Phosphatidylinositol-3-OH kinases are Rab5 effectors

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Phosphatidylinositol-3-OH kinases (PI(3)Ks) constitute a family of lipid-modifying enzymes that are involved in signal transduction, cytoskeletal organization and membrane transport[1]. Three different classes of PI(3)K have been described, which differ in their specificities for phosphatidylinositol, phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate substrates, as well as in their regulation. PI(3)Ks are needed for receptor trafficking in the endocytic pathway in mammalian cells and for transport from the Golgi to the vacuole in yeast (reviewed in ref. 1). The docking and fusion of early endosomes, which is regulated by the small GTPase Rab5 (ref. 2), requires PI(3)K activity[3]. In addition, PI(3)Ks regulate the recruitment to membranes of EEA1, an effector of the small GTPase Rab5 and a core component of the docking and fusion machinery[4,5] that binds phosphatidylinositol-3-phosphate (PtdIns(3)P) through a FYVE-finger motif[6]. We have recently identified over 20 cytosolic proteins that interact with the active form of Rab5 (ref. 5). Here we report the identification of two distinct PI(3)Ks, hVPS34 and p85α–p110β, among these cytosolic proteins. We suggest a new mechanism by which Rab5 can modify its membrane environment by coupling the local production of phosphoinositides to the selective recruitment of Rab5 effector proteins.

We used matrix-assisted laser-desorption-ionization mass spectrometry (MALDI-MS) to identify the proteins purified on the basis of a specific interaction with the active form of Rab5 (ref. 5). Surprisingly, among these proteins we identified the regulatory subunit of class-I PI(3)Ks, p85α, and confirmed its identity by western blotting (Fig. 1a). We therefore performed PI(3)K enzymatic assays to demonstrate the presence of catalytic activity in the Rab5-GTPγS column eluate. PI(3)K activity was markedly detected in this eluate and was inhibited >90% by 100 nM wortmannin (Fig. 1b). The activity was more than 100 times greater than the activity in the Rab5-GDP column eluate, and 900-fold greater than the background activity (Fig. 1b). The enrichment was ~10-fold higher than the corresponding value reported for Ras and PI(3)K[7]. PI(3)K therefore efficiently interacts with, and is recruited by, Rab5.

Given the presence of a class-I-PI(3)K regulatory subunit in the Rab5-GTPγS eluate, we would expect the catalytic subunit to be either p110α or p110β (ref. 1). By using isoform-specific antibodies we established that p110β, but not p110α, was present in the eluate

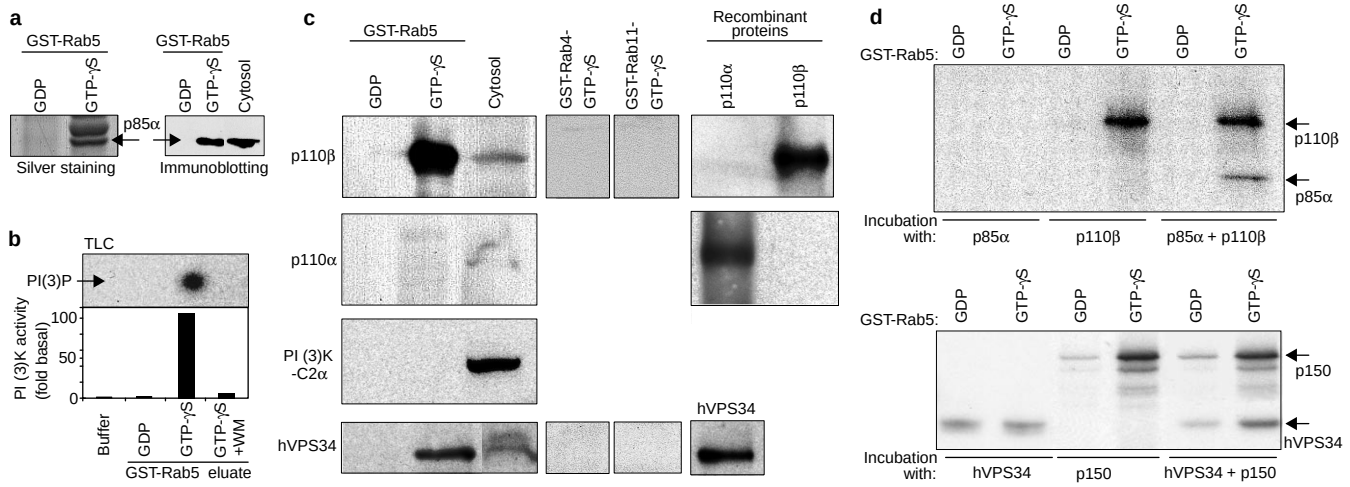

Figure 1 The PI(3)Ks p85α–p110β and p150–hVPS34 bind specifically to a Rab5-GTPγS affinity column. **a**, Identification of p85α in the Rab5-GTPγS column eluate. Eluates from GST–Rab5-GDP or GST–Rab5-GTPγS affinity columns were analysed by SDS–PAGE, silver staining and MALDI analysis (left) or immunoblotting (right). **b**, PI(3)K-activity assay on eluates from GST–Rab5-GDP or GST–Rab5-GTPγS affinity columns, using phosphatidylinositol as a substrate, in the absence or presence of 100nM wortmannin (WM); and quantification of thin-layer chromatography (TLC) spots using Phosphoimager analysis (basal: activity bound to Rab5-GDP was normalized to 1.0). The position of PtdIns(3)P (PI(3)P) was determined by co-migration with control PI(3)P produced by recombinant p110β using phosphatidylinositol as a substrate. **c**, Eluates from GST–Rab5-GDP, GST–Rab5-GTPγS, GST–Rab4-GTPγS and GST–Rab11-GTPγS affinity columns, cytosol and recombinant proteins (as controls) were analysed by western blotting using antibodies against p110β (S-19), p110α (C-17), PI(3)K-C2α and hVPS34, as indicated. **d**, Glutathione–Sepharose beads loaded with GST–Rab5-GDP or GST–Rab5-GTPγS were incubated with [^{35}S]methionine-labelled, *in vitro*-translated p85α, p110β or both (top) and hVPS34, p150 or both (bottom). Bound proteins were eluted and analysed by SDS–PAGE followed by autoradiography.

(Fig. 1c). We detected no interaction of p110β with fusion proteins consisting of glutathione-*S*-transferase (GST) fused to Rab4-GTPγS or Rab11-GTPγS. We also tested a class-II PI(3)K (PI3K-C2α), but failed to detect binding of this enzyme to Rab5-GTPγS or Rab5-GDP. These results indicate that Rab5 specifically and GTP-dependently interacts with the class-I PI(3)K p85α–p110β.

However, we were intrigued by these findings given that the membrane recruitment of EEA1 requires PtdIns(3)P, hence predicting the involvement of the class-III PI(3)K hVPS34 (ref. 8). Therefore, we repeated the affinity chromatography using cytosol from HeLa cells and analysed the presence of hVPS34, a PI(3)K catalytic subunit, by immunoblotting. A strong enrichment of this PI(3)K was obtained upon Rab5-GTPγS affinity chromatography as compared with cytosol (Fig. 1c). The specificity of this signal was shown first, by competition with an excess of the peptide against which the anti-hVPS34 antiserum was raised (data not shown); second, by co-migration with recombinant hVPS34; and third, by the lack of interaction with Rab4-GTPγS and Rab11-GTPγS. We conclude that hVPS34 is also a specific effector of Rab5. Using kinase-specific inhibitory antibodies[8], we determined that 52% of the total PI(3)K activity detected in the Rab5 column eluate prepared from HeLa cytosol was due to p110β and 48% to hVPS34. PI(3)K activity in a similar eluate from bovine brain cytosol, however, appeared to be mostly (~80%) due to p110β activity.

To determine whether the recruitment of the PI(3)Ks on the Rab5 affinity column resulted from a direct interaction of the PI(3)Ks with Rab5-GTP, we incubated *in vitro*-translated proteins with beads containing immobilized GST–Rab5-GDP or GST–Rab5-GTPγS. Whereas p110β bound exclusively to GST–Rab5-GTPγS, p85α failed to bind unless it was co-translated with p110β (Fig. 1d). Thus, the p110β catalytic subunit binds directly to Rab5-GTPγS. In contrast, hVPS34 showed no specific, direct interaction with Rab5-GTPγS, but only background staining (Fig. 1d) as also detected with GST-loaded beads (data not shown). Instead, the hVPS34-associated protein p150 showed preferential interaction with Rab5-GTPγS. Coexpression of the two subunits resulted in recruitment of hVPS34 to the Rab5-GTPγS column, indicating that the interaction with Rab5 may be mediated by p150. We could not analyse the recruitment of cytosolic p150 because there are no antibodies that detect the endogenous protein. However, as monitored

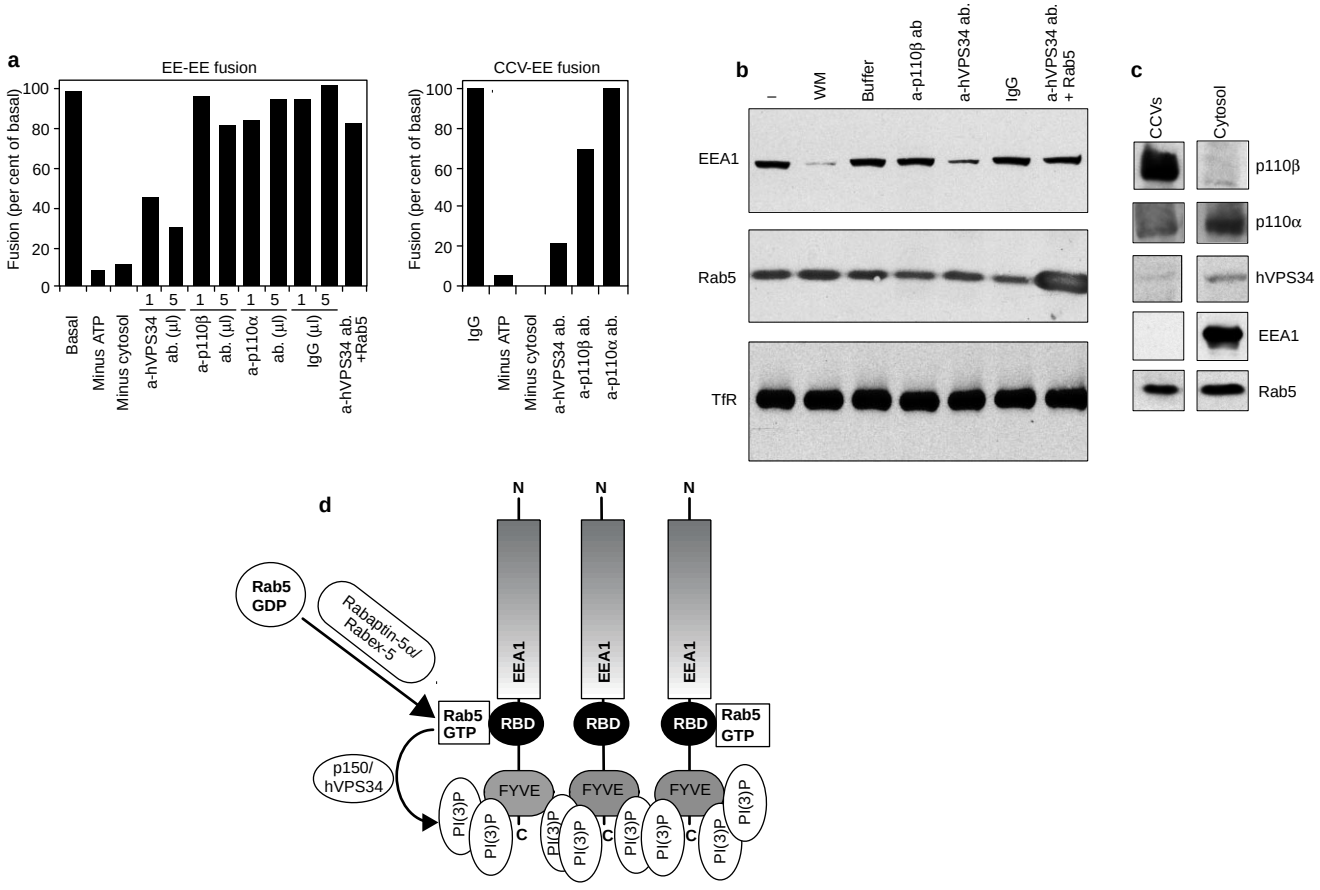

Figure 2 Role of PI(3)Ks in endosome fusion and recruitment of EEA1. **a**, Left, *in vitro* early-endosome (EE) fusion of early endosomes was studied in the presence of 3 mg ml^{-1} HeLa cytosol and in the absence or presence of the indicated affinity-purified activity-blocking antibodies (abs). In the last column, a complex of Rab5 with the Rab guanine-nucleotide-dissociation factor (rabGDI) was added at a final concentration of 75nM in the presence of 1μl anti-hVPS34 antibody. Right, *in vitro* fusion between CCVs and EEs was studied using HeLa cytosol and in the presence of the indicated activity-blocking antibodies. Values are expressed as percentages of the basal fusion reaction (done in the presence of cytosol and ATP). **b**, Recruitment of EEA1 onto early endosomes. EEs were incubated with HeLa cytosol for 30 min in the (from left to right) absence or presence of wortmannin (WM; 100nM), control buffer, anti-p110β and anti-hVPS34 affinity-purified function-blocking antibodies, control IgGs and anti-hVPS34 antibody together with Rab5–rabGDI complex. The presence of EEA1 and Rab5 on endosomes was detected by western blot using corresponding specific antibodies. The loading of membranes in each lane was monitored using transferrin receptor (TfR) as a membrane marker. **c**, Highly purified CCVs from bovine brain and HeLa cytosol were tested by immunoblotting for the presence of p110β (β3 antibody), p110α (α2 antibody), hVPS34, EEA1 and Rab5. **d**, Model of Rab5-dependent coupling of PI(3)K activity and EEA1 recruitment. The interaction of Rab5-GTP with hVPS34 would localize the production of PI(3)P to the site of recruitment of EEA1 specified by Rab5-GTP (see text). RBD, Rab-binding domain; FYVE, a motif that binds PI(3)P.

by the presence of hVPS34, the interaction of p150–hVPS34 with Rab5-GTPγS appeared to be more efficient when p150 and hVPS34 were present in cytosol rather than translated *in vitro*, indicating that post-translational modifications or other cytosolic factors may facilitate the association.

Rab5 regulates the ability of early endosomes to fuse either homotypically or with clathrin-coated vesicles (CCVs)[2]. The identification of two PI(3)Ks that interact with Rab5 raises the question of whether both enzymatic activities participate in endocytic membrane fusion. Using isoform-specific inhibitory antibodies against these PI(3)Ks[8] in an *in vitro* endosome-fusion assay, we found that only anti-hVPS34 antibodies were inhibitory (Fig. 2a). The inhibitory effect was dose dependent and neutralized by an excess of Rab5. Anti-hVPS34 antibodies also markedly and specifically inhibited the heterotypic fusion between CCVs and early endosomes. In this assay, anti-p110β antibodies had also a minor inhibitory effect. In light of these data, we conclude that, out of the two PI(3)Ks that interact with Rab5, only hVPS34 is required for the fusion activity of early endosomes. Consequently, these results imply that PtdIns(3)P is the most important 3′-phosphoinositide in this process. Given the role of EEA1 as a core component in endosome docking and fusion[5], we tested whether the inhibitory effect of anti-hVPS34 antibodies is due to a concomitant release of EEA1 from the membrane. Consistent with the fusion-assay data, only anti-hVPS34 antibodies caused a substantial decrease in amounts of EEA1 on the endosome (Fig. 2b). A large excess of Rab5 was necessary to retain endogenous levels of EEA1 on the membrane, thereby rescuing fusion activity (Fig. 2a), indicating that, in the absence of PtdIns(3)P, Rab5 alone does not efficiently recruit EEA1 to endosomes. We conclude that this GTPase recruits EEA1 not only by direct binding, but also through the activity of p150–hVPS34.

As seen by electron microscopy, EEA1 associates predominantly with early endosomes with only low amounts being detected on the plasma membrane[9], consistent with its function in specifying vesicle targeting to early endosomes[4,5]. In agreement with these morphological observations, we could not detect EEA1 on CCVs purified from bovine brain (Fig. 2c) and observed no recruitment of cytosolic EEA1 by these membranes *in vitro* (M. Rubino *et al.*, unpublished observations). Given the different substrate specificities of the PI(3)Ks recruited by Rab5, we tested whether the asymmetric distribution of EEA1 between the plasma membrane and early endosomes was paralleled by an asymmetric distribution of the PI(3)Ks p110β and hVPS34. We detected a marked enrichment of p110β on CCVs (Fig. 2c) as compared with the other PI(3)Ks and, most notably, hVPS34. Thus, unlike endosomes (Fig. 2a), CCVs appear to lack the lipid kinase required for the production of PtdIns(3)P and the binding of EEA1 to the membrane, but instead contain primarily p110β. These data indicate that the differential distribution of PI(3)Ks may correlate with the differential localization of EEA1 in the endocytic pathway.

Previous studies have placed PI(3)Ks upstream of Rab5 function as regulators of guanine-nucleotide exchange[3]. Our data instead support a different mechanism, in which PI(3)Ks are downstream effectors of Rab5. Previously, only Ras- and Rho-family GTPases were thought to regulate this enzymatic activity[1]. The two Rab5-associated PI(3)Ks belong to distinct classes and have different substrate specificities. The first, p85α–p110β, is a class-I enzyme that preferentially produces phosphatidylinositol-3,4-bisphosphate and phosphatidylinositol-3,4,5-trisphosphate. The function of this enzyme with respect to Rab5 is unclear at present, but one possibility is that it modulates the formation of CCVs at the plasma membrane, given that both 3′-phosphoinositides and Rab5 have been implicated in the assembly of the AP2 adaptor protein required for vesicle formation[10,11], and that Rab5 regulates the half-life of surface coated pits *in vivo*[12]. The catalytic activity of p110β has also been implicated in the translocation of glucose transporters from intracellular pools to the plasma membrane[13], and in lamellipodia formation and membrane ruffling in macrophages[14]. Rab5 may therefore participate in these events.

The second Rab5-interacting PI(3)K, p150–hVPS34, has a distinct function. VPS34 specifically produces PtdIns(3)P and is involved in vesicle formation at the Golgi apparatus[15]. Our results and others[8,16] now indicate that it is the major PI(3)K responsible for the recruitment of EEA1 to the endosome membrane. An important implication of our work is that Rab5 mediates the recruitment of EEA1 not only by binding directly to this protein but also catalytically, through an interaction with p150–hVPS34. In coupling active Rab5 to PtdIns(3)P production *in situ*, this mechanism ensures the concomitant presence of both binding sites for EEA1 at the point of recruitment on the endosome membrane (Fig. 2d). This may amplify the recruitment signal, overcoming the requirement for stoichiometric levels of Rab5 after the initial binding event. The fact that only a large excess of Rab5 can compensate for the effect of wortmannin and the consequent loss of EEA1 (refs 4, 17) can now be explained by a deficit of hVPS34 activity downstream of Rab5. This mechanism is appealing if one considers the dynamic GTP cycle of Rab5 on the membrane[18] and the multiplicity of effectors that this GTPase must bind[5].

The interaction of p150–hVPS34 with Rab5 may not necessarily involve a modulation of PI(3)K enzymatic activity, as Rab5 did not appear to stimulate the activity of hVPS34 (or p110β) *in vitro* (data not shown). A more likely role of Rab5 is to localize p150–hVPS34 to the appropriate site in the endosome membrane. The membrane association of hVPS34 in mammalian cells is regulated by membrane-bound p150 (M.D.W. and J.M.B., unpublished observations). We therefore propose that active Rab5 transiently interacts with p150–hVPS34 on the plane of the membrane to confine the production of PtdIns(3)P to a restricted region of the endosome, that is, a membrane microdomain, where the function of EEA1 in endosome tethering and membrane fusion can be coordinated with the function of other Rab5 effectors. Finally, the asymmetric distribution of the two PI(3)Ks described here, between CCVs and early endosomes, provides an explanation for the differential recruitment of EEA1 and its exclusive localization to early endosomes, thereby contributing to the biochemical and functional identity of this organelle. □

Methods

In vitro binding assays.

GST–Rab5 affinity chromatography was done as described[5]. *In vitro* transcription–translation of [^{35}S]methionine-labelled proteins was done according to the manufacturer's instructions (Promega). The transcribed and translated proteins (75μl of standard reactions) were incubated for 2 h at 4 °C with 30μl glutathione–Sepharose beads coupled with GST–Rab5-GDP or GST–Rab5-GTPγS and 330μl buffer containing 20 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT) and 1 mM GDP or GTP-γS. Beads were washed and proteins eluted as described[5]. Peptide-mass mapping of the eluted proteins was done on a Bruker REFLEX MALDI time-of-flight mass spectrometer (Bruker-Franzen, Bremen, Germany) using the fast evaporation technique for matrix preparation.

PI(3)K enzymatic assays.

Rab5-GDP and Rab5-GTPγS affinity-column eluates were treated appropriately to remove GST–Rab5 and to change the buffer composition[5]. 10μl eluate were then tested for PI(3)K activity essentially as described[8]. The labelled lipid products were visualized and quantified using bio-imaging analyser FUJI FILM FLA-2000.

Antibodies.

Activity-blocking antibodies against hVPS34 and p110α have been described[8]. Anti-p110β function-blocking antibodies were produced in rabbits against the p110β carboxy-terminal peptide (C)KVNWMAHTVRKDYRS and affinity purified. Western-blotting antibody against hVPS34 was raised against the peptide sequence (C)NFDDIKNGLEPTKK in rabbits and affinity purified before use. PI(3)K-C2α rabbit polyclonal antibodies were provided by J. Domin and anti-p110β (β3) and anti-p110α (α2) antibodies were provided by S. Roche. Commercially available antibodies were anti-p85α mouse monoclonal antibodies (Upstate Biotechnology); anti-p110β (S-19) rabbit and anti-p110α (C-17) goat polyclonal antibodies (Santa Cruz); and anti-tranferrin-receptor mouse monoclonal antibodies (Zymed).

Endosome recruitment of cytosolic proteins and endosome-fusion reactions.

Early endosomes, HeLa cytosol and ATP-regenerating system were incubated for 30 min at 37 °C in a total volume of 60μl essentially as for the *in vitro* fusion reactions[5]. The mixture was diluted with 100μl fusion-assay buffer, and pelleted using a TLA 100.2 rotor for 30 min, 80,000 r.p.m., 4 °C. The pellet was washed with 500μl PBS, resuspended in loading buffer and analysed by SDS–PAGE and immunoblotting.

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