An enzymatic cascade of Rab5 effectors regulates phosphoinositide turnover in the endocytic pathway

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Generation and turnover of phosphoinositides (PIs) must be coordinated in a spatial- and temporal-restricted manner. The small GTPase Rab5 interacts with two PI 3-kinases, Vps34 and PI3Kα, suggesting that it regulates the production of 3-PIs at various stages of the early endocytic pathway. Here, we discovered that Rab5 also interacts directly with PI 5- and PI 4-phosphatases and stimulates their activity. Rab5 regulates the production of phosphatidylinositol 3-phosphate (PtdIns[3]P) through a dual mechanism, by directly phosphorylating phosphatidylinositol via Vps34 and by a hierarchical enzymatic cascade of phosphoinositide-3-kinaseβ (PI3Kβ), PI 5-, and PI 4-phosphatases. The functional importance of such an enzymatic pathway is demonstrated by the inhibition of transferrin uptake upon silencing of PI 4-phosphatase and studies in weeble mutant mice, where deficiency of PI 4-phosphatase causes an increase of PtdIns(3,4)P2 and a reduction in PtdIns[3]P. Activation of PI 3-kinase at the plasma membrane is accompanied by the recruitment of Rab5, PI 4-, and PI 5-phosphatases to the cell cortex. Our data provide the first evidence for a dual role of a Rab GTPase in regulating both generation and turnover of PIs via PI kinases and phosphatases to coordinate signaling functions with organelle homeostasis.

Introduction

Phosphoinositides (Pis) play a key role in fundamental cell functions, such as signal transduction, cytoskeleton remodeling, cell migration, and membrane trafficking (De Camilli et al., 1999b). The latter kinase is responsible for the generation of phosphatidylinositol 3-phosphate (PtdIns[3]P) on the early endosome and the recruitment of a set of PtdIns[3]P binding Rab5 effectors such as EEA1 (Simonsen et al., 1998; Christoforidis et al., 1999b). The former kinase is responsible for the generation of phosphatidylinositol 3-phosphate (PtdIns[3]P) on the early endosome and the recruitment of a set of PtdIns[3]P binding Rab5 effectors such as EEA1 (Simonsen et al., 1998; Christoforidis et al., 1999b).

Whereas PtdIns(4)P on the TGN and PtdIns(3)P on endosomes exert an essential housekeeping function in organelle homeostasis and membrane transport, other PIs are produced in response to a variety of extracellular stimuli. Phosphatidylinositol 3,4-bisphosphate (PtdIns[3,4]P2) and phosphatidylinositol...
3,4,5-trisphosphate (PtdIns[3,4,5]P3) are produced by type I PI 3-K upon stimulation by growth factors or cytokines at the plasma membrane, where they induce morphogenetic changes via the reorganization of actin filaments (Vanhaesebroeck et al., 2001). However, these PI species accumulate only transiently. PtdIns(3,4,5)P3 peaks at 5–6 s after stimulation (Chung et al., 2001) and is rapidly degraded upon phagocytosis and macropinocytosis by PI phosphatases (Marshall et al., 2001; Rupper et al., 2001; Funamoto et al., 2002). Similar to PI kinases, PI phosphatases display exquisite substrate specificity. For example, PI 3-phosphatases such as PTEN (Cantley and Neel, 1999) dephosphorylate PtdIns(3,4,5)P3 to PtdIns(4,5)P2. PI 5-phosphatases such as SHIP, synaptojanin, and type II PI 5-phosphatase, dephosphorylate either PtdIns(3,4,5)P3 or PtdIns(4,5)P2 or both, respectively (Vanhaesebroeck et al., 2001; Mitchell et al., 2002). Two PI 4-phosphatases isoforms exist (types I and II) each having two alternative splicing variants (α and β), which preferentially dephosphorylate PtdIns(3,4)P2 to PtdIns(3)P (Norris et al., 1995, 1997). The functional importance of PI kinases and phosphatases in PI metabolism is underscored by the finding that mutations in genes encoding these proteins are associated with hereditary disorders in humans and induce severe developmental abnormalities in animal model systems, particularly affecting neural function. For example, the gene product deficient in the oculocerebrorenal syndrome of Lowe (OCRL) is an inositol polyphosphate 5-phosphatase (Attree et al., 1992; Zhang et al., 1995) associated with endosomes and Golgi membrane (Ungewickell et al., 2004; Choudhury et al., 2005). A targeted mutation of mouse synaptojanin-1 (Cremona et al., 1999) causes defects in vesicle trafficking and actin dynamics at the synapse. Conditional knockout of PTEN specifically in the brain induces severe alterations in the cerebellum, with decreased cell proliferation and degeneration of Purkinje cells in mice (Backman et al., 2001). The weebale mutant mice bearing a mutation in the gene encoding type I PI 4-phosphatase are characterized by early postnatal neuronal loss in the cerebellum and in the hippocampus, that ultimately results in the death of homozygous animals 2–3 wk after birth (Nystuen et al., 2001). Whereas the identification and characterization of several PI kinases and phosphatases has greatly advanced our understanding of the enzymology of PI metabolism, the mechanisms that coordinate the activity of these enzymes to link PIs function and turnover remain largely unknown. Here, we provide insights into this question through the discovery of novel effectors of Rab5.

Results

Compartmentalization of PIs

Rab5 interacts with two distinct types of PI 3-K, PI3Kβ, and hVps34 (Christoforidis et al., 1999b). To investigate whether such interaction serves to regulate the synthesis of PtdIns(3)P as well as other PIs in the early endocytic pathway we took advantage of the Rab5Q79L activated mutant, which causes the expansion of early endosomes (Fig. 1 A). We examined the intracellular localization of different PIs by expressing GFP fused to various PIs-binding motifs. The 2XFYVE domain of Hrs, which recognizes PtdIns(3)P (Gillooly et al., 2003), accumulated on early endosomes (Fig. 1 A, 2XFYVE). In contrast, even upon Rab5 activation the PH domain of Akt, a probe for PtdIns(3,4)P2 and PtdIns(3,4,5)P3 (Watton and Downward, 1999), localized to the plasma membrane in a polarized fashion and no significant staining was observed on early endosomes (Fig. 1 A, Akt1[PH]). The PH domain of phosphatidylinositol 4-phosphate adaptor protein 1 (FAPP1), which recognizes PtdIns(4)P (Dowler et al., 2000), was mainly present on the Golgi apparatus (Fig. 1 A, FAPP1[PH]), where it colocalized with β'-COP (Levine and Munro, 2002; De Matteis and Godi, 2004; unpublished data), whereas the PLCβ PH domain, which binds PtdIns(4,5)P2 (Stauffer et al., 1998) was detected on the plasma membrane and in the nucleus. Thus, among the PIs tested, PtdIns(3)P is the only one that accumulates on early endosomes bearing activated Rab5.

Early and recycling endosomes are structured as a mosaic of subcompartments that harbour distinct Rab GTPases and display a nonstochastic distribution (Sonnichsen et al., 2000). We next investigated whether PtdIns(3)P is not only specifically concentrated in the Rab5 domain within the early endo-
somes (Gillooly et al., 2003) but also depleted from the other Rab domains along the recycling pathway. Plasmids encoding myc–2XFYVE, GFP–Rab5, and YFP–Rab4 or myc–2XFYVE, YFP–Rab4, and GFP–Rab11 were microinjected into A431 cells (Fig. 1, B and D and E) and the overlap between Rab5, Rab4, and Rab11 and myc–2XFYVE was quantified (Fig. 1, C and E), as previously described (Sonichsen et al., 2000). The 2XFYVE fluorescent probe was expressed at levels that neither abolished in the presence of 50 nM wortmannin (unpublished data). Surprisingly, in addition to the aforementioned products, additional PI species were generated when PtdIns(4)P and PtdIns(4,5)P2 were used as substrates (Fig. 2 A, asterisks). In the presence of PtdIns(4)P, not only PtdIns(3,4)P2, but also PtdIns(3)P was detected. When PtdIns(4,5)P2 was used as substrate, in addition to the expected PtdIns(3,4,5)P3, also PtdIns(3,4)P2 and PtdIns(3,4)P2 were generated (Fig. 2 A, lane 3, asterisks). All TLC spots were 3’-phosphorylated PIs (3-PIs) as confirmed by HPLC analysis after excision from the TLC plate (unpublished data). The most straightforward explanation for such complex pattern of PIs is that, in addition to the two PI 3-Ks, the Rab5 affinity column eluate contains PtdIns(3,4,5)P3 5-phosphatase and PtdIns(3,4)P2 4-phosphatase activities. We thus performed PI 5-phosphatase and 4-phosphatase activity assays using PtdIns(3,4,5)P3 and PtdIns(3,4)P2 32P-labeled substrates (see Materials and methods). As shown in Fig. 2 B, PtdIns(3,4,5)P3 5-phosphatase and PtdIns(3,4)P2 4-phosphatase activities were indeed detected in the column eluate from the Rab5–GTPγS but not the Rab5–GDP affinity column. We therefore set out determine the identity of such phosphatases.

Identification of PI phosphatases as new Rab5 effectors

The Rab5–GTPγS column eluate was fractionated by Superose 6 gel filtration column chromatography (Christoforidis et al., 1999a; Fig. 2 C) and the PtdIns(3,4,5)P3 5-phosphatase and PtdIns(3,4)P2 4-phosphatase activities measured in every third fraction (Fig. 2, D and E). The phosphatase assay was performed in the presence of 50 nM wortmannin to inhibit the PI 3-K activity during the reaction. Peaks of PI 5- and 4-phosphatase activity were detected in fractions 26–29 and 34–36,
respectively, and protein bands from SDS-PAGE of these fractions were subjected to mass spectrometry sequencing. Among these proteins, we identified a type II inositol 5-phosphatase (5-Pase; Jefferson and Majerus, 1995) and a type I α PtdIns(3,4,5)P3 4-phosphatase (4-Pase; Norris et al., 1995). We cloned the cDNAs encoding both phosphatases, expressed them in Sf9 insect cells using the baculovirus system, purified the recombiant proteins and raised corresponding polyclonal antisera in rabbits. The antibodies against the 5- and 4-Pase detected protein bands corresponding to the expected molecular weight both in brain cytosol and selectively in the Rab5–GTP eluted protein bands corresponding to the expected molecular antisera in rabbits. The antibodies against the 5- and 4-Pase de- product, respectively.

To address this question, we used endosome enriched fractions from HeLa cells, incubated these membranes with [γ32P] cATP, in the presence or absence of either Rab5–GDI complex or RabGDI alone (see Materials and methods). Lipids were extracted, deacylated, and the PI isomers determined by HPLC analysis. Addition of RabGDI, which efficiently

**Direct and specific interaction between 5- and 4-Pases and Rab5**

The following three experiments demonstrated that both 5- and 4-Pase interact with Rab5 directly and specifically. First, we used a GST–Rab5 pull-down assay. Both recombinant 5- and 4-Pases bound to beads containing GST–Rab5 preloaded with GTPyS but not GDP (Fig. 3 C). Second, the specificity of such interaction was verified using the yeast two-hybrid system. Using the β-galactosidase replica filter assay (Fig. 3, D and E), blue colonies were obtained only upon cotransformation of prey plasmids (5- and 4-Pase) with bait vectors expressing wild-type Rab5 (WT) or activated Rab5 Q79L (QL) but neither from dominant negative Rab5 S34N (SN), nor Rab4 (WT, Q67L, S22N) transformants. Third, we tested the ability of Rab5–GTP to recruit 5- and 4-Pase onto endosomes in vivo. Whereas both endogenous and overexpressed 5- and 4-Pases were predominantly cytosolic, a fraction was recruited onto the membrane of enlarged endosomes specifically upon coexpression of the activated Rab5 Q79L mutant in HeLa cells (Fig. 3 F). Combined with the data of Fig. 2, these results suggest that the 5- and 4-Pases interact in a GTP-dependent manner, directly and specifically with Rab5 both in vitro and in vivo.

**Active Rab5 stimulates the catalytic activity of PI3Kβ, 5-, and 4-Pase in vitro**

Rab5 interacts with three different enzymes that can be ordered in a pathway to sequentially generate PtdIns(3,4,5)P3 from PtdIns(3,4)P2 (5-Pase) and PtdIns(3)P (4-Pase). To test the hypothesis that conversion of PtdIns(3,4,5)P3 into PI(3)P may indeed be regulated by Rab5, we first investigated whether the interaction of 5- and 4-Pases or PI3Kβ with this GTPase can result in a stimulation of their enzymatic activity. Recombinant 5-Pase, PI3Kβ, or the Superose 6 fraction containing the 4-Pase were incubated with recombinant Rab5 preloaded with GTPyS or GDP before determining the corresponding enzymatic activity. Nonprenylated Rab5 was used in this experiment as it binds these enzymes in vitro (Christoforidis et al., 1999b). PtdIns(4,5)P2 was used as substrate for PI3Kβ activity and 3’-32P-labeled PtdIns(3,4,5)P3 or PtdIns(3,4)P2 as substrates for 5- or 4-Pase activity, as described above (Fig. 2 B). Rab5–GTPyS but not Rab5–GDP dose dependently stimulated the activity of PI3Kβ, 5-Pase, or 4-Pase (Fig. 4, A–C). We conclude that the interaction with Rab5 stimulates the enzymatic activity of PI3Kβ, 5-, and 4-Pases in vitro.

**Rab5 modulates PtdIns(3)P production by two different mechanisms**

These findings prompted us to determine whether Rab5 actively regulates the production of PtdIns(3)P on membranes. To address this question, we used endosome enriched fractions from HeLa cells, incubated these membranes with [γ32P] cATP, in the presence or absence of either Rab5–GDI complex or RabGDI alone (see Materials and methods). Lipids were extracted, deacylated, and the PI isomers determined by HPLC analysis. Addition of RabGDI, which efficiently
Rab5 regulates PI(3)P production with its effectors. (A–C) The activity of recombinant protein of PI3K (p85γ–p110β), 150 nM or 5-Pase (25 nM) or 4-Pase fraction (1 μl fraction 36) were analyzed with recombinant Rab5–GDP or–GTPγS in the presence of GDP (white bar) or GTPγS (black bar), as indicated. The concentration of recombinant Rab5 is indicated in each bar. Bars indicate the stimulation of the enzymatic activity expressed in percent of the control (bars indicate the stimulation of the enzymatic activity expressed in percent of the control). The simplest explanation of these results is that 30% of the Rab5–GDI complex increases the fraction of Rab5 on the membrane and the recruitment of its effectors (Rubino et al., 2000) stimulated the synthesis of PtdIns(3)P more than 2.5-fold (Fig. 4 D, compare first bar with third bar).

In principle, Rab5 can generate PtdIns(3)P directly through phosphorylation of PI by hVps34. However, because PI3Kβ, 5-, and 4-Pase are also Rab5 effectors, these three enzymes could be functionally linked in the generation of PtdIns(3)P from PtdIns(3,4,5)P3. To test this hypothesis, we used function-blocking antibodies, as shown previously for hVps34 (Siddhanta et al., 1998; Christoforidis et al., 1999b), and to the sequential dephosphorylation of PI and PtdIns(3,4,5)P3 (p85γ–p110β) or 4-Pase in vitro (unpublished data).

Anti-hVps34 function blocking antibodies reduced the production of PtdIns(3)P in endosomal fractions by 70%, consistent with previous results showing that Vps34 is required for the Rab5-dependent recruitment of EEA1 and early endosome fusion (Siddhanta et al., 1998; Christoforidis et al., 1999b; Hill et al., 2000; Fig. 4 E). Interestingly, anti-p110β function-blocking antibodies also reduced generation of PtdIns(3)P by 30%, and a comparable degree of inhibition was obtained with anti–4-Pase antibodies (Fig. 4 E). The stimulatory effect of Rab5 on PtdIns(3)P production was also dependent on PI3Kβ (p85γ–p110β) and 4-Pase. The concomitant addition of anti-p110β or anti–4-Pase to Rab5GDI complex inhibited PtdIns(3)P by 30–40% (Fig. 4 D, compare third bar with fourth and fifth bar), supporting the idea that PI3Kβ and 4-Pase are downstream effectors of Rab5.

The simplest explanation of these results is that 30% of the PtdIns(3)P production in these membrane fractions, which contain early endosomes and some residual plasma membrane fragments, is due to PI3Kβ and to the sequential dephosphorylation of PtdIns(3,4,5)P3 via 5- and 4-Pase activities. Given the established activation of type I phosphoinositide-3-kinase (PI3-K) at the plasma membrane (Stephens et al., 1993; Fig. 1), the presence of PI3-Kβ on clathrin-coated vesicles (Christoforidis et al., 1999b) and the enrichment of PI(3)P on early endosomes (Gillooly et al., 2003), these results suggest that Rab5 regulates the maintenance of a gradient of PtdIns(3)P from the plasma membrane to endosomal membranes by a combination of direct synthesis and PtdIns(3,4,5)P3 dephosphorylation.
Down-regulation of the 4- and 5-Pase inhibits transferrin uptake

We next explored the functional role of the 4-Pase in Rab5-mediated endocytic transport, by measuring transferrin internalization. We established experimental conditions to knockdown the 4-Pase using specific small interfering RNA (siRNA) oligonucleotides. 72 h after transfecting the cells with the siRNAs, we observed a dramatic reduction (~70%) in 4-Pase protein levels as evidenced by Western blot (Fig. 5 A). Strikingly, we observed that knockdown of 4-Pase markedly inhibited transferrin internalization (Fig. 5 B). Silencing of the 5-Pase yielded a similar phenotype (unpublished data). These results therefore suggest a requirement of 4- and 5-Pases for Rab5-dependent receptor-mediated endocytosis.

Translocation of 4- and 5-Pase to cortical ruffles following serum stimulation

We next investigated whether the localization of endogenous 5- and 4-Pase were consistent with the aforementioned model. To this aim we used primary cultures of astrocytes, as pilot experiments revealed significant levels of both proteins in these cells (Fig. 6). PtdIns(3,4,5)P3 production is strongly stimulated by growth factors, which induce recruitment and activation of PI 3-K to the plasma membrane (Stephens et al., 1993). Thus, we examined the localization of 5- and 4-Pase both in serum-starved cells and in starved cells exposed to serum for 15 min. The 4-Pase and the 5-Pase were mainly diffuse in the cytoplasm of serum-starved astrocytes (Fig. 6). However, upon serum stimulation, both enzymes translocated to the cortical ruffles where they colocalized with the actin binding protein cortactin and with a pool of Rab5 also recruited to the cell surface (Fig. 6 A). The localization of Rab5 to the plasma membrane is in good agreement with its participation in the generation of membrane ruffles and lamellipodia (Spaargaren and Bos, 1999; Lanzetti et al., 2004; unpublished data). Furthermore, EEA1-positive early endosomes also accumulated underneath the regions of the ruffling plasma membrane that were enriched in the 5- and the 4-Pase (Fig. 6 B). However, the two phosphatases did not accumulate on these structures although we occasionally observed the presence of the 4-Pase.
The 4-Pase was found to be present at the highest level in cerebellar Purkinje cells (Fig. 7 B), where it had a diffuse distribution throughout the cell body, dendrites, axons, and axon terminals (Fig. 7, B and D; Fig. 7, F–H shows colocalization of 4-Pase with the nerve terminal marker VAMP2–synaptobrevin 2 in Purkinje cell nerve terminals in the deep cerebellar nuclei). This result is consistent with high expression of 4-Pase mRNA in Purkinje cells and with the finding that Purkinje cells are the most severely affected cell population of the cerebellum are the most severely affected cell population of the brain (unpublished data). The staining was completely absent in brain of weebles (unpublished data). The staining was completely absent in brain of weebles (Fig. 7, C, E, and J), indicating that the antibody specifically detects the 4-Pase protein, as confirmed by Western blot analysis (Fig. 7 A).

We investigated whether the absence of 4-Pase in weebles results in a pathway leading to the generation of PtdIns(3,4,5)P3 in vivo, we searched for potential alterations of PI metabolism in cells of weebles. Weebles carry a loss of function mutation in the gene encoding the 4-Pase (Nystuen et al., 2001). The 4-Pase was found to be present at the highest level in cerebellar Purkinje cells (Fig. 7 B), where it had a diffuse distribution throughout the cell body, dendrites, axons, and axon terminals (Fig. 7, B and D; Fig. 7, F–H shows colocalization of 4-Pase with the nerve terminal marker VAMP2–synaptobrevin 2 in Purkinje cell nerve terminals in the deep cerebellar nuclei). This result is consistent with high expression of 4-Pase mRNA in Purkinje cells and with the finding that Purkinje cells are the most severely affected cell population of the cerebellum are the most severely affected cell population of the brain (unpublished data). The staining was completely absent in brain of weebles (unpublished data). The staining was completely absent in brain of weebles (Fig. 7, C, E, and J), indicating that the antibody specifically detects the 4-Pase protein, as confirmed by Western blot analysis (Fig. 7 A).

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levels of PtdIns(3,4,5)P3 in stimulated cells was also observed (Fig. 7 L), possibly reflecting some compensatory feed-back mechanisms. Consistent with the data of Fig. 4 (D and E), the levels of PtdIns(3)P were also reduced under both resting and stimulated conditions (27% and 28%, respectively; Fig. 7 L, inset) in the cells lacking 4-Pase as compared with wt cells. Altogether, these data indicate that the absence of 4-Pase specifically leads to an accumulation of PtdIns(3,4)P2 and a reduction of PtdIns(3)P presumably due a defect of PtdIns(3,4,5)P3 turnover, in agreement with the measurements on HeLa cells in vitro (Fig. 4 E).

Discussion

In this study we report that Rab5 coordinates a cascade of PI enzymes including the two previously described PI 3-Ks (hVps34 and PI3Kβ) and two new Rab5 effectors, the type II inositol 5-Pase (Jefferson and Majerus, 1995) and the type I α PtdIns(3,4)P2 4-Pase (Norris et al., 1995). Our data uncover a mechanism whereby a single GTPase, Rab5, regulates both synthesis and turnover of PIs through an enzymatic cascade of effectors, thus coupling the function of PIs in signal transduction to the requirements of endocytic trafficking.

Rab5 regulates PI synthesis and turnover in the endocytic pathway

Rab5 regulates a complex network of effector proteins that are recruited on the early endosome membrane by binding to PtdIns(3)P (Simonsen et al., 1998; Christoforidis et al., 1999b; Nielsen et al., 2000; Schnatwinkel et al., 2004). PtdIns(3)P is an important molecular hallmark of the endocytic pathway (Wurmser and Emr, 1998). It is required for early endosome fusion and motility along microtubules (Christoforidis et al., 1999a; Hoepfner et al., 2005), phagosome maturation (Vieira et al., 2001), multivesicular body formation (Futter et al., 2001), and signaling (Tsukazaki et al., 1998). The findings that the lipid kinase that generates PtdIns(3,5)P, hVps34, is also a Rab5 effector (Christoforidis et al., 1999b) and that the effectors form large oligomeric complexes on the early endosome membrane (McBride et al., 1999), led us to propose that Rab5 regulates the formation of a membrane domain on the early endosome enriched in PtdIns(3)P and containing the various effector proteins required for early endosome tethering, fusion, and motility (Zerial and McBride, 2001). Our present results strengthen this model with the demonstration that the synthesis of PtdIns(3)P is regulated by Rab5 itself.

Among the PIs tested, PtdIns(3)P was the major 3-PI present on early endosomes, despite the generation of other PI species on the plasma membrane and a continuous membrane flow toward the early endosomes. Furthermore, PtdIns(3)P is enriched in the Rab5 domain and less abundant or absent from other subcompartments of early and recycling (Rab4- and Rab11-positive) endosomes. In addition to hVps34, Rab5 interacts also with PI3Kβ, a type I PI 3-K involved in the generation of PtdIns(3,4,5)P3 and PtdIns(3,4)P2 upon stimulation at the plasma membrane. Even in the presence of the constitutively active Rab5Q79L mutant, PtdIns(3,4,5)P3 was localized to the plasma membrane but not early endosomes, suggesting that turnover of this PI must occur early to maintain the specificity of PtdIns(3)P localization in the endosomal system. The discovery that Rab5 interacts with, and stimulates the enzymatic activity of, 5- and 4-Pase, provides an explanation for how such synthesis and turnover can be coordinated.

We propose that the synthesis of PtdIns(3,4,5)P3 at the plasma membrane is coupled either to the dephosphorylation of the 3’ position by PTEN, thus leading to PtdIns(4,5)P2, or to the sequential dephosphorylation by 5- and 4-Pase, leading to PtdIns(3)P. Such enzymatic cascade could initiate at the plasma membrane, where under certain stimulatory conditions pools of PtdIns(3)P can be detected (Maffucci et al., 2003), and continue along transport to the early endosomes, given that PI3Kβ is detected in clathrin-coated vesicles (Christoforidis et al., 1999b), presumably until PtdIns(3,4,5)P3 and PtdIns(3,4)P2 are depleted. Other 5-phosphatases, in particular SHIP, that prefers PtdIns(3,4,5)P3 as a substrate, may cooperate with 5-Pase in the first of the two dephosphorylation reactions. This model is consistent with a study by Ivetac et al. (2005), published while this manuscript was in revision, that reported the association of 4-Pase with early and recycling endosomes in COS-1 cells.

Interestingly, the 5-Pase was identified in a search for PtdIns(3,4,5)P3-binding proteins (Krügmann et al., 2002) and the 4-Pase was recovered in a complex with PI3-K (Munday et al., 1999) and binds PtdIns(3,4)P2 via its C2 domains (Ivetac et al., 2005). These observations raise the interesting possibility that, at the plasma membrane, Rab5 may regulate the recruitment of various effectors by a combinatorial principle similar to the one operating on early endosomes. Whereas on early endosomes Rab5 regulates the recruitment of FYVE proteins (e.g., EEA1) in combination with PtdIns(3)P, at the plasma membrane it may cooperate with PtdIns(3,4,5)P3 in the recruitment of other effector proteins. Specifically, activated Rab5 would bind and stimulate PI3Kβ activity, thus eliciting in a positive feedback mechanism the production of its “co-receptor” PtdIns(3,4,5)P3. Both Rab5 and PtdIns(3,4,5)P3 would then serve as binding sites to recruit the 5-Pase, resulting in dephosphorylation of PtdIns(3,4,5)P3 to PtdIns(3,4)P2. PtdIns(3,4)P2 would then recruit the 4-Pase, which, activated by Rab5-GTP, would dephosphorylate PtdIns(3,4)P2 to PtdIns(3)P.

The extent to which such enzymatic cascade operates along the pathway probably depends on cell type and growth conditions. Under steady-state, it may contribute only a lesser (~30%) fraction of PtdIns(3)P production in comparison with direct phosphorylation of PtdIns by hVps34. However, it may constitute an important regulatory system ensuring endocytic transport and organelle homeostasis under various PI 3-K-dependent signaling conditions. Rab5 itself is activated both at the plasma membrane and on EEA1-positive early endosomes upon EGF stimulation (Di Fiore and De Camilli, 2001). The stimulatory activity of Rab5 on PI3Kβ could thus contribute to the generation of 3-Pis at the cell surface in response to various signals, thus inducing morphogenetic changes (Spaargaren and Bos, 1999; Lanzetti et al., 2004). The finding that both 5- and 4-Pase are recruited along with Rab5 to the cell cortex upon se-
Unbalance in PI metabolism and neuronal degeneration in 4-Pase-deficient mice

That the aforementioned turnover of PIs is of high physiological importance is underscored by the phenotypic analysis of weeble mutant mice (Nystuen et al., 2001). When we inspected the PIs of cultured astrocytes of weeble mice, we detected an enhanced production of PI(3)P on endosomes by 4-Pase RNAi (unpublished data). By converting PtdIns(4,5)P2 into PtdIns(4)P, OCRL may contribute to the Rab5-dependent regulation of PIs on endosomal receptor trafficking and sorting (Ungewickell et al., 2004; Choudhury et al., 2005).

Although dephosphorylation at the 3’ position of the inositol ring by PI 3-Pases such as PTEN (Leslie and Downes, 2002) may contribute to termination of PI(3,4,5)P3 signaling, a distinguished feature of the enzymatic cascade described in this study is the generation of other 3-PIs that have signaling functions of their own. Unlike Ivetac et al. (2005), we could not consistently observe endosomal abnormalities in HeLa cells lacking 4-Pase or primary cultures of weebie neurons (unpublished data). The expression of inositol 4-phosphatase type II may partially compensate for the lack of 4-Pase (the type I isoform; Majerus et al., 1999) and the presence of Vps34 ensures the bulk of production of PtdIns(3)P. However, unexpectedly, we could detect alterations in receptor-mediated endocytosis. Because inhibition of PI3-K with wortmannin does not dramatically impair transferrin internalization (Martsys et al., 1996; Shpetner et al., 1996; Spiro et al., 1996), it is plausible that the accumulation of PtdIns(3,4,5)P3 and PtdIns(3,4)P2 rather than reduced production of PI(3)P on endosomes by 4-Pase RNAi may exert an inhibitory effect on the endocytic process.

Materials and methods

Reagents and cell lines

Phospholipids were purchased from Sigma-Aldrich, [γ-32P]ATP from Amer sham Biosciences, and Silica Gel 60 TLC plates (20 × 20 cm) from Merck. [3H]PtdIns, [3H]PtdIns(4)P, and [3H]PtdIns(4,5)P2 were purchased from NEN Life Science Products. Most HPLC grade organic chemicals and water were purchased from Fluka or Merck. GST–p85α baculovirus was a gift of Dr. Waterfield (Ludwig Institute for Cancer Research, London, UK), the polyclonal human anti-EEA1 antibody, GFP-2XFYVE (Hrs) and myc-2XFYVE plasmids of Dr. Stenmark (Norwegian Radium Hospital, Oslo, Norway). A431, NIH3T3, Hela cells, and astrocytes from weebie mice were cultured under standard conditions.

Antibodies and plasmids

Anti-p110β or HkVps34 function blocking antibodies were raised against synthetic peptides (C)KVNWMAHTVRKDYRS or AVVEQIHKFAQYWRK (Siddhanta et al., 1998; Hill et al., 2000) and anti-3 or -4 Pase antibodies against recombinant full length proteins (see Preparation of recombinant PtdKp, 5-Pase, 4-Pase, and Rab5). The affinity-purified anti-p110β and anti-4-Pase antibodies blocked >80% activity of the recombinant proteins in 1:2 or 1:4 antigen/antibody molar ratio. Anti-HkVps34 antibody was used as previously reported (Siddhanta et al., 1998; Christoforidis et al., 1999b). Mouse monoclonal antibody against Rab5a was 4F11, monoclonal mouse anti-cortactin, and rat anti-HA (3F10) antibodies, and HRP- and...
fluorescent-conjugated secondary antibodies were purchased from Upstate Biotechnology, Rock, Dianova, and Molecular Probes, respectively.

The human CDNA encoding human p110β, a gift of Dr. M. (Free University, Berlin, Germany), was subcloned into modified baculovirus expression vector pFastBacGST. The human CDNAs encoding 5- and 4-Pase obtained by RT-PCR were subcloned into pcdNA3 (In vitro), pGAD10, and pFastBacGST. Plasmids expressing ECFP- and EYFP-tagged Rab and Rab5-GFP/79L was as previously described [Stenmark et al., 1994; Sonnichsen et al., 2000]. CDNAs encoding the PH domain of human Akt was cloned by RT-PCR and mouse mGFP, human FAPP1 were purchased from IMAGE consortium and subcloned into EGF-p3 vectors [Clontech].

Yeast two-hybrid and pull down assay

Yeast transformation and two-hybrid analysis were performed according to the MATCHMAKER instructions [Clontech]. In brief, a yeast strain L40 was cotransformed with a PexA-based bait vector and a pGAD10-based prey vector and plated on a medium lacking Trp and Leu. After 2-3 of incubation colonies were tested for β-galactosidase activity by the replica filter assay. GST-Rab5 pull-down assay using recombinant 5- and 4-Pase proteins (5 µg each) was performed as previously described [Christofidis et al., 1999a].

Cell transfection, microinjection, and immunofluorescence analysis

Plasmids were transfected to NIH3T3 cells with FuGENE6 (Roche). Expression of proteins in HeLa cells grown to 60% confluence using the T7 RNA polymerase-dependent vaccinia virus was as in [Stenmark et al., 1994].

For the colocalization of 2XYFVY with Rab proteins, a mixture of 50 ng/µl plasmid DNA for myc-2XYFVY, EYFP-Rab4, and ECFP-Rab5 or ECFP-Rab11 was injected into the nucleus of A431 cells with an Eppendorf microinjection apparatus and transfection. Immunofluorescence analysis [Stenmark et al., 1994] was performed using an LSM 510 station confocal microscope [Carl Zeiss MicroImaging, Inc.]. Quantification of colocalization was performed as in Sonnichsen et al. (2000).

Cultured astrocytes serum-starved overnight were incubated in MEM with or without 10% FCS for 15 min at 37°C, fixed in 4% PFA in phosphate buffer, and then stained by immunofluorescence by standard procedures. Primary antibodies were visualized with goat anti-rabbit IgGs conjugated to Oregon green, anti-mouse IgGs conjugated to Alexa 594, and anti-human IgGs conjugated to Texas red.

Immunochemistry of brain sections

Weeble mice and wt 12- or 19-dold littermates were fixed by transcardiac perfusion with 1% PFA in 120 mM sodium phosphate buffer. Brains were collected, fixed for an additional 3 h in the same solution, cryoprotected with 30% sucrose in 120 mM sodium phosphate buffer, and then stained by immunofluorescence by standard procedures. Primary antibodies were visualized with goat anti-rabbit IgGs conjugated to Oregon green, anti-mouse IgGs conjugated to Alexa 594, and anti-human IgGs conjugated to Texas red.

Preparation of recombinant PI3Ks, 5-Pase, 4-Pase, and Rab5

Recombinant Pases were expressed as GST-tagged proteins in High Five insect cells according to the manufacturer’s instructions (BD Biosciences) with the use of pFast Bac GST-5-Pase or GST-4-Pase. To produce recombinant p85α and untagged p110β baculoviruses, and the proteins purified by a single-step on a glutathione-agarose column followed by cleavage of GST with Precision protease (GE Healthcare). Recombinant GST-Rab5 was purified as previously described [Christofidis et al., 1999a] and GST was cleaved with Factor Xa.

PI 3-K activity assay

PI 3-K activity in the GST-Rab5/GTP-γS column eluate of bovine brain cytosol [Christofidis et al., 1999a] or by recombinant PI3Kβ was assayed in buffer containing PtdIns (0.2 mg/ml), PtdIns(4P) (0.2 mg/ml), or PtdIns(4,5)P2 (0.23 mg/ml) in 50 µl of 20 mM Hepes, pH 7.4, 1 mM EGTA, 5 mM MgCl2, 50 µM ATP, 10 Ci [γ-32P]ATP, 0.23 mg/ml phosphatidylycerine, 0.2 mM adenine. The reaction was run for 10 min at 37°C (unless stated) and arrested with 10 µl of 1 N HCl, extracted with 100 µl of CHCl3/MeOH (1:1) and washed twice with 1 N HCl/MeOH (1:1). Dried lipids were resuspended in 15 µl of CHCl3/MeOH/H2O (75:25:2, vol/vol/vol), separated by TLC using CHCl3/acetone/MeOH/glacial acetic acid/H2O (80:30:26:24:14, vol/vol/vol), analyzed using BAS3000 bioimaging analyzer [Fuji] and the corresponding spots were quantified by Image Gauge software [Fuji]. TLC plates were pre-
labeled, sheep anti–human transferrin antibodies (SAFU) and subse-
quently analyzed using an ECL-Analyzer System from Igen Inc.
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