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Differential Activities of the Human Immunodeficiency Virus Type 1-Encoded Vpu Protein Are Regulated by Phosphorylation and Occur in Different Cellular Compartments

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The human immunodeficiency virus type 1 (HIV-1)-specific Vpu is an 81-amino-acid amphipathic integral membrane protein with at least two different biological functions: (i) enhancement of virus particle release from the plasma membrane of HIV-1-infected cells and (ii) degradation of the virus receptor CD4 in the endoplasmic reticulum (ER). We have previously found that Vpu is phosphorylated in infected cells at two seryl residues in positions 52 and 56 by the ubiquitous casein kinase 2. To study the role of Vpu phosphorylation on its biological activity, a mutant of the *vpu* gene lacking both phosphoacceptor sites was introduced into the infectious molecular clone of HIV-1, pNL4-3, as well as subgenomic Vpu expression vectors. This mutation did not affect the expression level or the stability of Vpu but had a significant effect on its biological activity in infected T cells as well as transfected HeLa cells. Despite the presence of comparable amounts of wild-type and nonphosphorylated Vpu, decay of CD4 was observed only in the presence of phosphorylated wild-type Vpu. Nonphosphorylated Vpu was unable to induce degradation of CD4 even if the proteins were artificially retained in the ER. In contrast, Vpu-mediated enhancement of virus secretion was only partially dependent on Vpu phosphorylation. Enhancement of particle release by wild-type Vpu was efficiently blocked when Vpu was artificially retained in the ER, suggesting that the two biological functions of Vpu are independent, occur at different sites within a cell, and exhibit different sensitivity to phosphorylation.

In addition to containing the common retroviral genes *gag*, *pol*, and *env*, human immunodeficiency virus type 1 (HIV-1) encodes the proteins Nef, Rev, Tat, Vif, Vpr, and Vpu. The accessory protein Vpu is encoded only by HIV-1 (6, 21, 40, 41). No functional homologs are known in the closely related primate lentiviruses HIV-2 and simian immunodeficiency virus (SIV) with the exception of the SIV_{CPZ} chimpanzee isolate, which has the capacity to encode a Vpu-like protein (17). Analysis of *vpu*-deficient HIV-1 mutants suggested that Vpu promotes virus release (18, 40, 41). Accumulation of intracellular viral proteins and cell-associated HIV-1 particles accompanied by increased cytopathogenicity were observed in the absence of Vpu (18). More recently, it was demonstrated that Vpu causes degradation of CD4 in the endoplasmic reticulum (ER) (45), resulting in enhanced intracellular transport and processing of gp160 (44). Since Vpu-facilitated release of HIV-1 capsid proteins is independent of Env and CD4 (48), Vpu-mediated degradation of CD4 and enhancement of virus particle release appear to be functionally distinct.

Biochemical investigations revealed that the 81-amino-acid Vpu is an amphipathic integral membrane phosphoprotein with an apparent molecular mass of 16 kDa in glycine-containing sodium dodecyl sulfate (SDS)-polyacrylamide gels (6, 39–41) or 9 kDa in Tricine-containing SDS-polyacrylamide gels (35–37). Twenty-seven highly hydrophobic amino acids at the N terminus of the amphipathic protein presumably constitute the membrane anchor domain (18, 20, 39). Vpu has the topology of a type I integral membrane protein and forms homo-oligomeric complexes (20). Oligomerization of Vpu is independent of its phosphorylation status and might be caused by the presence of two amphipathic α helices of opposite net charge which were identified in the hydrophilic C-terminal part of Vpu by ^1H nuclear magnetic resonance (NMR) and circular dichroism (CD) spectroscopy (16, 47).

In this study, we analyzed the biological significance of Vpu phosphorylation in two functional assays currently available: (i) enhancement of virus particle release and (ii) degradation of CD4. The results of our experiments suggest that phosphorylation of Vpu is absolutely required for Vpu-dependent CD4 degradation but has only a partial effect on the Vpu-enhanced release of virions. We also studied the effect of brefeldin A (BFA), a fungal metabolite that blocks protein traffic from the ER to the Golgi system, on Vpu function and found that BFA blocks the Vpu-dependent enhancement of virion release but not the Vpu-induced decay of CD4. This finding suggests that Vpu has two independent functions which show differential sensitivities to Vpu phosphorylation and require the presence of Vpu in different subcellular compartments.

MATERIALS AND METHODS

Site-directed mutagenesis and plasmid constructions. All plasmids containing HIV-1 sequences are derivatives of the infectious molecular clone pNL4-3 (1). A Vpu phosphorylation mutant carrying serine-to-asparagine transitions in positions 52 and 56 was constructed by PCR-based mutagenesis of the pNL4-3 *vpu* gene. Two pairs of oligonucleotide primers were used to amplify two fragments that overlap and share a *Bss*HII

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site at the location of the desired mutations (Fig. 1A). A 476-bp *Eco*RI-*Bss*HII fragment was amplified by using the sense primer VpuA (5′-CGGAATTCTGCAACAACTGCTG-3′) and the antisense primer VpuME (5′-CTGCGCGCTC TATTAGTCTATC-3′). A 135-bp *Bss*HII-*Kpn*I fragment was amplified by using the sense primer VpuMA (5′-CTAATA GAGCGCGCAGAAGACAACGGCAATGAGAACGA AG GAG-3′) and the antisense primer VpuE (5′-CAGGTAC CCCATAATAGACTG-3′). Both PCR products were digested with the indicated restriction enzymes and cloned into the *Eco*RI-*Kpn*I sites of plasmid pSP-6 (40), resulting in plasmid pSP-6/$U_{2/6}$. The mutations were confirmed by double-stranded DNA sequencing using the dideoxy-chain termination method (31). In a second step, a 1,507-bp *Eco*RI-*Nhe*I fragment of pSP-6/$U_{2/6}$ containing the entire mutated *vpu* gene was introduced both into pNL4-3, leading to pNL4-3/$U_{2/6}$, as well as into pNL-A1 (40), resulting in pNL-A1/$U_{2/6}$. A 610-bp *Eco*RI-*Kpn*I-fragment of plasmid pSP-6/$U_{2/6}$ was furthermore introduced into the Vpu expression vector pAR (10), leading to pAR-$U_{2/6}$. Plasmid pNL-A1 is a derivative of pNL4-3 that lacks *gag* and *pol* genes but has the capacity to express all other HIV-1 genes (40). The *vpu*-deficient plasmids pNL-U_{35} and pNL-A1/U_{35}, which were used as negative controls in our experiments, have been described elsewhere (40). Two plasmids, pHIV-CD4 and pHIV-CD4/Q421, which allow the expression of wild-type CD4 and an ER retention mutant, respectively, under the control of the HIV-1 long terminal repeat have been described before (45).

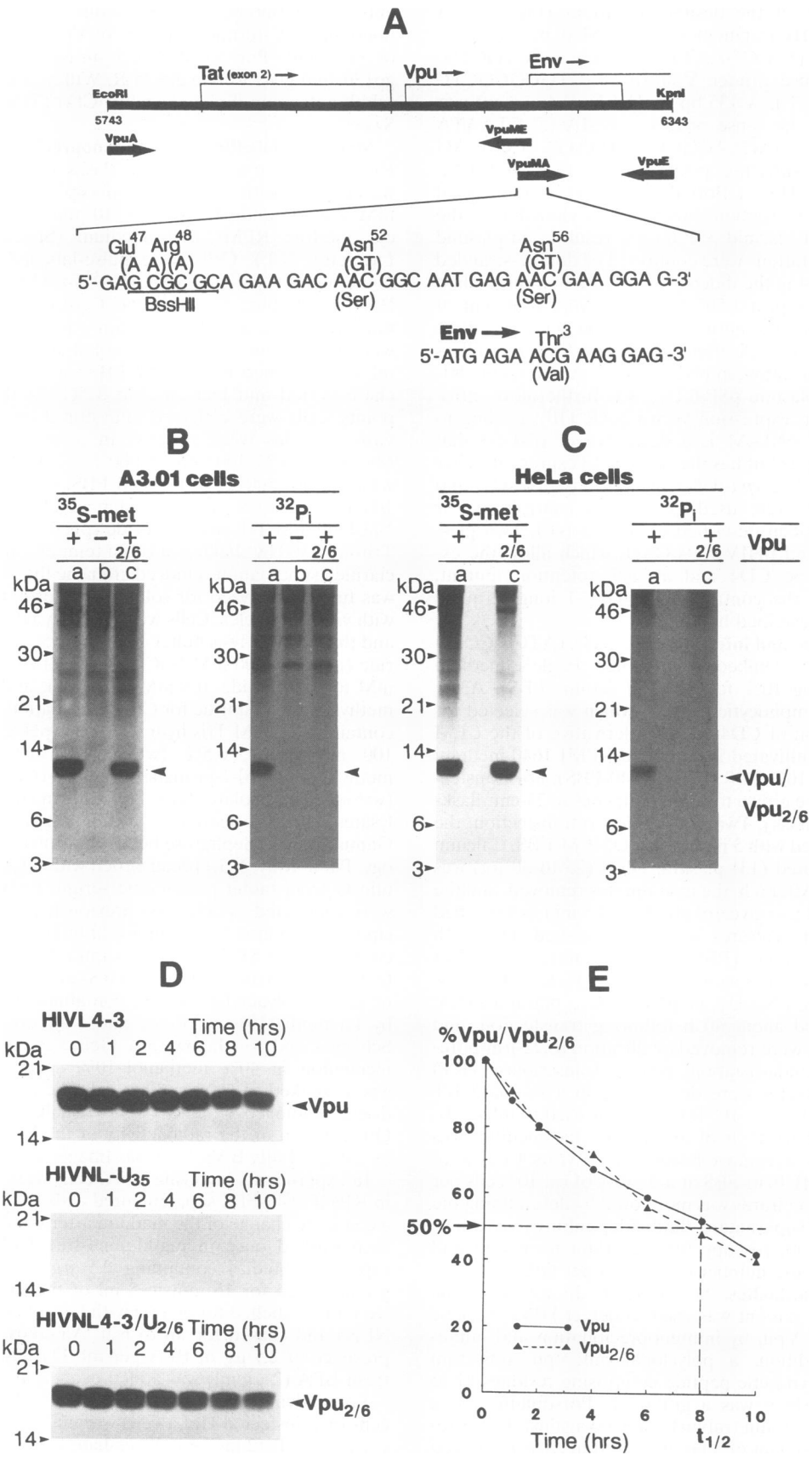
Cells, transfection, and infection. HeLa cells (ATCC CCL2) were propagated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). A3.01 cells, a human T-lymphocytic cell line which was selected for high-level expression of CD4 and is a derivative of the CEM cell line (8), were cultivated in complete RPMI 1640 medium supplemented with 10% FBS (RPMI 1640-FBS). For transfection, HeLa cells were grown to near confluency in 25-cm^2 flasks (5×10^6 cells per flask). Two hours prior to transfection, the medium was replaced with 5 ml of fresh DMEM-FBS. Calcium phosphate-precipitated (13) plasmid DNA (25 to 30 μg) was added to the cells. After 6 h, the medium was removed, and the cells were subjected to a glycerol shock for 2.5 min as described elsewhere (12). The cultures were then washed once with phosphate-buffered saline (PBS) and maintained in 5 ml of DMEM-FBS. Virus stocks were prepared in HeLa cells transfected with pNL4-3, pNL-U_{35} or pNL4-3/$U_{2/6}$ plasmid DNA. Virus was harvested about 40 h following transfection, and contaminating cells were removed by filtration (0.22-μm-pore-size filter). For standardization, reverse transcriptase (RT) activities in the filtrates were determined in a standard RT assay (42). Routinely, 2×10^6 RT units was used to infect 10^7 A3.01 cells. Following 15 h of absorption, the medium was changed completely to remove residual input virus. Cells were maintained in RPMI 1640-FBS at a density of ca. 10^6 cells per ml. Infection of the cultures was monitored by determining the RT activity in the supernatant fluid. The cultures were also examined by light microscopy for syncytium formation and scored by counting the number of syncytia per field.

Antisera and antibodies. Serum from an asymptomatic HIV-1-seropositive patient was used to detect HIV-1-specific proteins, including Vpu, by immunoprecipitation and immunoblotting. In addition, a polyclonal anti-Vpu antiserum (sheep) against a synthetic peptide comprising residues 41 to 58 of Vpu (16), which was a gift of T. Porstmann, and a polyclonal anti-Vpu serum (rabbit), directed against the hydrophilic C-terminal domain of Vpu expressed in *Escherichia coli* (20), were used for detection of Vpu. A monoclonal (mouse) anti-Vpu antibody, AT7C8, is directed against the immunodominant C-terminal epitope of Vpu (34, 35) and was a gift of G. Pauli. Polyclonal rabbit antisera recognizing gp160, gp120, and gp41 were a gift of R. Willey (44, 45). A polyclonal rabbit antiserum directed against CD4 (7) was a gift of R. W. Sweet.

Metabolic labelling and immunoprecipitation. Transfected HeLa cells were scraped off the flasks at 24 h posttransfection, washed once with PBS (10 mM phosphate buffer [pH 7.4], 100 mM NaCl), and starved for 10 min in methionine- and cysteine-free RPMI 1640 medium (Specialty Media, Inc., Lavalette, N.J.). Cells were pulse-labelled with [^{35}S]methionine and [^{35}S]cysteine (TRANS^{35}S-LABEL; 2 mCi/ml; ICN Biomedical, Inc., Costa Mesa, Calif.) for various times as indicated in the text. The medium was then removed, the cells were washed once in PBS, and equal aliquots were added to 1 ml of prewarmed RPMI 1640-FBS for each time point of the chase period and incubated at 37°C. At the indicated time points, cells were collected and stored on dry ice. Cell-free virus particles were pelleted in a refrigerated Eppendorf Microfuge (4°C, 100 min, 16,000 × *g*). The pelleted virus was washed with 500 μl of ice-cold PBS, centrifuged again (4°C, 100 min, 16,000 × *g*), and lysed in a buffer containing 300 mM NaCl, 50 mM Tris hydrochloride (pH 7.4), and 0.1% (vol/vol) Triton X-100 by shaking at room temperature for 20 min. The clarified supernatant removed after the first centrifugation step was further analyzed for soluble viral proteins not associated with virion particles. Cells were lysed by two cycles of freezing and thawing in a lysis buffer containing 50 mM Tris hydrochloride (pH 7.4), 300 mM NaCl, 0.5% (vol/vol) Triton X-100, 10 mM iodoacetamide, 0.5 mM leupeptin, and 0.2 mM phenylmethylsulfonyl fluoride for CD4 degradation or in a lysis buffer containing 50 mM Tris hydrochloride (pH 8.0), 5 mM EDTA, 100 mM NaCl, 0.5% (wt/vol) 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS), and 0.2% (wt/vol) deoxycholate (DOC) for studying particle release. Cell lysates were precleared by incubation at 4°C for 1 h with GammaBind G Sepharose beads (Pharmacia LKB Biotechnology, Piscataway, N.J.) preadsorbed with 20 μg of immunoglobulin G from rabbit preimmune serum. Immunoprecipitations were conducted as described previously (44, 45). Immunoprecipitated proteins were solubilized by boiling in sample buffer containing 2% SDS, 1% β-mercaptoethanol, 1% glycerol, and 65 mM Tris hydrochloride (pH 6.8) and separated on SDS–10 or 12.5% polyacrylamide gels containing glycine as described by Laemmli (19) or Tricine and 6 M urea as described by Schägger and von Jagow (33). Gels were fixed for 30 min by incubation in 40% methanol–10% acetic acid, rinsed with water, soaked in 1 M sodium salicylic acid for 30 min, and dried. Radioactive bands were visualized by fluorography. Quantitation of the radioactivity of the bands was performed by using a Fujix BAS 2000 Bio-Image analyzer.

In experiments involving BFA, cells were incubated for 4 h in RPMI 1640-FBS supplemented with BFA (2.5 μg/ml), with a complete change of the medium after the first 2 h. Cells were then washed once in methionine-free RPMI 1640 medium (Specialty Media) containing 2.5 μg of BFA per ml and preincubated for 10 min in 5 ml of the same medium. Cells were then labelled for 30 min with [^{35}S]methionine (2 mCi/ml; NEN) and chased for up to 6 h. All steps were done in the presence of 2.5 μg of BFA per ml. During the chase period, fresh BFA (2 μg/ml) was added every 2 h.

For studying in vivo phosphorylation of Vpu, infected A3.01 cells or transfected HeLa cells were labelled with ^{32}P-labelled carrier-free P_i (2 mCi/ml; Amersham Corp., Arlington Heights, Ill.) as described previously (37).


A
Tat (exon 2)
Vpu
Env
EcoRI
KpnI
5743
6343
VpuA
VpuME
VpuMA
VpuE
Glu47 Arg48
(A A)(A)
5'-GAG CGC GCA GAA GAC AAC GGC AAT GAG AAC GAA GGA G-3'
BssHII
Asn52
(GT)
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Asn56
(GT)
(Ser)
Env
Thr3
5'-ATG AGA ACG AAG GAG -3'
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Vpu
Vpu2/6

Immunoblotting. Transfected HeLa cells (5×10^6) were lysed 24 h posttransfection in 210 μl of lysis buffer containing CHAPS and DOC, diluted with 200 μl of sample buffer, boiled for 5 min, separated in SDS–10% polyacrylamide gels, and transferred to Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, Mass.). Membranes were incubated with the appropriate combination of antibodies as described previously (44, 45), and binding of the antibodies was identified by using ^{125}I-protein A (0.1 μCi/ml; New England Nuclear, Du Pont, Wilmington, Del.) followed by autoradiography.

RESULTS

Construction and characterization of an HIV-1 provirus encoding a phosphorylation mutant of Vpu. To analyze the role of Vpu phosphorylation in the biological activity of the protein, we used PCR-directed mutagenesis to construct a mutant, $Vpu_{2/6}$, carrying an exchange of two seryl residues by asparagine at amino acid positions 52 and 56, which are the previously identified phosphoacceptor sites within Vpu (16, 36, 37). We decided to introduce these conserved amino acid exchanges in the phosphorylation mutant $Vpu_{2/6}$ since asparagine and serine have similar effects on the structure of a protein backbone (25), which for the amino acid exchanges in $Vpu_{2/6}$ was experimentally confirmed by structural analysis of Vpu peptides by using ^{1}H NMR and CD spectroscopy (16, 46, 47).

The details of the mutagenesis procedure are described in Materials and Methods, and the strategy is schematically outlined in Fig. 1A. The mutant PCR fragment was subcloned into the infectious molecular clone of HIV-1, pNL4-3, resulting in plasmid pNL4-3/$U_{2/6}$, as well as into the subviral expression plasmids pNL-A1 (40) and pAR (10), resulting in plasmids pNL-A1/$U_{2/6}$ and pAR/$U_{2/6}$, respectively. Virus stocks were prepared from HeLa cells transfected with full-length proviral clones as described in Materials and Methods.

In an initial set of experiments, we analyzed the effects of the serine-to-asparagine transitions on the stability and phosphorylation of the mutant $Vpu_{2/6}$. For this purpose, parallel cultures of the human CD4$^+$ T-cell line A3.01 were infected with equivalent RT doses of wild-type HIVNL4-3, HIVNL-U_{35}, a *vpu*-deficient variant of HIVNL4-3 (40), and HIVNL4-3/$U_{2/6}$ virus stocks. Proteins were metabolically labelled at the time of peak virus production with [^{35}S]methionine or $^{32}P_i$. In parallel cultures, HeLa cells were transfected with the Vpu expression plasmid pAR or pAR/$U_{2/6}$. Cultures were metabolically labelled 24 h later with either [^{35}S]methionine or $^{32}P_i$ as described above. Cell lysates of the labelled A3.01 and HeLa cells were prepared, and Vpu was immunoprecipitated with a mixture of polyclonal and monoclonal Vpu-specific antisera and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). Similar amounts of ^{35}S-labelled wild-type Vpu and $Vpu_{2/6}$ proteins were produced in both A3.01 and HeLa cells (compare lanes a and c in the panels labelled [^{35}S]-met in Fig. 1B and C). As expected, no Vpu protein was produced in cells infected with the *vpu*-deficient HIVNL-U_{35} mutant (Fig. 1B, lane b). After labelling with $^{32}P_i$, only wild-type Vpu, not $Vpu_{2/6}$, was detectable (compare lanes a and c in the $^{32}P_i$ panels of Fig. 1B and C), indicating that the Ser-to-Asn exchanges in positions 52 and 56 of $Vpu_{2/6}$ had eliminated all phosphoacceptor sites in Vpu for endogenous protein kinases of A3.01 and HeLa cells.

To further rule out possible effects of the Ser-52 and Ser-56 mutations on Vpu stability, parallel cultures of A3.01 cells were infected with equal RT doses from virus stocks of either wild-type HIVNL4-3, the *vpu*-deficient mutant HIVNL-U_{35}, or the phosphorylation mutant HIVNL4-3/$U_{2/6}$. At the peak of virus production, cells were labelled with [^{35}S]methionine for 30 min and chased in the absence of radiolabelled amino acids for up to 10 h. Vpu was immunoprecipitated with Vpu-specific antisera, separated by SDS-PAGE, and analyzed by fluorography (Fig. 1D). Radioactive bands were quantitated by using an image analyzer (Fig. 1E). This analysis showed that wild-type Vpu and the phosphorylation mutant $Vpu_{2/6}$ are expressed at comparable levels and have a half-life ($t_{1/2}$) of ca. 8 h in A3.01 cells.

The effect of Vpu on particle release: phosphorylation is not essential but improves Vpu function. To evaluate the impact of Vpu phosphorylation on the Vpu-mediated enhancement of virus particle release, we studied the kinetics of processing and release of viral proteins in virus-producing cells. HeLa cells were transfected with pNL4-3, pNL-U_{35}, and pNL4-3/$U_{2/6}$ as described in Materials and Methods. Approximately 24 h later, cells were pulse-labelled with TRANS^{35}S-LABEL for 30 min and chased for up to 8 h. Viral particles released into the culture supernatants were pelleted by centrifugation and lysed in the presence of Triton X-100 as described in Materials and Methods. Viral proteins in the cell lysates, the viral pellet fractions, and the clarified supernatants were immunoprecipitated with an HIV-1-seropositive human serum, separated by SDS-PAGE, and analyzed by fluorography (Fig. 2A to C). Column I represents cell-associated viral proteins, column II depicts pelleted virions, and column III shows soluble viral

FIG. 1. Construction and characterization of the phosphorylation mutant $Vpu_{2/6}$ in infected A3.01 and transfected HeLa cells. (A) For construction of the phosphorylation mutant $Vpu_{2/6}$ with exchange of Ser-52 and Ser-56 for Asn, two overlapping fragments corresponding to the *Eco*RI-*Kpn*I fragment of the HIV-1 molecular clone pNL4-3 were amplified by PCR, using the primers VpuA, VpuE, VpuMA, and VpuME (for details, see Material and Methods). Seven nucleotide changes were introduced into the $vpu_{2/6}$ gene: three silent A→G/C transitions creating a unique *Bss*HII site and two GT→AC transitions resulting in the exchange of Ser-52 and Ser-56 for Asn in $Vpu_{2/6}$ as well as in the exchange of Val-3 to Thr in the signal sequence of gp160. The mutated bases and wild-type amino acids are indicated in parentheses. (B) A3.01 cells (5×10^6) were infected with equal RT doses of HIVNL4-3 (*vpu*$^+$; lanes a), HIVNL-U_{35} (*vpu*$^-$; lanes b), or HIVNL4-3/$U_{2/6}$ (*vpu*$^{+2/6}$; lanes c), harvested on day 12 postinfection, and labelled with either [^{35}S]methionine or $^{32}P_i$, as indicated. Vpu proteins were immunoprecipitated with a 1:1:1 mixture of the Vpu specific polyclonal (sheep, rabbit) and monoclonal (mouse) antibodies, separated in a Tricine-containing SDS–15% polyacrylamide gel in the presence of 6 M urea, and analyzed by fluorography. (C) Subconfluent cultures of HeLa cells (5×10^6) were transfected with 30 μg of Vpu expression vectors pAR (*vpu*$^+$; lanes a) and pAR/$U_{2/6}$ (*vpu*$^{+2/6}$; lanes c). Cells were labelled 24 h after transfection with either [^{35}S]methionine or $^{32}P_i$. Immunoprecipitation and SDS-PAGE analysis were performed as described for panel B. Vpu proteins migrating with a molecular mass of ca. 9 kDa are identified by arrowheads. (D) A3.01 cells were infected with HIVNL4-3 (*vpu*$^+$), HIVNL-U_{35} (*vpu*$^-$), and HIVNL4-3/$U_{2/6}$ (*vpu*$^{+2/6}$). Cells were pulse-labelled for 30 min with [^{35}S]methionine and chased for up to 10 h. Vpu proteins were immunoprecipitated as described for panel B, separated in a 12.5% glycine-containing SDS-polyacrylamide gel, and visualized by fluorography. The amounts of Vpu and $Vpu_{2/6}$ at the indicated time points of the chase are demonstrated. As a control, immunoprecipitation in the absence of Vpu (HIVNL-U_{35}) is illustrated in the middle. (E) Vpu proteins shown in panel D were quantitated with an image analyzer. The percentage of Vpu or $Vpu_{2/6}$ recovered relative to the amount present at the end of the pulse (0 h) was plotted as a function of time.

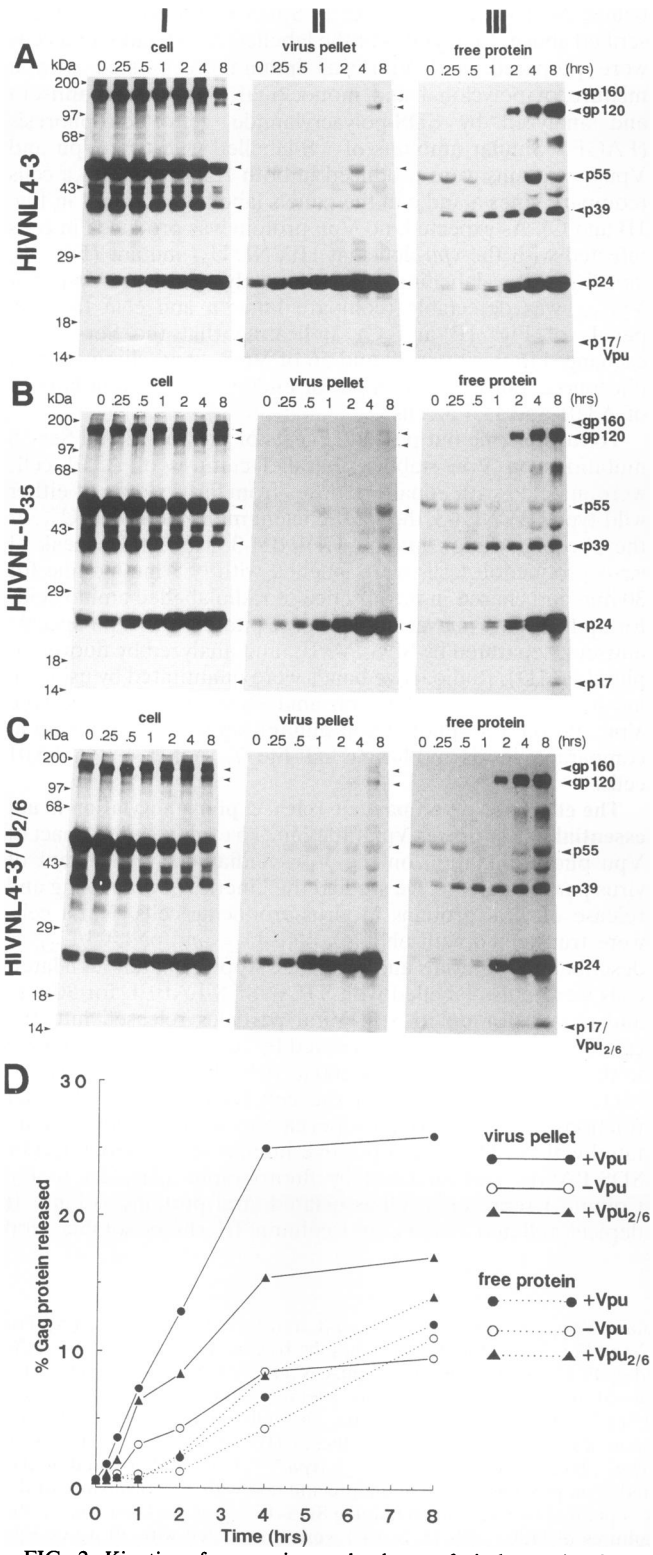

FIG. 2. Kinetics of processing and release of viral proteins from transfected HeLa cells. Parallel cultures of semiconfluent HeLa cells (5×10^6) were transfected with 30 μg of DNAs of the molecular clone pNL4-3 (A), pNL-U_{35} (B), or pNL4-3/$U_{2/6}$ (C). Cells were labelled for 30 min with TRANS^{35}S-LABEL and chased for up to 8 h. Cells were pelleted and lysed in lysis buffer containing CHAPS and DOC. Viral particles were pelleted from the supernatants by centrifugation and lysed in a buffer containing 0.1% Triton X-100 as described in Materials and Methods. For each time point, 50% of the cell lysate (column I), the virus lysate (column II), and the clarified supernatant (column III) was immunoprecipitated with an HIV-1-seropositive human serum, separated in an SDS–10.5% polyacrylamide gel, and analyzed by fluorography. To visualize minor viral components in addition to p24^{gag}, the fluorograms of columns II and III were exposed five times longer than the fluorograms corresponding to column I. (D) The p24^{gag} and p55^{gag} bands in each fluorogram were quantitated with an image analyzer. The percentage of Gag proteins found in the virus fraction (column II, solid lines) or soluble fraction (column III, dotted line) relative to the total amount of Gag proteins (the sum of proteins in columns I, II, and III) is plotted as a function of time.

proteins secreted into the culture supernatants. Radioactive bands corresponding to p24^{gag} and p55^{gag} were quantitated by using an image analyzer (Fig. 2D). Secretion of viral particles was calculated as the percentage of p24^{gag} present in the viral pellet (column II) relative to the sum of p24^{gag} and p55^{gag} detected intra- and extracellularly (columns I to III). We observed an increase of particle release for the first 4 h of the chase period, with a maximum of 26% for wild-type Vpu, 17% for $Vpu_{2/6}$, and 10% in the absence of Vpu after 8 h of chase (Fig. 2D). The two- to threefold increase in particle secretion in cultures expressing wild-type Vpu compared with cultures lacking Vpu is consistent with results from earlier studies in infected A3.01 cells (39). Unphosphorylated Vpu ($Vpu_{2/6}$) showed about half of the activity of wild-type Vpu, suggesting that phosphorylation of Vpu is not absolutely required for its function in virion release. However, phosphorylation clearly enhances the biological activity of Vpu. The release of soluble viral proteins was unaffected by the presence or absence of Vpu or the state of Vpu phosphorylation (represented by the dotted lines in Fig. 2D). This finding indicates that Vpu affects only the release of virions and not the general secretion of Gag proteins from the virus-producing cells.

No differences in the relative protein compositions of HIVNL4-3, HIVNL-U_{35}, and HIVNL4-3/$U_{2/6}$ virions were detected (compare Fig. 2A to C, column II), indicating that the mutations introduced into the *vpu*$_{2/6}$ gene in HIVNL4-3/$U_{2/6}$ had no effect on the expression or processing of viral proteins. In particular, an exchange of Val to Thr at position 3 in the leader peptide of gp160, which was caused by the change of Ser-56 to Asn in the overlapping Vpu sequence (Fig. 1A), did not affect synthesis, maturation, or secretion of Env glycoproteins (Fig. 2A and C, gp160 and gp120). The radioactive band at 17 kDa immunoprecipitated from the cell lysates of HeLa cells producing HIVNL4-3 or HIVNL4-3/$U_{2/6}$ virions (Fig. 2A and C, column I) is a mixture of p17^{gag} and Vpu or $Vpu_{2/6}$, which comigrate in this gel system. Because of the comigration with Vpu proteins, intracellular p17^{gag} can be identified only in the absence of Vpu (Fig. 2B, column I).

In a similar set of experiments, we analyzed the effect of Vpu phosphorylation on virus particle release in A3.01 cells. Parallel cultures of A3.01 cells were infected with equal RT doses of HIVNL4-3, HIVNL-U_{35}, and HIVNL4-3/$U_{2/6}$ virus stocks. Particle release from these cultures was evaluated in a pulse-chase experiment similar to that described for HeLa cells (Fig. 2). Cells were labelled at the time of peak virus production with TRANS^{35}S-LABEL and then chased for up to 4 h. Aliquots of radiolabelled viral proteins from the cell lysates and the cell-free supernatants were immunoprecipitated with an HIV-1-seropositive serum, separated by SDS-PAGE, and analyzed by fluorography (not shown). The amounts of intracellular and extracellular p24^{gag} and p55^{gag} proteins were determined with an image analyzer. The percentage of cell-

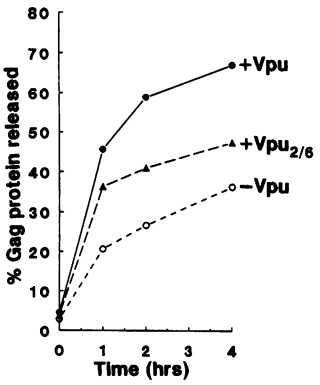

FIG. 3. Kinetics of virus particle release in infected T cells. Parallel cultures of A3.01 cells (5×10^6) were infected with equal RT units of either wild-type virus HIVNL4-3 (*vpu*$^+$) or the mutant viruses HIVNL-U_{35} (*vpu*$^-$) and HIVNL4-3/$U_{2/6}$ (*vpu*$^{+2/6}$). Virus production, measured as RT activity in the culture supernatants, was monitored every other day. Near the peak of virus production (day 12 postinfection), a pulse-chase experiment was conducted as described for Fig. 2. Cell lysates and culture supernatants of each time point were immunoprecipitated with an HIV-1-seropositive human serum, separated in an SDS–10.5% polyacrylamide gel, and analyzed by fluorography (not shown). $p24^{gag}$ and $p55^{gag}$ proteins were quantitated with an image analyzer. The amount of Gag proteins in the supernatants of the cultures was calculated as the percentage of total $p24^{gag}$ and $p55^{gag}$ for any given time point.

free Gag proteins was plotted as a function of time (Fig. 3). Similar to our observation in HeLa cells (Fig. 2), release of virus particles in A3.01 cells in the presence of wild-type Vpu was twice as great as in cultures lacking Vpu. Approximately 67% of the total Gag protein was found in the supernatant 4 h after synthesis in the presence of Vpu, compared with 36% in the absence of Vpu. Again, nonphosphorylated Vpu caused a partial enhancement of virus release, with ca. 47% of the Gag protein secreted into the culture supernatant during the 4 h of the chase period. Thus, in both HeLa and A3.01 cells, nonphosphorylated Vpu showed partial activity when tested for its capacity to enhance virion release. Similar results were obtained when the replication profiles of wild-type virus (HIVNL4-3), the Vpu-deficient mutant (HIVNL-U_{35}), and the phosphorylation mutant (HIVNL4-3/$U_{2/6}$) were analyzed upon infection of A3.01 cells. The RT activities detectable in cultures infected with the phosphorylation mutant were consistently higher than in cultures infected with Vpu-defective virus and reached about 50% of the levels observed for wild-type virus (data not shown). Nevertheless, infection of A3.01 cells with the phosphorylation mutant HIVNL4-3/$U_{2/6}$ resulted in more rapid onset of syncytium formation, usually 2 days earlier than in cultures infected with the wild-type virus, and approximately paralleled the cytopathic changes detected in HIVNL-U_{35}-infected cells. As discussed earlier (18, 39), the greater cytopathic effect in the absence of Vpu is likely the result of increased accumulation of viral proteins intracellularly, which was observed in the absence of Vpu and in the presence of nonphosphorylated Vpu (Fig. 2).

Phosphorylation of Vpu is essential for the Vpu-mediated degradation of CD4 in the ER. We have previously shown that Vpu induces degradation of CD4 in the ER (45). To assess the importance of Vpu phosphorylation for this process, we performed a series of analyses in HeLa cells. In an initial set of experiments, we compared the effects of wild-type Vpu and the Vpu phosphorylation mutant, $Vpu_{2/6}$, on the steady-state levels of CD4. Since we established earlier (45) that CD4 degradation occurs in the ER, we first used an ER retention mutant of CD4, CD4/Q421, as the target protein for Vpu (Fig. 4A, panel I). The expression plasmid pHIV-CD4/Q421 (45) was cotransfected into HeLa cells with pAR (*vpu*$^+$ [10]), pAR-U_{35} (*vpu*$^-$ [44]), or pAR-$U_{2/6}$ (phosphorylation mutant, *vpu*$^{+2/6}$) as described in the legend to Fig. 4. HeLa cells were harvested 24 h after transfection, cell lysates were prepared, and proteins were separated by SDS-PAGE. CD4 and Vpu proteins were identified by immunoblotting with CD4- and Vpu-specific antisera followed by incubation with ^{125}I-protein A. The amounts of CD4 were quantitated with an image analyzer. The percentage of CD4 was determined on the basis of amounts of CD4 detected in the absence of Vpu (lanes b), which was defined as 100%. As can be seen in Fig. 4A, panel I, the steady-state level of CD4 in the presence of wild-type Vpu (lane a) was significantly reduced (36%) relative to the level in Vpu-deficient cultures. In contrast, the presence of nonphosphorylated Vpu (lane c) did not result in any reduction of CD4 levels even though comparable amounts of Vpu protein were made (Fig. 4B).

Similarly, the role of Vpu phosphorylation was analyzed by using wild-type CD4 as a target (Fig. 4, panels II and III). To facilitate ER retention of CD4, the HIV-1 Env protein was coexpressed in those experiments since formation of complexes between gp160 and CD4 results in the retention of CD4 in the ER (45). In panel II, CD4, Vpu, and Env were independently expressed (in *trans*) from three different plasmids by cotransfecting pHIV-CD4 (44), pNL-A1/U_{35} (Env expressor [40]), and pAR (10) or one of its derivatives (Vpu expressors) into HeLa cells. Since it was previously demonstrated that Vpu can function in *cis* as well as in *trans* (44), we analyzed the phosphorylation mutant $Vpu_{2/6}$ in both configurations. In panel III, Vpu and Env were expressed in *cis* from a bicistronic mRNA, using pNL-A1 or one of its derivatives as the Env/Vpu expression plasmid. Details of the transfection protocols are given in the legend to Fig. 4. HeLa cells were transfected and processed for immunoblotting as described for panel I. For the detection of Vpu and Env, an HIV-positive patient serum was used in addition to CD4- and Vpu-specific antisera. As observed in panel I, wild-type Vpu caused a significant reduction in the steady-state level of CD4, while nonphosphorylated Vpu appeared to be completely inactive regarding CD4 degradation. The appearance of mature gp120 envelope glycoprotein in cultures expressing wild-type Vpu (Fig. 4, panel II) is indicative of the destabilization of gp160-CD4 complexes and reflects Vpu-mediated CD4 degradation in these cells (44, 45). The lack of gp160 processing to gp120 in panels II and III of Fig. 4, lanes b and c, suggests the presence of stable intracellular gp160-CD4 complexes and is additional evidence for the lack of Vpu activity in the absence of its phosphorylation.

In a second set of experiments, we directly determined the stability of CD4 in the presence of wild-type Vpu or nonphosphorylated Vpu by pulse-chase analysis. HeLa cells were cotransfected with pHIV-CD4 plasmid DNA and one of the following plasmids: pNL-A1 (*vpu*$^+$ *env*$^+$), pNL-A1/U_{35} (*vpu*$^-$ *env*$^+$), or pNL-A1/$U_{2/6}$ (*vpu*$^{+2/6}$ *env*$^+$). Approximately 24 h after transfection, cells were pulse-labelled with TRANS^{35}S-LABEL for 6 min and chased for up to 1 h in complete medium. Cells were harvested at 10-min intervals and lysed by freezing and thawing in a lysis buffer containing 0.5% Triton X-100 as described in Materials and Methods. Equal aliquots from precleared cell lysates were immunoprecipitated with a rabbit polyclonal serum raised against CD4, separated by SDS-PAGE (10% gel), and analyzed by fluorography (Fig.

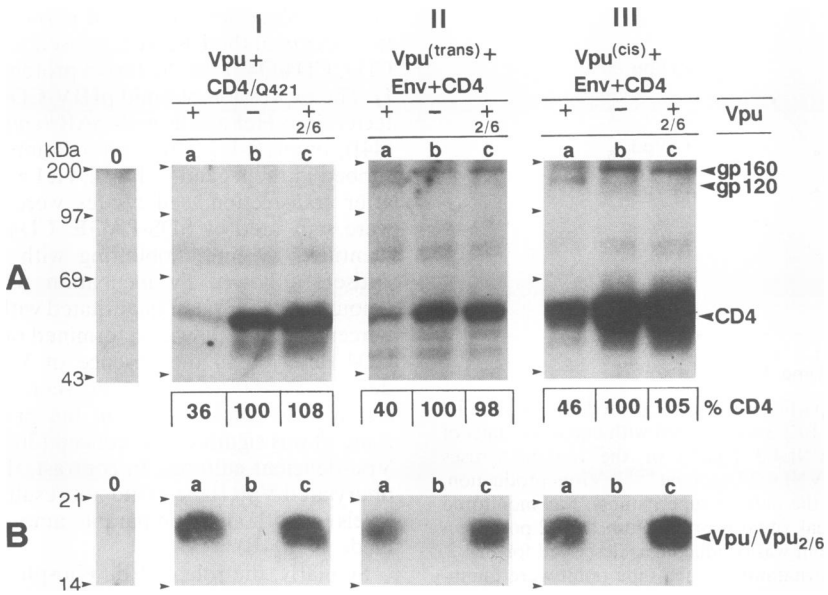

FIG. 4. Reduction of CD4 steady-state levels requires phosphorylation of Vpu. (A) HeLa cells (5×10^6) were cotransfected with 8 μg of either pHIV-CD4/Q421, encoding an ER retention mutant of CD4 (column I), or pHIV-CD4, encoding wild-type CD4 (columns II and III), and 24 μg of one of the following Env and Vpu expression plasmids: column I, pAR (*vpu*$^+$; lane a), pAR-U_{35} (*vpu*$^-$; lane b), and pAR-$U_{2/6}$ (*vpu*$^{+2/6}$; lane c); column II, 12 μg of pNL-A1/U_{35} (*env*$^+$ *vpu*$^-$) plus 12 μg of one of the pAR vectors as in column I; column III, 24 μg of pNL-A1 (*env*$^+$ *vpu*$^+$; lane a), pNL-A1/U_{35} (*env*$^+$ *vpu*$^-$; lane b), and pNL-A1/$U_{2/6}$ (*env*$^+$ *vpu*$^{+2/6}$; lane c). In column II, Vpu and Env were expressed from two separate plasmids (*trans*), while in column III, Vpu and Env were expressed from the authentic bicistronic mRNA (*cis*). Cells were lysed 24 h later in 210 μl of lysis buffer; 5% of the cell lysate were separated in an SDS–12.5% polyacrylamide gel and electrotransferred to PVDF membranes. The membranes were incubated with a 3:1 mixture of a rabbit polyclonal antiserum directed against human CD4 and an AIDS patient serum. Bound antibodies were identified by ^{125}I-protein A followed by autoradiography. The radioactive bands corresponding to CD4 and CD4/Q421 were quantitated with an image analyzer. The relative amount of CD4 protein detected in the absence of Vpu (lanes b) was defined as 100%. The relative amounts of total intracellular CD4 present in the cultures of each cotransfection experiment are indicated at the bottom. (B) In parallel, 20% of each cell lysate was separated in an SDS–12.5% polyacrylamide gel, electrotransferred to PVDF membranes, and incubated with a 1:1:1 ratio of Vpu-specific monoclonal (mouse) and polyclonal (sheep and rabbit) antibodies. Bound antibodies were identified with ^{125}I-protein A as in panel A followed by autoradiography. Reactions of antibodies with lysates from mock-transfected cultures are demonstrated in lane 0. The positions of Env-, CD4-, and Vpu/$Vpu_{2/6}$-specific proteins are indicated on the right.

5A). Gels were quantitated with an image analyzer, and the levels of CD4 present at different time points were calculated relative to the levels of CD4 present at the end of the pulse time point (0 min), which was defined as 100%. As described previously (45), wild-type Vpu reduced the $t_{1/2}$ of CD4 to about 10 min, with only 23% of the initial pulse-labelled 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 or in the presence of nonphosphorylated Vpu. The relative levels of CD4 remained constant throughout the pulse-chase experiment. In an independent experiment, the $t_{1/2}$ of CD4 in the presence of $Vpu_{2/6}$ was estimated to be about 6 h and was therefore comparable with the stability of CD4 in the absence of Vpu (data not shown). Thus, in contrast to Vpu-mediated enhancement of virion release, phosphorylation of Vpu is essential for its ability to direct degradation of CD4 in the ER.

Vpu acts at two separate locations intracellularly. The results from the experiments shown in Fig. 2 to 5 suggest that nonphosphorylated Vpu has differential effects on CD4 stability and particle release: degradation of CD4 is dependent on Vpu phosphorylation, while enhancement of particle release appears to be less phosphorylation sensitive. Since CD4 degradation occurs in the ER and release of HIV virions takes place at the plasma membrane, we investigated the possibility that Vpu is required at two different intracellular sites to perform its tasks.

We had previously demonstrated that treatment of cells with BFA leads to the retention of CD4 in the ER and, in the presence of Vpu, causes increased CD4 degradation (45). Since Vpu, like CD4, is an integral membrane protein (20), BFA treatment of cells should also retain Vpu in the ER. To assess the consequences of ER retention of Vpu, we analyzed the effect of Vpu on HIV-1 particle release in the presence or absence of BFA.

An Env-deficient plasmid, pNLenv1, was constructed by deleting a *Kpn*I-*Bgl*II fragment from the *env* region (positions 6343 to 7611) in the infectious molecular clone pNL4-3 (Fig. 6). Plasmid pNLenv1 therefore lacks the ability to express the Env protein but is able to produce all other viral proteins. A *vpu*-deficient variant of pNLenv1, pNLenv1/U_{35} was constructed by deleting a *Kpn*I-*Bgl*II fragment from pNL-U_{35} (40).

HeLa cells were cotransfected with pHIV-CD4 and pNLenv1 or pNLenv1/U_{35} plasmid DNAs. Approximately 20 h after transfection, cells were harvested, half of the cells were cultivated in RPMI 1640-FBS containing 2.5 μg of BFA per ml, and the remaining half were incubated in RPMI 1640-FBS without BFA for 4 h, with a complete change of medium after 2 h. Cells were then pulse-labelled with [^{35}S]methionine for 30 min and chased for up to 6 h in the presence or absence of BFA. At the indicated time points, cells were harvested and lysed in CHAPS-DOC buffer as described in Materials and Methods. Half of the cell lysates and cell-free supernatants were immunoprecipitated with an HIV-1-seropositive patient serum, and the other half were immunoprecipitated with an

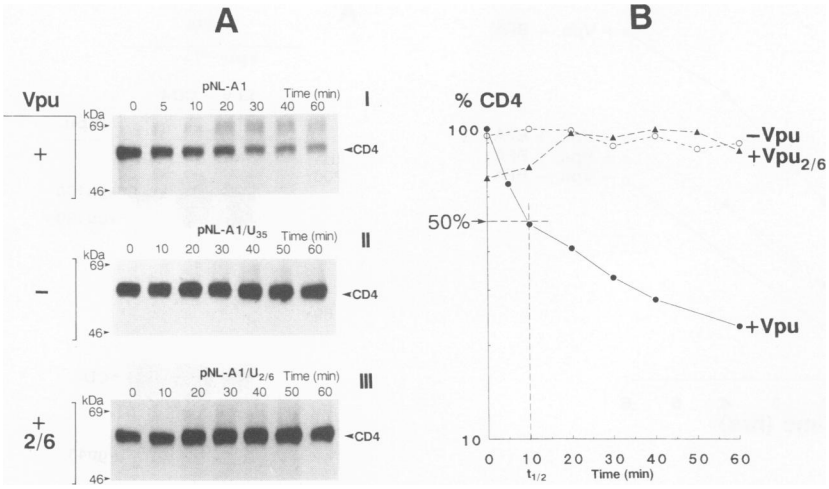

FIG. 5. Nonphosphorylated Vpu is unable to direct degradation of CD4. (A) HeLa cells (5×10^6) were cotransfected with the CD4 expression plasmid pHIV-CD4 (8 μg) in combination with either pNL-A1 (panel I, +), pNL-A1/U_{35} (panel II, −), or phosphorylation mutant pNL-A1/$U_{2/6}$ (panel III, +2/6) plasmid DNA (24 μg) as described previously (44, 45). HeLa cells coexpressing Env, CD4, and Vpu (panel I), Env and CD4 only (panel II), or Env, CD4, and the phosphorylation mutant $Vpu_{2/6}$ (panel III) were pulse-labelled with TRANS^{35}LABEL for 6 min and chased for the indicated times. Cell lysates were immunoprecipitated with a polyclonal rabbit antiserum against CD4, separated in SDS–10% polyacrylamide gels, and visualized by fluorography. The fluorogram of panel I was exposed four times longer than the fluorograms of panels II and III. (B) Quantitative analysis of CD4 degradation. CD4 was quantitated with an image analyzer, and the percentage of CD4 relative to the amount present at the end of the pulse time (0 min) was plotted as a function of time.

anti-CD4 antiserum. Proteins were separated in an SDS–12.5% polyacrylamide gel. Gels were quantitated by using an image analyzer, and the amounts of cell-free Gag proteins were determined as described for Fig. 3. As expected, particle release in the absence of BFA was Vpu dependent, resulting in the release of ca. 39% of cell-free Gag proteins in the presence of Vpu (Fig. 6, + Vpu, − BFA), compared with 20% in Vpu-deficient cultures (Fig. 6, −Vpu, − BFA). In contrast, particle release in BFA-treated cultures was not affected by Vpu (Fig. 6, + or − Vpu, + BFA) and yielded levels of cell-free Gag proteins that were similar to those observed in untreated Vpu-deficient cultures (Fig. 6, − Vpu, − BFA). Thus, BFA did not interfere with the secretion of HIV particles in Vpu-deficient cultures, in accordance with published observations (27). As expected, the rate of CD4 decay in the presence of Vpu was increased in the BFA-treated cultures (data not shown). Thus, Vpu was unable to enhance virion release from virus-producing cells when it was retained in the ER by BFA, suggesting that Vpu enhances virion release from an intracellular compartment that is different from the ER.

Retention of nonphosphorylated Vpu in the ER cannot restore Vpu-mediated degradation of CD4. Our finding that Vpu may act at two different sites within a cell raises the question of whether some or all of the effects observed with our phosphorylation mutant could be due to phosphorylation-dependent intracellular trafficking of Vpu. For example, it is conceivable that the lack of phosphorylation causes enhanced transport of Vpu out of the ER. This could explain the observed lack of CD4 degradation even though it would not explain the partial inhibition of Vpu-mediated enhancement of virus release described in Fig. 2 and 3. To further investigate a potential correlation between intracellular transport and phosphorylation of Vpu, we analyzed the effect of BFA on the steady-state levels of CD4 in the presence of wild-type Vpu and its phosphorylation mutant. Similar to the experiment shown in Fig. 4, HeLa cells were cotransfected with pHIV-CD4 and pNL-A1/$U_{2/6}$ plasmid DNAs. As a control, pNL-A1 (*env*$^+$ *vpu*$^+$) or pNL-A1/U_{35} (*env*$^+$ *vpu*$^-$) plasmid DNA was cotransfected with pHIV-CD4. After 24 h, transfected cells were scraped off the flasks, half of the cells were incubated for 9 h in RPMI 1640-FBS containing 2.5 μg of BFA per ml, and the remaining half were incubated in RPMI 1640-FBS without BFA. Cells were then harvested and lysed in CHAPS-DOC buffer, and proteins were separated in an SDS–10% polyacrylamide gel. CD4 and Env proteins were identified by immunoblotting with a mixture of polyclonal rabbit antisera against CD4 and Env followed by incubation with ^{125}I-protein A. Radiolabelled proteins were visualized by autoradiography (Fig. 7). Quantitation of the blots showed that retention of nonphosphorylated Vpu in the ER by BFA did not have any effect on the stability of CD4, since the steady-state levels of CD4 were indistinguishable in BFA-treated or untreated cultures (Fig. 7A and B, lanes c). The levels of CD4 in those cultures were comparable to the CD4 levels found in Vpu-deficient cultures (Fig. 7A and B, lanes b). In contrast, the presence of wild-type Vpu caused a significant reduction in the steady-state levels of CD4 (Fig. 7A and B, lanes a), which was significantly increased in the presence of BFA, as demonstrated by the virtual absence of CD4 in the BFA-treated culture (Fig. 7B, lane a). As expected, no processing of gp160 was observed in BFA-treated cultures because of the BFA-induced ER retention of the protein. Taken together, these results indicate that retention of nonphosphorylated Vpu in the ER does not restore its ability to direct CD4 into a degradative pathway. This finding suggests that the lack of CD4 degradation by nonphosphorylated Vpu is not the result of a phosphorylation-dependent intracellular distribution of Vpu but is due to the loss of its biological activity.

DISCUSSION

Protein phosphorylation is an important form of protein modification which is used in living systems to regulate biological processes. Depending on the specific protein, phosphory-

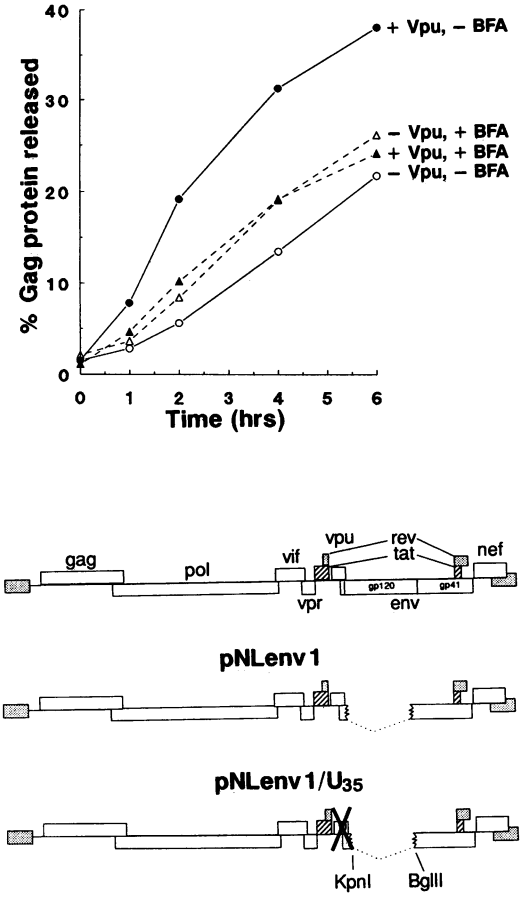

FIG. 6. Retention of Vpu in the ER blocks its ability to enhance the release of virions. HeLa cells (5×10^6) were cotransfected with 8 μg of pHIV-CD4 DNA and 24 μg of pNLenv1 (+ Vpu) or pNLenv1/U_{35} (– Vpu) plasmid DNA. Approximately 20 h after transfection, cells were scraped off the flasks and divided into two equal fractions. One fraction was incubated for 4 h in RPMI 1640-FBS containing BFA (2.5 μg/ml; + BFA), and the other fraction was incubated in RPMI 1640-FBS without BFA (– BFA). Cells were labelled for 30 min with [^{35}S]methionine and chased for up to 6 h in the presence or absence of BFA. Cell lysate and cell-free supernatants were immunoprecipitated with an HIV-1-seropositive patient serum and separated by SDS-PAGE (12.5% gel). Gels were quantitated with an image analyzer, and the percentage of cell-free Gag proteins relative to the total amount of Gag proteins was plotted as a function of time. The structures of pNLenv1 and pNLenv1/U_{35} are schematically shown at the bottom.

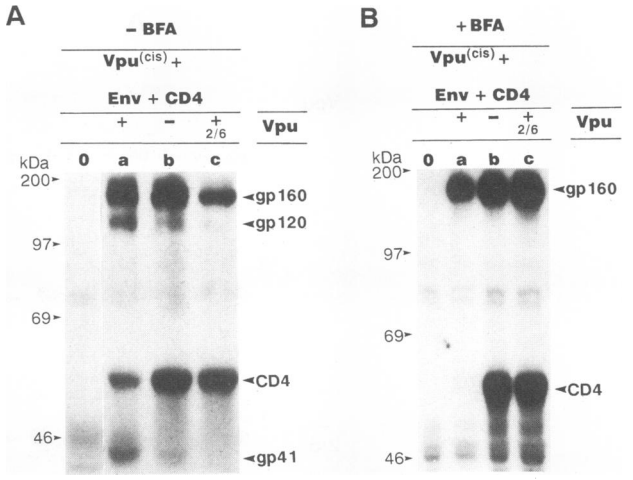

FIG. 7. Retention of nonphosphorylated Vpu in the ER by BFA does not restore CD4 degradation. Parallel cultures of HeLa cells (5×10^6) were cotransfected with 8 μg of pHIV-CD4 and 24 μg of one of the following plasmid DNAs: pNL-A1 (*vpu*$^+$ *env*$^+$; lanes a), pNL-A1/U_{35} (*vpu*$^-$ *env*$^+$; lanes b), and pNL-A1/$U_{2/6}$ (*vpu*$^{+2/6}$ *env*$^+$; lanes c). Lanes 0 represent a mock-transfected control. Cells were harvested 24 h after transfection. Half of each culture was further incubated in RPMI 1640-FBS in the absence (A) or presence (B) of 2.5 μg of BFA per ml. Cells were lysed after 9 h of BFA treatment in 110 μl of lysis buffer containing CHAPS and DOC. Aliquots (10 μl) of the cell lysates were separated by SDS-PAGE (10% gel) and electrotransferred to PVDF membranes. The membranes were incubated with a 1:1 mixture of a polyclonal rabbit anti-CD4 serum and polyclonal rabbit sera against gp160, gp120, and gp41. Binding of antibodies was identified by using ^{125}I-protein A followed by fluorography. The positions of gp160, gp120, gp41 and CD4 are indicated on the right.

lation can either activate or inactivate its biological function, quite often in a reversible way. A well-studied example is the cell cycle kinase cdc2, whose activity is reversibly regulated through phosphorylation and dephosphorylation (9). Other important processes that are regulated through protein phosphorylation include the initiation of protein synthesis (e.g., the regulation of eIF-2α [32]), signal transduction (e.g., activation of NF-κB by phosphorylation of its inhibitor IκB [3]), regulation of neurotransmitter function (23), or the expression of cell surface receptors (e.g., the phosphorylation dependent downmodulation of CD4 [38]). Phosphorylation of proteins is regulated by an array of protein kinases and phosphatases which are either regulated themselves or, like the Vpu-phosphorylating casein kinase 2 (CK-2), are constitutively active in a cell. Because of the importance of protein phosphorylation in the regulation of cellular processes, it is not surprising that virus-encoded proteins have adopted this mode of regulation as well. For example, a poliovirus-associated protein kinase has been implicated in the phosphorylation-dependent destabilization of the viral capsid during the uncoating process (30). Furthermore, phosphorylation of the phosphoprotein P of vesicular stomatitis virus by CK-2 is essential for its transcriptional function (2). For several HIV-1 proteins, including Gag (22), Rev (5, 15), Nef (14), and Vpu (39), phosphorylation has been described but no functional correlations have been made.

We have previously demonstrated that Vpu is phosphorylated by endogenous CK-2 at two seryl residues in positions 52 and 56 (16, 37), which are the only phosphoacceptor sites in Vpu. Phosphorylation of Ser-56 is favored over phosphorylation of Ser-52 by a fivefold-lower K_m value for CK-2, suggesting that both closely neighboring phosphoacceptor sites are phosphorylated through a positive cooperative mechanism (36). Both CK-2 recognition sites are located within a conserved dodecapeptide of Vpu which is likely to represent an active site of the protein, since a deletion in that region completely abolishes Vpu activity (4).

We previously showed that phosphorylation of Vpu in HIV-1-infected cells can be inhibited by treating cells with the ATP analog DRB [5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole] (37), which was shown to inhibit CK-1 and CK-2 but not other cellular kinases (28). While in theory, the importance of Vpu phosphorylation could be tested by treating cells expressing wild-type Vpu with DRB, such an approach is not feasible, since the ubiquitous protein kinase CK-2 plays a vital role during cell proliferation, cell differentiation, and cell

activation (24, 26, 28, 49), and inhibition of these processes would be cytotoxic. As a consequence, long-term studies involving CK-2-specific inhibitors which would be required for functional studies on Vpu phosphorylation are not possible.

To investigate the significance of Vpu phosphorylation, we therefore decided to eliminate the two phosphoacceptor sites in Vpu. One of our main concerns was to minimize any potential structural changes to Vpu which could result from the mutation of two amino acids in the 81-amino-acid sequence of Vpu. We decided to exchange Ser-52 and Ser-56 in Vpu with Asn, since serine and asparagine have similar effects on the secondary structure of a protein according to the structure-derived correlation matrix described by Niefind and Schomburg (25). In addition to these theoretical predictions, molecular structural analyses including ^{1}H NMR and CD spectroscopy of synthetic peptides comprising the hydrophilic C-terminal part of Vpu provide experimental evidence that the change of Ser-52 and Ser-56 to Asn does not affect the structure of the protein backbone in this region (46, 47). More recent studies involving in vitro-translated Vpu as well as synthetic peptides indicated that the exchange of Ser-52 and Ser-56 with Asn does not affect the folding, membrane integration, or formation of homo-oligomeric complexes of Vpu (47). Taken together, these data provide strong evidence that the biological effects observed with our Vpu phosphorylation mutant are not due to a change in the primary amino acid sequence of Vpu but are caused by the lack of a phosphorylation-dependent biological activity of Vpu.

Even though the exact intracellular localization of Vpu is still obscure, we now have good evidence that Vpu is required at two different sites within a cell. We have demonstrated in the past that degradation of CD4 occurs from the ER (45) and that treatment of cells with BFA, which blocks transport of proteins from the ER, therefore does not inhibit but actually enhances Vpu-mediated CD4 degradation. On the other hand, we now show that retention of Vpu in the ER by BFA treatment leads to a complete loss of the Vpu-mediated enhancement of particle release, which implies that this function of Vpu requires its presence in some other cellular compartments. The identity of the compartment from which Vpu controls particle release is still not clear and will require further investigation. However, the fact that regulation of particle release by Vpu did not require Env or CD4 suggests that the effects of Vpu on particle release or CD4 stability are functionally independent, in agreement with previous observations (48).

The molecular mechanisms of Vpu function are still not resolved. From our observation that retention of Vpu in the ER completely abolishes the ability of Vpu to enhance virion release, we could speculate that the partial loss of this Vpu function in the absence of phosphorylation is caused by a partial inhibition of its intracellular trafficking. This would imply that, in contrast to CD4 degradation, enhancement of virion release is actually completely independent of Vpu phosphorylation and suggest that the two biological activities of Vpu are regulated by two independent molecular mechanisms. Alternatively, it is possible that phosphorylation of Vpu modulates both of its biological activities, albeit to different degrees. This scenario would not rule out the possible existence of two independent molecular mechanisms, but it would also be consistent with the possibility that differences in the intracellular milieu require phosphorylation of Vpu in the ER but not in other subcellular compartments for proper function of the protein.

The existence of two distinct catalytic activities of Vpu could provide an explanation for an apparent paradox that comes from the investigation of the two biological functions of Vpu. We have reported in the past that Vpu-induced degradation of CD4 is highly specific (4, 45), since there are no other known proteins that are targeted by Vpu. Furthermore, we recently identified a short sequence in the cytoplasmic domain of CD4 which is targeted by Vpu (43). The specific recognition of a short protein signal by Vpu is in stark contrast to the proposed general mechanism through which Vpu enhances virion release (11): recent work by Goettlinger et al. (11) suggests that Vpu is capable of enhancing the release not only of HIV-1 virions but of HIV-2, SIV, and murine leukemia virus virions as well. It is therefore unlikely that this function of Vpu involves the recognition of a specific signal within Gag but would be compatible with a more generalized activity of Vpu such as an ion channel. Such a possible mechanism was discussed earlier and is based on the similarity of Vpu with small membrane-associated, amphipathic, and oligomeric proteins of other viruses (18, 20, 37). One of these proteins, the influenza virus M_2 protein, which has recently been identified as an ion channel (29), is a phosphoprotein, like Vpu, and was predicted to be phosphorylated by CK-2 because of the presence of CK-2 consensus sequences in its cytoplasmic domain (36, 37). However, the importance of M_2 phosphorylation for ion channel function has not been determined. In addition, there is no experimental evidence to date suggesting an ion channel-like function for Vpu. Additional experiments will be needed to elucidate the precise mechanism for the different functions of Vpu.

The contributions of the two phospho groups in Vpu to its biological mechanism(s) remain unresolved. Nevertheless, some insight into the molecular structure of Vpu has been provided recently with the identification of an α helix-flexible-α helix-turn secondary structural motif covering the hydrophilic part of Vpu (16, 36, 47). Using a combination of ^{1}H NMR and CD spectroscopy, the solution structures of synthetic Vpu peptides were analyzed in the presence of trifluorethanol, which provides a membrane-like situation as a prerequisite for stabilization of the secondary structure in this part of Vpu. The results of those studies suggest that the cytoplasmic domain of Vpu consists of two amphipathic α helices, with helix 1 comprising amino acids 30 to 50 and helix 2 extending from positions 57 to 69 (16, 46, 47). The two α helices are separated by an unstructured, highly acidic region containing both phosphoacceptor sites of Vpu. Phosphorylation of Vpu within an unstructured, flexible part of Vpu supports our earlier argument that the amino acid exchanges introduced into our phosphorylation mutant do not affect the structure of the protein. The function of Vpu phosphorylation could be to add acidic charges near the membrane where Vpu is inserted. Consequently, binding of bivalent cations to Vpu could be important for its function. More detailed structural analyses of Vpu will be required to determine the precise structure of oligomeric Vpu in a membrane and to study the consequences of Vpu phosphorylation.

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