

Minireview

Structure–function correlates of Vpu, a membrane protein of HIV-1

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Abstract Vpu, a membrane protein from human immunodeficiency virus-1, folds into two distinct structural domains with different biological activities: a transmembrane (TM) helical domain involved in the budding of new virions from infected cells, and a cytoplasmic domain encompassing two amphipathic helices, which is implicated in CD4 degradation. The molecular mechanism by which Vpu facilitates virion budding is not clear. This activity of Vpu requires an intact TM helical domain. And it is known that oligomerization of the VPU TM domain results in the formation of sequence-specific, cation-selective channels. It has been shown that the channel activity of Vpu is confined to the TM domain, and that the cytoplasmic helices regulate the lifetime of the Vpu channel in the conductive state. Structure–function correlates based on the convergence of information about the channel activity of Vpu reconstituted in lipid bilayers and on its 3-D structure in membranes by a combination of solution and solid-state nuclear magnetic resonance spectroscopy may provide valuable insights to understand the role of Vpu in the pathogenesis of AIDS and for drug design aimed to block channel activity.

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Key words: AIDS; Ion channel; Transmembrane helical bundle; Lipid bilayer; Reconstitution

1. Introduction

The lifecycle of human immunodeficiency virus-1 (HIV-1), the retrovirus that causes AIDS, is intimately associated with membranes [1]. Infection starts with the viral envelope recognizing and fusing with susceptible cells, and ends with the budding of new virus particles from the membranes of infected cells. The genome of HIV is only about 10 kb and encodes all the proteins necessary to enter, replicate within, and bud from susceptible helper T cells. This minimal set of proteins must therefore provide the essential components to efficiently complete the life cycle of HIV within cells. Vpu is unique to HIV-1 and is one of five accessory or regulatory proteins encoded by the HIV genome. A hallmark of AIDS progression is the gradual depletion of CD4⁺ helper T cells. This is, at least in part, the consequence of a rapid down-modulation of CD4 following HIV infection and involves the activities of Env, Nef, as well as Vpu [1,2]. Each of these

proteins acts through a different mechanism: Env and Vpu prevent transport of de novo synthesized CD4 to the cell surface through the formation of stable complexes in the endoplasmic reticulum (ER) and the targeting of CD4 to the ubiquitin-dependent proteasome pathway, respectively; Nef, in contrast, induces down-modulation of pre-existing CD4 from the cell surface with subsequent degradation in the lysosomes. Aside from its involvement in CD4 down-modulation, Vpu is also essential for the budding of new virions from infected cells. These two biological activities of Vpu have been correlated with its two distinct structural domains, an N-terminal transmembrane (TM) helix and a C-terminal cytoplasmic domain [2–5]. There is compelling evidence implicating the cytoplasmic domain of Vpu in CD4 degradation. In the ER, Vpu binds to CD4 and targets it for proteolysis in the cytosolic ubiquitin-proteasome pathway. Vpu mediates this process by binding to β -transducin repeats-containing protein (β -TrCP), which in turn binds to the proteasome-targeting factor Skp1p [6]. Indeed, a ternary complex consisting of CD4–Vpu– β -TrCP has been identified, and binding of Vpu to β -TrCP requires the two phosphoserine residues 52 and 56 that are essential for CD4 degradation [2,6]. In its phosphorylated form, Vpu enhances the degradation of the CD4/gp160 complexes that form in the ER of infected cells. The gp160 proteins made available by the action of Vpu are subsequently processed by viral protease to form gp41 and gp120, large amounts of which are required for the production of new virus particles. The integrity of the TM domain is required for progeny virus release [4,7,8]. Without active protein, newly formed virions accumulate in the cells resulting in increased cytotoxicity [9]. The molecular mechanism by which the Vpu TM domain facilitates the budding of new viral particles is not clear. Nonetheless, oligomerization of the Vpu TM domain has been shown to result in the formation of ion channels [5,10,11]. Several models have been postulated for the role of a Vpu channel in the budding process [11]. At the ER, oligomerization of Vpu through the cytoplasmic domain could induce the formation of channels and result in the collapse of the membrane potential across the ER cisternae, thereby accelerating membrane fusion and protein traffic in the exocytic pathway. Alternatively, at the ER/mitochondrial junctions, Vpu may contribute to the collapse of the mitochondrial membrane potential and thereby promote apoptosis or compromise the energetic homeostasis of the cell. Finally, at the plasma membrane, insertion of Vpu channels may attenuate the cell resting potential and thereby promote fusion and release of new virions.

What do we know about viral channel proteins in general,

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and why is Vpu a prime candidate for structure–function–drug design analysis? Today, there are only five virus-encoded proteins considered to have ion channel activity: influenza virus M2 protein [12–17] (97 amino acids [aa]), influenza virus B NB protein [12,18] (100 aa), the Chlorella virus PBCV-1 Kcv protein [19] (94 aa), and the HIV-1 proteins Vpr [12,20,21] (96 aa) and Vpu [4,5,10,22,23] (81 aa). All these five virus-encoded channel proteins share common structural features: they are small, ≤ 100 residues, type III integral membrane proteins [24] displaying a predicted N-terminal extracellular stretch, a TM domain and a C-terminal cytoplasmic domain; they exhibit a propensity to oligomerize, which is required for the assembly of conductive channels. There is compelling evidence for the role of the M2 protein in the life cycle of the influenza virus: an M2 oligomer is assembled in the endosomal lumen and forms a proton channel that lowers the intra-virion pH and thereby weakens the interactions between the matrix protein M1 and the ribonucleoprotein (RNP) core, releasing it for entry into the nucleus to propagate the infection [12]. The anti-influenza drug amantadine blocks the M2 proton channel [16,17,25,26] and precludes the dissociation of the M1 protein from the RNP core preventing virus uncoating and RNP entry into the nucleus, and thereby abrogates infection. In contrast, there is no specific blocker for the NB protein, and its role as an ion channel remains controversial [12]. The Kcv protein is a highly selective K^+ channel thought to be critical for virus replication. Remarkably, millimolar concentrations of amantadine block the Kcv channel and inhibit virus plaque formation [19]. This concentration is ~ 1000 -fold higher than that required to block the influenza virus M2 protein. The HIV Vpr protein is known to arrest cell proliferation and has recently been shown to disrupt and break the integrity of the nuclear envelope allowing the mixing of cytosolic and nuclear contents [27]. A Vpr channel may participate in the formation or propagation of such nuclear membrane defects. For Vpu, the ion channel activity thought to be associated with the ability of Vpu to facilitate the budding of new virus particles is likely to result from the oligomerization of the TM helix to form a water-filled helical bundle [4,5,28–31]. The cytoplasmic domain is associated with the ability of Vpu to affect the CD4/gp160 complex on the cell surface [3,6]. While M2 is the target of amantadine, the only drug against influenza, its 3-D structure is not available. In contrast, the only partial high resolution structure available thus far is for Vpu [11], and it clearly shows that Vpu folds into two distinct domains: an N-terminal TM helix and a cytoplasmic domain encompassing two amphipathic in-plane helices. Further, the structural motifs are highly correlated with the biological functions of Vpu known to affect the virulence of the infections. We turn now to summarize the progress achieved on the molecular dissection of the ion channel domain of Vpu and the analysis of its function in the context of the structure of Vpu.

2. Molecular dissection of the ion channel domain of Vpu

Our focus has been on the TM domain of Vpu based on two main findings:

1. Vpu-mediated enhancement of virus release is strictly dependent on the integrity of the TM domain. We demonstrated that a mutant of Vpu, Vpu_{RD}, encoding a TM domain with wild-type amino acid composition but

scrambled primary sequence predicted to retain the propensity to fold into a helical structure was unable to enhance virion release from infected or transfected cells, and virus encoding Vpu_{RD} had replication characteristics in T cells indistinguishable from that of a Vpu-deficient HIV-1 isolate [4]. Vpu_{RD} integrated normally into membranes, formed homo-oligomers, and exhibited expression levels, protein stability, and intracellular transport characteristics similar to those of wild-type Vpu. Vpu_{RD} binds to CD4 and induces its degradation with wild-type efficiency in accordance with proper membrane topology, indicating that the alteration of the Vpu TM domain did not interfere with this function of Vpu.

2. Expression of full-length Vpu in amphibian oocytes induces a cation-selective conductance with marginal permeability to divalent cations [5]. And the peptide Vpu^{1–27}, with a sequence encompassing the Vpu N-terminal segment and TM domain (MEPIQIAIVA LVVAIIIAIV VWSIVII)-forms ion channels in lipid bilayers [5]. Randomization of such sequence [Vpurd (MIPIVIAVILAVAVQAIVIVISWIIIE)] abolished ion channel activity in lipid bilayers and, when introduced into the *vpu* gene, abrogated the induction of membrane conductance by the expressed protein in oocytes.

The results led to the notion that Vpu forms ion channels, that the channel activity is confined to the TM domain and is pivotal for the enhancement of progeny virus release from infected cells. Independent support for this view has been amply documented [7,10,22,23], and reservations based on incomplete analysis were expressed [32].

A systematic approach to establish structure–function correlates was launched [11,33,34]. This involved the design of a number of recombinant constructs that would make it possible to posit questions about the function of Vpu as an ion channel, and to provide incremental structural information towards the complete determination of the 3-D structure of Vpu. The high resolution structure of Vpu was deemed necessary also as a solid framework for the rigorous formulation of a potential prophylactic or therapeutic approach to AIDS based on Vpu-specific channel blockers.

The approach we developed to produce pure Vpu in *Escherichia coli* relies on CNBr cleavage of the polypeptide corresponding to Vpu from a fusion protein. The main advantage of CNBr is its high specificity for cleavage on the C-terminus of methionine residues. Since the N-terminal residue of native Vpu is Met (MQPIQIAIVA LVVAIIIAIV VWSIVIIIEYR KILRQRKIDR LIDRLIERAE DSGNESEGEI SALVEMGVEM GHAPWDVDD L, isolate BH10, accession number P05920), this enables the release of an 80 residue polypeptide that differs from the native protein only in the absence of Met-1. However, this also requires replacement of two internal Met at positions 66 and 70 with Leu, a change which was shown not to affect the two biological activities of Vpu, facilitation of virus particle release and induction of CD4 degradation [33]. Vpu and other constructs are expressed as fusion proteins with a portion of the trp. Δ LE 1413 polypeptide under the control of the *trp* promoter [35]; they form inclusion bodies when expressed in *E. coli*, and are thus protected from proteolysis. The fusion protein is not toxic to the *E. coli* host cell, and is expressed at levels up to 20% of total cellular protein in *E. coli* strain BL21(DE3). All of the variants of recombinant Vpu, which include polypeptides encompassing

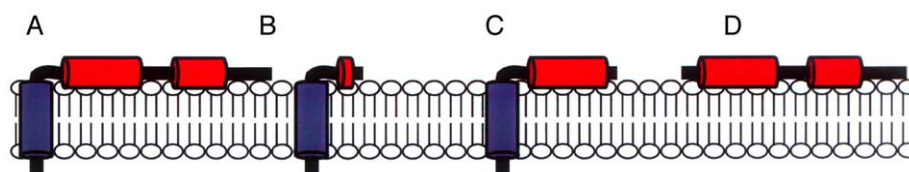


Fig. 1. Schematic representation of the overall architecture of four Vpu constructs in the context of a membrane lipid bilayer, with the hydrophobic TM helix in blue and the two amphipathic helices in red. (Modified from [11].)

residues 2–81, 2–51, 2–37 or 28–81, were produced with this approach, and they are referred to as Vpu_{2–81}, Vpu_{2–51}, Vpu_{2–37} and Vpu_{28–81}, respectively. The fusion proteins are isolated using nickel chelate chromatography. After cleavage with CNBr and reverse phase high performance liquid chromatography (HPLC), proteins of the predicted molecular mass are isolated in a single HPLC fraction, as detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Mass spectrometry confirms that each one is a single protein. The yield of purified Vpu polypeptides is routinely in the range of 1 mg/l of minimal medium, except for Vpu_{28–81} which is ~10-fold higher.

The following recombinant Vpu polypeptides were characterized in parallel by single channel recordings in lipid bilayers and by solution and solid-state nuclear magnetic resonance (NMR) spectroscopy (Fig. 1): Vpu_{2–81} corresponds to full-

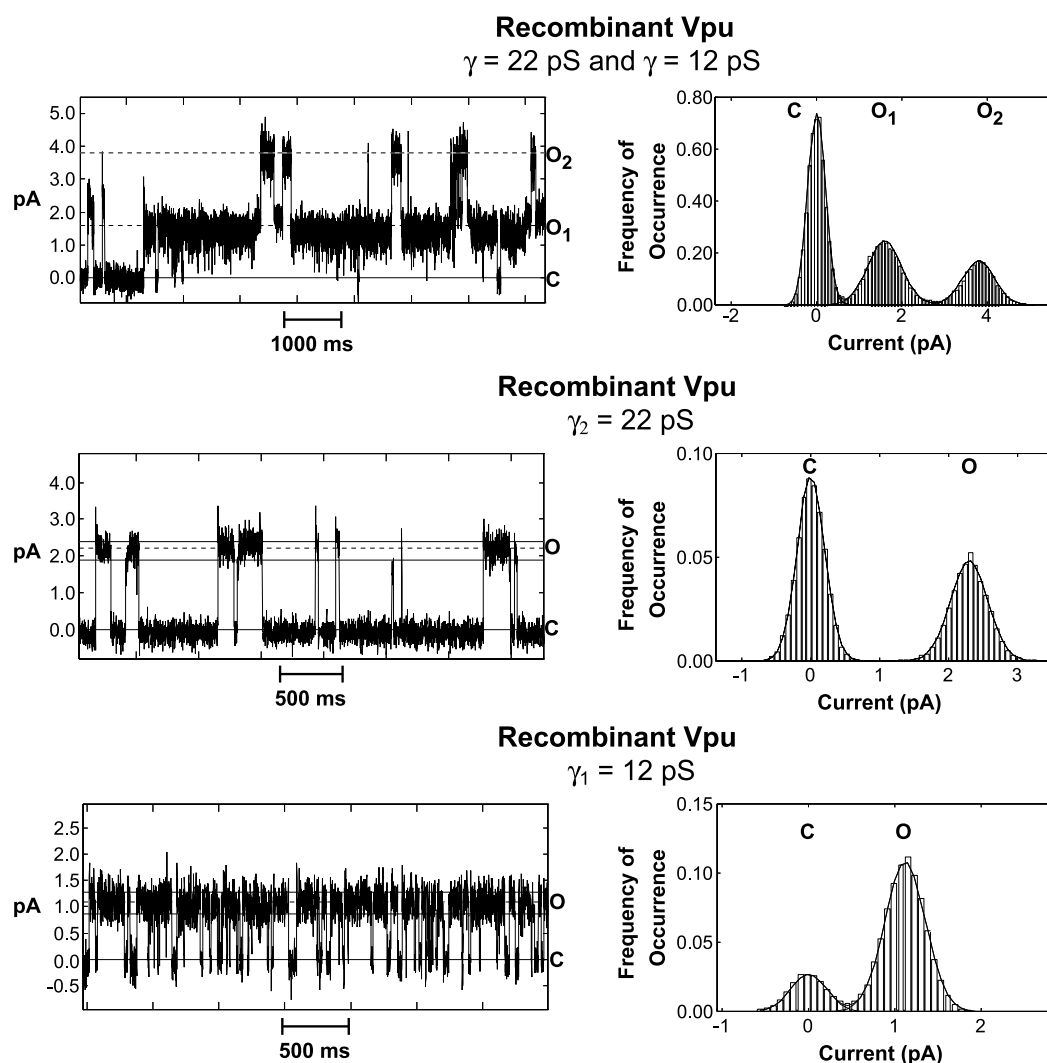


Fig. 2. Single channel currents from purified full-length recombinant Vpu reconstituted in lipid bilayers. Lipid bilayers are formed at the tip of patch pipettes by apposition of two monolayers spread from a lipid solution in hexane; the lipids used are diphytanoylphosphatidylethanolamine and diphytanoylphosphatidylcholine (1:1) (Avanti, Alabaster, AL, USA). Recombinant proteins are dissolved in trifluoroethanol and added to the aqueous subphase (0.5 M KCl and 5 mM HEPES, pH 7.4) after bilayer formation to a final concentration of 0.5–1.0 μ M. The applied voltage was 100 mV. The currents of closed (C) and open (O) states are indicated by the dotted lines. Solid lines define the range set to discriminate the transitions between states, based on signal to noise measurements. Conductances were calculated from the corresponding Gaussian fits to current histograms; the respective probabilities of the open (O) versus closed (C) channel states are indicated (right panels). (Unpublished results of M. Oblatt-Montal.)

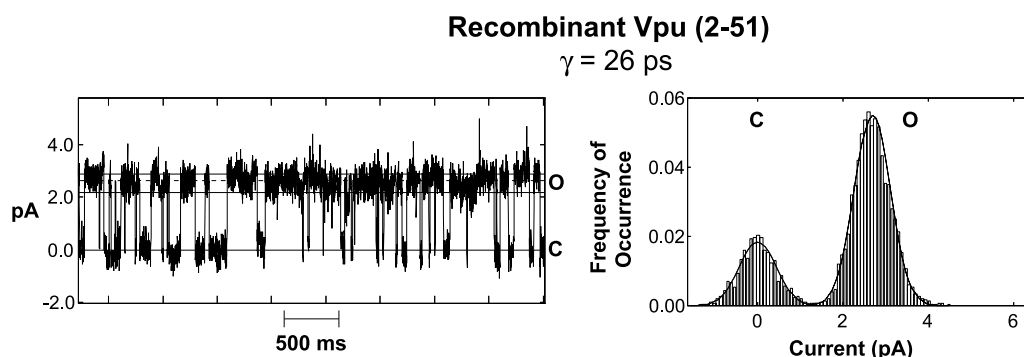


Fig. 3. Single channel currents from purified Vpu_{2–51} reconstituted in lipid bilayers. Other conditions as in Fig. 2. (Unpublished results of M. Oblatt-Montal.)

length Vpu (Fig. 1A); Vpu_{2–51}, a truncated form containing only the TM helix and the entire first amphipathic helix of the cytoplasmic domain (Fig. 1C); Vpu_{2–37}, a truncated form encompassing the TM helix and only the six N-terminal residues of the first amphipathic helix of the cytoplasmic domain (Fig. 1B); and Vpu_{28–81}, representing the entire cytoplasmic domain with two amphipathic helices (Fig. 1D). We focus here on function.

2.1. Full-length recombinant Vpu

Purified recombinant Vpu was reconstituted in lipid bilayers. Vpu forms discrete ion-conductive channels of heterogeneous conductance [11]. At pH 7.4, the most frequent events detected exhibited a single channel conductance (γ) of 22 ± 3 pS (Fig. 2, top left panel) in 0.5 M KCl. In addition to the primary conductances, a second discrete channel conductance of 12 ± 3 pS occurred at high frequency (Fig. 2, top left panel). The occurrence of these two channel activities is independent, as clearly indicated in the records displayed on a compressed time scale. Bursts of channel activity in which the predominant species is the 22 pS channel or the 12 pS channel are displayed at higher time resolution in the middle and bottom left panels (Fig. 2). Smaller and larger channel conductances were also detected with progressively lower frequency of occurrence, implying step-wise formation of smaller and larger conductive oligomers in the membrane. The heterogeneity of single conductive events is characteristic of amphipathic ion channel-forming peptides and proteins. This pattern of channel activity is conjectured to arise from the self-assembly of non-covalently bonded oligomers, the conductance of which increases with the number of subunits [5,11,36–39]. The most frequent conductance observed for full-length Vpu (22 pS in 0.5 M KCl) is compatible with a structure consisting of a water-filled, four helix bundle, arising from the self-assembly of Vpu monomers [5,11,39,40].

2.2. Truncated Vpu_{2–51}

Purified Vpu_{2–51}, encompassing the TM and the first cytosolic helix (Fig. 1C), shows channel activity which is similar to that of full-length Vpu. The single channel conductance in 0.5 M KCl is 26 ± 3 pS with a $P_o = 0.76$, slightly higher than that of Vpu ($P_o = 0.56$), under otherwise identical conditions (Fig. 3) [11].

2.3. Truncated Vpu_{2–37}

Purified Vpu_{2–37}, encompassing the TM helix and only the six N-terminal residues of the first cytosolic helix (Fig. 1B),

displays similar activity to Vpu and Vpu_{2–51}; however, the most frequent openings display a $\gamma = 12 \pm 2$ pS, and a $P_o = 0.39$ at $V = 100$ mV in 0.5 M KCl (Fig. 4) [33].

2.4. Truncated Vpu_{28–81}

Purified Vpu_{28–81}, representing the entire cytoplasmic domain with two amphipathic helices (Fig. 1D), does not show channel activity. Only stray, erratic fluctuations in membrane current were discerned, indicating that Vpu_{28–81} interacts with membranes in a non-specific manner. Together, this systematic analysis provides compelling evidence for the conclusion that the channel activity of Vpu is confined to the TM domain [11,33], in agreement with our earlier study that utilized synthetic peptides [5].

2.5. Structure–function correlates

It is instructive to compare the activity of Vpu_{2–81}, Vpu_{2–51} and Vpu_{2–37}. For all three, channel activity appears in bursts characterized by relatively long segments in which a single channel undergoes transitions between closed and open states. During the bursts of activity the most frequent opening for Vpu_{2–37} shows a $\gamma = 12 \pm 2$ pS, and a $P_o \approx 0.4$ (Fig. 4). Full-length Vpu exhibits the same pattern of activity, albeit the two most frequent openings have $\gamma = 22 \pm 2$ pS and 12 ± 2 pS; Fig. 2, bottom panel displays a burst in which only the openings with $\gamma = 12 \pm 2$ pS occur. Display of segments of the recordings at faster time resolution shows the high signal to noise ratio of these records (~ 5). Note, however, that the P_o for Vpu_{2–81} and Vpu_{2–51} is ~ 0.8 , indicating that during the burst the channels are preponderantly in the open state. In contrast, for Vpu_{2–37}, with a similar $\gamma = 12 \pm 2$ pS, the channel resides longer in the closed state. In 0.5 M NaCl, for Vpu, $P_o = 0.75$ recorded at $V = 50$ mV; for Vpu_{2–51}, $P_o = 0.56$ recorded at $V = 50$ mV, and for Vpu_{2–37}, $P_o = 0.29$ at $V = 200$ mV. Thus, the probability of channel opening decreased progressively with the removal of the cytoplasmic helices. Moreover, the frequency of occurrence of the openings with $\gamma = 22$ pS decreased with the removal of the cytosolic helices and was compensated by an increment in the occurrence of openings with $\gamma = 12$ pS. The channels with $\gamma = 12$ pS or $\gamma = 22$ pS are likely to arise from the assembly of tetramers or pentamers. This inference is based on our studies of covalently tethered tetramers and pentamers of the M2 channel lining segment of the acetylcholine receptor [37–40]. Given the similar single channel conductance of Vpu_{2–81}, Vpu_{2–51} and Vpu_{2–37}, it is reasonable to surmise that the cytoplasmic domain modulates the number of units in the conductive oligomer and its stabil-

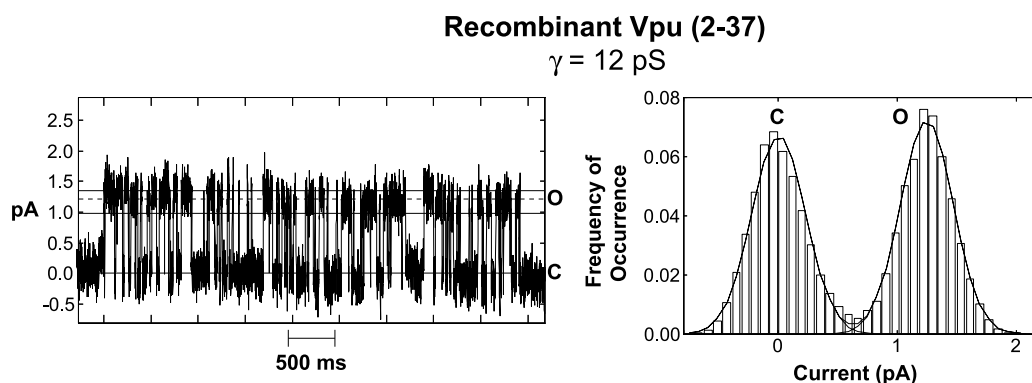


Fig. 4. Single channel currents from purified Vpu_{2–37} reconstituted in lipid bilayers. Other conditions as in Fig. 2. (Unpublished results of M. Oblatt-Montal.)

ity, and that in full-length Vpu, under these experimental conditions, the cytoplasmic domain promotes the formation of a pentamer and the residence of the channel in the open state. These findings therefore identify a novel functional role for the two cytoplasmic helices of Vpu, in addition to their involvement in CD4 degradation, namely the regulation of the assembly of the conductive oligomer and of the probability of the channel residing in the open or closed states.

This inference is in line with the domain structure of Vpu as determined from the NMR structural analysis (Fig. 1). Given that the TM α helix, residues 6–27, is the channel-forming domain of Vpu it would be expected to retrieve channel activity from Vpu_{2–37}, Vpu_{2–51} and Vpu_{2–81} with similar conduction characteristics. The cytoplasmic domain in Vpu_{2–81} may therefore confer to the TM domain features conducive to regulation of the probability of the channel being open. This is significant because the functional Vpu channel is oligomeric and the oligomeric state may be subject to regulation by cytosolic events in infected cells. The two serine residues in the linker between the two cytosolic helices are phosphorylated by protein kinases. The cytoplasmic domain in phosphorylated Vpu may, as a result of electrostatic repulsion, maintain the two amphipathic helices aligned with the TM domain and, therefore, stabilize a patent conductive pathway of the oligomer. This is reminiscent of the structure of *MscL* – a mechanosensitive channel of *Mycobacterium tuberculosis*: it is a pentameric oligomer in which the TM channel is formed by a bundle of five helices, one helix contributed by each subunit, and the cytoplasmic domain consists of five helices from each subunit that impart modulation properties on the gating of the channel, namely the probability of the channel to be open or closed [41–43].

There is a strong correlation between the structural features of the four kinds of Vpu polypeptides determined by NMR and their ion channel activity measured after reconstitution in lipid bilayers. Briefly, all Vpu polypeptides containing the TM α -helix – full-length Vpu, Vpu_{2–51}, and Vpu_{2–37} – have ion channel activity [11,33]. In contrast, the cytoplasmic domain construct, Vpu_{28–81}, devoid of the TM helix, has no recognizable ion channel activity [11]. Solution NMR experiments show that Vpu folds into two distinct domains: a TM hydrophobic α -helix, and a cytoplasmic domain with two in-plane amphipathic α -helices separated by a linker region. Solid-state NMR spectra of Vpu and the three truncated versions support this structural model of Vpu in the membrane. What's more, solid-state NMR data indicate that the TM α -helix has

a tilt of $\sim 15^\circ$ from the bilayer normal [11]. The results thus far indicate that the TM and cytoplasmic domains of Vpu fold independently in the membrane, adopt the same 3-D structures irrespective of which other segments are present, and do not interact strongly with each other.

3. Self-assembly of Vpu and the oligomeric state of the conductive channel

Vpu exhibits a strong propensity to dimerize and oligomerize in vivo [44] and in vitro [5,11]. It is precisely this proclivity that may be at the root of the self-assembly of the Vpu channel. The precise number of monomers which participate in structuring the channel is not yet known. However, molecular modeling calculations have been extremely valuable in so far as they have suggested that at least five parallel helices corresponding to the TM of Vpu would suffice to form a channel compatible with the conductance properties measured for full-length Vpu [28–31,38]. Detailed molecular dynamics (MD) simulations of the Vpu pentameric bundle placed in a hydrated lipid bilayer were recently conducted independently

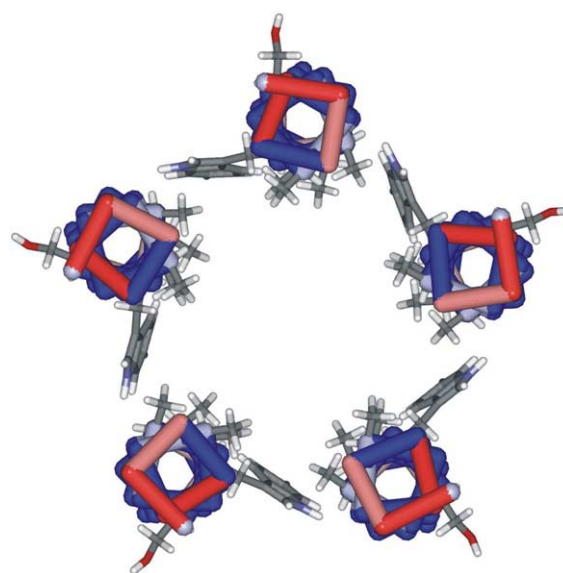


Fig. 5. A homopentameric helical bundle of the Vpu TM domain is a structural model of the Vpu channel. Top view of a pentameric helix bundle of Vpu^{1–27}, placed in a fully solvated POPE lipid bilayer; the N-terminus in front. (Modified from [34].)

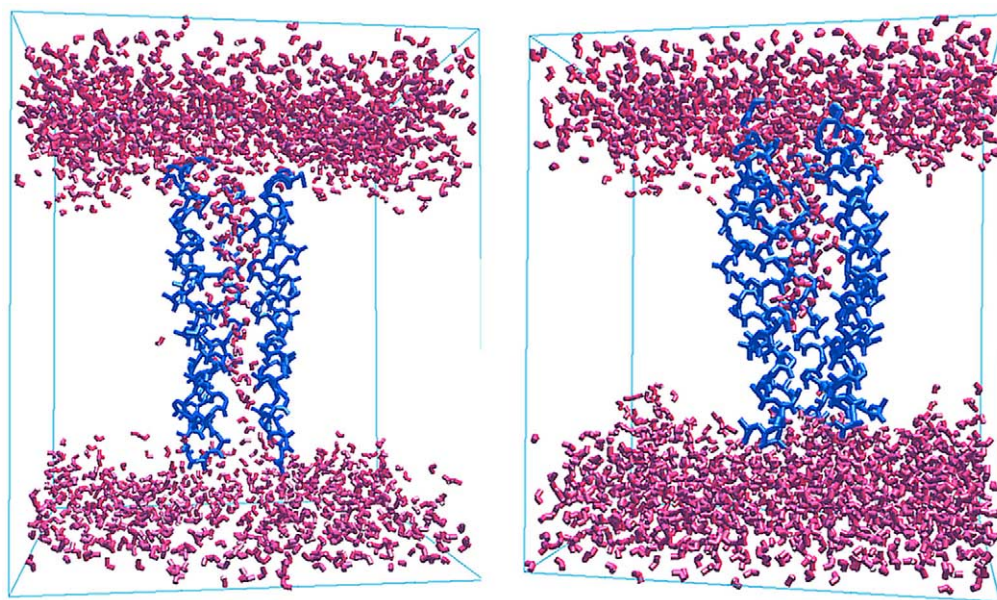


Fig. 6. Side view of the homopentameric helical bundle of the Vpu TM domain and its structural properties calculated from a 3 ns MD run. Only the bilayer profile – not the lipid molecules – is displayed. The two panels represent snapshots of the evolution of the MD simulation at the beginning of the run (left) and after 3 ns of MD (right). The N-terminus of the pore is at the top; water molecules are shown in purple and the Vpu TM bundle in blue. (Modified from [34].)

by two groups [31,34]. Cordes et al. [31] used the Fourier transform infrared spectroscopic data on the orientation of the Vpu TM [29]. Lopez et al. [11] used the NMR structural information. In particular, the findings of Lopez et al. [34] started with an unconstrained pentameric helix bundle [5,40] of Vpu^{1–27}, placed in a fully solvated palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphatidylethanolamine (POPE) lipid bilayer and its structural properties calculated from a 3 ns MD run. Fig. 5 shows the top view of the bundle with the N-terminus in front. The entire system contained five Vpu helices, 64 POPE molecules and 2085 water molecules. The total simulation time resulted in ~60 000 CPU hours of single processor or computer time [34]. Fig. 6 shows the side view of the bundle: left and right panels display snapshots of the evolution of the MD simulation at the beginning of the run and after 3 ns of MD, respectively. The N-terminus of the pore is at the top; water molecules are shown in purple and the Vpu TM bundle in blue. Some water molecules initially inside the channel lumen (left panel) were extruded halfway through the simulation and the bundle adopted a conical structure with the narrow end pointing towards the C-terminus of Vpu (right panel). The pore constriction with a diameter of 4 Å was generated by a ring of tryptophans, Trp-22. This structure may correspond to the closed state of the channel and is stabilized by interactions between the Vpu TM helices and the carbonyl oxygen atoms of the lipid molecules, particularly at the level of Gln-5 and Ser-23. In the calculations of Cordes et al., using a 2 ns simulated annealing via a restrained MD simulation run of Vpu in a hydrated lipid bilayer of palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphatidylcholine, a closed structure with Trp-22 facing the pore lumen was also obtained. A stable open structure shows a ring of five serines, Ser-23, exposed to the lumen of the channel. The combined results suggest a dynamic model for gating involving rotations around the helix axis such as to bring the bulky hydrophobic Trp residues into the pore lumen to close a channel which would otherwise

expose the polar Ser and thereby create a hydrophilic, water-filled column connecting the two aqueous compartments separated by the bilayer (Fig. 6). Overall, the structural features of the five helix bundle model of Vpu shown in Figs. 5 and 6 are similar to those identified in the crystal structures of the tetrameric pore of the K channels KcsA [45] and MthK [46], the pentameric pore of the mechanosensitive channel *MscL* [41], and the heptameric pore of the mechanosensitive channel *MscS* [47]. The ion-conducting pathway is formed by helical bundles packed around a central pore; the pores have a funnel-like architecture; the occurrence of a pore constriction formed by hydrophobic residues in the presumed closed channel may be critical for channel opening if that were to involve a rotation of helices thereby repositioning the occluding hydrophobic residues to a permissive location. These models have predictive power in assigning key roles to the indicated Vpu residues which may be tested by site-directed mutagenesis.

4. Evolutionary relationship of functional motifs in channel proteins

Virus-encoded ion channels emerge as a family of primordial proteins that predated the highly selective and regulated channel proteins of bacteria and eukarya. For the family of voltage-gated channels, a canonical structure of the membrane domain for each channel repeat or subunit considers six TM α -helices, segments S1–S6. There is now general agreement that a voltage-gated channel subunit or repeat consists of two distinct, tandemly arranged, functional modules: a voltage sensor module and a pore module [48–52]. Many of the voltage-dependent properties of K⁺ channels have been attributed to the N-terminal half of the protein, the cluster comprising S2, S3, and S4 [48–51], which may thus represent a voltage sensor module responsible for detecting changes in TM potential. And extensive mutagenesis studies of the S5–

P-S6 region have been largely consistent with the view that it represents a pore module within the larger protein [52]. The crystal structure of the K⁺ channel KcsA from *Streptomyces lividans* supports the modular organization of channels since it displays a canonical topology consisting of two TMs and a linker with closest kinship to S5-P-S6 [45]. Phylogenetic analysis indicates that the *Chlorella* Kcv channel is a very primitive K⁺ channel, and may represent the ancestor of the archetypal KcsA channel [45], namely the ancestor of the pore module with two TMs and a linker [19]. From a protein evolution viewpoint, the Kcv channel is therefore more complex than M2 of influenza or Vpu of HIV-1. These two channel proteins may represent a limit of simplicity: a single TM helix per polypeptide with an inherent propensity to oligomerize. The beauty of this essential design resides in the fact that the channel activity is a thermodynamic consequence of the natural tendency of the membrane-embedded, amphipathic TM helix to aggregate in order to optimize its interactions within the hydrophobic interior of the bilayer thereby generating a helical bundle, i.e. the structural blueprint for the channel [38]. This is fascinating in so far as it allows one to conjecture if functional diversity was initially acquired by evolving from a single TM motif, as in Vpu (non-selective channel), to the two TMs with a linker motif as in Kcv and KcsA (K⁺-selective channel), and much later into the fusion with a voltage sensor module to generate the six TM motif found in voltage-gated channels of prokarya and eukarya (voltage-gated and cation-selective channels).

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