Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 111–115, January 1996
Biochemistry

Vpr protein of human immunodeficiency virus type 1 forms cation-selective channels in planar lipid bilayers

(acquired immunodeficiency syndrome/ion channels)

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Communicated by Frank Fenner, Australian National University, Canberra, Australia, September 25, 1995

ABSTRACT **A small (96-aa) protein, virus protein R (Vpr), of human immunodeficiency virus type 1 contains one hydrophobic segment that could form a membrane-spanning helix. Recombinant Vpr, expressed in *Escherichia coli* and purified by affinity chromatography, formed ion channels in planar lipid bilayers when it was added to the cis chamber and when the trans chamber was held at a negative potential. The channels were more permeable to Na^+ than to Cl^- ions and were inhibited when the trans potential was made positive. Similar channel activity was caused by Vpr that had a truncated C terminus, but the potential dependence of channel activity was no longer seen. Antibody raised to a peptide mimicking part of the C terminus of Vpr (AbC) inhibited channel activity when added to the trans chamber but had no effect when added to the cis chamber. Antibody to the N terminus of Vpr (AbN) increased channel activity when added to the cis chamber but had no effect when added to the trans chamber. The effects of potential and antibodies on channel activity are consistent with a model in which the positive C-terminal end of dipolar Vpr is induced to traverse the bilayer membrane when the opposite (trans) side of the membrane is at a negative potential. The C terminus of Vpr would then be available for interaction with AbC in the trans chamber, and the N terminus would be available for interaction with AbN in the cis chamber. The ability of Vpr to form ion channels *in vitro* suggests that channel formation by Vpr *in vivo* is possible and may be important in the life cycle of human immunodeficiency virus type 1 and/or may cause changes in cells that contribute to AIDS-related pathologies.**

Small proteins of influenza A and B viruses, M2 and NB, have been shown to form cation-selective ion channels (1–4). M2 is thought to form amantadine-sensitive proton channels that are essential for replication of the influenza A virus (5–8). It is possible that similar virus-encoded ion channels may have important roles in the life cycles of other enveloped viruses. Human immunodeficiency virus type 1 (HIV-1) encodes a small (96-aa) protein, virus protein R (Vpr), which, like M2 and NB, contains a single hydrophobic segment that could span a phospholipid bilayer as a transmembrane helix.

The *vpr* gene is highly conserved in human and primate immunodeficiency viruses. Since Vpr is found in the mature virus particle in multiple copies, it has been suggested that it plays a role in the early phase of virus replication (9–11). Although whether Vpr is essential for virus replication remains controversial, it has been demonstrated that it increases the rate of HIV replication and accelerates cytopathic effects in T cells (12, 13).

We report here that purified Vpr forms cation-selective channels in planar lipid bilayers. This raises the possibility that channel formation by Vpr *in vivo* may play a role in the life cycle of the virus or contribute to pathological conditions caused by the virus in AIDS. Drugs that block the channels may provide a useful new kind of anti-HIV agent.

MATERIALS AND METHODS

Construction of Recombinant Plasmid p2GexVPR. A cDNA clone of part of the HIV-1 genome (isolate HXB2) containing the *vpr* gene was obtained from Nick Deacon (McFarlane Burnet Centre, Melbourne, Australia). The open reading frame encoding Vpr was amplified by PCR using *Pfu* DNA polymerase and the following oligonucleotides and conditions: 5′ primer, 5′-CAGAGGATCCATGGGAACAAGCC-3′; 3′ primer, 5′-CCAGGAATTCAGTCTAGGATCT-3′; 30 cycles of 94°C for 45 sec, 55°C for 1 min, and 72°C for 1 min. For cloning, the PCR product was digested with *Bam*HI restriction endonuclease (site incorporated in 5′ primer is underlined) and ligated with plasmid prepared by double digestion with *Bam*HI and *Sma* I. The extra thymidine residue in the 72nd codon of *vpr* that results in a reading frame shift and subsequent early termination of Vpr in this HIV-1 isolate (14) was removed by oligonucleotide-directed mutagenesis and the repaired *vpr* gene was cloned as a *Bam*HI–*Sma* I fragment into our modified version of the bacterial expression vector pGEX-4T (Pharmacia), resulting in p2GexVPR, in which the *vpr* open reading frame is fused in-frame to the 3′ end of one copy of the glutathione *S*-transferase (GST) gene (Fig. 1*A*). The nucleotide sequence of the final plasmid construct was confirmed across the gene-fusion site and the entire Vpr coding region.

Expression and Purification of Vpr. Vpr was expressed as a GST fusion protein in *Escherichia coli* using the plasmid p2GexVPR (Fig. 1*A*). Expression of the GST-Vpr fusion protein was induced by addition of isopropyl β-D-thiogalactopyranoside (IPTG, 0.1 mM) to *E. coli* cultures at a cell density of 150 Klett units (blue filter no. 42) and the cells were harvested after a further 2 hr of growth at 37°C. Cells were washed and resuspended in MTPBS/glycerol [16 mM Na_2HPO_4/4 mM NaH_2PO_4/150 mM NaCl/20% (vol/vol) glycerol, pH 7.3] and disrupted by two passes through a French pressure cell. In initial trials, the crude lysate was centrifuged (18000 × *g*, 30 min) to remove particulate matter and the cleared supernatant was centrifuged again (125,000 × *g*, 1 hr) to separate soluble proteins from the membrane fractions. The majority of the GST-Vpr fusion protein was found associated with the membrane fraction (data not shown). In subsequent purifications, the crude cell lysate was treated directly (2 hr, stirred at 4°C) with the zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate [CHAPS, 2% (wt/vol) in MTPBS/glycerol], and the solubilized GST-Vpr protein was purified by affinity chromatography on glutathione-agarose resin (Sigma). The GST was

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; GST, glutathione *S*-transferase; HIV, human immunodeficiency virus; IPTG, isopropyl β-D-thiogalactopyranoside.

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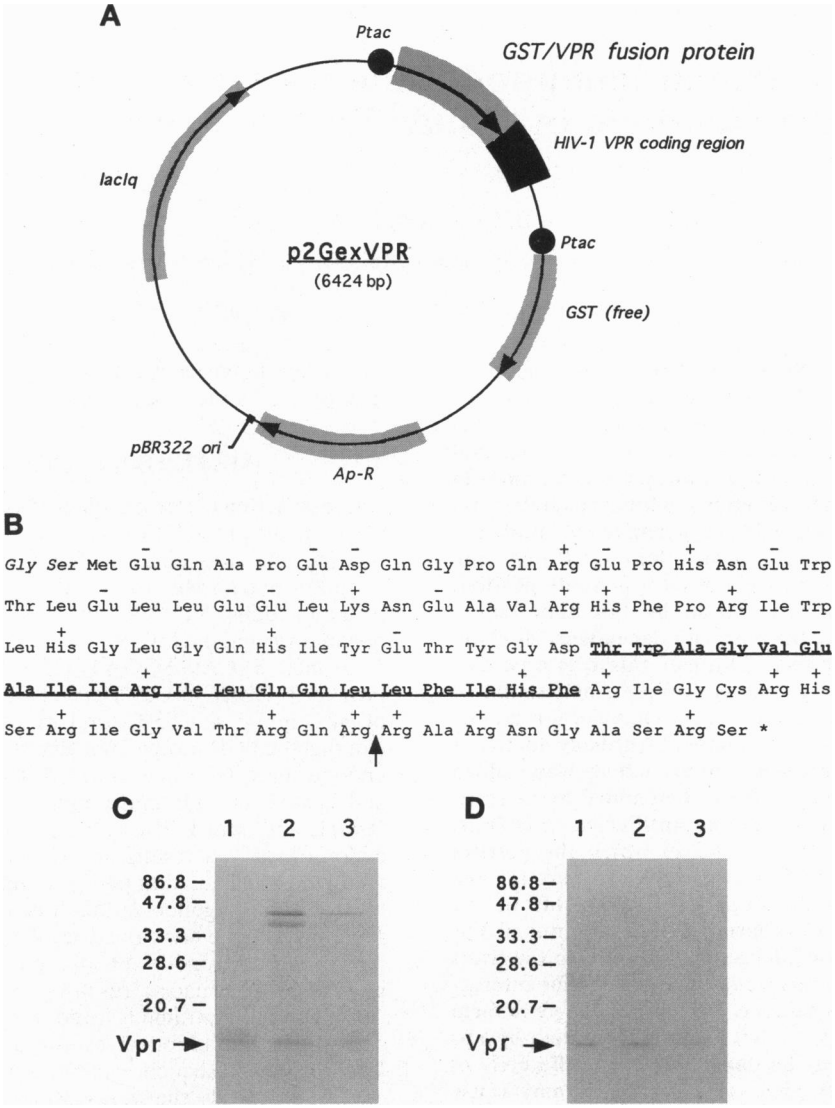

FIG. 1. (*A*) Map of the recombinant plasmid p2GexVPR. *Ptac*, *tac* promoter; *ori*, origin of replication; Ap-R, ampicillin-resistance gene. (*B*) Deduced amino acid sequence of Vpr isolated as described in the text. Italicized amino acids are remnants of the thrombin cleavage site. Charges are shown above appropriate amino acids. The predicted α-helical transmembrane region is underlined. The arrow indicates the C terminus of the truncated Vpr species. (*C*) Coomassie blue-stained SDS/18% polyacrylamide gel of three HPLC fractions containing full-length Vpr (see arrow). (*D*) Western blot probed with AbN. The same bands were seen with AbC (data not shown). Fraction 1 was used for bilayer experiments depicted in Fig. 2*A*.

cleaved from the Vpr protein with thrombin (Sigma; 20 units/ml, 2 hr at 37°C) by virtue of the high-affinity thrombin recognition site engineered to be at the C terminus of the GST in the pGEX-4T-based vector. The liberated Vpr protein was desalted into HPLC buffer (20 mM Tris·HCl/0.5% CHAPS/20% glycerol, pH 7.0) and further purified by cation-exchange (MA7S resin; BioRad) HPLC with elution by a linear NaCl gradient (0–1 M).

Raising of Polyclonal Antibodies to N- and C-Terminal Domains of Vpr. Peptides corresponding to the N (MEQAPEDQGPQREPHNEWTLC) or C (CRHSRIGVTRQRRARNGASRS) termini of Vpr were synthesised with an Applied Biosystems model 477A machine in the Biomolecular Resource Facility at the John Curtin School of Medical Research, Australian National University. Multiple Antigenic Peptides (MAPs) were prepared (15) by coupling the N- or C-terminal peptides to a polylysine core via their terminal cysteine residues. The MAPs were used to immunize rabbits for production of polyclonal antisera recognizing the N (AbN) or C (AbC) termini of Vpr.

Identification of Vpr on Acrylamide Gels and Western Blots. SDS/PAGE in homogeneous 18% polyacrylamide gels was run on a MiniProtean apparatus (BioRad). Samples were boiled for 2 min in the presence of SDS before they were loaded on the gels. Protein bands were detected either with Coomassie brilliant blue R250 or by silver staining. For Western blotting, proteins were transfered from polyacrylamide gels to poly(vinylidene difluoride) (PVDF) membranes with a semi-dry apparatus (Pharmacia LKB). Nonspecific protein binding sites on the PVDF membranes were blocked in a 10% (wt/vol) solution of skim milk proteins and the blots were incubated in diluted rabbit sera containing either AbN or AbC. Vpr was detected after reaction with goat anti-rabbit alkaline phosphatase conjugate and color development using the Western Blue stabilized substrate for alkaline phosphatase (Promega).

Recording of Ion Channel Activity. Purified Vpr was tested by standard techniques (16) for its ability to induce channel activity in planar lipid bilayers. Bilayers were formed from a mixture of 1-palmitoyl-2-oleoyl phosphatidylethanolamine and

1-palmitoyl-2-oleoyl phosphatidylcholine (8:2 weight ratio) (Avanti Polar Lipids) dissolved in *n*-decane (50 mg/ml). The lipid mixture was painted onto a 150- to 200-μm-diameter aperture in the wall of a 2-ml Delrin cup separating cis and trans chambers containing salt solutions. The cis chamber was connected to ground and the trans chamber to the input of an Axopatch 200 amplifier (Axon Instruments) via Ag/AgCl/agar bridges. The cis chamber normally contained 150 or 500 mM NaCl and the trans chamber 50 mM NaCl; both solutions contained 10 mM Mes adjusted to pH 6. Voltages were measured in the trans chamber with respect to the grounded cis chamber. Bilayer formation was monitored electrically from the amplitude of the current pulse generated by a voltage ramp. An aliquot (40–80 μl) of HPLC fractions containing Vpr (in 20 mM Tris·HCl/20% glycerol/0.5% CHAPS, pH 7.0, and up to 415 mM NaCl for the fraction containing full-length Vpr) was added to the cis chamber, which was stirred until channel activity was seen. Currents were filtered at 500 Hz, digitized at 44 kHz (Sony PCM 100), and stored on magnetic tape by a video cassette recorder. For analysis, currents were replayed through the same system in reverse and digitized at 1 or 2 kHz by using an A-to-D converter interfaced with an IBM-compatible computer.

Computer Modeling. A computer-generated model of Vpr was constructed with a Silicon Graphics Iris 4D/310GTX workstation using the programs INSIGHT and DISCOVER (Biosym Technologies, San Diego). Residues 53–71 were predicted to comprise a transmembrane helix and these residues in the extended structure were converted to an α-helix. The resulting structure was minimized by steepest descents and then by conjugate gradients with a Fujitsu VP220 supercomputer until the maximum derivative was <0.001 kcal·mol^{-1}·Å^{-1}.

RESULTS

Purification of Vpr. Initial characterization studies (data not shown) revealed that (*i*) expression of the GST-Vpr fusion protein from p2GexVPR after IPTG induction, but not expression of GST alone, quickly stopped growth of the *E. coli* cultures and (*ii*) the fusion protein was largely associated with the membrane fraction (125,000 × *g* pellet), whereas the GST molecule itself was very soluble. The fusion protein was readily solubilized by treatment of the membranes with the zwitterionic detergent CHAPS (2%) (this did not prevent subsequent binding of the GST portion to glutathione-agarose beads) and enriched by affinity chromatography. The Vpr portion could be liberated directly from the fusion protein bound to glutathione-agarose beads by incubation in the presence of thrombin followed by centrifugation to remove the beads and bound GST.

During further purification by cation-exchange HPLC, two Vpr species were obtained that were eluted at different salt concentrations. The full-length protein was eluted at 415 mM NaCl (Fig. 1 *C* and *D*). A lower molecular weight species of Vpr was also detected (data not shown) which bound poorly to the cation-exchange column. This latter species was purified by cation-exchange chromatography using conditions of lower ionic strength, and N-terminal sequencing revealed that its N terminus was intact. Mass spectroscopy (matrix-assisted laser desorption/ionization–time-of-flight method) gave a molecu-

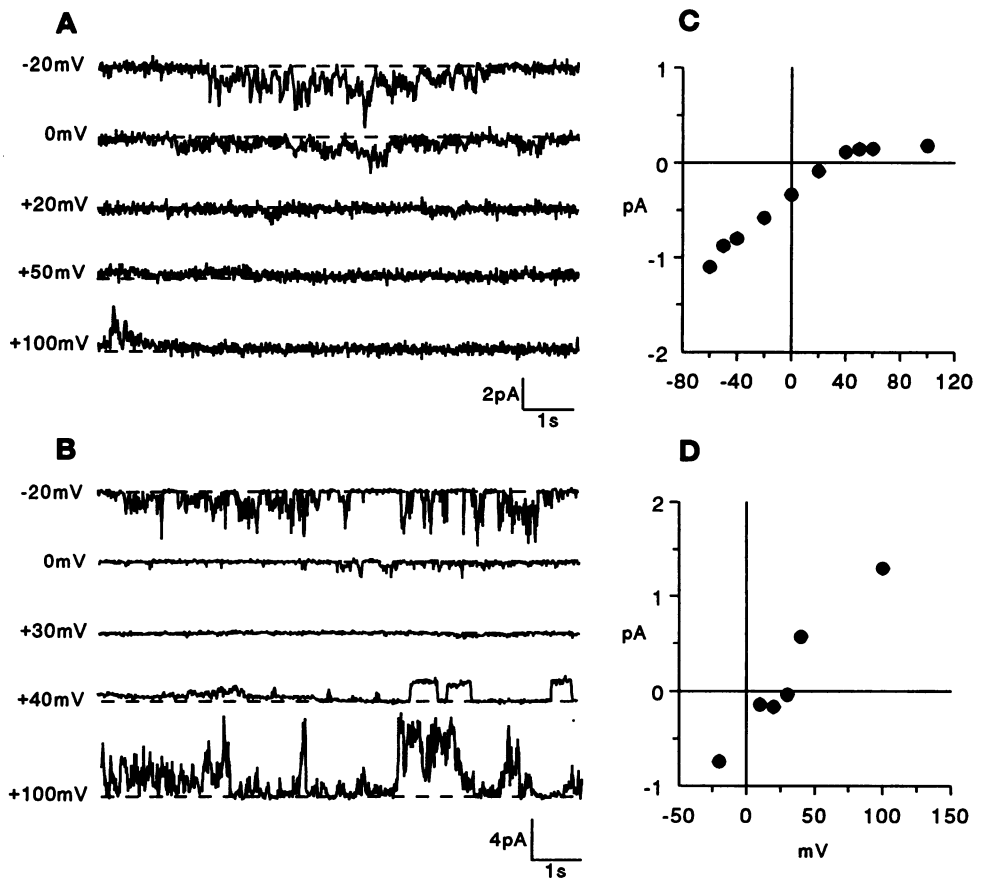

FIG. 2. Currents generated in planar lipid bilayers by full-length Vpr (*A* and *C*) and truncated Vpr (*B* and *D*) at different potentials. Vpr was added to the cis chamber and the chamber was stirred. The average current per second, the current integral of a 14-sec record/14, is plotted against potential in *C* and *D*. Voltages are given as trans chamber with respect to the grounded cis chamber. Broken lines show the closed channel current levels in *A* and *B*.

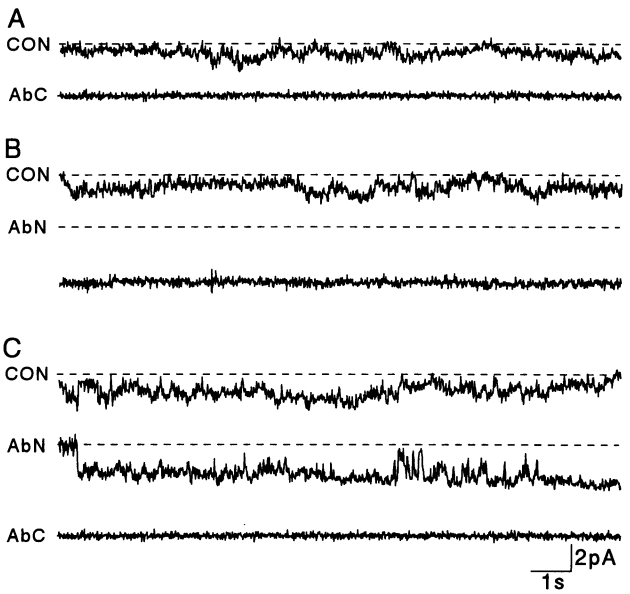

FIG. 3. Effects of AbC and AbN on currents generated by full-length Vpr. (*A*) Currents generated by Vpr at a potential of 0 mV (control, CON) are inhibited following addition of 40 μl of AbC to the trans chamber (AbC). (*B*) The open probability of currents generated by Vpr at a potential of 0 mV (CON) is increased following addition of 20 μl of AbN to the cis chamber (AbN). (*C*) Currents generated by Vpr at a potential of 0 mV (CON) are potentiated by 60 μl of AbN added to the cis chamber (AbN) and then blocked following addition of 40 μl of AbC to the trans chamber (AbC). Currents in *A–C* were from different bilayers. Broken lines show the closed-channel current levels. Calibration bars apply to *A–C*.

lar mass of 10,560 Da, consistent with the loss of 9 aa from the C terminus.

Vpr Forms Ion Channels. Both the full-length and the truncated Vpr species were investigated for their ability to form ion channels in planar lipid bilayers. Channel activity was produced by full-length Vpr in bilayers in 21 experiments. An example of typical channel activity produced by full-length Vpr added to the cis chamber is shown in Fig. 2*A*. This activity was seen only when the trans chamber was held at negative potentials and was quickly suppressed at positive potentials (Fig. 2*A*). Following the suppression of channel activity at positive potentials, it was necessary to return to very negative potentials to reactivate channels. The currents were negative (downward) at potentials of −20, 0, and +20 mV and positive (for short periods only) after the voltage was switched to +50 or +100 mV (Fig. 2*A*). A plot of average current against potential from another experiment (Fig. 2*C*) shows the depression of channel activity at positive potentials. The current passes through zero at a potential between +30 and +40 mV, indicating that the channels formed by Vpr were more permeable to Na^+ than to Cl^- ions (P_{Na}/P_{Cl} = 5–12).

In contrast to the effects of potential on the channel-forming ability of full-length Vpr, currents were seen at both negative and positive potentials following addition of truncated Vpr to the cis chamber (Fig. 2*B*): similar results were seen in 17 experiments. These currents also reversed close to the Na^+ equilibrium potential (+26 mV in the 150/50 mM NaCl solutions used) but did not turn off at positive potentials (Fig. 2 *B* and *D*).

Aliquots of HPLC buffer alone did not produce any channel activity at any potential, nor did HPLC fractions that did not show Vpr on Western blots, even those adjacent to fractions containing Vpr.

Antibodies to Vpr Modulate Channel Activity. When the antibody to the C terminus (AbC) was added to the trans chamber, channel activity was rapidly and completely inhibited (Fig. 3*A*). However, this antibody had no effect when added to the cis chamber (data not shown). This chamber-selective effect of AbC showed that the C terminus of Vpr had crossed the bilayer and was exposed in the trans chamber, where it was involved in channel activity.

Conversely, when antibody to the N terminus (AbN) was added to the cis chamber, there was a great increase in the fraction of time that channels were open (Fig. 3*B*), but the antibody had no effect when added to the trans chamber (data not shown), presumably because the N terminus was not exposed in the trans chamber. The heightened activity caused by adding AbN to the cis chamber was depressed when AbC was added to the trans chamber (Fig. 3*C*). Neither antibody produced any detectable current when added without Vpr to either chamber (data not shown). These effects of the antibodies on channel activity strengthened our conclusion that the channels were indeed formed by Vpr.

DISCUSSION

We have shown that Vpr forms cation-selective channels in planar lipid bilayers. The voltage dependence of channel activity and the selective effects of antibodies can be interpreted in terms of a computer-generated model (Fig. 4). The C terminus has positively charged side chains whereas the N terminus has a majority of negatively charged residues (Fig. 1*B*). The computer-predicted structure (Fig. 4) is very compact and shows a hydrophobic surface (not indicated in Fig. 4) that

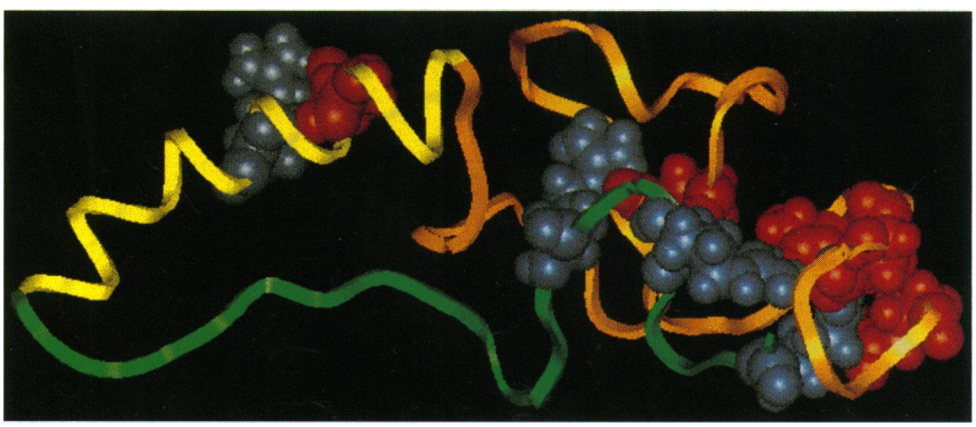

FIG. 4. Computer-generated model of Vpr. Salt bridges were formed between Glu-58 and Arg-62 in the helical region and between glutamate residues in the N-terminal region and arginine residues at the C terminus. The figure shows the backbone structure, the ribbon in the predicted helix region, in yellow; the N terminus in orange; and the C terminus in green. The residues shown as Corey–Pauling–Koltun models (Glu, red; Arg, blue) participate in salt bridge formation.

could stabilize interaction between Vpr and a lipid bilayer. The formation of salt bridges between arginine residues in the C terminus and glutamate residues in the N terminus is predicted (see Fig. 4). To allow channel formation, we propose that a negative potential on the opposite side of the membrane to Vpr may be needed to break salt bridges and drive the C terminus across the membrane. The channel structure would then be stabilized by hydrophobic interactions between the lipid environment and the segment predicted to be a membrane-spanning α-helix (Fig. 1*B*). Conversely, a positive potential would tend to drive the C terminus back across the membrane, disrupting the channel structure and switching off the channel currents. The truncated Vpr has lost three arginine residues from the C terminus (Fig. 1*B*), and the observation that channel activity caused by this form of Vpr is not voltage-dependent provides support for the computer model and the functional importance of the structural interactions proposed.

The M2 protein of influenza A was the first virally encoded protein shown to form a cation-selective channel (1) that has a vital role in the replication of a virus (5, 6). Our demonstration that Vpr forms cation-selective channels in planar lipid bilayers raises the question whether Vpr also forms channels that have an important role in the life cycle of HIV-1. Certainly, the HIV-1 particle contains multiple copies of Vpr (10), and Vpr is found in the nuclear and plasma membrane fractions of peripheral blood mononuclear cells infected with HIV-1 (11) and may well play a role in virus replication. Furthermore, Vpr has been shown to increase replication and cytopathogenicity of HIV-1 in $CD4^+$ T lymphocytes (12, 13) and primary monocytes (17), to cause growth inhibition and morphological differentiation in rhabdomyosarcoma cells (18), to play a role in the control of productive versus nonproductive infection (19), and to influence nuclear localization of viral nucleic acids in nondividing cells (20).

Vpr may have an important influence on the development of HIV-induced pathologies when released from infected cells. It is found in the blood and cerebrospinal fluid of HIV-positive people (21), and antibodies to Vpr have been detected in the serum of HIV-infected patients (22). When Vpr purified from serum was added to the extracellular environment of latently infected cell lines and resting peripheral blood mononuclear cells, HIV replication was activated (17).

HIV-induced alterations in the host cell membrane have been proposed to play a crucial role in AIDS pathology (23). The wide range of effects of HIV in a variety of cell types (24) may be due in part to formation of channels by Vpr in membranes. Our results suggest that the negative internal potential of cells would favor channel formation by Vpr approaching the cell from the extracellular environment. Biological cells normally have an intracellular negative potential, and this may promote insertion of Vpr into cell membranes and formation of channels. These channels, by allowing a steady leak of ions across the plasmalemma, would disrupt normal ion concentration gradients essential for normal cell function, and this would be expected to cause significant pathological changes—e.g., in neurons. Although it remains to be proven that Vpr does form ion channels across cell membranes *in vivo*, the role of Vpr in the HIV-1 life cycle and in AIDS pathology should now be reconsidered in light of our finding that it can form ion channels in phospholipid bilayers.

We thank Professor Frank Gibson for doing the energy-minimization calculations on the Fujitsu VP of the Australian National University Supercomputer Facility and Dr. Peter Milburn for performing the mass spectrometry.

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