INSIGHTS INTO THE MOLECULAR MECHANISMS OF MEMBRANE GEOMETRY GENERATION AND RECOGNITION

A Dissertation in Biochemistry and Molecular Biology

by

Richard L. Gill, Jr.

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The dissertation of Richard L. Gill, Jr. was reviewed and approved by the following:

**Fang Tian**  
Assistant Professor of Biochemistry and Molecular Biology  
Dissertation Advisor  
Chair of Committee

**John M. Flanagan**  
Professor of Biochemistry & Molecular Biology

**Faoud Ishmael**  
Assistant Professor of Pulmonary Medicine

**Ira J. Ropson**  
Associate Professor of Biochemistry and Molecular Biology

**James Broach**  
Professor and Chair of the Department of Biochemistry and Molecular Biology

*Signatures are on file in the Graduate School.*
ABSTRACT

Membrane remodeling is an essential process in cell growth, division, intracellular vesicle transport, and many other essential biological phenomena. Furthermore, membrane geometry has recently been shown to regulate biological activity and determines the final cellular localization of some proteins. The aim of this thesis is to provide structural and molecular insights into the mechanism governing membrane geometry induction and recognition.

SpoVM is a small peptide that was recently found to recognize and preferentially localize to the slightly curved outer surface of the forespore (diameter of curvature, R, ~1 μm) during Bacillus subtilis spore development. However, little was known about how this was accomplished. We determined that the SpoVM molecule adopted an atypical amphipathic helical structure in bilayer-like bicelles and that the helix is deeply embedded into the membrane. Our study challenges the current accepted model, which is based on circular dichroism and biochemical data, and provides structural support that SpoVM uses a new molecular mechanism to sense small membrane curvature.

Recent studies have linked protein intracellular trafficking to human diseases. In Alzheimer’s Disease (AD), a type I membrane protein referred to as LR11 (SorLA) is a key regulator of the amyloid precursor protein (APP) trafficking. LR11 diverts APP away from amyloidogenic processing to Aβ peptides. The accumulation of Aβ in the brain is generally accepted as the primary cause of AD. We discovered a membrane proximal amphipathic α-helix in LR11 C-terminal domain and, moreover, this helix deforms the membrane mimic liposomes. Since changes in membrane
geometry are inherent to trafficking events, we postulate that this helix may sense and/or induce the bending of the membrane during vesicle biogenesis to facilitate LR11 intracellular transport.

Amphipathic helices play a myriad of functions in mediating protein-lipid interactions. Despite their structural simplicity, several key properties including charge, the physiochemical characteristics of the hydrophobic vs. hydrophilic face, hydrophobicity, helical length, membrane orientation, and insertion depth, can be fine-tuned for their biological functions. Our study highlights the importance to quantitatively dissect these parameters in order to mechanistically understand their functions in membrane remodeling and ultimately to be able to manipulate these properties for therapeutic uses such as in designing antimicrobial and cell penetrating peptides.
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<th>Full Form</th>
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<tr>
<td>1,2-DOG</td>
<td>1,2-Dioleoyl-sn-glycerol</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s Disease</td>
</tr>
<tr>
<td>AHs</td>
<td>Amphipathic Helices</td>
</tr>
<tr>
<td>ALPS</td>
<td>Amphipathic Lipid Packing Sensor</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid Precursor Protein</td>
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<tr>
<td>ArfGAP1</td>
<td>ADP-Ribosylation Factor GTPase-Activating Protein 1</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<td>B. subtilis</td>
<td>Bacillus subtilis</td>
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<tr>
<td>BAR domain</td>
<td>Bin-Amphiphysin-Rvs domain</td>
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<tr>
<td>CD</td>
<td>Circular Dichroism Spectroscopy</td>
</tr>
<tr>
<td>COPI/II</td>
<td>Coat Protein I/II</td>
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<tr>
<td>CT</td>
<td>Cytoplasmic Tail</td>
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<tr>
<td>DHPC</td>
<td>1,2 dihexanoyl-sn-glycero-3-phosphocholine</td>
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<td>DLS</td>
<td>Dynamic Light Scattering</td>
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<td>DMPC</td>
<td>1,2-dimyristoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DOPE</td>
<td>Dioleoyl-phosphatidylethanolamine</td>
</tr>
<tr>
<td>DSA</td>
<td>Doxyl-stearic acid</td>
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<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
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<td>EGF</td>
<td>Epidermal Growth Factor</td>
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<td>EM</td>
<td>Electron Microscopy</td>
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<tr>
<td>Gd(DPTA)</td>
<td>Gadolinium-(diethylenetriaminepentaacetic acid)</td>
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<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
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<tr>
<td>GTP</td>
<td>Guanosine-5’-triphosphate</td>
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<tr>
<td>GUV</td>
<td>Giant Unilamellar Vesicle</td>
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<tr>
<td>His</td>
<td>Histidine tag</td>
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<td>HSQC</td>
<td>Heteronuclear Single Quantum Coherence</td>
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<td>ITC</td>
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<td>LR11</td>
<td>Lipoprotein receptor 11 Repeats</td>
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<td>LUV</td>
<td>Large Unilamellar Vesicle</td>
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<td>MBP</td>
<td>Maltose Binding Protein</td>
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<td>MCS</td>
<td>Multiple Cloning Site</td>
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<td>MCS</td>
<td>Membrane Curvature Sensor</td>
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<td>Ni-NTA</td>
<td>Nickel-Nitrilotriacetic Acid</td>
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<td>NMR</td>
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<td>PC</td>
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<td>pMTBP</td>
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sortilin-related receptor, L(DLR class) A repeats-containing
SSLB Spherically Supported Lipid Bilayer
SUV Small Unilamellar Vesicle
TM Transmembrane
TGN trans-Golgi Network
TOCSY Total Correlation Spectroscopy
TROSY Transverse Relaxation Optimized Spectroscopy
VHS domain VPS-27, Hrs and STAM domain
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Chapter 1: Introduction

1.1 Membrane Geometry

1.1.1 Diverse and Dynamic Cellular Membrane

The cellular membrane provides the physical separation of the interior of the cell from the external environment. Cellular membranes are selectively permeable to ions and small organic molecules controlling the movement of small substances in and out of the cell. Inside eukaryotic cells, membranes further partition the cell into different compartments and organelles. Cell membranes are involved in a variety of cellular processes such as ion conductivity, cell adhesion, cell signaling and serve as the attachment surface for extracellular structures, including the cell wall, glycocalyx, and in intracellular cytoskeleton [1].

Cellular membranes come in a diverse array of shapes in both prokaryotes and eukaryotes (Figure 1-1). For many years, membrane geometry was thought of as a passive feature of membrane function, but in recent years the important role membrane geometry plays in biological function has become apparent [2]. Active membrane shaping is critical for such processes as cell growth, cell division, cell movement, intracellular vesicle transport, endocytosis, and exocytosis [1] [3] [4] (Figure 1-2). It has also been discovered that membrane geometry can itself provide a cue for protein localization, function, and trafficking [5, 6]
Figure 1-1: The beautiful and complex shapes of cells and cell organelles. a) These panels show the prokaryotic shapes of cocci, rods and a spirochete. b) The large image shows the stained endoplasmic reticulum of a 3T3 fibroblast cell as recorded by confocal microscopy. From top to bottom, the three smaller images show a part of a tubular-saccular Golgi ribbon, an intracisternal connection of the Golgi (labeled Golgi bypass) and fenestrated Golgi forms (labelled trans-Golgi). Figure adapted from [1].
That membrane geometry can be a cue for protein localization, function, and trafficking was first discovered in the intracellular vesicle transport system of the trans-Golgi Network (TGN). In the TGN, transport vesicles are constantly budding from the Golgi and fusing with membrane bound organelles through the action of protein coats and SNARE proteins. Dozens of different cytosolic proteins arrive and cause the membrane to gradually deform and eventually pinch off into transport vesicles. Studying the assembly and disassembly cycle of the COPI coat, which mediates the formation of vesicles from the Golgi apparatus, it was noticed that the activity of certain proteins were quite sensitive to the size of the artificial vesicles, liposomes, used in *in vitro* reconstitution studies [8].
One particular enzyme, ArfGAP1, was found to be 100 times more active when the coat was reconstituted on liposomes with the size of authentic transport vesicles (radius, \( R = 35 \text{ nm} \)) than on larger liposomes (\( R = 150 \text{ nm} \)) \([7, 9]\). This hypersensitivity to membrane curvature suggested a feedback loop mediated by membrane morphology. As shown in Figure 1-3, a young COPI coat (Coatomer + Arf1-GTP) remolds a weakly curved membrane in a safe manner because it is protected from the disassembly effect of ArfGAP1. Later as it matures into an old coat surrounding a small vesicle, curvature-activated ArfGAP1 quickly disassembles it. ArfGAP1 is the first protein to be classed as a membrane curvature sensor, a protein whose binding affinity, activity, or localization for membranes is dependent on the geometry and/or extent of curvature of the membrane \([10]\).

![Figure 1-3: Control of COPI coat dynamics by membrane curvature. ArfGAP1 “centric view” of COPI coat dynamics. Once a bud forms, ArfGAP1 eliminates most Arf1 molecules except at the neck where membrane curvature is negative. After fission, ArfGAP1 hydrolyzes the last Arf1-GTP molecules, and the coat disassembles. Figure adapted from \([11]\).](image)

Since the discovery of ArfGAP1 as a membrane curvature sensor, a wide variety of proteins have been identified as being sensitive to membrane geometry in both eukaryotes and prokaryotes. Membrane curvature sensors help to organize very diverse reactions, such as lipid transfer between membranes, the tethering of
vesicles at the Golgi apparatus [12], cell division in gram positive bacteria [13], and has been even implicated in Parkinson’s Disease [14].

Understanding the mechanisms of membrane geometry induction and recognition has also important implications in understanding biomedically relevant processes. For example, entry of enveloped viruses requires formation and expansion of a fusion pore allowing entry of the viral nucleoprotein or core into the cytoplasm [15].

Membrane shape and cellular signaling processes are interdependent [16]. In a recent study by Rangamai, et al., they demonstrated that information contained in the cell’s shape could be transformed into measurably different MAPK phosphorylation levels in growth factor receptor pathways. This curvature-dependent mechanism now adds another possible level of organization in cell signaling pathways.

One more example of membrane-curvature-enhanced interactions comes from recent studies of the inner mitochondrial membrane. This membrane is morphologically defined by repeated infolding that forms the cristae. When researchers made alternations of cristae shape, they found that these morphological changes impacted the assembly and activity of the respiratory chain complexes (RCC) that reside in this membrane. The efficiency of mitochondrial-dependent cell growth appears dependent on cristae morphology, providing an organellar example of membrane shape influencing cellular physiology.

Lastly, a recent article has identified membrane curvature as contributing factor in the sorting and targeting of certain transmembrane proteins to particular
regions of the eukaryotic cell [17]. Using lipid nanotubules they demonstrated that the potassium channel KvAP, in the presence of nanotubes with controlled radii were more highly enriched in the regions of highest curvature as compared to the flat regions. This is contrast to the water channel AQP0, which showed no preference for flat or curved membranes. Using a thermodynamic sorting model, the authors were able to determine the factors contributing to the difference in localization of KvAP and AQP0 and concluded that interaction of membrane shape and the effect of transmembrane proteins on the stiffness and shape of the membranes can provide a mechanism for transmembrane protein targeting within the cell. Together these studies demonstrate that membrane shape/curvature once thought to be a passive feature of biological membranes contributes significantly to cellular function.

1.1.2 Geometric Principles of Membrane Curvature

Current study of membrane geometry mainly focuses on a concept, namely membrane curvature. When we wish to describe biological membrane curvature mathematically, it is often treated as a two-dimensional surface, which spans a three-dimensional space (Figure 1-4).
The curvature of a biological membrane can be mathematically described by the principle radii of curvature $R_1$ and $R_2$. $R_1$ and $R_2$ are derived from considering two perpendicular planes of the curved surface and taking the radii of these two perpendicular planes. The principle curvatures, $c_1 = 1/R_1$ and $c_2 = 1/R_2$, are used to describe the various geometric shapes biological membranes form. Figure adapted from [1].

If you cross-section the membrane surface at a point under consideration using two planes that are perpendicular to the surface and orientated in two special directions called the principle directions, the principle curvatures are the curvatures of the two lines of intercepts between the planes and the surface, which have almost circular shapes in close proximity to the point under consideration. The radii of these two circular fragments, $R_1$ and $R_2$, are called the principle radii of curvature, and their inverse values, $c_1 = 1/R_1$ and $c_2 = 1/R_2$, are referred to as the two principle curvatures.

The principal curvatures $c_1$ and $c_2$ can vary arbitrarily and thereby give origin
to different geometrical shapes, such as cylinders, planes, spheres and saddles. Analysis of the principal curvature is important, since a number of biological membranes possess shapes that are analogous to these common geometry staples. For instance, prokaryotic cells such as cocci display the shape of a sphere, while rods and spirochette resemble the shape of a cylinder. Erythrocytes commonly referred to as red blood cells, have the shape of a saddle. Figure 1-5 below lists common geometric shapes, an analysis of their two principal curvatures, and a corresponding biological membrane shape.

**Figure 1-5:** Geometric shapes with their principle of curvature $C_1$ and $C_2$ and biological membrane shape. Each combination of positive and negative values of $C_1$ and $C_2$ give rise to a particular geometric shape. For a flat shape both principles of curvature have no value. a) a membrane fragment under a flat clathrin lattice b) Bacillus c) Erythrocyte d) Cocci. Figure partially adapted from [1].
Another geometric description of curvature that must be also considered is the direction of curvature, which can be described as being zero (planar), positive (convex), or negative (concave). For a cell membrane the curvature by convention is characterized in respect to the volume contained by the membrane (Figure 1-6). Positive (convex) curvature corresponds to the membrane bulging towards the outside medium. For negative (concave) curvature the bulge is in the direction of the inner medium.

![Diagram of curvature types](image)

**Figure 1-6:** Geometric description of surfaces in vesicles and tubules. Positive curvature (convex), negative curvature (concave), and zero curvature (planar). Figure adapted from [2].

The final geometric consideration for the description of curvature is whether the bend of the curvature is sharp (high curvature) or shallow (low curvature). For a more semi-quantitative analysis of this concept, in Figure 1-7a, the curvature is considered shallow if the radii \( R_1, R_2 \) are much larger than the thickness of the
membrane $d$, $R_1 \gg d$, $R_2 \gg d$. In Figure 1-7b, it is considered to be high curvature when one of the radii is comparable or somewhat larger than the membrane thickness: $R_1 \geq d$ or $R_2 \geq d$. Taking into account that the lipid bilayer is ~4-7 nm thick, intracellular tubules or vesicles with radii of ~25-30 nm have relatively high curvatures.

1.1.3 Generation of Membrane Curvature

To generate curved membrane a cell has to perform some work. The reason for this is the intrinsic tendency of a lipid bilayer (composed of cylindrical lipids) to

\begin{align*}
\text{Figure 1-7: The scale used to assess the values of membrane curvature is based on membrane thickness, } d. \text{ a) The curvature, } c, \text{ is considered to be small if the corresponding radius of curvature, } R, \text{ is much larger than the membrane thickness, } R \gg d \text{ (that is, the absolute value of the curvature, } |c|, \text{ should satisfy the equation } |c| \cdot d \ll 1) \text{. b) In cases in which the curvature radius is close to the membrane thickness, } R \approx d, \text{ (that is, } |c| \cdot d \approx 1), \text{ the membrane curvature is regarded as being high. Figure adapted from [1].} \\
\end{align*}
adopt and keep a flat shape. The energy cost for bending a bilayer can be determined from the elastic model of lipid membranes [18]. Generation of a fragment of lipid tubule with a 60 nm diameter and a length of 60 nm requires ~70 kcal/mol whereas formation of a spherical vesicle of the same diameter requires ~300 kcal/mol [19]. Both energies are much larger than the characteristic thermal energy of fluctuation (~0.6 kcal/mol), which means these shapes cannot be generated simply by thermal fluctuations. However, these bending energies are well within the energy generated through the hydrolysis of a few tens of adenosine triphosphate (ATP) (10 kcal/mol each) suggesting consistent with the idea that metabolic energy can be used to shape membranes [19]. This readily suggests that interplay between lipids and proteins are required to generate, regulate, and maintain the dynamic behavior of cellular membranes.

What are the specific mechanisms and the related molecular machineries a cell can use to produce and maintain the required curvatures of its membranes? Four primary mechanisms have been identified (which are not mutually exclusive) 1) asymmetric lipid composition and shape 2) extrinsic membrane deformation 3) scaffolding 4) hydrophobic insertion [2, 20, 21]. In this section, a brief summary of each mechanism will be given highlighting our current understanding of how biological membrane curvature are produced.

1.1.3.1 Lipid Composition

In principle, lipids alone can generate membrane curvature if the lipid composition of the membrane monolayers is asymmetric. This is because, depending on their chemical structures, lipids tend to curve with a slight spontaneously
negative or positive curvature. For a better understanding of this mechanism, it helpful to understand the classification of lipid molecules by shape. In Figure 1-8, when the cross-sectional area of the head group and acyl chain are the same, the lipid can be envisioned as a cylinder due to their shape and these lipids will be able to pack well and pack into a planar bilayer. These lipids are known simply as type 0 lipids. Phosphatidylcholines (PC) are representative of type 0 lipids. When the head group is larger than the acyl chain this lipid resembles a cone or wedge. These lipids, such as lyso-phosphatidylcholine are referred to as type I lipids and prefer convex curvature when mixed with bilayer lipids. Lipids with a smaller head group and larger volume acyl chain can be conceptualized as an inverted cone shape, are Type II lipids. These lipids, represented by phosphatidylethanolamine (PE), prefer to form inverted micelles and prefer negative curvature.

**Figure 1-8:** Curvature in membranes arises through lipids of differing shapes. Left is the type II phosphatidylethanolamine (PE), which gives rise to negative curvature. In the middle is the type 0, cylindrical, phosphatidylcholine (PC) that does not induce any curvature. On the right is lyso-phosphatidylcholine that brings about positive curvature in membranes.
In the early 80’s lipid composition was seen as the primary means of generating membrane geometry, however theoretical calculations show that generation of strongly bent membranes with a curvature of radii of a few tens of nanometers requires fairly strong monolayer asymmetry, which is not expected, based on the lipid composition of the cell membranes. Therefore the current consensus in the field is that high membrane curvature is generated through the interaction of protein and lipid.

1.1.3.2 Extrinsic Membrane Deformation

Extrinsic membrane deformation by proteins is the physical exertion of force on the bilayer causing the membrane to be pulled, pushed, or bent by mechanical force. This mechanism primarily involves the actin or cytoskeleton filaments. Cytoskeletal assembly and disassembly is intimately linked with membrane-shape changes of the plasma membrane and of organelles. Branching, bundling and treadmilling of actin filaments are involved in the generation and remodeling of many areas of high membrane curvature, including filopodia, pseudopodia, phagocytic cups and axonal growth cones [20]. The ability of the cytoskeleton to influence membrane shape is affected by membrane tension, and decreases in tension can help the generation of local curvature (for example, membrane trafficking events). The cytoskeleton has a large role in maintaining membrane tension (Figure 1-9) and conversely actin rearrangements are responsive to changes in tension [22].
Figure 1.9: Cytoskeletal mechanisms for membrane deformation. Cytoskeletal elements may have multiple roles in membrane deformation. Above left: cytoskeleton-dependent formation and maintenance of tubular organelle structures; middle: formation of membrane tubules pulled by a cytoskeletal motor protein; below right: external cytoskeletal forces abutting the membrane and causing deformation. Figure adapted from [20].

1.1.3.3 Scaffolding

The two primary mechanisms for protein-lipid induced membrane curvature are scaffolding and insertion. Membrane scaffolding is one of the multiple functions of protein coats found on the surface of membrane invaginations and buds. The role of protein coats serving as scaffolding can be seen with COPI, COPII, and clathrin-adaptor complexes providing scaffolding for spherical curvature, whereas dynamin and BAR domain containing proteins wrap around membranes and provide scaffolds
for cylindrical curvature. Scaffolding can take on many forms - either through the action of proteins with an intrinsic shape or a networking of proteins, which work as a whole to mold the shape of the membrane bilayer. The clearest examples of an assembly of proteins working as a scaffold are dynamin-containing proteins. In Figure 1-10, dynamin binds to lipid membranes and forms cylindrical coats that resemble "split interlinked washers" [23]. The rigidity of the dynamin coat is greater than that of the lipid bilayer and it has been reported that dynamin binding to lipids is sufficiently strong enough to allow the scaffold mechanism to work in membrane shaping and membrane fission [24].

![Figure 1-10](image.png)

**Figure 1-10:** Polymerized dynamin coat proteins stabilize membrane curvature [1].

The BAR domain has a banana-like shape and its concave surface binds the lipid membrane [25] (Figure 1-11). In addition, the domain has multiple positively charged residues on its concave surface, which allows its concave surface to interact strongly with the negatively charged polar head groups of the lipid molecules [26].
The curvature of many tubes that are covered by these domains is close to the curvature of the concave BAR-domain surface [27].

![Figure 1-11: The scaffold mechanism of BAR domain. A rigid protein, or protein domain (for example, the BAR (Bin, amphiphysin, Rvs) domain), that has an intrinsic curvature binds to the membrane surface and bends the membrane beneath it [1].](image)

1.1.3.4 Hydrophobic Insertion

The third mechanism of membrane bending is hydrophobic insertion (wedging). Here, proteins insert hydrophobic domains into the upper part of the membrane monolayer. This results in the perturbation of the packing of the lipid head groups and generates local curvature. The two most common motifs for hydrophobic insertion are amphipathic helices and loops. A brief description of each is provided below.

Amphipathic helices are stretches of alpha-helices, one side of which is polar and the other hydrophobic (Figure 1-12a). Unstructured before insertion, the helices
are predicted to sit on the membrane surface with the hydrophobic residues dipping into the hydrophobic phase of the membrane and the polar residues interacting with the lipid head groups resulting in displacement of lipid head groups and a reorientation of acyl chains. Given the asymmetric insertion it acts like a wedge inserted into one leaflet of the membrane bilayer causing an asymmetry of the membrane bilayer surface area (Figure 1-12b).

![Figure 1-12: Amphipathic helix insertion into membrane bilayer induces membrane deformation. a) Helical wheel plot of a hypothetical amphipathic helix. b) Schematic representation of lipid monolayer bending (lipid molecules shown in light shading) by insertion of a cylindrical inclusion (shown in dark shading), where L is the half-distance between the inclusions, h is the monolayer thickness, and r is the inclusion radius. (a) The monolayer is flat before the inclusion insertion; (b) the monolayer bends as a result of inclusion insertion. Figure 1-12b adapted from [28].](image)

The first evidence for the hydrophobic insertion mechanism was through the study of Epsins, which were shown to induce membrane curvature by amphipathic helix insertion. Upon interaction with phosphatidylinositol-4,5-biphosphate polar groups, amphipathic α-helices fold and embed into the lipid monolayer matrix, transforming the flat membrane into tubules of ~20 nm diameter [29]. Further
evidence for amphipathic helix induced curvature induction is provided by small G-proteins Arf1 and Sar1 which expose amphipathic alpha-helices upon exchange of GDP to GTP, and results in the anchoring of these proteins into the lipid bilayer and subsequent bilayer bending. [30]

In addition to amphipathic helices, some proteins contain a short peptide loop composed of hydrophobic residues that insert into the leaflet of the lipid bilayer. The primary example of this mechanism is the synaptotagmins. The synaptotagmins are a family of proteins with an N-terminal transmembrane domain and two cytoplasmic C2 domains. Many C2 domains mediate calcium-dependent binding to negatively charged membranes. Synaptotagmin-1 is localized to synaptic vesicles and is the trigger for their calcium-induced exocytosis, the two C2 domains of synaptotagmin-1 insert into the membrane upon calcium binding [31]. When bound to calcium the two C2 domains insert into the membrane, Figure 1-13, shallow insertions of the loops into the membrane cause the induction of positive membrane curvature seen as tubulation[32].
**Figure 1-13**: Calcium binding to the C2 domains promotes the shallow insertion of two loops into the membrane causing the induction of positive membrane curvature. Figure adapted from [32].

### 1.1.4 Recognition of Membrane Curvature

Preferential binding of proteins on curved membranes (membrane curvature sensing (MCS)) is emerging as a new mechanism whereby cells effect protein localization and trafficking [11]. The ability to locate and preferentially bind areas of high membrane curvature has so far been attributed to two protein motifs: BAR domains and amphipathic helices.

BAR domains are crescent-shaped homodimers (~20 nm in length), the monomers are composed of coiled-coil association of a 3-helix bundle structure. BAR domain subclasses can be distinguished according to the shape of their quaternary structure. Classical BAR domains are highly curved, whereas F-BAR and I-BAR domains have shallow curvature. In addition, N-BAR domains have N-terminal amphipathic helix preceding the BAR domain.

The overall curved structure of the dimer and the net positive charge along its concave surface suggests features maximized for interaction with a negatively charged membrane of complementary curvature. Thus, it has been suggested that classical BAR domains with highly curved surfaces interact with highly curved vesicles whereas shallow curvature is recognized by F-BAR and I-BAR domains that bind to correspondingly shallow curved dimer surfaces (Figure 1-14) [21].
The MCS mechanism for amphipathic helices was hypothesized from the study of ArfGAP1 selective binding to high curvature liposomes. Mesmin, et al. showed that this selectivity was attributed to a sequence of about 40 amino acids in ArfGAP1, known as the ALPS motif (ArfGAP1 Lipid Packing Sensor). The ALPS motif binds avidly to small liposomes, shows the same hypersensitivity to liposome curvature as full-length ArfGAP1 and was necessary for coupling ArfGAP1 activity with membrane curvature. Analysis of site-directed mutagenesis, limited proteolysis and circular dichroism experiments suggested that the ALPS motif, which is unstructured in solution, forms an amphipathic helix on highly curved membranes.
The features of this helix differed from classical amphipathic helices by the abundance of serine and threonine residues on its polar face and bulky hydrophobic residues on its hydrophobic face (Figure 1-15).

**Figure 1-15**: ALPS motif composition promotes membrane curvature sensing. Helical Wheel Plot of ALPS Motif. Hydrophobic face features residues with bulky hydrophobic groups for insertion into lipid packing defects, while the hydrophilic face is rich in polar serine and threonine residues reduce electrostatic interaction with charged lipid head groups. Yellow = polar residues, Blue = charged residues, Purple = bulky hydrophobic residues, Green = aliphatic hydrophobic residues, arrow indicates direction of hydrophobic moment.

In addition to its exquisite sensitivity to membrane curvature, the ALPS motif is also very sensitive to lipid membrane composition. Lipids with a cylindrical shape (type 0) and saturated acyl-chains limit the adsorption of the ALPS motif. In contrast, lipids with a small polar head group and with unsaturated acyl-chains such as DOPA or DOG favor the adsorption of ArfGAP1. At high mol%, these conical lipids can even
bypass the need for curving the membrane [9].

The key feature linking high membrane curvature sensitivity and lipid composition is that both create lipid packing "defects" (Figure 1-16). For membrane geometry - lipid-packing defects are created when the surface area of one bilayer leaflet is increased relative to the other leaflet. The higher the curvature the greater the surface area difference between leaflets, this difference creates voids "bilayer defects". Lipid composition creates packing defects due to the difference in lipid shape and membrane surface shape. These bilayer defects are thought to provide binding pockets (sites) for which the ALPS motif to bind [12].

**amphipathic helix (AH)**

Figure 1-16: Amphipathic helices binding to lipid packing defects generated by highly curved vesicles. The binding of these helices alleviates energy costs associated with the strain of phospholipid head groups moving apart created by curving membranes.
The unique features of the ALPS motif and its sensitivity to lipid packing defects have lead to a model in which the hydrophobic effect causes ALPS motif binding, in contrast to electrostatic interactions associated with classical AHs. The current model suggests that the lack of charged residues on the polar face of ALPS weakens its interaction with planar membranes. In the presence of lipid packing defects of highly curved vesicles, the bulky hydrophobic residues of the ALPS motif insert into packing defects and decrease the energy costs associated with the strain of phospholipid head groups moving apart created by curving membranes.

Consistent with this model, increasing packing stress by introducing lipids with small head groups into small vesicles resulted in the increased binding of ArfGAP1 [9]. Conversely, alleviating this lipid packing stress by incubating highly curved vesicles with molecules that insert into this space abrogated the preferential adsorption of another alpha helix-bearing curvature sensing protein. Thus, lipid-packing defects on the binding surface provide one mechanism for the preferential insertion of amphipathic alpha helices into highly convex membranes.

So what is the driving force for the ability of amphipathic motifs to bind preferentially to highly curved membranes? The discussion can be based on the Langmuir–Hill isotherm, a well-accepted formalism used to describe molecular binding equilibria (Eq. (1)):

\[ B(C) = \frac{B_{\text{max}}}{1 + (K_D/C)^H} \]

In this context, as illustrated in Fig. 1-17, an increase in binding density \(B_C\) from a low curvature (black line) to a high curvature can arise either as a result of a
relative decrease in the dissociation constant ($K_D$, blue curve), an increase in cooperative interactions, represented by the Hill coefficient ($H$, red curve), or by an increase in saturation density ($B_{\text{max}}$, grey curve).

![Graphical evaluation of the parameters that may govern membrane curvature selective binding. Langmuir–Hill isotherm illustrating binding on a flat membrane (black line) compared to a curved membrane due to a relative increase of the $H$ coefficient (red line), a decrease in the $K_D$ value (blue line) or an increase in the $B_{\text{max}}$ (grey line). Membrane curvature sensing by amphipathic motifs is an interplay of these components but is currently believed to be dominated by $B_{\text{max}}$ [33].](image)

Bhatia, et al. [33], studied two AHs (Alps motif and $\alpha$-synuclein) and measured binding curves as a function of curvature. Fitting the binding curves for the full concentration range revealed that there was no significant change in the Hill coefficient as a function of curvature. Hatzakis et al. next evaluated whether affinity was responsible for mediating curvature selective binding of the two AHs, as had been previously proposed. The fitted binding curves revealed only a modest (2–2.5-fold) increase in affinity for binding to smaller vesicle diameters, an observation that was independently confirmed by other groups. Finally, examination of the binding curves revealed a strong increase ($\sim$35 fold) of $B_{\text{max}}$ with decreasing vesicle diameter.
This suggested that membranes of different curvature exhibit a different effective density of binding sites for AHs. Thus, a mechanistic model (Figure 1-18) was proposed that curvature selective binding is mediated by higher density of binding sites ($B_{\text{max}}$) on curved membranes rather than increased affinity or cooperative interactions.

**Figure 1-18:** Model explaining sensing and recruitment to high membrane curvature by $B_{\text{max}}$. Lipid packing defects introduced upon bending a membrane become binding sites for amphipathic motifs and alkyl-chain anchored proteins. The density of binding sites scale as $r^{-1}$ for a membrane of thickness $l$ curved to a radius $R$. $B_{\text{max}}$ is proportional to the density of defects. The model serves as a unifying mechanism explaining membrane curvature sensing by BAR domains, AHs, membrane-anchored proteins. Figure adapted from [33].

In conclusion, although presented as two separate functions; membrane curvature recognition and membrane remodeling are not mutually exclusive phenomena. Bar domains, for example, have been shown to efficiently tubulate liposome *in vitro* [25], but as discussed in this section, have high avidity for more highly curved liposomes [26]. Recent evidence suggests that membrane remodeling and curvature recognition appear to use the same mechanisms and whether a protein functions as an inducer or sensor depends on the complex interplay between
the protein and lipid environment [21].

1.2 Nuclear Magnetic Resonance Spectroscopy

1.2.1 Introduction

Membrane proteins constitute about 30% of the genes of any genomes and these proteins are involved in a wide variety of functional activities that are essential for life [34]. Membrane proteins, especially the membrane-bound receptors and ion channels are the targets for over half of the currently used drugs. However, the structural investigation of membrane proteins lags far behind that of soluble proteins. X-Ray Crystallography of membrane proteins has been proven difficult due to the difficulties to crystallize membranes in the presence of lipids and the instability of the proteins in the absence of lipid bilayers. Nuclear Magnetic Resonance Spectroscopy (NMR) has become a powerful tool in understanding membrane protein structure, protein-lipid interaction, and dynamics [35]. The ability to study membrane proteins in a wide variety of mild detergents, lipid bilayer mimetics, and even native-like membrane environments has made NMR the premier technique for understanding membrane protein biology. In recent times, solution NMR spectroscopy methods has been able to determine several large structures of both α-helical and β-barrel membrane proteins like OmpG, VDAC-1, UCP2, DsbB, DAGK, pSRII, KcsA, and proteorhodopsin [36-42].

1.2.2 Heteronuclear Single Quantum Coherence (HSQC)

One-dimensional NMR techniques yield extremely useful information in small molecules, but are of limited applicability to the complex, highly overlapped spectra
of biological macromolecules. Another limitation for complex molecules is the low inherent sensitivity of the technique from large complex molecules. The $^{15}$N HSQC experiment is one of the most frequently recorded experiments in protein NMR. The HSQC experiment can be performed using the natural abundance of the $^{15}$N isotope [43] but normally for protein NMR, isotopically labeled proteins are used. $^1$H-$^{15}$N HSQC spectra are considered the "fingerprint" of a protein. This experiment correlates the nitrogen atom of NH$_2$ group with a directly attached proton. In the spectrum, each resonance (peak) represents a proton that is bound to a nitrogen atom (amide-proton pair). Therefore each resonance will represent every residue in a protein with an exception to proline (Figure 1-19). In addition to the backbone amide resonances, sidechains with nitrogen-bound protons will also produce peaks. If the protein is folded, the peaks are usually well dispersed, and most of the individual peaks can be distinguished. In a typical HSQC spectrum, the NH$_2$ peaks from the sidechain of glutamine and asparagine appear as doublets on the top right corner, and a smaller peak may appear on top of each peak due to deuterium exchange from the D$_2$O normally added to an NMR sample, giving these sidechain peaks a distinctive appearance. The sidechain amine peaks from tryptophan are usually shifted downfield and appear near the bottom left corner. The backbone amide peaks of glycine normally appear near the top of the spectrum.
The assignment of the HSQC spectrum cross-peaks requires other experiments, ideally. This is usually done with triple resonance experiments with $^{15}$N and $^{13}$C-labelled proteins. The assignment of the spectrum is essential for a meaningful interpretation of more advanced NMR experiments such as structure determination and relaxation analysis.

The HSQC experiment is also useful for detecting binding interface in protein-protein interaction, as well the interactions with drugs. By comparing the HSQC of the protein in the presence and absence of ligand, changes in the chemical shifts of some peaks may be observed. These peaks are likely to lie on the binding surface where the binding perturbed their chemical shifts. The $^{15}$N HSQC may also be used in
relaxation analysis in the studies of molecular dynamics of proteins, the determination of ionization constant, and other studies.

The basic scheme of this experiment involves the transfer of magnetization on the proton to the second nucleus, which may be $^{15}$N or $^{13}$C, via an INEPT (Insensitive Nuclei Enhanced by Polarization Transfer) step. After a time delay ($t_1$), the magnetization is transferred back to the proton via a reverse INEPT step and the signal is then recorded. In HSQC, a series of experiments is recorded where the time delay $t_1$ is incremented. The $^1$H signal is detected in the direct dimension in each experiment, while the chemical shift of $^{15}$N or $^{13}$C is recorded in the indirect dimension.

The pulse sequence for a basic HSQC experiment is shown in Figure 1-20:

![Pulse sequence for a typical HSQC experiment.](image)

Figure 1-20: Pulse sequence for a typical HSQC experiment. Narrow and wide pulses correspond to 90° and 180° flip angles, respectively, with the indicated phase. The delay $\tau$ is set to $1/(2J_{IS})$.

The product operator treatment of this HSQC pulse sequence, begins with 1) an INEPT transfer from a $^1$H atom (spin I) to the attached heteronucleus (spin S, e.g. $^{15}$N or $^{13}$C): $-2I_zS_y$
b. During the period $t_1$, the single quantum $2I_zS_y$ magnetization evolves under the chemical shift of spin $S$. The coupling is refocused by the central $180^\circ$ pulse on spin $I$ after $t_{1/2}$.

\[
-2I_zS_y \xrightarrow{(\Omega_S t_1)S_z} 2I_zS_y \cos \Omega_S t_1 - 2S_x \sin \Omega_S t_1
\]

c. After $t_1$ a reverse INEPT pulse sequence transfers the magnetization back to the $^1H$ atom, which may be detected during $t_2$ to give a 2D HSQC spectrum with cross peaks at $(F_1, F_2) = (\Omega_I, \Omega_S)$.

\[
2I_zS_y \xrightarrow{1^{80}_y} 2I_zS_y \cos \Omega_S t_1 - 2S_x \sin \Omega_S t_1
\]

d. \[
2I_zS_z \xrightarrow{2J_{IS}S_z} I_y \cos \Omega_S t_1 - 2S_x \sin \Omega_S t_1
\]

Several key advances in NMR spectroscopy have pushed the limits of protein structure elucidation; the first improvement has been the shift to higher magnetic field strengths. Field strengths have increased continually from $\sim 30$ MHz in the 1950's to $\sim 400$ MHz in the 1980's to 1GHz in 2009 [44]. The increasing field strength led to increase signal to noise and improved spectral resolution resulting in
less signal overlap. Another major advancement in biomolecular NMR was development of Transverse Relaxation Optimized Spectroscopy (TROSY) in 1997 enabled by the availability of high magnetic field strengths [45] (Figure 1-21).

![a) HSQC  b) TROSY](image)

**Figure 1-21:** Spectral improvements by the TROSY effect. a) An NMR experiment without TROSY results in large line-widths and therefore much signal overlap. b) Exploiting the TROSY effect results in small line-widths and much less signal overlap. The experiments were carried out using identical samples of the 45kDa gyrase-45 from *Staphylococcus aureus* on a 750 MHz spectrometer [46].

### 1.2.3 Paramagnetic Relaxation Enhancement (PRE)

In studies of membrane proteins knowledge of the protein topology within the membrane environment can provide useful insights into protein function. Membrane protein topology includes the immersion depth and orientation of the protein in the membrane bilayer, and the extent of exposure of each residue to solvent. Computational studies have shown that depth of hydrophobic peptide insertion is critical for inducing or sensing curvature [47, 48]. Shallow insertion of hydrophobic moieties is optimal for inducing curvature, whereas deep insertion into the hydrophobic core promotes protein self-assembly and curvature sensing [49, 50].
In solution state NMR, the use of paramagnetic additives has become a technique to probe protein topology through depth specific relaxation effects or chemical shift perturbations [51]. These additives may include spin labels anchored to lipids or detergents within a given region of the membrane or be small freely diffusing paramagnetic species. In the presence of spin labels the rate of transverse relaxation of a protein nuclear spin is dramatically enhanced if it interacts with a nearby spin of an unpaired electron. This paramagnetic relaxation enhancement depends on the inter-spin distance to the sixth power $r^6$. This technique has the highest accuracy determining distances within the 15-25 Å.

In a typical PRE experiment the use of water-soluble and lipid soluble spin labels are used to provide complementary information. In Figure 1-22, a water-soluble probe such as Gd(DPTA) is titrated into a $^1$H-$^{15}$N labeled protein and a 2D TROSY spectrum obtained for each titration step. Upon addition of water soluble Gd(DPTA), protein residues that are exposed to the solvent at the surface of the membrane will show relaxation enhancement that is manifested through line broadening and intensity reduction of the resonance peak. Titration of lipid soluble paramagnetic compounds such as 5-DSA (5-doxyl stearic acid) or 16-DSA will enhance the relaxation of protein residues that are buried within membrane bilayer, while the intensity of resonance peaks of residues near the surface will not be significantly reduced.
Figure 1-22: Water-soluble Gd(DPTA) and lipid soluble 5-doxyl stearic acid use in characterizing membrane topology (example G_{M1}). Using water-soluble Gd(DPTA), for G_{M1} residues exposed to the water-soluble environment, a reduction in intensity of the corresponding resonance peak will occur. The converse assessment can be made using lipid soluble 5-doxyl stearic acid for lipid embedded G_{M1} residues. Figure adapted from [52].

1.3 Circular Dichroism Spectroscopy (CD)

Circular dichroism exploits the fact that chiral carbon atoms in protein secondary structure elements have differential absorption of left- and right-handed circularly polarized light. CD measures this difference. Secondary structural elements have characteristic CD spectra (Figure 1-23) and thus are used to identify and estimate secondary structure, as well as large-scale protein dynamics. For example, highly alpha helical proteins have a characteristic large negative transition at 222 nm and 208 nm and a positive transition at 190 nm, while predominantly beta sheet protein has a CD spectrum with a negative transition at 218 nm and a positive transition at 195 nm. A protein composed of mostly random coil will have a CD spectrum consisting of a positive transition at 230 nm and 195 nm. As CD is a
spectrally additive quantity, these elements sum in the spectrum of a protein with a mix of secondary structure elements.

![CD spectra for secondary structures](image)

**Figure 1-23:** Representative CD spectra for secondary structures.

1.4 Isothermal Titration Calorimetry (ITC)

ITC is a quantitative technique that can determine the binding affinity ($K_a$), enthalpy changes ($\Delta H$), and binding stoichiometry (n) of the interaction between two or more molecules in solution. From these initial measurements, Gibbs energy changes ($\Delta G$) and entropy changes ($\Delta S$) can be determined using the relationship:

$$\Delta G = -RT \ln K_a = \Delta H - T \Delta S$$
(where $R$ is the gas constant and $T$ is the absolute temperature).

An isothermal titration calorimeter (Figure 1-24) is composed of two identical cells made of a highly efficient thermal conducting and chemically inert material such as Hastelloy alloy or gold, surrounded by an adiabatic jacket. Sensitive thermopile/thermocouple circuits are used to detect temperature differences between the reference cell (filled with buffer or water) and the sample cell containing the macromolecule. Prior to addition of ligand, a constant power (<1 mW) is applied to the reference cell. This directs a feedback circuit, activating a heater located on the sample cell. During the experiment, ligand is titrated into the sample cell in precisely known aliquots, causing heat to be either taken up or evolved (depending on the nature of the reaction). Measurements consist of the time-dependent input of power required to maintain equal temperatures between the sample and reference cells.

In an exothermic reaction, the temperature in the sample cell increases upon addition of ligand. This causes the feedback power to the sample cell to be decreased (remember: a reference power is applied to the reference cell) in order to maintain an equal temperature between the two cells. In an endothermic reaction, the opposite occurs; the feedback circuit increases the power in order to maintain a constant temperature (isothermic/isothermal operation).
Figure 1-24: ITC instrument diagram. The whole instrument, including the syringe (VP-ITC microcalorimeter), sample cell and reference cell, needs to be cleaned thoroughly. In general, the stirring syringe is filled with compound solution and the sample cell is filled with protein solution. The reference cell is filled only with buffer. Figure adapted from [56].

Observations are plotted as the power needed to maintain the reference and the sample cell at an identical temperature against time. As a result, the experimental raw data consists of a series of spikes of heat flow (power), with every spike corresponding to one ligand injection. These heat flow spikes/pulses are integrated with respect to time, giving the total heat exchanged per injection. The pattern of these heat effects as a function of the molar ratio [ligand]/[macromolecule] can then be analyzed to give the thermodynamic parameters of the interaction under study (Figure 1-25).
A standard ITC trace consists of two panels. The upper panel shows the heat trace of the thermostat over the time of the experiment with the individual injections of ligand as peaks. By integrating the area of the peaks and plotting them against the molar ratio of ligand and protein one obtains the points depicted in the lower panel. By fitting a quadratic binding curve to the data, one obtains the binding isotherm. From the shape of this curve one can easily estimate the enthalpy and stoichiometry of the reaction as well as the affinity (which is the inverse of the dissociation constant). These are the parameters of the fit algorithm and are also used to calculate the entropy of the reaction.

Once data is obtained it can be analyzed by fitting the data to a specific binding model; the one-site binding model will be used to illustrate the analysis of ITC data through a binding model. The one-site binding model assumes that \( n \) ligands bind per macromolecule with identical thermodynamics and best-fit values of \( n \), the reaction stoichiometry, \( K \) and \( \Delta H \) are determined. The mathematical treatment for the one site-binding model is as follows:

For a system of one set of identical binding sites, the total heat evolved (or absorbed) during the binding process at the end of the \( i \)th injection, \( Q(i) \), is given by Equation (1):
where \( n \) is the number of binding sites, \( P_t \) is the total protein concentration, \( X_t \) is the total ligand concentration, \( V \) is the cell volume, \( K \) is the binding constant and \( \Delta H \) is the binding enthalpy. The heat corresponding to the \( i \)th injection only, \( \Delta Q(i) \), is equal to the difference between \( Q(i) \) and \( Q(i-1) \) and is given by Equation (2), which involves the necessary correction factor for the displaced volume (the injection volume \( dV_i \)):

\[
\Delta Q(i) = Q(i) + \frac{dV_i}{V_o} \left[ \frac{Q(i) + Q(i-1)}{2} \right] - Q(i - 1)
\]

The ITC unit measures \( \Delta Q(i) \) value for every injection. These values are then fitted to Equations (1) and (2) by a nonlinear least squares method. The fit process involves initial guess of \( n \), \( K \) and \( \Delta H \) which allows calculation of \( \Delta Q(i) \) values as mentioned above for all injections and comparing them with the corresponding experimentally determined values. Based on this comparison the initial guess of \( n \), \( K \) and \( \Delta H \) is improved and the process is repeated till no further significant improvement in the fit can be obtained.
1.5 Research Work and Significance

The complexity and diversity of biological membranes remains a significant hurdle in gaining a more quantitative understanding. The aim of this work is to provide insight into the mechanisms governing membrane geometry induction and recognition.

Two main mechanisms for shaping membranes have been identified: scaffolding and hydrophobic insertion. However, our understanding of these mechanisms remains qualitative and rudimentary. For instance, it is not clear how hydrophobic insertion detects differences in membrane curvature and how the geometry and insertion depth of the hydrophobic region affect curvature sensing. In this thesis two proteins are investigated that are ideal for gaining an understanding of the membrane curvature sensing and induction. The first project examines SpoVM, the first protein to demonstrate membrane curvature sensing in vivo. SpoVM is an ideal subject to study membrane curvature sensing due to its small size which makes it amiable to NMR spectroscopy study as well as other biophysical approaches such as isothermal titration calorimetry (ITC), circular dichroism spectroscopy (CD), and capture assays. The structural and thermodynamic data presented in thesis shows that SpoVM senses curvature using a novel mechanism.

LR11 (sorLA) is a type I membrane protein that mediates the trans-Golgi Network to endosome sorting of multiple growth factors. LR11 is a central player in the amyloidogenic processing of the amyloid precursor protein that is implicated in development of Alzheimer’s disease. We discovered an amphipathic α-helix in LR11
C-terminal domain, and moreover, this helix deforms liposomes. Since changes in membrane curvature are inherent to trafficking events, we speculate that this helix might sense and/or induce the bending of the membrane to facilitate intracellular transport. Taken together these two projects provide insight into the mechanisms of membrane geometry induction and recognition.

1.6 Literature Cited

Chapter 2: Structural and Mechanistic Studies of SpoVM Membrane Curvature Sensing

2.1 Introduction

In times of environmental stress gram-positive bacteria form a spore, a resistant structure used for survival under unfavorable conditions. During spore formation (sporulation) (Figure 2-1), the cell divides asymmetrically to create mother cell and forespore compartments. Next, the mother cell engulfs the forespore, enveloping it with inner and outer membranes. Following engulfment, a protein coat is deposited around the outer forespore membrane. This coat is a complex, multilayered assemblage of over fifty proteins that encases the spore and helps to protect it from noxious environmental agents [1]. SpoVM, a short 26-residue (3 KDa) peptide, initiates the proteinaceous spore layer that assembles around the forespore of gram-positive Bacillus subtilis [2].

![Fig. 2-1: Subcellular localization of SpoVM. a) A Bacillus subtilis sporangium is depicted, in which asymmetric division (left) creates a mother cell and a forespore. The mother cell then engulfs forespore (middle panel). Eventually, the forespore is pinched off as a double membrane-bound organelle (right). The site of SpoVM localization, corresponding to the region of highest positive (convex) membrane curvature, is indicated in red.](image-url)
So how is SpoVM recruited to the forespore? Initially the recruitment of SpoVM was attributed to the presence of a pre-localized protein at the forespore surface, but traditional biochemical and genetic approaches failed to find an a priori SpoVM localization factor. Ultimately, three lines of evidence suggested that SpoVM localizes to the surface of the forespore by sensing its convex membrane curvature \[3\]. First, in a \textit{B. subtilis} mutant (Δ\textit{spoIID/M/P}) in which sporulation is arrested at a stage before the start of engulfment (thereby blocking the formation of a convex surface; Figure 2-2A, top panel), SpoVM was mislocalized. While the peptide still adsorbed onto membranes, it no longer discriminated between sites of different membrane curvature and, instead, localized promiscuously. Upon elaboration of an artificial convex surface in the same genetic background, however, SpoVM resumed its localization to the newly created positively curved surface, Figure 2-2A(C). Second, in Figure 2-2B, production of SpoVM in heterologous cells that harbored internal structures unrelated to forespores resulted in the accumulation of SpoVM on the convex membrane surfaces of these structures, whereas a SpoVM variant harboring alanine instead of proline at position 9 (SpoVM\textit{P9A}) localized indiscriminately in these cells. Finally, in Figure 2-2C, purified SpoVM, when incubated in buffered solution with heterogeneously sized large membrane vesicles, preferentially adsorbed onto smaller, forespore-sized vesicles and was largely excluded from binding very large vesicles. In contrast, purified SpoVM\textit{P9A} no longer specifically adsorbed onto forespore-sized vesicles. Taken together, these experiments suggested that positive curvature is at least sufficient to localize wild-type SpoVM to the surface of the forespore.
How does SpoVM recognize the gentle positive curvature of the forespore surface? An analysis of SpoVM primary amino acid sequence in a helical wheel plot...
(Figure 2-3) suggests that SpoVM may adopt an amphipathic helix with all six of its positively charged residues clustered along one longitudinal face of the helix, while the opposite face is largely hydrophobic. Circular dichroism data shows that SpoVM was largely unordered in aqueous solution but assumed an alpha-helical conformation in the presence of lipid vesicles [4].

![Fig. 2-3: SpoVM helical wheel plot](image)

Modeling SpoVM at the surface of the forespore illustrates the difficulty of SpoVM membrane curvature sensing in comparison with molecules that recognize the highly curved membranes of small lipid vesicles. In Figure 2-4, if the SpoVM peptide is assumed to be perfectly alpha helical, it would form a ~40 Å long rod (26 aa-long peptide - 7.2 turns at 5.4 Å per turn). In Figure 2-4A, modeling SpoVM binding tangential of SpoVM to the forespore surface with a radius of curvature of 250 Å the deflection of SpoVM from the surface is ~3 Å, approximately three times the length of a typical covalent bond. However, Figure 2-4B, assuming a tangential
binding to the forespore surface with a radius of curvature 500 nm the deflection of SpoVM from the surface along the length of the 40 Å-long peptide would be less than 0.2 Å - less than the length of a typical covalent bond at 1 Å. Thus, SpoVM sits on a "flat" surface and is unable to directly sense such a small membrane curvature.

**Figure 2-4:** Comparison of macromolecule and surface dimensions on very small vesicles and bacterial membranes. A 26 amino acid-long perfectly alpha helical peptide is depicted as a 40 angstrom-long rod lying tangentially on the surface of either a small vesicle of 50 nm diameter a) or a large organelle of 1 micron diameter b) The calculated distance between the central axis of the rod and the surface of the vesicle is shown on the right in the magnified inset. The length of the rod, circumference of the spheres, and the inflection of the membranes are all drawn to scale [4].

SpoVM functions solely as a curvature-sensing molecule and does not deform membranes [5], unlike some other molecules (e. g. EPSINS, DP1/Yop1 families, and dynamin family proteins) that have dual functionality [6-8]. This well-defined biological function facilitates dissecting the molecular mechanism of membrane curvature recognition from curvature generation. Due to its small size, SpoVM is amenable to detailed structural and mechanistic studies. **The aim of this study was to provide a structural and mechanistic understanding of SpoVM binding to**
shallow curvature through high-resolution structural analysis and characterization of its thermodynamic parameters governing its binding to membranes.

The experiments conducted in this chapter have provided valuable insight into the mechanism of SpoVM recognition of membrane geometry: Our mechanistic study provides evidence that \( \beta_{\text{max}} \) does not govern SpoVM binding to high curvature vesicles. Our structural and topological study of SpoVM indicates SpoVM is not a long straight amphipathic helix that has a shallow insertion depth, but instead has a short deeply imbedded amphipathic helix that is disrupted by the proline at position 9 to form an N-terminally loop. This structure is in contrast to the structure of SpoVM\(^{P9A}\). Taken together, our mechanistic and structural data is used to develop a working model that supports SpoVM senses membrane curvature using a novel mechanism.

2.2 Experimental Procedures

2.2.1 Expression of unlabeled His\(_6\)-Sumo-SpoVM and His\(_6\)-Sumo-SpoVM\(^{P9A}\)

Plasmids for recombinant His\(_6\)-Sumo-SpoVM and His\(_6\)-Sumo-SpoVM\(^{P9A}\) were generously provided by Dr. Ramamurthi’s lab. His\(_6\)-Sumo-SpoVM and His\(_6\)-Sumo-SpoVM\(^{P9A}\) were expressed in \( E. \ coli \) BL21 (DE3) pRIL and Gold, respectively. LB starter cultures for His\(_6\)-Sumo-SpoVM \( E. \ coli \) BL21 (DE3) pRIL inoculated with 100 µg/ml ampicillin and 25 µg/ml chloramphenicol. His\(_6\)-Sumo-SpoVM\(^{P9A}\) \( E. \ coli \) BL21 (DE3) Gold LB starter cultures were inoculated with 100µg/ml ampicillin. Starter cultures were grown at 37 °C for 12 hours. Typically, recombinant protein was produced by inoculating 250 mL M9 media with 50:1 LB starter culture and inducing
at OD<sub>600</sub> 0.6-0.9 with 1 mM isopropyl β-D-1thiogalactopyranoside (IPTG) at 16 °C for ~20 hours. Cells were harvested by centrifugation and stored the pellets at −80 °C until use.

2.2.2 Expression of isotopically labeled proteins

For the production of labeled proteins, M9 media (3 g/L KH<sub>2</sub>PO<sub>4</sub>, 6 g/L Na<sub>2</sub>HPO<sub>4</sub>, 0.5 g/L NaCl, 0.2 mM MgSO<sub>4</sub>, 7 mg/L (NH<sub>4</sub>)<sub>2</sub>Fe(SO<sub>4</sub>)<sub>2</sub>·6H<sub>2</sub>O, and 0.01 mg/L thiamine hydrochloride) was supplemented with <sup>15</sup>NH<sub>4</sub>Cl (1 g/L) for <sup>15</sup>N labeled samples or D-glucose (or D-glucose-<sup>13</sup>C<sub>6</sub>) (4 g/L) and <sup>15</sup>NH<sub>4</sub>Cl (1 g/L) for <sup>15</sup>N/<sup>13</sup>C labeled samples.

2.2.3 Purification of SpoVM constructs

All labeled and unlabelled SpoVM and SpoVM<sup>P9A</sup> cells followed the same purification protocol. Cell pellets were homogenized with 50mM phosphate buffer pH 7.4, 500mM NaCl (lysis buffer). Cells were lysed using French press at 1500 psi (single pass) followed by sonication for 5 sec on/5 sec off intervals for 3 minutes total duration. Cell debris was removed by centrifugation at 15,000xg 10 °C for 20 min. Supernatants were collected and 0.8 % dodecyl maltoside (final concentration) added to solubilize cell membrane and protein. 30mM imidazole was added to the supernatants, which were agitated for 1 hour at room temperature. Detergent treated lysates were applied to columns containing 3.5mL Ni-NTA PerfectPro Agarose (5 Prime). Detergent treated lysate was reapplied to the columns twice to maximize product yield and then the columns were washed with 40mL lysis buffer with the addition of 40 mM imidazole and 0.2% DM. Excess detergent was removed with 5 mL lysis buffer. Proteins were eluted with 20 mM Tris-HCl pH 8.0, 500 mM
NaCl, 500 mM imidazole, 0.4 % DM into tubes prepared with 20 mM Tris pH 8.0, 0.4% DM. Final elution buffer concentration for each column was 20 mM Tris pH 8, 250 mM NaCl, 250 mM imidazole, 0.4% DM.

The N-terminal His<sub>6</sub>-Sumo tag was removed by the addition of H-Ulp1-His<sub>6</sub>-Sumo Protease gift from Dr. Ramamurthi at a 5:1 (protein:protease) mass ratio overnight at 25 °C. The digest was followed by SDS-PAGE until completion. 20 mM Tris pH 8.0 was added to reduce imidazole concentration of the protease reaction from 250 mM imidazole to 50 mM imidazole.

This solution was re-applied to the Ni-NTA column and washed first with 20 mM Tris-HCl pH 8.0, 500 mM NaCl, 80 mM Gdn-HCl, 0.8% DM and then 20 mM Tris-HCl pH 8.0, 2 M NaCl, 80 mM Gdn-HCl, 0.8 % DM. Each wash was incubated 20min at room temp. Wash 1 and 2 were collected and contained SpoVM and SpoVM<sup>P9A</sup>, which did not bind to the column. H-Ulp1-His<sub>6</sub>-Sumo Protease and His<sub>6</sub>-Sumo tag were eluted from the column with 20 mM Tris-HCl pH 8.0, 500 mM imidazole, 500 mM NaCl. SpoVM and SpoVM<sup>P9A</sup> were visualized by SDS-PAGE and prepared in the appropriated buffer by ultracentrifugation using an Amicon Ultra centrifugal filter device 3K MWCO (Millipore) at 4000xg 16°C.

2.2.4 Preparation of bicelles

16% q=0.3 ([DMPC]/[DHPC]) bicelles were prepared by reducing DM concentration below CMC 1.8mM through successive buffer exchange with 16 mM phosphate buffer pH 6.0, 80 mM NaCl (NMR Buffer). Bicelle solutions were prepared by dissolving DHPC in NMR buffer and resuspending DMPC in solution. DMPC suspension underwent at least three dry ice bath freeze thaw cycles until suspension
was homogenous. DHPC was added to DMPC suspension briefly vortexed then freeze-thawed again. Bicelle solution added to protein solution and spun down to a concentration of ~1 mM in 180 μl NMR buffer and 10 μl D₂O. Bicelle concentrations were checked utilizing ³¹P NMR.

2.2.5 DMPC SUV Liposomes

DMPC in chloroform was dried to a thin film by condenser rotor speed vac and further dried by lyophilization overnight. Lipids were re-hydrated and incubated in 40 °C water bath 1 hr with vigorous vortexing every 15 minutes. Rehydrated lipids underwent 5 cycles of freeze-thaw in dry ice bath and a 40 °C water bath. Lipids were sonicated in a pre-warmed water bath sonicator (Branson ultrasonic cleaner) until solution became transparent. Sonicated liposomes were stored at room temperature and usually utilized within 2 days of preparation.

2.2.6 SpoVM peptides

Full length SpoVM and SpoVM²⁹⁷A were synthesized by Genescript at >95% purity. Peptides were lyophilized in order to further remove trace organic solvents. Peptide stock solutions were prepared at a concentration of 1 mg/mL by weighing lyophilized peptide and dissolving the peptide in a 2.5% glacial acetic acid/H₂O solution. Peptides were stored at -20 °C before use.

2.2.7 Preparation of Spherically Supported Bilayers (SSLBs)

50 nm and 1 μm SiO₂ beads were obtained from Cospheric, LLC. DMPC sonicated SUVs were prepared as described above. SSLBs were prepared for DMPC adsorption by a series of wash steps, after each wash step the glass beads were centrifuged in a desktop centrifuge for sedimentation. The glass beads were washed
initially by methanol followed by a series of H₂O washes. The suspended beads were then incubated with prepared DMPC SUVs at 40 °C for 1 hr with the addition of CaCl₂. After incubation DMPC coated glass beads were washed with H₂O and resuspended three times.

2.2.8 SSLB Capture Assay

Using SSLBs, a capture assay was developed using 50 nm, 100 nm, and 1 μm DMPC coated silica beads. Peptide was added to beads at a concentration of 0 - 2 μM. Incubated for 1 hr at 37 °C. Beads were washed twice to remove non-binding peptide. Beads were then visualized on SDS-PAGE with Coomassie stain.

2.2.9 Isothermal Titration Calorimetry (ITC)

All measurements were performed using a MicroCal VP-ITC instrument equipped with a rotator-stirrer-syringe. For each experiment the cell contained 11 μM SpoVM peptide or 7 μM SpoVMP9A, and 30 consecutive injections of 5 μl aliquots of DMPC SUV liposomes at a total lipid concentration of 11.1 mM were added. To minimize the contribution of heat of dilution to the measured heat change, the protein and lipid vesicles were prepared in the same buffer. Injections were made at intervals of 10 minutes and a constant stirring speed of 307 rpm was maintained during the experiment. To account for the heat of dilution due to DMPC liposome injection experiments were performed by injecting DMPC SUV liposomes into the buffer solution in an identical manner and the resulting heat changes subtracted from the measured heats of binding. Buffer for this experiment consisted of 25 mM CH₃COONa (sodium acetate buffer) pH 4, 80 mM NaCl.
The data obtained from calorimetric titrations for SpoVM and SpoVM<sup>P9A</sup> were analyzed using Origin ITC data analysis software provided by the manufacturer using a one-site binding model, which provided the best fit for the data.

2.2.10 Circular Dichroism Spectroscopy

CD spectroscopy was performed on a Jasco 710 J-spectropolarimeter. CD spectra in the far UV (200-250 nm) were recorded at 25 °C using a 0.2 mm path length cuvette. SpoVM and SpoVM<sup>P9A</sup> were analyzed at a concentration of 0.1 mg/mL in 11.25 mM HEPES buffer pH 7.5. Each spectrum was obtained by averaging scans with a scan rate of 50 nm/min and 1 nm interval. The instrument was set at 2 nm bandwidth and 2 sec response time. For each sample, 6 scans were collected and averaged before buffer subtraction. For DMPC liposome titration experiments, scattering limited measurements to wavelengths greater than ≥200 nm.

2.2.11 Differential Scanning Calorimetry (DSC)

DSC measurements were performed using a Microcal VP-DSC calorimeter (MicroCal, Inc.) after the preparation of the samples. A 0.5 mL liposome suspension incorporated with peptide was placed in the sample cell and the same volume of buffer was placed in the reference cell. Samples were heated at a rate of 0.3 °C/min over a temperature range of 10–90 °C and were equilibrated for 15 min at 10°C before each scan. Blank experiments with buffer on both cells were also performed for subsequent baseline correction. The expected experimental errors in temperature and enthalpy values were ± 0.01 °C and ± 5%, respectively. The calorimetric cell was filled with DMPC coated 50 nm SSLBs at concentrations of 738 μM, 1.1 mM, and 1.5 mM (total lipid) in H<sub>2</sub>O. Measurements taken in the presence of
SpoVM peptide were also made using 738 μM DMPC coated 50 nm SSLBs and 5.5 μM (1:2.5 protein:lipid molar ratio (assuming 60 DMPC lipids associated per SpoVM peptide) or 11.1 μM peptide (1:1.1 protein:lipid molar ratio).

Buffer subtraction and baseline correction were performed using Microcal Origin software (MicroCal, Northampton, MA, USA). The DSC profiles were analyzed by decomposition of the main transition peaks into two individual components with Lorenz lineshape and fitted as the sum of the components using the non-linear least square curve fitting procedure supplied with the Origin 8.0 software.

2.2.12 NMR spectroscopy, Backbone Resonance Assignment, Structure Determination, and Dynamics

NMR data were collected on a ~1 mM on $^{15}$N, $^{13}$C labeled SpoVM or SpoVM$^{P9A}$ in 16 mM phosphate buffer pH 6, 80 mM NaCl (NMR buffer) reconstituted in q=0.3 ([DMPC]/[DHPC] 16% bicelle using Bruker 600 or 850 MHz spectrometer equipped with a cryoprobe. All NMR spectra were recorded at 37°C. Backbone resonance assignments were carried out using TROSY-based triple resonance experiments: HNCO, HNCA, HN(CO)CA, HN(CA)CB, and HN(CO)CACB. NMR data were processed using NMRPipe and analyzed using NMRView software. The symmetric H$^N$-H$^N$-NOEs obtained from 3D $^{15}$N-edited NOESY data were used to resolve and validate the connectivities when available. Distance restraints for structure calculations were from 3D $^{15}$N-edited NOESY and 3D $^{13}$C-edited NOESY. The chemical shifts of C$^\alpha$, C$^\beta$, N, and C$'$ were used to predict backbone torsion angles by TALOS.

Initial NOE assignment was performed manually and initial structures generated with the program CYANA 3.0. The final structures were calculated using
XPLOR-NIH. Pro-check-NMR was used to validate the ten lowest energy structures and the statistics for these are listed in Table 2-3.

$^{15}$N-labeled SpoVM and SpoVM$^{P9A}$ samples were used for $^1$H-$^{15}$N backbone relaxation data collection on a 600 MHz Bruker spectrometer equipped with a TCI cryoprobe. Backbone $^{15}$N longitudinal relaxation $T_1$ values were determined from a series of $^1$H-$^{15}$N correlation spectra with 50, 150, 250, 350, 500, 650, 750, 950 and 1300 ms relaxation evolution delays. Backbone $^{15}$N transverse relaxation $T_2$ values were obtained from the spectra with 16.1, 32.3, 48.4, 64.5, 80.7, 96.8 and 1130 ms delays. Steady-state $^1$H-$^{15}$N NOE values were determined from peak ratios observed between two spectra collected with or without a 3 s power presaturation in the proton channel.

2.2.13 Paramagnetic Probe-Induced Line Broadening Experiment

$^1$H-$^{15}$N-TROSY spectra for SpoVM and SpoVM$^{P9A}$ were collected on 600 MHz Bruker spectrometer equipped with a TCI cryoprobe of ~ 1 mM $^{15}$N-labeled SpoVM WT and SpoVM$^{P9A}$ in NMR buffer in 16% q=0.3 [DMPC/DHPC] bicelle. The titrations were either performed with 16-doxylstearic acid (16-DSA) or Gd(III)-diethylenetriaminepentaacetic acid (Gd-DPTA). 16-DSA was titrated over a concentration 0-0.8 mM. Gd-DPTA was added over a concentration range of 0-11 mM to a 1 mM U-$^{15}$N SpoVM & P9A sample.
2.3 Results

2.3.1 Thermodynamic Parameters Governing SpoVM binding to SUVs

Since our current understanding of membrane curvature sensing is built on molecules that recognize highly curved membrane. We first set up to examine if SpoVM is sensitive to highly curved membranes. ITC experiments were performed to determine the binding parameters of SpoVM and SpoVM\textsuperscript{P9A} binding on 60 ± 20 nm DMPC composed liposomes. The titration curves for both peptides (Figure 2-5) displayed typical sigmoidal shapes and for specific protein-ligand interactions and were evaluated assuming independent saturable binding sites in the outer surface of the lipid vesicles [9]. The measured thermodynamic parameters are given in Table 2-1. Both SpoVM and SpoVM\textsuperscript{P9A} binding to the liposomes were enthalpy driven as expected for AH binding to SUVs [10]. SpoVM affinity to liposomes of ~ 60 nm was $K_d$ of 2 μM appeared to be comparable to that observed in a free vesicle system of ≥ 1 μm ($K_d$ ~1 μM) [3]. Interestingly, SpoVM appears to bind these liposomes with weaker affinity than the SpoVM\textsuperscript{P9A} variant $K_d$ of 0.5 μM (with respect to peptides), but the wild-type protein associates with fewer lipid molecules than the variant, 28 vs. 42. Thus there are slightly more “binding sites” for SpoVM than for P9A within a given surface area of lipid vesicles (i.e. larger $B_{max}$). Due to technical reasons ITC analysis of free liposomes > 1 μm was not possible, but the thermodynamic parameters from this ITC analysis suggest SpoVM high curvature vesicles compared to the non-curvature discriminating mutant SpoVM\textsuperscript{P9A}. 
Figure 2-5: SpoVM ITC measurement of the peptide binding to liposomes of ~60 nm. 11 mM liposomes were injected into 11 μM WT and 7 μM P9A peptides with an injection volume of 5 and 7 μl, respectively. Inset thermogram of respective ITC experiment.

<table>
<thead>
<tr>
<th>Protein</th>
<th>n</th>
<th>$K_d$ (μM)</th>
<th>$\Delta G$ (kcal/mol)</th>
<th>$\Delta H$ (kcal/mol)</th>
<th>$\Delta S$ (kcal/mol/k)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SpoVM</td>
<td>28(+0.32)</td>
<td>2.2 (+0.7)</td>
<td>-5.39</td>
<td>0.42(+0.07)</td>
<td>16.5</td>
</tr>
<tr>
<td>SpoVM&lt;sup&gt;P9A&lt;/sup&gt;</td>
<td>42(+0.35)</td>
<td>0.52(+0.1)</td>
<td>-6.01</td>
<td>0.68(+0.01)</td>
<td>17.7</td>
</tr>
</tbody>
</table>

Table 2-1: Thermodynamic Parameters derived from SpoVM and SpoVM<sup>P9A</sup> binding DMPC SUVs

2.3.2 Validation of SSLBs as a model system for slightly curved membranes

To determine the biochemical basis for the preferential adsorption of SpoVM onto slightly convex (positively curved) membrane surfaces model membrane systems with precise curvature is required. The current use of giant unilamellar vesicles (GUVs) presents two primary difficulties. First, is the difficulty of controlling precisely the size and distribution of the vesicles [11]. Second, GUVs are not uniformly stiff, which makes it difficult to separate membrane stiffness effects from strictly geometric effects. Therefore spherical supported lipid bilayers (SSLBs) are being developed for use in membrane curvature studies. SSLBs were first introduced...
in the early 1990’s as a model system for membrane bilayers [12, 13], but have not been utilized for the study of membrane curvature recognition. SSLBs provide a more strictly defined radius of curvature to the membrane (dependent on the diameter of the silica bead), and the use of a supported bilayer system also ensures that the differently curved surfaces would be uniformly stiff.

SSLBs use as a model for membrane bilayers has been validated in that:

- Preserve the characteristic of cell membranes such as lateral fluidity, impermeability to ionic species, and flexibility along the lipid acyl chain. This is due to a thin hydration layer (~20 Å) surrounding the solid support, separating the support from the bilayer [12] (Figure 2-6).
- SSLBs form a single lipid bilayer
- SSLBs have been used in ssNMR experiments and to probe melittin binding to lipids using $^{31}$P NMR [14]

**Figure 2-6**: Representation of SSLBs and water layer between silica support and lipid bilayer.
To ensure SSLBs were coated with a lipid bilayer and suitable for membrane curvature studies differential scanning calorimetry (DSC) was used to measure the phase transition melting temperature ($T_m$) of $50 \pm 5$ nm SSLBs coated with DMPC lipids (Figure 2-7a). Increasing the concentration of coated SSLBs 738 μM to 1.4 mM (total lipid) resulted in an increase in the heat capacity ($C_p$), while the DMPC coated SSLB $T_m$ of $24.98 \pm 0.03^\circ$C closely matches the reported pure DMPC $T_m = 24^\circ$C.

Upon addition of 5.5 μM SpoVM peptide to SSLBs 738 μM a decrease (1:2.25 protein to lipid molar ratio - assuming SpoVM associates with $n = 60$ DMPC lipid molecules) in $T_m = 22.65 \pm 0.02^\circ$C was observed. The insertion of the peptide within the hydro-carbon region of the membrane bilayer is expected to increase the disorder of the surrounding lipids when the system is below the gel-liquid crystal transition temperature. Consequently, the DSC peak of this peptide-membrane system when compared to pure DMPC is expected to lower the $T_m$ and reduce the $C_p$. At near saturating peptide conditions 11.1 μM peptide to 738 μM SSLBs (1:1.1 protein:lipid molar ratio) two peaks are observed the first peak $T_m = 20.04 \pm 0.01^\circ$C and the second peak $T_m = 23.78 \pm 0.02^\circ$C. The first peak can be explained by the increased disorder of the surrounding lipids upon additional peptides, while the second peak is often observed at saturating concentrations of peptide as high concentration of peptides acts to increase the local lipid order adding rigidity to the membrane through cooperative lipid-peptide hydrophobic forces; as a consequence, $T_m$ is expected to increase relative to pure lipid.

CD analysis was further performed using SpoVM peptide in the absence and presence of 50 nm DMPC SSLBs (Figure 2-7b). In the absence of the beads, SpoVM
was unfolded (random coil) as indicated by the minima at 200 nm. Addition of 50 nm DMPC coated beads to SpoVM resulted in the folding of peptide into a structure with α-helical structure as indicated by the minima at 208 nm and maxima at 200 nm. These observations are similar to SpoVM in the presence and absence of DMPC liposomes. Together these experiments validate the use of SSLBs in membrane curvature studies.

Figure 2-7: SpoVM interacts with DMPC coated SSLBs.  a) DSC plots for DMPC coated 50 nm SSLBs alone and with SpoVM peptide (Black) 738 μM DMPC-coated (total lipid) 50 nm SSLBs (Red) 1.1 mM DMPC-coated 50 nm SSLBs (Green) 1.4 mM DMPC-coated 50 nm SSLBs (Blue) 5.5 μM SpoVM peptide incubated with 738 μM DMPC-coated SSLBs (1:2.5 protein:lipid molar ratio (assuming 60 DMPC lipids associated per SpoVM peptide) (Cyan) 11.1 μM SpoVM incubated with 738 μM 50nm DMPC-coated SSLBs (1:1.1 protein:lipid molar ratio) (b) CD spectrum SpoVM in the presence of 50 nm SSLBs (Blue) SpoVM peptide 33 μM (Red) SpoVM peptide 33 μM incubated with 1.4 mM (total lipid) DMPC-coated 50 nm SSLBs.
2.3.3 Preferential adsorption of SpoVM in vitro onto positively curved surfaces

Using SSLBs, our collaborator, Ramamurthi lab at NCI, SSLBs examined the binding parameters of SpoVM-GFP and SpoVM<sup>P9A</sup>-GFP using quantitative fluorescence microscopy. Their data are summarized below. 2 μm and 8 μm silica beads were coated with E. coli polar lipid extract (composed of approximately 67% phosphatidylethanolamine, 23% phosphatidylglycerol, and 10% cardiolipin to produce SSLBs. The ratio of 2 μm and 8 μm SSLBs were adjusted to produce approximately equal total surface area of membrane. Next, the distribution of the fluorescence signal of an increasing concentration of SpoVM-GFP and SpoVM-P9A-GFP with the SSLBs was measured by fluorescence microscopy (Figure 2-8). At the lowest concentrations of SpoVM-GFP, 0.3 μM, SpoVM bound to the surface of 2 μm vesicles while largely absent from 8 μm beads, demonstrating the preferential binding to SUVs as seen in previous studies of SSLBs coated entirely with phosphatidylcholine. As the concentration of SpoVM-GFP increased the fluorescence intensity increased on both beads, but more rapidly on the 2 μm beads. The protein bound approximately equally well at approximately 13 μM for both 2 μm and 8 μm SpoVM<sup>P9A</sup>-GFP, in contrast, bound to both beads at nearly equal distribution even at the lowest concentration of protein tested, suggesting that SpoVM<sup>P9A</sup>-GFP did not discriminate between differently curved surfaces in vitro.

To obtain the parameters for SpoVM-GFP and SpoVM<sup>P9A</sup>-GFP adsorption onto SSLBs, a saturation-binding curve was generated and fitted to an allosteric sigmoidal model. The parameters (Table 2-2) revealed differences in the parameters that described the adsorption of SpoVM-GFP onto 2 μm or 8 μm SSLBs. Examining $B_{max}$
values for SpoVM onto either size of SSLB, the values were not appreciably different, suggesting the number of binding sites per square micron on both types of surfaces for SpoVM-GFP were roughly similar and therefore did not contribute to the preferential binding of SpoVM-GFP onto smaller beads. In contrast, examining the Hill coefficient for SpoVM-GFP binding onto 8 μm beads was 1.1 (± 0.1) suggesting little or no cooperativity, whereas binding onto 2 μm beads suggests a slight increase in cooperativity among SpoVM-GFP molecules as seen by the modestly higher Hill coefficient of 1.7 (± 0.2). The binding curve also indicated that SpoVM-GFP displayed an approximately three-fold higher affinity for more convex surfaces as seen by a difference in the half-maximal concentration of SpoVM-GFP that adsorbed onto the differently curved beads (K_{half} = 1.18 μM ± 0.31 for 2 μm beads; K_{half} = 3.64 ± 0.52 for 8 μm beads). Conversely, SpoVM^{P9A} -GFP consistent with its inability to discriminate between the two differently curved surfaces in vitro; there were no significant differences in adsorption onto either 2 μm or 8 μm beads. In comparing SpoVM-GFP and SpoVM^{P9A}-GFP, the P9A variant appears to bind with an almost-two-fold higher intrinsic affinity to membranes in general than SpoVM-GFP towards 2 μm beads, and also occupied a slightly higher number of binding sites on the membrane.

Taken together, the data suggests that SpoVM preferentially inserts into slightly curved membranes by a small increase in binding affinity and cooperativity. In contrast, SpoVM^{P9A} largely did not discriminate between differently curved surfaces, and did not display appreciable cooperativity in membrane adsorption. Since previous studies [7, 14] describing the insertion of amphipathic helices into highly curved membrane surfaces reported a B_{max} -driven (increased number of
binding sites) mechanism by which smaller vesicles are preferentially recognized, the adsorption behavior of SpoVM onto slightly curved membrane surfaces (driven by increases in affinity and cooperativity) represents a fundamentally different mechanism of membrane curvature discrimination.

Figure 2-8: SpoVM-GFP preferential adsorbs onto 2 μm DMPC coated SSLBs, SpoVM$^{P9A}$ does not discriminate between positive curved surfaces in vitro. (Top panels) Confocal fluorescence micrographs of increasing concentrations of purified VM-GFP or SpoVM$^{P9A}$-GFP incubated with 2 μm and 8 μm SSLBs. (Bottom panels) Differential Interference Contrast (DIC) micrographs of SpoVM-GFP and SpoVM$^{P9A}$-GFP incubated with 2 μm and 8 μm SSLBs. (Unpublished data provided by Ramamurthi lab)
Table 2-2: Parameters for the allosteric sigmoidal fit for SpoVM-GFP and SpoVMP9A-GFP adsorption onto differently curved surfaces: $B_{\text{max}}$, maximal binding value; h, Hill coefficient; $K_{\text{half}}$, concentration of protein producing half maximal binding. Errors are SEM. (Unpublished data provided by Ramamurthi lab)

<table>
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<tr>
<th>Protein</th>
<th>Bead diam. (μm)</th>
<th>2</th>
<th>8</th>
<th>2</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>$B_{\text{max}}$ (units/μm$^2$)</td>
<td>143125 (±4487)</td>
<td>139618 (±12491)</td>
<td>167977 (±6014)</td>
<td>197454 (±7520)</td>
<td></td>
</tr>
<tr>
<td>h</td>
<td>1.7 (±0.2)</td>
<td>1.1 (±0.1)</td>
<td>1.2 (±0.2)</td>
<td>1.4 (±0.2)</td>
<td></td>
</tr>
<tr>
<td>$K_{\text{half}}$ (μM)</td>
<td>1.18 (±0.31)</td>
<td>3.64 (±0.52)</td>
<td>0.56 (±0.14)</td>
<td>0.77 (±0.23)</td>
<td></td>
</tr>
</tbody>
</table>

2.3.4 SSLB capture at 50 nm and 1 μm further supports SpoVM uses novel mechanism

To further investigate if the $B_{\text{max}}$ parameter plays a role in curvature recognition an SSLB capture assay was utilized in which 1 μm (shallow) and 50 nm (high) curvature vesicles coated with DMPC (at a fix total lipid concentration of 40 μM) were titrated with peptide until saturation. Surprisingly, 50 nm and 1 μm beads captured similar amount of peptides at saturation concentration (Figure 2-9). If $B_{\text{max}}$ were the governing parameter for SpoVM curvature recognition it would be expected that at saturation more peptides should be captured by 50 nm SSLBS. This result further supports SpoVM curvature recognition was not driven by $B_{\text{max}}$. 
2.3.5 Secondary Structural Analysis

Using circular dichroism spectroscopy, we examined SpoVM and SpoVM\textsuperscript{P9A} binding to vesicles composed of saturated cylindrical DMPC vesicles and DPC micelles. As shown in Figure 2-10, both WT and P9A are unfolded in aqueous solution (random coil). Upon addition of micelles or liposomes, the spectra change to that more characteristic of a \(\alpha\)-helix with minima at 208 nm and 222 nm present in both DPC micelles and DMPC liposomes. Interestingly, the CD spectra indicated P9A had more helical content than the WT peptide as indicated by the greater minima at 222 nm.
Figure 2-10: CD spectra of (left) SpoVM & P9A mutant

A structural difference between SpoVM and SpoVM$^{P9A}$ is further evidenced by extensive and large chemical shift perturbations of SpoVM$^{P9A}$ in comparison with that of SpoVM, shown in Figure 2-11. Thus we pursued NMR structures for both SpoVM and SpoVM. 
Figure 2-11: Assigned $^1$H-$^{15}$N TROSY-HSQC spectra of SpoVM and SpoVM$_{P9A}$. Overlay of $^1$H-$^{15}$N TROSY spectra of SpoVM (red) and SpoVM$_{P9A}$ (blue). Resonance assignments for SpoVM residues 3 to 26 are labeled red. For SpoVM$_{P9A}$ only most shifted resonances for residues 3 to 14 are labeled blue.

We obtained backbone resonance assignments for both SpoVM and SpoVM$_{P9A}$ using the TALOS program, secondary structures were predicted from backbone $^1$H$_\alpha$ and $^{13}$C$_\alpha$ chemical shifts. Shown in Figure 2-12 are secondary shifts for $^1$H$_\alpha$Δδ and $^{13}$C$_\alpha$Δδ for P9A. A negative value indicates propensity for α-helical structure, as does a positive $^{13}$C$_\alpha$ [15]. Clearly, residues 3-20 form an α-helix.
2.3.6 3D structure

Structural determination was done using standard techniques. A total of 365 NOE distance constraints and 16 dihedral angle constraints were used to define the final NMR ensemble (Figure 2-13), the r.m.s.d. values of all structures was 0.3 Å in regions of secondary structure. Ramachandran statistics and other validation
criteria listed in Table 2-3 indicate that the determination of structure is of high quality.

<table>
<thead>
<tr>
<th>NMR constraints</th>
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<th>SpoVM$^{\text{P9A}}$</th>
</tr>
</thead>
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<tr>
<td>NOE distances</td>
<td>391</td>
<td>365</td>
</tr>
<tr>
<td>intra-residue</td>
<td>96</td>
<td>100</td>
</tr>
<tr>
<td>sequential (</td>
<td>i-j</td>
<td>= 1)</td>
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<tr>
<td>medium range (2 ≤</td>
<td>i-j</td>
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<td>long range (</td>
<td>i-j</td>
<td>≥ 5)</td>
</tr>
<tr>
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</tr>
<tr>
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<td>10</td>
<td>16</td>
</tr>
<tr>
<td>psi</td>
<td>10</td>
<td>16</td>
</tr>
</tbody>
</table>

| Structure Statistics                 |       |                      |
| RMSD from the average atomic coordinates* | 0.2   | 0.3                  |
| backbone (Å)                         | 0.8   | 0.7                  |
| Ramachandran analysis*              |       |                      |
| most favored                         | 90%   | 99.4%                |
| additionally allowed                 | 10%   | 0.8%                 |
| generally allowed                    | 0.0   | 0.0                  |
| disallowed                           | 0.0   | 0.0                  |
| Violations (mean ± s.d)              |       |                      |
| distance constraints (Å)             | 0.075 ± 0.004 | 0.070 ± 0.004        |
| dihedral angle constraints (°)       | 0.721 ± 0.144 | 0.307 ± 0.170        |
| Deviations from idealized geometry   |       |                      |
| bond lengths (Å)                     | 0.009 ± 0.000 | 0.008 ± 0.000        |
| bond angles (°)                      | 0.336 ± 0.019 | 0.720 ± 0.021        |
| improper (°)                         | 0.525 ± 0.045 | 0.449 ± 0.033        |

*Evaluated by ProCheck for ordered residues, 11-23 SpoVM and 3-23 of SpoVM$^{\text{P9A}}$.

Table 2-3: NMR constraints and structure statistics for SpoVM and P9A variant.

The structure of SpoVM$^{\text{P9A}}$ consists of a long α-helix (F3-F23) of 5.8 turns flanked by the N-terminal (residues 1,2) and C-terminal region (residues 24-26), which are unstructured. The surface of the SpoVM$^{\text{P9A}}$ helix has a clear boundary between the polar and hydrophobic faces typical for an amphipathic helix.
Figure 2-13: SpoVM\textsuperscript{P9A} structure long straight amphipathic helix. (Top) Overlay of 10 lowest energy structures SpoVM\textsuperscript{P9A}. (Bottom) The hydrophobicity surface preset shows amino acid hydrophobicity in the Kyte-Doolittle scale with colors ranging from blue for the most hydrophilic to white at 0.0 to orange red for the most hydrophobic.

The structure of SpoVM in isotropic bicelles has also been determined. It is comprised of a short loop (residues 5-8) followed by a short 3.6 turn helix (residues 11-23) (Figure 2-14a). As reported by NMR relaxation measurements (Figure 2-14c) the loop is flexible, in contrast to SpoVM\textsuperscript{P9A} which has increased backbone steady-state \textsuperscript{1}H-\textsuperscript{15}N NOEs for the same residues.

SpoVM helix is not a typical amphipathic helix: its hydrophobic face is wide (~2/3 of the helix surface) and the hydrophilic side contains only one positively charged residue, arginine 17, and three glycines. On the other hand the substitution of the known helix breaker proline with alanine at residue 9 results in a straight
amphipathic helix. This result is consistent with the CD spectrum of SpoVM<sup>P9A</sup> showing higher helical content than SpoVM. One result of this structural change is that the number of positively charged residues at the polar face of the helix increases from one in SpoVM to three in SpoVM<sup>P9A</sup> (Figure 2-14a and 2-14b), raising the possibility of increased electrostatic interactions between SpoVM<sup>P9A</sup> and phospholipid head groups. It is plausible that this possible increase in electrostatic interaction may disrupt the delicate balance between electrostatic and hydrophobic interactions that recognition of highly curved membrane relies upon as suggest by previous studies [16] [17] [18].
Figure 2-14: SpoVM and SpoVM\textsuperscript{P9A} distinct structures. (a) and (b) SpoVM and SpoVM\textsuperscript{P9A} structures in isotropic DMPC/DHPC bicelles (q=0.3) determined by solution NMR and the helical wheel plots of their \(\alpha\)-helices, respectively. Gray: non-polar residues; green: glycines and polar residues; blue: positively charged residues, red: negatively charged residues. (c) \(^{1}H\text{-}^{15}N\) NOE SpoVM (Blue) and SpoVM\textsuperscript{P9A} (Red).
2.3.7 *SpoVM* and *SpoVM*<sup>P9A</sup> topology by PRE

For membrane proteins, their membrane insertion depth and orientation are also important. Thus they are determined in bicelles using NMR paramagnetic relaxation enhancement (PRE) from a water soluble probe Gd(DTPA). In this experiment, residues accessible by the probe molecule at the membrane surface experience large PRE effects while residues embedded in the membrane will have small PREs.

As shown in Figure 2-15, for SpoVM, residues at the N- and C-terminus show profound PREs, while residues 15 to 22 in the helix have small perturbations. The small perturbations of these residues are indicative of the helix being deeply inserted into the membrane. The estimated insertion depth for residues M19 and L20 is more than 10 Å into the membrane assuming residue K25 is located close to the bilayer surface assuming Gd(DPTA) is inert toward the protein and freely diffusing in solution. The small perturbations of these residues are indicative of the helix being deeply inserted into the membrane. In addition, the uniformity of small PRE effects indicates the helical residues 15-22 are oriented approximately parallel to the plane of the membrane. PRE experiments were also performed on *SpoVM*<sup>P9A</sup>. The *SpoVM*<sup>P9A</sup> C-terminus displayed similar PRE perturbations to that of SpoVM helix, suggesting a comparable depth and orientation of the C-terminus. The N-terminus, however, experienced a large PRE broadening, wherein the N-terminal part of *SpoVM* P9 helix became solvent accessible and likely interacted non-specifically with the Gd(DPTA) probe through Lys2 and Lys7, thereby precluding a quantitative comparison of membrane insertion depth between SpoVM and *SpoVM*<sup>P9A</sup>.
Nevertheless, the orientation of SpoVM and SpoVM^{P9A} parallel to the plane of the membrane can be concluded, and that compared to the reported surface association of amphipathic helices that sense highly curved membranes, SpoVM, is instead deeply inserted into the membrane bilayer.

Figure 2-15: PRE effects on a) SpoVM with 0.5 mM Gd(DTPA). b) SpoVM^{P9A} with 0.5 mM Gd(DTPA).
c) SpoVM$^{\text{P9A}}$ with 1.5 mM Gd(DTPA).

Molecular dynamic simulations by our collaborators (Huang lab at Stanford) using our NMR structures for SpoVM and SpoVM$^{\text{P9A}}$ were conducted using long-timescale molecular dynamic simulations on two protein-membrane systems (Figure 2-16) in the both simulations, SpoVM and SpoVM$^{\text{P9A}}$ remained associated with the membrane and were submerged below the headgroups of the top bilayer (Figure 2-16A-B) consistent with the results indicated by the PRE measurements. Furthermore, the molecular dynamic simulations confirm that SpoVM and SpoVM$^{\text{P9A}}$ amphipathic helices are oriented parallel to the plane of the membrane.
Figure 2-16: Molecular dynamics simulations of (A) SpoVM and (B) SpoVM^{P9A}. (Unpublished data from Huang lab)
2.4 Discussion

The aim of this study was to provide insight into the mechanism of membrane curvature recognition of SpoVM using a biochemical and structural approach. Our initial experiments examined the binding parameters governing SpoVM preferential adsorption onto slightly convex surfaces. Ramamurthi et al. previously reported that preferential binding resulted from SpoVM cooperatively binding to membranes with a radius of curvature of \( \sim 1 \mu m \), but not to membranes with a radius of curvature larger than \( 4 \mu m \). However, due to experimental difficulties, one key question was left unanswered: does the number of peptide “binding sites” for a given membrane surface area, \( B_{max} \), depend on the size of lipid vesicles? \( B_{max} \) is a critical parameter for protein-lipid interaction. As discovered recently, it was \( B_{max} \) as opposed to the binding affinity that is responsible for the sensing of highly curved membranes by several peptides.

SSLBs were developed and validated to determine the binding thermodynamic parameters. CD, DSC, and SSLB capture assay showed that SSLBs were suitable membrane mimics. Using 2 \( \mu m \) and 8 \( \mu m \) SSLBs, Ramamurthi lab generated a saturation binding-curve with GFP-labeled SpoVM and SpoVM\( ^{P9A} \). The study suggested that \( B_{max} \) values were similar for SpoVM between the differently curved vesicles, but showed a small increase in cooperativity and three-fold increase in affinity for more convex surfaces. In contrast, functionally impaired P9A mutant did not show any significant difference in these parameters. The \( B_{max} \) parameter was further evaluated using 50 nm and 1 \( \mu m \) SSLBs in a preliminary SSLB Capture Assay. Surprisingly, with fixed total concentration of lipids, each bead captures similar
amount of peptides further supporting the conclusion that SpoVM preferential curvature recognition is not $B_{max}$ driven. Taken together, the results suggest SpoVM preferentially inserts into slightly curved membranes by a cooperative mechanism and by an increased intrinsic affinity for positively curved surfaces, and that this preferential insertion did not depend on an increase in the number of binding sites on more convex surfaces. The adsorption behavior of SpoVM onto slightly curved membranes represents a fundamentally different mechanism of membrane curvature discrimination.

This conclusion is further supported by our structural analysis of SpoVM and SpoVM$^{P9A}$. We found that SpoVM does not form a long amphipathic helix as predicted, but instead has a flexible N-terminal loop (residues 5-8) with a short amphipathic helix. The structure of SpoVM$^{P9A}$, however, consists of a long amphipathic helix. Furthermore, the topology of SpoVM and SpoVM$^{P9A}$ indicate they are likely deeply embedded into the membrane. This is very different from the membrane topology suggested for those molecules sensitive to highly curved membrane.

While we still do not fully understand the molecular details of SpoVM membrane curvature recognition, we propose a working model that may provide explanation to the origin of cooperativity. Our experimental data suggests that the number of lipids associated with each peptide, ~30, strongly indicates a possible membrane mediated, or even direct, SpoVM-SpoVM interaction. A simple analysis provides a starting point for thinking about this system: assuming SpoVM binding sites are evenly distributed on the surface of lipid vesicles and each SpoVM molecule
covers a surface area of ~400 Å (~7 lipids), at ~30 lipids per peptide, SpoVM molecules are close enough to indirectly or/and directly interact with each other (Figure 2-17). For instance, they could form a head-to-head oligomer using the N-terminal loop, which harbors several residues critical for SpoVM localization.

Figure 2-17: SpoVM molecules are within distance of each other to support direct or indirect interactions. This model assumes that SpoVM binding sites are evenly distributed and each peptide (dark blue) is associated with ~30 lipids (light blue).

We propose SpoVM molecules may form high order structures in the membranes and these high order structures are responsible for SpoVM membrane curvature sensing. Many membrane proteins form (transient) high-order structures for their biological function. For instance, Rhodopsin self-associates in the membrane [19] and, as reported by Cui et al., F-BAR and N-BAR domain containing proteins self-organize into lattices that induce membrane tube formation [20]. Recent experimental and computational studies support the notion that membrane curvature-mediated attraction could complement specific interactions for protein
self-associations [21-23].

2.5 Literature Cited


Chapter 3: Membrane Deformation by a Membrane Proximal Helix in the Cytosolic Domain of the Human APP Interacting Protein LR11/SorLA

3.1 Introduction

3.1.1 Alzheimer’s Disease and Intracellular Trafficking

Alzheimer’s Disease (AD) is the leading cause of dementia, and currently affects more than 36 million people worldwide. According to the Amyloid Hypothesis, accumulation of (Aβ) peptide in the brain is the primary cause of AD [1-3]. As illustrated in Figure 3-1, Aβ peptide is produced by the sequential cleavage of the amyloid precursor protein (APP) by β- and γ-secretase, respectively.

Figure 3-1: Sequential proteolytic processing of APP by β- and γ-secretase generates Aβ40/42 peptide.
Although the generation of Aβ peptide is well understood, the underlying cause of Aβ overproduction is more complex [4]. Only approximately 1% of AD cases can be directly attributed to genetic factors (familial AD) whereas the cause of sporadic AD (late onset) remains unknown. Recent studies have established a link between aberrant subcellular trafficking and localization of APP with increased Aβ production [4-7]. In Figure 3-2, non-amyloidogenic (non-plaque forming) processing by α-secretase and γ-secretase primarily occurs at the cell surface and produces the neurotrophic factor sAPPα whereas the amyloidogenic (plaque forming) pathway occurs mainly in the late endosomal stage by β- and γ- secretase, respectively [8-10]. Aberrant subcellular trafficking has been linked to abnormal levels of Aβ and alterations in intracellular trafficking protein/adaptors lead to Aβ accumulation [11-13]. Variants associated with endocytosis and retromer sorting pathways have been identified as potential AD risk factors [14]. Thus it appears that the spatial and temporal subcellular localization of APP is critical for AD development.
Figure 3-2: Intracellular trafficking of APP and proteolytic processing. 1) Non-amyloidogenic pathway: APP brought to the cell surface through the constitutive exocytosis pathway where it is sequentially cleaved by $\alpha$-secretase and $\gamma$-secretase to produce neurotrophic factor sAPP$\alpha$ (approximately 90% of APP cleaved at surface) [6] 2) Amyloidogenic pathway: non-processed APP is endocytosed by the clathrin-mediated endocytic pathway where it is subsequently cleaved by $\beta$-secretase and $\gamma$-secretase to produce A$\beta$ peptide.
3.1.2 LR11 intracellular trafficking and AD

LR11/SorLA, a key intracellular receptor of APP, has been shown to be a critical regulator of APP trafficking and localization [15-17]. The importance of LR11 in the pathophysiology of AD is highlighted by the observations of poor LR11 expression in the brain of patients suffering from sporadic AD [18,19]. Comprehensive meta-analysis of studies on SORL1 (LR11 gene) SNPs, including approximately 12,000 cases of AD and 17,000 controls, substantiated the involvement of the SORL1 gene variants in AD and further suggested multiple causative gene variants in distinct regions of SORL1 [20]. Recently a study described a significant association between lower cerebrospinal fluid Aβ levels and specific SORL1 SNPs found in AD patients [21]. Thus LR11 has been recognized as a major genetic risk factor.

LR11 is a 250 kDa, highly conserved type-1 transmembrane receptor that is predominately expressed in the neurons of the cortex and hippocampus, regions of the brain that are associated with memory [22]. LR11 contains a vacuolar protein sorting 10 protein (Vps10p) homology domain, β-propeller and epidermal growth factor (EGF) domains, a cluster of 11 complement-type repeat domains, six fibronectin type III repeats, a single transmembrane domain (TM), and a cytoplasmic domain (CT) [23,24], shown in Figure 3-3.
Current model suggests that LR11 regulates APP trafficking between the trans-Golgi Network (TGN) and early endosomes by sequestering APP in the TGN, and consequently reduces the amount of APP that can be processed to Aβ and other products in post-Golgi compartments and at the cell surface as indicated in Figure 3-4 [25]. In addition, LR11 also shuttles APP from early endosomes back to the TGN, further reducing the amount of APP in late endosomes where most Aβ peptides are produced [27,28]. These regulatory roles of LR11 require its proper localization to the TGN, which is critically dependent on multiple motifs in its CT and the interactions of these motifs with cytosolic adaptor proteins [29].
Figure 3-4: LR11 sequesters APP within Golgi apparatus, thereby reducing Non- and Amyloidogenic processing of APP. LR11 also reduces proteolytic processing of APP through the late endosome by transporting APP back to the Golgi apparatus. This process is mediated through interaction with cytosolic factors such as GGA1 (anterograde) and PACS-1 (retrograde).

These cytosolic adaptor proteins include: PACS1, AP1, GGAs, and the retromer complex. They mediate the shuttling of LR11 through the TGN and endosomes by interacting with specific motifs in the cytoplasmic domain (Figure 3-5) [30]. For
example, the acidic cluster-based motif (DEDLGEDDED) in LR11-CT binds to PACS1, which mediates retrograde and anterograde Golgi-to-endosome transport [31]. The GGA family of monomeric-clathrin adaptors GGA1-3 and AP1 bind to the acidic-dileucine-like motif (DDVPMVIA) element and are required for the shuttling of LR11-APP between endosomal vesicles and the Golgi apparatus [32,33]. Lastly, the retromer complex through the VPS26 subunit binds to the FANSHY motif and facilitate retrograde transport of LR11 from the early endosome to Golgi [34]. The importance of these interactions between the LR11 cytosolic domain and cytosolic adaptors have been supported by the fact that mutations in these binding motifs lead to the aberrant sorting of LR11 and APP and enhanced processing of APP [31-34].

KHRLQSSFTA FANSHYSSLGSAIFSSG DDLGEDDED APMITGFS DDVPMVIA

Figure 3-5: LR11 CT putative cytosolic adaptor binding sites. Red: nuclear localization signal, Green: PACS-1 binding site, Blue: GGA binding site/AP-1 binding site, Purple: retromer complex VPS26 subunit binding site.

In this study, we designed a strategy that successfully expressed and purified LR11-CT in a membrane-mimicking environment. We found LR11-CT strongly interacts with the lipid membrane and further identify the interacting region as an amphipathic helix. Using CD, ITC, EM, and DLS we show that this α-helical region binds to vesicles mimicking lipid composition of the Golgi membrane, and furthermore it deforms liposomes. We suggest that this helix may play an active role in remodeling membrane structure during vesicular trafficking and facilitate the transport of LR11 between subcellular compartments.
3.2 Experimental Procedures

3.2.1 Cloning of LR11-CT

LR11-CT refers to residues 2161-2214 of the membrane protein LR11. The plasmid pTBMBP (His₆-MBP-TEV cleavage site-MCS1), which encodes amino acid residues 2161-2214 of LR11, was a kind gift from Dr. Cross’s lab. pLR11-CT was obtained by PCR using LR11-LR11-TMCT-his6 plasmid and the following primers:

Primer 1: 5’-GCACCTAGTTATATCGATACGAATAAC-3’
Primer 2: 5’-ACCAAGATCTTTACGCAATCACCATCGGCA-3’

The PCR product was inserted into the BamHI/HindIII site of the pTBMBP vector to create pLR11-CT and transformed into DH5α E. coli cells. The correct sequence of the insert was confirmed by DNA sequencing.

For recombinant LR11-CT protein two additional residues at the N-terminal region are derived from the TEV protease cleavage site. A mutation, HQ4, was not corrected in the primary sequence of the recombinant LR11-CT due to the proximity of mutation to the membrane proximal region of LR11-CT.

LR11-CT primary sequence:

S₁AKQRRLQSSFTAFANSHYSSRLGSAIFSSGDDLGEDDEDAPMITGFSDDVPMVIA˚⁵⁶

3.2.2 Expression of unlabeled LR11-CT

pLR11-CT was transformed into E. coli BL21 CodonPlus (DE3) RIPL competent cells (Stratagene) for protein expression and freezer stocks made. Starter cultures were grown overnight at 37 °C from glycerol stocks in 5 mL of LB media supplemented with 100 μg/mL ampicillin and 25 μg/mL chloramphenicol. Typically, recombinant protein was produced by inoculating 250 mL M9 media with 1:50 LB
starter culture and inducing at OD\(_{600}\) 0.6-0.9 with 1 mM isopropyl \(\beta\)-D-1 thiogalactopyranoside (IPTG) at 16 °C for ~27 hours. Cells were harvested by centrifugation and stored the pellets at −80 °C until use.

### 3.2.3 Expression of isotopically labeled LR11-CT

For the production of labeled proteins, M9 media (3 g/L KH\(_2\)PO\(_4\), 6 g/L Na\(_2\)HPO\(_4\), 0.5 g/L NaCl, 0.2 mM MgSO\(_4\), 7 mg/L (NH\(_4\))\(_2\)Fe(SO\(_4\))\(_2\)-6H\(_2\)O, and 0.01 mg/L thiamine hydrochloride) was supplemented with \(^{15}\)NH\(_4\)Cl (1 g/L) for \(^{15}\)N labelled samples or D-glucose (or D-glucose-\(^{13}\)C\(_6\)) (4 g/L) and \(^{15}\)NH\(_4\)Cl (1 g/L) for \(^{15}\)N/\(^{13}\)C labeled samples and induced with IPTG, as for unlabeled protein.

### 3.2.4 Expression of GGA1 GST-VHS

The expression plasmid pGGA1 GST-VHS (corresponding to residues 1–147 of human GGA1) was obtained and the recombinant plasmid was transformed into BL21 (DE3) Gold (Stratagene). Cells were grown overnight at 37 °C in LB media supplemented with 100 μg/mL ampicillin and stored at -80 °C until further use.

### 3.2.5 Purification of the LR11-CT/VHS binary complex

Cell pellets of either unlabelled or isotopically labeled His\(_6\)-MBP-LR11-CT and unlabelled GGA1-GST-VHS were resuspended in 25 mM phosphate buffer pH 7.4, 500 mM NaCl (Lysis Buffer), containing Roche EDTA free protease inhibitors and combined to give a single solution. This was lysed by sonication for 10 sec on/10 sec off cycles for 6 minutes. Sonicated lysate was further treated with egg white lysozyme (20 μg per mL lysate/Fisher Scientific) and benzonase (250 U/L Novagen) for 30 min at room temperature prior to centrifugation at 15,000xg 10 °C for 30 min.
The supernatant was collected and applied to a Ni-NTA agarose (5 Prime) column pre-equilibrated with lysis buffer at 4 °C and incubated on column for 30 min. Columns were washed with 20 column volumes of lysis buffer supplemented with 20 mM imidazole and eluted in a step-gradient with lysis buffer containing 500 mM imidazole. Fractions containing the eluted protein were identified using SDS-PAGE. His6-MBP was cleaved from the complex by the addition of TEV protease at a 1:5 (protease:protein) ratio. Optimal digestion was determined by visualization of 5 μL aliquots of protein using Coomassie stained SDS-PAGE. Free His6-MBP and TEV protease were removed from the mixture by application to a GST affinity column (GE Healthcare). GST-VHS:LR11-CT complex bound to the column allowing cleaved His6-MBP to pass through the column. The GST column was washed with 10 column volumes of 5mM reduced glutathione and the GST-VHS:LR11-CT complex eluted with 20mM reduced glutathione. Fractions containing GST-VHS:LR11-CT were identified through SDS-PAGE and pooled for further purification.

The GST tag was removed by thrombin digestion at a 1:10 (protease:protein) ratio and followed by SDS-PAGE. When the digestion was complete this solution was concentrated in ultrafiltration devices (Millipore Amicon 20 mL MWCO 3000 kDa) so that it has a volume of <1 mL. The LR11-CT:VHS complex was further purified by SEC to remove the GST tag and other impurities. The concentrated digest was loaded onto a S-75 16/60 superdex column (GE Healthcare) pre-equilibrated with lysis buffer and run at a rate of 0.4 mL/min at 25 °C. Fractions containing LR11-CT: VHS complex were identified by SDS-PAGE, pooled, concentrated to ~1 mg/mL, and stored at 4 °C until needed.
3.2.6 Preparation of bicelles

8% [DMPC]/[DHPC] q = 3 and q = 0.5 bicelles were prepared by dissolving DHPC in 16 mM phosphate buffer pH 6.0 in 80 mM NaCl (NMR buffer 7) and keeping solution on ice. DMPC was prepared by suspension of DMPC powder in NMR buffer 7 and placing the DMPC suspension in a CO₂ dry ice bath for freezing, followed by thawing the suspension in a 37 °C water bath and 15 secs of vortexing after thaw. Five cycles of freeze-thaw were performed, after final thaw cycle, DMPC suspension solution added to DHPC solution and mixed by brief vortexing. DMPC/DHPC mixture underwent three freeze-thaw cycles followed by 5 secs of vortexing. After final freeze-thaw cycle, bicelle preparation kept on ice and pipetted into NMR tube kept on ice and combined with concentrated protein.

3.2.7 TGN-mimetic Liposome Preparation

Egg PC, DOPE, 1,2-DOG, and brain PS in chloroform was purchased from Avanti Polar Lipids. Liposomes were produced by water bath sonication. A dried film of the lipid was prepared by evaporation of a mixture of the selected lipids in chloroform, and resuspended in water and allowed to hydrate at 42 °C for 1 hr. After five steps of freezing and thawing in a dry ice bath, the liposome suspension was sonicated at 42 °C in a water bath until transparent. Liposomes used for dynamic light scattering and CD measurements were extruded through polycarbonate filters using a hand extruder (Avanti Polar Lipids) sequentially (pore size) – 1, 0.2, 0.1, 0.05, and 0.03 µm. Remaining debris was removed by centrifugation at room temperature at 14,000xg for 10min. The supernatant contained the liposomes and it was harvested and liposomes were stored at room temperature. All liposome
preparations were used within 3 days of preparation. TGN-mimetic liposomes composition was (mol%) egg PC (50), DOPE (30) 1,2-DOG (10) and Brain PS (10) [36,37].

3.2.8 LR11-CTDD peptide

The LR11-CTDD peptide corresponding to residues 2161-2191 (residues K3-D33 of the recombinant LR11-CT) of the full-length protein was synthesized by Genscript (>95% purity) and lyophilized to remove trace organic solvents. The stock solutions were prepared at a concentration of 1 mg/mL in a 2.5% glacial acetic acid/H2O solution and stored at -20 °C until required.

3.2.9 NMR measurements

Samples of the binary complex of isotopically labelled LR11-CT and unlabelled GGA1-VHS domain were prepared by buffer exchange of the purified protein, described above, in 16 mM phosphate buffer pH 6.0, 80 mM NaCl (NMR buffer 7) prepared with 10% D2O. All experiments were conducted at 37 °C on a Bruker 600 MHz equipped with a TCI cryoprobe. 1H-15N TROSY spectra were recorded for 15N LR11-CT:VHS alone or in the presence of either q = 3 [DMPC/DHPC] 8% bicelles or q = 0.3 [DMPC/DHPC] bicelles. NMR spectra were processed by NMRPipe and analyzed by NMRView.

3.2.10 Circular Dichroism Spectroscopy

CD spectroscopy was performed on a 710J-spectropolarimeter (Jasco Inc). Spectra were recorded at Far UV (200-250nm) in a 0.02 cm optical path length cuvette. LR11-CTDD peptide was monitored at a concentration of 0.1 mg/mL. Experiments were performed in 75 mM Tris-HCl buffer pH 7.5 buffer at 25 °C. Each
spectrum was obtained by averaging scans with a scan rate of 50 nm/min and 1 nm interval. The instrument was set at 2 nm bandwidth and 2 sec response time. For each sample, 6 scans were collected and averaged before buffer subtraction. For DMPC liposome and TGN-mimetic liposome titration experiments, scattering limited measurements to wavelengths greater than ≥ 200 nm.

3.2.11 Dynamic Light Scattering (DLS)

Scattering data were collected at 25 °C with a Viscotek 802 Dynamic Light Scattering instrument (Malvern Instruments). Free and protein-bound vesicle solutions were illuminated and the photons that were scattered by the vesicles were collected at 90° on 1-5s acquisition times (depending on protein concentration). Translational diffusion coefficients (D) were determined from scattering data with the DYNAMICS autocorrelation analysis software (version 5.25.44; Protein Solutions). D was converted to a hydrodynamic radius \( R_H \) using the Stokes-Einstein equation \( R_H = kT/\pi\eta 6D \), where \( \eta \) is the solvent viscosity, \( k \) is the Boltzmann constant, and \( T \) is the temperature).

3.2.12 Electron Microscopy

Samples of 2mM sonicated TGN-mimetic liposomes were prepared in buffer. The LR11CTDD peptide was introduced at 87 μM. Negatively stained grids were prepared by applying 3 μl drops of 2% PTA pH 7.0 to copper-coated Formvar 400-mesh copper grids, blotting away excess sample after 2 min. A small drop of 1% (w/v) PTA pH 7.0 was applied to the grid and excess liquid removed with Whatman filter paper. This wash step was repeated two additional times. Micrographs were
taken at initial magnification of 28,500 with a Jeol 1400 transmission electron microscope (Phillips) operating at 80 kV.

3.3 Results

3.3.1 Protein Expression of LR11-CT

Initial attempts to purify LR11-CT alone in *E. coli* were unsuccessful as no recombinant protein could be detected after expression. Therefore, we investigated the use fusion constructs with maltose binding protein (MBP), which has been successful for expressing other low molecular weight transmembrane-containing proteins as they express well and are frequently located in the membrane fraction [38]. This fusion construct containing MBP fused to LR11-CT expressed at high levels. However, during protein purification it showed proteolytic degradation in (Figure 3-6) resulting in drastically reduced amounts of full-length proteins of suitable quality required for biophysical studies.

3.3.2 Purification of the LR11-CT:GGA1-VHS binary complex

To reduce the proteolytic cleavage of LR11-CT, we investigated the possibility of co-purifying MBP-LR11-CT with a binding partner that might protect the LR11-CT from proteolytic digestion. The C-terminal region LR11-CT is known to bind to the VHS domain of GGA1 [32]. Therefore we decided to purify the binary complex of LR11-CT with GGA1-VHS.
Figure 3-6: Coomassie stained SDS-PAGE of purified MBP-fused to LR11-CT showing proteolytic degradation of LR11-CT. Lane 1) Top band - full Length His$_6$-MBP-LR11-CT (48 kDa); lower band degraded protein, Lane 2) N-terminal His$_6$-MBP tag (42 kDa).

The purification scheme is summarized in Figure 3-7. MBP-LR11 and GST-VHS constructs expressed separately in *E. coli*, and mixed prior to lysis. The first purification step exploited the poly-histidine tag on MBP. The His$_6$-MBP-LR11-CT:GST-VHS complex eluted from a nickel affinity column, visualized through SDS-PAGE and the relevant peaks were pooled. To remove the N-terminal His$_6$-MBP tag of LR11-CT from the complex TEV protease was added, typically incubated for 4-5 hours depending on protease efficiency, and applied to a GST affinity column. The flow-through contained His$_6$-MBP, TEV and any unbound LR11-CT. The LR11-CT:GST-VHS complex was eluted and the GST tag of GGA1-VHS removed by activated thrombin. Finally, the LR11-CT:VHS complex was separated from protein contaminants using size exclusion chromatography (SEC, Figure 3-8). Isotopically labelled MBP-LR11-CT expressed as well as unlabelled protein and was used in NMR.
experiments. The presence of the protein complex was confirmed by NMR spectroscopy.

**Figure 3-7:** Purification scheme for LR11-CT:VHS domain complex. 1) His<sub>6</sub>-MBP-TEV-LR11-CT was mixed with *E. coli* GST-GGA1-VHS domain and lysed together. 2) The mixed lysate was flowed through an Ni-NTA column - bound complex was eluted off the column along with free His<sub>6</sub>-MBP-TEV-LR11-CT. 3) Protease treated Ni-NTA eluate was further purified through GST column and LR11-CT:VHS.
Domain complex eluted from the column. 4) GST tag was removed from LR11-CT:VHS Domain complex through final SEC chromatography step.

Step 4: Superdex 75 16/60 Sizing Column

![SEC chromatography and SDS-PAGE](image)

- **Figure 3-8**: The SEC chromatograph along with an SDS-PAGE of the peak fractions of the SEC. 1) GST (26 kDa) dimer at 52 kDa. 2&3) The LR11-CT:VHS complex elutes as a 22kDa complex (6 kDa + 16 kDa, respectively). 4) Excess VHS domain 16 kDa.

### 3.3.3 NMR characterization of LR11-CT:VHS

In order to characterize the LR11-CT:VHS complex we used NMR 2D $^1$H-$^{15}$N HSQC experiment. $^{15}$N labeled recombinant LR11-CT was over-expressed as for unlabeled protein and the labeled LR11-CT:unlabeled VHS complex purified as before.

The $^1$H-$^{15}$N TROSY spectrum of LR11-CT:VHS complex in solution (Figure 3-9) exhibited a dense cluster of cross-peaks over a narrow range, suggesting that LR11-CT either has α-helical secondary structure and/or is unfolded. The number of visible peaks (50) was close to expected (56). Since the VHS domain was unlabeled
there are no resonances for it. Missing resonances are likely due to fast chemical exchange between amide groups and the solvent.

The initial $^1$H-$^{15}$N TROSY indicated that the protein was suitable for further study. Therefore, 3D $^{15}$N TOCSY-HSQC and $^{15}$N-NOESY HSQC spectra were collected for backbone assignment, 38 out of 56 peaks were assigned.

![Figure 3-9: Backbone assignment of cross-peaks in $^1$H-$^{15}$N TROSY spectrum of LR11-CT:VHS complex. Only LR11-CT was $^{15}$N labeled.](image)

### 3.3.4 LR11-CT interacts with bilayer-like bicelles
We next tested whether LR11-CT:VHS complex interacts with lipid bilayers. $^1$H-$^{15}$N TROSY spectra were collected in the presence of large bicelles q=3 or small bicelles q=0.5. As shown in Figure 3-10a, LR11-CT cross-peaks corresponding to the membrane proximal residues L7 to F28 disappeared in the absence of bicelles (Figure 3-10a). This loss of cross-peaks occurred because the large size of the bicelle effectively broadened the resonances of the interacting amide groups of LR11-CT beyond detection. Analysis of $^1$H-$^{15}$N TROSY spectrum of LR11-CT:VHS in the presence of smaller q=0.5 bicelles (Figure 3-10b) indicated that residues of LR11-CT R6 to A15 were a subset of residues involved in binding the bicelles. The strongest cross-peaks observed in the TROSY spectrum of LR11-CT:VHS alone did not show a reduction in signal (line broadening) or a shift in position in the presence of q=3 or q=0.5 bicelles, indicating that these cross-peaks do not participate in binding to bicelles. Thus, NMR analysis suggested that residues S29 to A56 were not involved in bicelle binding.
Figure 3-10: LR11-CT interacts strongly with membrane mimetic bicelles.  a) 2D $^1$H-$^{15}$N TROSY overlay of $^{15}$N-LR11-CT:VHS Domain (red) vs. 15N-LR11-CT:GGA1 VHS Domain (blue) $q = 3$, 8% [DMPC]/[DHPC] bicelle - LR11-CT sequence below: Red residue - no reduction/shift, Black residue - no assignment, Green residue - peak missing, Blue residue - peak shift, Purple residue - reduced intensity b) 2D $^1$H-$^{15}$N TROSY overlay of $^{15}$N-LR11-CT:GGA1-VHS Domain (red) vs. 15N-LR11-CT:VHS
Domain blue q = 0.5 8% [DMPC]/[DHPC] bicelle LR11-CT sequence below with peak labels: Black residue - no assignment, Red residue - no change, Green residue - peak missing, Blue residue - peak shift, Purple residue - reduced intensity. Green, Blue, and Purple residues are likely involved in interacting with bicelles as indicated by (___).

3.3.5 LR11-CT^{DD} peptide adopts a helical conformation

In a separate study of LR11-TMCT in our laboratory, the region identified to interact with the bilayer-like bicelles forms an amphipathic helix [39]. To further characterize this membrane-binding region a peptide that corresponds from K3 to D33 was synthesized (Figure 3-11). A few charged residues on either side of this region were included to increase solubility.

$$K^3HRRLQSSFTAFANSHYSSRLGSAIFSSGDD^{33}$$

**Figure 3-11:** LR11-CT^{DD} peptide. Numbering of peptide corresponds to recombinant LR11-CT protein.

We used CD spectroscopy to follow the folding of LR11-CT^{DD} peptide. Bicelles are unsuitable for CD spectroscopy since they absorb and scatter in the far-UV region of the electromagnetic spectrum. Liposomes are spherical bilayer vesicles that are more curved than bicelles. They are commonly used in structural biology as a membrane mimetic, because they do not scatter significantly in the far UV wavelengths (>200 nm). 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC) liposomes were selected for their ease of production and neutral charge.

CD measurements were recorded in the far UV region (200-250 nm). Measurements below 200 nm were not recorded due to the absorption and scattering properties of liposomes at shorter wavelengths. Since we were unable to
measure data below 200 nm, prediction of percentage of secondary structure elements within the peptide based on the CD spectrum could not be made.

CD spectra of LR11-CT\textsuperscript{DD} in solution and in the presence of DMPC sonicated liposomes (Figure 3-12a) had a strong minimum at 200nm, indicating that LR11CT\textsuperscript{DD} resembles an intrinsically disordered protein (IDP) in solution. On the other hand since LR11 is inserted into the membrane of the \textit{trans}-Golgi network, \textit{in vivo}, we reasoned that lipid composition might affect the folding of LR11-CT\textsuperscript{DD} peptide. Vesicles with a lipid composition resembling those of the \textit{trans}-Golgi network (mol \%), egg PC (50), DOPE (30) 1,2 DOG (10) and Brain PS (10), were prepared through extrusion. The TGN-mimetic liposomes were homogeneous, with an average mean diameter of 70 ± 20 nm. As seen in Figure 3-12b, the disappearance of the minimum at 200 nm and the appearance of two minima at 208 nm and 222 nm indicate that LR11-CT\textsuperscript{DD} transitions from a random coil structure in solution to an alpha-helical conformation in the presence of these TGN-mimetic liposomes. Thus, this membrane interacting region of LR11-CT can form a $\alpha$-helical structure in the absence of LR11-TM and the rest of LR11-CT residues.
**Figure 3-12**: LR11-CTDD interacts with TGN mimetic liposomes. a) Far UV CD spectra of 33 μM LR11 CT<sup>30-60</sup> alone or in the presence of DMPC liposomes (total lipid concentration) 0 mM, 1 mM, 5 mM. b) Far UV CD spectra of 33 μM LR11CT<sup>DD</sup> peptide alone (blue) or in the presence of TGN mimetic liposomes (total lipid) 2.5 mM (green) and 5 mM (red). The folding of LR11 CT<sup>DD</sup> to a helical conformation is sensitive to lipid environment.

A helical wheel analysis of this membrane proximal region provided some molecular insights into its interactions with the lipid bilayer. As shown in Figure 3-13, the majority of this region (residues L7 to S21) adopts an amphipathic α-helix structure. The hydrophilic face of this helix mainly consists of polar residues, while the hydrophobic face includes non-polar amino acids and a histidine. When it is uncharged, the histidine residue prefers a hydrophobic environment. Amphipathic helices are particularly suited for effectively interacting with the membrane. As they bind to the membrane, the hydrophilic side of amphipathic helices can interact with the lipid head groups while the hydrophobic surface interacts with lipid acetyl
chains. Together, our data suggest that the N-terminal membrane proximal region of LR11-CT forms an amphipathic α-helix and interacts with the lipid bilayer.

Figure 3-13: Helical wheel plot of LR11-CT (Top). Yellow = hydrophobic residue Green = Basic residues Blue = polar (Below) Sequence of LR11-CT with membrane interacting residues L7 to F28 highlighted.

3.3.6 LR11-CTDD peptide induces membrane deformation

We noticed some precipitation in the LR11-CTDD peptide and TGN-mimetic liposome solution over time. There are several reports in the literature that suggest...
such precipitation might indicate the formation of larger vesicles [40-45]. To investigate this possibility, a dynamic light scattering (DLS) study was undertaken to examine the size and size distribution of liposomes incubated with and without LR11-CTDD. TGN-mimetic liposomes had an average mean diameter of 70 ± 20 nm (Figure 3-14). At the initial high lipid to peptide ratio of 54.8:1 (molar ratio) the size distribution is twice as broad as that for liposome alone. As lipid to peptide ratios decrease to 13.7:1 and 3.4:1, respectively, the liposome vesicles became polydisperse, indicating that LR11-CTDD can alter the shape and size of liposomes.

**Figure 3-14:** LR11-CTDD deforms TGN mimetic liposomes.

### 3.3.7 Electron Microscopy

We directly visualized the effect of LR11 CTDD binding on liposomes using
negative staining and EM. Extruded liposomes show vesicles of relatively similar sizes with an average size of about 60 nm (Figure 3-15 (Top)). By contrast, at a lipid-to-peptide molar ratio of 23:1 these liposomes are clearly deformed into significantly smaller vesicles coexisting with some tubule-like particles as seen in Figure 3-15 (Bottom). These tubule-like particles are likely responsible for the observed increase in vesicle sizes seen in Figure 3-14 since DLS measurements are more sensitive to larger particles. Together, our results indicate that the LR11-CTDD peptide may efficiently reshape membrane structures in vitro.
Figure 3-15: LR11-CT^{30-60} peptide remolds TGN mimetic liposomes. (Top micrograph) TGN-mimetic liposomes alone (bottom micrograph) LR11-CT^{30-60} interaction with TGN mimetic liposomes.
3.4 Discussion

This chapter described studies to characterize the interaction of LR11-CT with membrane bilayer mimetics. Our initial NMR study in bilayer-like bicleles showed that LR11-CT strongly interacts with the membrane environment, as judged by the loss of signal in large bicleles (q =3). From backbone resonance assignment of the $^1$H-$^{15}$N TROSY spectrum we identified the membrane proximal portion of LR11-CT (residues K3 to F28) as the primary site of interaction.

Interestingly, separate studies in our lab indicated that residues L7 to I27 adopt an amphipathic helix while the remainder of the residues S29 to A56 lack stable regular secondary structure [39]. To further characterize this membrane proximal region of LR11-CT, a peptide corresponding to residues K3 to D33 of the recombinant LR11-CT, LR11-CT$^{DD}$ was synthesized. CD data indicate the peptide folds into a α-helical structure in the presence of liposomes with lipid composition that resemble those of TGN transport vesicles. Thus we conclude this membrane proximal region of LR11-CT has an intrinsic propensity to adopt a helical conformation.

Amphipathic helices are a common motif that mediates protein-lipid interactions. Some of its classical roles include anchoring proteins to membrane surface and destabilizing membrane [46-50]. Recently, the amphipathic helix is increasingly being recognized as a common motif for membrane remodeling. For instance the amphipathic helices in the N-BAR containing proteins amphiphysin and endophilin are critical for the generation of tubule-like structures in the TGN [40]. In this study we identified a membrane proximal amphipathic helix in LR11-CT that
deforms liposomes *in vitro*. This helix is short and has a small hydrophobic face, similar to the membrane bending H0 helix in Epsin, a protein that contributes to the formation of clathrin-coated vesicles [51]. However, the hydrophilic face of LR11-CT does not contain charged residues, unlike the H0 helix, but contains numerous polar residues, a feature that resembles the ALPS motif, a class of amphipathic helices that senses, but not induces, membrane curvature {52}. Further study will be needed to determine if the LR11-CT helix can also function as a membrane curvature sensor.

While the basic process governing vesicular biogenesis has been described, little is known about intracellular sorting receptors to this process [53-55]. Membrane remodeling by LR11-CT seen in our study suggests a possibility that this sorting receptor may actively participate in altering membrane structure for vesicle formation and/or vesicle maintenance. This idea is supported by a recent study where mutations in the F^{14}ANSHY^{19} motif, particularly F14A, within the helix of LR11-CT disrupted its interaction with the retromer complex and resulted in altered LR11 subcellular localization [34]. Aromatic residues are known to be critical for mediating protein-lipid interactions. Hence, it is reasonable to speculate that F14 may play a role in the binding of LR11-CT helix to the membrane bilayer as well as its interaction with retromer complex.

In summary, a membrane proximal helix at the N-terminal region of LR11-CT has been identified that interacts with liposomes mimicking the composition of the TGN. Furthermore, this helix has characteristic features of an amphipathic helix and upon interaction with liposomes transforms them into small vesicles and tubule-like particles. Since the change in membrane geometry is an inherent part of vesicular
transport, we speculated that LR11 might play an active role in remodeling membrane structure and facilitating the intracellular trafficking process.

3.5 Literature Cited


Chapter 4: Summary of Thesis, Significance of Work, Future Direction

4.1 Summary and significance of this work

4.1.1 Introduction

Biological membranes adopt diverse and dynamic geometries. For example, bacteria display a wide range of shapes, ranging from spheres to rods and spirals; the internal membranes of eukaryotic cells are stunning arrays of tubules, sheets, vesicles, and cisternae. Many essential cellular processes such as endocytosis, vesiculation, organelle synthesis and cell division require transient membrane deformations, and the activity of some proteins (e.g. human ArfGAP1) dramatically increases with the curvature of the membrane bilayer [1, 2]. Recently, it was discovered that membrane curvature could serve as a geometric cue for the subcellular localization of some proteins (e.g. SpoVM and DivIVA) [3]. Membrane geometry is increasingly viewed as a critical component for creating microenvironments for membrane fusion and fission, protein localization, trafficking and signaling [1, 4]. This underscores the importance of understanding the molecular mechanisms responsible for the generation, recognition, maintenance, and regulation of membrane architecture.

The next paragraphs will briefly present the prevailing models prior to my studies and summarize the major results of my work using two amphipathic helices with different chemical properties to probe the mechanism of membrane geometry recognition (SpoVM; Chapter 2) and remodeling (LR11; Chapter 3). This work has resulted in a new model for shallow curvature recognition. Finally, I will describe additional experiments that could be performed to test my model.
Since membrane geometry recognition is a relatively new field of study, very few structural studies have been conducted on membrane curvature sensing. Before this work, membrane curvature recognition was studied *in vitro* using eukaryotic proteins such as ARFGAP1 (ALPS motif), amphiphysin (N-BAR domain), and α-synuclein that recognized the highly curved surfaces of the intracellular membrane environment [5-7]. Using liposomes vesicles (≤ 50nm (SUVs)) *in vitro* binding studies by Bhatia et al., and Antonny et al., showed that membrane curvature recognition was based on the surface density at saturation, $B_{\text{max}}$, of the protein rather than affinity or cooperativity. Furthermore, these studies showed that curvature recognition was highly sensitive to the lipid composition of the vesicles. Based on these observations a mechanism was proposed on the recognition of highly curved vesicles. In this model, lipid-packing defects, caused by the high curvature of the vesicles provide binding sites for these proteins (Figure 4-1a) [8]. The higher the curvature, the more binding sites are created, thus higher $B_{\text{max}}$ (more molecules per surface area at saturating concentrations) is observed for smaller vesicles. Primary sequence and secondary structure analysis of these proteins revealed the amphipathic helical (AH) binding motifs of these curvature sensitive AHs were quite distinct from classical amphipathic helices [9]. These curvature sensitive motifs featured bulky hydrophobic residues for the hydrophobic face and polar residues instead of charged residues at the hydrophilic face. Theoretical and MD simulations provided a model in which curvature recognition was based on surface association of a long monotonous amphipathic helix inserting its bulky hydrophobic residues
within the lipid bilayer defects of highly curved vesicles and relieving packing stress associated with the defects (Figure 4-1b) [8].

Figure 4-1: Proposed mechanism for recognition of high membrane convex curvature for amphipathic helices. a) Highly curved vesicles ≤ 50 nm have more lipid packing defects due to increased surface area of the outer bilayer leaflet relative to the inner leaflet. Amphipathic helices may bind to these defects and alleviate packing stress. Hence, the higher the curvature the more binding sites are available, thus at saturating concentration of protein more highly curved vesicles, have greater $B_{\text{max}}$. b) Based on theoretical and MD simulations - high membrane curvature sensors are proposed to be long monotonous amphipathic helices that lay on the surface of the membrane bilayer with bulky hydrophobic residues embedded into the defects and polar residues interacting with the lipid head groups and hydration shell. (Reviewed in section 1.1.4)
We wished to find out whether the model for high curvature applied to shallow curvature therefore, SpoVM, a 3 KDa peptide that can sense convex curvature *in vivo* was used to study shallow curvature recognition because of its insensitivity to lipid composition, the solubility of *E. coli* expressed recombinant protein, and small size made it suitable for NMR and other biophysical analysis. In addition a mutant, SpoVM$^{P9A}$, which does not discriminate between vesicles of different curvature was studied in order to identify structural, topological, physiochemical features of SpoVM that result in shallow curvature recognition. In order to provide a more quantitative analysis of SpoVM recognition of both high and shallow curvature vesicles a new membrane model system was introduced, Spherical Supported Lipid Bilayers (SSLBs), which are more robust, homogenous, and easier to prepared as compared to LUVs. Based on *in-vitro* SSLB Capture Assays, a slight increase in cooperativity and a threefold increase in affinity for 2 μm vs 8 μm DMPC coated SSLBs with no increase in $B_{max}$. These results were in contrast SpoVM$^{P9A}$, which showed no difference in affinity, $B_{max}$, or cooperativity between 2 μm vs 8 μm DMPC coated SSLBs. These results indicated SpoVM must use a different mechanism for membrane curvature recognition. Structural and topological analysis of SpoVM and P9A in bicelles by NMR and MD simulations using our structures supported a model of deeper insertion of SpoVM within the bilayer; approximately parallel orientation to the bilayer surface, and flexible N-terminal region (Figure 4-2b). The structure and topology of SpoVM is significantly different than that proposed for molecules sensitive to high curvature (Figure 4-2a).
This work has identified a novel mechanism for curvature recognition that is in contrast to the previous model proposed for SpoVM interaction with the membrane bilayer. The structural and binding data has lead to a new hypothesis for shallow curvature recognition for SpoVM (Section 2.4 for review), in which a higher order structure of SpoVM either through direct or indirect interaction mediates the slight cooperativity and increase in affinity observed in the SSLB capture assays (Figure 4-3).
Figure 4-3: SpoVM may directly or indirectly interact with other SpoVM molecules to form higher order structures for membrane curvature recognition (Reviewed in section 2.4).

4.1.2 Future studies of SpoVM higher-order structural model

To determine whether SpoVM clusters exist in the membrane and play a role in curvature sensing, the intermolecular distances of SpoVM could be measured in lipid vesicles of various sizes. These distances are likely long-range. PREs from NMR are sensitive to distances up to ~25 Å and have the advantage that multiple signals can be detected simultaneously, which allows for the collection of numerous constraints for structural characterization [10]. Intermolecular PREs can be measured in liposomes or SSLBs using a mixture of $^{13}$C labeled SpoVM and unlabeled protein carrying a spin label. For longer distances, EPR or FRET can be exploited as measurement tools [11, 12]. The labels used in EPR are usually smaller and more rigid than the chromophores commonly used in FRET. Thus, they have fewer potential perturbations on protein-lipid interactions and the data are easier for structural interpretation. Distance constraints in the range of 20 to 60 Å have
recently been obtained from EPR measurements. With the SpoVM monomer in hand, a few long-range distance measurements would allow for the construction of the cluster structure of SpoVM and lipids in conjunction with molecular dynamic simulations. In addition, the SpoVM clusters may be visible in image reconstruction from stained or cryo-EM [13].

4.1.3 A membrane proximal helix in LR11-CT

The work in chapter 3 further extended the structural and biophysical characterization techniques applied to SpoVM to the cytoplasmic domain of APP intracellular receptor, LR11, and identified an amphipathic helix which requires lipid composition similar to the composition of vesicles and tubules found in the trans-Golgi-network (TGN) for binding and folding [14]. Further characterization of this binding revealed LR11-CT is capable of inducing membrane deformation at high protein/lipid ratio. We speculate that this phenomenon may be related to inducing or maintaining vesicle transport vesicles.

As noted in the last paragraph of section 1.1.4 although this work has shown LR11-CT is capable of remodeling membrane structure, remodeling and curvature recognition are not mutually exclusive phenomena (as evident by the N-BAR domain) [15, 16]; due to the proximity of LR11 to the highly curved vesicles of the TGN it would be reasonable to speculate LR11 might show preferential binding to higher curvature vesicles, thus future experiments (such as competitive SSLB capture assay) for the LR11-CT project could be directed to address this question.
4.2 Future Studies

4.2.1 Introduction

Very few studies have examined the mechanism of membrane curvature recognition using high-resolution structural and quantitative biophysical analysis. Using this approach we examined whether SpoVM utilized the same structure, topology, and mechanism for recognition of shallow curvature as has been proposed for high membrane curvature recognition. In our study, we found SpoVM utilizes a novel mechanism of curvature recognition based on an increase in affinity and slight increase in cooperativity, not on $B_{\text{max}}$, which has been proposed as the primary parameter for membrane curvature recognition. In addition, the structure and topology of SpoVM within the membrane bilayer was not a long monotonous amphipathic helix shallowly sitting at the surface of the membrane, but was a short amphipathic helix with a flexible-N-terminal loop deeply inserted parallel to the bilayer. Ultimately, we propose that SpoVM utilizes a higher order structure (through direct or indirect interaction) that is critical for recognition of shallow curvature. In the next section, I will discuss the key structural features of SpoVM that may be critical for shallow curvature recognition, how these features affect interaction with the membrane, and how these features support our model for SpoVM higher order structure. In addition, I will suggest future experiments that could be performed to test the validity of our observations and proposed mechanism. Many of the future experiments suggested for the SpoVM project can be readily adapted for membrane remodeling studies such as for LR11-CT.

Through our study of SpoVM and SpoVM$^{P9A}$ we have identified key structural
and biochemical differences that result from proline 9. It is clear that proline 9 shortens the amphipathic helix observed in P9A and the N-terminal region forms a flexible loop. Proline has long been recognized as a helix-breaking amino acid, as a result of its lack of a hydrogen-bonding donor group and its steric hindrance of hydrogen bonding of the adjacent residue [17]. Despite these unfavorable properties, proline has been found in the helices of membrane bilayer-spanning regions of integral membrane proteins although its function is not clear. However, it has been suggested proline regulates ion transport channels by cis±trans isomerization [18].

Prolines appear frequently in helical peptides that display membrane-penetrating activity, including toxins such as mellitin [19], which is found in bee venom, margatoxin [20], a component of scorpion venom, antimicrobial peptides, such as cecropin [21] from the cecropia moth, and the amphibian proteins gaegurin [22], buforin [23], and brevinin [24].

Studies of the central proline of melittin provide an example of its critical function in membrane active peptides. Mellitin is a 26 amino acid peptide with a single proline at position 14. Structural and dynamics studies have shown that it separates the peptide into two distinct helical regions and that the unstructured region surrounding P14 acts like a flexible hinge (Figure 4-1) [25]. Substitution at P14 with alanine creates a longer, stable, less flexible structure with resultant reduction in pore-forming function and antimicrobial activity. These results are strikingly similar to the loss of flexibility and function seen with SpoVM\textsuperscript{P9A}.

For SpoVM, is it the loss of flexibility of P9A or the increase in helical structure that changes its membrane curvature recognition and possible higher order
structure? We propose that the primary differences between SpoVM and SpoVM\textsuperscript{P9A} stem from the helical length/stability, the flexible N-terminal loop, and charge distribution/hydrophobicity.

4.2.2 Helix length/stability

One of the striking structural features of SpoVM is the short length of the amphipathic helix compared to the P9A variant and other membrane curvature sensors such as the ALPS motif and GMAP210 [9]. The initial proposal that the amphipathic helix of SpoVM extended through the length of the peptide was based on the model of proteins that bind to the outer surface of the highly curved vesicles and tubules found within the eukaryotic intracellular environment composed primarily of neutral PC lipids. However based on SpoVM\textsuperscript{P9A} variant, an extended stable amphipathic helix actually abolishes SpoVM function and curvature recognition. Thus, it is proposed that the short amphipathic helix is a critical feature for the curvature recognition properties of SpoVM.

This assertion is supported by several studies of penetrating peptides of bacterial membranes: melittin, piscidin, and gaegurin. In each case substitution of their central proline with an alanine, resulted in the formation of extended stable helices, compared to their wildtype peptides [19, 22, 26, 27] and functionally reduced in antibacterial activity. In gaegurin, an antimicrobial peptide from the skin of a Korean frog, Rana rugosa, and altered membrane binding properties were observed [22]. Both peptides (WT/P14A) showed comparable binding affinities for negatively charged lipids, while wildtype had a considerably reduced affinity for
neutral lipids [22]. These results are similar to SpoVM, which P9A variant has two times higher affinity for 2 μm SSLBs coated with neutral PC lipids than WT.

How does helix length affect membrane curvature recognition mechanistically? While an exact mechanism still requires further study, one possibility is that the extended amphipathic helices of P to A mutants are thermodynamically more stable than the WT peptides. As suggested by Ladhkhin et al, >50% of the binding energy for binding to membrane bilayer can be attributed to the folding of the peptide. It is conceivable that the stability of the alanine mutants is such that they are not as sensitive to the subtle differences in membrane properties between membranes of different curvature. In several studies, including that of melittin and gaegurin, helix stability was noted as a possible contributing factor in modulating peptide binding to membranes that are driven by electrostatic interaction [22, 28]. To test this hypothesis the length of SpoVM$^{P9A}$ amphipathic α-helix can be shortened systemically from the N-terminus or C-terminus and its binding properties evaluated through SSLB Capture Assays or ITC. It would be expected decreasing the length of the peptide would decrease the stability of SpoVM$^{P9A}$ with a concomitant increase in sensitivity for lower curvature vesicles.

4.2.3 Flexible N-terminal loop

As seen in both the molecular dynamic simulations and NMR, substitution of proline with alanine resulted in a flexible and solvent accessible N-terminal region. We propose that the N-terminal loop and its flexibility are crucial for mediating higher order structure through self-association. Consistent with this idea is the observation that the N-terminal region can interact with cytosolic sporulation
proteins. It is our hypothesis that the flexible N-terminal region may be conducive for facilitating interaction with cytosolic SpoVM or facilitating interaction within the hydrophobic core of the bilayer. As observed through MD simulations and NMR studies of melittin and other AMPs, the flexibility of the peptide is often a significant requirement for mediating protein-protein interaction and deep insertion into the membrane bilayer [19, 27, 29]. Flexibility is thought to induce self-association in several ways. For melittin, flexibility allows for the peptide to insert into the bilayer with a kink that promotes tetramer formation (figure 4-4A) whereas the alanine mutant’s non-flexible amphipathic helix is thought to form a peptide bilayer (Figure 4-4B). This peptide bilayer has been shown to favor a parallel orientation and therefore shallower insertion depth as opposed the kinked tetramers [19].

![Figure 4-4: Melittin P14A substitution decreases flexibility resulting in aggregates with shallow insertion depth. a) The flexibility of melittin allows kinking of the peptide which in turn allows for tetramers to form can penetrate deeply into the bilayer to form pores b) Melittin P14A forms a long stable amphipathic α-helix. This structure forms pairs of anti-parallel or parallel (not shown) helices which tend to have shallow depths of insertion. Figure adapted from [John et al. 1992].](image-url)
In order to test the hypothesis that the flexible N-terminal loop of SpoVM is required for function, several experiments could be performed. SpoVM mutants with increasingly deleted N-terminal loops and/or lacking the N-terminal loop completely could be generated. Due to the small size of SpoVM, synthesized peptides and recombinant-SpoVM mutants, expressed in *E. coli*, are easily prepared and economically feasible. Measurement of membrane insertion depth using NMR PRE experiments as utilized for SpoVM and SpoVM<sup>P9A</sup> would provide information on the orientation and depth of insertion of the shorter peptides, along with EPR and fluorescence quenching. If the flexible N-terminal loop is required for deep insertion into the membrane bilayer and/or interaction with other SpoVM molecules, it would be expected that truncating the N-terminal loop will result in shallower insertion into the membrane bilayer.

**4.2.4 Charge distribution/hydrophobic surface**

Substitution of the proline with alanine changes the electrostatic character of the peptide surface such that three charged residues: K7, K10, and R17 are present in the P9A amphipathic helix instead of the single charge, R17, of SpoVM.
Figure 4-5: Charge distribution of SpoVM and SpoVM\textsuperscript{P9A} amphipathic helix. a) The amphipathic helix of SpoVM contains only 1 charged residue, R17 (highlighted in red box) b) SpoVM\textsuperscript{P9A} with an extended amphipathic helix has three charged residues: K7, K10, R17 (highlighted in red box). Blue = cationic residues, Green = polar residues, Grey = hydrophobic residues.

We propose that this difference in charge (charge distribution) has a profound affect on depth of insertion and on the sensitivity of curvature recognition. Recent studies of anti-microbial peptides melittin and piscidin have demonstrated the importance of charge distribution of peptides within the membrane bilayer [27]. Piscidin is a 22-residue antimicrobial cationic peptide isolated from mast cells of a hybrid striped bass. The cationic residues play an important role in the interaction of the amphipathic helix with the membrane bilayer. Substitution of K14 with D resulted in a deeper insertion depth in 3:1 PE:PG lipid mix [27] suggesting that the positive charge of lysine facilitated the interaction with the negative charge of the phosphate lipid head group. In the case of both SpoVM and P9A, MD simulations suggest that the amphipathic helices inserted deeply into the bilayer, but P9A was found to have 0.5 Å shallower insertion depths. With three cationic residues within the
amphipathic helical region of P9A, the shallower depth of insertion may be the result of increased electrostatic interactions with the lipid phosphate groups. In the study of the ALPS motif, the balance between electrostatic and hydrophobic interactions is thought to be critical in its curvature sensing [4]. Increasing the electrostatic charge of the ALPS in the polar face or substituting less bulky hydrophobic residues in the non-polar face disrupts the selectivity of the peptide to highly curved membranes [2]. Weak electrostatic interactions with the lipid bilayer are proposed to enhance ALPS motif requirement for membrane lipid packing defects found in highly curved membranes [30]. Thus, it may be reasonable to infer that the increase of exposed/accessible positively charged residues of SpoVM<sup>P9A</sup> may disrupt the balance between electrostatic and hydrophobic interactions required for SpoVM curvature discrimination.

A further examination of SpoVM amphipathic helix indicates that it is not a typical amphipathic α-helix: its hydrophobic face is very wide (~2/3 helix surface) in comparison to the ALPS motif (Figure 4-5).
Figure 4-6: SpoVM is not a typical amphipathic α-helix a) ALPS motif hydrophobic face covers ~1/2 helical surface (black lines denote approximate separation of hydrophilic and hydrophobic surfaces) b) SpoVM has a very wide hydrophobic face (~2/3 of surface) which could account for deeper insertion of SpoVM into the membrane bilayer hydrophobic core. (36 residue ALPS motif sequence derived from ARF-GAP1137-257). Red = anionic residues, Blue = cationic residues, Green = polar residues Grey = hydrophobic residues.

We speculate that this wide hydrophobic face may be a feature necessary for deeper insertion into the membrane hydrophobic core. As it stands to reason a greater hydrophobic surface area will provide greater stability within the hydrophobic core of the bilayer. To test this hypothesis substitutions of polar or charged residues in the hydrophobic face of SpoVM or conversely substituting a hydrophobic residue within hydrophilic face of SpoVM could be performed and changes monitored in the insertion depth through PRE experiments or molecular dynamic simulations. If our hypothesis correct then it should be expected that decreasing the surface area of the hydrophobic face will result in decreased insertion depth and a possible loss of membrane curvature discrimination.
4.2.5 Protein-lipid interactions-membrane insertion depth

The primary focus of this thesis was on the structural and biophysical properties of SpoVM and LR11-CT, but as membrane proteins, they are surrounded by a shell or annulus of lipid molecules, equivalent to the solvent layer surrounding a water-soluble protein. As the peptide binds and reorients either partially or fully into the membrane interior, the phospholipid molecules also undergo a structural reorganization. In the next two sections we will examine how insertion depth affects protein-lipid interactions and therefore provide a pathway for mediating higher order structure for proteins and then how the membrane environment itself can facilitate protein-protein interaction through a concept known as membrane-mediated interactions.

Membrane insertion depth is a primary factor in mediating protein-lipid and protein-protein interaction. Peptide - lipid interaction can be characterized in three levels. Surface associated-peptides (shallow insertion) interact with the solvent environment and the lipid head group region. Deeper insertion into the interfacial zone - peptides interact with the hydration shell, lipid head-group, and shallow insertion into hydrophobic acyl chain core. Finally, for deep inserting peptides the interaction is primarily with the acyl chains. Thus, for each level of depth within the bilayer a peptide must have structural and physiochemical properties that match its environment and favor interaction with neighboring lipids and peptides. In the following section the role insertion depth may play in promoting higher order structure will be examined.

SpoVM and SpoVM\textsuperscript{P9A} were found to be deeply imbedded within the
membrane bilayer with their amphipathic helices aligned with the long axis of the membrane bilayer. This deep insertion is contrary to the current shallow insertion depth model of high curvature membrane recognition [8]. In a study by Li et al., MD simulations of model peptides at various insertion depths, the authors obtained a surprising result that deep insertion within the bilayer resulted in promoting protein-aggregation and this protein-aggregate was even sensitive to membrane curvature as opposed to shallow inserting proteins, where only a weak ability to self-assemble was observed [31]. How does deep insertion promote self-aggregation? MD simulations suggest deep insertion within the acyl chains of the lipid bilayer disrupt the local lipid packing order, which-in-turn lowers the transitional energy cost of insertion of peptide into a rigid membrane bilayer [31]. Eventually the assembly of peptides produces a local curvature, which can facilitate binding of even more molecules. In contrast shallow inserting peptides, have the tendency to have the opposite effect, in that shallow insertion within the interfacial zone of the bilayer does not cause a disordering of the lipid molecules and enhances packing of the local lipid bilayer. Furthermore, shallow insertion of peptides creates wedges to further increase the surface area of the single leaflet, therefore promoting remodeling of membrane environment, without enhancing protein self-assembly [32].

In order to further examine the role R17 plays in the anchoring and penetration of the membrane bilayer, our collaborators in the Ramamurthi lab, generated a new mutant R17D. As shown in Figure 4-6, initial results with SpoVM$^{R17D}$-GFP show that SpoVM is mis-localized in *B. subtilis*, aggregating in the cell cytoplasm. In addition, CD results (data not shown) indicate SpoVM$^{R17D}$ is not mis-
folded, suggesting R17 plays a critical role in the interaction of SpoVM anchoring and penetration into the membrane bilayer.

![Figure 4-7](image)

**Figure 4-7:** SpoVM<sup>R17D</sup>-GFP in sporulating *B. subtilis* cells. (A,C) Whereas SpoVM-GFP almost exclusively localized to the forespore surface (B,D) substantial SpoVM<sup>R17D</sup>-GFP fluorescence was detected in the mother cell cytosol suggesting that substitution of the positive charge at position 17 with a negative charge affected the adsorption of SpoVM onto the membrane. C and D overlay of GFP signal with membranes visualized with FM4-64 (Red). (Unpublished data provided by Ramamurthi lab)

Recent MD simulations indicate the critical role arginine and lysine play in the deep insertion of membrane peptides due to a phenomenon known as the snorkeling effect [33]. In this effect membrane active peptides containing lysine and arginine can enhance the hydrophobic interaction by extending (lengthen) the side chains of lysine and arginine to bury more deeply in the hydrophobic core (Figure 4-7). This
result further supports the importance of electrostatic charge interaction of the polar face of the helix and charge distribution in membrane topology.

Figure 4-8: Long charged side chains (lysine and arginine) can snorkel, thereby stretching towards the water phase. In the figure, the dark gray regions are the hydrophobic interior of the membrane, and the light gray regions are the more polar lipid head group regions. Peptides or membrane bound segments of proteins are outlined in black with charges attached on side chains. A: Peripheral membrane segments or amphipathic peptides, lying on the surface of the membrane, can place hydrophobic residues deeper in the lipid bilayer if the side chains are allowed to stretch. Figure adapted from [33].

To further extend these initial results with SpoVM$^{R17D}$, mutants of SpoVM$^{P9A}$ substituting the positively charged residues in the amphipathic helix with aspartate, polar residues, or alanine could be analyzed. The interaction of these peptides with membranes of different curvatures could be monitored through the SSLB capture assay to observe whether any type of SpoVM$^{P9A}$ mutant re-gains partial functionality (curvature discrimination). Insertion depth resulting from SpoVM$^{P9A}$ mutations could be modeled in silico to determine the effect of decreasing cationic charge on insertion depth.
4.2.6 Membrane-mediated interactions

The composition and physical properties of the membrane bilayer can drastic effects on binding properties. LR11-CT requires for binding and folding a lipid composition resembling that of the trans-Golgi network, while SpoVM is capable of binding a bilayer composed of tightly packed PC lipids. In the case of SpoVM it remains to be seen what properties the 2 μm DMPC-coated beads have compared to the 8 μm DMPC beads that facilitates higher SpoVM binding? One of the most significant properties that is altered between vesicles of significant curvature differences is lipid packing; compared with lipids in the outer surface of highly curved membranes, lipids in the outer surface of slightly curved membranes are more tightly packed [1]. The insertion of hydrophobic moieties into one leaflet of the membrane alters lipid packing and results in a significant increase of lipid mobility. As shown previously, the binding of an AH to LUVs is entropy-driven [34]. These changes in lipid entropy and membrane thickness have been cited as major causes of membrane-mediated protein oligomerization and membrane remodeling [35, 36][37]. To examine if our hypothesis is correct we can employ $^{31}$P and $^2$H NMR to determine perturbations on the orientation and mobility of the lipid headgroups and on the motion of the lipid acyl chains by the binding of SpoVM and its functionally impaired mutants [38-41]. These NMR measurements reflect the behavior of all lipids and will uncover if and how changes in the packing of the lipid headgroup and acyl chains contributes to SpoVM membrane curvature recognition. Alternatively, EPR experiments with spin-labeled lipids may allow the detection of lipids in direct contact with the protein [42].
4.3 Conclusion

This thesis has examined the structure and physiochemical properties of two peptides that utilize amphipathic helices for insertion into the membrane bilayer using different mechanisms. Their difference in structure result in distinct functional and membrane binding characteristics. Ultimately, using the approach in this paper, a more detailed examination of the properties of these peptides will aid in gaining a more definitive understanding of membrane curvature remodeling and recognition and the delicate balance between both phenomena. This research has created a template for studying the effects of different amphipathic helices on the curvature of the membrane. Future projects using solid state and in-situ NMR will examine peptide-lipid interactions in a more native-like environment and build on the data presented in this thesis [43, 44].

4.4 Literature Cited


Curriculum Vitae
Richard L. Gill Jr.

Education:
2007-present  PhD Biochemistry & Molecular Biology
Penn State University, Hershey, PA

2002-2007  Post-Baccalaureate General Studies
University of Pennsylvania, Philadelphia, PA

1995-2001  B.S. Chemistry
Penn State University, Erie, PA

Awards:
2001  Penn State Erie – Undergraduate Researcher of the Year

Work Experience:
2007 – present  PhD Student, Department of Biochemistry and Molecular Biology, Penn State University, College of Medicine, Hershey, PA 17033, USA.
Supervisor: Fang Tian, PhD, Assistant Professor of Biochemistry & Molecular Biology
Project: Molecular Insights into the Generation and Recognition of Membrane Geometry.

Publications:

