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Membrane interactions and alignment of structures within the HIV-1 Vpu cytoplasmic domain: effect of phosphorylation of serines 52 and 56

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Abstract The cytoplasmic domain of the HIV-1 accessory protein Vpu is involved in the binding and degradation of the viral receptor CD4. In order to analyze previous structural models in the context of membrane environments, regions of Vpu^{CYTO} incorporating particular conformational features have been synthesized and labelled with ^{15}N at selected backbone amides. Well-oriented proton-decoupled ^{15}N solid-state NMR spectra with ^{15}N chemical shifts at the most upfield position indicate that the amphipathic helix within [^{15}N-Leu 45]-Vpu$^{27–57}$ strongly interacts with mechanically aligned POPC bilayers and adopts an orientation parallel to the membrane surface. No major changes in the topology of this membrane-associated amphipathic helix were observed upon phosphorylation of serine residues 52 and 56, although this modification regulates biological function of Vpu. In contrast, [^{15}N-Ala 62]-Vpu$^{51–81}$ exhibits a pronounced ^{15}N chemical shift anisotropy.

Key words: Solid-state nuclear magnetic resonance; Oriented lipid bilayer; Viral membrane protein; Amphipathic helix; CD4 degradation; Nuclear magnetic resonance structure determination

1. Introduction

In addition to the typical structural proteins, human immunodeficiency virus-1 encodes several small regulatory proteins [1]. One of them, viral protein U (Vpu), fulfils important accessory functions during the viral life cycle [1]. Biochemical experiments indicate that Vpu is an integral oligomeric membrane protein of 81 amino acids. Whereas the N-terminus serves as a hydrophobic membrane anchor, the polar C-terminal domain is located in the cytoplasm [2]. Several functions of Vpu have been identified and correlated to structural domains of the protein. Whereas cation-selective channel formation and virus release functions are associated with the hydrophobic N-terminus [3–5], other processes that occur in the endoplasmic reticulum are tightly connected to the cytoplasmic C-terminus [6]. The best characterized of these is the down regulation of CD4 [7]. This degradation of CD4 requires regulatory control by casein kinase 2-mediated phosphorylation of both serines 52 and 56 [6,8].

Solution-state circular dichroism (CD) and nuclear magnetic resonance (NMR) data indicate that in trifluoroethanol (TFE)/water mixtures or in high salt solutions, the polypeptide chain of Vpu forms several α-helices (numbered in this work as previously [9]) that are interconnected by turns [9–12]. In TFE/water solution, the cytoplasmic domain (32–81) consists of helix 2 (residues 37–51), a loop region involving two phosphorylation sites at serines 52 and 56, helix 3 (residues 57–72) and a C-terminal turn (74–78) [11]. NMR studies in 620 mM salt solutions without TFE indicate that these C-terminal helices are shortened and helix 3 appears irregular [13]. In addition, the short loop at the C-terminus is characterized by Ramachandran angles in the α-helical region. Under these conditions, the protein adopts a tertiary fold in which a few additional long-range contacts between the loop regions and the C-terminus indicate an antiparallel alignment of helix 2 and 3 [13]. CD and NMR structural data obtained with peptide fragments in TFE/water are in good agreement with the conformations of the full length cytoplasmic domain, thereby indicating that the individual helical domains of Vpu act as independent units [11–13].

Solid-state NMR spectroscopy can provide important complementary structural information of the polypeptide in the membrane-associated state (reviewed in [14]). Static preparations, which are oriented with respect to the magnetic field, exhibit chemical shifts, dipolar and quadrupolar interactions that are dependent on the alignment of chemical bonds and molecular domains [14,15]. These interactions can, therefore, be analyzed to obtain structural and topological information [14–16].

Proton-decoupled ^{15}N solid-state NMR spectroscopy has previously been used to investigate the membrane interactions of the most N-terminal helix in oriented phospholipid bilayers where it has been shown to assume a transmembrane orientation [9]. Most recently, data have been presented for uniformly ^{15}N-labelled Vpu and its cytoplasmic domain that allow some features of the topology to be suggested and imply both cytoplasmic helices are bound to the membrane surface [17]. However, more details are still required regarding the orientation and strengths of the interactions with the membrane of the individual secondary structures in Vpu. In particular, helix 2 (residues 37–51) in contrast to helix 3 has a pronounced amphipathy suggesting that its hydrophobic face will insert into the membrane while the behavior of helix 3

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Abbreviations: HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; TFE, trifluoroethanol; Vpu, viral protein U

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and the C-terminal section should be markedly different (Fig. 1).

In order to assess the individual characteristics of the structural features encompassed in the cytoplasmic domain of Vpu, we have investigated the interactions of helix 2 and helix 3 independently of one another, using ^{15}N-labelled peptides prepared by solid-phase peptide synthesis reconstituted into oriented phosphatidylcholine membranes [11]. The ^{15}N labels were introduced into the central regions of helices 2 and 3 [11,13], which have been identified to be essential for CD4 degradation by site-directed mutagenesis [6]. The proton-decoupled ^{15}N chemical shift of samples aligned with the membrane normal parallel to the magnetic field direction has provided valuable information on the orientation of the polypeptides with respect to the bilayer surface [14]. Furthermore, these techniques have been used for the first time to investigate the important effects of phosphorylation on the topological order of membrane-associated helices.

2. Materials and methods

Polypeptides [^{15}N-Leu 45]-Vpu$^{27-57}$, the corresponding diphosphorylated derivative at residues 52 and 56, and [^{15}N-Ala 62]-Vpu$^{51-81}$ of the cytoplasmic sequence IEYR KILRQRKID RLIDR-LIERA EDSGNESEGE ISALVEMGVE MGHHAPWDVD DL (residues 27–81) were prepared on a PE Biosystems 433A Synthesizer [10]. The crude peptides were purified by preparative high performance liquid chromatography (HPLC) and tested by analytical HPLC and mass spectrometry [10]. Oriented samples were prepared and equilibrated at 100% r.h. [14] and investigated by proton-decoupled solid-state NMR spectroscopy using a Bruker DSX 400 wide bore NMR spectrometer as described previously [9].

3. Results and discussion

Although recently evidence is becoming available for the orientation of the various domains of Vpu with regard to the membrane, considerably more work is required to show the membrane propensities of the individual structural features found in the solution studies of Vpu. Similarly, no data have been published on the effect of phosphorylation of serines 52 and 56 on the conformational and topological properties of Vpu, although this modification has a profound influence on the biological activity of Vpu. Mass spectrometry and HPLC analysis indicate that doubly phosphorylated Vpu$^{27-57}$, as well as non-phosphorylated fragments, are obtained at good yields and high quality and remain stable during several days of NMR spectroscopic analysis.

Proton-decoupled ^{15}N solid-state NMR spectroscopy provides valuable information on the alignment of α-helical polypeptides in oriented phospholipid membranes and has been used to analyze the secondary structure of membrane-associated peptides [14,15]. Using this approach, the hydrophobic N-terminus of Vpu has been shown to adopt a transmembrane alignment with a tilt angle of the α-helix being close to 20° [9].

A quantitative analysis of the ^{15}N solid-state NMR data requires a detailed description of the size and anisotropy of the ^{15}N chemical shift interaction. These can be graphically represented by an ellipsoid where the three main axes of the ellipsoid represent the main tensor elements σ_{11}, σ_{22} and σ_{33} [14]. The chemical shift that is measured at a given orientation of the molecule corresponds to the intersection of the magnetic field vector with the ellipsoid. The σ_{33} component of the ^{15}N chemical shift tensor is oriented close to parallel to the NH bond vector and, therefore, also the helix axis (Fig. 1C). As a consequence, at orientations of the helix parallel to the magnetic field direction, the molecules exhibit ^{15}N backbone resonances at values approaching 230 ppm [9]. This is the case for transmembrane helical peptides reconstituted into phospholipid bilayers oriented with their normal parallel to the magnetic field direction. In contrast, the σ_{22} and σ_{11} main tensor elements have values around 85 ppm and 65 ppm, respectively [18–20]. Chemical shifts of this order of magnitude, therefore, indicate that the magnetic field direction is oriented within the plane defined by the σ_{11} and σ_{22} vectors, as found for in-plane oriented peptide helices [21]. For samples in which all orientations in space are random, the signals of individual molecules add up to broad 'powder pattern' resonances.

Proton-decoupled ^{15}N solid-state NMR spectra of Vpu cytoplasmic polypeptides in oriented 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) membranes are shown in Fig. 2. At orientations of the bilayer normal parallel to the magnetic field direction, a narrow resonance at 68 ppm is observed for [^{15}N-Leu 45]-Vpu$^{27-57}$. The ^{15}N label is posi-

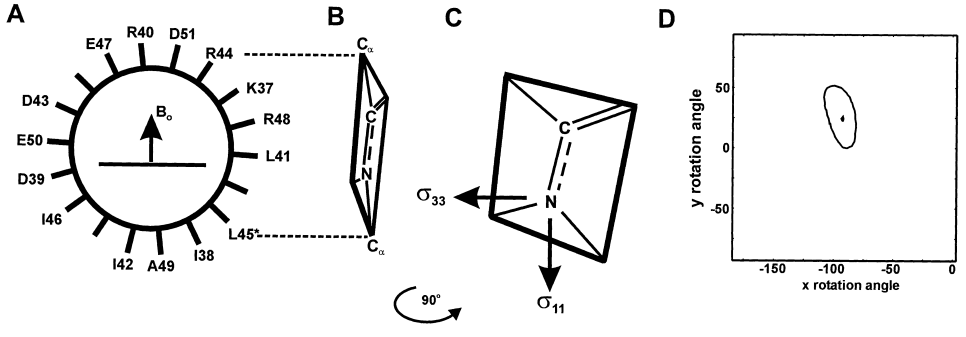

Fig. 1. A: Edmundson helical wheel diagram of residues 37–51 of Vpu [11]. The ^{15}N-labelled position is marked with a star. The wheel is oriented to agree with the ^{15}N chemical shift of Leu 45 when the lipid interface is running horizontal. The directions of the bilayer planes and the magnetic field are shown inside the helical wheel. B and C: The ^{15}N-labelled peptide bond with an orientation of the main tensor element σ_{11} approximately antiparallel to the magnetic field direction. σ_{22} is perpendicular to σ_{11} and σ_{33}. D: Contour plot representing orientations of Leu 45 that agree with a chemical shift measurement of 68 ppm as well as 5 ppm deviations of the chemical shift measurement or of the σ_{11} tensor element. The size and orientations of the main tensor elements used during these calculations are 68, 93 and 227 ppm as given in [20] (cf. text for details).

tioned in the central portion of helix 2 [11,13]. The chemical shift resonance close to σ_{11} indicates considerable restrictions of the possible helix orientations with respect to the magnetic field direction and hence the membrane normal. In order to evaluate the possible helix orientations in a quantitative manner, the peptide was initially oriented within an orthogonal co-ordinate system in such a manner that the *z*-axis is parallel to the helix axis and the *x*-axis runs parallel to the hydrophobic/hydrophilic interface of an ideal amphipathic helix as defined by the positions of the respective C_α atoms. The *y*-axis is perpendicular to the *x*–*z* plane. Thereafter, the molecule was rotated around the *x*- and *y*-axes to test for all possible orientations in space [9]. For every alignment, the ^{15}N chemical shift was calculated and compared to the experimental value as well as uncertainties in the chemical shift determination. With a ^{15}N chemical shift tensor as defined by [20] for hydrated alanine in a polypeptide, the alignment of the σ_{11} tensor element parallel to the magnetic field direction (bilayer normal) results in a helix orientation within a few degrees from perfect in-plane alignment and a tilt around the helix axis by 15–20° (Fig. 1D). Assuming a perfect amphipathic helix formed by residues K37 to D51, the helical region from solution NMR studies, such a rotation moves the C_α position of D39 into the membrane and that of R48 away from the bilayer surface (Fig. 1A). This orientation is in excellent agreement with the size and the direction of the hydrophobic moment calculated for this helix [22]. It should be kept in mind, however, that the direction and values of the main tensor elements are subject to some variation depending, for example, on the secondary structure of the polypeptide [19,20,21,23,24]. The range of possible helix orientations is increased when a 5 ppm deviation of the chemical shift measurement, of the main tensor element σ_{11} or, to a lesser extent, of σ_{22} is taken into consideration (Fig. 1D). In addition, it has been assumed during the previous considerations that residues K37 to D51 are part of an ideal right-handed α-helix. Although the peptide structure has been determined in membrane-like as well as in aqueous solutions [12,13], subtle differences in the α-helical conformation when interacting with a bilayer might exist.

Studies of small soluble proteins have shown that phosphorylation can result in a wide variety of effects ranging from small localized (e.g. [25,26]) to large long-range conformational changes (e.g. [27,28]). When the serine residues 52 and 56 of Vpu are phosphorylated, the position of the main peak remains unaltered, indicating the absence of major changes in conformation and topology of the membrane-associated peptide (Fig. 2B). An additional broad component between 50 and 200 ppm represents various other amide orientations (Fig. 2B) and suggests that the interactions with the membrane are weakened. This is possibly due to electrostatic repulsion of some of the peptide, which now carries a large negative charge surplus at its C-terminus. A 20 times decreased affinity to phospholipid membranes has also been observed when three phosphate groups were added to the basic MARCKS polypeptide [29].

As a comparison, the Vpu^{51-81} peptide was labelled with ^{15}N at position 62 and reconstituted into oriented lipid bilayers using a similar protocol (Fig. 2C). The proton-decoupled ^{15}N solid-state NMR spectra show a wide distribution of molecular orientations covering the full ^{15}N chemical shift anisotropy typically observed for backbone labelled ala-

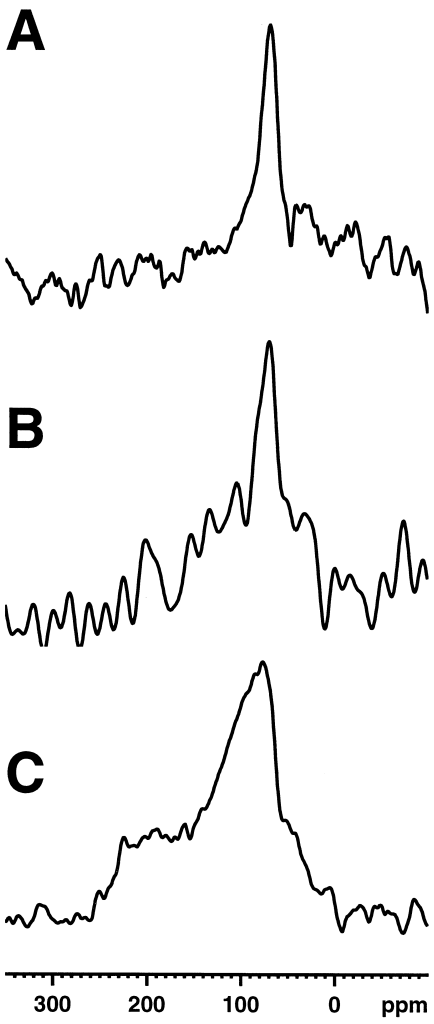

Fig. 2. Proton-decoupled solid-state NMR spectra of Vpu polypeptides (1.1 mol%) reconstituted with oriented POPC bilayers. A: [^{15}N-Leu 45]-Vpu^{27-57}, B: [^{15}N-Leu 45]-Vpu^{27-57} phosphorylated at serines 52 and 56, C: [^{15}N-Ala 62]-Vpu^{51-81}.

nine residues [19–21,23,24]. Tilting the sample by 90° results in a similar line shape (not shown). The solid-state NMR spectra, therefore, suggest that strong interactions of helix 3 with oriented POPC membranes are absent, thereby reflecting the hydrophilic side chain composition and the lack of a pronounced hydrophobic moment [22]. The line shape of Ala 62 is in sharp contrast to the oriented spectra recorded of Leu 45 of helix 2 (Fig. 2A,B). It is also different from the decrease of the ^{15}N chemical shift anisotropy in solid-state NMR investigations under similar conditions when fraying ends of α-helices or fast domain reorientation are present [30]. The lack of a preferred polypeptide orientation and mobility implies immobilized structures in the intermembrane space. Previous studies indicate that in the absence of TFE only a few residues of the C-terminal region of Vpu exhibit unstable and irregular secondary structure [11–13]. Therefore, both side chain and backbone functional groups are available for intermolecular interactions.

The present data provide a guideline for assembling structural models of Vpu in the proximity of lipid bilayers and act as a test for the details of previous models. In a first step, the oriented solid-state NMR data of helix 2 (Figs. 1 and 2A)

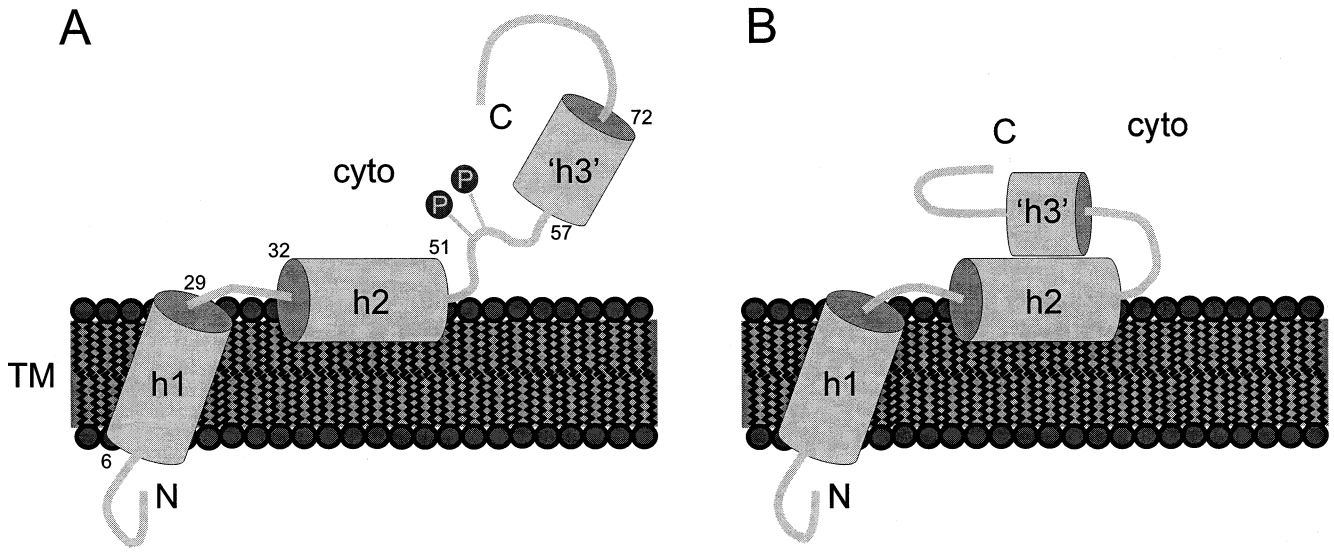

Fig. 3. Two models of Vpu in phospholipid bilayers, which for helix 1 and 2 take into consideration NMR spectroscopic measurements in isotropic solutions and in oriented membranes (cf. text for details). 'h3' indicates structured regions in TFE/water or high salt solutions, which might be different under physiological conditions. The exact structure, location and topology of the C-terminal portion of this protein, therefore, remain speculative.

were used to align the previously published three-dimensional structure of the partial Vpu cytoplasmic domain obtained in 620 mM salt solutions [13]. With a lipid bilayer surface running parallel to the hydrophobic/hydrophilic interface of helix 2, many charged and polar amino acids beyond residue 64 of this structural model are immersed in the membrane interior in an unfavorable hydrophobic environment. However, minor modifications within the flexible regions at the C-terminal of helix 2 would overcome this difficulty and allow solvation of most, if not all, charged amino acids in an aqueous environment (Fig. 3). Although our data preclude a strong interaction of helix 3 with the membrane, it remains possible that helix 2 adopts antiparallel arrangements with other structured regions further toward the C-terminus also in the presence of phospholipid bilayers (Fig. 3B). Clearly further work is necessary on the influence of phosphorylation on the entire cytoplasmic domain in order to verify details of the structures present.

Although the ^{15}N chemical shift of the membrane-associated helix 2 (Leu 45) is unaltered upon phosphorylation, it can be expected that the association equilibrium of helix 2 with the bilayer is modified due to electrostatic interactions and would be compatible with the appearance of an additional broad component in the ^{15}N spectrum. This effect would be more pronounced when acidic phospholipids and a high local negative surface charge density of the membrane are present. Electrostatic interactions can also play a role in peptide association [31], or in a refolding of the tertiary arrangement of the cytoplasmic Vpu domain thereby making accessible interaction sites of Vpu [32]. Such conformational rearrangements may be involved in the regulation of Vpu's activity mediated by phosphorylation.

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