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Human Immunodeficiency Virus Type 1 Vpu Protein Induces Degradation of Chimeric Envelope Glycoproteins Bearing the Cytoplasmic and Anchor Domains of CD4: Role of the Cytoplasmic Domain in Vpu-Induced Degradation in the Endoplasmic Reticulum

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The human immunodeficiency virus type 1 (HIV-1) Vpu protein is a transmembrane phosphoprotein which induces rapid degradation of CD4 in the endoplasmic reticulum (ER). To identify sequences in CD4 for Vpu-induced degradation, we generated four chimeric envelope glycoproteins having the ectodomain of HIV-1 gp160, the anchor domain of CD4, and 38, 25, 24, and 18 amino acids (aa) of the CD4 cytoplasmic domain. Using the vaccinia virus-T7 RNA polymerase expression system, we analyzed the expression of chimeric proteins in the presence and absence of Vpu. In singly transfected cells, the chimeric envelope glycoproteins having 38, 24, and 18 aa of the CD4 cytoplasmic domain were endoproteolytically cleaved and biologically active in the fusion of HeLa CD4$^+$ cells. However, one of the chimeras having 25 aa of the CD4 cytoplasmic tail was retained in the ER using the transmembrane ER retention signal and was defective in membrane fusion. Furthermore, biochemical analyses of the coexpressing cells revealed that the Vpu protein induced degradation of the envelope glycoproteins having 38, 25, and 24 aa of the CD4 cytoplasmic tail and degradation occurred in the ER. Consequently, the fusion-competent glycoproteins did not induce the formation of syncytia in HeLa CD4$^+$ cells expressing Vpu. However, the HIV-1 gp160 and chimeric envelope glycoprotein having the membrane-proximal 18 aa of the CD4 cytoplasmic tail were stable and fusion competent in cells expressing Vpu. In addition, we examined the stability of CD4 molecules in the presence of Vpu. Coexpression analyses revealed that the Vpu protein induced degradation of CD4 whereas mutant CD4 having the membrane-proximal 18 aa of the cytoplasmic domain was relatively stable in the presence of Vpu. Taken together, these studies have elucidated that the Vpu protein requires sequences or sequence determinants in the cytoplasmic domain of CD4 to induce degradation of the glycoproteins in the cell.

Human immunodeficiency virus type 1 (HIV-1) is the causative agent of AIDS. HIV-1 is a complex retrovirus whose genome encodes a number of regulatory and accessory proteins in addition to the structural components, Gag, Pol, and Env, that are common to all retroviruses (6, 17). The *env* gene product, gp160, undergoes endoproteolytic cleavage to generate gp120-gp41 complexes in the secretory pathway of mammalian cells (9, 41, 45, 64). The gp120-gp41 complex is primarily responsible for the cell tropism by virtue of its high-affinity binding to CD4 at the cell surface and delivering the HIV genome into the cytoplasm of helper T lymphocytes or other susceptible cells (37, 39, 54). One of the hallmarks of AIDS pathogenesis is the selective depletion of helper T lymphocytes, and this could predominantly be due to the envelope-mediated fusion of membranes and cell killing by the lysis of HIV-1-infected cells (1, 33, 37, 54). However, HIV-1 might employ other mechanisms to render the host immune system dysfunctional (31, 46, 55, 56). The cell surface expression of CD4 is greatly diminished in HIV-1-infected cells, and viral components have been implicated in the down-regulation of CD4 (7, 11, 21, 27, 57, 66). We and others have shown that newly synthesized gp160 and CD4 interacted with each other, forming gp160-CD4 complexes in the endoplasmic reticulum (ER), and CD4 in the complex was not able to reach the plasma membrane partly because of the defective transport of gp160 (2, 5, 23, 26). Recently, it has been shown that the regulatory protein Nef was capable of down-regulating CD4 from the cell surface, a process requiring the cytoplasmic domain of CD4 (11, 12).

One of the accessory proteins of HIV-1, Vpu, is a transmembrane phosphoprotein with a hydrophobic region at the N terminus (4, 58, 59). It is localized in the perinuclear region of the cell, presumably the ER or Golgi region (30), and is coordinately regulated with the envelope glycoprotein precursor, gp160, as both Vpu and gp160 proteins are synthesized from the same bicistronic mRNA in HIV-infected cells (48). The Vpu protein is unique to HIV-1, not being encoded by either the simian immunodeficiency virus or the HIV-2 genome. Viruses lacking the functional Vpu protein exhibited severe defects in the assembly and release of virus particles that accumulated predominantly in the intracellular vacuoles of infected cells (30, 62). In addition, it has been shown that the Vpu protein can selectively degrade CD4 in the ER (65, 66). Vpu is also involved in the export of capsid proteins independently of both gp160 and CD4, suggesting that the Vpu protein might have more than one target in the cell (67).

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In the present study, we have obtained evidence that a sequence or sequence determinant in the cytoplasmic domain of CD4 was required for Vpu-induced degradation in the ER. Using chimeric envelope glycoproteins bearing the anchor and cytoplasmic domains of CD4, we have demonstrated that a chimeric protein having 18 amino acids (aa) of the CD4 cytoplasmic domain was stable and fusion competent in cells expressing Vpu whereas the other chimeras having 38, 25, and 24 aa of the CD4 cytoplasmic tail were degraded in the presence of Vpu. Furthermore, the syncytium inhibition assay would be useful for analyzing the functional activity of Vpu in HeLa $CD4^+$ cells.

MATERIALS AND METHODS

Cells, virus, and expression plasmids. HeLa cells were used for expression studies. For the syncytium assay, HeLa T4 cells (39) were maintained and propagated in Dulbecco's modified Eagle's medium–10% newborn calf serum containing G418 (1 mg/ml). The recombinant vaccinia virus vTF7-3 (10) was used at a multiplicity of infection 10. The genes encoding envelope glycoproteins and Vpu were cloned behind the T7 promoter in pGem-3 (Promega, Madison, Wis.) or pcDNA1 (Invitrogen, San Diego, Calif.), respectively.

Construction of the genes encoding chimeric HIV-1 envelope glycoproteins and Vpu. The extracellular domain of gp160 was amplified by using primers NdeI and Env-BamAnc in polymerase chain reactions as described previously (45). Similarly, primer CD4BglAnc was combined with CD4wt.XbaI, K1, K2, or K3 to generate gene fragments encoding the anchor and cytoplasmic domains of wtCD4, CD4K1, CD4K2, and CD4K3, respectively (45). In separate reactions, the *Bgl*II-*Xba*I fragment (CD4s) and *Nde*I-*Bam*HI fragment (Env) were ligated into pGenv-7 (23), which was digested with *Nde*I and *Xba*I, to generate the chimeric HIV envelope glycoproteins. Recombinant clones were identified and sequenced through the junction to verify the in-frame joining of protein domains. The chimeric proteins, Env-CD4AC (Env-CD4.38), Env-CD4K3AC (Env-CD4.25), Env-CD4K2AC (Env-CD4.24), and Env-CD4K1AC (Env-CD4.18), have 38, 25, 24, and 18 aa, respectively, of the CD4 cytoplasmic tail. The following primers were used to create the chimeric HIV envelope glycoproteins (the boldface nucleotides represent specific restriction sites introduced in gene segments for the generation of chimeras): K-1, 5′ GACGC**TCTAGA**TTA GAGTCTCTTGATCTG 3′; K-2, 5′ GACGC**TCTAGA**TTAG GTCTTCTTCTCACT 3′; K-3, 5′ GACGC**TCTAGA**TTAG CAGGTCTTCTTCTC 3′; CD4BglAnc, 5′ GTCCACCCCGG TG**AGATCT**AAGGCCCTGATTGTGCT 3′; Env-BamAnc, 5′ TATCATTATGAATAT**GGATCC**ATACCACAGCCAG 3′; CD4wt.XbaI, 5′ GACGC**TCTAGA**CTAACCCAGGAGAA AC 3′; and NdeI, 5′ GCTAGTG**CATATG**ATACAGA 3′.

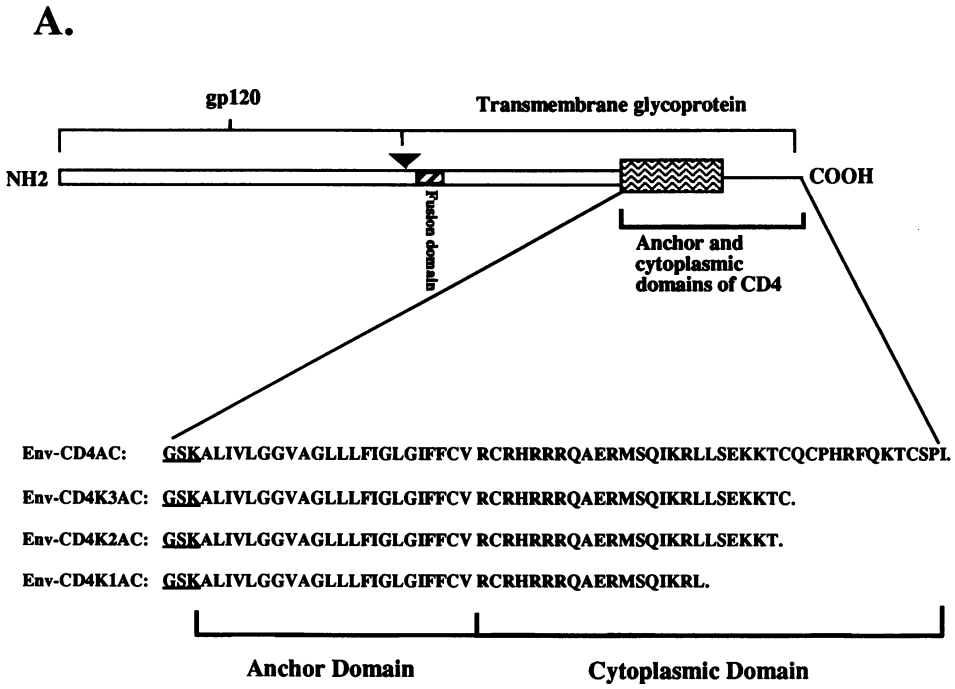
Plasmid pSP6 (59) (a gift from Klaus Strebel) and the two primers vpEcoR1 (5′ CTTCTCTAT**GAATTC**AGTAAGTA CATGTA 3′) and vpXba1 (5′ GACGC**TCTAGA**CACACAG GTACCCCATAA 3′) were used in polymerase chain reactions to amplify the gene encoding Vpu (the boldface sequences represent restriction sites *Eco*RI and *Xba*I, introduced at the 5′ and 3′ ends, respectively, of the Vpu gene fragment). Polymerase chain reaction products were cut with *Eco*RI and *Xba*I to release the Vpu fragment before being ligated into pcDNA1. A recombinant plasmid containing the Vpu gene was identified and named pcDN-VPU. Figure 1 schematically represents the chimeric HIV-1 envelope glycoproteins with the primary sequence of anchor-cytoplasmic domains and the Vpu protein (HIV-1_{NL4-3} [59]).

Infections and transfections. Transfections of vTF7-3-infected HeLa cells were performed as described previously (23). Briefly, 3 to 12 μg of expression vectors and 10 μl of Lipofectin (Bethesda Research Laboratories) were added separately to 0.1 ml of Opti-MEM in polystyrene tubes. Plasmid DNA and Lipofectin were mixed together for 15 min and added to 0.8 ml of Opti-MEM. The DNA-Lipofectin mix was layered onto infected cells and incubated for 3 h under 5% CO_2. At 3 h posttransfection, 1 ml of Dulbecco's modified Eagle's medium–10% fetal bovine serum was added to dishes, and incubation continued for 12 h at 37°C in the CO_2 incubator.

Western blot (immunoblot) analysis. Transfected HeLa cells were lysed in 0.5 ml of the lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl, 100 mM NaCl, and 5 mM $MgCl_2$), and protein was measured with the Bio-Rad (Richmond, Calif.) protein analysis kit. About 30 μg of protein was subjected to sodium dodecyl sulfate–8% polyacrylamide gel electrophoresis (SDS–8% PAGE), and immunoblot analysis was done as follows. Proteins were transferred to nitrocellulose papers by using the Bio-Rad semidry transfer apparatus for 45 min at 15 V, and blots were soaked in 5% milk (the blocking solution) for 30 min at 37°C before being washed twice with 0.1% Tween 20 in phosphate-buffered saline (PBS) for 5 min each. Ten milliliters of the antibody solution (20 μl of anti-$gp120_{SF}$ [α$gp120_{SF}$] in 10 ml of PBS containing 130 mg of sodium azide) was added, the mixture was incubated overnight at 4°C, and washings were done as described above. For the detection of antigen-antibody complexes, the biotin-avidin reaction was performed according to the manufacturer's instructions (ABC kit; Vector Labs, Burlingame, Calif.) by using biotinylated anti-goat antibodies. 4-Chloro-1-naphthol (0.5 mg/ml in a methanol-PBS mix [1:5]) was used as a substrate for avidin-conjugated horseradish peroxidase.

In vitro transcription and translation of the HIV-1 Vpu protein. Five micrograms of plasmid pcDN-VPU was linearized by digestion with *Xba*I (50 U), and in vitro transcription (10 μl) was performed with the T7 RNA polymerase according to the manufacturer's instructions (Promega). The synthetic RNA (1 μg) was translated by using rabbit reticulocytes (Promega) in a 15-μl reaction. Five microliters of the translation reaction mixture was mixed with 20 μl of acetone, and the mixture was incubated on ice for 15 min before being spun at 12,000 rpm (model 5415C; Eppendorf) for 5 min. The protein pellet was resuspended in 30 μl of sample buffer and heated at 100°C for 3 min. A fraction of the protein sample (30%) was analyzed by SDS–15% PAGE. For fluorography, gels were soaked in 1 M sodium salicylate containing 10% glycerol for 15 min, dried, and autoradiographed by using Kodak X-Omat-AR films.

Metabolic labeling, immunoprecipitation, endo-H treatment, and densitometry. Transfected cells were pulse-labeled for 30 or 10 min with 100 or 250 μCi of ^{35}S-Express-methionine (NEN) per ml (1,200 Ci/mmol; 0.5 ml of the labeling mix) and chased at different times in unlabeled methionine and cysteine as described previously (23). In experiments involving brefeldin A (BFA) (Boehringer Mannheim, Indianapolis, Ind.), the drug (5 μg/ml) was present during starvation (30 min), pulse-labeling (10 min), and chase periods. Immunoprecipitations, endo-β-*N*-acetylglucosaminidase H (endo-H) treatment, and fluorography were



B.

The HIV-1 vpu protein:

MQPIIVAIVALVVAIIIAIVVWSIVIIEYRKILRQRKIDRLIDRLIERAEDSGNESEGEVS

ALVEMGVEMGHHAPWDIDDL

FIG. 1. Schematic maps of chimeric HIV-1 envelope glycoproteins and primary structure of Vpu. (A) Chimeric HIV envelope glycoproteins were generated by the strategy described in Materials and Methods. Because of restriction sites, the extracellular amino acids Ile and Lys in gp160 (47) and Met in CD4 (40) were changed to Gly, Ser, and Lys, respectively, and thus the chimeric HIV envelope glycoproteins have Gly-Ser-Lys (GSK) at the boundary of extracellular and anchor domains. "AC" in the designations denotes the anchor and cytoplasmic domains of CD4 molecules. Positions of the lysine residues (indicated in the text) in the cytoplasmic domains are numbered from the carboxyl terminus (24). (B) Primary structure of the HIV-1 Vpu protein (59). The N-terminal hydrophobic amino acids are underlined.

performed as described previously (22, 45). Proteins were quantified by scanning autoradiograms (34) in a Bio-Rad model 620 densitomer.

Syncytium assays. For fusion assays, plasmids (3 μg each) encoding the chimeric envelope glycoproteins were transfected into HeLa T4 cells, and syncytia were observed 15 to 20 h posttransfection. For coexpression studies, plasmid DNAs (3 μg each) encoding the envelope glycoproteins and the Vpu protein (3 to 12 μg) were transfected together at different ratios (1:0, 1:1, 1:2, and 1:4). Transfected cells were photographed by using Polaroid 55 or 57 films at 20× in a phase-contrast microscope (Olympus).

RESULTS

HIV-1 Vpu protein induces degradation of CD4 in the ER. We employed the vaccinia virus-T7 RNA polymerase expression system to study the effect of Vpu in the intracellular metabolism of CD4. The plasmid encoding CD4 was introduced into HeLa cells alone or in combination with Vpu, and expression was analyzed. As shown in Fig. 2, CD4 made at the 10-min pulse-labeling point had acquired the endo-H-resistant modification after 3 h of chase (lanes 1 to 4, arrowhead a). However, a substantially smaller amount of CD4 was precipitated with the OKT4 antibody (αCD4) in the presence of Vpu, but the CD4 molecule made in coexpressing cells was transport competent, as evidenced by the acquisition of endo-H-resistant oligosaccharides (Fig. 2, lanes 5 to 8 [arrowhead b]). Thus, the pulse-chase analysis revealed that the amount of CD4 was considerably reduced in cells expressing Vpu, and this was perhaps due to the instability of CD4 in the presence of Vpu.

The Vpu protein has been shown to induce degradation of CD4 in the ER (65). We therefore used BFA to block the transport of proteins out of the ER (8, 38), and BFA has

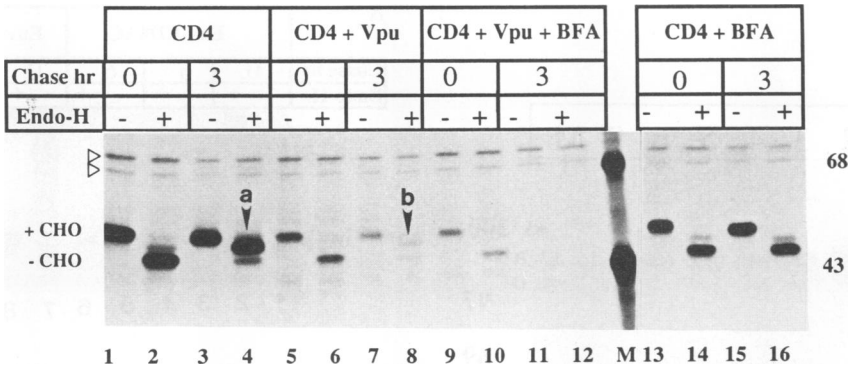


FIG. 2. Intracellular stability of CD4 with and without Vpu in BFA-treated cells. The plasmid (3 μg) encoding wtCD4 was transfected into HeLa cells alone or in combination with that expressing Vpu (12 μg). To block the ER to Golgi transport, BFA (5 μg) was present during starvation, pulse, and chase periods. Cells were pulse-labeled for 10 min and chased for 3 h in media containing excess unlabeled methionine. Detergent lysates were immunoprecipitated with the OKT4 antibody. The immunoprecipitates were treated with endo-H (+), or an equal portion was left untreated (−). Arrowheads a and b, endo-H-resistant CD4 made in the absence and presence of Vpu, respectively. +CHO, glycosylated CD4; −CHO, deglycosylated CD4. Viral proteins (internal controls) are indicated (triangles). Lane M, standard protein markers.

profound effects in the membrane trafficking apparatus of cells as well (28). The maturation of CD4 was effectively blocked in BFA-treated cells as expected (Fig. 2, lanes 13 to 16). Furthermore, the immunoprecipitation analysis revealed that CD4 made in the presence of both Vpu and BFA represented only about 50% of that synthesized in the presence of Vpu alone (Fig. 2, lanes 9 and 5, respectively). Thus, CD4 appeared to have already undergone Vpu-induced degradation during the 10-min pulse-labeling time. After 3 h of chase, the OKT4 antibody precipitated only a barely detectable amount of CD4 (Fig. 2, lanes 11 and 12), indicating that the Vpu protein induced degradation of CD4 in the ER.

The Vpu protein anchors to the membrane through the N-terminal hydrophobic region with little or no ectodomain and a cytoplasmic domain of 54 aa (58). We reasoned, therefore, that interactions, if any, between CD4 and Vpu are presumably mediated through the cytoplasmic or anchor domains. To test this hypothesis, we generated chimeric HIV-1 envelope glycoproteins bearing the anchor and cytoplasmic domains of CD4 and analyzed the intracellular stability of these proteins in cells expressing Vpu.

Expression of chimeric HIV-1 envelope glycoproteins and Vpu. Figure 3A shows the immunoblot analysis of chimeric HIV-1 envelope glycoproteins and gp160 expressed in HeLa cells (lanes 2 to 6). A fraction of envelope precursors was cleaved to generate gp120, which comigrated with the gp120 made in HeLa cells expressing gp120 (Fig. 3A, lanes 1 to 6, arrowhead b). To analyze expression of the Vpu protein in transfected cells, detergent lysates were immunoprecipitated with human HIV immunoglobulins and subjected to SDS–15% PAGE (Fig. 3B). A protein with the relative molecular mass of 16 kDa was synthesized (Fig. 3B, lane 2), and it comigrated with the Vpu made in vitro (Fig. 3B, lane 6). During the chase period, the amount of the 16-kDa Vpu protein was gradually reduced (lanes 2 to 4), and little or no Vpu was present after the 6-h chase (Fig. 3B, lane 5). The pooled serum did not precipitate proteins in the range of 14 to 16 kDa in vTF7-3-infected control cells (Fig. 3B, lane 1), demonstrating the specificity of HIV sera in recognizing the 16-kDa Vpu protein from the transfected cell lysates. Thus, Vpu exhibited a half-life of approximately 2 to 3 h, as previously reported (62, 65).

Vpu induces degradation of the HIV-1 envelope glycoprotein bearing the anchor and cytoplasmic domains of CD4. We then wanted to study the effect of Vpu in the intracellular metabolism of chimeric envelope glycoproteins. Accordingly, the plasmid encoding Env-CD4AC (Env-CD4.38) was transfected into HeLa cells alone or in combination with that expressing Vpu. Detergent lysates were immunoprecipitated with αgp120 antibodies, and proteins were resolved on SDS–8% polyacrylamide gels. As shown in Fig. 4A, the gp120 antibody recognized a major protein with the relative molecular mass of 140 to 150 kDa from the detergent lysate of pGEnv-CD4AC-expressing cells. Unlike wtCD4 (Fig. 2), comparable amounts of the envelope glycoprotein precursors were made in the absence and presence of Vpu during the 30-min pulse-labeling period (Fig. 4A, lanes 1 and 7). The envelope precursors were endo-H sensitive, indicating that they had undergone high-mannose sugar modifications in the ER (13, 32, 36). Sensitivity to the endo-H digestion did not change appreciably during the chase period, but the level of intracellular glycoprotein precursors was gradually reduced (30 to 15% at 3 and 6 h of chase) in the absence of Vpu (Fig. 4A, lanes 3 to 6). This appears predominantly due to the endoproteolytic cleavage, transport, and gp120 shedding into the medium (Fig. 4C, lanes 1 and 2). In our assays, the intracellular gp120 (Fig. 4A, arrowhead) could not be quantitatively precipitated with αgp120 antibodies alone. Others have used a combination of gp160- and gp120-specific antibodies to reveal the presence of both gp120 and gp160 in the cell (9, 65, 66). However, the detection of gp120 in the medium had indicated that gp120-TM glycoprotein complexes were transported to the plasma membrane (Fig. 4C).

Furthermore, the amount of intracellular Env-CD4AC was considerably reduced in the presence of Vpu during the chase period (<2% at 3 h of chase [Fig. 4A, lanes 9 to 12]). Consequently, only a small fraction of gp120 appeared to be transported to the cell surface and shed into the medium (Fig. 4C, lanes 3 and 4). This was clearly due to degradation of the envelope precursor in the intracellular compartment (Fig. 4A, lanes 9 and 11). Under the same condition, the HIV-1 gp160 remained very stable (data not shown) and was biologically active in the fusion of HeLa T4 cells (see below). Thus, the Vpu protein appears to target envelope glycopro-

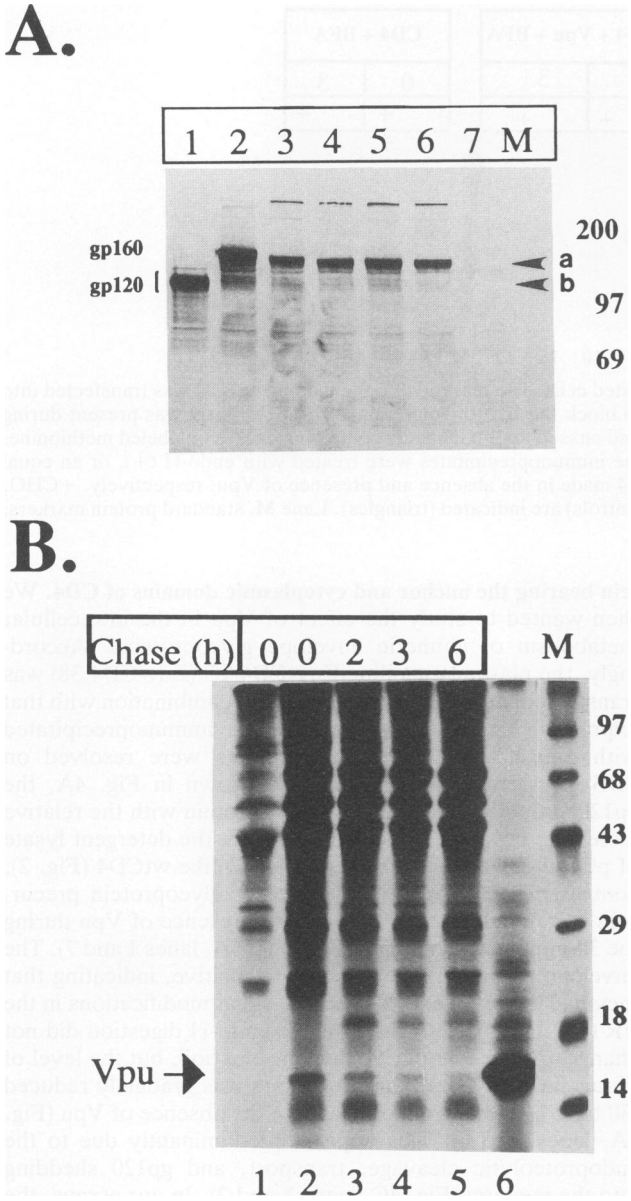


FIG. 3. (A) Expression of HIV-1 envelope glycoproteins. HeLa cells were transfected with plasmids encoding gp160, gp120, or each one of the chimeric envelope glycoproteins. Cytoplasmic lysates were prepared as described in Materials and Methods. Lanes: 1, gp120; 2, gp160; 3, Env-CD4AC; 4, Env-CD4K1AC; 5, Env-CD4K2AC; 6, Env-CD4K3AC; 7, vTF7-3-infected HeLa cells; M, protein markers. Positions of the chimeric envelope glycoprotein precursors and gp120 are indicated (arrowheads a and b, respectively). (B) Expression of the HIV-1 Vpu protein in vitro and in vivo. The synthetic RNA was made and translated by using rabbit reticulocytes as described in Materials and Methods. For in vivo expression studies, 12 μg of plasmid pcDN-VPU was transfected into vTF7-3-infected HeLa cells. The transfected cells were pulse-labeled for 30 min with 250 μCi of [^{35}S]methionine per ml and chased at different times in unlabeled methionine-cysteine, and lysates were immunoprecipitated with the HIV-1 antibody. Similarly, control lysates were made from vTF7-3-infected HeLa cells and immunoprecipitated. Proteins were analyzed by SDS–15% PAGE. Lane 1, vTF7-3-infected HeLa cells (pulse-labeling, 30 min); lanes 2 to 5, pcDN-VPU-transfected cells. Lane 2, 30-min pulse-labeling; lanes 3 to 5, chase times as indicated at the top. Lane 6, Vpu protein made in vitro from the synthetic RNA (6 ng). M, protein markers.

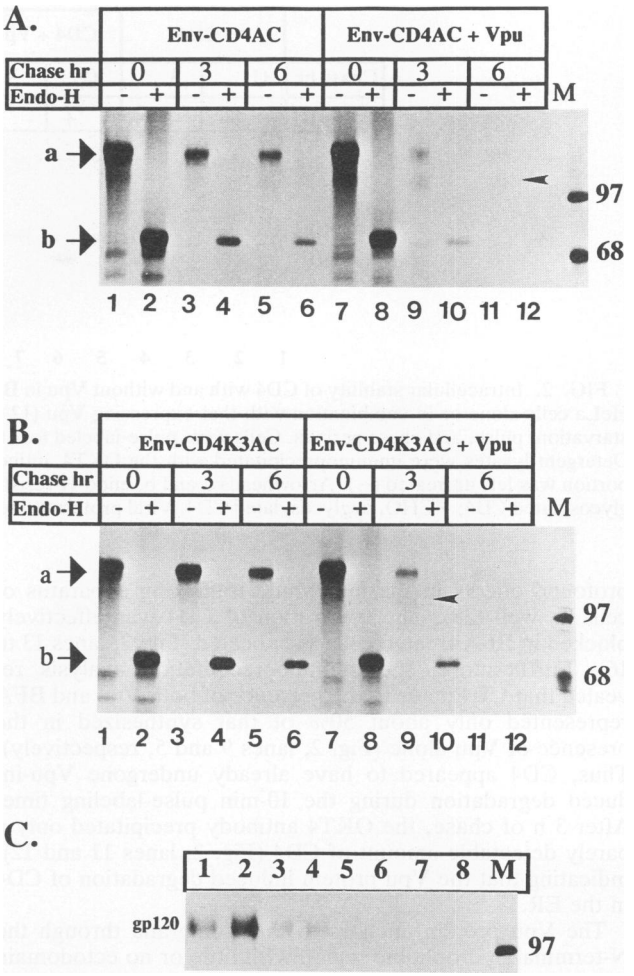


FIG. 4. Vpu induces degradation of Env-CD4AC and Env-CD4K3AC. The plasmid (3 μg) encoding Env-CD4AC or Env-CD4K3AC was transfected into HeLa cells infected with vTF7-3. In cotransfection experiments, 12 μg of pcDN-VPU was added to 3 μg of the plasmid encoding Env-CD4AC or Env-CD4K3AC. At 12 h posttransfection, the cells were pulse-labeled with 100 μCi of [^{35}S]methionine per ml and chased at the indicated times in media containing excess unlabeled methionine-cysteine. Subsequently, the cells were lysed in a radioimmunoprecipitation assay buffer (22), and the detergent lysates were immunoprecipitated with the αgp120 antibody. The immunoprecipitates were treated with endo-H (+) or left untreated (−) as described previously (23). To analyze cell-free gp120, media were collected from cells chased at 3 and 6 h and immunoprecipitated with the αgp120 antibody. (A and B) Expression and intracellular processing of Env-CD4AC and Env-CD4K3AC, respectively, in the absence or presence of Vpu. Arrows a and b, glycosylated and deglycosylated precursor molecules, respectively; arrowheads, gp120. (C) Kinetics of gp120 shedding into the medium. Lanes 1 and 2, gp120 from cells expressing Env-CD4AC alone; lanes 3 and 4, gp120 from cells expressing Env-CD4AC and Vpu; lanes 5 and 6, gp120 from Env-CD4K3AC alone; lanes 7 and 8, gp120 from Env-CD4K3 and Vpu; lane M, protein markers.

teins (the precursor and TM glycoproteins) bearing the anchor-cytoplasmic domains of CD4 for degradation in the cell. It is, therefore, likely that the extracellular gp120 (noncovalently associated with the TM glycoprotein) might have escaped Vpu-induced degradation and a fraction of gp120 was shed into the medium (Fig. 4C, lanes 3 and 4).

Vpu-induced degradation occurs in the ER. To examine the intracellular compartment(s) in which envelope glycoproteins could undergo Vpu-induced degradation, we have generated a chimeric envelope glycoprotein, Env-CD4K3AC (Env-CD4.25), which has the transmembrane ER retention signal (KKTC) in the carboxyl terminus (45, 51). The plasmid encoding Env-CD4K3AC was introduced into HeLa cells alone or in combination with Vpu, and expression was analyzed. As shown in Fig. 4B, Env-CD4K3AC acquired endo-H-sensitive oligosaccharide modifications, and comparable amounts of Env-CD4K3AC were made at the 30-min pulse-labeling point in both singly transfected and cotransfected cells (lanes 1 and 7). During the chase period, a substantial amount (70 to 50%) of Env-CD4K3AC was cell associated in the absence of Vpu (Fig. 4B, lanes 3 to 6), indicating that the chimeric glycoprotein was efficiently retained in the ER by the cellular machinery (24, 25). As a consequence, very little of gp120 was delivered to the plasma membrane, as evidenced by the profound lack of gp120 shedding in the medium (Fig. 4C, lanes 5 and 6). Furthermore, Env-CD4K3AC underwent degradation in cells expressing Vpu, as only a fraction of the glycoprotein was immunoprecipitated with the αgp120 antibody after 6 h of chase (10 to <1% at 3 and 6 h of chase [Fig. 4B, lanes 9 to 12]). Thus, these analyses have demonstrated that Vpu-induced degradation of Env-CD4K3AC had occurred predominantly in the ER.

Sequence specificity of Vpu-induced degradation. To determine the sequence requirement for Vpu-induced degradation process, we have generated two more envelope glycoproteins having progressive deletions (14 and 20 aa) in the cytoplasmic domain (Fig. 1A). Plasmids encoding Env-CD4K2AC (Env-CD4.24) and Env-CD4K1AC (Env-CD4.18) were transfected into HeLa cells in the absence and presence of Vpu, and expression was analyzed. Env-CD4K2AC and Env-CD4K1AC both were synthesized and translocated into the ER, undergoing endo-H-sensitive oligosaccharide modifications at the 30-min pulse-labeling point (Fig. 5A and B, lanes 1 and 2). In the absence of Vpu, the intracellular level of Env-CD4K2AC was gradually reduced predominantly because of the endoproteolytic cleavage and secretion of gp120 into the medium (Fig. 5A, lanes 3 and 5, and 5C, lanes 5 and 6). However, Env-CD4K2AC was unstable in the presence of Vpu, and the majority of intracellular envelope glycoprotein precursors were degraded after the 3- and 6-h chase periods (Fig. 5A, lanes 9 to 12).

Interestingly, Env-CD4K1AC was very stable in the absence and presence of Vpu (Fig. 5B). Approximately 60% of envelope glycoprotein precursors remained cell associated even after 6 h of chase in singly transfected cells (Fig. 5B, lane 5). This was in contrast to cells expressing Env-CD4AC and Env-CD4K2AC, in which the intracellular levels of envelope precursors were reduced because of the cleavage-secretion of gp120 (Fig. 3A and 5A, lanes 5). We have demonstrated here and elsewhere that 50 to 60% of mutant CD4 (CD4K1) having the lysine residue at the −3 position was retained in the ER (45) (see below). Env-CD4K1AC bearing the anchor and cytoplasmic domains of CD4K1 was endoproteolytically cleaved, and gp120 was shed into the medium (Fig. 5C, lanes 1 and 2). However, the majority of the envelope glycoprotein precursor was localized in the intracellular compartment, presumably in the ER using the motif KRL in the carboxyl terminus. Note that only 30 to 40% of gp120 was recovered from the medium of cells expressing Env-CD4K1AC and Vpu, and thus, to some extent, the Vpu protein appeared to have interfered with the

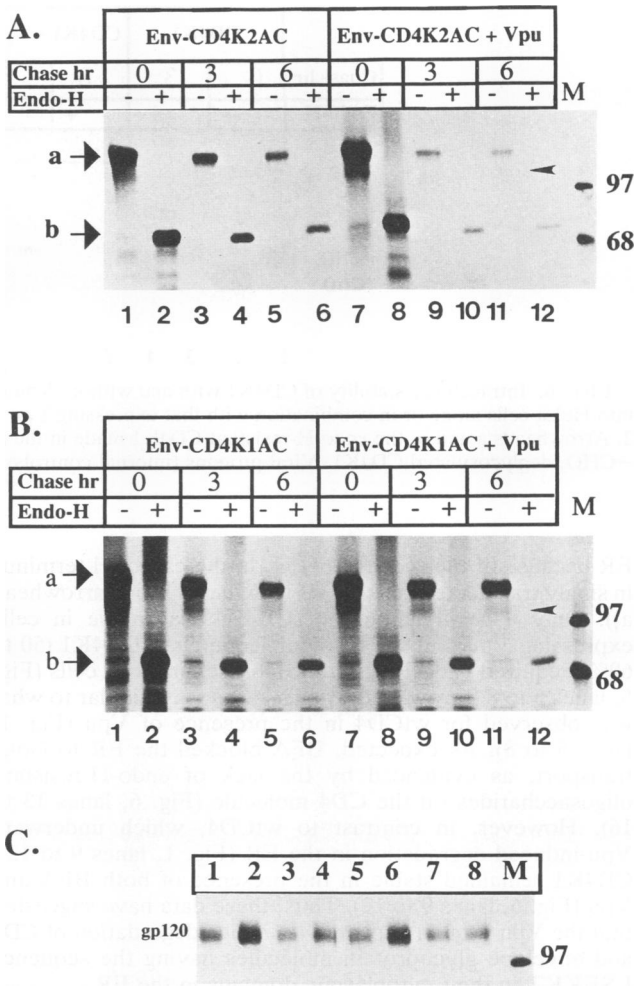


FIG. 5. Vpu did not induce degradation of Env-CD4K1AC, but Env-CD4K2AC underwent Vpu-induced degradation. The experimental protocol was identical to the one described in the legend to Fig. 3. (A and B) Expression and intracellular processing of Env-CD4K2AC and Env-CD4K1AC, respectively, in the absence or presence of Vpu. Envelope glycoprotein precursors (arrows a) and the deglycosylated precursor molecules (arrows b) are indicated. Arrowheads, gp120. (C) Kinetics of gp120 shedding into the medium. Lanes 1 and 2, gp120 from cells expressing Env-CD4K1AC alone; lanes 3 and 4, gp120 from Env-CD4K1AC with Vpu; lanes 5 and 6, gp120 from Env-CD4K2AC alone; lanes 7 and 8, gp120 from Env-CD4K2AC with Vpu; lane M, protein markers.

transport and/or shedding of gp120. However, more than 50% of the intracellular Env-CD4K1AC was stable and biologically active in the presence of Vpu (Fig. 5B) (see below).

These experiments have demonstrated that the chimeric envelope glycoprotein having 18 aa of the CD4 cytoplasmic domain was stable in cells expressing Vpu, compared with the other chimeras that underwent Vpu-induced degradation. Therefore, CD4K1 having the membrane-proximal 18 aa of the cytoplasmic domain (45) would also be stable in cells expressing Vpu. To examine this possibility, we expressed CD4K1 in BFA-treated cells with and without Vpu, and the stability of the protein was analyzed. As shown in Fig. 6, approximately 40 to 50% of CD4K1 was transport competent, and the rest of the molecule was retained in the

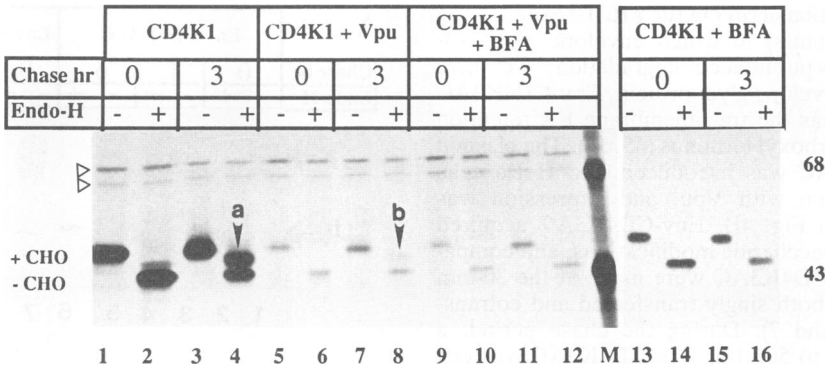


FIG. 6. Intracellular stability of CD4K1 with and without Vpu in BFA-treated cells. The plasmid (3 μg) encoding CD4K1 was transfected into HeLa cells alone or in combination with that expressing Vpu (12 μg). The protocol was the same as that described in the legend to Fig. 2. Arrowheads a and b, the endo-H-resistant CD4K1 made in the absence and presence of Vpu, respectively. +CHO, glycosylated CD4K1; −CHO, deglycosylated CD4K1. Viral proteins (internal controls) are indicated (triangles). Lane M, standard protein markers.

ER because of the sequence KRL in the carboxyl terminus in singly transfected cells (45) (Fig. 6, lanes 1 to 4 [arrowhead a]). Only a small amount of CD4K1 was made in cells expressing Vpu, but the transport-competent CD4K1 (50 to 60%) acquired endo-H resistance in coexpressing cells (Fig. 6, lanes 5 to 8 [arrowhead b]). This was very similar to what was observed for wtCD4 in the presence of Vpu (Fig. 1, lanes 5 to 8). As expected, BFA blocked the ER to Golgi transport, as evidenced by the lack of endo-H-resistant oligosaccharides on the CD4 molecule (Fig. 6, lanes 13 to 16). However, in contrast to wtCD4, which underwent Vpu-induced degradation in the ER (Fig. 1, lanes 9 to 12), CD4K1 remained stable in the presence of both BFA and Vpu (Fig. 6, lanes 9 to 12). Thus, these data have suggested that the Vpu protein appeared to induce degradation of CD4 and envelope glycoprotein molecules having the sequence LSEKKT in their cytoplasmic domains in the ER.

Sequence-specific Vpu-induced degradation occurs in the ER. Our analyses thus far indicated that Vpu-induced degradation of chimeric envelope glycoproteins, with the exception of Env-CD4K1AC, appeared to occur in the ER. It is, therefore, possible that this chimera was sorted to a membrane compartment to which Vpu cannot have access in the secretory pathway. To test this possibility, we analyzed the intracellular fate of Env-CD4K1AC in Vpu-expressing, BFA-treated cells. Single and cotransfected HeLa cells were pulse-labeled with [^{35}S]methionine and chased at different times in the presence or absence of BFA. Clarified cytoplasmic lysates were immunoprecipitated with αgp120 antibodies and analyzed by SDS-PAGE (Fig. 7). Envelope glycoproteins made during the pulse-labeling period remained relatively stable in the absence of both BFA and Vpu after 5 h of chase (Fig. 7A, lanes 1 to 3). The BFA treatment did not appreciably alter the stability of envelope glycoproteins (Fig. 7A, lanes 4 to 6), but the intracellular levels were increased after the 5-h chase because of the transport block by BFA (Fig. 7B, lanes 2, 6, and 10).

Env-CD4AC (Env-CD4.38) and Env-CD4K2AC (Env-CD4.24) both underwent degradation in BFA-treated (Fig. 7A, lanes 10 to 12) as well as untreated cells (Fig. 7A, lanes 7 to 9) in the presence of Vpu, suggesting that the majority of newly synthesized envelope glycoprotein precursors were susceptible to Vpu-induced degradation in the ER. In BFA-treated cells, the amounts of Env-CD4K1AC made in the absence and presence of Vpu (Fig. 7A, lanes 4 to 6 and 10 to 12, respectively) appeared comparable to those of the other two chimeric envelope glycoproteins synthesized without Vpu in the cell (Fig. 7A, lanes 1 to 6). In untreated cells, however, the envelope glycoprotein precursor was made at lower levels in the absence and presence of Vpu (Fig. 7A, lanes 1 to 3 and 6 to 8, respectively). Hence, the amount of gp120 shed into the medium was also proportional to the intracellular level of Env-CD4K1AC (Fig. 7A, lanes 1 to 3, and 7B, lane 5). Interestingly, Env-CD4K1AC was stable in both untreated and BFA-treated cells in the presence of Vpu (Fig. 7A, lanes 7 to 12). Thus, these data have suggested that the lack of Vpu-induced degradation was not due to the sorting of Env-CD4K1AC (Env-CD4.18) to a membrane compartment where the Vpu protein cannot have access to this envelope glycoprotein but rather due to the deletion of sequences or sequence determinants in the cytoplasmic domain.

Syncytium assay of cells expressing chimeric envelope glycoproteins. It has been shown that the anchor and cytoplasmic domains of HIV-1 gp160 are involved in the envelope-mediated syncycium formation (19, 44). We therefore tested whether the chimeric envelope glycoproteins bearing the anchor and cytoplasmic domains of CD4 would be biologically active. Accordingly, plasmids (3 μg each) encoding the chimeric glycoproteins were transfected into HeLa CD4$^+$ cells, and the formation of syncytia was observed 15 to 20 h postinfection (Fig. 8A). No syncytia were developed in cells expressing Env-CD4K3AC (Env-CD4.18) because of the retention of this protein in the ER (Fig. 4B and C, lanes 5 and 6). However, the other chimeric envelope glycoproteins were able to engage in membrane fusion, as evidenced by the formation of syncytia in HeLa CD4$^+$ cells (Fig. 8A). Thus, these studies have demonstrated that the anchor and cytoplasmic domains of CD4 could functionally substitute for the corresponding domains of gp160.

Membrane fusion ability of HIV-1 gp160 and chimeric envelope glycoproteins in cells expressing Vpu. We then performed experiments to study the effect of Vpu on the membrane fusion ability of HIV-1 envelope glycoproteins. Increasing concentrations of Vpu plasmids (3, 6, and 12 μg) and 3 μg each of the plasmid encoding HIV-1 gp160, Env-CD4AC, or Env-CD4K1AC were cotransfected into HeLa CD4$^+$ cells, and the formation of syncytia was observed 15 to 20 h postinfection. The HIV-1 gp160 induced syncytia in HeLa CD4$^+$ cells, and the formation of syncytia

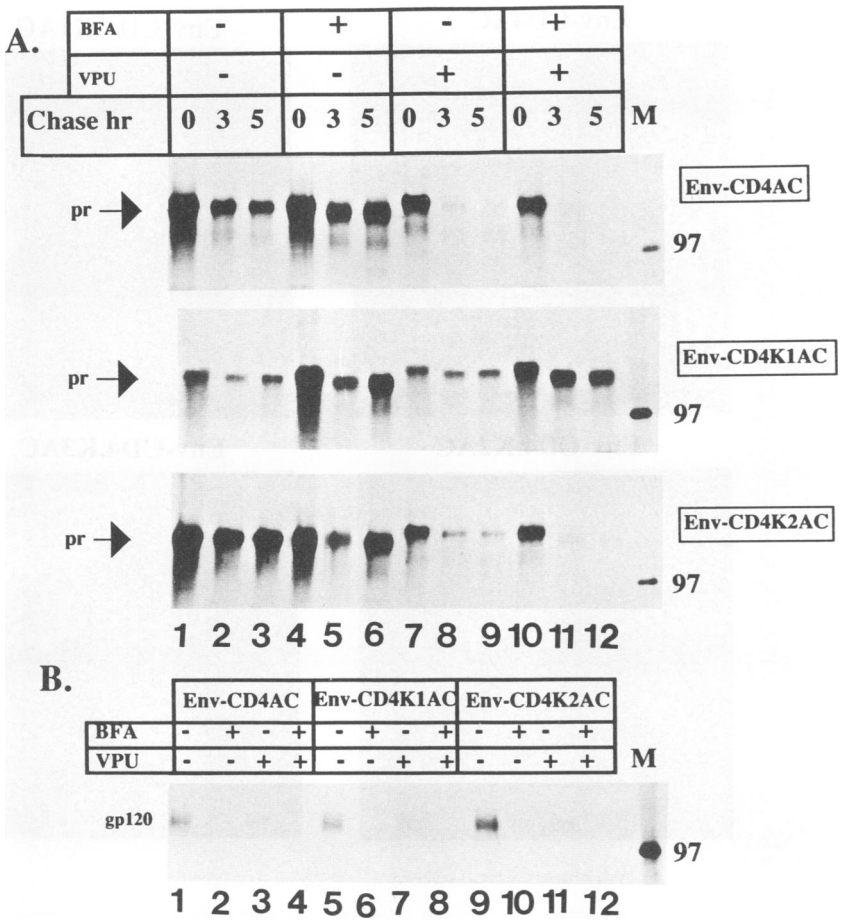


FIG. 7. Env-CD4K1AC was resistant to Vpu-induced degradation. The transfected HeLa cells were pulse-labeled with [^{35}S]methionine for 10 min in the presence (5 μg) or absence of BFA. The experimental protocol was the same as that described in the legend to Fig. 4. (A) Intracellular stability or degradation of the envelope glycoproteins in the presence and absence of the Vpu protein in BFA-treated or untreated cells. The precursor (pr) molecules synthesized in cells expressing Env-CD4AC, Env-CD4K1AC, and Env-CD4K2AC are indicated. (B) gp120 shedding into the medium. Cell-free media were collected at the 5-h chase point from cells expressing the chimeric envelope glycoproteins and immunoprecipitated with $\alpha gp120_{SF2}$ antibodies to reveal the presence or absence of gp120. M, protein markers.

was not significantly affected in the presence of Vpu (Fig. 8B). However, membrane fusion did not occur in cells expressing Env-CD4AC and Vpu; this was primarily due to degradation of the glycoprotein in the ER (Fig. 4A and 7A). Importantly, the chimeric envelope glycoprotein, Env-CD4K1AC, was able to induce the formation of syncytia in HeLa CD4$^+$ cells expressing Vpu, a finding consistent with the biochemical stability of this glycoprotein in the presence of Vpu (Fig. 5A and 7A). Thus, the syncytium inhibition assay can be employed to assess the functional activity of Vpu proteins from various HIV-1 isolates.

DISCUSSION

In the present study, we carried out experiments to assess the role of anchor and cytoplasmic domains of CD4 in a Vpu-induced degradation process. We prepared four chimeric HIV-1 envelope glycoproteins bearing the ectodomain of gp160 and the anchor-cytoplasmic domains of CD4. The chimeric envelope glycoproteins were biologically active in the fusion of HeLa T4 cells with the exception of one chimera that had the transmembrane ER retention signal. Biochemical analyses revealed that the chimeric envelope glycoproteins having 38, 25, and 24 aa of the CD4 cytoplasmic tail were degraded in the presence of Vpu and degradation occurred in the ER. However, the chimeric envelope glycoprotein having the membrane-proximal 18 aa of the CD4 cytoplasmic tail was stable in cells expressing Vpu. We have also demonstrated that wtCD4, but not CD4K1, underwent Vpu-induced degradation in the ER. Thus, these analyses have elucidated that the Vpu protein required the sequence (LSEKKT) in the cytoplasmic domain of CD4 and chimeric envelope glycoproteins to target them for degradation in the ER. Furthermore, these studies have also revealed that the ectodomain of CD4 was apparently not required for Vpu-induced degradation in the cell.

The mechanism by which the Vpu protein induced the degradation of CD4 and chimeric envelope glycoproteins is not clearly understood. The intracellular protein degradation as a regulatory mechanism has been described for both prokaryotic and eukaryotic systems (15). Recently, it has been shown that distinct signals (charged amino acids) in the anchor domain appear to determine the intracellular fate of T-cell antigen receptor α and β subunits (29, 53). It is, therefore, tempting to speculate that interactions between the cytoplasmic domain of CD4 and Vpu might perhaps

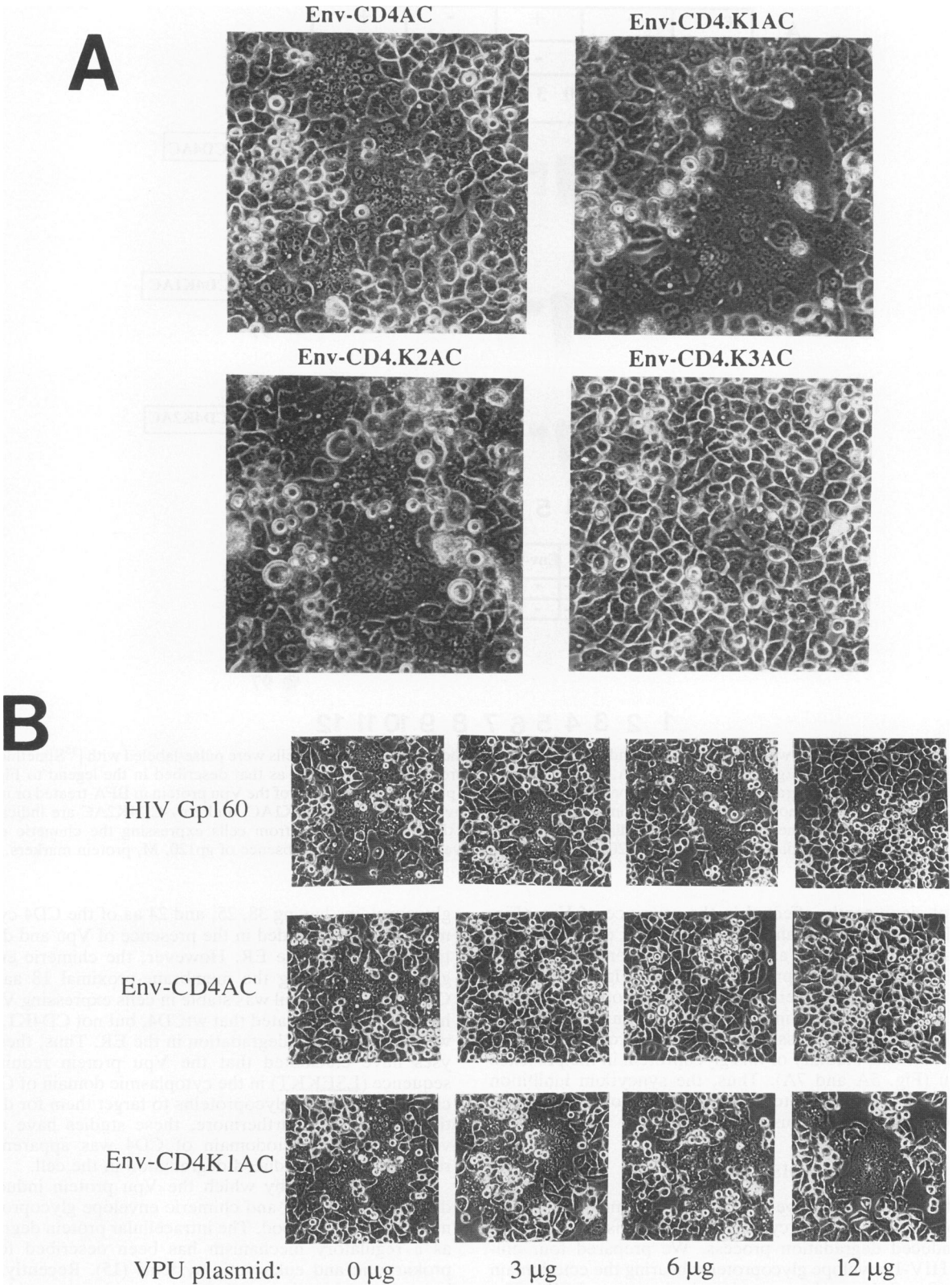


FIG. 8. (A) Membrane fusion abilities of chimeric envelope glycoproteins. Plasmids (3 μg each) encoding Env-CD4AC, Env-CD4K1AC, Env-CD4K2AC, and Env-CD4K3AC were transfected into vTF7-3-infected HeLa $CD4^+$ cells, and syncytia were observed at 15 to 20 h posttransfection. (B) Formation of syncytia in cells expressing Vpu. For the membrane fusion assay, plasmids (3 μg each) encoding gp160, Env-CD4AC, and Env-CD4K1AC were transfected into HeLa $CD4^+$ cells alone and in combination with the Vpu-expressing plasmid (3, 6, or 12 μg).

activate or expose degradation signals similar to those described for the α and β subunits. However, we have thus far failed to detect any physical association between the chimeric HIV-1 envelope glycoproteins and Vpu. In addition, the Vpu protein has been shown to play a role in the export of viral capsids to the cell surface independently of CD4 and gp160, suggesting that Vpu might have multiple targets in the cell (67). In experiments involving CD4 molecules, only a small amount of CD4 was immunoprecipitated from the detergent lysate of cells expressing Vpu and CD4 at the 10-min pulse-labeling time (Fig. 2 and 6). This was perhaps due to destabilization of free CD4 in cells expressing Vpu. Interestingly, Willey et al. (65) have reported that the steady-state levels of CD4 were reduced in the presence of Vpu when the transport of CD4 was blocked in the secretory pathway. In addition, we tested the stability of an unrelated glycoprotein, VSV-G, in cells expressing Vpu. The VSV-G was stable and transported to the Golgi apparatus in the presence of Vpu (data not shown), demonstrating the specificity of Vpu effects on the steady-state levels of CD4 molecules. Since the glycoproteins were expressed under control of the T7 promoter in vaccinia virus-infected cells, it is unlikely that the differences were due to transcriptional effects. However, we cannot rule out other translational or translocational effects of the Vpu protein in the intracellular metabolism of CD4.

A number of studies have elucidated that the cytoplasmic domain of CD4 contains information crucial for the function of CD4 in the immunoregulation and membrane trafficking in human and mouse cells (14, 49, 50, 52, 63). The membrane-proximal region of the cytoplasmic domain is predicted to form an α-helical conformation and can function as an endocytic uptake signal (52). Furthermore, the two cysteine residues in the cytoplasmic domain (KKTCQC) have been shown to interact with the $p56^{lck}$ kinase forming CD4-$p56^{lck}$ complexes, and such interactions appeared critical in the T-cell antigen receptor-mediated signaling pathway (14, 49, 50, 63). Recently, it has been shown that the HIV-1 Nef down-regulates CD4 from the cell surface through cytoplasmic interactions (11, 12). We have shown here that the Vpu protein requires the sequence LSEKKT in the cytoplasmic domain to target proteins to a degradative pathway in the ER. Thus, the sequence overlaps with those recognized by the $p56^{lck}$ kinase (49, 50). The $p56^{lck}$ protein can associate with CD4 or chimeric proteins bearing the CD4 tail in the ER and at the cell surface (49, 63). The ER association has been shown to retard the movement of proteins in the secretory pathway (49). It is not known how the Nef protein interacts with the cytoplasmic domain, triggering the uptake of CD4 from the plasma membrane (12). Mutational analyses would reveal novel interactions between CD4 and other regulatory or accessory proteins of HIV-1.

Furthermore, the Vpu-induced degradation of CD4 has been shown to relieve a block in the transport and processing of HIV-1 gp160 in cells expressing Vpu, gp160, and CD4 (66). Mutants defective in the functional Vpu protein accumulate virus particles in intracellular vacuoles and exhibit severe defects in the assembly and release of mutant particles from infected cells (30, 58, 62). The phenotype of Vpu mutants (e.g., defective assembly and release) might be related to Vpu function(s) that are elaborated in the membrane transport pathway (30, 65, 66). Interestingly, the HIV-1 Vpu protein shares some of the structural features with the influenza virus M_2 protein, the best characterized type III transmembrane protein (18, 20, 35, 42, 61). Biochemical and genetic analyses of the M_2 protein have revealed that it could modulate luminal milieu (e.g., pH) of the trans-Golgi compartment, thereby protecting the viral hemagglutinin from undergoing premature acid-sensitive conformational changes in the secretory pathway (3, 16, 60). Furthermore, the elegant experiments of Lamb and coworkers (43) have established that M_2 is an ion channel protein, which forms monovalent ion channel pores by its transmembrane domain in *Xenopus* oocytes (43). However, it is not known whether the HIV-1 Vpu protein is functionally related to the M_2 protein of influenza viruses. Clearly, further genetic and biochemical characterizations of Vpu will define the roles that this protein might play in the HIV-1 life cycle.

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