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The Vpu Protein of Human Immunodeficiency Virus Type 1 Forms Cation-Selective Ion Channels

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Vpu is a small phosphorylated integral membrane protein encoded by the human immunodeficiency virus type 1 genome and found in the endoplasmic reticulum and Golgi membranes of infected cells. It has been linked to roles in virus particle budding and degradation of CD4 in the endoplasmic reticulum. However, the molecular mechanisms employed by Vpu in performance of these functions are unknown. Structural similarities between Vpu and the M2 protein of influenza A virus have raised the question of whether the two proteins are functionally analogous: M2 has been demonstrated to form cation-selective ion channels in phospholipid membranes. In this paper we provide evidence that Vpu, purified after expression in *Escherichia coli*, also forms ion channels in planar lipid bilayers. The channels are approximately five- to sixfold more permeable to sodium and potassium cations than to chloride or phosphate anions. A bacterial cross-feeding assay was used to demonstrate that Vpu can also form sodium-permeable channels in vivo in the *E. coli* plasma membrane.

Vpu is an integral membrane protein encoded by human immunodeficiency virus type 1 (HIV-1). It is associated with the Golgi and endoplasmic reticulum membranes in infected cells but has not been detected in the plasma membrane or in the viral envelope (8, 13). The protein is 80 to 82 amino acids long depending on the viral isolate, with an N-terminal transmembrane anchor and a hydrophilic cytoplasmic C-terminal domain. The C-terminal domain contains a 12-amino-acid sequence that is conserved in all isolates and two serine residues that are phosphorylated (3, 23).

Two independent physiological activities in which Vpu is involved have been characterized to date: control of the release of virus particles (9) and degradation of the CD4 molecule in the endoplasmic reticulum (1, 35). Mutations to serine residues at positions 52 and 57, which are phosphorylated by casein kinase II in vivo (22, 23), abolish Vpu-dependent CD4 degradation but do not affect the virus release function (3). On the other hand, mutations in the transmembrane anchor domain knock out enhancement of virus release by Vpu but do not affect the CD4 degradation activity (21). The molecular mechanisms employed by Vpu to mediate both of these functions are unknown.

Structural similarities between Vpu and another small viral protein, M2, encoded by influenza A virus were first noted soon after the discovery of Vpu (9, 26). Since then, M2 has been shown to form cation-selective ion channels when expressed in *Xenopus* oocytes (17) or mammalian cells (34) and also when purified and reconstituted into planar lipid bilayers (2, 31). The physiological significance of M2 channel activity to replication of influenza A virus is indicated by the effects of the anti-influenza A virus drug amantadine: amantadine blocks channels formed by M2 proteins encoded by influenza virus strains whose replication is sensitive to the drug, but channels formed by variant M2 proteins, with mutations affecting specific amino acid residues in the putative transmembrane domain, are not blocked by amantadine (6, 17, 19, 28).

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The structural similarity of Vpu to M2 has caused a number of investigators to consider whether Vpu is a functional analog of M2 in HIV-1 (3, 9, 22, 23, 32, 35). In this paper we present evidence that Vpu forms cation-selective channels in planar lipid bilayers and also in the plasma membrane of *Escherichia coli* cells expressing Vpu.

MATERIALS AND METHODS

Construction of recombinant plasmids p2GEXVpu and pPLVpu. The open reading frame encoding Vpu (Fig. 1A) was amplified by PCR from a cDNA clone of an *Nde*I fragment of the HIV-1 genome (isolate HXB2, a gift from N. Deacon, McFarlane Burnet Centre, Melbourne, Australia). Native *Pfu* DNA polymerase (0.035 U/μl; Stratagene) was chosen to catalyze the PCR to minimize possible PCR-introduced errors by virtue of the enzyme's proofreading activity. The 5′ sense primer (AGTAGGATCCA**T**GCAACCTATACC) introduces a *Bam*HI site (underlined) for cloning in frame with the 3′ end of the glutathione *S*-transferase (GST) gene in p2GEX (16). This primer also repairs the start codon (**T** [boldfaced] replaces a C) of the *vpu* gene, which is a threonine codon in the HXB2 isolate. The 3′ antisense primer (TCTGGAATTCTAC AGATCAT CA AC) introduces an *Eco*RI site (underlined) to the other end of the PCR product to facilitate cloning. After 30 cycles of 94°C for 45 s, 55°C for 1 min, and 72°C for 1 min in 0.5-ml thin-walled Eppendorf tubes in a Perkin-Elmer thermocycler, the 268-bp fragment was purified, digested with *Bam*HI and *Eco*RI, and ligated to p2GEX prepared by digestion with the same two enzymes. The resultant recombinant plasmid is illustrated in Fig. 1B. The entire Vpu open reading frame and the *Bam*HI and *Eco*RI ligation sites were sequenced by cycle sequencing, by using the Applied Biosystems dye-terminator kit, to confirm the DNA sequence.

To prepare the Vpu open reading frame for insertion into the pPL451 expression plasmid (11), p2GEXVpu was first digested with *Bam*HI, and the 5′ base overhang was filled in with Klenow DNA polymerase in the presence of deoxynucleoside triphosphates. The fragment encoding Vpu was then liberated by digestion with *Eco*RI, purified from an agarose gel, and ligated into pPL451 which had been digested with *Hpa*I and *Eco*RI. Western blotting (immunoblotting) subsequently confirmed that the pPLVpu construct (Fig. 1C) expressed Vpu after induction of cultures at 42°C to inactivate the *c*I857 represser of the P_R and P_L promoters.

Raising polyclonal antibodies for immunoidentification of Vpu. A peptide (CALVEMGVEMGHHAPWDVDDL) corresponding to the C-terminal 20 amino acid residues of Vpu was synthesized in the Biomolecular Resource Facility (Australian National University, Canberra City, Australia) with an Applied Biosystems model 477A machine. A multiple antigenic peptide was prepared (12) by coupling the peptide to a polylysine core via the N-terminal cysteine residue. The multiple antigenic peptide was used to immunize rabbits for production of polyclonal antisera recognizing the C terminus of Vpu. For immunizations, 1 mg of the multiple antigenic peptide was dissolved in 1.25 ml of MTPBS (16 mM Na_2HPO_4, 4 mM NaH_2PO_4, 150 mM NaCl [pH 7.3]), emulsified with 1.25 ml of Freund's complete adjuvant, and injected at multiple subcutaneous sites on the rabbit's back. Booster injections of Freund's incom-

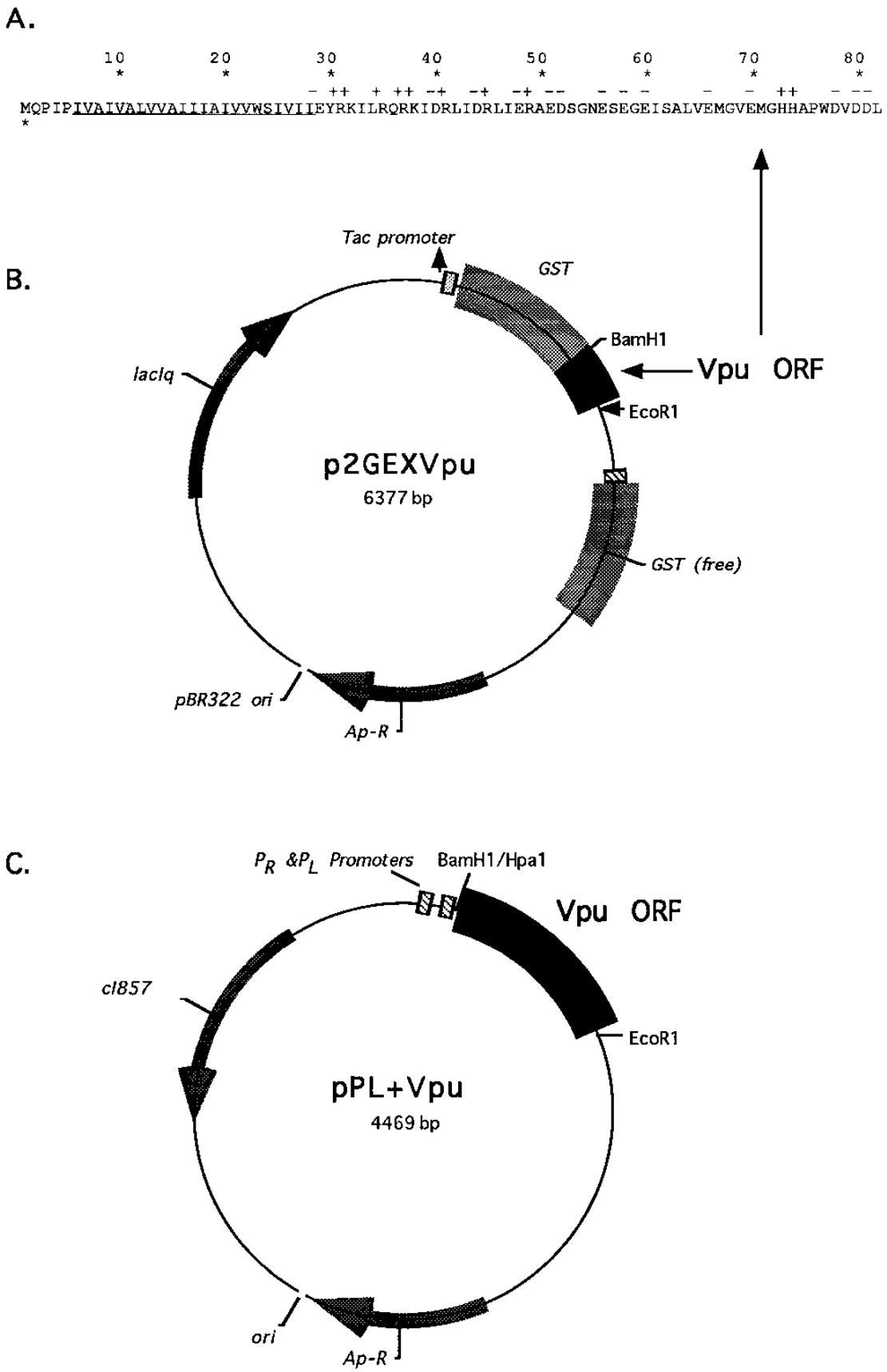

FIG. 1. Plasmids used for expression of Vpu in *E. coli*. (A) The amino acid sequence encoded by the *vpu* open reading frame (ORF) generated by PCR from an HIV-1 strain HXB2 cDNA clone, as described in Materials and Methods. (B) The *vpu* ORF was cloned in frame at the 3′ end of the GST gene in p2GEX to generate p2GEXVpu. (C) It was subsequently cloned into pPL451 to produce the plasmid pPLVpu.

plete adjuvant were spaced at least 4 weeks apart, and serum was sampled 10 to 14 days after each injection.

Other antibody techniques. Peptide-specific antibodies were purified from rabbit sera by using an Immunopure antigen-antibody immobilization kit from Pierce. The synthetic peptide was cross-linked via its N-terminal cysteine to the matrix of a 5-ml Sulfo Link column according to the kit's instructions. Vpu immunoreactive serum (2.5 ml) was added to 20 ml of Tris buffer (10 mM; pH 7.4) and passed through the peptide column three times to maximize exposure of the antibodies to the peptide. The column was washed with 20 ml of 10 mM Tris (pH 7.4) and then with 20 ml of the same buffer supplemented with 500 mM NaCl. The bound antibodies were eluted in 5 ml of 100 mM glycine–150 mM NaCl (pH 2.5), and eluants were immediately neutralized by the addition of 250 μl of 1 M Tris (pH 9.0) and dialyzed overnight against MTPBS.

An anti-Vpu immunoaffinity column was constructed by covalently cross-linking 200 μg of purified antibody to 100 μl of protein A-agarose beads (Schleicher & Schuell) with the bifunctional cross-linking reagent dimethylpimelimidate as described previously (5).

Immunoprecipitation of Vpu was done by incubation of the samples in the presence of an approximately fivefold excess of purified antibody (at room temperature for 1 h) followed by the addition of excess protein A-agarose, incubation for 30 min, and centrifugation to pellet the Vpu-antibody complexes. The supernatant, which was subsequently used as a control in the electrophysiological bilayer experiments, was tested by Western blotting to confirm that Vpu had been completely removed.

Polyacrylamide gel electrophoresis (PAGE) and Western blotting. Protein samples were electrophoresed on homogeneous sodium dodecyl sulfate (SDS)–18% polyacrylamide gels by using a minigel apparatus and prepoured gels (Novex). Samples were treated with SDS (3.2% final) and mercaptoethanol (0.8% final) at 60°C for 5 min before being loaded onto the gels. Protein bands were visualized either with Coomassie brilliant blue R250 or by silver staining.

For Western blotting, proteins were transferred from acrylamide gels to polyvinylidene difluoride membranes with a semidry transfer apparatus (Pharmacia LKB). Vpu was detected after consecutive reactions of the blots with polyclonal antiserum or purified antibodies, a goat anti-rabbit alkaline phosphatase conjugate, and a Western Blue stabilized substrate (Promega).

Purification of recombinant Vpu from *E. coli*. Cultures of *E. coli* XL1-Blue cells containing p2GEXVpu were grown at 30°C with vigorous aeration in Luria-Bertani medium supplemented with glucose (6 g/liter) and ampicillin (50 mg/liter) to a density of approximately 250 Klett units, at which time IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 0.01 mM, and growth was allowed to continued for a further 4 h. The final culture density was approximately 280 Klett units. Since early experiments revealed that the majority of expressed GST-Vpu fusion protein was associated with both the cell debris and membrane fractions (data not shown), we adopted the method of Varadhachary and Maloney (33) to isolate osmotically disrupted cell ghosts (combining both cell debris and membrane fractions) for the initial purification steps. Cells were harvested, washed, weighed, and resuspended to 10 ml/g (wet weight) in MTPBS containing dithiothreitol (DTT) (1 mM) and $MgCl_2$ (10 mM). Lysozyme (0.3 mg/ml; chicken egg white; Sigma) was added and incubated on ice for 30 min with gentle agitation and then for 5 min at 37°C. The osmotically sensitized cells were pelleted at 12,000 × *g* and resuspended to the original volume in water to burst the cells. The suspension was then made up to 1× MTPBS-DTT with a 10× buffer stock, and the ghosts were isolated by centrifugation and resuspended in MTPBS-DTT, to which glycerol (to 20% [wt/vol]) and 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS) (to 2% [wt/vol]) were then sequentially added to give a final volume of one quarter the original volume. This mixture was stirred on ice for 1 h and then centrifuged at 400,000 × *g* for 1 h to remove the insoluble material. The GST-Vpu fusion protein was purified from the detergent extract by affinity chromatography on a glutathione agarose resin (Sigma). The resin was thoroughly washed in 50 mM Tris (pH 7.5) containing glycerol (5%), DTT (1 mM), and CHAPS (0.5%) (buffer A), and then the Vpu portion of the fusion protein was liberated and eluted from the resin-bound GST by treatment of a 50% (vol/vol) suspension of the beads with human thrombin (100 U/ml; 37°C for 1 h). Phenylmethylsulfonyl fluoride (0.5 mM) was added to the eluant to eliminate any remaining thrombin activity. This Vpu fraction was further purified on a column of MA7Q anion-exchange resin attached to a Bio-Rad high-performance liquid chromatograph (HPLC) and eluted with a linear NaCl gradient (0 to 2 M) in buffer A.

Vpu was purified to homogeneity, as determined on silver-stained gels, in an immunoaffinity column as follows: HPLC fractions containing Vpu were desalted on an NAP-25 column (Pharmacia) into buffer A and then mixed with the antibody-agarose beads for 1 h at room temperature. The beads were washed thoroughly, and Vpu was eluted by increasing the salt concentration to 2 M. Protein was quantitated by the Bio-Rad dye-binding assay.

Reconstitution of Vpu in phospholipid vesicles. Proteoliposomes containing Vpu were prepared by the detergent dilution method (15). A mixture of lipids (phosphatidylethanolamine-phosphatidylcholine-phosphatidylserine [5:3:2]; 1 mg of total lipid) dissolved in chloroform was dried under a stream of nitrogen gas and resuspended in 0.1 ml of potassium phosphate buffer (50 mM; pH 7.4) containing DTT (1 mM). A 25-μl aliquot containing purified Vpu was added, and the octylglucoside was added to a final concentration of 1.25% (wt/vol). This mixture was subjected to three rounds of freezing in liquid nitrogen, thawing, sonication in a bath-type sonicator (20 to 30 s), and then rapidly diluted into 200 volumes of the potassium phosphate buffer. Proteoliposomes were collected by centrifugation at 400,000 × *g* for 1 h and resuspended in approximately 150 μl of phosphate buffer.

Assaying ion channel activity. Purified Vpu was tested for its ability to induce channel activity in planar lipid bilayers by standard techniques described elsewhere (14, 16). The solutions in the chambers labeled CIS and TRANS were separated by a Delrin plastic wall containing a small circular hole (diameter, approximately 100 μm) across which a lipid bilayer was painted to form a high-resistance electrical seal. Bilayers were painted from an 8:2 mixture of palmitoyl-oleoly-phosphatidylethanolamine and palmitoyl-oleoly-phosphatidylcholine (Avanti Polar Lipids, Alabaster, Ala.) in *n*-decane. The solutions in the two chambers contained 10 mM MES buffer (morpholineethanesulfonic acid [pH 6.0]) to which various NaCl or KCl concentrations were added. Currents were recorded with an Axopatch 200 amplifier. The electrical potential between the two chambers could be manipulated between ± 200 mV (TRANS relative to grounded CIS). Aliquots containing Vpu were added to the CIS chamber either as a detergent solution or after incorporation of the protein into phospholipid vesicles. The solution in the chamber was stirred until currents were observed.

NADH-dependent quinacrine (Atebrin) fluorescence quenching. Cultures of *E. coli* cells containing pPLVpu were grown at 30°C to 150 Klett units, and then the temperature was changed to 42°C for a further 2 h. To prepare everted membrane vesicles, cells were harvested, resuspended in STEM buffer {110 mM TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; pH 7.0], 20 mM magnesium acetate, 0.25 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 8.6% [wt/vol] sucrose}, disrupted by passage through a French press, and centrifuged at 25,000 × *g* for 30 min to remove cell debris. The supernatant was recentrifuged at 400,000 × *g* for 1 h to pellet the membrane vesicles. The vesicles were washed once and resuspended in TES buffer (50 mM TES [pH 7.0], 15% [wt/vol] glycerol). All buffers contained the proteinase inhibitors 6-amino-*n*-hexanoic acid (0.5% [wt/vol]) and *p*-aminobenzamidine (0.12% [wt/vol]). The NADH-dependent quinacrine fluorescence-quenching assay was then used to determine the permeability of the membrane vesicles to hydrogen ions, as described previously (4). Membrane vesicles containing the influenza virus strain B protein NB linked to a six-histidine C-terminal tag were prepared in the same way from cells containing the plasmid pQE+NB (29) after induction of cultures for 2 h at 37°C in the presence of IPTG (0.1 mM).

RESULTS

Vpu can form cation-selective channels in planar lipid bilayers. (i) Expression and purification of Vpu in *E. coli*. The plasmid p2GEXVpu (Fig. 1B) was constructed as described in Materials and Methods to create an in-frame gene fusion between the GST and Vpu open reading frames. This system enabled IPTG-inducible expression of the Vpu polypeptide fused to the C terminus of GST and allowed purification of the fusion protein by affinity chromatography on glutathione agarose.

Cell growth was stopped and only relatively low levels of GST-Vpu fusion protein were expressed after the addition of IPTG to cultures of cells containing p2GEXVpu. These results presumably reflect an extreme toxicity of the recombinant protein to *E. coli* metabolism. Optimal levels of expression were obtained by growing the cultures at 30°C to a cell density of approximately 250 to 300 Klett units and inducing the cells with low levels of IPTG (0.01 mM). By SDS-PAGE of whole-cell lysates and Western blotting with the Vpu C-terminal-specific antibody, a band corresponding to the fusion protein at approximately 36 kDa was detected. On fractionation of the cells, a small amount of the fusion protein could be detected in the cytoplasmic fraction, but the majority of the fusion protein was detected in the cell debris and plasma membrane fractions, which were pelleted by sequential centrifugation of mechanically disrupted cells at 12,000 × *g* and 400,000 × *g*, respectively (data not shown). This result is in contrast to that for unfused GST, which is soluble.

A combined cellular fraction containing the cell debris and plasma membrane was prepared by lysozyme treatment of the induced cells followed by a low-speed centrifugation (see Materials and Methods). Approximately 50% of the GST-Vpu protein could be solubilized from this fraction with the zwitte-

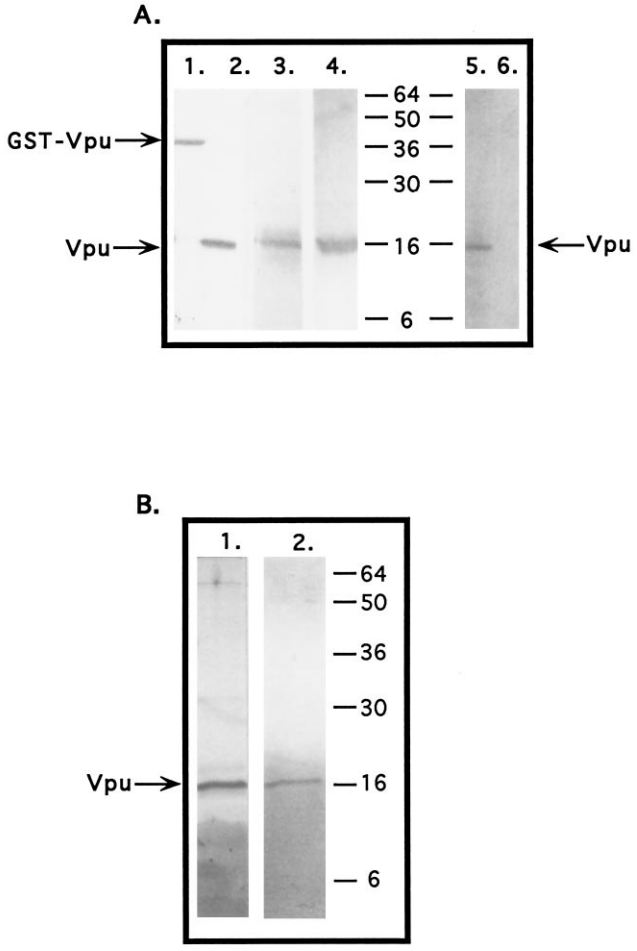

FIG. 2. Expression and purification of Vpu in *E. coli*. (A) Western blotting after SDS-PAGE was used to detect expressed Vpu in *E. coli* extracts. Lanes: 1 to 4, samples, at various stages of purity, of Vpu expressed from p2GEXVpu: GST-Vpu fusion protein isolated by glutathione-agarose affinity chromatography, Vpu liberated from the fusion protein by treatment with thrombin, Vpu purified by HPLC anion-exchange chromatography, and Vpu after passage through the immunoaffinity column, respectively; 5 and 6, membrane vesicles prepared from 42°C induced cells containing pPLVpu or pPL451, respectively. (B) Silver stained SDS-PAGE gel. Lanes: 1, Vpu purified by HPLC anion-exchange chromatography; 2, Vpu after passage through the immunoaffinity column. Molecular size markers (in kilodaltons) are indicated.

rionic detergent CHAPS. Affinity chromatography with glutathione-agarose beads was used to enrich the fusion protein, and thrombin was used to cleave the fusion protein at the high-affinity thrombin site between the fusion partners, liberating Vpu (Fig. 2A). In fractions eluted from the anion-exchange column, Vpu was the major protein visible on silver-stained gels (Fig. 2B, lane 1). Finally, Vpu was purified to apparent homogeneity on an immunoaffinity column (Fig. 2B, lane 2). The N-terminal amino acid sequence of the protein band (excised from SDS-PAGE gels) corresponding to the immunodetected protein confirmed its identity as Vpu.

(ii) Vpu forms ion channels in lipid bilayers. When samples of Vpu (containing between 7 and 70 ng of protein) purified to homogeneity on the immunoaffinity column (e.g., see Fig. 2B, lane 2) were added to 1 ml of buffer in the CIS chamber of the bilayer apparatus, current fluctuations were detected after periods of stirring that varied from 2 to 30 min (Fig. 3). The time taken to observe channel activity correlated approximately with the amount of protein added to the chamber.

No channels were detected when control buffer aliquots or control lipid vesicles were added to the CIS chamber. In those control experiments the chambers could be stirred for more than an hour without the appearance of channel activity.

When aliquots of partially pure Vpu obtained after anion-exchange chromatography (e.g., Fig. 2B, lane 1) were used, either directly or after reconstitution into lipid vesicles, the gross properties of the channels detected (discussed below) were similar to those generated with the pure Vpu. To confirm that the channels were due to the presence of Vpu in the partially pure preparations, purified anti-Vpu antibody was used in immunoprecipitation experiments (as described in Materials and Methods). The supernatant after immunoprecipitation of Vpu did not induce channels in the bilayer assay.

The specificity of the purified antibody preparation to Vpu was confirmed by Western blotting (Fig. 2A), which revealed that the Vpu protein was the only immunoreactive species present in the fractions tested for channel activity. In addition, immunoprecipitation with a rabbit antibody that does not recognize Vpu did not remove the channel-forming species from solution. These two observations negate the possibility that removal of the channel-forming species in the immunoprecipitation experiments was due to nonspecific binding between the antibodies and a contaminating protein.

Since the channels generated by the pure and partially pure fractions were similar, subsequent discussion will not differentiate between data generated with Vpu of either level of purity.

(iii) Properties of Vpu channels. Channel activity was observed in more than 40 individual experiments with Vpu samples prepared from five independent purifications. In different experiments, the amplitude of the currents varied over a large range and, again, seemed to correlate approximately with the amount of protein added. The smallest and largest channels measured had conductances of 14 and 280 pS, respectively. The channels were consistently smaller when lipid vesicles containing Vpu were prepared and fused to the bilayer rather than when purified protein in detergent solution was added. This result may be because the former method included treatment with high concentrations of detergent and a dilution step that may have favored the breakdown of large aggregates into monomers.

The relationship between current amplitude and voltage was linear, and the reversal potential in solutions containing a 10-fold gradient of NaCl (500 mM CIS; 50 mM TRANS) was 30 mV (Fig. 3B). A similar reversal potential was obtained when solutions contained KCl instead of NaCl. In five experiments with either NaCl or KCl in the solutions on either side of the membrane, the mean reversal potential ± standard error of the mean was 31.0 ± 1.2 mV. This result is more negative than expected for a channel selectively permeable for the cations alone. Using ion activities in the Goldman-Hodgkin-Katz equation gives a P_{Na}/P_{Cl} ratio of about 5.5, indicating that the channels are also permeable to chloride ions. An attempt was made to reduce the anion current by substituting phosphate ions for chloride ions. When a sodium phosphate gradient (150 mM Na^+ and 100 mM phosphate for CIS and 15 mM Na^+ and 10 mM phosphate for TRANS [pH 6.8]) was used instead of the NaCl gradient, the reversal potential was 37.1 ± 0.2 (n = 2), again indicating a cation/anion permeability ratio of about 5. (For calculations involving the phosphate solutions, the summed activities of the mono- and bivalent anions were used, and it was assumed that the two species were equally permeable.) The current-voltage curve now showed rectification (Fig. 3D) that was not seen in the NaCl solutions (Fig. 3B); we have no explanation for this difference. It can be concluded that the channels formed by

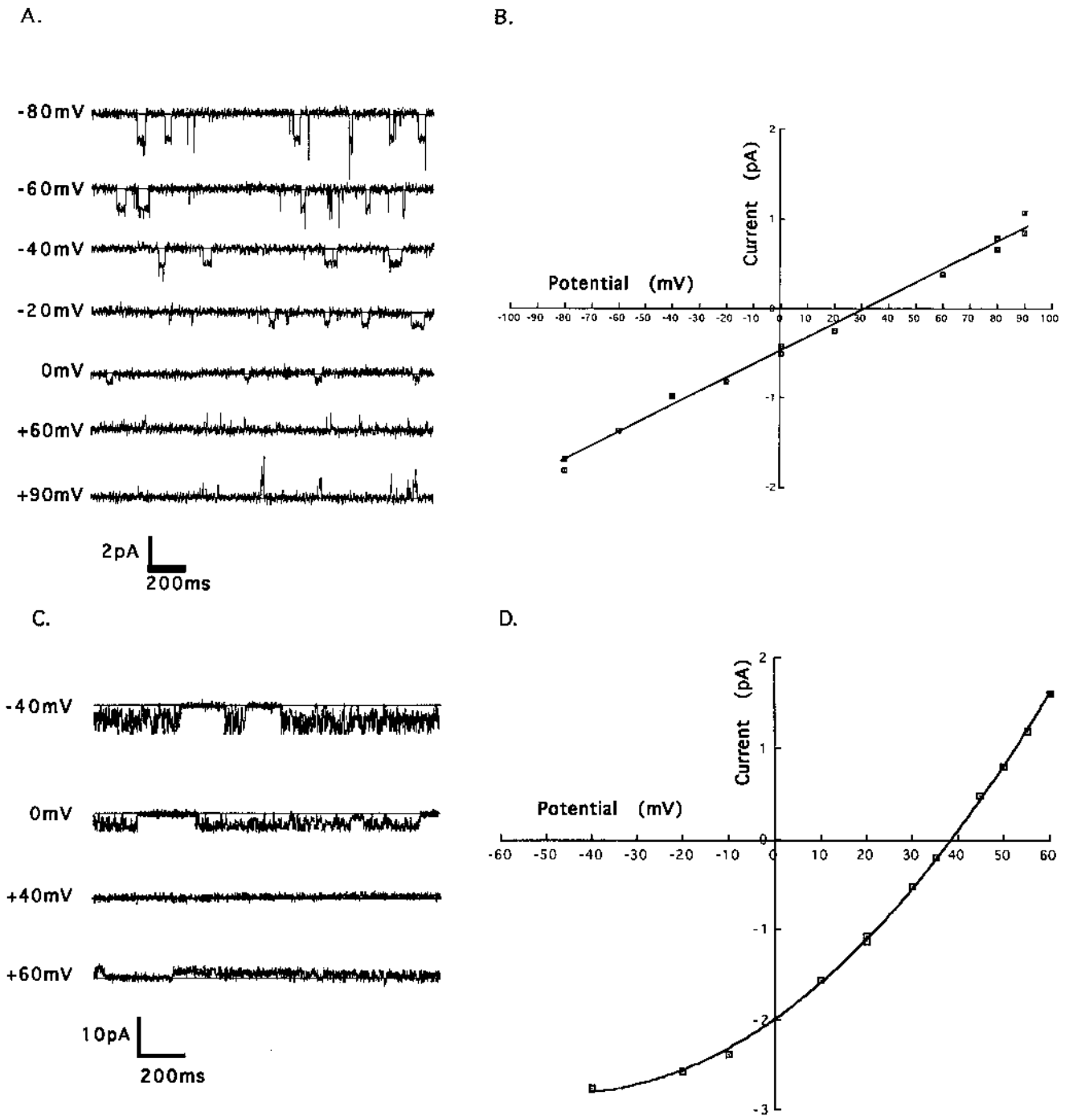

FIG. 3. Ion channel activity observed after exposure of lipid bilayers to aliquots containing purified Vpu. (A and B) The CIS chamber contained 500 mM NaCl and the TRANS chamber contained 50 mM NaCl; both solutions were buffered at pH 6.0 with 10 mM MES. (B) Current-voltage curve generated from data similar to that shown in panel A. (C and D) The CIS chamber contained a sodium phosphate solution at pH 6.8, which was prepared by mixing solutions of Na_2HPO_4 (49 mM final concentration) and NaH_2PO_4 (51 mM final concentration). The TRANS chamber contained the same solution diluted 1:10 with water. (D) Current-voltage curve generated from channels obtained in the presence of sodium phosphate buffers.

Vpu are equally permeable to Na^+ and K^+ and are also permeable, though to a lesser extent, to chloride ions as well as phosphate ions.

Vpu can also form sodium channels in the *E. coli* plasma membrane. To express unfused Vpu in *E. coli*, the Vpu open reading frame was cloned into the plasmid pPL451 (11) as described in Materials and Methods to create the recombinant plasmid pPLVpu (Fig. 1B). In this vector the strong P_L and P_R lambda promoters are used to drive expression of Vpu under control of the temperature-sensitive *c*I857 repressor, such that with growth at 30°C, expression is tightly repressed and can be induced by raising the temperature to between 37 and 42°C.

(i) Growth and expression characteristics of pPLVpu. On agar plates, cells containing pPLVpu grew at 30 and 37°C but

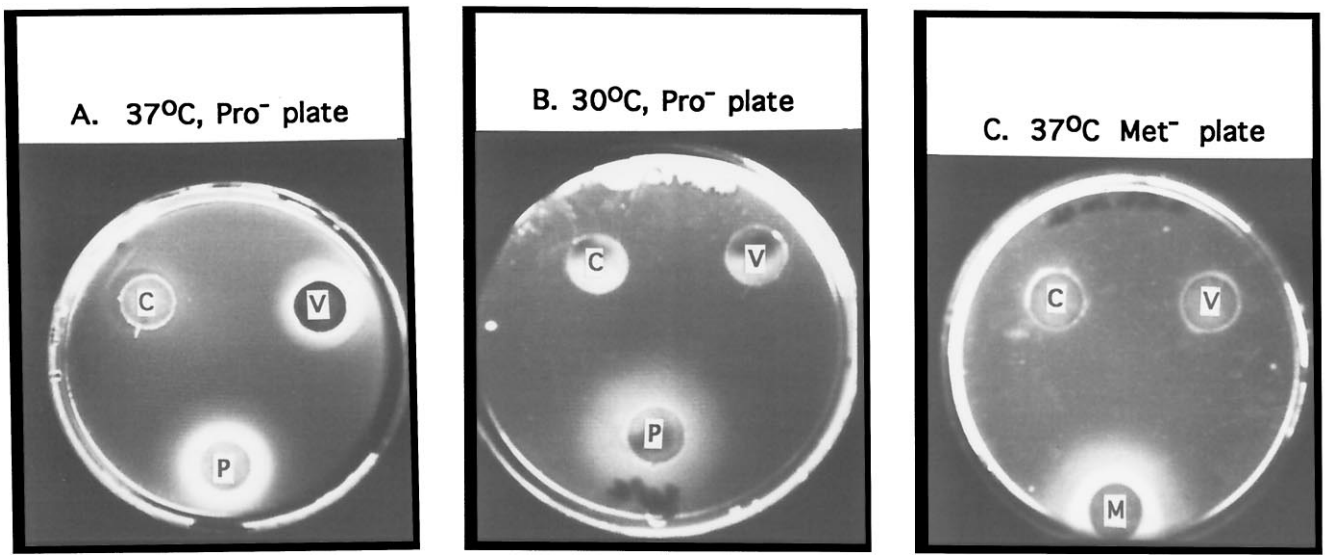

FIG. 4. Bacterial cross-feeding assays. Refer to Materials and Methods for a full description of this assay. For all plates, the Met^- Pro^- auxotrophic strain was used to seed a soft agar overlay. (A and B) Minimal medium supplemented with methionine; (C) medium supplemented with proline. To control for viability of the cells in the background lawn, the discs labelled P and M contained added proline or methionine, respectively. The discs labelled C and V were inoculated with Met^+ Pro^+ *E. coli* cells containing the plasmids pPL451 or pPL+Vpu, respectively. Plates were incubated at 37°C (A and C) or 30°C (B) for 2 days and photographed above a black background with peripheral illumination from a fluorescent light located below the plate. The images were recorded on a Novaline video gel documentation system. Light halos around the discs labelled P or M on all plates and around the disc labelled V on plate A indicate growth of the background lawn strain.

not at 42°C, while control strains grew well at 42°C. Liquid cultures of cells containing pPLVpu were grown at 30°C to an optical density at 600 nm of 0.84 and then at 42°C for 2 h (the final cell density was 0.75). The plasma membrane fraction was prepared, and a single band at approximately 16 kDa was detected by Western blotting, indicating that Vpu was expressed and associated with the membranes (Fig. 2A, lane 5).

(ii) Cross-feeding experiments reveal that proline leaks out of cells expressing Vpu. Uptake of proline by *E. coli* has been well characterized, and active transport of the amino acid into the cells is known to use the sodium gradient as the energy source (36). We predicted that if the sodium gradient was dissipated by a sodium channel in the plasma membrane, then proline synthesized in the cytoplasm would diffuse out of the cells. To detect whether this proline leakage occurred, the following cross-feeding assay was used: a lawn of an *E. coli* strain auxotrophic for proline and methionine (Met^- Pro^-), was seeded and poured as a soft agar overlay on minimal medium plates lacking proline but containing methionine. Sterile porous filter discs were inoculated with a Met^+ Pro^+ strain (XL1-Blue) containing either the pPL451 control plasmid or pPLVpu and placed onto the soft agar. The plates were then incubated at 37 or 30°C for 2 days. After that time a halo of growth of the Met^- Pro^- strain was clearly visible surrounding the disc inoculated with the cells containing pPLVpu incubated at 37°C (Fig. 4A). This growth can only be due to the leakage of proline from the Vpu-expressing cells on the disc. No such leakage was apparent from the control strain at 37°C or around either strain on plates grown at 30°C (Fig. 4B).

(iii) Methionine does not leak out of cells expressing Vpu. In contrast to proline transport, *E. coli* methionine permease is known to belong to the ABC transporter family (18) and hence is energized by ATP. Cross-feeding experiments identical to those described above were set up except that the Met^- Pro^- strain was spread on minimal medium plates lacking methionine but containing proline. No growth of this strain was evident around any of the discs (Fig. 4C), indicating that methionine was not leaking out of the XL1-Blue cells even when Vpu was being expressed.

(iv) Proton permeability of membrane vesicles is unaffected by the presence of Vpu. To investigate whether the Vpu sodium-conductive channel expressed in *E. coli* membranes was also permeable to H^+, the NADH-dependent quinacrine fluorescence-quenching assay (4) was used. This technique can be used to determine the ability of *E. coli* membrane vesicles to maintain a proton gradient generated by the electron transport chain during oxidative phosphorylation. The fluorescent quinacrine molecule contains two protonatable nitrogen atoms. The unprotonated form is electrically neutral and is able to equilibrate between the interior and exterior of the vesicles. The increased internal concentration of protons, generated in the presence of NADH and oxygen, results in protonation of quinacrine molecules that are inside the vesicles, and the subsequent net accumulation of quinacrine inside the vesicles results in quenching of its fluorescence. Vesicles leaky to protons, and hence unable to maintain a high H^+_{in}/H^+_{out} ratio, do not quench quinacrine fluorescence as efficiently as do control vesicles.

In this study, membrane vesicles prepared from *E. coli* cells expressing Vpu from pPLVpu were not more proton permeable than control vesicles prepared from the background strain (Fig. 5A and B). The Vpu protein was present in the membranes (Fig. 2A, lanes 5 and 6), and it can therefore be concluded that it had not formed a channel permeable enough to protons to be detected by the fluorescence-quenching technique.

The NB protein of influenza B virus has been shown to form cation-selective channels in bilayers (29) and may be equivalent to M2 of influenza A virus, which has been shown to be a hydrogen ion channel (20). Membrane vesicles were prepared from a strain containing the plasmid pQE+NB. These vesicles contained the NB protein by Western blot analysis (data not shown) and had clearly reduced quinacrine fluorescence-quenching activity compared with that of the control strain

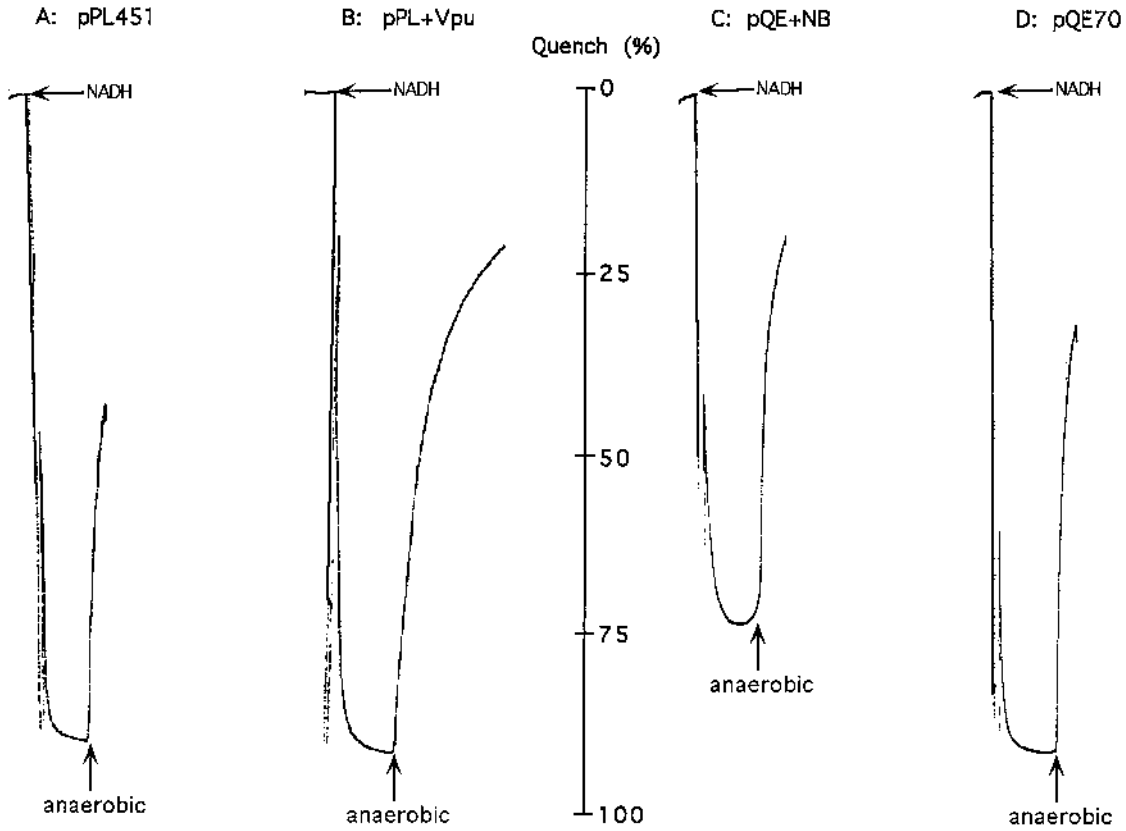

FIG. 5. NADH-dependent quinacrine fluorescence quenching from everted plasma membrane vesicles prepared from *E. coli* cells expressing Vpu (B) or influenza B virus protein NB (C). Control vesicles were prepared from strains containing the appropriate expression vectors (A and D). NADH addition and the time at which the cuvette solution becomes anaerobic are indicated by the arrows.

(Fig. 5C and D), confirming that the NB channels are permeable to hydrogen ions. The fluorescence-quenching technique is clearly capable of detecting the presence of proton-conducting channels, and this control experiment provides support for the conclusion that the Vpu protein does not form a proton-conducting channel when expressed in *E. coli.*

DISCUSSION

Whether the Vpu protein might form an ion channel, analogous to the M2 protein of influenza A virus, is a question that has been raised a number of times (3, 9, 22, 23, 32, 35). The results presented in this paper provide experimental evidence that Vpu forms sodium channels in phospholipid membranes. Work from two experimental approaches supports this conclusion. Firstly, exposure of planar lipid bilayers to purified Vpu protein results in ion currents, indicating that ion channels are present in the bilayer. These channels are more permeable to monovalent cations (Na^+ and K^+ tested) than to anions (Cl^- and phosphate tested). The conclusion that the channels are due to Vpu is based on the high purity of the immunoaffinity-purified protein: Vpu is the only protein species detected on silver-stained SDS-PAGE gels of such fractions. This procedure and Western blotting of less-pure fractions show that the antibodies recognize Vpu specifically and exclusively. Consequently, the ability of the purified antibodies to immunoprecipitate the channel-forming species from the less-pure HPLC fractions establishes that Vpu is necessary in those fractions for channel formation to occur.

Secondly, Vpu expressed in *E. coli* associates with the plasma membrane and affects the permeability of the bacterial cells to proline. The altered membrane permeability induced by Vpu is not nonspecific since methionine does not leak out of the transformed cells. The simplest explanation of these results, and an explanation consistent with the results from the bilayer assay, is that Vpu forms Na^+-permeable channels in the plasma membrane of *E. coli*. The proline transporter of *E. coli* has been well characterized and proline uptake is known to be coupled to the Na^+ electrochemical gradient (36). We propose that, in the cross-feeding assay, dissipation of the Na^+ gradient by Vpu in the XL1-Blue cells allows the proline transporter to function in reverse, driven by the outwardly directed proline concentration gradient, so that proline moves out of the cells to the proline-free external medium.

We have previously shown (29) that the NB protein of influenza B virus also forms cation-selective channels in planar lipid bilayers, but attempts to demonstrate hydrogen ion movement by using the bilayer assay requires the use of very low-pH buffers in order to have H^+ concentrations sufficiently high to give detectable channel currents. At these extremes of pH, NB channels become Cl^- selective (29), presumably because of the protonation of acidic residues in the selectivity filter of the channel protein. The effect, presented in this paper, of the presence of the NB protein on the NADH-dependent fluorescence-quenching of membrane vesicles represents the first direct evidence that the NB channels are H^+ permeable. This evidence strengthens the argument that NB is the equivalent of influenza A virus M2. However, it is clear that Vpu in the *E. coli* plasma membrane does not conduct H^+ ions at a rate

detectable by the fluorescence-quenching assay, despite the fact that the protein is present at levels sufficient to dissipate the sodium gradient and to affect proline retention in the cross-feeding assay, as discussed above.

The H^+ channels (M2 and NB) in the influenza viruses are proposed to be involved in pH-dependent viral capsid disassembly during early stages of infection and also in dissipating the pH gradient across the Golgi membrane to prevent hemagglutinin from assuming its low-pH conformation (7, 24, 27). The Vpu protein is not present at detectable levels in the virus envelope (25, 30) and so is probably not involved in virion disassembly. Furthermore, HIV-1 infects cells by direct fusion (10) rather than via the pH-dependent endocytosis pathway used by influenza virus. It would seem, therefore, that Vpu channels are unlikely to be functionally analogous to the M2 and NB channels of the influenza viruses.

On the other hand, like M2, Vpu is found in the Golgi and endoplasmic reticulum membranes. From these cellular locations Vpu mediates the degradation of CD4 and controls the budding and release of virus particles (9, 35). It is possible that one or both of these functions may be dependent on the movement of monovalent cations across the internal cellular membranes.

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