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Phosphoinositides as Regulators in Membrane Traffic

Pietro De Camilli,* Scott D. Emr, Peter S. McPherson, Peter Novick

Phosphorylated products of phosphatidylinositol play critical roles in the regulation of membrane traffic, in addition to their classical roles as second messengers in signal transduction at the cell surface. Growing evidence suggests that phosphorylation-dephosphorylation of the polar heads of phosphoinositides (polyphosphorylated inositol lipids) in specific intracellular locations signals either the recruitment or the activation of proteins essential for vesicular transport. Cross talk between phosphatidylinositol metabolites and guanosine triphosphatases is an important feature of these regulatory mechanisms.

In eukaryotic cells, the distinct composition of the different intracellular compartments is maintained despite continuous intercompartmental transport of membrane and lipid components. This homeostasis depends on vesicular carriers that mediate traffic by means of vectorial transfer of selected membrane and lumenal cargoes. A general framework has been proposed to explain the selective incorporation of proteins from donor membranes into carrier vesicles as well as the specific targeting and fusion of each class of vesicle with the appropriate target component (*1*). Membrane and lumenal proteins are incorporated into vesicles through direct or indirect interactions with coat proteins that are assembled on the cytoplasmic surface of the donor membrane (*1*, *2*). Self-assembly of the coat forces an increase in membrane curvature in a localized region until a coated vesicle bud, anchored by a narrow stalk, has formed. In at least some cases, additional factors are required to sever the vesicle neck and generate a free vesicle (*3*). After transport, the vesicle sheds its coat proteins and the uncoated vesicle docks with the target membrane through a cascade of molecular interactions, including the binding of membrane proteins of the vesicle (termed v-SNARES) with membrane proteins of the target membrane (termed t-SNARES) (*1*). Formation of the v- and t-SNARE complex is then followed by the fusion event that completes the transport reaction.

This framework raises a number of questions, many of which can be viewed as problems of regulation. For example, what regulates vesicle formation? What makes this process vectorial? What regulates coat assembly and disassembly? Because v-SNAREs and other membrane proteins must be recycled back to the donor compartment, what distinguishes the forward vesicles from the recycling vesicles that carry the same proteins but are directed toward a different target compartment?

Any model of vesicular traffic must include mechanisms to guarantee temporal and spatial specificity, because without such regulators, vesicular traffic would result in the rapid homogenization of all cellular compartments. Both proteins and lipids participate in this regulation. Among proteins, a major role is played by guanosine triphosphatases (GTPases). Among lipids, growing evidence suggests a key function for phosphatidylinositol (PtdIns) and its phosphorylated derivatives, collectively referred to as phosphoinositides (PIs). Unlike the head group of other phospholipids, the inositol ring is a highly versatile substrate that can be modified at several positions. Phosphorylation of the inositol ring of PtdIns at one or a combination of positions (3′, 4′, or 5′) generates a set of five unique stereoisomers that appear to function as regulators of vesicular transport reactions, the cytoskeleton, and cell growth. Here, we review results that implicate PIs and their metabolites in vesic-

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ular traffic and discuss the links that have emerged between their function and the function of GTPases.

Phosphatidylinositol Metabolism and Secretion

A role for PtdIns metabolism in the secretory process was first suggested by experiments performed in the mid-1950s (*4*). Pharmacological stimulations of the exocrine pancreas that lead to secretion were also found to produce an increased incorporation of ^{32}P into phospholipids, primarily because of an increased turnover of PtdIns and its phosphorylated products. A similar correlation between evoked secretion and increased PtdIns metabolism was later observed in a variety of other secretory cells (*4*). The discovery of the agonist-mediated cleavage of PtdIns-4,5-bisphosphate [PtdIns(4,5)P_2] by the activation of phospholipase C led to the widely accepted hypothesis that the increased PtdIns metabolism represents a signal transduction event leading to the generation of diacylglycerol (DAG) and inositol-1,4,5-trisphosphate [Ins(1,4,5)P_3] (*5*) (Fig. 1). However, in recent years, evidence obtained from a variety of experimental systems supports a direct role for inositol phospholipids and inositol polyphosphates in vesicular traffic events, distinct from their role in classical signaling pathways. New roles have been established for PtdIns(4,5)P_2 and its metabolites. Moreover, the discovery of kinases that phosphorylate the 3′ position of the inositol ring of PtdIns and PIs has shed light on a new class of PIs that are not targets of phospholipase C, yet play an important role in cellular regulation and vesicular traffic.

One of the first indications that inositol phospholipids are directly involved in Ca^{2+}-regulated exocytosis came from studies of permeabilized adrenal chromaffin cells. The observed requirement of adenosine triphosphate (ATP) for secretion could be partially explained by the need to generate PtdIns(4,5)P_2 (*6*). These observations were strongly corroborated by the identification and characterization of cytosolic factors from the brain, PEPs (priming of exocytosis proteins), which are required for the ATP-dependent priming of secretory granule exocytosis from broken PC12 cells (*7*). Two of the three protein factors isolated, PEP1 and PEP3, are enzymes involved in the synthesis of PtdIns(4,5)P_2. PEP3 is the mammalian PtdIns transfer protein (PITP). PITP was originally identified as the factor that exchanges PtdIns and phosphatidylcholine between lipid bilayers and thus can transfer PtdIns from its site of synthesis (in the endoplasmic reticulum and Golgi complex) to other membranes. PITP also participates in the synthesis of PIs by presenting PtdIns to PtdIns-4 kinase and to PtdIns-4-phosphate [PtdIns(4)P]–5 kinase (*8*) (Fig. 1). PEP1 is a PtdIns(4)P-5 kinase [that is, the last enzyme in the synthesis of PtdIns(4,5)P_2 (*7*)], and preliminary evidence suggests that PtdIns-4 kinase, which is associated with secretory vesicles, is also involved in exocytosis (Fig. 2 and Table 1) (*9*). Thus, PITP—in concert with a membrane-bound PtdIns-4 kinase and a cytosolic PtdIns(4)P-5 kinase—appears to function in the generation of PtdIns(4,5)P_2, which is needed for a critical step in exocytosis.

Vesicular Traffic in Yeast

Genetic studies in yeast provided initial support for a role of PIs not only in Ca^{2+}-regulated exocytosis, but more generally in fundamental aspects of vesicular transport. Cloning of the *SEC14* gene revealed that it encodes a PITP (*10*); mutations in this gene block post-Golgi secretory traffic. The phospholipid-binding and exchange activity of Sec14p appears to enable it to function as a sensor of Golgi membrane phospholipid composition. By regulating the cytidine diphosphate–choline pathway, one of the pathways in phosphatidylcholine biosynthesis, Sec14p helps to maintain the appropriate lipid composition in the Golgi complex membranes, which is essential for normal Golgi complex function (*11*). The precise site of action of Sec14p in vesicular transport is not known. However, recent cell-free studies of the biogenesis of immature secretory granules from the trans-Golgi network (TGN) have led to the isolation of mammalian PITP as a factor required for vesicle budding. Yeast Sec14p can functionally substitute for mammalian PITP in this assay (*12*). Because mammalian PITP is also implicated in exocytosis, the requirement for PITP in Golgi complex function may reflect a general role for this protein in cytoplasmic transport and phosphorylation of PtdIns and its metabolites.

Direct evidence for an essential role of PIs in vesicle-mediated transport was provided when the yeast *VPS34* gene was found to encode a PtdIns-3 kinase (*13*). Yeast *vps* mutants define a set of nearly 50 genes whose functions are required for the sorting and delivery of soluble vacuolar hydrolases from the late Golgi to the vacuole

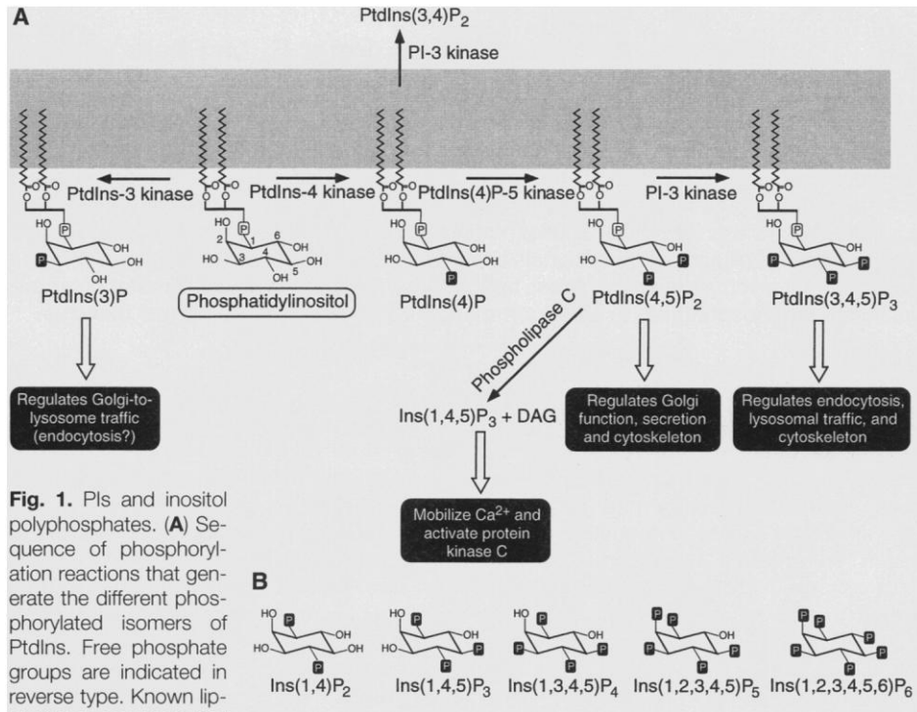

Fig. 1. PIs and inositol polyphosphates. (**A**) Sequence of phosphorylation reactions that generate the different phosphorylated isomers of PtdIns. Free phosphate groups are indicated in reverse type. Known lipid kinase enzymes that catalyze these reactions are shown. Phospholipase C hydrolyzes PtdIns(4,5)P_2 to generate the second messengers DAG and water-soluble Ins(1,4,5)P_3. This enzyme does not act on PIs modified at the 3′ position of the inositol ring, and it is presumed that PIs phosphorylated at this position execute their function at the membrane site where they are synthesized. The cellular processes that are thought to be regulated by different PIs are indicated in reverse type. The distinct and highly charged PI isomers appear to function at specific membrane sites by selectively recruiting or activating proteins, including proteins essential for vesicle-mediated protein transport (see Fig. 2). (**B**) Structures of inositol polyphosphates described in the text. Specific phosphatases like the inositol-5-phosphatase have been characterized that selectively remove phosphate from specific positions on the inositol ring (for example, the 5′ position). Certain of these enzymes also act on PI substrates and can catalyze the inactivation of inositide signaling pathways or convert one PI into another, which then may trigger additional downstream events.

(the yeast equivalent of the lysosome) (*14*). Yeast cells expressing a temperature-conditional allele of *VPS34* exhibit a rapid defect in both protein sorting to the vacuole and PtdIns-3 kinase activity when the mutant cells are shifted to a nonpermissive temperature (*15*). Thus, an immediate consequence of the inactivation of Vps34p is a defect in a specific vesicle-mediated transport process.

Vps34p is found in a complex with another *VPS* gene product, Vps15p, which both activates and recruits Vps34p to the membrane (*16*). Vps15p is a serine-threonine protein kinase that associates with the Golgi complex and possibly with endosomal membranes. Mutational inactivation of the Vps15p kinase prevents its association with Vps34p, which in turn blocks activation of Vps34p PtdIns-3 kinase activity. Vps15p appears to regulate the sorting of proteins to the vacuole from the Golgi complex by selectively recruiting Vps34p to the appropriate membrane site, where vacuolar hydrolases are packaged into vesicular carriers. An attractive possibility is that the localized production of PtdIns-3-phosphate [PtdIns(3)P] by Vps34p may either recruit or activate effector molecules, such as vesicle coat proteins, that catalyze the transport reaction (*14*). Inactivation of the Vps34p PtdIns-3 kinase also has been shown to alter a late stage of the endocytic pathway (*17*). However, it is not yet clear if this effect is direct. Further studies with the temperature-conditional allele of *VPS34* should help to resolve this question. A complex that resembles Vps34p–Vps15p and was recently identified in mammalian cells is hypothesized to play a similar role in the TGN → lysosome vesicular transport pathway (*18*). Indeed, the PI-3 kinase inhibitor wortmannin was recently

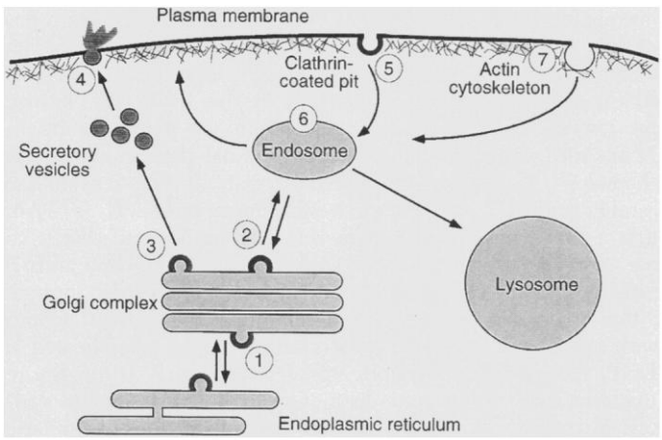

Fig. 2. Membrane traffic in eukaryotic cells and sites where a function of PtdIns metabolites has been implicated. Numbers refer to traffic stations discussed in Table 1.

Table 1. Proteins, directly or indirectly implicated in vesicular traffic, that participate in PI metabolism or bind PI metabolites.

Protein or protein complex	Function		Traffic station (Fig. 2)
	Link to PtdIns metabolism	Putative function in traffic	
Coatomer (COP I)	Binds InsPPs (*52*)	Generation of vesicle buds at the endoplasmic reticulum–Golgi complex boundary (*1*)	1
ARF1 (GTPase)	Activates phospholipase D (*44*); activity regulated by PtdIns(4,5)P_2 (*42*)	Promotes recruitment of coatomer and TGN clathrin coat (*1, 2*)	1, 2
Vps34p (PtdIns-3 kinase)	Synthesis of PtdIns(3)P (*13*)	Essential for Golgi → vacuole transport in yeast (*13, 14*)	2
Vps15p (protein kinase)	Recruits Vps34p to membrane and activates it (*15*)	Serine-threonine kinase essential for Golgi → vacuole transport in yeast (*14, 16*)	2
Sec14p (PITP)	Senses PtdIns-phosphatidylcholine content of membranes (*11*)	Required for TGN → plasmalemma transport in yeast (*10*)	3
Mammalian PITP (PEP3)	Mediates PtdIns exchange and phosphorylation (*8*)	Role in budding from the TGN and in exocytosis of secretory granules (*7, 12*)	3, 4
PtdIns(4)P-5 kinase (PEP1)	Synthesis of PtdIns(4,5)P_2	Role in exocytosis of secretory granules (*7*)	4
PtdIns-4 kinase	Synthesis of PtdIns(4)P	Present on secretory vesicles (*9*)	4
Synaptotagmin	C2b domain binds InsPPs (*53*)	Putative role in exocytosis and endocytosis (*54*)	4, 5
AP2	Binds InsPPs and PIs (*50, 57*)	Recruits clathrin coats at the plasmalemma (*41*)	5
AP180 (AP3)	Binds InsPPs (*51*)	Neuron-specific clathrin adaptor (*41, 51*)	5
Dynamin	PH domain binds Ins(1,4,5)P_3 and PtdIns(4,5)P_2 (*74*)	Fission reaction of plasmalemma-derived clathrin-coated vesicles (*3, 33*)	5
Synaptojanin (5-phosphatase)	Substrates include PtdIns(4,5)P_2, Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 (*32*)	Neuron-specific protein with putative role in synaptic vesicle recycling (*31, 32*)	5?
p85-p110 PI-3 kinase	Synthesis of PtdIns(3,4,5)P_3 and other PIs (*20*)	Implicated in recycling through early endosomes (*22, 24, 25*)	6
Rab5 (GTPase)	Regulated by PI-3 kinase (*25*)	Role in endosome fusion (*49*)	6
Actin-binding proteins (profilin, gelsolin, α-actinin, cofilin, β-spectrin, and others)	Bind PtdIns(4,5)P_2 (*68*)	Regulate function of actin-based cytoskeleton (*68*)	7
Rho family (GTPases)			
Rho	Binds and activates PtdIns(4)P-5 kinase (*72*)	Regulate function of actin-based cytoskeleton implicated in clathrin-independent endocytosis (67)	7
Rac	Activated by p85-p110 PI-3 kinase (*70*)		
Rac, Cdc42	GTP-bound forms bind and activate p85-p110 PI-3 kinase (*71*)		

shown to induce the missorting and secretion of the mammalian lysosomal hydrolase cathepsin D (*19*).

The Endocytic Pathway in Mammalian Cells

The identification of a role for the Vps34p PtdIns-3 kinase in vesicular transport prompted a search for a function of the mammalian p85-p110 PI-3 kinase in membrane traffic. The main known function of this enzyme is to transduce signals of tyrosine-phosphorylated receptors into a variety of intracellular responses, including mitogenic responses (*20, 21*). Its enzymatic p110 subunit, unlike Vps34p and its mammalian homolog (*14, 18*), can use PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2 as substrates (*20*). Thus, it is a PI-3 kinase rather than a PtdIns-3 kinase.

A role for PI-3 kinase in the endocytic pathway is indicated by the observation that it is cointernalized with the platelet-derived growth factor (PDGF) receptor (a tyrosine kinase receptor) in clathrin-coated vesicles, and that mutations in the PDGF receptor that block its association with PI-3 kinase result in a defect in postendosomal sorting and degradation of this receptor, but not of other proteins (*22*). The receptor internalization step, however, is not affected, which indicates that PI-3 kinase is not involved in clathrin-coated vesicle formation at the plasmalemma. Wortmannin markedly decreases the rate of down-regulation and degradation of the wild-type PDGF receptor, which suggests that the catalytic activity of the enzyme is required for these effects (*23*). Wortmannin also affects fluid phase uptake as well as recycling of transferrin receptors, although different effects are observed in different experimental systems (*24, 25*). These actions are seen at the low concentrations of the drug that are thought to act on the p85-p110 PI-3 kinase, but the involvement of other enzymes that are inhibited by wortmannin, for example a PtdIns-4 kinase (*26*) cannot be excluded.

Recent evidence suggests that inositol-5-phosphatases (5-phosphatases) may play a role in vesicular transport. The family of 5-phosphatases—that is, the enzymes that remove the phosphate in the 5′ position of the inositol ring—includes several members, each of which has its own unique substrate preference. Type I 5-phosphatase selectively dephosphorylates soluble inositol polyphosphates including Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 (*27*). Type II 5-phosphatase dephosphorylates inositol polyphosphates as well as PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 (*27, 28*). Additional 5-phosphatases that dephosphorylate both Ins(1,3,4,5)P_4 and PtdIns(3,4,5)P_3 (*29*) or selectively dephosphorylate PtdIns(3,4,5)P_3 (*30*) have been identified.

A nerve terminal–enriched protein, synaptojanin, which was identified on the basis of its interaction with the Src homology 3 (SH3) domains of Grb2, is closely related to type II 5-phosphatase (*31, 32*). Synaptojanin colocalizes with dynamin in nerve endings, is associated in part with endocytic vesicles, is rapidly dephosphorylated in parallel with dynamin after the stimulation of neurotransmitter release, and (with dynamin) is one of the two major brain proteins that bind the SH3 domain of amphiphysin (*31, 32*). Dynamin is a GTPase that functions in the fission of clathrin-coated vesicles from the plasmalemma, including nerve terminal clathrin-coated vesicles that participate in the recycling of synaptic vesicle membranes (*3, 33*). Amphiphysin is a neuronal protein that is colocalized with dynamin and synaptojanin in nerve terminals (*32, 34*) and also interacts with the α subunit of the clathrin adaptor AP2 (the clathrin adaptor implicated in synaptic vesicle endocytosis) through a region distinct from its SH3 domain (*34*). Amphiphysin contains regions of similarity to the yeast proteins Rvs161p and Rvs167p, and mutations in these proteins affect the actin-based cytoskeleton and produce endocytosis defects (*34, 35*). Synaptojanin may function with dynamin and amphiphysin in synaptic vesicle endocytosis through regulation of the amounts of specific PtdIns metabolites (see below). Strikingly, another type II 5-phosphatase, the OCRL protein, is concentrated in the region of the Golgi complex (*36*), which raises the possibility that synaptojanin and the OCRL protein have corresponding functions in vesicular traffic at the plasmalemma and at the Golgi complex, respectively.

Relation Between Phosphoinositides and GTPases

Several different families of GTPases play important regulatory roles as switches in membrane traffic (*37*). GTPases bind both guanosine diphosphate (GDP) and GTP, and they have different conformational states depending on the nucleotide bound. In their GTP-bound state, they bind specific effectors, which results in the activation of the effector or its recruitment to certain sites. The interconversion between the GDP- and GTP-bound forms is controlled by interaction with accessory proteins, including guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) (*38*). Members of the Sar and ADP ribosylation factor (ARF) branches of the Ras GTPase superfamily participate in vesicle coat recruitment and are required for the formation of carrier vesicles (*39*), whereas members of the Rab branch of the superfamily are required for a later event, perhaps to facilitate the docking of vesicles with the target membrane (*37, 40*). Additional GTPases of the dynamin and heterotrimeric protein families are also implicated in vesicle fission and budding, respectively (*33, 37*).

Several interconnections have emerged between PI metabolism and some of these GTPases or their accessory proteins, which suggests that at least some of the actions of PIs may involve GTPases. ARF1 is a myristylated GTPase that plays a critical role in the assembly of the COP I coat as well as the clathrin coat at the TGN (*2, 39*). Clathrin mediates budding from the TGN of vesicles destined to the lysosome (and possibly to peptide-containing secretory granules), whereas the COP I coat (coatomer) mediates vesicle budding at the interface between the endoplasmic reticulum and the Golgi complex (*1, 2, 41*). Although ARF1 is soluble in its GDP-bound form, it associates with membranes in its GTP-bound form, which indicates that the attachment reaction is coupled to nucleotide exchange. PtdIns(4,5)P_2 stimulates guanine nucleotide exchange and activates ARF (*42*); thus, it could play a role as a cofactor in coat assembly, because the GTP-bound form of ARF1 triggers the attachment of the coat proteins (*43*). During coat assembly, activated ARF1 appears to function in concert with PtdIns(4,5)P_2 to activate an isoform of phospholipase D. This enzyme generates phosphatidic acid (PA), primarily from phosphatidylcholine; the PA then activates a PtdIns(4)P-5 kinase to generate additional PtdIns(4,5)P_2 (*44*). Such a mechanism could be the basis for a local feedback amplification loop in the generation of PtdIns(4,5)P_2 and PA at the expense of phosphatidylcholine, which could result in locally elevated amounts of PtdIns(4,5)P_2 and PA in a membrane bud or vesicular carrier generated by an ARF1-dependent mechanism (*8*). PtdIns(4,5)P_2, together with PA, also stimulates the activity of an ARF GAP (*45*) and hence may provide a system to interrupt the loop by inactivation of ARF. GTP hydrolysis is necessary for uncoating, which in turn is necessary for fusion with the target compartment (*1*).

In this model, a putative key function of ARF, which is required for the assembly of both the COP I coat and the TGN clathrin coat (*1*), is the generation of a high local concentration of PtdIns(4,5)P_2. ARF would therefore act on coat assembly in a catalytic fashion rather than [as previously proposed (*1*)] in a stoichiometric fashion. This possibility is supported by the recent finding that in a cell-free assay, the requirement for exogenous ARF in COP I binding can be replaced by overexpression of phospholipase D (*46*). Howev-

er, these hypothetical models are based exclusively on biochemical studies and must be validated by cell-free vesicle budding studies or studies in vivo. Further consideration of these models will require evidence that vesicle buds have a PI composition that is distinct from the surrounding membrane.

Studies of yeast PtdIns-3 kinase (Vps34p) have suggested that a main function of this enzyme is to generate a membrane patch in the TGN that is enriched in PtdIns(3)P (*14*). Thus, if future studies confirm that the feedback mechanism outlined above plays a physiological role, it will be of interest to further elucidate the functional interplay of PtdIns(4,5)P_2 and PtdIns(3)P in budding from the TGN. Moreover, it will be important to further investigate the involvement of proteins of the Arf family in clathrin-mediated budding from the plasmalemma. Such a role for Arf family proteins has not yet been conclusively proven; ARF6 has been implicated in recycling at the cell surface, but its link to clathrin function remains unclear (*47*).

The Rab proteins are unique within the Ras GTPase superfamily because of their large number (more than 30 in mammalian cells) and their localization to a specific stage of the exocytic or endocytic pathways (*40*). An interaction between PIs and at least one Rab protein, Rab5, has been suggested by a study that showed that a PI-3 kinase may act upstream of this GTPase (*25*). As with the ARF proteins, Rabs are also found in the cytoplasm and the membrane attachment reaction is coupled to nucleotide exchange (*48*). Rab5 participates in the early stages of the endocytic pathway. In its GTP-bound form, it activates fusion between early endosomes and stimulates horseradish peroxidase (HRP) uptake and transferrin endocytosis (*49*). The PI-3 kinase inhibitor wortmannin inhibits HRP and transferrin uptake as well as endosome fusion in vitro, in a pattern similar to the inhibition produced by dominant negative Rab5 mutants. Conversely, the stimulatory effect of constitutively activated forms of Rab5 on endocytosis and endosome fusion is not blocked by wortmannin (*25*). This is consistent with a model in which PtdIns(3,4,5)P_3 acts, either directly or indirectly, to stimulate nucleotide exchange on Rab5. It will be interesting to see whether other Rab proteins, acting on other pathways, are also activated by PtdIns metabolites. To date, there are no data that suggest a direct role for PI metabolites in Rab effector or GAP function. However, clues from the ARF field and several data concerning other members of the Ras superfamily of GTPases (see below) indicate that additional regulatory cross talk might be expected to occur between the Rab GTPases and PIs.

Inositol Polyphosphate–Binding Proteins

On the basis of the prediction that soluble inositol polyphosphates other than $InsP_3$ may have regulatory roles in cell function, possibly through regulation of Ca^{2+} channel subtypes, several groups attempted to identify $InsP_4$, $InsP_5$, and $InsP_6$ (InsPPs) receptors by affinity chromatography. Surprisingly, this search led to the identification of proteins with a role in membrane traffic, although these in vitro results must be interpreted with caution because of the high negative charge of InsPPs. The first InsPP-binding protein identified by this approach was the clathrin adaptor complex AP2, more specifically its α subunit, which was later found to bind Ins(1,4,5)P_3 as well (*50*). The function of AP2 is to recruit clathrin and promote its polymerization at the membrane (*41*). InsPPs also bind to the nerve terminal–specific clathrin adaptor AP180 (*51*) and to the coatomer of the COP I coat (*52*). Moreover, InsPP binding inhibits the clathrin assembly properties of both AP2 (*50*) and AP180 (*51*). It remains to be seen whether InsPPs also bind the AP1 complex, which recruits clathrin to the TGN and is structurally related to AP2.

Another high-affinity InsPP-binding protein was identified in rat cerebellar membranes by affinity chromatography and was found to be identical to synaptotagmin (*53*), a synaptic vesicle protein with a putative role in exocytosis and in clathrin-dependent endocytosis (*54*). Synaptotagmin, a type I transmembrane protein with two cytoplasmic C2 domains, participates in exocytosis through multiple interactions with components of the SNARE complex, including binding to β-SNAP (whose interaction with synaptotagmin is modulated by InsPPs) (*55*) and a Ca^{2+}-dependent interaction with syntaxin (*54*). Synaptotagmin also binds in vitro to AP2 (*54*). The binding of InsPPs to synaptotagmin is mediated by the C2b domain (*53*), which is also the AP2-binding domain and is thus the region of the molecule that is thought to be involved in endocytosis (*54*). Consistent with these findings, InsPPs potently blocked neurotransmitter release when injected presynaptically at the squid giant synapse, and injection of antibodies to the C2b domain blocked endocytosis (*56*).

These affinity chromatography studies imply that soluble phosphorylated inositol metabolites may regulate both vesicle budding and fusion through multiple interactions. It is also possible that these in vitro binding reactions may reflect interactions that in situ are mediated by the head groups of PIs. AP2 binds not only InsPPs but also PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 (*50, 57*). Thus, the physiological ligands for both AP2 and AP180 coatomer and synaptotagmin may be (or may include) PIs.

A new chapter in the elucidation of PI function has been opened by the discovery that PtdIns(4,5)P_2, and possibly other PIs, interact with modules that are present in a variety of proteins, including pleckstrin homology (PH) domains (*58*), phosphotyrosine-binding (PTB) domains (*59*), and Src homology 2 (SH2) domains (*60*). Both PTB and SH2 domains can also bind to protein regions containing phosphorylated tyrosine residues (*59, 60*). Some of these domains are present in proteins that have been implicated in vesicular traffic [for example, dynamin contains a PH domain (*33*)] or in protein transport along the secretory and endocytic pathway (for example, SH2 domains are found in proteins that regulate endocytosis) (*61*). However, the significance of these interactions is clearly not limited to cellular traffic. A consensus binding motif for PI binding (lysine and arginine–rich region) (*62*) has also been identified in a variety of actin-binding proteins (see below).

Nuclear magnetic resonance and crystallographic studies have yielded considerable information about the interaction between the head group of PtdIns(4,5)P_2 and the PH domain (*63, 64*). The PH domain has an electrostatic sidedness with a positively charged face that accommodates the negatively charged phosphate groups and presumably faces the membrane (*63*). The PTB domain appears to have a related structure, which suggests that it may bind in a similar way to the polar group of PIs (*59*). A point that has emerged from structural studies of the PH domain is that its interaction with the head group of the PI appears to be mediated exclusively by its phosphorylated inositol ring (*64*). Thus, the same protein may bind both to InsPPs and to PIs, and (as discussed above) it is possible that in vitro binding reactions of InsPPs may reflect in situ binding of PIs. Physiologically, a competitive interplay of soluble and lipid-bound phosphorylated metabolites of inositol may regulate the association and dissociation of specific proteins with membranes.

A Consensus Theme of Phosphoinositide Action

The finding that a major class of binding molecules for the phosphorylated inositol ring consists of coat proteins that are implicated in the generation of vesicular carriers (the AP2 and AP180 adaptors of clathrin coats and the coatomer COP I coat) is convergent with some of the yeast genetic studies and biochemical studies reviewed above, which indicate local production of specific PIs at sites of vesicle budding. The possibility that repulsive forces between the highly neg-

ative polar heads of PIs may play a role in forcing the membrane to curve into a bud cannot be excluded. However, it seems logical to propose that specific interactions between the phosphorylated inositol ring of PIs and some proteins may represent a mechanism by which certain coat proteins can be recruited to membranes. Although the affinities involved are not thought to be very high (*63*, *64*), these interactions may cooperate with protein-protein interaction mechanisms. The presence of kinases (for example, Vps34p) and phosphatases (for example, synaptojanin) that can alter the phosphorylation of the inositol ring in specific subcellular regions and in specific membranes could provide a mechanism for the generation of membrane patches of unique PI composition, and therefore could account for spatial specificity.

Phosphorylated lipid derivatives of PtdIns may cooperate with membrane proteins in the recruitment of at least certain vesicle coats (COP I and the clathrin coat). The binding of a coat protein to the head group of a phospholipid may also help to orient the coat protein and to facilitate its side-to-side association through homophilic or heterophilic interactions with other proteins to generate the coat. Alteration of the lipid composition of vesicular carriers may also play a role at the fusion step of the transport reaction. Here again, the head groups of PIs may serve to recruit, activate, or modulate the activity of the factors necessary for membrane fusion, including Rab proteins. In addition to its effect on peripheral proteins, the head groups of PIs may also bind to integral membrane proteins. For example, in the case of synaptotagmin, the C2b domain may fold back to bind a PI on the vesicle membrane. This binding may control other interactions of the cytoplasmic domain of the protein by an allosteric mechanism. InsPPs may have a potent inhibitory effect on exocytosis (*56*) because they may interfere with this conformation.

At least in the case of exocytosis and endocytosis, it is also possible that some of the effects of PtdIns metabolites may be indirect and may be mediated by effects on the peripheral cytoskeleton. Strong evidence implicates PIs in the regulation of the actin cytoskeleton, which in turn has been found to regulate exocytosis-endocytosis in a variety of systems. In yeast, mutations in actin and in actin-binding proteins affect both secretion and endocytosis (*35*, *65*). In mammalian cells, stimulation of exocytosis correlates with changes in the organization of the peripheral actin cytomatrix (*66*); conversely, experimental manipulations that affect the peripheral cytomatrix also affect endocytosis (*67*). Several accessory proteins of actin, including profilin, gelsolin, and cofilin, interact with PtdIns(4,5)P_2, and there is evidence that modulation of the amount of PtdIns(4,5)P_2 affects actin function (*68*). Moreover, several studies have suggested a bidirectional control between the Rho family of Ras-like GTPases, which regulate the function of the actin cytoskeleton (*69*), and PI-metabolizing enzymes. This family includes Rho, Rac, and Cdc42. PI-3 kinase activates nucleotide exchange on Rac (*70*). Conversely, the p85 subunit of the p85-p110 PI-3 kinase is a target for the GTP-bound forms of Cdc42 and Rac (*71*), whereas a PtdIns(4)P-5 kinase was reported to be stimulated by Rho (*72*), which in turn can be activated by a GTPase cascade involving Cdc42 and Rac in series (*69*). Local changes in the actin-based cytomatrix may regulate vesicle → target membrane interactions. Moreover, perturbation of the subplasmalemma cytoskeleton to produce membrane ruffles, as produced by manipulations of several Ras superfamily members, may have direct effects on fluid phase endocytosis (*67*) by enhancing the formation of membrane invaginations that give rise to intracellular vacuoles.

Concluding Remarks

Results from a variety of experimental approaches, including biochemistry, cell-free vesicle transport studies, genetics, and immunocytochemistry, suggest that PIs and possibly their soluble metabolites function as important regulators of vesicular traffic. The elucidation of the precise molecular mechanisms by which this regulation is accomplished is still at a preliminary stage, and many in vitro results await validation by experiments on living cells. However, it appears that the inositol ring is a versatile module that can be modified in a combinatorial fashion to generate numerous stereospecific isomers, which in turn can be used in a variety of ways to recruit or activate key transport factors. The possibility of regulating cellular functions by means of phosphorylation and dephosphorylation of lipids or lipid-derived molecules is reminiscent of the regulatory role of the phosphorylation-dephosphorylation of proteins, and the homology between certain lipid kinases and protein kinases indicates that at least some of the enzymes may be evolutionarily related (*73*). The important interrelations that have already emerged between the regulatory role of GTPases in traffic and PI metabolism suggest that these mechanisms may be quite general and point to interesting avenues for future research.

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Targeting of Motor Proteins

Richard B. Vallee and Michael P. Sheetz

Microtubules are responsible for chromosome segregation and the movement and reorganization of membranous organelles. Many aspects of microtubule-based motility can be attributed to the action of motor proteins, producing force directed toward either end of microtubules. How these proteins are targeted to the appropriate organellar sites within the cell, however, has remained a mystery. Recent work has begun to define the targeting mechanism for two well-studied motor proteins, kinesin and cytoplasmic dynein.

A role for microtubules in cell motility has been evident for nearly 50 years since these structures were identified as major components of cilia and flagella. That they function in the sorting of intracellular constituents was first suggested by the presence of these hollow polymers within the mitotic spindle. Depolymerization of microtubules was also found to block the segregation of chromosomes, and there is ample additional evidence that microtubules and their associated proteins are actively involved in many aspects of mitosis.

Evidence for a role for microtubules in sorting and transport of membranous organelles has been slower to emerge. The physiological importance of microtubules in some cases, such as axonal transport, is now evident, where the requirement for rapid, directed transport is obvious (*1*). However, the importance of microtubules and microtubule-based motor proteins in secretion and endocytosis, as well as in other aspects of membrane traffic, is still being elucidated. Certainly, microtubules are required for proper positioning of most, if not all, of the membranous components of the cytoplasm, presumably to facilitate their orderly interaction (*2*). However, depolymerization of microtubules allows most sorting activities to continue, albeit in some cases at slower rates. Such results may reflect a curious circularity inherent in experiments of this type: organelles that may normally be attached to microtubules and require microtubule motors for their transport become free to diffuse throughout the cytoplasm after microtubule depolymerization. Thus, microtubules may be required for aspects of subcellular sorting that have not been clearly revealed by microtubule disassembly experiments.

Additional roles for microtubules and motor proteins are beginning to emerge in concert with our view of membranous organelles as dynamic structures. For example, removal of microtubule motors from cell lysates has been found to inhibit fusion between early and late endosomes (*3*, *4*). There is also direct evidence for a role for microtubule motors in tubular network formation from endoplasmic reticulum membranes in vitro (*5*, *6*). Finally, elongation of membrane tubules of both Golgi and endocytic origin in cells treated with brefeldin A was found to be microtubule-dependent (*7*). Thus, microtubules may have both direct and indirect roles in the sorting of subcellular constituents.

The general principles of how microtubules and their associated motor proteins account for the distribution and redistribution of intracellular structures seem clear. Microtubules are polar polymers, the minus, or slowly polymerizing ends, of which tend to be located toward the cell center, whereas the plus, or rapidly polymerizing ends, tend to be located toward the cell periphery. Microtubules provide a directional track for organelle movement, whereas motor proteins provide the motive force. The first cytoplasmic motor proteins identified were kinesin and cytoplasmic dynein, which produce force toward the plus (*8*) and minus (*9*) ends of microtubules, respectively. Together with knowledge of the organization of microtubules within a given cell, it appeared that microtubule-based motility might be simply explained by the action of these two proteins. It has since become clear that both proteins are members of larger families and that microtubule-based movements within any given cell probably require a variety of different motor proteins. An additional factor critical to microtubule-dependent transport is the proper targeting and activation of motor proteins. Recent research has provided the first in-

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