Journal of Virology, June 1997, p. 4452–4460
0022-538X/97/$04.00+0
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Vol. 71, No. 6

Putative α-Helical Structures in the Human Immunodeficiency Virus Type 1 Vpu Protein and CD4 Are Involved in Binding and Degradation of the CD4 Molecule

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Received 12 November 1996/Accepted 3 March 1997

The human immunodeficiency virus type 1 (HIV-1) *vpu* gene encodes a 16-kDa class I integral membrane phosphoprotein with an N-terminal membrane-spanning region and a C-terminal cytoplasmic domain. In the cytoplasmic domain, two amphipathic α-helices joined by a flexible turn containing two phosphoacceptor sites have been predicted. Previous studies have shown that Vpu downregulates CD4 molecules by inducing their specific degradation in the endoplasmic reticulum. Phosphorylation of serine residues 52 and 56, present within the cytoplasmic domain of the Vpu protein, has been shown to be essential to this Vpu function. However, the contribution of these two phosphoacceptor sites in the mechanism of CD4 degradation remains undefined. Interestingly, a specific interaction between Vpu and CD4 was recently demonstrated in coimmunoprecipitation experiments. Binding of Vpu was shown to be necessary but not sufficient to mediate CD4 degradation, indicating that interaction between Vpu and CD4 represents an early step critical in triggering a process leading to CD4 degradation. To delineate the sequence(s) and/or structural determinant(s) involved in this Vpu-CD4 interaction and in the Vpu-mediated CD4 degradation, we performed a mutational analysis of the cytoplasmic domain of CD4 and Vpu. Coimmunoprecipitation experiments reveal that disruption of the putative α-helical structure in the membrane-proximal cytoplasmic domain of CD4 affects the binding to Vpu, suggesting that this structure may act as an interface for the CD4-Vpu interaction that mediates CD4 degradation. Vpu proteins containing mutations in either or both of the phosphoacceptor sites (Ser52 or/and Ser56) were inactive in regard to CD4 degradation yet retained the capacity to interact with the cytoplasmic domain of CD4. In an attempt to define the minimal region responsible for this interaction, we tested a panel of mutations which were designed to affect the integrity of the putative α-helices present in the cytoplasmic domain of Vpu. Our results indicate that although both C-terminal α-helices are required for degradation of CD4, only α-helix I, located in the membrane-proximal cytoplasmic region of Vpu, is involved in the interaction between Vpu and CD4. Taken together, these results demonstrate that α-helical structures in the HIV-1 Vpu and CD4 proteins are involved in binding and degradation of CD4 molecules.

The human immunodeficiency virus type 1 (HIV-1) *vpu* gene encodes an 81-amino-acid protein that is not expressed by the closely related HIV-2 and most simian immunodeficiency viruses (5, 17, 26). Vpu has the topology of a class I integral membrane protein, with an N-terminal hydrophobic region of 27 amino acids, which constitutes the membrane-spanning domain, and a charged C-terminal hydrophilic domain facing the cytoplasmic side (15). Vpu has been shown to be phosphorylated at two seryl residues at positions 52 and 56 by the ubiquitous casein kinase II (7, 21, 23). In studies using a combination of circular dichroism and ^{1}H nuclear magnetic resonance spectroscopy, the cytoplasmic domain of Vpu has been predicted to form two amphipathic α-helices joined by a flexible turn containing the two phosphoacceptor sites of the protein (34).

Numerous functional studies have indicated that Vpu possesses several biological activities (reviewed in reference 27). Expression of this protein during HIV-1 infection in CD4^{+} cells delays cytopathic effects of the virus by reducing the rate of syncytium formation (12, 28, 36). Also, this protein has been shown to induce CD4 degradation in the endoplasmic reticulum (ER) and to regulate the transport of glycoproteins (soluble gp120, gp160 and vesicular stomatitis virus G protein) through the intracellular secretary pathway (30, 32, 33). In addition, Vpu facilitates the release of HIV-1 viral particles from the surface of infected cells in a manner which is independent of the expression of Env glycoproteins and CD4 receptor molecules (9, 19, 25, 28, 37). Recent data have demonstrated that the CD4-degrading capability of Vpu is modulated by sequences present in the cytoplasmic domain of the protein and requires its phosphorylation (20). Conversely, the virus release function of Vpu seems to be modulated by the N-terminal region of the protein (22). These observations have emphasized the possibility that Vpu promotes differential biological activities during HIV-1 replication and may in fact have more than one target in infected cells (7, 20, 22).

The efficient Vpu-induced CD4 degradation has been shown to require the retention of CD4 molecules in the ER either through formation of complexes with the Env precursor gp160 or by the treatment of cells with brefeldin A (BFA), a fungal metabolite known to block protein sorting from the ER to the Golgi apparatus. By mutational analysis, several studies have identified sequences and/or structural determinants in the transmembrane domain and cytoplasmic tail of CD4 and in the cytoplasmic domain of Vpu which are involved in the degradation of CD4 (2, 3, 13, 18, 20, 29, 31, 35). However, the

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molecular mechanism involved in the degradation of CD4 by Vpu has yet to be defined. Recently, coimmunoprecipitation experiments have shown that Vpu can specifically bind CD4 molecules in the ER (1). The weak affinity of these two proteins and/or the high rate of Vpu-induced CD4 degradation may have previously impeded the detection of Vpu-CD4 complexes in whole-cell systems. Interestingly, the use of chimeric proteins and deletion mutants of CD4 indicated that the cytoplasmic tail of the protein previously shown to be important for Vpu-induced degradation also contains determinants important for the binding of Vpu. Moreover, substitution mutations of Vpu phosphoacceptor sites as well as deletion of five amino acids between residues 47 and 52 which abrogate the capacity of Vpu to promote CD4 degradation were shown to have no effect on the capacity of the protein to interact with CD4 (1, 3, 20). On the basis of these results, it was proposed that the binding of Vpu appears necessary but not sufficient to induce CD4 degradation. However, a recent study using a two-hybrid assay in yeast cells has shown that GAL4 fusion proteins containing a cytoplasmic domain of Vpu carrying a double mutation in the phosphorylation sites could no longer interact with GAL4 fusion proteins containing the cytoplasmic tail of CD4 (16).

To gain further insight into the mechanism of Vpu-mediated CD4 degradation, we proceeded with a mutational analysis of both the CD4 and Vpu proteins. Coimmunoprecipitation experiments show that α-helical structures predicted to be formed in the cytoplasmic domains of CD4 and Vpu are necessary for both binding and degradation of CD4 molecules. Moreover, we show that Vpu-mediated degradation requires phosphorylation of both Vpu phosphoacceptor sites and the presence of a second α-helical structure in the distal cytoplasmic region of the protein. Finally, evidence presented here indicates that the Vpu C terminus is not involved in the degradation of CD4 mediated by Vpu.

MATERIALS AND METHODS

DNA constructions. The HIV-1 Vpu-expressing plasmid SVCMVVpu$^+$ and the negative control plasmid SVCMVVpu$^-$ used in this study were described previously (37). Vpu substitution mutants were generated by using a two-step PCR-based method (11). Complementary oligonucleotide primers containing the desired mutations were used to generate the Vpu mutated fragments by PCR. The nucleotide sequences of the mutagenic sense oligonucleotides are as follows: VpuYRK, sense, 5′-GTAATCATAGAATTTTGGCCAATATTAAGAC-3′; VpuY29A, sense, 5′-AATCATAGAAGCTAGGAAAATA-3′; VpuDRLI, sense, 5′-AACGGGTTAATTAATGGA-3′; VpuA65-70, sense, 5′-GAAATATCAGCACCTGTGGCGTTGGGGATGGCGTTG-3′; VpuH72D, sense, 5′-GAGATGGGGGACCATGCTC-3′; VpuΔDVDDL, sense, 5′-CATGCTCCTTAGCATGTTGATGAT-3′; and VpuL63P, sense, 5′-TATCAGCACCTGTGGAGAT-3′. A 5′ primer (5′-AAGCCACCTTTGCCTAGT-3′) located 23 bp upstream from the Vpr initiation codon and a 3′ primer (5′-GGCTACACAGGCATGTGT-3′) located 148 bp downstream from the Vpu termination codon were used for DNA amplification of the mutated fragments. The Vpu mutated fragments were then digested at *Sal*I and *Kpn*I sites located at positions 5331 and 5893 (+1 is the site of transcription initiation of the HXBH10 molecular clone), respectively, and cloned into plasmid pSP64 (Promega). The resulting Vpu mutants were then amplified by PCR using a second pair of primers. The 5′ primer (5′-CAGTCTAGAGTACATGTAATGCAACCT-3′) creates an *Xba*I site upstream of the initiation codon of Vpu, and the 3′ primer (5′-CACACAGGTACCCCATAA-3′) is located in the 3′ region following the stop codon of Vpu and creates a *Kpn*I site. Amplified fragments were subsequently digested and cloned into the linearized (*Xba*I-*Kpn*I) expression plasmid SVCMVVpu to yield VpuY29A, VpuYRK, VpuDRLI, VpuL63P, VpuA65-70, VpuH72D, and VpuΔDVDDL. The Vpu cDNA fragments corresponding to VpuS52G, VpuS56G, and VpuSS52/56 were PCR amplified as previously described (11), using HIV-1 proviruses HxBH10VpuS52G, HxBH10VpuS56G, and HxBH10VpuSS52/56 as templates (7). The resulting PCR fragments were then inserted into the expression plasmid SVCMVexpa to generate expression plasmids encoding Vpu phosphorylation mutants. All of the mutant constructs described above were confirmed by the dideoxynucleotide chain termination sequencing method by using a Sequenase kit (United States Biochemical Co., Cleveland, Ohio).

SVCMVCD4 was constructed by inserting a cDNA fragment encoding CD4 into the corresponding sites of the expression vector SVCMVexpa as described before (35). The CD4 cDNA was derived from the pT4B CD4 expressor, which was obtained from Richard Axel through the AIDS Research Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (14). The construction of CD4 deletion mutants CD4Δ7, CD4Δ14, CD4Δ23, and CD4Δ32 and of CD4 substitution mutants CD4LL413,4AA, CD4KK417,8IG, CD43S, CD4EMKL, and CD4MK407,11PP was also described earlier (35).

Cell lines and transfections. COS-7, an African green monkey kidney cell line transformed by an origin-defective mutant of simian virus 40, was cultured in Dulbecco's modified Eagle medium (DMEM; GIBCO Laboratories). For transfection, one million COS-7 cells were seeded into a 100-mm-diameter petri dish and cultured overnight in DMEM containing 10% fetal calf serum. Cells were then cotransfected with a mixture of 5 μg of CD4 expressor and 12.5 μg of Vpu expressor (SVCMVVPU$^+$, SVCMVVPU$^-$, or a Vpu mutant; molar ratio of 1:3) by the calcium phosphate method (6).

Antibodies and chemical compound. The rabbit anti-Vpu serum was raised by immunizing rabbits with a synthetic peptide corresponding to amino acids 73 to 81 of the BH10 Vpu protein (5, 37). The anti-CD4 (OKT4) monoclonal antibodies were derived from ascitic fluids of BALB/c mice that were injected with an OKT4 hybridoma which was obtained from the American Type Culture Collection, Rockville, Md. BFA was obtained from Sigma Chemical Co. and was stored as a 10 mM stock solution in ethanol at −20°C. Digitonin was purchased from the Aldrich Chemical Co.

Metabolic labeling and radioimmunoprecipitation. CD4 degradation experiments were all performed 48 h posttransfection. Transfected cells were pretreated with 10 μM BFA for 30 min, and then metabolically labeled with 200 μCi of [^{35}S]methionine (New England Nuclear) for 5 h in the presence of BFA (10 μM) and lysed on ice with radioimmunoprecipitation assay (RIPA) lysis buffer (140 mM NaCl, 8 mM $NaHPO_4$, 2 mM NaH_2PO_4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.05% sodium dodecyl sulfate [SDS], 0.2% phenylmethylsulfonyl fluoride). Pulse-chase experiments were processed as follows. Transfected cells were starved in the methionine-free DMEM medium containing 10% dialyzed fetal calf serum in the presence of 10 μM BFA for 30 min prior to labeling. Then cells were pulsed-labeled for 20 min with [^{35}S]methionine (400 μCi/sample) and chased in complete DMEM supplemented with 10 μM BFA. At the indicated time periods, radiolabeled cells were lysed in RIPA buffer.

CD4-Vpu binding experiments were all performed at 48 h posttransfection. Transfected cells were first starved in methionine-free DMEM for 30 min in the presence of 10 μM BFA. Then cells were pulse-labeled for 20 min with [^{35}S]methionine (400 μCi/sample) and chased in complete DMEM supplemented with the same concentration of BFA. At the indicated time periods, labeled cells were lysed in a digitonin lysis buffer, consisting of 1% digitonin, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, and 0.2% phenylmethylsulfonyl fluoride, as described before (1).

Following lysis, the labeled cell lysates were subjected to centrifugation at 14,000 rpm at 4°C. Cleared lysates were first immunoprecipitated with anti-CD4 (OKT4) monoclonal antibodies and subsequently immunoprecipitated with a rabbit anti-Vpu serum. Immunoprecipitates were analyzed on an SDS–12.5% polyacrylamide gel followed by autoradiography. Densitometric analysis of autoradiograms was performed with a Molecular Dynamics Personal densitometer using an Image QuantTM software version 3.22.

Protein secondary structure analysis by computer. The effects of mutants on the secondary structure of the Vpu protein were analyzed on a PowerMac computer, using the MacVector sequence analysis software (International Biotechnologies Inc., New Haven, Conn.). The secondary structure prediction of proteins is based on the Robson-Garnier and Chou-Fasman methods (4, 8).

RESULTS

Deletion of amino acids 411 to 419 in the cytoplasmic tail of CD4 abrogates Vpu binding. Several studies have shown that the cytoplasmic domain of CD4 is necessary and sufficient to confer sensitivity to Vpu (3, 13, 29, 31, 35). Moreover, a recent study demonstrates that a specific sequence between amino acids 402 to 420 in the cytoplasmic domain of CD4 contributes to Vpu binding (1). To identify precisely the Vpu binding site, we tested a series of CD4 truncation mutants, CD4Δ7, CD4Δ14, CD4Δ23, and CD4Δ32 (35) (Fig. 1A). Each of these CD4 mutants was cotransfected with the SVCMVVpu$^+$ expressor in COS-7 cells. Forty-eight hours posttransfection, cells were pulse-labeled with [^{35}S]methionine for 20 min and immediately lysed with 1% digitonin lysis buffer. Cell lysates were then immunoprecipitated first with an anti-CD4 antibody and subsequently with a Vpu antiserum. The immunoprecipitated proteins were analyzed on an SDS–12.5% polyacrylamide gel followed by autoradiography (Fig. 1B). Since the transfected

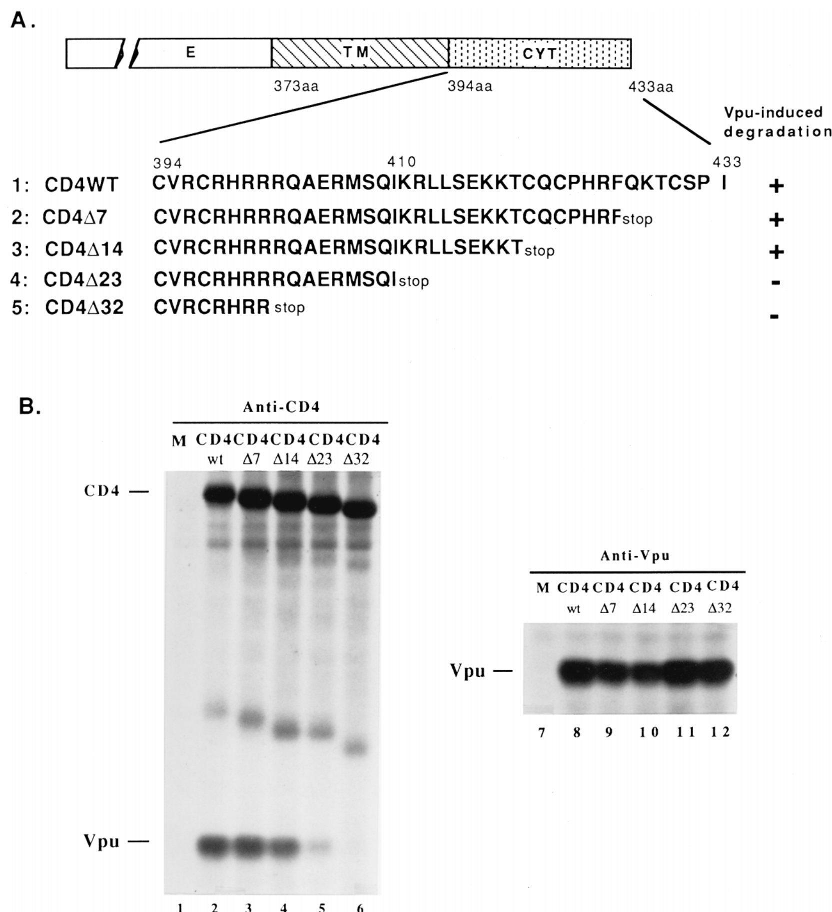

FIG. 1. Effects of C-terminal deletions of the cytoplasmic tail of CD4 on Vpu binding. (A) Schematic structure of the CD4 molecule and amino acid (aa) sequence of its cytoplasmic tail are shown at the top. E, TM, and CYT indicate the ectodomain, the transmembrane domain, and the cytoplasmic domain, respectively. Each deletion was made by introducing a stop codon at the indicated position in the cytoplasmic domain of the CD4 as described before (35). The sensitivity of each truncated CD4 to Vpu-mediated degradation is indicated on the right. Susceptibility to Vpu-mediated degradation is indicated by a +, whereas resistance is indicated by a −. (B) COS-7 cells cotransfected with the SVCMVVpu$^+$ expressor and each CD4 expression plasmid were labeled with [^{35}S]methionine for 20 min in the presence of 10 μM BFA and lysed with 1% digitonin lysis buffer. Labeled cell lysates were immunoprecipitated first with the monoclonal anti-CD4 antibody (OKT4) (left) and subsequently with the anti-Vpu serum (right). Immunocomplexes were separated by electrophoresis on an SDS-polyacrylamide gel and revealed by autoradiography. M indicates mock-transfected cells. CD4 and Vpu proteins are indicated.

cells were lysed immediately after a very short labeling period in these experiments, the degradation of wild-type CD4, CD4Δ7, and CD4Δ14 was not detected. However, under these conditions, it is clear that wild-type CD4 can efficiently bind wild-type Vpu (Fig. 1B, lane 2). CD4 mutants harboring a deletion of the last 7 or 14 C-terminal amino acids were also capable of binding Vpu (Fig. 1B, lanes 3 and 4). Conversely, when the last 23 or 32 amino acids were deleted, both of the resulting truncation mutants (CD4Δ23 and CD4Δ32) were affected in the ability to bind to Vpu (Fig. 1B, lanes 5 and 6), even though similar amounts of Vpu were detected in all of the cotransfected cell lysate samples (Fig. 1B, lanes 8 to 12). Faster-migrating bands were also detected upon transfection of CD4 and Vpu (Fig. 1B, lanes 2 to 6). These bands appear to represent proteolytic cleavage products of CD4. However, they do not correspond to CD4 degradation products resulting from the action of Vpu since they were detected even with deletion mutants of CD4 (CD4Δ23 and CD4Δ32) which are resistant to the effect of Vpu (Fig. 1B, lanes 5 and 6). In agreement with a previous study (1), these results indicate that the cytoplasmic domain of CD4 contributes to Vpu binding and that the sequence KRLLSEKKT, located between amino acids 411 and 419 in the cytoplasmic domain of CD4, may harbor the binding determinant. All of our attempts to coprecipitate CD4 with Vpu by using the antibodies directed against the C-terminal nanopeptide (HAPWDVDDL) of Vpu were unsuccessful (data not shown). It appears that upon binding of Vpu to CD4, the C-terminal region of Vpu may be masked, rendering the HAPWDVDDL epitope unavailable to the antibodies.

Disruption of the putative α-helical structure located in the membrane-proximal cytoplasmic domain of CD4 abrogates Vpu binding. To identify the Vpu binding determinant in the cytoplasmic domain of CD4, we first investigated whether specific amino acids present within the KRLLSEKKT segment were required for the interaction. We proceeded by using three previously described substitution mutants, CD43S, CD4LL413,4AA, and CD4KK417,8IG (35). Each of these CD4 substitution mutants was cotransfected with the Vpu double phosphorylation mutant (VpuSS52/56) plasmid. This biologically inactive mutant of Vpu was previously shown to increase the stability and detection of the CD4-Vpu interaction (1). Following labeling and cell lysis with digitonin lysis buffer, Vpu-CD4 complexes were immunoprecipitated with the anti-CD4 monoclonal antibody. The results of this experiment revealed

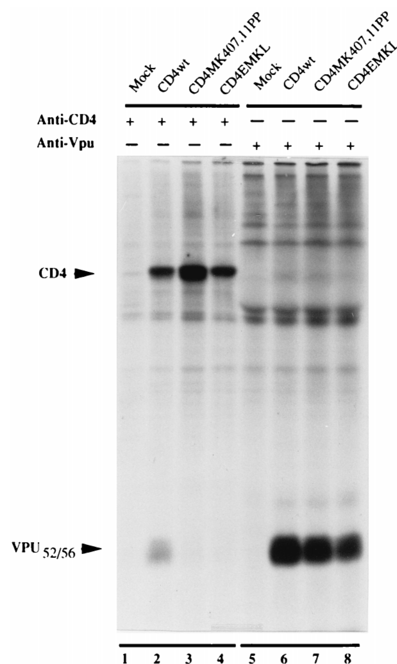

FIG. 2. Disruption of the predicted α-helical structure in the cytoplasmic tail of CD4 affects Vpu binding. COS-7 cells were cotransfected with 5 μg of plasmid encoding either wild-type CD4 (CD4wt), CD4EMKL, or CD4MK407,11PP (35) and 12.5 μg of the Vpu52/56 plasmid expressor. Forty-eight hours posttransfection, cells were labeled with [^{35}S]methionine for 20 min in the presence of 10 μM BFA and lysed with 1% digitonin lysis buffer. Cell lysates were first immunoprecipitated with the anti-CD4 monoclonal antibody, and the Vpu-CD4 complexes were analyzed on an SDS–12.5% polyacrylamide gel (lanes 1 to 4). Cell lysates were subsequently immunoprecipitated with the anti-Vpu serum. Immunoprecipitated Vpu proteins from each sample are shown in lanes 5 to 8. The positions of CD4 and Vpu are indicated.

that the substitution of individual amino acids within the KRLLSEKKT segment did not impair the binding of CD4 to Vpu (data not shown).

The KRLLSEKKT segment has been suggested to be part of a putative α-helical structure which is predicted to be present in the membrane-proximal cytoplasmic domain of CD4 (24, 35). We have previously shown that removal of this segment disrupted this α-helix and abolished the CD4 sensitivity to Vpu-induced degradation (35). To further verify whether this predicted α-helix is also involved in the Vpu-CD4 interaction, we used two previously described CD4 mutants, CD4MK407, 11PP and CD4EMKL, which have lost their inherent ability to form the α-helical structure as predicted by our computer-assisted structural analysis (35). After cotransfection of wild-type CD4, CD4MK407,11PP, or CD4EMKL with the expressor plasmid encoding VpuSS52/56 in COS-7 cells, binding experiments were performed as described in Materials and Methods. The results shown in Fig. 2 indicate that in contrast to wild-type CD4, both CD4EMKL and CD4MK407,11PP lost the ability to coprecipitate Vpu (Fig. 2; compare lanes 3 and 4 to lane 2). To rule out the possibility of variation of VpuSS52/56 expression among the different transfections, each sample was subsequently immunoprecipitated with the Vpu antiserum. Results show that similar amounts of VpuSS52/56 were expressed in each cotransfection (Fig. 2, lanes 6 to 8), while no CD4 or VpuSS52/56 was detected in mock-transfected cells (Fig. 2, lanes 1 and 5). These results clearly indicate that the conformation of the putative α-helix in the membrane-proximal cytoplasmic region of CD4 is critical for the binding of CD4 to Vpu. On the basis of these results, it can be speculated that this putative α-helix may act as an interface for the CD4-Vpu interaction that mediates CD4 degradation.

Phosphoacceptor sites Ser52 and Ser56, present in the cytoplasmic domain of Vpu, contribute equally to CD4 degradation. Several studies have demonstrated the importance of Vpu phosphorylation in the process of CD4 degradation (3, 20, 30). Mutations of Vpu phosphoacceptor sites and deletion of five amino acids between residues 47 and 52 in Vpu, which removed the Ser52 phosphorylation site, were shown to abolish the ability of Vpu to promote CD4 degradation (3, 20). However, it was not clear whether both phosphoacceptor sites, present within the cytoplasmic domain of Vpu, contribute equally to this process. To address this question, we tested the effect of single phosphorylation mutants (VpuS52G or VpuS56G) as well as double phosphorylation mutant (VpuSS52/56) on the ability of Vpu to bind and degrade CD4 molecules. COS-7 cells were cotransfected with wild-type CD4 expressor and either SVCMVVpu$^-$, SVCMVVpu$^+$, or each of the SVCMVVpu phosphorylation mutant expressors, as indicated in Fig. 3A. Forty-eight hours posttransfection, cells were labeled with [^{35}S]methionine for 5 h in the presence of 10 μM of BFA and lysed in RIPA buffer as described in Materials and Methods. Equal aliquots of cell lysates were first immunoprecipitated with an anti-CD4 antibody and subsequently immunoprecipitated with the anti-Vpu serum. The immunoprecipitated proteins were separated on an SDS–12.5% polyacrylamide gel and quantified by densitometry. The levels of CD4 detected in the presence of different Vpu mutants were calculated relative to the level of CD4 in the absence of Vpu (Vpu$^-$ sample), which was defined as 100% (Fig. 3A, lanes 6 to 10). The results show that expression of wild-type Vpu efficiently induced CD4 degradation (Fig. 3A; compare lane 7 to lane 6). All of the phosphorylation mutants, including single phosphorylation mutants VpuS52G and VpuS56G as well as the double phosphorylation mutant VpuSS52/56, lost the ability to degrade CD4. Levels of CD4 in these three samples were comparable to the CD4 levels found in the Vpu$^-$ sample (Fig. 3A; compare lanes 8 to 10 to lane 6). The absence of CD4 degradation in the presence of these Vpu phosphorylation mutants does not appear to be a consequence of Vpu expression variation since abundant Vpu protein was detected in each transfection (Fig. 3A, lanes 8 to 10).

In an attempt to verify whether these Vpu phosphorylation mutants could equally bind to CD4, Vpu mutants VpuS52G, VpuS56G, and VpuSS52/56 were tested in the coprecipitation assay described in Materials and Methods. The results clearly reveal that each of these Vpu phosphorylation mutants can efficiently bind to wild-type CD4 (Fig. 3B, lanes 2 to 4). Quantification of Vpu levels that are coprecipitated with CD4 by densitometric analysis demonstrate that no significant differences can be detected among the mutants (Fig. 3B, right panel). Taken together, these results indicate that both of Ser52 and Ser56 are dispensable for the binding of Vpu to CD4 but are equally critical for the induction of the proteolytic activity associated to Vpu since mutations affecting either of these two sites abrogate the ability of Vpu to degrade CD4 molecules.

The two amphipathic helices present in the Vpu cytoplasmic domain contribute to CD4 degradation. The cytoplasmic domain of Vpu has been predicted to form two amphipathic α-helices joined by a flexible turn containing two phosphoacceptor sites (Ser52 and Ser56), as shown in Fig. 3A (34). However, the relevance of these two amphipathic α-helices in Vpu function is still undefined. To verify whether these two α-helical structures contribute to the CD4-degrading capability of Vpu, we proceeded with a mutational analysis spanning the

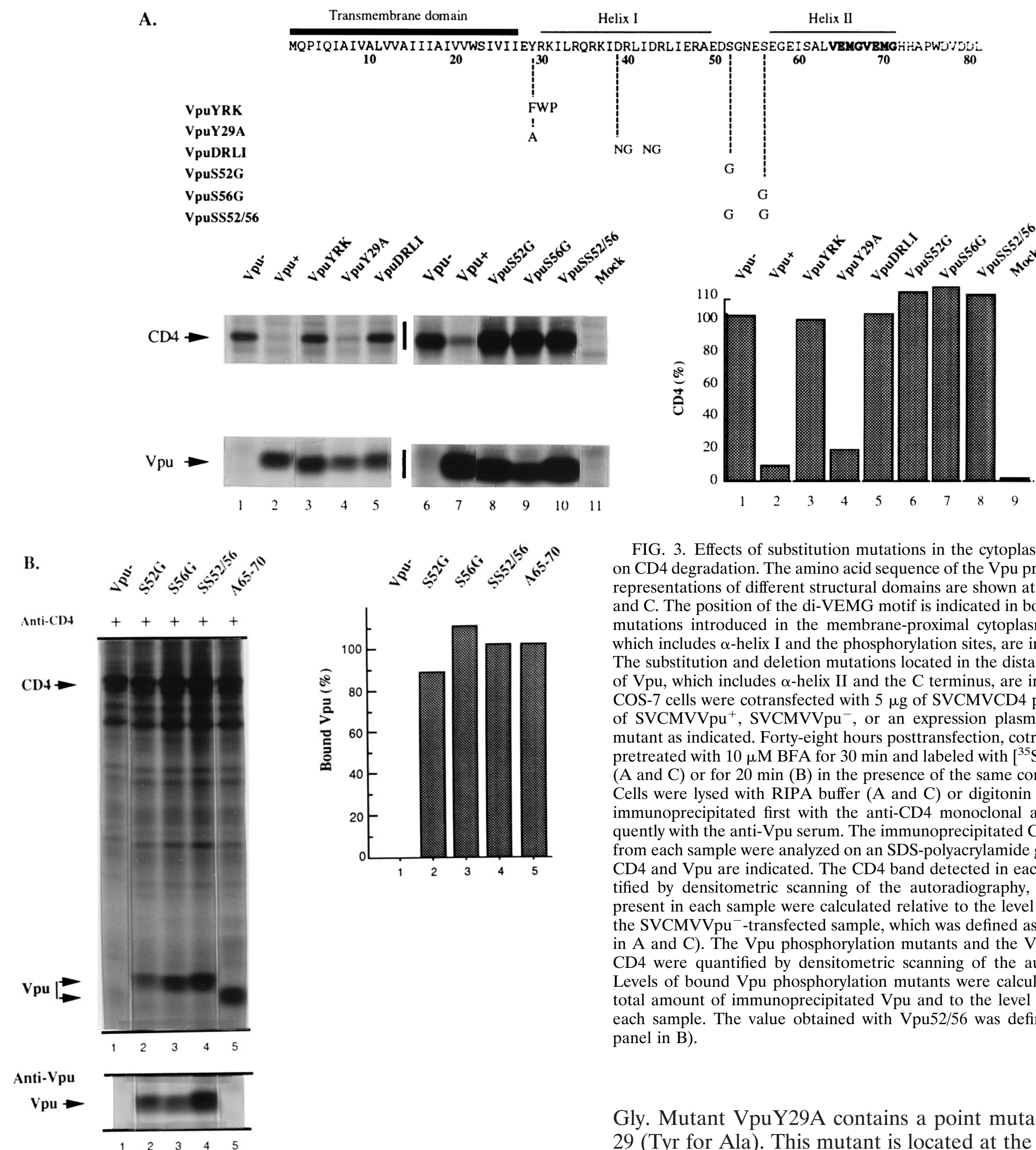

FIG. 3. Effects of substitution mutations in the cytoplasmic domain of Vpu on CD4 degradation. The amino acid sequence of the Vpu protein and schematic representations of different structural domains are shown at the top of panels A and C. The position of the di-VEMG motif is indicated in boldface. Substitution mutations introduced in the membrane-proximal cytoplasmic region of Vpu, which includes α-helix I and the phosphorylation sites, are indicated in panel A. The substitution and deletion mutations located in the distal cytoplasmic region of Vpu, which includes α-helix II and the C terminus, are indicated in panel C. COS-7 cells were cotransfected with 5 μg of SVCMVCD4 plasmid and 12.5 μg of SVCMVVpu$^+$, SVCMVVpu$^-$, or an expression plasmid encoding a Vpu mutant as indicated. Forty-eight hours posttransfection, cotransfected cells were pretreated with 10 μM BFA for 30 min and labeled with [^{35}S]methionine for 5 h (A and C) or for 20 min (B) in the presence of the same concentration of BFA. Cells were lysed with RIPA buffer (A and C) or digitonin lysis buffer (B) and immunoprecipitated first with the anti-CD4 monoclonal antibody and subsequently with the anti-Vpu serum. The immunoprecipitated CD4 or Vpu proteins from each sample were analyzed on an SDS-polyacrylamide gel. The positions of CD4 and Vpu are indicated. The CD4 band detected in each sample was quantified by densitometric scanning of the autoradiography, and levels of CD4 present in each sample were calculated relative to the level of CD4 detected in the SVCMVVpu$^-$-transfected sample, which was defined as 100% (right panels in A and C). The Vpu phosphorylation mutants and the VpuA65-70 bound to CD4 were quantified by densitometric scanning of the autoradiography (B). Levels of bound Vpu phosphorylation mutants were calculated relative to the total amount of immunoprecipitated Vpu and to the level of CD4 detected in each sample. The value obtained with Vpu52/56 was defined as 100% (right panel in B).

cytoplasmic domain of the protein, as indicated in Fig. 3A and C. Generally, substitutions were designed to either disrupt the conformation of the α-helical structure or substitute conserved amino acids. The secondary structure prediction of mutant proteins was based on the Robson-Garnier and Chou-Fasman methods (4, 8) and was obtained using the MacVector analysis software (International Biotechnologies Inc.). Mutants VpuYRK and VpuDRLI were designed to disrupt the conformation of the first α-helix structure of Vpu (Fig. 3A). Plasmid VpuYRK contains a three-amino-acid substitution in which Tyr29, Arg30, and Lys32 were replaced by Phe, Trp, and Pro, respectively. Mutant VpuDRLI harbors a four-amino-acid substitution in the middle region of the first α-helix, where Asp39, Arg40, Asp43, and Arg44 were replaced by Asn, Gly, Asn, and Gly. Mutant VpuY29A contains a point mutation at position 29 (Tyr for Ala). This mutant is located at the junction region between the transmembrane domain and the first predicted α-helix (Fig. 3A). Mutants VpuL63P and VpuA65-70 were designed to affect the integrity of the second α-helix of Vpu (Fig. 3C). VpuL63P introduces a proline residue in the middle region of the predicted second α-helix. VpuA65-70 carries a five-amino-acid substitution and was designed to replace the highly conserved di-VEMG motif found in this region. Finally, two Vpu mutants, VpuΔDVDDL and VpuH72D, contained mutations in the C-terminal end of the protein. VpuH72D carries a negatively charged Asp to replace a conserved positively charged His72, while VpuΔDVDDL has a deletion of the last C-terminal five amino acids (Fig. 3C, top panel).

Each Vpu mutant was tested for its ability to degrade wild-type CD4 by using the CD4 degradation assay described in Materials and Methods. Figures 3A and C (left panels) show the steady-state levels of CD4 detected in each cotransfection. The amount of CD4 detected were also quantified by densito-

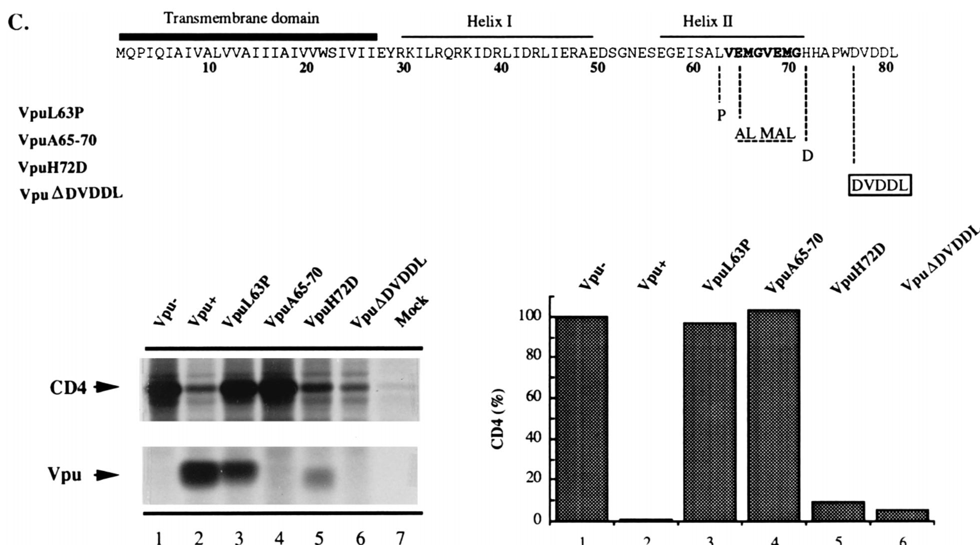

FIG. 3—*Continued.*

metric scanning analysis and expressed as a percentage of the CD4 level obtained when SVCMVCD4 was cotransfected with SVCMVVpu$^-$ (right panels). As expected, wild-type Vpu caused a significant reduction in the steady-state level of CD4 (Fig. 3A and C, lanes 2). The same effect was also observed with mutants VpuY29A, VpuH72D, and VpuΔDVDDL (Fig. 3A, lane 4; Fig. 3C, lanes 5 and 6). In contrast, all Vpu mutants located in either α-helix I or α-helix II, including VpuYRK, VpuDRLI, VpuL63P, and VpuA65-70, lost their inherent ability to degrade CD4 (Fig. 3A, lanes 3 and 5; Fig. 3C, lanes 3 and 4). The loss of CD4 degradation ability by these mutants was not the result of a decrease in their expression levels or stability since most of the mutants analyzed in this study were shown to be stable and adequately expressed (Fig. 3A and C, left panels). It should, however, be noted that the anti-Vpu peptide antiserum could not detect Vpu mutants VpuA65-70 and VpuΔDVDDL (Fig. 3C, lanes 4 and 6). Lack of reactivity of the anti-Vpu antiserum with VpuΔDVDDL is expected since this antiserum was raised against a peptide, HAPWDVDDL, derived from the C-terminal end of Vpu (5). Failure to detect the VpuA65-70 mutant with the Vpu antiserum (Fig. 3C, lane 4; Fig. 4, bottom panel) suggests that the substitutions intro-

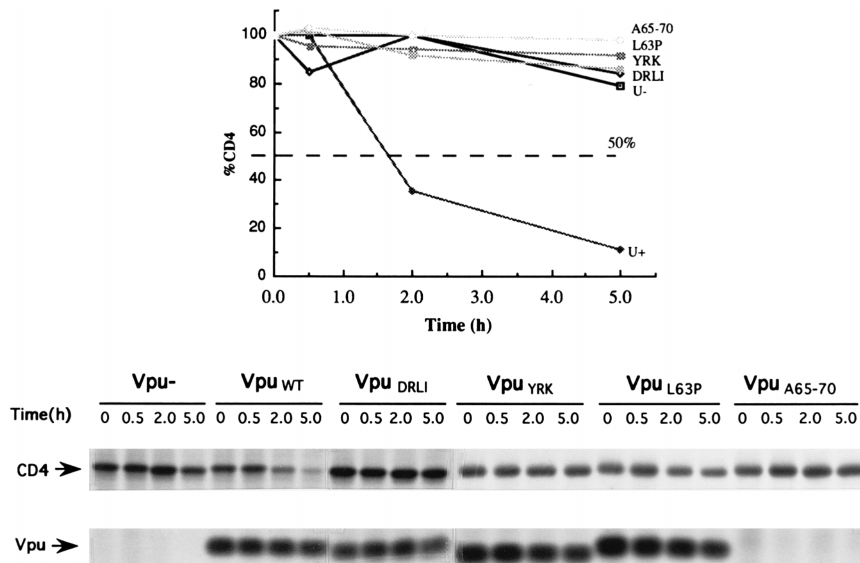

FIG. 4. Analysis of the rate of CD4 decay in presence of different mutated Vpu. COS-7 cells were cotransfected with 5 μg of SVCMVCD4 plasmid and 12.5 μg of SVCMVVpu$^+$, SVCMVVpu$^-$, or a plasmids encoding a Vpu mutant as indicated. Forty-eight hours posttransfection, cells were starved in methionine-free DMEM medium containing 10 μM BFA for 30 min prior to labeling. Cells were then pulse-labeled for 20 min with [^{35}S]methionine (400 μCi/sample) and chased in complete DMEM supplemented with 10 μM BFA. At the indicated time periods, radiolabeled cells were lysed on ice in RIPA buffer and immunoprecipitated first with the anti-CD4 monoclonal antibody and subsequently with the anti-Vpu serum, as indicated. The immunoprecipitated CD4 and Vpu proteins were then analyzed on an SDS–12.5% polyacrylamide gel (bottom). The rate of CD4 decay was quantified by densitometric scanning. CD4 bands from the autoradiogram are shown in the bottom panel. As shown in the top panel, the levels of CD4 present at different times were calculated relative to the amounts detected at the beginning of the chase (which was defined as 100%).

duced affected the conformation of the C-terminal end, which contains the antibody epitope. Indeed, abundant amount of VpuA65-70 can be coimmunoprecipitated with CD4 by using the anti-CD4 antibody, indicating that this mutant is stable and retains its affinity to CD4 (Fig. 3B, lane 5).

To confirm and quantitatively determine the effects of the different Vpu mutants on CD4 degradation, the turnover of wild-type CD4 in the presence of wild-type or mutated Vpu, (VpuYRK, VpuDRLI, VpuL63P, or VpuA65-70) was evaluated by pulse-chase experiments as shown in Fig. 4. As expected, the half-life of CD4 was significantly reduced in the presence of wild-type Vpu (less than 2 h). In contrast, the half-life of CD4 in the absence of Vpu or in the presence of VpuDRLI, VpuYRK, VpuL63P, or VpuA65-70 was more than 5 h (Fig. 4, upper panel). The result of this experiment also confirmed that VpuDRLI, VpuYRK, and VpuL63P were stable in COS-7 cells (Fig. 4, bottom panel), with calculated half-life of more than 5 h for each of the mutants and wild-type Vpu (data not shown). Interestingly, none of these α-helix mutations impaired the phosphorylation status of the protein (data not shown).

Overall, the data indicate that Vpu mutants carrying mutations affecting the conformation of α-helix I and α-helix II lost the ability to degrade CD4. This result strongly suggests that in addition to phosphoacceptor sites (Ser52 and Ser56), the overall structure of the cytoplasmic domain of the protein is required for Vpu-mediated CD4 degradation. In addition, the ability of VpuH72D and VpuΔDVDDL to mediate CD4 degradation suggests that the C-terminal domain of Vpu is not necessary for the protein's biological activity.

The first α-helical structure, located in the membrane-proximal region of the cytoplasmic domain of Vpu, is involved in the interaction with CD4. To determine whether the two putative α-helical structures in the C-terminal hydrophilic region of Vpu contribute equally to the interaction between Vpu and CD4, we tested the CD4-binding abilities of Vpu mutants VpuYRK, VpuDRLI, and VpuL63P by pulse-chase analysis as described in Materials and Methods. Since the VpuS52G mutant lost its ability to degrade CD4 but retained its CD4-binding capacity (Fig. 3A and B), we used VpuS52G as a positive control. In agreement with our previous results, none of the mutants tested were able to degrade CD4 (Fig. 5A). In contrast, the CD4-binding capacities of these mutants varied. While VpuS52G was shown to coimmunoprecipitate efficiently with CD4 (Fig. 5, lanes 1 to 3), only traces of α-helix I mutants VpuDRLI and VpuYRK were found to interact with CD4 (Fig. 5A, lanes 4 to 6 and lanes 7 to 9). Densitometric analysis of autoradiograms of Fig. 5A shows that approximately 20% of VpuYRK and 25% of VpuDRLI were bound to CD4 compared to VpuS52G (100%) (Fig. 5A). Conversely, mutations introduced in α-helix II did not greatly affect the ability of the protein to interact with CD4. Mutant VpuA65-70, which carries five amino acid substitutions in this α-helix, retained a strong ability to interact with CD4 (Fig. 3B, lane 5). Likewise, mutant L63P retained 50% of CD4-binding ability even though a proline, which provokes a strong distorting effect and has very low helix propensity, was introduced in the middle region of α-helix II (Fig. 5A, lanes 10 to 12). Results in Fig. 5B clearly indicate that all mutated Vpu proteins were expressed at comparable levels in this coprecipitation experiment. Taken together, these results indicate that α-helix I present in the membrane-proximal cytoplasmic region of Vpu is necessary to mediate CD4 degradation since it may provide a binding interface that allows interaction between Vpu and CD4. Following this binding process, an intact α-helix II structure as well as phosphorylation of Vpu appear necessary for the mechanism responsible for degradation of the CD4 molecule.

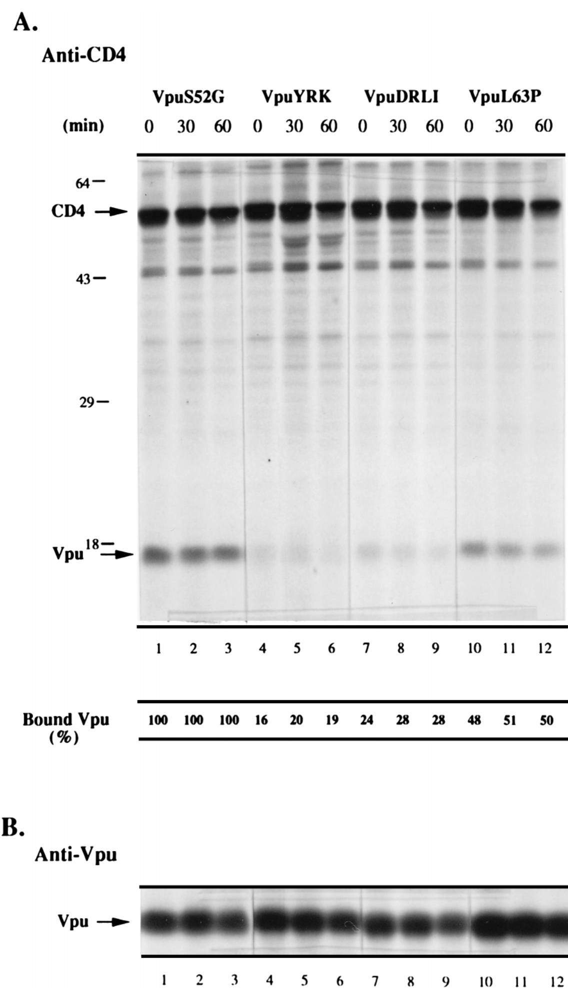

FIG. 5. CD4-binding abilities of Vpu cytoplasmic domain mutants. COS-7 cells were cotransfected with 5 μg of wild-type CD4 expression plasmid and 12.5 μg of either VpuS52G (lanes 1 to 3), VpuYRK (lanes 4 to 6), VpuDRLI (lanes 7 to 9), or VpuL63P (lanes 10 to 12) expression plasmid. Forty-eight hours posttransfection, cells were pulse-labeled for 20 min with [^{35}S]methionine and chased for 0, 30, or 120 min in complete DMEM in the presence of BFA. After lysis with 1% digitonin lysis buffer, cell lysates were immunoprecipitated first with the anti-CD4 monoclonal antibody (A) and subsequently with the anti-Vpu serum (B). Immunoprecipitates were separated on an SDS–12.5% polyacrylamide gel and visualized by autoradiography. The positions of CD4, Vpu, and molecular weight markers (in kilodaltons) are indicated on the left. Quantitation of Vpu bound to CD4 is shown at the bottom of panel A. These values represent the levels of bound Vpu mutants relative to the total amount of immunoprecipitated Vpu and to the level of CD4 detected in each sample. The value obtained with VpuS52G was defined as 100%.

DISCUSSION

Previous studies have shown that the CD4 degradation induced by Vpu is a specific phenomenon since Vpu is unable to degrade CD8 or gp160 (13, 31, 33). Indeed, the presence of specific amino acid residues located in the CD4 cytoplasmic domain appears to confer sensitivity to Vpu-mediated degradation (3, 13, 29, 35). Moreover, a specific interaction between Vpu and CD4 was recently demonstrated by coimmunoprecipitation (1). Binding of Vpu was shown to be necessary but not sufficient to mediate CD4 degradation, indicating that the interaction between CD4 and Vpu represents an early step critical in triggering a process leading to CD4 degradation (1). In the present study, we further analyzed the sequence and structural requirements necessary for the binding between Vpu

and CD4. The results indicate that α-helical structures predicted to be formed in the cytoplasmic domain of CD4 and Vpu are required for both binding and degradation of the CD4 molecules. Moreover, we show that phosphorylation of either or both of the Vpu phosphoacceptor sites is dispensable for binding to CD4 but is required for CD4 degradation. Finally, evidence presented here indicates that the Vpu C terminus is not required in the processes leading to CD4 degradation.

Apart from retaining determinants essential for the Vpu-mediated CD4 degradation, sequences located between amino acids 402 and 420 in the cytoplasmic tail of CD4 were shown to be necessary and sufficient to allow Vpu binding (1). Using CD4 cytoplasmic tail deletion mutants that were described previously (35), we showed that the deletion of the sequence KRLLSEKKT, located between amino acids 411 and 419 in the cytoplasmic domain of CD4, prevented CD4 binding to Vpu. However, the abrogation of Vpu binding resulting from the deletion of this sequence does not appear to be a consequence of the removal of specific amino acids or motifs, since several substitution mutations in this segment still retain the ability to bind Vpu (data not shown). One possibility that could explain the lack of Vpu binding to these CD4 deletion mutants is that deletions disrupted structural element(s) in the cytoplasmic tail of CD4 which may be critical for interaction with Vpu. In fact, a report has predicted a putative α-helical structure in the membrane-proximal region of CD4 (24). In an attempt to verify whether this helical structure also contributes to Vpu binding, we tested two CD4 substitution mutations (CD4EMKL and CD4MK407,11PP), which were predicted to disrupt the α-helix and were shown to abolish CD4 sensitivity to Vpu (35). Results from this experiment show that both of these helix-disrupting mutants are unable to bind Vpu, suggesting that this putative α-helical structure in the membrane-proximal cytoplasmic region of CD4 may act as a binding interface for interaction with Vpu. Interestingly, CD8, which is not sensitive to Vpu action, lacks this putative α-helix region in its cytoplasmic domain (24, 31). Since the lack of Vpu binding by the helix-disrupting mutants correlated with their resistance to degradation, it is likely that in the case of CD4, a major requirement for degradation is the ability to interact with Vpu. However, we do not rule out at present the possibility that upon binding to Vpu, other sequences in the cytoplasmic tail of CD4 are necessary for degradation to occur.

The cytoplasmic domain of Vpu has been predicted to form a structure of α-helix-turn-α-helix in which a flexible and strongly acidic turn region contains the phosphoacceptor sites, Ser52 and Ser56, of the protein (10, 21, 34). Although functional studies have demonstrated that phosphorylation of Vpu is important for the degradation of CD4 (20), the relative importance of these two phosphoacceptor sites (Ser52 and Ser56) in Vpu function has not been defined. In the present study, we now demonstrate that the two Vpu phosphoacceptor sites are equally required for the specific event leading to the degradation of the CD4 molecule, even though both do not contribute to the ability of Vpu to bind CD4. These results are not in agreement with a recent study which showed that single and double phosphorylation mutants of Vpu (S52R and SS52/56NN) lost the ability to bind CD4 in a two-hybrid assay using yeast cells (16). These conflicting results could conceivably be due to the nature of the substitution mutations used in each study, to the different testing systems (COS versus yeast cells) used, or to the different contexts in which Vpu or CD4 were presented (native proteins versus GAL4 fusion proteins containing either the cytoplasmic tail of CD4 or the cytoplasmic domain of Vpu). In contrast, our results are in agreement with the data of Bour et al. which showed that a deletion mutant of Vpu (Vpu52) as well as a double phosphorylation mutant (Vpu2/6), both unable to degrade CD4, retained the ability to interact with the CD4 cytoplasmic domain (1).

Another important feature of Vpu is the presence of two amphipathic α-helices (residues 30 to 50 and 57 to 72) of opposite polarity in its cytoplasmic domain (21, 34). Several pieces of indirect evidence suggested that these structures played a role in the ability of Vpu to degrade CD4. (i) Substitution of the phosphoacceptor sites did not affect the helical structures of the Vpu cytoplasmic domain, yet binding between CD4 and Vpu phosphorylation mutants still occurred, indicating that other elements within Vpu were involved in the interaction with the cytoplasmic tail of CD4 (20, 34) (Fig. 3A and B). (ii) Bour et al. reported that a synthetic peptide comprising the cytoplasmic domain of Vpu from residue 32 to 81 was not found to bind CD4 in an in vitro assay (1). However, this peptide was shown previously to form α-helical structures in aqueous solution only when trifluoroethanol, which mimics a membrane-like environment, was present (10). Results from this study clearly indicate that both amphipathic α-helices present in the cytoplasmic region of Vpu are involved in CD4 degradation. Surprisingly, the contribution of each α-helix to the process of CD4 degradation appears to be distinct. α-Helix I is located in the membrane-proximal cytoplasmic region upstream of the phosphorylation sites of Vpu. Mutations introduced within this α-helix, including VpuYRK and VpuDRLI, severely affected the capacity of Vpu to mediate the degradation of CD4. Impairment of Vpu function by these mutations was not the result of the instability of the mutant proteins since their turnover was comparable to that of wild-type Vpu (Fig. 4). Moreover, these mutations did not affect Vpu intracellular localization or Vpu phosphorylation (data not shown). In fact, our results clearly show that these mutations, which were designed to affect the conformation of α-helix I, impaired the CD4-binding ability of Vpu. In contrast, the data show that α-helix II, which is located distal to the phosphorylation sites of Vpu, may not be the primary binding site for CD4. Indeed, although a five-amino-acid substitution (VpuA65-70) or a substitution by a proline (VpuL63P) in the middle of this helical region inhibited CD4 degradation, these mutations did not significantly affect the capacity of Vpu to interact with CD4 compared with α-helix I mutants. Interestingly, in the case of VpuA65-70, the mutant protein was still able to bind CD4 even though a conformational change of the C terminus was suggested by the inability of the antipeptide serum to precipitate the protein. In this regard, all of our attempts to immunoprecipitate CD4-Vpu complexes with our anti-Vpu peptide serum have been unsuccessful. Since the antibodies are directed against a peptide corresponding to the last nine amino acids of Vpu, it appears that this region of Vpu may be masked or may undergo a conformational change upon binding to CD4. The exact role of α-helix II in Vpu-mediated CD4 degradation remains to be defined. It is possible that the mutations introduced in this α-helix disrupt the optimal conformation of the Vpu protein which is required for CD4 degradation. Alternatively, this α-helix may be directly involved in an interaction with other unidentified cellular factor(s) which contribute to the processes leading to CD4 degradation. Experiments aimed at the identifying potential Vpu cellular partners are currently being pursued.

The carboxyl-terminal region of Vpu has previously been shown to be required for Vpu-mediated CD4 degradation since a deletion of six amino acids prevented the degradation of CD4 in an in vitro assay using rabbit reticulocyte lysate (3). In contrast, our results indicate that the introduction of a substitution mutation (VpuH72D) or a deletion mutation that truncates the last five amino acids of the Vpu C terminus did

not affect the activity of the protein. This apparent discrepancy is likely due to the different deletion mutations used (deletion of six versus five amino acids) and may possibly indicate the importance of the highly conserved tryptophan residue located at position 76 for Vpu function. However, we cannot rule out the possibility that these deletions have different effects on the overall structure of the protein.

Overall, in this study, we present evidence that structural elements in the cytoplasmic domain of CD4 and Vpu contribute to their specific interaction. It is conceivable that both the putative helical structures in the cytoplasmic tail of CD4 and those in the membrane-proximal cytoplasmic region of Vpu mediate the interaction between the two molecules and allow the subsequent steps leading to CD4 degradation. Also, our study demonstrates that this proteolytic activity mediated by Vpu requires the phosphorylation of both phosphoacceptor sites Ser52 and Ser56 and the presence of a second helical structure in the distal cytoplasmic region of Vpu.

ACKNOWLEDGMENTS

The first two authors contributed equally to this work.

We thank Ramu Subbramanian for critical reading of the manuscript.

N.D. is a recipient of a studentship from the National Health Research and Development Program (NHRDP), Health and Welfare Canada. E.A.C. is a recipient of an NHRDP AIDS career award. This work was supported by grants from NHRDP, the Medical Research Council of Canada, and the Fonds pour la formation de chercheur et l'aide à la recherche to E.A.C.

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