Published in final edited form as:
Curr Top Microbiol Immunol. 2012 ; 362: 111–126. doi:10.1007/978-94-007-5025-8_6.

Molecular Analysis of Protein–Phosphoinositide Interactions

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Abstract

Diverse biological processes including cell growth and survival require transient association of proteins with cellular membranes. A large number of these proteins are drawn to a bilayer through binding of their modular domains to phosphoinositide (PI) lipids. Seven PI isoforms are found to concentrate in distinct pools of intracellular membranes, and this lipid compartmentalization provides an efficient way for recruiting PI-binding proteins to specific cellular organelles. The atomic-resolution structures and membrane docking mechanisms of a dozen PI effectors have been elucidated in the last decade, offering insight into the molecular basis for regulation of the PI-dependent signaling pathways. In this chapter, I summarize the mechanistic aspects of deciphering the 'PI code' by the most common PI-recognizing domains and discuss similarities and differences in the membrane anchoring mechanisms.

Introduction

Phosphoinositides (PIs), phosphorylated derivatives of phosphatidylinositol (PtdIns), are essential components of eukaryotic cell membranes.[1] They are involved in regulation of various fundamental biological processes, including cell growth and survival, membrane trafficking and cytoskeletal dynamics (reviewed in Di Paolo and De Camilli 2006; Hurley 2006; Lemmon 2008; Roth 2004). Although PIs comprise approximately 1 % of cellular lipids, they play pivotal roles in major signaling pathways, serving as docking sites for protein effectors and as precursors of secondary messengers. The inositol headgroup of PIs can be reversibly phosphorylated at three positions, D3, D4, and D5, and all seven PI isoforms, including three mono-phosphorylated [PtdIns(3)P, PtdIns(4)P, and PtdIns(5)P], three bis-phosphorylated [PtdIns(3,4)P_2, PtdIns(3,5)P_2, and PtdIns(4,5)P_2], and one tris-phosphorylated PtdIns(3,4,5)P_3 species have been identified in eukaryotic cells.

The amount and spatial and temporal distribution of PIs in the cell vary substantially (Vanhaesebroeck et al. 2001). The largest pool of these lipids comprises PtdIns(4)P and PtdIns(4,5)P_2, whereas PtdIns(3,4,5)P_3 is undetectable in unstimulated cells. Some PIs, such as PtdIns(4)P and PtdIns(4,5)P_2, are constitutively present in membranes, others are transiently produced in response to the activation of cell surface receptors and other stimuli. The level and turnover of PIs are tightly controlled by a set of PI-specific enzymes. Found exclusively in the cytosolic leaflet of membrane bilayers, PIs are readily accessible to PI kinases and phosphatases capable of attaching and removing phosphate groups, respectively, and to phospholipases that cleave the lipids. Because PI-modifying enzymes are heterogeneously localized in the cell, PIs are clustered in distinct intracellular membranes

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[1]This chapter is adapted from Kutateladze (2010).

and thus each PI essentially serves as a marker of an organelle. For example, the plasma membrane is enriched in PtdIns(4,5)P_2, whereas PtdIns(4)P and PtdIns(3)P are detected primarily in the Golgi and early endosomes, respectively. The unique distribution of PIs may provide the mechanism for fine-tuning the membrane trafficking flow and controlling the proper sequence of signaling events.

A major breakthrough in understanding the significance of PI signaling was the identification of protein effectors that recognize individual PIs (Fig. 6.1). The pleckstrin homology (PH) domain was the first effector found to associate with PIs (Harlan et al. 1994). The list of PI-binding domains has since grown rapidly and at present contains 15 modules that display a wide range of affinities and selectivities toward lipid membranes. It includes the AP180 *N*-terminal homology (ANTH), Barkor/Atg14(L) autophagosome targeting sequence (BATS), conserved region-2 of protein kinase C (C2), Dock homology region-1 (DHR-1), epsin *N*-terminal homology (ENTH), 4.1, ezrin, radixin, moiesin (FERM), Fab1, YOTB, Vac1 and EEA1 (FYVE), Golgi phosphoprotein 3 (GOLPH3), PtdIns(4)P binding of SidM/DrrA (P4M), postsynaptic density 95, disk large, zonula occludens (PDZ), β-propellers that bind PIs (PROPPINs), phosphotyrosine binding (PTB), Phox homology (PX), SH3YL1, Ysc84p/Lsb4p, Lsb3p and plant FYVE proteins (SYLF), and Tubby modules (reviewed in Kutateladze 2010; Moravcevic et al. 2012). The recognition of a unique arrangement of phosphate groups around the inositol ring, which can be referred to as a ‘PI code’, by these domains results in the recruitment of the host proteins to specific intracellular compartments (Kutateladze 2010. Many of these proteins are modular in architecture and contain other lipid- and protein-binding domains or possess catalytic activities. These in turn trigger phosphorylation/dephosphorylation of other membrane-associated complexes and adaptors and promote the interconversion of PIs, subsequently leading to the activation or termination of signaling cascades. A crosstalk between PIs, PI-modifying enzymes, and effectors capable of ‘reading’ the PI code constitutes one of the most intriguing and complex signaling networks in the cell, which we have only begun to understand. This chapter focuses on the structural and mechanistic aspects of deciphering the PI code by the most common PI-binding protein effectors.

The FYVE Domain Targets PtdIns(3)P

The FYVE domain is a ~70-residue zinc-binding finger which is found in 53 human proteins (SMART database) (reviewed in Kutateladze 2006). It binds PtdIns(3)P with high specificity and affinity and bridges a number of cytosolic proteins with PtdIns(3)P-enriched early endosomes, multivesicular bodies (MVB), and phagosomes (Burd and Emr 1998; Gaullier et al. 1998; Patki et al. 1998). A small fraction of PtdIns(3)P has been identified in the nucleus and the Golgi apparatus, and FYVE domain-containing proteins DFCP1 and Alfy preferentially localize to these sites (Ridley et al. 2001; Simonsen et al. 2004). The FYVE domain is defined by the three conserved sequences: the WxxD, RR/KHHCR and RVC motifs that form a highly positively charged binding site for PtdIns(3)P. Whereas topologically the FYVE domain belongs to a larger family of zinccoordinating RING fingers, it can be distinguished from other DNA- and protein-binding members of the RING superfamily by the presence of these three signature motifs.

FYVE domain-containing proteins have diverse biological functions. One of the largest subsets of FYVE proteins is involved in the regulation of endocytic trafficking and fusion of endosomal membranes with transport vesicles and other organelles (Gillooly et al. 2001). This subset includes well-characterized mammalian proteins EEA1, Endofin, FENS-1, FYCO1, Hrs, Rabenosin-5, Rabip4 and WDFY2, and yeast proteins Vac1p and Vps27p. Another fast growing subset plays a critical role in signal transduction and TGFβ/Smad activation (Hrs and SARA), adipocyte differentiation (ProF), leukocyte signaling (FGD2),

cytoskeletal reorganization (EhFP), autophagosome formation (DFCP1), and apoptosis (Phafin1/2). A number of enzymes such as kinases (Fab1 and PIKfyve), phosphatases (MTMR3 and MTMR4), and ubiquitin ligases (Pib1p) contain FYVE domain, and their localization to endosomal membranes and catalytic activities require binding to PtdIns(3)P.

Molecular Mechanism of the FYVE Domain Association with Membranes

While specific recognition of PtdIns(3)P is a major characteristic of the FYVE finger, it localizes to membranes through a multivalent mechanism that also involves nonspecific electrostatic contacts with acidic lipids other than PtdIns(3)P (Diraviyam et al. 2003; Kutateladze et al. 2004; Stahelin et al. 2002), activation of a histidine switch (He et al. 2009; Lee et al. 2005; Mertens et al. 2007), hydrophobic insertion into the bilayer (Blatner et al. 2004; Brunecky et al. 2005; Diraviyam et al. 2003; Kutateladze et al. 2004; Kutateladze and Overduin 2001; Misra and Hurley 1999; Sankaran et al. 2001; Stahelin et al. 2002), and in some cases dimerization of the host protein (Callaghan et al. 1999; Dumas et al. 2001; Hayakawa et al. 2004; Lawe et al. 2000). Each of these components uniquely contributes to the FYVE domain specificity and increases the binding affinity for PtdIns(3)P embedded in membranes to the low nM level (Blatner et al. 2004; Ridley et al. 2001; Stahelin et al. 2002).

The FYVE domain contains a variable-length loop next to the PtdIns(3)P binding pocket. Upon binding to PtdIns(3)P, the hydrophobic residues at the tip of this loop [termed the turret loop or membrane insertion/interaction loop (MIL)] insert into the bilayer. The MIL is flanked by a set of basic and polar residues that are positioned at the level of the lipid headgroups when the protein penetrates the membrane. These residues make nonspecific electrostatic contacts with acidic phospholipids, such as phosphatidylserine (PS) and phosphatidic acid (PA). The strong positive potential around the MIL can also drive the initial membrane docking and facilitate association with PtdIns(3)P. It has recently been shown that interaction of the FYVE domain with PtdIns(3)P is pH-dependent and can be regulated by a histidine switch comprising a pair of adjacent His residues in the RR/KHHCR motif. The FYVE domain binds PtdIns(3)P when both histidine residues are positively charged and releases the lipid upon their deprotonation. Membrane association of FYVE domain-containing proteins can be further enhanced by bivalent or multivalent interactions with PtdIns(3)P. For example, a central region of EEA1 forms a parallel coiled coil homodimer that juxtaposes two *C*-terminal FYVE domains, allowing for the simultaneous interaction with two PtdIns(3)P headgroups (Dumas et al. 2001).

Structural Basis of PtdIns(3)P Recognition by the FYVE Domain

The three-dimensional structures of the FYVE domain of human EEA1 bound to inositol 1,3-bisphosphate (Dumas et al. 2001) and dibutanoyl PtdIns(3)P (Kutateladze and Overduin 2001) and the ligand-free FYVE domains of EEA1 (Kutateladze and Overduin 2001), human Endofin, fly Hrs (Mao et al. 2000), Leishmania Major Lm5-1 (Mertens et al. 2007), human RUFY and yeast Vps27p (Misra and Hurley 1999) have been determined by X-ray crystallography and NMR spectroscopy. The structures reveal a similar overall fold that consists of two double-stranded antiparallel β sheets and a *C*-terminal α helix (Fig. 6.2). An additional *N*-terminal α-helical turn is seen in the structures of the FYVE domain of EEA1 and Endofin, and a short α helix connecting β2 and β3 is present in EEA1, Endofin, Lm5-1, and RUFY. The functionally important β1 strand spans three residues of the RR/KHHCR motif and pairs with the β2 strand, which links two zinc-binding clusters. The zinc ions are bound by four CxxC motifs in a cross-braced topology. One zinc ion is coordinated by the first and third cysteine motifs, whereas another zinc ion is bound by the second and fourth motifs in all human proteins. In yeast Vps27p, the fourth Cys residue is replaced by a His.

Structural insight into PtdIns(3)P recognition by the FYVE domain is provided by the crystal structure of the EEA1 FYVE domain in complex with inositol 1,3-bisphosphate (Dumas et al. 2001) (Fig. 6.3a). The structure reveals that the WxxD, RR/KHHCR, and RVC motifs are centrally involved in coordination of the inositol headgroup. Critical hydrogen bonds are formed between the 3-phospate group of PtdIns(3)P and the RR/KHHCR motif, particularly the guanidino moiety of the last arginine of this motif (R1375), the imidazole ring of the first histidine (H1372), and the backbone amide of the second histidine (H1373). The 1-phosphate group of PtdIns(3)P is bound by the first arginine of the motif (R1370) and through a water-mediated contact with the backbone carboxyl group of the second arginine (R1371). R1400 of the RVC motif, positioned between H1372 and R1375, forms another water-mediated hydrogen bond to the 3-phosphate group. Coordination of the 4-, 5-, and 6-hydroxyl groups of the inositol ring is crucial for stereospecificity and the exclusion of alternatively phosphorylated PIs. The 4- and 5-hydroxyl groups are hydrogen bonded to the imidazole ring of H1373, whereas the carboxylate of D1352 in the *N*-terminal WxxD motif makes contacts with the hydroxyl groups at the 5 and 6 positions.

The PH Domain Binds Various PIs

The PH domains comprise one of the largest families of signaling modules and are the most thoroughly characterized among the PI binding domains (reviewed in DiNitto and Lambright 2006; Lemmon and Ferguson 2000). The PH domain was identified within a set of human proteins in 1993 and derives its name from the two homologous regions of pleckstrin, the major protein kinase C substrate in platelets (Haslam et al. 1993; Mayer et al. 1993). Since then, it has been found in 561 human proteins involved in intracellular signaling, membrane trafficking, cytoskeletal structure, and lipid modifications (SMART). The PH domain contains ~120 residues that are folded in a highly conserved three-dimensional structure despite little sequence similarity between the family members. As a result of high sequence variability, the PH domains have diverse functions and interact with numerous ligands, including proteins, acidic phospholipids, inositol polyphosphates, and PIs. Many of those able to recognize PIs do so weakly and promiscuously, however, a subset of PH domains (about 10–20 % of all PH modules) binds individual PIs specifically and strongly, most commonly PtdIns(3,4,5)P_3, and PtdIns(4,5)P_2, as well as PtdIns(3,4)P_2. The binding affinity of the PH domains for PIs varies significantly, ranging from low nM to low μM (DiNitto and Lambright 2006). Among the best characterized PtdIns(3,4,5)P_3 effectors are the PH domains of ARNO, Btk, Gap1, Grp1, and cytohesin-1 (Cronin et al. 2004; Fukuda et al. 1996; Klarlund et al. 1997). The PLCδ1 PH domain is specific for PtdIns(4,5)P_2, whereas PH domains of TAPP1, Centaurin β2, PEPP1, and FAPP1 prefer PtdIns(3,4)P_2, PtdIns(3,5)P_2, PtdIns(3)P, and PtdIns(4)P, respectively, and Akt/PKB, PDK1, and DAPP1 bind both PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (DiNitto and Lambright 2006; Lemmon 2004; Lemmon and Ferguson 2000).

PH domains are present in GTP/GDP exchange factors (ARNO, cytohesin, FGD2, Grp1), GTPase activating proteins (Centaurin β2, Gap1), lipid-metabolizing (PLCδ1, PLD1/2), lipid-transport (FAPP2) and cytoskeletal (dynamin, β-spectrin) proteins, kinases (Akt/PKB, Btk/Itk, CERK), phosphatases (PHLPP1), and other macromolecules that are implicated in vital biological processes including growth, proliferation, metabolism, cell polarization and migration, receptor endo- and exocytosis, membrane budding and trafficking, actin rearrangement, immune responses, and apoptosis.

Molecular Mechanism of Membrane Docking by the PH Domain

The PI-binding site of the PH domain is formed by three variable loops flanking the open end of a β barrel (described below and shown in Fig. 6.2c). Like the binding sites of all other PI-binding modules, it contains a cluster of basic lysine and arginine residues that make direct contacts with the phosphate groups of the lipid. The PH domain is electrostatically polarized and displays a strong positive electrostatic potential around the binding site that contributes to both specific PI binding and nonspecific electrostatic interactions with other anionic lipids in membranes (DiNitto and Lambright 2006; He et al. 2008; Lemmon and Ferguson 2000; Manna et al. 2007; Singh and Murray 2003). Single-molecule fluorescent studies reveal that the Grp1 PH domain can interact with PS via one or more secondary binding sites and that the electrostatic search mechanism speeds its association with PtdIns(3,4,5)P_3 (Knight and Falke 2009).

PtdIns(3,4,5)P_3 binding of the Grp1 PH domain can be further enhanced by acidification of the media and subsequent protonation of the histidine residue (He et al. 2008) that forms a critical hydrogen bond to the 4-phosphate group of the PI (Ferguson et al. 2000; Lietzke et al. 2000). The pH-dependence is less pronounced for the PH domain than for the FYVE domain—unlike FYVE domains which all contain two invariable histidine residues in the binding pocket, a single histidine is present in only a small set of PH domains (He et al. 2008). Additionally, PH domains of ARNO, DAPP1, Fapp1, Grp1, PLCδ1, and TAPP1 have been demonstrated to penetrate PI-containing monolayers to various degrees (Flesch et al. 2005; He et al. 2008; Lumb et al. 2011; Manna et al. 2007; Stahelin et al. 2007), and, at least in the case of Grp1, the membrane insertion is triggered by specific recognition of the inositol headgroup and is increased in an acidic environment (He et al. 2008).

Several PH domains have been found to interact with two ligands. The PH domains of Grp1, Fapp1, and oxysterol binding protein recognize not only PIs but also membrane-attached Arf GTPases (Balla et al. 2005; Cohen et al. 2007; Godi et al. 2004; Levine and Munro 2002). Recent biochemical and structural analysis of the Fapp1 PH domain reveals that the PtdIns(4)P and Arf1 binding sites are separate, which allow for the association with both ligands simultaneously and independently (He et al. 2011a, b). This mode of ‘coincidence detection’ increases affinities and specificities of the PH domains toward membranes enriched in unique PIs and Arfs. The membrane recruitment is further augmented at the sites where Arfs are active. A phospholipid or sphingolipid molecule can also serve as a second ligand in coincidence detection as reported for the PH domains of Akt1 and Slm1 (Gallego et al. 2010; Huang et al. 2011).

Structural Basis of the PH Domain-PtdIns(3,4,5)P_3 Interaction

Over 100 three-dimensional crystal and NMR structures of various canonical PH domains have been deposited in the PDB. Of them, two are complexes with PtdIns(3,4,5)P_3 [Btk and PDK1 (Komander et al. 2004)], 13 are complexes of eight PH domains with inositol 1,3,4,5-tetrakisphosphate (IP4) [ARNO (Cronin et al. 2004), Btk (Baraldi et al. 1999), DAPP1/PHISH (Ferguson et al. 2000), Grp1 (DiNitto et al. 2007; Ferguson et al. 2000; Lietzke et al. 2000), Kindlin (Liu et al. 2011), PDK1 (Komander et al. 2004), PEPP1, and PKB/Akt (Carpten et al. 2007; Milburn et al. 2003; Thomas et al. 2002)], two are complexes with inositol 1,4,5-trisphosphate (IP3) [ARNO (Cronin et al. 2004) and PLCδ1 (Ferguson et al. 1995)], two are complexes with inositol 1,2,3,5,6-pentakisphosphate [pleckstrin (Jackson et al. 2007)], and one is a complex with inositol 1,3,4,5,6-pentakisphosphate [Grp1 (Ferguson et al. 2000)]. Additionally, PH domains of ArhGAP9 (Ceccarelli et al. 2007) and β-spectrin (Hyvonen et al. 1995) have been shown to non-canonically interact with IPs. The canonical PH domain folds into a seven-stranded β-barrel, capped by an amphipathic α helix at one

open end, while the opposite end is framed by three variable loops (Fig. 6.2c). The variable loops form a large PI-binding pocket, and their length and primary sequence define the specificity of the PH domain.

Details of how the PH domain recognizes the pattern of sequential 3-, 4-, and 5-phosphate groups in PtdIns(3,4,5)P_3 are revealed by the crystal structure of the Grp1 PH domain in complex with IP4, an isolated headgroup of PtdIns(3,4,5)P_3 (Ferguson et al. 2000; Lietzke et al. 2000) (Fig. 6.3b). The inositol ring lies in the center of a deep positively charged pocket formed by the β1–β2, β3–β4, and β6–β7 loops and the strands they connect. The distal phosphates are buried the furthest in the pocket, whereas the 1-phosphate group is positioned near the tips of the loops. A network of hydrogen bonds, formed between conserved lysine and arginine residues in β2, β3, β4, and β7 and all four phosphate groups of IP4, fully restrains the inositol molecule. A unique β-hairpin in the long β6–β7 loop of Grp1 is involved in additional hydrogen bonding contacts with the 5-phosphate group of IP4. These additional hydrogen bonds with the 5-phosphate account for the high specificity of the Grp1 PH domain toward PtdIns(3,4,5)P_3. In general, the β1–β2 loop of PH domains functions as a platform for the interaction with PIs (Lemmon 2008). It contains the sequence motif Kx_n(K/R)xR, which makes the most critical contacts with the phosphate groups of the lipid (Lemmon 2008).

The PX Domain Prefers PtdIns(3)P

The PX domain consists of ~130 residues and is found in 83 human signaling and regulatory proteins (SMART) (reviewed in Seet and Hong 2006). It is named after the two phagocyte NADPH oxidase (phox) subunits $p40^{phox}$ and $p47^{phox}$, in which it was first identified in 1996 (Ponting et al. 1996). Of all PIs, PtdIns(3)P appears to be the primary target of PX domain-containing proteins, as the majority of them associate with PtdIns(3)P-enriched endosomes and vacuoles [KIF16B, $p40^{phox}$, PXK, sorting nexins (SNXs), Vam7p], although binding to other PIs has also been reported for Bem1, CISK, CPK, FISH, NOXO1, $p47^{phox}$, PI3K-C2α, PLD1, and SNXs. All yeast PX domains bind PtdIns(3)P, however, only four with relatively high affinity (K_d ~ 2–3 μM) (Yu and Lemmon 2001).

PX domain-containing proteins play essential roles in endocytosis, protein sorting, membrane trafficking, transcription, cell polarity, and signaling (Seet and Hong 2006). SNXs, found in both yeast and mammalian cells, comprise the largest subset of proteins harboring this domain. Human SNXs are involved in endosomal sorting and recycling, in internalization, transport and lysosomal degradation of epidermal growth factor receptor, and in membrane tubulation. Yeast SNXs are required for regulation of protein retrieval and recycling traffic from prevacuolar/late endosomes to late Golgi. The t-SNARE Vam7p mediates fusion of multiple transport intermediates with the vacuole. The subunits of neutrophilic NADPH oxidase complex, $p40^{phox}$ and $p47^{phox}$ are implicated in phagocyte-mediated destruction of ingested microbes. The cytokine-independent survival kinase (CISK), PI 3-kinases, and the adaptor protein FISH play roles in cell signaling.

Molecular Mechanism of the PX Domain Association with Membranes

Similar to the FYVE and PH modules, the PX domain associates with PtdIns(3)P- or other PI-containing membranes via multiple interactions. Specific recognition of the inositol headgroup is often facilitated by nonspecific electrostatic contacts with acidic membrane surfaces and is accompanied by a hydrophobic insertion into the bilayer. The PX domain was identified as a PI binding module independently by several groups in 2001 (Cheever et al. 2001; Ellson et al. 2001; Kanai et al. 2001; Song et al. 2001; Xu et al. 2001; Yu and

Lemmon 2001). The $p40^{phox}$, SNX3, and Vam7p PX domains were found to recognize PtdIns(3)P, whereas the $p47^{phox}$ PX domain was shown to prefer PtdIns(3,4)P_2.

Several mechanistic studies have demonstrated that the PI binding induces membrane penetration of the hydrophobic residues in the variable loop α1–α2 (MIL). X-ray reflectivity experiments show that the $p40^{phox}$ PX domain penetrates 9Å into the lipid layer, with the side chains of a tyrosine and a valine inserted most deeply (Malkova et al. 2006). The corresponding hydrophobic residues in the $p47^{phox}$ and Vam7p PX domains also penetrate membrane-mimetics (Cheever et al. 2001; Lee et al. 2006; Stahelin et al. 2003). Alignment of the PX domain sequences reveals some conservation of the hydrophobic residues despite the fact that overall the α1–α2 loop is highly variable. Bem1, CISK, CPK, FISH, Grd19p, $p40^{phox}$, $p47^{phox}$ and SNX3 contain VPYV, IFG, MVLG, VYVGV, ILF, ILL, WFDG, and LPF sequences, respectively, in place of the hydrophobic residues in $p40^{phox}$, $p47^{phox}$ and Vam7p, and the MIL occupies analogous conformations in the PX domain structures (Bravo et al. 2001; Hiroaki et al. 2001; Karathanassis et al. 2002; Kutateladze 2007; Lu et al. 2002; Xing et al. 2004; Zhou et al. 2003).

Basic residues located in and around the PI binding pocket and the MIL of the $p40^{phox}$, $p47^{phox}$ and Vam7p PX domains are involved in nonspecific electrostatic contacts with the negatively charged lipids (Karathanassis et al. 2002; Lee et al. 2006; Stahelin et al. 2003). Electrostatic interactions have been shown to alleviate recognition of the PI lipid, enhance affinity, and induce hydrophobic insertion of the PX domains (Malkova et al. 2006; Stahelin et al. 2004, 2003, 2006). Coincidence detection of PtdIns(3,4)P_2, and another lipid (PS or PA) in a separate well-defined binding site is essential for membrane targeting of the $p47^{phox}$ PX domain (Karathanassis et al. 2002; Stahelin et al. 2003).

Structural Basis of PtdIns(3)P Recognition by the PX Domain

The atomic-resolution crystal and solution structures of three PX domains bound to PtdIns(3)P [Grd19p (Zhou et al. 2003), $p40^{phox}$ (Bravo et al. 2001), and SNX9 (Pylypenko et al. 2007)] and 17 PX domains in the ligand-free form [Bem1p (Stahelin et al. 2007), CISK (Xing et al. 2004), Grd19p (Zhou et al. 2003), KIF16B (Blatner et al. 2007), Nischarin, NOXO1b, $p40^{phox}$ (Honbou et al. 2007), $p47^{phox}$ (Hiroaki et al. 2001; Karathanassis et al. 2002), PI3K-C2α (Parkinson et al. 2008; Stahelin et al. 2006), PI3K-C2γ, SNX1 (Zhong et al. 2005), SNX7, SNX9 (Pylypenko et al. 2007), SNX12, SNX17, SNX22 (Song et al. 2007) and Vam7p (Lu et al. 2002)] have been determined. The structures show a similar fold that consists of a three-stranded β sheet, packed against a helical subdomain composed of three to four α-helices (Fig. 6.2d). An additional 3_{10} helix is present in the structures of the Bem1p, CISK, $p40^{phox}$ and PI3K-C2α PX domains and another helix α0 is formed by the residues *N*-terminal to the β sheet in $p40^{phox}$ (Bravo et al. 2001; Stahelin et al. 2006, 2007; Xing et al. 2004). The α1 and α2 helices are connected by a long variable loop (MIL), which in Bem1p, CISK, $p40^{phox}$, $p47^{phox}$ and PI3K-C2α contains a type II polyproline helix (Bravo et al. 2001; Karathanassis et al. 2002; Stahelin et al. 2006, 2007; Xing et al. 2004). The β1 strand has a β-bulge that twists the β sheet, forming one wall of the lipid binding pocket.

In the $p40^{phox}$ PX complex PtdIns(3)P is bound in a relatively narrow and deep (7Å) groove formed by the three elements: the loop connecting β3 and α1, a part of MIL closest to α2, and the *N*-terminal halves of β2 and α2 (Bravo et al. 2001) (Figs. 6.2d and 6.3c). The 3-phosphate group of the lipid is restrained through the formation of hydrogen bonds with the guanidino moiety of Arg58 in the β3/α1 loop and with backbone amides of Tyr59 and Arg60. The side chains of Lys92 and Arg60 are involved in the hydrogen bonding contacts with the 1-phosphate, whereas the 4- and 5-hydroxyl groups of PtdIns(3)P are hydrogen

bonded to Arg105. The three motifs essential for PI binding, RRYx_2Fx_2Lx_3L of β3/α1, Px_2PxK of the MIL, and RR/Kx_2L of α2 are present in the majority of PX domain sequences.

Other PI Effectors

The number of PI effectors is increasing rapidly and, in addition to the FYVE, PH and PX domains described above, includes the ANTH, BATS, C2, DHR-1, ENTH, FERM, GOLPH3, P4M, PDZ, PROPPINs, PTB, SYLF, and Tubby modules. The ANTH domain and its structural relative ENTH bind strongly and specifically to PtdIns(4,5)P_2 in the plasma membrane (Itoh and De Camilli 2006). The BATS domain localizes to highly curved membranes enriched in PtdIns(3)P (Fan et al. 2011). Although the majority of C2 domains associate with the most common anionic and zwitterionic lipids such as PS and phosphocholine (PC), some show preference for PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 (Cho and Stahelin 2006). Arecently characterized atypical C2 module, the DHR-1 domain, binds PtdIns(3,4,5)P_3 and is required for targeting of the Rho family guanine exchange factor, Dock1, to the plasma membrane and for triggering cell polarization (Premkumar et al. 2010). Another effector of PtdIns(4,5)P_2 is the FERM domain (Hamada et al. 2000). GOLPH3 and P4M target specifically PtdIns(4)P in the Golgi apparatus (Dippold et al. 2009; Schoebel et al. 2010; Wood et al. 2009), whereas PDZ recognizes PtdIns(4,5)P_2 in the plasma membrane (Zimmermann 2006). Human and yeast PROPPINs bind PtdIns(3,5)P_2 in membranes of endosomes, lysosomes and vacuoles (Dove et al. 2009), and the fly PROPPIN Dm3 associates with both PtdIns(3,5)P_2 and PtdIns(3)P (Lemmon 2008). Interaction with PtdIns(4,5)P_2 and PtdIns(4)P has been reported for the PTB domain that normally binds phosphotyrosine peptides (DiNitto and Lambright 2006). The SYLF domain interacts preferentially with PtdIns(3,4,5)P_3 and is involved in the regulation of dorsal ruffle formation (Hasegawa et al. 2011). The Tubby domain localizes to the plasma membrane through binding to PtdIns(4,5)P_2 and to a lesser degree to PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 (Santagata et al. 2001; Szentpetery et al. 2009). Several distinct proteins, for example, AKAP79, CAP23, GAP43, MARKS, NHE3, PAR-3, profilin and WASP, recognize PIs via clusters of basic residues, however, the molecular details of these interactions have not been characterized (Caroni 2001); Dell'Acqua et al. 1998; Goldschmidt-Clermont et al. 1990; Horikoshi et al. 2011; Mohan et al. 2010; Rohatgi et al. 2000; Wang et al. 2001).

Although PI effectors have diverse and unrelated structures, their membrane docking and PI binding mechanisms share many similarities. First, all effectors possess a highly basic binding site composed of at least three positively charged residues [three basic residues and a histidine in CALM ANTH (Ford et al. 2001)], and as many as six positively charged residues [six basic residues and a histidine in Epsin1 ENTH (Ford et al. 2002) and Grp1 PH (Ferguson et al. 2000; Lietzke et al. 2000)] (Fig. 6.3). Second, membrane binding involves some or all components of multiple anchoring. Association with PI-containing membranes can be augmented by nonspecific electrostatic interactions, hydrophobic insertion, protonation of a histidine switch, coincidence detection and increased avidity, with each component contributing to binding energetics. Cooperation of multiple interactions is particularly essential for the recruitment of less selective PI-binding modules, including many PH domains. The affinity and specificity can be further increased due to cooperative binding of multiple PI-recognizing domains present in the same protein and association of the adjacent regions with various membrane elements and membrane-attached proteins.

Concluding Remarks

Phosphoinositide-binding domains have emerged as a family of 'PI code' readers, and considerable effort has been put forth by many groups to determine their role in mediating

acute and constitutive membrane signaling. This chapter focuses on the mechanistic aspects of single PI effectors; however, these domains are often found next to other PI-binding modules and PI-modifying catalytic domains. Of those discussed here, pleckstrin and Centaurin contain two and five PH domains, respectively; FGD1 contains two PH domains separated by a FYVE finger; PLCγ1 has two PH domains, a catalytic phospholipase module and a C2 domain; PLD1C contains PX, PH and phospholipase domains; DFCP1 contains tandem FYVE fingers; and PDZ and PX domains are present in SNX27. Depending on the specificities, these modules may act either in concert or compete for targeting their host proteins to particular subcellular membranes and regions. The location and duration of membrane association by proteins containing multiple distinct PI effectors can be mediated by activities of organelle-specific PI kinases, phosphatases and lipases. The crosstalk between PIs can provide a mechanism for the regulation of temporal and spatial membrane localization of these proteins and may be essential for controlling signaling cascades. The link between dysregulation of the PI signaling network and numerous diseases suggests a strong therapeutic potential (Engelman et al. 2006; McCrea and De Camilli 2009; Prestwich 2004; Xu et al. 2006; Zhang et al. 2010), and further mechanistic studies will be essential to fully understand and exploit this potential.

Acknowledgments

The research in TGK laboratory is supported by the NIH grants GM096863 and CA 113472.

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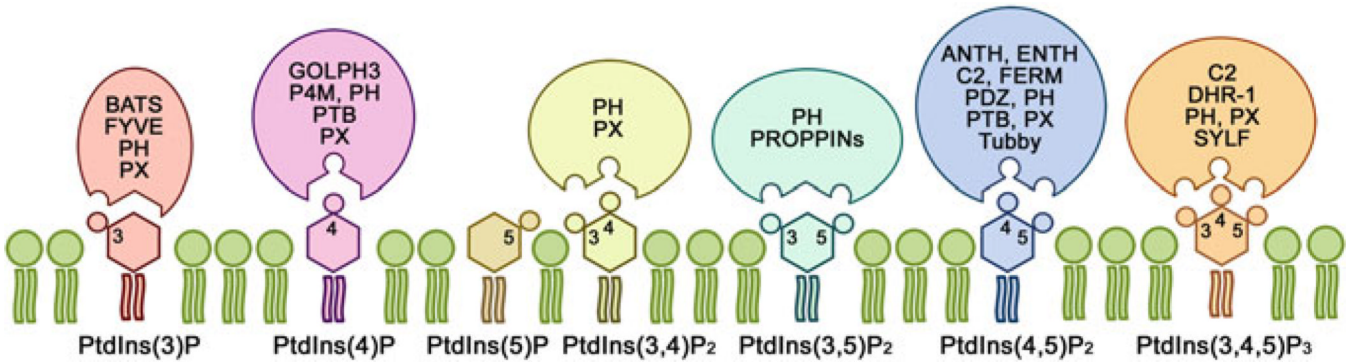

Fig. 6.1.
PI-recognizing effectors. Signaling domains and their target PIs are shown

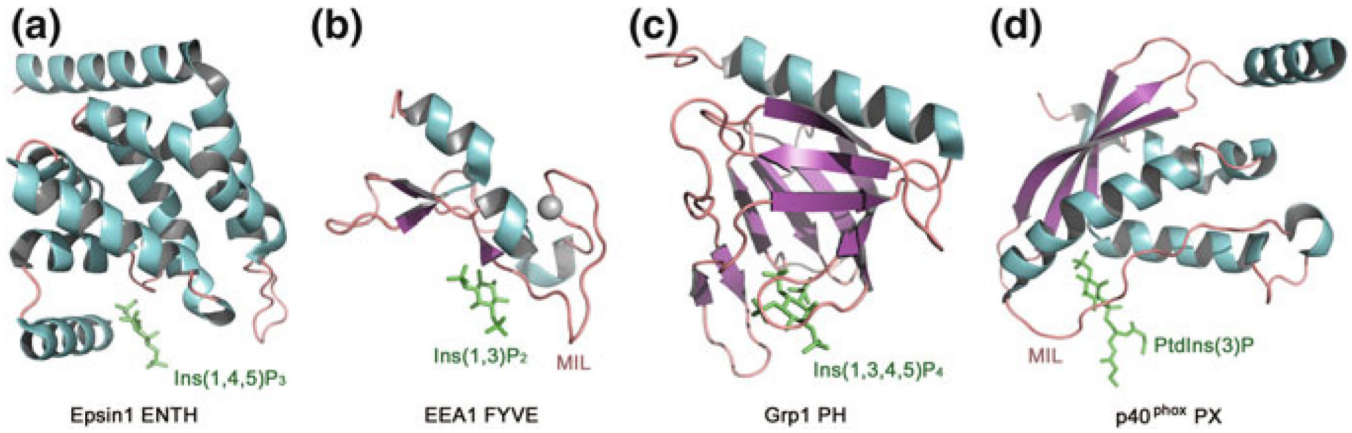

Fig. 6.2.

The crystal structures of **a** the ENTH domain of Epsin1 in complex with inositol 1,4,5-trisphosphate, a headgroup of PtdIns(4,5)P_2 (1H0A) (Ford et al. 2002), **b** the EEA1 FYVE domain in complex with inositol 1,3-bisphosphate, a headgroup of PtdIns(3)P (1JOC) (Dumas et al. 2001), **c** the Grp1 PH domain in complex with inositol 1,3,4,5-tetrakisphosphate, a headgroup of PtdIns(3,4,5)P_3 (1FGY) (Lietzke et al. 2000) and **d** the $p40^{phox}$ PX domain in complex with dibutanoyl PtdIns(3)P (1H6H) (Bravo et al. 2001)

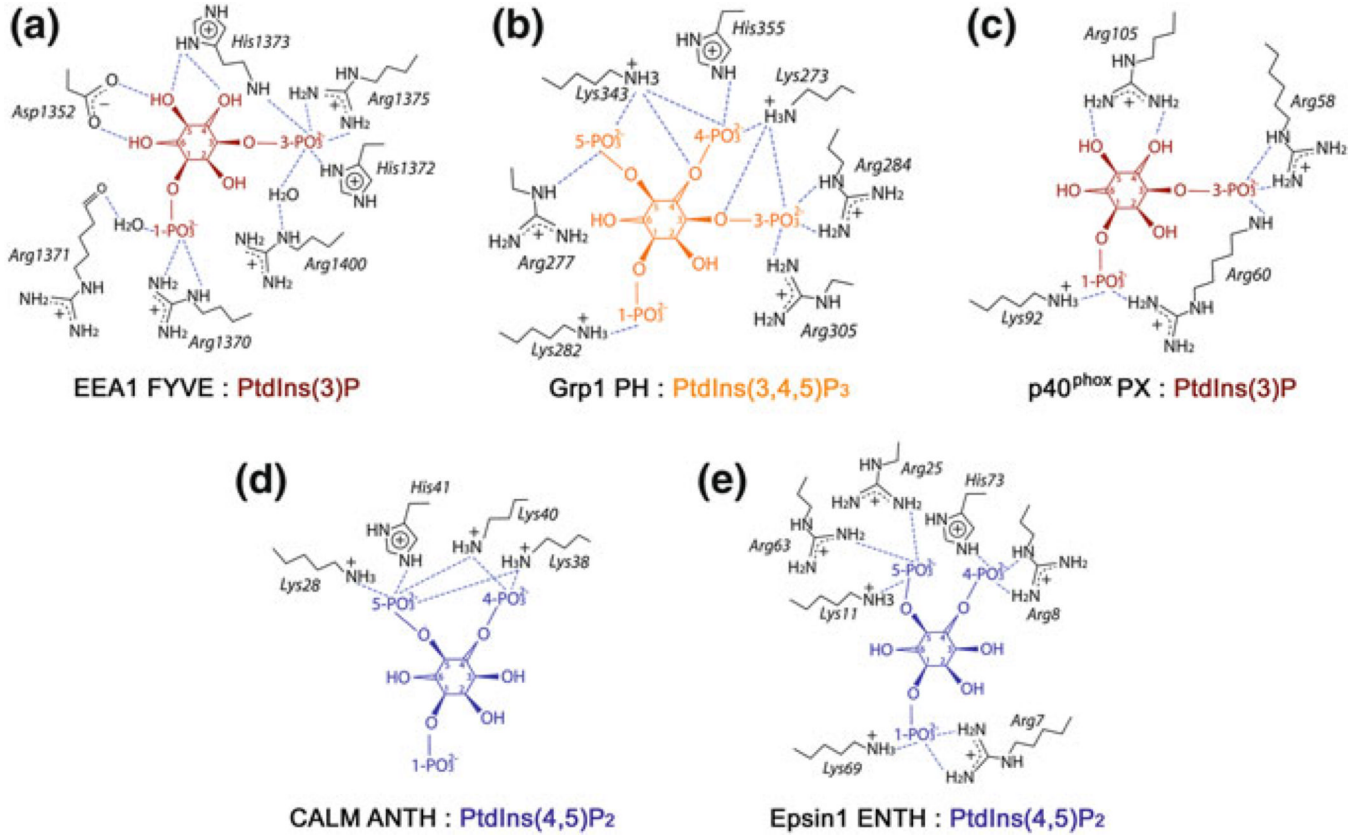

Fig. 6.3.

Schematic diagrams showing PtdIns(3)P headgroup coordination by **a** EEA1 FYVE (1JOC) and c $p40^{phox}$ PX (1H6H) domains; PtdIns(3,4,5)P_3 headgroup coordination by **b** Grp1 PH domain (1FGY); and PtdIns(4,5)P_2 headgroup coordination by **d** CALM ANTH (1HFA) and **e** Epsin1 ENTH (1H0A) domains. Only charged residues of the proteins are depicted for clarity