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Human Immunodeficiency Virus Type 1 Vpu Protein Is an Oligomeric Type I Integral Membrane Protein

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The human immunodeficiency virus type 1 Vpu protein is a 16-kDa phosphoprotein which enhances the efficiency of virion production and induces rapid degradation of CD4, the cellular receptor for human immunodeficiency virus. The topology of membrane-inserted Vpu was investigated by using in vitro-synthesized Vpu cotranslationally inserted into canine microsomal membranes. Proteolytic digestion and immunoprecipitation studies revealed that Vpu was a type I integral membrane protein, with the hydrophilic domain projecting from the cytoplasmic membrane face. In addition, several high-molecular-weight proteins containing Vpu were identified by chemical cross-linking. Such complexes also formed when wild-type Vpu and a Tat-Vpu fusion protein were coexpressed. Subsequent analysis by one- and two-dimensional electrophoresis revealed that these high-molecular-weight complexes consisted of homo-oligomers of Vpu. These findings indicate that Vpu is a type I integral membrane protein capable of multimerization.

Human immunodeficiency virus type 1 (HIV-1) is a member of the lentivirus subfamily of *Retroviridae*. The genomes of lentiviruses encode structural (*gag*), enzymatic (*pol*), and surface envelope (*env*) proteins, as well as several additional proteins. One accessory protein of HIV-1, Vpu, is a membrane-bound phosphoprotein composed of an amphipathic sequence of 81 amino acids, with a hydrophobic N-terminal portion, and a hydrophilic C-terminal portion. Alignment of Vpu sequences from HIV isolates shows that Vpu is a highly conserved protein and contains a sequence of 10 amino acids that are invariant among all Vpu-containing HIV isolates (13). Although Vpu is synthesized in amounts similar to the Env glycoprotein in infected cells, it has not been detected in cell-free virions (21). Vpu is expressed after HIV infection in vivo; antibody to Vpu can be detected in approximately 30% of infected persons as early as 3 months following infection (15, 22).

The function of Vpu remains unclear; in T-cell lines, comparison of replication of *vpu*$^+$ and *vpu* mutant infectious molecular clones suggested that Vpu is involved in late steps in virus replication; decreases in the yield of extracellular virus with increases in levels of cell-associated virus proteins were documented (21, 22, 29). In addition, increased intracellular budding and aberrant budding structures at the plasma membrane were observed (11). Perhaps as a result of these phenomena, cells infected with *vpu* mutant virus demonstrate cytopathic effects earlier in the course of infection than cells infected with isogenic *vpu*$^+$ HIV-1 (11). In addition to the effect of Vpu on HIV particle production, recent studies have demonstrated that Vpu also induces intracellular degradation of the CD4 molecule. Kinetic analyses have revealed that the stability of CD4 protein in the presence of Vpu is reduced 30- to 40-fold (27, 28). The findings that Vpu may affect the efficiency of particle production and CD4 degradation suggest that Vpu may serve several independent functions in the course of HIV-1 infection.

The mechanism by which Vpu affects CD4 stability and virion particle production remains unclear; it is not known whether Vpu interacts directly with other cellular or viral proteins or exerts its effects indirectly by altering the intracellular milieu. Because Vpu is a membrane protein, the interactions between Vpu and other proteins are critically dependent on the orientation of the Vpu protein in membranes. To understand the function of Vpu, we characterized the structure of Vpu in membranes and the possible interactions of Vpu with other proteins.

Orientation of Vpu in membranes. Previously (21) we demonstrated that the Vpu protein synthesized in vitro in rabbit reticulocyte lysates was cotranslationally inserted into canine microsomal membrane vesicles (CMM). We have recently demonstrated that Vpu protein synthesized in this system is biologically active and is able to degrade CD4 in vitro (1). CMM have a defined sidedness, with the inside of the vesicle corresponding to the endoplasmic reticulum luminal or extracellular face and the outside of the vesicle representing the cytoplasmic face (26). This property of CMM enabled us to determine the membrane topology of in vitro-synthesized Vpu in a series of protease protection and antibody-binding studies.

In the protease protection experiments, we determined the sensitivity of Vpu to digestion by the nonspecific protease proteinase K. Exposure to protease will degrade proteins protruding from the exterior face of microsomal membranes, while proteins oriented into the membrane lumen are protected because the membrane excludes the protease. As an internal control for the integrity of microsomal membranes after limited proteolytic digestion, the human CD4 gene was included in the experiment. CD4 is inserted into membranes with its extracellular hydrophilic portion facing into the lumen of the membrane vesicle and the cytoplasmic C-terminal domain facing the outside of the microsomal membrane; protease treatment of membrane-bound CD4 should result in degradation of not more than 40 amino acids from its cytoplasmic tail.

Vpu was translated in a rabbit reticulocyte lysate in the presence of CMM by using in vitro run-off RNA transcripts of the Vpu-expressing plasmid pSP-9 (Fig. 1) and digested with proteinase K. As shown in Fig. 2, the 16-kDa Vpu protein was rapidly and completely degraded within 1 min of proteinase K digestion (Fig. 2A, Vpu). No distinct proteo-

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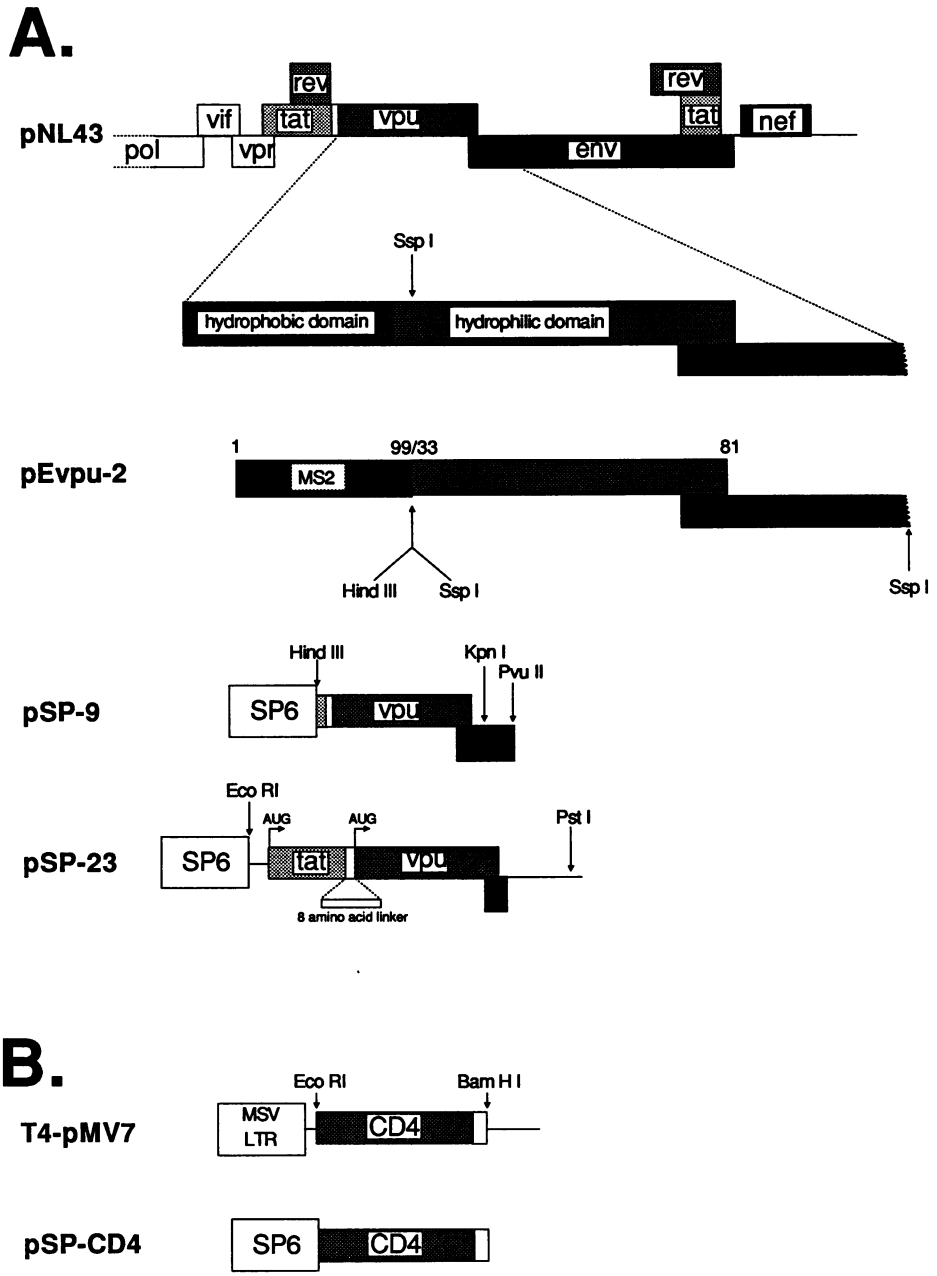

FIG. 1. Vpu and CD4 expression plasmids. (A) The bacterial expression plasmid pEvpu-2 expresses a fusion protein composed of amino acids 1 to 91 of the MS2 replicase and amino acids 33 to 81 of Vpu and was constructed by cloning the Vpu-containing *Ssp*I fragment of pNLA-1 (20) downstream of a filled-in *Hin*dIII site of the *Escherichia coli* expression plasmid pPLc24 (16). The MS2-Vpu fusion protein was expressed in *E. coli*, purified (19), and used to inoculate rabbits. The in vitro transcription plasmid pSP-9 contains the *Hin*dIII-*Pvu*II fragment (positions 6026 to 7073, by the numbering of Myers et al. [13]) of pNL43 downstream of the bacteriophage SP-6 promoter sequence and was linearized with *Kpn*I prior to in vitro transcription. PSP-23 encodes a Tat-Vpu fusion protein consisting of 43 N-terminal amino acids of Tat and the entire 81-amino-acid Vpu protein; the Tat and Vpu sequences were linked via an 8-amino-acid spacer with the nucleotide sequence 5′-CGA GGG GAT CGA TCC GGC CAA GCT-3′ inserted at the *Bsu* 36I site (position 5958). pSP-23 was linearized with *Pst*I prior to transcription. (B) pSP-CD4 contains the *Eco*RI-*Bam*HI fragment of T4-pMV7 cloned into the *Eco*RI-*Bam*HI sites of pSP65 downstream of the SP6 promoter and was linearized with *Bam*HI prior to in vitro transcription.

lytic fragment of Vpu was detected, although a smear of radioactivity was present, suggesting that the membrane anchor for Vpu contains less than approximately 30 residues; the mobility of a peptide fragment of that size (3,000 to 3,500 Da) is at the limit of resolution in the 12.5% acrylamide gel.

In vitro translation of the CD4 RNA from plasmid pSP-CD4 (Fig. 1) in the presence of microsomal membranes revealed two proteins with estimated molecular sizes of 48,000 and 55,000 Da (Fig. 2A, CD4). Studies by Tifft and coworkers (24) revealed that the higher-molecular-weight species is a glycosylated (+CHO) form of CD4; since

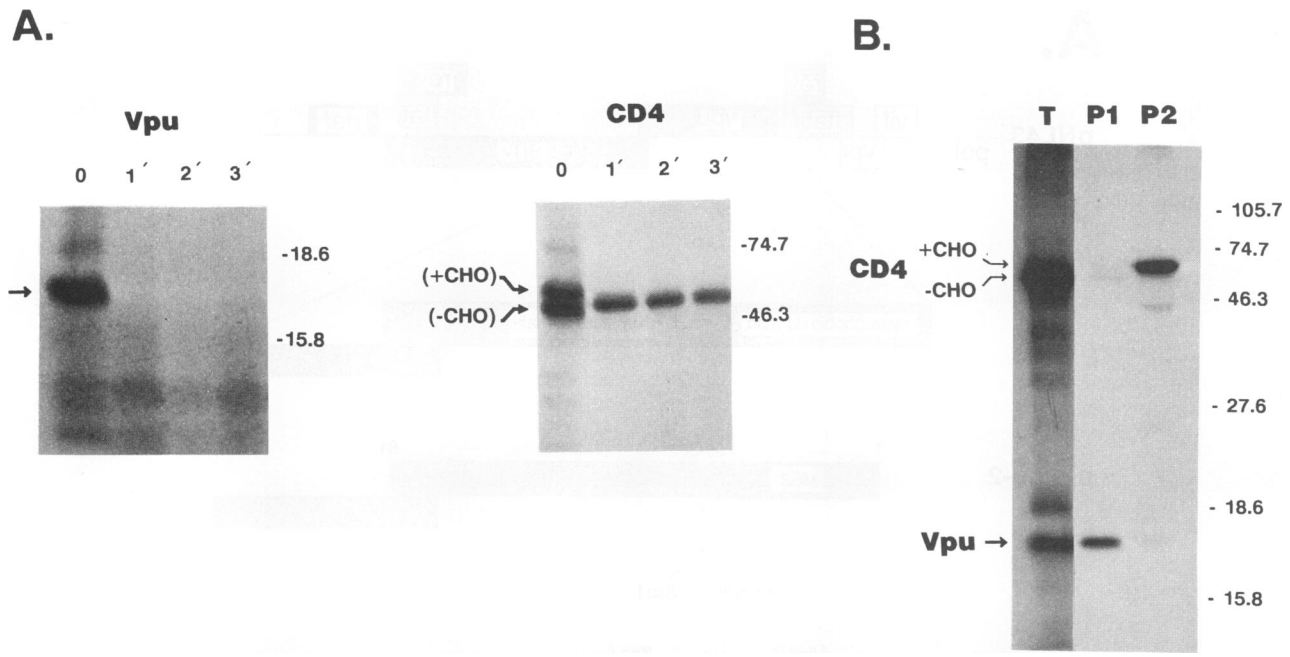

FIG. 2. Analysis of Vpu topology. (A) Protease protection studies. Run-off transcripts of pSP-9 and pSP-CD4 were translated in vitro in the presence of CMM and [^{35}S]methionine as described before (21) and mixed together, and proteinase K (Boehringer Mannheim) was added to a final concentration of 0.7 mg/ml. Aliquots of the translation mixture were removed after 1, 2, and 3 min of incubation at room temperature; samples were then diluted 1:13 in phosphate-buffered saline and precipitated with trichloroacetic acid (TCA; 10% final concentration). The precipitated proteins were pelleted, washed twice with 2.5% TCA, and then dissolved in sample buffer (4% SDS, 10% 2-mercaptoethanol, 10% glycerol, 125 mM Tris-HCl [pH 6.8]; 5 to 10 μl of 0.5 N NaOH was added to each sample to neutralize TCA). The TCA precipitates were subjected to SDS-PAGE (21) on 12.5% acrylamide gels to identify Vpu or 10% acrylamide gels to resolve glycosylated (+CHO), nonglycosylated (−CHO), and proteinase K-resistant forms of CD4. (B) Antibody recognition studies. Vpu and CD4 were translated in the presence of microsomal membranes and then incubated with anti-Vpu (1:5) or anti-CD4 (1:20) antiserum for 1 h at 4°C. Anti-Vpu antiserum was elicited in rabbits by using a bacterially expressed MS2-Vpu fusion protein composed of amino acids 1 to 91 of the MS2 replicase and amino acids 33 to 81 of Vpu. A monospecific, polyclonal antiserum to the extracellular portion of CD4 was raised by immunizing rabbits with purified sCD4-IgG (gift of Genentech). After antibody binding, membranes were purified by centrifugation at 100,000 × *g* for 30 min through a 30% sucrose layer onto a 60% sucrose cushion in an SW 50.1 rotor. The 30%-60% sucrose interface was disrupted in TNTN (0.01 M Tris [pH 7.4], 300 mM NaCl, 0.05% Nonidet P-40, 0.3% Tween 20), and antigen-antibody complexes were isolated with protein A-Sepharose (P1). To detect membrane-associated Vpu and CD4 which were not accessible to the C-terminal Vpu antiserum or the N-terminal CD4 antiserum, the supernatants from the first round of immunoprecipitation were incubated with CD4 and Vpu antisera, and antigen-antibody complexes were isolated by protein A-Sepharose (P2) and subjected to SDS-PAGE. As a control, an aliquot of the total lysate was immunoprecipitated with a mixture of Vpu and CD4 antisera to identify the proteins (T). The positions of molecular size markers are indicated (in kilodaltons).

glycosylation occurs after cotranslational insertion, this larger glycosylated form represents CD4 which is inserted into membranes. The shorter, nonglycosylated form of CD4 (Fig. 2A, −CHO) either is not inserted into the membrane or is inserted aberrantly and unable to undergo glycosylation. Analysis of proteinase K digestion results revealed that the glycosylated form of CD4 was protected (Fig. 2A, CD4, +CHO), while the nonglycosylated form of CD4 was completely degraded (Fig. 2A, −CHO); as expected, a slight increase in the mobility of the glycosylated, membrane-inserted form of CD4 was detected (Fig. 2A, CD4), indicating that the cytoplasmic portion of CD4 was removed by proteinase K. When membranes were disrupted in 0.5% Nonidet P-40 detergent prior to proteinase K treatment, CD4 was completely sensitive to digestion and was degraded within 1 min (data not shown), demonstrating that the CD4 protein, in the absence of membrane insertion, is highly sensitive to proteinase K degradation. These data confirm the reported membrane orientation of CD4 and demonstrate that the lumen of CMM was inaccessible to proteinase K.

We confirmed these findings and identified the C-terminal hydrophilic domain as the portion of Vpu projecting from the membrane surface in a series of antibody-binding studies. From the amphipathic nature of Vpu, we would predict that the hydrophilic domain projects from the face of CMM and should therefore be accessible to antisera directed against this portion of Vpu. Again, we used CD4 as a control for membrane integrity; the extracellular domain of CD4 is expected to be oriented into the lumen of the membrane vesicles and should therefore be inaccessible to antiserum raised against the CD4 extracellular domain. Intact Vpu and CD4 were translated independently in the presence of microsomal membranes, mixed, and incubated with rabbit polyclonal antiserum to the C-terminal portion of Vpu (amino acids 33 to 81) and rabbit polyclonal antiserum to the extracellular domain of CD4. After binding, membranes and bound antibody were isolated from the bulk reaction mix by centrifugation on a two-step sucrose gradient: the 30% sucrose layer excluded free protein and antigen-antibody complexes, while the 60% sucrose layer provided a cushion on which to pellet intact membranes. The membrane pellet from the 30%-60% sucrose interface was detergent disrupted, and antigen-antibody complexes present were immo-

bilized by protein A-Sepharose and processed for electrophoresis (Fig. 2B, P1).

The proteins in the disrupted membrane fraction which were not present as antigen-antibody complexes and which were not immobilized by protein A-Sepharose were subjected to a second round of immunoprecipitation with anti-Vpu and anti-CD4 sera (Fig. 2B, P2). As shown in Fig. 2B, Vpu was found almost exclusively in the P1 fraction, demonstrating that the C-terminal portion of Vpu protruded from the membrane face and was accessible to antibody binding. Only trace amounts of Vpu were detected in the second round of immunoprecipitation. In contrast, the glycosylated membrane-associated form of CD4 was detected exclusively in P2 but not in P1, demonstrating that the extracellular portion of CD4 was inaccessible to the CD4-specific antibodies until after the membrane was disrupted by detergent (Fig. 2B, P2). Nonglycosylated, non-membrane-bound CD4 was not detected in P2 because free protein and antigen-antibody complexes not present in the membranes were separated from the membrane pellet during centrifugation through the 30% sucrose layer. The result from this experiment is consistent with those from the proteinase K digestion studies and confirms that Vpu is an integral membrane protein, with its hydrophilic C-terminal domain directed into the cytoplasm; Vpu thus has the topology of a type I integral membrane protein.

Vpu forms high-molecular-weight complexes in vivo and in vitro. The finding that Vpu projects from the cytoplasmic face of microsomal membranes suggests that Vpu affects CD4 degradation and HIV particle production by protein interactions on the cytoplasmic side of membranes. We investigated whether Vpu does associate with other proteins in HIV-1-infected T-lymphocytic cells. HIV-1-infected A3.01 cells were labeled with [^{35}S]methionine and disrupted by freeze-thawing; pelleted lysates containing heavy membranes were immunoprecipitated by Vpu antiserum and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) without reducing agents or prior boiling. An 80-kDa complex was detected in addition to Vpu monomers (Fig. 3A), suggesting that Vpu formed an oligomeric structure. We attempted to stabilize the 80-kDa complex by chemical cross-linking; freeze-thaw supernatants from HIV-1-infected A3.01 cells were incubated with the cross-linker EGS [ethylene glycol bis(succinimidylsuccinate)], and Vpu-containing proteins were immunoprecipitated with Vpu antiserum and analyzed by SDS-PAGE. Two complexes with approximate sizes of 28 and 45 kDa (Fig. 3B, marked by arrows) were detected in addition to monomer Vpu (Fig. 3B, Vpu). We were unable to stabilize the 80-kDa protein complex visible in Fig. 3A even when various concentrations of EGS or DTSSP [dithiobis(sulfosuccinimidylpropionate)], a different cross-linker, were used (data not shown). It is conceivable that sites critical to cross-link the 80-kDa complex are inaccessible to the cross-linker.

To avoid the potential interference of membrane insertion with accessibility to cross-linking sites, we investigated whether membrane-free Vpu can form higher-molecular-weight structures by using Vpu translated in vitro in a rabbit reticulocyte lysate containing [^{35}S]methionine. Cross-linking with EGS stabilized a complex of approximately 80 kDa (Fig. 3C), which corresponds in size to the 80-kDa complex shown in Fig. 3A. A larger complex of about 85 kDa was also present, and minor amounts of Vpu-specific complexes of 28 and 45 kDa were detected (Fig. 3C, arrows), which are similar in size to the complexes detected after cross-linking membrane-associated Vpu (Fig. 3B). These data indicate

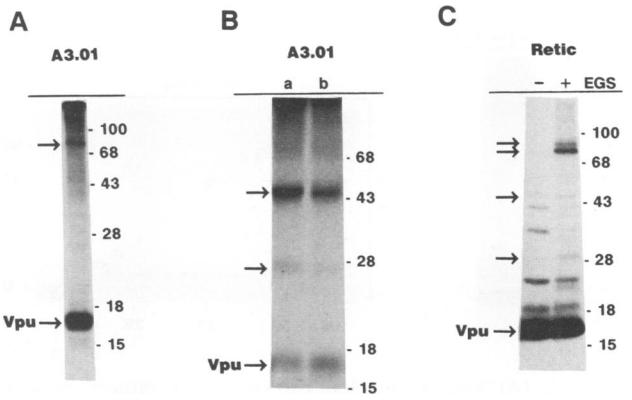

FIG. 3. Vpu forms oligomeric structures. (A) A3.01 cells were infected with HIV-1 and labeled with [^{35}S]methionine as described previously (21). Cells were lysed in PBS by five cycles of freeze-thawing. The lysate was centrifuged in a microcentrifuge for 3 min at 15,000 × *g*. An aliquot from the pellet fraction was extracted with CHAPS buffer (50 mM Tris [pH 8.0], 5 mM EDTA, 100 mM NaCl, 0.5% CHAPS [3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate]) and immunoprecipitated with Vpu-specific antiserum. The immunoprecipitate was suspended in nonreducing sample buffer (2% SDS, 62.5 mM Tris-HCl [pH 6.8], 5% glycerol) and incubated for 5 min at 37°C prior to electrophoresis on an SDS–12.5% polyacrylamide gel (A3.01). (B) Proteins in the supernatant fraction of lysates prepared as in panel A were cross-linked for 30 min at ambient temperature with EGS (Pierce Biochemicals) at a final concentration of 25 mg/ml (a) or 5 mg/ml (b). The cross-linking reaction was stopped by adding an equal volume of 1 M glycine to the reaction mix. Samples were immunoprecipitated with a polyclonal antiserum to Vpu and analyzed on an SDS–12.5% polyacrylamide gel. (C) Vpu was translated in rabbit reticulocyte lysate (Retic) in the presence of [^{35}S]methionine for 1 h at 30°C. The lysate was split into two equal fractions, and one fraction was treated with EGS (6 mg/ml) for 30 min (+) at ambient temperature; the other fraction was left untreated (−). The cross-linking reaction was stopped by adding an equal volume of 1 M glycine. Vpu was immunoprecipitated with a Vpu-specific antiserum and analyzed on an SDS–12.5% polyacrylamide gel. Proteins were visualized by fluorography. Arrows indicate the locations of Vpu-specific proteins. Sizes are shown in kilodaltons.

that Vpu forms oligomeric structures even in the absence of a membrane environment. Attempts to cross-link other in vitro-synthesized proteins, including CD4, Vif, and Nef, in the absence of CMM did not reveal any discrete oligomeric structures. Cross-linking reactions with mixtures of in vitro-translated Vpu and CD4 or Vpu and HIV-1 Gag did not result in any oligomeric structures in addition to the 80-kDa Vpu oligomer (data not shown), demonstrating that cross-linking of Vpu was specific and not the result of aggregation of Vpu in the absence of membranes.

The observation of high-molecular-weight complexes after cross-linking of Vpu indicates the formation of either homo-oligomeric complexes of Vpu or stable complexes with other cellular or viral factors. To demonstrate directly that Vpu forms homo-oligomeric complexes, we constructed a Tat-Vpu chimeric protein which differs in size from Vpu and is easily distinguished from wild-type Vpu by electrophoretic mobility. The fusion protein contains the 43 N-terminal amino acids from the first coding exon of Tat in frame with the full open reading frame of Vpu (Fig. 1). Expression of pSP-23 in a reticulocyte lysate revealed comparable synthesis of the Tat-Vpu fusion and of wild-type Vpu (Fig. 4A, a and b). Since the original initiation codon for Vpu is main-

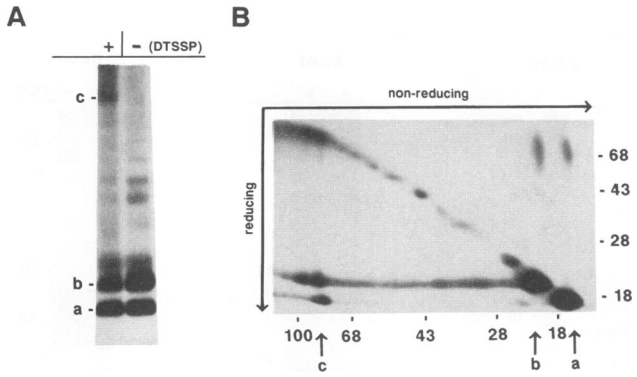

FIG. 4. (A) Vpu (a) and the Tat-Vpu fusion protein (b) were translated from pSP-23 transcripts in a reticulocyte lysate supplemented with [^{35}S]methionine for 2 h at 30°C. The lysate was then split in half, and one portion was cross-linked with 5 mg of the reversible cross-linker DTSSP (Pierce Biochemicals) per ml for 30 min at ambient temperature (+). The cross-linking reaction was stopped with an equal volume of 1 M glycine. Untreated and DTSSP-treated lysates were immunoprecipitated with a Vpu-specific polyclonal antiserum and analyzed by SDS-PAGE under nonreducing conditions. c, High-molecular-weight complex of Vpu detectable after cross-linking. (B) A lane containing DTSSP-treated lysate from panel A was cut from the gel, boiled for 20 min in 50 mM dithiothreitol, and subsequently separated in the second dimension by PAGE. Arrows point to the locations of Vpu-specific proteins as identified in panel A.

tained in pSP-23, expression of wild-type Vpu presumably occurs by internal initiation.

To determine whether Vpu can form homo-oligomers, Vpu and Tat-Vpu expressed from pSP-23 in a reticulocyte lysate were treated with the reversible cross-linker DTSSP. The Vpu-specific products were immunoprecipitated with Vpu antiserum and analyzed by nonreducing SDS-PAGE. As shown in Fig. 4A, cross-linking of the Vpu/Tat-Vpu mixture with DTSSP reveals a complex (c) of 80 to 85 kDa which is not detected in the absence of DTSSP. To demonstrate that both Vpu and the Tat-Vpu fusion protein are present in complex c, the DTSSP-treated gel strip was excised, reduced by boiling in dithiothreitol, and subjected to electrophoresis in the second dimension (Fig. 3B). The high-molecular-weight complex c that was detectable after cross-linking with DTSSP contained both wild-type Vpu and the Tat-Vpu hybrid, providing direct evidence that Vpu can self-associate. The exact stoichiometry of proteins in the complex is not certain; assuming that no cellular proteins are incorporated into the complex, it must contain at least four subunits of Tat-Vpu and Vpu to account for the observed molecular size.

The data presented in this article demonstrate two new structural characteristics of Vpu, membrane orientation and self-assembly, which may have important functional implications. The finding that Vpu has the topology of a type I integral membrane protein implies that the hydrophobic domain provides the anchor which retains the protein in part, within the membrane. The structure of the membrane anchor remains unknown; the length of the hydrophobic domain (27 to 30 amino acids) is sufficient to span a lipid membrane one time, but it is not clear whether this domain is completely embedded within the membrane or folded back so that the N-terminal methionine is at the cytoplasmic membrane face. Previous studies (21) indicated that Vpu does not have a cleavable signal sequence. These cumulative data suggest that the hydrophobic domain contains both the signal and the anchor sequence (S-A) for Vpu. Type I integral membrane proteins with S-A sequences are uncommon, and the mechanism by which such proteins are inserted and retained in membranes remains controversial (5, 8, 9, 18). In that regard, Vpu may be of general interest as a model system to study the requirements for membrane insertion and retention of type I integral membrane proteins.

The results of our experiments provide direct evidence that Vpu forms oligomers in vivo as well as in vitro and that oligomerization takes place even in the absence of membranes. While cross-linking predominantly stabilized dimeric and trimeric forms of Vpu, higher-order complexes are likely, because of our observation of an 85-kDa complex (Fig. 3A), which was also present as the predominant oligomeric structure in vitro. The exact number of subunits in a membrane-associated Vpu complex remains to be determined by more detailed physicochemical analyses.

Our observation that Vpu can self-associate into oligomers raises the question of which domains in Vpu are critical for this process. Calculations of the two-dimensional structure of Vpu with the Chou-Fasman algorithm (2) indicated that the hydrophilic region of amino acids 28 to 50 has a high α-helical character and predicts a line of charged residues on two faces of the α-helix. These charges are potentially interlocking and allow neighboring Vpu molecules on the same membrane to interact via salt bridges; one face of the helix has the arrangement ^{28}E-R-R (− + +), while the three-eights-turn face has the arrangement ^{31}K-D-E (+ − −). These predictions are useful in suggesting what specific mutations may be used to define the minimal requirements for multimerization and the role of oligomerization in the biological activity of Vpu. We have previously discussed the possibility that Vpu forms an ion channel (27); such a function would be compatible with the dual activity of Vpu in CD4 degradation (28) and enhancement of particle release (29).

A number of enveloped RNA and DNA viruses encode small, amphipathic proteins (3, 4, 6, 7, 10, 12, 14, 17, 23, 25, 30). In general, most are similar in structure to Vpu, with a strongly hydrophobic N-terminal domain and a hydrophilic C-terminal domain; several are known to oligomerize, and two, Vpu and M2, are known to be phosphorylated. A common function for these proteins has not been detected; in fact, a number of different activities have been identified. Ongoing studies will determine whether the activity of Vpu is similar to a mechanism identified for small proteins of other viruses or represents an entirely new biochemical function.

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