Regulation of membrane traffic by phosphoinositide 3-kinases

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Summary

Phosphoinositide (PI) 3-kinases control essential cellular functions such as cytoskeletal dynamics, signal transduction and membrane trafficking. FYVE, PX and PH domains mediate the binding of effector proteins to the lipid products of PI 3-kinases. Recent studies have provided significant insights into the roles of PI 3-kinases, their catalytic products and their downstream effectors in membrane trafficking. Class I and II PI 3-kinases trigger receptor-induced trafficking processes, such as phagocytosis, macropinocytosis and regulated exocytosis. Class I PI 3-kinases also function to inhibit autophagy. By contrast, class III PI 3-kinases mainly mediate receptor-independent trafficking events, which mostly are related to endocytic membrane traffic, phagosome maturation and autophagy.

Key words: Autophagy, Endocytosis, Exocytosis, Macropinocytosis, Phagocytosis, PI 3-kinase

Introduction

Phosphoinositides (PIs), which are phosphorylated derivatives of phosphatidylinositol (PtdIns), are essential in eukaryotic cells, regulating nuclear processes, cytoskeletal dynamics, signalling and membrane trafficking. Among the enzymes involved in PI metabolism, PI 3-kinases have attracted special attention because of their oncogenic properties and potential as drug targets (Luo et al., 2003). PI 3-kinases phosphorylate PtdIns or PIs at the 3-position of the inositol ring (Fig. 1). Mammalian cells contain multiple PI 3-kinases, which can be divided into three main classes (Table 1). Class I PI 3-kinases use phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2] as their main substrate, yielding the product phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3]. Their activity is acutely controlled by agonist stimulation of cells, resulting in dramatic fluctuations in the cellular levels of PtdIns(3,4,5)P_3. Whereas subclass IA PI 3-kinases are typically activated by receptor tyrosine kinases (RTKs) or receptors for immunoglobulin G (FcγRs), subclass IB enzymes are activated by G-protein-coupled receptors (GPCRs). Class II PI 3-kinases are thought to use PtdIns as their in vivo substrate, yielding phosphatidylinositol 3-phosphate [PtdIns(3)*P*]. Some evidence has been presented that class II enzymes, similarly to class I PI 3-kinases, can be activated by external stimuli. By contrast, the class III PI 3-kinases, represented by a single species (hVps34) in humans, have a relatively high activity even in resting cells. Nevertheless, even the activity of class III kinases can be upregulated in some situations (Table 1). Like class II PI 3-kinases, the class III enzymes use PtdIns as a substrate and thus produce PtdIns(3)*P*. The class III PI 3-kinases are the only ones conserved from lower eukaryotes to plants and mammals, and represent the most ancient form of PI 3-kinases. The differential substrate preferences and activation modes of the distinct PI 3-kinase classes have important consequences for their functions. Recent reviews have provided excellent overviews of their functions in the regulation of cytoskeletal and enzyme functions and signal transduction (Vanhaesebroeck et al., 2001; Perisic et al., 2004; Cantley, 2002). In this Commentary, we highlight the functions of PI 3-kinases in membrane trafficking.

Inhibitors of PI 3-kinases

Inhibitors have been very useful for characterizing the functions of PI 3-kinases in mammalian cells. The classical PI 3-kinase inhibitor is the fungal metabolite wortmannin, a very potent inhibitor that has an IC_{50} of ~10 nM (Woscholski et al., 1994). Another very useful PI 3-kinase inhibitor is the compound LY294002 (Vlahos et al., 1994; Knight et al., 2004). This inhibitor is much less potent than wortmannin (its IC_{50} is ~1000-fold higher), but it has a similar specificity and the advantage of being more stable than wortmannin. Therefore, LY294002 might be the inhibitor of choice in experiments that involve incubation times longer than 2-3 hours. A third PI 3-kinase inhibitor is 3-methyladenine, a compound originally established as an inhibitor of autophagy (Petiot et al., 2000; Seglen and Gordon, 1982) (see below). This inhibitor is much less potent than wortmannin and LY294002 (its IC_{50} is ~1000-fold higher than that of LY294002). It is possible that 3-methyladenine preferentially inhibits class III PI 3-kinases, although its inhibition of class I and II enzymes has not been examined in detail (Petiot et al., 2000). By contrast, the specificities of wortmannin and LY294002 have been tested quite thoroughly. In general, these compounds inhibit all PI 3-kinases to the same extent, with one important exception. The ubiquitously expressed class II PI 3-kinase, PI3K-C2α, is at least tenfold more resistant to wortmannin and LY294002 than other PI 3-kinases (Vanhaesebroeck et al., 2001). Like other kinase inhibitors, PI 3-kinase inhibitors compete for ATP binding in the active site of the kinase domain. Because ATP-binding sites of distinct kinases are structurally similar, this

means that PI 3-kinase inhibitors might inhibit additional kinases, especially when used at high concentrations. This has to be taken into account when interpreting results from inhibitor studies.

Effectors and localizations of the lipid products of PI 3-kinases

The main class I PI 3-kinase product, PtdIns(3,4,5)P_3, interacts with a subset of proteins that contain PH (pleckstrin-homology) domains (Lemmon and Ferguson, 2000; Cozier et al., 2004). This domain is rare in lower eukaryotes but the most widespread PI-binding protein module in higher eukaryotes. Examples of PH-domain-containing effectors of PtdIns(3,4,5)P_3 include GRP1, a guanine nucleotide exchange factor (GEF) for Arf GTPases that regulates the actin cytoskeleton, and PDK1 and Akt/PKB, two protein kinases that function in anti-apoptotic and growth-promoting signalling cascades. Because of its high affinity and specificity for PtdIns(3,4,5)P_3, the GRP1 PH domain has been used extensively as a cellular probe for this PI. Such studies have shown that PtdIns(3,4,5)P_3 is formed rapidly at the plasma membrane during agonist stimulation of cells, and that its levels decrease within a few seconds (Cozier et al., 2004).

The product of class II and class III PI 3-kinases, PtdIns(3)P, also has several known protein effectors. The first PtdIns(3)P-specific domain to be identified was the FYVE (for conserved in Fab1, YOTB, Vac1 and EEA1) zinc finger domain (Stenmark et al., 2002). This domain is more highly evolutionarily conserved but much less abundant than the PH domain and has a narrower substrate preference. In fact, all FYVE domains tested bind to PtdIns(3)P, although some bind with low affinity and show some binding to the related PI, PtdIns(5)P. The other known PtdIns(3)P-binding domain is the conserved Phox-homology (PX) domain, whose abundance is a little higher than that of the FYVE domain (Ellson et al., 2002). Even though most PX domains bind preferentially to PtdIns(3)P, a few can bind to other PIs, such as PtdIns(3,4)P_2.

Similarly to PH domains, FYVE and PX domains can be used as probes for PIs. In particular, a tandem FYVE domain (2×FYVE) derived from the endosomal protein Hrs (see below) has been used extensively as a PtdIns(3)P probe (Gillooly et al., 2000). Electron and fluorescence microscopy using this probe has shown that PtdIns(3)P is found constitutively on the limiting and intraluminal membranes of endosomes. The fact that the regulatory p150 subunit of the class III PI 3-kinase hVps34 binds to the active (GTP-bound) form of the early-endosomal GTPase Rab5 probably explains the localized formation of PtdIns(3)P on early endosomes (Christoforidis et al., 1999; Murray et al., 2002). The hVps34-p150 complex also co-immunoprecipitates and partially colocalizes with the late-endosomal GTPase Rab7 (although, surprisingly, the nucleotide-bound state of Rab7 appears to be of minor importance for this interaction), which suggests that the formation of PtdIns(3)P on late endosomes could be controlled by this GTPase (Stein et al., 2003). Evidence has also been presented that PtdIns(3)P accumulates transiently at the plasma membrane in cells stimulated with insulin or lysophosphatidic acid (LPA). This pool of PtdIns(3)P appears to be generated by the class II PI 3-kinase PI3K-C2β and might play a role in cell migration (Maffucci et al., 2005; Maffucci et al., 2003).

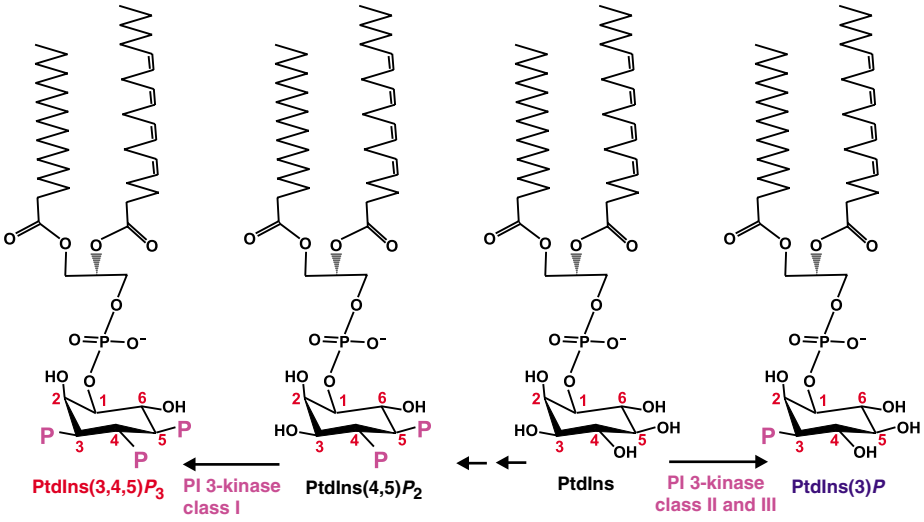

Fig. 1. Biochemical activities of PI 3-kinases. The substrates and catalytic products of class I, II and III PI 3-kinases are indicated.

Regulation of endocytic membrane traffic

The first realization that PI 3-kinases can regulate membrane traffic came with the identification of two vacuolar protein sorting (Vps) proteins in yeast, Vps34 and Vps15, as the catalytic and regulatory subunits, respectively, of a class III PI

Table 1. Classes of PI 3-kinases

PI 3-kinase	Subunit composition	In vivo substrate	Activated by
Class IA	Catalytic (p110α,β,δ) Regulatory (p85α,β, p55γ)	PtdIns(4,5)P_2	RTKs, FcγR, Ras
Class IB	Catalytic (p110γ) Regulatory (p101)	PtdIns(4,5)P_2	GPCRs, Ras
Class II	Catalytic (PI3K-C2α,β,γ)	PtdIns	LPA, insulin receptors
Class III	Catalytic (Vps34/hVps34) Regulatory (Vps15/p150) Accessory (Vps30/Beclin1) Accessory (Vps38 or Vps14)*	PtdIns	Constitutive†

FcγR, receptor for immunoglobulin G; RTK, receptor tyrosine kinase; GPCR, G-protein-coupled receptor; LPA, lysophosphatidic acid.

*Vps38 is part of the PI 3-kinase complex that mediates vacuolar protein sorting, whereas Vps14 is part of the PI 3-kinase complex that mediates autophagy. So far, mammalian homologs of these proteins have not been identified.

†Stimulated to some extent by amino-acid-rich medium (Byfield et al., 2005). The hVps34/p150 complex is also activated by the small GTPase Rab5, which can be activated by RTKs (Christoforidis et al., 1999; Murray et al., 2002; Barbieri et al., 2000).

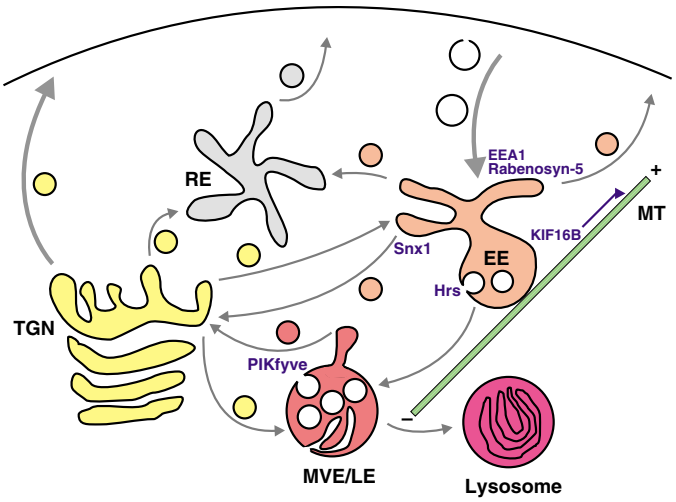

Fig. 2. Regulation of endocytic membrane trafficking by PI 3-kinases. The figure gives an overview of membrane trafficking in the endocytic pathway. Effectors of PtdIns(3)*P* are indicated in blue. EE, early endosome; MVE/LE, multivesicular endosome/late endosome; MT, microtubule; RE, recycling endosome; TGN, trans-Golgi network. Arrow thickness corresponds to the magnitude of various trafficking pathways indicated.

3-kinase (Schu et al., 1993; Stack et al., 1995). Subsequent studies in many organisms have revealed that class III PI 3-kinases play multiple roles in endocytic membrane trafficking (summarized in Fig. 2).

Endosome fusion

One of the early ties between PI 3-kinase and endocytic traffic in mammalian cells was the observation that wortmannin inhibits homotypic endosome fusion in vitro (Jones and Clague, 1995). Since then, several FYVE-domain-containing PtdIns(3)*P* effectors in endocytic membrane fusion have been identified, including EEA1 [early-endosomal antigen 1; a protein originally identified as an autoantigen in systemic lupus erythematosus (Simonsen et al., 1998)], Vac1/Rabenosyn-5 (Nielsen et al., 2000) and Rabankyrin-5 (Schnatwinkel et al., 2004). Interestingly, these proteins share not only the ability to bind PtdIns(3)*P* through a FYVE domain but also the ability to bind Rab5-GTP. This suggests that their membrane recruitment might rely on coincident recognition of Rab5-GTP and PtdIns(3)*P*, which could explain their specific localization to early endosomes. Detailed analyses of these three Rab5/PtdIns(3)*P* effectors have revealed some functional differences. Although all three proteins are required for efficient homotypic endosome fusion in vitro, EEA1 appears to be the most important for the heterotypic fusion of endocytic vesicles with early endosomes. Rabankyrin-5 is a potent regulator of macropinocytosis in non-polarized cells and clathrin-independent fluid-phase endocytosis in polarized epithelial cells (Schnatwinkel et al., 2004). The latter process has been proposed to be controlled by the class I PI 3-kinase p110β, raising the question of how PtdIns(3)*P* effectors may be activated by PtdIns(3,4,5)P_3. The answer is probably a localized conversion of PtdIns(3,4,5)P_3 into PtdIns(3)*P* by PI 5- and 4-phosphatases. Interestingly, not only are class III PI 3-kinase effectors of Rab5-GTP, but so are p110β, a PI 5-kinase and a PI 4-kinase (Shin et al., 2005; Christoforidis et al., 1999). Thus Rab5, which is present at the plasma membrane as well as on early endosomes, is central to 3-PI metabolism in endocytosis.

How do the Rab5/PtdIns(3)*P* effectors control endocytic membrane fusion? Intracellular membrane docking and fusion in general are facilitated by the formation of SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) complexes that force two membranes tightly together through the formation of energetically favorable tetrahelical bundles (Jahn et al., 2003; Ungermann and Langosch, 2005). EEA1 interacts with at least two endosomal SNARE proteins, syntaxin6 and syntaxin13; syntaxin13 appears to co-assemble with EEA1 in high-molecular-weight oligomers during membrane docking (Simonsen et al., 1999; McBride et al., 1999). Likewise, Rabenosyn-5 interacts with hVps45, a member of the Sec1 family of SNARE regulators (Nielsen et al., 2000). Rab5/PtdIns(3)*P* effectors may thus act in concert with SNAREs during membrane docking, although the mechanisms are not understood in detail. Prior to the docking between two membranes, tethering is thought to take place. Such longer-distance recognition should require elongated membrane-associated proteins. EEA1 is a good candidate, since it forms long coiled-coil homodimers that contain a Rab5-binding site at the N-terminus and another Rab5-binding site plus a FYVE domain at the C-terminus (Callaghan et al., 1999; Dumas et al., 2001). EEA1 could thus tether two Rab5-positive membranes, such as two early endosomes or an endocytic vesicle and an early endosome. Further studies using purified proteins and isolated endosomes will have to be performed to test these models.

Even though in vitro endosome fusion assays have proven extremely useful to characterize the functions of EEA1, Rabenosyn-5 and Rabankyrin-5, it is still not clear how important these proteins are in vivo. An indication that EEA1 and Rabenosyn-5 might be required for endocytosis comes from studies of the μ-opioid receptor, a GPCR whose endocytosis is negatively controlled by p38 MAP kinase (Mace et al., 2005). This kinase phosphorylates Rabenosyn-5 and EEA1 within their FYVE domains, which impedes their membrane recruitment and function. Moreover, short interfering (si)RNA-mediated depletion of EEA1 inhibits endocytosis of the μ-opioid receptor, suggesting a link between endosome fusion and endocytosis. One scenario is that fusion of endocytic vesicles with early endosomes could be required for the formation of new endocytic vesicles at the plasma membrane by liberation of rate-limiting components of the endocytosis machinery (Mace et al., 2005). Nevertheless, the general requirements for the Rab5/PtdIns(3)*P* effectors in endocytosis remain to be determined.

Endosome motility and recycling

In order to perform their functions in recycling and degradative trafficking, early endosomes undergo dynamic movements along microtubules and actin filaments. Even though the net movement of endosomes along microtubules is towards their minus ends (and thus the microtubule-organizing center), early endosomes also move towards their plus ends. This can be reconstituted in vitro, and in vitro assays have revealed that both plus- and minus-end-directed motility of early endosomes is dependent on Rab5 and hVps34 (Nielsen et al., 1999). A

kinesin motor that binds to PtdIns(3)*P* through a PX domain and mediates plus-end-directed early-endosome motility, KIF-16B, has recently been identified (Hoepfner et al., 2005). Whereas overexpression of KIF16B causes early endosomes to relocate to the cell periphery, depletion of KIF16B causes their perinuclear clustering. Overexpression of KIF16B thus promotes transferrin recycling and inhibits epidermal growth factor (EGF) receptor degradation, whereas KIF16B depletion has the opposite effect. This shows that the KIF16B-mediated positioning of endosomes is important for their functions and might give a clue as to why inhibition of hVps34 interferes with endocytic recycling (Siddhanta et al., 1998). However, the identification of KIF16B provides, at best, only half of the answer to why hVps34 is needed for endosome motility along microtubules. The putative PtdIns(3)*P* effector(s) that mediates minus-end-directed endosome motility remains to be identified.

Endosome-to-Golgi trafficking

Retrograde trafficking is important for recycling of sorting receptors, and several of its mechanisms have been conserved from lower to higher eukaryotes. Thus, the retrograde trafficking of the vacuolar protein sorting receptor Vps10 has been studied extensively in yeast, and trafficking of the functionally related mannose 6-phosphate receptors has been the subject of many studies in mammalian cells. The finding that two of the accessory proteins for the class III PI 3-kinase Vps34 in yeast, Vps30 and Vps38, are essential for endosome-to-Golgi trafficking of Vps10 illustrates the need for PI 3-kinase in this trafficking step (Burda et al., 2002). Studies in yeast have also led to the identification of the so-called 'retromer' complex consisting of Vps5, Vps17, Vps26, Vps29 and Vps35 as an important mediator of endosome-to-Golgi retrieval of Vps10 (Seaman et al., 1998). Vps26, Vps29 and Vps35 appear to be involved in cargo recognition, whereas Vps5 and Vps17 assemble onto endosome membranes to mediate vesicle budding. Interestingly, both Vps5 and Vps17 contain PX domains and bind specifically to PtdIns(3)*P* (Burda et al., 2002). These proteins are thus likely to be major PtdIns(3)*P* effectors in endosome-to-Golgi trafficking in yeast.

The retromer complex is evolutionarily conserved, and Snx1 and Snx2 have been assigned as mammalian orthologs of sorting nexin 1 (Snx1) (Haft et al., 1998). Individual knockouts of these proteins in mice cause minor phenotypes, whereas the double knockout causes embryonic lethality (Schwarz et al., 2002). This suggests that these proteins act redundantly to perform an essential function. Like Vps5, Snx1 and Snx2 bind to PtdIns(3)*P*, but they also bind PtdIns(3,5)P_2 in vitro (Cozier et al., 2002). It is thus not entirely clear whether the requirement for PI 3-kinase in retrograde trafficking reflects a need forPtdIns(3)*P* directly, or simply as a precursor for PtdIns(3,5)P_2. In any event, it is interesting to note the localization of Snx1 and Snx2 to tubular regions of early endosomes (Cozier et al., 2002; Gullapalli et al., 2004). The fact that these proteins contain a banana-shaped BAR domain that can sense curved membranes has led to the hypothesis that Snx1 and Snx2 are targeted specifically to endosomal tubules through coincident detection of PtdIns(3)*P* [or PtdIns(3,5)P_2] and the high membrane curvature found in endosomal tubules (Carlton et al., 2004). The mammalian retromer complex has been shown to be involved in the retrograde trafficking of mannose 6-phosphate receptors (Arighi et al., 2004), which indicates that it functions similarly to the yeast retromer.

Degradative sorting

The earliest connection between PI 3-kinases and membrane trafficking was the observation that class III PI 3-kinase is required for trafficking of membrane proteins from endosomes to the lumen of lysosomes/vacuoles (Schu et al., 1993). Most of the proteins that enter this degradative pathway are ubiquitylated, often at multiple lysine residues (Hicke and Dunn, 2003; Haglund et al., 2003), and sorted into vesicles that bud inwards into endosomes. Endosomes containing intraluminal vesicles, multivesicular endosomes (MVEs), fuse with lysosomes/vacuoles, which results in the degradation of the intraluminal vesicles and their content (Katzmann et al., 2002; Raiborg et al., 2003; Gruenberg and Stenmark, 2004). The class E subset (grouped on the basis of vacuole morphology and sorting defects) of yeast Vps proteins and their mammalian orthologs are required for degradative membrane trafficking downstream of class III PI 3-kinase. The vacuoles of yeast class E *vps* mutants show strongly reduced levels of intraluminal vesicles, and wortmannin treatment of mammalian cells results in swollen endosomes that contain few intraluminal vesicles (Katzmann et al., 2002; Fernandez-Borja et al., 1999).

Although several PtdIns(3)*P*-binding proteins may be involved in degradative trafficking, a highly conserved protein, Vps27/Hrs (hepatocyte-growth-factor-regulated tyrosine kinase substrate), is clearly a master effector of PtdIns(3)*P* in this pathway (Raiborg and Stenmark, 2002). Hrs binds to PtdIns(3)*P* through a FYVE domain, and this is required for its targeting to early endosomes (Raiborg et al., 2001). Here, Hrs recognizes ubiquitylated cargo, selecting it for degradation (Raiborg et al., 2002; Shih et al., 2002; Lloyd et al., 2002). However, Hrs is more than a sorting component: it facilitates the assembly of the machinery responsible for the formation of the intraluminal endosomal vesicles ESCRT (endosomal sorting complex required for transport)-I, ESCRT-II and ESCRT-III on endosome membranes (Katzmann et al., 2002; Raiborg et al., 2003). Hrs recruits ESCRT-I through a direct interaction with its ubiquitin-binding subunit Vps23/Tsg101, and presumably delivers the ubiquitylated cargo to this complex (Bache et al., 2003). ESCRT-I is thought to deliver its cargo to ESCRT-II, which also contains a ubiquitin-binding subunit, Vps36/Eap45 (Babst et al., 2002b). Interestingly the PH-domain-related GLUE domain of mammalian Eap45 binds not only ubiquitin but also 3-PIs (Slagsvold et al., 2005). Hence, the activity and/or recruitment of ESCRT-II might be regulated by PI 3-kinase, although this remains to be studied.

By a poorly understood mechanism, ESCRT-III, which acts downstream of ESCRT-II, mediates the invagination of the endosomal membrane to form intraluminal vesicles (Babst et al., 2002a). The ESCRT-III subunits form high-molecular-weight multimers that assemble transiently on endosomal membranes, and their disassembly requires the function of an AAA-type ATPase, Vps4 (Babst et al., 1998). ESCRT-III contains a 3-PI-binding subunit, Vps24, which interacts with PtdIns(3,5)P_2 (Whitley et al., 2003). This is formed from PtdIns(3)*P* by the kinase Fab1/PIKfyve (Odorizzi et al., 1998; Sbrissa et al., 1999), which contains a FYVE domain that

probably targets it to membrane domains containing high levels of its substrate. Inhibition of Fab1/PIKfyve function prevents degradative sorting of some (but not all) cargoes, and results in the accumulation of abnormally large vacuoles or late endosomes (Odorizzi et al., 1998; Ikonomov et al., 2001). However, the functional relationship between Fab1/PIKfyve, Hrs and the ESCRT proteins remains to be established. It is worth noting that even though Hrs is clearly a crucial component of the degradative sorting pathway, it also appears to be required for efficient endocytic recycling of certain non-ubiquitylated cargoes, such as transferrin receptors and some GPRCs (Yan et al., 2005; Hanyaloglu et al., 2005). This opens the possibility that the observed requirement of hVps34 for efficient transferrin recycling (Siddhanta et al., 1998) could in part be related to Hrs recruitment.

Regulation of phagocytosis and macropinocytosis

Phagocytosis of microorganisms can occur by two main mechanisms (Meresse et al., 1999). Adsorptive phagocytosis involves the association between a phagocyte and a microorganism that binds to receptors on the phagocyte surface. By contrast, triggered phagocytosis is induced by factors injected from the microbe into the host cell (typically a non-phagocytic cell) that promote internalization by macropinocytosis, a membrane ruffling process that can also be caused by certain growth factors (Fig. 3). Both require profound reorganization of the subcortical actin cytoskeleton, which is controlled by class I PI 3-kinases (Stephens et al., 2002). Specifically, the class IA catalytic subunit p110β has been found to mediate Fcγ-induced phagocytosis and apoptotic-cell phagocytosis in macrophages and fibroblasts (Leverrier et al., 2003). The PI 3-kinases may be activated by several distinct mechanisms during phagocytosis. For instance, initiation of adsorptive phagocytosis of IgG-opsonized particles through Fcγ receptors triggers the protein kinase Syk, which causes recruitment and activation of class IA PI 3-kinase at the plasma membrane (Stephens et al., 2002). Alternatively, activation of macropinocytosis by RTKs might involve Rab5-dependent recruitment and activation of class IA PI 3-kinases (Lanzetti et al., 2004).

Since class I PI 3-kinases generate PtdIns(3,4,5)P_3, effectors of this lipid might regulate the actin cytoskeleton. The best candidates so far are a group of PH-domain-containing GEFs for the small GTPases Rac1, Arf6 and Cdc42 (Franco et al., 1999; Han et al., 1998; Fleming et al., 2004; Klarlund et al., 1998), which are well-known regulators of the cortical actin cytoskeleton and plasma membrane dynamics during phagocytosis. Rac1 is activated by the GEF Vav downstream of PI 3-kinase class IA (Patel et al., 2002) and has been reported to function upstream of Arf6 and Cdc42 in FcγR-mediated phagocytosis (Niedergang et al., 2003). The function of Arf6 is particularly important for membrane recycling at phagocytic sites, and its activation is thought to be mediated by GEFs such as EFA6 and GRP1 (Niedergang et al., 2003). Whereas Rac1 appears to be active throughout the phagocytic cup, Cdc42 activation is restricted to the leading tip of the pseudopod (Hoppe and Swanson, 2004). Members of the zizimin family are good candidates for GEFs for Cdc42 during phagocytosis (Meller et al., 2005). It is interesting to note that a PtdIns(3,4,5)P_3-binding GTPase-activating protein (GAP) for Arf6 and Rho GTPases, Arap3, is also a possible mediator of PI 3-kinase responses to the actin cytoskeleton, which could balance the effects of the GEFs (Krugmann et al., 2002). Much less is known about the relationship between PI 3-kinase and GEFs during macropinocytosis. Thus, in dendritic cells, Rac1 is required for constitutive macropinocytosis, but whether this is controlled through Vav or other GEFs is not known (Nobes and Marsh, 2000; West et al., 2000).

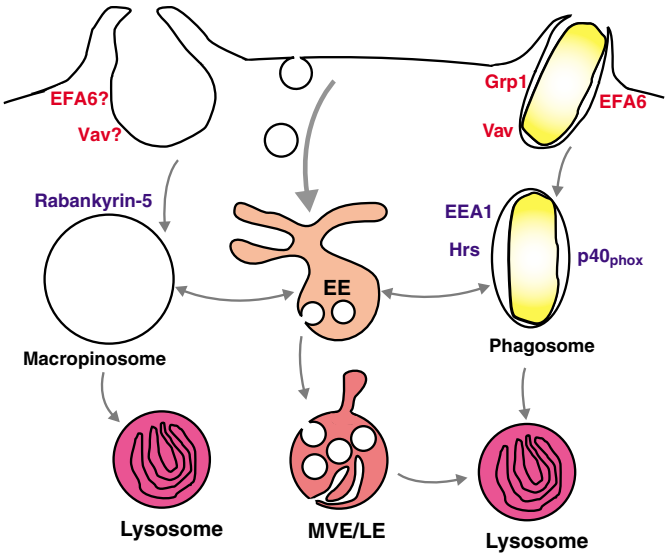

Fig. 3. Regulation of phagocytosis and macropinocytosis by PI 3-kinases. The involvement of PtdIns(3,4,5)P_3 effectors in macropinocytosis (left) and phagocytosis (right) are indicated in red. PtdIns(3)*P* effectors needed for macropinocytosis or phagosome maturation are indicated in blue. Arrow thickness corresponds to the magnitude of various trafficking pathways indicated.

The requirement for class I PI 3-kinase in adsorptive phagocytosis is restricted to the stage at which the phagocytic cup seals to form a phagosome (Marshall et al., 2001). Following this, PtdIns(3,4,5)P_3 levels in the newly formed phagosome decline rapidly, probably owing to the activity of PI phosphatases such as SHIP (Vieira et al., 2001). However, once the phagosome has been sealed from the plasma membrane, another PI 3-kinase, hVps34, comes into play. Maturation of the phagosome, which involves its acquisition of late-endosome markers and low intraluminal pH, is crucial for its eventual fusion with lysosomes, which degrade the ingested material. Among the components needed for phagosome maturation are Rab5 and hVps34 (Vieira et al., 2001; Fratti et al., 2001). Even though the exact functions of PtdIns(3)*P*-binding proteins in phagosome maturation have not been clarified, there is evidence that both EEA1 and Hrs are involved in this process (Fratti et al., 2001; Vieira et al., 2004). Moreover, the phagosomes of neutrophilic granulocytes play a special role in microbial killing. In addition to their acquisition of low pH and lysosomal enzymes, the mature neutrophilic phagosomes contain reactive oxygen species (ROS), which are highly toxic to pathogens. PtdIns(3)*P* has been found to stimulate ROS production in neutrophils by activating the PX-domain-containing protein $p40^{phox}$, a core component of the phagocyte oxidase (Phox) complex (Ellson et al., 2001). Interestingly, PtdIns(3)*P* itself is upregulated during neutrophil activation, but whether this is a result of activation of class II or class III PI 3-kinases is not known (Condliffe et al., 1998).

Pathogenic microorganisms in general and intracellular pathogens in particular subvert host defense systems in various ways (Meresse et al., 1999). Given the important role of PI 3-kinases in phagocytosis and microbial killing, it is not surprising that PIs are targets for several invading microorganisms. One important example is *Mycobacterium tuberculosis*. Acquisition of PtdIns(3)*P*, as well as its effectors Hrs and EEA1, by endosomes is required for efficient killing of phagocytosed mycobacteria (Fratti et al., 2001; Vieira et al., 2004). *Mycobacterium tuberculosis* evades this defense mechanism by secreting a phosphatase, SapM, that dephosphorylates PtdIns(3)*P* and thus halts phagosomal maturation (Vergne et al., 2005). As we learn more about the interactions between microorganisms and host cells, many more examples of how microbes reprogram PI 3-kinase-dependent defense pathways are likely to emerge.

Positive and negative regulation of autophagy

Autophagy is a process that shuttles cytoplasmic content to lysosomes for degradation (Klionsky, 2005). Studies in yeast indicate that autophagy initiates from a cytoplasmic pre-autophagosomal structure (PAS) that contains lipids as well as early autophagic effector proteins such as Atg5 and Atg8/LC3. Upon appropriate stimuli, the PAS elongates to form a double-membraned isolation membrane. When this is sealed, it forms a so-called autophagosome. The autophagosome then fuses with the lysosome and/or the late endosome, releases its inner vesicle and forms a degradative compartment known as the autolysosome (Fig. 4).

Autophagy is important for cytoplasmic homeostasis and continuously degrades long-lived proteins and damaged organelles. In addition, it can be induced, for instance, by starvation (Seglen and Bohley, 1992). Autophagy is initiated in a cell when amino acid levels are low, to recycle proteins and organelles and thereby to free amino acids. The sensing of low amino acid levels might involve inhibition of the TOR (target of rapamycin) kinase complex (Scott et al., 2004; Colombani et al., 2003). Autophagy is also important in freeing the cell from protein aggregates, which can lead to cell death and neurodegeneration (Ravikumar and Rubinsztein, 2004; Ravikumar et al., 2004). In addition, it can function as a tissue degradative process. During development, it plays a role in remodeling and degrading tissues (Baehrecke, 2005) such as the *Drosophila* salivary gland, gut and fat body during metamorphosis (Baehrecke, 2003; Rusten et al., 2004).

The PI 3-kinase Vps34, its regulatory subunit Vps15, as well as the two accessory proteins Vps14 and Vps30/Atg6, are all required for autophagy in yeast (Kihara et al., 2001). In mammalian cells, the PI 3-kinase inhibitors 3-methyladenine, wortmannin and LY294002 inhibit autophagy (Blommaart et al., 1997). Treating cells with synthetic PtdIns(3)*P* can reverse the effect of wortmannin, and 3-methyladenine has been shown to inhibit autophagy by targeting hVps34 (Petiot et al., 2000). The human Vps30/Atg6 ortholog Beclin-1, an accessory protein for hVps34, is also required (Liang et al., 1999). In plants, autophagy is induced in the hypersensitive response programmed cell death (HR PCD). Autophagy levels are reduced in plant Beclin-1 mutants, and the restriction of HR PCD also requires other plant orthologs of these proteins, including Vps34 (Liu et al., 2005). Together, these findings indicate that class III PI 3-kinase, and by extension PtdIns(3)*P*, is universally required for autophagy. The specific function of

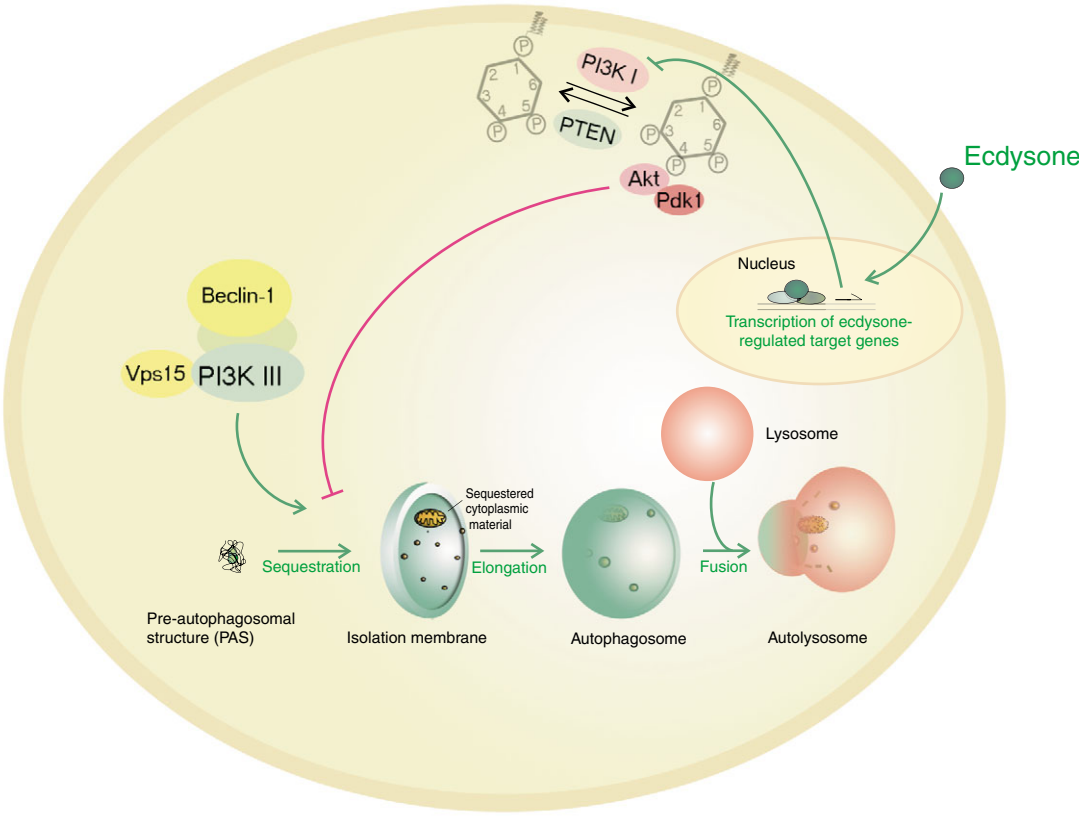

Fig. 4. Regulation of autophagy in *Drosophila* by PI 3-kinases. Autophagy is known to be regulated by both the class I and the class III PI 3-kinases. The class III PI 3-kinase is required for autophagy through producing PtdIns(3)*P* via the autophagy-specific complex containing Beclin-1, Vps15 and hVps34. (In yeast, an additional accessory protein, Vps14, has been shown to be part of the complex.) By contrast, the class I PI 3-kinase inhibits autophagy by activating the Akt/PKB pathway. The insect hormone ecdysone acts through nuclear receptors and triggers programmed autophagy in the *Drosophila* fat body by downregulating the class I PI 3-kinase pathway through an unknown mechanism. The overexpression of the PI 3-phosphatase PTEN mimics this effect.

PtdIns(3)*P* in autophagy has not been clarified, but it could function at an early stage, perhaps during PAS elongation (Noda et al., 2002). Clearly, it will be important to identify the effectors of PtdIns(3)*P* in this process.

Whereas class III PI 3-kinase is needed for the autophagic process, class I PI 3-kinases inhibit it, probably by activation of the kinases Akt/PKB and TOR (Jacinto and Hall, 2003). Insulin, a potent activator of class IA PI 3-kinases (Saltiel and Pessin, 2002), is a well-established inhibitor of autophagy (Pfeifer, 1977). Likewise, stimulation of the class I PI 3-kinase pathway by synthetic PtdIns(3,4,5)P_3 inhibits autophagy (Petiot et al., 2000), as does overexpression of a constitutively active form of PKB (Arico et al., 2001). PI 3-kinase also represses autophagy in *Caenorhabditis elegans* (Melendez et al., 2003), and the PI 3-phosphatase PTEN triggers autophagy when overexpressed. In *Drosophila melanogaster*, programmed autophagy is induced in the late larval stages by the hormone ecdysone through downregulation of the class I PI 3-kinase. This induction can be triggered even in the presence of a dominant-negative ecdysone receptor, by overexpression of PTEN (Rusten et al., 2004).

A surprising connection between class I and class III signaling in autophagy has recently emerged. Whereas class I PI 3-kinase mediates the inhibitory effects of insulin on autophagy through TOR activation, hVps34 appears to mediate the amino-acid-mediated activation of the TOR complex (Byfield et al., 2005; Nobukuni et al., 2005). At present, it is difficult to understand how hVps34 can be required for autophagy on the one hand and function as a central component of an autophagy-inhibitory pathway on the other hand. The explanation could be related to a modulatory effect of accessory proteins such as Beclin-1, a topic that clearly deserves further investigation.

Regulated exocytosis

Given the roles of class I and II PI 3-kinases in agonist-induced processes, it is not surprising that these kinases have also been implicated in regulated exocytosis, such as regulated degranulation of mast cells and basophils (Ali et al., 2004; Ito et al., 2002), insulin-induced membrane translocation of GLUT4 glucose transporters (Thong et al., 2005), and neurosecretory granule exocytosis (Meunier et al., 2005). Class IA PI 3-kinases play a pivotal role in the translocation of GLUT4-containing vesicles from endosomal compartments to the plasma membrane (Thong et al., 2005). The binding of insulin to its RTK causes tyrosine phosphorylation of insulin receptor substrate (IRS) proteins, which in turn causes recruitment of the regulatory p85 subunit of class IA PI 3-kinase through its SH2 domain, followed by enzyme activation and PtdIns(3,4,5)P_3 production. The mechanistic coupling between PtdIns(3,4,5)P_3 and GLUT4 translocation is not understood yet, but the PtdIns(3,4,5)P_3-activated kinases PDK1 and PKB appear to play a role in this process. In addition, there is evidence that the atypical protein kinase C (aPKC) forms PKC-ζ and PKC-λ/ι may be involved (Thong et al., 2005). Interestingly, even though these lack PH domains, they do contain regulatory domains that bind PtdIns(3,4,5)P_3 as well as other acidic phospholipids in vitro. However, it remains to be clarified how the activation of PDK1, PKB and aPKCs can be translated into GLUT4 vesicle translocation. There is evidence that GLUT4 translocation is actin dependent and, as mentioned previously, PtdIns(3,4,5)P_3 controls the cortical actin cytoskeleton through activation of GEFs for Arf6, Rac1 and Cdc42. Even though it seems paradoxical that PtdIns(3,4,5)P_3-mediated control of the actin cytoskeleton would mediate such highly distinct processes as phagocytosis and GLUT4 translocation, it is quite possible that the proper coordination between kinases and GEFs could in fact help orchestrate the remodeling of actin required for GLUT4 translocation.

In contrast to other PI 3-kinase-dependent exocytic processes characterized, neurosecretory granule exocytosis requires a class II PI 3-kinase, PI3K-C2α. This kinase and its reaction product PtdIns(3)*P* appear to be involved in the ATP-dependent priming of mature neurosecretory granules to make these competent for fusion with the plasma membrane (Meunier et al., 2005). It will be interesting to identify the

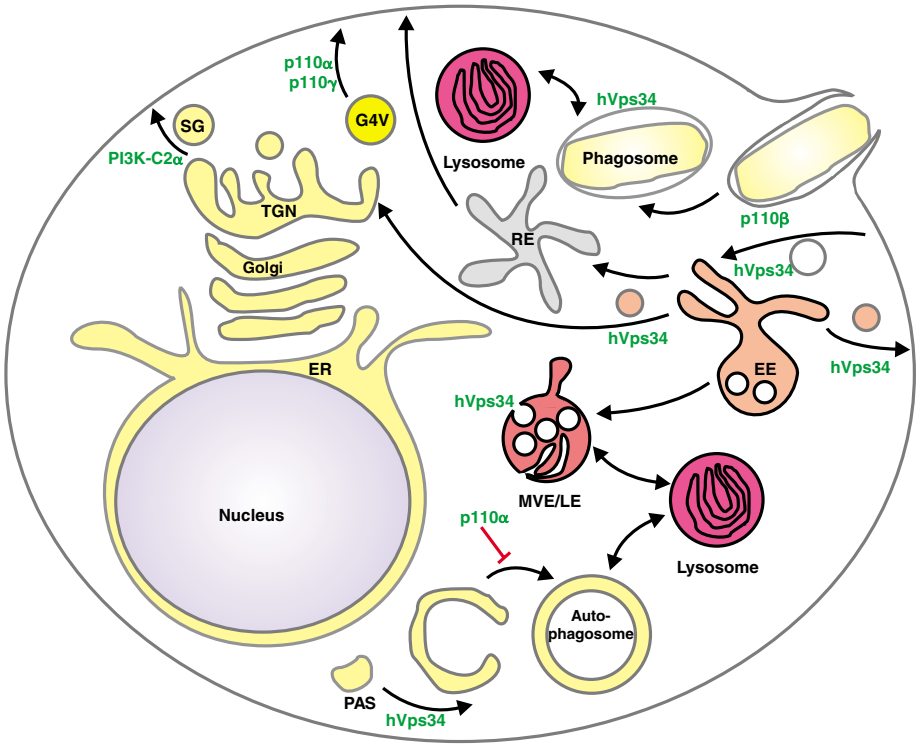

Fig. 5. PI 3-kinases in membrane trafficking. The figure summarizes the involvement of PI 3-kinases in trafficking pathways discussed in this review. PI 3-kinases (catalytic subunits) are indicated in green. ER, endoplasmic reticulum; EE, early endosome; G4V, GLUT4-containing vesicle; LE, late endosome; MVE, multivesicular endosome; PAS, pre-autophagosomal structure; RE, recycling endosome; SG, secretory granule.

PtdIns(3)*P* effector(s) on neurosecretory granules that regulate this process. An especially challenging question in this context is how such proteins can be recruited preferentially to neurosecretory granules and not to endosomes, which contain high amounts of PtdIns(3)*P*.

Conclusions and perspectives

PI 3-kinases regulate a variety of intracellular trafficking events that include cargo selection, vesicle formation, vesicle movement and membrane fusion. The evolutionarily conserved class III PI 3-kinase hVps34 mediates most of the trafficking events studied, through its catalytic product PtdIns(3)*P* and the FYVE- and PX-domain-containing effectors of this lipid. Most of these events can be regarded as housekeeping functions that occur constitutively. By contrast, the class I and II PI 3-kinases appear to have evolved as regulators of agonist-induced processes, such as phagocytosis and regulated exocytosis (summarized in Fig. 5). It is easy to envision a role for class III PI 3-kinase and PtdIns(3)*P* in constitutive membrane trafficking, and class I PI 3-kinases and PtdIns(3,4,5)P_3 in acute agonist-stimulated trafficking events, but the role of the class II enzymes remains more enigmatic. These latter enzymes appear to be agonist activated but yield PtdIns(3)*P*, and the reaction product is generated on membranes other than the 'typical' localization of PtdIns(3)*P* (i.e. endosomes). This raises the question of whether different PtdIns(3)*P* effectors can distinguish between PtdIns(3)*P* formed by class II and class III PI 3-kinases. It is not inconceivable that such a distinction can take place. As discussed herein, some PI effectors appear to require a coincident detection of the PI and another structural determinant, such as membrane curvature or binding to a small GTPase. It is thus possible that a subset of PtdIns(3)*P* effectors are recruited to the plasma membrane or neurosecretory granules through coincident detection of additional determinants on these membranes.

Even though PI 3-kinases mediate their functions through the formation of 3-PIs, another activity of these enzymes should not be neglected. These kinases have a dual substrate preference – they can also phosphorylate proteins. Because the protein kinase activity of PI kinases is generally weak, it has usually been dismissed as a nonspecific in vitro artifact of questionable biological relevance. However, recent results have shown that it is possible to make mutations in class IB PI 3-kinase that differentially affect its protein and PI kinase activities. Importantly, the protein kinase activity of class IB PI 3-kinase has been shown to be essential for endocytosis of β-adrenergic receptors (a type of GPCR) through wortmannin-sensitive phosphorylation of non-muscle tropomyosin, a protein required for agonist-induced endocytosis of β-adrenergic receptors (Naga Prasad et al., 2005). This intriguing finding means that we should revisit several previous studies in order to exclude the possible involvement of proteins rather than PIs as direct mediators of various PI 3-kinase functions. In any case, the finding that PI 3-kinases can exert part of their function as protein kinases does not preclude the conclusion that PtdIns(3)*P*, PtdIns(3,4,5)P_3 and their effectors mediate the vast activities of PI 3-kinases in membrane trafficking. The embedding of these lipids in the cytoplasmic leaflets of biological membranes, combined with their highly regulated generation and turnover, make these PIs ideal for the spatiotemporally-controlled recruitment of protein complexes that mediate various steps of intracellular membrane trafficking. Future studies will tell us more about the regulation of PI 3-kinases, identify new effectors of PtdIns(3)*P* and PtdIns(3,4,5)P_3, and explain how these act together to control the intricate mechanisms of membrane trafficking.

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