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Dimerization of the Transmembrane Domain of Human Tetherin in Membrane Mimetic Environments

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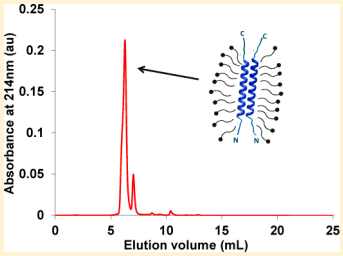
Supporting Information

ABSTRACT: Tetherin/Bst-2 is a cell surface protein that can act as a restriction factor against a number of enveloped viruses, including HIV-1. It acts by tethering new virus particles to the host cell membrane, promoting their internalization and degradation. Tetherin is a type II membrane protein, with an N-terminal transmembrane domain, an extracellular coiled-coil domain, and a C-terminal GPI anchor. This double membrane anchor is important for anti-HIV activity, as is dimerization of the coiled-coil domain, but despite recent crystal structures of the coiled-coil ectodomains of human and mouse tetherin, the topology of tetherin with respect to host and viral membranes has yet to be determined. The tetherin transmembrane domain is also thought to mediate interactions with the HIV-1 encoded integral membrane protein Vpu, which is an antagonist of tetherin, through direct binding to the transmembrane region of Vpu. Using a combination of SDS-PAGE, size exclusion chromatography, and pyrene excimer fluorescence, we show that in the absence of the coiled-coil domain the transmembrane domain of human tetherin forms parallel homodimers in membrane mimetic environments. Transmembrane domain dimerization does not require disulfide bond formation and is favored in TFE, SDS micelles, and POPC liposomes. This observation has implications for functional models of tetherin, suggesting that both transmembrane domains in the dimeric molecule are inserted into the same lipid bilayer, rather than into opposing membranes.



The innate human immune system has developed specialized defense mechanisms to fight viral infections. These innate restriction factors, which are up-regulated by the type I interferon response induced by viral infection, act to reduce the replication and infectivity of the virus. Recently, it has been shown that the interferon-induced human protein tetherin (also known as bone marrow stromal cell antigen 2 protein or BST-2) acts as a restriction factor, providing host cell resistance against certain viruses, most notably against human immunodeficiency virus type 1 (HIV-1).[1−4] The proposed mechanism of HIV-1 restriction by tetherin is through an inhibition of release of mature virus particles by directly tethering them to the surface of an infected cell, thus preventing the normal budding process.[5,6] The tethered virions remain linked to the host cell surface by a protease-sensitive bridge comprised of one or more tetherin molecules that span the gap between the host plasma membrane and the membrane of the viral particle.[5,7] As Perez-Caballero et al. point out, this differs from the effect of mutations in late domains required for budding of enveloped RNA viruses, which instead cause the viral envelope to remain fused with the host cell membrane, as reviewed by Morita and Sundquist.[7]

Tetherin is a 180-residue, type II single pass membrane protein with structural features that include a short N-terminal cytoplasmic tail followed by a single helical transmembrane domain, a coiled-coil ectodomain, and a C-terminal glycosylphosphatidylinositol (GPI) anchor.[8,9] The latter feature is thought to target tetherin to sites of viral budding and may also play a more direct role in tethering.[5] Tetherin also contains two N-linked glycosylation sites (N65 and N92) which have been shown to play an important role in correct cellular trafficking of the mature protein.[9−11] Three cysteine residues (C53, C63, and C91) found in the coiled-coil domain facilitate the formation of stable disulfide-linked homodimers, and crystal structures of this domain support a parallel dimer architecture.[6,12−14] Mutagenesis of these three cysteine residues has shown that formation of covalently cross-linked dimers is required for tethering of HIV-1, but not Lassa or Marburg viruses.[3,5,10,15]

While the architecture of the tetherin bridge between the viral membrane and the cell surface has not yet been elucidated, the structures of isolated coiled-coil domains strongly suggest that parallel homodimers are present and span the gap, with either the GPI anchors or transmembrane domains of both monomers embedded in the opposing membranes. It has been shown that at least one of these domains must be inserted into the viral envelope in order to inhibit budding.[5] Alternative models suggest a tetramerization of tetherin via the coiled coil domain, allowing one homodimer to be present in each membrane. However, while tetramers are observed in crystals of the tetherin ectodomain, mutagenesis suggests that this

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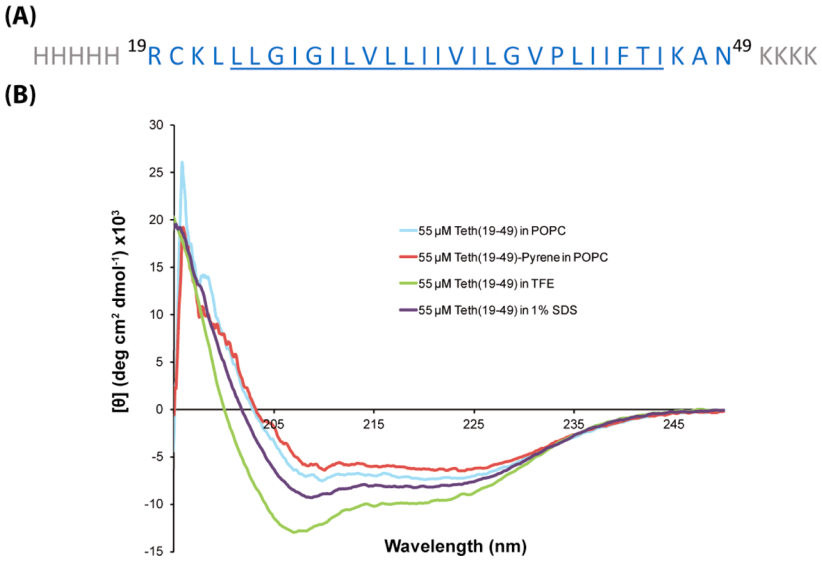

Figure 1. Circular dichroism of Teth(19–49) in membrane mimetic environments. (A) Primary structure of the 4541 Da tetherin transmembrane peptide (19–49),with N- and C-terminal solubility tags added as shown. The predicted transmembrane domain of tetherin, as determined by TM-Finder, is underlined. (B) CD spectra are shown for Teth(19–49) at 55 μM dissolved in TFE, incorporated into SDS micelles, or reconstituted into POPC liposomes. The CD spectrum for Teth(19–49) labeled at Cys20 with pyrene maleimide is also shown. For samples in POPC, the peptide to lipid ratio was 4 mol %. All spectra were recorded at 25 °C and are an average of three scans.

association is not essential for antiviral activity.[6,13] Recent electron microscopy data have observed tetherin-rich tubular structures of unknown origin connecting the cell surface with the viral envelope.[16] While these are unlikely to be composed solely of multimeric tetherin, they suggest a potential functional role for higher order oligomers of this protein.[14]

In order to evade the tetherin defense mechanism, HIV-1 has evolved a countermeasure in the form of the viral accessory protein Vpu.[2,4] This small integral membrane protein is encoded by the virus, and expressed in infected cells,[17−19] resulting in an enhanced release of newly formed virions from the host cell membrane.[19−22] This activity was linked to the transmembrane domain of Vpu; however, the link to virus release was not clear until it was shown that Vpu expression leads to a loss of cell-surface tetherin.[2,4] It has been proposed that Vpu causes internalization and ubiquitin-mediated degradation of tetherin, but the precise mechanism remains unclear.[23−28]

Mutagenesis of the transmembrane domain of tetherin showed that several residues are important for inhibition by Vpu. In particular, either mutation of T45 or deletion of both G25 and I26 is capable of impeding virion release,[29,30] although the double deletion required further transmembrane domain mutations toward nonhuman tetherin sequence to resist antagonism by Vpu. Likewise, a native Vpu transmembrane domain is required for downregulation of tetherin. Additionally, it has been shown that Vpu is capable of preventing the transmembrane domain of tetherin, but not the GPI anchor, from inserting into HIV-1 membranes.[5] When these data are combined with fluorescence microscopy showing colocalization of Vpu and tetherin within internal membranes, they suggest a direct interaction of these two proteins, mediated by their transmembrane domains, and resulting in a reduction of cell-surface tetherin and enhanced virus release.[24,29−34] This has recently been supported by a combined biophysical and cell biology study in which NMR was used to demonstrate the formation of transmembrane Vpu−tetherin heterodimers.[35]

To obtain further insight into the membrane bound architecture of tetherin, the physical mechanisms underlying viral tethering, and the interplay of this protein with Vpu, it is important to extend structural studies of tetherin to include its transmembrane domain. In the present study we show that a peptide corresponding to the transmembrane domain of human tetherin, Teth(19–49), forms stable homodimers in membrane mimetic environments, including detergent micelles and phospholipid liposomes. By performing SDS-PAGE and gel filtration under both reducing and nonreducing conditions, we show that dimer formation is independent of disulfide formation. Furthermore, the use of pyrene excimer fluorescence provides evidence that dimers are formed in a head-to-head orientation. These data support a parallel arrangement of tetherin dimers, as suggested by recent crystal structures of the coiled coil domains, with implications for the architecture of the tether formed between the host cell and viral membranes.

EXPERIMENTAL PROCEDURES

Peptide Synthesis, Purification, and Labeling. The putative transmembrane segment for human tetherin was identified using the program TM-Finder (The Hospital for Sick Children, Toronto, Ontario). Based on this analysis, a tetherin transmembrane domain peptide Teth(19–49), corresponding to residues 19–49 (Figure 1A) of the full length human protein, was synthesized via automated solid phase Fmoc chemistry using PalPEG resin (Applied Biosystems). As a negative control for size exclusion chromatography a similar peptide containing a scrambled transmembrane domain sequence, Teth(Scr), was synthesized, while a peptide containing the transmembrane domain of glycophorin A (GpATM) was synthesized for use as a positive control (Figure S1). GpA has been shown to form SDS-resistant dimers through specific transmembrane-domain interactions.[36,37] In order to minimize hydrophobic interactions and prevent aggregation or truncation, 50 mg of lithium chloride was added to each of the synthesis vessels during the coupling of

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residues 19−46. Two tags were introduced to facilitate solubility and aid in purification, as described by Melnyk et al.[38,39] A polar tag comprised of five histidines was added to the N-terminus, and the C-terminus was capped with four lysines. Peptide concentration in solution was estimated using a Bradford assay whenever appropriate.

Purification was achieved by reverse-phase high performance liquid chromatography (HPLC) on an 11 × 300 mm C4 column (Vydac), using a 10 to 100% gradient of HPLC A (10% acetonitrile, 0.1% TFA) to B (90% acetonitrile, 0.1% TFA). HPLC fractions were lyophilized and peptide identity was confirmed by MALDI mass spectrometry at the APTC (The Advanced Protein Technology Centre, The Hospital for Sick Children, Toronto, ON).

Labeling with pyrene−maleimide (Invitrogen) was achieved by reconstitution of Teth(19−49) in TFE, followed by the addition of an equal volume of 100 mM Tris pH 7.5, giving a final protein concentration of 4 mg/mL. Tris(2-carboxyethyl)-phosphine (TCEP) was added at a final concentration of 3 mM. A 20 mM stock of pyrene−maleimide was prepared in DMF, and a total of 250 μL added for each 1 mg of protein to be labeled as follows: 100 μL additions of dye were done dropwise every half an hour, and the reaction was then topped off with nitrogen gas and stirred in the dark; these steps were repeated up to complete addition of the desired volume. The reaction was then allowed to stir overnight while in the dark. Upon completion of incubation, quenching was achieved by the addition of β-mercaptoethanol, followed by purification of labeled peptide on a C4 column as described for unlabeled peptide. Peptide labeling was confirmed by mass spectrometry. The degree of labeling was estimated based on the molar extinction coefficient of the dye ε (40 000 M^{-1} cm^{-1}), the absorbance of pyrene at 338 nm (A_{338}), and protein mass (4541.7 Da).

$$\frac{A_{338}}{\varepsilon} \times \frac{\text{MW of protein}}{\text{mg of protein/mL}} = \frac{\text{moles of dye}}{\text{moles of protein}}$$

Tetherin TM Peptide Incorporation into Liposomes. Lyophilized Teth(19−49) peptide was dissolved in a 2:1 (v/v) mixture of methanol:TFE. Palmitoyl-2-oleoylphosphatidylcholine (POPC) in chloroform was added to the peptide, giving a final protein concentration of 4 mol % relative to phospholipid. The mixture was incubated to ensure complete dissolution and then dried under $N_{2(g)}$. To ensure complete removal of solvent, the sample was resuspended in water, frozen, and lyophilized. The final peptide-containing liposomes were reconstituted in 50 mM Tris, pH 7.5, followed by four cycles of freeze−thawing. To reduce light scattering in spectroscopic measurements, SUVs (small unilamellar vesicles) were produced by 12 cycles of sonication, 1 s each, with sample cooling. The resulting SUV samples were clarified via centrifugation to remove any residual large liposomes prior to experiments.

SDS-PAGE. A stock solution of peptide was made at 1 mg/mL in Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% Bromophenol Blue), at an SDS concentration of 2% (w/v). Subsequent dilutions for SDS-PAGE were done in Laemmli buffer. For samples containing 5% SDS, a stock of 10% SDS was titrated into the sample in order to achieve the desired detergent concentration. Samples were heated at 45 °C for 20 min and then loaded onto a 12% Bis-Tris gel (Invitrogen) and electrophoresis performed at 175 V. PinkPlus (GeneDirex), Precision Plus (BioRad), and Mark12 (Invitrogen) were used as molecular weight markers.

Circular Dichroism. All CD measurements were performed with a Jasco J-810 spectropolarimeter, using a 1 mm path length quartz cuvette (Hellma Analytics). Spectra were measured from 190 to 250 nm at a scan rate of 100 nm/min and were recorded as an average of three measurements. CD spectra were recorded for samples containing 55 μM peptide in trifluoroethanol (TFE), 1% sodium dodecyl sulfate (SDS), or 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) (at a 4 mol % peptide relative to lipid).

Fluorescence Spectroscopy. Pyrene fluorescence emission measurements were performed on a Photon Technology International C60 spectrofluorimeter with scans between 370 and 550 nm and an excitation wavelength of 340 nm. Each emission spectrum was recorded as an average of three scans, acquired with integration set to 0.3 s, at a step size of 1 nm, and excitation and emission slit widths of 5 nm. Experimental values were corrected for solvent background using appropriate blanks, and the area under the monomer (371−440 nm) and excimer (441−549 nm) peaks was calculated. These values were then used to calculate the excimer-to-monomer ratios, which was corrected based on the degree of Teth(19−49) labeling obtained as described above.

Size Exclusion Chromatography. SEC (size exclusion chromatography) samples were prepared by dissolving lyophilized protein in TFE to create a protein stock. Concentration was estimated using a Bradford assay. TFE was evaporated from a volume containing 1 mg of protein, which was then reconstituted in 1 mL of running buffer (10 mM Tris pH 7.5, 1% SDS) to create a 1 mg/mL stock. Final samples were then made by serial dilution of the 1 mg/mL stock. Reduced samples were prepared by incubating overnight in running buffer containing 1 mM DTT or 1 mM TCEP. SEC was performed on a Phenomenex Biosep-SEC-S-2000 column. The column was equilibrated with 3 column volumes of running buffer. Samples were loaded at a volume of 100 μL, and the column was run at a flow rate of 1 mL/min. Protein was detected based on UV absorbance at 214 nm.

Calibration of the SEC-2000S column was carried out using a low molecular weight gel filtration calibration kit purchased from GE Healthcare, which contained the following standards: Blue dextran (>2000 kDa), used for void volume; conalbumin (75 kDa); ovalbumin (43 kDa); carbonic anhydrase (29 kDa); ribonuclease A (13.7 kDa); aprotinin (6.5 kDa). Insulin chain B (3.5 kDa from Sigma) was also used to provide a lower molecular weight calibration. Each protein standard was dissolved in 10 mM Tris buffer (pH 7.5) containing 0.3% SDS and 5% β-mercaptoethanol, and boiled for 5 min, then incubated overnight at room temperature. After incubation, 0.1 mg (100 μL) of each was applied individually to the equilibrated SEC-2000S column. A calibration curve was created by plotting the K_{av} (gel-phase distribution coefficient) versus log(MW) for each protein. K_{av} was calculated using the equation $K_{av} = (V_e - V_0)/(V_c - V_0)$, where V_e is the elution volume of a protein, V_0 is the column void volume, and V_c is the geometric column volume. Three replicate runs were used to obtain each data point.

RESULTS

Secondary Structure of Teth(19−49). While the C-terminal coiled-coil domain of human tetherin has been shown to form dimers, little is known about the potential role of the

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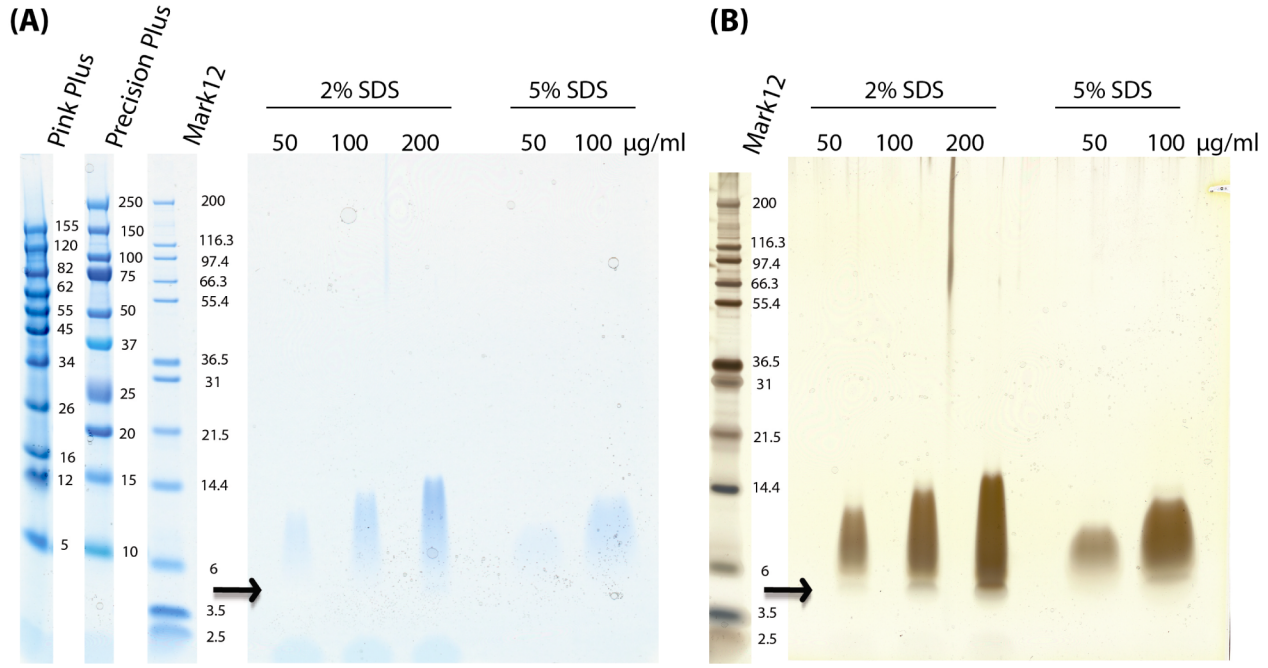

Figure 2. SDS-PAGE analysis of Teth(19−49). (A) SDS-PAGE of peptide loaded at indicated concentrations in 2 or 5% SDS. Stained with Coomassie Blue. The arrow points to apparent migration of the monomer, which is in equilibrium with the oligomer. Three different sets of molecular weight markers are shown to illustrate variations in estimated migration of similarly sized proteins under the same conditions. All lanes shown were run on the same gel and only cut out for clarity and representation of markers' sizes. (B) Equivalent gel stained with silver stain.

transmembrane domain in oligomerization of this protein. In order to provide further insight into the modes of assembly accessible to tetherin in cellular membranes, the ability of its transmembrane domain to self-assemble in the absence of the coiled-coil domain was examined. Further motivation for examining the structure and assembly of the transmembrane domain alone has been provided by several studies that demonstrated a critical role for this region in mediating interactions with the HIV-1 accessory protein Vpu.[24,26,29,30,32,35] The latter protein colocalizes with tetherin *in vivo* and leads to reduction of cell-surface tetherin, potentially through targeting it for degradation. This antagonism facilitates the release of new virions from the surface of an infected host cell.

The synthetic peptide Teth(19−49), containing the transmembrane domain of human tetherin (Figure 1A), was incorporated into several membrane mimetic environments. CD spectra recorded for this peptide in TFE solution, SDS micelles, and POPC liposomes all show the characteristic minima at 208 and 222 nm, indicating that the peptide adopts a predominantly helical structure in all cases (Figure 1B). The molar ellipticity at these wavelengths is significantly larger in TFE. This solvent is known to induce helix formation, and thus it is likely that the helical structure extends closer to the ends of the Teth(19−49) peptide under these conditions than in micellar or liposomal environments. These results demonstrate that Teth(19−49) is likely to adopt a native conformation in SDS or POPC, validating the use of these as membrane mimetics for study of this peptide.

Oligomerization of Teth(19−49) in SDS Micelles. SDS-PAGE analysis was used as an initial screen for oligomerization of the tetherin transmembrane domain. As shown in Figure 2A,B, the mobility of Teth(19−49) on SDS-PAGE gels strongly suggests the formation of dimers by this peptide in SDS. As has been previously reported for several transmembrane peptides, Teth(19−49) migrates as a relatively diffuse band, although this is mitigated at lower concentrations and in the presence of higher concentrations of SDS. For instance, the silver stained gel in Figure 2B shows a relatively narrow band of approximately 8−10 kDa molecular weight for this peptide at 50 μg/mL in 5% SDS—approximately twice the monomer weight of 4.5 kDa.

Determination of molecular weights using SDS-PAGE is not necessarily accurate, especially at the lower mass ranges required for this study. As can be seen in Figure 2, three different sets of molecular weight standards exhibit different relative mobilities for the lower molecular weight protein, highlighting this effect. Similarly, it has recently been reported that hydrophobic proteins, especially those that retain helical structure in detergent micelles, do not bind quantitatively the same amount of SDS as denatured globular proteins.[40] This can lead to significant deviation from ideal electrophoretic separation.

Therefore, SEC was used to confirm the oligomerization of Teth(19−49) in SDS micelles (Figure 3). As shown in Figure 3A, Teth(19−49) in SDS micelles migrates as a single peak on an S-2000 column. No change in peptide retention is observed from 100 to 10 μg/mL, which is the lower limit of detection for this method. Comparison with a standard curve prepared using proteins dissolved in an SDS containing buffer suggests a molecular weight for tetherin of ∼9955 Da. This is consistent with the formation of a strong dimer by Teth(19−49) in SDS micelles. Note that incubation with the reducing agents DTT or TCEP did not alter the mobility of Teth(19−49) (Figure S2), demonstrating that formation of the transmembrane domain dimer does not require a disulfide linkage, although the presence of such a modification may have a stabilizing effect on dimers. Likewise, matching the SDS concentration used for the standard curve (0.3% SDS) had no effect on the mobility of Teth(19−49) (Figure S2); therefore, 1% SDS was used in all

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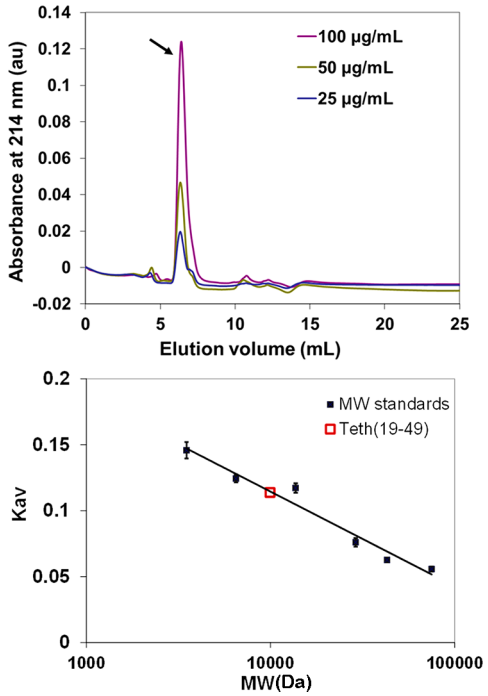

Figure 3. Size exclusion chromatography (SEC) analysis of Teth(19–49) in SDS micelles. (A) Chromatograms of Teth(19–49) in 10 mm Tris (pH 7.5) containing 1% SDS are shown for three different concentrations of peptide. The peak of interest is indicated by an arrow, while other minor peaks are associated with small molecule contaminants in the sample and are present in solvent blanks. Note that the latter peaks do not change in volume with varied peptide concentration. (B) A molecular weight calibration curve for the S-2000 column, obtained by running standard proteins in the presence of SDS, is shown (line, black squares). The relative retention time of Teth(19–49) is also indicated on the plot (red square).

other experiments to avoid artificially concentrating the hydrophobic peptides and inducing nonspecific self-association.

As an additional control, a peptide containing a scrambled tetherin transmembrane domain, Teth(Scr), was used for SEC experiments. As shown in Figure S3, this peptide had a dramatically reduced self-association in SDS micelles relative to Teth(19–49). This supports the presence of specific self-association of the tetherin transmembrane domain, although a portion of the observed dimer may be due to nonspecific association of hydrophobic helices in detergent micelles. Further support for specific dimers of Teth(19–49) in SDS micelles comes from comparison with the SEC elution profile of a peptide containing the GpA transmembrane domain (Figure S3). As expected, this peptide is a constitutive dimer on SDS-PAGE (Figure S4) and elutes from the SEC column at the same position as Teth(19–49).

Dimerization of Teth(19–49) Probed by Pyrene Excimer Fluorescence. In order to extend the observation of Teth(19–49) dimerization to a bilayer membrane, a pyrene moiety was attached to Cys20. When two pyrene molecules are within 3.5 Å of each other they form an excited dimer, or excimer, giving rise to a characteristic fluorescence emission spectrum. The emission maximum of the excimer at 470 nm is readily distinguished from that of the monomeric fluorophore, which occurs at 376 and 396 nm.[41] Pyrene excimer fluorescence has been used to probe assembly and conformational changes in both soluble and membrane embedded proteins.[38,42,43]

When incorporated into POPC liposomes, the pyrene labeled Teth(19–49) gave a CD spectrum which closely matched that of the unlabeled peptide (Figure 1B), indicating similar secondary structure in a bilayer membrane environment. Likewise, the labeled peptide exhibited identical mobility by SDS-PAGE and SEC (not shown), therefore validating the use of this labeled peptide to investigate the oligomeric state of the tetherin transmembrane domain and also supporting the argument against a role for Cys20 in dimerization.

Fluorescence emission spectra recorded for pyrene-labeled Teth(19–49) incorporated into membrane mimetic environments are shown in Figure 4, along with the calculated

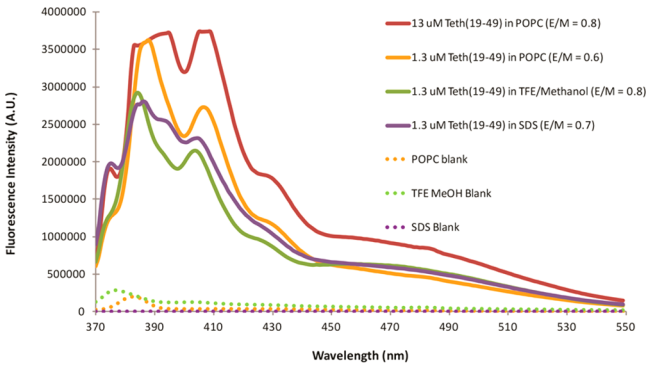

Figure 4. Pyrene fluorescence emission spectra of Teth(19–49). Fluorescence emission spectra for Teth(19–49) labeled with pyrene at Cys20, recorded in different membrane-mimetic environments. The excitation wavelength was 340 nm, with monomer and excimer emission maxima measured at 376/396 and 470 nm, respectively. Emission spectra recorded for blank samples containing only buffer and membrane mimetics are indicated by dashed lines and have been subtracted from the experimental values plotted. The excimer to monomer ratio (E/M) indicated for each experiment was calculated as described in the Experimental Procedures section.

excimer:monomer (E/M) ratios for each sample. In each case, the E/M ratio is 0.6–0.8, indicating that approximately 30–40% tetherin transmembrane domain dimer is present at these concentrations in TFE/methanol solution, SDS micelles, and POPC liposomes. This observation agrees with the SDS-PAGE and SEC data and supports a role for the transmembrane domain of tetherin in oligomerization.

It is important to note, however, that SEC data obtained for samples containing 10–100 μM Teth(19–49) indicated 100% dimerization of this peptide in 1% SDS, while the E/M ratios obtained by fluorescence indicate a significantly lower dimer population. Since the degree of peptide labeling is already accounted for when calculating the E/M ratios, and only a small change in E/M is observed when the peptide concentration is increased to 13 μM (shown for POPC in Figure 4), the lower than anticipated excimer fluorescence likely stems from geometric considerations. The formation of excimers by pyrene requires extremely close approach of the two fluorophores, with high intensity excimer fluorescence only being observed for molecules within 3.5 Å of each other. Pyrene molecules separated by more than that distance will give rise to apparent monomer signal. Several possibilities could therefore account for the fluorescence observed for the tetherin transmembrane domain. A poorly defined dimer interface could allow flexible association of two monomers or permit several slightly differing

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conformations to exist. Alternatively, Cys20 from each monomer may either not be optimally positioned relative to the dimer interface. Similarly, rotation of the Cys20 $\chi 1$ or $\chi 2$ bonds could place some or all of the pyrene moieties outside of the optimal distance for excimer formation. The presence of some antiparallel dimers may explain this result, although this has rarely been observed for homo-oligomeric membrane proteins.

DISCUSSION

The role of tetherin in preventing the release of newly formed virions from the surface of an infected host cell requires that it make strong contacts with both the host cell membrane and the nascent virus particle. Several recent crystal structures of the dimeric C-terminal ectodomain from human and murine tetherin support the formation of an extended coiled-coil formed by two head-to-head monomers.[6,12−14] Structures of the reduced form of the ectodomain show a more relaxed structure, allowing formation of a tetrameric assembly, although the biological relevance of the putative tetramer has not been established.[6,13] Based on these structures, several models of the active form of full-length tetherin have been developed in an effort to identify the mechanism through which it tethers viral particles to the host cell membrane.[6,14,27] The simpler models assume a head-to-head dimer, with both transmembrane domains embedded in either the host or viral membrane and the GPI anchors of both tetherin molecules in the other membrane. Likewise, depending on the proximity of the two membranes, it is possible for one transmembrane domain and one GPI anchor to be inserted into each bilayer. In the case of a tetrameric assembly, more potential interactions with both membranes become possible, depending on the relative arrangement of the two tetherin dimers. A tetrameric assembly composed of one tetherin dimer in each membrane has also been suggested, which would lead to a very close approach of the viral envelope and the cell membrane. Other potential roles for multimeric tetherin have been suggested, based on observed clustering of tetherin at the cell surface.[14,16] These may include stabilization of filamentous membrane-bound structures observed between the host cell and the viral envelope or inhibition of membrane fission through a local stabilization of the bilayer.

Here we have shown that the transmembrane domain of tetherin forms parallel homodimers in the absence of the ectodomain and that dimerization does not rely on disulfide bond formation. The observation that the isolated transmembrane domain of human tetherin is capable of forming strong dimers in several membrane mimetic environments, including liposomal membranes, suggests that this domain may not solely act as an anchor but may also play a role in formation of tetherin oligomers *in vivo*. This interaction likely acts to further stabilize the parallel dimer formed by the ectodomain or could play a role early in folding and assembly of the mature protein. On the basis of our current data, we cannot exclude the possibility that the transmembrane domain could serve to link two dimers, forming a tetramer, although this seems unlikely. Increased dimer stability in the absence of disulfide bonds may in part account for the nonessential nature of the ectodomain cysteines in tethering of Lassa and Marburg viruses.

It is important to note that the tetramer packing observed in two recent crystal structures required removal of the Cys53 and Cys63 disulfide bonds or a reducing environment, which leads to a fraying of the coiled-coil structure and allowing interleaving of two adjacent dimers.[6,13] While high-resolution structural details of the transmembrane domain dimer have not yet been reported, close packing of these domains would require a close approach of the N-terminal ends of the coiled-coil ectodomains in the dimer and would likely inhibit formation of the tetramers previously reported. Likewise, our data suggest that models of tetherin architecture that place the two transmembrane domains in different membranes are highly unlikely.

Finally, the self-association of the tetherin transmembrane domain has implications for the interaction of this domain with HIV-1 Vpu. Based on mutagenesis, the native transmembrane domains of both proteins are required for Vpu to antagonize tetherin and prevent tethering of nascent HIV-1 virions.[24,26,29,30,32,35,44−46] This has been supported by a recent NMR study which provided evidence for a direct and specific association of these two proteins in liposomes and in detergent micelles.[35] While the complex was modeled as a heterodimer, *in vivo* tetherin is likely to interact with Vpu as a dimer given the extent of the tetherin homodimer interface, which we have shown to extend through the transmembrane domain. While Vpu itself forms an oligomeric helical bundle in model membranes,[47−52] it is likely that the Vpu pentamer disassociates into free monomer prior to binding the tetherin transmembrane domain, in a similar manner to the proposed interaction of phospholamban with the sarcoplasmic reticulum Ca^{2+} ATPase.[53−55] Further study will be required to elucidate both the structure of the full-length tetherin dimer and its role in virus tethering and the interplay with viral antogonists.

ASSOCIATED CONTENT

Supporting Information

Figures S1−S4. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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REFERENCES

(1) Jouvenet, N., Neil, S. J. D., Zhadina, M., Zang, T., Kratovac, Z., Lee, Y., McNatt, M., Hatziioannou, T., and Bieniasz, P. D. (2009) Broad-spectrum inhibition of retroviral and filoviral particle release by tetherin. *J. Virol. 83*, 1837−1844.

(2) Neil, S. J. D., Zang, T., and Bieniasz, P. D. (2008) Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. *Nature 451*, 425−430.

(3) Sakuma, T., Noda, T., Urata, S., Kawaoka, Y., and Yasuda, J. (2009) Inhibition of Lassa and Marberg virus production by tetherin. *J. Virol. 83*, 2382−2385.
(4) Van Damme, N., Goff, D., Katsura, C., Jorgenson, R. L., Mitchell, R., Johnson, M. C., Stephens, E. B., and Guatelli, J. (2008) The interferon-induced protein BST-2 restricts HIV-1 release and is downregulated from the cell surface by the viral Vpu protein. *Cell Host Microbe 3*, 245−252.
(5) Perez-Caballero, D., Zang, T., Ebrahimi, A., McNatt, M. W., Gregory, D. A., Johnson, M. C., and Bienasz, P. D. (2009) Tetherin inhibits HIV-1 release by directly tethering virions to cells. *Cell 139*, 499−511.
(6) Yang, H., Wang, J., Jia, X., McNatt, M. W., Zang, T., Pan, B., Meng, W., Wang, H.-W., Bienasz, P. D., and Xiong, Y. (2010) Structural insight into the mechanisms of enveloped virus tethering by tetherin. *Proc. Natl. Acad. Sci. U. S. A. 107*, 18428−18432.
(7) Morita, E., and Sundquist, W. (2004) Retrovirus budding. *Annu. Rev. Cell Dev. Biol 20*, 395−425.
(8) Ishikawa, J., Kaisho, T., Tomizawa, H., Lee, B. O., Kobune, Y., Inazawa, J., Oritani, K., Itoh, M., Ochi, T., Ishihara, K., and Hirano, T. (1995) Molecular cloning and chromosomal mapping pf a bone marrow stromal cell surface gene, BST2, that may be involved in pre-B-cell growth. *Genomics 26*, 527−534.
(9) Kupzig, S., Korolchuk, V., Rollason, R., Sugden, A., Wilde, A., and Banting, G. (2003) Bst-2/HM1.24 is a raft-associated apical membrane protein with an unusual topology. *Traffic 4*, 694−709.
(10) Andrew, A. J., Miyagi, E., Kao, S., and Strebel, K. (2009) The formation of cysteine-linked dimers of BST-2/tetherin is important for inhibition of HIV-1 virus release but not for sensitivity to Vpu. *Retrovirology 6*, xxxx.
(11) Ohtomo, T., Sugamata, Y., Ozaki, Y., Ono, K., Yoshimura, Y., Kawai, S., Koishihara, Y., Ozaki, S., Kosaka, M., Hirano, T., and Tsuchiya, M. (1999) Molecular cloning and characterization of a surface antigen preferentially overexpressed on multiple myeloma cells. *Biochem. Biophys. Res. Commun. 258*, 583−591.
(12) Hinz, A., Miguet, N., Natrajan, G., Usami, Y., Yamanaka, H., Renesto, P., Hartlieb, B., McCarthy, A. A., Simorre, J.-P., Gottlinger, H., and Weissenhorn, W. (2010) Structural basis of HIV-1 tethering to membranes by the Bst2/tetherin ectodomain. *Cell Host Microbe 7*, 314−323.
(13) Schubert, H. L., Zhai, Q., Sandrin, V., Eckert, D. M., Garcia-Maya, M., Saul, L., Sundquist, W., Steiner, R. A., and Hill, C. P. (2010) Structural and functional studies on the extracellular domain of BST2/tetherin in reduced and oxidized conditions. *Proc. Natl. Acad. Sci. U. S. A. 107*, 17951−17956.
(14) Swiecki, M., Scheaffer, S. M., Allaire, M., Fremont, D. H., Colonna, M., and Brett, T. J. (2011) Structural and biophysical analysis of BST-2/tetherin ectodomains reveals an evolutionary conserved design to inhibit virus release. *J. Biol. Chem. 286*, 2987−2997.
(15) Sakuma, T., Sakurai, A., and Yasuda, J. (2009) Dimerization of tetherin is not essential for its antiviral activity against Lassa and Marburg viruses. *PLoS One 4*, 1−6.
(16) Hammonds, J., Wang, J.-J., Yi, H., and Spearman, P. (2010) Immunoelectron microscopic evidence for tetherin/Bst2 as the physical bridge between HIV-1 virions and the plasma membrane. *PLoS Pathog. 6*, e1000749.
(17) Cohen, E. A., Terwilliger, E. F., Sodroski, J. G., and Haseltine, W. A. (1988) Identification of a Protein Encoded by the Vpu Gene of Hiv-1. *Nature 334*, 532−534.
(18) Strebel, K., Klimkait, T., Maldarelli, F., and Martin, M. A. (1989) Molecular and Biochemical Analyses of Human Immunodeficiency Virus Type-1 Vpu Protein. *J. Virol. 63*, 3784−3791.
(19) Strebel, K., Klimkait, T., and Martin, M. A. (1988) A Novel Gene of Hiv-1, Vpu, and Its 16-Kilodalton Product. *Science 241*, 1221−1223.
(20) Klimkait, T., Strebel, K., Hoggan, M. D., Martin, M. A., and Orenstein, J. M. (1990) The Human Immunodeficiency Virus Type 1-Specific Protein Vpu Is Required for Efficient Virus Maturation and Release. *J. Virol. 64*, 621−629.
(21) Terwilliger, E. F., Cohen, E. A., Lu, Y. C., Sodroski, J. G., and Haseltine, W. A. (1989) Functional-Role of Human Immunodeficiency Virus Type-1 Vpu. *Proc. Natl. Acad. Sci. U. S. A. 86*, 5163−5167.
(22) Neil, S. J. D., Eastman, S. W., Jouvenet, N., and Bieniasz, P. D. (2006) HIV-1 Vpu promotes release and prevents endocytosis of nascent retrovirus particles from the plasma membrane. *PLoS Pathog. 2*, 354−367.
(23) Mangeat, B., Gers-Huber, G., Lehmann, M., Zufferey, M., Luban, J., and Piguet, V. (2009) HIV-1 Vpu neutralizes the antiviral factor tetherin/BST-2 by binding it and directing its beta-TrCP2-dependent degradation. *PLoS Pathog. 5*, e1000574.
(24) Douglas, J. L., Viswanathan, K., McCarroll, M. N., Gustin, J. K., Fruh, K., and Moses, A. V. (2009) Vpu directs the degradation of the HIV restriction factor BST-2/tetherin via a {beta}TrCP-dependent mechanism. *J. Virol. 83*, 7931−7947.
(25) Goffinet, C., Allespach, I., Homann, S., Tervo, H.-M., Habermann, A., Rupp, D., Oberbremer, L., Kern, C., Tibroni, N., Welsch, S., Krijnse-Locker, J., Banting, G., Krausslich, H.-G., Fackler, O. T., and Keppler, O. T. (2009) HIV-1 antagonism of CD317 is species specific and involves Vpu-mediated proteasomal degradation of the restriction factor. *Cell Host Microbe 5*, 285−297.
(26) Mitchell, R., Katsura, C., Skasko, M. A., Fitzpatrick, K., Lau, D., Ruiz, A., Stephens, E., Margottin-Goguet, F., Benarous, R., and Guatelli, J. C. (2009) Vpu antagonizes BST-2-mediated restriction of HIV-1 release via b-TrCP and endo-lysosomal trafficking. *PLoS Pathog. 5*, e1000450.
(27) Andrew, A., and Strebel, K. (2010) HIV-1 Vpu targets cell surface markers CD4 and BST-2 through distinct mechanisms. *Mol. Aspects Med. 31*, 407−417.
(28) Miyagi, E., Andrew, A. J., Kao, S., and Strebel, K. (2009) Vpu enhances HIV-1 virus release in the absence of Bst-2 cell surface down-modulation and intracellular depletion. *Proc. Nat. Acad. Sci. U. S. A. 106*, 2868−2873.
(29) Gupta, R. K., Hue, S., Schaller, T., Verschoor, E., Pillay, D., and Towers, G. J. (2009) Mutation of a single residue renders human tetherin resistant to HIV-1 Vpu-mediated depletion. *PLoS Pathog. 5*, e1000443.
(30) Rong, L., Zhang, J., Lu, J., Pan, Q., Lorgeoux, R.-P., Aloysius, C., Guo, F., Liu, S.-L., Wainberg, M. A., and Liang, C. (2009) The transmembrane domain of BST-2 determines its sensitivity to down-modulation by human immunodeficiency virus type 1 Vpu. *J. Virol. 83*, 7536−7546.
(31) Jia, B., Serra-Moreno, R., Neidermyer, W., Rahmberg, A., Mackey, J., Ben Fofana, I., Johnson, W. E., Westmoreland, S., and Evans, D. T. (2009) Species-specific activity of SIV Nef and HIV-1 Vpu in overcoming restriction by tetherin/BST-2. *PLoS Pathog. 5*, 1−17.
(32) McNatt, M., Zhang, T., Hatziioannou, T., Bartlett, M., Ben Fofana, I., Johnson, W. E., Neil, S. J. D., and Bieniasz, P. D. (2009) Species-specific activity of HIV-1 Vpu and positive selection of tetherin transmembrane domain variants. *PLoS Pathog. 5*, e1000300.
(33) Iwabu, Y., Fujita, H., Kinomoto, M., Kaneko, K., Ishazaka, Y., Tanaka, Y., Sata, T., and Tokunaga, K. (2009) HIV-1 accessory protein Vpu internalizes cell-surface BST-2/tetherin though transmembrane interactions leading to lysozomes. *J. Biol. Chem. 284*, 35060−35072.
(34) Dube, M., Roy, B. B., Guiot-Guillain, P., Mercier, J., Chiasson, A., and Cohen, E. A. (2010) Antagonism of tetherin restriction of HIV-1 release by Vpu involves binding and sequestration of the restriction factor in a perinuclear compartment. *PLoS Pathog. 6*, e1000856.
(35) Skasko, M., Wang, Y., Tian, Y., Tokarev, A., Munguia, J., Ruiz, A., Stephens, E. B., Opella, S. J., and Guatelli, J. (2012) HIV-1 Vpu antagonizes the innate restriction factor BST-2 via lipd-embedded helix-helix interactions. *J. Biol. Chem. 287*, 58−67.
(36) Furthmayr, H., and Marchesi, V. T. (1976) Subunit structure of human erythrocyte glycophorin A. *Biochemistry 15*, 1137−1144.
(37) Lemmon, M. A., Flanagan, J. M., Hunt, J. F., Adair, B. D., Bormann, B.-J., Dempsey, C. E., and Engelman, D. M. (1992)

Gycophorin A dimerization is driven by specific interactions between transmembrane helices. *J. Biol. Chem. 267*, 7683−7689.
(38) Melnyk, R. A., Partridge, A. W., and Deber, C. M. (2002) Transmembrane domain mediated self-assembly of major coat protein subunits from Ff bacteriophage. *J. Mol. Biol. 315*, 63−72.
(39) Melnyk, R. A., Partridge, A. W., Yip, J., Wu, Y., Goto, N. K., and Deber, C. M. (2003) Polar residue tagging of transmembrane peptides. *Peptide Sci. 71*, 675−685.
(40) Rath, A., Glibowicka, M., Nadeau, V. G., Chen, G., and Deber, C. M. (2009) Detergent binding explains anomalous SDS-PAGE migration of membrane proteins. *Proc. Natl. Acad. Sci. U. S. A. 106*, 1760−1765.
(41) Lehrer, S. S. (1997) Intramolecular pyrene excimer fluorescence: a probe of proximity and protein conformational change. *Methods Enzymol. 278*, 286−295.
(42) Hammarstrom, P., Persson, M., Freskgard, P.-O., Martensson, L.-G., Andersson, D., Jonsson, B.-H., and Carlsson, U. (1999) Structural mapping of an aggregation mucleation site in a molten globule intermediate. *J. Biol. Chem. 274*, 32897−32903.
(43) Liu, W., Chen, Y., Watrob, H., Bartlett, S. G., Jen-Jacobson, L., and Barkley, M. D. (1998) N-termini of EcoRI restriction endonuclease dimer are in close proximity on the protein surface. *Biochemistry 37*, 15457−15465.
(44) Hout, D. R., Gomez, M. L., Pacyniak, E., Gomez, L. M., Inbody, S. H., Mulcahy, E. R., Culley, N., Pinson, D. M., Powers, M. F., Wong, S. W., and Stephens, E. B. (2005) Scrambling of the amino acids within the transmembrane domain of Vpu results in a simian-human immunodeficiency virus ($SHIV_{TM}$) that is less pathogenic for pig-tailed macaques. *Virology*,in press.
(45) Marassi, F. M., Ma, C., Gratkowski, H., Straus, S. K., Strebel, K., Oblatt-Montal, M., Montal, M., and Opella, S. J. (1999) Correlation of the structural and functional domains in the membrane protein Vpu from HIV-1. *Proc. Natl. Acad. Sci. U. S. A. 96*, 14336−14341.
(46) Paul, M., Mazumder, S., Raja, N., and Jabbar, M. A. (1998) Mutational analysis of the human immunodeficiency virus type 1 Vpu transmembrane domain that promotes the enhanced release of virus-like particles from the plasma membrane of mammalian cells. *J. Virol 72*, 1270−1279.
(47) Bour, S., and Strebel, K. (2003) The HIV-1 Vpu protein: a multifunctional enhancer of viral particle release. *Microbes Infect. 5*, 1029−1039.
(48) Fischer, W. B. (2003) Vpu from HIV-1 on an atomic scale: experiments and computer simulations. *FEBS Lett. 552*, 39−46.
(49) Hussain, A., Das, S. R., Ranwar, C., and Jameel, S. (2007) Oligomerization of the human immunodeficiency virus type 1 (HIV-1) Vpu protein - a genetic, biochemical and biophysical analysis. *Virol. J. 4*, 1−11.
(50) Lu, J., Sharpe, S., Ghirlando, R., Yau, W.-M., and Tycko, R. (2010) Oligomerization state and supramolecular structure of the HIV-1 Vpu protein transmembrane segment in phospholipid bilayers. *Protein Sci. 19*, 1877−1896.
(51) Park, S. H., Mrse, A. A., Nevzorov, A. A., Mesleh, M. F., Oblatt-Montal, M., Montal, M., and Opella, S. J. (2003) Three-dimensional structure of the channel-forming trans-membrane domain of virus protein "u" (Vpu) from HIV-1. *J. Mol. Biol. 333*, 409−424.
(52) Schubert, U., FerrerMontiel, A. V., OblattMontal, M., Henklein, P., Strebel, K., and Montal, M. (1996) Identification of an ion channel activity of the Vpu transmembrane domain and its involvement in the regulation of virus release from HIV-1-infected cells. *FEBS Lett. 398*, 12−18.
(53) Becucci, L., Cembran, A., Karim, C. B., Thomas, D. D., Guidelli, R., Gao, J., and Veglia, G. (2009) On the function of pentameric phospholamban: Ion channel or storage form? *Biophys. J. 96*, L60−L62.
(54) Cornea, R. L., Autry, J. M., Chen, Z., and Jones, L. R. (2000) Reexamination of the role of the leucine/isoleucine zipper residues of phospholamban in inhibition of the Ca2+ pump of cardiac sarcoplasmic reticulum. *J. Biol. Chem. 275*, 41487−41494.
(55) Kimura, Y., Kurzydlowski, K., Tada, M., and MacLennan, D. H. (1997) Phospholamban inhibitory function is activated by depolymerization. *J. Biol. Chem. 272*, 15061−15064.