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The p10 FAST protein fusion peptide functions as a cystine noose to induce cholesterol-dependent liposome fusion without liposome tubulation

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ABSTRACT

The reovirus p10 fusion-associated small transmembrane (FAST) proteins are the smallest known membrane fusion proteins, and evolved specifically to mediate cell–cell, rather than virus–cell, membrane fusion. The 36–40-residue ectodomains of avian reovirus (ARV) and Nelson Bay reovirus (NBV) p10 contain an essential intramolecular disulfide bond required for both cell–cell fusion and lipid mixing between liposomes. To more clearly define the functional, biochemical and biophysical features of this novel fusion peptide, synthetic peptides representing the p10 ectodomains of ARV and NBV were analyzed by solution-state NMR spectroscopy, circular dichroism spectroscopy, fluorescence spectroscopy-based hydrophobicity analysis, and liposome binding and fusion assays. Results indicate that disulfide bond formation promotes exposure of hydrophobic residues, as indicated by bis-ANS binding and time-dependent peptide aggregation under aqueous conditions, implying the disulfide bond creates a small, geometrically constrained, cystine noose. Noose formation is required for peptide partitioning into liposome membranes and liposome lipid mixing, and electron microscopy revealed that liposome–liposome fusion occurs in the absence of liposome tubulation. In addition, p10 fusion peptide activity, but not membrane partitioning, is dependent on membrane cholesterol.

1. Introduction

Fusion of two cell membranes is an important step in many essential biological processes, such as fertilization, formation of the syncytiotrophoblast layer of the placenta, myoblast fusion during muscle development, and formation of osteoclasts for bone resorption [1]. Membrane fusion is also an essential step in the infection of host cells by enveloped viruses, such as HIV and influenza [2]. Such membrane merger events are energetically unfavorable processes and require protein fusogens to mediate fusion of biological membranes under physiological conditions. In the case of enveloped virus fusion proteins, the process involves triggered, complex structural rearrangements of the fusion proteins. These conformational changes expose a hydrophobic sequence known as the fusion peptide (FP), which is normally sequestered from solvent within the pre-fusion structure. Formation of an extended intermediate projects the FP toward the target membrane for membrane insertion. Folding back of this extended intermediate into a compact trimeric hairpin structure is believed to provide energy to pull the apposed membranes together and drive membrane merger [3]. Exactly how membrane fusion proteins mediate the actual merger of membranes, and the precise role of FPs in this process, is still unclear.

While extensive structural remodeling of enveloped virus fusion proteins is clearly a key event in the fusion process, it seems likely that FPs serve a greater role in promoting membrane merger than just serving as membrane anchors [4]. There are two general classes of enveloped virus FPs. The first are N-terminal FPs in proteins such as influenza virus hemagglutinin (HA) and HIV gp41, which form amphipathic α-helices that are frequently kinked or in a helical hairpin conformation, exposing hydrophobic faces for membrane insertion [5–7]. The

Abbreviations: Δδ, secondary chemical shift; ARV, avian reovirus; bis-ANS, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid; Chol, cholesterol; CM, conserved motif; DTT, dithiothreitol; DMSO, dimethyl sulfoxide; DOPC, 2-dioleoyl-*sn*-glycero-3-phosphocholine; DPC, dodecylphosphocholine; FAST, fusion-associated small transmembrane; FP, fusion peptide; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; NBD-DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl); NBV, Nelson Bay reovirus; p10ecto, p10 ectodomain peptide; p10ectoTr, truncated p10 ectodomain peptide; NOESY, nuclear Overhauser enhancement spectroscopy; Rho-DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-4(lissamine rhodamine B sulfonyl); Sph, sphingomyelin; TCEP, tris(2-carboxyethyl)phosphine; TOCSY, total correlation spectroscopy.

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second class are referred to as fusion loops, and are present in proteins such as dengue virus E1 and Ebolavirus gp2 [8,9]. These loops are flanked by elongated, anti-parallel β-strands and expose hydrophobic residues at the apex of the loop for membrane insertion. The prevailing view is that FPs shallowly insert into the outer leaflet of membrane bilayers, inducing membrane bending that results in formation of a dimple. Dimple formation would promote close membrane apposition, with membrane bending stresses in the highly curved lipidic cap of the dimple being relieved by membrane merger [10]. Support for this model of FP function derives from studies of cellular proteins involved in vesicle fusion. For example, shallow insertion of an amphipathic helix in the endophilin N-BAR domain or hydrophobic loops in the C2 domains of synaptotagmin and Doc2b proteins induce extensive membrane curvature resulting in liposome tubulation and generation of highly fusogenic lipidic end caps on these tubules [11–15]. Whether viral FPs induce liposome fusion via a similar tubulation mechanism has not been determined.

In addition to enveloped viruses, a single family of nonenveloped viruses also encodes membrane fusion proteins. The fusion-associated small transmembrane (FAST) proteins are encoded by the fusogenic *Aquareoviruses* and *Orthoreoviruses*, two genera in the family *Reoviridae*, a large diverse group of nonenveloped viruses with segmented, dsRNA genomes [16]. FAST proteins are nonstructural viral proteins expressed inside virus-infected cells, where they traffic to the plasma membrane to induce cell–cell membrane fusion and syncytium formation [17]. All FAST proteins are integral membrane proteins, with a single transmembrane domain separating very small (~20–40 residues) N-terminal ectodomains from equal-sized or considerably larger C-terminal cytoplasmic endodomains [18–23]. The homologous p10 FAST proteins of avian (ARV) and Nelson Bay (NBV) orthoreoviruses, and the unrelated p14 and p15 FAST proteins of reptilian (RRV) and baboon orthoreoviruses, respectively, all contain motifs in their small ectodomains that share features with canonical viral FPs [17]. These FP motifs are essential for cell–cell fusion, and synthetic peptides based on these motifs induce liposome–liposome lipid mixing. Structurally, these motifs differ dramatically from each other, and from enveloped virus FPs. An intramolecular disulfide bond in p10 creates an 11-residue cystine loop FP, the p14 FP contains a 7-residue proline-hinged loop, while the 19-residue p15 ectodomain FP comprises a polyproline type II helix flanked by short, amphiphilic, unstructured regions [24–26]. Aside from being essential for cell–cell fusion and inducing lipid mixing between liposomes, these atypical FAST protein FPs remain poorly characterized.

At 95–98 residues in size, the p10 FAST proteins encoded by avian and bat reoviruses are the smallest known viral or cellular membrane fusion proteins. We recently reported the presence of a cystine loop FP in ARV and NBV p10, and showed stringent sequence constraints within and flanking the cystine loop affect formation of the intramolecular disulfide bond [27]. Specific features of the p10 cystine loop are quite distinct from enveloped virus fusion loops. For example, the enveloped virus fusion loops are located at the tips of disulfide-stabilized structures comprising anti-parallel β-strands [9,28] or in order-turn-order structures [29,30]. In some cases, such as Ebola virus gp2, the fusion loop is actually partly helical [31,32]. Enveloped virus FPs that function as fusion loops are therefore components of larger structures, and the role of disulfide bonds is to stabilize the overall fusion domain rather than the FP directly. In contrast, the p10 ectodomain is only 36–40 residues in size, and the 11-residue fusion loop is self-contained within a 15–19 residue sequence that is solely required for formation of the cystine loop FP [27]. We now show that formation of the p10 intramolecular disulfide bond forces solvent exposure of hydrophobic residues, suggesting the cystine loop functions as a cystine noose [33]. Cystine noose formation is required for membrane partitioning, structural transitions and fusion activity. Additionally, we demonstrate that p10-induced liposome-liposome fusion occurs in the absence of liposome tubulation, and that cholesterol is required for the post-binding lipid mixing stage of p10-mediated membrane fusion.

2. Materials and methods

2.1. Synthetic peptides

Peptides corresponding to the ectodomain (residues 1–40) of the ARV p10 protein (ARV p10ecto) were synthesized by Genscript to contain an intramolecular disulfide bond between Cys9 and Cys21. Peptides corresponding to a partially truncated (residues 4–36) ARV p10 ectodomain (p10ectoTr) and to the NBV p10 ectodomain (residues 1–35, NBV p10ecto) were synthesized by United Peptide to contain a similar intramolecular disulfide bond. A similar NBV p10ecto peptide containing a Ser substitution of Cys5 (NBV p10C5ecto) to prevent formation of the disulfide bond was also synthesized by United Peptide. All peptides were purified to 95% purity by HPLC and confirmed by mass spectrometry.

2.2. Hydrophobicity and aggregation analysis

Hydrophobicity predictions were performed with Protscale on the ExPASy server, using a sliding window average of 5 residues. Relative hydrophobicity was measured in aqueous conditions (10 mM $NaPO_4$, 100 mM NaF, pH 7.4) using 4.7 μM 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid (bis-ANS, Invitrogen) and 80 μM of a given p10 ectodomain peptide with and without 20 mM tris(2-carboxyethyl)phosphine (TCEP). Steady-state fluorescence spectroscopy was performed using a Varian Cary Eclipse spectrofluorometer at 35 °C in a 3 mm quartz cuvette (Varian), with excitation and emission slit widths of 5 nm. Emission spectra were measured from 460 to 600 nm with an excitation wavelength of 360 nm. Time-dependent aggregation of the p10ecto peptide (0.5 mM), in aqueous conditions (10 mM $NaPO_4$, 100 mM NaF, pH 7.4) with and without 5 mM dithiothreitol (DTT), was followed by OD350 measurements using a Varian Cary 50 UV/Vis spectrophotometer. All spectra were background subtracted. Bis-ANS experiments were repeated in quadruplicate, while peptide aggregation experiments were performed in triplicate.

2.3. Solution-state nuclear magnetic resonance (NMR) spectroscopy

Solution-state NMR experiments were performed on the p10ectoTr peptide in the organic solvents dimethyl sulfoxide (DMSO-d_6) and hexafluoro-2-propanol (HFIP-d_2), and in the sodium dodecylsulfate (SDS-d_{25}) and dodecylphosphocholine (DPC-d_{38}) membrane-micellar mimetic environments. Organic solvent samples were prepared by dissolving the peptide (~0.5 mM) in 100% DMSO-d_6 or 50% HFIP-d_2 plus 40% H_2O and 10% D_2O. Micelle samples were prepared by dissolving the peptide in SDS-d_{25} or DPC-d_{38} (150 mM) in 90% H_2O and 10% D_2O, and adjusting the pH to 5.0. The HFIP, SDS and DPC samples also contained 0.5 mM sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) and 0.2 mM sodium azide. The SDS and DPC samples were buffered with 20 mM sodium acetate (pH 5.0). NMR data were acquired using a Bruker Avance III 700 MHz spectrometer equipped with a 5 mm triple-resonance inverse cryoprobe and processed using Bruker Topspin 3.1. 1D ^1H, 2D ^1H–^1H total correlation spectroscopy (TOCSY; number of scans 16, size of fid 2048/512 in F2(^1H)/F1(^1H), sweep width 12/12 ppm in F2/F1, recycle delay 2 s, mixing time 60/120 ms, DIPSI2 isotropic mixing sequence) and 2D ^1H–^1H nuclear Overhauser enhancement spectroscopy (NOESY; number of scans 16, size of fid 2048/512 in F2(^1H)/F1(^1H), sweep width 12/12 ppm in F2/F1, recycle delay 2 s, mixing time 200–400 ms) spectra were acquired for the p10ectoTr peptide under all four solvent conditions at 22 and 37 °C. Spectra collected in HFIP, SDS and DPC were referenced to internal DSS at 0 ppm. Spectra collected in DMSO were indirectly referenced to 0 ppm for aqueous DSS using the intermediate trimethylsilane (TMS) shift. Spin system assignment was performed, to the extent possible, in Sparky 3.110 (Goddard, T.D., and Kneller, D.G. Sparky 3, University of California, San Francisco). Secondary chemical shifts ($\Delta\delta$) for H^α resonances of unambiguously identified residues were calculated by