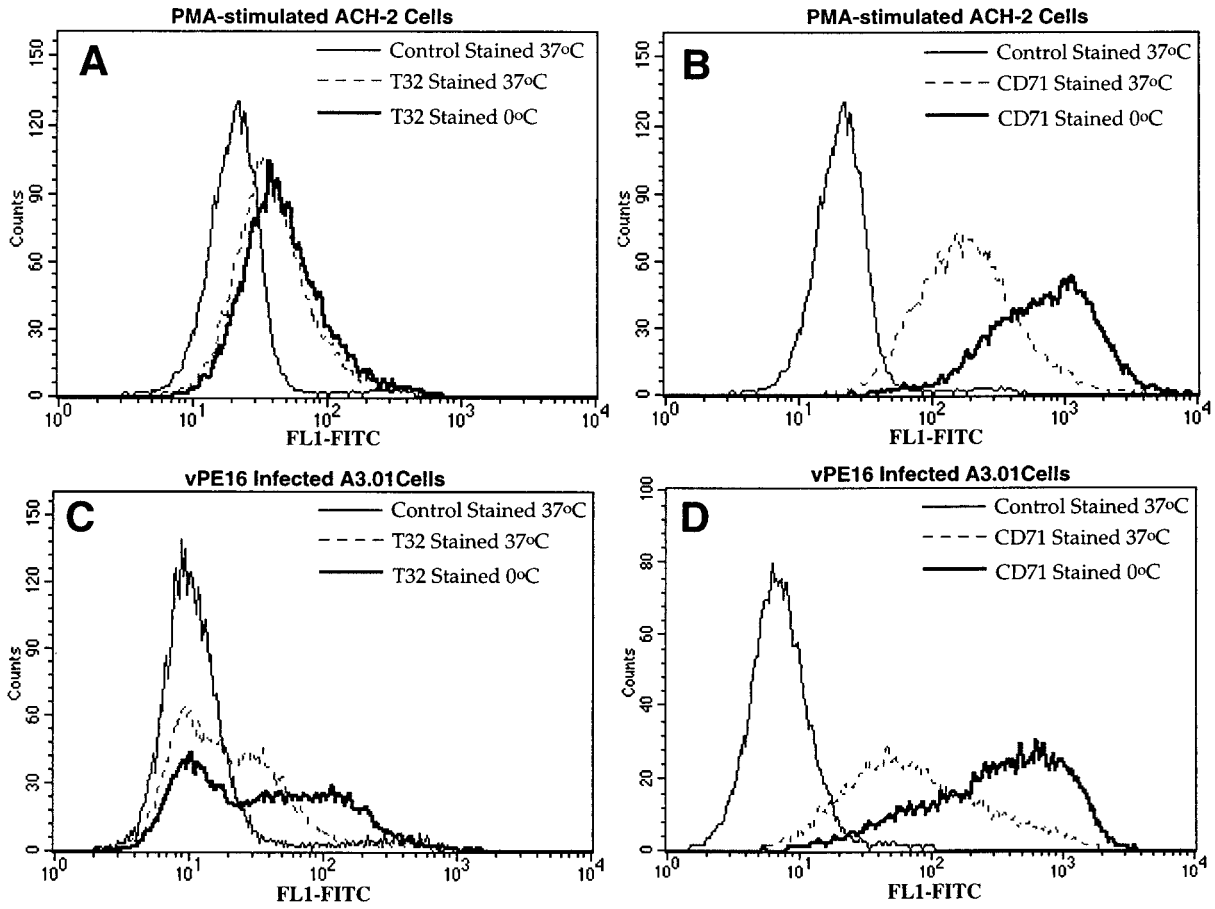

FIG. 2. Internalization of the HIV-1 Env protein and the TfR from the surfaces of PMA-stimulated ACH-2 and A3.01 cells. Chronically infected ACH-2 cells (A and B) or uninfected parental A3.01 (C and D) were stimulated with PMA 2 days prior to assay. One day before assay, A3.01 cells were infected with a vaccinia virus vector (vPE16) carrying the wild-type HIV-1 *env* gene. HIV-1-infected ACH-2 cells and vPE16-infected A3.01 cells were then incubated at 0°C with the T32 antibody to gp41 (A and C) or the anti-CD71 antibody to the TfR (B and D). Cells were then washed and incubated at 37 or 0°C for 60 min. The cells were then switched to 0°C, stained with an FITC-conjugated secondary antibody, and analyzed by flow cytometry as described in Materials and Methods. Each cell population was also stained with an isotype-matched control primary antibody followed by FITC-conjugated secondary antibody.

Internalization of the HIV-1 Env protein from the surfaces of HIV-1-infected CD4$^+$ T cells. Rapid endocytosis of the Env protein would be expected to interfere with incorporation of Env protein into virions budding from the plasma membrane. We therefore sought to determine whether productively infected human T cells show rapid internalization of the Env protein. To generate a uniformly infected population of T cells, we made use of the ACH-2 cell line, derived by infection of the CD4$^+$ human T cell line A3.01 with HIV-1_{LAI} (7). ACH-2 cells carry one integrated copy of HIV-1 provirus. Previous studies have shown that expression of HIV-1 genes in ACH-2 cells can be upregulated by PMA and by cytokines such as tumor necrosis factor alpha (7). Expression of Env protein on unstimulated ACH-2 cells was undetectable (not shown). After stimulation with PMA for 16 h, a high level of cell surface Env protein was detected (Fig. 2A). Surprisingly, analysis of the rate of internalization of surface Env protein showed that even after a 60-min incubation at 37°C, all of the Env protein on the cell surface at the beginning of the assay remained on the surface (Fig. 2A). Fluorescence histograms for cells populations incubated at 37 and 0°C were virtually superimposable. The failure of Env protein to undergo endocytosis was not due to a generalized defect in internalization or clathrin-dependent endocytosis in HIV-1-infected cells, since the TfR was readily internalized from the surface of PMA-stimulated ACH-2 cells. This is indicated by the dramatic shift to the left of the 37°C histogram in Fig. 2B. Rather, there was selective defect in endocytosis of the Env protein in these cells. The uninfected parental cell line A3.01 showed rapid endocytosis of Env protein expressed by using the vaccinia virus vector vPE16 (Fig. 2C) and rapid endocytosis of endogenous TfR (Fig. 2D). The experiment shown is representative of several experiments with this and other chronically infected cells lines which have never shown Env internalization. Taken together, these results demonstrate that in HIV-1-infected cells, most of the cell surface Env protein is not subject to rapid internalization, suggesting interference with the function of the intrinsic internalization signal in this context.

Env internalization in acutely infected primary CD4$^+$ lymphoblasts. To determine whether the suppression of endocytosis of Env protein also occurred in primary, nontransformed CD4$^+$ T lymphoblasts, CD4$^+$ T-lymphoblast cell lines derived from seronegative donors were infected at high multiplicity with HIV-1_{LAI}. Eight days after infection, the cells were uniformly positive for surface expression of the Env protein (Fig. 3A). However, no internalization of Env protein from the surfaces of HIV-1-infected T cells was detectable (Fig. 3A). As with ACH-2 cells, the failure of the Env protein to undergo

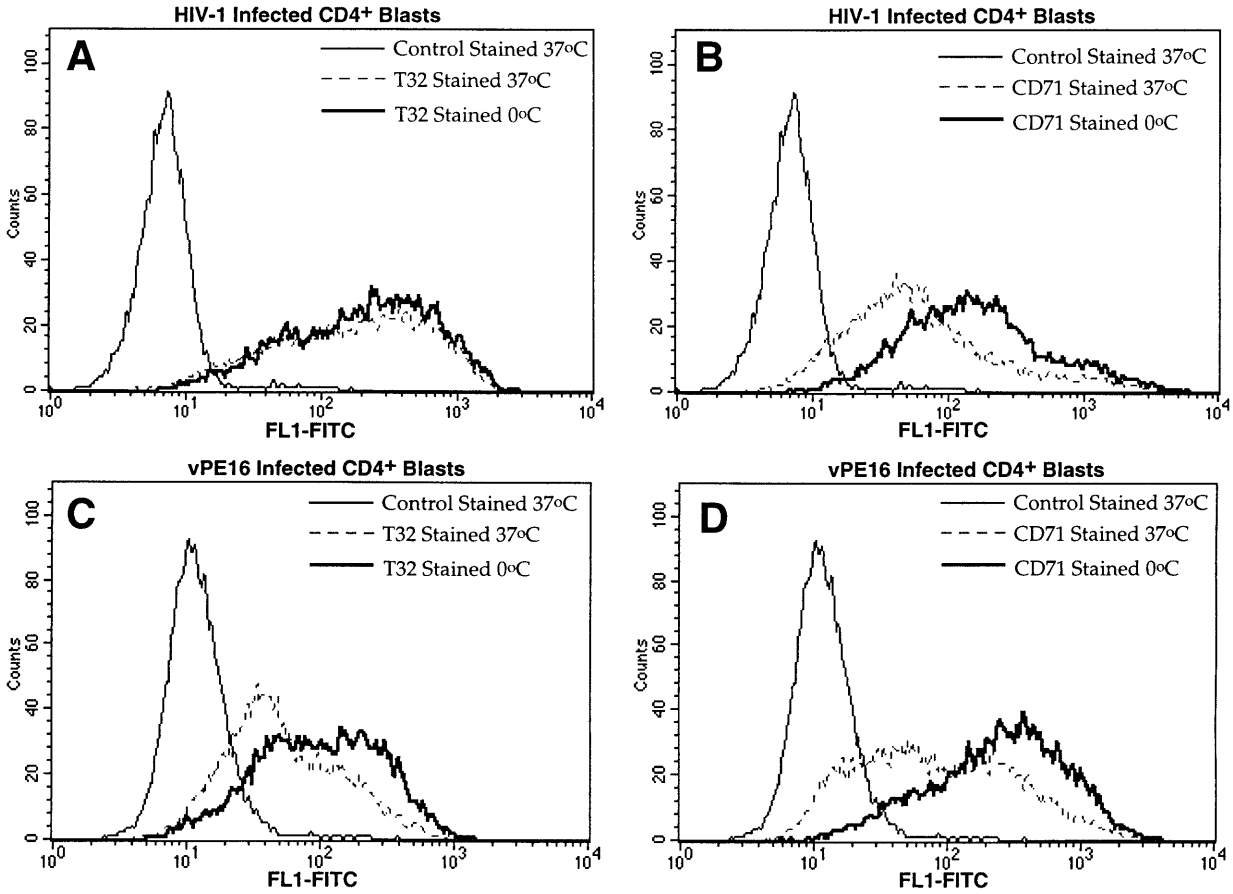

FIG. 3. Internalization of the HIV-1 Env protein and the TfR from the surfaces of HIV-1-infected and uninfected $CD4^+$ lymphoblasts. Primary $CD4^+$ lymphoblast cultures acutely infected with HIV-1 (A and B) or with a vaccinia virus vector carrying the wild-type HIV-1 *env* gene (C and D) were incubated at 0°C with antibodies to gp41 (A and C) or the TfR (B and D). Cells were then washed and incubated at 37 or 0°C for 60 min. The cells were then switched to 0°C, stained with a FITC-conjugated secondary antibody, and analyzed by flow cytometry as described in Materials and Methods. Each cell population was also stained with an isotype-matched control primary antibody followed by FITC-conjugated secondary antibody.

internalization was not due to a global defect in endocytic processes in HIV-1-infected cells, since the TfR was rapidly internalized in the same cells (Fig. 3B). When the *env* gene was expressed in parallel uninfected control cultures of $CD4^+$ lymphoblasts by using a vaccinia virus vector, rapid internalization of Env protein and of the TfR was observed (Fig. 3C and D). These results confirm in primary cells the conclusion that in the context of HIV-1 infection, the intrinsic internalization signal in the cytoplasmic domain of gp41 is not functional in promoting Env internalization.

Env internalization is inhibited in the presence of $Pr55^{gag}$. Because internalization of the Env protein is rapid when the protein is expressed in T cells in the absence of other HIV-1 proteins but essentially undetectable when it is expressed in the context of HIV-1 infection, it is reasonable to assume that another HIV-1 protein may be responsible for inhibiting the endocytosis of the Env protein in HIV-1-infected cells. To identify the HIV-1 protein involved, individual HIV-1 proteins were coexpressed with Env in B-LCL and in $CD4^+$ T lymphoblasts by using vaccinia virus vectors, and rates of Env protein internalization were measured. Coexpression of Pol, Nef, and the Gag p24 domain had no effect on levels of Env expression or rates of Env internalization (data not shown). However, coexpression of the $Pr55^{gag}$ precursor protein with Env resulted in a significant and highly reproducible increase in steady-state levels of Env protein and inhibition of Env protein internalization (Fig. 4 and Table 1). Inhibition of internalization by $Pr55^{gag}$ was observed in each of eight independent experiments (29 to 100% inhibition; mean, 55%). Thus, the Gag precursor protein, which may interact with the cytoplasmic domain of gp41 during virus assembly (3, 8, 28, 31, 35, 49, 50), may prevent the internalization of the Env protein.

To confirm that the $Pr55^{gag}$ protein is involved in the inhibition of Env protein endocytosis observed in HIV-1-infected cells, we have examined the rates of endocytosis of Env protein in cells infected with a provirus containing a Gag mutation that blocks Env incorporation into virions, namely, a WA→RP substitution at positions 36 and 37 (48b). Because this mutation prevents the formation of infectious virions, Jurkat cells were transfected with a plasmid carrying the mutant provirus (HXB2 WA). In addition to the Gag mutation, a neomycin resistance gene was inserted in place of the *nef* gene in this plasmid (48a). A plasmid carrying the HXB2 provirus with a wild-type Gag sequence was also generated. Stable cells lines carrying the wild-type (Jurkat B2) and mutant (Jurkat B2WA) proviruses were derived by selection in G418 and then assayed for endocytosis of Env protein as described above. Importantly, this procedure allows the generation of cells lines in which 100% of the cells carry provirus. Total amounts of cell-associated Gag and Env proteins are comparable in the wild-

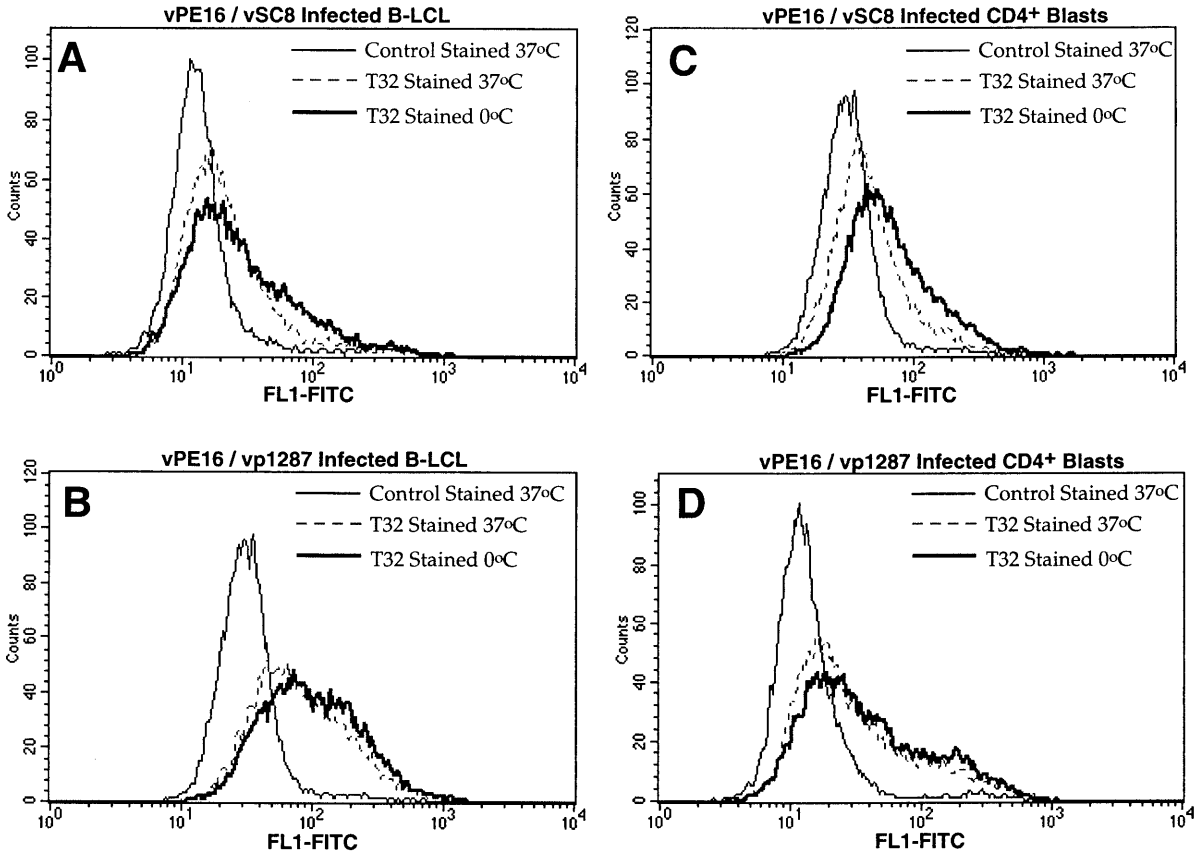

FIG. 4. Env internalization is inhibited by Gag-Env interactions. The HIV-1 Env protein was expressed in B-LCL (A and B) or primary $CD4^+$ lymphoblasts (C and D) by using vPE16 (multiplicity of infection of 5). These cells were simultaneously coinfected with the control vaccinia virus vector vSC8 (A and C) or the vP1287 vector, which expresses $Pr55^{gag}$ (B and D). Env internalization was measured by flow cytometry. Quantitation of the changes in mean linear fluorescence for $CD4^+$ lymphoblasts is shown in Table 1.

type and mutant transfectants. Pulse-chase experiments revealed that the rates of Env synthesis in both cells lines were identical, as measured at a 15-min chase time (48a). High-level surface expression of Env protein was detected on Jurkat B2 cells carrying the wild-type provirus (Fig. 5A). Confirming results obtained with acutely infected $CD4^+$ lymphoblasts and chronically infected ACH-2 cells, Jurkat B2 cells showed minimal internalization of Env protein after 1 h at 37°C (<15%). This was not due to any generalized defect in endocytic pathway or saturation of any of the critical components, since the TfR was rapidly internalized in the same cells (Fig. 5B). In contrast, Jurkat B2WA cells carrying mutant provirus defective in Env incorporation into virions showed extremely low steady-state levels of Env protein and, more importantly, readily detectable endocytosis of the small pool of Env protein on the cell surface (Fig. 5C). Expression and internalization of the TfR were also normal in these cells (Fig. 5D). The low steady-state level of Env expression on Jurkat B2WA cells may in part reflect effects of the Gag protein on transport of Env to the cell surface, but our results show that the rapid internalization of the surface Env protein in these cells maintains the low steady-state levels of Env on the surface. The kinetics of internalization of Env protein and the TfR on Jurkat cells carrying the wild-type and Gag mutant proviruses are shown in Fig. 6. In the case of the Jurkat B2WA cells expressing mutant Gag protein with a WA-RP substitution in the MA domain, over 50% of the cell surface Env protein was internalized within 5 min at 37°C as was the case with the TfR. In contrast, Env internalization was extremely slow in cells carrying the wild-type proviral construct. Comparable results were obtained in a completely independent set of transfectants in which the HXB2 and HXB2 WA constructs were introduced in the H9 cell line. These results show that in the presence of wild-type Gag protein, Env protein endocytosis does not occur and that a two-amino-acid substitution in the Gag protein that abrogates Env incorporation into virions can restore the function of the intrinsic internalization signal in the Env protein.

TABLE 1. Inhibition of Env internalization by Gag-Env interactions

Cells[a]	Antibody	Temp (°C)	MF (linear scale)	% Internalization[b]
vPE16/vSC8	Control	37	14	
	T32	37	23	66.7
	T32	0	41	
vPE16/vp1287	Control	37	13	
	T32	37	52	23.5
	T32	0	64	

[a] Primary $CD4^+$ blasts were coinfected overnight at a multiplicity of infection of 5:1 with the indicated vaccinia virus vectors.
[b] Calculated as [MF(0°C) − MF(37°C)]/[MF(0°C) − MF(control)] × 100.

The endocytosis signal in gp41 allows internalization of excess surface Env protein that is not protected from endocytosis by $Pr55^{gag}$. The results presented above indicate that the

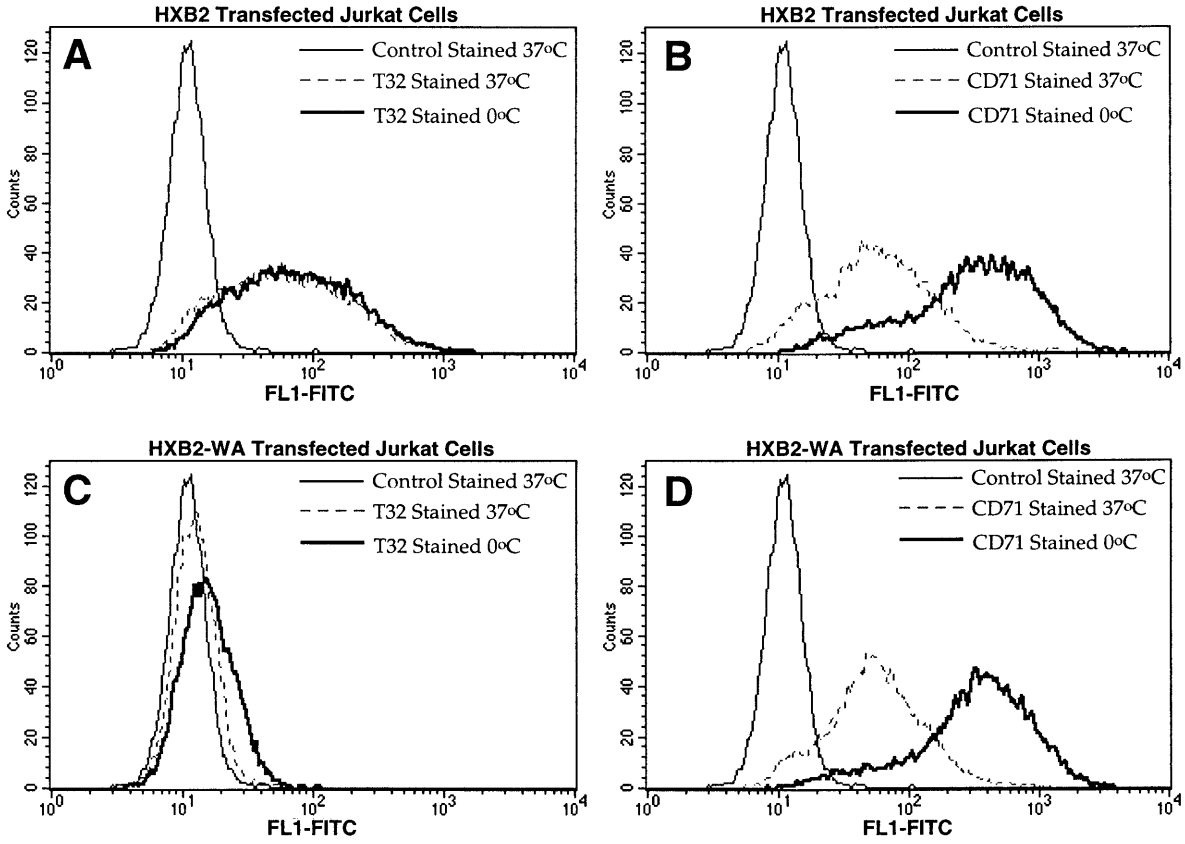

FIG. 5. Internalization of the HIV-1 Env protein in cells carrying a mutant provirus with substitutions in Gag that block Env incorporation into virions. Jurkat cells transfected with full-length HXB2 proviral constructs carrying a wild-type *gag* gene (A and B) or mutant forms of the *gag* gene (C and D) were assayed for internalization of the Env protein (A and C) or the TfR (B and D).

MA domain of the $Pr55^{gag}$ protein is critical for interactions that allow accumulation of Env protein on the cell surface. The much higher steady-state levels of cell surface Env protein on Jurkat B2 cells than on Jurkat B2WA cells result in part from the inhibition of Env protein endocytosis by wild-type $Pr55^{gag}$.

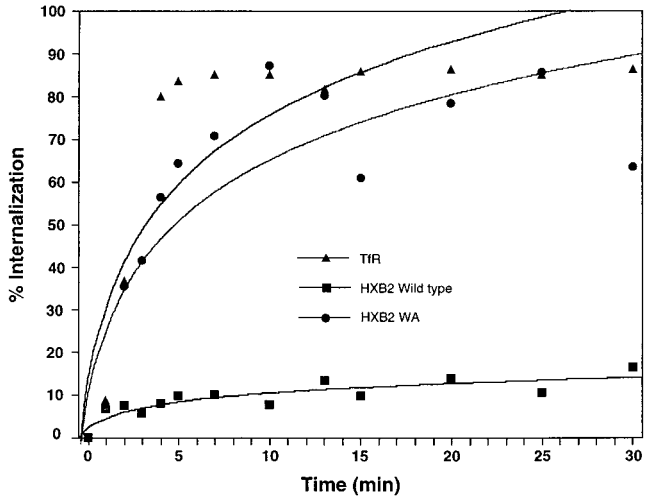

FIG. 6. Kinetics of internalization of TfR and Env protein on Jurkat cells transfected with a wild-type (HXB2 Wild type) or Gag mutant (HXB2 WA) proviral construct. Data from a single representative experiment are shown.

These observations raise the interesting question of what selective pressures favor the conservation of the internalization motif in gp41, given that internalization is inhibited in productively infected cells by $Pr55^{gag}$. One obvious explanation is that this motif allows internalization of any excess Env protein that is present on the cell surface in a form that is not protected from endocytosis by direct or indirect interactions with $Pr55^{gag}$ and that is therefore not destined for incorporation into virions. Removal from the cell surface of this unneeded excess Env protein could prevent the premature death of infected cells from Env-dependent cytopathic effects and from Env-specific cytolytic effector mechanisms. To explore this notion, we overexpressed wild-type Env protein or the Y707A mutant form of the Env protein in Jurkat B2 cells which carry wild-type HXB2 provirus. Overexpression was accomplished by using vaccinia virus vectors which readily infect this Jurkat cell line and alter the normal balance of Gag and Env proteins seen in HIV-1-infected cells. Previous pulse-chase studies have shown that the rates of biosynthesis and export of the wild-type and the Y707A mutant Env proteins are identical (37). Infection of Jurkat B2 cells with the vPE16 vector carrying the wild-type *env* gene resulted in a marked increase in the amount of cell-associated Env protein over levels expressed from the HXB2 provirus, as shown by Western blotting (Fig. 7A). Levels of Gag expression were unaffected by infection of cells with vaccinia virus expression vectors. Strikingly, infection of Jurkat B2 cells with vPE16 caused absolutely no change in surface levels of Env protein (Fig. 7B) relative to Jurkat B2 cell that were mock infected or infected with a control vaccinia virus vector

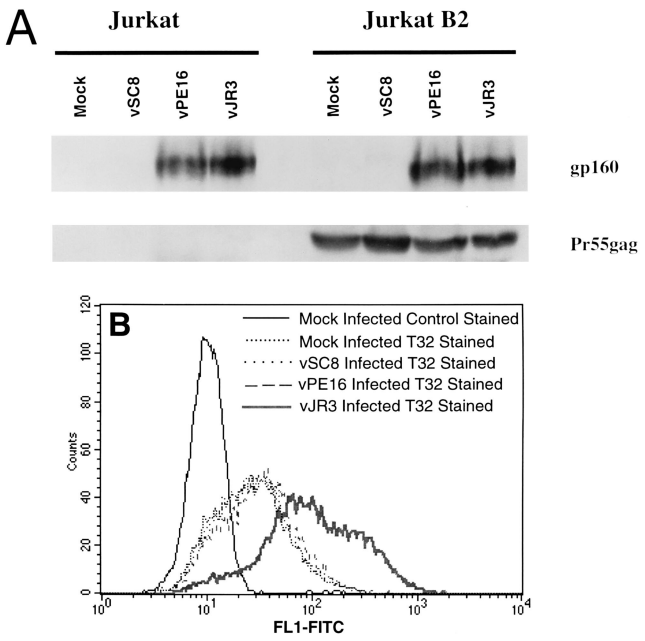

FIG. 7. Overexpression of wild-type and mutant forms of the Env protein in chronically infected cells. (A) Western blot analysis of Env and Gag expression in parental Jurkat cells and in Jurkat B2 cells. The indicated cell lines were either mock infected or infected with a control vaccinia virus vector (vSC8), a vaccinia virus vector carrying the wild-type *env* gene (vPE16), or a vaccinia virus vector which expresses the Y707A Env mutant (vJR3). Cell lysates were subjected to Western blotting analysis using an HIV-1-seropositive patient serum with strong reactivity against epitopes in gp41 and $Pr55^{gag}$. Bands for gp160 and $Pr55^{gag}$ are shown. Note that expression of Env protein from the HXB2 provirus is barely detectable under conditions in which Env protein expressed from vaccinia virus vectors gives very strong bands. (B) Flow cytometric analysis of Env expression on Jurkat B2 cells. Jurkat B2 cells carrying the HXB2 provirus were mock infected or infected with the vaccinia virus vector vSC8, VPE16, or vJR3. After an overnight incubation, cells were stained with an isotype-matched control antibody or with the anti-gp41 antibody T32.

(vSC8). Measurement of the mean linear fluorescence demonstrated that vPE16 infection resulted in only a 1.18-fold increase in Env expression relative to the mock-infected cells or the vSC8-infected cells. Thus, vPE16 infection failed to increase surface expression of Env protein even though levels of total cell-associated Env protein were greatly increased in vPE16-infected cells. In contrast, when Jurkat B2 cells were infected with the vJR3 vaccinia virus vector carrying the Y707A Env mutation, there was a dramatic (6.17-fold) increase in cell surface levels of Env protein. Comparable results were obtained in a completely independent set of transfectants in which the HXB2 and HXB2 WA constructs were introduced in the H9 cell line (data not shown). Thus, surface levels of wild-type Env protein are strictly controlled in HIV-1-infected cells through a mechanism that is critically dependent on the endocytosis motif in gp41.

DISCUSSION

We show here that the HIV-1 Env protein undergoes rapid internalization when expressed in $CD4^+$ T cells in the absence of other HIV-1 proteins. In contrast, if the same cells are infected with HIV-1, no internalization is observed. This was demonstrated in three different cell lines, including acutely infected primary $CD4^+$ lymphoblasts. The absence of internalization is not due to a global defect in internalization in infected cells, since the TfR was readily internalized in these cells. Rather, the slow internalization phenotype of productively infected cells is dependent on some function of the Gag protein. We found that internalization was inhibited in the cells coexpressing Env and $Pr55^{gag}$ and that internalization of Env was rapid in ells infected with an HIV-1 provirus that expresses a mutant Gag protein incapable of mediating Env incorporation. Taken together, these results indicate that some form of Gag-Env interaction inhibits the function of the intrinsic internalization motif in the HIV-1 Env protein. This motif functions to limit the amount of Env protein on the cell surface by clearing excess Env protein that is not protected from endocytosis by Gag-Env interactions.

The mechanism by which the intrinsic internalization signal in the gp41 subunit of the Env protein directs endocytosis of Env protein is unknown, but given that rapid Env protein endocytosis is dependent on a tyrosine residue in a motif resembling internalization motifs in host cell membrane proteins, it is likely that internalization involves the accumulation of the Env protein in clathrin-coated pits. We propose that the rapid internalization of Env from the surfaces of cells infected with a vaccinia virus vector carrying the wild-type *env* gene results from the interaction of the cytoplasmic domain of gp41 with host adaptins involved in receptor-mediated endocytosis (2, 34, 42). Binding of the medium (μ2) subunit of the AP2 adaptin complex to tyrosine-containing internalization motifs is an important initial step in the internalization of many cell membrane proteins. The dramatic effect of a tyrosine-to-alanine substitution in the context of this internalization motif strongly supports the notion that incorporation of Env protein into clathrin-coated pits is an important step in this internalization process. Work presented here demonstrates that in contrast to cells expressing the Env protein in the absence of other HIV-1 proteins, HIV-1-infected T cells do not internalize the Env protein. The failure of the Env protein to undergo endocytosis from the surface of HIV-1-infected cells may reflect the fact that the proposed interaction of the MA domain of the Gag protein with Env during assembly prevents the interaction of Env with host adaptin molecules that recruit plasma membrane molecules such as the TfR into clathrin-coated pits. As discussed below, this conclusion allows for a synthesis of divergent results in the literature regarding the nature of Gag-Env interactions and the mechanism of Env incorporation into virions.

Several studies suggest that interactions between the MA domain of the $Pr55^{gag}$ precursor polyprotein and the cytoplasmic domain of gp41 are important for Env incorporation into virions (3, 8, 28, 31, 35, 49, 50). Deletions within MA can affect Env incorporation into virions (3, 49). Similarly, some truncation mutations of the cytoplasmic domain of gp41 result in reduced Env incorporation in virions (4, 50). The presence of the MA domain of HIV-1 Gag is sufficient to mediate the incorporation of HIV-1 Env protein into hybrid HIV-1–visna virus particles (3). In addition, although the Gag protein can drive the formation of virus-like particles in the complete absence of the Env protein (12, 14, 15, 39), the basolateral localization of the Env protein in polarized epithelial cells dictates the polarized budding of virus in these cells (28, 35). Taken together, these studies suggest that some form of interaction between the MA domain of Gag and the cytoplasmic domain of gp41 mediates Env incorporation. Interestingly, for Gag mutants unable to incorporate Env protein, incorporation of truncated Env mutants can still occur (8, 31). This finding suggests that Gag-Env interactions are essential for the incorporation of the wild-type Env protein with its large cytoplasmic tail into virions. In the absence of Gag-Env interactions, wild-type Env protein may be sterically excluded from virions or

excluded by a mechanism similar to that proposed for the exclusion of host proteins, namely, as a result of interactions with other cellular factors that interfere with incorporation (16, 31). Truncated Env mutants can be incorporated through a nonspecific, passive mechanism. A logical extension of our results is that Gag-Env interactions are important for Env incorporation because they disrupt Env interactions with specific host proteins, namely, those involved in endocytosis, as was originally suggested by Hunter (16). In addition to increasing the steady-state level of Env protein on the cell surface, the inhibition of Env interactions with the host endocytosis machinery frees the Env protein to enter the constricted environment of the assembling viral envelope, a process that may be facilitated by direct Gag-Env interactions.

The endocytosis and assembly characteristics of wild-type virus and three classes of mutants are explained by this hypothesis. In the case of wild-type Env protein, interactions between the cytoplasmic tail of gp41 and the endocytosis machinery allow rapid endocytosis in the absence of Gag (37). In the presence of Gag, these interactions are disrupted, presumably by direct Gag-Env interactions. Thus, the Env protein is not readily internalized, as shown here, and can be readily incorporated into virions. Env mutants with defects in the endocytosis motif in gp41 will not associate with adaptins and are therefore internalized poorly as shown here and previously (37). These mutants can nevertheless be incorporated into virions (2a). Certain truncation mutants of the cytoplasmic domain of gp41 can be incorporated into virions even in the absence of Gag-Env interactions (8, 31). This may reflect the fact that the mutations disrupt interactions with the endocytosis machinery even though the endocytosis motif is still present. We have previously shown that some truncation mutants of the cytoplasmic tail of gp41 fail to undergo rapid endocytosis even though the motif is present (37). In this case, it appears that the truncation alters the accessibility of the motif. Finally, as shown here, Gag mutants that do not mediate Env incorporation show rapid env endocytosis because the interactions between Env protein and the adaptins are not disrupted.

Recent studies have shown that the mutations in the SIV Env protein at a position comparable to Y-707 result in higher levels of expression of Env protein on the surfaces of chronically infected human T-cell lines (22, 23). The most dramatic effects were observed with variants that were derived in in vitro culture systems and that have large truncations of the cytoplasmic tail. For these variants, immunofluorescence microscopy was used to show that the Env protein accumulated in intracellular vesicles, presumably as a result of internalization. The fact that the SIV Env protein can be internalized from the surface of chronically infected cells may reflect the fact that the Gag-Env interactions that block endocytosis can not occur in the case of the truncated Env mutants. Viruses containing a Tyr→Cys mutation in the endocytosis motif show increased replication rates and cytopathic effects in human cells (38). Interestingly, a mutant of HIV-1 Env with a corresponding Tyr→Cys change has recently been isolated from a patient with rapid $CD4^+$ T-cell loss (10). Increased levels of surface expression of Env protein were also noted on human cells chronically infected with mutants of SIVmac239 in which the relevant tyrosine residue was mutated to other residues. However, in the case of these viruses, which have full-length cytoplasmic tails, the increase in surface expression was not as dramatic. More recent studies have shown that the relevant region of SIV gp41 can function as an endocytosis motif when transplanted to a heterologous protein (38). Overall, studies in the SIV system support the notion that the Env proteins of these lentiviruses contain a highly conserved intrinsic internalization signal.

Endocytosis of wild-type and mutant forms of other viral glycoproteins has been observed. Ng et al. have shown that most of the hemagglutinin (HA)-neuraminidase glycoprotein of the paramyxovirus simian virus 5 that reaches the surface of infected cells undergoes rapid endocytosis (33). These investigators proposed that endocytosis and subsequent degradation of this viral antigen might reduce its immunogenicity. Introduction of a tyrosine residue at position 543 in the short cytoplasmic domain of influenza virus HA allows rapid endocytosis of HA in clathrin-coated pits (25). Rapid envelope glycoprotein endocytosis has also been observed for some temperature-sensitive mutants of Semliki Forest virus (47). Intrinsic sorting signals, which are distinct from but partially overlap endocytosis signals, allow segregation of influenza virus HA (13, 36) and vesicular stomatitis virus G protein (13) to the apical and basolateral plasma membrane domains, respectively, in polarized epithelial cells.

The finding that the HIV-1 Env protein contains a highly conserved motif that can mediate rapid endocytosis is surprising in light of the fact that accumulation of Env protein on the cell surface is important for the assembly of infectious virions. Although the inhibition of interaction between gp41 and adaptin proteins by Gag provides a plausible explanation for the low rate of endocytosis of Env protein in infected cells, there remains the issue of why the region of gp41 containing the endocytosis motif shows such a high degree of conservation. One possibility is that the interaction of gp41 with host proteins involved in endocytosis is important for some aspect of virus assembly. However, HIV-1 mutants with alanine substitutions at critical positions within the internalization motif are replication competent (2a), suggesting that a functional internalization motif is not critical for virus assembly per se. Another possibility is that high-level expression of Env protein is selected against in vivo as a result of Env-dependent cytopathic effects including syncytium formation (26, 40) and the toxic effect of Env protein on individual cells (19, 41). In addition, Env-specific host immune responses such as antibody-dependent cell-mediated cytotoxicity may be more effective against cells expressing high levels of Env protein. Our results indicate that physiological function of this highly conserved sequence is to remove from the cell surface any excess Env protein that is not associated with Gag and that is therefore not destined for incorporation into virions. This may prevent premature death of infected cells from cytopathic effects or immune surveillance.

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