The Human Immunodeficiency Virus Type 1 Vpu Protein Enhances Membrane Permeability†

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ABSTRACT: Infection of T lymphocytes by the 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 *υpu* in *Escherichia 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.

The *υpu* 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 or 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 *υpu* gene.

The N terminus of the 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 ref *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, the 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 viroporins, that modify membrane permeability upon expression in intact cells (*13*). This is the case for poliovirus proteins 2B and 3A, the 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 the 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, in *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, in both prokaryotic and eukaryotic cells.

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EXPERIMENTAL PROCEDURES

Materials. L-[^{35}S]Methionine (1000 Ci/mmol) and [5-^{3}H]-uridine (29 Ci/mmol) were purchased from Amersham Corp. Cationic liposomes containing dimethyldioctadecylammonium 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 Escherichia coli. The construct for the production of the native HIV-1 Vpu protein in bacteria was made by standard PCR and cloning techniques. The genome from the 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 the *vpu* gene. The host for initial cloning and analysis of recombinant plasmids was the *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 0.5A_{600nm}, they were induced to synthesize Vpu by addition of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG).

Expression in Mammalian Cells. The *vpu* gene was amplified by the 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 the pTM1 plasmid (*35*). The 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 the *vpu* gene (*35*). The Vpu protein was also expressed from the SV40 early promoter using the pBJ3 plasmid in COS cells (*36*). The *vpu* gene was amplified by PCR using the 5′-primer: 5′-GGGCCCAAGCTTATG-CAACCTATACAAATAG-3′ containing a *Hin*dIII site (underlined) and the same 3′-primer described above. The amplified fragment was cloned in *Hin*dIII and *Bam*HI restriction sites of the polylinker of the pBJ3 plasmid. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine (DMEM/10% FCS).

Rabbit Antiserum against the vpu Gene. The Vpu protein from the 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 adyuvant. Serum was collected from immunized animals and adsorbed to acetone powders of MBP-producing DH5 cells to remove the 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 of 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 the A_{420nm} of the supernatant was measured.

Subcellular Fractionation. Cell fractionation was performed essentially as described previously (*37*). Eighty milliliters of bacterial culture was harvested after 3 h of induction by centrifugation at 5000*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, and 1 mM dithiothreitol (TKMD) and frozen. After the solid was thawed in cold water, the cell extract was sonicated at 12 Hz and centrifuged at 15000*g* for 20 min at 4 °C. Clarified cell lysate was centrifuged at 120000*g* for 3 h at 4 °C. The supernatant (cytosolic fraction) was mixed with an equal volume of sample buffer 2X. The pellet (crude membrane fraction) was resuspended in half of the original volume of TKMD. The 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 X-100, or 8 M urea in a final volume of 250 μL, for 30 min. Each fraction was centrifuged at 120000*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. Ten microliters of each fraction was loaded onto a 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 provided by B. Moss, National Institutes of Health, Bethesda, MD) in serum-free DMEM. After adsorption for 1 h, cells were transfected with a mixture of 0.5 μg of pTM1 construct DNA and 1 μL of liposomes per well. For each transfection experiment with pBJ3 constructs, 10^7 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 Bio-Rad cuvette. A mixture of 5 μL of 1.5 M NaCl, 20 μg of pUC19 carrier DNA, and 5 μg of the expression plasmid was added. Cells were electroporated using a Bio-Rad gene pulser set at 200 V and 960 μF for 40 s. Then cells were diluted in 10 mL of fresh medium (DMEM/10% FCS with 10 mM Hepes) and plated.

SDS Gel Electrophoresis and Western Blot and Immunoprecipitation Assays. After being labeled with [^{35}S]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 (Bio-Rad) and reacted with a 1:5000 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 of [^{35}S]methionine for 1 h. Cell monolayers were scraped and collected in a phosphate-buffered saline solution (PBS). The pelleted cells were lysed in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1% Nonidet P40, and 0.2 mM phenylmethanesulfonyl fluoride. Cell lysates were incubated with 10 μL of 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 being washed with PBS, cells were incubated in PBS with a 1:400 dilution (v:v) of polyclonal Vpu antiserum for 1 h at 37 °C. Cells were washed with PBS three times and incubated in PBS with 30 μg/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 μg/mL neurobiotin in fresh medium, washed with PBS, and analyzed for polyamine uptake as described previously (*39*). Briefly, cells were fixed with 4% (w:v) 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 μg/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 for testing 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 can be induced 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 the 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 2 h after addition of IPTG, bacteria continued to synthesize 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 mass of 16 kDa is immunologically related to Vpu (Figure 1). Therefore, the Studier system is very suitable for the efficient expression of the HIV-1 *vpu* gene.

Vpu Enhances the 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 the optical density of the culture at different times after IPTG induction of Vpu. As a control, cells bearing the 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 [^{3}H]-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 would not precipitable with trichloroacetic acid, suggesting that it corresponded to low-molecular mass 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 in the level 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 of hygromycin B. Figure 2D shows that synthesis of Vpu permeabilizes cells to hygromycin B to such an extent that the antibiotic at 0.5 mM completely blocked protein synthesis in these cells. In conclusion, expression of Vpu

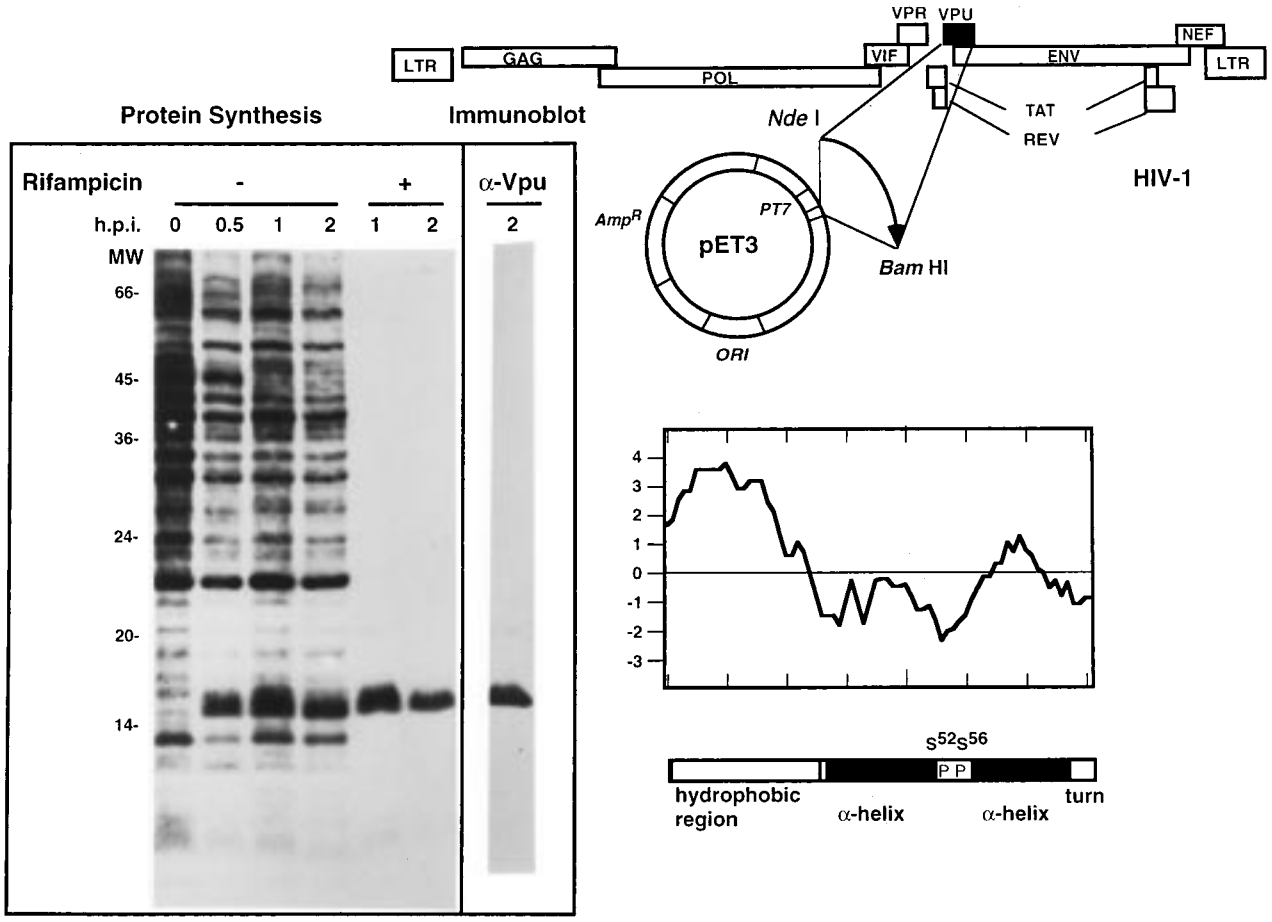

FIGURE 1: Cloning of the HIV-1 *vpu* coding sequence into the pET3 plasmid. (Top right) Schematic representation of the *vpu* cloning strategy: P_{T7}, T7 promoter; ORI, origin of replication; and Amp_R, ampicillin resistance gene. (Bottom right) Hydrophobicity profile of HIV-1 Vpu (Kite Doolittle) and structural elements of the Vpu protein. (Left) Time course of Vpu synthesis in BL21(DE3)pLysS cells. Cells carrying the 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 postinduction time, cells were metabolically labeled with [^{35}S]methionine for 10 min, and the proteins were separated by SDS−20% PAGE and detected by autoradiography. After IPTG induction for 2 h, the proteins from cell lysates were separated by SDS−20% PAGE, transferred to nitrocellulose membrane, and probed with rabbit anti-Vpu polyclonal serum.

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 (Figure 3A). After induction for 3 h, sufficient amounts of Vpu were achieved in *E. coli* cells (Figure 3A, lane T) to allow subcellular fractionation and biochemical analysis of the recombinant protein. The 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. 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 concentrations (Figure 3A, lanes A), or high pH (Figure 3A, lanes B), suggesting the integral membrane localization of the Vpu protein. The membrane fraction was also treated with 1% Triton X-100 that solubilizes the inner membrane proteins of *E. coli* cells or denaturated with 8 M urea. Our results agree well with previous observations on the partial solubilization by urea (Figure 3A, lanes D) and detergents (Figure 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 Experimental Procedures. Synthesis of Vpu is clearly detected from the 8 h after vaccinia virus infection. Vpu synthesis continued until 24 h postinfection (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 (Figure 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

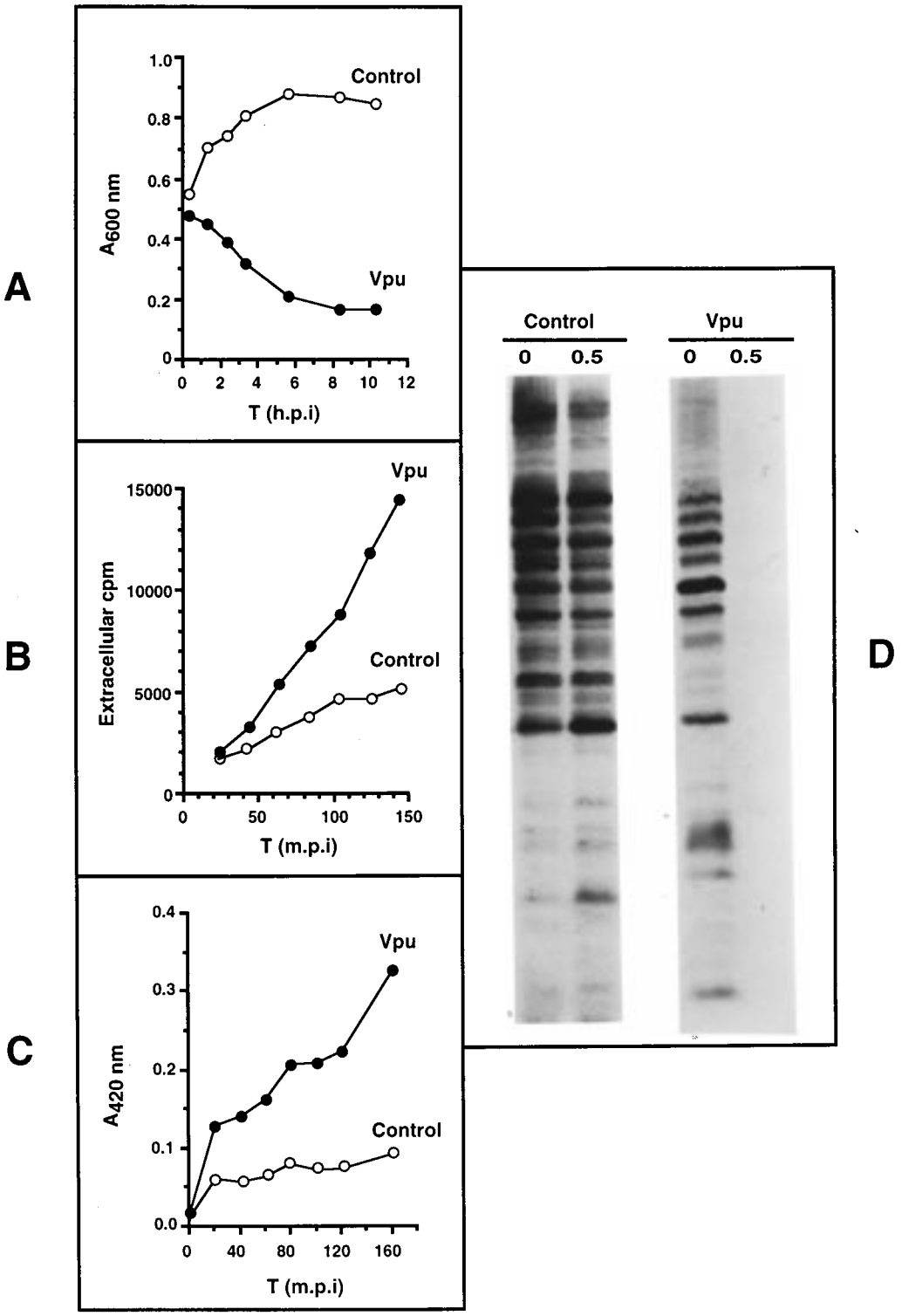

FIGURE 2: Modifications of membrane permeability by the Vpu protein in prokaryotic cells. (A) Cell growth analysis. *E. coli* BL21(DE3)-pLysE cells carrying the pET3 Vpu plasmid were induced with 1 mM IPTG. A_{600nm} was measured at different postinduction times. Control represents cells containing the native plasmid. (B) Nucleotide release from cells. BL21(DE3)pLysS cells synthesizing the Vpu protein were preloaded with 2 μCi/mL [^{3}H]uridine for 90 min and induced with 1 mM IPTG. At the indicated postinduction times, 0.2 mL aliquots of supernatant were removed from cultures and radioactivity was measured. Control represents cells containing the native plasmid. (C) ONPG entry into cells. BL21(DE3)pLysS cells transformed with the pET3 plasmid were induced with IPTG. After the indicated postinduction times, 2 mM ONPG was added and 10 min later β-galactosidase activity of the cells was determined by measuring A_{420nm}. Control represents cells containing the native plasmid. (D) Entry of translation inhibitors into cells. BL21(DE3)pLysS cells transformed with the pET3-Vpu plasmid were induced with IPTG, and 30 min postinduction, hygromycin B was added at the concentrations (millimolar) indicated. Fifteen minutes later, proteins were metabolically labeled with [^{35}S]methionine for 15 min and cell extracts were analyzed by SDS−20% PAGE.

of Vpu were achieved 48 h after transfection (Figure 3C). This second eukaryotic expression system is very appropriate for studies of Vpu activity in the absence of vaccinia virus infection.

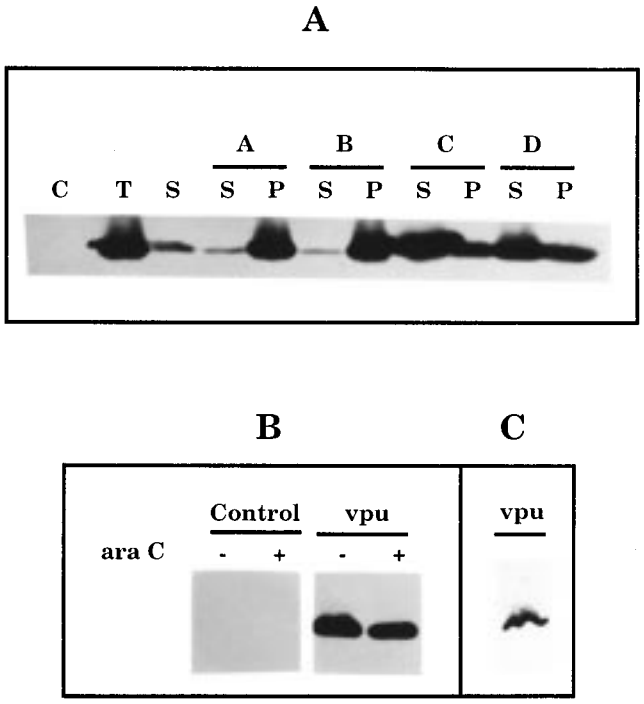

FIGURE 3: Western blot analysis of the Vpu protein. (A) Subcellular localization of Vpu in *E. coli* cells. Induced BL21(DE3)pLysS cells carrying the pET-3vpu plasmid were grown for 3 h, and a cell extract was prepared and subjected to cellular fractionation as described in Experimental Procedures. The soluble fraction was analyzed, and the insoluble fraction was further treated with 1 M NaCl (lanes A), 0.2 M Na_2CO_3 (lanes B), 1% Triton X-100 (lanes C), or 8 M urea. Treated membrane extracts were fractionated and soluble (S) and insoluble (P) extracts 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 the 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). Ara-C (40 μg/mL) was (+) or was not (−) added to transfected cells. Cells were collected 20 h postinfection and cell lysates probed with rabbit anti-Vpu polyclonal serum. (C) Detection of the Vpu protein in COS cells transfected with pBJ3-Vpu. Proteins of cell lysates were probed with anti-Vpu polyclonal antiserum 48 h after transfection.

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 the permeability of membranes to hygromycin B while poliovirus 2C has no effect (*45*). Both proteins 2BC and 2C were used as positive and negative controls, respectively, in our experiments on hygromycin B entry (Figure 4B,C). 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,D). 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 through 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, an increased extent of 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 such as impaired Ca^{2+} fluxes, increased intracellular concentrations of K^+ and Na^+, decreased membrane potential, or inhibition of phospholipid synthesis are hallmarks of HIV-infected cells (*52*−*55*). Moreover, it was recently 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 the 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

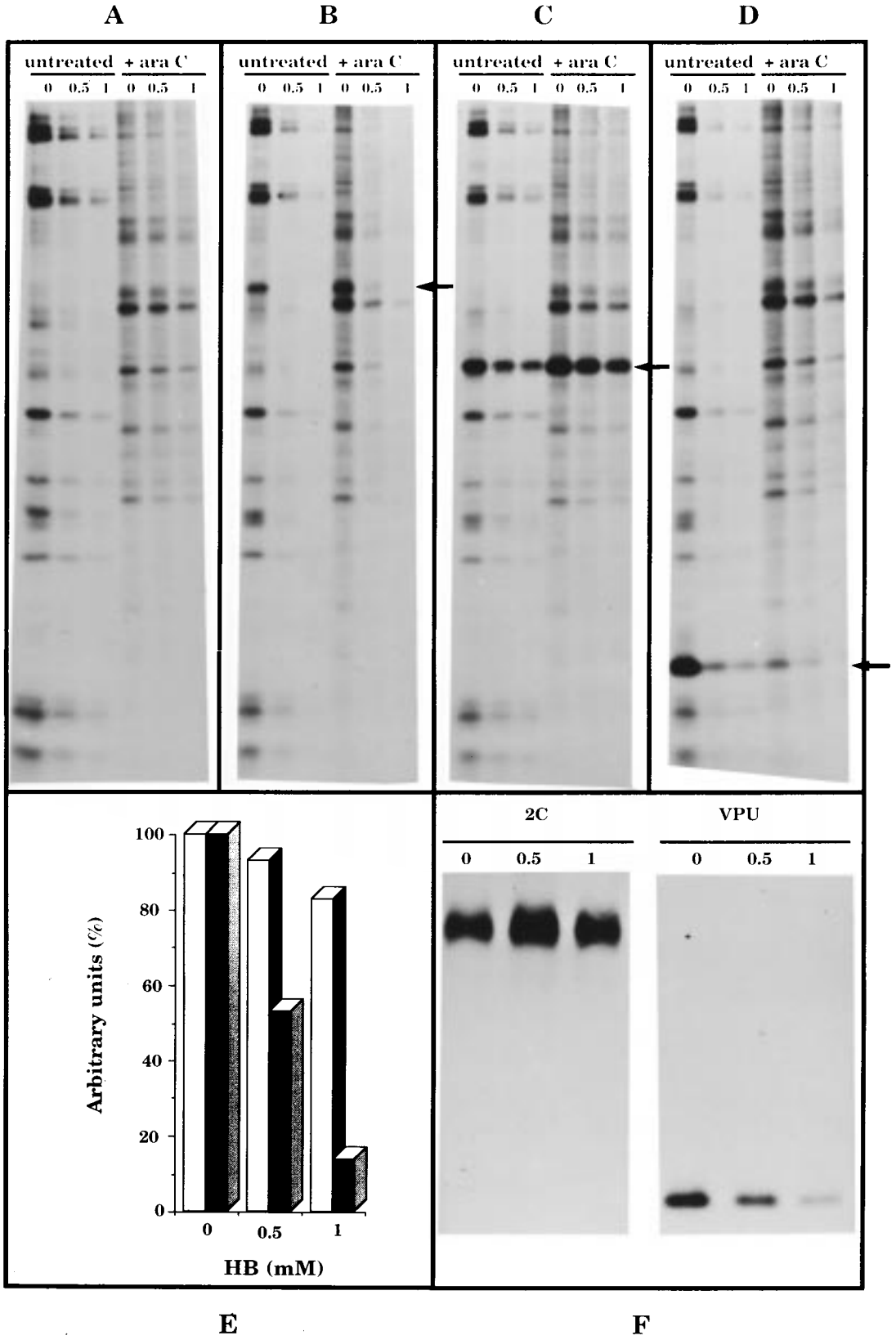

FIGURE 4: Hygromycin B entry into COS cells. COS cells were infected with recombinant vaccinia virus VT7 and then transfected with pTM1 (A), pTM-1 poliovirus 2BC (*45*) (B), pTM1-poliovirus 2C (*45*) (C), or pTM1-Vpu (D), in the absence (untreated) or presence of 40 μg/mL ara-C. At 9 h postinfection, 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 panel D. (F) Immunoprecipitation analysis of the Vpu protein. COS cells were infected with the VT7 virus and transfected with pTM1-2C or pTM1-Vpu constructs. At 7 h posttransfection, 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.

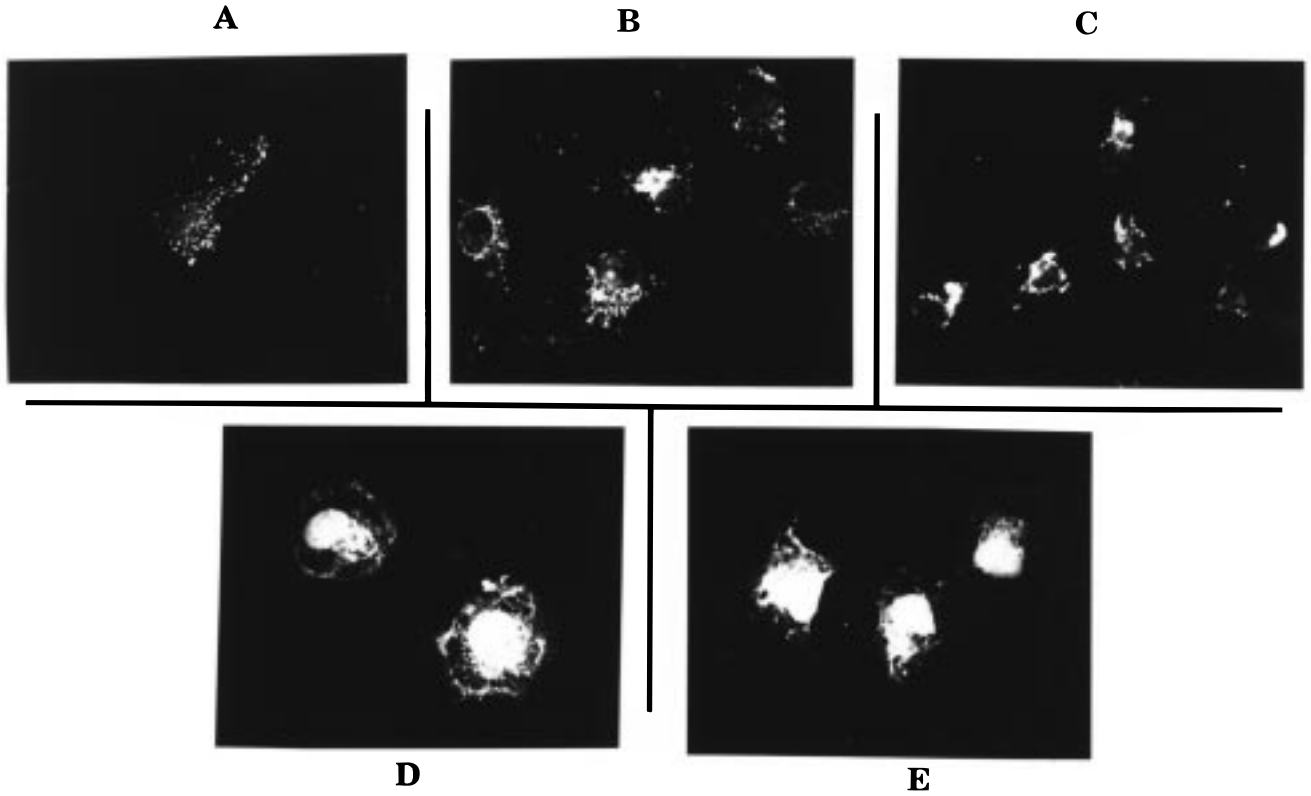

FIGURE 5: Fluorescence detection of intracellular neurobiotin. COS cells were infected with the recombinant VT7 virus and transfected with the pTM1 construct. Cells were incubated with 1 μg/mL neurobiotin, fixed, and FITC−streptavidin stained: (A) mock-infected cells, (B) untransfected but infected cells,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

eukaryotic cells, using different expression systems. The membrane permeability to lysozyme, uridine, ONPG, and hygromycin B was enhanced in bacteria cells. We also detected an increase in the level of hygromycin B and neurobiotin entry into mammalian cells. Undoubtedly, membrane permeability to ions should be modified too. These permeability changes induced by Vpu may account for 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 to be mainly associated with the 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 X-100, 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 the 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 among them. To this list of proteins can be added the picornavirus 3A and 2B and adenovirus E3 11.3K proteins (*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 a diminished level of virus formation (*58*). Moreover, blockade of influenza M2 by amantadine inhibits not only 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*) supports 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 extent of 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 (this 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 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 the M2 protein of the influenza virus, changes in ion content in cellular compartments may lead

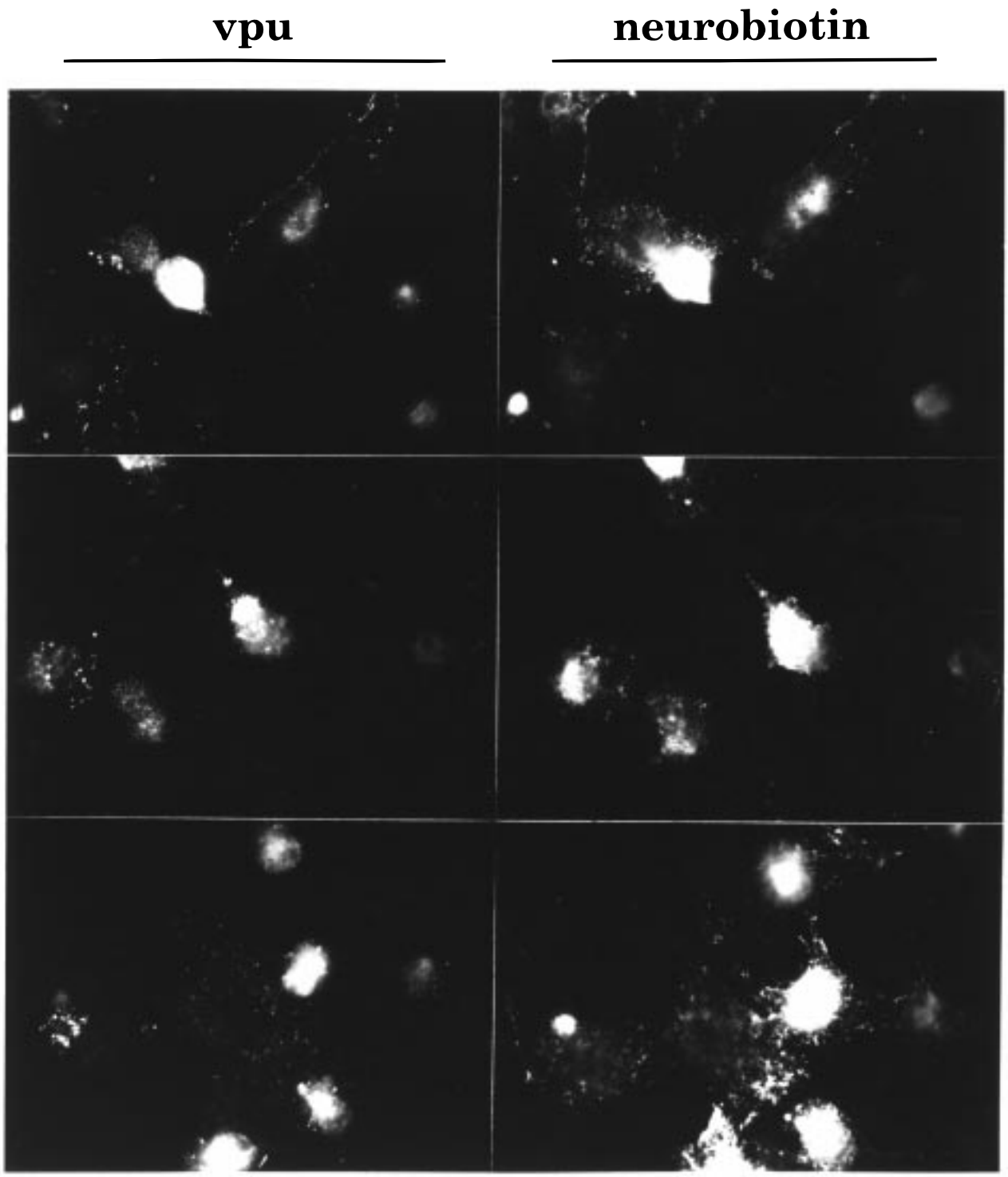

FIGURE 6: Immunofluorescence localization of intracellular neurobiotin in Vpu-expressing cells. COS cells were electroporated with pBJ3-Vpu. At 48 h posttransfection, cells were incubated with 1 μg/mL neurobiotin. Fixed cells were stained with FITC−streptavidin for neurobiotin detection and labeled with rabbit anti-Vpu serum/rhodamine-goat anti-rabbit IgG.

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 interfere 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 connection between these phenomena.

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