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Human Immunodeficiency Virus Type 1 Vpu Protein Induces Rapid Degradation of CD4

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CD4 is an integral membrane glycoprotein which is known as the human immunodeficiency virus (HIV) receptor for infection of human cells. The protein is synthesized in the endoplasmic reticulum (ER) and subsequently transported to the cell surface via the Golgi complex. HIV infection of CD4$^+$ cells leads to downmodulation of cell surface CD4, due at least in part to the formation of stable intracellular complexes between CD4 and the HIV type 1 (HIV-1) Env precursor polyprotein gp160. This process "traps" both proteins in the ER, leading to reduced surface expression of CD4 and reduced processing of gp160 to gp120 and gp41. We have recently demonstrated that the presence of the HIV-1-encoded integral membrane protein Vpu can reduce the formation of Env-CD4 complexes, resulting in increased gp160 processing and decreased CD4 stability. We have studied the effect of Vpu on CD4 stability and found that Vpu induces rapid degradation of CD4, reducing the half-life of CD4 from 6 h to 12 min. By using a CD4-binding mutant of gp160, we were able to show that this Vpu-induced degradation of CD4 requires retention of CD4 in the ER, which is normally accomplished through its binding to gp160. The involvement of gp160 in the induction of CD4 degradation is restricted to its function as a CD4 trap, since, in the absence of Env, an ER retention mutant of CD4, as well as wild-type CD4 in cultures treated with brefeldin A, a drug that blocks transport of proteins from the ER, is degraded in the presence of Vpu.

Human immunodeficiency virus type 1 (HIV-1) infection of CD4$^+$ T lymphocytes leads to reduced cell surface expression of CD4 (6, 14). Several mechanisms involving either the virus-encoded gp160 envelope precursor glycoprotein or the Nef protein have been reported to cause downmodulation of CD4 (3, 5, 8, 11–13, 29). In the case of gp160, several studies have demonstrated that the formation of intracellular complexes between CD4 and gp160 "traps" CD4 in the endoplasmic reticulum (ER) (3, 5, 12), preventing its transport to the cell surface. We have recently shown (39) that complex formation also reduces the processing of gp160 into the mature gp120 and gp41 envelope components. However, we found that expression of the HIV-1-encoded Vpu protein, an integral membrane protein that is present in HIV-1-infected cells but is not found in virions, could disrupt gp160-CD4 complexes. In addition, a consistently lower amount of CD4 was recovered from cells expressing Vpu, suggesting that reduced complex formation was the result of Vpu-induced destabilization of CD4.

In the present study, we have examined the effect of Vpu on CD4 stability and show that Vpu induces rapid degradation of CD4 in the ER. A prerequisite for Vpu-induced CD4 degradation is that CD4 be prevented from leaving the ER, which is accomplished through its binding to gp160. The role of gp160, however, is restricted to its function as a CD4 trap, since in the absence of Env, both an ER retention mutant of CD4 and wild-type CD4 in cultures treated with brefeldin A (BFA), a drug that blocks transport of proteins from the ER, are degraded in the presence of Vpu.

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MATERIALS AND METHODS

Cells and transfections. HeLa cells (ATCC CCL2) were propagated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). For transfection, HeLa cells were grown to near confluency in 25-cm^2 flasks (3 × 10^6 cells per flask). Before transfection, the medium was replaced with 5 ml of fresh DMEM containing 10% FBS. Calcium phosphate-precipitated (10) plasmid DNA (4 to 30 μg) was added to the cells. After 3 h, the medium was removed, and the cells were subjected to a glycerol shock for 2.5 min (9). The cultures were then washed once with phosphate-buffered saline (PBS) and maintained in 5 ml of DMEM containing 10% FBS.

Cloned DNAs. All plasmids containing HIV-1 sequences are derivatives of the infectious molecular clone pNL4-3 (1). The plasmid pNL-A1 (*vif*$^+$ *vpr*$^+$ *tat*$^+$ *rev*$^+$ *vpu*$^+$ *env*$^+$ *nef*$^+$) was constructed as described previously by substituting a *Bss*HII-*Eco*RI fragment from the *vif* cDNA clone pUC1-11 (30). The Vpu-deficient plasmid pNL-A1/U$_{35}$ (*vif*$^+$ *vpr*$^+$ *tat*$^+$ *rev*$^+$ *env*$^+$ *nef*$^+$) was derived from pNL-A1 by inserting an 8-bp *Xho*I linker sequence into the *vpu* gene (32) but is otherwise isogenic to pNL-A1. The pNL-A1(CD4$^-$) and pNL-A1/U$_{35}$(CD4$^-$) plasmids express a mutant gp160 which is unable to bind CD4. These plasmids are derivatives of pNL-A1 and pNL-A1/U$_{35}$, respectively, and were constructed by substituting a 580-bp *Bgl*II fragment from the *env* gene of a CD4-binding mutant plasmid which contains a 12-codon in-frame deletion between residues 410 and 421 of gp120 (16). Plasmid pNL-A1/dEnv, which does not express the gp160 envelope protein, was derived from pNL-A1 by introducing a frameshift mutation at codon 41 of the *env* reading frame. This was accomplished by digesting the plasmid DNA with *Kpn*I (New England Biolabs) and removing the 3′ overhanging nucleotides with *Escherichia coli* DNA polymerase I (New England Biolabs). Finally, a *vpu*-

and *env*-deficient variant of pNL-A1, pNL-A1/U_{35}/dEnv, was constructed by exchanging a 600-bp *Eco*RI-*Kpn*I fragment from pNL-A1/U_{35} in pNL-A1/dEnv. The CD4 expression plasmid pHIV-CD4 (39) was constructed by inserting a 3.0-kb *Eco*RI restriction fragment which was isolated from the plasmid T4-pMV7 (19) and contains the entire coding sequence for the human CD4 gene, between the *Bss*HII and *Xho*I sites (nucleotide positions 711 and 8887) of the molecular HIV-1 clone pNL4-3 (1). An ER retention mutant of CD4, pHIV-CD4/Q421, was constructed by replacing a 1.0-kb *Nhe*I-*Bam*HI fragment from CD4.Q421stop (27). CD4 expressed from this construct is completely retained in the ER (27).

Metabolic labelings and immunoprecipitations. Transfected HeLa cells were scraped off the flasks at 18 to 20 h posttransfection, washed, and then starved for 10 min in methionine-free RPMI 1640. Cells were pulse-labeled for 6 min in 250 μl of methionine-free RPMI 1640 containing 500 μCi of [^{35}S]methionine (New England Nuclear). The medium was then removed, and the cells were resuspended for the chase in 1 ml of complete RPMI 1640 containing 10% FBS and incubated at 37°C for 1 h. Individual cell aliquots were harvested immediately after labeling and at 10-min intervals, washed in cold PBS (pH 7.0), and lysed by freezing and thawing in buffer containing 50 mM Tris-hydrochloride (pH 7.4), 300 mM NaCl, 0.5% Triton X-100, 10 mM iodoacetamide, 0.5 mM leupeptin, and 0.2 mM phenylmethylsulfonyl fluoride. Cell lysates were precleared by incubation at 4°C for 1 h with protein A-agarose beads (30 μl of packed beads) (Bethesda Research Laboratories) in the absence of antiserum.

Immunoprecipitations were conducted as described previously (39) with either polyclonal rabbit antisera which recognize gp160, gp120, and gp41 (38) or a polyclonal rabbit antiserum directed against CD4 (gift of R. W. Sweet [7]). When multiple antisera were used, lysates were split into aliquots prior to immunoprecipitation. Immunoprecipitated proteins were solubilized by boiling the samples for 5 min in sample buffer (2% sodium dodecyl sulfate [SDS], 1% 2-mercaptoethanol, 1% glycerol, 65 mM Tris-hydrochloride [pH 6.8]) and separated on 10% acrylamide–AcrylAide (FMC) gels (38). Gels were fixed in a solution of 40% methanol and 10% acetic acid for 2 h, rinsed with water, soaked in 1 M sodium salicylic acid for 2 h, and dried. Radioactive bands were visualized by fluorography.

In experiments involving BFA, cells were pretreated for 1 h at 37°C with BFA (2 μg/ml). All subsequent steps of the labeling procedure, including starvation and pulse-chase labeling, were done in the presence of BFA (2 μg/ml).

Endo H analysis. Endo-β-*N*-acetylglucosaminidase H (endo H) from *Streptomyces griseus* (Genzyme) was used to study the oligosaccharide composition of CD4. All digestions were performed directly on immunoprecipitated CD4 proteins bound to protein A-agarose beads as described previously (38). Five milliunits of endo H was added to 25 μl of packed beads resuspended in 30 μl of 50 mM sodium phosphate buffer (pH 6.1) containing 0.03% SDS, 0.1% Triton X-100, and 0.6 μg of aprotinin (Boehringer Mannheim), and the mixture was incubated at 37°C for 16 h. Samples were then analyzed on 10% acrylamide–AcrylAide gels as described above.

Immunoblotting. Transfected HeLa cells were lysed 24 h after transfection in 200 μl of lysis buffer (50 mM Tris-hydrochloride [pH 8.0], 5 mM EDTA, 100 mM NaCl, 5% CHAPS [3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate], 0.2% deoxycholate). The lysates were run in duplicate on a 12.5% polyacrylamide–SDS gel and electrotransferred to nitrocellulose membranes. The membranes were incubated with either serum from an AIDS patient to detect HIV-specific proteins or a polyclonal rabbit antiserum against human CD4 (gift of R. W. Sweet), and binding of antibodies was identified by using ^{125}I-protein A (0.1 μCi/ml; New England Nuclear) and autoradiography.

RESULTS

Vpu induces rapid degradation of CD4. CD4 stability was initially evaluated in the presence of gp160 and Vpu by cotransfecting HeLa cells with HIV-1 subgenomic and CD4 expression plasmids which were previously used to evaluate the interactions of CD4, gp160, and Vpu (39). The structures and characteristics of the individual plasmids used are shown in Fig. 1. The CD4 expression plasmid pHIV-CD4 was transfected in combination with either the Env- and Vpu-producing plasmid pNL-A1 or the analogous Vpu-deficient plasmid pNL-A1/U_{35} (Vpu$^-$ Env$^+$). Expression of Vpu from pNL-A1 has been demonstrated previously (32, 39). The cells were pulse-labeled at 20 h after transfection with [^{35}S]methionine for 6 min and chased for up to 1 h in complete medium lacking the labeled amino acid. Cells were harvested at 10-min intervals and lysed in detergent, and aliquots from precleared supernatants were immunoprecipitated with either a polyclonal rabbit antiserum specific for gp160, gp120, or gp41 (to assess the amount of CD4 associated in a complex with gp160) or a rabbit polyclonal serum raised against CD4 (to analyze the total amount of labeled intracellular CD4 at the various times). With the Env-specific antiserum, the amount of CD4 which coprecipitated with gp160 in the absence of Vpu initially increased but then remained constant (Fig. 2A), indicating the formation of stable complexes, whereas in cells expressing Vpu (Fig. 2B), the amount of CD4 associated with gp160 diminished during the 1 h of chase. The Vpu-induced reduction of gp160-CD4 complex formation was accompanied by a marked decrease in the amount of CD4 detectable with the anti-CD4 serum during the same time period; in contrast, the level of CD4 detected in the absence of Vpu was virtually unchanged throughout the experiment (compare anti-CD4 panels in Fig. 2A and B).

The rate of CD4 decay in the presence and absence of Vpu was quantitated by densitometric scanning of the anti-CD4 lanes in Fig. 2A and B, and the half-life ($t_{1/2}$) of CD4 was determined (Fig. 2D). In the presence of Vpu, the $t_{1/2}$ of CD4 was approximately 12 min, with only about 15% of the initial pulse-labeled CD4 remaining at the end of the 1-h chase period; no decay of CD4 was observed during the 1-h chase in the absence of Vpu. The $t_{1/2}$ of CD4 in the absence of Vpu was estimated to be approximately 6 h in an independent experiment (data not shown). Similar results were obtained when different antisera to CD4 (either a second polyclonal antiserum to CD4 or a monoclonal antiserum, OKT4) were used in replicate experiments (data not shown), suggesting that the "disappearance" of CD4 in the experiments shown in Fig. 2 is due to degradation rather than to an antibody recognition problem.

Binding of gp160 to CD4 is required for Vpu-induced degradation of CD4. To investigate whether CD4 binding to gp160 is required for efficient Vpu-induced CD4 degradation, a 12-codon in-frame deletion mutation, which has been shown previously to prevent the binding of gp120 to CD4 (16), was introduced into the *env* gene of the pNL-A1 plasmid, generating the mutant Env expression plasmid

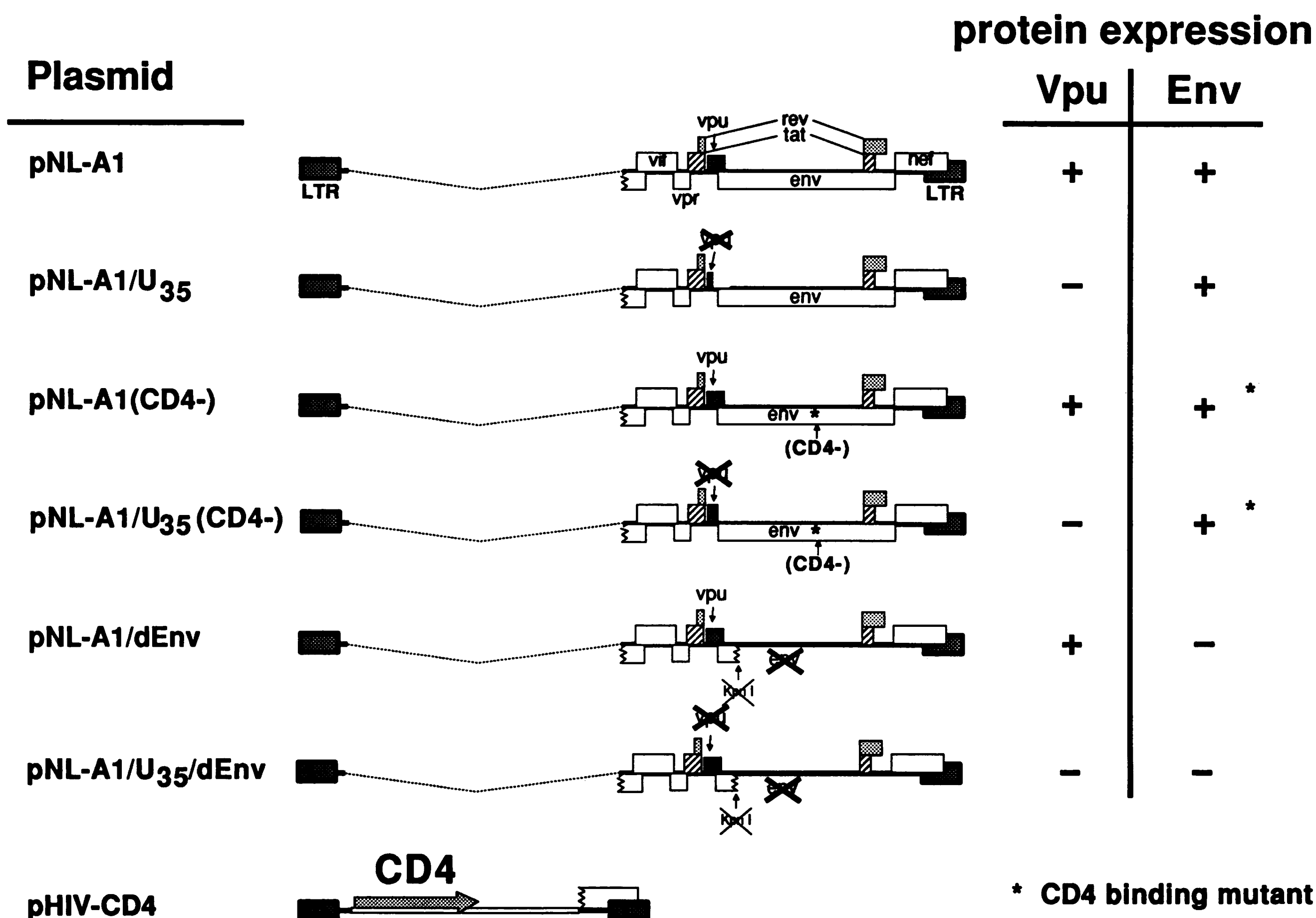

FIG. 1. Plasmids used to express Env, Vpu, and CD4. Construction of the HIV-1 subgenomic pNL-A1 (Vpu$^+$ Env$^+$) and pNL-A1/U$_{35}$ (Vpu$^-$ Env$^+$) expression plasmids and the HIV-1 long terminal repeat (LTR)-driven CD4 expression plasmid pHIV-CD4 has been described previously (39). The pNL-A1(CD4$^-$) and pNL-A1/U$_{35}$(CD4$^-$) plasmids express a mutant gp160 which is unable to bind CD4. These plasmids are derivatives of pNL-A1 and pNL-A1/U$_{35}$, respectively, and were constructed by substituting a 580-bp *Bgl*II fragment from the *env* gene of a CD4-binding mutant plasmid which contains a 12-codon in-frame deletion between residues 410 and 421 of gp120 (16). Plasmids pNL-A1/dEnv and pNL-A1/U$_{35}$/dEnv, which do not express the gp160 envelope protein, were derived from plasmids pNL-A1 and pNL-A1/U$_{35}$ by introducing a frameshift mutation at codon 41 of the *env* reading frame as described in the Materials and Methods section. The pNL-A1 plasmid and its derivatives also express Vif, Vpr, Tat, Rev, and Nef proteins, as indicated.

pNL-A1(CD4$^-$) (Fig. 1). The rate of synthesis and the half-life of Vpu (approximately 2 h) expressed from pNL-A1(CD4$^-$) were determined from pulse-chase experiments and found to be indistinguishable from those for pNL-A1 (data not shown). The pNL-A1(CD4$^-$) plasmid was cotransfected into HeLa cells with the CD4 expression plasmid pHIV-CD4, and pulse-chase labelings and immunoprecipitations were performed. As shown in Fig. 2C, gp160 and CD4 did not coprecipitate following incubation of cell lysates with either the Env- or CD4-specific antiserum, confirming the inability of CD4 to bind to the mutant gp160. Interestingly, the failure to form gp160-CD4 complexes was associated with greater CD4 stability in the presence of Vpu (compare middle and bottom curves in Fig. 2D). After 1 h of chase, 70% of the CD4 protein present at the pulse time point was recovered, compared with 15% recovery when gp160-CD4 complexes were formed. This result suggests that degradation of CD4 requires not only the presence of Vpu but also the formation of gp160-CD4 complexes.

Vpu induces degradation of endo H-sensitive CD4. Endo H is an enzyme capable of hydrolyzing N-linked high-mannose oligosaccharides which are cotranslationally added to glycoproteins in the ER but will not cleave complex oligosaccharides which can be acquired as the glycoprotein traverses the Golgi complex. Because of this characteristic, analysis of endo H sensitivity has been widely used to identify the intracellular localization of N-linked glycoproteins. Several groups have reported that CD4 normally acquires partial resistance to endo H. However, when gp160 is coexpressed with CD4, CD4 remains fully endo H sensitive, leading to the conclusion that it is trapped in the ER (5, 12). Since all of these studies were done in the absence of Vpu, we decided to analyze the endo H profile of CD4 in the presence of Vpu and either the wild-type or CD4-binding-deficient Env protein. Experiments similar to those in Fig. 2B and C were performed, and the CD4 protein, after immunoprecipitation with anti-CD4 antiserum, was subjected to endo H digestion prior to gel analysis as described in the Materials and Methods section.

The results, shown in Fig. 3, indicate that in the presence of Vpu and wild-type Env, endo H-sensitive CD4 was rapidly degraded (Fig. 3, left panel). A small amount of

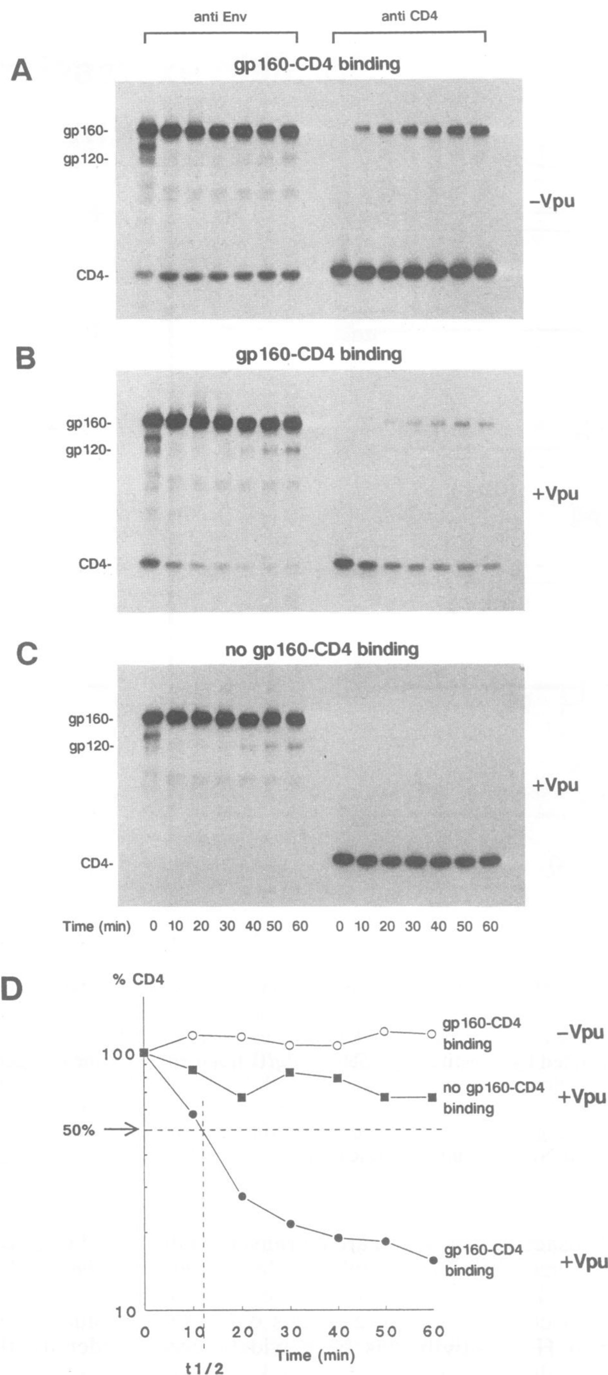

FIG. 2. Effect of Vpu on the stability of CD4 and significance of gp160-CD4 complexes. HeLa cells were transfected with the pHIV-CD4 expression plasmid (8 μg) in combination with either wild-type pNL-A1, Vpu mutant pNL-A1/U_{35}, or CD4-binding mutant pNL-A1($CD4^-$) plasmid DNA (24 μg) as described previously (39). HeLa cells coexpressing Env and CD4 only (A), Env, Vpu, and CD4 (B), or the Env^{CD4^-} binding mutant, Vpu, and CD4 (C) were pulse-labeled with [^{35}S]methionine for 6 min and chased for the indicated time intervals. Cell lysates were immunoprecipitated with a polyclonal rabbit antiserum against CD4 (anti-CD4) as described in the Materials and Methods section. Immunoprecipitates were solubilized by boiling in sample buffer, resolved on 10% acrylamide–AcrylAide–SDS gels, and visualized by fluorography. (D) Quantitative analysis of CD4 degradation. The percentage of CD4 remaining relative to the amount present at the end of the pulse (0 min) was determined by densitometric scanning of the anti-CD4 lanes in panels A, B, and C and is plotted as a function of time.

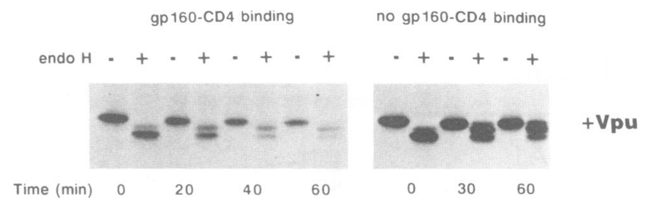

FIG. 3. Endo H analysis of CD4 in the presence of Vpu. HeLa cells were transfected with pHIV-CD4 (8 μg) in combination with either the wild-type pNL-A1 (gp160-CD4 binding) or the CD4-binding mutant pNL-A1($CD4^-$) (no gp160-CD4 binding) plasmid DNA (24 μg), and pulse-chase labelings were performed as described in the legend to Fig. 2. Cell lysates were immunoprecipitated with a polyclonal rabbit antiserum against CD4 (anti-CD4), and the immunoprecipitates were split and either not treated (−) or treated with endo H (+) as described in the Materials and Methods section. Samples were analyzed on a 10% acrylamide–AcrylAide gel. The two bands in the endo H-treated lanes represent partially resistant (top) or fully sensitive (bottom) CD4. The band in the non-endo H-treated lanes represents undigested CD4.

partially endo H-resistant CD4, present immediately after the pulse, appeared to be unaffected during the 1 h of chase (Fig. 3, left panel). In contrast, in the presence of the CD4-binding-deficient Env and despite the presence of Vpu, the majority of CD4 acquired partial endo H resistance within 60 min after synthesis (Fig. 3, right panel), and its stability appeared to be unaffected by Vpu, as observed before (see Fig. 2C). Our observation that endo H-sensitive CD4 is targeted by Vpu indicates that Vpu-induced degradation of CD4 occurs before the protein can acquire complex carbohydrates in the Golgi complex and therefore may take place in the ER.

BFA bypasses the requirement for gp160-CD4 complexes for CD4 degradation. Previous studies (26) have shown that the downmodulation of cell surface CD4 during the normal cell cycle in uninfected T cells is associated with CD4 degradation in lysosomal vesicles. In contrast, Vpu-induced CD4 degradation requires the formation of gp160-CD4 complexes (Fig. 2) and occurs prior to CD4 export to the Golgi complex (Fig. 3), suggesting that CD4 degradation in this case was occurring in the ER. Further evidence that the ER is the site of CD4 degradation comes from our observation that treating cells with ammonium chloride, chloroquine, or methylamine, weak bases which inhibit lysosomal protein degradation (17), has virtually no effect on Vpu-induced decay of CD4 (data not shown). This issue was examined further by using BFA, a fungal metabolite which blocks protein export out of the ER (18, 22) without affecting ER degradation (2, 22). HeLa cells were transfected with the CD4 expression plasmid pHIV-CD4 in combination with either wild-type pNL-A1 (Vpu^+ Env^+), Vpu-deficient pNL-A1/U_{35} (Vpu^- Env^+), or CD4-binding mutant pNL-A1($CD4^-$) (Vpu^+ Env^{CD4^-}) plasmid DNA. To control for the possibility that BFA treatment itself might affect the stability of CD4, a Vpu-deficient CD4-binding mutant plasmid, pNL-A1/U_{35}($CD4^-$) (Vpu^- Env^{CD4^-}; Fig. 1), was included as a control. At 20 h after the transfection, the cultures were divided, and pulse-chase labelings were conducted in the presence or absence of BFA (2 μg/ml) as described in the Materials and Methods section. Immunoprecipitations were performed on cell lysates with the CD4-specific antiserum.

The results of this experiment demonstrated that in the absence of Vpu, CD4 stability was unaffected by the BFA treatment whether CD4 was capable of binding to gp160 or

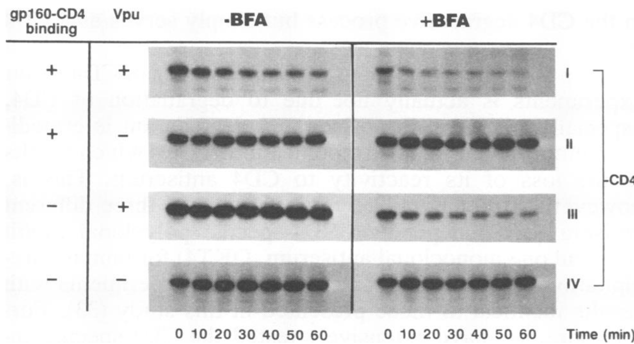

FIG. 4. Vpu induces ER degradation of CD4. HeLa cells were transfected with the CD4 expression plasmid pHIV-CD4 (8 μg) plus 24 μg of either the wild-type pNL-A1, the Vpu-deficient pNL-A1/U_{35}, the CD4-binding mutant pNL-A1(CD4$^-$), or the Vpu-deficient CD4-binding mutant pNL-A1/U_{35}(CD4$^-$) plasmid DNA (Fig. 1). At 20 h posttransfection, monolayers were scraped off, split into equal fractions, and pulse-chase labeled, in either the absence or presence of BFA, as described in the Materials and Methods section. Cell aliquots were removed at the indicated time points, and CD4 protein was immunoprecipitated from cell lysates with the CD4-specific polyclonal rabbit antiserum. Immunoprecipitates were resolved on 10% acrylamide–AcrylAide–SDS gels and visualized by fluorography.

not (Fig. 4, panels II and IV). This indicates that simple retention of CD4 in the ER does not promote its degradation. In contrast, in the presence of Vpu (Fig. 4, panels I and III), BFA treatment of cells led to enhanced degradation of CD4 even in the absence of gp160-CD4 complex formation (Fig. 4, panel III). These experiments clearly demonstrate two points: (i) Vpu induces degradation of CD4 in the ER and (ii) degradation of CD4 in BFA-treated cultures does not require the formation of gp160-CD4 complexes, suggesting that Vpu does not selectively target CD4 bound to gp160 but can induce degradation of "free" CD4 as long as it is retained in the ER.

HIV-1 Env protein is not directly involved in CD4 degradation but acts as a CD4 trap. All of the Vpu-induced CD4 degradation experiments described thus far were conducted in the presence of wild-type or mutated gp160. If the function of wild-type gp160 is to serve as a CD4 trap by sequestering CD4 in the ER, we should also be able to demonstrate efficient Vpu-induced degradation of CD4 in the absence of gp160 if CD4 is retained in the ER by BFA treatment. For this purpose, an Env-deficient variant of pNL-A1, pNL-A1/dEnv (Fig. 1), was constructed by introducing a 4-bp deletion into the *env* gene, which causes a translational frameshift mutation at codon 11 of gp160. This plasmid is otherwise isogenic to pNL-A1. The pNL-A1/dEnv plasmid DNA was cotransfected with pHIV-CD4 into HeLa cells, and pulse-chase experiments were conducted in the presence or absence of BFA as described above.

In the absence of any Env protein, Vpu promoted the rapid degradation of CD4 only when it was retained in the ER (Fig. 5). In fact, the kinetics of CD4 decay in the presence of BFA were indistinguishable from those observed when Vpu was present with wild-type gp160 (see Fig. 2B); in both cases, the $t_{1/2}$ of CD4 was calculated to be 12 min (compare Fig. 2D and Fig. 5). In the absence of BFA, very little CD4 decay was observed, which is analogous to the result obtained when the CD4-binding mutant of gp160 was present with Vpu (compare Fig. 2D and Fig. 5). Analysis of CD4 in these experiments with endo H confirmed that in

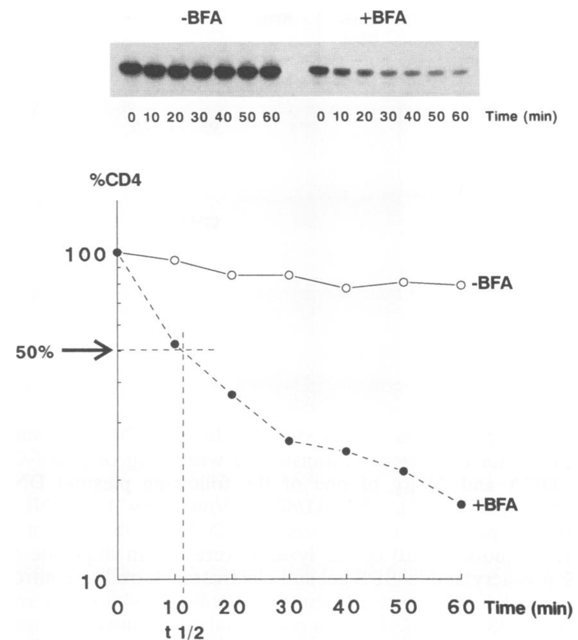

FIG. 5. Vpu-induced degradation of CD4 requires ER retention but not gp160. Plasmid DNA (24 μg) from the Env-deficient plasmid pNL-A1/dEnv was cotransfected into HeLa cells with pHIV-CD4 plasmid DNA (8 μg) as described in the Materials and Methods section. Pulse-chase labelings were conducted in the absence or presence of BFA, and CD4 was immunoprecipitated with the CD4-specific polyclonal rabbit antiserum. Immunoprecipitates were resolved on 10% acrylamide–AcrylAide–SDS gels and visualized by fluorography. The top portion of the figure shows the amount of CD4 present at the indicated time points. The bottom portion is the quantitative analysis of the CD4 bands with a Fujix Bas 2000 Bio-Image Analyzer. The percentage of CD4 recovered relative to the amount present at the end of the pulse (0 min) is plotted as a function of time.

the presence of BFA, CD4 is blocked in its transport out of the ER; CD4 remained endo H sensitive in the presence of BFA, while in its absence, most of the CD4 acquired partial resistance to endo H (data not shown).

Vpu reduces steady-state levels of CD4 when it is retained in the ER. In the previous experiments, the effect of Vpu on CD4 was analyzed under conditions in which CD4 was retained in the ER either through its interaction with gp160 or because of the effect of BFA on intracellular protein trafficking. To further substantiate our conclusions that ER retention is sufficient for Vpu to induce CD4 decay, we studied the steady-state levels of a CD4 mutant, CD4.Q421-stop (27), which lacks 13 C-terminal amino acids of CD4 and contains the C-terminal sequence KKTC, which has been shown to act as an efficient ER retention signal (27). This mutant was introduced into the CD4 expression vector pHIV-CD4 as described in the Materials and Methods section, resulting in plasmid pHIV-CD4/Q421. Plasmid DNA from pHIV-CD4/Q421 was cotransfected into HeLa cells with either pNL-A1 (Vpu$^+$ Env$^+$), pNL-A1/dEnv (Vpu$^+$ Env$^-$), or pNL-A1/U_{35}/dEnv (Vpu$^-$ Env$^-$). Cells were harvested 20 h after transfection, and cell lysates were analyzed by immunoblotting with the CD4-specific polyclonal antiserum (Fig. 6, anti-CD4) or serum from an HIV-positive patient (APS). It is apparent from Fig. 6 that in the presence of Vpu, independent of the presence or absence of

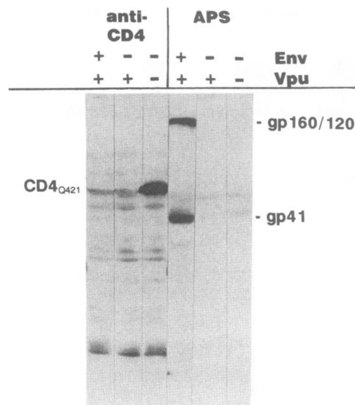

FIG. 6. Vpu reduces steady-state levels of a CD4 ER retention mutant. HeLa cells were cotransfected with 8 μg of pHIV-CD4/Q421 DNA and 24 μg of one of the following plasmid DNAs: pNL-A1 (Vpu^+ Env^+), pNL-A1/dEnv (Vpu^+ Env^-), or pNL-A1/U_{35}/dEnv (Vpu^- Env^-). Cells were lysed 20 h later in 200 μl of lysis buffer. Aliquots (30 μl) of the lysates were run in duplicate on a 12.5% polyacrylamide–SDS gel and electrotransferred to a nitrocellulose membrane. The membrane was incubated with either serum from an AIDS patient (APS) or a polyclonal rabbit antiserum against human CD4 (anti-CD4), and binding of antibodies was identified by using ^{125}I-protein A (0.1 μCi/ml) and autoradiography.

Env, only marginal levels of CD4 were detectable. However, in the absence of Vpu, high levels of CD4 were found. This confirms the results of the BFA pulse-chase experiments (Fig. 5), which indicated that retention of CD4 in the ER in the presence of Vpu is sufficient to cause a major reduction in the CD4 level.

DISCUSSION

It has previously been shown that HIV-1 downregulates its receptor following infection of $CD4^+$ cells. Work by several groups (3, 5, 12) suggests that the reduced cell surface expression of CD4 in productively infected $CD4^+$ T lymphocytes is due to the trapping of CD4 in the ER by gp160. In addition, a more recent study reported that the HIV-1 *nef* gene product can also interfere with the cell surface expression of CD4 (8). We now present evidence that a third HIV-1-encoded protein, Vpu, in conjunction with gp160, targets CD4 and as a result may cause reduced levels of cell surface CD4. While the *env* and *nef* gene products have not been shown to affect the stability of the CD4 protein (3, 5, 8, 12), the results of our study clearly demonstrate that Vpu induces the rapid degradation of newly synthesized CD4 in the ER. The half-life of CD4 in our experiments was calculated to be 12 min. However, the half-life of CD4 in the presence of Vpu may actually be even shorter, since degradation of CD4 has presumably already occurred during the 6 min of the pulse labeling, as indicated by the lower absolute amounts of CD4 detectable at the end of the pulse (compare zero time points of the anti-CD4 panels in Fig. 2A and B). The trapping of CD4 in the ER by gp160 evidently plays a critical role in this process, since it is through gp160-facilitated retention of CD4 in the ER that Vpu directs CD4 into a degradative pathway. The observation that Vpu, even in the absence of gp160, promotes degradation of CD4 when it is sequestered in the ER following exposure to BFA or due to the presence of an ER retention signal indicates that gp160 is not directly involved in the CD4 degradative process but simply serves as a CD4 trap.

The possibility exists that the disappearance of CD4 in our experiments is actually not due to degradation of CD4, especially since we do not see any degradation intermediates, but to a Vpu-induced modification of CD4 which results in the loss of its reactivity to CD4 antiserum. This is, however, extremely unlikely, since we used three different antisera (two independent CD4-specific polyclonal rabbit sera and one monoclonal antiserum, OKT4) for immunoprecipitation of CD4 in replicate degradation experiments with results identical to those presented in this study (33). Furthermore, we have extensively tested the CD4-specific serum used for the experiments shown in this study and found that this serum recognizes native CD4 (Fig. 2, 4, and 5), denatured CD4 (Fig. 6), free (noncomplexed) CD4 (Fig. 2 and 4), CD4 in a complex with gp160 (Fig. 2), glycosylated CD4 (Fig. 2, 4, 5, and 6) and unglycosylated CD4 (33). The absence of any degradation products could either be due to rapid degradation of CD4 or result from random proteolytic degradation, resulting in an invisible "smear" rather than distinct intermediates.

The exact mechanism underlying Vpu-mediated CD4 degradation in the ER is not presently understood. We have reported previously that Vpu is structurally and biochemically similar to the influenza A virus M2 protein (15, 32). This protein has recently been shown to possess an ion channel-like function (23, 34, 35), and it is conceivable that Vpu has a similar type of activity. M2 is thought to function during the entry phase of infection by regulating pH during the release of influenza virus particles from endosomes. In contrast, HIV-1 entry is pH independent (20, 28), and Vpu has not been found to be incorporated into virus particles (31). Vpu is a cell-associated protein which is active at a much later step in the virus life cycle (15, 31, 32, 36). Nonetheless, the association of Vpu with intracellular membranes (31, 33) suggests that it might affect the ionic milieu within the cytosol or the lumen of the ER. In this regard, it has recently been reported that the depletion of intracellular calcium accelerates the degradation of proteins in the ER (37), possibly reflecting increased proteolysis due to misfolding. In view of its biochemical similarity to the influenza virus M2 protein, the observed Vpu-induced degradation of CD4 may also represent an example of a virus-encoded protein which acts indirectly. However, if Vpu functions as an ion channel, it might be expected to induce the degradation of other ER proteins. No experimental evidence currently supports such an activity; at present, Vpu-induced degradation appears to be specific for CD4. Both gp160, as shown in Fig. 2A to C, and the T-cell surface antigen CD8 (33) are stable when coexpressed with Vpu in HeLa cells after treatment of the cells with BFA.

Assuming that Vpu specifically affects CD4, one would predict that the two proteins would interact. We have so far been unable to detect a Vpu-CD4 complex, even after the chemical cross-linking of cell lysates (33), although a transient Vpu-CD4 interaction, resulting in a chemical modification of CD4, cannot be excluded. For example, phosphorylation, which has been shown to regulate protein degradation in other systems, such as 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase) (21), could specifically "target" CD4 as a substrate for proteolytic degradation. Ubiquitination, another type of protein modification, results in the degradation of tagged proteins via the ubiquitin-dependent protease system (for a review, see reference 4). This pathway has recently been suggested to be operative

for the papillomavirus E6 protein-induced degradation of the cellular p53 tumor suppressor factor (25). However, ubiquitination has been described mainly for soluble proteins, and its potential involvement in ER degradation has not yet been demonstrated. Finally, the possibility that Vpu itself might have proteolytic activity with substrate specificity for CD4 has not been ruled out. Elucidation of the biochemical basis of Vpu function may not only help to explain the role of Vpu in HIV-1 replication but also provide insight into the generalized mechanism of ER degradation.

It is tempting to speculate why HIV-1, in contrast to the HIV-2 and simian immunodeficiency virus (SIV) group of primate lentiviruses, encodes a gene product which causes rapid degradation of its own receptor. We have previously noted that the affinities of the HIV-2 and simian immunodeficiency virus Env proteins for CD4 are generally lower than that of HIV-1 Env and suggested that HIV-1 might have acquired Vpu to prevent the formation of stable intracellular gp160-CD4 complexes, thereby relieving the block to gp120 production (39). Interestingly, Env processing in HIV-2-infected cells is extremely slow (24). Whether this also reflects CD4 complex formation or is an inherent property of the precursor envelope glycoprotein itself is unknown. Nonetheless, the capacity to express a functional *vpu* gene is clearly advantageous to the replication of HIV-1 in vitro, not only for its effects on cell surface expression of CD4 and Env proteins, but also because it enhances the release of particles from infected cells, as described in previous work (15, 31, 32, 36). It remains to be investigated whether the Vpu-induced enhancement of virus release and the degradation of CD4 described in this study are mechanistically linked or whether they represent two functionally independent activities of Vpu.

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