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Vpu-Induced Degradation of CD4: Requirement for Specific Amino Acid Residues in the Cytoplasmic Domain of CD4

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Two functions have been attributed to the product of the human immunodeficiency virus type 1 *vpu* open reading frame: it increases virion release from infected cells and induces rapid degradation of CD4 shortly after its synthesis. In the absence of Vpu, newly synthesized gp160 and CD4 associate in the endoplasmic reticulum (ER), forming a complex whose further maturation is blocked and which is eventually degraded. In studies using NL4-3-based expression vectors, it has been previously shown that Vpu induces the release of gp160 from the complex that it forms with CD4 in the ER. This release, which appears to be due to the rapid degradation of CD4 induced by Vpu, allows gp160 to transit to the Golgi, where it matures further. We investigated which regions of CD4 are important for its susceptibility to Vpu-induced degradation by transfecting HeLa cells with isogenic *vpu*-positive and *vpu*-negative proviruses and vectors expressing various truncated or mutated CD4 molecules. The results suggested that the cytoplasmic domain of CD4 contains a determinant lying within amino acids 418 to 425 that is critical for susceptibility to Vpu-induced degradation. Neither the phosphorylation sites in the cytoplasmic domain nor the Lck interaction region was required for the effect. Vpu-induced degradation was specific for CD4, since CD8, even when retained in the ER, was not degraded. In addition, under conditions of high-level Vpu expression, CD4 degradation could be observed in the absence of gp160 or other means of retaining CD4 in the ER.

The *vpu* open reading frame of human immunodeficiency virus type 1 (HIV-1) is located 3′ to the first exon of *tat* and overlaps with the 5′ end of *env*. It encodes an 81-amino-acid, serine-phosphorylated protein (13) consisting of a hydrophobic amino terminus of 28 amino acids and a charged carboxy terminus of 53 amino acids (3, 18). *vpu* is largely restricted to HIV-1; HIV-2 and simian immunodeficiency virus do not encode an analogous product (with the exception of a single simian immunodeficiency virus isolate [5a]). Although Vpu, like the virion structural components, is encoded by an incompletely spliced mRNA (14) and produced late in replication, it has not been found in virions (3, 17). Instead, the protein appears to be localized to a perinuclear compartment and anchored by its hydrophobic amino-terminal region to membranes (6, 17). Vpu and gp160 are translated from a single spliced mRNA, making this transcript the only known lentivirus bicistronic mRNA (14). It is possible that this arrangement ensures synthesis of a fixed ratio of Vpu to gp160. Translation of the two proteins in *cis* may also increase the efficiency at which Vpu functions (24).

vpu is not essential for HIV-1 replication in cell lines (6, 20) or in peripheral blood monocytes (10). Some common laboratory HIV-1 infectious molecular clones, such as HXB2, are *vpu* negative because of mutations that block Vpu synthesis. *vpu* does cause a significant increase in the amount of reverse transcriptase activity released into the supernatant by infected T cells (6, 20) and primary macrophages (22). T cells infected with *vpu*-positive virus released three- to fivefold more virions than did those infected with an isogenic *vpu*-negative virus (6). Electron microscopic studies have shown that virions produced from *vpu*-negative provirus accumulate at the cell surface and in endosomes (6, 20). In infected T-cell cultures, *vpu*-positive virus also resulted in less cytolysis and a delay in the appearance of syncytia (6, 20, 26). This decrease in syncytium formation could be due to more efficient release of virus from the cell surface, resulting in less gp160 at the cell surface.

Vpu has a second, apparently independent function in infected cells: it appears to induce rapid degradation of CD4 following its synthesis in the endoplasmic reticulum (ER) (25). It has been previously shown that following synthesis in the ER, CD4 and gp160 become associated (4, 23). This gp160-CD4 complex is blocked from further maturation and is eventually degraded in an intracellular compartment. Vpu-induced degradation of CD4 molecules in the complex may serve to release the gp160 molecules, freeing them to transit through the Golgi apparatus and to mature to functional gp120-gp41 on the cell surface. Support for this hypothesis comes primarily from two findings. (i) When gp160 and CD4 were expressed in HeLa cells in the absence of Vpu, processing of gp160 into gp120 and gp41 was largely blocked (24). In the presence of Vpu, processing of gp160 molecules was restored to levels comparable to those observed in the absence of CD4. (ii) In cells expressing CD4 and gp160, the half-life of CD4 was significantly decreased in the presence of Vpu (from 6 h to 12 min) (25).

Degradation of CD4 appears to occur in the ER (25). In infected cells, this is probably accomplished by association of CD4 with gp160 in the ER. Experimentally, the requirement for gp160 can be bypassed if CD4 is retained in the ER by an ER retention signal or by treatment of cells with brefeldin A (BFA) (25). There is no evidence that Vpu itself degrades CD4 or, instead, indirectly induces CD4 degradation. Whether there is a direct interaction of Vpu and CD4 or whether Vpu interacts with other cellular factors is also unknown. Interestingly, the effects of Vpu on gp160 processing and virion release appear to be independent of one another. Even with gp160-

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deficient HIV, and in CD4-negative cells, *vpu*-positive HIV-1 released more virions than did *vpu*-negative virus (27).

We report here results of our studies on Vpu-induced degradation of CD4. Our interest was to determine which domains of CD4 are critical for its susceptibility to Vpu-induced degradation. Our results suggest that Vpu-induced degradation is specific for CD4 and requires amino acid residues of the cytoplasmic domain. For these studies, we used HIV-1 provirus HXB2. Since this molecular clone does not express *vpu*, because of mutation, we restored the open reading frame by mutagenesis. The *vpu* product produced by this provirus proved to be fully functional.

MATERIALS AND METHODS

Proviral DNAs. HIV-1 provirus HXB2 was transferred into pBS-KS (Stratagene) by excising it from HXB-gpt with *Hpa*II and *Xba*I and ligating it to pBS-KS cleaved with *Xba*I and *Hin*cII. The *vpu* reading frame of this provirus was restored by changing the ACG codon (*vpu* amino acid 1, nucleotides 5,608 to 5,610) to ATG by oligonucleotide-directed mutagenesis (8). The mutation was confirmed by determining the *vpu* nucleotide sequences of both proviruses.

Plasmids. pSV-CD4 contains the human CD4 cDNA cloned into simian virus 40 (SV40)-based expression vector pSV-7d, a derivative of pHS210 (19), and has been previously described (9). pSV-CD8 contains the human CD8α cDNA F1.1 (12) and was obtained from Dan Littman. pSV-sCD4 and pSV-sCD4-KDEL (2) were constructed by excising the inserts of pBS-sCD4 and pBS-sCD4-KDEL (provided by John Rose) and ligating them to pSV-7d cleaved with *Eco*RI and *Bam*HI. Vectors expressing CD4 truncations and missense mutations were constructed by excising the inserts of pSP65-CD4.stop401, pSP65-CD4.stop403, pSP72-CD4.stop418, pSP72-CD4.ala410, and pSP72-CD4.leu417 (1) with *Eco*RI and *Xba*I and ligating them to similarly cleaved pSV-7d. pSV-CD4.stop425 was constructed by polymerase chain reaction with a 5′ primer which inserted an *Eco*RI site 48 bp upstream of the initiator methionine and a 3′ primer which replaced CD4 amino acid 425 with a termination codon followed by an *Xba*I site. pSV-CD4.ala422 was constructed by polymerase chain reaction mutagenesis with the 5′ primer described above and a 3′ primer which changed Cys-422 to Ala and inserted an *Xba*I site after the CD4 termination codon. Both polymerase chain reaction products were cleaved with *Eco*RI and *Xba*I and ligated into pSV-7d. Vpu expression vector pc-U^{atg} was constructed by polymerase chain reaction amplification of the HXB2 *vpu* reading frame with a 5′ primer that changed the ACG at amino acid 1 to an ATG and changed the surrounding sequence to that corresponding to a translation initiation consensus sequence (7) (CCACC ATG) and a 3′ primer that hybridized at the *vpu* termination codon (nucleotide 5,854). The primers contained terminal *Eco*RI and *Xba*I sites for cloning into pcDNA.I-amp (Invitrogen).

Cell culture and transfections. HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C and 5% CO_2. Cells were plated for transfection in six-well dishes (0.5×10^6 per well). On the next day, we prepared calcium phosphate DNA coprecipitates (5) containing a total of 10 μg of DNA (2.5 μg of HXB.U$^+$ or HXB2 and 7.5 μg of the CD4 expression vector) as described previously (21). Each precipitate was divided among three wells of cells. All transfected DNA was purified by double banding in cesium chloride density gradients. The cells were incubated overnight in the presence of 50 μM chloroquine, and the medium was changed on the next day.

Pulse-chase metabolic labeling and immunoprecipitation. On the day after transfection, the cells were washed in methionine- and cysteine-free RPMI and then starved in 0.9 ml of methionine- and cysteine-free RPMI for 10 min at 37°C. Labeling was initiated by adding 250 μCi of EXPRESS label (approximately 85% [^{35}S]methionine and 15% [^{35}S]cysteine; Dupont, NEN) in 100 μl of methionine- and cysteine-free RPMI. After 1 h, the medium was replaced with 2.0 ml of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The cells were then incubated at 37°C for 10, 90, or 180 min; washed once with cold phosphate-buffered saline; and lysed by addition of 0.5 ml of cold lysis buffer (150 mM NaCl, 0.5% Nonidet P-40, 2 mM EDTA, 10 mM Tris [pH 7.5], 50 μg of phenylmethylsulfonyl fluoride per ml). Lysates were incubated on ice for 5 min and then clarified by centrifugation in a microcentrifuge at 16,000 × *g* and 4°C for 5 min. For pulse-chase experiments in which only one time point was tested, cells were labeled as described above and processed as for the 10-min chase. When BFA (Epicenter Technologies) was included, it was added at 2 μg/ml at 1 h prior to labeling and maintained through the experiment.

For immunoprecipitation, lysates were precleared by adding 500 μl of RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 50 mM Tris [pH 7.5]) and 50 μl of protein A-agarose beads and incubating them for 30 min at 4°C. The beads were removed by brief centrifugation. Labeled proteins were immunoprecipitated by adding 0.5 μl of AIDS patient serum, rabbit anti-CD4 (obtained from R. Sweet through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases), monoclonal antibody OKT8 (Ortho), rabbit anti-recombinant gp120 (Genentech), or rabbit anti-recombinant Vpu. Anti-Vpu serum was prepared by injecting a rabbit with a glutathione *S*-transferase–Vpu fusion protein purified from *Escherichia coli*. Lysates were incubated for 1 to 3 h on ice. Antibody-antigen complexes were collected by adding 25 μl of protein A-agarose for 30 min at 4°C. Immunoprecipitates were washed three times with 1.0 ml of RIPA buffer and then solubilized in sample buffer containing 2-mercaptoethanol. Immunoprecipitated proteins were separated on SDS-polyacrylamide gels, fluorographed by treatment with En^{3}Hance (Dupont, NEN) in accordance with the manufacturer's instructions, dried, and exposed to X-ray film (Kodak) at −80°C with an intensifying screen.

RESULTS

HIV-1 infectious molecular clone HXB2 encodes a functional *vpu* product after restoration of its initiation codon. The HXB2 infectious molecular clone of HIV-1 has an ACG codon, instead of ATG, at amino acid 1 of *vpu* and therefore cannot express Vpu. To generate a *vpu*-positive variant of HXB2, we reverted the ACG codon to ATG (Fig. 1A; HXB-U$^+$). The predicted product of HXB-U$^+$ *vpu* is similar to that of NL4-3, which encodes a functional Vpu, but differs by a single amino acid insertion (Pro at position 6) and two conservative substitutions (Ile-61 to Val and Val-79 to Ile). To verify that HXB.U$^+$ expresses Vpu, we used the proviral DNA to transfect HeLa cells, metabolically labeled them, and then immunoprecipitated them with anti-Vpu serum (Fig. 2). HXB-U$^+$, but not HXB2, expressed a protein that comigrated with the Vpu produced by NL4-3, a *vpu*-positive provirus, and with that of Vpu expression vector pc-U^{atg} (Fig. 1B). The amount of Vpu produced by HXB.U$^+$ was similar to that of NL4-3. These results confirm that HXB-U$^+$ expresses Vpu, while HXB2

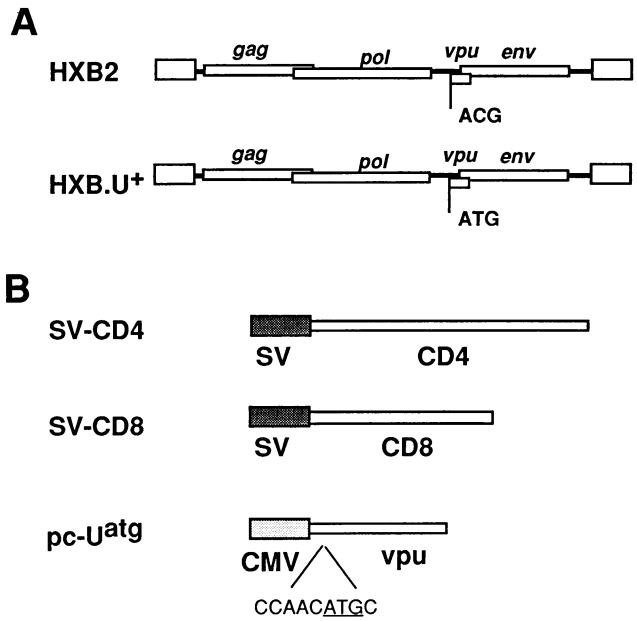

FIG. 1. Provirus and expression vectors. HXB2 is *vpu* negative because of the presence of ACG instead of the ATG initiation codon. In HXB.U$^+$, the *vpu* initiation codon was restored by mutagenesis. SV-CD4 (9) contains a human CD4 cDNA linked to the SV40 promoter and enhancer of expression vector pSV7-d. pc-U^{atg} contains the *vpu* sequence of HXB2 linked to the cytomegalovirus promoter in pcDNAI.amp. The ACG at amino acid 1 was converted to ATG, and the surrounding nucleotides were changed to reflect a sequence compatible with the translation initiation consensus sequence (7).

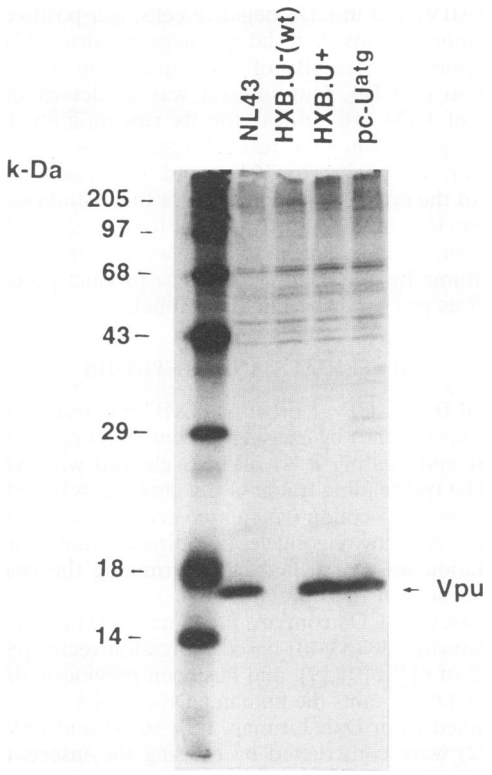

FIG. 2. Immunoprecipitation of Vpu expressed by HXB-U$^+$. HeLa cells (0.5×10^6) were transfected with 10.0 μg of NL4-3, HXB2, HXB.U$^+$, or pc-U^{atg}. The cells were metabolically labeled with a mixture of [^{35}S]methionine and [^{35}S]cysteine for 1 h, solubilized in Nonidet P-40-containing buffer, and immunoprecipitated with rabbit anti-Vpu serum. Proteins were separated by electrophoresis on a 15% polyacrylamide gel and visualized by autoradiography. Molecular size markers are indicated on the left.

does not produce Vpu or any antigenically related product of the *vpu* open reading frame.

Because we did not know whether Vpu produced by HXB.U$^+$ would be functional or whether HXB2 gp160 would be responsive to Vpu, we characterized the *vpu* phenotypes of the two proviruses. First, we determined whether the HXB.U$^+$ Vpu affects virion release. To do this, HeLa cells were transfected with each provirus and the amounts of cell-associated and supernatant p24^{gag} were measured. The results, averaged over several experiments, showed that cells transfected with HXB-U$^+$ released three- to fivefold more virions than did HXB2 (in one representative experiment, the results were 8.0 ng/ml for HXB2 and 34.0 ng/ml for HXB-U$^+$, with intracellular levels of 1,300 and 600 ng/ml, respectively). These results are consistent with those reported previously for other strains of HIV-1 (6, 20, 26) and suggest that Vpu encoded by HXB2-U$^+$ functions to increase virion release.

We next determined whether the Vpu encoded by HXB-U$^+$ restores gp160 processing in the presence of CD4. To do this, HeLa cells were cotransfected with HXB2 or HXB-U$^+$ and pSV-CD4 at a ratio previously determined to reduce gp160 processing significantly. The cells were pulse-labeled and chased, and the newly synthesized viral proteins were detected by immunoprecipitation with AIDS patient serum. The results showed that in the absence of CD4, the gp160 molecules produced by the *vpu*-negative and *vpu*-positive proviruses were processed (as measured by the amounts cleaved to gp120) with similar efficiencies (Fig. 3, lanes 1 to 6). In the presence of CD4, gp160 processing was blocked in cells transfected with HXB2 but proceeded unhindered in those transfected with HXB.U$^+$ (Fig. 3, lanes 7 to 12). The somewhat inefficient processing of gp160 we observed, even in the presence of Vpu (most of the gp160 remained unprocessed after a 3-h chase), is consistent with previous reports (23). Also visible on the gel were HIV-1 proteins p24^{gag}; the Gag precursor, pr55^{gag}; and a Gag processing intermediate, pr41^{gag}. The amounts of these proteins present in the transfected cells did not appear to be affected by Vpu, suggesting that Vpu does not affect processing of other HIV proteins. Furthermore, the differences in gp160 processing that we observed cannot be attributed to variability of transfection or labeling efficiency, since this would have been reflected in the amount of Gag products detected in this analysis. Taken together, these results suggest that Vpu produced by HXB.U$^+$, like that of NL4-3 (24), functions to increase the efficiency of gp160 processing in the presence of CD4.

Vpu-induced degradation requires sequences in the transmembrane or cytoplasmic tail of CD4. To define a region of CD4 that might be involved in determining susceptibility to Vpu-induced degradation, we tested the stability of truncated CD4 molecules in the presence of Vpu (Fig. 4A). One such molecule, sCD4, consisted of the four extracellular domains of CD4 but lacked the transmembrane and cytoplasmic domains. A second was similar but contained the ER retention signal, KDEL, at the carboxy terminus. This molecule has been previously shown to be retained in the ER, where it effectively blocks gp160 processing in a vaccinia virus expression system (2). The susceptibility of this molecule to Vpu-induced degra-

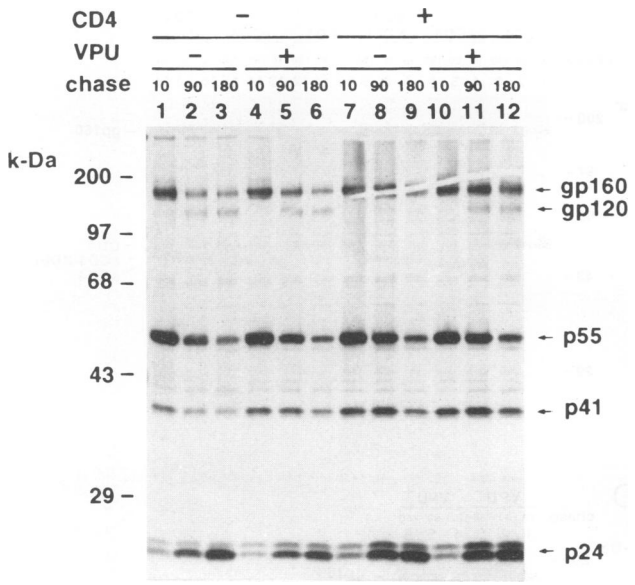

FIG. 3. Pulse-chase analysis of gp160 processing in the presence of CD4 and Vpu. HeLa cells were transfected with HXB.U$^+$ or HXB2 (2.5 μg) and pSV-CD4 or control plasmid DNA (7.5 μg). Transfected cells were metabolically labeled with [^{35}S]methionine for 1 h, chased with normal medium for 10, 90, or 180 min, and solubilized in Nonidet P-40-containing buffer. Lysates were immunoprecipitated with AIDS patient serum, and proteins were separated by electrophoresis on a 12.5% polyacrylamide gel. Labeled proteins were visualized by fluorography. Molecular size markers are indicated on the left.

dation was of interest because of previous data showing that a nearly full-length CD4 molecule containing an ER retention signal was degraded when coexpressed with Vpu, even in the absence of gp160 (24). To test the susceptibility of the truncated CD4 molecules to Vpu-induced degradation, HeLa cells were cotransfected with vectors expressing sCD4, sCD4-KDEL, or, as a positive control, wild-type CD4 and HXB2 or HXB.U$^+$. The cells were pulse-labeled and chased, and newly synthesized CD4 molecules were detected by immunoprecipitation with anti-CD4 serum (Fig. 4B). The results showed that in the presence of Vpu, the levels of wild-type CD4 were significantly reduced compared with those seen in its absence (by densitometric scanning of the autoradiogram, about fivefold less CD4 in cells cotransfected with HXB.U$^+$ compared with those cotransfected with HXB2). In contrast, sCD4 and sCD4-KDEL levels appeared not to be affected by the presence of Vpu, suggesting that the molecules were resistant to Vpu-induced degradation. The finding that sCD4-KDEL, a molecule that is retained in the ER, was not degraded in the presence of Vpu, suggests that the transmembrane or cytoplasmic domain of CD4 plays a role in Vpu-induced degradation. This conclusion is consistent with the finding that a nearly full-length CD4 molecule containing an ER retention signal was degraded in the presence of Vpu (24).

We also tested whether the efficiency of gp160 processing in the presence of sCD4-KDEL could be increased by the presence of Vpu. To do this, the lysates used in the experiment shown in Fig. 4B were immunoprecipitated with AIDS patient serum (Fig. 4C). In this analysis, wild-type CD4 blocked gp160 processing in the absence but not in the presence of Vpu. In contrast, sCD4, did not block gp160 processing in the presence or absence of Vpu. sCD4-gp160 complexes that form in the ER appear to remain competent for transport through the Golgi and are therefore processed normally, as reported previously (2). sCD4-KDEL, in contrast to CD4 or sCD4, blocked gp160 processing in the presence and absence of Vpu. sCD4-KDEL is not degraded in the presence of Vpu, nor is gp160 transport restored (as shown above). These two effects could not be experimentally separated. Therefore, these results support the hypothesis that it is the ability of Vpu to induce CD4 degradation that results in the release of gp160 from the block to its processing in the presence of CD4.

The lack of degradation of sCD4 molecules in the presence of Vpu could reflect a requirement for a specific amino acid sequence in the cytoplasmic or transmembrane domain of CD4. Alternatively, susceptibility of CD4 to Vpu-induced degradation may simply require anchoring in the ER membrane. To distinguish between these two possibilities, we used a chimeric CD4 molecule consisting of the CD4 extracellular domain fused to the transmembrane and cytoplasmic domains of the low-density lipoprotein (LDL) receptor (Fig. 4A). This chimeric protein has been previously shown to be transported to the cell surface and to bind to gp160 (1). Pulse-chase analysis showed that CD4-LDL was resistant to Vpu-induced degradation (Fig. 4D, compare lanes 1 to 3 with lanes 4 to 6). Thus, anchoring of CD4 in the ER membrane through heterologous transmembrane and cytoplasmic domains does not render the molecule susceptible to Vpu-induced degradation. This result supports a role for a specific amino acid sequence in the transmembrane or cytoplasmic domain of CD4 in Vpu-induced degradation.

Throughout the pulse-chase experiments described here, the level of CD4 present at the earliest time point was already decreased. Therefore, the results do not distinguish between a degradative mechanism for the CD4 down-regulation observed and a block at the level of translation. However, it is likely that the effect is due largely to CD4 degradation. In very short pulse-chase experiments, Willey et al. (24) have shown, and we have confirmed in this system (data not shown), that CD4 is synthesized in the presence of Vpu but rapidly degraded. Because the very short pulse-labelings (5 to 6 min) that are required for detection of CD4 before its degradation were, in our experiments, prone to variability, we used relatively long pulse-labeling times that yielded more reliable data and allowed us to observe the effect of Vpu on gp160 processing.

Susceptibility of CD4 cytoplasmic domain mutants to Vpu-induced degradation. Because it appeared that Vpu-induced degradation required specific amino acid sequences in the transmembrane or cytoplasmic domain, we next tested several truncations and missense mutants in this region. The cytoplasmic domain of CD4 contains 37 amino acids (Fig. 4A and 5A, positions 398 to 435). It contains two serine residues (positions 410 and 417) that are sites of protein kinase C phosphorylation and a Cys-His box (positions 420 to 422) that is the site for interaction with the tyrosine kinase, Lck (21). We first tested CD4 vectors containing termination codons at various locations in the cytoplasmic domain (Fig. 5A). SV-CD4.stop425, SV-CD4.stop418, SV-CD4.stop403, and SV-CD4.stop401 express molecules truncated by 10, 17, 32, and 34 amino acids, respectively. To determine the susceptibility of these proteins to Vpu-induced degradation, HeLa cells were cotransfected with each CD4 expression vector and either HXB.U$^+$ or HXB2. The susceptibility of these molecules to Vpu-induced degradation was assessed after a 1-h pulse-label and a 10-min chase (Fig. 5B). The results showed that truncation of the carboxy-terminal 10 amino acids had no effect on the susceptibility of CD4 to Vpu-induced degradation; however, further truncation of the 17 carboxy-terminal amino acids (CD4.stop418) or removal of nearly the entire cytoplasmic

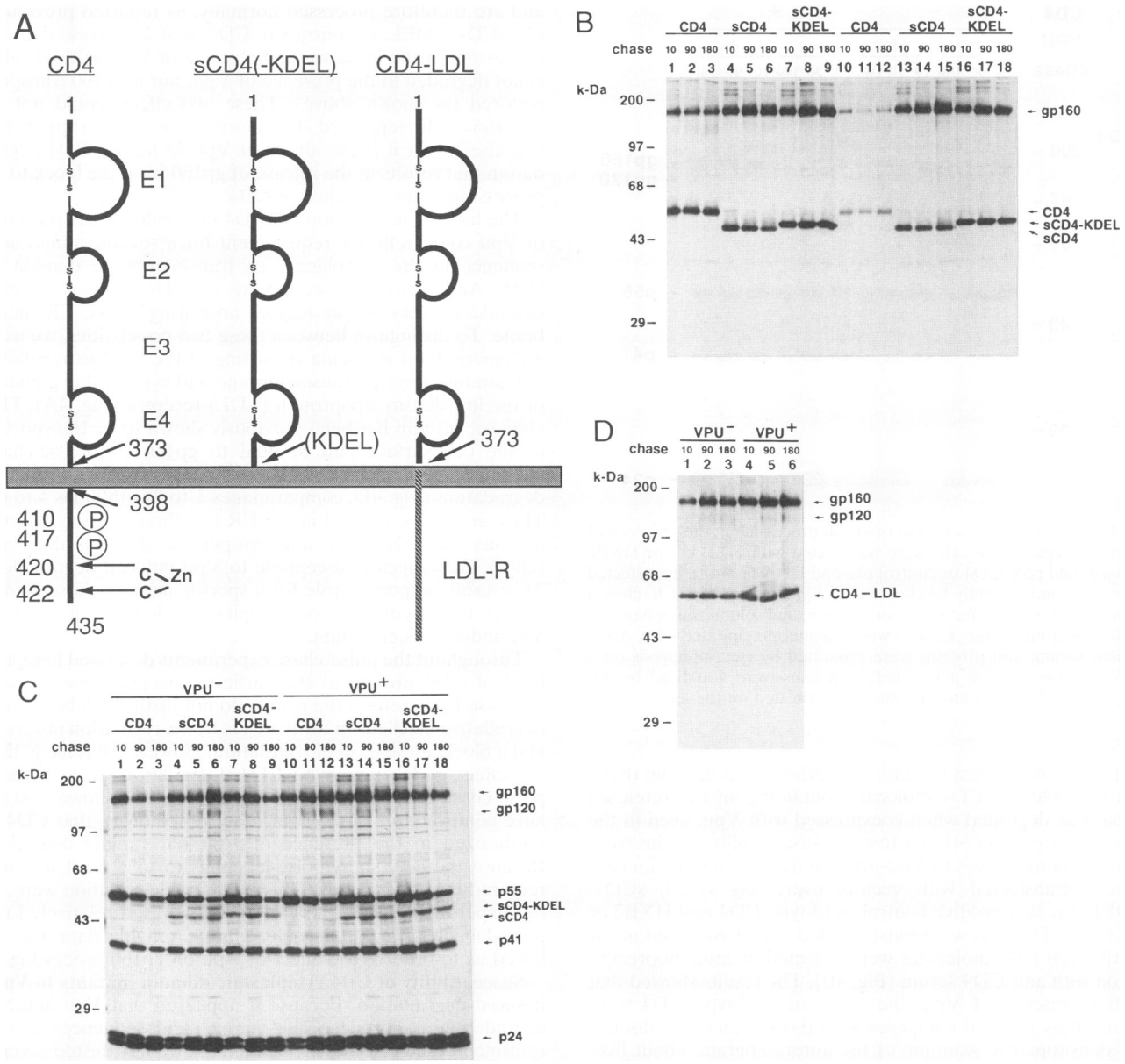

FIG. 4. Mutant CD4 molecules: susceptibility to Vpu-induced degradation and ability to block gp160 processing. (A) Structures of CD4, sCD4, sCD4-KDEL, and CD4-LDL. sCD4 and sCD4-KDEL are truncated at amino acid 373, immediately preceding the transmembrane domain (2). CD4-LDL contains a substitution of the cytoplasmic and transmembrane domains with the analogous region of the LDL receptor (1). sCD4 contains the four extracellular domains of CD4 but is truncated at the first amino acid of the transmembrane domain. sCD4-KDEL is similar, except that it contains the ER retention signal, KDEL, at its carboxy terminus. (B) HeLa cells were transfected with 7.5 μg of the truncated CD4 expression vector and 2.5 μg of HXB2 (lanes 1 to 9) or HXB.U$^+$ (lanes 10 to 18) and then pulse-labeled and chased as described in Materials and Methods. Proteins were immunoprecipitated with rabbit anti-CD4 serum or (C) AIDS patient serum. (D) HeLa cells were cotransfected with pSV-CD4-LDL (2.5 μg) and HXB2 or HXB.U$^+$ (7.5 μg) and analyzed as described for panel C. Immunoprecipitates were separated by SDS–10% polyacrylamide gel electrophoresis and visualized by fluorography. Molecular size markers are indicated to the left of panels B to D.

domain (CD4.stop401 and CD4.stop403) resulted in proteins that were not susceptible to Vpu-induced degradation. Therefore, the amino acids between positions 418 and 425 appear to contain a sequence necessary for Vpu-induced degradation.

We also tested three CD4 molecules containing missense mutations in the cytoplasmic domain (Fig. 5B). SV-CD4.ser410ala and SV-CD4.ser417ala contain mutations that remove protein kinase C phosphorylation sites. Both of these molecules remained susceptible to Vpu-induced degradation. SV-CD4.cys422ala, which contains a mutation within the region that was required for degradation, alters the Cys-His box such that Lck no longer interacts with the cytoplasmic domain (21). This molecule also remained susceptible to Vpu-induced degradation. These results suggested that Vpu-induced degradation requires neither phosphorylation of the cytoplasmic domain nor the presence of the LCK interaction site of CD4.

Because this analysis is dependent on consistent transfection and labeling efficiencies across each of the samples, each lysate

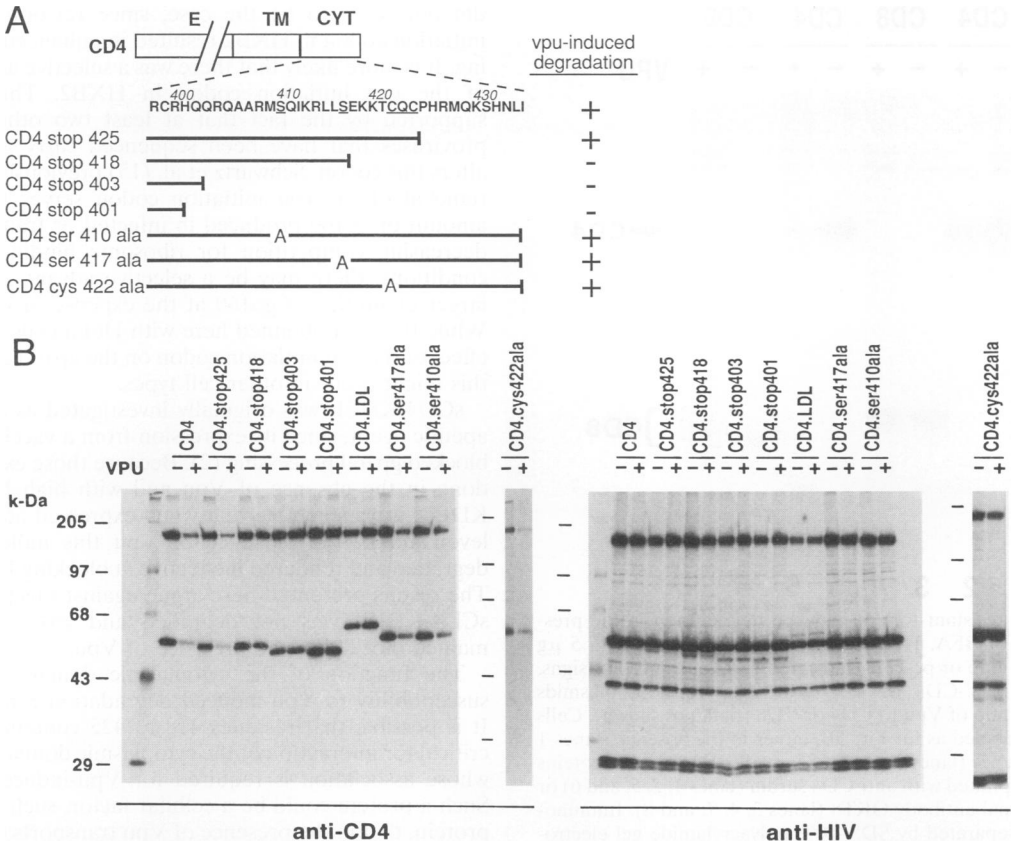

FIG. 5. Susceptibility of CD4 molecules with cytoplasmic domain truncations or missense mutations to Vpu-induced degradation. Plasmids expressing CD4 molecules with cytoplasmic domain truncations or missense mutations (A) were used together with HXB2 or HXB.U$^+$ to cotransfect HeLa cells. Cells were metabolically labeled for 1 h and chased for 10 min. (B) Labeled proteins were immunoprecipitated with polyclonal anti-CD4 serum or AIDS patient serum as described in Materials and Methods. Immunoprecipitates were separated by SDS–10% polyacrylamide gel electrophoresis and visualized by fluorography. Results are from a single experiment, except those for CD4.cys422ala, which was transfected in a separate experiment. E, extracellular; TM, transmembrane domain; CYT, cytoplasmic domain. Molecular size markers are indicated to the left of panel B.

was immunoprecipitated with AIDS patient serum to monitor the amounts of HIV proteins detected (Fig. 5B). Although there was some variability in band intensity, labeling was similar for each of the *vpu*-negative and *vpu*-positive pairs.

Vpu-induced degradation is specific for CD4 and can be observed without ER retention. To explore the specificity of Vpu-induced degradation further, we tested CD4-related cell surface protein CD8 for its susceptibility to Vpu-induced degradation. This experiment was complicated by the likelihood that efficient degradation of CD4 normally requires its retention in the ER (25). In the experiment presented above, gp160 produced from the cotransfected proviral vectors served to retain CD4 in the ER. Because CD8 does not associate with gp160 in the ER, it was necessary to establish experimental conditions in which Vpu-induced degradation did not depend on the presence of gp160. This was accomplished by two methods. In the first, HeLa cells were transfected with Vpu expression vectors pc-U^{atg} and SV-CD4 or SV-CD8 under conditions that resulted in overexpression of Vpu (Fig. 6, lanes 1 to 4). The results of such a transfection showed that coexpression of Vpu with CD4 in the absence of gp160 resulted in significant degradation of CD4. In contrast, the amount of CD8 did not decrease in the presence of Vpu.

In the second, perhaps more sensitive method of bypassing the requirement for gp160, the transfected cells were incubated with BFA prior to and during pulse-chase labeling. This drug, which blocks transport from the Golgi to the ER (11), has been shown to bypass the requirement for gp160, presumably by causing retention of CD4 in the ER (24). In the presence of BFA, CD4 degradation was slightly enhanced while the amount of CD8 remained relatively unchanged (Fig. 6, lanes 5 to 8). Results from both approaches suggest that CD8 is resistant to Vpu-induced degradation. Vpu-induced degradation is therefore specific for CD4.

DISCUSSION

The findings reported here on the susceptibility of various truncated or mutant CD4 molecules to Vpu-induced degradation suggest that Vpu-induced degradation requires amino acid sequences in the cytoplasmic tail of CD4. Analysis of truncated CD4 molecules showed that the region of the cytoplasmic tail between positions 418 and 425 contained a determinant critical for this effect. Other functionally important amino acid residues in the cytoplasmic domain that we tested, including the Cys-His motif in the cytoplasm domain that serves as the LCK-binding site and the two serine phosphorylation sites, were not required for Vpu-induced degradation. The region of the CD4 cytoplasmic domain from amino acids 418 to 425 contains the sequence KKTC, which can act as

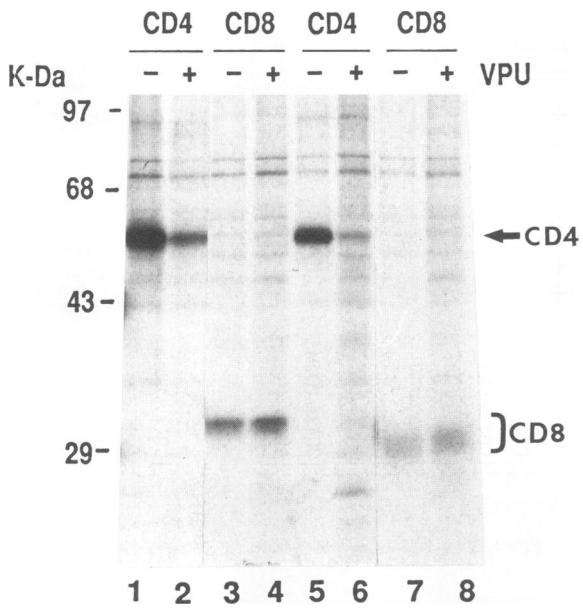

FIG. 6. CD8 is resistant to Vpu-induced degradation in the presence and absence of BFA. HeLa cells were cotransfected with 5 μg each of pc-DNA.I-amp or pc-U^{atg} (indicated by minus and plus signs, respectively) and pSV-CD4 or pSV-CD8. This ratio of plasmids resulted in a high ratio of Vpu to CD4 or CD8 (data not shown). Cells were pulse-chase labeled as for Fig. 5B, either in the absence (lanes 1 to 4) or in the presence (lanes 5 to 8) of 2.0 μg of BFA per ml. Proteins were immunoprecipitated with anti-CD4 serum (lanes 1, 2, 5, and 6) or anti-CD8 monoclonal antibody OKT8 (lanes 3, 4, 7, and 8). Immunoprecipitates were separated by SDS–10% polyacrylamide gel electrophoresis and visualized by fluorography. CD8 had a slightly lower apparent molecular weight in the presence of BFA. Molecular size markers are indicated on the left.

an ER retention signal (16); however, it is unlikely that this sequence plays a role in Vpu-induced degradation since its mutation to KKTA in SV-CD4cys422ala had no effect.

Vpu-induced degradation was specific for CD4, since the related T-cell surface protein CD8 was not affected by Vpu. It is possible that proteins in addition to CD4 are degraded; however, there is sequence specificity to this effect. These results argue against the possibility that Vpu causes general disruption of the ER, resulting in massive degradation of ER-resident proteins and abnormalities in protein transport. Furthermore, we have shown that secretion of human growth hormone from HeLa cells transfected with a human growth hormone expression vector was not affected by the presence of Vpu (10).

Our results differ from those of Willey et al. (25) in that we observed CD4 degradation in the absence of gp160 (Fig. 6). It may be that the amount of Vpu produced in our experiments was high enough to override the requirement for ER retention. In addition, some dependence on gp160 was observed in our experiments, since CD4 degradation appeared to be more efficient when Vpu was expressed from HXB2-U^+ rather than pc-U^{atg}.

The significance of the loss of the *vpu* initiation codon in HXB2 is unclear. It is possible that the *vpu* open reading frame had accumulated mutations that rendered it nonfunctional or that the gp160 encoded by this virus had acquired the ability to be efficiently processed even in the presence of CD4. Loss of the ability to express *vpu* would then have been due to lack of selection for the product of this reading frame. However, this did not seem to be the case, since restoration of the *vpu* initiation codon in HXB2 resulted in enhanced gp160 processing. It is more likely that there was a selective advantage to loss of the *vpu* initiation codon in HXB2. This possibility is supported by the fact that at least two other independent proviruses that have been sequenced carry a mutation that alters this codon. Schwartz et al. (15) previously suggested that removal of the *vpu* initiation codon serves to increase the amount of gp160 produced in infected or transfected cells by decreasing competition for ribosome binding. Under some conditions, there may be a selective advantage to producing larger quantities of gp160 at the expense of eliminating *vpu*. While the data obtained here with HeLa cells do not show an effect of the *vpu* initiation codon on the gp160 expression level, this might occur in other cell types.

sCD4-KDEL was originally investigated as a possible therapeutic agent, since its expression from a vaccinia virus vector blocked gp160 processing (2). Because those experiments were done in the absence of Vpu and with high levels of sCD4-KDEL, it was possible that when expressed at a physiological level and in the presence of Vpu this molecule might be degraded and rendered ineffective at blocking HIV replication. The results presented here argue against this possibility, since sCD4-KDEL was not degraded and gp160 processing remained blocked in the presence of Vpu.

The function of the cytoplasmic domain in determining susceptibility to Vpu-induced degradation remains unknown. It is possible that residues 418 to 425 contain a determinant critical for interaction of the cytoplasmic domain with a protein whose association is required for Vpu-induced degradation. Such a protein could be a cellular factor, such as a chaperone protein, that in the presence of Vpu transports CD4 to a site at which it is degraded. This factor could be Vpu itself, although interaction of Vpu with CD4 has not been reported. Alternatively, it is possible that amino acids 418 to 425 of the CD4 cytoplasmic domain contain a cleavage site for a protease that mediates the degradation. It will be of interest to define further which residues in the cytoplasmic domain of CD4 are critical for susceptibility to Vpu-induced degradation and to understand how these residues contribute to this effect.

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