Inositol-lipid binding motifs: signal integrators through protein-lipid and protein-protein interactions

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Accepted 16 March 2005
Journal of Cell Science 118, 2093-2104 Published by The Company of Biologists 2005
doi:10.1242/jcs.02387

Summary

Inositol lipids have emerged as universal lipid regulators of protein signaling complexes in defined membrane compartments. The number of protein modules that are known to recognise these membrane lipids is rapidly increasing. Pleckstrin homology domains, FYVE domains, PX domains, ENTH domains, CALM domains, PDZ domains, PTB domains and FERM domains are all inositide-recognition modules. The latest additions to this list are members of the clathrin adaptor protein and arrestin families. Initially, inositol lipids were believed to recruit signaling molecules to specific membrane compartments, but many of the domains clearly do not possess high enough affinity to act alone as localisation signals. Another important notion is that some (and probably most) of these protein modules also have protein binding partners, and their protein- and lipid-binding activities might influence one another through allosteric mechanisms. Comparison of the structural features of these domains not only reveals a high degree of conservation of their lipid interaction sites but also highlights their evolutionary link to protein modules known for protein-protein interactions. Protein-protein interactions involving lipid-binding domains could serve as the basis for phosphoinositide-induced conformational regulation of target proteins at biological membranes. Therefore, these modules function as crucially important signal integrators, which explains their involvement in a broad range of regulatory functions in eukaryotic cells.

Key words: Phosphoinositide, Pleckstrin homology domain, Intracellular signaling, Inositol lipid kinase, Inositol lipid phosphatase, Phosphorylation

Introduction

One of the most impressive advances in signal transduction research over the past 30 years was the deciphering of the molecular details and importance of the inositide-Ca^{2+} second messenger system (Berridge, 1984). The receptor-mediated activation of the hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] with the resultant production of inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG), the two second messengers linked to Ca^{2+} signaling and protein-kinase-C-mediated phosphorylations, respectively, had become textbook knowledge by the early 90s. Yet, we could not have foreseen the wealth and diversity of the biochemical processes subsequently proven to be regulated by membrane phosphoinositides. Identification of multiple inositol lipid kinases and phosphatases that modify the -OH groups at various positions of the inositol ring in membrane PtdIns revealed that inositol lipids not only are precursors for second messengers but also serve as membrane-bound signaling molecules in their own right (Toker and Cantley, 1997; Martin, 1997; Wenk and De Camilli, 2004). This, together with the discovery of protein motifs that recognise inositol lipid headgroups with high affinity and often remarkable specificity (Hurley and Meyer, 2001; Lemmon, 2003; DiNitto et al., 2003), led to the current notion that production of specific inositol lipids in well-defined membrane compartments upon stimulation helps recruit signaling molecules to membranes and hence contributes to the organization of signaling complexes. This principle was an important basis for the development and wide use of protein modules [mostly pleckstrin-homology (PH) domains] fused to the green fluorescent protein (GFP) for monitoring the dynamics and spatial organization of inositol lipid signals in single living cells (Stauffer et al., 1998; Balla et al., 2000; Balla and Varnai, 2002; Irvine, 2004; Walker et al., 2004).

Since the introduction of these methods, however, it has become apparent that these smart molecular probes may not ‘see’ all pools of a specific inositide species equally well (Várnai and Balla, 1998). Moreover, recruitment of multiple proteins containing PH domains binding to the same inositol lipid would create a large degree of information ‘spreading’ and it is hard to see how cells could maintain signaling specificity if this were the case. This conundrum is further complicated by the fact that several other domains also act as docking sites for phosphoinositides. These include FYVE domains (Simonsen et al., 1998; Misra and Hurley, 1999; Kutateladze et al., 1999), PX domains (Ellson et al., 2001; Cheever et al., 2001; Kanai et al., 2001; Hiroaki et al., 2001), ENTH (Ford et al., 2002; Legendre-Guillemin et al., 2004) and ANTH/CALM (Mao et al., 2001; Ford et al., 2001) domains, PTB domains (Forman-Kay and Pawson, 1999; Yan et al., 2002), FERM domains (Christy et al., 1998; Pearson et al., 2000; Mangeat et al., 1999), PDZ domains (Nourry et al., 2003; Jelen et al., 2003) and the heterotetrameric clathrin adaptors AP-2 and AP-1 (Collins et al., 2002; Wang et al., 2003; Owen et al., 2004; Heldwein et al., 2004). The most important structural features and physiology associated with each of these

Journal of Cell Science

domain families have been amply covered by several excellent recent reviews cited above. Here, I focus on the structural and functional similarities between these modules, emphasising their proven or potential protein-protein interactions in combination with their lipid-binding properties. These comparisons reveal notable general principles about inositide regulation and provoke new insights into how phosphoinositides can control several biochemical and signaling pathways simultaneously and yet specifically.

PH-, PTB- and FERM domains

The pleckstrin homology domain was first described in the platelet protein pleckstrin and shown to be a module that binds PtdIns(4,5)P_2 (Harlan et al., 1994). This discovery was followed by a large number of studies identifying and characterizing PH domains, often with unique inositide-binding specificities, in a variety of signaling proteins (Lemmon and Ferguson, 2000; Cozier et al., 2004). PH domains are present in protein kinases, guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) for small GTP-binding proteins, lipid transport proteins and phospholipases – just to name a few major groups of proteins (Cozier et al., 2004). They are probably the most studied protein domains and in most (but not all) cases show prominent inositide binding with various degrees of specificity (Yu et al., 2004). PH domains specific for a variety of phosphoinositides, including PtdIns(4,5)P_2, PtdIns(4)P, PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, have been described, and the features that determine their lipid-binding specificities have been revealed by structural studies (DiNitto et al., 2003; Cozier et al., 2004). Some PH domains have also been found to bind to proteins (see below), but until very recently, they were not considered to be protein-binding modules.

PTB domains, which have a remarkable structural resemblance to PH domains, are present in certain adaptor proteins, such as IRS-1 or Shc, and are known to interact specifically with tyrosine-phosphorylated peptide sequences found in the cytoplasmic membrane-adjacent regions of receptor tyrosine kinases (Forman-Kay and Pawson, 1999). Regulation of PTB domains by phosphoinositides is uncommon, but the PTB domains of the clathrin adaptor protein Dab1 and its relative the autosomal hypercholesterolemia protein (ARH), both of which bind to the internalization sequence of lipoprotein receptors, have been shown to bind PtdIns(4,5)P_2 (Stolt et al., 2003; Yun et al., 2003; Mishra et al., 2002a; Mishra et al., 2002b).

Another group of inositide-binding domains, the FERM domains, were first described in the N-termini of proteins that link the actin cytoskeleton to the plasma membrane (hence the acronym FERM from 4.1, ezrin/radixin/moiesin) (Christy et al., 1998). FERM domains bind to the cytoplasmic sequences of integrins as well as phosphoinositides (Hamada et al., 2000; Mangeat et al., 1999). The highly conserved structures of FERM domains, show that one of their three subdomains, subdomain C, contains a PTB fold that bears all the hallmarks of PH/PTB domains.

The common structural feature of these domains is that they contain two nearly orthogonal β sheets formed by seven β strands arranged in groups of four and three, flanked by a C-terminal helix that 'holds' the structure together (Fig. 1). The structural similarities between the three domains are quite obvious, but even the motifs within the structures that are involved in lipid and peptide binding, respectively, show striking similarities. In PH domains, the inositide-binding site is almost always formed by the β1-β2 and β3-β4 strands and the variable loops connecting them (this section of the domains is shown in light blue in Fig. 1). One exception is the PH domain of spectrin, in which Ins(1,4,5)P_3 (mimicking the PtdIns(4,5)P_2 headgroup) binds between the loops connecting the β1-β2 and the β5-β6 strands just outside the usual lipid-binding pocket (Macias et al., 1994). A unique variant is the binding of PtdIns(3,4,5)P_2 by the PH domains of members of the Grp1/ARNO family, in which the lipid binds between the β1-β2 loops and the long insertion between what would correspond to the β6 and β7 strands in typical PH domains. This long insertion (colored brown in Fig. 1) replaces the β3-β4 strands and their connecting loop in forming the inositide-binding site (Lietzke et al., 2000; DiNitto et al., 2003).

Comparing binding of the inositides to the PTB domain Dab-1 with that to PH domains, one can recognise the structural conservation: in Dab-1, a helix is inserted between the β1 and β2 strands in place of the shorter loop found in PH domains (colored salmon in Fig. 1). This helical insertion (which can be of various lengths) is a feature of PTB domains. In the case of Dab-1, one side of the inserted helix forms the phosphoinositide-binding site (Stolt et al., 2003). The Shc PTB domain, which binds PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 (Rameh et al., 1997), also contains a cluster of positive residues on the same side of this helix (Zhou et al., 1995), although the inositide-binding site within this latter structure has not been identified.

The crystal structure of the radexin FERM domain reveals an apparent deviation from the inositide binding described above (Hamada et al., 2000). Here, Ins(1,4,5)P_3 (presumably mimicking the PtdIns(4,5)P_2 headgroup) binds to a region formed by the terminal helix of the radexin C-subdomain that has the PTB/PH superfold and the adjacent loops of subdomain A. However, mutagenesis studies have indicated that phosphoinositide binding and cellular localization of ezrin are altered by mutation of basic residues within the FERM domain that, on the basis of the radexin structure, are not predicted to be involved in inositide binding (Barret et al., 2000). The positions of some of these residues are in the region corresponding to the inositide-binding region of PH and PTB domains, suggesting the presence of an additional inositide-binding site. Similarly, two inositide-binding regions within the FERM domain of the protein tyrosine phosphatase PTPL1 (Bompard et al., 2003) that also map to the same areas have been described. Intriguingly, the C-terminal tail of moesin, which acts as an autoinhibitory domain, interacts with the FERM domain, competing with the inositide-binding site in subdomain C that corresponds to the inositide-binding region of PH domains (Edwards and Keep, 2001). This, together with the reported role of PtdIns(4,5)P_2 binding in priming and activation of ezrin (Fievet et al., 2004) and moesin (Edwards and Keep, 2001) suggests that the inositide-binding site corresponding to those found in PH domains is an important feature of FERM domains and that the additional site located between subdomains C and A may represent yet another regulatory site that, given its position, could represent a binding site for soluble inositol phosphates.

Journal of Cell Science

The protein/peptide binding mechanisms of these domains also show remarkable similarities. The tyrosine-phosphorylated peptide always binds between the C-terminal α helix and the β5 strand within PTB domains (Forman-Kay and Pawson, 1999) (Fig. 1). In the case of the PTB domains of Dab1, Dab2, Numb and AHR, the tyrosine within the FxNPxY motif does not have to be phosphorylated (Mishra et al., 2002a; Mishra et al., 2002b; Li et al., 1998). Exactly the same region within the C subdomain of FERM domains binds the peptide tail of integrins in radexin and talin (Garcia-Alvarez et al.,

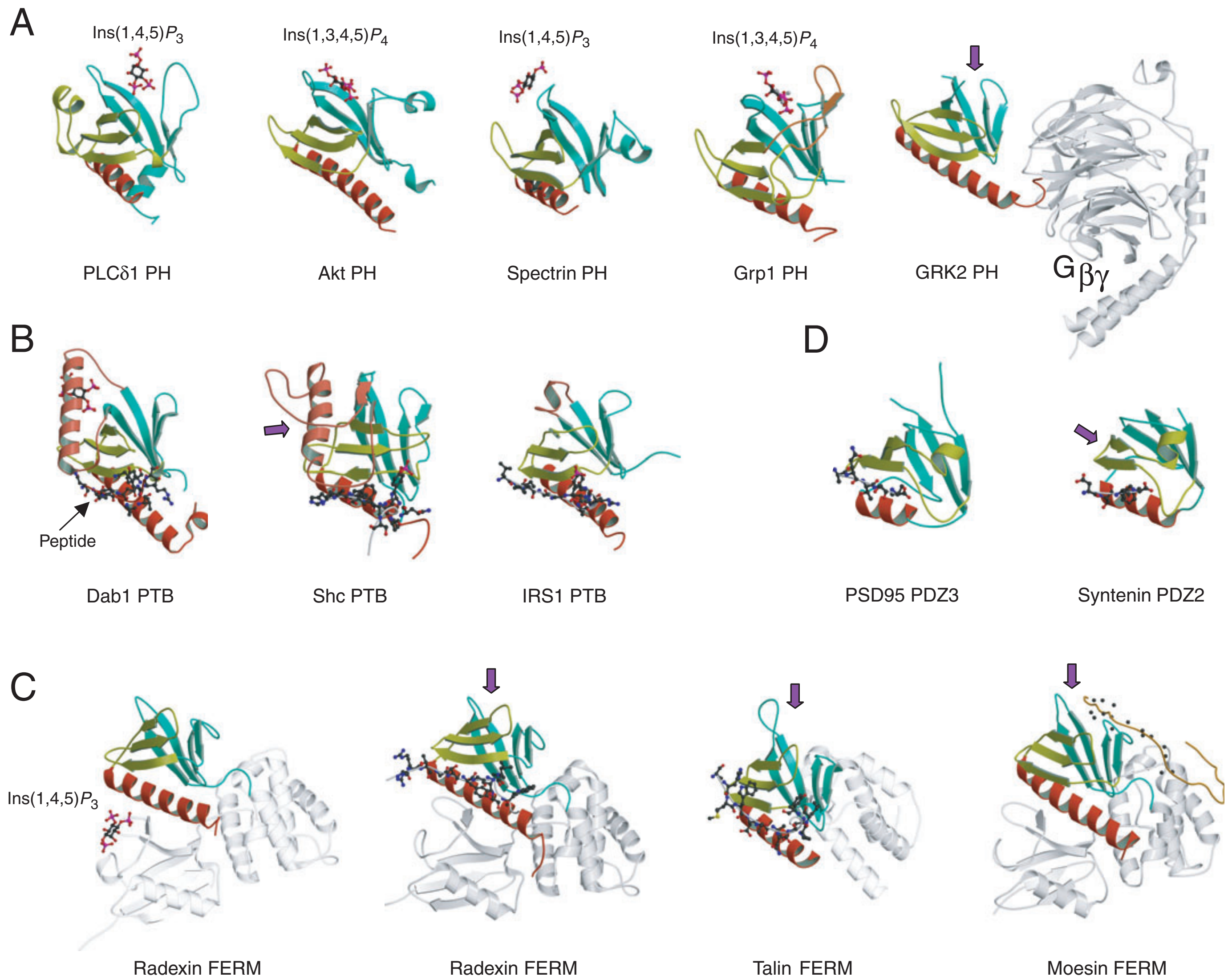

Fig. 1. Similarities and differences between the inositide and peptide binding of PH, PTB, PDZ and FERM domains. (A) Inositide binding of PH domains occurs in a pocket formed by the β1-β4 sheets and connecting loops (light blue). Exceptions are the spectrin PH domain, which binds the lipid outside this position, and the Grp1/ARNO PH domain, in which a long insertion (brown) replaces the β3-β4 sheets in forming the pocket. The position of Gβγ (gray) bound to the GRK2 PH domain shows that it involves a surface distinct from the putative lipid-binding site (purple arrow). (B) PTB domains often contain a helix inserted between their β1-β2 strands (salmon). Inositol lipids bind at the outer surface of this helix (the purple arrow indicates the presumed lipid-binding site of Shc), whereas the peptide always binds between the C-terminal helix and the parallel β7 strand. (C) The C-subdomain of FERM domains is a PTB fold that binds peptide sequences found within integrin tails. The inositide binding site of radexin is quite far from the inositide-binding site of PH domains, but mutagenesis studies indicate an additional binding site in radexin talin and moesin (purple arrows) that corresponds to the region where PH domains bind inositides. This putative lipid-binding site is masked by the C-terminal tail in the inactive conformation of moesin. (D) The PDZ domain binds its peptide binding-partners at a position similar to that in the PTB domains. The kink in the loop between the β1 and β2 strand explains why PDZ domains usually bind C-terminal peptides. The only reported inositide binding of the Syntenin PH domain maps to a surface pointed to by the purple arrow. The PDB accession numbers used were: 1MAI, PLCδ1PH (Ferguson et al., 1995); 1H10, AktPH (Thomas et al., 2002);1BTN, SpectrinPH (Hyvonen et al., 1995); 1FHX, Grp1PH (Ferguson et al., 2000); 1OMW, GRK2PH (Lodowski et al., 2003); 1NU2, Dab1PTB (Stolt et al., 2003); 1SHC, ShcPTB (Zhou et al., 1995); 1IRS, IRS1PTB (Zhou et al., 1996); 1BE9, PSD95PDZ3 (Morais-Cabral et al., 1996); 1OBX, SyntheninPDZ2 (Kang et al., 2003); 1GC6, Radixin-FERM-InsP_3 (Hamada et al., 2000); 1J19, Radixin-FERM-ICAM2pept (Hamada et al., 2003); 1MK7, Talin-FERM (Garcia-Alvarez et al., 2003); 1SGH, Moesin-FERM (Finnerty et al., 2004). Pictures were created by Molscript (Kraulis, 1991).

Journal of Cell Science

2003; Hamada et al., 2003). Here again, the tyrosine residue within the NPxY consensus sequence does not have to be phosphorylated for binding.

Binding of proteins to PH domains has been reported in a few cases (Yao et al., 1997; Tsukada et al., 1994; Carman et al., 2000; Tanaka et al., 1999) but only documented at the structural level in the case of the PH domain of GRK2 (Lodowski et al., 2003). Here, the βγ subunit of heterotrimeric G proteins binds to the PH domain at a site formed by sequences that follow the C-terminal helix, and the two loops connecting the β4-β5 and the β6-β7 strands. Other PH domains, such as those of the PLCβ proteins are probably also regulated by Gβγ by similar interactions (Barr et al., 2000; Wang et al., 1999). In addition, given the structural similarities of these domains, some PH domains might bind specific peptide sequences at the site PTB and FERM domains use. A protein interaction site at this location has been suggested on the basis of theoretical considerations (Alberti, 1998). Such peptide sequences might belong to the cytoplasmic regions of integral membrane proteins, which would explain the difficulties in identifying protein binding partners for PH domains in yeast two-hybrid screens.

Although such speculation currently lacks experimental support, recent work suggests that binding of PH domains to lipid or membrane targets is affected by residues that do not contribute to the formation of the lipid-binding pocket. These include the recent identification of a threonine residue within the PH domain of Akt whose phosphorylation by PKCζ affects its membrane recruitment (Powell et al., 2003), or the identification of a tyrosine and/or histidine residue within the β7 strand of PtdIns(4)*P*-specific PH domains that appears to determine their interaction with putative protein binding partners (Roy and Levine, 2004). It is also very possible that intramolecular interactions involving the PH domains of some proteins will be proven to be important for keeping proteins in an inactive conformation by analogy with how the C-terminal tail of some FERM-domain-containing proteins competes with the inositide for its binding site (Fig. 1).

Notice that, the PH/PTB fold is also found in the Ran-binding domain of RanBP2 (Vetter et al., 1999) and in the Enabled/VASP homology domain (EVH-1) (Prehoda et al., 1999), but neither inositide interaction nor regulation of these domains has been reported. By contrast, the structure of the recently described GRAM domain (Doerks et al., 2000), which also shows the PH/PTB fold (Begley et al., 2003), has been shown to bind phosphoinositides (Berger et al., 2003).

PDZ domains

PDZ (postsynaptic density protein, disk large, zonula occludens) domains are small ~85-residue modules that bind short peptide sequences, generally the last 4-5 residues of the C-terminal tails of transmembrane proteins. They often appear with other modular domains or additional PDZ domains, and organize protein signaling complexes at specific cellular locations (Nourry et al., 2003). The structures of PDZ domains are vaguely reminiscent of those of PH/PTB domains in that six antiparallel β strands and an internal α helix are arranged in a configuration similar to that in the PH/PTB domains. Interestingly, the peptide is bound in the groove formed by the α helix and the parallel β2 strand (Fig. 1D). PDZ domains are classified into three classes on the basis of the peptide sequences they prefer to bind (Songyang et al., 1997; Jelen et al., 2003; Nourry et al., 2003). These always have a hydrophobic (valine, leucine or isoleucine) C-terminal residue, and a serine or threonine (Class I), a hydrophobic (tyrosine, phenylalanine or valine; Class II) or an acidic (Class III) residue at the –2 position. There are several basic residues in the third PDZ domain of PSD95 (many of which are conserved in some, but not all, PDZ domains) that form a basic pocket analogous to the inositide-binding site of PH domains relative to the peptide-binding site. However, phosphoinositide binding or regulation of PDZ domains has not been reported (and perhaps widely examined), and only one report has indicated the PtdIns(4,5)P_2-dependent association of the tandem PDZ domain of syntenin with the plasma membrane (Zimmermann et al., 2002). Nevertheless, this possibility should be explored further in domains that contain basic clusters in the putative lipid-binding region.

FYVE domains

FYVE domains were initially described as ~70-residue sequence motifs that localize several proteins that participate in vacuolar sorting or endocytosis to membranes by interacting with PtdIns(3)*P* (Stenmark et al., 1996; Simonsen et al., 1998; Burd and Emr, 1998). FYVE domains are cysteine-rich motifs that contain a conserved (R/K)(R/K)HHCR sequence surrounding their third zinc-coordinating cysteine. The crystal structures of FYVE domains revealed their great similarity to the C1b domain of PKCδ and the cysteine-rich region of rabphilin 3A (Misra and Hurley, 1999; Kutateladze et al., 1999). FYVE domains contain several long loops at the N-terminus, two short antiparallel β sheets and a C-terminal α helix. The structure is stabilized by two Zn^{2+}-binding clusters, and the PtdIns(3)*P*-binding site is formed by the basic residues in the conserved (R/K)(R/K)HHCR sequence (Fig. 2A). Importantly, the structure contains a prominent hydrophobic protrusion that allows the domain to penetrate the membrane bilayer (Misra and Hurley, 1999). Because of the relatively shallow PtdIns(3)*P*-binding pocket and a single phosphate interaction, isolated FYVE domains are not sufficient for membrane localization and require adjacent sequences that interact with Rab5 proteins or allow dimerisation for efficient membrane recruitment (Lawe et al., 2000; Dumas et al., 2001). There is no indication that FYVE domains per se should interact with protein binding partners. However, several proteins, mostly transcription factors, contain Zn^{2+} finger motifs, such as the PHD zinc finger (Pascual et al., 2000) or the ZZ finger (Legge et al., 2004), that greatly resemble FYVE domains, and some bind both proteins and phosphoinositides (Gozani et al., 2003). Given these similarities, FYVE domains might originate from an ancient protein fold that mediated protein-protein interactions and some FYVE domains could contact protein binding partners in the membrane.

PX domains

PX domains were initially identified as ~120-residue motifs in the $p40^{phox}$ and $p47^{phox}$ subunits of the neutrophil NADPH oxidase complex (Ponting, 1996). These domains were soon recognised as binding modules for 3-phosphorylated

Journal of Cell Science

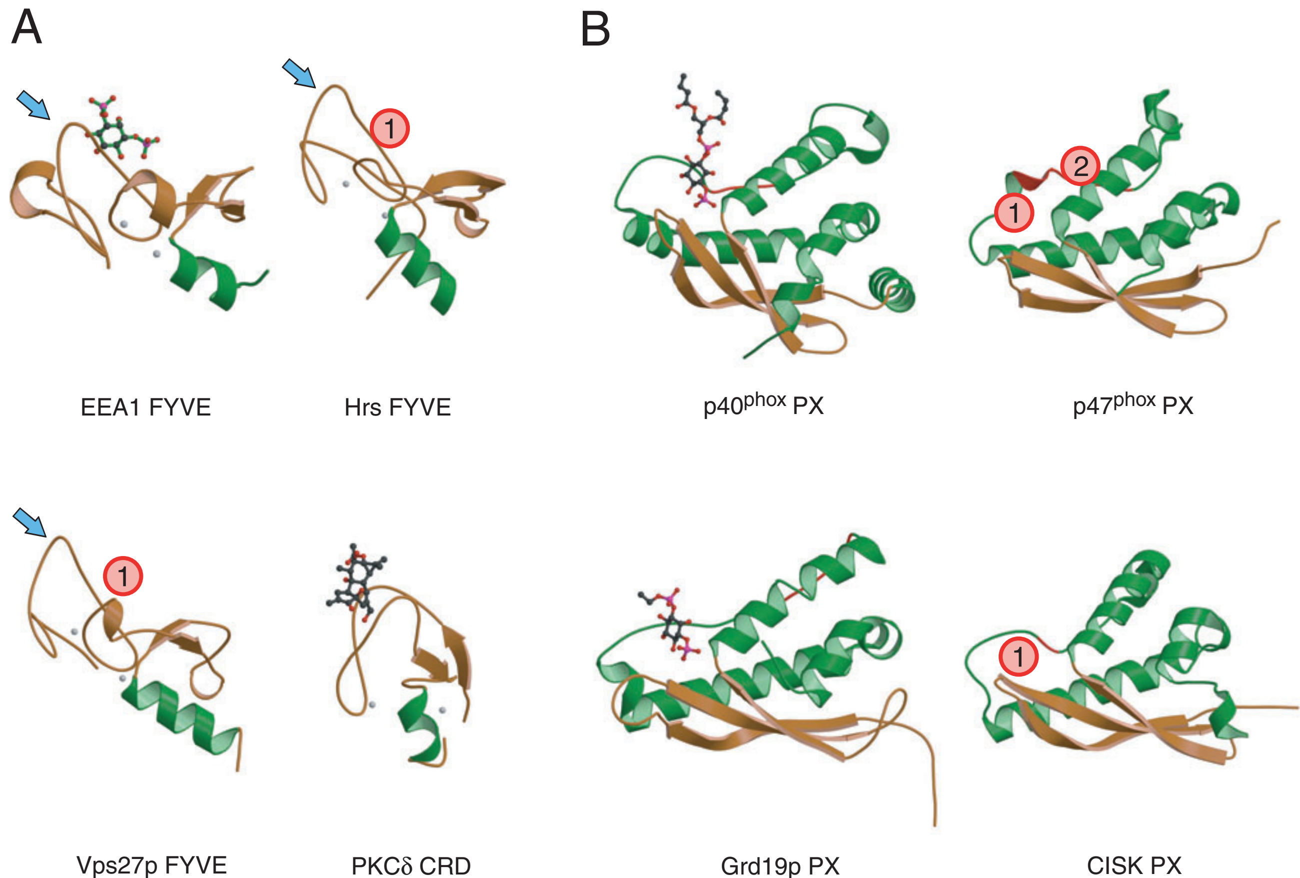

Fig. 2. Inositide binding by FYVE and PX domains. (A) The Ins(1,3)P_2 (mimicking PtdIns(3)*P*) binding of the EEA1 FYVE domain occurs in a relatively shallow groove and involves the conserved R(R/K)HHCRxCG sequence. The binding pocket occupies the same position within the Hrs FYVE and Vps27p FYVE domains (red circle labeled 1). A hydrophobic tip facing the membrane is important for membrane penetration (blue arrows). The corresponding area within the PKCδ cysteine-rich domain (CRD) recognises the hydrophobic phorbol esters. (B) PX domains bind 3-phosphorylated inositides in the pocket formed by two helices and the loop connecting the β1 and β2 strand. An additional binding site that binds PA or other acidic phospholipids is located in the p47phox PX domain (red circle labeled 2). The long coil containing the PxxP consensus sequence (red) in many PX domains (only a single proline in the CISK PX domain) is the site of interaction with the SH3 domain in the p47phox PX domain. The PDB accession numbers used are: 1JOC, EEA1 FYVE (Dumas et al., 2001); 1DVP, Hrs FYVE (drosophila) (Mao et al., 2000); 1VFY, Vps27p FYVE (Misra and Hurley, 1999); 1PTR, PKCδ CRD (Zhang et al., 1995); 1H6H, $p40^{phox}$ PX (Bravo et al., 2001); 1O7K, $p47^{phox}$ PX (Karathanassis et al., 2002); 1OCU, Grd19p PX (Zhou et al., 2003); 1XTE, CISK PX (Xing et al., 2004).

phosphoinositides (Ellson et al., 2001; Cheever et al., 2001; Kanai et al., 2001). PX domains have been identified in numerous proteins with a wide spectrum of functions, including protein sorting, vesicular trafficking and phospholipid metabolism (Sato et al., 2001). The crystal structures of the $p47^{phox}$ (Karathanassis et al., 2002) and $p40^{phox}$ (Bravo et al., 2001) PX domains show an N-terminal three-stranded β-sheet, followed by a helical subdomain made up from four α helices (Fig. 2B). Two of the helices are linked by a polyproline loop. The inositide-binding pocket is located between the bulge formed by the loop linking the β1-β2 strands and one of the helices. An additional lipid-binding site has been described within the $p47^{phox}$ PX domain. It is adjacent to the PtdIns(3)*P*-binding site and binds phosphatidic acid. This contributes to the efficient membrane recruitment of the domain (Karathanassis et al., 2002). Most PX domains, including all of the PX domains identified in *Saccharomyces cerevisiae* proteins, bind PtdIns(3)*P* (Yu and Lemmon, 2001), but some show binding preference for other inositides, such as the $p47^{phox}$ PX domain, which preferentially binds PtdIns(3,4)P_2 (Karathanassis et al., 2002), the PX domain of CISK (cytokine-independent survival kinase), which prefers PtdIns(3,4,5)P_3 (Xing et al., 2004) and the PX domain of the Class II PI-3-kinase, which prefers PtdIns(4,5)P_2 (Song et al., 2001).

The best-known and structurally characterized PX-domain–protein interaction is the intramolecular interaction between the C-terminal SH3 domain and a PxxP motif within the PX domain of $p47^{phox}$ (Hiroaki et al., 2001; Karathanassis et al., 2002). This motif is conserved in most (but not all) PX domains and the region shows a large chemical shift-change upon PtdIns(3)*P* binding in the Vam7 protein (Cheever et al., 2001). Protein binding and PtdIns(3)*P* binding could therefore affect one another. However, interaction of SH3 domains with other PX domains, including that of $p40^{phox}$, has not been shown to be a general principle, and the putative interacting

proteins for other PX domains remain elusive. Importantly, among the PX domains identified in yeast, significant differences in PtdIns(3)P-dependent membrane recruitment have been found despite strong sequence conservation within their PtdIns(3)P-headgroup-binding regions (Yu and Lemmon, 2001). Although the role of additional interactions between lipids and hydrophobic residues within the PX domain or by the adjacent PA-binding sites can explain some of these differences, it is also possible that PX domains in general also serve as 'dual detectors' that bind to inositides and proteins. Indeed, the insulin-induced membrane translocation of the p47^{phox} PX domain has been reported to be only partially dependent on PtdIns(3)P binding (Zhan et al., 2002). In a recent study several PX domains of yeast were found to have protein binding partners in a genome-wide two-hybrid screen (Vollert and Uetz, 2004). Many of the interacting proteins are membrane proteins involved in vesicular trafficking and it will be important to determine whether a common PX-domain-interacting motif can be identified in these proteins.

Clathrin adaptor proteins

The first indication that phosphoinositides and clathrin adaptors functionally interact came from the identification of the soluble InsP_6 'receptor' as the tetrameric clathrin adaptor protein AP-2 (Voglmaier et al., 1992). Tetrameric adaptors bind to membrane-adjacent, short conserved sequences (sorting signals) in the cytoplasmic regions of transmembrane proteins and tie them to the clathrin-dependent vesicular sorting machinery (Bonifacino and Traub, 2003; Owen et al., 2004). Several studies have indicated that membrane PtdIns(4,5)P_2 regulates recruitment of AP-2 and its interaction with the sorting motifs. The structural basis for this was finally revealed following the crystallization of the heterotetrameric trunk domain of AP-2 (Collins et al., 2002). AP-2, however, is not the only clathrin adaptor that binds to and is regulated by phosphoinositides. The N-terminal regions of the monomeric clathrin adaptors AP-180 (Mao et al., 2001) and its non-neuronal homologue, CALM (Ford et al., 2001) (hence, the name ANTH or CALM domains), have also been recognised as inositide-binding motifs. These motifs show a great degree of sequence similarity to the N-terminal sequence of Epsin1, a protein that contains additional binding regions that interact with ubiquitin, clathrin and the ear domain of AP-2 (Ford et al., 2002). ENTH domains (dubbed from epsin N-terminal homology) have since been recognised in several proteins that participate in clathrin-mediated endocytosis or vesicle budding (Legendre-Guillemin et al., 2004). Not all ENTH domains bind PtdIns(4,5)P_2: the ENTH domain of EpsinR binds PtdIns(4)P at the trans-Golgi network (Mills et al., 2003; Hirst et al., 2003), whereas that of yeast Ent3p has been reported to bind PtdIns(3,5)P_2 in multivesicular bodies (Friant et al., 2003).

A common feature of binding of inositides to ANTH/CALM domains, ENTH domains and AP-2 is that it occurs at a region formed by multiple helices (Fig. 3A,B). In the case of AP-2 and AP-180, there is no real binding pocket, and the inositol phosphate [in this case InsP_6, presumably mimicking PtdIns(4,5)P_2] interacts with a cluster of positive residues in the terminal α-helix as if being balanced on a fingertip (Collins et al., 2002). Mutagenesis studies indicate that PtdIns(4)P binds to a similar peripheral site in the AP-1 trunk (Heldwein et al., 2004), but it is not clear how stereospecific interaction with a monophosphorylated inositide could happen at this location. By contrast, in the ENTH domain, a groove is formed by the so-called helix 0 and the adjacent helices in which the lipid interacts with the basic residues. The N-terminal helix 0 is unstructured in the absence of the phosphoinositide (Ford et al., 2002). One possibility is that in the cases of AP-1, AP-2 and AP180, an interacting protein donates a complementary binding surface that mimics helix 0 of the CALM/ANTH domain.

There is another phosphoinositide-binding site in AP-2, which is formed by the β-strands within the μ2 domain (Fig. 3). Binding of lipid to this second site is claimed to induce a conformational change that enhances the affinity of the μ2 domain for the tyrosine-based sorting motif, YxxΦ (Collins et al., 2002). Interactions of the μ2 domain with tyrosine-based sorting signals and phosphoinositides greatly resemble the regulation of β-arrestins by phosphoinositides. Arrestins uncouple G-protein-coupled receptors (GPCRs) from heterotrimeric G-proteins following phosphorylation of the GPCR tails and link the receptors with the internalization machinery (Luttrell and Lefkowitz, 2002). Recently, arrestins have also been shown to act as scaffolds that link GPCRs to the activation of effectors such as Src (Luttrell, 2003).

Although arrestins have been regarded as specific regulators of GPCRs, they could equally be considered to be monomeric clathrin adaptors (Owen et al., 2004) because they can interact with clathrin (Krupnick et al., 1997) and AP-2 (Laporte et al., 2000; Kim and Benovic, 2002) in addition to the receptor tails. Mutagenesis studies have indicated that phosphoinositides regulate light-induced translocation of arrestin to the outer segment in the *Drosophila* eye (Lee et al., 2003). Similarly, β-arrestin-1 and β-arrestin-2 (but not mammalian visual arrestin) are also regulated by phosphoinositides (Gaidarov et al., 1999). The inositide-interaction regions in both map to a basic groove close to the hinge region of the molecule, facing the same direction as the postulated binding site for the phosphorylated receptor tail and are distinct from the clathrin- and AP-2-binding region (Fig. 3C). Interaction of the *Drosophila* visual arrestin with the NINAC myosin III protein depends on PtdIns(4,5)P_2 (Lee and Montell, 2004). By contrast, mutated β-arrestins that cannot bind phosphoinositides can still bind to receptors, clathrin and AP-2 in vitro, but fail to support endocytosis of GPCR and do not move to clathrin-coated pits (Gaidarov et al., 1999). Given the location of the inositide-binding site relative to the other binding regions of arrestins, it is not difficult to see how lipid binding could affect the interaction of arrestins with their other protein-binding partners in an in vivo situation.

Other phospholipid-binding proteins

There are several other examples of motifs that interact with membrane phosphoinositides or other acidic phospholipids, such as PS or PA. Many of these have not been characterized at the structural level. Some of the interactions are believed to involve stretches of clustered basic residues that do not necessarily form a specific lipid-binding pocket, although the distinction between these and the above-detailed interactions may just reflect the lack of structural information about the proteins. Examples are the MARCKS proteins (Wang et al.,

Journal of Cell Science

2001), the A-kinase-anchoring protein AKAP79 (Dell'Acqua et al., 1998) and profilin (Chaudhary et al., 1998). The structure of the Ca^{2+}-regulated phospholipid-binding proteins annexins (Rescher and Gerke, 2004) and their inositol-lipid-binding activity (Rescher et al., 2004) indicate that their interactions with inositides should resemble those involving the AP-2 trunk domain and the ENTH or ANTH domains in their helical regions. No structural information is currently available to substantiate these speculations. Nevertheless, it is very possible that more protein domains will be shown to be inositide-binding modules and it will be interesting to compare their structural features with the known inositide-binding motifs.

General conclusions

The major benefit of these structural comparisons is that, by recognizing the analogies among structures, one can define basic residues that are candidates for inositide-binding residues by performing sequence alignments with known structures. However, a question also inevitably arises: can all of this information reveal a general theme to how inositides regulate countless biological processes? Phosphoinositides undoubtedly contribute to the reversible binding of signaling proteins to the plasma membrane. However, in most cases, this probably complements protein-protein interactions with additional membrane proteins (Fig. 4). The main puzzle is how

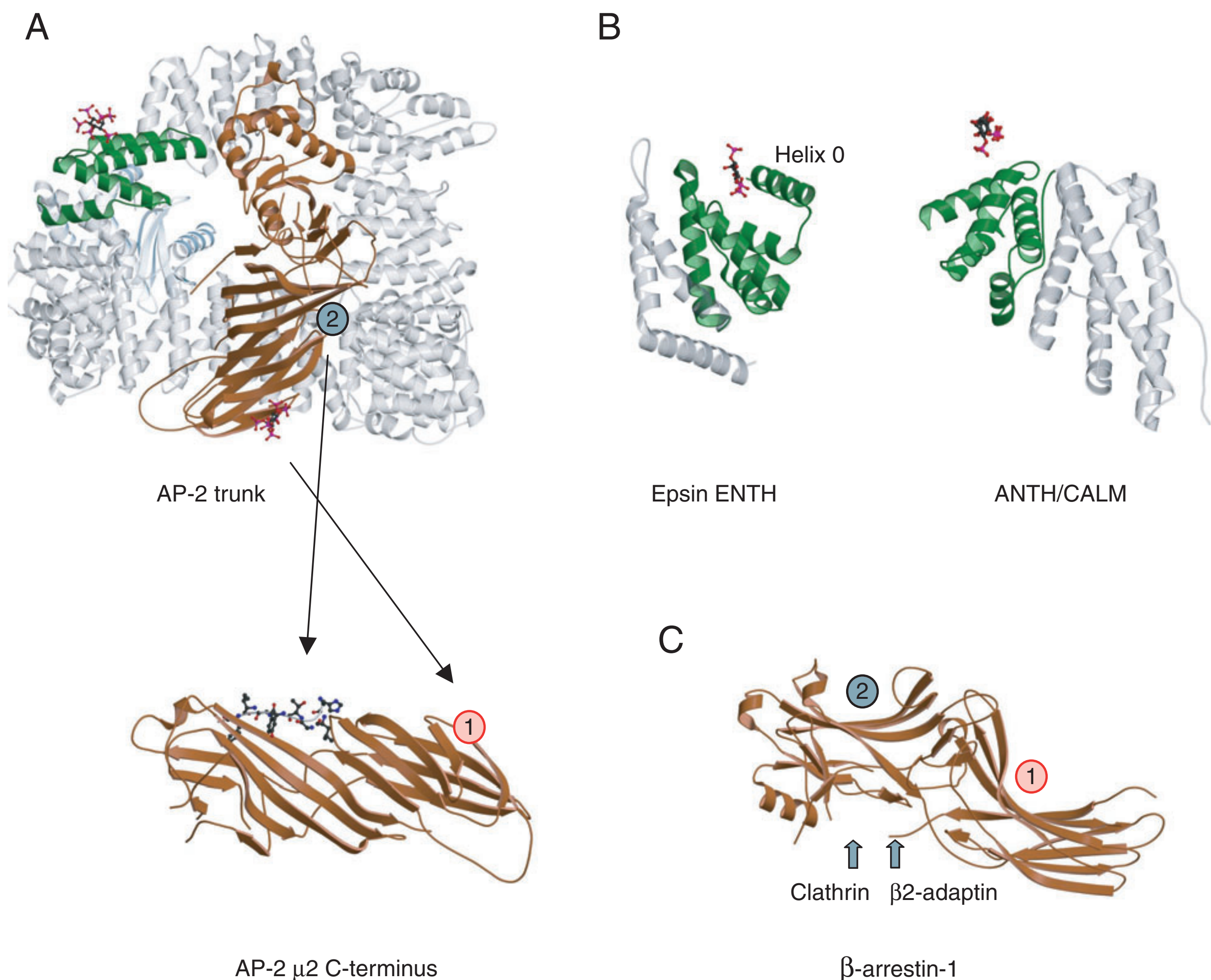

Fig. 3. Inositide and peptide recognition by adaptor proteins. (A) The trunk domain of the tetrameric clathrin adaptor AP-2 binds phosphoinositides in two locations. One of the binding sites is at a peripheral position in the N-terminus of the α2 subunit (green). The other site (red circle labeled 1) is within the μ2 subunit (brown). Binding of the lipid to this site is believed to be one of the factors inducing a conformational change that allows binding of the internalization motif YxxΦ to μ2 at a site that is otherwise blocked by interaction with the β2 subunit (gray circle labeled 2). (B) The binding of Ins(1,4,5)P_3 [mimicking PtdIns(4,5)P_2] to CALM occurs at a helical domain (green) in a similarly peripheral position to that seen in AP-2. By contrast, in the ENTH domain of epsin, an additional helix (helix 0), which is disordered in the structure without the inositide, contributes to the coordination of the lipid headgroup within the multi-helical structure that is otherwise very similar to CALM/ANTH domains. (C) The structure of β-arrestin-1 resembles that of the C-terminal multi-stranded domain of the AP-2 μ2 subunit. The electropositive groove that interacts with phosphorylated tails of G protein-coupled receptors (gray circle labeled 2) and the adjacent inositide-binding pocket (red circle labeled 1) are very reminiscent of the peptide- and lipid-binding sites of the AP-2 μ2 domain. Gray arrows indicate clathrin- and β2-adaptin-binding sites. The PDB accession numbers used are: 1GW5, AP-2 core (Collins et al., 2002); 1HES, μ2-C-terminal (Owen and Evans, 1998); 1H0A, Epsin-ENTH (Ford et al., 2002); 1HG2, CALM-ANTH (Ford et al., 2001).

Journal of Cell Science

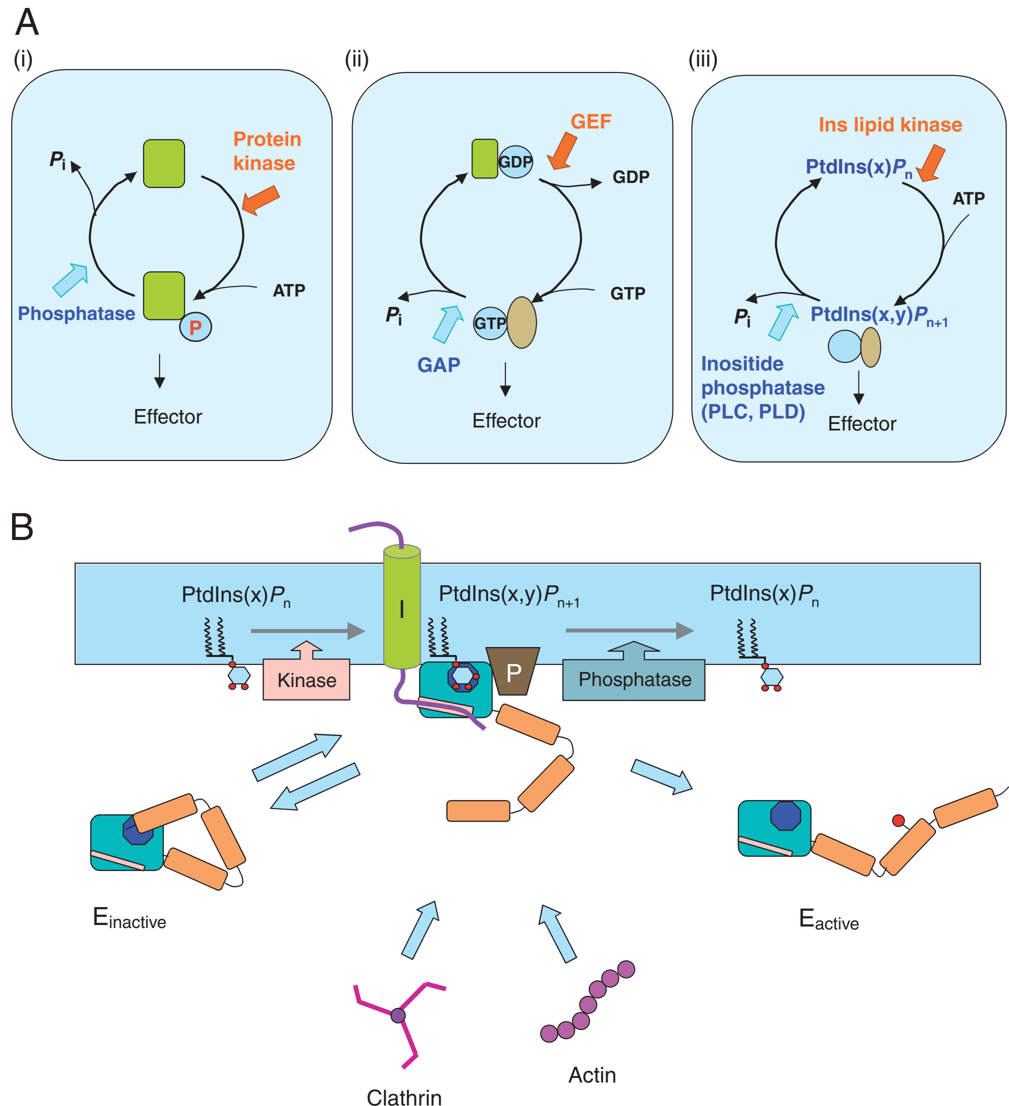

Fig. 4. General principles of regulation by phosphoinositides. (A) Phosphorylation and dephosphorylation cycles control signaling cascades either directly (i) by phosphorylation of proteins, or indirectly (ii) by altering the phosphorylation state of GDP/GTP bound to GTP-binding proteins. Phosphorylation of membrane inositides (iii) offers a unique regulatory feature in that the phosphate acceptor molecule is membrane bound and can be phosphorylated in multiple ways. The phosphorylated lipid species interacts with proteins that recognise the lipid. It thereby contributes to the recruitment of these proteins to the membrane and probably also evokes conformational changes that affect their functions. (B) Phosphoinositides can control several kinds of signaling processes. Many proteins that contain phosphoinositide-recognition domains assume an inactive conformation ($E_{inactive}$) in the cytosol in which the inositide-binding site is masked by intra-molecular or inter-molecular interactions. Production of the appropriate phosphoinositide (PtdIns(x,y)P_{n+1}) recruits the protein to the membrane, and specificity is imparted through interaction with integral (I) or peripheral (P) membrane proteins. The complex can remain active at the membrane and recruit additional proteins, such as actin or clathrin or, after modification, the protein can return to the cytosol in an activated (i.e. phosphorylated) form (E_{active}). The localization of both the lipid kinase and phosphatases that act on phosphoinositide adds to the precise control of individual effectors.

the same lipid is used by the many effectors to which it can bind. This question is even more tantalizing for protein motifs interacting with PI(4,5)P_2, the most abundant phosphoinositide in the plasma membrane.

An important clue might come from the old observation that the phosphomonoester groups of PtdIns(4,5)P_2 (i.e. those in positions 4 and 5) have a very high turnover rate (Hawkins et al., 1984) because of what had been conceived to be ‘futile’ cycles of phosphorylation and dephosphorylation. What this tells us is that PtdIns(4,5)P_2-binding proteins also have to continuously engage-disengage and, hence, could rapidly react to local changes in inositide levels. The existence of these on-off cycles is experimentally supported by FRAP studies showing that PH domains, even when membrane-bound, rapidly cycle between the cytosol and the membrane phosphoinositides (van Der Wal et al., 2001).

The key to specificity might therefore be the localized production and elimination of the inositide in close molecular proximity to the effector molecules. The numerous inositide kinases and phosphatases, and their recruitment together with the effectors are means by which signaling specificity can be greatly increased, and by which the same lipid species could regulate multiple effectors simultaneously and, to some extent, independently. Therefore, specificity of the inositide signal may come from the lipid species per se – as indeed is the case with many effectors – but also, perhaps equally important, from the inositide kinases and phosphatases present in a signaling complex. The multiplicity of inositide phosphatases (Majerus et al., 1999) and the fact that impairments in their function are very often associated with human disease – much more so than those of the kinases – tell us that the phosphatases are just as important as the kinases.

Experimental findings also support the idea of production or elimination of inositide lipids in close molecular proximity to the effectors: inositide kinases and also phosphatases associate with protein domains that could bind phosphoinositides. The

FERM domain of talin has been found to associate and regulate PIP kinase type 1γ (Di Paolo et al., 2002) and the PH domain of the Bruton's tyrosine kinase is able to associate with and regulate the mouse PIP kinase type-Iβ (Saito et al., 2003). The inositide 5-phosphatase synaptojanin-2 (Nemoto and De Camilli, 1999) and the tumor suppressor inositide phosphatase PTEN (Leslie et al., 2001) associate with PDZ domains – with functional consequences. All PLC enzymes contain PH domains and also act upon and hydrolyze inositol lipids. In essence, this may mean that distinct functional pools of inositides are assigned to effectors, and it is easy to see why protein-protein interactions of the inositide-binding module are an important factor. The inositide-binding PH domain and the peptide-binding PTB domain may represent two extremes; most of these domains may fall in between, showing binding of various strength to both lipids and proteins (Yu et al., 2004).

These ideas are offered with the hope that they will provoke further thoughts and discussion about the way phosphoinositides control almost every signaling process associated with cellular membranes. This regulatory system, however, reaches even beyond membranes, because increasing evidence supports the idea that highly phosphorylated inositol phosphates are important for regulating proteins in the cytosol or in the nucleus (Odom et al., 2000), and the nuclear phosphoinositide system is now also firmly established (Irvine, 2003). Such work is now complemented by new reports showing PH domains (Lu et al., 2004; Ray and Strott, 1981) or inositide-recognizing PHD domains (Gozani et al., 2003) in proteins known to be transcriptional regulators. Recent observations have also revealed that certain orphan receptors could bind phosphoinositides (Krylova et al., 2005). The increasing number of inositide-binding protein motifs and their conservation at the structural and functional level together with their diversity are vivid reminders that Nature has found something fundamentally useful in these molecules that appeared in eukaryotes even before tyrosine phosphorylation evolved.

The author would like to thank Kevin J. Catt (NICHD, NIH, Bethesda, MD) and James H. Hurley (NIDDK, NIH, Bethesda) for reading the manuscript and their valuable comments, and Frederick Dyda (NIDDK, NIH, Bethesda, MD) for his help with generating the figures in Molscript

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