**THE HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 VPU PROTEIN
ENHANCES MEMBRANE PERMEABILITY**

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Running title: Vpu enhances membrane permeability

SUMMARY

Infection of T lymphocytes by Human Immunodeficiency virus causes drastic alterations in the intracellular cation content of the infected cells. The Human Immunodeficiency virus type 1 genome encodes several accessory proteins, including Vpu, an integral membrane protein that forms ion channels in planar lipid bilayers. The effect of Vpu on the permeability of the plasma membrane to several molecules has been analyzed. Expression of *vpu* in *E. coli* cells increases membrane permeability to a number of molecules such as 2-nitrophenyl β -D-galactopyranoside, uridine, the impermeable translation inhibitor hygromycin B and lysozyme. In addition, transient expression of Vpu in eukaryotic COS cells enhances entry of charged molecules such as hygromycin B and neurobiotin into these cells. The effect of Vpu on cell membrane permeability resembles that reported for other membrane-active proteins from different animal viruses including influenza M2, Semliki Forest virus 6K and poliovirus 2B and 3A proteins.

INTRODUCTION

The *vpu* gene of human immunodeficiency virus type 1 (HIV-1) encodes an integral membrane protein of 81 amino acid residues that forms oligomers (1). Vpu is unique to HIV-1; it is not encoded by HIV-2 nor by the majority of simian immunodeficiency virus (SIV) isolates with the exception of SIV_{cpz} (2). However a recent study showed that the envelope glycoprotein of HIV-2 is a Vpu-like factor capable of substituting for Vpu in HIV-1 infections (3). The sequence of Vpu is highly conserved in most HIV-1 isolates but this protein is dispensable for the replication of HIV-1 in cultured cells (4). However, HIV-1 isolated from infected individuals usually contains an intact *vpu* gene.

The N-terminus of HIV-1 Vpu protein contains a hydrophobic 27 amino acid region that constitutes the membrane anchoring domain. In contrast, the cytoplasmic C-terminal part of the protein is highly hydrophilic. This region has been characterized by CD and NMR spectroscopy and consists of two α -helices joined by a flexible region, which contains two phosphoacceptor sites (5). The second α -helix is followed by a reverse turn within the flexible C-terminus (6). Vpu is expressed from the same bicistronic mRNA and in the same amount as the Env glycoprotein in the infected cells (7), but the protein has not been detected as forming part of the virus particle.

Two activities have been attributed to the Vpu protein: enhancement of virus particle release from infected cells and degradation of CD4 in the endoplasmic reticulum (ER) (Reviewed in (8)). The majority of virus particles bud from internal membrane compartments, but are poorly released to the culture medium in Vpu-deficient viruses (9,10). These particles have the appearance of mature virions with condensed nucleoids (11). Thus, Vpu protein seems either to prevent virus assembly in inappropriate internal membranes or to target the virus to the plasma membrane. Furthermore, Vpu enhances virus

particle production from such divergent retroviruses as Visna virus and Mo-MLV (12). Together these data suggest that Vpu has an important role in the virus life cycle.

A number of animal virus gene products which are involved in cytopathogenicity act at the level of the plasma membrane to exert their toxic effect (13). Thus, infection of cells by cytolytic animal viruses leads to alteration in membrane permeability (13,14). Membrane leakiness at late stages of infection requires virus gene expression and involves the diffusion of ions and small molecules through the plasma membrane (13,14). Different proteins encoded by the HIV genome have been shown to be involved in membrane alterations (15-17). As occurs with the majority of animal viruses, HIV enhances cellular membrane permeability in the infected cells (18,19). Structural comparison of animal virus proteins has led to the suggestion of a new family of small hydrophobic proteins, the vioporins, that modify membrane permeability upon expression in intact cells (13). This is the case for poliovirus proteins 2B and 3A, Semliki forest virus 6K protein and influenza M2. In addition to enhancing membrane permeability, these proteins also interfere with glycoprotein processing (20-22). Both, structural and functional similarities between Vpu and influenza M2 protein have been noted (11,23). Influenza M2, which is thought to play a role in hemagglutinin transport by regulating intracellular pH (24,25), forms ion channels in planar lipid bilayers, *Xenopus laevis* oocytes and in CV-1 monkey cells (26-28). Notably, Vpu also forms cation-selective ion channels in planar lipid bilayers (29). In this study we show that Vpu enhances membrane permeability upon inducible expression, both in prokaryotic and eukaryotic cells.

EXPERIMENTAL PROCEDURES

Materials.

L[³⁵S]methionine (1,000 Ci/mmol) and [5-³H]uridine (29 Ci/mmol) were purchased from Amersham Corp. Cationic liposomes containing dimethyldioctadecyl ammonium bromide (DDAB) were prepared as recommended (30). Mowiol mounting medium was prepared as indicated (31). 2-nitrophenyl β-D-galactopyranoside (ONPG) and hygromycin B were from Boehringer Mannheim. Rifampicin and cytosine β-D-arabinofuranoside (ara-C) were from Sigma.

Expression in *E. coli*

Construct for the production of native HIV-1 Vpu protein in bacteria was made by standard PCR and cloning techniques. The genome from BH10 clone of HIV-1 (32) was the template used for PCR amplification of the Vpu encoding sequence, using oligonucleotides containing appropriate restriction sites. The original pET3Xa plasmid (33) was digested with *Nde* I and *Bam* HI and the fragment was replaced with the PCR product corresponding to *vpu*. The host for initial cloning and analysis of recombinant plasmids was *E. coli* DH5 strain. Single clones containing the corresponding plasmid were grown at 37°C in LB medium with 100 µg/ml ampicillin. Once plasmids were obtained, T7 lysozyme producer strains of *E. coli*, BL21 (DE3)pLysS or BL21 (DE3)pLysE (34), were transformed with pET3 constructs. Single clones were grown in LB medium until saturation. For the induction of Vpu synthesis, cultures were diluted 100 fold, in M9 medium supplemented with 0.2 % glucose, 100 µg/ml ampicillin and 34 µg/ml chloramphenicol. When the cultures reached 0.5 A_{600nm} they were induced to synthesize Vpu by addition of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG).

Expression in mammalian cells.

vpu gene was amplified by standard PCR technique using the 5'- and 3'-primers: 5'-GGGGCCCATGGAACCTATACAAATAG-3' and 5'-GGGCCCGGATCCTACTACAGATCATCAA-3', containing *Nco* I and *Bam* HI sites (underlined sequences). The resulting PCR product was cloned into *Nco* I and *Bam* HI polylinker sites of pTM1 plasmid (35). Vpu protein was efficiently expressed, under the control of the bacteriophage T7 promoter, in COS cells infected with T7-recombinant vaccinia virus and transfected with the pTM1 plasmid containing *vpu* (35). Vpu protein was also expressed from the SV40 early promoter using pBJ3 plasmid in COS cells (36). The *vpu* gene was amplified by PCR using the following 5'- primer: 5'-GGGCCCCAAGCTTATGCAACCTATACAAATAG-3' containing a *Hind* III site (underlined) and the same 3'- primer described above. The amplified fragment was cloned in *Hind* III and *Bam* HI restriction sites of the polylinker of pBJ3 plasmid. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine (DMEM/10% FCS).

Rabbit antiserum against vpu.

Vpu protein from BH10 clone was amplified by PCR and cloned into pMalC2 (New England Biolabs) fused to the maltose-binding protein (MBP). Transformed *E. coli* DH5 cells were grown in the presence of glucose. To induce the synthesis of the hybrid protein MBP-Vpu, 1 mM IPTG was added to exponentially growing bacteria. The fusion protein was purified by affinity chromatography, following the directions of the manufacturer (New England Biolabs). Rabbits were injected subcutaneously with the MBP-Vpu protein, using Freund's adjuvant. Serum was collected from immunized animals and adsorbed to acetone powders of MBP-producing DH5 cells to remove nonspecific binding capacity.

β-galactosidase assay.

To measure the entry of ONPG into bacterial cells, aliquots of 1 ml were removed from cultures at different times, as indicated in each experiment. After centrifugation, cells were resuspended in 1 ml fresh medium with 2 mM ONPG, a β-galactosidase substrate. Cells were incubated for 10 min at 30°C and the β-galactosidase reaction was stopped by addition of 0.4 ml of 1 M sodium carbonate. Samples were centrifuged and A_{420 nm} of the supernatant was measured.

Subcellular fractionation.

Cell fractionation was performed essentially as described previously (37). 80 ml of bacterial culture were harvested after 3 h of induction by centrifugation at 5,000 x g for 15 min at 4°C. The pellet was resuspended in 1 ml of ice-cold 10 mM Tris acetate (pH 7.6), 50 mM KCl, 10 mM magnesium acetate, 1 mM dithiothreitol (TKMD) and frozen. After thawing in cold water, the cell extract was sonicated at 12 Hz and centrifuged at 15,000 x g for 20 min at 4°C. Clarified cell lysate was centrifuged at 120,000 x g for 3 h at 4°C. The supernatant (cytosolic fraction) was mixed with equal volume of sample buffer 2X. The pellet (crude membrane fraction) was resuspended in half of the original volume of TKMD. Membrane extract was split in four aliquots of 125 µl followed by corresponding treatment on ice with 1 M sodium chloride, 0.2 M sodium carbonate, 1% triton X100 or 8 M urea in a final volume of 250 µl, for 30 min. Each fraction was centrifuged at 120,000 x g for 30 min at 4°C. Collected supernatants were mixed with sample buffer 2X. Pellets were resuspended in 250 µl of TKMD and then mixed with equal volume of sample buffer 2X. 10 µl of each fraction were loaded on SDS-20% polyacrylamide gel and subjected to electrophoresis and western blot analysis using polyclonal Vpu antiserum.

Transfections and viral infections of mammalian cells.

COS cell monolayers were grown to 30% confluency in 24-well plates. The cells were infected with recombinant vaccinia virus (5 pfu/cell) encoding the bacteriophage T7 RNA polymerase (VT7) (Kindly given by Dr. B. Moss, NIH, Bethesda, MD) in serum free DMEM. After 1 h of adsorption cells were transfected with a mixture of 0.5 µg of pTM1 construct DNA and 1 µl liposomes per well. For each transfection experiment with pBJ3 constructs, 10⁷ COS cells were used. Cells were resuspended in 0.2 ml of DMEM/10% FCS with 10 mM Hepes pH 7.4 and placed in a 0.4 cm BioRad cuvette. A mixture of 5 µl 1.5 M NaCl, 20 µg pUC19 carrier DNA and 5 µg of the expression plasmid was added. Cells were electroporated using a BioRad gene pulser set at 200 V and 960 µF for 40 sec. Then cells were diluted in 10 ml fresh medium (DMEM/10% FCS with 10 mM Hepes) and plated.

SDS gel electrophoresis, Western blot and immunoprecipitation assays.

After labeling with [³⁵S]methionine, cells were resuspended in sample buffer (62.5 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 0.1 M dithiothreitol (DTT), 17% glycerol and 0.024% bromophenol blue), sonicated at 18 Hz, heated to 100°C for 4 min and applied to a polyacrylamide gel. Electrophoresis was performed with glycine containing buffer. Densitometric analysis of the protein bands was carried out by scanning the exposed X-ray films with a Computing densitometer (Molecular Dynamics). For immunoblot analysis, proteins were electrotransferred to a nitrocellulose membrane (BioRad), reacted with a 1:5,000 dilution of antiserum and proteins detected by chemiluminescence reactions as previously described (38). For immunoprecipitation, cells were grown in 60 mm diameter plates and labeled with 50 µCi [³⁵S]methionine for 1 h. Cell monolayers were scraped and collected in phosphate-buffered saline solution (PBS). The pelleted cells were lysed in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X100, 1% Nonidet

P40 and 0.2 mM phenylmethylsulfonyl fluoride. Cell lysates were incubated with 10 μ l specific polyclonal antiserum and the immune complexes were collected on protein A sepharose (Pharmacia Biotech), as described previously (31).

Immunofluorescence assay.

Very diluted cell monolayers were grown on glass coverslips. Prior to cell staining, cell monolayers were washed with PBS and fixed with chilled methanol. After washing with PBS, cells were incubated in PBS with 1:400 dilution (vol/vol) of polyclonal Vpu-antiserum for 1 h at 37°C. Cells were washed with PBS three times and incubated in PBS with 30 μ g/ml goat anti-rabbit IgG conjugated to fluorescein or rhodamine (Pierce). The coverslips were placed on a glass slide, mounted with Mowiol solution and viewed with a Zeiss Axiovert 35 microscope.

Neurobiotin entry assay.

Cells were cultured on glass coverslips and transfected with the indicated plasmid. Cell monolayers expressing Vpu were incubated for 40 min at 37°C with 1 μ g/ml neurobiotin in fresh medium, washed with PBS and analyzed for polyamine uptake as described previously (39). Briefly, cells were fixed with 4% (wt/vol) paraformaldehyde for 10 min at 22°C. Then, cells were permeabilized for 15 min at 22°C with PBS, 2% bovine serum albumin, 0.25% Triton X-100 and 0.02% sodium azide. The coverslips were washed twice with PBS and incubated for 30 min at 37°C in permeabilization solution with 10 μ g/ml fluorescein isothiocyanate-conjugated streptavidin (Molecular Probes). Samples were washed twice again with PBS and cells were examined with a fluorescence microscope. When indicated, cells were doubly stained with rabbit anti-Vpu serum using rhodamine-conjugated goat anti-rabbit antibodies.

RESULTS

Cloning and inducible expression of HIV-1 Vpu in *E. coli* cells.

As a first approach to test membrane permeabilization by HIV-1 Vpu, the prokaryotic system described by Studier et al. (33) was used. This system has proven to be very efficient for the synthesis of viral membrane proteins that are cytotoxic (40-42). Host cells used for Vpu synthesis were *E. coli* BL21(DE3)pLysS and BL21(DE3)pLysE. Both DE3 lysogens carry integrated in the chromosome the T7 RNA polymerase gene under the control of the lac UV5 promoter that is inducible by IPTG. In addition, these *E. coli* strains carry pLysS or pLysE plasmids that synthesize the T7 phage lysozyme, a natural inhibitor of the T7 RNA polymerase, at low or high levels respectively (33,34). Using standard PCR cloning procedures the *vpu* sequence of HIV-1 BH10 was placed under the control of the T7 promoter in the pET3 expression vector as depicted in Figure 1.

Several regions have been defined in HIV-1 Vpu (Figure 1). This protein contains a membrane spanning stretch of hydrophobic amino acids at the N-terminus that modulates viral particle release and a C-terminal hydrophilic cytoplasmic domain that mediates CD4 degradation. To analyze the Vpu interaction with bacterial membranes, the Vpu gene was cloned in pET3 expression vector. Figure 1 shows that high levels of Vpu synthesis were achieved in *E. coli* BL21(DE3)pLysS as soon as 30 min after IPTG-induction. Even two hours after addition of IPTG, bacteria continued synthesizing the viral protein at high levels. Analysis of Vpu synthesis in *E. coli* cells is facilitated by addition of rifampicin to bacterial cultures already induced with IPTG. Since rifampicin inhibits only transcription by *E. coli* RNA polymerase, the expression of Vpu is not affected by this antibiotic. A single protein band of approximately 16 KDa is synthesized in the presence of rifampicin, suggesting that this protein is encoded by the recombinant plasmid. Immunoblot analysis reveals that the

major protein synthesized with an apparent molecular weight of 16 KDa is immunologically related to Vpu (Figure 1). Therefore, the Studier system is very suitable for the efficient expression of HIV-1 *vpu* gene.

Vpu enhances membrane permeability in *E. coli* cells.

Changes in the permeability of the bacterial membrane to various molecules upon synthesis of Vpu were assayed. First, the passage of constitutively expressed lysozyme through the membrane of bacterial cells was tested. Under normal conditions, *E. coli* tolerates high levels of cytoplasmic lysozyme because the protein is unable to pass through the inner membrane to reach the peptidoglycan layer (33). But, when the inner membrane is disrupted even small amounts of cytosolic T7 lysozyme give rise to rapid cell lysis. Membrane disruption by lysozyme was tested by measuring optical density of the culture at different times after IPTG induction of Vpu. As a control, cells bearing pET3-Xa plasmid (33) that encoding 260 amino acids of the T7 S10 protein (control) did not cause lysis of bacteria. To assay the release of nucleotides, bacteria were loaded with [³H]uridine and the radioactivity released to the medium was analyzed after induction of Vpu (Figure 2B). Nucleotides do not easily pass across the membrane of control cells. However, substantial amounts of radioactivity were released to the medium after induction of Vpu synthesis. This radioactivity was not precipitable by trichloroacetic acid, suggesting that it corresponded to low molecular weight molecules.

In addition to the study of molecules leaking from Vpu-expressing cells, the entry of compounds into these cells was analyzed. Entry of ONPG into bacterial cells is easily monitored by measuring its conversion to the colored compound by the β -galactosidase activity present in the culture. As shown in Figure 2C, induction of Vpu synthesis caused a clear increase of ONPG entry into cells. Another probe used to study the influx of molecules through the

membrane was hygromycin B. This aminoglycoside antibiotic is an inhibitor of protein synthesis to which cells are normally nonpermeable (14). IPTG-induced cells were metabolically labeled with [^{35}S]methionine 15 min after addition hygromycin B. Figure 2D shows that synthesis of Vpu permeabilizes cells to hygromycin B to such an extent that 0.5 mM of the antibiotic completely blocked protein synthesis in these cells. In conclusion, expression of Vpu modifies the passage of molecules (both exit and entry) through the bacterial membrane.

Localization of Vpu by subcellular fractionation.

BL21(DE3)pLysS cells were used to analyze subcellular localization of recombinant Vpu (Fig. 3A). After 3 h of induction sufficient amounts of Vpu were achieved in *E. coli* cells (Fig. 3A lane T) to allow subcellular fractionation and biochemical analysis of the recombinant protein. Cellular lysate was clarified and then fractionated in the soluble (S) and insoluble fraction (P). Only a small amount of Vpu was detected in the soluble cytoplasmic fraction. In order to characterize the interaction of Vpu with bacterial membranes the insoluble fraction was treated with 1 M sodium chloride, or 0.2 M sodium carbonate and the new solubilized proteins were separated from the insoluble fraction. Vpu was totally resistant to extraction by high salt concentration (Fig. 3A, lanes A), or high pH (Fig. 3A, lanes B), suggesting the integral membrane localization of Vpu protein. The membrane fraction was also treated with 1% Triton X100 that solubilize the inner membrane proteins of *E. coli* cells or denaturated with 8 M urea. Our results agree well with previous observation on the partial solubilization by urea (Fig. 3A, lanes D) and detergents (Fig. 3A, lanes C) of Vpu from *E. coli* cells (43).

Cloning and expression of Vpu in mammalian cells.

To analyze the effects of Vpu in mammalian cells, the sequences encoding this protein were cloned in the pTM1 expression vector as described in Materials and Methods. Synthesis of Vpu is clearly detected from the eight hour after vaccinia virus infection. Vpu synthesis continued until 24 h post-infection (results not shown). Ara-C, an inhibitor of vaccinia virus DNA polymerase, blocks the replication of vaccinia virus allowing the synthesis of cellular proteins and of the recombinant proteins dependent on T7 RNA polymerase (44). Therefore, addition of ara-C to vaccinia virus-infected cells bearing the pTM1-Vpu construct facilitates the analysis of membrane permeabilization by Vpu, avoiding the interference of vaccinia virus late proteins. Moreover, addition of ara-C to COS cells has only a slight effect on the levels of Vpu protein reached inside cells, as shown by immunoblot assay (Fig. 3B).

When Vpu was transiently expressed under the control of the SV40 promoter in COS cells, using pBJ3 plasmid, only approximately 20% of cells were efficiently transfected (Results not shown). Consequently, the radioactively labeled protein was hardly distinguishable in cell extracts (Results not shown). However, immunoblot analysis of the transfected cell monolayers demonstrated that detectable levels of Vpu were achieved 48 h after transfection (Fig. 3C). This second eukaryotic expression system is very appropriate for studies of Vpu activity in the absence of vaccinia virus infection.

Vpu increases membrane permeability in mammalian cells.

The modification of membrane permeability in COS cells, 9 h after infection with vaccinia virus encoding the T7 RNA polymerase and transfection with plasmid pTM1-Vpu, was tested. Recently, it has been reported that poliovirus protein 2BC enhances permeability of membranes to hygromycin B

while poliovirus 2C has not effect (45). Both proteins 2BC and 2C were used as positive and negative controls respectively in our experiments on hygromycin B entry (Figure 4B and 4C). This antibiotic efficiently blocks protein synthesis in vaccinia virus infected cells in the absence of ara-C (Figure 4A), due to the permeabilizing capacity of vaccinia virus infection (46). However, in cells that express 2BC or Vpu protein synthesis is strongly inhibited by hygromycin B, even in the presence of ara-C (Figure 4B and 4D). Protein synthesis in cells expressing the negative control protein 2C is less affected by the antibiotic (Figure 4C). Entry of the aminoglycoside antibiotic was quantitated by densitometric analysis of protein labeling (Figure 4E). Hygromycin B reduces the synthesis of poliovirus 2C protein by 15%, while Vpu synthesis is diminished to 85% of control suggesting a drastic enhancement of membrane permeability to hygromycin B by Vpu. In addition, both proteins 2C and Vpu were radioactively labeled, immunoprecipitated with specific antiserum and analyzed by SDS-PAGE. Clearly, synthesis of Vpu, but not 2C, was profoundly inhibited by hygromycin B (Figure 4F).

Neurobiotin is a compound that does not pass the membrane of mammalian cells. Hence, it has been used recently to assay changes in membrane permeability (39). Uptake of the polyamine depends on specific connexin channels present on cells (47). Therefore, increased import of the cationic molecule was assayed after synthesis of Vpu in VT7-infected cells. In COS cells mock-infected, infected but untransfected or infected and transfected with plasmid alone the detection of neurobiotin is restricted to a punctate pattern in the cytoplasm, suggesting some entry of the polyamine into cells mediated by endocytosis (Figure 5A-C). Clearly, entry of neurobiotin into cells was enhanced by poliovirus 2BC (Figure 5D) or Vpu (Figure 5E) synthesis. The cytoplasm of these cells was loaded with the compound that now is not located in the endosome-like structures, but is dispersed throughout the cell interior.

To avoid potential interference with membrane permeabilization by vaccinia virus proteins, further analysis of the Vpu-induced modification of neurobiotin transport was carried out using the pBJ3 expression vector. This Vpu expression system directed by the SV40 promoter, although less efficient than expression with the VT7 system produces significant levels of Vpu protein (Figure 6). As indicated above, only a small percentage of cells (about 20%) synthesize the protein as shown using the polyclonal Vpu antiserum (Figure 6). After incubation with neurobiotin the cytoplasm of the cells that do not synthesize Vpu contained a punctate pattern of fluorescence, as indicated above for vaccinia-infected COS cells. Notably, the cytoplasm of Vpu-synthesizing cells showed enhanced labeling with neurobiotin. Thus, these experiments suggest that Vpu expression enhances neurobiotin entry into mammalian cells.

DISCUSSION

The action of Vpu as well as the other HIV-1 accessory proteins, during the viral life cycle remains a matter of intensive research (8,23). A detailed understanding of the role of Vpu in membrane permeabilization could provide a new target for the design of anti-HIV agents (48). HIV infection produces a number of cytopathic effects leading to cell ballooning, syncytia formation and cell lysis (49). These phenomena are clearly related to cell membrane perturbation (19). Thus, expression of the HIV-1 envelope glycoprotein at the cell surface triggers formation of syncytia (50), while lysis of HIV-infected cells is preceded by cell swelling (51). In addition, events like impaired Ca^{2+} fluxes, increased intracellular concentration of K^{+} and Na^{+} , decreased membrane potential or inhibition of phospholipid synthesis, are hallmarks of HIV-infected cells (52-55). Moreover, recently it was reported that HIV-1 infection decreases intracellular pH in T-lymphoblastoid cells (56). The mechanism by which HIV reduces intracellular pH is currently unknown. Our present findings indicate that

synthesis of HIV-1 Vpu protein in cells leads to enhanced membrane permeability to various molecules. Both entry and exit of molecules were affected by Vpu in bacterial and in eukaryotic cells, using different expression systems. Membrane permeability to lysozyme, uridine, ONPG and hygromycin B was enhanced in bacteria cells. We also detected an increase in hygromycin B and neurobiotin entry into mammalian cells. Undoubtedly, membrane permeability to ions should be modified too. These permeability changes induced by Vpu may give account of the reported modifications of intracellular cations in HIV-infected T cells. Our results are consistent in showing interaction of Vpu with membranes. Indeed, Vpu produced in *E. coli* was found mainly associated with crude membrane fraction. Only small amounts of the overexpressed Vpu were presented as a soluble cytosolic protein in *E. coli* cells. A substantial fraction of membrane-associated Vpu was solubilized by detergent Triton X100 suggesting that Vpu has an inner membrane location but might have some association with components of the outer membrane. Furthermore, the majority of this protein was resistant to extraction with a high salt concentration or high pH. Together these results suggest that Vpu is integrally associated with membrane. Also proteolytic digestion and immunoprecipitation studies, using Vpu protein synthesized in canine microsomal membrane vesicles, demonstrated that Vpu was an integral membrane protein. Besides, immunofluorescence microscopic studies in mammalian cells showed perinuclear localization of Vpu (11,57) as well as on the cell membrane in the later stages of infection (43). Therefore we conclude that Vpu produced in *E. coli* and mammalian cells is mostly associated with membranes.

The structure of Vpu, a small hydrophobic protein, that forms oligomers, resembles a number of virus-encoded proteins, that in addition share with Vpu the characteristic of being an integral membrane protein (6). As previously noted, HIV-1 Vpu, the alphavirus 6K and influenza M2, share structural

similarities between them. To this list of proteins can be added the picornavirus 3A and 2B and adenovirus E3 11.3K (13). The similarities between Vpu and the alphavirus 6K protein extend beyond their physical characteristics, since deletion of the 6K gene from the alphavirus genome leads to diminished virus formation (58). Moreover, blockade of influenza M2 by amantadine, not only inhibits the entry, but also the exit of new virions from cells (59,60). These findings lead us to speculate that the release of animal virus particles could be mediated by specialized viral proteins that disturb the plasma membrane by forming pores. The recent finding that Vpu forms ionic channels in planar lipid bilayers (23,29), argues in favor of such a function. Formation of ionic pores by an isolated animal virus protein was demonstrated for influenza M2 (26-28). Synthesis of this protein both in *Xenopus* oocytes and in mammalian cells increased the passage of sodium and potassium ions through the plasma membrane. Expression of M2 in bacterial cells enhances membrane permeability to a number of compounds (42), similar to that reported for the alphavirus 6K (41) or Vpu (The present report).

Two activities have been reported for Vpu in the infected cells: enhancement of viral particle release and CD4 degradation (8). It is not known whether Vpu interacts with viral or cellular proteins in order to regulate virion release or if Vpu indirectly modifies the intracellular ionic environment close to the membranes of the ER or the exocytic pathway (61-63). As reported for M2 protein of influenza virus, changes of ion content in cellular compartments may lead to conformational changes or activation of proteolytic enzymes (27). The transmembrane domain of Vpu plays a crucial role in virus release (64), while the cytoplasmic domain has elements necessary for CD4 degradation (61,65). These functions occur in spatially separated cellular compartments, the plasma membrane and the ER. The mechanism of Vpu-mediated modification of the secretory pathway is presently unknown. Also modification of glycoprotein trafficking has been reported for the poliovirus proteins that enhance

membrane permeability, such as 2B and 2BC (20). Moreover, influenza virus M2 and alphavirus 6K also interferes with the correct processing of glycoproteins (21,22). Thus, several membrane-active proteins from different animal viruses including HIV-1 Vpu, poliovirus 2B and 2BC and influenza virus M2 also have adverse effects on the ionic content and the pH of different cellular compartments which could give rise to these modifications in glycoprotein trafficking. Further experiments are needed to elucidate the potential connexion between these phenomena.

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FIGURE LEGENDS

Figure 1. Cloning of HIV-1 vpu coding sequence into pET3 plasmid. **Upper right panel:** Schematic representation of *vpu* cloning strategy. P_{T7}, T7 promoter; ORI, origin of replication; Amp_R, ampicillin -resistance gene. **Lower right panel:** Hydrophobicity profile of HIV-1 Vpu (Kite Doolittle) and structural elements of Vpu protein. **Left panel:** Time course of Vpu synthesis in BL21(DE3)pLysS cells. Cells carrying pET3-vpu plasmid were induced with 1 mM IPTG; 30 min later 150 µg/ml rifampicin was added (+) or not added (-) to bacterial cultures. At the indicated h post induction, cells were metabolically labeled with [³⁵S]methionine for 10 min, the proteins were separated by SDS-20%PAGE and detected by autoradiography. After 2 h of IPTG-induction the proteins from cell lysates were separated by SDS-20%PAGE, transferred to nitrocellulose membrane and probed with rabbit anti-Vpu polyclonal serum.

Figure 2. Modifications of membrane permeability by wild type and truncated variants of Vpu protein in prokaryotic cells. **A. Cell growth analysis.** *E. coli* BL21(DE3)pLysE cells carrying the pET3 Vpu plasmid were induced with 1 mM IPTG. A_{600 nm} was measured at different h post induction. "Control" represents cells containing native plasmid. **B. Nucleotide release from cells.** BL21(DE3)pLysS cells synthesizing Vpu protein were preloaded with 2 µCi/ml [³H]uridine for 90 min and induced with 1 mM IPTG. At the indicated times post induction 0.2 ml aliquots of supernatant were removed from cultures and radioactivity measured. "Control" represents cells containing native plasmid. **C. ONPG entry into cells.** BL21(DE3)pLysS cells transformed with pET3 plasmid were induced with IPTG. After the indicated min post induction 2 mM ONPG was added and 10 min later β-galactosidase activity of cells was determined by measuring A_{420 nm}. "Control" represents cells containing native plasmid. **D. Entry of translation inhibitors into cells.** BL21(DE3)pLysS cells transformed with pET3-Vpu plasmid were induced with

IPTG and 30 min post induction hygromycin B was added at the concentrations (mM) indicated. 15 min later proteins were metabolically labeled with [^{35}S]methionine for 15 min and cell extracts were analyzed by SDS-20%PAGE.

Figure 3. Western blot analysis of Vpu protein. A: Subcellular localization of Vpu in E. coli cells. Induced BL21(DE3)pLysS cells carrying the pET-3vpu plasmid were grown for 3 h and a cell extract was prepared and subjected to cellular fractionation as described in Materials and Methods. Soluble fraction was analyzed, and insoluble fraction was further treated with 1 M NaCl (lanes A), 0.2 M Na_2CO_3 (lanes B), 1% Triton X100 (lanes C) or 8 M urea. Treated membrane extracts were fractionated and soluble (S) and insoluble (P) analyzed by immunoblot using anti-Vpu polyclonal serum. Control total protein extracts of uninduced (C) or induced (T) cells were also analyzed. **B: Detection of Vpu protein in COS cells transfected with pTM1-Vpu.** COS cells were infected with recombinant vaccinia virus VT7 and then transfected with pTM1 (Control) or pTM1-vpu (Vpu). 40 $\mu\text{g}/\text{ml}$ ara-C was (+) or not (-) added to transfected cells. Cells were collected 20 h post-infection and cell lysates probed with rabbit anti-Vpu polyclonal serum. **C: Detection of Vpu protein in COS cells transfected with pBJ3-Vpu.** Proteins of cell lysates were probed with anti-Vpu polyclonal antiserum 48 h after transfection.

Figure 4. Hygromycin B entry into COS cells. COS cells were infected with recombinant vaccinia virus VT7 and then transfected with pTM1 (**A**), pTM-1 poliovirus 2BC (45) (**B**), pTM1-poliovirus 2C (45) (**C**) or pTM1-Vpu (**D**), in absence (untreated) or presence of 40 $\mu\text{g}/\text{ml}$ ara-C. At 9 h post-infection 0, 0.5 or 1 mM hygromycin B was added and 15 min later cells were metabolically labeled with [^{35}S]methionine for 1 h. Cell extracts were subjected to SDS-15%PAGE and autoradiographed. (**E**): Hygromycin B entry into cells was quantified (arbitrary units) by densitometric scanning of Vpu (black bars) and

negative control 2C (white bars) protein bands from ara-C lanes of **(D)**. **(F)**: Immunoprecipitation analysis of Vpu protein. COS cells were infected with VT7 virus and transfected with pTM1-2C or pTM1-Vpu constructs. At 7 h post transfection hygromycin B was added in methionine-free medium and 10 min later cells were pulse labeled for 1 h in the presence of hygromycin B. Cell lysates were immunoprecipitated with anti-2C (66) or anti-Vpu polyclonal sera. Samples were analyzed by SDS-15%PAGE followed by fluorography.

Figure 5. Fluorescence detection of intracellular neurobiotin. COS cells were infected with recombinant VT7 virus and transfected with pTM1-construct. Cells were incubated with 1 μ g/ml neurobiotin, fixed and FITC-streptavidin stained. **(A)**: mock-infected cells. **(B)**: untransfected but infected cells. **(C)**: pTM1 plasmid transfected cells. **(D)**: pTM1-2BC transfected cells (45). **(E)**: pTM1-Vpu transfected cells.

Figure 6. Immunofluorescence localization of intracellular neurobiotin in Vpu expressing cells. COS cells were electroporated with pBJ3-Vpu. At 48 h post transfection cells were incubated with 1 μ g/ml neurobiotin. Fixed cells were stained with FITC-streptavidin for neurobiotin detection and labeled with rabbit anti-Vpu serum/rhodamine-goat anti-rabbit IgG.

Figure 1

Figure 2

A

B

C

D

Figure 3

A

B

C

Figure 4

Figure 5

Figure 6

vpu

neurobiotin