PTEN, phosphatase and tensin homologue deleted on chromosome 10, is a tumor suppressor that is commonly lost or mutated in many different diseases, including cancer, Bannayan Zonana syndrome, heart disease and cancer. The mechanism of the effects of the loss or mutation of PTEN is not known, other than the effects on the regulation of downstream effector molecules. While many other research groups have studied the effects of PTEN loss or mutation on tumor formation in mouse models, no group has undergone the daunting task of characterizing the protein biophysically, in order to determine how these mutations affect the actual action of the protein.

In this study, we have shown that PTEN binds specifically to PI(4,5)P$_2$ over all other phosphoinositides, including its substrate, PI(3,4,5)P$_3$. This binding event is also associated with a conformational change, which is believed to activate the protein and increase its rate of turnover of PI(3,4,5)P$_3$. Additionally, we shown in these studies that the binding specificity of PTEN is dependent on the presence and identity of the N-terminus, which contains a PI(4,5)P$_2$ binding domain. Removal or mutation of the protein’s N-terminus abrogates the binding and thus activity of the protein. One particular amino acid which is often mutated in many forms of cancer, the lysine in position 13, has
been shown to be extremely important in the interaction of PTEN with PI(4,5)P₂ containing membranes. We show that even while maintaining overall charge of the N-terminus and even overall identity, mutation of the lysine in this position results in a loss of binding specificity. We have also studied the binding of an autism related mutation of PTEN, which results in an increased binding to phosphatidylinerine, which may play a role in the ability of the protein to turnover its substrate \textit{in vivo}.

We have also studied the effects of cholesterol on the binding of proteins to phosphoinositide containing membranes, which has proven to have different effects on the binding of different proteins. Finally, we show that phosphoinositides form domains in model membranes in the absence of proteins and the presence of cholesterol appears to enhance these formations.
CHARACTERIZATION OF THE BINDING OF PTEN AND ITS DISEASE RELATED MUTANTS TO PHOSPHOLIPID MODEL MEMBRANES

A Dissertation submitted
to Kent State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Phosphoinositides are lipids with large, negatively charged headgroups that compose a surprisingly small portion of the lipid membrane with respect to the functions they mediate. Phosphoinositides are different from other acidic lipids, which bind to proteins in an often non-specific, electrostatic fashion. Unlike these lipids, phosphoinositides bind to proteins in a very specific manner, which is dependent on the phosphorylation pattern of the inositol headgroup. The inositol ring has three positions (the 3, 4, and 5 positions) that are found phosphorylated in nature, and the phosphorylation of these sites is controlled by a variety of lipid kinases and phosphatases. The regulation of the phosphorylation of the inositol ring is important, as phosphoinositides mediate processes in the cell such as proliferation, apoptosis, cell motility and vesicle trafficking (Deleris 2006). These diverse functions obviate the necessity for control over the activity of the various lipid kinases and phosphatases, and how dysregulation of these proteins can have devastating effects on the cell.

Figure 1.1. Naturally occurring phosphatidylinositol
Some time ago, it was found that some portions of the outer leaflet of the plasma membrane were not homogenous, and that some structures seemed to form within the membrane that were insoluble in detergents such as TritonX-100 at 4°C (Chamberlain 2004). These formations, termed lipid rafts, are preferentially enriched with sphingomyelin, cholesterol, and saturated acyl chain phospholipids and are thus in a liquid-ordered state, which is thought to limit the diffusion of molecules within these entities. Furthermore, proteins that are modified with a lipid anchor like myristoyl or palmitoyl chains, were also found to be preferentially enriched within these formations. This association with many signaling proteins suggested that these small lipid domains may be scaffolds for signal transduction components. It should be noted, however, that much controversy has been associated with methods of studying lipid rafts, as it is thought that the use of detergents such as Triton X-100 could cause the clustering of lipid molecules into non-preexisting domains (Mayor 1994; Kenworthy 2000).

Since the discovery of lipid rafts on the outer leaflet of the plasma membrane, it has also been found that lipid components of the inner leaflet exist in a non-homogeneous fashion, which helps to explain why some lipid molecules such as phosphoinositides, which make up such a small portion of the lipid membrane, are nevertheless able to play such great roles in signal transduction. It is thought that this is possible because they may be preferentially enhanced in lipid domains within the inner leaflet of the membrane, thus greatly increasing the local concentration of those lipid molecules and providing a scaffolding on the interior, cytosolic facing portion of the membrane for signaling events to take place. Inner leaflet lipid domains are in all likelihood quite different from outer
leaflet lipid rafts, as the composition is different (sphingomyelin does not exist on the inner leaflet of the plasma membranes). Lipid domains are a very difficult subject of study, because their size is smaller than the diffraction limit of light, so they can not be visualized using conventional light microscopy. Another interesting question which has been an area of intense study since the discovery of lipid rafts and domains is how the two leaflet domains are coupled to one another. Because it is hypothesized that both exist for the purpose of signal transduction, it is important to understand how the signal is sent from the outer leaflet of the plasma membrane to the cytosolic poriton, so that the signal may be conducted inside of the cell (or vice versa). While this aspect is still not understood and efforts are being made to study the coupling of each of the components of the bilayer, it is known that the fatty acyl chains of the lipids do not intercalate and thus this is not the coupling mechanism for signal transduction across the membrane.

Importantly, because proteins were found to be associated with lipid rafts,
especially those which contained a lipid modification anchoring them to the membrane, it was hypothesized that protein association may be a necessary factor in the formation of such domain structures. This idea carried over to the formation of lipid domains within the inner leaflet of the plasma membrane. Several studies have shown, however, that the presence of proteins is not necessary for a lateral separation of lipid molecules in a fluid/fluid bilayer consisting of two or more lipid components, and lateral separation of lipid molecules occurs in a pH dependent manner. It is thought that while proteins can induce domain formation, this process may be similar to a nucleation event, in which a small domain exists prior to interaction with proteins, but upon interaction the protein may help to recruit more lipid molecules to the area, increasing the local concentration of those lipids and the size of the lipid domain in question.

One important aspect of the study of lipid rafts is that the canonical raft mixture is DPPC, sphingomyelin and cholesterol, all of which are necessary for raft formation. Of particular interest is need for cholesterol, as it is possible to deplete lipid bilayers which contain cholesterol using compounds such as β-cyclodextrin. Upon cholesterol depletion, it was found that the lipid rafts ceased to exist, and were therefore cholesterol dependent (Simons 1997; Simons 2000). Because it seemed that lipid rafts were dependent on the presence of cholesterol, it became clear that cholesterol could also have an effect on the formation of domains within the inner leaflet of the membrane. The effects that cholesterol exerts on the formation of these structures are much more difficult than understanding the interactions of cholesterol with the lipid molecules which make up lipid rafts. Rafts in the outer leaflet of the membrane consist of saturated lipids (e.g.
DPPC) or lipids with a trans unsaturated alkyl chain (e.g. sphingomyelin), which have rigid fatty acyl chains that pack rather well. Cholesterol is a bulky hydrophobic sterol, which interacts well with saturated fatty acyl chains, but interrupts the packing ability of these lipids with one another. The cholesterol molecules are able to intercalate between the lipid molecules and form a new phase which is less ordered. Domains also form in the absence of cholesterol but are considered to be a gel phase domain. The addition of cholesterol and its disordering effects give rise to a new liquid ordered phase. This is the mechanism which gives rise to the formation of lipid rafts.

The role of cholesterol in the formation of non-raft lipid domains is much more difficult to assess because the natural composition of the fatty acyl chains of lipids which make up the inner leaflet of the plasma membrane usually consist of one saturated fatty acyl chain, typically a stearoyl chain, and one unsaturated fatty acyl chain, typically an oleoyl or arachidonoyl chain. While cholesterol may be able to interact easily with the stearoyl chain, it is unlikely that cholesterol molecules would interact well with the arachidonoyl chains, which have several double bonds, and are thus kinked, preventing tight packing of these unsaturated lipid molecules. Because the interactions between cholesterol and the type of lipid molecules that make up significant amounts of the inner leaflet are not clear, many research groups have studied the effects of the presence of cholesterol on the formation of domains and the lateral separation of lipid components in model membrane systems. While several groups have concluded that cholesterol can induce lipid domain formation and enhances the ability of proteins to induce lipid domain formation (London 2000; Epand 2004), still others are wary of the possibility that
cholesterol plays such a role (Silvius 2001).

Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is a dual specificity phosphatase that was originally identified as a protein phosphatase, specific for tyrosine residues of the target protein, but also active against serine and threonine residues (Maehama 1998). While the protein contains motifs found in many other protein tyrosine phosphatases, the structure of the protein is different from those phosphatases in a very important way, which led to the discovery that PTEN functions chiefly as a lipid phosphatase (Lee 1999). PTEN consists of several different domains, including a phosphatase domain, a C2 domain, and a PDZ binding domain. The phosphatase domain contains the conserved sequence of amino acids necessary for phosphatase activity that is present in other phosphotyrosine phosphatases (PTPs). PTEN also has two lysine residues within this motif, which lends a more positive overall charge to the region, possibly increasing its ability to associate with negatively charged membranes (Myers 1997). This HCXXGXXR motif forms a loop (the P loop, residues 123-130), which is located at the bottom of the active site pocket (Lee
The conserved residues are necessary for the conformation of the loop, are essential for catalysis, and are targeted by mutations in cancer (Maehama 1999). The active site pocket of the phosphatase domain in PTEN has been found to be much wider than active sites of similar PTPs; this is consistent with PTEN’s function as a PI(3,4,5)P₃ phosphatase, as the PI(3,4,5)P₃ would require a wider active site opening (Lee 1999). The loop of PTEN at the active site may play a very important role in its activity; it has been found for several other phosphatases that a similar loop must go through a series of conformational changes, opening and closing in order for catalysis to occur (Schubert 1995).

The phosphatase domain interacts with the C2 domain at an extensive interface. Hydrogen bonding by nine residues that occur in the interface between the two domains is necessary for the stability of the protein, while seven hydrophobic and aromatic residues form a buried core. These structural features are highly conserved across species, almost as much as the residues of the P loop. The residues that are involved in the interfacial contact of the C2 and phosphatase domains are the most highly conserved residues of the C2 domain. The interface is very important for the function and structure of PTEN, as mutations of the residues involved in the hydrogen bonding network are often found to be mutated in cancer. Mutation of these residues affects phosphatase activity, reducing the PI(3,4,5)P₃ phosphatase activity of PTEN by up to 85% (Lee 1999).

The presence of the C2 domain was recognized only upon solving the crystal structure of PTEN. The C2 domain is thought to be involved in PTEN’s association with the membrane; PTEN is known to bind to pure phosphatidylcholine vesicles (Lee 1999),
but to a lesser extent than to membranes containing anionic lipids (Das 2003). Because the C2 domain helps to recruit PTEN to the membrane, it has been suggested that it may also position and orient the catalytic domain with respect to its substrate, which is membrane bound (Lee 1999). Unlike other C2 domains, PTEN lacks Ca$^{2+}$ ligands, and does not bind to membranes in a Ca$^{2+}$-dependent manner. PTEN does, however, contain a CBR3 loop that is similar to those of the Ca$^{2+}$ dependent C2 domains, which contains 5 solvent exposed lysine residues, accounting for the C2 domain’s binding to anionic lipid membranes. Mutations of these residues reveals no stabilizing role for the loop they form (Lee 1999).

The PDZ binding domain of PTEN is located at the C-terminus and is thought to help recruit PTEN to the membrane through interactions with membrane-bound PDZ proteins. PTEN is phosphorylated at the C-terminus, which deactivates the protein and prevents the interaction of PTEN with the membrane through the PDZ binding domain. It is thought that the phosphorylation of PTEN may affect the conformation of the protein, causing a closed conformation that prevents binding to PDZ proteins, but also enhances protein stability in the cell (Vazquez 2000; Vazquez 2001). It has also been proposed that this closed conformation may cause a shielding of amino acids that interact with the membrane (Iijima 2004). PTEN has been shown to be primarily phosphorylated by Casein Kinase 2 (CK2), which is a serine/threonine kinase (phosphorylating serines 370 and 385) (Miller 2002). The possibility of PTEN being phosphorylated by glycogen synthase kinase 3β has also been investigated, which poses an interesting possible negative feedback loop, as GSK 3β is inhibited by insulin and other activators of the
PI3K pathway (Al-Khour 2005).

PTEN’s N-terminus is known to bind to PI(4,5)P2, and enhancement of PTEN’s activity against its substrate, PI(3,4,5)P3, by its product, PI(4,5)P2, has been observed by several groups (Campbell 2003; McConnachie 2003). From these results, it has been suggested that binding of PI(4,5)P2 results in a conformational change in the protein that activates the phosphatase domain (Campbell 2003). It has also been suggested that the PI(4,5)P2 binding domain of the N-terminus may block the phosphatase active site and that binding of PI(4,5)P2 may cause a conformational change that allows substrate access to the active site pocket (Iijima 2004). Still another hypothesis is that because the C2 and phosphatase domains interact so extensively at their interface, a conformational change that occurs in one of the domains might propagate to the other domain (Wishart 2002). The existence of so many models without a clear forerunner necessitates further investigation into the conformational changes that occur in the protein upon binding to membranes of differing compositions. It has also been observed that PTEN has activity against multiple phosphoinositides with phosphate groups at the 3 position of the inositol ring (Campbell 2003). PTEN’s activity is greatest against PI(3,4,5)P3, followed by PI(3,4)P2. Like PI(4,5)P2, PI(5)P has also been shown to be a potent activator of PTEN activity towards PI(3,4,5)P3, while both PI(3,4)P2 and PI(3,5)P2 have little or no activating abilities (Campbell 2003). PTEN’s activity against phosphoinositides seems to be quite dependent on the number and position of phosphate groups on the inositol ring. It is important to confirm the findings of activity assays and characterize the binding of PTEN with each of the phosphoinositide derivatives.
PTEN is known to act as a hopping enzyme, because it associates with the membrane only transiently, disassociates, and moves to another site (Berg 2001; McConnachie 2003). The enzyme was shown to associate with the membrane for several hundred milliseconds at a time; long enough to dephosphorylate several PI(3,4,5)P$_3$ molecules per association (Vazquez 2006). Many studies have focused on the localization of PTEN in the cell and its association with the membrane. PTEN is present in both the nucleus and cytoplasm, and while the enzyme interacts transiently with the plasma membrane, it seems as though PTEN also interacts with cytoplasmic structures, possibly tethering the protein and placing it in the vicinity of its substrate or even preventing its interaction with the plasma membrane (Liu 2005). However, because the subcellular localization of PI(3,4,5)P$_3$ is not known, both of these hypotheses are still viable.

PTEN plays an important role in the PI3K pathway, by acting as an antagonist of PI3K, which phosphorylates PI(4,5)P$_2$ to form PI(3,4,5)P$_3$. PI(3,4,5)P$_3$ plays roles in many different cellular processes, including cell proliferation, differentiation, and survival, as well as carbohydrate metabolism (Deleris 2006). It is therefore very important to regulate the levels of PI(3,4,5)P$_3$ in the cell, as dysregulation of the levels of this lipid is associated with many different diseases, such as diabetes, cancer, and heart disease (Furnari 1997; Deleris 2006). The PI3K pathway is not the same in every type of cell, but is very similar in most. Insulin binds at a tyrosine receptor kinase, which dimerizes upon binding its substrate. This phosphorylates and thus activates PI3K, which phosphorylates PI(4,5)P$_2$ to produce PI(3,4,5)P$_3$. This product drives the phosphorylation of Thr$^{308}$ on Akt by phosphoinositide-dependent kinase1 (PDK1). The phosphorylation of
Akt affects the phosphorylation and activation of a number of downstream molecules, the overall effect being an increase in cell survival signal, a decrease in apoptosis, and increased cell cycle entry. PTEN acts to regulate this process by controlling the production of PI(3,4,5)P₃ and thus the phosphorylation and activation of Akt and the downstream molecules which prevent apoptosis and increase the cell survival signal (Datta 1999). Importantly, while PTEN controls the basal levels of PI(3,4,5)P₃, the
phosphatase specific for the 5 position of the inositol ring reduces the acute levels of PI(3,4,5)P3 back to basal levels.

PTEN is known to be deleted or inactivated in many types of tumors (Simpson 2001), especially gliomas (Li 1997). Loss of one copy of PTEN results in tumor formation, while complete loss of PTEN is embryonic lethal. PTEN’s tumor suppressor activity has been found to be associated with its phosphatase activity. Therefore, any mutation that confers a loss of phosphatase activity may be involved in tumorigenesis. Some of the most important mutations occur at the interface between the phosphatase and C2 domains, such as Ser-170 and Arg-173, which are among the most frequently mutated cancer relevant residues of PTEN (Lee 1999). Because phosphatase activity is associated with tumor suppressing activity of the protein, mutations of the active site pocket and residues nearby are also very important and often implicated in PTEN-related cancers. One of the most important of these mutations is G129E, which is a residue near the pocket that disrupts PTEN’s PI(3,4,5)P3 activity, while the tyrosine phosphatase activity of the protein remains (Furnari 1997; Myers 1998). Other residues of the active site that are essential for catalysis and the structure of the P loop in similar tyrosine phosphatases are Cys-124, Arg-130, and Gly-127 (Barford 1994; Stuckey 1994). Mutations of the C2 domain have also been implied in cancer, such as Asp-331. The C2 domain has been shown to have some role in the binding of PTEN to anionic lipids, due to a group of five positively charged lysines within residues 327 to 335. Because the C2 domain’s binding to these lipids is thought to position the phosphatase domain near its substrate, mutations that interrupt this binding may affect the phosphatase activity of the protein (Lee 1999). It
is also thought that mutations in one domain may propagate and affect the structure and functions of other domains in the protein (Wishart 2002). Other mutations have been implicated in diseases such as Cowden disease, Bannayan-Zonana syndrome, autism, and heart disease (Oudit 2004; Pilarski 2004; Butler 2005). Importantly, mutations of the N-terminus have been implicated in cancer, and are thought to act by abrogating binding to PI(4,5)P₂, thus preventing the protein’s ability to bind and turnover its substrate. These mutations, such as PTENK₁₃E, have been shown to profoundly reduce PTEN’s catalytic activity (Walker 2004). Further study of the effects of disease-associated mutations on PTEN’s binding to anionic lipids, its substrate, PI(3,4,5)P₃, and PI(4,5)P₂, and the effects of these mutations on the protein’s structure, is necessary to determine the cause of each disease state.

Gelsolin is a 82-84 kD protein which is the founding member of the gelsolin superfamily of proteins, a group of proteins present in mammalian and non-mammalian organisms. All of the members of the gelsolin superfamily of proteins contain three or six homologous repeats of a domain named the gelsolin-like domain. Gelsolin is expressed in many different cell types and is often found with a signaling peptide within the cytoplasm. The protein’s main function in the cell is to bind, sever and cap actin filaments, as well as to control the polymerization of barbed ends of actin. Importantly, gelsolin is also able to bind to two separate actin monomers and initiate the polymerization of an actin filament (Su 2007).

Gelsolin is regulated in many different manners, including by Ca²⁺, pH, tyrosine phosphorylation and interaction with phosphoinositides. Phosphoinositides, particularly
PI(3,4)P_2 and PI(4,5)P_2, are known to inhibit gelsolin by preventing gelsolin’s severing actions and causing the dissociation of gelsolin from actin. Because gelsolin plays such a great role in the regulation of actin polymerization, its effects on cytoskeletal properties are of interest because these parameters control cell motility and morphology. Furthermore, gelsolin also plays roles in the regulation of phagocytosis and apoptosis. Additionally, mice that lack gelsolin develop and reproduce normally, but have an increase in bleeding due to the lack of platelet activation because of gelsolin’s role in cell motility, and inflammatory responses are affected as neutrophils are unable to migrate normally, resulting in slower wound healing (Silacci 2004). Notably, gelsolin has been recently discovered to interact with lipopolysaccharide molecules, which coat the surfaces of some bacteria as a protective measure for the prevention of recognition within a host organism. In this way, gelsolin allows the host to recognize the bacteria and respond appropriately, thus treating the infection indirectly. Gelsolin has begun to be used in the treatment of sepsis due to the nature of its interactions with the protective layers of bacteria usually involved in those processes.

Profilin is a protein that is also associated with the reorganization of the cell’s cytoskeleton and affects cell motility and morphology. Profilin acts in a manner that is antagonistic to some of the functions of gelsolin, as profilin is a protein that promotes polymerization (Disanza 2005). This is accomplished by the protein interacting with actin filaments which have barbed ends, and it has been observed that overexpression of profilin in CHO cells causes an increase in filamentous actin, which provides evidence that profilin promotes actin polymerization (Finkel 1994). Furthermore, profilin is known
to bind to G-actin to form a complex in a 1:1 ratio, then acting as a nucleotide exchange factor, which charges the actin molecules with ATP. The only known molecule able to release the profilin protein from the G-actin molecules are phosphoinositides, and it is known that PI(4,5)P$_2$ and profilin interact with a very high affinity (Lassing 1985).

Profilin is also known to interact with a variety of protein partners which are also involved in actin recruitment and cytoskeletal restructuring, particularly in the tails of *Listeria monocytogenes*, such as the Arp2/3 complex, VASP, MENA, and dynamin. It is thought that these interactions play a role in bringing the profilin-actin complex to a site which requires actin polymerization in the host cell as well as in the bacteria (Witke 1998). Though the role of profilin in signal transduction is not clear at this time, it is known that profilin plays a role in endocytosis and synaptic recycling, and because of its interactions with several different proteins and signaling molecules such as Rac, Rho and PI(4,5)P$_2$, it is thought profilin may act as a negative regulator in signal transduction. It has also been speculated that because profilin interacts strongly with phosphoinositides that it may function to cause domain formation at the membrane, recruiting many different signaling molecules such as receptors and kinases, forming a “signaling membrane site”, which would exist transiently but could be necessary to induce endocytosis (Witke 1998).
1.1 Objectives and Methodologies

In the first part of this study, we will examine the interaction between several different proteins and phosphoinositides, in order to determine the specificity of binding of those proteins to lipid membranes. We will use a fluorescence quenching method to determine whether these proteins (PTEN, PTEN mutants, gelsolin, and profilin) bind to specific phosphoinositides characterized by the number and position of phosphates present on the inositol head group. We will also use a fluorescence resonance energy transfer method to investigate phospholipid/protein binding which utilizes the fluorescent tryptophan residue and a pyrene labeled lipid within vesicles of varying lipid composition. In this way, we can study the extent of binding between proteins and non-labeled lipids, including lipids such as phosphatidylserine. Additionally, we will also use Isothermal Titration Calorimetry (ITC) to measure and quantitate the extent of binding of these proteins to lipid vesicles. Using Attenuated Total Reflectance Infrared Spectroscopy (ATR-FTIR), we will also investigate to what extent PTEN, Gelsolin and Profilin undergo conformational changes upon interaction with phosphoinositide containing membranes that may be important in the regulation of their function. We hypothesize that proteins such as PTEN bind in a specific manner to different phosphoinositide derivatives and undergo conformational changes as a result of this interaction. In particular, we hypothesize that PTEN binds specifically to PI(4,5)P₂ as this molecule acts as an activator of PTEN activity and that conformational changes occur as a result which are likely to make the active site more accessible to the lipid substrate, PI(3,4,5)P₃.
The second part of this study will examine the effects of varying amounts of cholesterol on the domain formation of phosphatidylcholine / phosphoinositide mixtures. To accomplish this, we will use a FRET method designed to study fluid / fluid demixing of lipids as previously described by Redfern et al. Because it is believed that not only the number of phosphate groups present on the inositol ring, but also their position, is important for their mutual interaction and therefore for the formation of domains and specific binding to proteins, we will also study the zeta potential of each of the bisphosphate substituted phosphoinositides of identical chain composition, while also determining the effects of the presence of cholesterol on this aspect of the lipid vesicles. We hypothesize that due to its different interactions with phosphatidylcholine and phosphoinositides, cholesterol will enhance the formation of domains in a fluid/fluid system. We will also use Differential Scanning Calorimetry (DSC) and Fourier Transform Infrared Spectroscopy to examine the phase behavior of dimyristoylphosphatidylcholine in the presence of dipalmitoylphosphatidylinositol in differing amounts to gain further insight into the phase behavior of phosphatidylinositol.
# Table 1.1. List of chemicals

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Abbreviation</th>
<th>Chemical Formula</th>
<th>Company</th>
<th>Formula Weight</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dipalmityl phosphatidylinositol</td>
<td>DPPI</td>
<td>C$<em>{41}$H$</em>{78}$O$_{13}$PNH$_4$</td>
<td>Matreya Inc. (Pleasant Gap, PA)</td>
<td>828</td>
<td>98+%</td>
</tr>
<tr>
<td>1,2-Dipalmitylphosphatidyl inositol-4,5-bisphosphate</td>
<td>DPPI (4,5)P$_2$</td>
<td>C$<em>{41}$H$</em>{77}$O$_{19}$P$_3$Na$_4$</td>
<td>Cayman Chemical (Ann Arbor, MI)</td>
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<td>L-α-Phosphatidylinositol</td>
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<td>C$<em>{45}$H$</em>{94}$N$<em>3$O$</em>{19}$P$_3$</td>
<td>Avanti Polar Lipids, Inc. (Alabaster, AL)</td>
<td>1074.17</td>
<td>99+%</td>
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<tr>
<td>1,2-Dioleoyl-sn-3-Glycerol-Phosphatidylinositol-3,5-bisphosphate</td>
<td>DOPI(3,5)P$_2$</td>
<td>C$<em>{45}$H$</em>{94}$N$<em>3$O$</em>{19}$P$_3$</td>
<td>Avanti Polar Lipids, Inc. (Alabaster, AL)</td>
<td>1074.17</td>
<td>99+%</td>
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<td>1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphatidylcholine</td>
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<td>Avanti Polar Lipids, Inc. (Alabaster, AL)</td>
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<td>1-Palmitoyl-2-Oleoyl-sn-Glycerol-3-Phosphatidylserine</td>
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<td>Chemical Name</td>
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<td>Company</td>
<td>Formula Weight</td>
<td>Purity</td>
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<td>1-Palmitoyl-2-Oleoyl-sn-Glycero-3-[Phospho-rac-1-glycerol] sodium salt</td>
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<td>BODIPY-PI TMR</td>
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<td>BODIPY-PI (3)P TMR</td>
<td>C_{66}H_{116}BF_{2}N_{6}O_{19}P_{2}</td>
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<td>D(1)-sn-1-O-[1'-6'']-6'[-[(4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl) phenoxy] acetyl] amino[hexanoyl][amino][hexanoyl]-2-hexanoylglyceryl D-myo-phosphatidylinositol 4-phosphate</td>
<td>BODIPY-PI (4)P TMR</td>
<td>C_{66}H_{116}BF_{2}N_{6}O_{19}P_{2}</td>
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<td>BODIPY-PI (5)P TMR</td>
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<td>BODIPY-PI (3,4)P_{2} TMR</td>
<td>C_{78}H_{147}BF_{2}N_{6}O_{22}P_{3}</td>
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<td>BODIPY-PI</td>
<td>C_{78}H_{147}BF_{2}N_{9}O_{22}P_{3}</td>
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<td>BODIPY-PI(4,5)P_{2} TMR</td>
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<td>BODIPY-PI(3,5)P_{2} TMR</td>
<td>C_{78}H_{147}BF_{2}N_{9}O_{22}P_{3}</td>
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<td>BODIPY-PI(3,4,5)P_{3} TMR</td>
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<td>BODIPY-PI(4)P TR</td>
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Table 1.1. List of chemicals (continued)

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<th>Company</th>
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<td>D(1)-sn-1-O-[1-6’-6-[(4-(4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-y) phenoxy) acetyl) amino]hexanoyl]amino]hexanoyl]-2-hexanoylglyceryl D-myophosphatidylinositol</td>
<td>BODIPY-PI(3,4,5)P&lt;sub&gt;3&lt;/sub&gt; TR</td>
<td>C&lt;sub&gt;42&lt;/sub&gt;H&lt;sub&gt;74&lt;/sub&gt;N&lt;sub&gt;5&lt;/sub&gt;O&lt;sub&gt;11&lt;/sub&gt;P</td>
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<td>C&lt;sub&gt;40&lt;/sub&gt;H&lt;sub&gt;80&lt;/sub&gt;N&lt;sub&gt;10&lt;/sub&gt;O&lt;sub&gt;8&lt;/sub&gt;P</td>
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<td>Ethylenediamine tetraacetate disodium salt</td>
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<td>C&lt;sub&gt;10&lt;/sub&gt;H&lt;sub&gt;14&lt;/sub&gt;O&lt;sub&gt;8&lt;/sub&gt;Na&lt;sub&gt;2&lt;/sub&gt;N&lt;sub&gt;2&lt;/sub&gt;·2H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>Fisher Scientific (Fair Lawn, NJ)</td>
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<td>N-(2-Hydroxyethyl) piperazine-N’-(2-ethanesulfonic acid)</td>
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<td>C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;18&lt;/sub&gt;N&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;4&lt;/sub&gt;S</td>
<td>Fisher Scientific (Fair Lawn, NJ)</td>
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<td>99%</td>
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<td>Tris Hydroxymethyl Aminomethane Hydrochloride</td>
<td>TRIS</td>
<td>NH&lt;sub&gt;3&lt;/sub&gt;C(CH&lt;sub&gt;2&lt;/sub&gt;OH)&lt;sub&gt;3&lt;/sub&gt;HCl</td>
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<td>99.8 atom% D</td>
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<td></td>
<td>Aldrich Chemical Co. (Milwaukee, WI)</td>
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Table 1.1. List of chemicals (continued)

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<th>Purity</th>
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CHAPTER 2: TECHNIQUES APPLIED IN THIS STUDY

2.1) Preparation and Expression of Recombinant PTEN Protein

All PTEN proteins were expressed and purified by the Ross lab, University of Massachusetts Medical School, Worcester, MA. The cDNA encoding full-length (1-403) human PTEN was cloned into the NdeI and XhoI sites of the pET30b vector (Novagen), thereby, introducing a 6-histidine tag at the C-terminus. Point mutations were introduced with the Quick-Change site-directed mutagenesis kit from Stratagene. The deletion mutant 16-403 was prepared by PCR with the Phusion DNA polymerase (New England Biolabs). The 5’ primer included an NdeI restriction site and a met initiation codon. The 3’ primer included an XhoI site. The PCR product was blunt end cloned into the EcoRV site of the pcDNA3 plasmid. This insert was released from the pcDNA3 plasmid and cloned in the NdeI and XhoI sites of pET30b.

PTEN proteins were expressed in Eschericia coli BL21(DE3) cells. To increase protein solubility, cells were grown at 37°C until the OD at 560 nm reached 0.5-0.6. The culture was shifted to 21°C and after 30 min, protein expression was induced with 0.05 mM isopropyl-β-D-thiogalactoside. After 20-22 hrs, the culture was harvested. His6-proteins were purified with a HisTrap HP Kit from GE Healthcare using buffers with 10 mM mercaptoethanol. The PTEN proteins were further purified with a Superdex 200 16/60 column in 100 mM NaCl, 10 mM Tris, pH 7.4, 1 mM dithiothreitol. The final purification was done with a MonoQ 5/5 anion-exchange column in 10 mM Tris, pH 7.4.
with a linear gradient of 50-600 mM NaCl. Fractions were analyzed by SDS-PAGE and Coomassie Blue staining. Those fractions containing >95% pure protein were pooled. Dithiothreitol was added to 10 mM, and the proteins were stored on ice because initial experiments revealed that freeze-thawing resulted in protein aggregation. The yield of recombinant protein was typically 1.5-2.0 mg/ml. Phosphatase activity was confirmed as described (Campbell 2003). All protein samples were checked for quality assurance purposes using Dynamic Light Scattering (DLS), to be sure that the proteins were not aggregated before use in any of the following experiments. PTEN proteins typically display a distribution around 10nm in size when not aggregated in solution.

2.2) Quenching of Fluorescence

2.2.1) Fluorescence Quenching Introduction

Fluorescence is a phenomenon in which a molecule is excited by the absorption of a photon from an electronic, vibrational ground state to an excited electronic, excited vibrational state. The Franck-Condon principle states that because the nuclei are so much more massive than the electrons, an electronic transition occurs much more quickly than the nuclei can respond, thus all transitions are vertical and the internuclear distance is maintained. The upper vibrational excited states then undergo a series of radiationless decay steps by giving up energy to their surroundings, typically as heat. After reaching a vibrational ground state of the electronic state, the molecule then undergoes a radiative transition from the vibrational ground, excited electronic state to a vibrationally excited, electronic ground state, which results in the emission of a photon. Because the change in
energy of absorption is greater than the change in energy due to emission, the emission band of a fluorescent molecule is always found at longer wavelengths than the absorption band, which is described as a Stokes shift. It should be noted that the absorption and emission spectra of a fluorescent molecule are dependent on the structure of the molecule in question. Therefore, the absorption spectrum will be characteristic of the vibrational upper state and the emission spectrum will be characteristic of the vibrational lower state. Because these are similar, the absorption and emission spectra are almost like a mirror image (Lakowicz 2006).

Fluorescence quenching refers to any process which results in an overall decrease in the fluorescence intensity of any fluorescent molecule. There are different types of fluorescence quenching, which consist of collisional quenching and static quenching, both of which require that the quencher and the fluorophore come into physical contact with one another. There are many different types of molecular interactions that can be studied which result in an overall quenching of fluorescence intensity, including the formation of a ground state complex, molecular rearrangements, and energy transfer. Because both collisional and static quenching require that the quencher and fluorophore come into contact with one another, it is obvious that concentration, temperature and pressure are quite important parameters in these types of studies. Additionally, collisional quenching, which can be either dynamic or static, requires that the molecule which acts as a quencher come into contact with the fluorescent molecule of interest during the lifetime of the fluorophore’s excited state. These types of interactions only bring the fluorophore back into an electronic ground state, without emitting a photon, and the
fluorophore undergoes no other molecular changes (Lakowicz 2006).

Many different types of molecules act as quenchers of fluorescence, including molecular oxygen, which is one of the most flexible quenchers of fluorescence, though the molecular mechanisms of this quenching are not fully understood. Other molecules which act as quenchers include but are not limited to: aromatic and aliphatic amines, heavy atoms such as iodide and bromide, some halogenated compounds, acrylamide, disulfides, ethers and peroxides. It is important to note that not all quenchers will quench all fluorophores and the types of quenchers used experimentally must be appropriately paired with the fluorescent molecule of interest.

Many types of information can be gathered by doing experiments which monitor fluorescence quenching; importantly by using the Stern-Volmer equation,

$$\frac{F_0}{F} = 1 + k_q \tau_0 [Q] = 1 + K_D [Q],$$

where $F_0$ and $F$ are the initial and final fluorescence intensities, respectively, $k_q$ is the bimolecular quenching constant, $[Q]$ is the concentration of quencher and $K_D$ is the dissociation constant, one can derive the Stern-Volmer constant and find the slope of the $F_0/F$ vs. $Q$. Analysis of these data allow the investigator to determine the effects of many different experimental parameters, such as temperature, and also allows the determination of type of quenching, whether the mechanism is dynamic or static in nature. The analysis of such data can become quite complex, because observed quenching can also be due to a combination of both static and dynamic quenching mechanisms. However, simple examination of the Stern-Volmer plots and the absorption spectra allow for fairly simple distinction between dynamic and static quenching (Lakowicz 2006).
While it is interesting to study the nature of the mechanisms of quenching, more important is the application to biomolecular systems. Fluorescence quenching can be used to study the properties of many different biomolecules, including lipids, proteins, and DNA. Because contact between quencher and fluorescent molecule is a requirement, this technique can be used to study the accessibility of a fluorescent molecule to the quencher (and thus solvent), which is especially important in the study of DNA, and can accordingly also be used to monitor the interaction of two molecules which are labeled with a fluorophore and quencher molecule. Fluorescence quenching can also be used to examine conformational changes within a protein by monitoring the tryptophan fluorescence intensity, or can be used to study mutual interactions of lipids in a membrane, as well as their lateral diffusion within membranes. Taking advantage of the flexible and widely applicable nature of this technique can allow one to gain a plethora of knowledge about a variety of biomolecules and their interactions with one another and members of their environments.

2.2.2) Aim of Fluorescence Quenching Measurements

The fluorescence measurements we will perform will help to establish the affinity of binding of several different proteins to lipid bilayers containing one of the naturally occurring phosphoinositide derivatives. Because each of the phosphoinositide derivatives is commercially available with a fluorescent bodipy label attached, and this type of fluorophore self-quenches upon association with another bodipy fluorophore, we can titrate different protein solutions into vesicles which contain the labeled
phosphoinositides. It has been shown that binding of positively charged proteins (peptides) leads to a clustering of bodipy labeled phosphoinositides and therefore quenching (Epand 2004; Ghambir 2004). By monitoring the fluorescence intensity of the sample, we can determine the extent of clustering of phosphoinositide molecules that occurs upon interaction with the different proteins, which can be related to the extent of binding to each of the proteins. In this way, we can analyze and compare the data to determine whether a protein of interest binds specifically to a particular phosphoinositide.

2.2.3) Fluorescence Quenching Materials and Methods

Lipid stock solutions were dissolved in mixtures of chloroform and methanol (3:1 v/v) and stored in amber glass vials to protect them from UV light. Lipid solutions were then dried at about 50°C under a stream of nitrogen to produce a lipid film. This is done so that the sample dries as quickly as possible to ensure homogeneity. Samples were then
kept overnight at about 45°C in high vacuum to remove any residual solvent. The lipid films were resuspended in buffer solution and vortexed for 60s three times, waiting approximately 5 minutes between vortexing cycles. Vesicles comprised of 99.9% POPC and 0.1% bodipy labeled PI(x,y,z)Pn were then extruded through a 100 nm polycarbonate filter, in order to produce unilamellar vesicles. Dynamic Light Scattering was performed on the vesicles to ensure correct size distribution, usually narrowly centering around 112 nm. The fluorescence of the lipid vesicles was then monitored using a Varian Eclipse fluorescence spectrometer, using the excitation wavelength 542 nm, and scanning the emission spectrum from 550 nm to 600 nm. The bodipy-labeled lipid emits at 574 nm, and the maximum intensities of this peak were recorded. The vesicles were then titrated with a 1µM solution of protein, adding protein in fixed amounts until the protein to lipid ratio equaled 0.5. PTEN and its mutant proteins were supplied by Alonzo Ross, University of Massachusetts Medical School, Worcester, MA (see section 2.1 for PTEN expression). Profilin and gelsolin proteins were provided by Paul Janmey, University of Pennsylvania, Philadelphia, PA. At each titration point, the bodipy fluorescence was measured and recorded. The fluorescence intensity was corrected for changes due to dilution (based on total volume of the protein solution added). The initial intensity of fluorescence was set to be 100 percent, and the rest of the data was normalized based on the initial fluorescence. The titration experiments were repeated a minimum of three times for each of the eight naturally occurring phosphoinositides.
2.3) Fluorescence Resonance Energy Transfer

2.3.1) Introduction

Fluorescence Resonance Energy Transfer (FRET) is the radiationless decay of energy with the transfer of energy from one molecule in an excited state (donor) to another molecule (acceptor), which does not require the emission and reabsorption of photons (as is the case in radiative transfer). FRET is dependent on three main conditions. First, the molecules must be close to one another (about 10 nm), which makes the technique useful as a “spectroscopic ruler”. As depicted in Fig. 2.2, the emission spectrum of the donor must overlap with the excitation spectrum of the acceptor, and the transition moments for emission (donor molecule) and absorption (acceptor molecule) must be approximately parallel. The Förster distance, which is different for each molecule, is defined as the distance at which the FRET efficiency is 50%, and is typically in the range of 20 to 60 Å. The FRET efficiency is dependent on the inverse sixth power
of separation between the two molecules, which makes the technique ideal for use in investigating distance dependent phenomena in many biological systems. FRET has been used in the study of many types of biological molecules and their properties, such as the folding of proteins, binding of receptors and ligands, and even in the imaging of intracellular RNA. Additionally, FRET can be combined with techniques that take advantage of fluorescence lifetimes for imaging several different systems and study association reactions (Lakowicz 2006).

2.3.2) Aim of FRET Measurements

We have used FRET to investigate phosphoinositide domain formation in binary (PC/phosphoinositide) and ternary (PC/phosphoinositide/cholesterol) mixtures. For this

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**Figure 2.3. Schematic representation of the FRET experiment used to monitor the lateral organization of a phospholipid model membrane.** Experiments were done using POPC, Brain PI(4,5)P₂ and either 0%, 20% or 40% cholesterol. Emission spectra of fluorescently labeled PC and phosphoinositide molecules were monitored over a range of pH values as an indicator of domain formation.
experiment, small amounts of labeled PC and labeled phosphoinositide are added to the respective lipid mixture. The two labeled lipids are forming a FRET pair; colocalization (mixing) results in a pronounced fluorescence transfer while demixing (domain formation) is indicated by a reduced energy transfer. Previous experiments carried out in our lab characterized the domain formation in fluid / fluid systems of phosphoinositides and phosphatidylcholine, which were found to be pH dependent (Redfern 2004; Redfern 2005). Using similar systems, that would utilize lipids with unsaturated chain compositions leading to fluid/fluid demixing, we wished to highlight not only the pH dependence of domain formation in these systems, but also to determine the effect of the presence of different amounts of cholesterol on the domain formation within those systems. Additionally, we wanted to examine the effects of salt on domain formation in these systems, and whether the effect of salt was the same in systems which contained varying amounts of cholesterol.

We used a different type of FRET experiment to characterize the extent of binding of proteins to lipid vesicles with differing compositions. This experiment allowed us to use non-labeled phosphoinositides as well as other lipids, such as phosphatidylserine and a combination of lipids for our binding studies. This experiment involves the use of pyrene-PE in the lipid mixture, which forms a FRET pair with tryptophan. Upon association of the protein with pyrene-PE labeled vesicles the tryptophan fluorescence is reduced, which can be used to evaluate the extent of binding. In a related experiment, lipid molecules which are labeled with bromine atoms at different positions on the acyl chain were added to the vesicle forming lipid mixture in
place of the fluorescently labeled PE molecule. The bromine atoms act as a fluorescence quencher, and so cause the overall tryptophan fluorescence of the protein to decrease upon interaction (Prieto 2007). As these lipid molecules contain the bromine atoms in different positions of the acyl chains, the lipids can be used as a molecular ruler to determine how deeply a protein inserts into the lipid membrane and investigate the extent of interaction between a particular protein and lipid vesicles of differing compositions.

2.3.3) FRET Materials and Methods

Experiments were carried out using a Varian Eclipse Fluorescence Spectrometer. For the studies investigating the effects of cholesterol on the fluid / fluid demixing of phosphoinositides with phosphatidylcholine, lipid stock solutions were dissolved in mixtures of chloroform and methanol (3:1 v/v) and stored in amber glass vials to protect them from UV light. Large unilamellar vesicles were prepared by drying lipid solutions at about 50°C under a stream of nitrogen to produce a lipid film. The samples were then kept overnight at about 45°C in high vacuum to remove any residual solvent. The lipid films were resuspended in buffer solution and vortexed for 60s three times, waiting approximately 5 minutes between vortexing cycles. The resulting multilamellar vesicles were then extruded through a polycarbonate film with a 100-nm pore size (Avestin, Ottawa, ON). The size of the obtained unilamellar vesicles was checked using dynamic light scattering (HPPS and Nanosizer, Malvern Instruments, Southborough, MA) before and after experiments. Typically, these unilamellar vesicles exhibited a narrow size distribution centered at about 112 nm diameter. The resulting unilamellar vesicles were
then combined with a solution of fluorescently labeled lipids, and the pH was raised to a value between 9 and 10. The two labeled lipids (e.g. NBD-PC and Bodipy PI) were added as an aqueous solution and were then allowed to insert into the outer leaflet of the bilayer of the unilamellar vesicles for approximately one hour. The fluorescence emission spectra of the fluorophores were monitored repeatedly until the emission maxima of each fluorophore remained constant, indicating that the fluorescently labeled lipids had inserted completely into the membrane.

Subsequent to the insertion of the fluorescently labeled lipids into the unilamellar vesicles, the solution was titrated with a 0.1M solution of HCl. The pH was lowered by about 0.3 pH units before obtaining the next emission spectrum. The emission maximum of each fluorophore was recorded and the ratio of the two calculated. This method of titration was repeated until the vesicle solution reached a pH between 3 and 4. DLS was used after the titration was completed to assure the integrity of the vesicles had been maintained.

For the experiments monitoring the binding of lipid vesicles to different proteins, lipid samples were prepared in the same manner, except that fluorescently labeled lipid or brominated lipid molecules were added as part of the stock solution before the sample was dried and resuspended. Thus, insertion of the fluorophore was not necessary and did not have to be monitored. DLS was used to monitor vesicle size which were found to be about 100 nm. A 1.5 µM solution of protein was then prepared with buffer in a quartz fluorescence cuvette and also placed into the DLS to assure that the protein was not aggregated before the titration. The fluorescence of the protein’s tryptophan residues was
then monitored using the fluorescence spectrometer at an excitation wavelength of 290 nm, scanning the emission spectrum from 300 nm to 380 nm. The solution of vesicles containing 2% pyrene-PE or brominated PC was then titrated into the protein solution over 20 steps, adding 25 µL per titration step. After each addition of lipid vesicles, the fluorescence emission spectrum of the protein’s tryptophan residue was collected and its maximum recorded. This was repeated until the total lipid concentration reached 1mM. Each experiment was repeated at least three times. Also, the fluorescence intensity was corrected for changes due to dilution (based on total volume of protein added) and the same experiments of protein and lipid vesicles were carried out using vesicles which were not labeled with pyrene-PE to correct for changes in natural fluorescence, as described by Qin et al.

2.4) Isothermal Titration Calorimetry

2.4.1) Introduction to Isothermal Titration Calorimetry

ITC is a method that allows for the thermodynamic characterization of lipid/protein interactions. The instrument is equipped with two cells, one is a reference cell in which the investigator injects a buffer or water, and the other is a sample cell, in which the sample is placed. An automated syringe with the biomolecule that is to be titrated into the sample is placed in the sample cell. The machine allows the temperature to increase very slowly, where the heat evolved from the reaction is balanced by a change in the rate of heating. The two cells are kept at the same temperature using a feedback thermocouple/thermopile system. Small amounts of the titrant are added to the sample,
and the temperature of the sample cell will change based on the type of reaction that takes place. If adding the titrant to the sample is an endothermic process, then the sample cell needs to be heated to keep the sample and reference cell at the same temperature. The input electrical energy that is required to maintain the temperature of the sample cell is then converted to yield the heat of the interaction (for the binding process of the biomolecules used). Since the titration is carried out at constant pressure, this heating is the enthalpy of the binding process. Multiple titration steps characterized by decreasing

**Figure 2.4. Schematic drawing of the MicroCal VP-ITC.** Also shown is an up-close view of the cells (2007).
heat values are carried out until the sample is saturated with the ligand and subsequent injections yield the heat of dilution of the ligand. Usually, enough sample is added to make sure that all ligand is bound in the first titrations steps, which allows for determination of the total enthalpy (O'Brien 2001).

Each individual injection of titrant into the sample will then be integrated with respect to time and plotted against the molar ratio, which allows one to calculate enthalpy, and from this, one can also calculate the K_B or K_D, ΔG and ΔS (using well-
known equations \( \Delta G = -RT\ln K_B \) and \( \Delta G = \Delta H - T\Delta S \). One can also determine the change in heat capacity using the equation \( \Delta C_p = \frac{d(\Delta H)}{dT} \). This property is temperature independent and for a rigid, bi-molecular interaction, \( \Delta H \) should increase linearly. If the \( \Delta H \) value increases nonlinearly, it is evidence of a coupled equilibria with complex formation. \( \Delta C_p \) has been shown to correlate with the amount of surface area buried from the solvent when binding occurs (O'Brien 2001).

The enthalpy which is determined using ITC is quite precise, but is like many other scientific techniques, prone to error. The \( \Delta H \) determined is referred to as \( \Delta H_{\text{obs}} \), where \( \Delta H_{\text{obs}} = \Delta H_{\text{bind}} + \Delta H_{\text{conf}} + \Delta H_{\text{ion}} \). This is important because changes in \( \Delta H_{\text{obs}} \) may not only be due to the binding of the biomolecules under study (and the rearrangement of non-covalent bonds between the biomolecules), but might also be due to the effects of solvent and co-solute interactions in the free and bound states. \( \Delta H_{\text{ion}} \) is important when using such interactions that show pH dependence are studied and is related to the heat of ionization. Much structural data can be obtained by using this information to determine the \( pK_a \)'s of the free and bound states and relating the ionization enthalpy, which can then be compared to literature values to assist in determining some of the structure of a protein and the amino acid groups that take part in binding events (O'Brien 2001). Finally, in mixed lipid systems, lipid mixing/demixing will be associated with \( H_{\text{mix}} \).

Lipid-peptide interactions, unlike many chemical interactions that are studied today, do not form or break covalent bonds. Rather, a peptide which becomes associated with a membrane first does so as an adsorption process, possible because most peptides will carry a positive charge while the membrane usually carries a negative charge (this is
not always the case, and different models have been formulated to account for the partitioning of charged and uncharged peptides into the membrane. The penetration of particular peptides into the membrane is thought to be caused by the “hydrophobic effect”, which is driven by entropy requirements, and may include a change in the conformation of the peptide. Usually, when a peptide is integrated into the membrane, it takes on an alpha helical conformation, but can also aggregate to form structures that resemble beta sheets. The study of the interactions between peptide and lipid can be carried out in two ways. First, the lipid vesicles can be the sample, into which the peptide would be titrated. In this case, the lipid amount will strongly exceed the amount of peptide, which will allow $\Delta H$ to be determined (it is assumed for each titration step that

![Diagram of peptide binding to lipid membranes]

**Figure 2.6. Two-step binding for peptide binding to lipid membranes.** Represented are different modes of insertion of peptides into lipid membranes (Seelig 1997).
all peptide will bind). Second, the peptide can be placed in the sample cell and the lipid vesicles will be titrated into this until all peptide is bound, i.e. a binding curve as shown in Fig. 2.5 is obtained. In order to correct the \( \Delta H \) which is calculated from the first of these experiments, the peptide can be titrated into buffer (the same as that which is injected into the reference cell) and the heat of dilution can be subtracted from the initial \( \Delta H \) in order to calculate the final \( \Delta H \). The \( \Delta H \) can also be determined using the second type of experiment, and the two calculated \( \Delta H \) values should be the same (Seelig 1997). As mentioned before, this can then be manipulated to find the values of \( \Delta G \), \( \Delta S \), \( K_B \) and \( \Delta C_p \).

Using this technique, it is possible to determine whether the binding equilibrium is a single, fast binding step, or if there are multiple state binding equilibria, which would mean that a conformational change occurs or there is a clustering or a phase transition of lipids. Titration calorimetry is also able to detect pH shifts at the membrane surface and changes in pK’s of the peptides when binding to a lipid. All of these physical characteristics are important in understanding how proteins (or peptide portions of them) interact with membranes of cells in the body (Seelig 1997).

2.4.2) Aim of Isothermal Titration Calorimetry Measurements

While it is possible to characterize the binding of a protein to lipid vesicles using fluorescence techniques, and the binding constants of those interactions can be determined from the information gathered using that technique, it is not possible to gather all of the thermodynamic parameters associated with the binding event. Using ITC allows
us to use lipid vesicles that have not been altered in any way for the study of binding to the proteins of interest. Furthermore, ITC gives information about the enthalpy and entropy changes associated with the binding event, but more importantly can be used to find the stoichiometry of the protein/lipid binding event. This aspect makes ITC a particularly useful tool for us, especially in the study of mutant proteins, which may cause the loss or addition of binding sites for particular lipids.

2.4.3) Isothermal Titration Calorimetry Materials and Methods

ITC experiments were carried out using a MicroCal VP-ITC Microcalorimeter (Northampton, MA). Unilamellar POPS vesicles were produced as previously described, using DLS to test for the correct size. A 3mM solution was then titrated into a 1.5μM solution of protein in pH 7.0 HEPES buffer (10 mM Hepes, 100 mM NaCl, 0.1 mM EDTA). All experiments were carried out at 25°C, over the course of 40 separate injections, of 5μL each. The duration of each titration was 15s, using 900s spacing. The initial delay used was 4000s and stir speed was set as 300 rpm. The protein was dialyzed against pH 7.0 buffer to remove interfering DTT using Pierce dialysis cartridges with a MWCO of 10,000 (Fisher Scientific, Chicago, IL). Protein concentration was confirmed by UV-VIS spectroscopy using a SPECTRAmax spectrometer (Molecular Devices, Sunnyvale, CA). DLS was used to ensure the integrity of the lipid vesicles after titration.
2.5) **Transmission Fourier Transform Infrared Spectroscopy**

2.5.1) **Introduction to Fourier Transform Infrared Spectroscopy**

It is well known that molecules are constantly undergoing many types of movement, regardless of its state of matter, given that the temperature is not absolute zero. Study of different movements of molecules can be exploited in order to determine the structural differences between many kinds of molecules. Vibrational spectroscopy is one such method, which takes advantage of the non-translational and non-rotational movements that molecules constantly undergo. It has long since been established that particular types of bonds vibrate in very characteristic ways, making the investigation of

![Figure 2.7. Comparison between the ordered and disordered acyl chains and its effect on the methylene stretching vibration.](image)

The hydrogen that moves into the C-C-C plane is marked in red.
those bonds within a molecule a useful tool for probing not only the chemical composition of a sample, but also the conformation of larger molecules by monitoring the characteristic bands associated with the functional groups of said molecules.

Vibrational spectroscopy is a type of absorption spectroscopy; a molecule is excited by absorbing light energy, and the energy required to excite a particular molecule is related to its frequency through the equation $E=\hbar \nu$, where $\hbar$ is Planck’s constant. There are many types of vibrational transitions which can occur, which are expressed with respect to the initial vibrational state of the molecule being excited. For example, a fundamental transition is defined as the absorbance of one quantum of energy by a molecule in its ground state. Additional quanta may be absorbed which will lead to overtone bands. Infrared spectroscopy is typically used to study vibrational transitions, because the energy required to vibrationally excite most molecules corresponds to the infrared region of the spectrum, however, other techniques, such as Raman spectroscopy, can also be used to probe the vibrations of molecules, but requires a change in the overall polarizability of a molecule. For example, we are interested in the thermotropic behavior of different lipid species. The acyl chains of lipids are long hydrocarbon chains with several methylene groups which can exist in different conformations. The presence and ratio of gauche and trans conformers can be used as an indicator of the conformation of the lipid molecule, and gives information as to the state of the packing of the lipids with regard to one another. By probing the CH$_2$ symmetric or antisymmetric stretching modes and the temperature-induced changes of the position of these bands, we are able to investigate the order-disorder transition of the lipid bilayer. The conformational melting
of the fatty acyl chains which are packed in a hexagonal lattice in the gel phase results in a liquid crystalline phase, which is a more fluid phase characterized by more gauche conformers preventing tight packing of the lipid molecules. It should be noted that there are other vibrational modes which can be used as a probe of conformation of lipid molecules, as well as to investigate the extent of hydrogen bonding and hydration of headgroups, such as the CH$_2$ scissoring and wagging modes, the phosphate stretching modes, and the carbonyl stretching mode (Casal 1987; Blume 1988).

FTIR has been extensively used to characterize the thermotropic behavior of

![Figure 2.8. Comparison of FTIR results for the phase transition of DPPC and DPPC-d$_{62}$ multilamellar vesicles at pH 7.4](image)

Left: Infrared spectrum and corresponding 2nd derivative in red. Right: Methylene stretching band frequencies vs. temperature
many lipid molecules in pure systems (and to study the interactions of various ions with these lipids), and has been proven a useful and robust technique for characterizing the effect of headgroup and acyl chain composition on the transition temperatures of lipid systems. It can also be used to investigate mixed lipid systems. This technique is a reliable way of reproducing data that may be gathered about mixed lipid systems by using Differential Scanning Calorimetry, by giving information about the effects of the presence of each lipid on the other’s transition temperature. Importantly, the technique can also be exploited to gain information about the state of mixing of the system. To do this, one takes advantage of the isotope effect on the characteristic modes of the molecule’s functional groups. Particularly, the hydrogen atoms of a methylene group can be replaced by deuterium, and the resulting CD$_2$ stretching modes give rise to bands in different spectral regions than do the CH$_2$ stretching modes (about 2854 and 2193 cm$^{-1}$ for the antisymmetric modes, respectively). In this way, it is possible to use two separate lipid components, one of which has been labeled with deuterium atoms, and analyze the thermotropic behavior of each lipid component of the mixture separately. The resulting band position versus temperature plots can then be overlaid and differences in the behaviors of the two lipids will give information about the extent of mixing of the two lipid components. While DSC provides thermodynamic data but can usually not distinguish between individual lipid components, FTIR makes the investigation of each lipid component and not only the entire lipid system possible and gives greater information about the presence of lateral heterogeneity in binary lipid system.

After the initial spectra of the lipid samples are obtained, it is necessary to
calculate the second derivative of each spectrum, which is followed by peak picking of the CH$_2$ and CD$_2$ bands. The use of second derivative spectra makes the peak picking much more robust than is found for the original spectra. The position of the CH$_2$ and CD$_2$ stretching modes is dependent on the conformation of the fatty acyl chains of the lipid molecules, and will shift to higher wavenumbers with an increasing number of gauche conformers present within the chains. Using this, we can correlate the temperature with the positions of the bands of the CH$_2$ and CD$_2$ stretching modes in order to determine the transition temperatures of each of the lipids within the system. As previously stated, the thermotropic behavior of each lipid component is then compared to determine the extent of demixing which occurs in the sample.

2.5.2) Aim of Transmission FTIR Spectroscopy Measurements

Utilizing FTIR Spectroscopy allows us to confirm that DMPC/DPPI demixing can occur in the absence of protein. These data will also confirm studies conducted using DSC, allowing us to determine the transition temperatures of the mixture of DMPC and DPPI while affording a luxury that DSC cannot provide; we use FTIR to monitor the phase behavior of each lipid component in the mixture separately, in order to detail the effects of the presence of one lipid on the thermotropic behavior of the other. These data will help to give a greater understanding of the dynamics of the interactions of the two lipids over a range of temperatures and lipid compositions.
2.5.3) Fourier Transform Infrared Spectroscopy Materials and Methods

Lipid stock solutions were prepared as previously described, however, IR transmission experiments can be carried out using multilamellar vesicles and thus extrusion is not necessary. Because both of the lipid components used contained saturated acyl chains, the temperature of the lipid solution had to be kept above the transition temperature of the lipid mixture. During the process of making the vesicles for all mixtures, the lipid solutions were kept above 55°C during vesicle formation. Due to the nature of the experiment, one of the lipid components must be deuterated, in order to be able to separately observe the phase behavior of the two lipid components. Thus, we have used DPPI and a deuterated form of PC, DMPC-d54. After the lipid solutions and vesicles were prepared, the 30µL sample was placed between BaF2 salt plates, using a 25 µm Teflon spacer. After being placed within the Bruker Tensor Spectrometer (Billerica, MA) housing unit, the sample holder is connected to a Haake water bath which is controlled by a computer program. The sample is first allowed to equilibrate at 70°C for 6 hours, then is brought down to 2°C. Upon reaching this temperature, the temperature is then raised 0.5°C and a spectrum is obtained. This is repeated until the temperature reaches 35°C (i.e. until the gel/liquid crystalline phase transition is completed). The temperature is then raised 1°C between each spectrum collected, until the final temperature of 90°C is reached. The spectra are then processed using OPUS software by obtaining the second derivative of each of the spectra followed by peak picking, at 5% sensitivity.
2.6) Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy

2.6.1) Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy

Introduction

Infrared spectroscopy, particularly Attenuated Total Reflectance Infrared Spectroscopy (ATR) is a technique that is often used to study the secondary structure of proteins, especially in the presence of lipids (Corbalan-Garcia 2003; Tatulian 2003). ATR uses an evanescent wave, in which the infrared light is passed through an internal reflection plate which usually consists of germanium or ZnSe, and is reflected at least once (see Fig. 2.9). The resulting evanescent wave, the total internal reflectance, extends by at least a few microns into the sample, which is much greater than the size of even very large proteins. The method is also ideal for use with lipid systems because the use of infrared light prevents the interference of light scattering which occurs in circular dichroism spectroscopy (Tatulian 2003).

Study of protein structure using ATR requires that the protein be dialyzed against

Figure 2.9. Schematic diagram of ATR-FTIR. Shown is the resulting evanescent wave and its penetrance into the sample (Perkin Elmer 2005).
a D$_2$O buffer, as the H$_2$O scissoring mode ($\delta$(H$_2$O)) interferes with our analysis of the bands that occur due to vibrational transitions in the protein, most notably the amide I band. While amino-acid side chains also absorb infrared light and can provide information about the proteins, there are two regions in which proteins most strongly absorb the infrared energy that can be used a probe of the protein’s conformation. The amide I band, which appears at about 1600-1700 cm$^{-1}$, and the amide II band, which occurs from about 1500-1575 cm$^{-1}$. The amide I band is more sensitive to changes in secondary structure of the protein and is thus more often used as an indicator of structural changes within proteins of study. Additionally, it is important that the amide I band is not affected to any great extent by the identity of the amino acid side groups of the protein.

The use of the amide I vibrations to study the secondary structure of protein is possible because different secondary structures give rise to bands which occur at different frequencies. The amide I band is sensitive to the conformation of the protein because its existence is due to the vibrations that occur in the amide backbone of the protein. These vibrations occur differently when the protein is in different conformations, which causes a shift of this amide I band. The carbonyl stretching vibration of the protein contributes most greatly to the amide I vibration, and the out-of-phase CN stretching vibration, CCN deformation and the NH in plane bend also contribute to this absorption band (Barth 2002). The assignment of structure can still be quite complicated, though, as the frequencies associated with these secondary structures are not strict and some of the bands that are associated with different structural elements of the proteins overlap.

Though ATR-FTIR spectroscopy is not an ideal technique for assigning the
secondary structure of proteins in absolute terms, it is an excellent tool for monitoring changes in protein secondary structure, especially in the presence of lipid bilayers, which is very difficult if not impossible, using other comparable techniques.

2.6.2) Aim of ATR-FTIR Measurements

ATR-FTIR measurements of the protein samples of interest are used to determine whether a conformational change accompanies the interaction between the protein and lipids we are studying. Studies of PTEN’s tryptophan fluorescence upon addition of lipid vesicles suggested that structural changes are occurring, but that technique is not able to give information as to what types of conformational changes are occurring. Using ATR-FTIR spectroscopy, we can confirm and identify membrane induced conformational changes that have been suggested based upon results from other methods. In particular, we are able to carry out these experiments for a myriad of PTEN proteins, including all the mutant proteins of interest. Additionally, study of the mutant proteins in the absence of lipid vesicles can be compared to the wild type protein’s infrared spectrum to confirm proper folding of the mutant proteins of interest.

2.6.3) ATR-FTIR Spectroscopy Materials and Methods

ATR-FTIR experiments were carried out using a Bruker Tensor Spectrometer (Billerica, MA) equipped with a narrow band MCT detector and a Bruker BioATRII unit. Interferograms were collected at 2 cm$^{-1}$ resolution (512 scans, 20°C), apodized with a Blackman-Harris function, and Fourier transformed with one level of zero-filling to yield
spectra encoded at 1 cm\textsuperscript{-1} intervals. Protein samples were concentrated using 10,000 MWCO Centricon tubes obtained from Fisher Scientific (Chicago, IL), reaching a concentration of 8.33mg/ml. Protein samples were then exchanged against D\textsubscript{2}O buffer (10mM TRIS, 100mM NaCl, 10mM DTT) at room temperature as described above, using Pierce dialysis cartridges with a MWCO of 10,000 (Fisher Scientific, Chicago, IL). Dialysis against buffer was done in three steps, each 20 min in length. 125µg of the D\textsubscript{2}O exchanged protein sample was placed in the ATR unit and the protein only spectra were analyzed using Bruker OPUS software. Mixed multilamellar lipid vesicle/protein samples were obtained by resuspending the appropriate lipid mixtures in a protein solution. IR spectra of this kind do not require the use of unilamellar vesicles, because the protein will also be present in the water space between the layers of the MLVs. Lipid samples varied, but followed the form 95 mol% POPC, 5 mol% PI(x,y,z)P\textsubscript{n}, or 90 mol% POPC, 5 mol% POPS, and 5 mol% PI(4,5)P\textsubscript{2}. The protein-bearing samples included 1 mole protein per 8 moles of phosphoinositide lipid. After adding the protein solution to the dried lipid samples, they were vortexed for 60s, three times, waiting approximately 5 min between vortexing cycles. The resulting solution of protein and multilamellar vesicles was placed in the BioATR II unit, spectra were obtained and then analyzed using Bruker OPUS software. The vesicles did not form an anisotropic, ordered film on the ATR crystal but remained isotropic (which was checked by IR measurements using polarized radiation). We obtained buffer spectra using the buffers against which the proteins were exchanged. The D\textsubscript{2}O buffer samples were adjusted with respect to their H\textsubscript{2}O (HOD) content so that the intensities of the H\textsubscript{2}O and HOD bands matched between the respective protein and
buffer solutions. Subsequently, the buffer spectra were subtracted from the protein/(lipid) samples to yield a flat baseline between 1600 – 1900 cm\(^{-1}\). All subtraction values were 1.0000\(\pm\) 0.0005. The resulting spectra were exported to Origin software, where the lipid/protein spectra were normalized to the protein only spectrum, using the maximum value of the amide I band (\(\approx 1638\, cm^{-1}\)).

2.7) Zeta Potential

2.7.1) Introduction to Zeta Potential

Zeta potential is the potential at the solid-liquid interface, and has become a very important parameter in the study of colloidal suspensions. In aqueous colloidal suspensions within an aqueous suspension, a net charge density exists which causes the

Figure 2.10. Schematic representation of zeta potential. Shown are the Stern Layer and Diffuse double layer and their relationship to zeta potential (Malvern Instruments 2005).
formation of an electrical double layer, or a shielding layer, as counterions in solution will preferentially localize with the oppositely charged surface of the particle. In the Gouy-Chapman-Stern model, the electrical double layer consists of two layers, a diffuse layer and a Stern layer. The Stern layer refers to the layer of counterions which concentrate at the surface of the charged particle. The measurement of the zeta potential is helpful in quantifying the electrical charge which exists at this double layer, but is not equal to the Stern potential or electric surface potential. A boundary exists in the diffuse layer where ions and particles are stable and upon treatment with an electrical field, the ions in the boundary move the particle, and the potential at this boundary is the zeta potential. Essentially, the zeta potential can be described as the potential difference between the dispersion medium and the stationary layer of fluid associated with the particles of interest (Kirby 2004).

Zeta potentials were determined by measuring the electrophoretic mobility of the respective lipid vesicles in the presence of an electrical field, the net density charge which gives rise to the electrical double layer is also responsible for an electroosmotic response. The charge density creates an electric field which causes the oppositely charged counterions to be drawn to the particle. The potential and ion concentration in the diffuse portion of the electrical double layer lead directly to electroosmosis and are thus the most important parameters in this type of study. Overall, the application of an electric field causes the particles, which are highly charged, to move in the sample cell based on their associated charges, potentials, and concentrations of counterions. The electroosmotic mobility of the particles can be monitored and related to the zeta potential, which is
essentially a measure of the stability of the colloidal particles. Generally, the greater the magnitude of zeta potential of a suspension, the more stable it is, while the closer the value is to zero, the less stable the suspension and flocculation becomes more likely. Typical zeta potential values of a stable suspension are +/- 30mV. Comparison of the zeta potential of like molecules can also give insight as to the distribution of charge on the surface of the particles of interest, which is the objective for this study.

2.7.2) Aim of Zeta Potential Measurements

The zeta potential measurements that we carried out in the lab were first done using a DPPC/DPPG system. This allowed us to check the accuracy of the method by reproducing data presented in a technical note from the manufacturer of the instrument we were using, to be sure that our method of measurement was satisfactory. We then wished to move to a similar system with unsaturated chains to determine whether the

Figure 2.11. Representation of a folded capillary cuvette used in measuring zeta potential. (Malvern Instruments 2005)
chain composition of the lipids of interest would have an effect on the measured zeta potential. This is necessary background information for our future studies. Ultimately, the aim of using zeta potential in conjunction with our study of lipid bilayers was to test whether bisphosphate phosphoinositides, which have the same number of charges in different positions on their inositol headgroups, would have different surface charge densities, which could perhaps account for differences in binding affinities of proteins to these bisphosphate phosphoinositides. Additionally, we wished to study the effects of cholesterol on the surface charge density, in order to better understand the effects of cholesterol on protein binding to membranes containing phosphoinositides, and to determine if the presence of cholesterol would have an effect on the surface charge densities of each of the bisphosphate phosphoinositides.

2.7.3) Zeta Potential Materials and Methods

A solution of unilamellar lipid vesicles was formed as previously described. In preliminary experiments, lipid vesicles were made using varying percentages of the saturated lipids DPPC and DPPG, and all samples used contained a total of 0.5 mg of lipid. Because the saturated lipids must be above their transition temperatures in order to form vesicles, upon rehydration the lipid suspensions were kept above 55°C between cycles of vortexing. The lipid suspensions were kept at this temperature for 10 minutes rather than 4 minutes to ensure that the samples’ temperatures reached 55°C and were equilibrated before removing them from the water bath. Furthermore, extrusion of these lipid vesicles to obtain ~100nm unilamellar samples was carried out in a water bath
which maintained a temperature of 70°C. The fabrication of POPC/POPG multilamellar vesicles and their extrusion can be carried out at room temperatures because the gel/liquid-crystalline phase transition temperature is significantly lower due to the unsaturated acyl chains. However, aside from the temperature, samples were prepared in the same form, following the same percentages and totaling 0.5 mg of lipid in each separate sample. In the study of each of the bisphosphate phosphoinositides, the vesicles followed the form 95% POPC, 5% DOPIP2 and 55% POPC, 40% cholesterol, 5% DOPIP2. DLS of the lipid samples were taken before and after the zeta potential measurements to ensure that the integrity of the lipid vesicles was maintained.

A disposable folded capillary cuvette purchased from Malvern Instruments was rinsed with the same buffer in which the lipid sample was suspended, taking care not to scratch any surface of the cell. The lipid sample was then injected via syringe into the folded capillary cell and luer tips were placed within the openings of the cuvette. The cuvette was placed within the Nanosizer instrument (Malvern Instruments, Southborough, MA) and five runs were completed, each consisting of 100 scans using the 40V setting. Only runs exhibiting a satisfactory phase plot were used for analysis. Due to the high salt content of our buffer, each cuvette was only used about 3 times and samples were not able to be retained for further analysis as corrosion of the electrodes had occurred. The size of the vesicles was recorded using DLS before and after each experiment to ensure the integrity of the vesicle samples.
2.8) Differential Scanning Calorimetry

2.8.1) Introduction to Differential Scanning Calorimetry

Differential Scanning Calorimetry is a technique which measures the excess apparent specific heat during a controlled change in temperature. This is particularly useful in investigating biomolecules and biologically relevant structures as these molecules are often stabilized by many different types of weak forces, such as hydrogen bonding, which can be defeated, and thus the molecules undergo conformational or transitional changes based on heating or cooling of the system (Chowdry 1989).

The instrument consists of two cells which are found within an adiabatic jacket: a sample cell, in which the molecules of interest are placed, and a reference cell, in which the dispersant used to suspend the molecule of interest is placed. Both cells are then heated in a continuous manner over a temperature range defined by the user. When a process that is temperature dependent takes place in the sample cell, such as a phase transition in a lipid system, the calorimeter will have to supply either more or less electrical power to the sample cell in order to maintain the same temperature as the reference cell. The system will have to provide more power to keep the temperature the same as the reference cell if the process occurring within the sample cell is endothermic and will provide less power to the sample cell if the process is exothermic. Typically, the difference in temperature between the reference and sample cells is detected by a thermopile/thermocouple system, which will then activate appropriate heaters to maintain the temperatures of the cells. The calorimeter then records the difference in power that was supplied to the reference and sample cells as a function of the temperature. This
difference in power supplied to each of the cells is proportional to the heat capacity of the sample.

Many thermodynamic parameters of a particular system can be determined by the information gathered from DSC, such as the calorimetric enthalpy, $\Delta H_{\text{cal}}$, the van’t Hoff enthalpy, $\Delta H_{\text{vH}}$ and the transition temperature, $T_m$. These parameters can then be associated with the conformational or transitional changes occurring in the sample system (Chowdry 1989). Of particular interest in this study is the phase transition temperature, as it allows us to determine how the phase transition temperature of one lipid is affected by the presence of another lipid component. By determining the transition temperatures of a system of mixed lipids over a range of ratios of the lipid components in the mixture, one can construct a phase diagram of the lipid system in question. This information allows us to examine the miscibility of the two lipids in the system; that is to say, we can use this technique to probe the presence of lateral heterogeneity within a mixed lipid system. While we can only gather transition temperature information about the system as a whole using this method, one particular advantage of this technique is that neither of the lipid components need to be labeled in any way for us to gather this information. Additionally, this technique can be complemented by using other techniques, such as FTIR to study the thermotropic behavior of each lipid component of the system separately.

It should also be noted that the shape and width of the transition seen using DSC is important in the analysis of the data gathered. The solidus and liquidus phase boundaries are estimated by finding the onset and completion temperatures from the DSC thermograms for increasing ratios of the two lipid components, and $T_m$ values of the lipid
components can be used to construct lipid melting curves (Leidy 2001).

### 2.8.2) Aim of Differential Scanning Calorimetry Measurements

DSC was used in our study of lipid model membranes in order to help us determine whether or not a mixed lipid system of DMPC and DPPI would mix homogenously over a range of molar ratios. DSC is particularly useful in gathering this type of information because the technique does not require that any component be altered in any fashion (such as labeling, which is necessary for FTIR and fluorescence experiments). Using this technique allows us to gather information about the transition temperature of this lipid mixture over a range of different compositions of each lipid component; taken together, these data allow for the construction of a phase diagram and melting curves of the DMPC/DPPI system. Additionally, DSC is an uncomplicated method for determining the effects of ions such as Mg$^{2+}$ and Ca$^{2+}$ on the phase behavior of a lipid bilayer system. Of particular interest to us was how Mg$^{2+}$ and Ca$^{2+}$ would affect the phase transition temperatures of DPPI as well as the mixing behavior of DPPI/DMPC bilayer systems. These data enable us to examine how these ions interact with this type of bilayer and how these interactions interrupt intermolecular hydrogen bonding between lipid molecules and the extent of hydration of the lipid molecule headgroups (Casal 1987).
2.8.3) **Differential Scanning Calorimetry Materials and Methods**

Lipid stock solutions were prepared as previously described and lipid samples were prepared. The lipid samples were then either resuspended in buffer (10mM HEPES, 1mM EDTA, 100mM NaCl) or in aqueous solutions of CaCl₂ or MgCl₂. Because the lipids used contained at least one saturated lipid, the temperature of the solution was kept above 55°C during vesicle formation. Due to the nature of the experiment, unilamellar vesicle formation is not necessary, and thus extrusion of these samples was not performed. The lipid sample was then injected into the MicroCal VP-DSC (Northampton, MA) microcalorimeter’s sample cell, while the solution in which the lipid sample is suspended (buffer, CaCl₂ or MgCl₂) was injected into the reference cell of the machine, using Hamilton (Reno, NV) syringes. The pressurized cap was placed over the cells, and the pressure was monitored. A pressure of at least 42 psi is necessary to be sure that bubbles do not exist within the sample. The solidus and liquidus phase boundaries are estimated by finding the onset and completion temperatures from the DSC thermograms for increasing DMPC/DPPI ratios and used to construct a phase diagram as described previously (Leidy 2001).
CHAPTER 3: BINDING OF PTEN TO MODEL MEMBRANES

3.1) Characterization of Binding of PTEN to Lipid Model Membranes

PTEN is known to bind to pure phosphatidylcholine membranes, but has been found to bind more strongly to membranes which contain anionic lipids. PTEN contains several different domains, and it is thought that the C2 domain may serve to bind to anionic lipids in order to position the active site of the protein to the membrane to aid in the turnover of its substrate, PI(3,4,5)P$_3$ (see Figure 3.1). It has also been suggested that the PDZ domain of the protein may serve to bring the protein into proximity of its lipid substrate (Vazquez 2000; Vazquez 2001). It is thought that PTEN may undergo conformational changes upon binding to phospholipid membranes, but the changes that occur within the protein are not known at this time. It has been observed by several groups that the addition of PI(4,5)P$_2$ to a lipid membrane will increase the rate of turnover of its substrate, PI(3,4,5)P$_3$ (Campbell 2003). The N-terminal end of the phosphatase domain has been identified as the PI(4,5)P$_2$ binding motif. At the onset of our studies, it was highly controversial whether the binding of PTEN’s N-terminal end to PI(4,5)P$_2$ simply enhances PTEN binding and therefore activity or whether in addition to increased binding, PI(4,5)P$_2$ also induces a conformational change that leads to activation. For example, it has been hypothesized that the N-terminal domain is folded in such a way that it may block the active site of the protein. Upon binding to a membrane containing PI(4,5)P$_2$, the N-terminal domain is thought to then move away from the active site of the protein, allowing access of the substrate to the active site and
thus increasing the rate of its turnover. Interestingly, other bisphosphate substituted phosphoinositides do not increase the rate of turnover as PI(4,5)P₂ does. We hypothesize that PTEN’s N terminal domain binds specifically to PI(4,5)P₂ over other phosphoinositides and that PTEN will also bind synergistically to anionic lipids such as phosphatidylserine, due to the binding site within the C2 domain. We also hypothesize that binding of PTEN to PI(4,5)P₂ results in conformational changes that may be responsible for the increase in turnover rate of PI(3,4,5)P₃ (see Figure 3.1). In order to test these hypotheses, we have carried out a broad array of studies, utilizing fluorescence
spectroscopy, Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR), and Isothermal Titration Calorimetry (ITC).

First, we will study the specificity of PTEN binding to membranes containing differently phosphorylated phosphoinositide derivatives. In order to do this, we made vesicles that were comprised of 99.9% POPC and 0.1% of a Bodipy labeled PI-x,y,zP_n, as described in the materials and methods section. The fluorescence emission spectra of the vesicles were repeatedly collected and protein was titrated into the solution until a ratio of 1:2 protein to lipid ratio was reached. Each experiment was repeated a minimum of three times. If interacting extensively with protein, the lipid molecules are thought to be

![Figure 3.2. Raw data of bodipy quenching experiment. PTEN protein is titrated into vesicles comprised of 99.9% POPC and 0.1% Bodipy PI-4,5P_2 (▲), Bodipy PI-3,4P_2 (♦) and Bodipy PI-3,5P_2 (●). Decrease in overall fluorescence intensity indicates PTEN binding and suggests the possibility of resulting domain formation.](image)
brought within close proximity of one another in order to bind to the protein. It has been shown previously that binding of positively charged proteins/peptides leads to a clustering of the Bodipy labeled phosphoinositides which results in a reduced fluorescence intensity due to self quenching (Epand 2004; Ghambir 2004). It is not known at this time whether phosphoinositide enriched domains in cell membranes usually form before binding to protein and thus target the proteins to them or whether the protein binding to multiple lipid molecules is responsible for the formation of these domains, but it is thought that both mechanisms may play a role in the formation of some domains. Upon titrating the full length PTEN into these vesicles solutions, it became clear that PTEN bound preferentially to PI(4,5)P2 over other lipids, as the total fluorescence decreased to less than 80% of its initial value (See Figs. 3.2 and 3.3). Interestingly, PTEN also bound somewhat more preferentially to PI(5)P over the other phosphoinositides. It has been found that PI(5)P is also an activator of PTEN, though to a lesser extent than PI(4,5)P2. PTEN did not bind well to PI(3,4,5)P3, even though this lipid is the protein’s natural substrate. This may be due to the fact that because we are using vesicles, the protein’s active site is not available for binding to the substrate because the N-terminus may be blocking that site.

Because the binding of PTEN proved to be specific for PI(4,5)P2 over other phosphoinositides, including the other bisphosphates and trisphosphate substituted phosphoinositides, the evidence is clear that binding of this protein is not governed by electrostatic interactions alone. Though electrostatic interactions most likely play some role in the binding of PTEN to these model membranes, if the binding event were not
specific, all of the bisphosphate substituted phosphoinositides would have decreased in fluorescence intensity by about the same amount, and fluorescence intensity of the Bodipy PI(3,4,5)P₃ containing vesicles would have decreased the most. In other words, PTEN binding to phosphoinositide containing vesicles would increase with increased charge density: PI(x)P < PI(x,y)P₂ < PI(x,y,z)P₃.

PTEN’s specific binding to PI(4,5)P₂ must be investigated further, as PTEN contains a PI(4,5)P₂ binding motif within its N-terminus. In order to further study the role of this domain in PTEN’s binding to phospholipids membranes, we purchased a peptide made up of the amino acids of PTEN’s N-terminus. We originally purchased the first 16 amino acids of the protein’s sequence, as this would just include the PI(4,5)P₂ binding domain. This peptide, however, proved very difficult to work with, as it surprisingly

Figure 3.3. Binding specificity of PTEN. Titration of protein into 99.9% POPC vesicles labeled with 0.1% Bodipy PI(x,y,z)Pₙ. Full length PTEN shows specific binding, preferring PI(4,5)P₂ and PI(5)P over other PIs (a). The N-terminal end of PTEN, PTEN₁₋₂₁, also shows preference for PI(4,5)P₂ (b). PTEN₁₆₋₄₀₃, lacking PTEN’s N-terminus shows a lack of preference for any phosphoinositide (c).
became quite aggregated in buffer solution, but remained in monomeric form when brought into a solution of water alone as studied using DLS. Therefore, we purchased PTEN1-21 (MTAIKEIVSRNKRYYQEDGF), in order to include the entire PI(4,5)P$_2$ binding domain and a few extra amino acids to prevent the aggregation which occurred in the PTEN1-16 peptide. This peptide was then titrated into vesicles that were labeled with 0.1% of the bodipy labeled phosphoinositide derivatives, and experiments were completed using all eight of the possible phosphoinositide derivatives in order to determine whether this peptide would bind with the same specificity as the full length protein. We found that the PTEN1-21 peptide also bound specifically to PI(4,5)P$_2$, as the fluorescence intensity of the bodipy labeled PI(4,5)P$_2$ decreased to about 87% of its original intensity, as shown in Figure 3.3. In contrast, for the other phosphoinositide derivatives the fluorescence intensity dropped by less than 5 percent. The decrease observed for PI(4,5)P$_2$ is not as great as the decrease in the fluorescence intensity of the same labeled phosphoinositide titrated with the full length protein, but this may be due to the size of the protein added and the number of lipid molecules it is then able to interact with. It is important to observe that the PTEN1-21 peptide did not bind to any significant extent to any of the other labeled phosphoinositides (Figure 3.3).

To further study the specificity of PTEN binding to PI(4,5)P$_2$, we also created a mutant PTEN protein which lacked the N-terminal sequence, in order to completely remove the PI(4,5)P$_2$ binding domain (PTEN$_{16-403}$). This protein was titrated into vesicles which contained each of the eight labeled phosphoinositide derivatives in the same manner as the experiments conducted using full length PTEN and PTEN$_{1-21}$. As expected,
the PTEN\textsubscript{16-403} protein did not bind specifically to any of the phosphoinositide derivatives (Figure 3.3); the binding specificity was completely abrogated. Additionally, we saw that the protein did not bind very strongly to any of the other phosphoinositide derivatives.

Because of this specific binding of PTEN to bodipy labeled PI(4,5)P\textsubscript{2}, we wished to further investigate the binding of PTEN to lipid vesicles containing the natural unlabeled phosphoinositides as well as the binding to other lipids, such as phosphatidylserine. In order to this, we are able to take advantage of the two tryptophan residues that lie at positions 111 and 274 of the protein. It is possible to monitor the tryptophan fluorescence of the protein while titrating vesicles into the protein solution that are composed primarily of POPC, plus a certain amount of anionic lipid of interest, which is unlabelled. The respective lipid mixture also contains a small amount of phosphatidylethanolamine (PE) labeled with either a dansyl or pyrene group which forms a FRET pair with the tryptophan residues of proteins, and is used in the vesicles only as an acceptor for the energy transferred from the protein’s tryptophan residues. Upon binding of the protein to the lipid vesicles, the tryptophan residue will act as an energy donor, and its fluorescence intensity will decrease, while the fluorescence intensity of the labeled PE will increase. In this way, one would monitor only the fluorescence intensity of the protein’s tryptophan residues as a measure of the extent of binding to a lipid of interest, as the fluorescence intensity of the labeled lipid is often far too great to monitor concomitantly and increases continually with addition of the lipid vesicles.

We have carried out a number of experiments using this method, and have been able to use the result to determine the $K_d$ values of several different combinations of
PTEN proteins and lipids (Tables 3.1 and 3.2). Initially, we titrated vesicles into the protein that were composed of 2% pyrene labeled PE, and either 1%, 5%, or 10% brain lipids.

<table>
<thead>
<tr>
<th>LIPID</th>
<th>$K_d$ ($\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>585 ± 15</td>
</tr>
<tr>
<td>PC/PS</td>
<td>444 ± 15</td>
</tr>
<tr>
<td>PC/PI(4,5)P$_2$</td>
<td>163 ± 6</td>
</tr>
<tr>
<td>PC/PS/PI(4,5)P$_2$</td>
<td>41.4 ± 4.8</td>
</tr>
<tr>
<td>PS</td>
<td>22.0 ± 0.5</td>
</tr>
<tr>
<td>PI(4,5)P$_2$</td>
<td>8.14 ± 0.29</td>
</tr>
<tr>
<td>PS/PI(4,5)P$_2$</td>
<td>2.07 ± 0.24</td>
</tr>
</tbody>
</table>

Table 3.1. $K_d$ values of PTEN binding to mixed vesicles. $K_d$ values were determined by fitting the data collected using the Trp/pyrene PE FRET assay using the equation $y = (k[L])/(1 + k[L])$.

We found that the greater the amount of PI(4,5)P$_2$ that we added to the system, the greater the extent of binding between PTEN and those vesicles (Figure 3.4). The results obtained using 5% brain PI(4,5)P$_2$ were found to be optimal, considering the extent of binding and relative cost. Thus, we carried out the same experiments using vesicles that were composed of 2% pyrene labeled PE, 5% POPS, and 93% POPC. We observed that the extent of binding between PTEN and vesicles containing POPS was significantly less than the extent of binding between the PTEN protein and the equivalent amount of PI(4,5)P$_2$, as shown in Figure 3.4. We then increased the amount of POPS in the system to 10% and 20%, again varying the amount of POPC in the system while the pyrene labeled PE remained 2%. While the binding of the vesicles containing greater amounts of POPS increased the binding of PTEN to these vesicles, the extent of binding was still lower than that observed with PI(4,5)P$_2$.
membranes, the extent of binding did not reach the same level as those vesicles that contained both 5% PI(4,5)P₂. Because it is thought that PTEN may have two separate binding sites for these lipids (the N-terminal domain containing a PI(4,5)P₂ binding motif and the C2 domain containing a binding site for PS), we also used vesicles that were comprised of 5% PI(4,5)P₂ as well as 5% POPS. Incredibly, we saw a tremendous increase in the extent of binding between the protein and these vesicles, which was even greater than the amount of binding observed for the vesicles containing 10% PI(4,5)P₂ (i.e. vesicles that exhibit a higher surface charge density). This evidence suggests that PTEN does contain two separate binding sites for PI(4,5)P₂ and PS, and that these two

**Figure 3.4. Binding of PTEN to PI(4,5)P₂ containing lipid vesicles.** Tryptophan fluorescence lost to pyrene labeled PE through FRET was monitored using vesicles containing 2% pyPE and 93% POPC with 0% PI(4,5)P₂ (▼), 1% PI(4,5)P₂ (●), 5% PI(4,5)P₂ (▲) or 10% PI(4,5)P₂ (■). These results show that the greater the percentage of PI(4,5)P₂ in the system (the greater the charge density), the more strongly PTEN binds to the vesicles.
lipids do not compete for the same binding site. Based on our results, the binding of these two lipids may act synergistically to bring the protein to the membrane.

It should be noted that experiments were conducted to determine whether PS and PI(4,5)P\(_2\) colocalize within a membrane of this type or whether demixing between the two components occurs. This was done using the FRET experiment previously described with bodipy labeled PS or bodipy labeled PI(4,5)P\(_2\) and NBD labeled PC. The results of this experiment indicate that domain formation does occur in membranes that contain PS and PI(4,5)P\(_2\) (see Figure 3.6), which may affect the overall concentration of PI(4,5)P\(_2\) near PTEN, and could be a partial explanation for the enhanced binding of the protein to

Figure 3.5. Binding of PTEN to mixed lipid vesicles. Tryptophan fluorescence lost to pyrene labeled PE through FRET was monitored using vesicles containing 2% pyPE and 93% POPC with 5% PI(4,5)P\(_2\) (▲), or 5% POPS (●). This was also done with 88% POPC, and 5% PI(4,5)P\(_2\) with 5% POPS (■), or 98% POPC (♦). These results show that PTEN binds most strongly to vesicles containing both PS and PI(4,5)P\(_2\), and binds more strongly to vesicles containing PI(4,5)P\(_2\) than those comprised of only POPC and POPS. PTEN also binds to a lesser extent to pure POPC vesicles.
these membranes. It was found that PS mixes with the PC lipids, while PI(4,5)P₂ forms a domain.

In order to determine whether the effects of increased binding to PI(4,5)P₂ containing vesicles and vesicles that were comprised of both PS and PI(4,5)P₂ was due to an increase in the number of negative charges contained within the vesicle, we increased the percentage of PS to 25%, to compare with the results obtained for 5% PI(4,5)P₂ containing vesicles. When we increased the concentration of PS within the vesicles, there was a slight increase in the binding, but the amount of binding observed for PTEN to vesicles containing 25% PS was still not as great as that seen for PTEN binding to

**Figure 3.6. Demixing of POPS and Brain-PI(4,5)P₂ in POPC vesicles.** PI(4,5)P₂ and POPS are labeled with Bodipy and NBD fluorophores, respectively. Energy transfer from NBD-PS to Bodipy PI(4,5)P₂ is monitored by recording emission spectra of both fluorophores and comparing the ratio of the maximum intensity of their emissions over a range of pH values. This shows that the vesicles containing PS and PI(4,5)P₂ are demixed at high pH value, and mixes at lower pH value. At physiological pH, there is some extent of demixing present in the vesicles.
vesicles containing 5% PI(4,5)P₂. Interestingly, when the percentage of PS contained within the vesicle was increased to 25% in addition to the presence of 5% PI(4,5)P₂, the binding is decreased in comparison to the binding of PTEN to vesicles containing only 5% PS and 5% PI(4,5)P₂. While we have done studies to show that fluid/fluid demixing does occur between PS and PI(4,5)P₂ vesicles of equal composition, it is not known what

![Plot](image)

**Figure 3.7. Binding of PTEN to PI(4)P and PI(4,5)P₂ containing vesicles.** Tryptophan fluorescence lost to pyrene labeled PE through FRET was monitored using vesicles containing 2% pyPE and 93% POPC with 5% PI(4,5)P₂ (■), or 5% PI(4)P (●). These results show that though PTEN is not activated by PI(4)P, it binds significantly to vesicles containing 5% PI(4)P.

the effect of such great percentages of PS in excess over PI(4,5)P₂ would be for lipid organization in the membrane.

This type of experiment was found to be quite useful, as the $K_d$ values could be fairly easily determined and the extent of binding of the protein to different natural lipids
could be compared. We used other lipids as part of the vesicles to compare to the binding of PTEN to PI(4,5)P₂ and PS. Most interestingly, PTEN bound to vesicles containing 5% PI(4)P to nearly the same extent as to vesicles containing 1% PI(4,5)P₂, though this lipid does not act as an activator of the PTEN tumor suppressor (Figure 3.7).

The binding of PTEN to membranes is known to occur with any pure phosphatidylcholine vesicle and increases upon the addition of any anionic lipid. Therefore, we also investigated the effects of phosphatidylethanolamine on binding of PTEN to PC lipid vesicles. Not surprisingly, we did see enhanced binding to vesicles containing POPE, though the binding to this lipid is not as great as the binding to either PS or PI(4,5)P₂ alone. It should also be noted that the percentage of PE used in these vesicles was extremely high, 35% as compared to the 5% PS and PI(4,5)P₂ that are typically used in this type of experiment. While it may be rather difficult to interpret the binding, because it is not easy to separate the effects of each of the lipids when using more than a ternary system, we also investigated the binding of PTEN to vesicles that contain PC/PS/PE/PI(4,5)P₂. As can be seen in Figure 3.8, PTEN binding to vesicles of this composition is quite strong, but the effects of possible lipid demixing associated with the binding event also makes the interpretation difficult. It is possible that in such a mixture PI(4,5)P₂ domain formation is enhanced and that PTEN binding to these domains is increased due to the increase in local charge density, which could account for the slight increase in binding of the protein to these membranes. Despite the problems in interpreting these results, it is important to look at these effects, because the membranes in physiological systems contain a variety of phospholipids, which may result in a distinct
lateral membrane organization in the cell.

Overall, these results indicate that PTEN does bind well to vesicles that contain 5% PI(4,5)P$_2$. As part of these experiments, we were also required to carry these experiments out for vesicles that were not labeled with pyrene PE, in order to be able to account for the changes in Trp fluorescence that occurred as a result of a conformational change (or change in the Trp environment), rather than the interaction of the fluorescent tryptophan residues with the fluorescently labeled PE molecules within the vesicles. These studies showed that there were in fact some changes in the natural fluorescence of the protein upon titration with unlabeled lipid vesicles, which suggests that a conformational change may be occurring within the protein upon interaction with PI(4,5)P$_2$ and PS containing vesicles.

In an attempt to further exploit this very useful method, we also created mutant proteins, in which one of the two tryptophan residues (at positions 111 or 274) within the enzyme had been replaced with a phenylalanine residue. This substitution is ideal, because the phenylalanine residues will not interfere with the Trp fluorescence, but are still structurally very similar to the tryptophan residues, having a somewhat large hydrophobic ring structure as the side chain. We replaced the tryptophan residues systematically and carried out titrations using vesicles containing the pyrene labeled lipid and either PI(4,5)P$_2$, PS or a combination of the two. To our surprise, we did not see a decrease in the tryptophan fluorescence of either the PTEN$_{W111F}$ or PTEN$_{W274F}$ mutants. Though this was discouraging, because we had hoped to determine more closely where in the protein the lipid vesicles are docking and perhaps also determine where the
Figure 3.8. Relative fluorescence intensity of PTEN tryptophan residues titrated with PC/PS/PE/PI(4,5)P₂ containing vesicles. The extent of binding of PTEN to vesicles containing POPC, 38%; POPS, 20%; Brain PE, 35%; Brain PI(4,5)P₂, 5%; was monitored as a function of the tryptophan fluorescence intensity. Shown are experiments in which the vesicles contained either 0% pyPE (▲) or 2% pyPE (■). Results show that these vesicles bind well to PTEN, but it is likely that the protein undergoes large conformational changes which complicate analysis of the data.

Conformational changes might be occurring, we were able to determine that the protein’s fluorescence increased to almost 250 percent of its original value upon titration with the lipid vesicles, apparently the labeled lipid is not coming close enough to either of these buried tryptophan residues, however, the strong increase in fluorescence indicates a conformational change within the protein.
The same types of experiments were carried out using each of these tryptophan mutant proteins and titrating in vesicles which contain instead of a fluorescently labeled lipid, a brominated lipid molecule. Each of the brominated lipid molecules contain two bromine atoms upon their lipid chains, at either the 6 and 7, 9 and 10, or 11 and 12 positions of their acyl chains. Depending on how deeply the protein inserts into the membrane upon interaction, these differently brominated lipid molecules will quench the tryptophan fluorescence to different extents. For example, if the protein is inserting very deeply into the lipid membrane, the PC molecule which is brominated at the 11 and 12

![Graph showing the binding of truncated PTEN16-403 to lipid vesicles.](image)

**Figure 3.9. Binding of truncated PTEN\textsubscript{16-403} to lipid vesicles.** Full length PTEN (▲) and truncated PTEN (■) binding to lipid vesicles of POPC with 5\% PI(4,5)P\textsubscript{2} was measured by monitoring tryptophan fluorescence loss to pyrene labeled lipids (FRET). PTEN binding to POPC vesicles is shown for comparison (♦). These results show that PTEN\textsubscript{16-403} binds less strongly to vesicles containing PI(4,5)P\textsubscript{2} than does full length PTEN. Binding of the truncated PTEN to PI(4,5)P\textsubscript{2} containing vesicles is slightly stronger than binding of full length PTEN to pure POPC vesicles, due to the removal of PTEN’s N-terminal PI(4,5)P\textsubscript{2} binding domain.
positions of the acyl chain will quench the tryptophan fluorescence most efficiently, while the lipid molecule which is brominated at the 6 and 7 positions of the acyl chain will have the least effect. In this way, these brominated lipids can be used systematically to help determine how deeply a protein is inserted into a particular lipid membrane (Prieto 2007).

The results of these studies were similar to those of the pyrene labeled vesicles. For both of the tryptophan mutant proteins, none of the three brominated lipid molecules we used as a molecular ruler was able to quench the tryptophan fluorescence of the residues at positions 111 or 274. Again, this indicates that the tryptophan residues which

![Figure 3.10. Binding of PTEN_{K13E} to PI(4,5)P_2 containing vesicles. Binding of PTEN_{K13E} to PI(4,5)P_2 containing vesicles, as studied by monitoring loss of tryptophan fluorescence to pyrene labeled PE. PTEN’s binding to PI(4,5)P_2 containing vesicles is abrogated by the mutation of lysine at position 13, as depicted by black squares (■). Wild type PTEN binding to PI(4,5)P_2 (▲) and POPC (♦) vesicles is shown for comparison.]

are known to be somewhat buried and located at interfaces within the protein are not accessible to the fluorescence quenchers that we are using. The fluorescence of the mutated proteins again increased to nearly 250 percent of their initial values, again indicating that a conformational change is indeed occurring within these proteins upon interaction with membranes containing either PI(4,5)P$_2$, PS or a combination of the two. Additionally, it is known that one tryptophan in a protein molecule can transfer energy to another tryptophan molecule within a protein. This can be evidenced by mutation of a tryptophan residue within the protein and observing the changes in natural fluorescence of the protein. The differences seen in the fluorescence of the PTEN tryptophan mutants used in our studies suggest that the tryptophan molecules of the wild type PTEN may interact with one another (Loewenthal 1991).

Because we had found in other types of experiments that the PTEN$_{16-403}$ mutant no longer bound phosphoinositides specifically and that the binding to PI(4,5)P$_2$ seemed to be abrogated, it was desirable to quantitate the extent of binding of this protein to PI(4,5)P$_2$. Using the tryptophan residues which are still present within this protein, we were able to show that the binding of PTEN$_{16-403}$ is nearly the same as the binding of the full length protein to vesicles comprised of only PC (Figure 3.9). This further highlights the necessity of the presence of the N-terminal PI(4,5)P$_2$ binding domain in the specific binding of PTEN to PI(4,5)P$_2$. Unfortunately, because the peptide contained only one tyrosine residue, similar experiments to determine the $K_d$ of binding of the N-terminal derived PTEN$_{1-21}$ to vesicles containing PI(4,5)P$_2$ were unsuccessful (data not shown). Additionally, we studied the binding of PTEN$_{16-403}$ to vesicles that contained 5% POPS
rather than PI(4,5)P₂, which gave evidence that the protein bound to nearly the same extent as the wild type protein. This further supports our belief that the protein contains two separate and distinct binding sites for PI(4,5)P₂ and PS, and that these lipids do not compete for binding to either of these sites.

Our results suggest that the presence of the N-terminus is necessary for the binding of PTEN to membranes that contain PI(4,5)P₂, while not required for the binding of the protein to lipid membranes that contain PS. As such, we continued to study the binding of the N-terminus to vesicles containing 5% PI(4,5)P₂ and thus used another PTEN mutant that has been found to be quite important in the formation of tumors. This protein contains a single mutation of the lysine at residue 13 to a glutamic acid residue. This lysine is part of the putative PI(4,5)P₂ binding motif and is thought to be very important in the binding of the protein to PI(4,5)P₂. Thus, as a comparison for the PTEN mutant lacking the entire N-terminus, we conducted this same experiment using PTENK₁₃E protein. Figure 3.10 shows that the binding of PTEN to vesicles containing PI(4,5)P₂ is dependent on the lysine at position 13, as mutation of this protein has the overall same effect on binding of the protein to these vesicles as does removing the entire

Table 3.2. K<sub>d</sub> values of mutated PTEN proteins binding to vesicles containing PC, PS and PI(4,5)P₂. K<sub>d</sub>s were calculated by fitting data collected by using the tryptophan/pyrene PE experiment using the equation  \[ y = \frac{k[L]}{1 + k[L]} \].

<table>
<thead>
<tr>
<th></th>
<th>PC/PS</th>
<th>PC/PI(4,5)P₂</th>
<th>PC/PS/PI(4,5)P₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTEN&lt;sub&gt;16-403&lt;/sub&gt;</td>
<td>1211.5 ± 95</td>
<td>469.6 ± 14.2</td>
<td>643.9 ± 4.4</td>
</tr>
<tr>
<td>PTEN&lt;sub&gt;K₁₃E&lt;/sub&gt;</td>
<td>901 ± 118.7</td>
<td>501.9 ± 32.2</td>
<td>670 ± 71.2</td>
</tr>
<tr>
<td>PTEN&lt;sub&gt;H₉₃R&lt;/sub&gt;</td>
<td>90.6 ± 6.7</td>
<td>407.8 ± 20</td>
<td>45.2 ± 6.0</td>
</tr>
</tbody>
</table>

Our results suggest that the presence of the N-terminus is necessary for the binding of PTEN to membranes that contain PI(4,5)P₂, while not required for the binding of the protein to lipid membranes that contain PS. As such, we continued to study the binding of the N-terminus to vesicles containing 5% PI(4,5)P₂ and thus used another PTEN mutant that has been found to be quite important in the formation of tumors. This protein contains a single mutation of the lysine at residue 13 to a glutamic acid residue. This lysine is part of the putative PI(4,5)P₂ binding motif and is thought to be very important in the binding of the protein to PI(4,5)P₂. Thus, as a comparison for the PTEN mutant lacking the entire N-terminus, we conducted this same experiment using PTENK₁₃E protein. Figure 3.10 shows that the binding of PTEN to vesicles containing PI(4,5)P₂ is dependent on the lysine at position 13, as mutation of this protein has the overall same effect on binding of the protein to these vesicles as does removing the entire

Table 3.2. K<sub>d</sub> values of mutated PTEN proteins binding to vesicles containing PC, PS and PI(4,5)P₂. K<sub>d</sub>s were calculated by fitting data collected by using the tryptophan/pyrene PE experiment using the equation  \[ y = \frac{k[L]}{1 + k[L]} \].
N-terminus. The binding of this protein and other PTEN proteins that contain a mutation of the lysine at position 13 will be discussed in further detail in Chapter 4.

3.2) Conformational Changes of PTEN as a Result of Binding to Lipid Membranes

The addition of soluble PI(4,5)P$_2$ to soluble PI(3,4,5)P$_3$ substrate solutions (i.e. the solutions did not contain micelles or vesicles) used in activity assay by our collaborator showed that the addition of PI(4,5)P$_2$, but not the addition of the other bisphosphate phosphoinositide derivatives substantially increased the turnover of the lipid substrate PI(3,4,5)P$_3$ by PTEN. Because the protein contains a PI(4,5)P$_2$ binding motif and we found that the protein’s N-terminus regulates specific binding to lipid vesicles that contain PI(4,5)P$_2$, we hypothesized that the binding of PTEN to PI(4,5)P$_2$ may cause a conformational change that allows the N-terminus of the protein to swing away from the active site, allowing better access of the substrate, PI(3,4,5)P$_3$. In order to test this hypothesis, we have conducted a series of ATR-FTIR experiments to characterize the conformation of the protein in its bound and unbound states. While using this technique does not enable us to pinpoint the localization of the protein undergoing conformational change, it does allow us to determine an overall effect of the binding of lipid membranes on the secondary structure of the protein of interest.
Protein and lipid samples were prepared as described in Materials and Methods. The protein sample was applied to the ATR crystal and the cell was closed to prevent the absorption of H₂O from the atmosphere. Sample spectra were collected (512 scans), and corresponding buffer spectra were collected and subsequently subtracted from the protein spectra. The spectra were exported from OPUS and the files were then imported into Origin software for further analysis. The maximum of the amide I band spectra of the protein without lipid was found and the data for the spectra of the protein with lipid were normalized to this value by manual mathematical manipulation of the dataset within the

**Figure 3.11. Structural changes of PTEN in the presence of PI(4,5)P₂ containing vesicles.** Using Attenuated Total Reflectance Infrared Spectroscopy, the amide I band of PTEN is monitored in the presence of vesicles containing 5% PI(4,5)P₂ (—), or PTEN alone (—). PTEN adopts a more alpha helical conformation upon binding to PI(4,5)P₂ containing vesicles. Spectra were normalized to the protein only amide I band peak maximum.
Origin software. The baselines of the two spectra were also set to the same value in order to allow for comparison of the amide I bands. These manipulations allow us to overlay the spectra in a manner that is conducive to examining the somewhat subtle differences between the spectra without changing the actual shape of them at all.

The vesicles that were added to the protein samples were comprised of 95% POPC and 5% PI(4,5)P₂, which was 8 times the molar amount of protein in the sample. The overall concentration of the lipid in the sample is thus 8.33mM, so that the sample

**Figure 3.12. Structure of PTEN in the presence of PI(3,5)P₂ containing vesicles.** Monitoring the protein secondary structure by ATR-FTIR, we see that the structure of PTEN (—) does not change in the presence of 5% PI(3,5)P₂ containing vesicles (—). This helps to confirm that PTEN binds to PI(4,5)P₂ specifically, and that this interaction alone results in a conformational change. Spectra were normalized to the amide I peak maximum of the protein only spectrum.
has a very milky, white consistency. When we compared the conformationally sensitive amide I band of the PTEN protein alone with the corresponding band obtained for PTEN in the presence of vesicles containing 5% PI(4,5)P₂, we found a significant increase in the overall alpha helical secondary structure content of the protein (Figure 3.11). Adding vesicles comprised of 100% POPC did not alter PTEN’s secondary structure as can be seen from the amide I band envelope. In order to test whether this effect was specific to the PI(4,5)P₂ containing vesicles, we also used vesicles that were comprised of 95% POPC and 5% PI(3,5)P₂ in the presence of PTEN. Interestingly, we did not see any change in the secondary structure of the protein in the presence of PI(3,5)P₂ containing vesicles, as shown in Figure 3.12 (Redfern 2008).

It is known that wild type PTEN binds to PI(3,4,5)P₃, but it is not known to what extent this occurs in actual membranes. Because activity assays are carried out using the soluble substrate, it would be easy for the lipid to move past the N-terminus if it were blocking the active site of the protein so that the lipid could be turned over. Though we did not observe a great extent of binding between PTEN and PI(3,4,5)P₃ in other assays that we carried out (see section 3.1), we felt it necessary to determine whether the interaction of PTEN with vesicles that contained PI(3,4,5)P₃ would have an effect on the conformation of the protein. Using the same protocol as that used with the vesicles containing the bisphosphate substituted phosphoinositides, we examine the secondary structure of the protein in the presence of vesicles comprised of 95% POPC and 5% PI(3,4,5)P₃ (see Figure 3.13). Upon analysis of the spectra, we found that the interaction of PTEN with vesicles containing the trisphosphate phosphoinositide did not have an
effect on the overall secondary structure of the protein (Redfern 2008).

Our studies have shown that PTEN’s N-terminus is responsible for the specificity of binding to PI(4,5)P₂. Because we were able to show that the PTEN protein lacking the N-terminus does not bind to PI(4,5)P₂ containing vesicles, it was desirable to study the conformational effects of the interaction of PI(4,5)P₂ with PTEN₁₆₋₄₀₃ on the protein. Without the intact N-terminal PI(4,5)P₂ binding domain, the protein not only does not bind to the vesicles containing PI(4,5)P₂, but undergoes no conformational change upon interaction with those vesicles, as studied by ATR-FTIR (Figure 3.14). Additionally, studies of the conformational effects of PI(4,5)P₂ containing vesicles on the PTEN protein containing the mutation of lysine 13 to glutamic acid show a slight increase in alpha helical secondary structure content, which is much less pronounced than the effects

![Figure 3.13. Structure of PTEN in the presence of PI(3,4,5)P₃ containing vesicles.](image)

Monitoring the protein secondary structure by ATR-FTIR, we see that the structure of PTEN (—) does not change in the presence of 5% PI(3,4,5)P₃ containing vesicles (—). This is surprising, as PI(3,4,5)P₃ is PTEN’s specific substrate. Spectra were normalized to the amide I peak maximum of the protein only spectrum.
of PI(4,5)P$_2$ on the wild type protein. These results support our hypothesis that the binding of the N-terminus to PI(4,5)P$_2$ is necessary for activity by causing a change in conformation of the protein to increase accessibility of the phosphatase active site to the membrane (see Figure 3.14).

It should also be mentioned that some had postulated that varying salt concentration might have an effect on the conformation of PTEN. Using CD spectroscopy, which allows us to quickly and easily investigate the conformation of the protein when no lipid is being added to the system, we investigated the PTEN secondary structure for different salt concentrations. These data indicate that in the absence of any type of salt, the structure of PTEN does not change relative to the spectra of the protein

![Figure 3.14. Structure of PTEN$_{16-403}$ in the presence of PI(4,5)P$_2$ containing vesicles.](image)

Monitoring the protein secondary structure by ATR-FTIR, we see that the structure of PTEN$_{16-403}$ (—) does not change in the presence of 5% PI(4,5)P$_2$ vesicles (—). This is expected, as this protein lacks the N-terminal PI(4,5)P$_2$ binding domain. Spectra were normalized to the amide I peak maximum of the protein only spectrum.
obtained in buffer containing 100mM KCl (KCl must be used in CD spectroscopy as NaCl interferes with the rotation of light by the molecules of interest). Also, data have been obtained using ATR-FTIR of the protein in pure D₂O rather than salt containing buffer, and no conformational differences in secondary structure can be seen between the amide I bands of the proteins in each of the different solutions. Therefore, salt has no effect on the conformation of the PTEN protein (Redfern 2008).

![Figure 3.15. Structure of PTEN<sub>K13E</sub> in the presence of PI(4,5)P₂ containing vesicles.](image)

Monitoring the protein secondary structure by ATR-FTIR, we see that the structure of PTEN<sub>K13E</sub> (—) does not change in the presence of 5% PI(4,5)P₂ containing vesicles (—) to as great an extent as the wild type PTEN in the presence of PI(4,5)P₂ containing vesicles (—). This helps to confirm that the N-terminus of PTEN binds to PI(4,5)P₂ specifically, and that this interaction and its resulting conformational change requires the conservation of the lysine at position 13. Spectra were normalized to the amide I peak maximum of the protein only spectrum.
Figure 3.16. Structure of PTEN_{W111F} compared to structure of PTEN. Using ATR-FTIR, the amide I band of PTEN_{W111F} is monitored (—), and compared to the amide I band of PTEN (—). The overlay of these two bands shows that there evidently no difference in secondary structure upon mutation of the tryptophan in position 111 to phenylalanine.

Figure 3.17. Structure of PTEN_{W274F} compared to structure of PTEN. Using ATR-FTIR, the amide I band of PTEN_{W274F} is monitored (—), and compared to the amide I band of PTEN (—). The overlay of these two bands shows that there is evidently no difference in secondary structure upon mutation of the tryptophan in position 274 to phenylalanine.
Furthermore, we felt it necessary to conduct studies on the conformation of proteins which we have mutated for other uses. Of particular interest for these types of experiments are the proteins in which we have systematically mutated the tryptophan residues contained within the protein. These proteins, PTEN\textsuperscript{W111F}, PTEN\textsuperscript{W274F} and the double mutant, PTEN\textsuperscript{W111FW274F} were examined by ATR-FTIR in the absence of any lipid and compared to the amide I band of the wild type PTEN protein. This was done to

![Figure 3.18. Structure of PTEN\textsuperscript{W111FW274F} compared to structure of PTEN and in the presence of PI(4,5)P\textsubscript{2} containing vesicles. Using Attenuated Total Reflectance Infrared Spectroscopy, the amide I band of PTEN\textsuperscript{W111FW274F} is monitored (—), and compared to the amide I band of PTEN (—). The overlay of these two bands shows that there is evidently no difference in secondary structure upon mutation of the tryptophans in positions 111 and 274 to phenylalanine. Additionally, the conformation of PTEN\textsuperscript{W11FW274F} is monitored in the presence of vesicles comprised of 95% POPC, 5% PI(4,5)P\textsubscript{2}, which shows that this doubly mutated protein undergoes conformation changes in the presence of PI(4,5)P\textsubscript{2} which are similar to those in the native protein.](image-url)
assure us that the mutation of these residues does not alter the secondary structure of the protein. Upon comparison of these spectra, we see that the amide I bands of all of these mutated proteins overlay with the wild type protein’s amide I band quite nicely, indicating that no structural change occurred within the protein upon mutation of one or both of the tryptophan residues in question (Figures 3.16, 3.17, and 3.18). We also examined the effects of interaction with vesicles containing PI(4,5)P2, in order to help us conclude whether the proteins are still interacting with the vesicles in the same manner as the wild type protein, and whether they undergo the same conformational changes found in the wild type protein. This is particularly important, as the experiments carried out based on the changes in tryptophan fluorescence indicate that some structural changes are occurring within these proteins upon interaction with both PI(4,5)P2 and PS. ATR-FTIR studies show that PTENW111F undergoes a conformational change which is quite similar to that found within the wild type protein upon interaction with vesicles containing PI(4,5)P2; that is to say that the amide I band shifts toward 1654 cm⁻¹, indicating an increase in the alpha helical structure content of the protein. The double tryptophan mutant, PTENW111FW274F also undergoes a similar conformational change in the presence of vesicles containing PI(4,5)P2, wherein the alpha helical content of the secondary structure increases fairly dramatically (See Figure 3.18). At this point, the PTENW274F protein has proven more difficult to work with than the other tryptophan mutants, and the conformational changes observed within the other proteins has not been detectable within this mutant protein. This is interesting, because the double mutant has been shown to be in the same conformation as the wild type protein and undergoes the same
conformational changes as the wild type protein in the presence of PI(4,5)P₂ (See Figure 3.11). Additionally, the tryptophan fluorescence data obtained for the PTEN<sub>W274F</sub> mutation show that a conformational change is occurring, which makes us confident that the mutation of the tryptophan in position 274 to phenylalanine does not actually affect the conformation or conformational changes that occur in the protein.

It is known that PTEN binds to vesicles containing PS, and our previous studies suggested that PTEN may undergo a conformational change upon binding to these vesicles. Therefore, we continued our studies of the effects of lipid model membranes on PTEN’s structure by examining the secondary structure of the protein in the presence of

![Figure 3.19. Structural changes of PTEN in the presence of PS containing vesicles. Using Attenuated Total Reflectance Infrared Spectroscopy, the amide I band of PTEN is monitored in the presence of vesicles containing 5% PS (—), or PTEN alone (—). PTEN’s secondary structure contains more β-sheet upon binding to PS containing vesicles. Spectra were normalized to the protein only amide I peak maximum.](image)
vesicles containing 5% POPS. Though the effect of the PS was slight, the vesicles comprised of 5% POPS did cause a shift in the amide I band of the protein to lower wavenumbers, which suggests a small increase in the overall beta sheet content of the protein. Additionally, vesicles that contained both 5% PI(4,5)P\(_2\) and 5% PS also had an effect on the conformation of the protein (Figure 3.19). It seems that the effects of each of the lipids is additive within the spectra; while PI(4,5)P\(_2\) causes an overall shift towards higher wavenumbers giving evidence of an increase in alpha helical content of the

![Graph showing structural changes of PTEN in the presence of PS/PI(4,5)P\(_2\) containing vesicles.](image)

**Figure 3.20. Structural changes of PTEN in the presence of PS/PI(4,5)P\(_2\) containing vesicles.** Using Attenuated Total Reflectance Infrared Spectroscopy, the amide I band of PTEN is monitored in the presence of vesicles containing 5% PS (—), or PTEN in the presence of vesicles containing both PS and PI(4,5)P\(_2\) (—). PTEN’s secondary structure contains more \(\beta\)-sheet upon binding to PS containing vesicles, and the amide I band shifts to also contain more \(\alpha\)-helix upon interaction with PS/PI(4,5)P\(_2\) containing vesicles, resulting in an overall widening of the amide I band. Spectra were normalized to the protein only amide I peak maximum.
protein, PS causes a shift towards lower wavenumbers suggesting increase in beta sheet content. When combined with vesicles that contain both PS and PI(4,5)P$_2$, both of these shifts occur, causing not an overall shift in the amide I band, but an overall widening of it. This effect shows that the PS and PI(4,5)P$_2$, which we believe bind at separate sites within the protein, most likely have different conformational effects on those sites, and do so synergistically (Redfern 2008).
CHAPTER 4: EFFECTS OF MUTATIONS IN THE N-TERMINUS ON BINDING OF PTEN TO PHOSPHOINOSITIDES

4.1) Characterization of N-terminally Mutated Proteins Binding to Lipid Model Membranes

Results of many of our studies indicate that the mutation of the lysine in position 13 to glutamic acid results in a decrease in binding of the protein to membranes that contain PI(4,5)P$_2$ as well as a decrease in the conformational changes that occur upon interaction of the protein with those membranes. We have also shown that the binding of PTEN to phosphoinositide containing membranes is specific; and thus not governed solely by electrostatic forces. Thus, we decided to study whether the effects of the K13E mutation on binding was due to a change in overall charge of the protein’s N-terminus or whether the position of the lysine is an important factor for binding.

Because we found that the protein binds to phosphoinositide containing membranes in a manner that was specific and not governed solely by electrostatic forces, we decided to test whether the K13E mutation abrogates binding of the protein’s N-terminus due to the overall change in charge of the N-terminus. First, we tested the binding of the PTEN$_{1-21}$ peptide (purchased from GenScript, Corp) containing the K13E mutation in order to be sure that the peptide did not bind specifically to any of the phosphoinositide containing membranes. To accomplish this, we employed the Bodipy quenching assay, as previously described. Indeed, our results indicate that the binding of the PTEN$_{1-21}$ peptide containing
the K13E mutation of the phosphoinositide binding domain did not bind specifically to PI(4,5)P₂, nor to any of the other phosphoinositide derivatives (Figure 4.1).

In order to test the hypothesis that PTEN binding specifically requires a lysine in position 13, we also mutated the lysine 13 to become an arginine which maintains the local charge but alters the molecular geometry and the ability of hydrogen bond formation. Again, we employed the Bodipy quenching experiment as previously described in order to test whether PTEN₁₋₂₁K₁₃R would bind specifically to membranes containing PI(4,5)P₂ or any of the other phosphoinositide derivatives. Remarkably, the N-terminal peptide containing the K13R mutation did not bind specifically to PI(4,5)P₂ or any of the other phosphoinositide derivatives (Figure 4.1).

Our results to this point were quite compelling for the argument that the binding of PTEN’s N-terminus to phosphoinositide containing membranes is specific and not

**Figure 4.1. PTEN peptide binding specificity.** Comparison of the phosphoinositide binding specificity of PTEN₁₋₂₁ (a), PTEN₁₋₂₁K₁₃E (b) and PTEN₁₋₂₁K₁₃R (c) as monitored by the decrease in fluorescence intensity (quenching of bodipy labeled phosphoinositides).
governed by electrostatic forces. That is to say, the binding of the protein to these membranes is not only dependent on the charge of the protein or the lipid involved in the interaction in question. To further convince ourselves that this binding event is specific and that the lysine at position 13 is absolutely necessary for the binding event to occur, we also tested mutations which not only maintained the overall charge of the peptide, but also maintained the identity of the amino acids contained within the N-terminus of the PI(4,5)P₂ binding domain. To achieve this, we switched the position of the lysine 13 with its neighbors, an asparagine in position 12 and an arginine in position 14. Again using the bodipy quenching assay, we found that neither of these mutated peptides binds specifically to PI(4,5)P₂, as can be compared to the binding of the wild type N-terminal peptide, PTEN₁₋₂₁. Surprisingly, however, it seems that there may be some binding preference for PI(3,4)P₂ over the other phosphoinositides (Figure 3.2). This is quite

![Figure 4.2 Effects of lysine position on specificity. Comparison of the phosphoinositiude binding specificity of PTEN₁₋₂₁ (a), PTEN₁₋₂₁N₁₂K₁₃N (b) and PTEN₁₋₂₁K₁₃R₁₄K (c) as monitored by the decrease in fluorescence intensity (quenching of bodipy labeled phosphoinositides).]
intriguing, as the amino acid composition is overall the same as that of the wild type peptide, suggesting that not only the charge of the amino acid in position 13 of the N-terminus is important for binding, but so is the identity. It is also quite evident from these studies that while the lysine must be present in the PI(4,5)P$_2$ binding domain, its position is also important. It is possible that the lysine in position 13 is important in determining the conformation of the N-terminus, which could account for the difference in binding to membranes of these peptides.

To further confirm the results of our studies, we have used the full length protein containing these mutations of the N-terminus in order to test whether the peptide results could be extended to help determine the effects of these mutations on binding and activity

![Graph showing effects of lysine mutation on specificity of PTEN binding](image)

**Figure 4.3. Effects of lysine mutation on specificity of PTEN binding.** Comparison of the phosphoinositide binding specificity of PTEN (a), PTEN$_{K13E}$ (b) and PTEN$_{K13R}$ (c) as monitored by the decrease in fluorescence intensity (quenching of bodipy labeled phosphoinositides).

*in vivo.* The bodipy quenching assay was used again with PTEN$_{K13E}$, and all phosphoinositides were studied. Interestingly, we found that the K13E mutation of the full length protein had the same overall effect on binding as that which occurred in the
peptide containing the same mutation. The protein no longer bound specifically to PI(4,5)P$_2$, and the binding of the protein to that phosphoinositide was abrogated by the mutation. Additionally, the protein showed no specific binding to any of the phosphoinositide derivatives, as was the case in the peptide containing the K13E mutation (Figure 4.3).

The K13R mutation of the N-terminus was also tested in the full length protein using the bodipy quenching assay to determine specificity. Again, the results were quite similar to those obtained for the N-terminal peptide containing the same mutation. PTEN$_{K13R}$ did not bind to PI(4,5)P$_2$, and did not bind specifically to any of the other phosphoinositides (Figure 4.3). This is quite important, as it confirms previous data suggesting that PI(4,5)P$_2$ binding occurs at the N-terminus and that another phosphoinositide binding site in the protein does not exist. If there were another binding site for PI(4,5)P$_2$, deletion of the N-terminus or mutation of one of its amino acids would not result in such drastic decreases in binding to phosphoinositide containing membranes.

We have also tested the effects of switching the position of lysine 13 with its arginine neighbor into position 14 of the N-terminus. The effects of this mutation in the peptide resulted in a decrease in binding of the protein to PI(4,5)P$_2$, but increased the binding of the peptide to PI(3,4)P$_2$. The full length protein containing this mutation showed a slight binding preference for PI(3,4)P$_2$. Unfortunately, the fluorescently labeled PI(3,4)P$_2$ molecule that had been used for the peptide binding assays was no longer commercially available upon beginning the assay utilizing the full length protein containing the K13R/R14K mutation. When the new fluorophore available for purchase
was tested with the peptide PTEN_{1-21K13RR14K}, we found that the extent of quenching of the new fluorophore was not as great as the extent of quenching of the old fluorophore. We hypothesize in light of this information that the PTEN_{K13RR14K} would quench the old bodipy labeled PI(3,4)P\(_2\) more strongly than it does with the newly available labeled PI(3,4)P\(_2\) molecule.

The data collected using the bodipy quenching assay in combination with a myriad of mutations of both the peptide derived from PTEN’s N-terminal end and the full length protein with the same mutations have confirmed our hypothesis that the binding of the PTEN protein to membranes containing PI(4,5)P\(_2\) occurs within the PI(4,5)P\(_2\) binding domain and that this interaction is specific. Though it is quite likely that electrostatic forces do contribute to the binding between proteins and lipid membranes, it is evident that in the case of PTEN, charge of both the protein and the lipids are not the only factor in determining whether binding will occur. The charge of the lipid headgroup and the position of the phosphate groups of the inositol ring determines the binding that occurs between the membrane and protein, as does the charge and placement of the charge within the protein itself. Furthermore, conserving the charge of the N-terminus of the protein does not guarantee that binding will occur; this study suggests that the identity of the amino acid in a specific position of the protein can also be necessary for the interaction to occur. Though it is not known how the mutation is affecting the binding of the protein to the membrane, it is possible that mutations of lysine in position 13 to any other amino acid, even one of the same charge, could be affecting structural geometry of the N-terminus, which could have devastating effects on the binding ability of the protein.
CHAPTER 5: BINDING OF PTENH93R TO MODEL MEMBRANES

5.1) Characterization of the Binding of PTENH93R to Phospholipid Model Membranes

While most of the PTEN mutations we have studied were aimed at understanding the effects of the respective mutation on the binding, other mutations of PTEN have been found to be prevalent and related to specific disease states. While most of the mutations

![Graph showing the binding of PTENH93 to PI(4,5)P2 containing vesicles.](image)

Figure 5.1. Binding of PTENH93R to PI(4,5)P2 containing vesicles. Loss of tryptophan fluorescence as transferred to pyrene labeled PE was monitored over the course of titration of lipid vesicles into the 1µM protein solution. Results show that PTENH93R does not bind as strongly to PI(4,5)P2 containing vesicles (▲) as the wild type PTEN does (▲). Binding of PTENH93R (●) and PTEN (●) to POPC vesicles is also shown for comparison.
of the N-terminus are related to a cancerous and tumorigenic state, mutation of other portions of the protein are related to a plethora of other diseases. Of particular interest is the mutation of the histidine residue in position 93 to an arginine. This mutation has been found to be related to autism, but at this point the manner in which the mutation affects PTEN function is not known. In order to help elucidate the mechanism of this mutation in disease, we have studied the effects of this mutation on the binding of the protein to different membranes.

Our first step was to use the tryptophan fluorescence assay to help determine the binding of PTEN$_{H93R}$ to vesicles of different composition. We have monitored the

![Figure 5.2. Binding of PTEN$_{H93R}$ to PS containing vesicles.](image)

Figure 5.2. Binding of PTEN$_{H93R}$ to PS containing vesicles. Loss of tryptophan fluorescence as transferred to pyrene labeled PE was monitored over the course of titration of lipid vesicles into the 1µM protein solution. Results show that PTEN$_{H93R}$ binds more strongly to PS containing vesicles (■) than the wild type PTEN does (■). Binding of PTEN$_{H93R}$ (■) and PTEN (●) to POPC vesicles is also shown for comparison.
tryptophan fluorescence of the PTEN_{H93R} while titrating lipid vesicles into the protein solution. The extent of binding of protein to lipid vesicles that contain small amounts of pyrene labeled lipids is studied by monitoring the decrease in tryptophan fluorescence, as energy is transferred from the tryptophan to the labeled lipid upon docking of vesicles.

![Graph showing binding of PTEN_{H93R} to PS/PI(4,5)P_2 containing vesicles](image)

**Figure 5.3. Binding of PTEN_{H93R} to PS/PI(4,5)P_2 containing vesicles.** Loss of tryptophan fluorescence as transferred to pyrene labeled PE was monitored over the course of titration of lipid vesicles into the 1µM protein solution. Results show that PTEN_{H93R} binds more strongly to PS/PI(4,5)P_2 containing vesicles (♦) than the wild type PTEN does (♦). Binding of PTEN_{H93R} (●) and PTEN (●) to POPC vesicles is also shown for comparison. The binding of PTEN_{H93R} to PS/PI(4,5)P_2 containing vesicles was the strongest binding event observed using this assay.

Vesicles of differing compositions were titrated into the PTEN_{H93R} solutions, but the overall composition follows the form POPC, 93%; pyrene-PE, 2%; anionic lipid, 5%. The changes in tryptophan fluorescence were followed as a function of lipid concentration. Experiments were also carried out by titrating vesicles into the protein solution that did not contain fluorescently labeled lipid, in order to account for changes in tryptophan
fluorescence that are due to conformational changes in the protein rather than an energy transfer associated with a binding event.

Previous studies of this type have given evidence that wild type PTEN binds \( \text{PI}(4,5)\text{P}_2 \) more strongly than other anionic lipids, and that the binding to \( \text{PI}(4,5)\text{P}_2 \) and PS is synergistic. That is to say that there are two separate binding sites for PS and \( \text{PI}(4,5)\text{P}_2 \) and that these two lipids do not compete for binding to the same site. Interestingly, PTEN\text{H}93\text{R} binds much more strongly to PS (5 mol %) than does the wild type protein (Figure 5.2), suggesting the possibility that the mutation of the amino acid in the 93 position of the protein may create another binding site for PS. The extent of binding of PTEN\text{H}93\text{R} to vesicles containing 5 mol % PS is similar to the extent of binding between the wild type protein and vesicles containing both PS and \( \text{PI}(4,5)\text{P}_2 \), which is the strongest binding pair we have studied to this point. In contrast, PTEN\text{H}93\text{R} binds less strongly to \( \text{PI}(4,5)\text{P}_2 \) (5 mol %) containing vesicles than does the wild type protein (Figure 5.1), but binds to almost the same extent to vesicles containing both PS and \( \text{PI}(4,5)\text{P}_2 \) (5 mol % each) as the wild type protein (Figure 5.3). See Table 3.2 for \( K_d \) values.

5.2) Conformational Changes of PTEN\text{H}93\text{R} as a Result of Binding to Phospholipid Model Membranes

Our studies provide evidence that the PTEN\text{H}93\text{R} binds to vesicles containing PS and \( \text{PI}(4,5)\text{P}_2 \) separately or together, and the changes in the natural tryptophan fluorescence suggest that conformational changes occur within the protein upon these binding events, which we explored further by using ATR-FTIR as previously described. In our previous
studies, wild type PTEN conformation exhibited more alpha helical secondary structure content in the presence of vesicles containing PI(4,5)P_2 as evidenced by a significant band intensity increase in the spectral region around 1654 cm\(^{-1}\). In the presence of PS, PTEN’s conformation seemed to contain more beta sheet structures, as the amide I band shifts to lower wavenumbers, giving evidence that PI(4,5)P_2 and PS have distinct effects on the conformation of PTEN.

Our FTIR results indicate that upon binding to vesicles that contain POPC and PI(4,5)P_2 (95:5 mol%), PTEN_{H93R}’s structure, rather than becoming more alpha helical in

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**Figure 5.4. Structural changes of PTEN_{H93R} in the presence of PI(4,5)P_2 containing vesicles.** Using Attenuated Total Reflectance Infrared Spectroscopy, the amide I band of PTEN_{H93R} is monitored in the presence of vesicles containing 5% PI(4,5)P_2 (---), and PTEN_{H93R} alone (--). PTEN_{H93R}’s secondary structure contains more β-sheet upon binding to PI(4,5)P_2 containing vesicles. This conformational change is different from that induced by PI(4,5)P_2 in wild type PTEN. Spectra were normalized to the protein only amide I peak maximum.
nature as does the wild type protein, the amide I band shifts slightly towards more beta sheet content (Figure 5.4). PTEN\textsubscript{H93R} protein undergoes conformational changes in the presence of vesicles comprised of POPC and POPS (95:5 mol%), increasing the beta sheet content of the protein very slightly in a manner similar to that which occurs in the wild type protein (Figure 5.5). While it is not surprising that PS induces secondary structure with an increased beta sheet content in PTEN\textsubscript{H93R} and wild type PTEN (PS binds to the C2 domain of the protein, which is rich in beta sheet) it is unexpected that PI(4,5)P$_2$ also induces enhanced beta sheet formation in PTEN\textsubscript{H93R}.

**Figure 5.5. Structural changes of PTEN\textsubscript{H93R} in the presence of PS containing vesicles.** Using Attenuated Total Reflectance Infrared Spectroscopy, the amide I band of PTEN\textsubscript{H93R} is monitored in the presence of vesicles containing 5% PS (—), or PTEN\textsubscript{H93R} in the absence of lipid vesicles (—). PTEN\textsubscript{H93R}’s secondary structure contains more β-sheet upon binding to PS containing vesicles, as is observed upon interaction of wild type PTEN with PS containing vesicles. Spectra were normalized to the protein only amide I peak maximum.
We also compared the spectra of PTEN\textsubscript{H93R} in the absence of lipid vesicles and in the presence of vesicles containing both PS and PI(4,5)P\textsubscript{2} (90:5:5 mol %). In this case, the amide I band is shifted to lower wavenumbers, indicating an overall increase in the amount of beta sheet present within the protein’s secondary structure (Figure 5.6). This shift is larger than those induced by vesicles containing only PS or PI(4,5)P\textsubscript{2} which supports our previous work, suggesting that PI(4,5)P\textsubscript{2} and PS bind at separate sites and induce conformational changes separately.

To test the binding differences between wild type PTEN and PTEN\textsubscript{H93R} more...
thoroughly, we have also used Isothermal Titration Calorimetry, in order to quantitate binding and the thermodynamic parameters associated with the binding event. For these studies, unilamellar vesicles comprised of only POPS were formed and placed within the syringe unit of the ITC. The size of the vesicles was determined using DLS and were found to be about 110 nm. This vesicle solution was then titrated into a 1.5µM solution of PTEN or PTEN<sub>H93R</sub>, which was suspended in the same buffer composition as the vesicles. POPS vesicles were also titrated into a solution of the buffer alone, in order to be able to subtract the heats of dilution from the resulting thermograms involving protein. The ITC studies showed that PTEN<sub>H93R</sub> bound well to the POPS vesicles, and was saturated at a molar ratio of about 50 molecules of POPS to protein (Figure 5.7). In contrast, while the wild type PTEN protein also bound to the POPS vesicles, the protein was saturated at a much high molar ratio, at over 100 molecules of PS to PTEN. Unfortunately, the software available in our lab has not proven useful for complete analysis of this data (given that PS binds in all likelihood at 2-3 distinct sites). In the future, collaborative efforts will be made to find the biophysical parameters associated with this binding event, in order to determine the stoichiometry of binding of the protein to the POPS vesicles.

Using several different techniques, we have demonstrated that PTEN<sub>H93R</sub> interacts more preferentially with PS than does the wild type PTEN, and conformational changes occurring as a result of this interaction result in an increase in beta sheet content of the protein’s secondary structure. Alternately, PTEN<sub>H93R</sub> interacts less strongly with PI(4,5)P<sub>2</sub> than does the wild type protein, and interestingly, this interaction results in a
conformational change which is different from that which occurs in the wild type protein. Rather than inducing an increase in alpha helical secondary structure content, as is the case in for wild type PTEN, PI(4,5)P₂ induces an increase in the overall amount of beta sheet of the protein’s secondary structure. In the presence of vesicles containing both PS and PI(4,5)P₂, we see again an additive effect of conformational changes. Upon interaction with these vesicles, the wild type protein’s overall amide I band broadened

**Figure 5.7. ITC comparing the binding of POPS to PTEN and PTEN₉₃R.** Experiments were carried out by titrating unilamellar 100nm 3mM POPS vesicles into a 1.5μM solution of protein at 25°C over a series of 40 titration steps, which are 5µL each. Results indicate that PTEN₉₃R binds more strongly to POPS than does PTEN as it is more quickly saturated with the vesicles.
due to an increase in both alpha helical and beta sheet secondary structure content. Similarly, the effect of the conformational changes found for PTEN\textsubscript{H93R} in the presence of both PS and PI(4,5)P\textsubscript{2} showed an additive effect, resulting in a much larger shift to lower wavenumbers. This increase in beta sheet induced by either lipid is not necessarily occurring within the same portion of the protein, but is more likely occurring at different sites, depending upon where the lipid is binding. These conformational changes do not occur only at the binding site, and may be propagated throughout the protein, which could also account for the large conformational changes observed upon interaction of PTEN\textsubscript{H93R} with vesicles containing both PS and PI(4,5)P\textsubscript{2}.

Based on our results, the $K_\text{d}$ for PTEN\textsubscript{H93R}’s binding to vesicles containing 5 mol\% PS in comparison to the wild type PTEN’s binding to PS suggests that the mutation of the amino acid at the 93 position from histidine to arginine may create a second binding site for PS in the protein (Table 3.2). Also, ITC results indicate that upon titration with POPS vesicles, PTEN\textsubscript{H93R} is saturated more quickly (i.e. binds more strongly) than the wild type PTEN, supporting the tryptophan quenching data and the hypothesis that PTEN\textsubscript{H93R} may have a second PS binding site.

The results obtained by our collaborator indicate that though PTEN\textsubscript{H93R} is active using an in vitro phosphatase assay, it is enzymatically inactive in the cell. In light of other data, this is particularly interesting because PTEN\textsubscript{H93R} is not binding as well to PI(4,5)P\textsubscript{2} as the wild type protein does, and binds much more strongly to PS. We hypothesize that the H93R mutation creates a second binding site for PS within the protein, which would account for the strength of binding and the quick saturation of the
protein with PS containing vesicles. Because PTEN is a hopping enzyme with limited scooting availability, it is important that the protein is able to bind to the membrane in order to come into proximity of its substrate, PI(3,4,5)P₃. It is also, however, important that the protein is able to move away from the membrane in order to be able to move to a new site and undergo another round of catalysis. Therefore, while it is important for the protein to be able to bind to the membrane with some strength, it is also important that the extent of binding is not too great, so as not to prevent the protein from being able to move to new sites within the membrane. The pronounced binding preference for PS may also result in a different subcellular localization of PTEN_{H93R}, which might result in a sequestration of the protein away from its substrate. These results also suggest a need for an in vitro phosphatase assay using lipids within membranes, such as vesicles, rather than using a soluble substrate assay alone to determine protein activity.
CHAPTER 6: THE EFFECT OF CHOLESTEROL ON LATERAL PHOSPHOINOSITIDE ORGANIZATION WITHIN A PHOSPHATIDYLCHOLINE MODEL MEMBRANE

While it is known that cholesterol exists within the membrane and affects the overall order of the lipids which it neighbors (increasing the order of unsaturated lipids and decreasing the order of a system of saturated lipids), much is left to be learned about the effect of cholesterol on the organization of membranes. Among the unknowns, the amount of cholesterol in the membrane and in particular the distribution between the two plasma membrane leaflets are the parameters which have not yet been satisfactorily determined. It is thought that the percentage of cholesterol within the membrane is most likely above 30 percent but no more than 50 percent, and it is known that cholesterol’s planar sterol structure is more likely to interact preferentially with saturated lipids rather than unsaturated lipids. It has been postulated that upon interaction with lipids of natural composition, containing one saturated stearoyl acyl chain and one unsaturated arachidonoyl chain, that the cholesterol is most likely to interact with the stearoyl chains of the surrounding lipids, which may affect how the neighboring lipids are organized and thus how they are able to interact with one another.

Previously, data was published using a FRET assay which suggested that POPC/phosphoinositide fluid/fluid demixing does occur within model membranes in a
In a pH dependent manner. This data showed that saturated lipids, both acyl chains of the lipids being palmitoyl chains, at temperatures above the phase transition temperatures of the lipids, are demixing quite strongly at high pH values (above pH 8), and continue to be somewhat demixed at physiological pH values (Redfern 2005). This is evidenced by a low donor to acceptor emission ratio in PC/phosphoinositide vesicles containing NBD-PC/bodipy phosphoinositide labeled lipids, meaning that the labeled lipids, PC and labeled phosphoinositide are too far from one another to be interacting, thus the phosphoinositide is residing within a domain (see Figure 2.3). At lower pH values, the phosphoinositides are mixing with PC, as made apparent by the increase in FRET ratio and thus decrease in distance between the labeled PC and PI molecules.

**Figure 6.1. Ratio of donor/acceptor emission of Bodipy PI and NBD-PC in the absence and presence of cholesterol.** Emissions of Bodipy-PI (0.7%) and NBD-PC (0.6%) were monitored as an indicator of domain formation in the presence of 0% cholesterol (■), 20% cholesterol (●), or 40% cholesterol (▲) in buffer (10mM HEPES, 100mM NaCl, 0.1mM EDTA). Results indicate that the presence of cholesterol enhances the formation of domains in this system.
In order to examine the effects of cholesterol on the mixing properties of fluid/fluid PC/phosphoinositide systems, we took advantage of this assay using instead unsaturated lipids. Both the labeled and unlabeled lipids are commercially available, but not all of the phosphoinositide derivatives are available with unsaturated or natural composition chains. Thus, experiments were carried out using at least one of each of the monophosphate, bisphosphate, and trisphosphate forms, as well as phosphatidylinositol. The experiments were done using vesicles that were made up mainly of 1-palmitoyl-2-oleoyl-phosphatidylcholine, which is the only lipid other than cholesterol whose

![Figure 6.2. Ratio of donor/acceptor emission of Bodipy PI(4)P and NBD-PC in the absence and presence of cholesterol.](image)

**Figure 6.2. Ratio of donor/acceptor emission of Bodipy PI(4)P and NBD-PC in the absence and presence of cholesterol.** Emissions of Bodipy-PI(4)P (0.7%) and NBD-PC (0.6%) were monitored as an indicator of domain formation in the presence of 0% cholesterol (■), 20% cholesterol (●), or 40% cholesterol (▲) in buffer (10mM HEPES, 100mM NaCl, 0.1mM EDTA). Results indicate that the presence of cholesterol enhances the formation of domains in this system.
composition percentage is varied. In addition to POPC, we also use 5% of either Brain PI, Brain PI-4(P), Brain PI(4,5)P₂, or SAPI(3,4,5)P₃, as well as 0.6% of the Bodipy-labeled PC derivative and 0.7% of the NBD-labeled phosphoinositide derivative and either 0%, 20% or 40% cholesterol. Brain phosphoinositides exhibit a stearoyl/arachidonoyl chain composition. Experiments were carried out in 10mM HEPES buffer, including 0.1mM EDTA and 100mM NaCl (Figures 6.1, 6.2, 6.3, and 6.4).

We found that also phosphoinositides with an unsaturated natural chain composition are forming domains in a pH-dependent manner, similar to the results obtained using their saturated chain counterparts. Additionally, upon the addition of 20% cholesterol...
cholesterol, the initial donor to acceptor ratio is lower than it was found for the systems not including cholesterol, which may suggest the existence of a greater number and/or larger domains. Likewise, the systems including 40% cholesterol began at an even lower initial donor to acceptor ratio, which suggests an even greater extent of domain formation. These results were seen in each of the systems, and the trend was similar regardless of the number of phosphate groups on the inositol headgroup. Because the initial ratio of donor to acceptor fluorescence was lower to start with in the systems that contained 20% cholesterol and even lower in those systems that contained 40% cholesterol, we investigated whether the amount of fluorescently labeled lipid which was inserting into the membrane was different, which would account for the differences in the

Figure 6.4. Ratio of donor/acceptor emission of Bodipy PI(3,4,5)P₃ and NBD-PC in the absence and presence of cholesterol. Emissions of Bodipy-PI(3,4,5)P₃ (0.7%) and NBD-PC (0.6%) were monitored as an indicator of domain formation in the presence of 0% cholesterol (■), 20% cholesterol (●), or 40% cholesterol (▲) in buffer (10mM HEPES, 100mM NaCl, 0.1mM EDTA). Results indicate that the presence of cholesterol enhances the formation of domains in this system.
ratios of the emissions of the two fluorophores. However, we found that the initial fluorescence intensity of each of the emissions peaks of both fluorophores, bodipy and NBD, were approximately the same for all of the systems, and was not dependent on the amount of cholesterol contained within the model membranes. Therefore, the ability of the fluorophores to insert into the lipid vesicle is not affected by the presence or the amount of cholesterol in the system, and this cannot account for the differences in donor to acceptor ratios observed in the different cholesterol containing systems. It should be noted, as well, that DLS was used before, during and after the pH titrations to ensure that the integrity of the vesicles was maintained throughout the experiments. In conclusion, cholesterol enhances domain formation for conditions (pH range) where domains would also be found in the absence of cholesterol, i.e., the pH dependence of the domain formation is maintained.

Because there has been some data that suggests that salt may have an effect on the organization of the phosphoinositides within the membrane, we carried out the same experiments using buffer devoid of salt (10mM HEPES, 0.1mM EDTA). Overall, the results remained very much the same; the domain formation of each of the phosphoinositides occurred in a pH dependent fashion, and the effect of cholesterol was the same. Increasing the concentration of cholesterol within the membranes causes a decrease in the initial donor to acceptor ratios, and an overall lower ratio of donor to acceptor at low pH as well (Figure 6.5). Again, DLS was employed throughout the experiments to ensure that the integrity of the vesicles was maintained throughout the experiments.
Further studies of the effects of cholesterol on the organization of phosphoinositides have been subsequently carried out in our lab. Using Giant Unilamellar Vesicles (GUVs), one can visualize clustering of a fluorescently labeled lipid using confocal microscopy. Results from these studies show that cholesterol did not induce phase separation in vesicles comprised of POPC, 60%; brain PI(4,5)P₂, 20%; cholesterol, 20% in conditions that contained no salt. However, when GUVs were formed under conditions of high salt concentration, using 50 or 100 mM salt, lateral separation of the POPC and PI(4,5)P₂ occurs in the presence of cholesterol. Additionally, further experiments of this type have shown that the extent of protein induced domain formation is increased in the presence of cholesterol (Z. Jiang, unpublished result).

Figure 6.5. Ratio of donor/acceptor emission of Bodipy PI(4,5)P₂ and NBD-PC in the absence and presence of cholesterol in salt free buffer. Emissions of Bodipy-PI(4,5)P₂ (0.7%) and NBD-PC (0.6%) were monitored as an indicator of domain formation in the presence of 0% cholesterol (■), 20% cholesterol (●), or 40% cholesterol (▲) in buffer lacking NaCl (10mM HEPES, 0.1mM EDTA). Results indicate that the absence of NaCl has no effect on the formation of domains in this system, and cholesterol’s effect on domain formation is retained.
CHAPTER 7: EFFECT OF CHOLESTEROL ON PROTEIN BINDING TO MODEL MEMBRANES

The binding of gelsolin to phosphoinositides, in particular PI(4,5)P₂ has not been thought to be a specific interaction; rather it is thought that the reason that gelsolin is binding strongly to PI(4,5)P₂ in a physiological setting is due to the fact that there is a

![Figure 7.1](image-url)

**Figure 7.1. Binding of gelsolin in the presence and absence of cholesterol.** The extent of binding of gelsolin to each of the naturally occurring phosphoinositide derivatives was monitored using the bodipy quenching assay. Shown are the binding of gelsolin to the phosphoinositides in the absence of cholesterol (blue) and in the presence of cholesterol (red). Results show that gelsolin does not bind specifically to any of the phosphoinositide derivatives.
local enhancement of the concentration of PI(4,5)P₂ near gelsolin. Using the bodipy phosphoinositide quenching assay described above, we show that gelsolin in fact does not bind preferentially to PI(4,5)P₂, and it does not seem to show a strong preference for binding to any of the phosphoinositide derivatives, as can be seen in Figure 7.1. We have also examined the binding of a gelsolin derived peptide, gelsolin₁₅₁₋₁₆₉ which contains the stretch of amino acids within gelsolin which is thought to interact with PI(4,5)P₂ in

**Figure 7.2. Binding of gelsolin₁₅₁₋₁₆₉ in the presence and absence of cholesterol.** The extent of binding of gelsolin₁₅₁₋₁₆₉ and each of the naturally occurring phosphoinositide derivatives was monitored using the bodipy quenching assay. Shown are the binding of gelsolin₁₅₁₋₁₆₉ to the phosphoinositides in the absence of cholesterol (blue) and in the presence of 40% cholesterol (red). Results show that gelsolin₁₅₁₋₁₆₉ does not bind specifically to any of the phosphoinositide derivatives.
membranes. Figure 7.2 shows that while this short stretch of amino acids derived from gelsolin does bind slightly more strongly to PI(4,5)P2 in the presence of cholesterol than does the full length gelsolin protein, it is also not binding to any phosphoinositide in a specific manner.

To further study the binding of gelsolin to membranes containing PI(4,5)P2, we have also utilized the tryptophan/pyrene-PE FRET assay. Here, we have varied the concentration of PI(4,5)P2 within the membrane, using 1 mol%, 5 mol% and 10 mol%. The binding of gelsolin to these membranes was not much stronger

Figure 7.3. Change in gelsolin tryptophan fluorescence upon binding to pyrene PE labeled vesicles. Loss of fluorescence intensity is normalized to the system with the largest change in fluorescence intensity for comparison. Shown is binding of vesicles that contain 10% PI(4,5)P2 (■), 5% PI(4,5)P2 (▲), 1% PI(4,5)P2 (●), 100% POPC (▼), or 5% PI(3,4,5)P3 (♦). The greater the mole percent of PI(4,5)P2 in the vesicle, the stronger gelsolin binds. Also, gelsolin binds more strongly to vesicles containing PI(4,5)P2 than vesicles containing only POPC or PI(3,4,5)P3.
than the binding to vesicles comprised of purely POPC and labeled PE. Increasing the percentage of PI(4,5)P₂ within the membrane did not have a great effect either, in contrast to what was found for PTEN. Interestingly, however, the binding of gelsolin to vesicles containing either PI(4,5)P₂ or even vesicles comprised purely of POPC was stronger than the binding of gelsolin to vesicles that contained 5% PI(3,4,5)P₃ (Figure 7.3). The data obtained using the bodipy quenching assay did not show a strong preference for binding of PI(4,5)P₂ over PI(3,4,5)P₃, but it is important to recognize that the tryptophan/pyrene PE FRET assay is likely to be a more sensitive assay for probing the actual interaction of a protein and vesicles with specific lipid compositions. These data help to stress the point that the interaction of proteins and lipids is not governed by electrostatic forces alone, and do occur in a specific manner, though the forces governing this specificity may be different for each protein. However, in highly charged proteins and peptides, electrostatic forces may actually be that which governs the interaction between that particular protein or peptide and lipid.

In addition to studying the binding specificity of gelsolin to membranes containing phosphoinositides, we have also studied the binding of profilin to each of the phosphoinositide derivatives using the bodipy quenching assay. Our results indicate that profilin binds to PI(4,5)P₂ specifically over all other phosphoinositides, as the fluorescence of labeled PI(4,5)P₂ was decreased to about 85% of its initial value upon titration with the profilin protein (Figure 7.4).

Because we have shown that cholesterol affects the lateral organization of phosphoinositides within the membrane, perhaps enriching the concentration of some of
these lipids and enhancing the formation of domains, and because it is thought that some proteins are binding to some lipids more strongly physiologically because of the local enrichment of these lipids, we have studied the effects of the presence of cholesterol within a phosphoinositide containing membrane upon the binding of proteins to those membranes. While cholesterol did seem to have an effect on the binding of proteins and peptides to the fluorescently labeled phosphoinositide derivatives, there seems to be no clear trend, and thus the data must be treated separately.

First, because we did not see any specific binding of gelsolin to phosphoinositides...
and it is known that gelsolin binds to PI(4,5)P₂ in a physiological setting due to the local enrichment of that lipid, we have tested the effects of 40% cholesterol on the binding of gelsolin to vesicles which contain each of the phosphoinositide derivatives. Using this assay, we see that the binding of gelsolin to PI(4,5)P₂ is indeed enhanced by the presence of 40% cholesterol within the membrane, however, the binding of gelsolin is still not specific; the binding of the protein to nearly all of the phosphoinositides was enhanced by the presence of cholesterol within the vesicles (Figure 7.1). In addition, we have investigated the effects of the presence of 40% cholesterol on the binding of the gelsolin derived peptide which is thought to be responsible for the binding to PI(4,5)P₂ within cells. This peptide also showed enhanced binding to PI(4,5)P₂ upon addition of cholesterol to the membranes, and also showed enhanced binding only to PI(3,4)P₂ as well as to membranes containing the unphosphorylated phosphoinositide (PI) derivative (Figure 7.2). Unlike its full length counterpart, gelsolin₁₅₁₋₁₆₉ did not show enhanced binding to all of the phosphoinositide derivatives in membranes containing 40% cholesterol in comparison to those membranes that contained no cholesterol.

The effects of cholesterol on the binding of profilin to the different phosphoinositide derivatives is perhaps the most interesting effect we have seen in this part of the study. Unlike gelsolin, profilin’s binding to phosphoinositides was not enhanced by the presence of cholesterol across the board. The effect of cholesterol on profilin binding was much more specific and unique. Upon addition of 40% cholesterol to the membranes containing each of the phosphoinositide derivatives, the binding specificity of profilin was switched; in the presence of cholesterol the binding to
PI(4,5)P₂ containing vesicles was decreased, while the binding to vesicles containing PI(3,4)P₂ was dramatically increased. In the presence of 40% cholesterol, profilin binds specifically to vesicles containing PI(3,4)P₂ rather than binding specifically to PI(4,5)P₂, as is the case in the absence of cholesterol. The binding of profilin to each of the other phosphoinositide derivatives was not significantly changed in the presence of 40% cholesterol, and in some cases the extent of binding decreased slightly for vesicles containing cholesterol.

In light of these data, it is also quite necessary to study the effects of the presence of cholesterol on the binding of profilin to these vesicles.

**Figure 7.5. Binding of PTEN to phosphoinositides in the presence and absence of cholesterol.** The extent of binding of PTEN to each of the naturally occurring phosphoinositide derivatives was monitored using the bodipy quenching assay. Shown are the binding of PTEN to the phosphoinositides in the absence of cholesterol (blue) and in the presence of 40% cholesterol (red). Results show that PTEN binds specifically to PI(4,5)P₂ and this binding is enhanced by the presence of 40% cholesterol.
of cholesterol within the membrane on the binding of the different PTEN proteins that we have previously described. First, we studied the binding specificity in the presence of cholesterol for the full length protein. The binding of the full length wild type PTEN is decreased in the presence of cholesterol to most of the phosphoinositides in question. The only lipids in which the binding was enhanced in the presence of cholesterol are those with which the protein had already shown specific binding; that is to say that in the presence of 40% cholesterol, the binding of PTEN to vesicles that contain PI(5)P and PI(4,5)P₂ were the only events which were enhanced (Figure 7.5).

Unlike the phenomenon observed in the full length protein, the presence of cholesterol in vesicles containing the different phosphoinositide derivatives had a much broader effect on the binding of PTEN₁⁻₂¹ to those membranes. The interaction of PTEN₁⁻₂¹ was enhanced for every one of the phosphoinositide derivatives. Taken together, however, the binding to PI(4,5)P₂ and PI(5)P are the strongest binding events still, though the specificity is not quite as strong in the presence of cholesterol as it was found in the absence of cholesterol (Figure 7.6). To complement this data, and because we have still seen no real trend in the effects of cholesterol on the binding of the proteins to phosphoinositide containing membranes, we also studied the effect of the presence of cholesterol within the membranes on the binding of PTEN₁⁻₄₀₃. These data show again that cholesterol does not have the same effect on the binding of every protein to each of the phosphoinositide derivatives; the binding of PTEN₁⁻₄₀₃ was slightly enhanced for several of the phosphoinositides in the presence of cholesterol, such as PI(5)P, but the interaction between the protein and other phosphoinositides was decreased in the
presence of cholesterol, such as PI(3,4)P_2 (see Figure 7.7). Overall, the data show that PTEN_{16-403} still does not bind specifically to any phosphoinositide in the presence of 40% cholesterol, while the binding to PI(4,5)P_2 and PI(5)P is enhanced for the full length protein and peptide PTEN_{1-21}, and the specificity of the interaction of those proteins remains in the presence of cholesterol. In fact, the specificity of binding to these phosphoinositides for the full length protein is actually enhanced in the presence of cholesterol, which suggests that perhaps the binding may be even stronger and more specific physiologically than we can observe in these simple systems.

![Figure 7.6. Binding of PTEN_{1-21} to phosphoinositides in the presence and absence of cholesterol.](image)

The extent of binding of PTEN_{1-21} to each of the naturally occurring phosphoinositide derivatives was monitored using the bodipy quenching assay. Shown are the binding of PTEN_{1-21} to the phosphoinositides in the absence of cholesterol (blue) and in the presence of 40% cholesterol (red). Results show that PTEN_{1-21} binds specifically to PI(4,5)P_2 and this binding is enhanced by the presence of 40% cholesterol.
The presence of cholesterol within the membrane obviously has a great effect on the binding of proteins to the vesicles we have studied, and we have also shown that cholesterol has an effect on the lateral organization of the phosphoinositides within these systems. For the lipid/lipid FRET assay, we must use both labeled phosphoinositides and phosphoinositide lipids with the natural chain composition in order to be sure that the system is behaving as we expect the natural lipids to do. Unfortunately, it is not possible to purchase each of the phosphoinositides with natural chain composition (1-stearoyl-2-

![Figure 7.7. Binding of PTEN16-403 to phosphoinositides in the presence and absence of cholesterol.](image)

The extent of binding of PTEN16-403 to each of the naturally occurring phosphoinositide derivatives was monitored using the bodipy quenching assay. Shown are the binding of PTEN16-403 to the phosphoinositides in the absence of cholesterol (blue) and in the presence of 40% cholesterol (red). Results show that PTEN16-403 does not bind specifically to any phosphoinositide in the presence and absence of 40% cholesterol.
arachidonoyl), which is why we have studied the effects of cholesterol only on Brain PI, Brain PI(4)P, Brain PI(4,5)P₂, and SAPI(3,4,5)P₃. However, because we have seen that cholesterol has effects on these systems and that cholesterol has different effects on the binding of proteins to the different phosphoinositides, most notably the switching of the specificity of binding of profilin to PI(3,4)P₂ from PI(4,5)P₂, we felt it necessary to study the effects of cholesterol on lateral organization of membranes containing each of the various bisphosphate substituted phosphoinositide derivatives. In order to achieve this, we needed to use chain compositions that are all exactly the same, to determine whether cholesterol’s effect could be different according only to the changes in the positions of the phosphates of the inositol ring.

For these studies, we have used zeta potential measurements of vesicles comprised of 95% POPC and 5% DO-PI(x,y)P₂. In our instrument, the zeta potential is somewhat similar to capillary electrophoresis; a folded capillary cell is used which has conducting plates at both ends (see Figure 2.11). A voltage is applied across the system and the molecules within the cell move according to their charge densities. The technique can be rather harsh, and thus has not been used extensively in studying protein solutions. The measurements must also be carefully designed to account for the effects of high salt concentrations on the electrodes in the cell. The data obtained using this method are also somewhat difficult to interpret at times. A zeta potential distribution is obtained, and the point around which the distribution centers is considered the zeta potential of the sample. This value can be positive or negative, and it is known that the further from zero the zeta potential of a sample is, the more stable the vesicular sample is considered to be. That is,
when the zeta potential is about +30mV or -30mV, the sample is thought to be stable and no longer likely to flocculate (in our case, vesicle fusion). Besides gaining information about the stability of the sample, the raw zeta potential data can be very useful for the characterization of surface charge when compared to another similar sample. This is why for us the technique is ideal for studying the effects of cholesterol on the membranes which contain the different bisphosphate substituted phosphoinositides. We can gather data for vesicles which are exactly the same, except for the position of the phosphates on the inositol ring of the phosphoinositolte component. Then we can compare these data with the exact same systems that are different only in the addition of cholesterol. This allows us to determine whether cholesterol has a different effect on the clustering of the

\[ \text{Figure 7.8. Zeta potential measurements for DPPC/DPPG or POPC/POPG mixed vesicles.} \]

Zeta potential measurements were taken for a vesicles containing 0.5 mg total lipid at differing concentrations. Shown for comparison is the data provided in a technical note by Malvern Instruments. Results show a small difference in zeta potential between lipid mixtures with saturated and unsaturated chains. All data points represent at least two separate trials of five runs each.
bisphosphate phosphoinositides, and will help elucidate the effects of cholesterol of the binding of different proteins to these membranes.

To be sure that our experiments procedures were satisfactory, we first attempted to reproduce data published as a technical note that accompanied the instrument we were using for the zeta potential measurements. This technical note used vesicles that were composed of DPPC and DPPG at differing percentages, but always equaling a total of 0.5 mg of lipid. Several of the concentrations that we measured produced zeta potential distributions that were quite similar to the data given within the technical note, but the greater the percentage of DPPG in the system, the greater the difference in zeta potential values we obtained versus the data provided by Malvern Instruments (Figure 7.8). Our results were reproduced several times, and we became quite confident that the errors that may exist were not due to our handling of the lipid samples, but were a result of a

**Figure 7.9. Representative graph of raw zeta potential data.** Shown is a sample of the zeta potential distribution with 3 runs included from one trial for POPC, 95%/DOPI(3,4)P2,5%. The distribution centers around nearly -20mV, which is then taken as the zeta potential value.
different sample history between our samples and the samples made by Malvern. It was also important, however, to determine whether the chain composition of the lipid components would have an effect on the zeta potential of the lipid vesicles if the headgroup identities were exactly the same. In order to test this, we used samples that were the same percentages of lipid with PC and PG headgroups, however we used lipid molecules containing one saturated and one unsaturated acyl chain, POPC and POPS (Figure 7.8). Interestingly, we found that the composition of the fatty acyl chains did have an effect on the zeta potential measurements of the lipid molecules, regardless of the fact that the headgroup identities and compositions were exactly the same. Most likely this can be attributed to the fact that the lateral density of the lipid molecule is lower in the case of lipids with unsaturated chains compared to lipids with saturated acyl chains. This was applied to later studies of each of the bisphosphate phosphoinositides; to be sure that any differences in zeta potential that we observed were due to the phosphate substitution pattern on the inositol headgroup it was imperative that we used lipid molecules with the same chain compositions. Thus, it is not possible to use lipids derived from natural sources for these studies, such as brain PI(4,5)P₂, which was used throughout most of our previous studies. Rather, we used DOPIP₂ molecules, which are the only lipid molecules commercially available for all bisphosphate phosphoinositides with the same fatty acyl chain composition.
We see from the data obtained that the zeta potential of pure POPC vesicles is about -9 mV, which agrees with data reported in the literature (Figure 7.10) (Carrion 1994). The addition of 5 mol% DOPI(x,y)P₂ gives rise to zeta potential values in the range of -20 to -25mV. Upon the addition of 40% cholesterol to the system, we see a change in zeta potential of several mV, suggesting that the addition of cholesterol leads to an increased charge density.

Interestingly, the results for the vesicles containing 5% DO-PI(x,y)P₂ show that

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{zeta_potential.png}
\caption{Zeta potential measurements of DOPIP₂ containing vesicles in the presence and absence of cholesterol. Zeta potential measurements were carried out in a pH 7.0 buffer (10mM HEPES, 100mM NaCl, 0.1mM EDTA) with vesicles comprised of POPC and 5% DOPI(x,y)P₂ and either 0% or 40% cholesterol. Results show no significant difference in the surface charge density of the bisphosphate substituted phosphoinositides in the absence or presence of cholesterol with respect to one another, but indicate that the presence of 40% cholesterol does affect the surface charge density of these vesicles.}
\end{figure}
all of the bisphosphate substituted phosphoinositides have very similar zeta potential values, and do not differ significantly when the measurement errors are taken into account. This is very important, because it tells us that the charge densities of the surfaces of these lipid vesicles do not change much with the phosphate substitution pattern at the inositol ring. Thus, the electrostatic forces which inevitably play some role in the interactions of proteins with lipids cannot be the governing force in the systems we have investigated, because the even differences in charge densities of vesicles containing the differently substituted phosphoinositides are roughly the same and would not account for differences in protein binding. Furthermore, the addition of cholesterol to these systems changes the zeta potential of all of these bisphosphate phosphoinositide containing vesicles in a similar manner. The addition of 40% cholesterol lowers the zeta potential by about 5-10 mV, bringing the value to about -30 mV for each of the vesicles containing one of the DO-PI(x,y)P₂ derivatives (see Figure 7.10). The zeta potential values of each of the different bisphosphate phosphoinositides are again not different enough from one another to account for the observed phosphoinositide binding preference of the respective proteins purely based upon electrostatic forces, and the changes induced herein by the addition of cholesterol certainly cannot account for a switch in the specificity of the profilin protein in the presence of 40% cholesterol.

Overall, we can conclude that cholesterol’s effects on vesicles comprised of POPC and DOPI(x,y)P₂ are stabilizing, that is because the zeta potential becomes more negative, indicating that the samples are less likely to flocculate. Furthermore, the zeta potential values for each of the different bisphosphate substituted phosphoinositides are
within the range of error of one another, showing that the substitution pattern does not significantly affect the packing and thus charge density of the lipids, and thus electrostatic forces alone cannot be considered responsible for the specificity of protein binding we have observed. Similarly, the addition of cholesterol to these systems affects the overall zeta potential of these systems in the same manner, so that this argument also does not apply for the specificity of binding to vesicles that contain phosphoinositides and cholesterol (Jiang et al, in preparation).
CHAPTER 8: LATERAL ORGANIZATION IN A DMPC/DPPI MIXED BILAYER

8.1) Lateral Organization of a DMPC/DPPI Mixed Bilayer System

Our lab has previously shown that in a fluid/fluid system, demixing of lipids does occur in a pH dependent fashion. These results were obtained using the FRET experiment previously described. It was also shown that gel phase demixing for each of the lipid

Figure 8.1. Thermogram of DMPC/DPPI mixed vesicles. Using DSC, thermograms were collected for DMPC/DPPI vesicles in concentration intervals of 5%. The results show demixing of DMPC and DPPI over a range of concentrations.
phosphoinositides (the unphosphorylated form, a monophosphate, bisphosphate and trisphosphate) occurred in the presence of DPPC (temperature dependent FTIR). FTIR allows us to follow the phase behavior of two lipid components at the same time, observing the effects of each of the lipids on the transition temperatures of the other lipid concomitantly, while also allowing us to determine if at the same time these two lipid components are mixing homogenously in the system at temperatures above their respective phase transition temperatures. This allows us to determine without doubt the existence of gel phase demixing. As mentioned, it has already been determined that in a system of DPPC with each of the phosphorylated phosphoinositides that gel phase demixing does occur. This data was complemented with FRET measurements which

Figure 8.2. Phase diagram of DMPC/DPII mixed vesicles. The phase boundaries (■) were defined by the onset and completion temperatures determine by DSC, while the melting curve (□) was defined by the position of the Tm as measured by DSC.
showed a similar demixing (domain formation) in the fluid phase. It should also be noted that in experiments such as the FRET assay described, one must have not only the labeled lipid in the system, but also the unlabeled lipid counterpart, otherwise the demixing of the lipids is impossible to observe (Fernandes 2006). In FTIR, the transition temperatures of each of the components can be examined separately, which is a real advantage over a technique such as DSC, which only allows one to observe the total shift in transition temperature of the entire system. On the other hand, DSC offers the advantage that the heat associated with the transition can be determined. Furthermore, repeated

![Figure 8.3](image)

**Figure 8.3. DSC thermogram of pure DPPI vesicles at high concentration.** Thermogram was taken of pure DPPI multilamellar vesicles at high concentrations. Results show that at lipid concentrations similar to those used in FTIR experiments, metastable structures are formed, resulting in multiple transitions, which occur at temperatures that are higher than for pure DPPI in a thermodynamically stable state.
heating/cooling scans allow for the investigation of hysteresis effects and the easier identification of metastable states. We are able to use the two techniques in combination, not only to validate the results from each, but also to determine the concentrations at which the lipids in a particular system are becoming demixed.

For this particular set of experiments, we have used dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylinositol (DPPI), with differing concentration ratios. In contrast to DPPC/DPPI, the phase transition temperatures of DPPI and DMPC are far enough apart that they can be readily distinguished in a DSC experiment. For the DSC experiment, we start with 100 percent DMPC, then the next experiment utilizes 5 percent DPPI, decreasing the percentage of DMPC to 95 percent (see Figure 8.1). We do this in steps of 5 percent until we reach 100 percent DPPI. The FTIR experiments were carried out in intervals of 10 percent (for cost reasons) to solve the phase diagram of a mixture of DMPC/DPPI (Figure 8.2). In addition to this, we have also examined the effects of CaCl₂ and MgCl₂ on the transition temperature of DPPI using DSC.
Figure 8.4 FTIR of mixed DMPC/DPPI vesicles. Experiments were done using mixed DMPC<sub>d54</sub>/DPPI multilamellar vesicles, the sample was placed between two CaF<sub>2</sub> plates and scans were taken over a range of temperatures from about 4°C to 90°C. Appropriate peaks were then chosen and plotted as a function of temperature. Results show the presence of gel phase demixing in the DMPC<sub>d54</sub>/DPPI system.
Our results indicate that DMPC and DPPI do exhibit fluid/fluid demixing (Figures 8.1 and 8.4). Our DSC results indicate that the transition temperature of pure multilamellar DMPC vesicles is at approximately 22°C. Upon addition of DPPI, the transition temperature of the mixture is increased, and continues to increase as the fraction of DPPI in the lipid vesicles becomes greater and greater. The transition temperature observed for pure DPPI vesicles is about 42°C. The FTIR data show similar trends; the two components are demixing, even above their transition temperatures at all concentrations which were studied. Interestingly, however, we found a very different transition temperature for pure DPPI vesicles using this technique, somewhat higher, close to 60°C. We found that the concentration difference between the experiments carried out in the DSC and FTIR techniques was an important factor in this discrepancy; in fact, upon using the same concentration of the DPPI lipid vesicles in the DSC, we found that the transition temperature was greatly increased and there were several other small transitions occurring which may have been contributing to the data we had collected previously (Figure 8.3). Taken together, we believe that at very high DPPI concentrations metastable structures are formed, which is the reason for the great increase in the transition temperature of this lipid in our FTIR studies. Overall, these data show that there is indeed gel phase demixing of these two components.
8.2) Effects of Mg$^{2+}$ and Ca$^{2+}$ on Transition Temperature of DPPI vesicles

Mg$^{2+}$ and Ca$^{2+}$ are both ions present in the cell which carry two positive charges; because of these charges, these cations are able to interact with the negatively charged head groups of many different lipid species. The effects of these ions on phospholipid bilayers of varying composition have been extensively studied and it has generally been

![Figure 8.5. DPPI vesicles in the presence of Ca$^{2+}$. Using DSC, thermograms of DPPI multilamellar vesicles were collected in the presence of differing ratios of DPPI:Ca$^{2+}$. The ratios change from bottom of graph as follows (DPPI:Ca$^{2+}$) 10:1, 5:1, 3:1, 2:1, 1:1, 1:2, 1:3, 1:5, 1:10. Results indicate that as the concentration of Ca$^{2+}$ increases, the phase transition temperature of DPPI increases as well.](image-url)
found that the cations act to shield the negative charges of the head groups as well as potentially bridge them, thus removing any repulsive forces that had prevented proximity of the lipid headgroups. The interaction with cations and the resulting shielding effects which they carry interrupt the hydrogen bonding network of the lipid headgroups in the system. Presence of Mg$^{2+}$ or Ca$^{2+}$ are expected to allow the headgroups to come within a closer proximity to one another because the repulsive forces have been balanced, resulting in lower extent of hydration of the lipid headgroups and a closer packing of the

Figure 8.6. DPPI vesicles in the presence of Mg$^{2+}$. Using DSC, thermograms of DPPI multilamellar vesicles were collected in the presence of differing ratios of DPPI:Mg$^{2+}$. The ratios change from bottom of graph as follows (DPPI:Mg$^{2+}$) 10:1, 5:1, 3:1, 2:1, 1:1, 1:2, 1:3, 1:5, 1:10. Results indicate that as the concentration of Mg$^{2+}$ increases, the phase transition temperature of DPPI increases as well.
lipid molecules. This effect of the ions on lipid bilayer structure is generally referred to as inducing lipid bilayer crystallization, because the lipid molecules can pack so tightly in these systems. This improved packing leads, intuitively, to a higher transition temperature of the lipid system in question.

We have studied the effects of Mg$^{2+}$ and Ca$^{2+}$ separately on lipid vesicles comprised of only DPPI. It is generally known that Mg$^{2+}$ interacts more strongly with negatively charged lipid headgroups than does Ca$^{2+}$, so we have tested the effects of both ions over a range of molar ratios (see Figures 8.5 and 8.6). As expected, both ions cause an increase in the transition temperature of DPPI vesicles. The phase transition temperature of pure DPPI vesicles is about 42°C. In the presence of Ca$^{2+}$, even at low ratios (DPPI concentrations ten times as great as Ca$^{2+}$ concentration), the phase transition temperature is shifted to about 44°C. Increasing the Ca$^{2+}$ concentration to be equal to that of DPPI in the system results in an increase of the phase transition temperature to about 49°C. Interestingly, a sharp increase in the phase transition temperature of the system occurs when the concentration of Ca$^{2+}$ in the system exceeds that of the DPPI, jumping quickly to about 58°C. Increasing the Ca$^{2+}$ concentration further leads to greater increases in the phase transition temperature, until we reach a ratio in which Ca$^{2+}$ exceeds DPPI concentration ten-fold. At these concentrations, the phase transition temperature is about 62°C (Figure 8.5).

The presence of Mg$^{2+}$ on the phase transition temperature of the DPPI vesicles has a similar effect, which is slightly less than that of Ca$^{2+}$. Upon addition of Mg$^{2+}$ to the system in concentrations exceeded ten-fold by DPPI, the phase transition temperature of
the system shifts to about 44°C, and the thermogram shows a small shoulder for this peak. Upon reaching equal concentrations of DPPI and Mg$^{2+}$, the phase transition temperature is shifted to about 48°C, which is very similar to the effects observed for Ca$^{2+}$ at this concentration. Again, there is a sharp jump in transition temperature as soon as the Mg$^{2+}$ concentration exceeds the amount of DPPI in the system, shifting to about 55°C. At concentrations in which Mg$^{2+}$ exceeds DPPI ten-fold, however, the transition temperature shifts to about 58°C, which is slightly lower than the same ratios of Ca$^{2+}$ with DPPI (see Figure 8.6).
CHAPTER 9: PROJECT SUMMATION

The lipid phosphatase and tumor suppressor PTEN has been shown to be more active against its lipid substrates in the presence of PI(4,5)P₂, and the presence of a PI(4,5)P₂ binding domain within the protein’s N-terminus suggest that PTEN may be interacting specifically with PI(4,5)P₂, and that a conformational change may be responsible for the increased rate of turnover of the substrate upon interaction with PI(4,5)P₂. Using a variety of biophysical techniques, we have shown that these hypotheses were indeed correct; we have shown that PTEN binds specifically to PI(4,5)P₂, in a manner that is not governed by electrostatic forces alone, and that this specificity is due to the PI(4,5)P₂ binding domain contained within the N-terminus. Furthermore, we have shown that mutation of the lysine in position 13 of the PI(4,5)P₂ binding domain to glutamic acid, which is highly associated with a tumorigenic state, abrogates the binding of both the protein and its N-terminally derived peptide to PI(4,5)P₂ containing vesicles. We have further tested the hypothesis that proteins and peptides bind to lipid vesicles electrostatically by maintaining the charge and the overall amino acid composition of the PI(4,5)P₂ binding domain, which also results in a loss of binding of PTEN and its N-terminally derived peptide to PI(4,5)P₂ containing vesicles.

We have also shown in these studies that PTEN does undergo conformational changes upon interaction with vesicles containing PI(4,5)P₂, and that mutation of the N-terminus also affects these conformational changes. The study of the binding of PTEN to
another lipid important to its function, phosphatidylserine, was also performed, to show that this weak binding cannot alone be responsible for the recruitment of PTEN to the lipid membrane. Interestingly, interaction of PTEN with this lipid molecule also induces a conformational change within the protein. Finally, an autism relevant mutation in PTEN was studied to determine whether binding of the protein to lipid membranes could be responsible for its role in disease. While the protein appears to be active in vitro, this is not the case against soluble (non-membranous) substrates in in vivo studies, and an increased binding to phosphatidylserine molecules may be responsible for this inactivity and for the effects of this mutation in disease.

The study of binding of proteins to lipid model membranes necessitates a greater understanding of the dynamics of the lipid systems under investigation. Thus, we have studied the zeta potential of the different bisphosphate phosphoinositides, in an attempt to understand the specificity of binding of proteins to lipid headgroups that have the same number of charges in different positions. The surface charge densities of all the different bisphosphate phosphoinositides did not vary significantly, giving greater evidence that proteins do bind specifically to phosphoinositide molecules by forming distinct hydrogen bonds. We have also studied the effects of cholesterol on the formation of domains in phosphoinositide containing membranes, because cholesterol is present within physiological membranes and it is thought that domains may be present prior to interaction with proteins, perhaps helping to recruit the proteins to the membrane. Our results indicate that cholesterol does enhance domain formation, which may account for the increased extent of binding we observed between several proteins and peptides and
phosphoinositide containing membranes which also contained 40% cholesterol.

Finally, we have also studied a mixture of DMPC and DPPI to better understand how phosphatidylcholine and phosphatidylinositol headgroups interact with one another in a gel phase system. We have shown that these two components do form a heterogeneous lipid bilayer, even above their phase transition temperatures, further confirming data suggesting that each of the phosphoinositides (whether containing saturated or unsaturated fatty acyl chains) are likely to form domains even in a fluid/fluid system. The presence of bivalent cations (Mg$^{2+}$/Ca$^{2+}$) increased the phase transition temperatures as a result of tighter packing of the acyl chains upon shielding of headgroup charges. This is also important for understanding how these lipid bilayers exist physiologically, as many types of bivalent cations are present in the cell. These data are only one small piece of a huge puzzle of understanding how lipid membrane structure and dynamics are affected by their environments and related to signal transduction within a cell.

9.1) Future Studies

As is typically the case when tackling a research project, the results usually end up taking the researcher in a completely different direction than what was originally planned, and this research project was no exception. It becomes a challenge then, to predict the direction the project will take in the future, knowing how endless the possibilities really are. There are several types of experiments that could be done in the somewhat near future, however, and the results will surely throw the research onto a
different track once again.

The number of mutations of PTEN whose binding properties to vesicles containing PI(4,5)P₂, PS or a combination of the two are endless. There are a plethora of mutations known to be associated with a myriad of different diseases, which may have very different characteristics from the mutations we have studied here. It would likely be very interesting to test the binding specificity of these mutations to each of the phosphoinositide derivatives, as done in this work.

One necessary step that need be taken is to develop a phosphatase assay that utilizes lipid vesicles that contain PI(3,4,5)P₃ with or without PI(4,5)P₂. The current assay uses a soluble substrate, the results of which may not be physiologically relevant, as evidenced by the difference in results we have seen using PTENH93R in our \textit{in vitro} and \textit{in vivo} studies. Attempts have been made at developing such an assay, all of which have failed. In the future, use of mass spectrometry might be the best way to study the protein’s activity, as the lipids would not have to be altered or labeled in any way and the results would be somewhat easily and accurately quantified.

Further study of PTEN’s interaction with lipid membranes should also be done using Giant Unilamellar Vesicles, which have a curvature that is somewhat more similar to the membranes found physiologically than the LUVs used in this study. Use of GUVs would be a fairly flexible technique, allowing us to alter the compositions of the vesicles and study the interactions of the protein with those vesicles. More complex compositions would also be easy to use with this technique, and future studies should include work on how PTEN interacts with Ceramide-1-Phosphate, which is proving to be a very relevant
lipid in the PI3K pathway.

Finally, the conformational changes that are occurring within the protein must be probed in more detail, in order to determine exactly where these conformational changes happen. Studies of the protein with systematic tryptophan mutations have laid the groundwork for mutation of both tryptophan residues from their positions to add new tryptophan residues at other positions within the protein to probe the conformations of different areas of the protein, one at a time. Of particular importance in this regard is examination of the conformation of the loops of the active site, as it has been suggested that in some phosphatases, these act almost as a lid to the active site to regulate activity of the enzyme.
REFERENCES


