

PHOSPHOINOSITIDE LIPIDS AS SIGNALING MOLECULES: Common Themes for Signal Transduction, Cytoskeletal Regulation, and Membrane Trafficking

T. F. J. Martin

Department of Biochemistry, University of Wisconsin, 420 Henry Mall, Madison,
Wisconsin 53706; e-mail: tfmartin@facstaff.wisc.edu

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ABSTRACT

Signaling roles for phosphoinositides that involve their regulated hydrolysis to generate second messengers have been well characterized. Recent work has revealed additional signaling roles for phosphoinositides that do not involve their hydrolysis. PtdIns 3-P, PtdIns 3,4,5-P₃, and PtdIns 4,5-P₂ function as site-specific signals on membranes that recruit and/or activate proteins for the assembly of spatially localized functional complexes. A large number of phosphoinositide-binding proteins have been identified as the potential effectors for phosphoinositide signals. Common themes of localized signal generation and the spatially localized recruitment of effector proteins appear to underlie mechanisms employed in signal transduction, cytoskeletal, and membrane trafficking events.

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CRITERIA FOR PHOSPHOINOSITIDE SIGNALING

A signaling role for inositol phospholipids was established in the 1980s when it became clear that phospholipase C-mediated hydrolysis of PtdIns 4,5-P₂ generates the intracellular signals Ins 1,4,5-P₃ and diacylglycerol for regulating Ca²⁺ mobilization and protein phosphorylation mechanisms, respectively (Berridge & Irvine 1984). More recently the inositol phospholipids have been found to have signaling roles that do not require their hydrolysis. In this role, phosphoinositides serve as site-specific signals on membranes that recruit and regulate protein complexes at the interface with the cytosol. Phosphoinositide signals are used in this way for signal transduction, cytoskeletal assembly, and membrane budding and fusion processes that are spatially restricted to specific membrane domains.

The concept of phosphoinositides as spatially localized membrane signals is relatively recent, and there remain many gaps in our understanding of the detailed mechanisms involved. There are considerable obstacles to establishing the full outline of membrane-based signaling processes. Many of the phospholipids are in low abundance in cells but at high local concentrations in membrane domains that are not readily detected by conventional biochemical approaches. Establishing the identity of the effector proteins regulated by phosphoinositide binding is also challenging because of the difficulty of extending *in vitro* findings into the intracellular environment. As was true for cytosol-based signaling pathways, it is useful to describe criteria for establishing a signaling role for phosphoinositides in a cellular process.

Firstly, it is essential to establish the nature of the lipid involved in the cellular process (e.g. PtdIns 3,4,5-P₃, PtdIns 4,5-P₂, or others). In some cases

it may be possible to detect changes in the levels of the phosphoinositide in response to cellular activation. In other cases, it may be necessary to use techniques for detecting localized phosphoinositides in membrane domains (e.g. immunocytochemistry with phosphoinositide antibodies; Voorhout et al 1992, Tran et al 1993). For some cellular processes, phosphoinositides may be essential constitutively produced cofactors rather than regulated signals whose levels change. The introduction of phosphoinositide phosphatases (Zhang et al 1998) and phospholipases (Rhee & Bae 1997) of defined specificity or of phosphoinositide-specific antibodies (Fukami et al 1988) and phosphoinositide-binding peptides (Hartwig et al 1995) into cells should inhibit cellular responses mediated by phosphoinositide signals and help to establish the identity of the lipid involved. Cell-permeant lipid kinase inhibitors with better specificities than those currently available will simplify this identification.

Secondly, overexpressing wild-type or constitutively active phosphoinositide kinases of defined substrate specificity (Shibasaki et al 1997) or sometimes even introducing the phosphoinositide itself (Franke et al 1997) should mimic the effects of cellular activation or enhance a phosphoinositide-dependent process. With the increasingly complete characterization of lipid kinases, the identification of their upstream activators, and the elucidation of the targeting mechanisms responsible for their membrane localization, a fuller repertoire of methods to selectively increase phosphoinositide levels will become available.

Lastly, it is important to identify the effector apparatus that is regulated by the phosphoinositide signal and is responsible for mediating changes in cellular events. Numerous phosphoinositide-binding proteins have been identified as candidate effector proteins for phosphoinositide signals (see below). An improved understanding of the molecular basis for phosphoinositide-protein interactions will facilitate the generation of mutations that abrogate effector-phosphoinositide binding to critically assess the role of such interactions in cellular events (Salim et al 1996). The possibility that localized phosphoinositide synthesis directly imparts new properties to the membrane leaflet (such as curvature for budding) will require testing by reconstituting cellular processes in artificial membranes.

In only a limited number of cases has the nature of the lipid, the role of its regulated synthesis by a defined lipid kinase, and the identity of a physiological downstream effector for the lipid been characterized for a cellular process. This article reviews the diversity of known phosphoinositide signals, the lipid kinases responsible for their synthesis, and the potential effectors of the phosphoinositide signals involved in signal transduction, cytoskeletal and membrane trafficking events. For related reviews of these topics see DeCamilli et al 1996 and Martin 1997.

A DIVERSITY OF SIGNALING MOLECULES IS GENERATED BY LIPID PHOSPHORYLATION

The dynamic phosphorylation at the D3, D4, and D5 hydroxyls of the inositol headgroup generates a diverse array of phosphoinositides that are well suited for signaling roles. Classical work (Grado & Ballou 1960) that characterized PtdIns 4-P and PtdIns 4,5-P₂ (referred to here as D4 phosphoinositides) provided the first view of the interconvertible diversity of these phospholipids. The discovery of phosphorylation at the D3 hydroxyl of the inositol headgroup (Whitman et al 1988) leading to the generation of PtdIns 3-P, PtdIns 3,4-P₂ and PtdIns 3,4,5-P₃ (referred to here as D3 phosphoinositides) increased this diversity. Recent findings have uncovered additional phosphoinositides generated by novel kinase reactions, and it is likely that the complete cellular complement remains to be characterized.

A summary of the synthetic pathways for the seven polyphosphoinositides identified as of 1998 derived by the phosphorylation of PtdIns is shown in Figure 1. The classical D4 phosphoinositides are primarily synthesized by the sequential phosphorylation of PtdIns by PtdIns 4-kinase and PtdIns 4-P 5-kinase

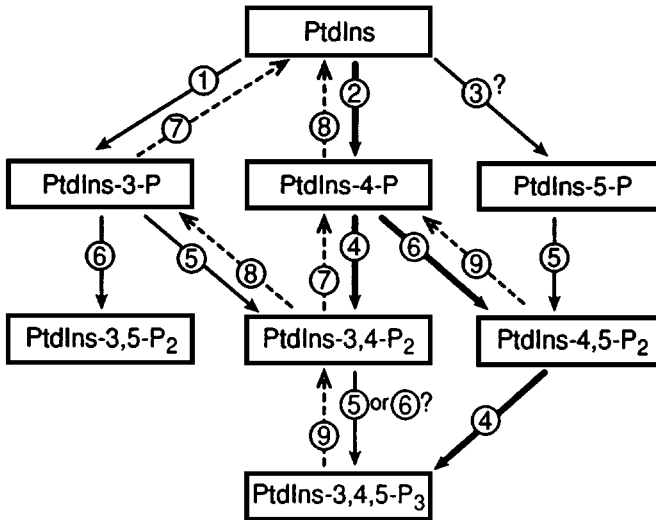


Figure 1 Synthetic pathways for phosphoinositides. *Solid arrows* represent kinase reactions with major pathways indicated by *bold arrows*. *Dotted arrows* represent phosphatase reactions. Numbers correspond to individual enzyme-catalyzed reactions: 1, PtdIns 3-kinase (type III); 2, PtdIns 4-kinase; 3, PtdIns 5-kinase (conjectured); 4, PtdIns 3-kinase (type I or II); 5, PtdIns 5-P 4-kinase; 6, PtdIns 4-P 5-kinase; 7, 3-phosphatase; 8, 4-phosphatase; 9, 5-phosphatase.

(2 followed by 4 in Figure 1). An alternative pathway for the synthesis of PtdIns 4,5-P₂ (3 followed by 5 in Figure 1) is suggested by the finding that the type II isoform of PtdIns 4-P 5-kinase may be a PtdIns 5-P 4-kinase (Rameh et al 1997b). PtdIns 5-P, which had not been previously identified, was found to be a minor lipid present in mammalian cells (Rameh et al 1997b), which suggests the existence of a yet-uncharacterized PtdIns 5-kinase (3 in Figure 1). The recent discovery of PtdIns 3,5-P₂ in yeast and mammalian cells (Whiteford et al 1997, Dove et al 1997) and the finding that the type I PtdIns 4-P 5-kinase also utilizes PtdIns 3-P as a substrate (Rameh et al 1997b) indicate an additional novel phosphoinositide synthesis pathway (1 followed by 6 in Figure 1). This work will undoubtedly be followed by efforts to elucidate the cellular role of PtdIns 3,5-P₂, whose levels change in response to osmotic stress (Dove et al 1997).

Synthesis of D3 phosphoinositides is catalyzed by several classes of PtdIns 3-kinases (see below) that phosphorylate PtdIns to form PtdIns 3-P (1 in Figure 1) or PtdIns 4-P and PtdIns 4,5-P₂ to form PtdIns 3,4-P₂ and PtdIns 3,4,5-P₃, respectively (4 in Figure 1). Recent work (Rameh et al 1997b, Zhang et al 1997) reveals that the type II PtdInsP kinase, now characterized as a PtdIns 5-P 4-kinase, also employs PtdIns 3-P as a substrate providing an alternative pathway for PtdIns 3,4-P₂ synthesis from a PtdIns 3-P precursor (Rameh et al 1997b, Zhang et al 1997) (1 followed by 5 in Figure 1). Novel dual substrate specificities for the PtdInsP kinases were recently reported (Zhang et al 1997), which would allow the sequential phosphorylation of PtdIns 3-P at the D4 and D5 hydroxyls by PtdInsP kinases to form PtdIns 3,4,5-P₃ (from 1 to 5 to 5/6 in Figure 1).

LIPID KINASES AND THEIR REGULATION

Because the major pathways for D3 and D4 phosphoinositide synthesis proceed through 4-phosphorylation of PtdIns (Figure 1), the distribution of PtdIns 4-kinase in membranes will largely dictate where localized D3 and D4 phosphoinositide synthesis can occur. PtdIns 4-kinase activity is principally membrane associated and localized to plasma membrane, nuclear membrane, lysosomes, Golgi, endoplasmic reticulum and constitutive secretory vesicles (Carpenter & Cantley 1990, Pike 1992, Olsson et al 1995) depending upon the tissue. In secretory tissues, PtdIns 4-kinase activity is present on glucose transporter vesicles, chromaffin granules, mast cell granules, coated vesicles, and in neural synaptosomes (Pike 1992). Biochemical studies characterized type II and type III forms of the enzyme that differed in sensitivity to adenosine and detergent activation (Carpenter & Cantley 1990, Pike 1992), but this classification is somewhat blurred by recent molecular cloning. cDNAs corresponding to two isoforms of PtdIns 4-kinase, an α isoform (Wong & Cantley 1994) and a β

isoform (Meyers & Cantley 1997), have been characterized. The α isoform, homologous to a yeast STT4 enzyme, exists as long (≈ 200 kDa) and short (≈ 100 kDa) forms derived from alternative splicing (Wong & Cantley 1994, Nakagawa et al 1996a, Gehrmann et al 1996, Balla et al 1997). The β isoform (≈ 100 kDa), homologous to the yeast PIK1 enzyme (Nakagawa et al 1996b, Meyers & Cantley 1997, Balla et al 1997), is inhibited by both wortmannin and LY294002 (Downing et al 1996), which were previously thought to be diagnostic for certain PtdIns 3-kinase enzymes (Carpenter & Cantley 1996).

Wortmannin inhibits the synthesis of PtdIns 4-P that is required for hormone receptor-mediated phosphoinositide turnover, which initially suggested that the wortmannin-sensitive β isoform of PtdIns 4-kinase had a dedicated role in phosphoinositide synthesis for signal transduction (Downing et al 1996). Recent studies, however, found that splicing isoforms of the type α PtdIns 4-kinase are also inhibited by wortmannin (Nakagawa et al 1996a, Balla et al 1997). Nonetheless, it is likely that the α and β isoforms play distinct cellular roles because they exhibit distinct intracellular distributions (Wong et al 1997). Additional isoforms of PtdIns 4-kinase, possibly with dedicated membrane trafficking roles, will likely be characterized. Neither the α nor β cDNAs encode the enzyme classified as the type II enzyme, a 55 kDa protein with a kinase activity inhibited by the 4C5G antibody (Carpenter & Cantley 1990). A 4C5G-sensitive PtdIns 4-kinase, a resident of glucose transporter vesicles (Del Vecchio & Pilch 1991), is involved in the phosphoinositide synthesis essential for ARF activation of phospholipase D (Liscovitch et al 1994) and differs in membrane localization and wortmannin-insensitivity from α and β isoforms (Wong et al 1997).

The characterized cDNAs for PtdIns 4-kinases encode hydrophilic proteins that lack transmembrane domains. The basis for the membrane association and targeting of PtdIns 4-kinase isoforms to specific membranes is poorly understood although several potential mechanisms for localization have emerged. The α isoform contains a PH domain that could mediate its membrane association (Wong & Cantley 1994, Nakagawa et al 1996a). β isoforms associate with the cytoplasmic domains of several transmembrane and receptor proteins (Kauffmann-Zeh et al 1994, Pertile & Cantley 1995, Berditchevski et al 1997). Additional studies are needed to determine the basis for the membrane recruitment of the PtdIns 4-kinases in order to understand how localized membrane domains of D3 and D4 phosphoinositides are formed.

PtdIns 4-P 5-kinase activity is principally cytosolic and is enriched in neural synaptosomes (Stubbs et al 1988). Two isoforms of PtdIns 4-P 5-kinase that are related in sequence and differ from members of the phosphoinositide 3- and 4-kinase families have been characterized (Boronenkov & Anderson 1995, Loijens & Anderson 1996, Ishihara et al 1996). The type I isoform (Loijens

& Anderson 1996, Ishihara et al 1996) is a true PtdIns 4-P 5-kinase and also phosphorylates the D5 hydroxyl of PtdIns 3-P (Rameh et al 1997b). The type II isoform, a PtdInsP 4-kinase, phosphorylates the D4 hydroxyl of PtdIns 5-P and PtdIns 3-P (Zhang et al 1997, Rameh et al 1997b). Although the type I isoform is likely to be a bona fide constituent in the classical biosynthetic pathway for formation of PtdIns 4,5-P₂ from PtdIns 4-P, the type II isoform may catalyze an alternative route of PtdIns 4,5-P₂ synthesis from PtdIns 5-P or catalyze PtdIns 3,4-P₂ synthesis from PtdIns 3-P (see Figure 1).

The central importance of PtdIns 4-P 5-kinase for the synthesis of PtdIns 4,5-P₂ and PtdIns 3,4,5-P₃ in specific membrane compartments implies that regulatory and targeting mechanisms can operate on this enzyme, but only preliminary accounts of this have been reported. The type II isoform contains a proline-rich domain that may be an SH3-binding site and thus able to mediate the coupling of type I PtdIns 3-kinases for the channeled synthesis of PtdIns 3,4,5-P₃ (Boronenkov & Anderson 1995, Zhang et al 1997). A splicing isoform of the type II enzyme associates with a TNF receptor (Castellino et al 1997). The bona fide PtdIns 4-P 5-kinase (type I) is uniquely stimulated by phosphatidic acid (Jenkins et al 1994), which is speculated to be an important element of a positive feedback circuit with PLD (phospholipase D) that generates increased PtdIns 4,5-P₂ and may operate in membrane budding or fusion events (Liscovitch & Cantley 1995) (see below). Nonhydrolyzable guanine nucleotides stimulate PtdIns 4-P phosphorylation, implying a G protein regulation of 5-kinase activity (Smith & Chang 1989). Rho stimulates PtdIns 4-P 5-kinase activity in cell lysates (Chong et al 1994) and associates with a type I enzyme, although this association may be indirect (Ren et al 1996). Rac1 also associates directly or indirectly with the type I PtdIns 4-P 5-kinase (Tolias et al 1995). It will be important to further characterize Rac/Rho protein regulation of the type I PtdIns 4-P 5-kinase because of the role of phosphoinositides and Rho family members in the regulation of the actin cytoskeleton (see below).

The characteristics of PtdIns 3-kinases have been discussed in several recent reviews (Carpenter & Cantley 1996, Vanhaesebroeck et al 1997, Domin & Waterfield 1997). These enzymes are classified into three groups according to structure and activity. Class I enzymes are heterodimers of catalytic and adaptor subunits that utilize PtdIns, PtdIns 4-P, and PtdIns 4,5-P₂ as substrates. The adaptor subunits of these enzymes contain SH2 domains that mediate recruitment to phosphotyrosine residues on the cytoplasmic domains of receptors, which results in activation of the 3-kinase. Class II enzymes preferentially phosphorylate PtdIns and PtdIns 4-P but not PtdIns 4,5-P₂ *in vitro* and contain C2 domains that could mediate membrane interactions. Class III enzymes, which phosphorylate only PtdIns *in vitro*, consist of heterodimers of a catalytic subunit (such as yeast Vps34p) associated with a serine/threonine protein

kinase adaptor subunit (such as yeast Vps15p) that is required for membrane recruitment (Stack et al 1993).

PHOSPHOINOSITIDE-BINDING PROTEINS: EFFECTORS FOR PHOSPHOINOSITIDE SIGNALS

The identification of protein constituents that exhibit stereoselective interactions with phosphoinositides is an important step toward defining proteins with effector roles in phosphoinositide-dependent signaling pathways. Proteins with characterized phosphoinositide-binding activities are summarized in Table 1.

Table 1 Phosphoinositide-binding proteins

Category	Example	Binding specificity ^a	Binding site ^b	Reference ^c
Protein kinases and phospholipases	PKB/Akt	D3	PH	Franke et al 1997
	PDK1	D3	PH	Stokoe et al 1997
	β ARK	D3	PH	Rameh et al 1997a
	BTK	D3	PH	Rameh et al 1997a
	PLC γ ₁	D4	PH	Garcia et al 1995
Cytoskeletal proteins	Gelsolin	D3	KR	Hartwig et al 1996
	Profilin	D3	KR	Lu et al 1996
	Cofilin	nd	KR	Yonezawa et al 1991
	α -actinin	nd	KR/PH	Fukami et al 1996
	Vinculin	nd	KR	Gilmore & Burridge 1996
	Spectrin	nd	PH	Hyvonen et al 1995
GTPases and accessory proteins	CapZ	D4	nd	Schafer et al 1996
	Dynamin	D4	PH	Salim et al 1996
	ARF	nd	nd	Randazzo 1997
	ARF GEFs	D3	PH	Klarlund et al 1998
	ARF GAPs	D3	PH	Tanaka et al 1997
	PLD	D3 = D4	nd	Hammond et al 1997
	Rac/Rho GEFs	D3	PH	Rameh et al 1997a
Membrane trafficking proteins	Clathrin adaptors	D3	KR	Hao et al 1997
	EEA1	PI3P	nd	Patki et al 1997
	CAPS	D4	PH	Loyet et al 1998
	Synaptotagmin	D3	KR	Schiavo et al 1996
	Mint	nd	PTB	Okamoto & Sudhof 1997

^aBinding preference for either D3 or D4 phosphoinositides if tested; not determined (nd) indicates PtdIns 4,5-P₂ binding without testing D3 phosphoinositide binding.

^bCharacterized binding sites consisting of PH (or PTB) domain or (KR) lysine/arginine-rich region (see text) or not determined (nd).

^cRepresentative reference for each protein; consult text for additional references.

Until recently, only D4 phosphoinositides were employed in protein binding studies (Janmey 1994) but the availability of D3 phosphoinositides has led to the characterization of phosphoinositide-binding specificity for a number of proteins. Many proteins exhibit a binding specificity that favors D3 over D4 phosphoinositides (Table 1). Proteins in this category are potential effectors that function downstream of PtdIns 3-kinases and may mediate the effects of PtdIns 3,4- P_2 and PtdIns 3,4,5- P_3 on protein phosphorylation, cytoskeletal regulation, GTP exchange reactions, and membrane coat recruitment. Proteins that bind PtdIns 3-P that may act downstream of type III PtdIns 3-kinase involved in membrane trafficking have recently been identified, such as AP-2 and EEA1 (Rapoport et al 1997, Patki et al 1997). A small group of proteins exhibit a specificity for binding D4 phosphoinositides in preference to D3 phosphoinositides (Table 1). This group includes proteins that function in cytoskeletal regulation, exocytosis, and endocytosis and are the potential effectors for the cellular roles of D4 phosphoinositides.

MOLECULAR BASIS FOR PROTEIN-PHOSPHOINOSITIDE INTERACTIONS

Basic and Hydrophobic Sequences

Two general classes of binding sites for the stereoselective interaction of phosphoinositides with proteins have been identified: The first consists of short (≈ 10 – 20 residues) colinear sequences that are rich in basic and hydrophobic residues, and the second consists of longer sequences (≈ 120 residues) that share a well-defined tertiary structure of the PH domain. Studies on gelsolin (Yu et al 1992) resulted in the assignment of phosphoinositide binding to two separate regions near the N terminus (CKSGLKYKKGGVASGF and KHVVP-NEVVVQRLFQVKGR). Peptides corresponding to these sequences exhibit PtdIns 4,5- P_2 -binding similar to that of gelsolin (Janmey et al 1992). The first of these lysine/arginine-rich sequences was suggested to constitute a motif (K/RXXXXXKK/RK/R) that was present in other phosphoinositide-binding cytoskeletal proteins such as gCap39, villin, cofilin, and profilin (Yu et al 1992). A simple electrostatic interaction between lysine/arginine and phosphoinositol phosphates cannot fully account for the phosphoinositide-binding properties of gelsolin, which does not bind deacylated or deacylated/deglycerinated phosphoinositides (Janmey 1994). However, these short sequences contain a high percentage of hydrophobic amino acids that may form the part of the binding site that interacts with the diacylglycerol moiety.

The phosphoinositide-binding sites for several other actin-associated proteins are not homologous to those of gelsolin. It was suggested that a short peptide sequence (FSMDLRTKST) in profilin was responsible for phosphoinositide

binding (Sohn et al 1995). A 12 residue sequence (WAPECAPLKSKM) of cofilin was reported to bind PtdIns 4,5-P₂ (Yonezawa et al 1991). A linear sequence in α -actinin (TAPYRNVNIQNFHLSWK) accounted for PtdIns 4,5-P₂ binding, which was eliminated by mutagenesis of the two arginine and lysine residues (Fukami et al 1996). Although a diverse array of nonhomologous sequences constitute phosphoinositide-binding sites in cytoskeletal proteins, each contains at least two basic residues in the context of a high percentage ($\approx 50\%$) of hydrophobic residues. These short sequences may provide hydrophobic contacts with phospholipid acyl chains as well as charge interactions between the basic residues and the phosphate groups on the inositol headgroup. A hexadecapeptide from neurogranin exhibiting these hallmark features (WAAK-IQASFRGHMARKK) interacts with high affinity and specificity with phosphoinositides preferring PtdIns 3,4,5-P₃ over other phospholipids, and acquires a structure upon binding to phosphoinositides (Lu & Chen 1997).

Several coat proteins have been isolated in an effort to identify inositol polyphosphate-binding proteins. Clathrin adaptor proteins AP-2 and AP-3, as well as coatamer protein, interact with inositol phosphates and with phosphoinositides (Beck & Keen 1991, Fleischer et al 1994, Ye et al 1995, Norris et al 1995). Phosphoinositide interactions with AP-2 are mediated by N-terminal sequences of the α subunit (Beck & Keen 1991, Gaidarov et al 1996) and the phosphoinositide-binding site of a homologous subunit of AP-3 was suggested to consist of PKKKHLDYLIQATNE (Ye et al 1995, Hao et al 1997), a sequence also rich in basic and hydrophobic residues.

Several inositol phosphate-binding proteins such as centaurin- α and synaptotagmin II were purified based on inositol polyphosphate-binding (Fukuda et al 1994, Cullen et al 1995, Theibert et al 1997). However, phosphoinositides are probably the natural ligands for these proteins (Hammonds-Odie et al 1996, Schiavo et al 1996, Mehrotra et al 1997). The inositol phosphate-binding domain on synaptotagmin consists of the highly basic sequence GKRLKKKK-TTVKKK in the C2B domain (Fukuda et al 1994, 1995a). This portion of the C2 domain is disordered (Sutton et al 1995) but adopts an ordered structure upon binding inositol phosphates (Mehrotra et al 1997). Phosphoinositides bind to the same or an overlapping site in the C2B domain (Schiavo et al 1996). These lysine-rich sequences probably represent the inositol phosphate-binding portions of larger phosphoinositide-binding domains. In synaptotagmin, this basic sequence is flanked by regions rich in hydrophobic residues that could mediate acyl chain interactions.

PH Domains

PH (and related PTB) domains are the best-characterized and possibly most widespread of phosphoinositide-binding motifs. PH domains, termed pleckstrin

homology from the sequences initially identified in the platelet protein kinase C substrate pleckstrin, comprise ≈ 120 amino acid colinear regions identified by sequence comparison in nearly 100 proteins (Musacchio et al 1993, Gibson et al 1994). PH domains are present in cytoskeletal components (spectrin, α -actinin), guanine nucleotide exchange proteins or GTPase-regulating proteins (Ras-GRF, Dbl, VAV, cdc24, SOS, Ras-GAP, Tiam-1, ARNO, GRP1/cytohesin-1) and GTPases (dynammin), phosphoinositide-regulated protein kinases (Akt/PKB, PDK1), other protein kinases (BTK, β ARK), and phospholipases (PLC). Although not well conserved at the primary sequence level, NMR and X-ray diffraction studies reveal a remarkable similarity between the structures for PH domains from pleckstrin, spectrin, PLC δ_1 , and dynammin (Yoon et al 1994, Macias et al 1994, Hyvonen et al 1995, Ferguson et al 1995, Zheng et al 1996a, Salim et al 1996). The PH domain consists of two nearly orthogonal β sheets of three or four strands that form a β sandwich closed off near its C terminus by an amphipathic α helix.

Harlan et al (1994) originally reported that the PH domains of pleckstrin, ras-GAP, β ARK, and T cell kinase interacted with PtdIns-4,5- P_2 . Subsequent studies confirmed that PH domains from a large number of proteins (PLC δ_1 , spectrin, dynammin, BTK, SOS, Tiam-1, OSBP, Akt/PKB, SOS, GRP1/cytohesin-1, ARNO) bind phosphoinositides but with different affinities and specificities (Zheng et al 1996a, Salim et al 1996, Rameh et al 1997b, Frech et al 1997, Franke et al 1997, Kubiseski et al 1997, Klarlund et al 1997, Paris et al 1997). A study of six PH domains (Rameh et al 1997a) found that four exhibit a selectivity for PtdIns 3,4,5- P_3 over other phosphoinositides, whereas two others bound PtdIns 3,4,5- P_3 and PtdIns-4,5- P_2 with similar affinities. The PH domain of Akt/PKB has a high affinity and specificity for PtdIns 3,4- P_2 over PtdIns 4,5- P_2 and PtdIns 3,4,5- P_3 (Franke et al 1997, Klippel et al 1997, Frech et al 1997), whereas the PH domain of a kinase that phosphorylates PKB/Akt, PDK1, prefers PtdIns 3,4,5- P_3 over PtdIns 3,4- P_2 (Stokoe et al 1997). A similar preferential selectivity for binding PtdIns 3,4,5- P_3 was characterized for a number of PH domains including ARF nucleotide exchange factors and GTPase-activating proteins (Tanaka et al 1997, Klarlund et al 1998). The PH domains of PLC δ , CAPS, and dynammin are distinct in preferentially binding PtdIns 4,5- P_2 rather than D3 phosphoinositides (Salim et al 1996, Loyet et al 1998). The majority of PH domains where binding specificity has been determined have characteristics expected for effector proteins for PtdIns 3-kinase signaling.

Phosphoinositide binding to PH domains occurs at an electrostatically polarized (basic) face of the domain with critical residues residing on the loops between the β sheets (Lemmon et al 1996, 1997). This is a highly variable region between individual PH domains, which accounts for the diversity of phosphoinositide-binding specificity and affinity. For the PH domain of PLC δ_1 ,

which exhibits the highest affinity for phosphoinositides among the PH domains, binding is mediated by a network of interactions between the 4 and 5 position phosphates of the inositol headgroup and amino acid side chains, mainly lysine and arginine, of the variable loops of the PH domain (Ferguson et al 1995). Weaker binding of phosphoinositides to other PH domains corresponds to a more superficial interaction of the inositol headgroup with PH domain loops establishing many fewer interactions (Lemmon et al 1997).

There is evidence that phosphoinositides are physiological ligands for the PH domains of some of the above proteins. Mutation of critical loop residues required for phosphoinositide binding by the BTK PH domain results in a loss-of-function agammaglobinaemia phenotype (Salim et al 1996). Increasing evidence implicates D3 phosphoinositides as physiological regulators of Akt/PKB activity (see below). Mutagenesis studies on many of the other phosphoinositide-binding proteins will be required to convincingly establish the role of such interactions in cell function. Whether phosphoinositides constitute the sole ligands for all PH domains is an unresolved issue. The fact that the structurally homologous PTB domain (Lemmon et al 1996) interacts with phosphotyrosyl peptides via a binding site that overlaps with that for phosphoinositides (Rameh et al 1997a) suggests that phosphoamino acid peptides could be the ligands, or co-ligands, for some PH domains. For certain PH domains, cooperative interactions with multiple ligands may occur such as for β ARK, where phosphoinositides and $\beta\gamma$ subunits exhibit a synergism in binding (Pitcher et al 1995). The ability of the dynamin-1 PH domain, but not other PH domains including that of PLC δ_1 , to interfere with rapid endocytosis in chromaffin cells (Artalejo et al 1997) suggests that phosphoinositides are not the endogenous ligand for this PH domain despite the fact that direct phosphoinositide binding (Salim et al 1996) as well as phosphoinositide activation of the dynamin GTPase (Lin & Gilman 1996) have been demonstrated.

PH domains are essential for the membrane recruitment of several proteins that contain them, such as PKB/Akt (Andjelkovic et al 1997), GAP1^{IP4BP} (Lockyer et al 1997), Dbl (Zheng et al 1996b), and β ARK (Pitcher et al 1995). It is possible that other phospholipids, including minor novel phosphoinositides such as PtdIns 3,5-P₂, are the ligands that mediate membrane recruitment of PH domain-containing proteins found to bind D3 or D4 phosphoinositides only weakly. The active pace of research on the PH domain will soon clarify the general role of this motif and lead to the identification of additional physiological ligands that mediate membrane recruitment. At present, the concept of the PH domain as a signal-dependent membrane adapter that functions in the focal assembly of protein complexes at the membrane interface is very useful (Hemmings 1997). The fact that PH domains are frequently accompanied by other motifs (SH2, SH3, proline-rich, GTP exchange) in proteins is consistent

with the notion that the recruitment of the PH domain protein would nucleate sites on the membrane for protein complex assembly.

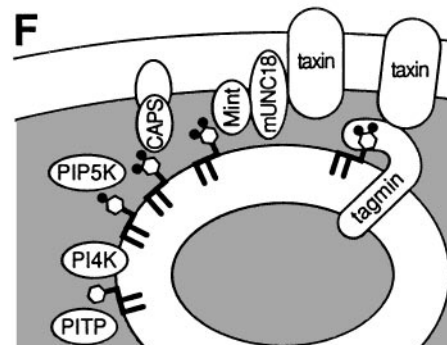
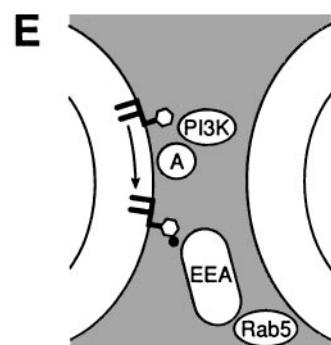
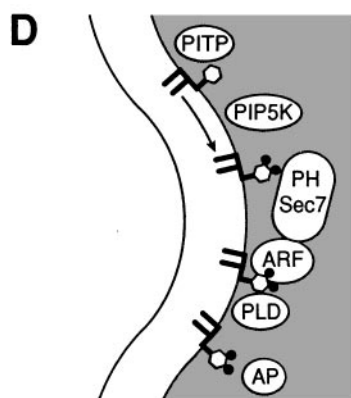
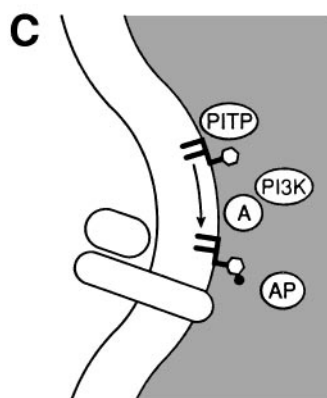
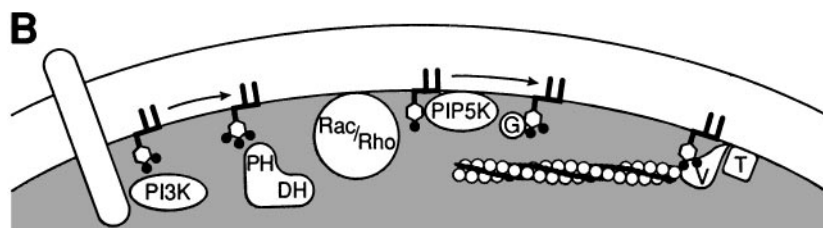
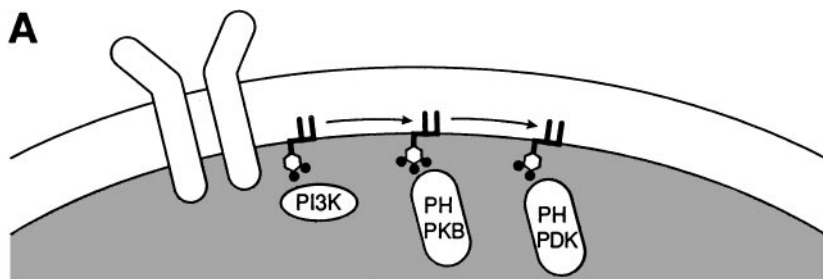
MEMBRANE MICRODOMAINS OF PHOSPHOINOSITIDES AND SITE-SPECIFIC PROTEIN COMPLEX ASSEMBLY

It is likely that phosphoinositides and the proteins they recruit are spatially localized at focal sites in specific membranes. Phosphoinositides and the phosphoinositide kinases are not uniformly distributed in cellular membranes (Pike 1992), and the phosphoinositides are metabolically compartmentalized (Monaco & Gershengorn 1992). Biochemical evidence for membrane domains enriched in receptor-regulated pools of PtdIns 4,5-P₂ and enzymes has been reported (Pike & Casey 1996, Hope & Pike 1996). Studies employing fluorescent PtdIns 4,5-P₂ in liposomes revealed that phosphoinositide-binding peptides can stabilize membrane microdomains in which both protein and phospholipid constituents are segregated (Glaser et al 1996).

Immunocytochemical studies with phosphoinositide-specific antibodies provide a preview of what may be a general concept of spatially localized domains of phosphoinositides in cellular membranes. PtdIns 4,5-P₂ colocalizes with α -actinin in focal contacts and membrane ruffles (Fukami et al 1994). A localized synthesis of PtdIns 4,5-P₂ on dense-core vesicles in neuroendocrine cells during a priming step for exocytosis can be detected by immunocytochemistry (K Loyet, personal communication). Spatially segregated membrane domains enriched for polyphosphoinositides would exhibit a positive membrane curvature (Chernomordik 1996) that could contribute to the remodeling of the bilayer for events such as membrane budding and fusion. The recruitment of phosphoinositide-binding proteins to such sites could allow for the assembly of signal transduction complexes, cytoskeletal-membrane attachments, coated membrane domains for bud formation, and scaffolds for membrane fusion reactions. Current efforts to define such mechanisms involving phosphoinositide-binding effector proteins are discussed below and summarized in Figure 2.

PROTEIN KINASES AS EFFECTORS FOR PHOSPHOINOSITIDES IN SIGNAL TRANSDUCTION

PtdIns 3,4,5-P₃ serves an essential signaling role in mediating the effects of a wide range of extracellular stimuli on cell proliferation, cell survival, and metabolism (Toker & Cantley 1997). Recent studies elucidated an important effector pathway that involves D3 phosphoinositide-mediated membrane recruitment and activation of several protein kinases (Figure 2*a*). The PKB/Akt



kinase is activated downstream of receptor-regulated PtdIns 3-kinase in part by direct interaction of PtdIns 3,4- P_2 with its PH domain (Franke et al 1997, Klippel et al 1997, Frech et al 1997, Cohen et al 1997). Recruitment of PKB/Akt to the membrane requires its PH domain (Andejkovic et al 1997), and possible dimerization of PKB/Akt at the membrane has been suggested (Franke et al 1997). PKB/Akt undergoes phosphorylation and activation by additional phosphoinositide-dependent protein kinases such as PDK1 (Alessi et al 1997, Stephens et al 1998). The phosphorylation of PKB/Akt by PDK1 requires D3 phosphoinositide binding to the PH domain of PKB/Akt (Stokoe et al 1997, Cohen et al 1997). PDK1 also contains a PH domain that binds PtdIns 3,4,5- P_3 ; however, its kinase activity may be constitutive, and phosphoinositide binding may serve to recruit PDK1 to a membrane site near its membrane bound substrate (Alessi et al 1997). This system represents the clearest example of phosphoinositide signaling where the identity of the lipid(s) involved, the basis of its regulated synthesis by a defined kinase, and the role of a physiologically relevant phosphoinositide-binding effector are relatively well defined (Figure 2a).

Several substrates of PKB/Akt that act downstream in signaling pathways regulating metabolism (Cohen et al 1997) and cell survival (del Peso et al 1997) have been identified. Expression of a constitutively active PtdIns 3-kinase

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Figure 2 Common themes in phosphoinositide signaling involving site-specific membrane recruitment. (A) Signal transduction mechanism employing receptor-regulated PtdIns 3-kinase (PI3K) to form D3 phosphoinositides for the recruitment of PH domain-containing kinases, PKB and PDK. (B) Cytoskeleton assembly mechanisms employing receptor-regulated PtdIns 3-kinase to form D3 phosphoinositides for the activation of PH domain-containing Dbl family members that promote guanine nucleotide exchange on Rho family members. Rho/Rac are depicted as potential activators of PtdIns 4-P 5-kinase (PIP5K) to form PtdIns 4,5- P_2 , which uncaps gelsolin (G) from actin to allow filament elongation, or promotes a conformational change in vinculin (V) to allow it to tether actin to talin (T). (C) Golgi membrane-budding reaction is depicted to proceed through cargo receptor activation of a PtdIns 3-kinase (PI3K) containing an adaptor (A) subunit. PtdIns transfer protein (PITP) enhances PtdIns 3-kinase-catalyzed phosphorylation to form PtdIns 3-P, which promotes the binding of clathrin adaptor proteins (AP) to cargo receptor tail. (D) Golgi membrane-budding reaction is depicted as proceeding through PITP enhancement of PtdIns 4-kinase phosphorylation along with PIP5K to form PtdIns 4,5- P_2 . This phosphoinositide recruits and activates the PH domain and Sec7 homology-containing exchange factors that promote guanine nucleotide exchange on ARF. ARF activates phospholipase D, which promotes further synthesis of PtdIns 4,5- P_2 via phosphatidic acid stimulation of PIP5K. Adaptor proteins (AP) are shown binding to acidic phospholipids. (E) Endosome fusion mechanism is depicted to proceed through activation of an adaptor-containing PtdIns 3-kinase (A and PI3K) that forms PtdIns 3-P, which recruits EEA for binding to Rab5. (F) Exocytotic fusion mechanism is depicted to involve PITP, PtdIns 4-kinase (PI4K), and PIP5K to promote formation of PtdIns 4,5- P_2 on the vesicle membrane. Potential effectors of this phosphoinositide are CAPS, Mint-mUNC18 complexes, and synaptotagmin (tagmin). Potential interactions of mUNC18 and tagmin with syntaxin (taxin) are depicted.

(Martin et al 1996, Frevert & Kahn 1997) or a PKB/Akt (Kohn et al 1996) enhances Glut4 translocation to the plasma membrane of 3T3-L1 adipocytes, suggesting that the PKB/Akt-catalyzed phosphorylation of an unidentified protein substrate regulates an aspect of the exocytotic pathway of glucose transporter vesicles. Delineation of this downstream pathway will provide interesting insights into the regulation of membrane trafficking by phosphoinositide-dependent protein phosphorylation.

PROTEIN EFFECTORS FOR CYTOSKELETAL REGULATION

Phosphoinositides have been implicated in the regulation of actin cytoskeleton assembly at several levels. Extensive connections between phosphoinositides and the Rho family of GTP-binding proteins, which mediate extracellular signal regulation of cytoskeletal rearrangements, have been identified (Hall 1998). A large family of Rho guanine nucleotide exchange factors has been characterized whose members contain Dbl homology (DH) domain as well as a PH domain that mediates the targeting of these proteins to the membrane (Cerione & Zheng 1996, Zheng et al 1996b). Constitutively active PtdIns 3-kinase activates Rac-dependent lamellipodia and Rho-dependent stress fiber assembly in fibroblasts (Reif et al 1996). The PH domain of some Dbl family members exhibits selectivity for binding D3 phosphoinositides (Rameh et al 1997a). This indicates that D3 phosphoinositide synthesis could promote recruitment of exchange factors to specific sites on the membrane for activation of Rho family GTPases (Carpenter et al 1997) (Figure 2*b*).

With the identification of numerous Rho/Rac-binding proteins (Tapon & Hall 1997), there are many options for downstream signaling to the cytoskeleton. One pathway for the Rho-dependent induction of stress fiber formation involves the activation of a Rho kinase (ROK) that phosphorylates both myosin light chain (Amano et al 1996) and myosin phosphatase (Kimura et al 1996), which leads to increased binding of myosin and the bundling of actin filaments. Another mechanism that promotes *de novo* actin polymerization involves D4 phosphoinositides as mediators of Rho/Rac regulation of the cytoskeleton (Figure 2*b*). In platelets, Rac activation results in increased actin polymerization for lamellae formation, which arises from an increase in the number of free actin barbed-ends available for filament elongation (Hartwig et al 1995). Rac activation causes an increased synthesis of PtdIns 4,5-P₂, which may be mediated by Rac stimulation of a type I PtdIns 4-P 5-kinase. The downstream effector proteins in this system are gelsolin and other capping proteins whose actin barbed-end capping activities are inhibited by phosphoinositides (Janmey 1994, Schafer et al 1996) (Figure 2*b*).

There are other potential downstream effectors among the phosphoinositide-binding proteins that regulate actin assembly (Table 1) (Janmey 1994). Polyphosphoinositides induce conformational changes in vinculin that allow vinculin to mediate the cross-linking of talin to actin, which plays a role in focal adhesion assembly (Gilmore & Burridge 1996) (Figure 2*b*). Consistent with a role for vinculin or other phosphoinositide-binding cytoskeletal proteins as important effectors, the Rho-dependent stimulation of focal adhesion formation in fibroblasts is blocked by microinjection of antibodies to PtdIns 4,5-P₂ (Gilmore & Burridge 1996).

In related efforts to assess physiological roles for phosphoinositides in regulating actin assembly, overexpression of the type I PtdIns 4-P 5-kinase in COS-7 cells was found to cause a dramatic increase in the assembly of short actin filaments (Shibasaki et al 1997). This depends upon a functional kinase domain and is counteracted by coexpression of a type II 5-phosphatase that decreases cellular PtdIns 4,5-P₂ levels (Zhang et al 1995). Conversely, overexpression of a type II 5-phosphatase in COS-7 cells reduces the number of actin stress fibers (Sakasaka et al 1997). Expression of a dominant-negative form of RhoA fails to suppress the formation of actin filaments induced by 5-kinase overexpression, consistent with a role for PtdIns 4-P 5-kinase downstream of Rho (Shibasaki et al 1997). These studies to alter cellular levels of polyphosphoinositides in cells provide an important link between *in vitro* studies of phosphoinositide-binding by cytoskeletal proteins and the regulation of actin assembly *in vivo*. Additional studies are required to determine which of the many candidate cytoskeletal proteins (Table 1) serve as essential effectors for phosphoinositide signaling in cytoskeletal rearrangements.

PROTEIN EFFECTORS FOR MEMBRANE COAT FORMATION AND BUDDING REACTIONS

Many intracellular transport events require the assembly of vesicle coats at specific sites on the membrane during budding (Rothman & Wieland 1996). Coat components bind to the cytoplasmic tails of transmembrane proteins that associate with transported cargo in the lumen of the budding vesicle, which serves to concentrate and sort cargo for transport (Kirchhausen et al 1997). Non-clathrin COPII- and COPI-coated vesicles mediate transport between the endoplasmic reticulum and Golgi and within the Golgi, respectively (Schekman & Orci 1996). Clathrin-coated vesicles mediate the endocytic trafficking of proteins to early endosomes and the trafficking of proteins from the *trans*-Golgi network to late endosomes. Clathrin assembly at the site of vesicle budding utilizes adaptor proteins, AP-1 for the Golgi and AP-2/AP-3 for the plasma membrane (Robinson 1997). Protein sorting at the *trans*-Golgi network

to multiple destinations involves the generation of numerous distinct transport vesicles for which coats and mechanisms of assembly have yet to be fully characterized (Traub & Kornfeld 1997).

As for other spatially localized, membrane-associated events requiring protein recruitment, there is evidence for an essential role for phosphoinositides in the assembly of vesicle coats. It remains to be determined whether phosphoinositides are required as constitutive cofactors for assembly or whether they act as regulatory signals that specify the site and timing of assembly. Within the variety of budding processes examined, coat components and adaptor proteins, as well as the enzyme PLD, have suggested roles as phosphoinositide effectors.

The initial indication that D3 phosphoinositides were critical for aspects of membrane trafficking was the discovery that a yeast vacuolar protein sorting gene *VPS34* encoded a PtdIns 3-kinase (Schu et al 1993). The *VPS34* gene product phosphorylates PtdIns, but not other phosphoinositides, and mammalian homologues exhibit a similar substrate specificity (Volinia et al 1995). Loss-of-function mutants of *VPS34* exhibit a defect in sorting newly synthesized proteins, as well as endocytosed proteins, to the vacuole, and it was suggested that the Vps34p protein executes a function essential for the formation of vesicles in the Golgi or their subsequent trafficking (Stack et al 1995). A serine/threonine-specific protein kinase (VPS15p) mediates the membrane recruitment and activation of the Vps34p lipid kinase (Stack et al 1993). The Vps15p/Vps34p complex has been suggested to be regulated by cargo receptors for vacuolar hydrolases (Marcusson et al 1994). In this model, cargo accumulation would specify the timing and location of budding by stimulating PtdIns 3-P formation (Horazdovsky et al 1995).

The immediate downstream effector for PtdIns 3-P remains to be identified. PtdIns 3-P could alter the structure of the bilayer to facilitate budding, recruit coat proteins or adaptors to the site of budding, or function as a vesicle membrane component essential for subsequent docking or fusion reactions (Stack et al 1995). The role of a dynamin homologue (Vps1p) in vacuolar sorting suggests that clathrin coat proteins may be involved (Conibear & Stevens 1995), and the sorting defects of conditional clathrin mutants are consistent with this (Stack et al 1995). Whether PtdIns 3-P functions to recruit clathrin adaptor proteins for bud formation remains to be determined.

In yeasts, PtdIns 3,5- P_2 synthesis was found to be dependent upon the Vps34p PtdIns 3-kinase (Dove et al 1997). This could indicate that the role of PtdIns 3-P in vacuolar sorting is to serve as a precursor to PtdIns 3,5- P_2 . The loss-of-function phenotype for *FABI*, which encodes one of two yeast PtdInsP 5-kinases (Yamamoto et al 1995, Loijens & Anderson 1996), includes a vacuolar morphology defect, which suggests a role for a 5-phosphorylated inositide in

aspects of vacuole membrane cycling. Genetic studies, however, did not reveal the epistatic interaction between *VPS34* and *FABI* alleles that is anticipated for a conversion of PtdIns 3-P to PtdIns 3,5-P₂ as essential for vacuolar targeting (Yamamoto et al 1995).

To investigate potential roles for the products of PtdIns 3-kinases in membrane trafficking in mammalian cells, extensive use has been made of wortmannin, a characterized irreversible inhibitor of some but not all PtdIns 3-kinases (Carpenter & Cantley 1996). The Vps34p lipid kinase is relatively insensitive to wortmannin (Stack & Emr 1994), whereas mammalian class III PtdIns 3-kinases that include Vps34p homologues exhibit a range of sensitivities to the drug (Stephens et al 1994, Volinia et al 1995). Wortmannin is not highly specific and is known to inhibit myosin light chain kinase, several phospholipases, and several PtdIns 4-kinases, albeit at higher concentrations (Cross et al 1995, Wong et al 1997). A chemically distinct inhibitor of PtdIns 3-kinases LY290042 (Vlahos et al 1994) is also used in membrane trafficking studies. Several mammalian PtdIns 3-kinase isoforms are inhibited by LY290042, but unfortunately so are isoforms of PtdIns 4-kinase. Thus studies that rely exclusively on wortmannin and LY290042 are difficult to interpret, and it is necessary to employ other methods such as dominant-negative mutants to assess the role of PtdIns 3-kinase in membrane trafficking reactions (Haruta et al 1995).

Wortmannin treatment of mammalian cells results in a limited repertoire of membrane trafficking defects that is restricted to the late Golgi-lysosomal-endosomal pathway (Brown et al 1995, Clague et al 1995, Davidson 1995, Reaves et al 1996, Shpetner et al 1996). Wortmannin treatment causes a striking swelling of late endosomal-prelysosomal compartments (Brown et al 1995, Reaves et al 1996); the inhibition of transport of endocytosed receptors from late endosomes to lysosomes (Shpetner et al 1996); the mis-sorting of cathepsin D, a lysosomal protease that is sorted via the mannose 6-phosphate receptor in the *trans*-Golgi (Brown et al 1995, Davidson 1995); an inhibition of transferrin receptor recycling (Shepherd et al 1996); the inhibition of GLUT4 transporter recycling (Yang et al 1996); and a partial inhibition of fluid phase endocytosis (G Li et al 1995).

In vitro reconstitution studies in mammalian systems of vesicle biogenesis from the *trans*-Golgi have identified several molecular requirements for budding and have begun to elucidate the role of phosphoinositides in budding reactions (Figure 2*c,d*). The formation of constitutive and regulated vesicles from the *trans*-Golgi of neuroendocrine cells in vitro depends upon at least two cytosolic protein fractions as well as ATP (Ohashi et al 1995). One of the required cytosolic factors is PtdIns transfer protein, which binds and transports PtdIns and PtdChol in vitro (Wirtz 1997). Mammalian PtdIns transfer proteins accelerate the phosphorylation of PtdIns through 4-phosphorylation and

3-phosphorylation reactions by providing PtdIns to PtdIns 4-kinase and PtdIns 3-kinase (Martin 1995, Wirtz 1997). A requirement for PtdIns transfer protein in Golgi budding reactions likely indicates a role for phosphoinositides in some aspect of vesicle formation.

In vitro reactions in which the formation of constitutive exocytic vesicles containing TGN38 are formed are inhibited by high concentrations of wortmannin, which correspond to the range that inhibits a Golgi-associated PtdIns 3-kinase (Jones et al 1993, Jones & Howell 1997). The PtdIns 3-kinase resembles the VPS34 lipid kinase in substrate specificity and low sensitivity to wortmannin, which led to the suggestion of a required role for PtdIns 3-P in vesicle formation (Jones & Howell 1997; see also Hickinson et al 1997). The enzyme resides in a membrane-bound complex with an adaptor protein (p62) that is related to, but immunologically distinct from, the p85a subunit of a type I PtdIns 3-kinase. Rab6 and several unidentified GTP-binding proteins co-reside in the complex in association with the cytoplasmic domain of TGN38 (Jones et al 1993, Jones & Howell 1997). The properties of a membrane and cytosolic p62-containing complex suggest a model in which a p62-Rab6 complex is recruited to TGN38 in the Golgi, thereby mediating the binding of PtdIns 3-kinase for the essential formation of PtdIns 3-P (Figure 2c). The effector system for PtdIns 3-P remains to be identified for this Golgi budding reaction, but may involve the p200 lace-like coats described for these vesicles (Ladinsky et al 1994).

Other experimental work on the biogenesis of vesicles in the *trans*-Golgi and the formation of COPI-coated vesicles within the Golgi suggests alternative or additional roles for the phosphoinositides (PtdIns 4,5-P₂ or PtdIns 3,4,5-P₃) in budding reactions (Figure 2d). ADP ribosylation factors (ARFs) comprise a family of proteins that play essential roles in Golgi membrane traffic by mediating the recruitment of coat proteins to the membrane (Rothman & Wieland 1996). COPI vesicle formation can be reconstituted in vitro with ARF and coatamer as the sole cytosolic factors (Orci et al 1993). An ARF guanine nucleotide exchange factor (ARF GEF) catalyzes GTP binding to ARF, and ARF-GTP promotes coatamer binding. An ARF GTPase-activating factor (ARF GAP) catalyzes uncoating prior to fusion of vesicles with an acceptor membrane. The recruitment of AP-1 clathrin adaptors to the Golgi membrane (Stamness & Rothman 1993) is ARF dependent, as is recruitment of AP-1 to immature secretory granules (Dittie et al 1996) and AP-2 to endosomal membranes (West et al 1997).

A potential key role for phosphoinositides in coat protein recruitment mediated by ARF is indicated by the dependence of ARF regulators and effectors on the presence of phosphoinositides in the membrane. ARF GEFs that contain Sec7 homology domains and PH domains that mediate phosphoinositide-stimulated exchange activity have been characterized (Paris et al 1997, Klarlund

et al 1997, 1998). ARF itself interacts with polyphosphoinositides (Randazzo 1997), which promote its nucleotide exchange (Terui et al 1994, Paris et al 1997) and its interaction with ARF GAP (Randazzo 1997). ARF GAPs also contain PH domains and exhibit phosphoinositide-regulated activity (Tanaka et al 1997). These observations suggest that phosphoinositides could play a central role as cofactors or regulators in recruiting ARF to the membrane and regulating its cycle of activity by affecting ARF GEFs and GAPs. Consistent with an essential role for phosphoinositides in membrane-coating reactions, the charged antibiotic neomycin, which binds polyphosphoinositides, inhibits *trans*-Golgi budding of regulated secretory granules and the membrane recruitment of AP-2 in vitro (Ohashi et al 1995, West et al 1997).

The finding that ARF is a GTP-dependent activator of PLD (Brown et al 1993, Cockcroft et al 1994) led to the suggestion that PLD functions as an effector for ARF in coat protein recruitment (Ktistakis et al 1996, Roth & Sternweis 1997). For COPI vesicle formation from the Golgi (Ktistakis et al 1996), for the budding of regulated secretory granules from the *trans*-Golgi involving an AP-1 adaptor (Chen et al 1996, 1997), and for AP-2 adaptor recruitment to endosomal membranes (West et al 1997), the ARF requirement can be bypassed by direct provision of PLD to the in vitro budding reaction. Diverting endogenous PLD catalysis with primary alcohols inhibits budding (Ktistakis et al 1996, Bi et al 1997, Chen et al 1997). The data are consistent with the possibility that PLD serves an essential effector role for ARF in budding.

PLD itself is an additional potential effector for phosphoinositides in membrane budding reactions because PLD activity and its stimulation by ARF stringently require polyphosphoinositides (Brown et al 1993, Liscovitch et al 1994). Either D4 or D3 polyphosphoinositides function equally effectively as cofactors for PLD. The phosphoinositide dependence of enzyme activity is mediated by direct interactions with the PLD, although the phosphoinositide-binding site on the enzyme has not been identified (Hammond et al 1997). The basis for an autocatalytic cycle for the ARF-dependent activation of PLD has been proposed (Liscovitch & Cantley 1995), which suggests that production of phosphatidic acid by PLD could result in the enhanced production of PtdIns 4,5-P₂ by activation of the type I PtdIns 4-P 5-kinase (Jenkins et al 1994). The PtdIns 4,5-P₂ formed would further enhance PLD activity, as well as ARF activation by ARF GEFs. Termination of this cycle might occur by deactivation of ARF through phosphoinositide stimulation of an ARF-GAP or by a phosphoinositide 5-phosphatase (Chung et al 1997). This mechanism encompasses some of the known in vitro properties of participant proteins and provides a basis for generating a spatially restricted membrane budding site (Figure 2d). The requirement for phosphoinositides in all aspects of the ARF activation/deactivation cycle and for PLD activation suggests a critical role for phosphoinositides in Golgi

budding and membrane coat recruitment; however, there is presently no evidence for this role in vivo and future work will need to address this.

The precise steps in late Golgi trafficking that are affected by wortmannin and presumed to require D3 phosphoinositides are uncertain. Such steps may involve sorting of proteins to appropriate vesicles, the formation of vesicles and the exit of a sorted protein from the *trans*-Golgi, the fusion of transport vesicles with endosomal intermediates, or recycling of sorted proteins back to the *trans*-Golgi. A recent study reported that wortmannin did not affect the recycling of the mannose 6-phosphate receptor to the *trans*-Golgi (Nakajima & Pfeffer 1997). However, there is evidence that wortmannin affects the sorting in the *trans*-Golgi of pro-cathepsin D by the mannose 6-phosphate receptor to clathrin-coated post-Golgi vesicles (Gaffet et al 1997). Wortmannin had little effect on levels of clathrin-coated vesicles; however, the vesicles from drug-treated cells exhibited reduced levels of the mannose 6-phosphate receptor, suggesting that cargo sorting rather than budding was being affected by wortmannin (Gaffet et al 1997).

A mechanism by which 3-phosphorylated inositides could regulate the sorting of cargo in the endosomal/lysosomal pathway was suggested by the observation that PtdIns 3-P enhances the affinity of the adaptor protein AP-2 complex for binding tyrosine-based signals present on membrane receptor cytoplasmic tails (Rapoport et al 1997, Kirchhausen et al 1997) (Figure 2c). In this mechanism, PtdIns 3-P would be a co-ligand in a protein complex that would modulate an interaction. By affecting the affinity of cytoplasmic tail interactions with adaptor proteins AP-1 and AP-2, D3 phosphoinositides could influence the routing of internalized proteins to recycling endosomes or to late endosomes and lysosomes (Marks et al 1997).

PROTEIN EFFECTORS FOR MEMBRANE FUSION AND RETRIEVAL

In addition to budding and membrane coat recruitment, phosphoinositides have been implicated in membrane fusion reactions. D3 and D4 phosphoinositides appear to be involved in endosome and exocytotic membrane fusion reactions, respectively. The nature of the effector proteins that mediate the requirement for phosphoinositides in fusion are beginning to be identified.

There is evidence for additional steps in the endosomal pathway at which PtdIns 3-kinases are required. Wortmannin and LY294002 inhibit the homotypic fusion between early endosomes that can be reconstituted in vitro (Jones & Clague 1995, G Li et al 1995). A clue to the underlying mechanism was provided by the observation that a constitutively active but not wild-type Rab5 reversed the inhibition by wortmannin (G Li et al 1995). A constitutively

active PtdIns 3-kinase also stimulated *in vitro* endosome fusion. These studies provide a convincing link between early endosome fusion and PtdIns 3-kinase activity and suggest that lipid kinases or their D3 phosphoinositide products function upstream of a fusion mechanism that involves activation of Rab5 (G Li et al 1995). An indication for a direct effect of D3 phosphoinositides and a tangible clue on a potential effector for D3 phosphoinositide regulation of Rab5 function were provided by the identification of EEA1 (early endosomal antigen) as a PtdIns 3-P-binding protein that resides in part on endosomes (Patki et al 1997). Treatment of cells with wortmannin caused a translocation of EEA1 from an endosomal to cytosolic distribution. Moreover, EEA1 was found to bind liposomes that contain PtdIns-3-P (Patki et al 1997), although its specificity for binding other D3 and D4 phosphoinositides is unknown. The sequence of the protein is homologous to that of Vps19p and contains a Zn^{2+} -binding domain that mediates interactions with Rab5 (Mu et al 1995). This work provides a model (Figure 2e) for the mechanism of action of D3 phosphoinositides in membrane fusion acting as a signal that recruits EEA1 to endosomes. EEA1 would then recruit or bind resident Rab5 to promote fusion. A number of components of the membrane-bound machinery that interact with activated Rab5, such as nucleotide exchange factors and effectors, have been identified (Horiuchi et al 1997), and this complex may constitute a protein scaffold that, acting with EEA1, allows membrane ligation and fusion.

That phosphoinositides are essential for exocytic fusion reactions was suggested by the discovery that PtdIns transfer protein is required for the reconstitution of Ca^{2+} -dependent neurotransmitter secretion in permeable PC12 cells (Hay & Martin 1993). PtdIns transfer protein was purified as one of three cytosolic factors required for an ATP-dependent priming step that precedes a Ca^{2+} -triggered membrane fusion step (Hay & Martin 1993). The ATP-dependence of priming and the role of PtdIns transfer protein were clarified by the finding that a type I PtdIns 4-P 5-kinase was one of the other cytosolic priming factors (Hay et al 1995) and that these two proteins synergistically stimulate priming. These results indicate a role for phosphoinositide phosphorylation in some aspect of regulated exocytic membrane fusion, which had been suggested by earlier studies of Holz and coworkers (Eberhard et al 1990). PtdIns 4-kinase is a resident on secretory vesicles from adrenal chromaffin, mast, and pancreatic β cells (Pike 1992), and this enzyme was shown to be essential for priming exocytosis (Wiedemann et al 1996). Priming is a reversible process, and the reversal of priming appears to be catalyzed by a phosphoinositide 5-phosphatase (Martin et al 1997).

These results indicate that synthesis of PtdIns 4,5- P_2 during priming is essential for regulated exocytosis. That additional phosphorylation by a PtdIns 3-kinase is not involved in priming was suggested by the failure of wortmannin

and LY294002 to interfere with priming (Martin et al 1997) and by the ability of phospholipase C δ to inhibit exocytosis from ATP-primed cells (Hay et al 1995). In addition, introduction of PtdIns 4,5-P₂ but not PtdIns 3,4,5-P₃ micelles into permeable cells selectively interfered with regulated exocytosis presumably by competing with endogenous effectors for the phosphoinositides (Martin et al 1997). The essential role of the intact PtdIns 4,5-P₂ lipid, rather than a derived metabolite, was suggested by the failure of all potential metabolites tested to significantly alter exocytosis in the absence or presence of Ca²⁺ (Hay et al 1995, Martin et al 1997) and the ability of a PtdIns 4,5-P₂ antibody to inhibit exocytosis from ATP-primed cells (Hay et al 1995).

A requirement for PtdIns 4,5-P₂ in regulated exocytosis raises a number of questions concerning the identity of the membrane where critical pools of phosphoinositides are generated and the function of these phosphoinositides in exocytosis. Recent immunocytochemical studies with PtdIns 4,5-P₂ specific antibodies conducted with permeable PC12 cells indicate that high concentrations of PtdIns 4,5-P₂ are synthesized on secretory granule membranes during ATP-dependent priming (K Loyet, personal communication). There have been few direct studies of the biophysical effects of PtdIns 4,5-P₂ in membrane bilayers, but it is likely that the highly charged hydrophilic headgroup would confer properties on a membrane that are antagonistic to fusion. The strong positive curvature imparted to the membrane might destabilize a stalk intermediate envisioned for bilayer fusion (Chernomordik 1996). It is likely that PtdIns 4,5-P₂ in the granule membrane would need to be segregated into domains in order to allow fusion to proceed. Such segregation could be mediated by protein binding to PtdIns 4,5-P₂, as was demonstrated for a lysine-rich peptide in liposomes (Glaser et al 1996). An analogous process on the secretory granule could recruit a PtdIns 4,5-P₂-binding protein to the membrane that is essential for subsequent Ca²⁺-triggered fusion events.

A small number of proteins, including dynamin, CapZ, and CAPS, have been characterized that exhibit a specificity for binding D4 phosphoinositides over D3 phosphoinositides (Table 1). The CAPS protein was discovered as a factor that reconstitutes regulated secretion in permeable PC12 cells at the Ca²⁺-dependent fusion step that follows ATP-dependent priming (Walent et al 1992, Ann et al 1997). Liposome binding and proteolysis studies indicate that CAPS interacts with D4 but not D3 phosphoinositides and that binding of PtdIns 4,5-P₂ promotes a conformational change (Loyet et al 1998). These observations suggest that CAPS may be an effector for PtdIns 4,5-P₂ in regulated exocytosis in neural and endocrine cells. CAPS copurifies with dense-core vesicles and plasma membrane from brain tissue (Berwin et al 1998). A model in which CAPS on vesicles and plasma membrane undergoes a conformational change in response to PtdIns 4,5-P₂ synthesis on the vesicle has been proposed (Loyet et al

1998). This could serve the purpose of segregating PtdIns 4,5-P₂ on the vesicle and promoting the close apposition of the vesicle to the plasma membrane for fusion (Figure 2*f*).

PtdIns 4,5-P₂ functions in the regulated exocytosis of granules in cells of hemopoietic origin as well. GTP γ S-stimulated hexosaminidase secretion in permeable HL60 cells requires the cytosolic factors ARF and PtdIns transfer protein (Fensome et al 1996). It was suggested that the synthesis of PtdIns 4,5-P₂, either activated by PtdIns transfer protein through a PtdIns 4-kinase/PtdIns 4-P 5-kinase pathway, or by ARF via PLD production of phosphatidic acid and stimulation of PtdIns 4-P 5-kinase, was the common factor essential for exocytosis. Phosphoinositide-binding proteins that mediate the essential role of PtdIns 4,5-P₂ in regulated exocytosis in hemopoietic cells, which do not express CAPS, have not been identified.

Based on *in vitro* phosphoinositide-binding studies, additional potential effectors for PtdIns 4,5-P₂ in regulated exocytosis have been suggested. Genetic and biochemical evidence indicates that synaptotagmin I, an abundant secretory vesicle protein, is an essential component of the Ca²⁺-sensing mechanism in the regulated exocytosis of synaptic vesicles in nerve cells (Sudhof 1995). Synaptotagmin I interacts with phosphoinositides via its membrane distal C2B domain, and Ca²⁺ increases binding to PtdIns 4,5-P₂ but decreases binding to PtdIns 3,4,5-P₃ (Schiavo et al 1996). It was suggested that the Ca²⁺-dependent switching of synaptotagmin from a preference for binding PtdIns 3,4,5-P₃ to binding PtdIns 4,5-P₂ may be part of a vesicle docking mechanism that is activated by Ca²⁺ at concentrations below those required for fusion (Schiavo et al 1996). The distribution of PtdIns 3,4,5-P₃ and PtdIns 4,5-P₂ in synaptic terminals will need to be established to evaluate this model. If synaptic vesicles, like dense-core vesicles, synthesize high concentrations of PtdIns 4,5-P₂, the effect of Ca²⁺ may be to promote the association of the membrane distal C2B domain of synaptotagmin with the vesicle membrane (Figure 2*f*). This folding of synaptotagmin might unmask the C2A domain for Ca²⁺-dependent interactions that occur with the presynaptic membrane proteins syntaxin and SNAP-25, which may be essential for fusion (Chapman et al 1995, C Li et al 1995, Schiavo et al 1997). The effects of InsP6 indicate an important role for the C2B domain of synaptotagmin in evoked neurotransmitter release. InsP6 inhibits evoked neurotransmitter release, but this can be prevented by C2B domain antibodies (Fukuda et al 1995b, Ohara-Imaizumi et al 1997). Synaptotagmin I binds InsP6 via its C2B domain (Fukuda et al 1994, 1995b) and InsP6 competitively inhibits C2B domain interactions with phosphoinositides (Schiavo et al 1996). It is possible that InsP6 inhibits evoked neurotransmitter release by interfering with synaptotagmin-phosphoinositide interactions.

An additional component of the exocytotic apparatus suggested to interact with phosphoinositides are the Mint proteins (Okamoto & Sudhof 1997). mUNC18 is a major soluble syntaxin-interacting protein required for neurotransmitter secretion. mUNC18-interacting proteins termed Mints, identified by yeast two-hybrid screening, contain a PTB domain that interacts with PtdIns 4,5-P₂. It was proposed that Mint complexed with mUNC18 may mediate vesicle docking through the binding of Mint to PtdIns 4,5-P₂ on the vesicle and of mUNC18 with syntaxin on the plasma membrane (Figure 2*f*).

PtdIns 4,5-P₂ formed on secretory vesicles during priming in neuroendocrine cells may also play a role in the endocytic retrieval of the vesicle membrane. The clathrin adaptors AP-2 and AP-3 and dynamin bind polyphosphoinositides (Beck & Keen 1991, Ye et al 1995, Norris et al 1995, Salim et al 1996, Gaidarov et al 1996, Hao et al 1997). Synaptojanin, a phosphoinositide 5-phosphatase, has been suggested to function in endocytosis but its role is unclear (Cremona & DeCamilli 1997). The dynamin GTPase is stimulated by PtdIns 4,5-P₂ binding (Lin & Gilman 1996), suggesting that phosphoinositide hydrolysis might occur following clathrin coating and scission of the coated vesicle by dynamin. Alternatively, because phosphoinositide binding to AP-2 and AP-3 inhibits adaptor-mediated clathrin coat assembly (Beck & Keen 1991, Ye et al 1995, Norris et al 1995), dephosphorylation of phosphoinositides could be a positive signal for clathrin recruitment if it occurred following AP-2 or AP-3 recruitment to the membrane. At present there is no direct evidence for either of these models, and additional studies are required to clarify the roles of phosphoinositides in endocytosis.

PERSPECTIVES

There are now many examples where phosphoinositides have been implicated as essential cofactors or regulators involved in signal transduction, cytoskeletal, and membrane trafficking events. A theme common to each is the role of phosphoinositides as site-specific signals that recruit and/or activate protein effectors to assemble a spatially localized machinery on the membrane that alters a cellular function. Critical gaps in our knowledge, particularly for membrane trafficking events, concern the mechanisms that activate phosphoinositide kinases that specify the site and timing of phosphoinositide signals. Cellular localization studies need to be undertaken to reveal what is predicted to be the highly localized properties of phosphoinositide signaling mechanisms. In vitro studies of protein-phosphoinositide binding and membrane-associated reactions have contributed heavily to our current view of phosphoinositides as site-specific membrane signals, and the major future challenge is to test the validity of these models on the role of phosphoinositides in vivo. Particularly

important will be the determination of whether phosphoinositide binding is critical for the function of numerous effector proteins that are proposed to function downstream of phosphoinositide kinase reactions.

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