Phosphoinositides in Constitutive Membrane Traffic

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I. INTRODUCTION

The organelles of the secretory and endocytic pathways in eukaryotic cells have distinct functions, molecular composition, and luminal environment. To achieve this diversity of structure and purpose, the membranes of these organelles must be kept separate for the most part, or they would rapidly fuse and become homogeneous (98, 263). Constitutive membrane traffic is the process by which membrane lipids, integral membrane proteins, and the soluble protein content of membrane organelles are moved from the endoplasmic reticulum (ER) where they are synthesized to the sites where they function. This process requires collecting proteins and lipids that should move from those that should remain behind and packaging them into a transport intermediate (usually thought of as a small vesicle, but this might not be the case in every instance). The transport intermediate fissions from its source membrane and is actively moved to its destination membrane where it fuses and delivers cargo. The process of forming transport intermediates is now understood in some detail (331). Constitutive membrane traffic is very
active, and in mammalian cells, many membrane proteins constantly cycle through multiple organelles on a time scale of tens of minutes. In addition to this continual traffic, there are many instances where the rates and direction of movement of membrane proteins and lipids are rapidly changed in response to signals from the extracellular environment. This “regulated” membrane traffic uses much the same machinery and principles as constitutive traffic. The history of research into the mechanisms of constitutive membrane traffic is that of continual discovery of unexpected levels of complexity and regulation (228). There are many more proteins that function for any one step in membrane traffic and many more levels of regulation than we would have thought necessary. In this aspect, research into membrane traffic resembles research into mechanisms of signal transduction. This is more than coincidence. Membrane traffic is controlled through intracellular signal transduction mechanisms that probably work by the same general principles as those that regulate gene expression in response to environmental cues.

Phosphoinositides were first recognized to be important as intermediates in signal transduction cascades where they serve as second messengers and signal integrators. Subsequently mutations that interfered with membrane traffic were mapped to genes encoding kinases and phosphatases that act on phosphatidylinositol (PtdIns) and phosphoinositides (the phosphorylated derivatives of PtdIns), and the role that these lipids play in membrane traffic began to be appreciated (reviewed in Refs. 53, 64, 66, 70, 74, 217, 219, 294, 295).1 Phosphoinositides (PIPs) are found in unicellular organisms and thus must have appeared quite early in evolutionary history. Whether the phosphorylation of PtdIns to multiple species first arose as part of a mechanism by which cells sensed their external environment, or as part of the machinery for moving membrane proteins between compartments, the molecular strategies for their use are probably similar. Thus it is likely that in membrane traffic PIPs contribute to a complex web of feedback pathways that generate a combinatorial control mechanism, just as they do in signal transduction. This ensures that actions such as vesicle budding or fusion do not occur unless multiple conditions are satisfied, and vesicles do not normally form unless they contain cargo and do not fuse unless they have reached the correct destination.

Currently there are abundant data indicating that phosphatidylinositol 3-phosphate [PtdIns(3)P], phosphatidylinositol 4-phosphate [PtdIns(4)P], phosphatidylinositol 3,5-bisphosphate [PtdIns(3,5)P2], and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P2] play important roles in constitutive membrane traffic. However, the number and diversity of roles that any lipid plays in any step in membrane traffic are not yet known precisely. Proteins known to be essential for membrane traffic bind to PIPs at defined locations in the cell, suggesting that one of the functions of these lipids is to establish membrane identity. The activity of some proteins is modified when they bind a particular PIP, indicating that these lipids can act as allosteric regulators. PIPs are also generated and consumed at locations where lipid bilayers are being sharply curved in the processes of membrane fission and fusion. It has been proposed that the changes in lipid shape that occur as PIPs are phosphorylated or dephosphorylated contribute to this process (43). To complicate matters, PIP species can be interconverted by kinases and phosphatases and can even act as regulators of their own production. Determining which, if any, of these processes is important for a particular step in membrane traffic has been a challenge. In addition, PIPs affect the membrane activity of a number of proteins that may have no direct impact on membrane traffic but are found on the organelles where membrane traffic occurs. There currently is little understanding of how potentially mobile lipids such as PIPs can be restricted to membrane subdomains or how competition among various cytosolic PIP-binding proteins is controlled.

Most of our current knowledge of the role of PIPs in constitutive membrane traffic is based on experiments that show that a particular enzyme activity is required (directly or indirectly) for a specific step in membrane traffic or on the discovery that a protein implicated as functioning in membrane traffic can bind PIPs. Relatively less is known about how the lipids themselves function in this process. Thus I will begin by summarizing what is known about the role in constitutive membrane traffic for enzymes that modify PIPs followed by a summary of the proteins known to be involved in membrane traffic that bind each lipid. I will summarize what is known about the distribution of PIPs in cells and finish with more speculative comments on the possible roles of the lipids. I will borrow concepts from our understanding of signal transduction processes to propose a hypothesis to explain how local production of PIPs might contribute to the generation of a transient membrane microdomain and how this might function in the process of constitutive membrane transport.

Superimposed on constitutive membrane traffic are many examples of regulated membrane transport in which PIPs probably serve multiple functions in both membrane traffic and signal transduction. The task of separating direct functions of PIPs in membrane traffic from indirect conse-

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1 In this review phosphatidylinositol is abbreviated PtdIns. The phosphorylated phosphatidylinositols are referred to as phosphoinositides, abbreviated as PIPs. Kinases that can act on either PtdIns or PIP are abbreviated as PIXK, where X refers to the position on the inositol head group that is modified. Kinases that act only on PIPs and not PtdIns are referred to as PIPXK. Kinases that only phosphorylate PtdIns are abbreviated as PtdInsXK.
quences of downstream signaling cascades is exceedingly difficult. For example, there is an extensive and interesting literature on the effects on membrane traffic that occur when peptide hormone or growth factor receptors activate phosphatidylinositol 3-kinases (63, 71), but it is still unclear if the lipid products of the hormone-activated 3-kinases have a direct effect on endocytosis. For the sake of simplicity, this review is focused on the discussion of aspects of membrane traffic not acutely controlled by extracellular signals. In addition, many genetic studies have identified mutations in proteins that make or bind to phosphoinositides that impact membrane traffic, as well as having pleotropic effects on cytoskeleton or other aspects of cell metabolism. Many of the proteins are undoubtedly interesting, but this review is limited to those for which there is some evidence that they directly affect membrane traffic.

II. ENZYMES THAT MODIFY PHOSPHOINOSITIDES ARE IMPORTANT FOR MEMBRANE TRAFFIC

Although it has been appreciated for 50 years that secretion stimulated by hormones is accompanied by changes in intracellular PIP pools (147), the realization that PIPs were also important for constitutive membrane traffic arose when a mammalian PI 3-kinase was cloned (140, 312) and found to have strong sequence homology to Vps34p, a yeast protein known to be essential for the delivery of proteins to the vacuole in Saccharomyces cerevisiae (137, 334). Subsequently, the PI 3-kinase inhibitor wortmannin was discovered to affect multiple steps in membrane traffic (reviewed in Refs. 36, 295). Although wortmannin inhibits multiple enzymes (68, 250, 253), complicating the interpretation of many experiments, its use pointed the way to more sophisticated investigations employing forward and reverse genetics. It is now clear that a number of enzymes that modify PIPs are required for constitutive membrane traffic and that others probably influence membrane traffic indirectly by modifying the actin cytoskeleton (Table 1). Most recently, some enzymes that participate in signal transduction events have been discovered to also participate in the rapid removal of hormone receptors by endocytosis.

A. PI 3-Kinases and PtdIns 3-Kinases

The kinases that phosphorylate PtdIns or PIPs on the D-3 position can be organized into three classes according to amino acid sequence relationships (82, 106). Although class I PI3Ks can phosphorylate PtdIns, PtdIns(4)P, PtdIns(3)P, PtdIns(3,4)P2, and PtdIns(3,4,5)P3, class II PI3Ks can only phosphorylate PtdIns(4)P, PtdIns(3,4)P2, and PtdIns(3,4,5)P3, and class III PI3Ks can only phosphorylate PtdIns(3)P, PtdIns(3,5)P2, and PtdIns(3,4,5)P3. The enzymes that modify PtdIns and PIPs that function in membrane traffic are listed in Table 1.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Location</th>
<th>Species</th>
<th>Molecular Weight</th>
<th>Substrate</th>
<th>Product</th>
<th>Reference Nos.</th>
</tr>
</thead>
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<tr>
<td>3-Kinases</td>
<td></td>
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<td></td>
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<td></td>
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<tr>
<td>PtdIns3KIII</td>
<td>Golgi, endosomes</td>
<td>Mammalian</td>
<td>101</td>
<td>PtdIns</td>
<td>PtdIns(3)P</td>
<td>106, 367</td>
</tr>
<tr>
<td>VPS34p</td>
<td>Golgi, endosomes</td>
<td>Saccharomyces cerevisiae</td>
<td>101</td>
<td>PtdIns</td>
<td>PtdIns(3)P</td>
<td>313, 333</td>
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<td>PtdIns3KiC2α</td>
<td>Clathrin-coated pits</td>
<td>Mammalian</td>
<td>170</td>
<td>PtdIns, PtdIns(4)P</td>
<td>PtdIns(3,4)P, PtdIns(3,4,5)P3</td>
<td>80, 81</td>
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<tr>
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<td>Mammalian</td>
<td>90</td>
<td>PtdIns</td>
<td>PtdIns(4)P</td>
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<td>S. cerevisiae</td>
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<td>PtdIns</td>
<td>PtdIns(4)P</td>
<td>102, 113</td>
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<td>Golgi, synapse</td>
<td>Mammalian</td>
<td>55</td>
<td>PtdIns</td>
<td>PtdIns(4)P</td>
<td>17, 123, 237</td>
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<td>55</td>
<td>PtdIns</td>
<td>PtdIns(4)P</td>
<td>17, 372, 377</td>
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<tr>
<td>LSB6/PIK2</td>
<td>?</td>
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<td>55</td>
<td>PtdIns</td>
<td>PtdIns(4)P</td>
<td>190, 315</td>
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<td>5-Kinases</td>
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<td>Mammalian</td>
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<td>PtdIns</td>
<td>PtdIns(5)P</td>
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<td>PM</td>
<td>Mammalian</td>
<td>61</td>
<td>PtdIns</td>
<td>PtdIns(5)P</td>
<td>157, 209, 357</td>
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<td>Synapse</td>
<td>Mammalian</td>
<td>87, 90</td>
<td>PtdIns</td>
<td>PtdIns(5)P</td>
<td>158, 382</td>
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<tr>
<td>Mss4</td>
<td>Nucleus, PM</td>
<td>S. cerevisiae</td>
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<td>PtdIns</td>
<td>PtdIns(4,5)P2</td>
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<td>PIKfyve</td>
<td>Late endosomes</td>
<td>Mammalian</td>
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<td>PtdIns</td>
<td>PtdIns(4,5)P2</td>
<td>156, 306, 308, 319</td>
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<td>Vacuole</td>
<td>S. cerevisiae</td>
<td>257</td>
<td>PtdIns</td>
<td>PtdIns(4,5)P2</td>
<td>5, 115, 258, 399</td>
</tr>
</tbody>
</table>

PM, plasma membrane; ER, endoplasmic reticulum; PtdIns, phosphatidylinositol; P, phosphate; P<sub>2</sub>, bisphosphate; P<sub>3</sub>, trisphosphate.
PtdIns(5)P, and PtdIns(4,5)P_2 in vitro, agonists that stimulate their activity mainly generate PtdIns(3,4)P_2 and phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3], two lipids present in very low amounts in quiescent cells. Thus the class I enzymes probably phosphorylate mainly PtdIns(4)P and PtdIns(4,5)P_2 in vivo and are important for signal transduction. Upon activation, platelet-derived growth factor (PDGF) receptors bind PI3K enzymes. Both PI3K binding and enzyme activity are required to sort the receptors into the degradative pathway after internalization (165). However, it is not clear whether this is a downstream effect of signaling through PtdIns(3,4,5)P_3 produced at the plasma membrane or a consequence of generating PIPs on endosomes. Low concentrations of wortmannin and LY294002 inhibit the endocytic traffic of transferrin receptors (220, 321, 329).

Because these concentrations of inhibitors inhibit PI3Ks but not the yeast Vps34p, it was proposed that a class I enzyme might be involved in endocytosis. However, mammalian PI3KIII is inhibited by wortmannin at low nanomolar concentrations (367) and probably was responsible for some of the effects observed. PI3K isoforms bind to regulatory subunits, and one of these, p85α, was identified as part of a protein complex required for the budding of vesicles containing the polyimmunoglobulin receptor from isolated Golgi membranes (170). This budding reaction was inhibited by micromolar wortmannin, a concentration much higher than required to inhibit known class I PI3Ks, and more consistent with inhibition of PI3KII. The identity of the PI3K that might interact with p85α on the Golgi is currently unknown. Thus there is currently no clear evidence for a direct role of PI3K enzymes in constitutive membrane traffic.

Class II PI3Ks can phosphorylate PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2 in vitro. Relatively little is known about the lipids that class II enzymes produce in vivo. However, PtdIns3KIIIC2α is found in clathrin-coated pits at the plasma membrane and on the Golgi (80) and may play a role in membrane traffic (112). The majority of PtdIns(3)P in mammalian cells is produced by class III PI3K (363), the mammalian counterpart of Vps34p (367). PI3KIII phosphorylates only PtdIns to PtdIns(3)P and is more properly called PtdIns3K. This enzyme is required for membrane traffic in the endocytic pathway and probably does not play a role in signal transduction.

1. PtdIns3K and Vps34p

Vps34p associates with a serine/threonine protein kinase, Vps15p, as a heterodimer (334). Binding to Vps15p is required for Vps34p to associate with membranes. Human PtdIns3K binds a 150-kDa homolog of Vps15p and in this heterodimeric form associates with, and is activated by, phosphatidylinositol transfer protein (PITP) (264). PITP plays a role in a number of different membrane traffic events (52, 179), perhaps by transferring substrates to the kinases. Vps34p was first identified as a protein required for the sorting of vacuolar enzymes into the pathway leading from the Golgi to the vacuole in S. cerevisiae (137, 312, 334). It was assumed that the site of action of the enzyme was at the Golgi, since in the absence of Vps34p vacuolar proteins were not sorted correctly and were secreted. Additional evidence supporting a Golgi location for Vps34p/PtdIns3K was provided by treating mammalian cells with wortmannin. Wortmannin inhibited the delivery of cathepsin D to lysosomes (28, 72) apparently through an effect on the sorting of cargo into vesicles rather than through an inhibition of vesicle budding. Wortmannin had only a minor effect on the production of small vesicles from trans-Golgi network (TGN) membranes but prevented the mannose 6-phosphate receptor from entering into vesicles (110). However, yeast Vps34p or mammalian PtdIns3K have not been shown to be located on Golgi or TGN membranes. As is described in more detail below, the proteins that have been discovered to bind PtdIns(3)P are all located on endocytic membranes. In fact, the phenotypes observed when PtdIns3K activity is inhibited do not require that the enzyme act at the TGN. If PtdIns3K generated on endosomes was necessary for the recycling of sorting receptors from endosomes to the Golgi, then loss of PtdIns3K function would cause the observed phenotypes at the Golgi as the sorting receptors became trapped in endosomes.

2. PtdIns3KIIIC2α

PtdIns3KIIIC2α has recently been reported to localize in clathrin-coated structures at the plasma membrane and TGN (80, 112). This enzyme binds to clathrin through amino-terminal sequences, and this derepresses enzymatic activity (112). Overexpression of PtdIns3KIIIC2α in COS cells inhibited internalization of transferrin receptors and prevented accumulation of mannose 6-phosphate receptors in the TGN, presumably by inhibiting the uncoating of clathrin-coated vesicles (112). Previously, it was observed that PtdIns(3)P and, to a lesser extent, PtdIns(3,4)P_2 enhanced the binding of AP2 adaptors to peptides containing internalization signals. Clathrin binding enhanced the affinity of AP2 for internalization signals to a similar extent, but the effect of clathrin and PtdIns(3)P were not additive (286). However, when fluorescent proteins that bind PtdIns(3)P are expressed in cells, they label endosomes and not the plasma membrane (32, 92, 318), suggesting that there is little free PtdIns(3)P at the cell surface. There is a recent report that PtdIns3KIIIC2α localizes to the nucleus rather than the plasma membrane (77). More work is required to determine how PtdIns3KIIIC2α functions in membrane traffic and to what extent PtdIns(3)P is involved in membrane traffic from the plasma membrane.
B. PtdIns 4-Kinases

Two distinct classes of kinases phosphorylate PtdIns at the D-4 position. These are called type II and type III (type I was discovered to be a PtdIns 3-kinase). Type III enzymes are homologous to two yeast PtdIns4Ks, PIK1 (102) and STT4 (405). The mammalian ortholog of STT4 is PtdIns4KIIα (251, 391) and the ortholog of PIK1 is PtdIns4KIIβ (12, 233, 250). In yeast, STT4 and PIK1 do not compensate for each other in deletion studies, with STT4 functioning in regulation of the actin cytoskeleton not compensating for each other in deletion studies, with STT4 functioning in regulation of the actin cytoskeleton (17, 233, 250). STT4 was identified in a screen for mutations in S. cerevisiae that prevent amino phospholipid transport to the Golgi (358), and its mammalian counterpart, PtdIns4KIIα, is reported to localize at the ER (251). However, currently there is no evidence that the effect of STT4 on lipid traffic is direct. By extension, it is possible that mammalian PtdIns4KIIα is not involved in membrane traffic.

Type II PtdIns4Kα and -β were only recently cloned (17, 237). These enzymes do not have the signature PIK domain and belong to a family of lipid kinases distinct from the other PtdIns3/PtdIns4 kinases. PtdIns4KIIα is a major contributor to cellular PtdIns(4)P levels and is located on the Golgi where it plays a role in membrane traffic from the TGN (372). PtdIns4KIIβ is a cytosolic protein that is recruited to plasma membrane and activated by Ras1 and may have no role in membrane traffic (377). In S. cerevisiae, Lsb6p is the ortholog of mammalian PtdIns4KII enzymes (130, 315).

1. PtdIns4KIIβ/PIK1

Pik1p is an essential 125-kDa enzyme of S. cerevisiae (102) found both in the nucleus and on Golgi membranes (370). The phenotype of pik1Δ yeast grown at nonpermissive temperature is similar to the phenotype produced by a conditional loss of ARF function (9). Overexpression of Pik1p suppresses the defect in secretion in yeast expressing a temperature-sensitive allele of Sec14, the yeast PTP (129). The mammalian Pik1p counterpart, PtdIns4KIIβ, is a 90-kDa enzyme that has been localized to Golgi membranes (120, 392). PtdIns4KIIβ enzyme activity is stimulated in vitro by ARF1 (120), suggesting that it is an effector of ARF for membrane traffic. Overexpression of mammalian frequentin, the homolog of an activator of Pik1p in S. cerevisiae (136), stimulates the delivery of a reporter protein to the apical surface in polarized cells (379). Both the PtdIns4KIIβ and Pik1p behave as soluble proteins (102, 392), suggesting that their association with membranes must be dynamic and regulated. PtdIns4KIIβ is inhibited by 50–100 nM wortmannin (233), but Pik1p is highly resistant to this inhibitor.

PtdIns4K activity is found on chromaffin granules and on small synaptic vesicles and is required for vesicle fusion (386, 387). PtdIns4K activity is also found on vesicles containing the Glut4 glucose transporter isolated from muscle (190). The identity of the enzymes responsible for these activities is not known.

2. PtdIns4KII/PIK2

Biochemical studies indicate that type II PtdIns4K is responsible for much of the PtdIns 4-kinase activity in response to extracellular signal transduction (106). There are two isoforms of the mammalian enzyme (17, 237, 372, 377). PtdIns4KIIα is located on perinuclear membranes that include the Golgi and on synaptic membranes (123, 372). Overexpression of PtdIns4KIIα in fibroblasts has no effect on transport of a reporter from the ER to the Golgi but does stimulate transport from the TGN to plasma membrane (372). Knockout of PtdIns4KIIα by small interfering RNA oligonucleotides causes AP1 clathrin coats to be released from Golgi membranes, and the Golgi to fragment. Export of a viral glycoprotein from the TGN is also inhibited. The binding of AP1 to Golgi in these cells can be rescued by replacing PtdIns(4)P but not by replacing PtdIns(4,5)P2. However, the glycoprotein transport defect is rescued by both lipids. Thus the effect of PtdIns4KIIα appears to be to produce PtdIns(4)P, which is directly recognized by coat proteins at the TGN and also serves as a precursor of the PtdIns(4,5)P2 required for membrane transport to the plasma membrane (372).

A PtdIns4KII activity coprecipitates with CD63, a tetrascarpin protein found mainly on lysosomes (50, 231), and is also found in plasma membrane lipid rafts, although it is not enriched in rafts that contain caveolin (376). The single yeast homolog of mammalian PtdIns4KII is Lsb6, now renamed PIK2. This gene is nonessential (130, 315), indicating that it is not required for the functioning of the secretory pathway, although it still might have a more specialized role in secretory processes. The functions of endosomes and the vacuole are not essential for yeast to grow in the laboratory, so a role of PIK2 in endocytic processes is still possible.

C. PIP 5-Kinases

PIP 5-kinases were originally purified as activities that phosphorylated PtdIns(4)P to PtdIns(4,5)P2 and subsequently cDNAs for six enzymes have been cloned (25, 37, 157, 159, 209). Based on sequence relationships, the PIP5Ks were grouped into two families called types I and II. Subsequently, it was realized that type II kinases phosphorylated the D-4 position and not D-5 and that both type I and II enzymes also phosphorylated the D-3 position (283, 409). Thus the PIP5KII proteins are more accurately called PIP4Ks. The α- and β-type I kinases were shown to...
phosphorylate PtdIns to PtdIns(5)P in vitro (357) and could properly be called PI5Ks. However, it is not clear if they produce PtdIns(5)P in vivo. Due to this uncertainty and because the type I enzymes are most often referred to as PI5Ks in the literature, that term will be used in this review. Three human PI5K enzymes have been identified, and all are related to the MSS4p kinase of *S. cerevisiae*. As major producers of PtdIns(4,5)P<sub>2</sub>, the PI5Ks play important roles in membrane traffic; however, the precise roles played by the various isoforms of these enzymes are not yet clear. *S. cerevisiae* has a second PI 5-kinase, FAB1, that has recently been shown to phosphorylate PtdIns(3)P to produce PtdIns(3,5)P<sub>2</sub> (57, 115). The mouse ortholog of this enzyme has been cloned and named PIKfyve (306, 320). PtdIns(3,5)P<sub>2</sub> is necessary for proper membrane traffic to the yeast vacuole; thus PIKfyve is very likely to play a similar role in mammalian cells.

1. **PI5K<sub>1</sub>α, PI5K<sub>1</sub>β, PI5K<sub>1</sub>γ, and MSS4p**

The mammalian PI5Ks α- and β-isoforms were cloned independently from human or mouse by two laboratories and named in a reciprocal manner (157, 209). A third isoform of PI5K<sub>1</sub>, γ, has also been cloned from both species (158). Unfortunately, the reciprocal nomenclature for isoforms α and β has been perpetuated in the genome sequence databases for human and mouse. This complication of the nomenclature means that one must be careful to note the species of enzyme used in any study. To simplify the process of specifying which gene product has a particular function, for the purpose of this review I will adopt the human genomic nomenclature where PIK5IA indicates human PI5K<sub>1</sub>α and murine PI5K<sub>1</sub>β, PI5KIB is human PI5K<sub>1</sub>β and murine PI5K<sub>1</sub>α, and PI5KIC indicates PI5K<sub>1</sub>γ.

PI5KIA and B are 61-kDa proteins, and C is larger and has two forms, 87 and 90 kDa, due to alternative splicing. The kinase domain of PI5Ks is comprised of ~400 amino acids located centrally and is 80% identical among all three proteins. Phosphatidic acid (PA) has been shown to stimulate these enzymes (164, 243), and all three of the PI5Ks are activated ~10-fold by this lipid (158). A major source of PA is through the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P<sub>2</sub>) by phospholipases D<sub>1</sub> and D<sub>2</sub>, enzymes that are themselves activated by PtdIns(4,5)P<sub>2</sub> (326). In vitro the PI5Ks can phosphorylate phosphoinositides other than PtdIns(4)P and will convert PtdIns(3,4)P<sub>2</sub> to PtdIns(3,4,5)P<sub>3</sub> and PtdIns(3)P to both PtdIns(3,5)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub> (106, 356, 409).

The regulation of PI5Ks is complicated. These enzymes can be precipitated in complexes that contain the small G proteins Rho and Rac (46, 289, 355), although it is not known if this is a direct interaction or involves additional proteins. PtdIns(4,5)P<sub>2</sub> is an important regulator of the actin cytoskeleton, and overexpression of PI5KIB causes massive actin polymerization that is not prevented by coexpression of a dominant negative form of RhoA, suggesting that the lipid kinase is downstream of Rho (316). Recently an activator of PI5KIB was purified and found to be the small G protein Arf1 (151). Arf1 has many activities that are important for membrane traffic (293). Purified Arf1, Arf5, and Arf6, but not RhoA or Rac1, could stimulate purified PI5KIB in vitro, and this stimulation required PA. However, only Arf6 colocalized with PI5KIB at the plasma membrane in vivo and is likely to be the regulator of PI5KIB relevant to regulation of the actin cytoskeleton (27, 39, 151). Although an activated Rac1 allele stimulated membrane ruffles and colocalized with Arf6 and PI5KIA, membrane ruffles were prevented when a dominant negative form of Arf6 was expressed (151). Thus current data suggest that Arf6 may be downstream of RhoA and Rac1 and interact directly with PI5KIB at the plasma membrane.

In addition to regulation by small G proteins, PI5KIB is regulated by phosphorylation (260). PI5K isolated from *Saccharomyces pombe* membranes is also regulated by phosphorylation by Ckt1, a casein kinase I ortholog (302). All three mammalian PI5Ks will auto-phosphorylate in vitro, and this is stimulated by PtdIns but not other phosphoinositides (160). In all cases documented so far, phosphorylation suppressed PI5K activity.

PtdIns(4,5)P<sub>2</sub> is important for many aspects of membrane traffic including endocytosis, synaptic vesicle fusion and recycling, regulated exocytosis, phagocytosis, and vesicle formation at the Golgi (53, 66, 119, 171, 217). In most cases, systematic comparisons of the PI5K and PI4K isoforms that might be responsible for producing PtdIns(4,5)P<sub>2</sub> for these activities have not been performed. Overexpression of wild-type PI5KIB, but not PI5KIB, stimulates internalization of the EGF receptor, and overexpression of a kinase dead mutant blocks EGFR endocytosis (14). However, overexpression of PI5KIB and to a lesser extent PI5KIA enhances endocytosis of the transferrin receptor in a different cell type (262). Knock-down of PI5KIB by siRNA, but not knock down of PI5KIA or PI5KIC, inhibits endocytosis of transferrin. Interestingly, expression of the C isoform was increased when transcription of either A or B was inhibited, but this did not result in rescue of endocytosis rates or a change in total cellular PtdIns(4,5)P<sub>2</sub> levels, suggesting that activity of the enzyme is regulated at a posttranscriptional level (262). PI5KIB can be recruited on to Golgi membranes where it is directly activated by Arf1 (168). PI5KIC is the major PI5K in synapses and is an important regulator of the recycling of synaptic vesicles (78, 382).

MSS4 encodes the PI5K of *S. cerevisiae* (76, 150) and is an essential gene (388). Acute loss of MSS4p func-
tion causes alterations in the actin cytoskeleton but not in secretion. Currently there are no data supporting a role for PtdIns(4,5)P$_2$ for secretion in S. cerevisiae, in contrast to a requirement for PtdIns(4)P (129, 370). There is indirect evidence for a role for PtdIns(4,5)P$_2$ in the internalization step in endocytosis in yeast (154), and by extension, a role for Mss4p in that process.

2. Fab1 and PIKfyve

Fab1p is the PI3P 5-kinase in S. cerevisiae and is required for maintenance of the vacuole, although not for membrane traffic to the vacuole (57, 115, 399). A mouse homolog of FAB1 has been cloned (306). Sequence homology searches reveal that the human Fab1 ortholog PIKfyve is encoded by a single gene on chromosome 2. Both PIKfyve and Fab1p contain a domain called a FYVE domain (see below) that binds to PtdIns(3)P, and in the case of PIKfyve, is required for the enzyme to locate on endosomes (308). PIKfyve will phosphorylate PtdIns or PtdIns(3)P to PtdIns(5)P or PtdIns(3,5)P$_2$, respectively, and also has intrinsic protein kinase activity (307). Like the PI5Ks, PIKfyve phosphorylates itself, and this inhibits its lipid kinase activity (307). Overexpression of a mutant PIKfyve able to produce PtdIns(5)P but unable to produce PtdIns(3,5)P$_2$ causes extensive vacuolization of cells that is rescued by microinjecting them with PtdIns(3,5)P$_2$ but not PtdIns(5)P (156). Thus PtdIns(3,5)P$_2$ appears to be a product of PIKfyve in vivo required for endosome function.

D. PIP 4-Kinasess

Three 48-kDa kinases have been identified that phosphorylate phosphatidylinositol 5-phosphate. PIP4K$_\alpha$ was originally purified as an activity that phosphorylated a commercial PtdIns(4)P preparation to PtdIns(4,5)P$_2$ (18, 208) and was named PIP5K type II. After this and a related enzyme, PIP5K type II$\beta$, were cloned (25, 37, 79), it was discovered that the enzymes actually phosphorylated the D-4 position (283). The actual substrate in the bovine brain PtdIns(4)P that was used for the original purification of the enzymes was a previously unidentified lipid, PtdIns(5)P. Thus these enzymes should be called PIP4Ks. PIP4K$_\alpha$ can phosphorylate PtdIns(3)P and PtdIns(5)P but does not make PtdIns(3,4,5)P$_3$ (283, 409) and will not phosphorylate PtdIns (106). The PIP4Ks are also not stimulated by PA (164). Currently little is known about the roles of these enzymes in cells or if they impact membrane traffic in any way. PIP4K$\beta$ partially localizes to the nucleus (49). A third member of this family, PIP4K$\gamma$, has been identified as resident in the ER (159).

E. Phosphatases Acting on Phosphoinositides

As one would expect, cells not only generate PIPs through phosphorylation but also consume or interconvert them through the action of phosphatases. Many different enzymes have been identified that specifically remove phosphate from one or more of the positions on the inositol ring (155, 213, 240). There is currently evidence for a role in membrane traffic for enzymes that remove phosphate from the D-5 position of PtdIns(4,5)P$_2$, the D-5 position of PtdIns(3,5)P$_2$, the D-3 position of PtdIns(3,5)P$_2$ and that remove the phosphate from both PtdIns(4)P and PtdIns(3)P (Table 2). It is likely that enzymes that specifically hydrolyze phosphate from PtdIns(3,4)P$_3$ or PtdIns(3,4,5)P$_3$ play roles in signal transduction, but not directly in membrane traffic. There is currently not much information about enzymes that remove phosphate only from the D-4 position of PtdIns(4)P, and S. cerevisiae does not have homologs to the currently identified mammalian enzymes. It is likely that the important function of degrading PtdIns(4)P in S. cerevisiae is performed by Sac1p.

1. Sac family phosphatases

The first lipid phosphatases shown to have a role in membrane traffic contain a domain originally recognized in the yeast protein Sac1p. One group of these enzymes contains only a NH$_2$-terminal Sac domain, and the second class contains an additional, 5-phosphatase domain in the center of the protein followed by various other domains (155). Sac1p was identified in a screen for mutations that relieved the block in secretion caused by loss of activity of the S. cerevisiae PITP, Sec14p (51, 385), and was subsequently discovered to have PIP phosphatase activity (47, 124). In S. cerevisiae there are two such proteins, Sac1p and Fig4p, and in humans three, KIAA0274, KIAA0966, and KIAA0851. Sac1p localizes to the ER and Golgi (311, 385), and sac1$\Delta$ cells contain 10-, 2.5-, and 2-fold increases in PtdIns(4)P, PtdIns(3,5)P$_2$, and PtdIns(3)P, respectively (124). In the ER, Sac1p is necessary for ATP import into the lumen (224), but it also has important phosphatase function antagonizing the PtdIns 4-kinase Pik1p in the Golgi. Fig4p is induced by pheromone and required for actin polarization during mating (96). KIAA0274 is probably the human ortholog to yeast Sac1p, and KIAA0851 is probably the Fig4p ortholog. KIAA0966 is a large protein of unknown function. The catalytic Sac domains of all these proteins except KIAA0966 hydrolyze PtdIns(3)P, PtdIns(4)P, and PtdIns(3,5)P$_2$, but not PtdIns(4,5)P$_2$ (155). KIAA0966 is a 5-phosphatase with preference for PtdIns(4,5)P$_2$ over PtdIns(3,4,5)P$_3$ (236).

The second group of Sac domain phosphatases includes the yeast proteins Ins51p (Slj1p), Imp52p (Slj2p),
and Inp53p (Slj3p) and mammalian proteins synaptojanin 1 and 2. The phenotypes of cells with null mutations in either INP51, INP52, or INP53 are relatively normal, but deletion of all three is lethal (332, 343). Ins51p is a 5-phosphatase specific for PtdIns(4,5)P2, and its Sac domain is inactive (124). INS51 has genetic interactions with PAN1, a protein required for endocytosis and regulation of actin (380). Inp52p and Inp53p have both 5-phosphatase activity as well as Sac domain phosphatase activity and are involved in regulating actin patches at the plasma membrane (PM) (260, 338). Disruption of INP53, but not INP51 or INP52, causes sorting defects at the TGN and increases the rate of transport of reporter proteins to the vacuole (19, 125). This is due to a defect in the pathway between the TGN and early endosomes that requires AP1/clathrin (126). This defect is more severe in an inp52Δ inp53Δ mutant and is complemented by a mutant inp52 lacking Sac phosphatase activity, indicating that it is the excess PtdIns(4,5)P2 that is the primary problem in these cells (338). Double mutants inp51Δ inp52Δ or inp52Δ inp53Δ, but not inp51Δ inp53Δ, have defects in endocytosis and disruption of the actin cytoskeleton (327, 332, 343). This suggests that Inp52p has overlapping function with the other two enzymes. In inp51Δ inp53Δ inp52 alleles at nonpermissive temperature, PtdIns(4,5)P2 is detected on internal membranes where it is normally not detected (338). Thus these enzymes control the location of PtdIns(4,5)P2 in S. cerevisiae.

Synaptojanin 1 and 2 are dual-function mammalian phosphatases that contain a 5-phosphatase activity as well as a Sac domain and convert PtdIns(4,5)P2 to PtdIns (124). This ability to decrease PtdIns(4,5)P2 without producing the potentially active intermediate PtdIns(4)P may be important for maintaining spatial segregation of PIPs (discussed below). Synaptojanin 1 is found in nerve terminals associated with membranes coated with clathrin (127, 227). Mice deficient in synaptojanin 1 (67, 181) and mutants in Unc26, the C. elegans synaptojanin ortholog (134), have neurological defects and nerve endings that accumulate coated vesicles, suggesting that there is a defect in vesicle uncoating. Synaptojanin 2 has a broader tissue distribution and differs from synaptojanin 1 at the COOH terminus (254). Synaptojanin 2 is reported to be an effector for the small GTPase Rac1, and overexpression

### Table 2. PIP phosphatases that function in membrane traffic

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Cellular Location or Ortholog</th>
<th>Species</th>
<th>Molecular Weight</th>
<th>Substrate</th>
<th>Product</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>5-Phosphatases</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OCRL</td>
<td>Golgi</td>
<td>Mammalian</td>
<td>75</td>
<td>PtdIns(4,5)P2</td>
<td>PtdIns(4)P</td>
<td>87, 344, 360, 407, 408</td>
</tr>
<tr>
<td>Synaptojanin 1</td>
<td>Clathrin coats (nerve terminals)</td>
<td>Mammalian</td>
<td>145</td>
<td>PtdIns(4,5)P2</td>
<td>PtdIns(4)P</td>
<td>124, 127, 227</td>
</tr>
<tr>
<td>Synaptojanin 2</td>
<td>Clathrin coats (ubiquitous)</td>
<td>Mammalian</td>
<td>138</td>
<td>PtdIns(4,5)P2</td>
<td>PtdIns(4)P</td>
<td>124, 254</td>
</tr>
<tr>
<td>Inp51p (Slj1p)</td>
<td>?</td>
<td>S. cerevisiae</td>
<td>108</td>
<td>PtdIns(4,5)P2</td>
<td>PtdIns(4)P</td>
<td>19, 124–126, 260, 338</td>
</tr>
<tr>
<td>Inp52p (Slj2p)</td>
<td>?</td>
<td>S. cerevisiae</td>
<td>118</td>
<td>PtdIns(4,5)P2</td>
<td>PtdIns(4)P</td>
<td></td>
</tr>
<tr>
<td>Inp53p (Slj3p)</td>
<td>TGN</td>
<td>S. cerevisiae</td>
<td>124</td>
<td>PtdIns(4,5)P2</td>
<td>PtdIns(4)P</td>
<td></td>
</tr>
<tr>
<td>In54p</td>
<td>ER</td>
<td>S. cerevisiae</td>
<td>48</td>
<td>PtdIns(4,5)P2</td>
<td>PtdIns(4)P</td>
<td>389</td>
</tr>
<tr>
<td><strong>Sac domain phosphatases</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KIAA0274</td>
<td>Sac1 ortholog</td>
<td>Mammalian</td>
<td>100</td>
<td>PtdIns(3)P, PtdIns(4,5)P2</td>
<td>PtdIns(4)P</td>
<td>155, 236</td>
</tr>
<tr>
<td>KIAA0966</td>
<td>?</td>
<td>Mammalian</td>
<td>100</td>
<td>PtdIns(4,5)P2</td>
<td>PtdIns(4)P</td>
<td></td>
</tr>
<tr>
<td>KIAA0851</td>
<td>Fig4 ortholog</td>
<td>Mammalian</td>
<td>65</td>
<td>PtdIns(4,5)P2</td>
<td>PtdIns(4)P</td>
<td></td>
</tr>
<tr>
<td>Sac1p</td>
<td>ER, Golgi</td>
<td>S. cerevisiae</td>
<td>71</td>
<td>PtdIns(4,5)P2</td>
<td>PtdIns(4)P</td>
<td>47, 51, 124, 385</td>
</tr>
<tr>
<td>Fig4p</td>
<td>Vacuole?</td>
<td>S. cerevisiae</td>
<td>102</td>
<td>PtdIns(3)P, PtdIns(4,5)P2</td>
<td>PtdIns(4)P</td>
<td>96, 114</td>
</tr>
<tr>
<td><strong>3-Phosphatases</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTM1</td>
<td>?</td>
<td>Mammalian</td>
<td>66</td>
<td>PtdIns(3)P</td>
<td>PtdIns(4)P</td>
<td>309, 350</td>
</tr>
<tr>
<td>MTMR2</td>
<td>?</td>
<td>Mammalian</td>
<td>71</td>
<td>PtdIns(3)P</td>
<td>PtdIns(4)P</td>
<td></td>
</tr>
<tr>
<td>MTMR3</td>
<td>?</td>
<td>Mammalian</td>
<td>128</td>
<td>PtdIns(3)P</td>
<td>PtdIns(4)P</td>
<td></td>
</tr>
<tr>
<td>MTMR6</td>
<td>?</td>
<td>Mammalian</td>
<td>68</td>
<td>PtdIns(3)P</td>
<td>PtdIns(4)P</td>
<td></td>
</tr>
<tr>
<td>Ymr1p (YJR110w)</td>
<td>?</td>
<td>S. cerevisiae</td>
<td>80</td>
<td>PtdIns(3)P</td>
<td>PtdIns(4)P</td>
<td>350</td>
</tr>
</tbody>
</table>

TGN, trans-Golgi network. Other definitions are as in Table 1.
of activated Rac1 or a synaptotagmin 2 targeted to membranes inhibits endocytosis (214). Depletion of synaptotagmin 2, but not synaptotagmin 1, by small interfering RNA decreased internalization of epidermal growth factor (EGF) and reduced numbers of coated pits in lung carcinoma cells (209).

2. Other 5-phosphatases

Inp54p, the fourth 5-phosphatase enzyme in S. cerevisiae, lacks a sac domain and has a single 5-phosphatase domain. It localizes to the ER, and its deletion increases the rate of secretion of a reporter, indicating that it functions directly or indirectly in secretion (389). In mammals there are a number of other 5-phosphatases, most of which have been investigated for roles in signal transduction or apoptosis, but not membrane traffic. However, the OCRL gene product that is defective in the human disease Lowe syndrome is a 5-phosphatase that localizes to the TGN (87, 344, 408). Kidney cells from patients with Lowe syndrome have elevated PtdIns(4,5)P2 levels (407) as well as elevated serum levels of lysosomal enzymes (360), raising the possibility that there is a defect in sorting proteins at the TGN.

3. 3-Phosphatases

PTEN/MMAC1, myotubularin, and myotubularin-related proteins are phosphatases specific for the D3 position of phosphoinositols (212). PTEN hydrolyzes PtdIns(3,4,5)P3 and is a negative regulator of signaling. It probably has no direct role in membrane traffic. Myotubularin (MTM1) was identified as the defective gene in X-linked myotubular myopathy, a defect in muscle development. Mutations in a related gene, MTMR2, cause type 4B Charcot-Marie-Tooth syndrome. There are eight myotubularin-related genes in humans and one in S. cerevisiae (YMR1). Recombinant MTM1, MTMR2, MTMR3 (KIAA0371), MTMR6, and Ymr1p proteins are active PtdIns(3)P phosphatases that also hydrolyze PtdIns(3,5)P2 (309, 350). The activity of MTM1 toward PtdIns(5)P in vitro is 200-fold less than for PtdIns(3)P, and other phosphoinositides are significantly poorer substrates (350). The physiological function of MTM family members is not known. However, MTMR3 contains a COOH-terminal FYVE domain for binding to PtdIns(3)P, as do a number of proteins that bind to endosomes, and may be a candidate for an enzyme responsible for controlling the amount and/or location of that lipid on endosomal membranes.

III. PHOSPHOINOSITIDE-BINDING MODULES THAT FUNCTION IN CONSTITUTIVE MEMBRANE TRAFFIC

The second discovery suggesting that phosphoinositides might be important for regulating constitutive membrane traffic was the realization that some proteins known to be required for membrane traffic contained a motif, the pleckstrin homology (PH) domain, known to bind PtdIns(4,5)P2. Subsequently, three additional motifs, the FYVE, ENTH, and PX domains, were identified in many proteins including some that had been identified through screens for mutations that affected membrane traffic in S. cerevisiae. These modules were subsequently shown to bind phosphoinositides. In certain proteins, these lipid-binding domains show great specificity for one phosphoinositide, but in most cases, they will bind to more than one species. A current unresolved problem is that in vitro many of these modules will show higher affinity for PtdIns(3,4,5)P3 than other PIPs. However, it is unclear if this difference in affinity is enough to be meaningful in the cell, where PtdIns(3)P, PtdIns(4)P, and PtdIns(4,5)P2 are 50–1,000 times more abundant than is PtdIns(3,4,5)P3.

A. PH Domains

The PH domain was first identified as a sequence motif of 100–120 amino acids that was found in many signaling proteins as well as in the cytoskeletal protein spectrin (135, 223, 246). In phosphoinositide-specific phospholipase C-81 (246), the PH domain was located in the region previously shown to bind to PtdIns(4,5)P2 (48). Subsequently the PH domains from several proteins were found to bind specifically to liposomes containing PtdIns(4,5)P2 (133). Primary sequence conservation among different PH domains is only 7–30%, but the structures that have been solved are quite similar. PH domains have a seven-stranded, antiparallel β-sheet that is twisted to fold back on itself as an orthogonal sandwich (100, 204, 288, 303). Most PH domains have strong charge polarity with one edge of the curved sheet much more positive than the other. The positive side interacts with the negative head group of the lipid. The affinity and specificity with which different PH domains bind phosphoinositides vary greatly. Most have only weak affinity (K_D of 30–40 μM) (133, 303), but some bind 10–1,000 times more tightly, especially those specific for PtdIns(3,4,5)P3 (200).

The concentration of PtdIns(4,5)P2 in neutrophil plasma membranes has been estimated as 3–5 mM and that of PtdIns(3,4,5)P3 at 5 μM (basal) to 200 μM (after stimulation) (341). Thus, unless a PH domain exhibits 25- to 1,000-fold greater affinity for the PtdIns(3,4,5)P3 than PtdIns(4,5)P2, it is unlikely to bind selectively in the cell. A K_D in the range of 30 μM means that stable binding to membranes by low-affinity PH domains should require additional interactions and experimental evidence supports this. In many if not most cases, stable membrane binding of proteins that contain PH domains involves interaction with other segments of the same protein, or...
interaction with additional proteins (184, 196–198). Binding to phosphoinositides has not been observed to alter the conformation of the PH domain (133), although it may change the interaction between the PH domain and another domain in the same protein (23).

Of the 251 PH domains identified in the human proteome, 20 or so have a conserved motif that allows high-affinity binding to PtdIns(3,4,5)P3 or PtdIns(3,4)P2 (100, 204) and probably function in signal transduction (200). Many of the remaining proteins do not yet have known functions, and some of these may contribute to membrane traffic. The proteins known to function in membrane traffic that contain PH domains include the three dynamin GTPases, some of the guanine nucleotide exchange proteins for Arf family members, some of the GTPase activating proteins for Arf, two kinesin motor proteins, and several lipid modifying enzymes (Table 3). Additional information on PH domains and the proteins that contain them can be obtained from recent review articles (23, 200, 278, 303).

### B. PX Domains

PX, or phox (phagocyte oxidase), homology domains are found in two subunits of NADPH-oxidase, in PI3KC2γ and in a family of small proteins called sorting nexins (SNX) (128, 277). They are also found in certain other yeast proteins known to be required for protein traffic between the Golgi and endosomes (91, 153, 368) and in phospholipase D1 (PLD1) and phospholipase D2 (PLD2) (105) (Table 4). The SNX proteins were key to understanding the function of the PX domain. The first sorting nexin family member, SNX1, had been identified as a protein that bound to the EGF receptor and that had sequence homology to a yeast protein known to function in membrane traffic to the vacuole (192). Subsequently, a number of SNX proteins have been shown to associate with specific membrane receptor proteins as part of an oligomeric complex that regulates sorting of receptors between recycling and degradation pathways (128, 153, 342). SNX proteins appear to associate into complexes with other SNX proteins, as well as with adaptor proteins that bind to receptors and to clathrin (193, 206, 211, 267).

The fraction of the genome devoted to SNX proteins in S. cerevisiae is threefold greater than it is in the human genome. Therefore, most SNX proteins probably function in evolutionarily conserved processes common to most cell types. Evidence for the functions of SNX proteins currently is dominated by results of experiments in which wild-type or mutant forms are overexpressed as dominant negative inhibitors of endocytosis. Because overexpression of one member of a family of proteins can affect processes unrelated to the normal function of that protein, more work is needed to determine where each human SNX protein functions.

There are 31 human genes predicted to encode proteins with PX domains and 15 in S. cerevisiae. PX domains from different proteins have been shown to

<p>| Proteins containing PH domains known or suspected to function in membrane traffic |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|</p>
<table>
<thead>
<tr>
<th><strong>Proteins</strong></th>
<th><strong>Lipid Bound</strong></th>
<th><strong>Function of Protein</strong></th>
<th><strong>Function of Domain</strong></th>
<th><strong>Reference Nos.</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Dynamins 1,2,3</td>
<td>PtdIns(4,5)P2</td>
<td>Required for budding of clathrin-coated vesicles</td>
<td>Modulates dynamin GTPase activity and protein binding</td>
<td>2, 16, 142, 171, 184, 196, 361, 402</td>
</tr>
<tr>
<td>B2-1/cytohesin-1/Sec7A/PSCD1</td>
<td>PtdIns(3,4,5)P3</td>
<td>Known and potential Arf GEFs</td>
<td>Stimulates activity and contributes to membrane location</td>
<td>163, 177, 183</td>
</tr>
<tr>
<td>Cytohesin-2/ARN01/Sec7B/PSCD2</td>
<td>PtdIns(3,4,5)P3</td>
<td>PtdIns(3,4,5)P3</td>
<td>PtdIns(4,5)P2(?)</td>
<td></td>
</tr>
<tr>
<td>GRP1/cytohesin-3/ARN03/Sec7C</td>
<td>Cytohesin-4/dj63g5.1</td>
<td>EFA6/PSD/cytohesin5</td>
<td>Tdic/cytohesin-6</td>
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<td>HCAA67/cytohesin-7</td>
<td></td>
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<tr>
<td>ASAP1/centaurin β4/DEF1</td>
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<td>ACAP1/centaurin β1</td>
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<td>ACAP2/centaurin β2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAPα,β/PAG3/centaurin β3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Centaurin 51/ARAP2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Centaurin 62/ARAP1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLD1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLD2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DGKδ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KIF1A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KIF1B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.**

DAG, diacylglycerol; PC, phosphatidylcholine; PA, phosphatidic acid; GEFs, guanine nucleotide exchange factors; GAPs, GTPase accelerating proteins.
bind preferentially to PtdIns(3)P, PtdIns(3,4)P₂, or PtdIns(4,5)P₂ (40, 94, 175, 328, 397, 398). However, PtdIns(3)P is much more abundant than other D-3 phosphoinositides and may represent the major lipid bound in the cell by PX domains that prefer a D-3 phosphate. Many PX domains also contain a binding motif for SH3 domains (143) or are found adjacent to coiled coil domains (351, 411). As is the case for PH domains, the membrane location of proteins containing PX domains is probably specified by protein-protein interactions as well as by lipid binding. Additional information on proteins containing PX domains can be obtained from recent reviews on this subject (93, 305, 393, 398).

### C. FYVE Domains

The FYVE domain, named as an acronym for the first four proteins in which it was recognized (340), is a type of zinc finger that binds to PtdIns(3)P (32, 117, 269). The signature of this domain is a defined spacing of cysteines that coordinate zinc and three additional blocks of residues that participate in lipid binding. Stenmark et al. (339) have identified 27 FYVE domain proteins in humans, 15 in *C. elegans*, and 5 in *S. cerevisiae* (339). Automated sequence analysis tools report larger numbers of FYVE domains in these organisms. EEA1, one of the four proteins in which the FYVE domain was first recognized, binds to early endosomes. The FYVE domain of EEA1 is necessary, but not sufficient, for this binding (35, 194). The COOH-terminal portion of EEA1 contains a coiled-coil domain and terminates in the FYVE domain. The crystal structure of this COOH-terminal fragment reveals a dimer held together through interactions between the two coiled coil domains and between one edge of each of the two FYVE domains. Together, the FYVE domains form a flat surface orthogonal to the coiled coil domain. Residues that contact the lipid head group are on the face of the protein opposite the coiled coil, and four hydrophobic residues near the phosphate binding residues form a loop that would dip down into the hydrophobic core of the bilayer (88). Thus, in EEA1, specificity of the head group-binding pocket is supplemented by nonspecific hydrophobic interactions as well as strengthened by being bivalent. A similar mode of binding has been reported for the FYVE domains of Vps27p and Hrs (335), and it is likely that FYVE domains, like PH and PX domains, use multiple contacts to achieve proper membrane location.

Proteins containing FYVE domains have been shown to function in endocytosis, in growth factor signaling, and in regulation of the actin cytoskeleton (62, 339, 396) (Table 5). Proteins with the first two functions are found on endosomes, and those of the last class, which also contain multiple PH domains, are found on the plasma membrane. The arrays of other domains found in proteins that contain FYVE domains suggest that many of them will assemble into multiprotein complexes. Table 5 lists proteins known or thought to function in membrane traffic that contain FYVE domains.

### D. ENTH/ANTH Domains

The ENTH (epsin 1 NH₂-terminal homology) domain was originally recognized as an NH₂-terminal sequence in epsin 1 and several other proteins that had known or suspected roles in endocytosis (41, 178). The COOH-terminal portion of EEA1 contains a coiled-coil domain and terminates in the FYVE domain. The crystal structure of this COOH-terminal fragment reveals a dimer held together through interactions between the two coiled coil domains and between one edge of each of the two FYVE domains. Together, the FYVE domains form a flat surface orthogonal to the coiled coil domain. Residues that contact the lipid head group are on the face of the protein opposite the coiled coil, and four hydrophobic residues near the phosphate binding residues form a loop that would dip down into the hydrophobic core of the bilayer (88). Thus, in EEA1, specificity of the head group-binding pocket is supplemented by nonspecific hydrophobic interactions as well as strengthened by being bivalent. A similar mode of binding has been reported for the FYVE domains of Vps27p and Hrs (335), and it is likely that FYVE domains, like PH and PX domains, use multiple contacts to achieve proper membrane location.

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### Table 4. PX domain proteins known to function in membrane traffic

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Lipid Bound</th>
<th>Function of Protein</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. cerevisiae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vam7p</td>
<td>PtdIns(3)P</td>
<td>Transport from Golgi to vacuole</td>
<td>40, 304, 328</td>
</tr>
<tr>
<td>Mvp1p</td>
<td></td>
<td>Golgi to endosome traffic</td>
<td>91</td>
</tr>
<tr>
<td>Grd10p</td>
<td></td>
<td>SNX3 homolog, retains proteins in TGN</td>
<td>397</td>
</tr>
<tr>
<td>Vps65p</td>
<td></td>
<td>Retrograde transport from endosomes to Golgi</td>
<td>153, 257</td>
</tr>
<tr>
<td>Vps17p</td>
<td></td>
<td>Retrograde transport from endosomes to Golgi</td>
<td>153</td>
</tr>
<tr>
<td>Mammalian</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SNX1</td>
<td>PtdIns(3)P</td>
<td>Sorting receptors (EGFR) for downregulation</td>
<td>65, 128, 192, 411</td>
</tr>
<tr>
<td>SNX2</td>
<td></td>
<td>Associates with growth factor receptors</td>
<td>128</td>
</tr>
<tr>
<td>SNX3</td>
<td>PtdIns(3)P</td>
<td>Retrograde transport from endosomes to Golgi</td>
<td>128, 397</td>
</tr>
<tr>
<td>SNX4</td>
<td></td>
<td>Sorting receptors (EGFR) for downregulation</td>
<td>128</td>
</tr>
<tr>
<td>SNX6</td>
<td></td>
<td>Associates with TGF-β receptors</td>
<td>267</td>
</tr>
<tr>
<td>SNX9</td>
<td></td>
<td>EGF receptor downregulation</td>
<td>206, 211</td>
</tr>
<tr>
<td>SNX15</td>
<td></td>
<td>Endosomal protein traffic</td>
<td>15, 272</td>
</tr>
<tr>
<td>SNX17</td>
<td></td>
<td>Endocytosis of LDL receptor family proteins</td>
<td>342</td>
</tr>
<tr>
<td>PLD1</td>
<td></td>
<td>Membrane localization</td>
<td>97, 278, 345</td>
</tr>
<tr>
<td>PLD2</td>
<td></td>
<td>Membrane localization</td>
<td>97, 278, 345</td>
</tr>
</tbody>
</table>

TGF, transforming growth factor; EGF, epidermal growth factor; LDL, low-density lipoprotein.
CALM have a NH₂-terminal domain with a distinct fold (ANTH domain). The ANTH domain binds the phosphoinositide head group via solvent-exposed basic residues (104), whereas the ENTH domain binds the lipid in a pocket that contacts the head group as well as the attached glycerol (103). Binding of PtdIns(4,5)P₂ by the ENTH domain causes the NH₂-terminal helix of the domain to penetrate into the lipid bilayer, slowing membrane dissociation of the domain and inducing membrane curvature (103, 336). Proteins that contain ENTH/ANTH domains also contain multiple recognition motifs for other types of protein-protein interaction modules and probably function as scaffold proteins that assemble protein complexes on membranes (73).

The ANTH domain proteins AP180 and CALM bind to clathrin and AP2 adaptors and are proposed to nucleate the polymerization of the clathrin coat (172). Epsin1 binds to the AP2 α- and β-ear domains, and another family member, EpsinR, binds to the AP1 γ-ear domain and to GGA1–3 at the TGN (144, 173, 235, 373). EpsinR contains an acidic phenylalanine motif found in two yeast proteins, Ent3p and Ent5p, that is necessary for them to bind to AP1 and Gga2p (89, 90, 235). EpsinR shows preference for binding to PtdIns(4)P and PtdIns(5)P in vitro (144, 235), which is consistent with the finding that PtdIns(4)P is abundant on Golgi membranes (372). Epsin and EpsinR contain a ubiquitin binding domain just COOH terminal to the ENTH domain and are themselves monoubiquitylated (146, 182, 259, 276). Ubiquitylation of hormone receptors (202, 210), as well as certain other membrane proteins (108), is necessary for sorting them in early endosomes from the recycling to the degradative pathway. It is less clear what role ubiquitylation would have for a protein that functions in TGN to endosome sorting, such as EpsinR (144, 235). In yeast, monoubiquitylation also serves as a signal for internalization from the plasma membrane (353). Internalization of the mammalian growth hormone receptor apparently requires that it be recognized by ubiquitylation machinery, but the actual ligation with ubiquitin is not necessary for internalization (300). The proteins that recognize ubiquitylated receptors on endosomal membranes, such as Hrs, are themselves monoubiquitylated (276, 280, 317). Although detailed knowledge of the precise interactions is lacking, it appears that the ENTH domain of the epsins links a membrane recognition event regulated by PtdIns(4,5)P₂ and perhaps PtdIns(4)P to another membrane recognition event regulated by PtdIns(3)P through the FYVE domain of Hrs.

Two other proteins that contain ENTH domains, HIP1 and HIP1R, and their counterpart in S. cerevisiae, Sla2p, interact with the actin cytoskeleton as well as play a role in endocytosis (95, 232, 238, 369, 383, 401). Phosphatidylinositides play a major role in regulating actin (404), and the ENTH domains of HIP1 and HIP1R may function to coordinate the assembly of endocytic coat proteins with changes in the actin cytoskeleton. A list of human proteins containing ENTH domains and thought to function in membrane trafficking is presented with their yeast orthologs in Table 6.

### Table 5. Proteins that contain FYVE domains and function in membrane trafficking

<table>
<thead>
<tr>
<th>Protein</th>
<th>Lipid Bound</th>
<th>Function of Protein</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. cerevisiae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vac1p</td>
<td>PtdIns(3)P</td>
<td>Rabenosyn-5 ortholog, Adaptor protein binding Rab5 and effectors</td>
<td>33, 255, 271, 348, 378</td>
</tr>
<tr>
<td>Vps27p</td>
<td>PtdIns(3)P</td>
<td>Hrs ortholog, required for sorting into multivesicular bodies</td>
<td>22, 188, 274</td>
</tr>
<tr>
<td>Fab1p</td>
<td>PtdIns(3)P</td>
<td>PtdIns(3)P 5-kinase required for formation of multivesicular bodies, PIKfyve ortholog</td>
<td>57, 115, 399</td>
</tr>
<tr>
<td>Mammalian</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EEA1</td>
<td>PtdIns(3)P</td>
<td>Rab 5 effector on early endosomes regulating endosome fusion</td>
<td>34, 116, 194, 195, 270, 325, 340</td>
</tr>
<tr>
<td>Hrs</td>
<td>PtdIns(3)P</td>
<td>Required for receptor sorting in endosomes to the degradative pathway, binds ubiquitylated receptors to clathrin-coated endosome membranes</td>
<td>45, 188, 280, 317, 347</td>
</tr>
<tr>
<td>PIKfyve</td>
<td>PtdIns(3)P</td>
<td>PtdIns 5-kinase producing PtdIns(3,5)P₂ required for endocytic organelle structure</td>
<td>156, 306, 308</td>
</tr>
<tr>
<td>Rabenosyn-5</td>
<td></td>
<td>Rab5 effector linking Rab 5 to hVPS45</td>
<td>255</td>
</tr>
<tr>
<td>Rabip4/RUFY1</td>
<td></td>
<td>Required for correct sorting of cathepsin D</td>
<td>61, 216, 400</td>
</tr>
<tr>
<td>FERG-1/DFCP1</td>
<td></td>
<td>Overexpression disturbs Golgi structure</td>
<td>290</td>
</tr>
<tr>
<td>MTMR3</td>
<td>PtdIns(3)P</td>
<td>PtdIns(3)P and PtdIns(3,5)P₂ phosphatase</td>
<td>371</td>
</tr>
</tbody>
</table>

### E. Basic Sequences and Other Motifs That Bind Phosphoinositides

In addition to the modular phosphoinositide binding motifs, a number of binding sites have been identified that have in common clusters of basic residues interspersed...
with hydrophobic residues and little other primary sequence conservation. These have been found in many cytoskeletal proteins (reviewed in Refs. 218, 404), clathrin adaptor proteins (55, 111, 132), C2 domains (38, 60), including in synaptotagmin (310) and in PLD (314, 410). PLD1 and PLD2 contain both PX and PH domains, but the site that binds PtdIns(4,5)P₂ that regulates enzyme activity is a conserved sequence of the basic/hydrophobic type found in PLD1, PLD2, yeast Spo14, and less conserved in plant PLDs. The lesson from this example is that the observation that a protein is regulated by a phosphoinositide and contains a known phosphoinositide-binding module does not necessarily lead to the conclusion that the module regulates protein activity. The number of examples of proteins with multiple phosphoinositide binding sites is increasing rapidly.

PHD domains are orphan zinc-finger domains found in a large number of nuclear proteins (1). ING2, a protein that associates with histone acetyltransferase and histone deacetylase complexes (99), was identified as a protein that bound to a phosphoinositide resin (121). ING2 bound preferentially to PtdIns(4,5)P₂ and PtdIns(3)P through its PHD domain, as did several other proteins with PHD domains. The PHD domain is structurally similar to a FYVE domain (239, 268), and basic residues in the ING2 PHD domain predicted to be on a surface analogous to the PtdIns(3)P binding surface of the FYVE domain were found to be necessary for binding PtdIns(5)P (121). No protein known to be important for membrane traffic has been identified that has a PHD domain.

### IV. INTRACELLULAR DISTRIBUTION AND FUNCTION OF PHOSPHOINOSITIDES FOR MEMBRANE TRAFFIC

It is likely that the PIPs important for constitutive membrane traffic are the more abundant ones (see Fig. 1). An exception to this may be PtdIns(3,5)P₂, which has been shown to be important for function of the vacuole in yeast, but about which very little quantitative data exist. Together, PtdIns and the PIPs are 10% of cellular lipids. Of this total, 5% is PtdIns(4)P (0.5% total lipids) and 5% is PtdIns(4,5)P₂ (292). Because all PIPs are confined to the inner leaflet, PtdIns(4)P and PtdIns(4,5)P₂ would each be 1% of inner leaflet lipids. Depending on the cell type, the plasma membrane can be from 2 to 10% of total membranes and the Golgi 7–12% (5, 122). Thus, if the major pool of PtdIns(4,5)P₂ is in the plasma membrane and PtdIns(4)P is on the Golgi (see below), these two lipids could be 5% or more of the cytoplasmic leaflet of the organelle where they are concentrated. Because these lipids are not uniformly distributed in the membranes that contain them, their local concentration is likely to be higher. Less than 0.25% of total inositol lipid is phosphorylated on D-3 (this equals 0.05% of inner leaflet lipid).
FIG. 1. The major phosphoinositide species are concentrated at distinct sites in intracellular membrane traffic pathways and may serve as organelle markers. The major concentration of phosphatidylinositol 4-phosphate \( [\text{PtdIns}(4)P] \) (blue) is at the Golgi complex, and very little free \( \text{PtdIns}(4)P \) is detected at the plasma membrane or on endosomes. \( \text{PtdIns}(3)P \) (green) is concentrated on early endosomes. The majority of phosphatidylinositol 4,5-bisphosphate \( [\text{PtdIns}(4,5)P_2] \) (red) is at the plasma membrane at steady state. \( \text{PtdIns}(3,5)P_2 \) (orange) is found on multivesicular endosomes and lysosomes. Some phosphoinositides are found in the endoplasmic reticulum and in the nucleus, but probably do not play major roles in membrane traffic.

Most of the \( \text{PtdIns}(4,5)P_2 \) in the cell is in the plasma membrane (11, 375). \( \text{PtdIns}(4,5)P_2 \) is enriched in detergent-resistant membranes (152), and \( \text{PtdIns}(4,5)P_2 \) that is turned over in response to hormone signaling may be enriched in caveolae or lipid raft membranes (273). However, recent studies by light or electron microscopy suggest that \( \text{PtdIns}(4,5)P_2 \) is not especially concentrated in caveolae at steady-state (364, 375). Thus the degree of subcompartmentalization of \( \text{PtdIns}(4,5)P_2 \) in the plasma membrane is currently uncertain. \( \text{PtdIns}(4,5)P_2 \) is generated during the process of fusion of regulated secretory vesicles or synaptic vesicles with the plasma membrane (66, 219), at the locations where actin rearrangements occur (59, 67, 404), and is required for clathrin-mediated endocytosis (67, 104, 161, 171, 262). Although required for vesicle fusion at the plasma membrane and for homotypic fusion of yeast vacuoles in vitro (222), the role of \( \text{PtdIns}(4,5)P_2 \) in vesicle fusion is not known precisely. In the vacuole fusion reaction, \( \text{PtdIns}(4,5)P_2 \) is required at the priming step where SNARE protein complexes are dissociated and also after the docking of the vesicle but prior to fusion (222). During dense-core vesicle secretion in mammalian cells, \( \text{PtdIns}(4,5)P_2 \) is detected mainly on the plasma membrane rather than on the vesicles (149, 219). In PC12 cells, the small GTP-binding protein Arf6 regulates the pool of \( \text{PtdIns}(4,5)P_2 \) required for dense-
core vesicle fusion by activating a PIP5KI enzyme in a calcium-dependent manner (4). The calcium dependence may be in part a protein kinase C (PKC)-regulated dephosphorylation of the PIP5KI, which activates it (4, 266, 382). In the same cell type vesicle fusion is inhibited in cracked cells incubated with recombinant C2A domains from various synaptotagmins, and this inhibition correlates with the ability of the domain to bind to PtdIns(4,5)P2 (359). Therefore, a synaptotagmin is likely to be one of the effectors for PtdIns(4,5)P2 for membrane fusion at the plasma membrane, at least for regulated secretion.

In the regulation of actin and the formation of clathrin-coated pits, it is clear that PtdIns(4,5)P2 increases the affinity of many mutually interacting proteins for membranes. As such, it may participate in defining the membrane location for the events that require these proteins. The AP2 adaptor, which binds membrane protein cargo to the clathrin lattice, contains two binding sites for PIPs. In the crystal structure of soluble AP2 core complex, these sites are orthogonal to each other and could not simultaneously bind to the membrane (55). In this structure, the binding pocket for internalization signals on receptors is occluded. Owen and colleagues (55) have proposed that binding to PIPs facilitates a conformational change that opens the signal-binding pocket and allows both PI binding sites to face the membrane. This change may be regulated by phosphorylation and the open conformation stabilized by binding to PIPs. Mutation of the PIP binding site on the AP2α subunit inhibits membrane binding, and mutation of the binding site on the AP2μ subunit prevents binding to cargo, demonstrating the importance of the interaction with PIPs (111, 291). AP2 can bind multiple PIPs, and in vitro those with D-3 phosphate increase the affinity of AP2 complexes for peptides that have internalization signals (286). This may be relevant to the observations that PtdIns3KIIC2α localizes in clathrin-coated pits (112) and that PtdIns(3,4,5)P3 is important for recruitment of AP2 to activated β-adrenergic receptors (248). However, given the relative scarcity of PtdIns(3,4,5)P3, PtdIns(3,4)P2, and PtdIns(3)P at the plasma membrane, it is likely that PtdIns(4,5)P2 is the major regulator of AP2 for constitutive endocytosis. The less abundant PIPs may be generated as part of the signaling of hormone receptors and function locally to accelerate the internalization of those receptors. Expression of PH domains specific for PtdIns(4,5)P2 inhibits both early and late stages of clathrin-coated vesicle formation (171), and increased or decreased expression of PIP5KIB causes more or less AP2 to bind to membranes (262). In addition to AP2, a number of other proteins important for endocytosis either have been shown to bind PIPs or are recruited to membranes in response to proteins that bind PIPs. The number of clathrin-coated pits at the plasma membrane and internalization of transferrin receptors increases or decreases in direct relation to the production of PtdIns(4,5)P2 in several cell types (262). This shows that the rates of constitutive endocytosis not only require PIPs, but also might be regulated through the control of PIP5KI activity. In HeLa cells, PIP5KIB is responsible for most of the cellular PtdIns(4,5)P2 with a minor contribution by PIP5KIA and no detectable contribution of PIP5KIC long or short isoforms (262). However, inhibition of PIP5KIA or PIP5KIC by small interfering RNA increases transcription of both of the remaining two isoforms, indicating that although isoforms A and C do not impact total cell PtdIns(4,5)P2 levels very much, their presence is sensed by the cell and all three lipid kinases are coordinately regulated (262). PIP5KIA has been shown to bind to the EGF receptor and accelerate EGF receptor internalization (14). PIP5KIC is the major producer of PtdIns(4,5)P2 in the synapse and probably responsible for the PtdIns(4,5)P2 required for endocytosis of synaptic vesicle components (382). Thus different PIP5KI enzymes may potentially regulate endocytic rates of different types of cargo.

There is a correlation in both time and space between the formation of endocytic vesicles and the reorganization of the actin cytoskeleton (107, 214, 327, 380, 382). In S. cerevisiae, where clathrin is not required for endocytosis, actin plays a crucial role (245). In mammalian cells, clathrin, AP2, and dynamin bind to proteins that also bind to actin (154, 180, 199, 229, 244, 279, 390). The functional consequence of these two events is not understood at present. It is possible that cortical actin is a barrier to the budding of a clathrin coat and must be reorganized so that a coated vesicle can move away from the cell surface. However, rather than depolymerize, actin polymerizes as coated vesicles move away from the membrane (229). In an early study of the distribution of clathrin coats on membranes of primary human fibroblasts, Anderson et al. (6) observed that clathrin-coated pits that contained low-density lipoprotein (LDL) receptors lined up over stress fibers. A more recent study by light microscopy of GFP-clathrin in live cells confirms that clathrin coats appear to organize in relation to cortical actin (302). However, a caveat to these experiments is that clathrin forms two structures on plasma membranes, flat lattices and curved pits. It has been assumed that flat clathrin is the precursor to curved clathrin pits, but this has never been proven. Recently, clathrin has been shown to interact dynamically, binding and releasing from the clathrin lattice (394). Thus the binding and release of GFP-clathrin from membranes cannot necessarily be equated with the formation and fission of clathrin-coated pits.

Overexpression of PIP5KIB causes actin tails to form on vesicles that contain lipid rafts (207). These actin structures require activation of Arp2/3 and contain dynamin 2 (261, 297). Although the actin “comets” produced in cells overexpressing PIP5KIB are exaggerated structures, a similar polymerization of actin has been observed on endosomes in Xenopus eggs and in cultured mast cells.
(230, 349). The polymerization of actin appears to move the organelles on which it is bound, but it is not clear if this process is used to move vesicles in cells or actin polymerization plays some other role.

Experiments in which the 5-phosphatase synaptotagmin is deleted prove that there must be turnover of the PtdIns(4,5)P2 on clathrin-coated vesicles for normal rates of vesicle uncoating to occur. As mentioned previously, synaptotagmins are dual function phosphatases capable of converting PtdIns(4,5)P2 to PtdIns without producing PtdIns(4)P. In fact, at steady-state, a GFP-oxysterol binding protein PH domain probe or anti-PtdIns(4)P antibody does not label the plasma membrane, suggesting that very little free PtdIns(4)P2 is present there (372). Because PtdIns(4)P is the precursor to PtdIns(4,5)P2, either it is generated from PtdIns by a plasma membrane PtdIns4K, or it might be generated at the Golgi but be rapidly converted to PtdIns(4,5)P2 upon arrival at the plasma membrane.

B. PtdIns(3)P on Endosomes

Clathrin-coated vesicles rapidly uncoat and fuse with each other or with early endosomes. Although PtdIns3KIIIC2α has been found in clathrin-coated pits and might still be present in vesicles when they uncoat, it is not known if PtdIns(3)P is generated on the membrane of incoming endocytic vesicles. In the presence of the PI3K inhibitor wortmannin, internalization is either unaffected or increased, and incoming endocytic vesicles continue to fuse into structures larger than small vesicles. Thus, in the absence of PtdIns(3)P, the incoming endocytic vesicles are still competent for fusion. The effect of wortmannin is to reduce the rate of entry of proteins into either the recycling pathway back to the plasma membrane or the pathway to late endosomes that leads to degradation in lysosomes (166, 220, 321, 329). A number of observations suggest that PtdIns(3)P is probably generated on the surface of early endosomes and not on the incoming vesicles and that this difference may be used to give directionality to the fusion reaction and to recruit the endosomal sorting machinery. Wortmannin concentrations that inhibit PtdIns3KIII inhibit the fusion of endosomes in vitro (166, 203, 329), a reaction that requires activated Rab5. Activated Rab5 binds to a complex of proteins that includes PtdIns3KIII, syntaxins, and accessory proteins that may play a role in activating SNARE protein complexes for membrane fusion (225, 348). The effect of wortmannin on endosome fusion in vivo is not bypassed by a constitutively active Rab5, so the inhibited step is downstream of the guanine nucleotide exchange on Rab5 required to deliver it to membranes (167). A number of the effectors of Rab5 (EEA1, rabenosyn-5) bind PtdIns(3)P and are released from membrane in the absence of this lipid (255, 270, 325). In an examination of the heterotypic fusion of uncoated endocytic vesicles with endosomes, Zerial and colleagues (298) found Rab5 on each population of vesicle but found the Rab5 effector EEA1 only on early endosomes. EEA1 binds to membranes through a combination of interactions with PtdIns(3)P and other proteins (88) but does not require a direct interaction with Rab5 for this (194). Thus a possible role of PtdIns(3)P on endosomes that is consistent with the data just described would be to identify the destination membrane for fusion of incoming vesicles to give directionality to the fusion reaction (298). Presumably, PtdIns(3)P would accomplish this through the assembly of a protein complex on the endosomes that would tether the incoming vesicle as well as participate in the following fusion reaction. If this is true, then the incoming endocytic vesicles should lack PtdIns(3)P and a key determinant of endosome identification would therefore be the activation of a PtdIns3K. Consistent with this proposal, PtdIns3KIII generates PtdIns(3)P on phagosomes only after they have budded off from the plasma membrane, and this is required for them to fuse with lysosomes (366).

PtdIns(3)P is also important for a second step in endocytic traffic, the formation of the internal vesicles of MVEs. The endocytic pathway delivers to lysosomes most of its luminal volume but only a minor fraction of its membrane. The delivery of membrane proteins to lysosomes is therefore an active event, and the delivery of fluid-phase cargo to lysosomes is the default outcome. Proteins destined to be degraded, such as the epidermal growth factor receptor (EGFR) bound to its ligand, are sorted into regions on MVEs that bud off as internal vesicles and therefore partition with luminal content to lysosomes. The formation of these internal vesicles has a topology opposite to the formation of other types of transport intermediates in the cell. Other transport vesicles form when a cytosolic coat binds to a membrane, collects cargo, and induces or stabilizes positive membrane curvature. After the vesicle fissions from the donor membrane (a process which is not understood), the peripheral membrane proteins that form the coat are released back into the cytosol to be reused (331). However, if the invaginations that form the internal vesicles in MVEs require a cytosolic coat to collect cargo and curve membrane, that coat will be incorporated in the interior of the vesicle and be delivered to lysosomes for degradation. As an alternative mechanism, protein coats may form on the limiting membrane of MVEs to stabilize it, and the internal vesicles might be formed by generating patches of lipid in the cytoplasmic leaflet of uncoated membrane that favor negative curvature and cause the membrane to invaginate (275). The internal vesicles of MVEs are enriched in cholesterol and lysobisphosphatidic acid (186, 187, 241), in-
indicating that sorting of lipids does occur during their formation.

Several laboratories have used wortmannin to investigate the participation of PtdIns(3)P in the process of forming MVE vesicles with somewhat contradictory results (26, 101, 109). A major problem for interpreting the results of experiments that use wortmannin (and most other inhibitors) in different cell types is that information about the sensitivity of that particular cell to the inhibitor is rarely provided. Because cells differ greatly in their ability to inactivate drugs, the fact that an inhibitor has a certain potency in vitro, or in a particular cell type, does not mean that it has a similar potency in a different cell type or over a different time course. A particularly instructive example of this is provided by Kundra and Kornfeld (191), who document that even a micromolar concentration of wortmannin is degraded in L cells fast enough that inhibition of membrane traffic over a 3-h time course requires addition of fresh drug at hourly intervals (191). A second problem is that, in a number of different cell types, at concentrations above 100 nM wortmannin inhibits PtdIns3KIII, PtdIns3KIC2α, and PtdIns4KIβ and at low nanomolar concentration inhibits a phospholipase A₂ activity in Swiss 3T3 cells (68).

With these caveats in mind, 100 nM wortmannin inhibited the formation of MVE internal vesicles in a melanoma cell line (101). However, the recycling of proteins from the limiting membrane of MVEs, but not the production of internal vesicles, was inhibited in NRK cells treated with 200 nM wortmannin (26). Futter et al. (109) investigated this question in experiments in which fusion of MVEs with lysosomes was prevented and the drug was added after a probe (EGFR) had passed through early endosomes so that the process of formation of MVE vesicles could be uncoupled from effects on earlier or later events. Under these conditions, HEP-2 cells treated with 200 nM wortmannin or microinjected with antibodies to PtdIns3KIII contained fewer MVE internal vesicles containing EGFR and increased limiting membranes of swollen vacuoles carrying EGFR. The increase in the limiting membrane was greater than would be predicted by the decrease in the formation of the internal vesicles (109).

This suggests that PtdIns(3)P is important both for the formation of internal MVE vesicles and for recycling membrane from multivesicular endosomes back to recycling endosomes or other destinations. Consistent with this interpretation, wortmannin has been reported to inhibit transport of mannose 6-phosphate receptors from endosomes to the Golgi, as well as transport between MVEs and lysosomes (191, 287). In contrast, Nakajima and Pfeffer (252) report no inhibition of mannose 6-phosphate transport from endosomes to the TGN by wortmannin or another inhibitor of PtdIns3K, LY294002 (252).

Three proteins that function for sorting endocytic cargo into the internal vesicles of MVEs have PtdIns(3)P binding modules. One of these is the protein defective in a S. cerevisiae class E vacuolar sorting mutant, Vps27 (274). Class E mutants inhibit the endocytic pathway at or downstream of the point where proteins internalized from the plasma membrane and proteins traveling from the Golgi to the vacuole converge. The mammalian counterpart to Vps27, Hrs (188), forms a complex with STAM1, STAM2, and eps15 (10). Hrs recruits clathrin to early endosome membranes (281), and areas of flat clathrin lattices are observed on endosomes that contain receptors destined to be degraded (281). Hrs interacts with the PX domain-containing protein SNX1 and functions to sort receptors from the recycling pathway into the internal vesicles in MVEs for delivery to lysosomes (45, 280, 281). Vps27p functions analogously in yeast (22). Thus Hrs plays an early role as part of the sorting event in which receptors are segregated from recycling proteins and membrane into coated regions on early endosomes as well as a later role when receptors are subsequently transferred to MVE vesicles. Hrs and Vps27p contain ubiquitin interaction domains and are themselves monoubiquitylated. Many cell surface receptors are ubiquitylated at the plasma membrane, and this serves as a signal that they should be sorted into the degradative pathway after internalization rather than recycling to the plasma membrane (138, 301, 337, 374). SNX1 binds to the EGFR, and overexpression of SNX1 increases the degradation of the receptor (192), whereas overexpression of Hrs inhibits EGFR degradation (45, 189). This suggests that Hrs might function to deliver a SNX1-EGFR complex into the STAM1, STAM2, eps15 complex necessary for sorting. The inhibition of EGFR degradation by overexpressed Hrs may be due to free Hrs binding SNX in competition with the Hrs that is part of the sorting complex. Three additional multiprotein complexes on the same endosome membrane are required for receptor degradation (176). The order in which these complexes act has been determined through yeast genetics, but their functions are still unclear.

C. PtdIns(3,5)P₂ on MVEs/Vacuoles

The third protein with a PtdIns(3)P₂ binding motif that is necessary for generating the internal vesicles of MVEs is the PtdIns(3)P 5-kinase, Fabp1/PKfyve. The phenotype of Δfab1 yeast is similar to the phenotype in mammalian cells in which PtdIns3KIII is inhibited (258), suggesting that the invagination of MVE vesicles requires production of PtdIns(3,5)P₂. Interestingly, the PX domain of SNX1 binds to both PtdIns(3)P and PtdIns(3,5)P₂ on liposomes (65); thus SNX1 may sort the EGFR into the membrane domain that will become an internal MVE vesicle.

PtdIns(3,5)P₂ was first discovered in yeast subjected to osmotic shock and then detected in mammalian cells...
(84, 384). Yeast (Fab1, Refs. 57, 226) and mammalian cells (PIKfyve, Ref. 306) contain a single PtdIns(3)P 5-kinase that produces PtdIns(3,5)P_2. The FYVE domain of PIKfyve is required for it to bind to endosomes (308). In S. cerevisiae, Vac14 is a regulator of Fab1 (24, 85). The mechanism of this regulation is not known; however, in large scale two-hybrid screens of the yeast genome, Vac14 has been shown to interact with 17 other yeast proteins, including proteins that interact with microtubules and components of an oligomeric complex that binds to the Golgi (details can be found at the Stanford Genomic Resources website).

PtdIns(3)P, and presumably PtdIns(3,5)P_2, incorporated into the internal vesicles of MVEs is hydrolyzed when these vesicles are transferred to the lumen of lysosomes or the yeast vacuole (395). However, there are several cytosolic phosphatases in yeast and mammalian cells capable of hydrolyzing PtdIns(3,5)P_2 to PtdIns. Such an activity would be needed if the spatial segregation of these two lipids were controlled dynamically by degrading excess or free PtdIns(3,5)P_2. Sac1 has PtdIns(3,5)P_2 phosphatase activity, but complementation studies in yeast suggest that it acts mainly on PtdIns(4)P (20). MTMR1, MTMR2, MTMR3, and MTMR6 hydrolyze both PtdIns(3)P and PtdIns(3,5)P_2 and are candidates for activities that control the levels of those lipids in the limiting membranes of organelles in mammalian cells (20, 309).

**D. PtdIns(3)P on the Golgi**

VPS34 was identified in a screen for genes that when mutated would allow vacuolar proteins to be secreted and was subsequently found to encode a new PtdIns3K (312, 333, 367). Since the branch point in the biosynthetic pathway separating vacuolar proteins and secreted proteins was the sorting event in the TGN, it was suspected that Vps34p and its mammalian orthologs would function at the Golgi. PtdIns3K inhibitors were found to disrupt the sorting of lysosomal proteins in the Golgi, and this was interpreted to mean that the site of PtdIns3K action was the TGN (31, 72). A PtdIns3K activity was found to be required for budding of vesicles containing the polyimmunglobulin receptor from isolated rat hepatocyte Golgi membranes (169), and wortmannin was observed to inhibit the sorting of mannose 6-phosphate receptors (M6PRs) into vesicles, but not the budding of vesicles, from the TGN (110). Wortmannin treatment also altered the morphology of the TGN (130). The conclusion that the effects of inhibiting PtdIns3Ks occurred on the Golgi began to be questioned when it was discovered that GFP-FYVE domains labeled endosomes but not the Golgi (32, 118), suggesting that there is little free PtdIns(3)P on the Golgi and the TGN. The observation that wortmannin inhibited the movement of M6PRs from endosomes back to the Golgi (191) raised the possibility that the inhibition of Golgi sorting was due to a failure to recycle important proteins back to the Golgi from endosomes. A second complication for interpretation of the result that wortmannin inhibits sorting in the Golgi was the discovery that overexpression of an enzymatically defective PtdIns3KIII blocks delivery of cathepsin D to lysosomes but does not cause it to be missorted at the Golgi, whereas treating HeLa cells with 100 nM wortmannin causes both (296). Wortmannin at that concentration might inhibit both PtdIns3KIIIC2α and PtdIns4KIIIβ (Table 1). Thus there is currently no conclusive evidence that PtdIns3KIII functions at the Golgi, and the preponderance of evidence suggests that this enzyme functions mainly on endosomes. A second PtdIns3K, PtdIns3KIIIC2α, has been reported to be associated with clathrin-coated vesicles on the TGN, and its overexpression disrupts the intracellular location of M6PR and lysosomal membrane proteins (80, 112). Clathrin/AP1 vesicles carry M6PRs from the Golgi to endosomes and back (83, 141), and it is not known where in this pathway PtdIns3KIIIC2α is active. A protein of novel function containing two FYVE domains binds to the Golgi and when overexpressed can disrupt Golgi structure (290). However, the Golgi binding domain of this protein is not found in the FYVE domain (44). The question of whether PtdIns(3)P plays any direct role in membrane traffic at the Golgi is currently unanswered.

**E. PtdIns(4)P and PtdIns(4,5)P_2 on the Golgi**

In a recent study of the cellular location of PtdIns(4,5)P_2, Watt et al. (375) used electron microscopy and a PLC51-PH domain probe to quantify PtdIns(4,5)P_2 on cellular membranes. After correcting for the fraction of total membrane represented by each compartment, the highest density of labeling was at the plasma membrane, and the density of labeling of the Golgi was detectable but ninefold lower than at the plasma membranes (375). Thus the pool of free PtdIns(4,5)P_2 on the Golgi is relatively small. Interest in a possible role for PtdIns(4,5)P_2 on the Golgi arose when it was discovered that nucleotide exchange on the important regulator of Golgi membrane traffic, Arf1, was increased by membranes containing PtdIns(4,5)P_2 (354) and that Arf is an activator of PLD (29). PLD produces PA, which is a potent activator of all of the PI(3)K enzymes, and PLD is itself potentially stimulated by PtdIns(4,5)P_2 (326). Thus the possibility that secretory events on the Golgi might be regulated through PtdIns(4,5)P_2 acting in positive and negative feedback mechanisms affecting Arf1 activity has received much attention. Arf1 is a major regulator of membrane traffic at the Golgi (293). There are six Arf proteins, and they can be grouped into three families according to their sequence relationships. Arfs 1, 2, and 3 are found at the Golgi (as
Overexpression of a dominant negative PI4KII 
its production is regulated through the production of PA.

Membranes and inhibition of PI4KII
PtdIns(4)P was the major lipid on mammalian Golgi membranes and by maintaining the Golgi structure both directly and through conversion to PtdIns(4,5)P2. Thus PtdIns(4)P functions in maintaining the structure of the Golgi, perhaps by interfering with the ability of spectrin to bind to membranes, although it is not clear if this is due to the lack of a precursor for PtdIns(4,5)P2 or to a direct effect of insufficient PtdIns(4)P. The release of secretory vesicles exporting hormones from the TGN requires PtdIns(4,5)P2 (42, 324).

The nature of the vesicle coat, if any, involved in this transport step is not known. AP1/clathrin coats are thought to sort and transport a number of proteins from the TGN. The AP1 clathrin adaptor has been shown to bind to liposomes in a manner that requires Arf1 and cargo with tyrosine signals and is facilitated by PtdIns(4,5)P2 more than by any other PI (69).

However, PtdIns(4)P is the major phosphoinositide on Golgi membranes and has functions independent of its use as a precursor for PtdIns(4,5)P2 (201, 372). In yeast, membrane traffic through the Golgi requires PtdIns(4)P generated by Pik4 (9, 338) and does not require PtdIns(4,5)P2. Yin and colleagues (372) found that PtdIns(4)P was the major lipid on mammalian Golgi membranes and inhibition of PI4KIIα by small interfering RNA caused the Golgi to fragment and inhibited the export of a viral glycoprotein from the TGN. The binding of AP1 to Golgi membranes was lost in these cells. AP1 binding and Golgi fragmentation were rescued by adding PtdIns(4)P back to cells but not by adding PtdIns(4,5)P2 (372). However, the inhibition of glycoprotein export was rescued by both lipids. Thus PtdIns(4)P functions in maintaining Golgi structure both directly and through conversion to PtdIns(4,5)P2, probably by recruiting cytoskeleton to Golgi membranes and by maintaining the flux of membrane moving through the organelle. One explanation for why PtdIns(4,5)P2 levels might be relatively low on Golgi membranes is that the 5-phosphatase OCRL on that organelle (87) might rapidly convert Golgi PtdIns(4,5)P2 back to PtdIns(4)P. Another possibility is the remarkable observation that a kinesin, unc104/KIF1A, binds preferentially to vesicles containing PtdIns(4,5)P2 (185). The ability of unc104/KIF1A to move vesicles is sharply dependent on the concentration of PtdIns(4,5)P2, and if that lipid is generated at the site of vesicle budding to recruit and activate a motor, PtdIns(4,5)P2 might be rapidly exported with the vesicle, keeping the pool of free PtdIns(4,5)P2 on the Golgi low. The concentration of PtdIns(4,5)P2 required to activate the motor is lowered when raft-forming lipids are added to the vesicles (185). This suggests that raft lipids, cholesterol and glycosphingolipids, may serve to concentrate PtdIns(4,5)P2 and enhance binding of the vesicle to the motor. Both polarized and nonpolarized cells contain separate raft and nonraft pathways from the Golgi (247, 406). It is an interesting possibility that raft and nonraft transport pathways may recruit different PI kinases, differ in the phosphoinositide that they employ, and perhaps the motors that associate with them.

F. Phosphoinositides on the ER

In contrast to the other organelles involved in membrane traffic, there is very little indication that PIPs play a direct role in constitutive traffic from the ER to the Golgi. The ER and nuclear membrane contain ~10% of the cellular PtdIns(4,5)P2 labeled with a PH domain probe (375). The COPII coat responsible for membrane protein export from the ER preferentially binds to acidic phospholipids but does not show a preference for specific PIPs (221). Several PI kinases and phosphatases are located on the ER, but the exact role that they play in secretion, if any, is not known (224, 389, 392). Membrane traffic between the ER and Golgi is inhibited when PtdOH production by PLD is blocked (21, 322), but it is not clear if this is an effect on the ER or the Golgi.

V. SUMMARY AND CONCLUSIONS

Previous sections have summarized data indicating that proteins that make, consume, and bind to PIPs are important for constitutive membrane traffic. Different PIPs are concentrated in different parts of the central vacuolar pathway, with PtdIns(4)P predominate on Golgi, PtdIns(4,5)P2 predominate at the plasma membrane, PtdIns(3)P the major PIP on early endosomes, and PtdIns(3,5)P2 found on late endocytic organelles. The reason for this spatial segregation is not known but may be the mechanism by which the direction of membrane traffic is controlled. One problem for organization within the central vacuolar system is to establish the identity of dynamic organelles that are constantly exchanging membranes. The integral membrane components that function for membrane traffic, including cargo receptors and mem-

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brane docking and fusion machinery, shuttle back and forth between organelles. To establish vectorial transport, this machinery must be activated only at the correct site and subsequently inactivated during recycling processes when proteins are transported back to the site where they function. The cytosolic coat proteins that recognize these components during the sorting process that loads them into transport intermediates must recognize them only at the correct membrane. A way to achieve this is to transiently increase the affinity of an area of membrane for these cytosolic proteins by modifying the lipids. In a process analogous to the phosphorylation of proteins, phosphorylation and dephosphorylation of PIPs could serve to switch a membrane from an active to an inactive state (Fig. 2).

If, for the sake of argument, one accepts that PIPs serve as organelle markers for membrane transport machinery, how do they become concentrated on the correct membrane? It is clear that the local concentration of PIPs is controlled dynamically by the balance between the kinases that make them and the phosphatases that hydrolyze them, since overexpression of the kinases or deletion of the phosphatases causes the distribution of PIPs to change. This raises the question of how the activity of the kinases and phosphatases is regulated at the correct location. We are currently far from understanding this process, but it is one of the most important problems for understanding how membrane traffic is organized.

Another important question is how PIPs serve as membrane identifiers. Here we have a few clear answers. One clue is the relatively weak binding between PIPs and the protein modules selective for them. PtdIns(3)P, PtdIns(4)P, and PtdIns(4,5)P_2 are each ~5 mol% of the cytoplasmic leaflet where they are concentrated, which is abundant enough to allow weak interaction with most of the proteins that bind to them, which have dissociation constants in the 10 μM range. This weak binding means that interactions will be extremely brief (less than milliseconds) unless stabilized by additional interactions. Many, if not most, PIP binding proteins have multiple PIP binding sites either on the same polypeptide or because they oligomerize, and these proteins usually also bind to other proteins on the membrane. Thus stable binding can be achieved through increases in avidity that occur as proteins binding weakly to the PIP on the membrane find and bind to each other. This allows a situation where once a critical concentration of PIP is generated a multimeric complex of proteins can assemble, and the stability of the complex can be controlled by the concentration of the PIP in the membrane. Since the cytosolic coats on transport intermediates need to assemble and then disassemble, some sort of cycling between active and inactive states is required. This occurs at multiple levels, including GTP hydrolysis on Rab and Arf proteins as well as phosphorylation of the membrane protein components and the lipids. Thus the formation of a vesicle coat or the docking and fusion of a vesicle are similar to a logical coincident gate. If multiple events do not coincide on the relevant time scale (which is determined by the affinity of mutually interacting components and their local concentration), then the traffic machinery will disassemble and start over again. This combinatorial approach is used in membrane traffic as a proofreading function that preserves the individual identities of highly fluid membranous organelles.

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