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The Two Biological Activities of Human Immunodeficiency Virus Type 1 Vpu Protein Involve Two Separable Structural Domains

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The human immunodeficiency virus type 1 (HIV-1) Vpu protein is an integral membrane phosphoprotein that induces CD4 degradation in the endoplasmic reticulum and enhances virus release from the cell surface. CD4 degradation is specific, requires phosphorylation of Vpu, and involves the interaction between Vpu and the CD4 cytoplasmic domain. In contrast, regulation of virus release is less specific and not restricted to HIV-1 and may be mechanistically distinct from CD4 degradation. We show here that a mutant of Vpu, Vpu_{35}, lacking most of its cytoplasmic domain has residual biological activity for virus release but is unable to induce CD4 degradation. This finding suggests that the N terminus of Vpu encoding the transmembrane (TM) anchor represents an active domain important for the regulation of virus release but not CD4 degradation. To better define the functions of Vpu's TM anchor and cytoplasmic domain, we designed a mutant, Vpu_{RD}, containing a scrambled TM sequence with a conserved amino acid composition and α-helical structure. The resulting protein was integrated normally into membranes, was able to form homo-oligomers, and exhibited expression levels, protein stability, and subcellular localization similar to those of wild-type Vpu. Moreover, Vpu_{RD} was capable of binding to CD4 and to induce CD4 degradation with wild-type efficiency, confirming proper membrane topology and indicating that the alteration of the Vpu TM domain did not interfere with this function of Vpu. However, Vpu_{RD} was unable to enhance the release of virus particles from infected or transfected cells, and virus encoding Vpu_{RD} had replication characteristics in T cells indistinguishable from those of a Vpu-deficient HIV-1 isolate. Mutation of the phosphorylation sites in Vpu_{RD} resulted in a protein which was unable to perform either function of Vpu. The results of our experiments suggest that the two biological activities of Vpu operate via two distinct molecular mechanisms and involve two different structural domains of the Vpu protein.

Vpu is an 81-amino-acid oligomeric integral membrane phosphoprotein (8, 32, 33). The protein is found exclusively in human immunodeficiency virus type 1 (HIV-1), and no homologs of Vpu have so far been described for any other primate lentiviruses such as HIV-2 or simian immunodeficiency virus (SIV) except for the HIV-1-related chimpanzee isolate SIV_{CPZ} (17). Two distinct biological activities have been attributed to Vpu: enhancement of virus particle release from the plasma membrane (32–34) and induction of CD4 degradation in the endoplasmic reticulum (ER) (37, 38).

Like other so-called accessory genes of HIV-1, *vpu* can be deleted or mutated without completely abrogating virus replication in vitro (8, 19, 32, 33). However, Vpu consistently increases the efficiency of virus secretion both in T-cell lines and in primary lymphocyte and macrophage cultures (26). The importance of Vpu for HIV-1 pathogenicity and disease progression is difficult to assess in the absence of a tractable animal model. Nevertheless, the conservation of the *vpu* gene in most of the known HIV-1 isolates suggests an important role in the virus life cycle in vivo. One direct consequence of Vpu-mediated CD4 degradation in the ER is the facilitated transport and processing of the envelope glycoprotein gp160 (37), which would otherwise be trapped in the ER because of the formation of stable complexes with CD4 (4, 6, 9, 16, 18). The Vpu-mediated release of gp160 from the CD4 trap in the ER for cell surface expression is accompanied by an increased rate of particle secretion, which itself is independent of the presence of CD4 or Env (44). Thus, in HIV-1-infected cells, the dual function of Vpu could ensure that sufficient quantities of Env proteins reach the cell surface for incorporation into an increased number of secreted virions.

The Vpu-stimulated degradation in the ER is highly specific for CD4, requires the presence of the CD4 cytoplasmic tail (7, 21, 35, 36–38), and involves a physical interaction between Vpu and CD4 (5). In contrast, Vpu-mediated enhancement of virus release appears to be a more general phenomenon, since Vpu enhances the secretion of HIV-1 virions as well as HIV-1 chimeric viruses containing *gag-pol* sequences of HIV-2, visna virus, or murine leukemia virus (14). Furthermore, the two biological functions of Vpu occur within different cell compartments and appear to be mechanistically distinct. For example, the Vpu-mediated degradation of CD4 but not the Vpu-stimulated augmentation of virus particle release requires phosphorylation of Vpu (30). The molecular mechanism(s) underlying the multiple functions of Vpu is unclear. It is not known whether Vpu interacts with viral or cellular proteins in order to regulate virion release or whether it exerts its effect indirectly by altering the intracellular milieu near the membranes of the ER or the exocytic pathway.

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Vpu has two main structural domains, the membrane-spanning hydrophobic N-terminal transmembrane (TM) domain and the C-terminal polar cytoplasmic domain. While the TM domain has an important function in anchoring the protein to the membrane, catalytic activity has so far been attributed only to the cytoplasmic domain. This domain contains functional elements crucial for the induction of CD4 degradation (5, 7, 12, 27, 30, 36) but also for the regulation of virus secretion, as evidenced by the fact that truncation of the cytoplasmic domain severely impairs the ability of Vpu to enhance virus release (32).

In this study, we investigated the role of the Vpu TM domain for the two functions of Vpu. We found that the membrane anchor retained partial activity in enhancing virus particle secretion but was inactive with respect to CD4 degradation. Furthermore, a mutant of Vpu containing a randomized TM sequence was incapable of supporting virion release but retained wild-type activity for CD4 degradation. Together, these data suggest that Vpu has two distinct functional modules that correspond to the two structural domains of Vpu. The TM domain, which has no catalytic activity for CD4 degradation but merely serves as a membrane anchor, has a critical role in regulating virus secretion presumably through the formation of an ion channel (28). The cytoplasmic domain of Vpu, on the other hand, contains sequences that are critical for the induction of CD4 degradation in the ER but may also contain elements necessary to efficiently regulate the activity of the TM domain for its function in enhancing virus secretion.

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). The mutant vpu_{RD}, carrying the randomized TM sequence MIPIVIAIILAV AVQAIVIVIVSWIIE, is based on the sequence of a synthetic peptide, Vpu^{rd}, which scored negative for ion channel formation (28), and was constructed by PCR-based mutagenesis of the pNL4-3 *vpu* gene (28). For construction of the mutant vpu_{R26}, a 190-bp *Ssp*I-*Kpn*I fragment of plasmid pAR-$U_{2/6}$ (30) was introduced into the in vitro expression vector pSP8-U_{RD} (28), leading to pSP8-U_{R26}. Furthermore, a 610-bp *Eco*RI-*Kpn*I fragment of pSP8-U_{R26} was introduced into subgenomic expression vectors pAR (33) and pNL-A1 (33), leading to pAR-U_{R26} and pNL-A1/U_{R26}, respectively, as well as into the molecular HIV-1 clone pNL4-3 (1), leading to pNL-U_{R26}. A 610-bp *Eco*RI-*Kpn*I fragment of pSP8-U_{RD} (28) was introduced into subgenomic expression vectors pNL-A1 (33), leading to pNL-A1/U_{RD}, and into the molecular HIV-1 clone pNL4-3 (1), leading to pNL-U_{RD}. The in vitro transcription plasmids pSP-9/U_{35}, pSP-9/U_{RD}, pSP-9/U_{R26}, and pSP-9/U_{DEL-1} were constructed by introducing the *Hin*dIII-*Kpn*I fragments containing the *vpu* mutants from pNL-U_{35} (33), pSP8-U_{RD} (28), pAR-U_{R26}, and vpuDEL-1 (19), respectively, into the in vitro transcription plasmid pSP9 (32). Plasmid pHIV-CD4 (37), which allows the expression of wild-type CD4 under the control of the HIV-1 long terminal repeat, and plasmid pSP9-$U_{2/6}$ (27), which directs the expression of nonphosphorylated Vpu, have been described before.

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 derivative of the CEM human T-lymphocytic cell line which was selected for high-level expression of CD4 (11), were cultivated in complete RPMI 1640 medium supplemented with 10% FBS (RPMI 1640-FBS). Stocks of peripheral blood mononuclear cells (PBMCs) were prepared from gradient-isolated lymphocytes of a healthy HIV-seronegative individual, using Lymphocyte Separation Medium (Organon Teknika-Cappel, Durham, N.C.), and stored in liquid nitrogen. For each infection, 5×10^6 cells were stimulated for 2 days with phytohemagglutinin (1 μg/ml) in the presence of recombinant interleukin-2 (20 U/ml; Boehringer, Mannheim, Germany). PBMC cultures were maintained in RPMI 1640-FBS supplemented with recombinant interleukin-2. 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 (15) 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 (13). 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 vpuDEL-1 plasmid DNA. Virus-containing supernatants were centrifuged ($1,000 \times g$, 5 min) and filtered through a 0.45-μm-pore-size filter to remove residual cells and debris. Virions were pelleted by ultracentrifugation in a Beckman SW55 rotor (1 h, 35,000 rpm), resuspended in 1 ml of RPMI 1640-FBS, and sterilized by filtration. The virus stocks were assayed for reverse transcriptase (RT) activity by an assay using [^{32}P]TTP incorporation with an oligo(dT)-poly(A) template as described previously (39). Routinely, 2×10^6 RT units were 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 approximately 10^6 cells per ml and were fed by replacing 80% of the medium at 2-day intervals. Infection of the cultures was monitored by determining the RT activity in the supernatant fluid as described above. 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 Western blot (immunoblot) analysis. In addition, a polyclonal anti-Vpu antiserum (sheep) against a synthetic peptide comprising residues 41 to 58 of Vpu (27), which was a gift of T. Porstmann, as well as a polyclonal anti-Vpu serum (rabbit), directed against the hydrophilic C-terminal domain of Vpu expressed in *Escherichia coli* (23), were used for detection of Vpu. Polyclonal rabbit antisera recognizing gp160, gp120, and gp41 were a gift of R. L. Willey (37, 38). A polyclonal rabbit antiserum directed against CD4 (10) was a gift of R. W. Sweet.

Metabolic labeling, pulse-chase, and immunoprecipitation. Transfected HeLa cells or infected A3.01 cells were washed once with PBS (10 mM phosphate buffer [pH 7.4], 100 mM NaCl) and starved for 10 min in methionine- and/or cysteine-free RPMI 1640 medium (Specialty Media, Inc., Lavalette, N.J.). Cells were pulse-labeled with [^{35}S]methionine and [^{35}S]cysteine (Trans^{35}S-Label; 2 mCi/ml; ICN Biomedical, Inc., Costa Mesa, Calif.) or with [^{35}S]methionine (2 mCi/ml; Du Pont Inc., Boston, Mass.) for various times as indicated. The medium was then removed, the cells were washed once in PBS, and equal aliquots were added to 500 μl of prewarmed RPMI 1640-FBS for each time point of the chase period and incubated at 37°C while gently shaking. At the indicated time points, cells were collected and stored on dry ice. For studying virus particle release, the supernatants were centrifuged (2 min, $16,000 \times g$) and filtered through a 0.45-μm-pore-size Spin-X filter tube (Costar, Cambridge, Mass.). Cell-free virus particles were pelleted in a refrigerated Eppendorf Microfuge (4°C, 100 min, $16,000 \times g$) as described previously (30). The pelleted virions were 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. Cells were lysed in a buffer containing 50 mM Tris-hydrochloride (pH 8.0), 5 mM EDTA, 100 mM NaCl, 0.5% (wt/vol) CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, and 0.2% (wt/vol) deoxycholate. 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 (30, 36–38), using antibodies preadsorbed to GammaBind-G–Sepharose beads (Pharmacia LKB Biotechnology). Immunoprecipitated proteins were solubilized by boiling in sample buffer containing 2% sodium dodecyl sulfate (SDS), 1% β-mercaptoethanol, 1% glycerol, and 65 mM Tris-hydrochloride (pH 6.8) and separated on SDS–10.5 or 12.5% polyacrylamide gels as described by Laemmli (20). 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 was performed with a Fuji BAS 2000 Bio-Image Analyzer.

Immunoblotting. Virus particles were pelleted from 1 ml of culture supernatants by centrifugation (100 min, $16,000 \times g$, 4°C), lysed in 25 μl of sample buffer, boiled for 5 min, separated on an SDS–10.5% polyacrylamide gel, and transferred to Immobilon polyvinylidene difluoride membranes (Millipore Corp., Bedford, Mass.). Membranes were incubated with a 1:1 mixture of an HIV-1-reactive human serum and a polyclonal antiserum specific for HIV-1 Env proteins as described previously (30). 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.

Immunostaining. HeLa cells (5×10^6) were transfected with 20 μg of plasmid DNA, using the calcium phosphate procedure as described previously (13, 15) except that the cells did not undergo glycerol shock but were washed and refed with DMEM-FBS 3 h after DNA addition. Transfected cells were then scraped and replated onto glass coverslips; 24 to 48 h after transfection, cells were washed three times in Ca^{2+}-Mg-free PBS, fixed for 10 min at room temperature in 4% paraformaldehyde in PBS buffered to pH 7.4, and then washed three times in PBS. Cells were blocked in 3% bovine serum albumin (BSA) in PBS for 5 min and then incubated with a rabbit anti-Vpu serum (23) diluted 1:300 in PBS containing 3% BSA at 37°C for 30 to 45 min in a humidified chamber as described previously (42). Cells were washed three to four times in PBS and stained with a Texas red-conjugated donkey anti-rabbit serum (Jackson Laboratory, Bar Harbor, Maine) diluted 1:300 in 3% BSA–PBS for 30 to 45 min at 37°C. Stained cells were washed three to four times in PBS and mounted in Fluoromount (Virotech, Rockville, Md.) and examined on a Zeiss LSM-410 laser scanning confocal microscope, using an excitation wavelength of 568 nm and monitoring Texas red fluorescent emission with a 590-nm long-pass filter.

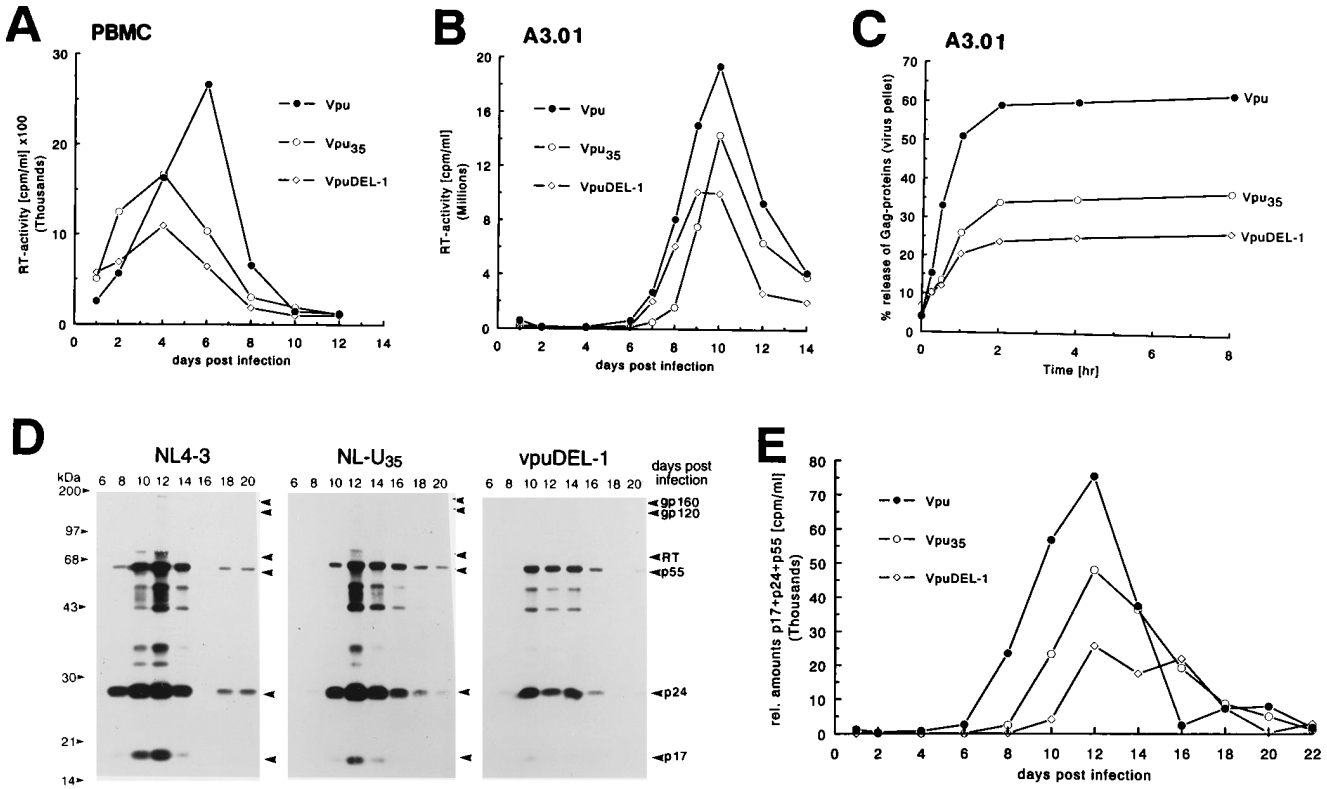

FIG. 1. The Vpu TM domain has partial activity for augmentation of virus particle release. Parallel cultures of either phytohemagglutinin–interleukin-2-stimulated PBMCs (A) or a $CD4^+$ T-cell line (A3.01) (B) were infected with equal RT doses of virus stocks encoding either wild-type Vpu, Vpu_{35}, or vpuDEL-1. Culture supernatants were collected at 2-day intervals and assayed for RT activity. (C) The kinetics of virus particle release were determined by pulse-chase analysis of infected A3.01 cultures conducted on day 10 postinfection. Secretion of viral particles was calculated as the percentage of Gag proteins (p24 and p55) present in the virus pellet relative to the total Gag proteins detected intra- and extracellularly. (D) In a parallel infection of A3.01 cells, virions from 1 ml of supernatants collected every other day were pelleted, separated on an SDS–10.5% polyacrylamide gel, and analyzed by immunoblotting with an HIV-1-reactive human serum and an HIV-1 Env-specific antiserum (rabbit). Bound antibodies were detected with ^{125}I-protein A. (E) Relative amounts of Gag proteins p17, p24, and p55 demonstrated in panel D were quantitated with an image analyzer and plotted in arbitrary units against the time of infection.

In vitro transcription, translation, and chemical cross-linking. Runoff transcripts were synthesized as described previously (32). In vitro translation was performed in a standard reaction mixture containing RNA derived from transcription of 5 μg of linearized template DNA, 40 μl of nuclease-treated rabbit reticulocyte lysate (Promega Biotec, Madison, Wis.), 100 μCi of [^{35}S]methionine (Du Pont Inc., Boston, Mass.), and 5 μl of canine microsomal membranes (1.2 eq/μl; Promega). As an internal control for translation efficiency and membrane integration, *Saccharomyces cerevisiae* α-factor mRNA (1.2 μg; Promega) was added in some reactions. Translation reactions were stopped by addition of RNase A (1 μg/ml, final concentration). The reaction mixtures were diluted in 1 ml of PBS, and membrane-associated proteins were pelleted by centrifugation onto a cushion of 55% (wt/vol) sucrose (100 μl) in an Eppendorf Microfuge (30 min, 14,000 rpm, 4°C). The sucrose phase containing the membrane fraction was resuspended in 1 ml of PBS, and membranes were repelleted (30 min, 14,000 rpm, 4°C). Pelleted material was solubilized in sample buffer and analyzed directly by SDS-polyacrylamide gel electrophoresis (PAGE) followed by fluorography. For immunoprecipitation of membrane-integrated Vpu, membrane pellets were lysed in 200 μl of CHAPS-deoxycholate buffer. Supernatants and wash and membrane fractions were subjected to immunoprecipitation with a mixture of polyclonal (rabbit and sheep) Vpu-specific antibodies. For in vitro binding studies, plasmid pSP-CD4 (7) was linearized with *Bam*HI and used as a template to synthesize CD4-specific runoff transcripts. Reaction mixes containing 40 μl of lysate, 5 μl of CD4 mRNA, and 5 μl of canine pancreatic membranes were translated for 15 min at 30°C, 5 μl of Vpu-specific RNAs was added, and the reaction was allowed to proceed for an additional 45 min. Samples were lysed in 350 μl of digitionin lysis buffer and subjected to immunoprecipitation with CD4-specific antisera as described recently (5). Fifty-microliter aliquots of lysates were loaded onto the gel for direct analysis of proteins present after translation.

For chemical cross-linking, Vpu or its mutants were translated in reticulocyte lysate in the presence of [^{35}S]methionine for 1 h at 30°C. The lysates were split into two equal fractions, RNase A (1 μg/ml) was added to stop the reaction, and one fraction was treated with DTSSP [3,3′-dithiobis(sulfo-succinimidylpropionate) 8 mg/ml; (Pierce Biochemicals, Rockford, Ill.] for 30 min at room temperature. The other fraction was left untreated. The reaction was stopped by addition of 100 μl of 1 M glycine. Vpu proteins were immunoprecipitated with a 1:1 mixture of polyclonal (sheep and rabbit) Vpu-specific antibodies and were separated on an SDS–12.5% polyacrylamide gel under nonreducing conditions.

RESULTS

The TM domain of Vpu exhibits partial activity in supporting virus secretion. To improve our understanding of the importance of structural domains in Vpu for its biological activities, we performed a side-by-side analysis of HIV-1 encoding wild-type or various mutant *vpu* genes and compared their replication profiles and virus release kinetics in infected cells. Our previous work on Vpu (5, 7, 23, 30, 32, 33, 36–38) involved the use of a mutant, Vpu_{35}, which carries a linker insertion resulting in a translational frameshift after the first 32 amino acids of Vpu followed by 3 nonspecific amino acids before translation terminates and has therefore the potential to express the entire N-terminal TM domain (32). In addition, we have constructed a second Vpu mutant, vpuDEL-1, which carries a deletion within the TM domain and is therefore unable to express any Vpu-specific sequences (19). To identify potential biological activities of the Vpu TM domain, we compared the replication profiles of viruses expressing Vpu_{35} and vpuDEL-1 mutants with that of wild-type HIV-1. For that purpose, PBMCs (Fig. 1A) or the $CD4^+$ human T-cell line A3.01 (Fig. 1B) were infected with the infectious molecular HIV-1 clone NL4-3 (1), carrying the wild-type *vpu* gene or

isogenic variants encoding the Vpu_{35} and vpuDEL-1 mutants. Virus particle secretion was assessed by measuring the release of virus-associated RT activity into the culture supernatants. Compared with wild-type Vpu, both Vpu mutants produced lower levels of progeny virus. However, virus release from infected cells expressing Vpu_{35} was consistently higher than from vpuDEL-1-infected cells, both in PBMCs and in A3.01 cells (Fig. 1A and B). In addition, vpuDEL-1-infected cultures exhibited higher cytopathic effects than cultures infected with the Vpu_{35} mutant (not shown). Since it was previously shown that expression of Vpu delays the onset of cytopathic effects in HIV-infected cultures, presumably because of the increased export of cytotoxic viral factors (19, 33, 43), the reduced cytopathic effects observed in Vpu_{35}-producing cultures are consistent with a partial activity of the TM domain expressed by the C-terminal truncation mutant Vpu_{35}.

To further examine the supposed residual activity of the Vpu TM domain, a pulse-chase analysis was conducted to study the kinetics of virus release from infected A3.01 cells. Parallel cultures of A3.01 cells were infected with NL4-3, NL-U_{35}, or vpuDEL-1 virus stocks. Cells were pulse-labeled at the time of peak virus production and chased for up to 8 h as previously described (30, 33, 36–38). Gag proteins (p24 and p55) present in cell lysates, virion preparations, or secreted as soluble proteins into the culture supernatants were immunoprecipitated with an HIV-1-reactive human serum, separated by SDS-PAGE (not shown), and quantitated with an image analyzer. Secretion of viral particles was calculated as the percentage of Gag proteins present in the viral pellet relative to the sum of Gag detected intra- and extracellularly (Fig. 1C). As expected, the rate of virus release from cultures expressing wild-type Vpu was significantly higher than that detected from cultures expressing vpuDEL-1 (Fig. 1C). Consistent with the replication profiles observed in PBMCs and A3.01 cells (Fig. 1A and B), the rate of virion secretion from cultures expressing Vpu_{35} was intermediate between the rates for wild-type Vpu and vpuDEL-1. Similar results were obtained when the amounts of cell-free virus particles present in the supernatants during an acute infection of A3.01 cells were quantitated by immunoblotting. In the experiment shown in Fig. 1D, A3.01 cells were infected with NL4-3, NL-U_{35}, and vpuDEL-1 virus stocks as described for Fig. 1B. Aliquots of culture supernatants were collected at various times during the infection as indicated in Fig. 1D, and viral particles were pelleted from 1 ml of supernatant each. Viral proteins were separated by SDS-PAGE and treated for immunoblotting with a 1:1 mixture of an HIV-1-reactive human serum and a polyclonal rabbit serum against HIV-1 Env as described in Materials and Methods. The relative amounts of the p17, p24, and p55 Gag products detected in Fig. 1D were calculated with an image analyzer and plotted as a function of time (Fig. 1E). Cultures infected with virus stocks encoding Vpu_{35} again revealed an intermediate phenotype in the amounts of viral particles detectable relative to cultures infected with wild-type virus or virus encoding vpuDEL-1. Thus, the replication profiles, as well as the results from the pulse-chase analysis and immunoblotting, are internally consistent and suggest that Vpu_{35} is a partially active form of Vpu. Since Vpu_{35} essentially lacks a cytoplasmic domain, these results are indicative of a biological activity of the Vpu TM domain on virus particle release.

Vpu_{35} stably expresses the Vpu TM domain in reticulocyte lysate. The experiments described so far suggest that Vpu_{35} is a partially active, C-terminally truncated Vpu protein. However, the existence of this truncated form of Vpu has not formally been demonstrated. Since antibodies to the TM domain of Vpu are not currently available, we used an in vitro system consisting of rabbit reticulocyte lysate supplemented with canine microsomal membranes to analyze the expression and membrane-association of Vpu_{35}. Wild-type Vpu, Vpu_{35}, and, as a control, vpuDEL-1 were expressed from pSP-9 (33), pSP-9/U_{35}, and pSP-9/U_{DEL-1}, respectively (Fig. 2B), and membrane-associated Vpu was pelleted and analyzed directly on an SDS–15% polyacrylamide gel (Fig. 2A) as described in Materials and Methods. As an internal control for translation efficiency and membrane integration, RNA encoding the α-mating factor of *S. cerevisiae* was included in each reaction. As expected, wild-type Vpu and the α-mating factor were associated with the membrane pellet (Fig. 2A). Expression of Vpu_{35} produced a 5- to 6-kDa protein corresponding to the size predicted for the TM domain of Vpu. Like wild-type Vpu, Vpu_{35} partitioned with the membrane fraction. In contrast, no Vpu product was detectable following translation of vpuDEL-1 (Fig. 2A). To verify that Vpu_{35} is cotranslationally integrated into membranes, Vpu_{35} was pretranslated in reticulocyte lysate in the absence of membranes. The reaction was then stopped

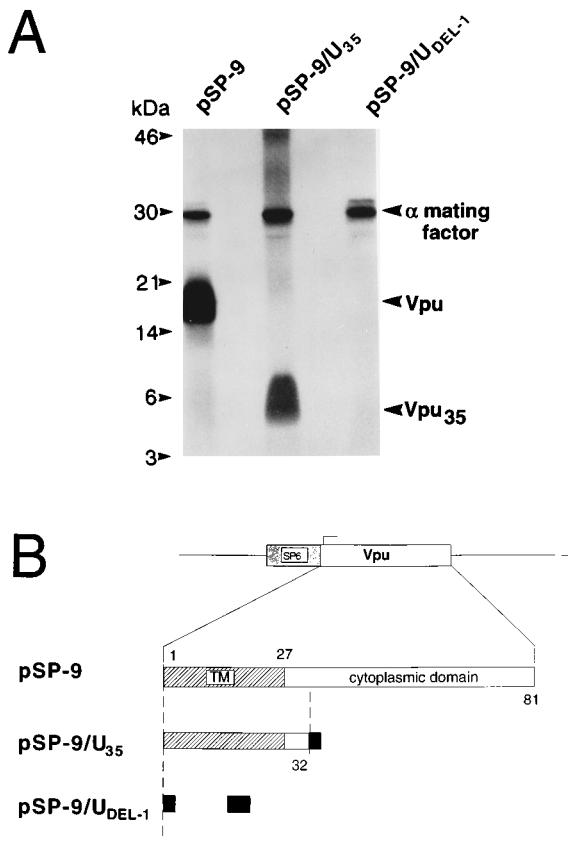

FIG. 2. In vitro translation and membrane integration of the Vpu TM domain encoded by the C-terminal truncation mutant Vpu_{35}. RNAs encoding full-length Vpu (pSP-9), the TM domain (pSP-9/U_{35}), or no Vpu-specific sequences (pSP-9/U_{DEL-1}) were translated in reticulocyte lysate supplemented with microsomal membranes in the presence of [^{35}S]methionine. Membranes were pelleted onto a sucrose cushion, separated on an SDS–15% polyacrylamide gel, and analyzed by fluorography. As a control, *S. cerevisiae* α-factor RNA was cotranslated in each reaction. The positions of full-length Vpu, the C-terminal truncation mutant Vpu_{35}, and *S. cerevisiae* α-mating factor in the gel are indicated on the right. The positions of molecular weight standard proteins are indicated on the left. (B) Structures of wild-type and mutant Vpu encoded by in vitro expression plasmids. Plasmid pSP-9/U_{35} (32) contains a stop codon at position 32, and pSP-9/U_{DEL-1} contains a 48-bp deletion within the TM domain of Vpu (19). Numbers refer to amino acid positions of Vpu (24). Solid bars represent nonspecific sequences which are in frame with the Vpu initiation codon.

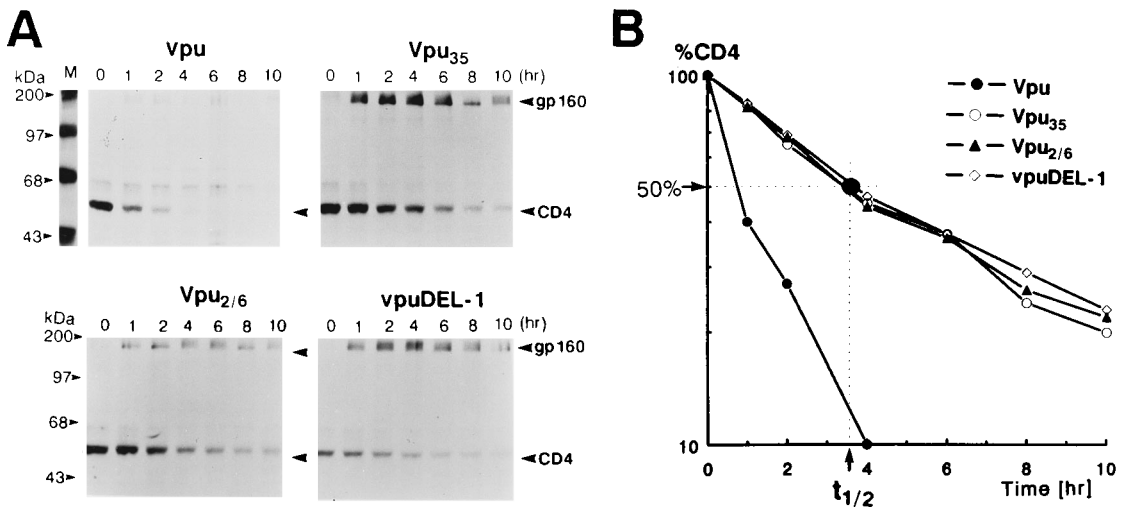

FIG. 3. The TM domain of Vpu does not induce CD4 degradation. (A) HeLa cells were cotransfected with the CD4 expression plasmid pHIV-CD4 in combination with either pNL-A1 (Vpu), pNL-A1/U_{35} (Vpu_{35}), pNL-A1/$U_{2/6}$ ($Vpu_{2/6}$), or pNL-A1/U_{DEL-1} (vpuDEL-1) plasmid DNA. Approximately 24 h after transfection, cells were pulse-labeled for 6 min and chased for the indicated times. CD4 was immunoprecipitated and separated on an SDS–10% polyacrylamide gel. Positions of CD4 and the coimmunoprecipitated gp160 are indicated on the right. Positions of marker proteins (M) are shown on the left. (B) CD4 bands from panel A were quantitated with an image analyzer, and the amounts of CD4 present at different times were calculated relative to the levels of CD4 present at the end of the pulse-labeling (0 h), which was empirically defined as 100%. $t_{1/2}$, half-life.

by addition of RNase, and the mixture was incubated with microsomal membranes for 1 h. No membrane association of Vpu_{35} could be detected under those conditions (data not shown), which is consistent with results obtained earlier for wild-type Vpu (33). Taken together, these results demonstrate that the 35-amino-acid Vpu_{35} peptide comprising the TM domain of Vpu is capable of cotranslationally integrating into membranes and is stably expressed in reticulocyte lysate.

Vpu_{35} does not induce CD4 degradation. We have previously reported (7, 36–38) and others have confirmed (12, 21, 35) that Vpu causes degradation of CD4 in the ER. This activity of Vpu is independent of its effect on virus release (44) and requires phosphorylation of Vpu by casein kinase II (12, 29, 30) at two conserved residues, Ser-52 and Ser-56, which are located in the cytoplasmic domain of Vpu (27). To assess if the Vpu TM domain nevertheless has a residual ability to induce CD4 degradation, we examined the stability of CD4 in the presence of wild-type Vpu, Vpu_{35}, and vpuDEL-1 by pulse-chase analysis in transfected HeLa cells as described previously (36–38). Retention of CD4 in the ER, which is required for Vpu-mediated CD4 degradation (37), was achieved by coexpression of the Env glycoprotein gp160, known to form stable complexes with CD4 that are retained in the ER (4, 6, 9, 16, 18, 37). The phosphorylation mutant $Vpu_{2/6}$, which is unable to induce degradation of CD4 (30), was included as a negative control. HeLa cells were cotransfected with pHIV-CD4 and either pNL-A1, pNL-A1/$U_{2/6}$, pNL-A1/U_{35}, or pNL-A1/U_{DEL-1} plasmid DNA. Approximately 24 h after transfection, cells were pulse-labeled for 6 min and chased for up to 10 h. CD4 from cell lysates was immunoprecipitated, separated by SDS-PAGE, and analyzed by fluorography (Fig. 3A). Gels were quantitated with an image analyzer, and the amount of CD4 present at different time points was calculated relative to the levels of CD4 present at the end of the pulse-labeling (0 h), which was empirically defined as 100% (Fig. 3B). As observed previously (38), wild-type Vpu caused a rapid decay of CD4. In contrast, none of the Vpu mutants, including Vpu_{35}, was able to induce CD4 degradation. The half-life of CD4 in cultures expressing Vpu_{35}, $Vpu_{2/6}$, or vpuDEL-1 was approximately 3.5 h in each case. Therefore, expression of the membrane anchor in Vpu_{35} had no effect on the stability of CD4. Thus, while retaining partial activity in mediating virus release, Vpu_{35} has no residual activity with regard to CD4 degradation.

Randomization of the Vpu TM domain does not affect protein stability, membrane association and topology, or intracellular localization of Vpu. To explore the potential catalytic role of the Vpu TM domain for Vpu-mediated enhancement of virus release, we constructed a mutant, Vpu_{RD} (Fig. 4), encoding a full-length protein with a randomized membrane anchor sequence as outlined in Materials and Methods. To minimize potential interference of the TM mutations with membrane integration of the resulting mutant protein, the hydrophobic nature of the TM domain was left unchanged. Instead, the primary structure was scrambled by rearranging amino acids of the TM domain. As a result, despite major rearrangements in the amino acid sequence of the TM domain, the overall amino acid composition of the mutant protein Vpu_{RD} remained the same as in wild-type Vpu, and the sequence was designed such that an α-helical structure in that region, which has recently been identified by using a combination of circular dichroism and ^1H nuclear magnetic resonance spectroscopy (40), was

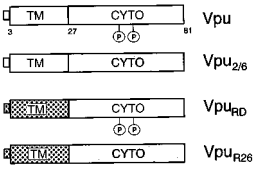

FIG. 4. Schematic structure of TM domain mutants of Vpu. The mutant Vpu_{RD} carries a randomized TM sequence which forms an α-helical structure similar to that of wild-type Vpu. The previously characterized phosphorylation mutant, $Vpu_{2/6}$ (27, 30), carries a change of phosphoacceptor sites Ser-52 and Ser-56 to Asn. The mutant Vpu_{R26} contains the TM domain from Vpu_{RD} and the cytoplasmic domain (CYTO) from $Vpu_{2/6}$. Numbers refer to Vpu amino acid positions of Vpu. P, phosphoseryl residues in positions 52 and 56. Symbols: ▭, wild type; ▭, nonphosphorylated cytoplasmic domain; ▩, randomized TM domain.

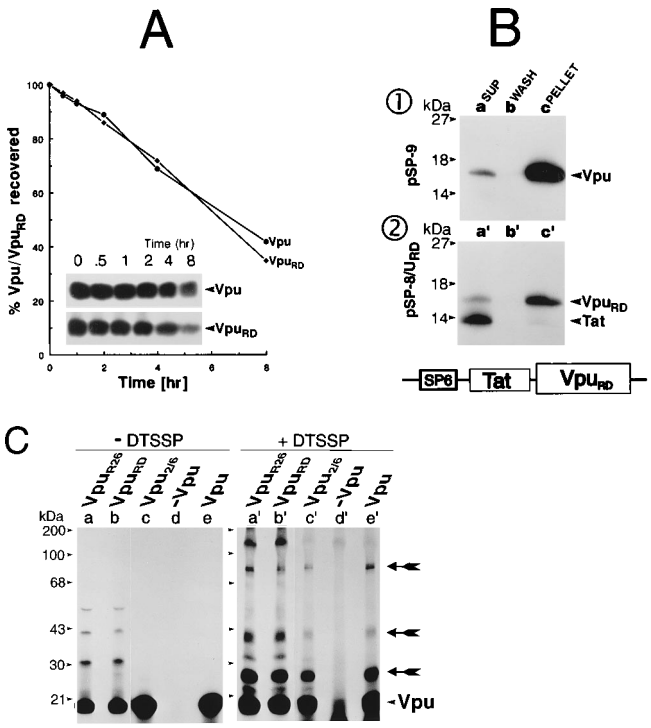

FIG. 5. Randomization of the membrane anchor sequence does not affect expression, stability, membrane integration, or oligomerization of Vpu. (A) A3.01 cells were infected with equivalent RT units of NL4-3 (Vpu) or NL-U_{RD} (Vpu_{RD}) virus stocks. At peak virus production, cells were pulse-labeled for 25 min with [^{35}S]methionine and chased for up to 8 h. Vpu proteins were immunoprecipitated with Vpu-specific polyclonal antibodies (sheep and rabbit), separated on an SDS–12.5% polyacrylamide gel, and visualized by fluorography (inset). Vpu proteins were quantitated with an image analyzer, and the percentage of Vpu or Vpu_{RD} recovered relative to the amount present at the end of the pulse (0 h) was plotted as a function of time. (B) Runoff transcripts of pSP-9 (Vpu) or pSP-8/U_{RD} (Vpu_{RD}) were synthesized in vitro. RNAs were translated in rabbit reticulocyte lysate supplemented with microsomal membranes. Following translation, membranes were pelleted, washed, and lysed. Vpu and Tat proteins were immunoprecipitated with an HIV-1-reactive human serum, separated on an SDS–15% polyacrylamide gel, and analyzed by fluorography. Lanes: a and a′, supernatant fraction; b and b′, wash fraction; lanes c and c′, membrane pellet fraction. The structure of the in vitro expression vector pSP-8/U_{RD} directing the expression of Vpu_{RD} as well as the first coding exon of Tat is outlined at the bottom. (C) Runoff transcripts of pSP-9 (Vpu), pSP-9/U_{DEL-1} (-Vpu), pSP-9/$U_{2/6}$ ($Vpu_{2/6}$), pSP-9/U_{RD} (Vpu_{RD}), and pSP-9/U_{R26} (Vpu_{R26}) were synthesized in vitro and used for in vitro translation in rabbit reticulocyte lysate. Lysates were either treated with DTSSP (lanes a′ to e′) or left untreated (lanes a to e). Vpu-specific products were immunoprecipitated, separated on a nonreducing SDS–12.5% polyacrylamide gel, and analyzed by fluorography. Positions of molecular weight marker proteins are indicated at the left; positions of oligomers stabilized by cross-linking are indicated at the right.

conserved. In addition, a derivative of Vpu_{RD}, Vpu_{R26}, lacking the two phosphoacceptor sites was constructed by replacing the cytoplasmic domain in Vpu_{RD} with that from the phosphorylation mutant $Vpu_{2/6}$ (Fig. 4).

The effects of the altered Vpu TM domain on protein stability and expression of the mutant proteins were assessed in infected T cells (Fig. 5A). Parallel cultures of A3.01 cells were infected with purified virus stocks of NL4-3 or NL-U_{RD}, and a pulse-chase experiment similar to that described in Fig. 1C was conducted at peak virus production. Cells were metabolically labeled and chased for up to 8 h. Vpu proteins were immunoprecipitated from cell lysates with Vpu-specific antisera, separated on an SDS–12.5% polyacrylamide gel, and visualized by fluorography (Fig. 5A, inset). The relative amounts of Vpu proteins recovered were quantitated by image analysis and plotted as a function of time (Fig. 5A). The results of the experiment suggest that wild-type Vpu and the mutant Vpu_{RD} were expressed at comparable levels and had similar turnover rates, with half-lives of approximately 7 h each. Similar results were obtained in pulse-chase analyses of HeLa cells transiently transfected with subgenomic expression vectors pNL-A1, pNL-A1/U_{RD}, and pNL-A1/U_{R26} (data not shown). Thus, randomization of the Vpu membrane anchor did not affect expression or the stability of the resulting mutant proteins in our assay systems.

To ensure that the randomization of the membrane anchor sequence did not alter the ability of the respective mutants to integrate into membranes, Vpu_{RD} was expressed in rabbit reticulocyte lysates in the presence of canine microsomal membranes (Fig. 5B). As a control, wild-type Vpu expressed from pSP-9 (32) was included. Unlike pSP-9, which encodes exclusively Vpu (Fig. 5B, panel 1), pSP-8/U_{RD} expresses the first coding exon of Tat in addition to Vpu_{RD} (Fig. 5B, panel 2). The soluble Tat product served as an internal marker for our fractionation studies. As shown in Fig. 5, the majority of Vpu_{RD} and wild-type Vpu were recovered from the membrane fractions (Fig. 5B, lanes c and c′), with only small amounts of the proteins remaining in the supernatant (Fig. 5B, lanes a and a′). In contrast, soluble Tat protein was almost exclusively detected in the supernatant (Fig. 5B, lane a′). Membrane association of the phosphorylation mutants $Vpu_{2/6}$ and Vpu_{R26} was analyzed in a parallel experiment (data not shown). In all cases, between 80 to 90% of Vpu proteins were consistently associated with membranes. Thus, neither the randomization of the TM domain in Vpu_{RD} and Vpu_{R26} nor the exchange of the phosphoacceptor sites in $Vpu_{2/6}$ and Vpu_{R26} interfered with membrane integration of the resulting protein product.

To assess the effects of randomization of the Vpu TM domain on the ability of Vpu to form homo-oligomeric complexes, wild-type Vpu, $Vpu_{2/6}$, Vpu_{RD}, and Vpu_{R26} were separately translated in rabbit reticulocyte lysates, and aliquots of each reaction were subjected to chemical cross-linking as described in Materials and Methods. Cross-linked and non-cross-linked proteins were immunoprecipitated with a mixture of Vpu-specific antisera, separated by nonreducing SDS-PAGE, and visualized by fluorography (Fig. 5C). Consistent with our previous findings (23), we were able to stabilize complexes with approximate sizes of 25, 40, and 80 kDa (Fig. 5C, panel +DTSSP, arrows), which were detected for wild-type Vpu and its mutants in addition to monomeric Vpu (Fig. 5C, lanes a′ to c′ and e′). These oligomeric forms of Vpu proteins were not detected in the untreated samples or in the absence of Vpu (Fig. 5C, lanes a to e and d′). Thus, the randomization of the TM domain did not affect the ability of the mutant Vpu proteins to form homo-oligomeric complexes.

One possible explanation for the altered biological activity of Vpu mutants carrying the randomized TM domain is a potential change in the subcellular localization of such Vpu mutants relative to wild-type Vpu. To address this question, HeLa cells were transfected with pNL-A1 encoding wild-type Vpu or isogenic variants directing the expression of $Vpu_{2/6}$, Vpu_{RD}, or Vpu_{R26}. Expression of Vpu in transfected cells was detected by immunofluorescence and analyzed by laser scanning confocal microscopy as described in Materials and Methods (Fig. 6). As observed previously (19), wild-type Vpu predominantly localized to the perinuclear region of the cell (Fig. 6A), which includes ER and Golgi membranes (22). Similarly, nonphosphorylated $Vpu_{2/6}$ (Fig. 6B), which is able to enhance virus particle release, as well as Vpu_{RD} (Fig. 6C) and Vpu_{R26} (Fig. 6D), both of which are unable to regulate virus secretion, predominantly localized to the perinuclear region. Thus, ran-

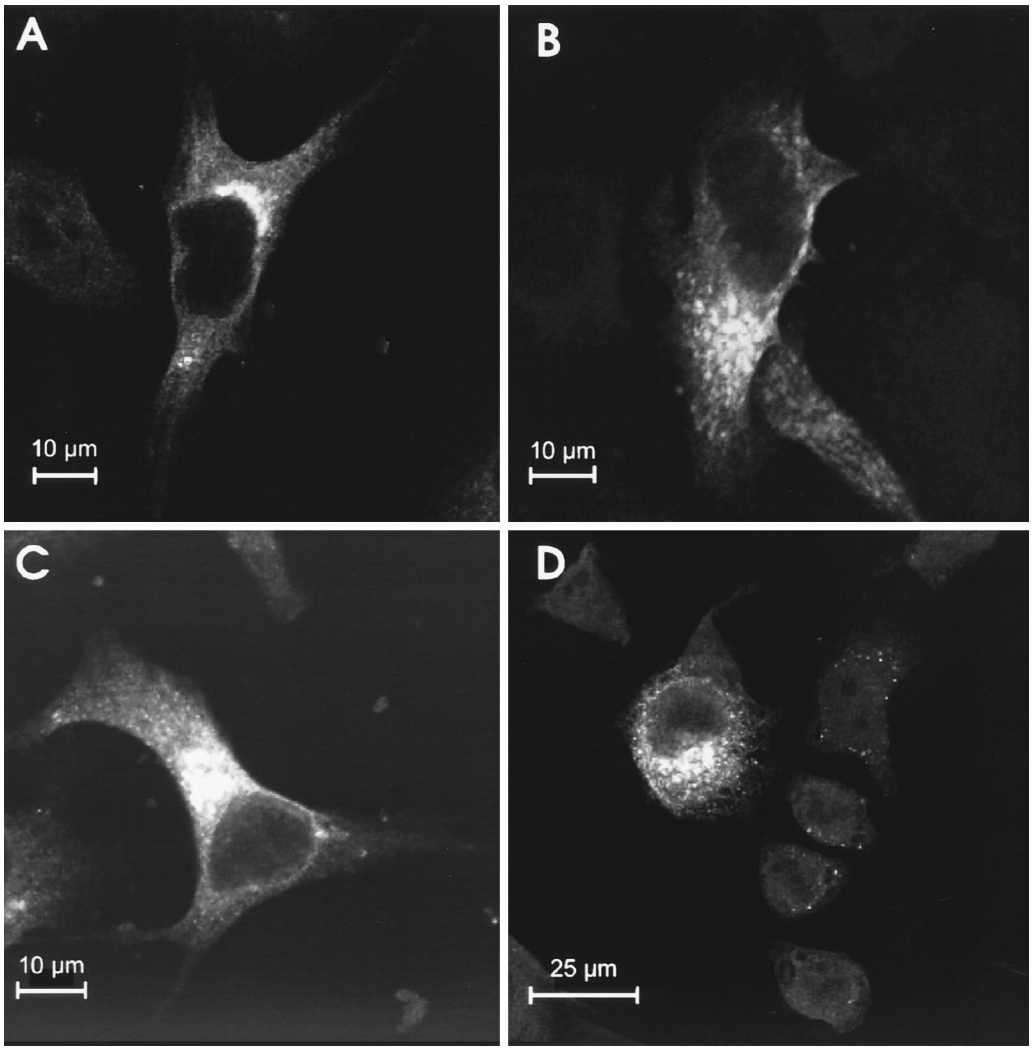

FIG. 6. Randomization of the TM anchor does not affect subcellular localization of Vpu. HeLa cells transfected with Vpu-expressing plasmids were fixed with 4% paraformaldehyde and sequentially stained with rabbit anti-Vpu and Texas red-conjugated donkey anti-rabbit sera. A largely perinuclear distribution of Vpu was observed in cells expressing wild-type Vpu (A), the phosphorylation mutant $Vpu_{2/6}$ (B), Vpu_{RD} carrying the randomized TM domain (C), and Vpu_{R26} containing both the randomized TM domain and the cytoplasmic domain of $Vpu_{2/6}$ (D).

domization of the Vpu TM domain did not have any apparent effects on the subcellular distribution of wild-type or mutant Vpu proteins.

The alteration of the Vpu TM domain does not affect CD4 degradation. To study the biological activity of Vpu_{RD}, we first evaluated its potential to induce degradation of CD4. For this purpose, we conducted a pulse-chase experiment with HeLa cells similar to that described for Fig. 3. HeLa cells were cotransfected with pHIV-CD4 and either pNL-A1/U_{RD}, pNL-A1/$U_{2/6}$, or pNL-A1/U_{R26}. Transfected HeLa cells were pulse-labeled for 6 min and then chased for up to 60 min. Cells were harvested at the indicated times. Cell lysates were immunoprecipitated with an anti-CD4 serum, separated by SDS-PAGE, and analyzed by fluorography (Fig. 7A). The kinetics of CD4 decay in the individual reactions were determined by calculating the levels of CD4 present at different times relative to the levels of CD4 present at the end of the pulse (0 min), which was defined as 100% (Fig. 7B). The result of this experiment demonstrates that Vpu_{RD} is able to induce degradation of CD4. The half-life of CD4 in the presence of Vpu_{RD} is 28 min, in contrast to a half-life of 3.5 h for CD4 in the absence of Vpu (compare with Fig. 3). As expected, $Vpu_{2/6}$ and Vpu_{R26} did not induce CD4 degradation, since these mutants lack the phosphoacceptor sites essential for this process (12, 30). We conclude that the capacity of Vpu to induce CD4 degradation in the ER is not compromised in mutants containing a randomized TM domain but is strictly dependent on the presence of the phosphorylated cytoplasmic tail. In addition, preliminary data suggest that chimeric Vpu molecules containing TM domains from heterologous type I integral membrane proteins are capable of inducing CD4 degradation as well (25). It is therefore likely that for this function of Vpu, the TM domain simply provides a membrane anchor, which can be substituted by heterologous TM domains, while the cytoplasmic tail contains the active domain.

One of the early steps in the Vpu-mediated degradation of CD4 involves the physical interaction between Vpu and the cytoplasmic tail of CD4 (5). We previously demonstrated that Vpu phosphorylation, while essential for CD4 degradation, is not required for the initial binding of Vpu to CD4 (5). The fact

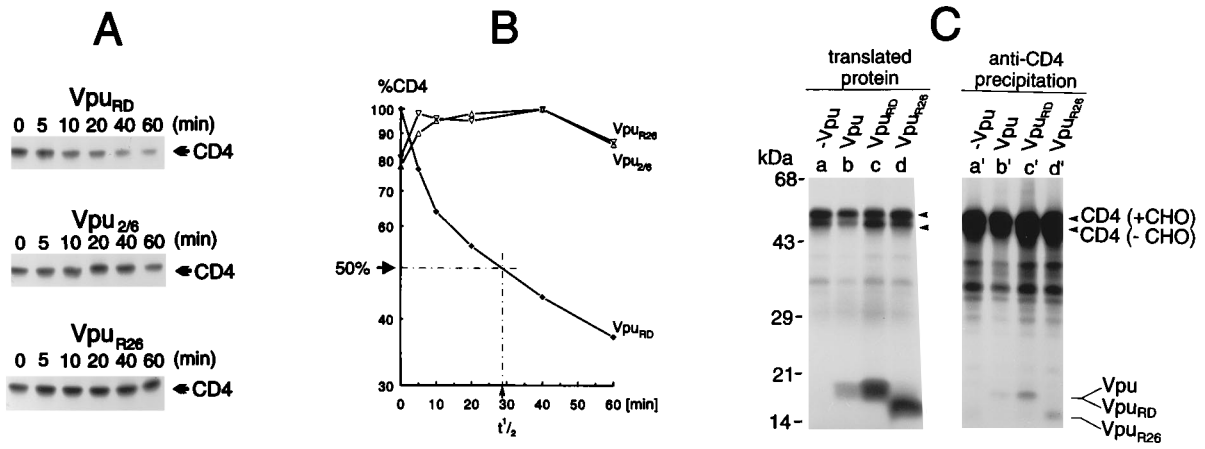

FIG. 7. Phosphorylated Vpu_{RD}, but not unphosphorylated Vpu_{R26}, induces degradation of CD4. (A) Parallel cultures of HeLa cells were cotransfected with the CD4 expression plasmid pHIV-CD4 in combination with either pNL-A1/U_{RD} (Vpu_{RD}), pNL-A1/$U_{2/6}$ ($Vpu_{2/6}$), or pNL-A1/U_{R26} (Vpu_{R26}) plasmid DNA as described for Fig. 3A. HeLa cells were pulse-labeled for 6 min and chased for the indicated times. Cell lysates were immunoprecipitated with a CD4-specific antiserum, separated in on an SDS–10% polyacrylamide gel, and analyzed by fluorography. Parts of the fluorograms containing radiolabeled CD4 are shown. (B) Quantitative analysis of CD4 degradation was done as described for Fig. 3B. (C) Vpu_{RD} and Vpu_{R26} retain the ability to interact with CD4 in vitro. CD4 RNA was translated in rabbit reticulocyte lysate either alone (lanes a and a′) or cotranslated with RNA encoding wild-type Vpu (lanes b and b′), the randomized TM mutant Vpu_{RD} (lanes c and c′), or the phosphorylation mutant Vpu_{R26} (lanes d and d′) in the presence of microsomal membranes. Aliquots were either directly analyzed (lanes a to d) or immunoprecipitated with the CD4 antiserum (lanes a′ to d′) prior to separation on an SDS–12.5% polyacrylamide gel and visualization by fluorography. The positions of glycosylated (+CHO) and nonglycosylated (−CHO) CD4 and the various Vpu species are indicated on the right.

that Vpu_{RD} is capable of inducing CD4 degradation suggests that such an interaction is also not critically dependent on the integrity of the Vpu TM domain, and it can be assumed that both Vpu_{RD} and Vpu_{R26} are capable of binding to CD4. To verify such a predicted interaction between CD4 and Vpu_{RD}, or Vpu_{R26}, we used our in vitro binding assay as recently reported (5, 7). Wild-type Vpu was included as a positive control in this experiment. Details of the in vitro translation reaction and the coimmunoprecipitation of Vpu and CD4 are described in the legend to Fig. 7C. Direct analysis of the translation products demonstrates the presence of comparable amounts of CD4 in individual reactions (Fig. 7C, left). Expression of wild-type Vpu was somewhat lower than that of Vpu_{RD} or Vpu_{R26} in this experiment. As predicted, immunoprecipitation with a CD4-specific antiserum revealed coimmunoprecipitation not only of wild-type Vpu but of Vpu_{RD} and Vpu_{R26} as well (Fig. 7C, right). The lower amount of wild-type Vpu coimmunoprecipitated with CD4 reflects the lower level of Vpu in the lysates. Thus, the insensitivity of the Vpu-CD4 interaction to alterations in the TM anchor of Vpu_{RD} and Vpu_{R26} suggests either that this domain of Vpu is not directly involved in the physical interaction with CD4 or, alternatively, that this interaction does not depend on the primary but rather depends on the secondary α-helical structure of this domain which is conserved in Vpu_{RD} and Vpu_{R26} (40).

Randomization of the Vpu TM domain abolishes Vpu-mediated enhancement of virus secretion. To evaluate the impact of the alterations introduced into the TM domain of Vpu on its ability to enhance virus particle release, we studied the kinetics of processing and release of viral proteins from HeLa cells transfected with NL4-3, vpuDEL-1, NL-U_{RD}, or NL-U_{R26}. A pulse-chase analysis was performed approximately 20 h after transfection essentially as described for Fig. 1C. Aliquots of cells were collected at the times indicated in Fig. 8A and B, and viral proteins present in the cell lysates, the viral pellet fractions, or the clarified supernatants were immunoprecipitated with an HIV-1-reactive human serum, separated on SDS–10.5% polyacrylamide gels, and analyzed by fluorography (Fig. 8A). The percentages of virus particle associated $p24^{gag}$ and $p55^{gag}$ proteins present in the supernatants at individual times during the chase were calculated by using an image analyzer and plotted as a function of time (Fig. 8B). We found that the release of virus particles in the presence of Vpu_{RD} or Vpu_{R26} was inefficient and comparable to that observed in the absence of Vpu. Both cultures released approximately four to five times less Gag proteins over an 8-h chase period than cultures expressing wild-type Vpu. The comparison of viral proteins immunoprecipitated from cell lysates (Fig. 8A, cell) revealed no difference in the relative protein compositions of NL4-3, vpuDEL-1, NL-U_{RD}, and NL-U_{R26}, indicating that randomization of the membrane anchor of Vpu_{RD} had no effect on the expression or processing of viral proteins. Also, the unspecific release of free, non-particle-associated viral proteins was not affected by the different Vpu proteins (not shown).

To further confirm the inability of Vpu_{RD} to support virus secretion, the replication profiles of wild-type NL4-3, vpuDEL-1, NL-U_{RD}, and NL-U_{R26} viruses were analyzed in A3.01 cells by measuring RT activities released into the culture supernatants during virus replication (Fig. 8C). The RT profiles of the NL-U_{RD}- and NL-U_{R26}-infected cultures were almost indistinguishable from those of the vpuDEL-1-infected cultures, confirming that mutants carrying the randomized membrane anchor are defective with respect to the augmentation of virus release. In addition, cytopathic effects in cells infected with NL-U_{RD}, NL-U_{R26}, or vpuDEL-1 were observed approximately 2 days earlier than in cultures expressing wild-type Vpu (data not shown), further supporting the conclusion that Vpu_{RD} and Vpu_{R26} are unable to support virus particle release. Thus, while Vpu_{RD} is capable of inducing CD4 degradation, the randomization of the membrane anchor in the mutants Vpu_{RD} and Vpu_{R26} completely abolished their ability to support virus particle secretion.

DISCUSSION

Previous studies have defined two distinct biological activities for the HIV-1 Vpu protein. One activity regulates the release of progeny virions from the surface of infected cells (8,

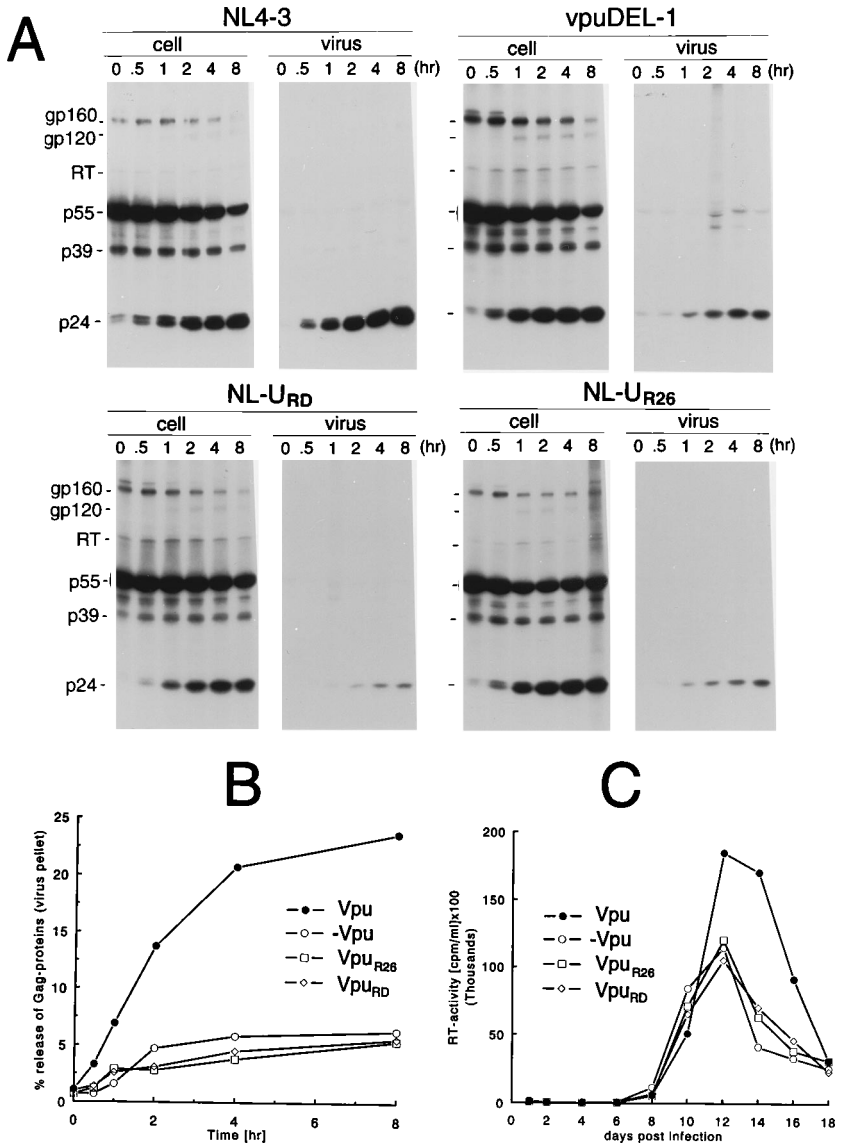

FIG. 8. Vpu_{RD} and Vpu_{R26} containing the randomized TM domain are unable to augment virus particle secretion. (A) Parallel cultures of HeLa cells (5×10^6) were transfected with 25 μg of DNAs of the molecular clone pNL4-3, vpuDEL-1, pNL-U_{RD}, or pNL-U_{R26}. Cells were labeled for 25 min with [^{35}S]methionine, and a pulse-chase experiment was conducted as described for Fig. 1C. Viral proteins from the lysates of cells and pelleted virions as well as the clarified supernatants were immunoprecipitated with an HIV-1-reactive human serum, separated on an SDS–10.5% polyacrylamide gel, and analyzed by fluorography. Fluorograms depicting cell lysates (cell) and virion fractions (virus) are shown. HIV proteins are indicated on the left. (B) $p24^{gag}$ and $p55^{gag}$ proteins detected in the cell lysates and virus pellet and the clarified supernatants (not shown) were quantitated with an image analyzer. The amounts of Gag proteins in the virus pellet were calculated as the percentage of total $p24^{gag}$ and $p55^{gag}$ for each time point and were plotted as a function of time. (C) Replication profiles of NL4-3 (Vpu), vpuDEL-1 (-Vpu), NL-U_{RD} (Vpu_{RD}), and NL-U_{R26} (Vpu_{R26}) viruses in A3.01 cells. Parallel cultures of A3.01 cells were infected with equal RT doses of virus stocks, and culture supernatants were assayed every other day for RT activity.

19, 32, 33, 34), while another activity results in the degradation of the HIV receptor molecule CD4 in the ER (36, 37). The prerequisites for these two functions of Vpu are substantially different. For example, Vpu-mediated CD4 degradation is highly specific, involves a physical interaction between the cytoplasmic domains of Vpu and CD4 (5), and is dependent on the phosphorylation of two highly conserved serine residues in the cytoplasmic domain of Vpu (30). In contrast, the effect of Vpu on particle release is not restricted to HIV-1 but has also been demonstrated for heterologous targets such as HIV-2, visna virus, or murine leukemia virus (14) and is relatively phosphorylation insensitive (12, 30). Finally, we demonstrated that the two functions of Vpu are regulated from different cellular compartments, with CD4 degradation occurring in the ER while particle release is regulated from a post-ER compartment (30). All of these findings are highly suggestive that the two functions of Vpu are controlled by independent molecular mechanisms. However, while there is good evidence that the interaction of Vpu with the cytoplasmic domain of CD4 leads to the activation of an energy-dependent, ER membrane-associated process that results in the rapid proteolysis of CD4 (5, 7, 31, 37, 38), little is known about how Vpu regulates the release of viral particles from the cell surface.

One of the key findings of the present work is that the two biological activities of Vpu can be correlated with two distinct structural domains of this protein and are mechanistically sep-

arable. We now have available mutants which are capable of performing only one of the two functions of Vpu. For example, Vpu_{RD} is able to induce CD4 degradation but has lost its ability to enhance virus release from the cell surface, while Vpu_{35} and $Vpu_{2/6}$ mutants are both unable to induce CD4 degradation but are at least partially capable of enhancing virus particle secretion. Finally, the scrambled and nonphosphorylated mutant Vpu_{R26}, with a secondary structure similar to that of the wild-type protein (30, 40, 41), has lost all of these activities.

The data presented in this work do not define the exact nature of the molecular mechanism controlled by the Vpu TM domain in the process of Vpu-facilitated virus particle release. However, preliminary results from studies designed to measure potential effects of Vpu on the ionic permeability of biological membranes suggest that the TM domain of wild-type Vpu but not that of Vpu_{RD} has the propensity to form ion-conductive membrane pores (28). The dependence of this phenomenon on the integrity of the Vpu TM domain and its correlation with the sensitivity of Vpu-mediated enhancement of virus release to alterations in the TM domain make it attractive to speculate that this function of Vpu is based on an ion channel activity. The apparent importance of the TM domain for Vpu's virus release function would be the formation of a membrane pore. Since Vpu has only one membrane-spanning domain, the formation of ion-conductive pores would depend on the formation of homo-oligomeric structures, the existence of which we have previously demonstrated (23). Even though, cross-linking data show that Vpu_{RD}, like wild-type Vpu, is principally capable of forming homo-oligomeric complexes, it is possible that the structure of such complexes, i.e., the number of subunits involved in each complex, is critical for Vpu function and dependent on the integrity of the TM domain. Thus, it cannot be ruled out that the inability of Vpu_{RD} to enhance virus secretion is due to improper assembly of Vpu homo-oligomers in vivo.

It is unclear if and how the cytoplasmic domain of Vpu could contribute to the proposed ion channel activity of the Vpu TM anchor. However, the fact that the presence of the cytoplasmic domain, although not essential for regulating virus release, significantly improves this function of Vpu (32) points to a potential role as a regulatory element that could modulate the opening and closing of the channel, regulate the topology or oligomerization of membrane-integrated Vpu, or influence the intracellular localization of the Vpu channel. Changes introduced into the active domains of membrane anchor and cytoplasmic tail did not seem to alter the subcellular distribution of the resulting proteins as assessed by immunocytochemical analyses, suggesting that the differential activities of the Vpu mutants analyzed in this study are unlikely to be due to differences in protein trafficking. However, since the precise intracellular site from which Vpu regulates virus secretion has not yet been identified, it is conceivable that the perinuclear site, where wild-type and mutant Vpu proteins were found to accumulate, is distinct from its site of action on virus release.

The question of how an ion channel could affect the release of progeny virions from the cell surface can presently not be answered. Nevertheless, depending on the precise intracellular localization of Vpu in a post-ER compartment, from which Vpu regulates virus release, an active or a passive role of Vpu can be envisioned. An active role of Vpu would involve the presence of Vpu at the cell surface, where it would actively support budding of virus particles. However, surface expression of Vpu has so far been observed only in a few cases in which Vpu was expressed at high levels in HeLa cells (22, 31). Also, its absence in virions could be an indication that Vpu is not present in large amounts, if at all, at the cell surface. As mentioned above, the majority of Vpu is found intracellularly (19, 22), making it attractive to speculate about a passive role of Vpu on virus release. In such a scenario, Vpu could enhance the release of virions from the cell surface by, for example, preventing budding from intracellular membranes, which has been previously observed in the absence of Vpu (19) and which results in the accumulation of virus particles in cellular vesicles.

The biological relevance of the two functions of Vpu is difficult to assess. It is apparent that the detrimental effect of Vpu on CD4 is redundant in the sense that at least two other viral mechanisms exist that result in cell surface down-modulation of CD4. For example, the accessory protein Nef has been shown to induce down-modulation of CD4 from the cell surface (2, 3). Most importantly, expression of the Env glycoprotein in HIV-infected cells allows the formation of stable complexes with CD4, resulting in the entrapment of CD4 in the ER (4, 6, 9, 18) and thus in its depletion from the cell surface. This mechanism, however, functions at the expense of Env, whose transport to the cell surface is blocked as well. It is possible that one of the roles of Vpu in the virus life cycle is to release Env from such intracellular entrapment by inducing degradation of CD4. The release of Env from its intracellular trap could be a means of providing sufficient levels of Env protein at the surface for incorporation into virions. Aside from that, it might be advantageous for HIV-1 to prevent CD4 from reaching the cell surface late in infection. One of the possible complications of CD4 expression at the time of progeny virion production could be the nonspecific packaging of CD4 into virions, thus potentially interfering with virus spread.

The relevance of Vpu-mediated enhancement of virus particle production for virus replication in vivo is unclear, not least because of the absence of an appropriate animal model. However, there are several possible advantages of such a Vpu function for HIV-1 replication. For example, the increased rate of virus release in the presence of Vpu is paralleled by a reduced budding of HIV-1 from internal membranes (19, 32–34). Such interference of Vpu with intracellular budding could prevent the accumulation of potentially toxic viral proteins in infected cells and thus explain the reduced cytopathic effects observed in HIV-1-infected cultures in the presence of Vpu (19, 43). The obvious benefit for HIV-1 would thus be not only an increased rate of virus production but also a prolonged output of progeny viruses from infected host cells, both of which would support virus spread in vivo.

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