Mechanistic Studies on the Membrane Recruitment and Function of 3’-Phosphoinositide Dependent Kinase-1

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THESIS
Submitted as partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry in the Graduate College of the University of Illinois at Chicago, 2012.

Chicago, IL

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ACKNOWLEDGEMENTS

I would first like to recognize Dr. Wonhwa Cho for his endless support and mentorship. His commitment to thorough scientific investigation has been an inspiration and has helped me develop my critical thinking skills to form rational conclusions from scientific experimentation. I would also like to thank the rest of my thesis committee, Dr. Richard Kassner, Dr. Lawrence Miller, Dr. Jung-Hyun Min, and Dr. John O’Bryan for their contributions and help which have enabled me to complete this journey.

A lot of what I have accomplished would not have been possible without the help of our collaborators. Dr. Richard Ye of the University of Illinois at Chicago was instrumental in assisting me to study the physiological function of 3’-phosphoinositide dependent kinase-1. Also, I would like to recognize Dr. Alex Toker from Harvard Medical School provided us with a generous gift which allowed me to complete my in vivo studies. I would also like to thank Dr. Michael Best from Vanderbilt University and Dr. Hui Lu from the University of Illinois at Chicago.

I have been fortunate over the last 5 years to work with some exceptional scientists in my research group. Debasis Manna was instrumental in my initial development in the laboratory. Youngdae Yoon has always been helpful as a sounding board for me to bounce ideas off of. His suggestions and insight have been greatly appreciated and I thank him. Shan Tao started working in the Cho lab about the same time as I did. She has been a great colleague and friend; her intelligence and generosity have been greatly appreciated. I could always count on her drawer of snacks when I felt my blood sugar starting to drop! If there were more people like her
the world would be a much friendlier place and I thank her for being my friend. To the rest of the Cho group members, both past and present, thank you for assisting me in my training, allowing me to work among you and learning about how to be a researcher that this lab can be proud to call its own.

None of this would have been possible without the love and support of my family. I want to thank my parents, Tom and Peggy, for providing me the opportunity to pursue my career goals and supporting me in my decisions over the years. My dad is probably the hardest working man I’ve ever met, and his example has been an inspiration to me over the years. My mom has always known exactly what to say and when to say it, I thank her for always believing in me and for giving me that extra push sometimes when I needed it. I cannot remember too many days in my life without my little brother Joe. I thank him for always being there to make me laugh and put things into perspective. My brother Jared has one of the biggest hearts of anyone I know. He always cared about what was going on in my life and has been a great example to me in treating others with dignity and respect. For that I thank him. I also want to thank my sister Natalie, who is so loving and sweet, for her support and her willingness to come to me for advice. She helped me with my struggles in teaching others as much as I helped her through obstacles in school.

Last, I want to thank the person who has stood by me through this journey, the love of my life, Sara Marchlewicz. She has been the one who I celebrate accomplishments with, the one who consoles me when things don’t work, the one who pushes me to believe in myself. Sara has a heart of gold; she has made life outside of
science that much more enjoyable and has given me a great companion to share the rest of my life with. Her patience with me has been astonishing, I am so grateful to have her in my life. I will do my best to provide her with the same support that she has so unselfishly given to me. I thank God for all of these blessings in my life and hope to someday repay Him for the gifts and talents He has bestowed upon me.
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<tbody>
<tr>
<td>AGC</td>
<td>Serine/Threonine kinase superfamily</td>
</tr>
<tr>
<td>Akt</td>
<td>Serine/Threonine kinase</td>
</tr>
<tr>
<td>BAD</td>
<td>Bcl-2-associated death promoter protein</td>
</tr>
<tr>
<td>BH3</td>
<td>Bcl-2 homology domain 3</td>
</tr>
<tr>
<td>BTK</td>
<td>Bruton’s tyrosine kinase</td>
</tr>
<tr>
<td>C1</td>
<td>Protein kinase C Conserved 1</td>
</tr>
<tr>
<td>C2</td>
<td>PKC Conserved region 2</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DAPP1</td>
<td>Dual adapter for phosphotyrosine and 3-phosphotyrosine and 3-phosphoinositide</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>FYVE</td>
<td>Fab1/YOTP/Vac1/EEA1</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>Grp1</td>
<td>General Receptor for 3-phosphoinositides 1</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>Ins(1,4,5)P₃</td>
<td>Inositol (1,4,5) trisphosphate</td>
</tr>
<tr>
<td>Ins(1,3,4,5)P₄</td>
<td>Inositol (1,3,4,5) tetrakisphosphate</td>
</tr>
<tr>
<td>Ins(1,3,4,5,6)P₅</td>
<td>Inositol (1,3,4,5,6) pentakisphosphate</td>
</tr>
<tr>
<td>Ins(1,2,3,4,5,6)P₆</td>
<td>Inositol (1,3,4,5,6) hexakisphosphate</td>
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<tr>
<td>Lact C2</td>
<td>Lactahedrin C2 domain</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>---</td>
<td>---</td>
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<tr>
<td>MAD</td>
<td>Selenomethionine-multiwavelength anomalous dispersion</td>
</tr>
<tr>
<td>MDM2</td>
<td>E3 Ubiquitin ligase</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
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<td>PDK1</td>
<td>3’-Phosphoinositide dependent kinase-1</td>
</tr>
<tr>
<td>PG</td>
<td>Phosphatidylglycerol</td>
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<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
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<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
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<tr>
<td>PIF</td>
<td>Hydrophobic motif binding pocket</td>
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<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>cAMP-dependent protein kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PKG</td>
<td>cGMP-dependent protein kinase</td>
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<tr>
<td>PLC</td>
<td>Phospholipase C</td>
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<td>PRK2</td>
<td>Protein kinase C-related kinase 2</td>
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<td>PS</td>
<td>Phosphatidylerine</td>
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<tr>
<td>PtdIns</td>
<td>Phosphatidylinositol</td>
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<tr>
<td>PtdIns(3)P</td>
<td>Phosphoinositide (3) phosphate</td>
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<tr>
<td>PtdIns(3,4)P₂</td>
<td>Phosphoinositide (3,4) bisphosphate</td>
</tr>
<tr>
<td>PtdIns(3,4,5)P₃</td>
<td>Phosphoinositide (3,4,5) trisphosphate</td>
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<tr>
<td>PtdIns(3,5)P₂</td>
<td>Phosphoinositide (3,5) bisphosphate</td>
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<th>Description</th>
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<tr>
<td>PtdIns(4)P</td>
<td>Phosphoinositide (4) phosphate</td>
</tr>
<tr>
<td>PtdIns(4,5)P₂</td>
<td>Phosphoinositide (4,5) bisphosphate</td>
</tr>
<tr>
<td>PtdIns(5)P</td>
<td>Phosphoinositide (5) phosphate</td>
</tr>
<tr>
<td>PX</td>
<td>Phox homology</td>
</tr>
<tr>
<td>Raf1</td>
<td>Serine/threonine protein kinase</td>
</tr>
<tr>
<td>SGK1</td>
<td>Serum-and glucocorticoid-induced protein kinase</td>
</tr>
<tr>
<td>SH2</td>
<td>Src-homology 2</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>S6K1</td>
<td>p70 ribosomal S6 kinase</td>
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SUMMARY

Cellular response is the product of a complex network of molecular interactions. Peripheral proteins capable of binding membrane surfaces are essential for efficient and proper transfer of signal from activated membrane receptors to downstream effectors. Therefore, understanding the mechanisms proteins utilize for membrane recruitment is immeasurably useful in deciphering how cells respond to the outside environment.

The strategy most often utilized by signaling and membrane-trafficking proteins for membrane recruitment is the presence of a modular lipid binding domain specialized in lipid binding. Lipid binding domains rank among the most common modular domains found in the eukaryotic proteome, consequently their structure and function have been the focus of intense investigation over the years. One lipid binding domain that has drawn particular interest over the years is the Pleckstrin homology domain. The PH domain is found in a number of different proteins and is best known for the ability to bind phosphoinositides. However, most PH domains show very low affinity for phosphoinositides and little to no selectivity, indicating that this module may have other roles inside the cell beyond phosphoinositide binding. The work presented in this thesis explores a unique lipid binding property associated with the PH domain-containing PDK1 protein. Using both in vitro biophysical measurements and in vivo imaging analysis, we identify a site that is separate from the canonical phosphoinositide-binding site which is required for proper membrane targeting and signaling function of PDK1.
CHAPTER 1: Cellular Lipids in Cell Signaling
1. **Cellular lipids in cell signaling**

   Living beings are coupled to their surrounding environment. Accordingly, apart from other qualities that distinguish living from non-living, organisms are data processing systems. They attach a meaning to exogenous stimuli based on inherited or amassed data that can be understood as an input signal. From there, they can properly respond with a calculated output signal and adjust their behavior which can include genetic readout, metabolism, reproduction, shape, and motility to any given environmental stimuli (1). This ability is an essential condition of survival, and can be attributed to all details of life, from cells to humans.

   Cellular responses to external stimuli are mediated by dynamic signal transduction pathways that involve many transmembrane receptors and a multitude of peripheral proteins forming large signaling complexes. Cells are defined by the presence of a lipid bilayer barrier composed of various glycerolipids, sphingolipids, and sterols used to separate the cell interior from its extracellular environment. Furthermore, intracellular membranes composed in a similar manner segregate the inner machinery from the cytosolic milieu. However, due to the non-random distribution of lipids in the cell, membranes maintain a unique composition with distinct physiochemical properties. This varied and complex composition permits each membrane to function in a greater capacity as a site for signal propagation.

   Cellular lipids are comprised of linear combinations of aliphatic chains and polar head groups that are covalently linked to a glycerol or sphingosine backbone. A closer look at phospholipids provides a clear example of the structural diversity found within cellular membranes. Phospholipids are made up of various combinations of fatty acids
esterified at the $sn$-1 and $sn$-2 positions of a glycerol backbone and a phosphoryl group attached at the $sn$-3 position (2). The phosphoryl group forms a phosphodiester linkage with various headgroups. The headgroups interact with the polar environment of the cytosol or extracellular space, while the hydrophobic tails form a non-covalent network that serves to keep the membrane from falling apart. The heterogeneity of lipid composition provides structural flexibility and mechanical stability, as well as an ability to recruit specific effectors. Different tissues as well as different cell types have distinct phospholipid compositions. Furthermore, lipid composition of cell membranes provides an additional layer of complexity because phospholipids are distributed asymmetrically between the two leaflets of the membrane bilayer. For example, the outer layer of the leaflet contains the majority (75-80%) of phospholipids with a choline group attached at the $sn$-3 position while the inner leaflet contains about 80% of the lipid phosphotidylethanolamine found in the entire cell and essentially all of the phosphatidylserine (3). This asymmetric distribution is not random and serves a functional role as well as a structural one. Thus, membrane composition not only provides integrity to the cell, but it also plays an important role by providing a molecular scaffold in signaling events.

Cell membranes consist of two types of lipids: bulk lipids and low abundance or signaling lipids. Bulk lipids include phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylinositol (PtdIns), cholesterol, and sphingomyelin. Their purpose is to grant the cell structural stability, although some have been shown to be essential in signaling events (4, 5). Low-abundance lipids, such as phosphoinositides, phosphatidic acid (PA), ceramides, and diacylglycerol function as
signaling units and recruit effectors to the membrane in an organized fashion. This
distribution is not random and plays a critical role both structurally and functionally, PC
and PE are characterized as neutral zwitterions, while PS, PtdIns, and PA have a net
negative charge (6, 7). Membranes which contain substantial levels of anionic lipids
render regions of the membrane with a distinct negative electrostatic potential at the
surface. The concentration of bulk lipids are normally maintained at a steady state,
while the concentration of low-abundance lipids can fluctuate dramatically in response
to stimuli. Both types of lipids have a profound effect on the spatiotemporal dynamics of
membrane binding of peripheral proteins.

Signals are transduced across cellular membranes by machinery which become
localized at the membrane (8). Peripheral proteins are cytoplasmic proteins that are
recruited to different cellular membranes during cell signaling and membrane trafficking
(9). These proteins exploit various approaches to reversibly interact with the target
membrane (10, 11, 12). The majority of peripheral proteins contain one or more
modular motifs that allow them to specifically recognize and interact with the appropriate
membrane restricted ligand. The lipid-binding structural modules, also known as
membrane-targeting domains, include protein kinase C (PKC) conserved C1, An
additional class of proteins uses covalently attached lipid anchors to insert into the lipid
bilayer and modify their biological activity. Conversely, other proteins utilize
unstructured basic motifs on their membrane contact surfaces and form nonspecific
interactions that contribute to membrane targeting of the peripheral protein (10, 13).
This thesis will focus on the mechanism membrane targeting domains utilize and the
result of membrane binding on subcellular localization and function.
Initially, it was thought that subcellular localization was regulated by protein-protein interactions, because many proteins are recruited by activated receptors and other membrane proteins (14). In recent studies, signaling proteins were shown to contain motifs that had the necessary affinity and specificity to interact with membrane lipids themselves. Known as lipid binding domains, these motifs are conserved throughout evolution and play a critical role in cellular signaling, membrane trafficking complexes, and membrane remodeling events such as vesicle budding, phagocytosis, and cell motility (8, 15, 16). The biological significance of reversible membrane targeting is exemplified in the fact that lipid binding domains rank as one of the most common modular domains in eukaryotes, and that the discovery of major proto-oncogene proteins and tumor suppressors contain essential lipid binding motifs which regulate membrane targeting (6). Thus, the principles that govern how these domains recognize their respective targets and the role they play in the function of the protein have been intensely investigated in the past several years.

A comparative analysis of numerous membrane binding domains reveals that many of them use similar approaches to recognize a wide range of different lipid species. Lipid binding domains such as PX, C1, and PH domains provide examples of domains that contain a pocket lined with cationic residues that stereospecifically recognize polar headgroups of membrane lipids. In some cases, these domains can also recognize soluble headgroups of the target membrane lipid (8, 17, 18). In other cases, hydrophobic residues lying outside the membrane binding pocket facilitate initial membrane interactions by penetrating into the lipid bilayer, increasing membrane affinity dramatically. Recent studies have also shown that nonspecific electrostatic interactions
with anionic phospholipids are capable of recruiting peripheral proteins to the membrane surface. Nonspecific interactions with anionic lipids are generally thought to cooperate with more well-defined targets at the membrane, thus increasing affinity and providing another level of specificity in subcellular localization.

Electrostatic interactions with negatively charged membranes are well known to contribute to the subcellular targeting of peripheral proteins containing polycationic motifs (9, 10, 19). Despite the fact that most studies focus on low-abundance lipids when defining the role membranes play in cell signaling, studies have clearly shown that bulk lipids are also involved in subcellular targeting of peripheral proteins. For instance, it was shown that in response to Ca\(^{2+}\) influx within the cytosol, PKC\(\alpha\) C2 domains can migrate to the plasma membrane and bind specifically to phosphatidylserine. PKC\(\alpha\) C2 domain is a prototypical Ca\(^{2+}\)-dependent plasma membrane targeting protein, where translocation is only triggered by increase in Ca\(^{2+}\) concentration in the cytosol. The aforementioned study concludes that although the domain is capable of binding both PS and phosphoinositides, PS binding is in fact the driving force in membrane recruitment and that PKC\(\alpha\) C2 domain can specifically bind PS through a structurally well-defined pocket that is spatially separate from the known phosphoinositide binding site (4). Thus, bulk anionic lipids can play a decisive role in membrane recruitment and signaling function of cytosolic proteins.

Determining the mechanisms proteins utilize to interact with the membrane can offer researchers insight into the formation of molecular complexes and signaling scaffolds that occur in response to certain environmental cues. This thesis examines the lipid binding properties of the pleckstrin homology (PH) domain of the 3’-
phosphoinositide dependent kinase-1 (PDK1) protein, a critical mediator of downstream phosphoinositide 3-kinase signaling. Surface plasmon resonance and in vivo subcellular localization studies reveal a novel lipid binding property in which the PDK1 PH domain has a high affinity for the phospholipid phosphatidylserine. Here we provide mechanistic insight into the role lipid binding plays for a key component in a major signaling pathway found in the cell. Evidence gathered from in vitro studies using model membranes is coupled with in vivo cellular data to provide structural and functional information into how lipid binding can regulate the spatiotemporal activity of proteins involved in cell signaling and membrane trafficking.
CHAPTER 2: Phospholipid Involvement in PI3K/PDK1/Akt signaling
2.1 Phosphoinositide 3-Kinases

Phosphoinositides are phosphorylated derivatives of phosphatidylinositol (PtdIns) constitutively present in cellular membranes (20). They were first discovered in the early 1950s, when Hokin and Hokin observed changes in membrane phospholipid content upon stimulation of exocrine tissues (21). Reversible phosphorylation on the inositol ring at positions 3, 4, and 5 gives rise to seven isoforms; three monophosphorylated (PtdIns(3)P, PtdIns(4)P, PtdIns(5)P; three bisphosphorylated (PtdIns(4,5)P$_2$, PtdIns(3,4)P$_2$, PtdIns(3,5)P$_2$); and one trisphosphorylated (PtdIns(3,4,5)P$_3$). These seven phosphoinositide species are concentrated in separate pools of cytosolic membranes of eukaryotic cells. Furthermore, their levels and turnover are tightly regulated. Although phosphoinositides together make up less that 1% of cellular lipids, they play very important roles as intermediates in major signal transduction pathways, serving as docking sites for signaling effectors and as second messengers for a multitude of signaling cascades (20).

Phosphoinositides serve as substrates for kinases, lipases, and phosphatases that are either membrane bound or are recruited to the membrane where they modify the target phosphoinositide. Some of these pathways are well-established, while others still remain elusive at the molecular level. One of the more well-defined pathways is that of the PI-specific phospholipase C (PLC), which cleaves PtdIns(4, 5)P$_2$ into membrane-bound diacylglycerol (DAG) and soluble Ins(1, 4, 5)P$_3$ (22). Another route involved in cellular inositol lipid metabolism utilizes a group of kinases known as phosphoinositide 3-kinases (PI3Ks). The emerging links between PI3K activity and many human diseases including cancer, diabetes, and heart disease have made them
the focus of many researchers, in hopes that elucidating their mechanism of regulation can aid in the development of therapeutic agents.

PI 3-kinase was discovered in 1984, when a group of researchers found an enzyme with minor inositol lipid kinase activity associated with immunoprecipitated oncogene products and could also reside in complexes with activated growth factor receptors at the membrane (i.e. EGF and PDGF receptors) (23). Future studies showed that this enzyme was capable of catalyzing the addition of a phosphate specifically to the 3’-hydroxyl group on the inositol ring of phosphoinositides. The PI 3-kinase superfamily consists of a large group of structurally related enzymes which are classified based on their PI substrate requirements and modes of regulation. According to data mining genome sequencing projects and cloning studies, there have been eight functional PI 3-kinase catalytic subunits identified which are able to phosphorylate inositol lipids (23). These isoforms have been divided into three distinct classes based on substrate specificity, protein domain structure, and regulatory subunits associated with them. They include class I enzymes p110α, p110β, p110δ, and p110γ; class II enzymes PI3K-C2α, PI3K-C2β, and PI3K-C2γ; and the one member of class III is Vps34 found in yeast (24, 25, 26). Class I and class III PI3Ks are made up of a catalytic subunit and a regulatory subunit, while class II PI3Ks are large proteins with catalytic subunits 45-50% homologous to that of class I PI3Ks. Class II PI3Ks also have a C-terminal region homologous to C2 domains that regulate lipid and calcium binding in protein kinase C isoforms (27).

These enzymes are classified according to their selectivity for various phosphoinositide species. The Class III PI3Ks are most specific for phosphotidylinositol
(PtdIns) and form the majority of phosphatidylinositol (3) phosphate (PtdIns(3)P) in the cell. Class II PI3Ks are the least characterized subclass of PI3Ks, yet they have been shown to preferentially phosphorylate PtdIns and phosphoinositol (4) phosphate (PtdIns(4)P) in vitro producing PtdIns(3)P and PtdIns(3,4)P₂ respectively. Class I PI3Ks are the most heavily researched class of PI 3-kinases because these isoforms are coupled with cellular growth factor responses. Although class I PI 3-kinases are capable of phosphorylating PtdIns, PtdIns(4)P, and PtdIns(4,5)P₂ in vitro, the preferred lipid substrate for class I in vivo is PtdIns(4,5)P₂ (PIP₂), leading to the production of PtdIns(3,4,5)P₃ (PIP₃). Generation of PtdIns(3,4,5)P₃ has a well-documented effect on the cell and leads to the activation of a host of cellular signaling processes.

The class I family is further subdivided into 2 groups based on their regulatory partners and mode of activation. The class IA subgroup consists of the catalytic subunits p110α, p110β, p110δ which all interact with a family of Src homology 2 (SH2)-domain containing regulatory adaptor proteins known as the p85 family. Isoforms from the p85 family are constitutively bound to the N-terminal region of the of p110 catalytic subunit through an inter-SH2 region and tightly regulate protein activation (28). Each regulatory subunit also contains two SH2 domains that are capable of recognizing phosphorylated tyrosine residues on growth factor receptors in a sequence specific manner. Upon growth factor stimulation, the membrane receptor becomes autophosphorylated on tyrosine residues which triggers translocation of the active p85/p110 complex to the plasma membrane surface. Thus, the catalytic subunit gets brought into close proximity with its membrane bound substrate, PIP₂, leading to the catalysis and formation of PtdIns(3,4,5)P₃. Studies are beginning to indicate that
individual isoforms have distinct, but possibly overlapping roles, which may vary between cell types. Knockout studies show that the p110α subunit has a specific role in cell survival, while p110β could be involved in cell proliferation signaling (29, 30). Overall the findings highlight the importance of why PI 3-kinases must be tightly regulated.

The p110γ catalytic subunit is the only member of class IB and provides versatility to PI 3-kinase signaling pathways activated by the extracellular stimuli. One difference is that p110γ lacks the extreme N-terminal site recognized by p85 regulatory subunits. Instead this enzyme forms a heterodimer complex with the p101 regulatory subunit, a novel protein that is unrelated to p85 regulatory subunits as well other known proteins. Two other regulatory subunits, p84 and p87 PI 3-kinase adaptor proteins, have also recently been identified to regulate p110γ (31, 32). Furthermore, as opposed to class IA PI 3-kinases, the p110γ isoform specifically responds to activation of G-protein coupled receptors (GPCRs). Activation of p110γ by GPCRs is mediated by the Gβγ subunit, which appears only after activation of the heterotrimeric G protein. Studies have shown that fully intact p101 is essential for the activation of p110γ catalytic subunit (33).

The various modes of activation of class I PI 3-kinases highlight their importance in the cell. These proteins are activated by a large variety of extracellular stimuli, which offers the cell versatility as well as flexibility in maintaining homeostasis. Furthermore, class I PI 3-kinases are coupled to receptor tyrosine kinases and G-protein coupled receptors, which adds a level of complexity to the PI 3-kinase story. Gaining a clearer understanding of the mechanisms by which each activated receptor is able to recruit
similar isoforms may provide valuable information on cross-talk between GPCRs and tyrosine kinase receptors.

Ultimately the recruitment by either pathway leads to an accumulation of PtdIns(3,4,5)P$_3$, and, with some delay, PtdIns(3,4)P$_2$, in the plasma membrane. This is a seminal event inside of the cell, because appearance of 3’-phosphorylated PtdIns in the plasma membrane is known to recruit a host of peripheral proteins which are activated upon membrane binding. Many of these proteins are known to harbor domains specific for these phosphorylated phosphoinositides and allow them to dock at discrete intracellular locations in which the lipid is produced. Membrane binding can spatially restrict the protein to a site of function and as well as limit interactions with other proteins. Determining how various effectors are able to properly respond to essentially the same signal will improve our understanding of mechanisms the cell utilizes to govern itself according to the extracellular stimuli.

Improper PI 3-kinase signaling often occurs in cancer. Although there are several levels where deregulation can arise, one in particular is overactivation of class IA PI 3-kinases. The gene that encodes for the p110α subunit, PIK3CA, is frequently mutated in human cancers. Most common mutations (>80%) occur within the helical domain (E543K/E545K), or the kinase domain (H1047R) (34). The high frequency of mutation within the same location is analogous to previously discovered oncogenes such a KRAS, where mutation leads to a gain-of-function phenotype. Mutation in either of the conserved regions on PIK3CA led to a constitutively active catalytic subunit which causes varying degrees of elevated levels in PtdIns(3,4,5)P$_3$ and was characterized as being tumorigenic. PtdIns(3,4,5)P$_3$ can recruit the serine/threonine kinase, Akt, as well
as an upstream kinase, PDK1, to the membrane where PDK1 phosphorylates Akt. Akt is believed to transduce the major downstream PI 3-kinase signal in cancer, and elevated levels of PtdIns(3,4,5)P$_3$ have been shown to augment Akt signaling in several systems (35, 36). Interestingly, some of the PIK3CA mutant cells show diminished levels of Akt phosphorylation (37). Furthermore, subcellular localization studies have shown that GFP-tagged Akt remains predominantly cytosolic in PIK3CA mutant cells with low p-Akt. It seems that mutations throughout the PI 3-kinase pathway are non-redundant and have distinct roles in the auto-proliferation of mutant cells.

Akt, also known as protein kinase B, is a well-established downstream effector of PI 3-kinase activation. Akt is thought to regulate a number of cellular processes, including glycogen metabolism, protein synthesis, apoptosis, and cell proliferation. The precise way in which membrane binding regulates the activity of Akt is not completely understood, consequently making it a focal point for rigorous studies of many laboratories.

### 2.2 Akt/Protein Kinase B

Akt was discovered independently by three research groups in 1991 based on its similarity to cAMP-dependent protein kinase 1 (PKA) and protein kinase C (PKC) and was subsequently cloned from an isolated retrovirus found in leukemic mouse T cells (38). At present, 3 isoforms have been identified, Akt1, Akt2, Akt3, which all share similar sequence homology and secondary structure (39, 40). Although tissue distribution of each isoform varies, the mode of regulation seems to be the same for all. Akt is a serine/threonine (Ser/Thr) kinase and belongs to a larger subfamily known as the AGC kinases. The term AGC kinase was established in 1995 to define a subgroup
of Ser/Thr kinases that contained catalytic domains similar to that of PKA, cGMP-dependent protein kinase (PKG), and PKC (41). There have been 60 AGC kinases identified in eukaryotic cells, with 42 of them containing functional regulatory domains independent of the conserved catalytic core. These regulatory domains define subgroups within the family, and play important roles in kinase activity and subcellular localization. AGC kinases respond to a wide range of extracellular stimuli and participate in a host of biological responses. Characterization of the AGC kinase family has shown many harbor lipid binding domains along with their catalytic core, suggesting that membrane binding could play a major role in the regulation of these proteins.

Akt consists of three conserved domains, an N-terminal pleckstrin homology (PH) domain, a central kinase domain, and a C-terminal extension which contains a hydrophobic motif (42, 43, 44). The pleckstrin homology domains are 80% identical in Akt isoforms, and 30% identical to PH domains found in other proteins. The catalytic domains of Akt isoforms are 90% identical and very closely related to other AGC family kinases, while the hydrophobic motif is only 70% identical among isoforms and is most closely related to a similar region found in PKC (42). It is hypothesized that the PH domain and the hydrophobic motif fold in upon the catalytic domain when the protein is in an inactive state (45). Only under conditions in which a signal would provoke a large conformational change could the protein be activated and serve its function inside the cell.

As discussed earlier, activation of PI 3-kinase induces a transient influx of PtdIns(3,4,5)P$_3$ and PtdIns(3,4)P$_2$ in local regions of the plasma membrane. The PH domain is known to bind among other ligands, PtdIns(3,4,5)P$_3$ and PtdIns(3,4)P$_2$. The
PH domain of Akt isoforms indeed has a high affinity for both PtdIns(3,4,5)P$_3$ and PI(3,4)P$_2$, and studies have shown that Akt activation is PI 3-kinase dependent. PtdIns(3,4,5)P$_3$ recruits Akt from the cytosol to the plasma membrane, where it undergoes a major change in conformation, exposing its catalytic core as well as a key residue found on the C-terminal hydrophobic motif. Phosphorylation of the activation segment, also known as the T-loop, at Threonine 308 (Thr$^{308}$) stimulates enzymatic activity at least 100-fold, while phosphorylation of the hydrophobic motif at Serine 473 (Ser$^{473}$) increases activity by another 10-fold (46). Taken together, dual phosphorylation initiated by the activation of PI 3-kinase leads to 1000-fold increase in the activity of Akt. Active Akt isoforms in turn act downstream of PI 3-kinase, phosphorylating effector proteins and setting into motion a number of signaling cascades inside the cell. Thus, Akt has emerged as the most proximal node downstream of PI 3-kinase activation.

The structure of the PH domain of Akt complexed with Ins(1,3,4,5)P$_4$, the soluble headgroup of PtdIns(3,4,5)P$_3$, was solved using selenomethionine-multiwavelength anomalous dispersion(MAD) (47). The crystal structure reveals a highly polarized structure, with a distinct patch of cationic residues forming a structured pocket which participate in hydrogen bonding with the D-1,-3,-4, and -5 phosphoryl groups on Ins(1,3,4,5)P$_4$. These residues structurally align with corresponding cationic amino acids found in the PH domain of Bruton’s tyrosine kinase (BTK), a known PIP$_3$-specific binding domain (48). Mutation of any of these residues to alanine abolishes the crystal structure of the domain interacting with the ligand. Furthermore, these mutants showed loss in ability to respond to growth factor stimulation as well as decreased specific activity (49). Failure to translocate suggests that membrane localization plays a critical
Figure 1. **Overview of the PI3K/Akt signaling pathway.** Activation of PI3K leads to the transient production of 3’ phosphoinositides, PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂, which in turn recruits Akt to localized regions of the membrane whereupon it is activated. Once active, Akt can control key cellular processes including apoptosis, protein synthesis, metabolism, and cell cycle progression.
role in Akt activation by growth factors.

The mode of regulation displayed here is not uncommon. At resting state, Akt is inactive, and the PH domain conformation is the predominant factor for intramolecular inhibition. The PH domain masks the activation loop of Akt, which keeps Akt from becoming improperly activated and participating in signaling cascades that would cause homeostatic instability. This intrinsic autoinhibition is released with the generation of 3'-phosphoinositides and their appearance has a two-fold effect on Akt activation. First, 3'-phosphoinositides induce a conformational change in Akt and expose the activation loop. A study has shown that if the Thr$^{308}$ is mutated to alanine, the protein can still translocate to the plasma membrane, but the protein remains inactive (49). So phosphorylation of Thr$^{308}$ is a key step in activating Akt. Second, the upstream kinase that phosphorylates Akt on its activation loop is also found at the membrane. 3'-phosphoinositide-dependent-kinase-1 (PDK1) has been identified as the upstream kinase capable of phosphorylating Akt upon its activation loop. This protein also contains a PH domain capable of binding 3'-phosphoinositides. So membrane binding also spatially restricts Akt to a place where it can co-localize with its upstream kinase partner and become activated.

Following the identification of a residue on the kinase GSK3 as the first direct target of Akt in cells (50), researchers have identified a minimum required motif for Akt recognition of R-X-R-X-S/T-B, where X stands for any amino acid, and B represents a bulky hydrophobic amino acid (46). While currently there are no other motifs rigorously tested and shown to be recognized by Akt, it remains possible that there are other sequential models which Akt interacts with. How phosphorylation of a specific site
drives a particular functional response is the most challenging aspect of characterizing the interaction between the kinase/substrate pair. In many cases, site directed mutagenesis where phosphorylation sites are mutated to glutamic acid or aspartic acid (phosphorylation mimic) or alanine (loss-of-function) are used on a substrate that has known function. Loss of function mutation should block the positive or negative effects of Akt on the substrate during a cellular process while phosphorylation mimics can induce activity of specific cellular processes, although in the latter case this is not always reliable.

Akt is probably most well-known for its involvement in promoting cell survival. Akt enhances cell proliferation by inhibiting the function of effectors that participate in promoting cell death. Akt blocks the expression of several Bcl-2 homology domain 3 (BH3)-only proteins, which function by binding prosurvival Bcl-2 family members and inducing apoptosis (51). Survival elements stimulate direct phosphorylation of the BH3-only protein BAD by Akt, which creates a binding site for proteins containing 14-3-3 motifs. When 14-3-3 proteins bind to BAD, they induce a conformational change that leads to the release of BAD from its target proteins (52). Now Bcl-2 proteins are free to form complex with Bax protein, thus inhibiting Bax-triggered apoptosis (53). Akt also inhibits apoptosis by regulating transcription factors responsible for expression of BH-3 only proteins. Akt phosphorylation of transcription factors occurs in the nucleus, and leads to the recruitment of 14-3-3 proteins which bind them and initiate nuclear export. This process blocks transcription of target genes involved in apoptosis, cell-cycle arrest, and metabolic processes (54). Finally, a third target of Akt that inhibits BH3-only proteins and triggers survival response is MDM2, and E3 ubiquitin ligase that triggers
the degradation of the transcription factor p53. p53 is a tumor suppressor that regulates cell cycle progression and has a critical involvement in cancer prevention. Upon phosphorylation by Akt, MDM2 translocates to the nucleus and binds to p53, which targets the complex to the proteosome for degradation.

Much work has gone into understanding how Akt functions inside the cell. Our understanding of the complex circuitry of Akt signaling has been extensively built upon over the past 15 years. A literature search has found over 100 reported potential substrates for Akt. Of those, 18 substrates have been identified by several published independent studies (51). Figuring out how all the pieces fit together will be a challenging obstacle in elucidating Akt function in the fates of so many signaling cascades.

2.3 3'-Phosphoinositide Dependent Kinase-1 (PDK1)

2.3.1 PDK1 role in 3' phosphoinositide signaling cascade

The event in which growth factors and insulin bind to their respective receptors leads to the activation of PI 3-kinase and the generation of PtdIns(3,4,5)P_3 in the plasma membrane. 3' Phosphoinositide dependent kinase-1 (PDK1) was discovered in 1997 in the field of insulin signal transduction research as the upstream kinase capable of phosphorylating and partially activating Akt in a PtdIns(3,4,5)P_3-dependent manner (55, 56). In the same way that activation of PI 3-kinase and influx of PtdIns(3,4,5)P_3 is the major apical signaling event, PDK1 plays a crucial role mediating the physiological response downstream to this important second messenger. PDK1 is a 60kDa serine/threonine kinase, composed of an N-terminal kinase domain and a C-terminal pleckstrin homology domain. It is known as the master regulator and member of the
AGC kinase superfamily, which are known as the primary transducers of the PI 3-kinase signal.

AGC kinases all share a structural similarity and a common mode of activation in which phosphorylation of 2 highly conserved residues is required. PDK1 phosphorylates all 23 agonist-stimulated AGC kinases upon serine/threonine residues found within their activation loop (17). The other required site for phosphorylation is in the hydrophobic motif. This site is regulated by a number of different kinases and is dependent upon the PDK1 target itself.

PDK1 is ubiquitously expressed in cells, and its activity is not affected by agonist stimulation. Researchers found that PDK1 immunoprecipitated from either unstimulated or growth factor stimulated cells possessed the same catalytic activity (55, 57, 58). In order for PDK1 to become active, it must be phosphorylated at the activation loop like all AGC kinases. Researchers found bacterially expressed PDK1 was fully active, indicating that this protein possesses the intrinsic ability to phosphorylate its own T-loop residue (59). Because of this quality regulation of PDK1 activity is distinct among most known enzymes. Biochemical, genetic, and structural data all conclude that in the absence of stimuli, the catalytic activity of PDK1 is mediated by its ability to access its substrates. Therefore, agonist stimulation is utilized to convert AGC kinases into forms which can be recognized and activated by PDK1.

Once it was clear that PDK1 was constitutively active, research shifted to how PtdIns(3,4,5)P$_3$ appearance enabled PDK1 to activate its substrates. For Akt it appears that the mutual ability of Akt and PDK1 to both interact with PtdIns(3,4,5)P$_3$ and PtdIns(3,4,)P$_2$ through their PH domains enables both proteins to co-localize together in
a spatial and temporally regulated way. In order for Akt to be efficiently activated by PDK1, 3’-phosphoinositides must be present, thus requiring the activation of PI 3-kinase inside the cell. Agonist stimulation has indeed shown to trigger translocation of Akt to the plasma membrane, an event that is prevented by treatment with PI 3-kinase inhibitors (60). This translocation is dependent upon the integrity of the Akt PH domain as mutation to the lipid binding pocket prevents Akt translocation (55). Whether or not PDK1 translocation to the plasma membrane requires PI 3-kinase activity remains a point of contention. Studies have shown that PDK1 translocates to the plasma membrane upon agonist stimulation, while another study identified a pool of PDK1 constitutively present at the plasma membrane in unstimulated cells (58, 61, 62).

More evidence supporting the hypothesis that PDK1 localization at the plasma membrane is PI 3-kinase independent comes from a study which utilizes a myristoylation sequence attached at the N-terminal end of Akt. The attachment of a plasma membrane targeting motif induced maximal T-loop phosphorylation in unstimulated cells, which could only have occurred if PDK1 was also present at the plasma membrane (44). In another study the PH domain of PDK1 was mutated and it was shown that its ability to bind phosphoinositides in vitro had been abolished. However, the study also revealed that insulin-induced activation of Akt was reduced, but not completely prevented (63). These points suggest that a factor other than phosphoinositides may be involved in regulating the co-localization and subsequent activation of Akt by PDK1.

Research has clearly shown Akt regulation is dependent upon membrane binding and the protein’s conformational state. However, most substrates of PDK1 are
found in the cytosol, thus requiring another mechanism through which they are recognized and activated by their common upstream kinase. All AGC kinase family members, excluding PDK1, contain a hydrophobic motif positioned C-terminal to the kinase domain (41). The hydrophobic motif contains a conserved Phe-Xaa-Xaa-Phe-Ser/Thr-Phe/Tyr sequence, where the Ser/Thr represents a site of phosphorylation. A yeast two hybrid screen found that the catalytic domain of PDK1 had a high affinity for the C-terminal fragment of PKC-related kinase-2(PRK2), which contained a similar sequence as the one found in AGC kinase hydrophobic motifs, only in PRK2, the Ser/Thr position was changed to Aspartic acid (17). This fragment was termed the PDK1-interacting fragment (PIF), and the idea was that the change from Ser/Thr to Asp was equivalent to the effect phosphorylation would have on the hydrophobic motif. Mutagenesis to alanine abolished PDK1 interaction, thus providing evidence that the hydrophobic motif could provide a docking site for PDK1 to interact with its other downstream effectors. Furthermore, mutagenesis of the hydrophobic pocket of p70 ribosomal S6 kinase (S6K1), a known substrate of PDK1, led to constitutively active protein (64). These studies offer evidence that this region has a bifunctional role for many PDK1-regulated kinases. First, it serves as a docking site for PDK1 when in a phosphorylated state; additionally, the hydrophobic motif interacts with a groove in the catalytic region of the same molecule which causes the transition of the AGC kinase into an active conformation. Interestingly, PDK1 itself does not contain a hydrophobic motif. Instead, it has a region in between the catalytic domain and the PH domain which forms a pocket. Known as the PIF pocket, this region has been shown to be
necessary for PDK1 to recognize and activate a vast number of its substrates inside the cell (64, 65).

Another study investigated the role of the hydrophobic PIF-binding pocket of PDK1 for effective activation AGC kinases S6K1, SGK1, and Akt (66). When the PIF pocket was mutated (L155E) and tested for its ability to phosphorylate these substrates, both S6K1 and SGK1 phosphorylation diminished drastically compared to wild type PDK1. In contrast, Akt phosphorylation was not affected by PIF pocket mutation and was only dependent upon addition of PtdIns(3,4,5)P3. Interestingly, a construct lacking the PH domain (ΔPH-Akt) was shown to be a poor substrate for L155E, with phosphorylation being similar to that of S6K1 and SGK1. Researchers concluded that lack of a PH domain converts Akt into a substrate similar to other AGC kinases, which require a functional PIF pocket for proper activation. These experiments establish that PDK1 recognizes Akt and other AGC kinases by distinctly different mechanisms, emphasizing the need for complete understanding of PDK1 regulation.

2.3.2 Structural Insights into PDK1

High resolution crystal structures of both the catalytic domain and the pleckstrin homology domain of PDK1 are both available for researchers to gather more insight into how each folded motif serves specific roles in the physiological function in cells.

The high resolution crystal structure of the kinase domain complexed with ATP was solved and is registered with the Protein Data Bank as entry 1H1W (67). The domain assumes a classic bilobal kinase fold, in which the catalytic core possesses an N-terminal lobe consisting mainly of β-sheet and a predominantly α-helical C-terminal lobe (68). It is similar to the catalytic domain of protein kinase A (PKA), which is the
only other AGC kinase that does not possess a traditional AGC kinase hydrophobic motif. Instead, PKA contains a FXXF terminating sequence, which shows similarity to the first part of the hydrophobic motif of S6K1 and SGK1. As stated earlier, unlike all other AGC kinases, PDK1 does not contain a hydrophobic motif. Structurally, this region normally forms an amphipathic α-helix which packs against the kinase core (69). However, in PDK1 this region remains mostly unstructured; suggesting the N-terminal region of PDK1 may perform a different function than the corresponding region found in other AGC kinase domains. Upon closer examination, it was determined that a hydrophobic groove approximately 5 angstroms deep did exist within the small lobe which was required for the binding of PDK1 to the hydrophobic motif of its substrates (17, 67). Also known as the PIF pocket, it was found to contain at its core a Leucine residue, which was shown to be essential for proper activation of representative AGC kinases (70). A second pocket was also identified adjacent to the PIF pocket that was occupied by a sulfate ion. This region was hypothesized to be the phosphate binding site for the phosphorylated residue of the substrate hydrophobic motif. Mutation of this pocket led to drastically reduced T-loop phosphorylation of S6K1 (67, 71). However, agonist stimulation led to Akt activation, yet another example that this kinase is activated differently than related kinases.

The crystal structure of the PDK1 PH domain (PDB:1W1D) was solved in 2004 complexed with the Ins(1,3,4,5)P$_4$, the soluble headgroup of PtdIns(3,4,5)P$_3$ (72). The structure of the PDK1 PH domain revealed structural variation with respect to the standard PH domain fold. N-terminal to the common fold has been termed the bud region, consisting of 2 antiparallel β strands along with an additional α-helix. The
proposed function of the bud region is to provide structural stability to the PH domain. The crystal structure indicated that the bud region can pack against the β barrel and form hydrophobic contacts as well as several non-covalent hydrogen bonds (72). Overall the bud buries approximately 30% of the PDK1 PH domain surface, creating a hydrophobic core between the helix in the bud and hydrophobic residues found within the β barrel (72). The second hydrophobic core is not standard for PH domains and suggests the bud region as an integral part of the overall fold for PDK1 PH domain. The PH domain itself is made up of two orthogonal β sheets, capped at one end by an amphipathic α-helix. At the other end lies the phosphoinositide-binding pocket, made up of basic residues found both in the loop regions connecting the β strands as well as the strands themselves. These residues form a shallow positively charged pocket which interacts with the phosphate groups of Ins(1,3,4,5)P₄ through electrostatics and hydrogen bonding. The phosphoinositide binding pocket is significantly more spacious than other PH domains, as illustrated by Figure 2. The variable loop 1 for PDK1 is considerably less constrictive than the corresponding loop regions found in DAPP1-PH and PKBα-PH. The larger ligand binding site could provide an explanation for why PDK1 PH domain shows promiscuous selectivity and has strong affinity for multiple phosphoinositol derivatives. PDK1 has the ability to interact with Ins(1,3,4,5,6)P₅ as well as Ins(1,2,3,4,5,6)P₆ with strong affinity, which has been used as evidence to describe a mechanism in which PDK1 is sequestered to the cytosol where it can properly regulate substrates that do not translocate to the plasma membrane (72). Mutational analysis has found that Lys465 is the primary residue involved in phosphoinositol interaction. This residue is found at the bottom of the lipid binding
pocket and is capable of forming hydrogen bonds with both D3 and D4 phosphates (45). As expected, when Lysine was mutated to Glutamic acid, PDK1 lost its ability to interact with phosphoinositides, and a decrease in the level of Akt phosphorylation \textit{in vivo} was observed (63).
Figure 2. Structural comparison of Ins(1,3,4,5)P$_4$ binding to PH domains.
2.4 Pleckstrin Homology Domains

The pleckstrin homology (PH) domain is the 11th most abundant domain class found in the human genome (73). It is the largest and most functionally diverse lipid binding domain that has yet been identified (8, 74). Originally identified in 1993 as a 100-120 residue stretch of amino acids with similar primary sequence that occurred twice in the protein pleckstrin, the PH domain has subsequently been identified in many effector proteins involved in cellular signaling, cytoskeletal organization, membrane trafficking and phospholipid metabolism (75). Over 100 three dimensional crystal and NMR structures of various PH domains have been registered in the Protein Data Bank (20), suggesting that not only is the domain structurally stable, it is also evolutionarily important. The canonical PH domain folds into a seven-stranded β-barrel, capped at one end by C-terminal α-helix (76, 77). The other end remains open and is framed by three loop regions that vary in length and sequence. Although originally functional characteristics had yet to be discovered, the domain had two distinct features. First, each domain has essentially the same structure yet the sequence homology can range from 7-23% (78, 79). Second, for many PH domains the electrostatic potential was highly dipolar, with high positive charge being localized to the open-ended loop region. Observers postulated that this region could be a ligand binding site based on the variable sequence homology and the electrostatic sidedness. The folding properties of PH domains closely resemble Src homology-2 (SH2) and SH3 domains, protein modules known to drive key protein-protein interactions. That led most researchers to focus on identifying a peptide target as the common ligand for PH domains. Subsequent work has shown that many PH domains direct membrane targeting of their
Figure 3. **High resolution crystal structure of the prototypical PH domain.** X-ray crystal structure of the PH domain of Akt1(PKBα) (*green*) bound to Ins(1,3,4,5)P₄ (*orange*).
host proteins, but do so through binding to phosphoinositides rather than proteins localized at the membrane. Although several PH domains have been shown to bind lipids, the mechanism by which most target the membrane within a physiological context has yet to be fully explained.

Studies followed which have shown a small subset (10-20%) of PH domains recognize particular phosphoinositides with high affinity and specificity (77). Proteins that contain high affinity PH domains utilize them as a tool to drive the protein to regions of interest in the membrane where it can then perform its physiological function. Phosphoinositide recognition is dependent upon electrostatic forces as well as stereoselective properties which allow the domain to discriminate between phosphoinositide species. PH domains that bind with high affinity tend to share a common sequence motif of Kxₙ(K/R)xR found within the first variable loop region which coordinate the acidic phosphate groups on the inositol ring and constrain the lipid within the pocket accordingly (79, 80). This subset tends to bind the corresponding polar headgroup with similar specificity and dissociation constants ranging from low nanomolar (nM) to low micromolar (µM) range. Because of this, many PH domains showing selectivity have been fused with green fluorescent protein (GFP) and used in vivo as probes for studying subcellular distribution of various pools of phosphoinositides (81). Although high affinity phosphoinositide binding has become the trademark for which PH domains are best known, studies have shown this is not exclusive to all. For instance, a genome wide analysis of 33 PH domains encoded in the S. cerevisiae species found that only one could bind PtdIns(4,5)P₂ with high affinity and selectivity (82). Six others were characterized as having moderate affinity and low selectivity while
the rest seemed to bind phosphoinositides very weakly or not at all. Studies have concluded that most PH domains require more than one binding target in cellular membranes, and that the most characterized aspect about them is also the least common.

PH domains have been the subject of many recent investigations due to their ability to bind with high affinity to PI 3-kinase products. As discussed earlier, PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ are barely detectable in resting cells, and upon agonist stimulation appear transiently in the plasma membrane (83). Even at maximal PI 3-kinase activity, peak levels of polyphosphorylated 3-phosphoinositides are considerably lower than their more abundant precursor, PtdIns(4,5)P₂ (79,84). Proteins such as Bruton’s tyrosine kinase (Btk), general receptor for 3-phosphoinositides (Grp1), and Akt, all contain PH domains that share similar high affinity for PI 3-kinase products while affinity for PtdIns(4,5)P₂ is between two and three orders of magnitude lower (80, 85). Thus, these proteins are capable of recognizing their target lipid amidst the high background associated with PtdIns(4,5)P₂ levels. PH domains that show high affinity and selectivity for phosphoinositides all share a common motif in which 3 basic residues are strategically placed to make up a rigid pocket. In PH domains that bind PI 3-kinase products with high affinity, this motif is expanded to ψ-X-K-(G/A/S/P)-Xm- ψ -X-(K/R)-X-R-X- ψ -X- ψ -Xn-Å-X-Y which spans from the β1 to β3 strands. The boldface residues are all conserved while the symbol ψ represents an essential hydrophobic residue. The hydrophobic residues not only stabilize the core of the domain, but also support the conserved residues which make contact with the phosphoinositide. Crystal structure analysis has shown that although the headgroup might be positioned differently, this
small subset of PH domains retains a common mechanism in which the conserved residues interact simultaneously with 3- and 4-phosphates (84). Furthermore, sequence alignment of various 3-phosphoinositide binding PH domains also shows that the length of the variable loop connecting β1 strand to β2 is not random and has three distinct sizes—a somewhat short, 6 to 7 residue strand, a very large 11 residue strand, and a third type that is usually 10 residues in length. There are no sequence requirements, however there is a clear preference for basic or hydrophobic residues. The hydrophobic residues interact with the interfacial region of the lipid bilayer while the positively charged residues form nonspecific contacts with acidic membranes (84). A similar model has been utilized by PtdIns(3)P specific FYVE domains (86). These nonspecific interactions contribute significantly to the overall binding affinity of these proteins. Since phosphatidylinerine is the most abundant anionic lipid found within the inner leaflet of the plasma membrane, it has been suggested that it is the membrane target participating in these interactions.

Most PH domains have low affinity for phosphoinositides and display the ability to interact with almost all anionic lipid species, including phosphatidylinerine, suggesting they have no stereospecificity. Furthermore, the binding strength in many of these domains correlates with the number of phosphate groups found on the inositol ring, rather than the spatial arrangement (80, 87). Therefore, the driving force behind membrane recruitment for promiscuous PH domains is a non-specific, delocalized, electrostatic selection that only recognizes the negative charge of the lipid surface. NMR studies show these domains still retain electrostatic sidedness and that lipid binding occurs on the positively charged face of the domain. However, many of the side
chains found in the lipid binding pockets of high affinity PH domains are missing in low affinity PH domains. Lack of conservation leads to the formation of a partial binding site, where loss of key basic residues causes a decrease in the number of hydrogen bonds formed between the lipid and the protein. Consequently, fewer interactions result in lower affinity and fewer constraints put upon the orientation of the polar headgroup. Under these conditions, a delocalized electrostatic attraction is the only tool the protein can utilize to drive itself to the membrane surface. Weak binding is often enhanced by avidity effects (i.e. oligomerization) as well as through interaction with ligands other than phosphoinositides found in the membrane (88). This suggests that PH domains may require a second factor distinct from phosphoinositides which enables the protein to relocate to regions abundant in both ligands. This idea of a dual key strategy has become quite popular over the past 10 years as an explanation for the physiological function of low affinity binding modules.

2.5 Role of Phosphatidylserine in biological processes

Research has shown that lipid composition of biological membranes plays a critical role in determining what signals are being transduced in addition to the subcellular localization of proteins capable of interacting with the bilayer surface. Years of research involving phosphoinositide signaling has proven that lipids make good second messengers which can be used to recruit cytosolic proteins to the membrane surface, ultimately leading to a binding reaction and the propagation of a lipid signal. What is less well understood is the role bulk phospholipids play in cellular signaling processes. Phosphatidylserine (PS) is a relatively minor component (>10%) of most biological membranes (3, 89). Yet its low abundance is outweighed by its physiological relevance,
which is attributed to its unique physical and biochemical properties. Scientists agree that the plasma membrane is the region where PS is most abundant, namely the inner leaflet of the lipid bilayer. Interestingly, a study looking at subcellular fractions of *S. cerevisiae* found that PS is the major phospholipid found in the plasma membrane, making up 34% of total phospholipids (90). The most distinctive feature about PS is the serine linkage found sn-3 position of the glycerol backbone. Other bulk phospholipids like phosphatidylcholine and phosphatidylethanolamine have cationic linkages at the sn-3 position which cancels out the negative charge of the phosphoryl group, rendering the lipid headgroup neutral essentially. In the case of PS, serine is neutral, so the headgroup retains a net negative charge. Therefore, membranes with substantial amounts of PS are known to be anionic in nature. Phosphoinositides found in the plasma membrane are normally thought of as the major lipid components required to attract a primary effector to the membrane surface through electrostatic forces. However, even at their highest levels in the plasma membrane, phosphoinositides only make up a very small percentage (~3%) of the total membrane lipid. PS is significantly more abundant within the inner leaflet, and has been shown to play an active role in the recruitment of proteins capable of binding phosphoinositides specifically. In some cases PS binding is the major driving force for membrane recruitment, while phosphoinositide binding only enhances the affinity (4, 91). These findings, along with its charge and the asymmetric distribution, make defining the role of PS in signal transduction pathways and the search for PS-binding proteins an important area of study.
Figure 4. **General structures for Phosphatidylserine and Phosphoinositides.** Phospholipids are generally made up of two apolar acyl chains bonded to a glycerol backbone and a polar headgroup, also bonded attached to the glycerol backbone. The polar headgroup for PS is substantially smaller than the inositol headgroup of phosphoinositides. Their structure is quite distinct from one another.
In addition to its presumed structural role, PS is actively involved in lipid signaling events that fall under two general categories; on the cytosolic leaflet it serves as an anchor for signaling proteins, while its appearance on the outer leaflet of the plasma membrane regulates significant extracellular events. Because of the impact on the organism, the functional roles of extracellular PS signaling have been more extensively studied and are better understood. For instance, during hemostasis, blood platelets are activated and expose PS on their outer surface. This event triggers the activation of blood clotting factors found in the extracellular space, which are recruited to the surface of blood platelets to form a complex. Specialized modules like the discoidin-type C2 domain and gamma-carboxyglutamic acid (Gla) domain found within coagulating factors recognize and bind PS with a high degree of selectivity and stereospecificity (89, 92). These proteins help to initiate the process of wound healing and limit blood loss.

Another cellular event in which PS exposure plays an active role is the process of apoptosis. When cells become damaged or reach a state in which they are no longer maintaining themselves properly, signals are triggered which induce programmed cell death, or apoptosis. One of the hallmarks of early apoptosis is the exposure of PS on the extracellular membrane of cells (93). PS exposure allows for selective targeting by phagocytes which rapidly engulf apoptotic cells and remove them. The exposed PS is believed to be targeted by the macrophage surface receptor encoded by the Psr gene that is specific to PS. Research supporting this model has shown that disruption of proper expression from the Psr gene in C. elegans and mice leads to severe developmental abnormalities (3, 94). This process enables the organism to undergo
renewal without potentially releasing harmful intracellular enzymes into the extracellular space. Another interesting aspect involving PS and programmed cell death is that increased synthesis of PS appears to protect cells from UV-induced programmed cell death. When cells overexpressing either of the known enzymes responsible for intracellular PS were exposed to UV light, the rate of PS synthesis was increased slightly, while the amount of PS externalized and percentage of cells undergoing apoptosis was reduced (95). Increased PS levels in plasma membrane also correlated with the enhanced translocation of Raf-1 kinase to the plasma membrane (96). Raf-1 kinase has been implicated in regulating apoptosis, and PS levels in the plasma membrane appear to dramatically impact its subcellular localization and function.

Unlike extracellular PS signaling, which is sporadic and circumstantial, intracellular PS functions continuously and in various capacities. Its abundance coupled with its negatively charged headgroup contributes to the notion that PS is involved in mostly nonspecific interactions with proteins inside the cell. This is normally the case because many specialized motifs show promiscuous selectivity toward acidic phospholipids (97). However, there are also examples of proteins selective towards PS which have been shown to localize to regions of the membrane rich with PS (10, 98, 99). Perhaps the best known role for PS inside the cell is as an enzyme cofactor for isoforms of protein kinase C (PKC).

PS involvement in the membrane recruitment of PKC was first discovered 20 years ago by Newton and colleagues. PKC normally is located in the cytosol but can translocate to the plasma membrane where it undergoes a conformational change and becomes active (100, 101). All PKC isoforms contain C2 domains, which bind PS with
varying degrees of specificity. C2 domains found in conventional isoforms PKCα and PKCβ, as well as novel PKCδ have the best characterized PS binding site (98, 102). Crystal structures analysis of conventional PKC isoforms shows that basic as well as polar residues found in Ca$^{+2}$- binding loops are responsible for stereospecific recognition of the serine headgroup found in PS (103, 104). For the conventional isoforms, PS binding is Ca$^{+2}$-dependent and essential for proper membrane recruitment (4). For novel PKCδ C2 domain, in vitro studies indicated that the protein was selective for PS-containing vesicles over other anionic lipids. Furthermore, studies also showed that the serine headgroup of PS may interact specifically with PKCδ and initiate membrane penetration of the protein (102).

Potential PS binding proteins make up a growing family of lipid binding proteins similar to other known effectors which selectively target membrane lipids. However, unlike other lipid signals such as phosphoinositides, PS is constitutively present in the membrane making it hard to be considered a true lipid signaling molecule. Because of this, the specificity of the protein-lipid interaction must be critically evaluated. Furthermore, distinguishing between lipid specificity as opposed to general features (i.e. anionic charged surface) must be established for each potential PS-binding candidate. This thesis aims to characterize a known lipid binding domain, the PDK1-PH domain, which is reported to have high affinity for 3'-phosphoinositides. Our results suggest that it not only binds these lipids, but also PS, and that PS binding is the driving force for plasma membrane recruitment.
CHAPTER 3: Phosphatidylserine Binding is Essential for Plasma Membrane Recruitment and Signaling Function of 3-Phosphoinositide-Dependent Kinase-1
3.1 Introduction

The activation of phosphoinositide 3-kinase (PI3K) in response to growth factors and insulin stimulation leads to the generation of 3'-phosphoinositides, phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P$_3$) and phosphatidylinositol-3,4-bisphosphate (PtdIns(3,4)P$_2$), that act as second messengers triggering diverse metabolic, proliferative, and survival responses (105, 106). The 3-phosphoinositide dependent kinase-1 (PDK1) is known as a master regulator of a subgroup of 3'-phosphoinositide responsive AGC protein kinase family members, including protein kinase B (PKB/Akt), p70 ribosomal S6 kinase (S6K), serum- and glucocorticoid-induced protein kinase (SGK), and atypical protein kinase C (PKC) (17, 63). Unlike other AGC family members, PDK1 is ubiquitously expressed and contains an intrinsic autophosphorylation mechanism consequently leaving the protein constitutively active in the cell (55, 59). Much effort has been devoted to understanding how an enzyme that is always active and is capable of inducibly phosphorylating its substrates in response to specific stimuli. Data suggests that agonists target the downstream kinases by converting them into substrates that can be recognized and phosphorylated by PDK1 (58). The most actively studied pathway PDK1 is involved with is the PI3K-PDK1-Akt pathway, due to its implications in disease such as cancer and diabetes. To briefly summarize, a transient increase in PtdIns(3,4,5)P$_3$ and PtdIns(3,4)P$_2$ in the plasma membrane (PM) by PI3K activation recruits Akt to the PM from the cytosol where it can be phosphorylated at Thr$^{308}$ by PDK1 and become partially activated (58). Akt and PDK1 both contain a pleckstrin homology (PH) domain capable of binding PtdIns(3,4,5)P$_3$ and PtdIns(3,4)P$_2$. It has been reported that binding of PtdIns(3,4,5)P$_3$
to their respective PH domains promotes co-localization of the two proteins at the PM (44). For Akt it also induces a conformational change of the protein exposing Thr<sup>308</sup> located on the T-loop (47) for phosphorylation by PDK1 (44, 56).

PtdIns(3,4,5)P<sub>3</sub> is known to recruit a multitude of effectors, including Akt, to the PM. However, whether or not PtdIns(3,4,5)P<sub>3</sub> recruits PDK1 to the PM remains controversial. Two studies reported that PDK1 translocated to the PM in response to PI3K activation (57, 61). However, another study found that a significant portion of the protein prelocalized at the PM and that growth factor stimulation had no effect on its localization (58). Also, Akt could be activated in cells expressing a PDK1 mutant the PH domain of which lacks phosphoinositide binding activity (107). Collectively, these results suggest the presence of another factor beside 3’-phosphoinositides that is important for PM recruitment of PDK1.

Many cytosolic proteins are recruited to the PM by directly interacting with membrane lipids present, although some simultaneously recognize and bind specific protein partners within the PM as well (14). The inner leaflet of the PM of mammalian cells is rich in anionic phospholipids, particularly phosphatidylserine (PS) (20-30 mole%) (15-17) and phosphatidylinositol-(4,5)-bisphosphate (PtdIns(4,5)P<sub>2</sub>) (ca. 1 mole%) (21, 19, 108, 109). Although much has been reported on the role of phosphoinositides in PM recruitment of peripheral proteins (19; 109), less has been known about the direct involvement of PS in their PM localization. Most lipid binding domains and proteins have cationic residues or patches on their membrane contact surfaces and non-specific electrostatic interactions between these residues and anionic lipids in cell membranes contribute significantly to the overall membrane binding of these proteins (9, 110). Since
PS is the most abundant bulk anionic lipid in the PM, it is generally thought that PS is involved in this type of non-specific interaction with cellular proteins. However, earlier *in vitro* membrane binding and cellular translocation studies of various proteins, including PKC (Stahelin & Cho 2004) and sphingosine kinase (9), as well as their isolated lipid binding domains (111), have indicated that they are targeted to the PM through direct and specific interactions with PS in the PM. More recently, Yeung et. al. (10) reported that PS binding is important for PM targeting of cytosolic proteins with polybasic motifs, including small G proteins. For the C2 domain of PKCα that can bind both PS (111, 103) and phosphoinositides (112, 113), it was shown that PS binding is essential for its PM recruitment while phosphoinositide binding augments the PS-dependent membrane binding (4).

It has been reported that PS enhances the binding of PH domains to phosphoinositide containing membranes. A single molecule study of the Grp1 PH domain showed that PS bound to an unidentified secondary binding site of the PH domain greatly enhanced its affinity for PtdIns(3,4,5)P3-containing vesicles and changed the diffusion behavior of the membrane-bound protein (114). More recently, binding of PS to basic residues near the PtdIns(3,4,5)P3-binding pocket of its PH domain was reported to be important for the activation of Akt (115). Here, we report that the PH domain of PDK1 specifically binds PS via a well defined site that is separate from its PtdIns(3,4,5)P3-binding pocket and this specific PS binding is essential for its PM localization and signaling function in response to physiological stimuli.
3.2 **Experimental Procedures**

*Materials*—1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoinositol (POPI), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (POPS) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P$_2$), phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P$_2$), and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P$_3$) were purchased from Cayman. The concentrations of the phospholipids were determined by a modified Bartlett analysis. Fatty acid-free bovine serum albumin was from Bayer, Inc. (Kankakee, IL).

*Expression Vector Construction and Mutagenesis*—A construct of PDK1-PH (residues 409–556) was generated from murine cDNA using PCR. The amplified PCR product was sub-cloned as a BglII and EcoRI fragment into pGEX4T-1 (Novagen) vector at the BamHI and EcoRI restriction site. The PDK1 mutants, K465A, R466A, K467A, R466A/K467A, as well as corresponding Glu mutants were produced using overlap PCR. The plasmids were transformed into Top10F’ cells for high throughput DNA isolation, which was sequenced for verification. For cellular imaging work with NIH 3T3 cells, wild type and mutants were sub-cloned again as a BglIII and EcoRI fragment between the EcoRI and BamHI site of pEGFP-C1 and mCherry-C1 vectors to generate PH domains tagged with an enhanced green fluorescence protein (EGFP) and a red fluorescence protein (mCherry), respectively, at their N termini. The N-terminal
mCherry-tagged lactadherin C2 (Lact-C2) was prepared from the EGFP-tagged Lact-C2 (Addgene). The mCerulean tagged Btk-PH was prepared as described (116).

Protein Expression and Purification—The GST fusion proteins were expressed in BL21 RIL cells. For expression of the PDK1-PH wild type (WT) and mutants, 2 liters of Luria broth containing 100 µg/mL ampicillin were inoculated with BL21 RIL colonies containing the construct. Cells were allowed to grow in medium at 37 °C until an absorbance of 0.6 at 600 nm was reached. Protein expression was induced with the addition of 200 µM isopropyl 1-thio-β-D-galactopyranoside (Research Products, Mount Prospect, IL), at which point cells were moved to a 25 °C shaker for 14-h incubation. Cells were harvested through centrifugation (2500 X g for 10 min at 4 °C), and the pellet was re-suspended in 20 ml of 20mM Tris buffer, pH 8, with 160 mM KCl, 50 µM phenylmethylsulfonyl fluoride, and 2 mM dithiothreitol. The solution was sonicated for 8 min (30 s of sonication followed by a 30-s pause) and then centrifuged for 30 min (39,000 X g at 4 °C). When centrifugation was complete, the supernatant was filtered into a 50-mL Falcon tube and 500 µL of glutathione S-transferase tag resin (Novagen, Madison, WI) was added. The supernatant was allowed to incubate with the resin for ~30 min at 4°C with moderate shaking. The supernatant was then poured onto a column, and the resin was washed with 20 mL of 20 mM Tris buffer, pH 8, 160 mM KCl to eliminate nonspecifically bound protein. After washing, the resin was resuspended in 1 mL of 20mM Tris buffer, pH 8.4, containing 160mM KCl, 25mM CaCl2, and 1 µL of thrombin added to cleave the glutathione S-transferase tag, and the column was sealed and incubated at 4°C for 12 h. The protein was eluted with three fractions of 1 mL
20 mM Tris buffer, pH 8, containing 160 mM KCl. Protein purity was checked using an 18% polyacrylamide gel, and the protein concentration was determined using the bicinchoninic acid method. Protein was frozen in liquid nitrogen and stored at -80 °C.

**Surface Plasmon Resonance (SPR) Measurements**— SPR measurements utilize total internal reflection as a robust means for determining macromolecular interactions (117). The most obvious advantages are the direct and rapid determination of association and dissociation rates in binding processes, label-free interaction analysis, and only small amounts of sample are needed. Briefly, monochromatic, p-polarized light traveling through an optical dense medium reaches an interface of lower optical density where it is reflected back into the denser medium. At the border of these two mediums lies a thin metal film, typically gold, where at a critical angle of incident light surface plasmons are generated. This absorbs some of the light and causes a decrease in the intensity of the reflected light. The critical angle is dependent upon the area within a few nanometers of the surface and will fluctuate when molecules bind. The gold film can be modified to attach a ligand of interest and as macromolecules travel across the evanescent wave and absorb to the ligand, an SPR signal is produced from changes in refractive index. The resulting sensorgram can be analyzed, revealing real time kinetics for biomolecular interactions.

The research and development team at Biacore (GE Healthcare) has led the way in SPR instrument development. They have generated a number of sensor chips available for use in their SPR instruments. Since the major theme of this thesis is the study of membrane-protein interactions, the L1 chip was utilized because its dextran coated gold surface provides a hydrophobic environment necessary for the attachment
of lipid vesicles. Vesicles at a final concentration of 0.5mM were prepared from lipid stock solutions in chloroform, and then dried under N₂ gas to remove the chloroform. Vesicles were re-suspended in the appropriate running SPR buffer (usually 20mM Tris, pH 7.4, 160mM KCl). The solution was then vortexed for 20 min and sonicated for another 2 min in a Branson 1200 sonifier. After sonication, vesicles were passed 20 times through a Liposofast microextruder containing a 100nm polycarbonate filter. After washing the sensor chip with SPR running buffer 20 µL of lipid vesicles at a given composition (e.g. POPC/POPS = 80:20) were injected at 5 µL/min to give a response of ~4000 resonance units (RU). Similarly, a control surface was prepared by injecting 100% POPC onto a chip in a separate channel. The immobilized lipid layer was washed several times with 90 µL of 50 mM NaOH until the change in resonance units after each wash was less than 10 RU. Both surfaces were allowed to stabilize until the drift in signal was less than 1 RU/min.

The equilibrium SPR measurements were performed at 23 °C using a Biacore X system at a flow rate of 5 µL/min. This allowed enough time for R values of the protein association phase to reach near equilibrium levels (R$_{eq}$). The surface was then regenerated with 50 µL of 50mM NaOH for subsequent protein injections. A minimum of five different protein concentrations were injected to collect data for K$_d$ determination. After each set of measurements, the immobilized vesicles were removed by injection of 20 µL of 40 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, followed by 25 µL of octyl glucoside at 5 µL/min, and the sensor chip was recoated with a fresh vesicle solution for the next set of measurements. All data was evaluated using BIAevaluation 3.0 software (Biacore) and Kaleidograph scientific graphing software.
(Synergy). For each trial, the control surface response was subtracted out in order to eliminate any refractive index changes due to buffer change. The $R_{eq}$ values were plotted against the protein concentrations ($P_0$), and the $K_d$ was established by nonlinear least squares analysis of the binding isotherm using the equation, $R_{eq} = R_{max}/(1 + K_d/P_0)$. The measurement was repeated at least three times to determine average and S.D. values. Kinetic measurements also were performed to determine selectivity as well as additive membrane-protein interactions. For kinetic measurements, protein (90µL) was injected at 30 µL/min and dissociation was monitored for 2 min after injection was complete. The surface was regenerated as previously described or removed for subsequent coating of another surface that differed in lipid composition.

**Cell Studies, Microscopic Imaging, and Data Analysis**—NIH 3T3 cells were seeded into eight wells of a sterile Nunc Lak-TeKIIITM chambered cover glass plate, which was filled with 400µL of Dulbecco’s modified Eagle’s medium (DMEM) and 10% (v/v) fetal bovine serum and incubated at 37 °C in a 5% CO$_2$ environment for 24 h. Only cells between the 5$^{th}$ and 20$^{th}$ passages were used for transfection and visualization. For transfection, NIH 3T3 cells were incubated for 4 h with EGFP-C1 vector containing each PH domain (0.5µg/mL) as well as mCerulean-C1 vector containing Btk-PH (1.0 µg/ml) in the presence of Lipofectamine reagent 2000 in DMEM. Cells were incubated overnight in DMEM with 10% fetal bovine serum, washed with DMEM, and then starved in DMEM without serum for another 4 h. Prior to imaging, cells were washed twice and resuspended in 300µl of Hanks’ balanced salt solutions. Subcellular localization of PDK1-PH WT and mutants was monitored at fixed time intervals (30 s) before and after stimulation with 50 ng/mL human platelet-derived growth factor (PDGF)-BB using a
custom-built, Zeiss LSM 510 laser scanning confocal microscope. A 40x, 1.2 numerical aperture immersion objective was used for all the experiments. For comparison, subcellular localization of a PtdIns(3,4,5)P$_3$ sensor, mCerulean-tagged Btk-PH (116), was monitored under the same conditions. A minimum of three experiments were done for all reported results, with ~80% of cells showing the same result as shown in each figure. All microscopic manipulation and data acquisition was controlled by the SimFCS program provided by Dr. Enrico Gratton. To calculate the time course of the degree of membrane localization of different proteins, cell images taken at regular intervals were analyzed using MATLAB. Lines were drawn in three places, crossing the PM and the cytosol, and photon count values were obtained along points on the lines. The values within the cytosol as well as at the PM were averaged. The relative PM distribution of a protein at a given time was then calculated by dividing the photon count average at PM by that at PM & cytosol.

Akt Activity Assay—Under appropriate conditions, fibroblastic 3T3-L1 cell lines can undergo differentiation to adipose like cells with an increase in lipogenic enzymes and heightened sensitivity towards hormones involved in lipid metabolism (118). In brief, 3T3-L1 fibroblasts were grown at 37 °C in a humidified atmosphere of 5% CO$_2$ in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Cells were plated onto 100 X 20mm polystyrene tissue culture dishes, and differentiated 2 days post-confluence with dexamethasone (0.4 mg/mL), 1-methyl-3-isobutylxanthine (0.5 mM), and 10% fetal bovine serum. Adipocytes were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, fed every 7 days, and used at approximately 10–30 days post-differentiation. NIH-3T3-L1 adipocytes were co-
transfected with Akt1 and PDK1-WT (or PDK1-R466A/K467A mutant). After a 16-h incubation to allow sufficient over-expression of proteins, cells were starved for a minimum of 3 h before they were stimulated with PDGF-BB (50 ng/mL) for 0, 10, or 20 min. Cell lysates were prepared using M-PER mammalian protein extraction reagent (Thermo Scientific) along with protease (Roche Applied Science) and phosphatase (Sigma-Aldrich) inhibitors, extracts were analyzed by SDS-PAGE, and the gel was transferred to polyvinylidene fluoride membranes (Millipore). Membranes were then incubated in blocking buffer TBS-T (50mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) with 5%(w/v) dry milk for 3 h. Immunoblots were washed, and probed with the following antibody: anti-Akt1, anti-Akt1-Thr(P)308, or anti-PDK1 at a 1:1000 ratio for each. Membranes were incubated with antibodies overnight and then washed with TBS-T with 1% milk a minimum of five times for 5 min. Blots were then incubated with anti-goat/anti-rabbit IgG secondary antibody for 2 h, washed five times, and prepared for development. Protein immunoblots were revealed using horseradish peroxidase.

Intensities of protein bands were quantified using ImageJ software. The relative phosphorylation index was calculated by dividing the intensity of an Akt-Thr(P)308 band by that of its protein gel band, and this parameter was plotted as a function of time for both WT and the mutant.

**Computer Modeling**—A glycerol molecule in the crystal structure PDK1-PH (Protein Data Bank code 1W1D) (72) was replaced with a PS molecule using AutoDock (version 4.0), and the energy minimization was performed using the Lamarckian genetic algorithm. Among models with the lowest mean energy, the most plausible model was
selected by considering the feasibility of embedding the hydrophobic tail of the PS molecule in the membrane.

3.3 Results

3.3.1 Membrane Binding Properties of the PDK1 PH Domain

Previous reports have shown that the PDK1 PH domain has strong affinity for 3'-phosphoinositides PtdIns(3,4,5)P$_3$ and PtdIns(3,4)P$_2$ while only a moderate affinity for PtdIns(4,5)P$_2$ (58). To explore the possibility that it binds other lipids, in particular PS, we measured the lipid binding properties of PDK1-PH through kinetic SPR analysis. As Fig. 5A shows, PDK1-PH has higher affinity for both PtdIns(3,4,5)P$_3$ (97:3) and PtdIns(3,4)P$_2$ (97:3) vesicles than for PtdIns(4,5)P$_2$ (97:3) vesicles which is in agreement with previous reports. It also shows no detectable affinity for vesicles containing 100% POPC. Interestingly, PDK1-PH shows high affinity for the surface made up of POPC/POPS (70:30) which is similar to its affinity for surfaces containing previously mentioned PtdIns(3,4,5)P$_3$ (97:3) and PtdIns(3,4)P$_2$ (97:3). Furthermore, when PS concentration was progressively increased from 0-30 mole%, the amount of protein bound in the associative phase also showed dramatic increase, indicating PS-dependence on protein binding (Fig. 5B). Recently a number of studies have shown that PH domains utilize global electrostatic properties to form weak nonspecific interactions with the membrane, only to tightly bind upon appearance of the target
Figure 5. **Kinetic membrane binding measurements of PDK1-PH domain.**  

A, phosphoinositide specificity of PDK1-PH. 90µL of PDK1 was injected into SPR and association/dissociation to representative surfaces was monitored.  

B, PDK1-PH membrane binding is PS-dependent. Concentration of POPS was varied from 0 to 30 mol%. Note that PDK1-PH shows much lower affinity for other anionic surfaces such as 20%POPG and 20%POPI.  

C, additive binding effect of PS and PtdIns(3,4,5)P₃. The molar ratio of PtdIns(3,4,5)P₃ was increased (0-3%) in vesicles containing a constant amount (20%) of POPS.  

D, molar ratio of PtdIns(3,4,5)P₃ was left constant and concentration of POPS was progressivel increased and effect was monitored. Protein concentration for all experiments was 0.2µM.
phosphoinositide (114, 115, 120). To determine if PS binding was specific or caused by non-specific electrostatic interactions, we measured PDK1-PH affinity for surfaces containing POPC/POPG (80:20) and POPC/POPI (80:20). Sensorgrams shown in Fig. 5B indicate that affinity for surfaces containing 20% POPG or POPI were much lower than affinity for the surface containing 20% POPS. These observations suggest that PDK1-PH can specifically bind PS. Next, we sought to find out if PDK1-PH utilized the known PtdIns(3,4,5)P$_3$-binding pocket to specifically interact with PS, or if there was another site capable of binding the lipid. To test this, we first measured the effect of adding PtdIns(3,4,5)P$_3$ to POPC/POPS (80:20) vesicles on membrane affinity of PDK1-PH. Fig. 5C shows that addition of PtdIns(3,4,5)P$_3$ to POPC/POPS (80:20) vesicles leads to concomitant association of PDK1-PH to the membrane surface in a concentration-dependent manner. Likewise, PDK1-PH shows enhanced affinity for POPC/PtdIns(3,4,5)P$_3$ (97:3) vesicles upon the addition of POPS (Fig. 5D). In both cases (Figs. 5C &5D), the positive effects of PS and PtdIns(3,4,5)P$_3$ on PDK1-PH binding when both are present in the membrane appear similar to their individual effects (e.g. see Fig. 5A). Together, these results support the notion that PDK1-PH utilizes separate binding pockets specific for PS and PtdIns(3,4,5)P$_3$ to tightly bind to the membrane and that the appearance of both lipids provides an additive rather than synergistic effect on protein-membrane interactions.

To quantitatively determine the lipid specificity of PDK1-PH, we performed equilibrium SPR measurements and determined $K_d$ values for its binding to vesicles containing different anionic lipids (see Table 1). Consistent with kinetic SPR data shown
Table I

Membrane binding properties of the PDK1 PH domain WT and mutants

Values represent the mean and S.D. from three determinations. All measurements were performed in 20mM Tris-Hcl, pH 7.4, containing 0.16 M KCl, and $K_d$ values were determined as shown in Fig. 6.

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<th>PDK1 PH domains</th>
<th>POPC/PtdIns(3,4,5)$P_3$ (97:3)</th>
<th>POPC/POPS (80:20)</th>
<th>POPC/POPS/PtdIns(3,4,5)$P_3$ (77:20:3)</th>
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<td>WT</td>
<td>$52 \pm 4$</td>
<td>$95 \pm 17$</td>
<td>$30 \pm 5$</td>
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<td>K465A</td>
<td>$1700 \pm 160$</td>
<td>$160 \pm 50$</td>
<td>$150 \pm 25$</td>
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<tr>
<td>R466A</td>
<td>$58 \pm 9$</td>
<td>$390 \pm 180$</td>
<td>$65 \pm 20$</td>
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<tr>
<td>K467A</td>
<td>$59 \pm 8$</td>
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<tr>
<td>R466A/K467A</td>
<td>$50 \pm 15$</td>
<td>$560 \pm 200$</td>
<td>$220 \pm 20$</td>
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Figure 6. **Determination of $K_d$ for PDK1-PH by SPR Equilibrium Measurements.** Binding Isotherm for PDK1-PH and POPC/PtdIns(3,4,5)$P_3$ (97:3) vesicles from equilibrium experiments. A solid line represents a theoretical curve constructed from $R_{\text{max}}$ (=1000 ± 40) and $K_d$ (52 ± 4 nM) determined by nonlinear least-squares analysis of the isotherm using $R_{eq} = R_{\text{max}}/(1+K_d/C)$. 
in Fig. 1A, PDK1-PH has high affinity for POPC/PtdIns(3,4,5)P₃ (97:3) (Kd = 52 nM) (see Fig. 6) and POPC/PtdIns(3,4)P₂ (97:3) vesicles (Kd = 70 nM) (data not shown). This affinity is comparable with that reported for other PH domains that have high affinity for PtdIns(3,4,5)P₃-containing membranes (116). We also found that the protein binds quite well to vesicles made up of POPC/POPS (80:20) with a reported Kₐ = 95nM, confirming our kinetic observations that PDK1-PH has a high affinity for vesicles containing PS. When physiological amounts of both PtdIns(3,4,5)P₃ (3 mol%) and PS (20 mol%) are present in the surface, we see a modest 3-fold increase in the membrane affinity of PDK1-PH. These results provide strong evidence that both PS and PtdIns(3,4,5)P₃ play distinctive roles in targeting PDK1-PH to the plasma membrane.

3.3.2 Identification of the PS-binding Site of the PDK1 PH Domain

To help determine the location of a PS-specific binding site in the PDK1-PH, we examined the crystal structure (17) and the surface electrostatic distribution of PDK1-PH and searched for cationic grooves on or near the putative membrane binding surface surrounding the PtdIns(3,4,5)P₃-binding pocket. Analysis of the surface found a cationic groove near the PtdIns(3,4,5)P₃-binding pocket made up of two basic residues, Arg⁴⁶⁶ and Lys⁴⁶⁷. Interestingly, a closer look at the crystal structure shows that these residues are capable of coordinating a glycerol molecule (Fig. 7A). This observation implies that these residues may directly coordinate the PS headgroup. Furthermore, molecular modeling studies and the shallow nature of the pocket show that the cationic groove can accommodate PS (Fig. 7B). Therefore, we mutated these residues to Ala and expressed the recombinant protein either as a single mutation or in combination.
Figure 7. **Identification of the PS-binding site of the PDK1-PH domain.**

A, The X-ray crystal structure of PDK1-PH complexed with inositol 1,3,4,5-tetrakisphosphate (IP4) shown in *red* and glycerol shown in *green* (Protein Data Bank code 1W1D) (Komander & Alessi 2004).  
B, a model representation of PDK1-PH-PS-IP4 complex. The PDK1-PH domain electrostatic surface representation is in the same orientation as in Fig. 7A.
We also mutated Lys\textsuperscript{465}, which has been documented to be essential for PtdIns(3,4,5)P\textsubscript{3} headgroup interaction (see Fig 7A). The effects of mutations on binding to vesicles of varying composition were measured by SPR analysis and are summarized in Table 1.

As expected, the K465A mutation substantially decreased PDK1-PH binding to vesicles made up of POPC/PtdIns(3,4,5)P\textsubscript{3} (97:3) while only having a modest effect (>2-fold) on binding to vesicles made up of POPC/POPS (80:20)(see Table 1). In addition, this mutant shows no increase in association to vesicles made up of POPC/POPS/PtdIns(3,4,5)P\textsubscript{3} (77:20:3) compared to vesicles made up of POPC/POPS (80:20). Clearly, evidence suggests that Lys\textsuperscript{465} is specifically engaged in phosphoinositide recognition and plays no role in binding to PS. Conversely, R466A and K467A show significant loss in affinity for POPC/POPS (80:20) vesicles compared to WT but display no loss in affinity to vesicles made up of POPC/PtdIns(3,4,5)P\textsubscript{3}.

Furthermore, the R466A/K467A double-site mutation exhibits ~6-fold reduction in affinity for POPC/POPS (80:20) vesicles but had no effect on binding POPC/PtdIns(3,4,5)P\textsubscript{3} (97:3) vesicles. Together, these results suggest that Arg\textsuperscript{466} and Lys\textsuperscript{467} form a putative PS-binding pocket but do not participate in binding to the PtdIns(3,4,5)P\textsubscript{3} headgroup. Furthermore, they support the claim that separate binding sites exist for both PS and PtdIns(3,4,5)P\textsubscript{3}.

### 3.3.3 Effects of PS Binding on the PM Localization of PDK1 PH Domain

To see how important PS binding of PDK1-PH was to its ability to localize at the plasma membrane, we transfected NIH 3T3 cells with PDK1-PH WT and mutants tagged with EGFP and monitored subcellular localization under several conditions. The cell populations expressing similar levels of EGFP-tagged protein were selected by
visual inspection of EGFP fluorescence intensity and used for translocation measurements. A minimum of three measurements were performed with each protein and >80% of the cell population showed similar behaviors with respect to subcellular localization and translocation.

Consistent with a previous report (58), we observe a significantly large proportion of cells expressing PDK1-WT display PM prelocalization in unstimulated NIH 3T3 cells (Fig. 8A). Conversely, the R466A/K467A mutant, which has significantly reduced PS affinity but still capable of PtdIns(3,4,5)P₃ binding, shows primarily cytosolic subcellular distribution under the same conditions (Fig. 8B). Interestingly, the K465A mutant, which is incapable of coordinating PtdIns(3,4,5)P₃, still shows a significant amount of PM prelocalization (Fig. 8C). Since PS is constitutively present on the cytosolic leaflet of the plasma membrane, these observations suggest that PS binding is responsible for PM localization of PDK1-PH in resting cells. Because of their reported robust sensitivity to PDGF stimulation (116), we utilized NIH 3T3 cells for the majority of our in vivo studies. However, we observe the same trend in other mammalian cell lines such as HEK 293 and HeLa which eliminates the argument that PDK1-WT PM prelocalization is specific only to NIH 3T3 cells (data not shown).

To further analyze the correlation between PS binding, PtdIns(3,4,5)P₃ binding and PDK1-WT PM localization, we measured the effects of suppressing PtdIns(3,4,5)P₃ and PS levels in NIH 3T3 cells. Reports have stated that the background PtdIns(3,4,5)P₃ levels in transformed cells may be higher than that of normal cells (109).
Figure 8. *Initial subcellular localization of EGFP-tagged PH domains in NIH 3T3 cells*. A, PDK1-PH wild type; B, PDK1-PH R466A/K467A; C, PDK1-PH K465A. *Bars* indicate 5µM.
Therefore, we treated cells with a PI3K inhibitor, wortmannin, and measured its effects on PDK1-PH WT. After treatment for 30min with 50nM wortmannin, NIH 3T3 cells transfected with a known PtdIns(3,4,5)P$_3$ sensor, Btk-PH, were monitored at 30 sec intervals for approximately 10 to 15 minutes. Results indicated PtdIns(3,4,5)P$_3$ appearance in the membrane was impeded, as mCerulean-tagged Btk-PH remained in the cytosol even after treatment with PDGF, which is known to activate PI3K (Fig 9A). In contrast, PDK1-PH still showed a significant degree of PM localization after the same wortmannin treatment as well as no significant increase in localization after growth factor treatment (Fig 9B). This control thus precluded any possibility that PDK1-PH PM prelocalization was due to residual PtdIns(3,4,5)P$_3$ levels in inactive NIH 3T3 cells. The lack of an inhibitor capable of reducing PS levels in eukaryotic cells led us co-express PDK1-PH domain with a domain that has at higher affinity for PS to see if this would have any effect on PDK1-PH PM prelocalization in NIH 3T3 cells. The C2 domain found in the protein Lactahedrin, a glycoprotein in milk, and has been reported to have high affinity for PS (10). Thus, we co-expressed mCherry-tagged Lact-C2 domain with EGFP-tagged PDK1-PH WT to see if Lact-C2 could mask PDK1-PH PS-binding capability. Indeed, co-expression of the two proteins in NIH 3T3 cells led to PDK1-PH WT localizing exclusively in the cytosol (Fig 10). This PS-masking effect was specific to proteins with PS-binding capability, as co-expression of Lact-C2 with the PtdIns(4,5,)P$_2$-specific phospholipase Cδ (PLCδ) PH domain had no effect on on PLCδ PM localization.
Figure 9. **Effect of PtdIns(3,4,5)P₃ depletion on subcellular localization of PH domains.** A, mCerulean tagged-Btk-PH domain treated with 50nM wortmannin and then stimulated with 50ng/mL PDGF; B, EGFP tagged-PDK1-PH wild type under same conditions as Btk-PH. Bars indicate 5μM.
Figure 10. Effect of co-transfecting PDK1-PH with PS-specific Lact-C2 domain on subcellular localization. Subcellular localization of EGFP-PDK1-PH (left) and mCherry-Lact-C2 (right) when co-transfected in NIH 3T3 cells. Bars indicate 5µM.
Figure 11. **Effect of co-transfecting PLCδ-PH with PS-specific Lact-C2 domain on subcellular localization.** Subcellular localization of EGFP- PLCδ-PH (*left*) and mCherry-Lact-C2 (*right*) when co-transfected in NIH 3T3 cells. *Bars* indicate 5µM.
These observations support our claim that PS binding and not PtdIns(3,4,5)P₃ binding, is responsible for PDK1-PH PM targeting in unstimulated NIH 3T3 cells.

Lastly, we monitored the effect of PI3K activation on subcellular localization of PDK1-PH WT and mutants. Figure 12A shows the time-lapse images of PH domains in representative cells in response to PDGF, while Fig. 12B illustrates the relative PM distribution of protein over time with respect to total protein concentration inside the cell. As expected, individual behaviors of PDK1-PH WT and the PtdIns(3,4,5)P₃ binding deficient K465A mutant show essentially the same trend in that the proteins are prelocalized in the PM and show little change in subcellular localization upon PDGF stimulation. However, R466A/K467A, which showed predominant cytosolic localization, abruptly translocated to the PM when resting cells were stimulated with PDGF. Strong signal from the membrane remained for approximately 10 to 15 minutes before the protein dissociated and translocated back to the cytosol, consistent with previous findings for PtdIns(3,4,5)P₃-selective proteins (116). Furthermore, mCerulean-tagged Btk PH domain showed similar behavior after cells were treated with growth factor. Collectively, the results show that PDK1-PH does not depend on transient influx of PtdIns(3,4,5)P₃ to localize at the PM.

**3.3.4 Effects of PS Binding on the Cellular function of PDK1 PH Domain**

To understand the physiological significance of PS binding, we examined the effect of mutating putative PS binding residues on the Akt phosphorylation activity of PDK1. PDK1 is known to co-localize with Akt at the PM in response to PI3K activation
Figure 12. Membrane translocation of EGFP-tagged PH domains in response to PDGF treatment. A, translocation of EGFP-tagged PDK1-PH, PDK1-PH-K465A, PDK1-PH-R466A/K467A, and mCerulean tagged-Btk-PH, each transfected in NIH 3T3 cells. Bars indicate 5µM. B, time-lapse changes in relative photon count PM distribution (i.e. photon count average at PM /total photon count average (PM + cytosol)) of PDK1-PH wild type, PDK1-PH-K465A, PDK1-PH-R466A/K467A, and Btk-PH.
and phosphorylate it at Thr\textsuperscript{308} found in the activation loop (55, 56). First off, we checked the subcellular distribution of full length EGFP-tagged PDK1 as well as the full length R466A/K467A mutant labeled with EGFP. Consistent with our observations of the PH domain alone, the full length PDK1 WT showed predominant PM prelocalization in resting NIH 3T3 cells, while R466A/K467A remained cytosolic (Fig. 13). This observation establishes that the PH domain directs PM localization of full length PDK1. To investigate effects of PS binding on activity, we performed immunoblot analysis on NIH 3T3 cells co-transfected with Akt1 and PDK1(WT or R466A/K467A). All cells co-transfected with PDK1 (WT and mutants) and Akt1, a similar level of background Akt1 phosphorylation was observed before PDGF stimulation, presumably due to transient protein co-localization of PDK1 and Akt1 (Fig. 14B) as reported previously (118). Strikingly, upon 10-min PDGF stimulation we observed >4-fold increase in Akt phosphorylation as measured by immunoblotting using phospho-Thr\textsuperscript{308} specific antibody (Fig 14A). Conversely, no significant increase in phosphorylation was observed for cells expressing PDK1 R466A/K467A or PtdIns(3,4,5)P\textsubscript{3} binding deficient K465A mutant (Fig. 14B). The results indicate PDK1 specifically recognizes and binds PS, and that PS binding is necessary for subcellular localization and signaling activity of PDK1.

3.4 Discussion

Since its original discovery years ago, PDK1 has emerged as a key regulator for PI3K signaling pathways that involve the activation of a number of downstream kinases. Because it remains constitutively active inside the cell, the mechanism by which PDK1
Figure 13. **Initial subcellular localization of EGFP-tagged full length PDK1 and mutant in NIH 3T3 cells.** Subcellular localization of EGFP-tagged full-length PDK1 wild type and R466A/K467A in unstimulated cells. *Bars* indicate 5µM.
Figure 14. **Akt phosphorylation activity of PDK1 WT, R466A/K467A, and K465A mutants.**  
*A*, immunoblot analysis of Akt1 Thr308 phosphorylation in NIH 3T3-L1 adipocytes co-transfected with Akt1 and PDK1(WT, R466A/K467A, or K465A).  
*B*, Comparative relative phosphorylation levels of Akt-Thr308 plotted as a function of time for PDK1 WT, R466A/K467A, and K465A.
activates its downstream substrates has been shown to involve PM recruitment and colocalization as the key regulatory steps necessary for downstream kinase activation. Evidence suggests that PtdIns(3,4,5)P$_3$ is important for the co-localization of PDK1 with its substrates (44). However, the mechanism by which PDK1 gets recruited to the PM remains unknown. The present study provides strong evidence for the notion that specific binding of PS to its PH domain is essential for the PM localization and hence signaling function of PDK1.

Our SPR studies of PDK1-PH WT clearly show that this domain can specifically bind PS using a site that does is independent of the canonical phosphoinositide binding pocket. Structural modeling and comparison of the in vitro membrane binding properties of wild type and respective mutants identified two basic residues, Arg$^{466}$ and Lys$^{467}$, which together compose a PS-specific binding pocket. Mutations of these residues significantly lower the affinity of PDK1-PH for PS-containing vesicles but not for POPC/PtdIns(3,4,5)P$_3$ (97:3) vesicles. In contrast, the mutation of a PtdIns(3,4,5)P$_3$ ligand, Lys$^{465}$, reduces the affinity for PtdIns(3,4,5)P$_3$-containing vesicles without affecting affinity for PS-containing vesicles. Arg$^{466}$ and Lys$^{467}$ are located within the highly variable loop regions that connect the β strands. These loop regions confer an electrostatic sidedness upon the PH domain and are responsible for PH domain binding to negatively charged membrane surfaces. Intriguingly, multiple sequence alignment (Fig. 15) shows that many PtdIns(3,4,5)P$_3$-binding PH domains have basic residues in this loop region, suggesting that these residues may also form similar cationic grooves and accommodate a PS headgroup. In fact, Arg$^{10}$ and Lys$^{15}$ of Akt1-PH were found to
Figure 15. **Partial amino acid sequence alignment of PH domains.** Selected N-terminal amino acid sequences of PH domains with high affinity toward PtdIns(3,4,5)P3. Shown in **bold** are basic residues that make up the canonical phosphoinositide binding pocket. Putative PS-binding cationic residues in the loop region (box) are shown in **italic, boldface** type. Note that PLCδ-PH which is PtdIns(4,5)P2-selective, lacks any cationic residues in the boxed region.
be involved in its PS binding (115). Conversely, the PtdIns(4,5)P$_2$-specific PLCδ-PH lacks corresponding basic residues which explains the lack of PS-binding capability. Thus, it would seem that specific PS-binding is a common property found among 3'-phosphoinositide-binding PH domains.

How does PS binding effect the physiological function of PH domains with high affinity for PtdIns(3,4,5)P$_3$? In the study of Grp1-PH, PS binding was shown to enhance its affinity for PtdIns(3,4,5)P$_3$-containing vesicles and slow the lateral diffusion of the membrane-bound domain; however, the physiological significance of PS binding of this PH domain was not investigated (114). For Akt-PH, PS binding not only augments the domain's affinity for PtdIns(3,4,5)P$_3$-containing membranes but also promotes a conformational change and activation of the full-length Akt (115). For both PH domains, it was speculated that PS binding is not strong enough to drive the PM recruitment of the domains (or intact proteins) and that the coincident interaction of the PH domain with PS and PtdIns(3,4,5)P$_3$ is necessary for effective PM localization (115, 119). In contrast, our SPR data (Fig. 5A) show that PDK1-PH has comparable affinity for POPC/POPS (70:30) and POPC/PtdIns(3,4,5)P$_3$ (97:3) vesicles. Given that the PS level in the inner leaflet of PM is estimated to be in the range of 20–30 mol %, PDK1-PH-PS binding may be strong enough to drive the PM translocation of the domain (hence the intact protein), at least partially, in the absence of PtdIns(3,4,5)P$_3$. Consistent with this notion, a significant portion of PDK1-PH (and intact PDK1) is prelocalized to the PM of unstimulated NIH 3T3 cells, and this PM prelocalization is disrupted either by mutation of PS-binding residues or by PS sequestration by another PS-binding protein but not by mutation of a PtdIns(3,4,5)P$_3$-binding residue or by
PtdIns(3,4,5)P₃ depletion. One could argue that the mutation of Arg⁴⁶⁶ and Lys⁴⁶⁷ may interfere with the interaction of PDK1-PH with other proteins present in the PM. Although this situation cannot be completely ruled out, the fact that the PS-binding Lact-C2 domain selectively blocks the PM localization of PDK1-PH but not the PtdIns(4,5)P₂-binding phospholipase Cδ-PH, strongly suggests that PS binding is mainly responsible for the prelocalization of PDK1-PH in the PM of quiescent cells.

In agreement with the critical role of PS binding in PM localization of PDK1-PH, a signature signaling activity of PDK1 (i.e. phosphorylation of Thr³⁰⁸ of Akt) depends on the PS binding activity of PDK1-PH. It should be noted that this activity also requires the presence of PtdIns(3,4,5)P₃ in the PM because it is necessary for co-localization of two proteins and the conformational change of Akt. In view of a recent report showing the role of PS in the membrane binding and the conformational change of Akt (115), it would seem that PS plays a more direct and crucial role in regulation of the PI3K-PDK1-Akt signaling pathway than previously thought.

Collectively, the present study establishes that PS plays a key role in PM localization of PDK1. As such, it adds to the growing evidence for the critical role of PS as a PM-specific marker that recruits many cytosolic proteins through specific interaction. Mammalian cells use complex mechanisms to maintain a high concentration of PS selectively in the inner leaflet of the PM (19), and it is therefore expected that membrane-binding cytosolic proteins should take advantage of its local abundance in their specific and reversible PM targeting. Genome-wide identification and characterization of the proteins that are targeted to the PM through specific interaction
with PS would be necessary to fully assess the significance of PS as a site-specific marker at the PM.
CHAPTER 4: Final Remarks
4. **Final Remarks**

Many phosphoinositide binding domains have been shown to require another ligand for proper subcellular localization and function and that these cooperative interactions are necessary in driving membrane targeting. The need for multiple ligands can add a level of complexity to the membrane targeting event, where specificity may not be defined by the precise nature of individual interactions, but rather the combination of multiple interactions. Recruitment to a specific membrane may require that two or more domain targets co-exist in that membrane. There are still many lipid binding domains being characterized each year that have unique lipid binding qualities that allow them to perform their known function inside the cell. This thesis has examined the mechanism by which a key signaling component in a major signaling pathway localizes to its target membrane and how specific interactions are required for proper signal propagation to occur.

Here we report that the PDK1 PH domain contains a second lipid binding pocket that forms a specific interaction with PS. This pocket is found near the canonical phosphoinositide binding pocket and its structural integrity must be maintained for proper signaling to occur. Using *in vitro* biophysical measurements, we find that physiological amounts of PS have similar associative characteristics to physiological amounts of 3'-phosphoinositides for PDK1 PH. Together these lipids increase the protein affinity for the membrane, thus producing a very strong interaction with the membrane surface. Our *in vivo* imaging studies show that PDK1 can localize within the PM in unstimulated cells, reflecting the high affinity the protein has for this bulk phospholipid. Our studies highlight the importance of utilizing biophysical
measurements to rigorously study lipid binding domains and highlight the significance of PS as an authentic signaling lipid.
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