JOURNAL OF VIROLOGY, Mar. 1995, p. 1510–1520
0022-538X/95/$04.00+0

Vol. 69, No. 3

The Human Immunodeficiency Virus Type 1 Vpu Protein Specifically Binds to the Cytoplasmic Domain of CD4: Implications for the Mechanism of Degradation

STEPHAN BOUR,* ULRICH SCHUBERT, AND KLAUS STREBEL

Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892

Received 29 September 1994/Accepted 29 November 1994

We have recently demonstrated that coexpression of Vpu and CD4 in HeLa cells results in the degradation of CD4 in the endoplasmic reticulum. The sensitivity of CD4 to Vpu-mediated degradation is conferred by the presence of specific sequences located between amino acids 402 and 420 in the CD4 cytoplasmic domain. Using an in vitro translation system, we also showed that degradation of CD4 by Vpu requires the two proteins to be present in the same membrane compartment. Although these results suggest that spatial proximity between CD4 and Vpu may be critical in triggering degradation, it remains unknown whether the two molecules have the ability to interact with each other. In order to better define the mechanisms involved in CD4 degradation, we investigated the existence and functional relevance of direct interactions between CD4 and Vpu. Coimmunoprecipitation experiments showed that Vpu specifically binds to the cytoplasmic tail of CD4. This phenomenon is relevant to the mechanism of CD4 degradation since the ability of CD8/CD4 chimeric molecules and various CD4 mutants to form complexes with Vpu correlates with their sensitivity to degradation. Accordingly, we found that amino acid residues in the CD4 cytoplasmic tail previously shown to be important for degradation are necessary for Vpu binding. We further demonstrate that a deletion mutant of Vpu as well as a phosphorylation mutant, both biologically inactive with regard to CD4 degradation, retained the capacity to interact with the CD4 cytoplasmic domain. Taken together, these results indicate that Vpu binding is necessary to trigger CD4 degradation. However, the binding to target molecules is not sufficient per se to cause degradation. Interaction between CD4 and Vpu is thus likely to be an early event critical in triggering a multistep process leading to CD4 degradation.

The *vpu* gene of human immunodeficiency virus type 1 (HIV-1) encodes a well-characterized accessory protein (11, 30, 44), which is translated from a bicistronic mRNA that also contains the downstream *env* open reading frame (ORF) (38). Vpu is an integral membrane phosphoprotein that forms homo-oligomeric structures both in vitro and in vivo (29, 36, 43). The *vpu* ORF is present in the majority of HIV-1 molecular clones but absent in the closely related viruses HIV-2 and simian immunodeficiency virus (SIV), with the notable exception of the SIV_{cpz} isolate, which encodes a Vpu-like protein (11, 22). Although not essential for virus replication in vitro, the expression of Vpu has been shown to enhance viral particle release from infected cells in tissue culture systems (43–45).

Infection of target cells by HIV-1 is dependent on surface expression of the CD4 molecule, which serves as a specific virus receptor (26, 28). In productively infected cells, intracellular complexes between CD4 and the HIV-1 envelope precursor glycoprotein gp160 have been shown to form in the endoplasmic reticulum, a process which results in the cell surface downregulation of CD4 (21). The formation of such complexes not only traps CD4 in the endoplasmic reticulum (ER) (7, 13, 24) but also blocks gp160 cleavage and maturation (7). Vpu has been shown to specifically induce degradation of CD4, thus allowing the envelope precursor to be released into its normal maturation pathway (49, 50). The contribution of gp160 to the degradation process was shown to be dispensable by trapping CD4 in the ER with brefeldin A, indicating that gp160 only serves to trap CD4 in the intracellular compartment where Vpu-mediated degradation takes place (49). The finding that envelope does not directly mediate CD4 degradation was confirmed by the ability of Vpu to destabilize CD4 when the two proteins were synthesized in a cell-free system independent of other viral components (10).

The presence of the CD4 cytoplasmic domain is necessary and sufficient for Vpu-mediated degradation (48). The half-life of the related CD8 cell surface molecule as well as a soluble form of CD4 remains unchanged in the presence or absence of Vpu (27, 48). However, CD8 could be sensitized for degradation by replacing its cytoplasmic tail with the corresponding region of CD4 (48). These results are in agreement with the identification of specific Vpu target sequences located between amino acids 402 and 420 in the CD4 cytoplasmic domain (10). The use of a panel of CD4 cytoplasmic tail deletion mutants further defined a discrete amino acid sequence, EKKTCQCP, removal of which suppressed CD4 sensitivity to Vpu-mediated degradation (27). In addition, work with gp160/CD4 chimeric molecules defined the amino acid residues 414-LSEKKT-419 as a minimal element in the CD4 cytoplasmic tail required to achieve efficient degradation by Vpu (47). Although both sequences overlap amino acids 402 to 420 of CD4, it is not known whether transfer of the LSEKKT minimal element is sufficient to confer Vpu sensitivity to heterologous proteins.

Several lines of evidence suggest that the mechanism of CD4 degradation requires a direct interaction with Vpu: (i) Vpu specifically targets CD4 (27, 48), (ii) the transfer of discrete CD4 amino acid sequences confers sensitivity to Vpu-mediated degradation to heterologous proteins (10, 48), and (iii) degradation in vitro requires CD4 and Vpu to be present in the same

* Corresponding author. Mailing address: NIH, NIAID, Building 4, Room 312, 9000 Rockville Pike, Bethesda, MD 20892-0460. Phone: (301) 496-3132. Fax: (301) 402-0226. Electronic mail address: StephaneBour@d4.niaid.pc.niaid.nih.gov.

membrane compartment (10). However, a direct interaction between CD4 and Vpu has yet to be demonstrated. Detection of CD4-Vpu complexes may have been impeded by a weak affinity of the two proteins and/or the high rate of CD4 degradation in the presence of Vpu. We reasoned that, by slowing down the rate of CD4 degradation, we would be able to increase the likelihood of detecting such complexes. In the present work, we made use of CD8 chimeric molecules bearing the CD4 cytoplasmic tail that are sensitive to Vpu-mediated degradation at a rate approximately 20 times slower than that of wild-type CD4 (48). Coimmunoprecipitation experiments performed in HeLa cells revealed the specific association of Vpu with the cytoplasmic tail of CD4 in the context of a CD8/CD4 chimeric molecule. Moreover, the ability of CD8/CD4 chimeric molecules to interact with Vpu correlated with their sensitivity to Vpu-mediated degradation. A detailed analysis of the specificity of this interaction showed that sequences between amino acids 402 and 420, previously defined as essential for CD4 degradation, contain a binding site for Vpu. Although complexes between wild-type CD4 and wild-type Vpu are difficult to detect in whole-cell systems, we were able to show the presence of such complexes using an in vitro translation system in which the rate of CD4 degradation is slower. To better define the role of Vpu binding in the mechanism of CD4 degradation as well as the Vpu sequence requirements for interaction with CD4, we examined the binding properties of two Vpu mutants unable to promote CD4 degradation. Interestingly, both mutants retained the ability to interact with CD4, indicating that binding of Vpu is necessary but not sufficient to cause degradation of CD4.

MATERIALS AND METHODS

Recombinant DNA constructs. Plasmids expressing CD8 and CD8/CD4 chimeras under the transcriptional control of the cytomegalovirus (CMV) promoter were described earlier (48). Briefly, pCMV-CD8, pCMV-CD8/TM4, and pCMV-CD8/4 encode wild-type CD8, CD8 bearing the transmembrane domain of CD4, and CD8 bearing both the transmembrane domain and cytoplasmic tail of CD4, respectively.

pHIV-CD4ΔBam expresses wild-type CD4 under the transcriptional control of the HIV-1 long terminal repeat (LTR). This plasmid was obtained from the previously described pHIV-CD4 construct (50) by deleting the *Bam*HI fragment of 3′ noncoding CD4 sequences. The pHIV-CD4/Q421 plasmid encodes a cytoplasmic tail truncation mutant which contains a stop codon terminating translation after amino acid 420, deleting the C-terminal 13 residues of the CD4 cytoplasmic tail (40, 49). The pHIV-CD4/Nar plasmid expresses a truncated CD4 molecule containing a stop codon that terminates translation at residue 401 in the cytoplasmic tail. pHIV-CD4/Nar was constructed as follows. In a first step, a 3-kb *Eco*RI fragment representing the full-length CD4 cDNA obtained from the pT4-pMV7 construct (28) was subcloned into the *Xha*I-*Msc*I sites of pCMV-CAT to generate pCMV-CD4. The pCMV-CD4/Nar plasmid containing a stop codon at the NarI site in the CD4 cytoplasmic domain was subsequently obtained by performing a partial NarI digest of pCMV-CD4 in order to introduce a PacI linker [d(pTTAATTAA); New England Biolabs, Beverly, Mass.]. Plasmid pHIV-CD4/Nar was obtained from pCMV-CD4/Nar by subcloning a 1.0-kb *Nhe*I-*Bam*HI fragment containing the mutation into pHIV-CD4ΔBam.

Subgenomic constructs derived from the infectious clone pNL4-3 (1) were used to express Vpu after transfection in HeLa cells. The pNL-A1 construct expresses all HIV-1 proteins except for those coded by the *gag* and *pol* genes (44). The pNL-A1/U_{35} plasmid was derived from pNL-A1 by inserting an 8-bp *Xho*I linker to disrupt the *vpu* gene (44). The pNL-A1/U_{52} construct encodes a mutant of Vpu carrying an internal in-frame deletion of five amino acids in the hydrophilic domain. pNL-A1/U_{52} was derived from the pSP9-52 construct (10) by replacing the *Eco*RI-*Nhe*I fragment in pNL-A1 with the corresponding fragment from pSP9-52. The $Vpu_{2/6}$ phosphorylation mutant, carrying serine to asparagine substitutions at amino acid positions 52 and 56 of Vpu, and its subgenomic expression vector, pNL-A1/$U_{2/6}$, have been previously described (37).

Plasmid pSP-9, used for in vitro transcription of wild-type Vpu, has been previously described (44). The pSP9-52 construct carries the same five-amino-acid deletion in the Vpu sequence of pSP-9 as pNL-A1/U_{52} (see above).

Cell culture and transfection. HeLa cells (ATCC CCL2) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 250 U of penicillin and 250 mg of streptomycin per ml. At 24 h prior to transfection, 3×10^6 HeLa cells were plated in 25-cm^2 (T25) culture flasks. Two T25 flasks per sample were transfected with 35 μg of total DNA by the calcium phosphate precipitation method as described elsewhere (49).

Antibodies. The anti-CD8 monoclonal antibody (MAb) OKT8 was purchased from Ortho Pharmaceuticals. CD4 was immunoprecipitated with the T4-4 CD4 antiserum obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, and was initially contributed by R. Sweet (15, 50). The TP human serum, reacting against all major HIV-1 proteins, including Vpu, was obtained from an asymptomatic HIV-seropositive individual.

Metabolic labeling and immunoprecipitation. Transfected HeLa cells were detached by scraping, centrifuged, and resuspended in 1 ml of methionine- and cysteine-free RPMI 1640 containing 2.5 μg/of brefeldin A (BFA) per ml (starving medium). After 10 min at 37°C, the cells were labeled for 30 min at 37°C in 200 μl of starving medium containing 200 μCi of [^{35}S]methionine and [^{35}S]cysteine (Tran^{35}S-Label; 10 mCi/ml; ICN Biomedical, Inc., Costa Mesa, Calif.). The medium was removed, and a chase was performed at 37°C in 500 μl of complete RPMI 1640 for the times indicated in the text. After removal of the chase medium, cells were gently suspended in 400 μl of digitonin lysis buffer, consisting of 1% digitonin, 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1 mM EDTA (23). Lysis was achieved by incubation for 30 min at 4°C on a rotating wheel. Lysates were cleared for 1 h at 4°C with 25 μl of packed protein A-Sepharose beads (Sigma Chemical Company, St. Louis, Mo.). At the same time, immune complexes were prepared: 25 μl of packed protein A-Sepharose beads per sample was resuspended in 400 μl of phosphate-buffered saline (PBS) and incubated with T4-4 (1 μl per sample), OKT8 (10 μl per sample), or TP serum (1 μl per sample) in the presence of 0.1% bovine serum albumin (BSA) for 1 h at 4°C on a rotating wheel. The precleared cell lysates were centrifuged for 5 min at 12,000 rpm in a microcentrifuge to pellet insoluble material and preclearing beads. Then 300 μl of the resulting supernatants was transferred to tubes containing either T4-4 or OKT8 immune complexes, and the remaining 100 μl was transferred to TP immune complexes. Immunoprecipitation was performed for 1 h at 4°C. Samples were rinsed twice in buffer consisting of 0.1% digitonin, 150 mM KCl, and 50 mM Tris-HCl (pH 7.4) (23), resuspended in 100 μl of electrophoresis sample buffer, boiled for 10 min, and analyzed on 12% polyacrylamide–sodium dodecyl sulfate (SDS) gels. Gels were fixed for 30 min in 30% methanol–7% acetic acid, soaked in Enlightning (DuPont) for 30 min, and dried. Radioactive bands were visualized by fluorography on X-Omat films.

In vitro transcription and translation. The pSP-CD4 plasmid (10) was linearized with *Bam*HI and used as a template to synthesize CD4 mRNA. The use of *Bam*HI removed the 3′ noncoding region of CD4. The pSP9 and pSP9-52 plasmids (10) were linearized with *Kpn*I and used as templates to synthesize wild-type and mutant Vpu, respectively. A total of 5 μg of linearized plasmid was used for in vitro transcription with 40 U of Sp6 RNA polymerase (Gibco-BRL Life Technologies) for 1 h at 37°C. RNA was purified by phenol-chloroform extraction followed by ethanol precipitation in the presence of 3 M ammonium acetate. The in vitro-transcribed RNA was resuspended in 50 μl of H_2O and assessed for purity and integrity by electrophoresis through 1% agarose in TBE buffer.

In vitro translation was performed with a nuclease-treated rabbit reticulocyte lysate (Promega Corp., Madison, Wis.). Canine pancreatic membranes from the same supplier were added to the translation mix to allow insertion of membrane-associated proteins. Reaction mixes containing 30 μl of lysate and 3 μl of CD4 mRNA were assembled as recommended by the manufacturer in the presence of 20 μCi [^{35}S]methionine (ICN Biochemicals) and 4 μl of canine pancreatic membranes. Following 15 min of translation at 30°C, 5 μl of Vpu or Vpu-52 mRNA was added, and the reaction was allowed to proceed for an additional 45 min. Samples were lysed in 400 μl of digitonin lysis buffer as described above. A total of 350 μl of lysate was used for immunoprecipitation as described above; the remaining 50 μl of lysate was directly loaded onto the gel for direct analysis of the proteins present after translation. Samples were separated on 12% polyacrylamide–SDS gels and analyzed by fluorography.

RESULTS

Vpu specifically interacts with the cytoplasmic tail of CD4 in the context of CD8/CD4 chimeric molecules. We took several experimental approaches to decrease the rate of Vpu-mediated degradation of CD4 and thus increase the likelihood of detecting CD4-Vpu complexes. Wild-type CD8 as well as CD8 chimeric molecules bearing the CD4 transmembrane domain are not affected by Vpu. However, CD8 chimeric molecules carrying the cytoplasmic tail of CD4 are sensitive to Vpu-mediated degradation, albeit at a slower rate than wild-type CD4 (48). We thus assessed whether direct interactions with Vpu could be detected in this context.

HeLa cells were cotransfected with the pNL-A1 plasmid, expressing wild-type Vpu and either wild-type CD8 or CD8

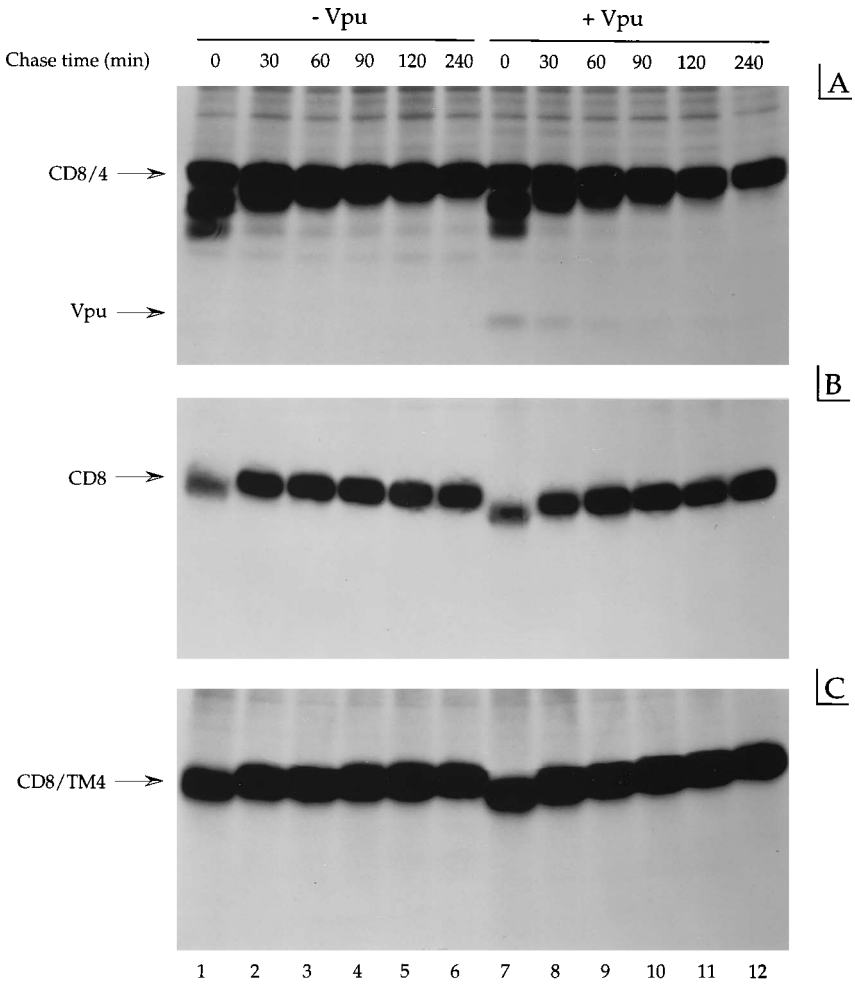


FIG. 1. Vpu interacts with CD4 cytoplasmic domain. Different CD8/CD4 chimeric molecules were transfected into HeLa cells in the presence of either the pNL-A1 plasmid, encoding wild-type Vpu (+Vpu), or the pNL-A1/U_{35} (−Vpu) isogenic plasmid. Cells were labeled for 30 min with Tran^{35}S-Label in the presence of BFA and chased for the indicated periods of time. Cell extracts obtained by digitonin lysis were subjected to immunoprecipitation with the anti-CD8 MAb OKT8. Immunoprecipitates were analyzed on 12% polyacrylamide–SDS gels and visualized by fluorography. The positions of the different chimeric molecules as well as coprecipitated Vpu (A) are indicated.

chimeras containing either the CD4 transmembrane domain (CD8/TM4) or both the transmembrane and cytoplasmic regions of CD4 (CD8/4). Cells were pulse labeled for 30 min with Tran^{35}S-Label in the presence of BFA, a drug that causes retention of Vpu and CD4 in the ER (50), and subjected to a chase for different periods of time. In cells coexpressing the CD8/4 chimera and wild-type Vpu, immunoprecipitation with the OKT8 MAb shows the coprecipitation of a band migrating at a molecular weight consistent with Vpu (Fig. 1A, lanes 7 to 12) No coprecipitation of the 16-kDa species with the OKT8 MAb was observed in cells cotransfected with pCMV-CD8/4 and the Vpu(−) pNL-A1/U_{35} plasmid (lanes 1 to 6). Immunoprecipitation with both a Vpu-positive anti-HIV serum (TP) and a rabbit polyclonal anti-Vpu serum confirmed that the coprecipitated 16-kDa species was indeed Vpu (data not shown). Under the same experimental conditions, we were not able to demonstrate association of Vpu with wild-type CD8 (Fig. 1B, lanes 7 to 12), indicating that association with Vpu is specifically mediated by CD4 sequences present in the CD8/4 chimeric molecule. In addition, analysis of the CD8/TM4 chimera demonstrates that the presence of the CD4 transmembrane domain alone does not confer on CD8 the capacity to interact with Vpu (Fig. 1C, lanes 7 to 12). Therefore, sequences present in the CD4 cytoplasmic tail, previously shown to be important for degradation (48), are also required for binding of Vpu. These results were confirmed by the capacity of a chimeric molecule containing only the CD4 cytoplasmic domain in a CD8 background to associate with Vpu (data not shown).

Since BFA was used in the previous experiments, we wished to verify that ER retention did not perturb the structure of Vpu or the chimera, creating an artifactual association between the two molecules. Cells cotransfected with pCMV-CD8/4 and either the pNL-A1 plasmid (+Vpu) or the pNL-A1/U_{35} plasmid (−Vpu) were pulse labeled for 30 min with Tran^{35}S-Label and subjected to a chase for the indicated periods of time (Fig. 2). Immunoprecipitation of CD8/4 with the OKT8 MAb showed coprecipitation of Vpu from cells transfected with pNL-A1 but not pNL-A1/U_{35}. Binding occurred in both the presence and absence of BFA, ruling out the contribution of the drug to the observed association between Vpu and the CD4 cytoplasmic domain. The maturation pattern of CD8/4 is clearly different in the absence of BFA, since the protein is not trapped in the ER. However, the kinetics of Vpu

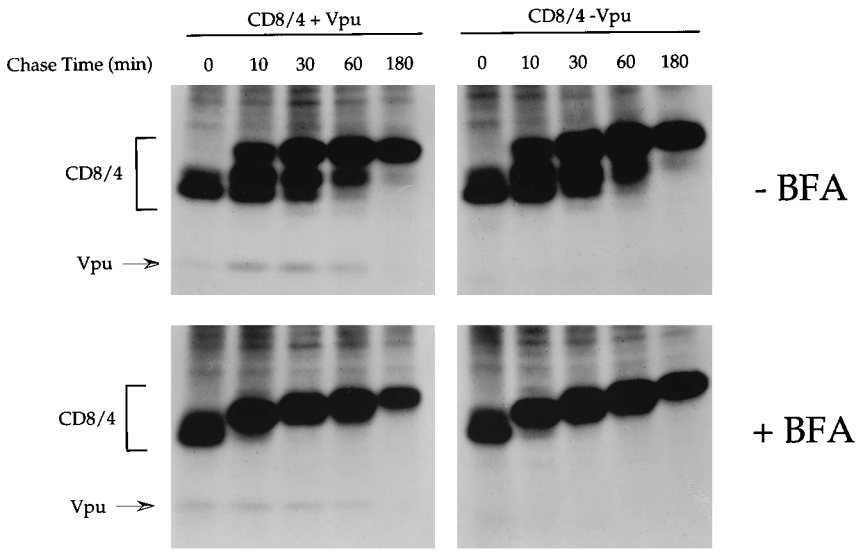


FIG. 2. Blocking CD8/4 in the ER does not inhibit or enhances association with Vpu. HeLa cells cotransfected with pCMV-CD8/4 (CD8/4) and either pNL-A1 (+Vpu) or pNL-A1/U_{35} (−Vpu) were labeled for 30 min with Tran^{35}S-Label in the presence or absence of BFA and then chased for the indicated periods of time. The samples were lysed in buffer containing 1% digitonin and immunoprecipitated with the anti-CD8 MAb OKT8. Immunoprecipitates were separated on 12% polyacrylamide gels and visualized by fluorography. The positions of CD8/4 and coprecipitated Vpu are indicated.

binding were not affected, indicating that Vpu and CD4 associate soon after their synthesis in a pre-Golgi subcellular compartment.

A Vpu mutant unable to degrade CD4 retains the capacity to interact with the cytoplasmic tail of CD4 and allows the detection of complexes with wild-type CD4. Mutagenesis performed in the *vpu* gene showed that conserved amino acid sequences in the hydrophilic domain are essential for the biological activity of Vpu (10). Hence, the Vpu_{52} mutant, carrying a deletion of five amino acids between residues 47 and 52, is no longer able to degrade CD4 (10). In order to clarify the role of CD4-Vpu interactions in the mechanism of degradation, we ascertained whether the inability of the Vpu_{52} mutant to degrade CD4 was due to impaired binding to the CD4 cytoplasmic tail. HeLa cells cotransfected with pCMV-CD8/4 and the pNL-A1/U_{52} plasmid, expressing Vpu_{52}, were pulse labeled for 30 min in the presence of BFA and subjected to a chase for the indicated periods of time (Fig. 3A). Cell lysates were immunoprecipitated with either the OKT8 MAb (lanes 1 to 4) or the TP anti-HIV serum, which recognizes most of the HIV-1 proteins, including Vpu (lanes 5 to 8). Despite the absence of amino acids important for the ability of Vpu to promote CD4 degradation, the Vpu_{52} mutant was still able to bind to CD8/4 (Fig. 3A, lanes 1 to 4). Immunoprecipitation of an aliquot of the lysates with TP serum confirmed that the protein coprecipitated with CD8/4 is Vpu (Fig. 3A, lanes 5 to 8). It is noteworthy that the anti-Vpu antibodies present in TP serum are unable to precipitate CD4-Vpu complexes, as shown by the absence of CD8/4 after TP immunoprecipitation (Fig. 3A, lanes 5 to 8). Similar results were obtained when a monospecific anti-Vpu serum was used (data not shown). Since Vpu antibodies generated in vivo are directed against the cytoplasmic domain (34), this result indicates that the immunodominant domain of Vpu and the region involved in CD4 binding may overlap, so that binding of CD4 and anti-Vpu antibodies is mutually exclusive. This notion is supported by structural considerations, as discussed later.

Although the ability of target molecules to interact with Vpu correlates with their sensitivity to degradation (Fig. 1), results obtained with the Vpu_{52} mutant indicate that binding is necessary but not sufficient to trigger degradation. The use of the biologically inactive Vpu_{52} mutant increased the stability of complexes with CD8/4 compared with wild-type Vpu (compare Fig. 1A, lanes 7 to 12, and Fig. 3A, lanes 1 to 4). However, even in the absence of degradation, only a small proportion of total Vpu_{52} coimmunoprecipitated with CD8/4, indicating the low affinity and/or transient nature of this interaction.

We took advantage of the binding properties of the Vpu_{52} mutant to test whether complexes could be detected not only with chimeric molecules but also with wild-type CD4 under conditions in which degradation does not occur. HeLa cells cotransfected to express wild-type CD4 and the Vpu_{52} mutant were pulse labeled for 30 min with Tran^{35}S-Label in the presence of BFA and subjected to a chase for various periods of time. Cell lysates were subjected to immunoprecipitation with either the T4-4 CD4 antiserum or the TP anti-HIV serum. As shown in Fig. 3B, Vpu_{52} was coprecipitated with CD4 by the T4-4 antibodies (lanes 1 to 4). Interactions between Vpu_{52} and CD4 were detected immediately after the pulse and at all times during the 3-h chase (Fig. 3B, lanes 1 to 4). The time course of complex formation between Vpu_{52} and CD4 was remarkably similar to that of Vpu_{52} and CD8/4 (compare lanes 1 to 4 in Fig. 3A and B). The presence of CD4 in the samples immunoprecipitated with the TP serum (lanes 5 to 8) likely reflects coimmunoprecipitation with the HIV-1 envelope precursor gp160, also expressed by the pNL-A1/U_{52} plasmid. Indeed, no such coprecipitation was observed with CD8/4, which is unable to bind to gp160, despite similar efficiency of complex formation (compare Fig. 3A and B, lanes 1 to 4). Cumulative results obtained with the Vpu_{52} mutant indicate that Vpu is able to specifically bind to CD4 in the context of either wild-type CD4 or a CD8 chimeric molecule bearing the CD4 cytoplasmic domain. The difficulty in detecting complexes between wild-type CD4 and wild-type Vpu in whole-cell systems is thus likely due to the high rate of CD4 degradation, although we cannot formally rule out that the mutation in Vpu_{52} enhanced its affinity for CD4 compared with wild-type Vpu.

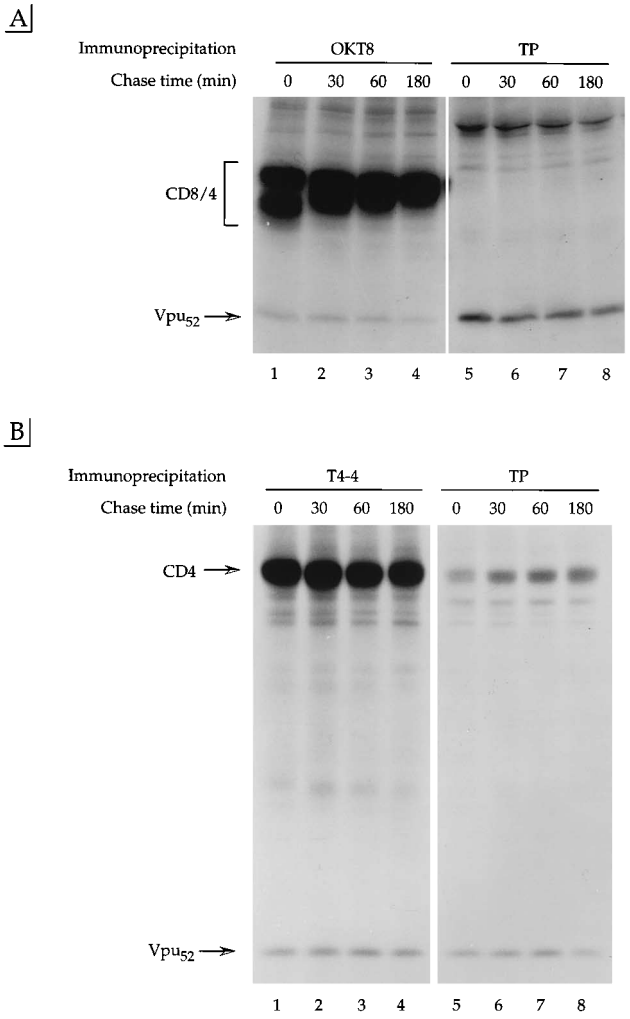


FIG. 3. Vpu_{52} mutant retains the capacity to interact with the cytoplasmic tail of CD4 and allows the detection of complexes with wild-type CD4. (A) HeLa cells cotransfected to express CD8/4 (pCMV-CD8/4) and Vpu_{52} (pNL-A1/U_{52}) were labeled with Tran^{35}S-Label for 30 min in the presence of BFA and chased for the indicated periods of time. Cells were lysed in buffer containing 1% digitonin and immunoprecipitated with either the anti-CD8 MAb (OKT8) or an HIV-positive human serum (TP). Immunoprecipitates were separated on a 12% polyacrylamide–SDS gel, and proteins were visualized by fluorography. The positions of the CD8/4 chimeric molecule and Vpu_{52} are indicated. (B) A similar experiment was performed in HeLa cells coexpressing wild-type CD4 (pHIV-CD4) and Vpu_{52} (pNL-A1/U_{52}). Lysates were immunoprecipitated with either the anti-CD4 polyclonal serum T4-4 or the anti-HIV TP serum. Immunoprecipitates were separated on a 12% polyacrylamide–SDS gel, and proteins were visualized by fluorography. The bands identified as Vpu_{52} are species coprecipitated with CD4 (lanes 1 to 4) or immunoprecipitated with the anti-HIV (TP) serum (lanes 5 to 8). The position of CD4 is shown on the left and identifies species that are either immunoprecipitated by the CD4-specific T4-4 antiserum (lanes 1 to 4) or immunoprecipitated by the TP serum as a complex with the envelope glycoproteins of HIV-1, also expressed by the pNL-A1 plasmid (lanes 5 to 8).

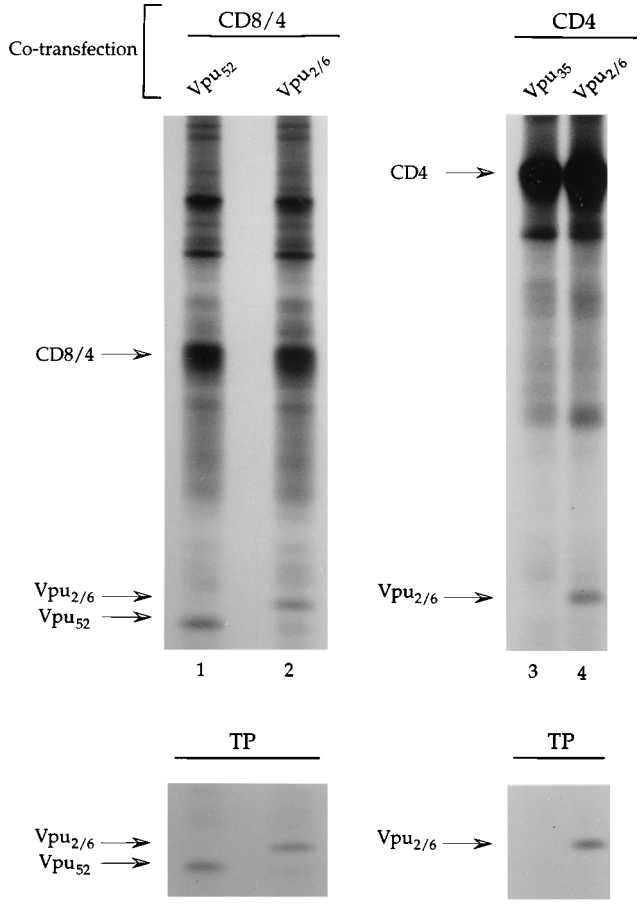
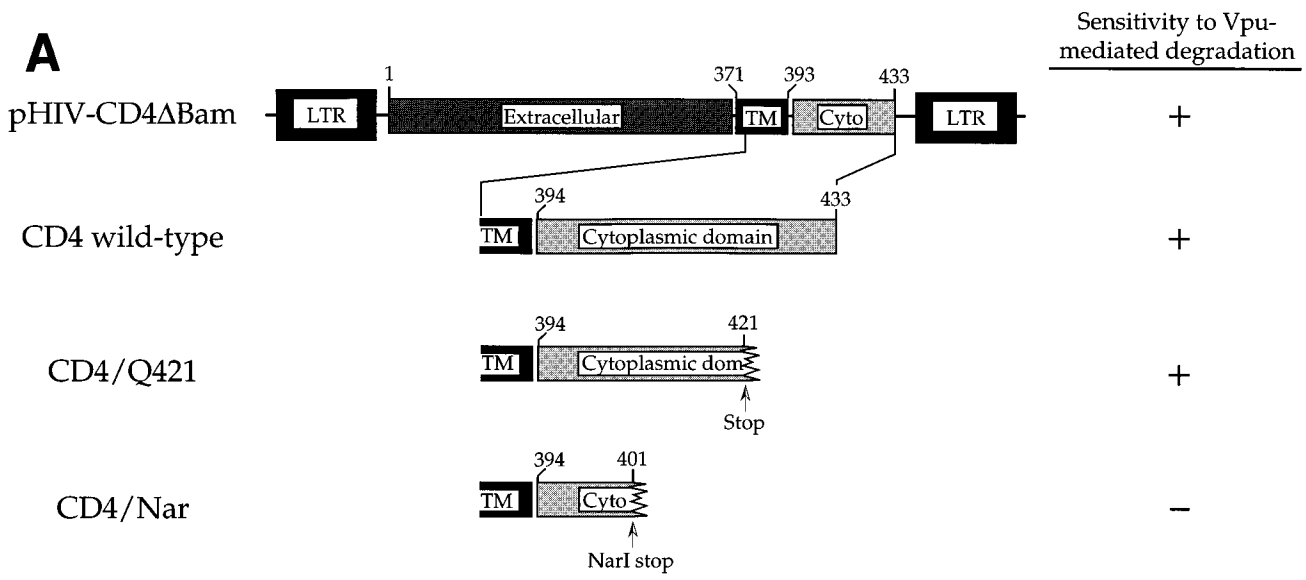


FIG. 4. Phosphorylation of Vpu is necessary for CD4 degradation but not for binding. HeLa cells were cotransfected with either pCMV-CD8/4 (CD8/4) or pHIV-CD4ΔBam (CD4) in the presence of either pNL-A1/U_{35} (Vpu_{35}), pNL-A1/U_{52} (Vpu_{52}), or the pNL-A1/$U_{2/6}$ plasmid, encoding the Vpu phosphorylation mutant $Vpu_{2/6}$. Cells were labeled for 30 min with Tran^{35}S-Label, lysed in 1% digitonin, and subjected to immunoprecipitation with either CD8/4 (OKT8), CD4 (T4-4), or HIV (TP) antibodies. Immunoprecipitates were separated on a 12% polyacrylamide gel, and proteins were visualized by fluorography. The positions of CD8/4 and wild-type CD4 are indicated on the left. Vpu_{52} and $Vpu_{2/6}$ are identified either as species that coprecipitate either with CD8/4 or CD4 (upper panel) or after immunoprecipitation with the anti-HIV TP serum (lower panel).

Phosphorylation of Vpu is necessary for CD4 degradation but not for binding. Although we found that binding of Vpu is required for degradation of target molecules, evidence provided by the Vpu_{52} mutant indicates that it is not sufficient to induce degradation. Functional domains of Vpu, distinct from the binding domain, are thus likely to participate in the biological activity of the protein. In this regard, we have previously shown the importance of Vpu phosphorylation in the process of CD4 degradation. Analysis of the stability of CD4 in the presence of a phosphorylation mutant of Vpu ($Vpu_{2/6}$) showed that phosphorylation is required for degradation (37). We now address whether the lack of degradation by $Vpu_{2/6}$ was due to its inability to bind to CD4 or to the perturbation of postbinding events important for degradation. HeLa cells were first transfected with pCMV-CD8/4 in the presence of either pNL-A1/U_{52} or the pNL-A1/$U_{2/6}$ plasmid, encoding the $Vpu_{2/6}$ phosphorylation mutant. Cells were labeled for 30 min with Tran^{35}S-Label, lysed in 1% digitonin, and subjected to immunoprecipitation with the anti-CD8 MAb OKT8 (Fig. 4, top, lanes 1 and 2). The result shows that, despite the absence of phosphorylation, the $Vpu_{2/6}$ mutant interacts with the CD4 cytoplasmic domain in a manner indistinguishable from that of the Vpu_{52} mutant (Fig. 4, lanes 1 and 2). When cells were cotransfected to express wild-type CD4 and $Vpu_{2/6}$, similar complexes were formed, as shown by the presence of $Vpu_{2/6}$ in the anti-CD4 immunoprecipitates (Fig. 4, lane 4). The identities of both Vpu_{52} and $Vpu_{2/6}$ were confirmed by immunoprecipitation of part of the lysates with the anti-HIV TP serum



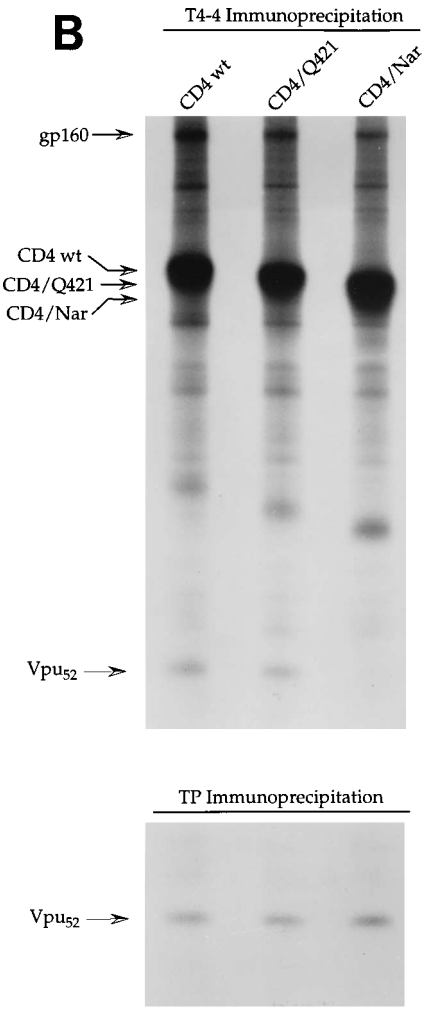


FIG. 5. Residues 402 to 420 in the CD4 cytoplasmic domain contain a binding site for Vpu. (A) Schematic representation of the CD4/Q421 and CD4/Nar deletion mutants. All constructs, including wild-type CD4, are under the transcriptional control of the HIV-1 LTR. The positions of the stop codons truncating the CD4 cytoplasmic domain at various places are indicated. The sensitivity of each mutant to Vpu-mediated degradation has been previously addressed in an in vitro system using similar constructs under the transcriptional control of the Sp6 polymerase promoter (10). (B) HeLa cells cotransfected with the pNL-A1/U_{52} plasmid, expressing Vpu_{52}, and constructs encoding either wild-type CD4, CD4/Q421, or CD4/Nar were metabolically labeled for 30 min with Tran^{35}S-Label, lysed in buffer containing 1% digitonin, and subjected to immunoprecipitation with either a monospecific polyclonal serum directed against CD4 (T4-4) or an HIV-positive human serum (TP). Immunoprecipitates were separated on a 12% polyacrylamide gel, and proteins were visualized by fluorography. The positions of the HIV-1 envelope precursor and the three different CD4 species are indicated on the left. The faster migration of CD4/Q421 and CD4/Nar than of wild-type (wt) CD4 reflects the truncation of cytoplasmic tail residues. Vpu_{52} is identified as species that coprecipitate either with CD4 (upper panel) or after immunoprecipitation with the anti-HIV TP serum (lower panel).

(Fig. 4, bottom). As shown after either TP immunoprecipitation or coprecipitation with the CD4 cytoplasmic tail, the Vpu_{52} mutant migrates more rapidly on an SDS-polyacrylamide gel than the $Vpu_{2/6}$ mutant because of the 5-amino-acid deletion in Vpu_{52}. As expected, no complexes were formed when CD4 was expressed in the presence of the Vpu(−) pNL-A1/U_{35} plasmid (Fig. 4, lane 3). Thus, like the Vpu_{52} mutant, the $Vpu_{2/6}$ phosphorylation mutant was able to interact with CD4 despite the loss of features important for degradation. This result suggests that CD4 degradation is a multistep process that requires phosphorylation of Vpu at a postbinding level.

Residues 402 to 420 in the CD4 cytoplasmic domain contain a binding site for Vpu. We have previously shown that residues 402 to 420 in the CD4 cytoplasmic tail are important for Vpu-mediated degradation in vitro (10). However, the role of these sequences in the mechanism of CD4 degradation is still obscure. To investigate whether residues 402 to 420 provide a binding site for Vpu, we analyzed the effect of removal of these residues on the ability of CD4 to form complexes with the Vpu_{52} mutant. Two deletion mutants of CD4 containing stop codons in the cytoplasmic domain that caused the deletion of either 13 (CD4/Q421) or 22 (CD4/Nar) C-terminal residues were used (Fig. 5A). HeLa cells cotransfected with the pNL-

A1/U_{52} plasmid and constructs encoding either wild-type CD4 or the CD4 truncation mutants were metabolically labeled for 30 min with Tran^{35}S-Label, lysed in buffer containing 1% digitonin, and subjected to immunoprecipitation with the T4-4 CD4 antiserum. A portion of each lysate was also immunoprecipitated with the anti-HIV (TP) serum to control for the amount of Vpu present in the samples. As shown earlier, Vpu_{52} coprecipitates with wild-type CD4, attesting to the presence of complexes between the two molecules (Fig. 5B, CD4 wt). Removal of the 13 C-terminal residues had no effect on the ability of CD4 to interact with Vpu (Fig. 5B, CD4/Q421). CD4 residues from positions 421 to 433 are thus dispensable for both degradation and binding of Vpu. In contrast, in the absence of the 33 C-terminal residues, no binding of Vpu to the CD4 cytoplasmic domain could be detected despite the presence of normal amounts of Vpu_{52} (Fig. 5B, CD4/Nar). Taken together, these results show that one role of CD4 residues 402 to 420 in the mechanism of degradation is to mediate recognition by Vpu.

Coprecipitation of Vpu with CD4 is specific and is not due to antibody cross-reactivity. Coprecipitation of Vpu via anti-CD4 or -CD8 antibodies is dependent on the presence of the CD4 cytoplasmic tail. In order to exclude the possibility that antibody cross-reactivity was leading to a nonspecific immunoprecipitation of Vpu, we addressed the antibody specificity of the coprecipitation protocol. HeLa cells transfected to express Vpu_{52} in the presence of either CD4 or CD8/4 were metabolically labeled with Tran^{35}S-Label for 30 min and lysed in digitonin buffer as described in Materials and Methods. Three equal parts of the cell lysates were subjected to immunoprecipitation with either the T4-4, OKT8, or TP antiserum (Fig. 6). In cells expressing CD4, coprecipitation of Vpu_{52} occurred via the CD4-specific T4-4 antibody but not via the CD8-specific OKT8 antibody (Fig. 6, lanes CD4 + Vpu_{52}). Conversely, complexes between CD8/4 and Vpu_{52} could be detected when the OKT8 but not the T4-4 antibody was used for immunoprecipitation (Fig. 6, lanes CD8/4 + Vpu_{52}). In all instances, Vpu_{52} could be immunoprecipitated with the anti-HIV TP serum. Control cells transfected with the pNL-A1/U_{52} plasmid alone were used to further rule out possible cross-reactivity of the T4-4 or OKT8 antibodies with Vpu_{52}. Neither antibody could precipitate Vpu_{52} in the absence of the relevant target protein, although Vpu_{52} was present in samples, as shown by the TP immunoprecipitation (Fig. 6, lanes Vpu_{52}). The coprecipitation of Vpu with the CD4 and CD8/4 molecules thus reflects specific interactions with the CD4 cytoplasmic domain.

Detection of complexes between wild-type Vpu and wild-type CD4 in an in vitro system. Although the use of the Vpu_{52} mutant allowed us to demonstrate the existence of complexes with wild-type CD4, we wished to verify interactions between wild-type molecules as well. As mentioned earlier, high rates of CD4 degradation in the presence of wild-type Vpu in HeLa cells could preclude the detection of even transient interactions between the two molecules. When assayed in an in vitro system, the degradation rate of CD4 is more than three times slower than in transfected cells, with a half-life of approximately 40 min (10). We thus investigated whether the slower rate of degradation in vitro would allow us to detect complexes between wild-type CD4 and wild-type Vpu. mRNA encoding wild-type CD4 was cotranslated with mRNA encoding either the wild-type or the Vpu_{52} mutant form of Vpu. Following 1 h of translation, the samples were lysed in buffer containing 1% digitonin. As shown in Fig. 7, direct analysis of aliquots of the cotranslation reaction mixes shows the presence of both CD4 and Vpu (lanes 2 and 3). CD4 alone as well as Vpu and Vpu_{52} alone were translated as controls (lanes 1, 4, and 5, respectively).

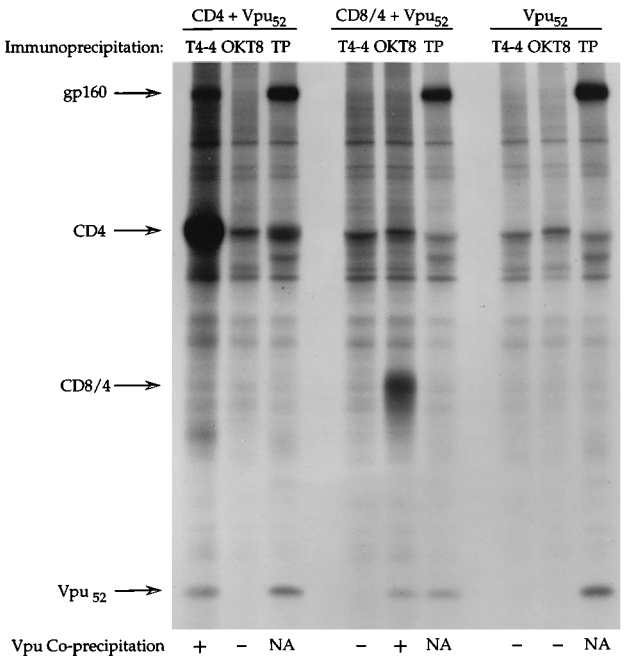


FIG. 6. Vpu coprecipitation reflects a specific association with the cytoplasmic domain of CD4 and is not due to antibody cross-reactivity. HeLa cells cotransfected with pHIVT4ΔBam (CD4) or pCMV-CD8/4 (CD8/4) and pNL-A1/U_{52} (Vpu_{52}) were labeled with Tran^{35}S-Label for 30 min. Cells transfected with pNL-A1/U_{52} alone are used as a control for antibody cross-reactivity. Following labeling, samples were chased for 15 min at 37°C, lysed in digitonin lysis buffer, and immunoprecipitated with the indicated antibodies. Immunoprecipitates were separated on a 12% polyacrylamide gel, and proteins were visualized by fluorography. The positions of the HIV-1 envelope precursor gp160, CD4, and the CD8/4 chimeric protein are indicated. The position of Vpu_{52}, either coprecipitated with CD4 and CD8/4 or specifically immunoprecipitated with the TP serum, is also indicated.

The ability of wild-type CD4 and Vpu to form complexes in vitro was addressed by immunoprecipitation of the same samples with the CD4 antiserum T4-4 (Fig. 7, lanes 6 to 10). The Vpu_{52} mutant was able to associate with CD4, as shown by the coprecipitation of the two molecules with T4-4 antiserum (Fig. 7, lane 8), confirming the results obtained in HeLa cells (Fig. 3B). When CD4 was translated in the presence of wild-type Vpu, similar complexes were detected, reflecting association between the two molecules (Fig. 7, lane 7). Vpu was not quantitatively coprecipitated with CD4 (compare lanes 2 and 3 with lanes 7 and 8). As mentioned above, this could indicate a transient nature of CD4-Vpu interactions. The coprecipitation of Vpu with CD4 in vitro reflects a specific association between the two molecules, since neither Vpu nor Vpu_{52} could be immunoprecipitated with the T4-4 antiserum in the absence of CD4 (Fig. 7, lanes 9 and 10).

In the presence of an excess of Vpu, the amount of CD4 molecules recovered after 1 h of cotranslation was significantly reduced compared with the control in the absence of Vpu (Fig. 7, compare lanes 1 and 2). This specific loss of CD4 is due to Vpu-mediated degradation of CD4, as shown previously (10). When CD4 was translated in the presence of the biologically inactive Vpu_{52} mutant, little if any CD4 loss was observed even though equivalent amounts of Vpu were synthesized (Fig. 7, lane 3). The destabilizing effect of Vpu was specifically targeted at CD4; the amount of Vpu recovered in the presence or absence of CD4 remained unchanged (Fig. 7, compare lanes 2

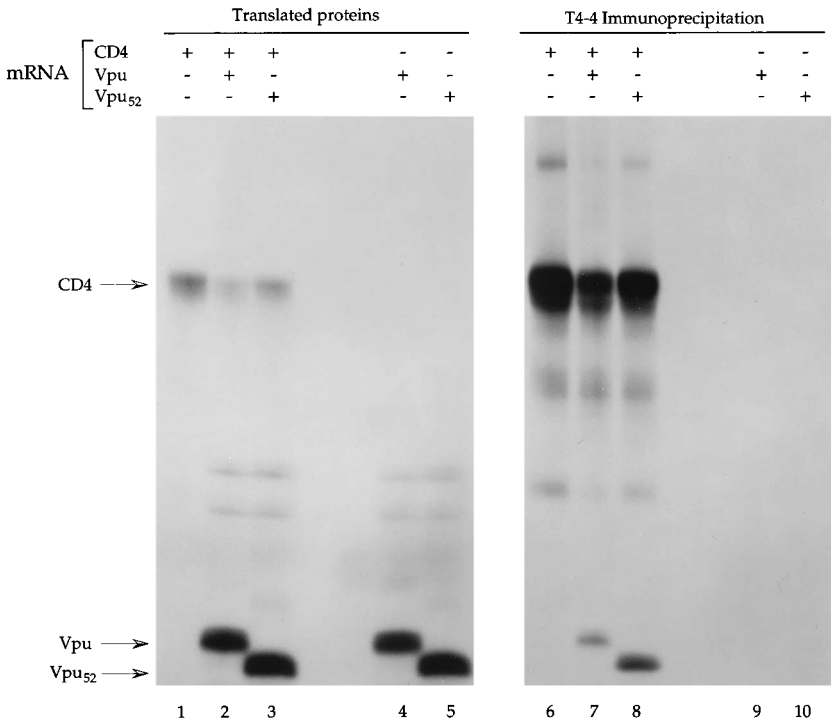


FIG. 7. Detection of complexes between wild-type CD4 and Vpu in vitro. mRNA obtained by transcription in vitro of the pSP-CD4 plasmid, encoding wild-type CD4, was cotranslated with mRNA encoding either wild-type Vpu or the Vpu_{52} mutant in the presence of canine microsomal membranes. Following 1 h of translation, digitonin lysis buffer was added. Samples were either directly analyzed on 12% polyacrylamide gels (lanes 1 to 5) or subjected to immunoprecipitation with the T4-4 monospecific CD4 antiserum. The positions of CD4 and Vpu are indicated on the left. The faster migration of Vpu_{52} than of the wild-type species reflects the 5-amino-acid deletion in this mutant.

and 3 with 4 and 5). Degradation of CD4 correlated with reduced coprecipitation of wild-type Vpu compared with the biologically inactive mutant Vpu_{52}. This correlation between the stability of CD4 and the stability of complexes with Vpu strongly suggests that the two events are mechanistically related. Taken together, these results demonstrate the existence of physical interactions between wild-type CD4 and wild-type Vpu in vitro. Even though such complexes are difficult to detect in transfected HeLa cells, the cumulative results of our experiments give a firm indication that they are also formed in cells expressing CD4 and Vpu.

DISCUSSION

The Vpu protein of HIV-1 has been shown to specifically induce the degradation of the CD4 receptor (10, 49, 50). While the exact contribution of this mechanism to the downregulation of cell surface CD4 in HIV-1-infected cells is presently unknown, it has been shown that steady-state levels of CD4 in cells expressing Vpu can be dramatically reduced (49). Under such conditions, cell surface expression of CD4 was found to be affected as well (42). The molecular mechanisms involved in CD4 degradation by Vpu are still obscure. Previous studies have shown that degradation occurs both in whole-cell systems and in vitro (10, 49, 50), and specific amino acid residues located in the CD4 cytoplasmic domain have been shown to confer sensitivity to Vpu-mediated degradation (10, 27, 47). We now demonstrate for the first time that an early step of CD4 degradation involves specific interactions with Vpu. Using a coprecipitation protocol, we were able to show binding of Vpu to wild-type CD4 as well as to CD8 chimeric molecules bearing the CD4 cytoplasmic tail. Our findings that decreased rates of CD4 degradation correlate with increased steady-state levels of CD4-Vpu complexes and that all proteins capable of binding to Vpu are sensitive while all proteins incapable of binding are insensitive to degradation strongly indicate that physical interactions between Vpu and CD4 precede or even initiate degradation. However, the fact that several Vpu mutants are capable of binding to CD4 but are unable to induce degradation suggests that binding in itself is not sufficient and requires additional catalytic activities, as discussed below. We cannot formally rule out the possibility that the interaction of CD4 and Vpu is not direct but involves additional cellular factors. However, we have no experimental evidence that would support such a possibility. Additional concern involves the possible interference of other cellular or viral proteins that are known to be, like Vpu, dependent on specific amino acid sequences in the CD4 cytoplasmic tail for their action. For instance, the Nef protein of HIV-1 has recently been shown to mediate internalization and lysosomal degradation of CD4 in a process that requires a specific dileucine motif at positions 413 and 414 (2). However, there is presently no experimental evidence for a physical interaction between Nef and CD4. In contrast, the $p56^{lck}$ protein tyrosine kinase has been shown to bind to the CD4 cytoplasmic domain in the ER and at the cell surface involving a discrete CQC motif at positions 420 to 422 (14, 46). Although this motif is not required for Vpu-mediated degradation of CD4 (10, 27), our finding that Vpu specifically interacts with CD4 residues 402 to 420 raises the question of whether the presence of $p56^{lck}$ could interfere with Vpu binding to CD4 and consequently affect the process of degradation. Preliminary results obtained in our laboratory indicate that the presence of $p56^{lck}$ in HeLa cells transiently expressing CD4 and Vpu has no effect on the efficiency of Vpu-mediated

degradation of CD4 (42). Moreover, complexes between Vpu and the CD8/4 chimeric molecule, as shown in the present work, could also be detected in the presence of $p56^{lck}$ (42). It remains to be elucidated whether CD4 is capable of forming heterotrimers with Vpu and $p56^{lck}$ or whether Vpu and $p56^{lck}$ competitively bind to CD4. In the latter case, Vpu might have a kinetic advantage over the cytoplasmic Lck protein, by virtue of its stable integration into the ER membrane in close proximity to de novo-synthesized CD4 molecules.

As mentioned earlier, we found that the presence of the CD4 cytoplasmic domain is necessary and sufficient to allow Vpu binding. Previous work demonstrated that sequences located between amino acids 402 and 420 in the CD4 cytoplasmic tail confer sensitivity to Vpu (10, 27, 47). However, the exact function of these sequences remained unknown. We now show that amino acid residues located between positions 402 and 420 contain a binding site for Vpu. Removal of the C-terminal 13 residues of CD4 had no effect on its ability to interact with Vpu. However, deletion of the C-terminal 22 residues, encompassing the region from 402 to 420, abrogated Vpu binding. Since the ability of the CD4 deletion mutants to bind Vpu correlated with their sensitivity to degradation, it is unlikely that, in the case of CD4, a major requirement for degradation is the ability to interact with Vpu. However, we do not rule out at present that these sequences have more than one role during Vpu-mediated degradation. In that regard, it should be mentioned that, in studies employing CD4/vesicular stomatitis virus protein G or CD4/HIV-Env chimeric molecules for the identification of Vpu-responsive sequences, the transmembrane (TM) domain of CD4 was also found to be required for Vpu-mediated degradation of the chimeric molecules (8, 31). These results are in contrast with our previous finding that, in the context of CD8/CD4 chimeric molecules, the cytoplasmic domain of CD4 was sufficient to confer sensitivity to Vpu-mediated degradation (48). It was therefore suggested that similarities between the CD4 and CD8 TM domains may explain our results (8, 31). However, it remains to be shown whether the CD8 TM can indeed substitute for the CD4 TM in the context of vesicular stomatitis virus G or HIV-Env chimeras or whether differences in the experimental design account for the contrasting results. Regardless of the potential involvement of the CD4 TM in the process of Vpu-mediated degradation, we now show that CD8/CD4 chimeric molecules containing the CD4 cytoplasmic domain in the presence or absence of the TM domain can associate with Vpu. Moreover, our inability to detect complexes between Vpu and the CD8/TM4 chimera that contained only the CD4 transmembrane domain further indicates that this region is unlikely to contribute to Vpu binding.

Although the ability to bind Vpu appears to be necessary and sufficient for CD4 to be sensitive to degradation, we found that, in the case of Vpu, binding to target molecules and degradation are separable events. We were able to show that a deletion mutant of Vpu (Vpu_{52}), as well as a phosphorylation mutant ($Vpu_{2/6}$), both unable to degrade CD4 (10, 37), retained the ability to interact with the CD4 cytoplasmic domain. Distinct domains of Vpu are thus likely to be involved in a multistep process leading to CD4 degradation. The binding domain is clearly located outside residues 47 to 52, which are deleted in Vpu_{52}. In addition, phosphorylation of Vpu at residues 52 and 56, which is critical for the catalysis of CD4 degradation (37), is dispensable for binding to target molecules. It is thus tempting to speculate that the inability of the Vpu_{52} mutant to degrade CD4 is due to the removal of the phosphorylation site at position 52. This notion reinforces the role of the phosphoseryl residues of Vpu in the mechanism of CD4 degradation. However, the results provided in this paper indicate that they are involved in postbinding events leading to CD4 degradation. The phospho groups of Vpu may be important for the actual proteolysis of CD4. For instance, they may interact with divalent cations, by virtue of their strong negative charges. Both magnesium- and calcium-dependent proteases have been involved in ER degradation of integral membrane proteins. In particular, the protease activity involved in the degradation of cytochrome P450 was shown to be activated by Mg^{2+} and ATP (17). Similarly, depletion of Ca^{2+} was shown to slow the degradation of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase in the ER (32). We are presently investigating the calcium dependence of Vpu-mediated CD4 degradation. In addition, the use of specific protease inhibitors will provide more information as to the type of enzymatic activity involved in Vpu-mediated degradation of CD4.

The ability of both Vpu_{52} and $Vpu_{2/6}$ to bind to the CD4 cytoplasmic domain may provide information on the functional domains of Vpu involved in interactions with CD4. We have shown that a CD8/CD4 chimera containing only the transmembrane domain of CD4 (CD8/TM4) was unable to bind Vpu. It is thus likely that the two molecules interact through their respective C-terminal cytoplasmic domains. The hydrophilic domain of Vpu has been predicted to form an α-helix–turn–α-helix structure, with the phosphorylation sites located between the two α-helices (35). This theoretical prediction was confirmed by studying the solution structure of synthetic Vpu fragments by a combination of circular dichroism and ^1H nuclear magnetic resonance spectroscopy (20, 51): amino acids 30 to 50 and 57 to 72 can each form an amphipathic α-helix. Both α-helices are of opposite polarity and are joined by a strongly acidic and flexible part that contains the two phosphoacceptor sites at Ser-52 and Ser-56. The amino acid changes in $Vpu_{2/6}$ do not affect the α-helical structure of the Vpu cytoplasmic domain (37, 51). Since $Vpu_{2/6}$ is still able to bind to CD4, it is possible that CD4-Vpu complexes are stabilized through interactions between the amphipathic α-helical domains of Vpu and the cytoplasmic domain of CD4, for which an α-helical secondary structure was predicated as well (39). In this regard, it is worth mentioning that a synthetic peptide comprising the cytoplasmic domain of Vpu from residues 32 to 81 (20) was not found to bind CD4 in an in vitro assay (6). However, previous results demonstrated that this peptide forms α-helical structures in aqueous solution only when trifluorethanol, which mimics a membrane-like environment, is present (20, 51). This result makes it tempting to speculate that structural constraints, in addition to specific amino acid sequences, are important for Vpu binding to CD4. The involvement of the Vpu cytoplasmic domain in the interaction with CD4 is further supported by our inability to immunoprecipitate CD4-Vpu complexes with Vpu-specific antisera. Since most Vpu antibodies present in serum are directed against the cytoplasmic immunodominant domain of Vpu (34), this result indicates that this region may be masked upon binding to CD4.

A number of cellular mechanisms have been implicated in ER degradation of misfolded or short-lived proteins (reviewed in references 12, 16, and 25). However, most of these mechanisms are primarily designed to assist in the proper conformation of unfolded or reversibly misfolded proteins. Vpu is unique in the sense that it induces the degradation of molecules that are already folded properly. Indeed, pulse-chase experiments performed in vitro have shown that CD4 molecules presynthesized into microsomal membranes are efficiently degraded by Vpu (10). The present work shows that the specificity of Vpu-mediated degradation is due to its ability to physically interact with the cytoplasmic domain of CD4. Al-

though the effect of Vpu binding on the conformation of CD4 is not known, it is conceivable that such interactions induce structural changes in CD4 so as to make the molecule behave as a misfolded species. Such a mechanism could resemble the selective ER retention and degradation of the alpha chain of the multicomponent T-cell receptor (TcRα) (5). Charged residues in the central part of the transmembrane region of the TcRα chain are responsible for rapid ER degradation in the absence of assembly with the other six components of the TcR (3, 4). However, there is evidence that the cytoplasmic tail of CD4 can stabilize chimeric molecules bearing the highly unstable transmembrane domain of the TcRα chain (41). The stabilizing effect of the CD4 cytoplasmic tail is apparently due to its highly organized structure (39), which could force the transmembrane domain of the TcRα into a more stable conformation (41). It is thus likely that, in the context of wild-type CD4, the cytoplasmic domain has an important role in the structure of the molecule. Binding of wild-type Vpu to this region may disturb the structure of the molecule and expose residues that trigger ER degradation.

Alternatively, other cellular mechanisms of ER degradation of proteins may be recruited by Vpu. We determined that Vpu-mediated degradation of CD4 was ATP dependent in a cell-free system (9), although we cannot exclude that ATP in this case is also required for Vpu phosphorylation. The ATP requirement for Vpu-mediated degradation of CD4 could point to the involvement of a more complex process, such as the ubiquitin-mediated proteolytic pathway. While most proteases do not require ATP, the hydrolysis of peptide bonds being thermodynamically favored, ATP hydrolysis has been involved in two major steps of the ubiquitin-mediated degradation pathway: the initial activation of ubiquitin for transfer to the protein substrate, and the hydrolysis of the ubiquitinated species by the multicatalytic 26S proteasome (18, 19). For example, the formation of complexes between the human papillomavirus E6 protein and the p53 tumor suppressor protein has been shown to induce the ubiquitin-dependent degradation of p53 in an ATP-dependent manner (33). Interestingly, E6 remained stable during this process. The facts that Vpu also forms specific complexes with its target protein (this paper), is unaffected by the degradation process (10), requires ATP for degradation (9), and, similarly to p53 degradation, functions both in whole-cell systems and in reticulocyte lysates (10, 33) may indicate some resemblance between the two mechanisms of degradation.

ACKNOWLEDGMENTS

We are indebted to Malcolm Martin for his support, and we thank Malcolm Martin and Ronald Willey for critical review of the manuscript.

REFERENCES

1. **Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin.** 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. **59:**284–291.
2. **Aiken, C., J. Konner, N. R. Landau, M. E. Lenburg, and D. Trono.** 1994. Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain. Cell **76:**853–864.
3. **Bonifacino, J. S., P. Cosson, and R. D. Klausner.** 1990. Colocalized transmembrane determinants for ER degradation and subunit assembly explain the intracellular fate of TCR chains. Cell **63:**503–513.
4. **Bonifacino, J. S., P. Cosson, N. Shah, and R. D. Klausner.** 1991. Role of potentially charged transmembrane residues in targeting proteins for retention and degradation within the endoplasmic reticulum. EMBO J. **10:**2783–2793.
5. **Bonifacino, J. S., C. K. Susuki, and R. D. Klausner.** 1990. A peptide sequence confers retention and rapid degradation in the endoplasmic reticulum. Science **247:**79–82.
6. **Bour, S.** Unpublished data.
7. **Bour, S., F. Boulerice, and M. A. Wainberg.** 1991. Inhibition of gp160 and CD4 maturation in U937 cells after both defective and productive infections by human immunodeficiency virus type 1. J. Virol. **65:**6387–6396.
8. **Buonocore, L., T. G. Turi, B. Crise, and J. K. Rose.** 1994. Stimulation of heterologous protein degradation by the Vpu protein of HIV-1 requires the transmembrane and cytoplasmic domains of CD4. Virology **204:**482–486.
9. **Chen, M. Y., and S. Bour.** Unpublished data.
10. **Chen, M. Y., F. Maldarelli, M. K. Karczewski, R. L. Willey, and K. Strebel.** 1993. Human immunodeficiency virus type 1 Vpu protein induces degradation of CD4 in vitro: the cytoplasmic domain of CD4 contributes to Vpu sensitivity. J. Virol. **67:**3877–3884.
11. **Cohen, E. A., E. F. Terwilliger, J. G. Sodroski, and W. A. Haseltine.** 1988. Identification of a protein encoded by the vpu gene of HIV-1. Nature (London) **334:**532–534.
12. **Craig, E. A., B. D. Gambill, and R. J. Nelson.** 1993. Heat shock proteins: molecular chaperones of protein biogenesis. Microbiol. Rev. **57:**402–414.
13. **Crise, B., L. Buonocore, and J. K. Rose.** 1990. CD4 is retained in the endoplasmic reticulum by the human immunodeficiency virus type 1 glycoprotein precursor. J. Virol. **64:**5585–5593.
14. **Crise, B., and J. K. Rose.** 1992. Human immunodeficiency virus type 1 glycoprotein precursor retains a CD4-p56^{lck} complex in the endoplasmic reticulum. J. Virol. **66:**2296–2301.
15. **Deen, K. C., J. S. McDougal, R. Inacker, G. Folena-Wasserman, J. Arthos, J. Rosenberg, P. J. Maddon, R. Axel, and R. W. Sweet.** 1988. A soluble form of CD4 (T4) protein inhibits AIDS virus infection. Nature (London) **331:**82–84.
16. **Doms, R. W., R. A. Lamb, J. K. Rose, and A. Helenius.** 1993. Folding and assembly of viral membrane proteins. Virology **193:**545–562.
17. **Eliasson, E., S. Mkrtchian, and M. Ingelman-Sundberg.** 1992. Hormone- and substrate-regulated intracellular degradation of cytochrome P450 (2E1) involving MgATP-activated rapid proteolysis in the endoplasmic reticulum membranes. J. Biol. Chem. **267:**15765–15769.
18. **Finley, D.** 1991. Ubiquitination. Annu. Rev. Cell Biol. **7:**25–69.
19. **Goldberg, A. L., and K. L. Rock.** 1992. Proteolysis, proteasome and antigen presentation. Nature (London) **357:**375–379.
20. **Henklein, P., U. Schubert, O. Kunert, S. Klabunde, V. Wray, K. D. Klöppel, M. Kiess, T. Portsmann, and D. Schomburg.** 1993. Synthesis and characterization of the hydrophilic C-terminal domain of the human immunodeficiency virus type 1-encoded virus protein U (Vpu). Peptide Res. **6:**79–87.
21. **Hoxie, J. A., J. D. Alpers, J. L. Rackowski, K. Huebner, B. S. Haggarty, A. J. Cedarbaum, and J. C. Reed.** 1986. Alterations in T4 (CD4) protein and mRNA synthesis in cells infected with HIV. Science **234:**1123–1127.
22. **Huet, T., A. Cheynier, A. Meyerhans, G. Roelants, and S. Wain-Hobson.** 1990. Genetic organization of a chimpanzee lentivirus related to HIV-1. Nature (London) **345:**356–359.
23. **Ishihara, K., W. Wood, Jr., M. Damore, G. G. Hermanson, R. Wall, and P. W. Kincade.** 1992. B29 gene products complex with immunoglobulins on B lymphocytes. Proc. Natl. Acad. Sci. USA **89:**633–637.
24. **Jabbar, M. A., and D. P. Nayak.** 1990. Intracellular interaction of human immunodeficiency virus type 1 (ARV-2) envelope glycoprotein gp160 with CD4 blocks the movement and maturation of CD4 to the plasma membrane. J. Virol. **64:**6297–6304.
25. **Jentsch, S.** 1992. The ubiquitin-conjugation system. Annu. Rev. Genet. **26:**179–207.
26. **Klatzmann, D., E. Champagne, S. Chamaret, J. Gruest, D. Guetard, T. Hercend, J.-C. Gluckman, and L. Montagnier.** 1984. T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. Nature (London) **312:**767–768.
27. **Lenburg, M. E., and N. R. Landau.** 1993. Vpu-induced degradation of CD4: requirement for specific amino acid residues in the cytoplasmic domain of CD4. J. Virol. **67:**7238–7245.
28. **Maddon, P. J., A. G. Dalgleish, J. S. McDougal, P. R. Clapham, R. A. Weiss, and R. Axel.** 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell **47:**333–348.
29. **Maldarelli, F., M. Y. Chen, R. L. Willey, and K. Strebel.** 1993. Human immunodeficiency virus type 1 Vpu protein is an oligomeric type I integral membrane protein. J. Virol. **67:**5056–5061.
30. **Matsuda, Z., M.-J. Chou, M. Matsuda, J.-H. Huang, Y.-M. Chen, R. Redfield, K. Mayer, M. Essex, and T.-H. Lee.** 1988. Human immunodeficiency virus type 1 has an additional coding sequence in the central region of the genome. Proc. Natl. Acad. Sci. USA **85:**6968–6972.
31. **Raja, N. U., M. J. Vincent, and M. A. Jabbar.** 1994. Vpu-mediated proteolysis of gp160/CD4 chimeric envelope glycoproteins in the endoplasmic reticulum: requirement of both the anchor and cytoplasmic domains of CD4. Virology **204:**357–366.
32. **Roitelman, J., S. Bar-Nun, S. Inoue, and R. D. Simoni.** 1991. Involvement of calcium in the mevalonate-accelerated degradation of 3-hydroxy-3-methylglutaryl-CoA reductase. J. Biol. Chem. **266:**16085–16091.
33. **Scheffner, M., B. A. Werness, J. M. Huibregtse, A. J. Levine, and P. M. Howley.** 1990. The E6 oncoprotein encoded by human papilloma virus types 16 and 18 promotes the degradation of p53. Cell **63:**1129–1136.

34. **Schneider, T., P. Hildebrandt, W. Rönspeck, W. Weigelt, and G. Pauli.** 1990. The antibody response to the HIV-1 specific "out" (vpu) protein: identification of an immunodominant epitope and correlation of antibody detectability to clinical stages. AIDS Res. Hum. Retroviruses **6:**943–950.
35. **Schubert, U., P. Henklein, B. Boldyreff, E. Wingender, K. Strebel, and T. Porstmann.** 1994. The human immunodeficiency virus type 1 encoded Vpu protein is phosphorylated by casein kinase-2 (CK-2) at positions Ser52 and Ser56 within a predicted alpha-helix-turn-alpha-helix-motif. J. Mol. Biol. **236:**16–25.
36. **Schubert, U., T. Schneider, P. Henklein, K. Hoffmann, E. Berthold, H. Hauser, G. Pauli, and T. Porstmann.** 1992. Human-immunodeficiency-virus-type-1-encoded Vpu protein is phosphorylated by casein kinase II. Eur. J. Biochem. **204:**875–883.
37. **Schubert, U., and K. Strebel.** 1994. Differential activities of the human immunodeficiency virus type 1-encoded Vpu protein are regulated by phosphorylation and occur in different cellular compartments. J. Virol. **68:**2260–2271.
38. **Schwartz, S., B. K. Felber, E. M. Fenyo, and G. N. Pavlakis.** 1990. Env and Vpu proteins of human immunodeficiency virus type 1 are produced from multiple bicistronic mRNAs. J. Virol. **64:**5448–5456.
39. **Shin, J., S. Dunbrack, Jr., S. Lee, and J. L. Strominger.** 1991. Phosphorylation-dependent down-modulation of CD4 requires a specific structure within the cytoplasmic domain of CD4. J. Biol. Chem. **266:**10658–10665.
40. **Shin, J., S. Dunbrack, S. Lee, and J. L. Strominger.** 1991. Signals for retention of transmembrane proteins in the endoplasmic reticulum studied with CD4 truncation mutants. Proc. Natl. Acad. Sci. USA **88:**1918–1922.
41. **Shin, J., S. Lee, and J. L. Strominger.** 1993. Translocation of TCRα chains into the lumen of the endoplasmic reticulum and their degradation. Science **259:**1901–1904.
42. **Strebel, K.** Unpublished data.
43. **Strebel, K., T. Klimkait, F. Maldarelli, and M. A. Martin.** 1989. Molecular and biochemical analyses of human immunodeficiency virus type 1 Vpu protein. J. Virol. **63:**3784–3791.
44. **Strebel, K., T. Klimkait, and M. A. Martin.** 1988. A novel gene of HIV-1, vpu, and its 16-kilodalton product. Science **241:**1221–1223.
45. **Terwilliger, E. F., E. A. Cohen, Y. C. Lu, J. G. Sodroski, and W. A. Haseltine.** 1989. Functional role of human immunodeficiency virus type 1 vpu. Proc. Natl. Acad. Sci. USA **86:**5163–5167.
46. **Veillette, A., M. A. Bookman, E. M. Horak, and J. B. Bolen.** 1988. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase $p56^{lck}$. Cell **55:**301–308.
47. **Vincent, M. J., N. U. Raja, and M. A. Jabbar.** 1993. 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. J. Virol. **67:**5538–5549.
48. **Willey, R. L., A. Buckler-White, and K. Strebel.** 1994. Sequences present in the cytoplasmic domain of CD4 are necessary and sufficient to confer sensitivity to the human immunodeficiency virus type 1 Vpu protein. J. Virol. **68:**1207–1212.
49. **Willey, R. L., F. Maldarelli, M. A. Martin, and K. Strebel.** 1992. Human immunodeficiency virus type 1 Vpu protein induces rapid degradation of CD4. J. Virol. **66:**7193–7200.
50. **Willey, R. L., F. Maldarelli, M. A. Martin, and K. Strebel.** 1992. Human immunodeficiency virus type 1 Vpu protein regulates the formation of intracellular gp160-CD4 complexes. J. Virol. **66:**226–234.
51. **Wray, V., T. Federau, P. Henklein, S. Klabunde, O. Kunert, D. Schomburg, and U. Schubert.** The solution structure of the hydrophylic region of the HIV-1 encoded virus protein U (Vpu) by CD and NMR spectroscopy. Int. J. Peptide Protein Res., in press.