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Role of Rab5 in the Recruitment of hVps34/p150 to the Early Endosome

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PI 3-kinases are important regulators of endocytic trafficking. We have previously proposed a model in which the Rab5 GTPase recruits EEA1 to the early endosome both directly, by binding to EEA1, and indirectly, through the recruitment of the p150/hVps34 PI 3-kinase and the production of PI[3]P in the endosomal membrane. In this study we have examined this model *in vivo*. We find that both endogenous hVps34 and p150 are targeted to enlarged endosomal structures in cells expressing constitutively activated Rab5, where they are significantly colocalized with EEA1. Recombinant fragments of p150 disrupt the endosomal localization of EEA1, showing that p150 is required for EEA1 targeting. We further analyzed the mechanism of GTP-dependent Rab5-p150 binding, and showed the p150 HEAT and WD40 domains are required for binding, whereas deletion of the protein kinase domain increases binding to Rab5. Overexpression of constitutively active Rab5 caused a redistribution of epitope-tagged hVps34 and p150 to Rab5-positive endosomes. However, subcellular fractionation showed that this was not due to a significant recruitment of hVps34 or p150 from the cytosolic to the particulate fraction. These data suggest that the binding of Rab5 to the HEAT/WD40 domains of p150 is important in regulating the localization of hVps34/p150. However, Rab5 does not appear to act by directly recruiting p150/hVps34 complexes from the cytosol to the endosomal membrane.

Key words: EEA1, endosome, p150, Rab5, Vps34

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The early endosome is a major sorting station in eukaryotic cells (1). This compartment initiates the separation of ligands from receptors, as well as the sorting of receptors for recycling, sequestration or degradation. The early endosome is also rich in signaling proteins. These include proteins that are associated with endosomes by virtue of their binding to internalized signaling receptors (2–4), as well as proteins such as SARA, RhoB and PRK1, which are targeted in a receptor-independent fashion (5–7).

The early endosome is itself a dynamic structure, capable of heterotypic fusion with incoming clathrin-coated vesicles, as well as homotypic fusion with other early endosomes (8). Endosomal dynamics are regulated by both small GTPases and lipid kinases (9,10). A major regulator of membrane transport through the early endosome is the small GTPase Rab5. In its activated, GTP-bound state, Rab5 mediates the membrane recruitment of various effector proteins, including the Rabaptin-5/Rabex-5 complex and the endosomal tethering protein EEA1 (11,12), which forms multiprotein complexes with syntaxin 13 (an endosomal t-SNARE) and NSF (13) on the early endosome membrane.

The transport activity of early endosomes also requires PI 3-kinase activity. The major lipid kinase in the early endosome appears to be the Class III PI 3-kinase hVps34 (14). The lipid product of hVps34, PI[3]P, produces a binding site for proteins containing FYVE domains, which act as modular PI[3]P receptors (15,16). The hVps34-catalyzed production of PI[3]P leads to the endosomal recruitment of FYVE domain-containing proteins, including EEA1 (17,18). hVps34 may also be important in the regulation of proteins containing PX domains, which also bind to PI[3]P (19).

How is hVps34 targeted to early endosomes? In yeast, membrane localization of Vps34p is dependent on association with Vps15p, a serine/threonine-specific protein kinase (20). The mammalian homologue of Vps15p, p150, also associates with mammalian hVps34 (21). We have previously identified both p150 and hVps34 among Rab5 effectors, and shown that *in vitro* association of Rab5 with hVps34 occurs via p150, which binds directly to Rab5 (14). These data suggest a model in which Rab5-GTP binds to p150, which in turn is bound to hVps34. Thus, Rab5 is hypothesized to recruit p150 and hVps34 to early endosomes, thereby regulating the production of PI[3]P and the recruitment of EEA1 to the endosomal membrane. Since Rab5 also binds to EEA1 directly, this means that Rab5 recruitment of EEA1 is a bivalent process, involving both direct and p150/hVps34-mediated interactions.

In the present study, we examine the role of Rab5 in the regulation of p150 and hVps34 in intact cells. Both p150

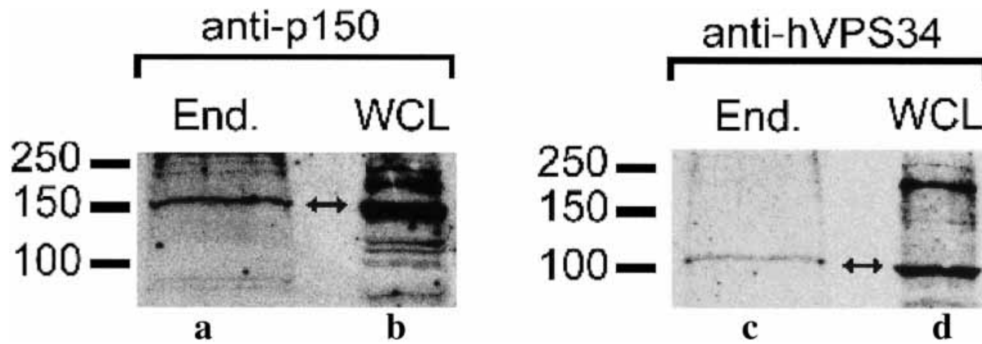


Figure 1: hVps34 and p150 are present in HeLa cell endosomal fractions. Early endosomes were isolated by sucrose sedimentation from HeLa cells and resolved by SDS-PAGE. Early endosomes (37.5 μg total protein/lane, lanes a, c) were probed with a rabbit pAb anti-p150 or a rabbit pAb anti-hVps34. HeLa whole-cell lysate was run for comparison (105 μg total protein/lane, lanes b, d).

and hVps34 are present in enlarged endosomes in cells expressing RabQ79L, where they significantly colocalize with EEA1, and recombinant fragments of p150 act as inhibitors of EEA1 endosomal targeting. The binding of p150 to Rab5 requires the WD40 domain and, to a lesser extent, the HEAT domain, but not the protein kinase domain. Co-expression of constitutively active Rab5 with recombinant hVps34 or p150 targets these proteins to enlarged endosomes, but does not lead to significant recruitment from the cytosol to cell membranes. Our data are consistent with a role for Rab5 in the regulation of endosomal p150/hVps34 targeting, but suggest that Rab5 does not act by directly recruiting p150/hVps34 complexes from the cytosol to the early endosome.

Results

Colocalization of p150 and hVps34 with activated Rab5 and EEA1

Inhibitory antibodies against hVps34 disrupt the targeting of the endosomal protein EEA1 *in vivo*, and p150/hVps34 complexes bind to the early endosomal GTPase Rab5 *in vitro* (14,22). These data predict that hVps34 and p150 are both present in early endosomes; this was tested by anti-hVPS34 and anti-p150 Western blots of purified endosomes from HeLa cells. As shown in Figure 1, both proteins can be detected in lysates from purified HeLa endosomes. We next examined the localization of endogenous p150 in HeLa cells expressing wild-type or activated (Q79L) Rab5. In control cells or cells expressing wild-type Rab5, anti-p150 antibodies produced an indistinct pattern, with some punctate staining but no apparent colocalization with Rab5 (data not shown). In contrast, as previously described (23), expression of Q79LRab5 caused the production of enlarged Rab5-positive endosomal structures (Figure 2B). Endogenous p150 was significantly, although not completely, colocalized to these Rab5-positive endosomal structures (Figure 2C). Activated Rab5 also leads to the recruitment of EEA1 to enlarged endosomes (24) (Figure 2E,H), and we found significant colocalization of EEA1 and p150 in cells expressing Q79LRab5 (Figure 2F,I).

We also looked at the effect of Rab5 on the localization of hVps34. As with p150, anti-hVps34 staining of control cells produced an indistinct pattern with some punctate staining (data not shown). However, expression of Q79LRab5 led to the production of enlarged endosomes in which both EEA1 and endogenous hVps34 were extensively colocalized (Figure 3C).

Recombinant fragments of p150 inhibit the targeting of EEA1

The colocalization of p150 with activated Rab5 and EEA1 in enlarged Rab5-positive endosomes is consistent with the proposed link between Rab5 and p150/hVps34 during EEA1 targeting. To demonstrate that p150 is functionally involved in EEA1 targeting, we injected HepG2 cells with recombinant maltose binding protein (MBP)-fusion proteins corresponding to the kinase, HEAT and WD-40 domains of p150. HepG2 cells were used because of their ease of injection relative to HeLa cells. Injection of MBP alone had no effect on the localization of EEA1 to early endosomes (Figure 4A,D). In contrast, injection of either the MBP–protein kinase domain (MBP-PKD) or the MBP-HEAT domain disrupted the localization of EEA1 by 50–70% (Figure 4B,C,D). Injection of an MBP-WD40 domain did not affect EEA1 localization, although we cannot rule out that the recombinant fusion protein may not have been correctly folded. When taken with our previous data showing that inhibitory antibodies to hVps34 block EEA1 targeting to early endosomes (22), these data show that the localization of EEA1 requires both hVps34 and p150.

Domain analysis of p150 binding to Rab5

To examine the mechanism by which p150 interacts with Rab5, we constructed deletion mutants of p150 (Figure 5A). These constructs were *in vitro* translated with [^{35}S]methionine, then incubated with GST-Rab5 in the presence of either GDP or GTP-γS. As shown in Figure 5B, full-length p150 and p150 lacking the protein kinase domain (ΔPKD-p150) both bound strongly to Rab5-GTP, whereas deletion of the HEAT domain (ΔHEAT-p150) reduced binding to Rab5 and deletion of the WD40 domain (ΔWD40-p150) abolished binding. Quantitation of the ^{35}S-labeled bands from 3 independent experiments (Figure 5C) confirmed that p150 binds to Rab5 in a GTP-dependent manner, as described previously (14). Deletion of the kinase domain caused a significant increase in binding to the GDP and GTP-bound forms of Rab5. In contrast, deletion of the HEAT or WD40 domains caused a complete loss of GTP-dependent Rab5 binding.

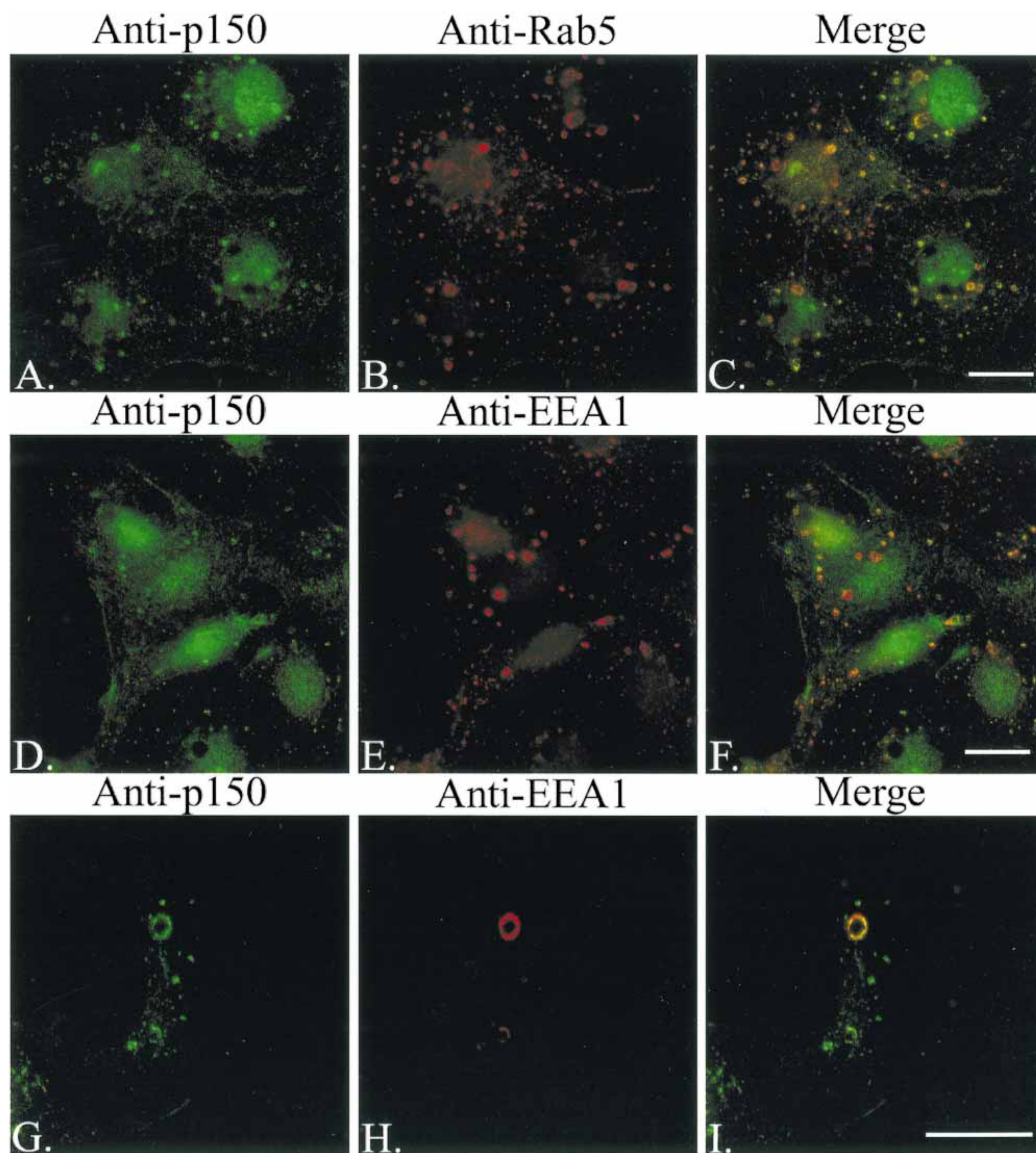
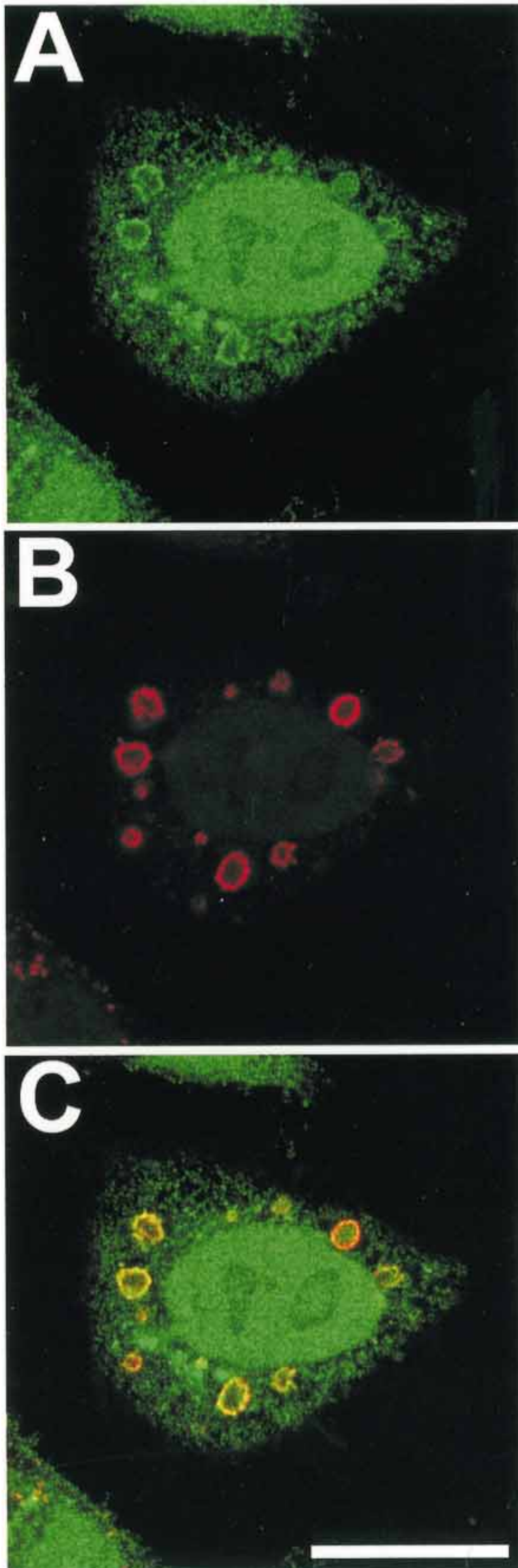


Figure 2: Endogenous p150 colocalizes with activated Rab5 and EEA1. HeLa cells were transfected with activated Q79LRab5. After 24 h the cells were fixed and stained with anti-p150 antibodies (A,D,G) and monoclonal anti-Rab5 (B) or anti-EEA1 (E,H) antibodies. Images were acquired using 60× (A–F) or 100× (G–I) objectives. Merged images are shown in the right-hand panels.

Mechanism of hVps34/p150 recruitment to endosomes

To test whether activated Rab5 alters the distribution of hVps34 and p150, we microinjected HeLa cells with cDNAs for EE-tagged p150 and wild-type or Q79LRab5. Overexpressed EE-p150 showed a punctate perinuclear distribution (Figure 6B), with little colocalization with wild-type Rab5 (Figure 6A). In contrast, in cells expressing Q79LRab5, p150 was prominently recruited to enlarged Rab5-positive endosomal structures (Figure 6C,D). Thus, constitutive activation of Rab5



was sufficient for the targeting of p150 to Rab5-positive endosomes. We also looked at the effect of Rab5 on the localization of hVps34. Myc-tagged hVps34 showed a mixed cytosolic-particulate distribution when co-expressed with wild-type Rab5 (Figure 7B). However, myc-hVps34 was recruited to large Rab5-positive endosomes in cells expressing Q79LRab5 (Figure 7D,F). This Rab5-mediated relocalization of hVps34 presumably utilizes endogenous free p150, which can be detected in ^{35}S-labeled cells (Figure 8, lane d) after exhaustive immunodepletion with anti-hVps34 antibodies (Figure 8, lanes a–c).

In order to biochemically analyze the recruitment of p150/hVps34 to early endosomes, we characterized the distribution of endogenous p150 and hVps34 in fractionated HeLa cells. p150 and hVps34 were primarily found in the particulate fraction from hypotonically lysed cells (Figure 9A). These data are somewhat different from studies in yeast, in which half of Vps34p and all of Vps15p were found in the particulate fraction (25,26). Mammalian hVps34 and p150 differed from each other in their susceptibility to extraction with non-ionic detergent. Whereas hVps34 was completely soluble in Triton X-100, approximately 50% of p150 was Triton X-100 insoluble (Figure 9B). Given that p150 is predicted to be N-terminally myristylated (21), we considered the potential association of p150 with cholesterol-rich lipid rafts. However, extraction of cells with Triton X-100 at 37 °C did not increase the solubility of p150 (Figure 9C), and p150 was insoluble in 1% saponin at 37 °C (Figure 9D). While the biochemical basis for the insoluble p150 pool is not yet clear, the differential solubility of p150 and hVps34 suggests that a significant fraction of p150 is present in an hVps34-free compartment.

In contrast to the endogenous proteins, both hVps34 and p150 show a mixed cytosolic and particulate distribution when overexpressed in HeLa cells (Figure 10A,B). We used this observation to test whether co-expression of Q79LRab5 with myc-hVps34 or V5-p150 was sufficient to recruit the proteins to the particulate fraction. When we compared the ratio of cytosolic to particulate myc-hVps34 in cells expressing only endogenous Rab5 vs. wild-type or Q79LRab5, we saw no significant recruitment of myc-hVps34 to the particulate fraction (Figure 10A). Similarly, the cytosol/particulate ratio of V5-p150 was unchanged in cells expressing only endogenous Rab5 vs. overexpressed WT or Q79L-Rab5 (Figure 10B). Finally, we tested whether overexpression of Rab5 could recruit V5-p150 from the triton-insoluble to the triton-soluble fraction. Wild-type Rab5 caused a slight decrease in Triton X-100 insoluble p150, whereas overexpression of Q79LRab5 had no effect on the partitioning of V5-p150 (Figure 10C). Thus, activated Rab5 is not sufficient to recruit cytosolic hVPS34 or p150 to cell membranes, perhaps due to a

Figure 3: Endogenous hVps34 colocalizes with activated Rab5. A–C: Cells were transfected with Q79LRab5 and stained with anti-hVps34 (A) or and EEA1 (B) antibodies. The merged confocal image is shown in (C).

requirement for additional endosomal factors that are limiting for recruitment.

Discussion

Recent work from a number of laboratories has focused on the targeting of the endosomal tethering protein EEA1 to the endosomal membrane. EEA1 binds directly to activated Rab5, and contains two discrete Rab5-binding domains (11,12). However, the physiological targeting of EEA1 also requires PI 3-kinase activity, as the EEA1 FYVE domain binds to PI[3]P in the endosomal membrane (27); the FYVE domain may also contribute to interactions with Rab5 (28). We have previously shown that hVps34 is the PI 3-kinase required for EEA1 targeting (22). Moreover, we have described *in vitro* experiments that suggest a mechanism for the targeting/regulation of hVps34 to the early endosome: hVps34 binds to the p150 protein kinase, which in turn binds to activated Rab5 (14). In this model, p150 is a critical link between hVps34 and Rab5.

The present studies examine the regulation of hVPS34/p150 by Rab5 *in vivo*. We find that endogenous p150 is localized to enlarged endosomal structures in cells expressing activated Rab5. p150 is significantly colocalized in these structures with EEA1. Similarly, hVps34 is colocalized with EEA1 in cells expressing activated Rab5. When hVPS34 and p150 are overexpressed, their distribution is markedly altered by co-expression with Q79LRab5. Finally, recombinant fragments of p150 act as inhibitors of EEA1 targeting. In aggregate, these experiments provide *in vivo* data suggesting that the regulation of p150/hVPS34 by Rab5 plays a critical role in the targeting of EEA1 to early endosomes.

Domain analysis of p150

The N-terminal half of p150 contains a serine/threonine protein kinase domain and a series of HEAT repeats. Numerous HEAT-repeat containing proteins have been reported, including Huntington, EF-3, PR65/A and mTOR (29). The individual HEAT motif forms a helical hairpin structure, and proteins containing HEAT repeats possess as few as 3 and as many as 22 individual repeats (29). Analysis of the p150 amino acid sequence against the REP database of structurally related repeats (http://www.embl-heidelberg.de/~andrade/papers/rep/search.html) suggested that possibly 7 HEAT repeats are present in p150 (30). The HEAT repeats in p150 are only weakly related to other HEAT-repeat containing proteins.

In yeast, the HEAT repeats of Vps15p are required but not sufficient for binding to Vps34p. We also find that deletion of the HEAT repeats in p150 reduces binding to Rab5. It is possible that the HEAT domain interacts with both proteins. However, crystallographic analysis of the 15 HEAT repeats of PR65/A indicates that they possess both conformationally rigid and flexible regions (31). HEAT repeats that serve as protein scaffolds and protein–protein interacting domains in proteins such as Importin-beta and XMAP215 are also thought to possess inherent flexibility (32–34). It is possible that deletion of the p150 HEAT repeats constrains p150 flexibility, interfering with the binding of the protein kinase and WD40 domains to hVps34 and Rab5-GTP, respectively.

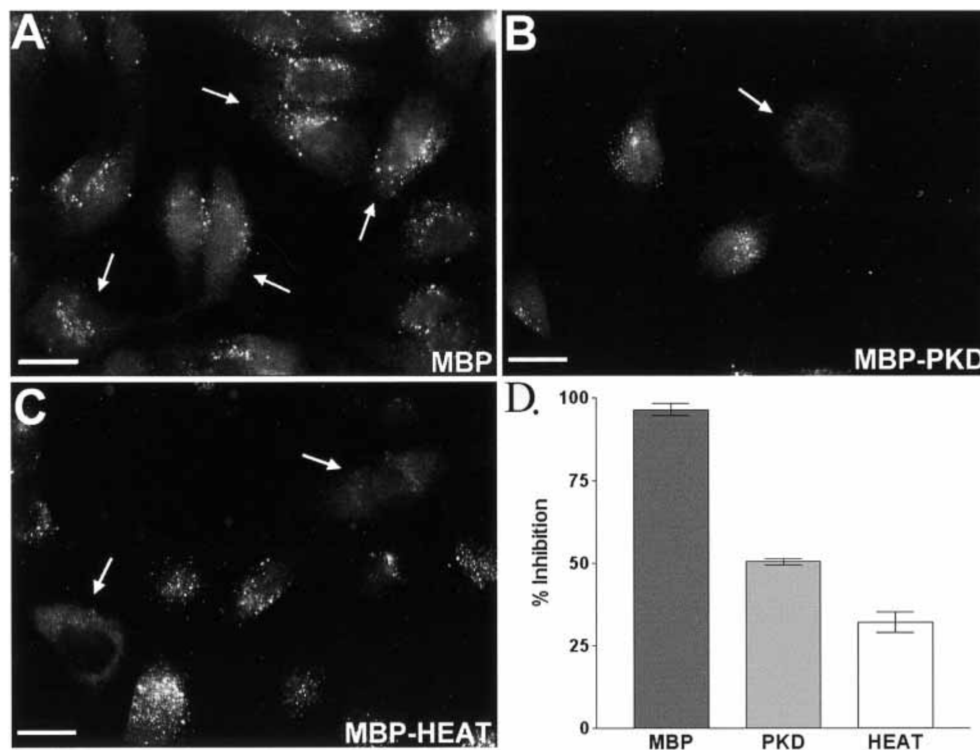


Figure 4: Recombinant fragments of p150 inhibit the targeting of EEA1 to the early endosome. HepG2 cells were microinjected with recombinant MBP-fusion proteins corresponding to: (A) MBP alone, (B) the protein kinase domain (PKD), or (C) the HEAT domain. Microinjected cells (77.6 ± 9 per coverslip) were identified with an FITC-dextran coinjection marker and are indicated by white arrows. (D) Injection data were quantified by counting the percentage of injected cells in which endosomal localization of EEA1 was detectable. Data are pooled from 3 to 4 experiments (error bars = SEM).

Analysis of the p150 protein sequence against the SMART database (http://smart.embl-heidelberg.de/) suggests that 6 WD40 repeats are present at the C-terminus of p150 (35,36). Comparison with this and other WD40 repeat-containing proteins suggests that the WD40 repeats of p150 are likely to form a structure similar to a beta-propeller. These structures are known protein–protein interacting domains (37), for example in the G-beta subunit of heterotrimeric G proteins (38).

We have demonstrated that the WD40 domain of p150 is required for Rab5-GTP binding. In addition, we present data that suggest the N-terminal protein kinase domain may regulate this interaction, since deletion of this domain increases basal and GTP-dependent binding to Rab5. Deletion of the protein kinase domain may release an intrinsic inhibitory conformation on p150, leading to increased Rab5 binding. If domain interactions between hVps34 and p150 are similar to that seen in yeast with Vps34p and Vps15p, then hVps34

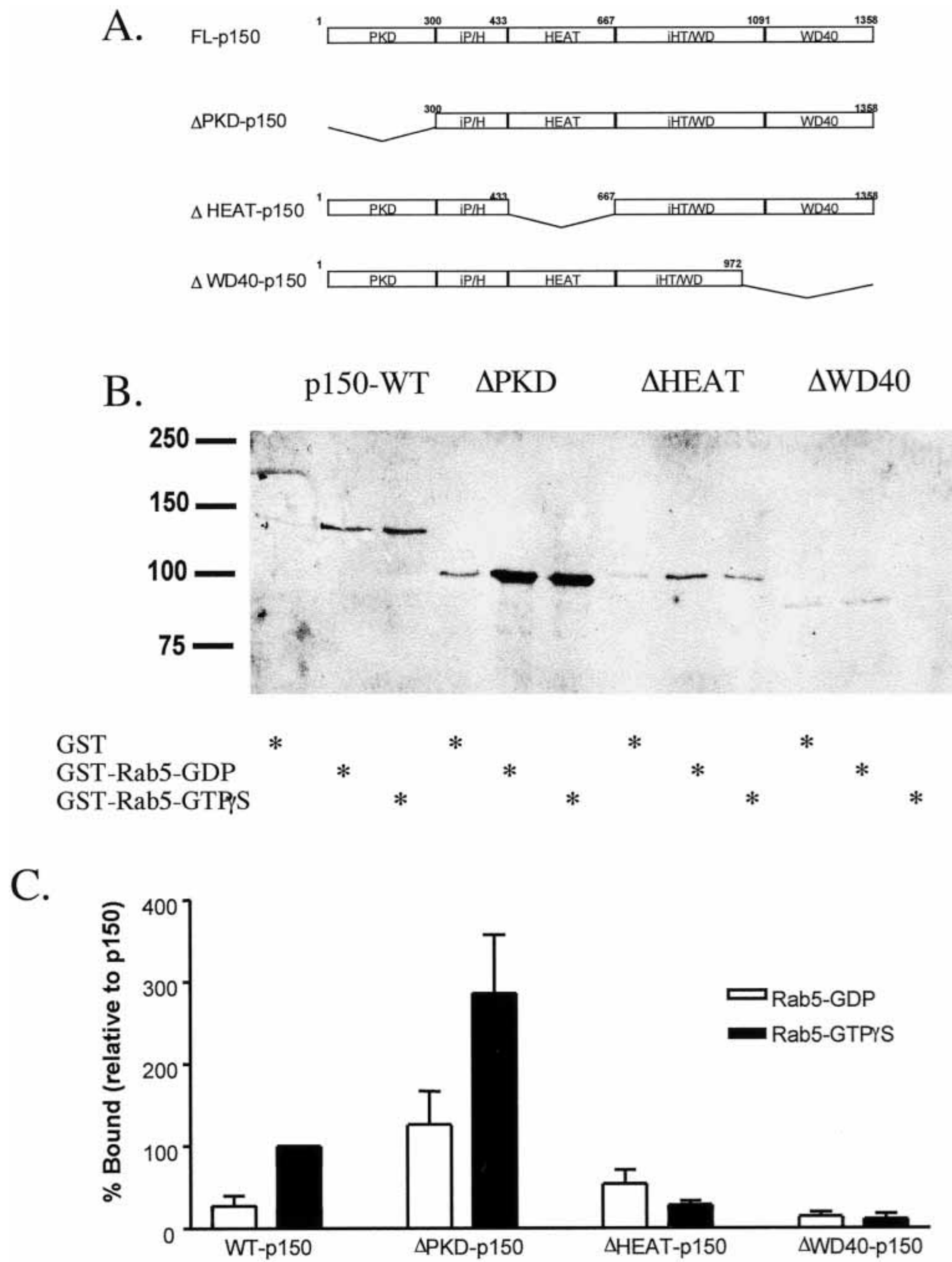


Figure 5: Domain analysis of p150–Rab5 interactions. A. Domain deletion mutants of p150 used in *in vitro* translation studies. B. Radiolabeled *in vitro* translated p150 was incubated with recombinant GST-Rab5 bound to glutathione Sepharose beads, or GST control. GST-Rab5 was preloaded with either GDP or GTPγS nucleotide prior to incubation with p150. Specifically associated p150 was eluted by incubation in EDTA, resolved by SDS-PAGE, and visualized by autoradiography. C. The association of recombinant p150 with GST-Rab5 was quantitated by densitometry from at least three independent experiments.

binding to the p150 protein kinase domain could affect the affinity of the p150 WD40 domain for Rab5.

Targeting of EEA1

Microinjection of either the protein kinase or HEAT domains of p150 disrupts EEA1 targeting. The mechanisms that target of EEA1 to early endosomes appear to involve binding to both PI[3]P and Rab5. EEA1 is displaced from early endosomes by wortmannin, but this displacement can be overcome by constitutively active Rab5 (11,17). However, the balance between these two binding events is complex. Thus, a C-terminal fragment of EEA1 containing an intact FYVE domain but a nonfunctional Rab5-binding domain is still targeted to early endosomes (39), which suggests that binding to PI[3]P is necessary and sufficient for endosomal targeting of EEA1. In contrast, native EEA1 is displaced from endosomes by dominant negative Rab5 (40); it is not clear if this is due to a loss of Rab5–EEA1 interactions, or a secondary effect via a loss of Rab5–p150/hVps34 interactions.

Given these complexities, it is likely that p150 fragments disrupt EEA1 targeting by competing with endogenous p150 for interactions with endosome-associated proteins. Interestingly, the domains that displace EEA1 are the same domains that are required for interactions between Vps34p and Vps15p in yeast (41). However, the kinase and HEAT domains of Vps15p are not sufficient to bind Vps34p by themselves (41), and it is unclear whether they would disrupt interactions between endogenous p150 and hVps34. On the other hand, data from *Saccharomyces cerevisiae* suggest that Vps15p and Vps34p exist in multiprotein complexes (42), and it is possible that the kinase and HEAT domains interfere with other p150-dependent interactions. Experiments are currently underway to determine the effects of p150 fragments on the subcellular distribution of endogenous hVps34, the localization of endogenous hVps34 and p150 in cells expressing Q79LRab5, and the pattern of associated proteins that co-immunoprecipitate with hVps34 and p150 in [^{35}S]-labeled cells.

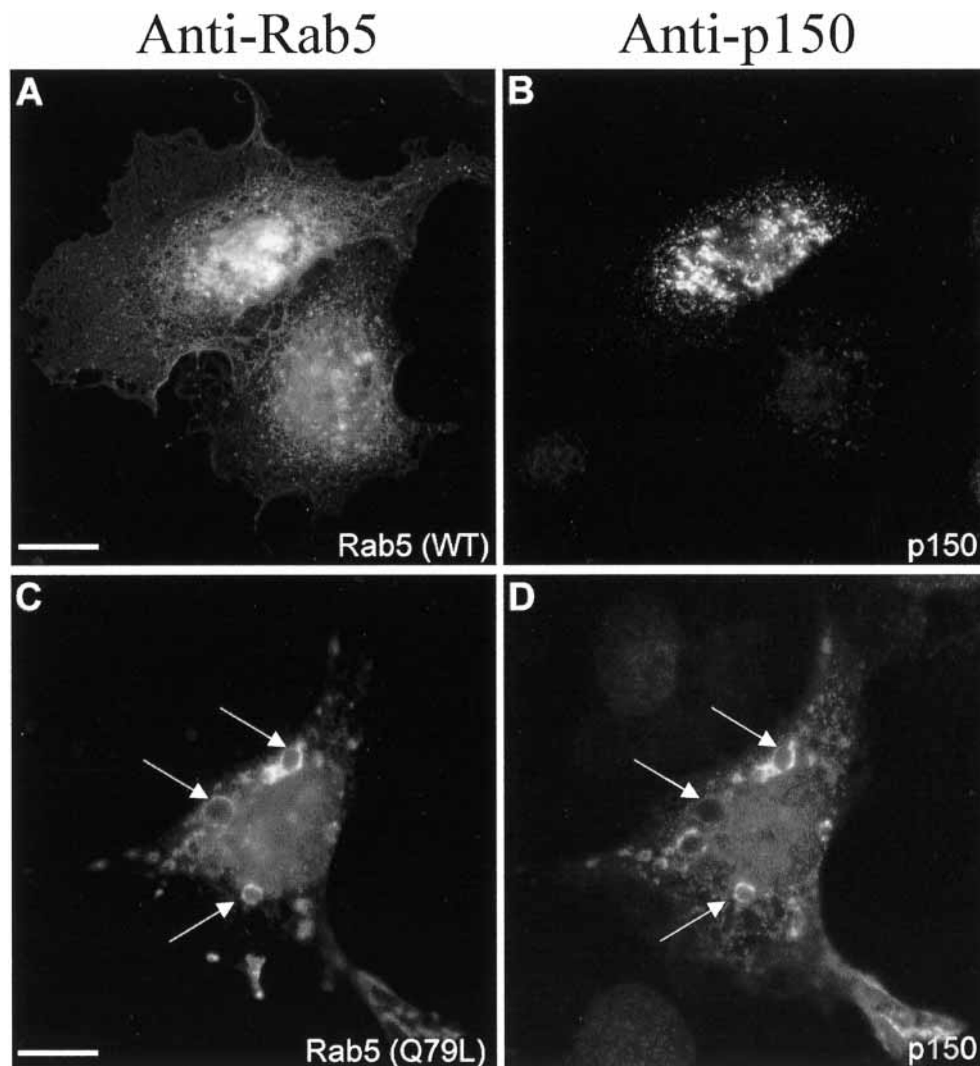


Figure 6: Recruitment of epitope-tagged p150 to Rab5 endosomes. HeLa cells were microinjected with cDNAs for EE-tagged p150 and wild-type (A,B) or Q79LRab5 (C,D). The cells were incubated for 24 h, fixed and stained with antibodies against Rab5 (A,C) or p150 (B,D). Arrows show examples of colocalization.

Regulation and targeting of hVps34/p150

Our data demonstrate that expression of activated Rab5 plays an important role in regulating the localization of p150 and hVPS34 to endosomes. Two aspects of hVPS34/p150 regulation by Rab5 remain unresolved. First, does Rab5 directly recruit hVPS34/p150 by binding to the p150 Heat-WD40 domains, or is the recruitment mediated through other Rab5 effectors? Our current data cannot distinguish between these possibilities. Second, is p150/hVPS34 recruited directly from the cytosol to the endosomal membrane, or does hVPS34/p150 redistribute between different membrane compartments? The latter possibility is consistent with the largely particulate localization of hVPS34 and p150 in fractionated cells. Moreover, we cannot detect a net recruitment of overexpressed hVps34 or p150 from cytosol to the particulate fraction in cells co-expressing activated Rab5.

In addition to regulating the recruitment of hVPS34/p150 to endosomal membranes, Rab5 or Rab5 effectors may also recruit p150/hVps34 to sites of concentration within the plane of the endosomal membrane. This model is consistent

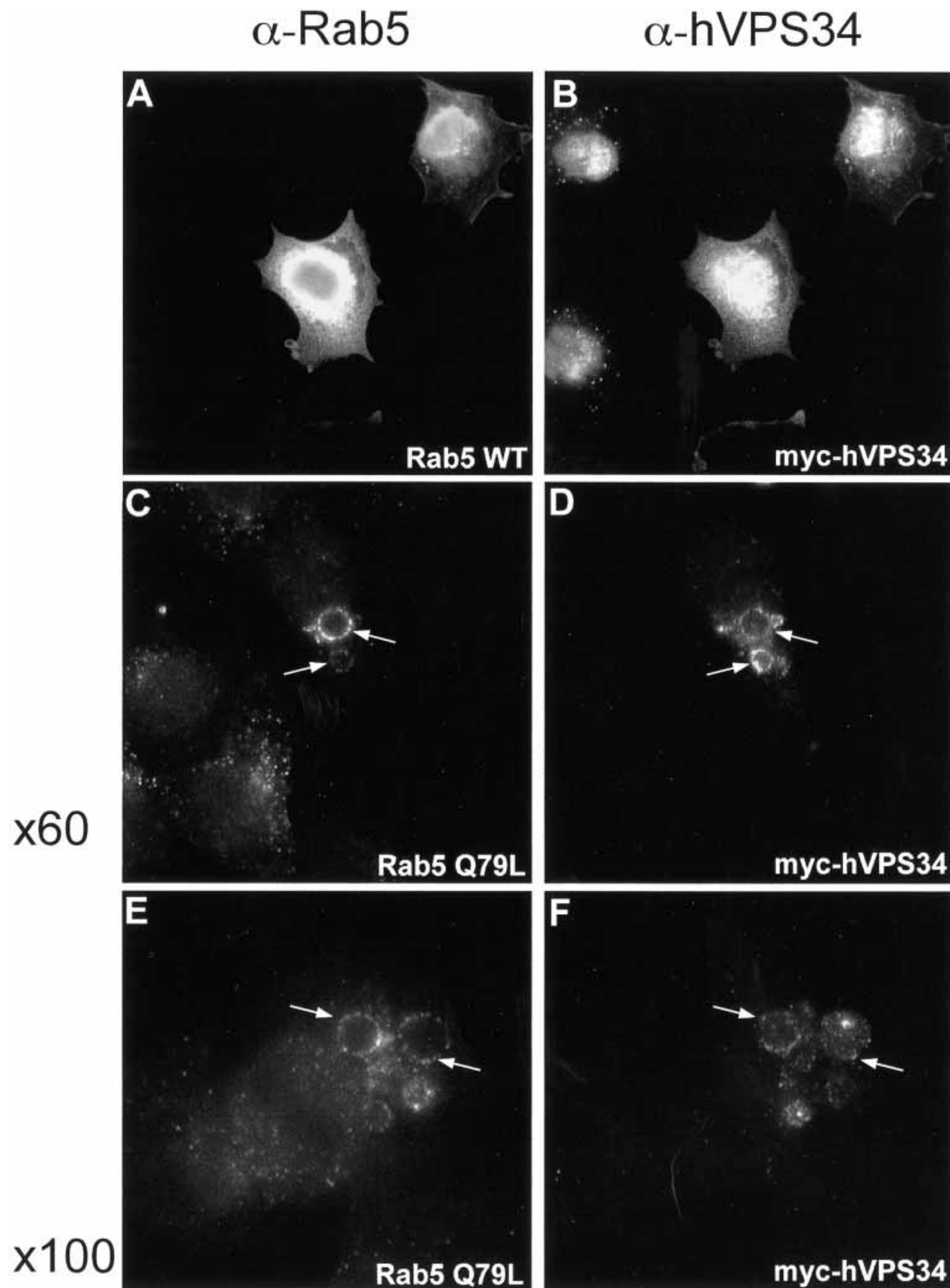


Figure 7: Recruitment of epitope-tagged hVps34 to Rab5 endosomes. A,B: Cells were transfected with myc-tagged hVps34 plus wild-type Rab5, and stained with anti-Rab5 (A) or anti-hVps34 (B) antibodies. C–F: Cells were transfected with myc-tagged hVps34 and Q79LRab5 and stained with anti-Rab5 (C,E) or anti-hVps34 (D,F) antibodies. Arrows show examples of colocalization.

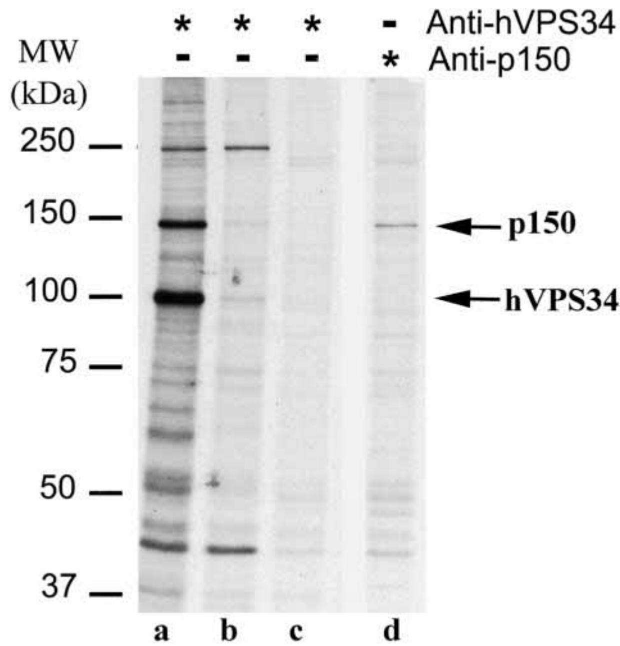


Figure 8: Immunodepletion of hVps34 from [^{35}S]-labeled cells. HeLa cells were labeled overnight with [^{35}S]methionine/cysteine, solubilized and subjected to three rounds of immunoprecipitation with anti-hVps34 antibodies (lanes a–c), followed by immunoprecipitation with anti-p150 antibody (lane d). The proteins were eluted, separated by SDS-PAGE (7.5% resolving), and visualized by autoradiography.

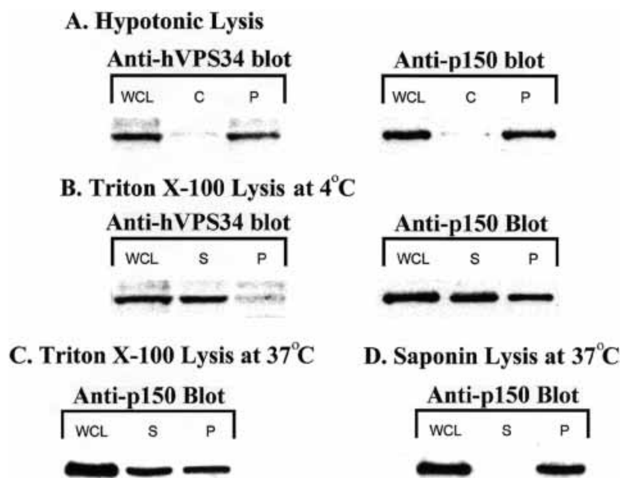


Figure 9: Subcellular localization of p150 and hVps34. A. HeLa cells were disrupted by hypotonic lysis and homogenization as described, and then subjected to sedimentation at $125\,000 \times g$. Normalized samples of whole cell lysate (WCL), cytosolic and particulate fractions were analyzed by Western blotting with anti-hVps34 (left panel) and anti-p150 (right panel) antibodies. B. HeLa cells were solubilized in 1% TX100 and insoluble material was collected by centrifugation. Normalized samples of WCL, soluble and particulate fractions were analyzed by Western blotting with anti-hVps34 (left panel) and anti-p150 (right panel) antibodies. C. HeLa cells were solubilized in buffer containing 1% Triton X-100 at 37 °C, fractionated as in (B) and analyzed by blotting with anti-p150 antibody. D. HeLa cells were solubilized in buffer containing 1% Saponin at 37 °C, fractionated as in (B) and analyzed by blotting with anti-p150 antibody.

with the demonstration of Rab5/effector microdomains in endosomal membranes (43), and is suggested by the patchy staining pattern for hVps34 and p150 in the enlarged endosomes in Figure 2(I) and Figure 3(C). The local production of PI[3]P at a confined region of the early endosome membrane might be necessary to promote a concentration of EEA1 molecules at a potential contact site, prior to endosome/endosome fusion.

Although we can clearly see colocalization of hVPS34 and p150 with EEA1 in cells expressing Q79LRab5, we see little colocalization in control cells or cells expressing wild-type Rab5. We presume that this is partially a function of the enlarged endosomes in Rab5Q79 L cells, which are easier to see. However, it is also likely that the lifetime of endosomally associated Rab5, hVPS34/p150, or both, is increased in cells expressing the activated mutant. Consistent with this, colocalization of endogenous Rab5 with EEA1 is also difficult to see, as compared to Rab5Q79 L.

If the localization of hVPS34/p150 is regulated by Rab effectors rather than by Rab5 itself, what is the function of the binding of activated Rab5 to the Heat-WD40 domains of p150? It is possible that once hVPS34/p150 is recruited to the early endosome, binding of p150 to activated Rab5 regulates the lipid kinase activity of the complex. This would be reminiscent of the effects of Ras GTPases on Class IA PI 3-kinases (44). In fact, Rab5 itself binds the Class IA p85/p110β *in vitro* (14), and expression of activated Rab5 *in vivo* increases the activation of Akt/PKB (45). It would not be surprising if Rab5 could regulate the activity of other classes of PI 3-kinase as well.

It is interesting to note that 50% of endogenous p150 is Triton X-100 insoluble, whereas hVps34 is completely solubilized by non-ionic detergents. Thus, approximately half of cellular p150 resides in a compartment that does not contain hVps34. This suggests that mammalian p150 may function differently than yeast Vps15p, since the latter protein is $>$95% soluble in Triton X-100 (26). Overall, these data suggest that the cellular locations, and by extension the cellular functions, of hVps34 and p150 overlap but are not identical. A characterization of the hVps34-independent functions of p150 will be an important future direction.

Materials and Methods

Recombinant proteins

Recombinant GST-Rab5 was produced in GDP or GTP-loaded forms as previously described (14). Individual domains of p150 (PKD, amino acids 1–300; HEAT, amino acids 433–667; and WD40, amino acids 1092–1358) were amplified from the human p150 cDNA (a gift from Dr Michael Waterfield, Ludwig Institute for Cancer Research, London, UK) by polymerase chain reaction (PCR) and cloned into pMAL-C2 expression vector (New England Biolabs, USA). Recombinant proteins were expressed in JM109 bacteria and purified by affinity chromatography on amylose resin as per manufacturer's protocol (New England Biolabs).

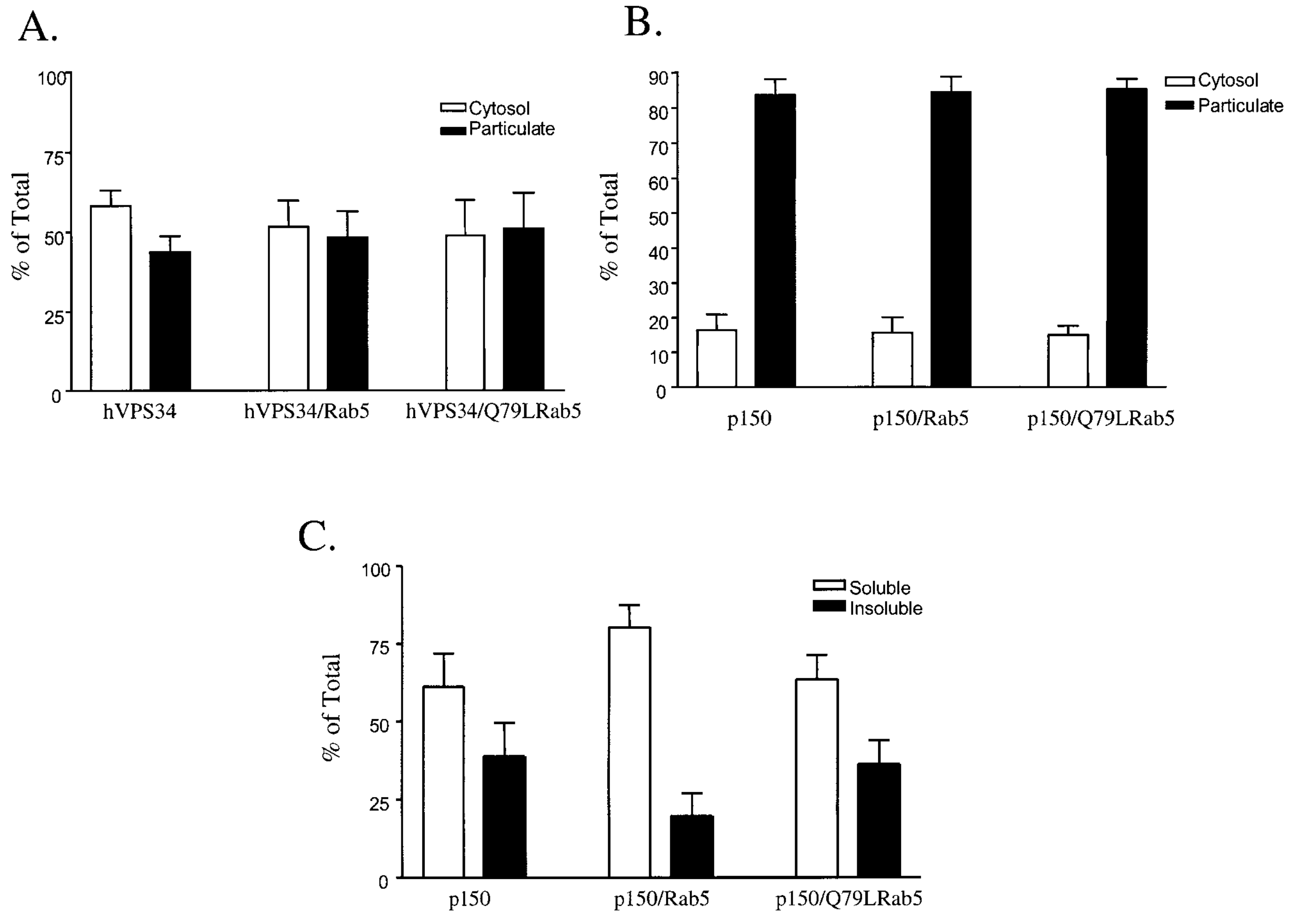


Figure 10: Effect of activated Rab5 on subcellular distribution of hVps34 and p150. HeLa cells were cotransfected with myc-tagged hVps34 or V5-tagged p150, along with empty vector, wild-type Rab5 or Q79LRab5. Twelve hours after transfection, the cells were fractionated into cytosolic and particulate fractions as described in Figure 9. Alternatively, the cells were fractionated into Triton X-100-soluble and insoluble fractions as described in Figure 9. A. Distribution of cytosolic and particulate myc-hVps34 as a percentage of total. B. Distribution of cytosolic and particulate V5-p150 as a percentage of total. C. Distribution of Triton X-100 soluble and insoluble V5-p150 as a percentage of total. Each panel represents the mean ± SEM from 3 to 4 separate experiments.

Antibodies

Antibodies against human Vps34 (hVps34) have been previously described (22). Endogenous EEA1 expression was detected with a rabbit polyclonal antibody (11) or monoclonal antibody E41120 (BD Biosciences, Palo Alto, CA, USA). Antibodies against p150 were prepared by immunizing New Zealand white rabbits with a recombinant MBP-fusion of the HEAT domain (residues 433–667) (Covance, Denver, PA, USA). The antibodies were affinity purified on a column of MBP-HEAT coupled to CNBr-activated Sepharose (Pharmacia, Piscataway, NJ, USA). The antibody recognized both endogenous p150 from HeLa cells and recombinant p150 produced in Sf-9 cells, and immunoprecipitated endogenous and recombinant p150 from HeLa cells (data not shown). Antibodies against p150 and hVps34 both immunoprecipitated a 150-kDa protein that was recognized by the anti-p150 antibody (data not shown). Thus, numerous criteria support the specificity of this antibody for mammalian p150.

Immunofluorescence analysis

Cells were fixed at 4 °C for 20 min in 3.7% formaldehyde/PBS. The coverslips were then washed twice with PBS, permeabilized with 0.1% Triton X-100/PBS for 15 min, followed by another three washes in PBS, before blocking in 3% BSA/PBS for 1 h. Cells were stained with primary antibodies diluted in blocking buffer for 1 h, washed three times and incubated with secondary antibody conjugated to Alexa^{594} and Alexa^{488} (Molecular Probes, Eugene, OR, USA) for 1 h. Coverslips were washed four times in PBS under low light conditions and mounted in Fluor-guard (Bio-Rad, Hercules, CA, USA). Specimens were examined on a Nikon E-400 upright fluorescence microscope, equipped with a Nikon 60X 1.4 N.A. plan-apo or Nikon 100X 1.25 N.A. oil-immersion infinity-corrected objectives. Images were acquired with a Cohu 4910 B/W CCD camera and NIH Image 1.62 analysis software. When indicated, images were acquired with a Bio-Rad Radiance 2000 confocal microscope using a Nikon 60X 1.4 N.A. plan-apo infinity-corrected objective, and Bio-Rad Lasersharp 2000 software. Scale bars on all micrographs are 20 μm.

Domain analysis of p150/Rab5 interactions

Individual domains of p150 (PKD, amino acids 1–300; HEAT, amino acids 433–667; and WD40, amino acids 972–1358) were deleted from the human p150 cDNA (a gift from Dr Michael Waterfield, Ludwig Institute for Cancer Research, London) by PCR and cloned into pcDNA3.1/V5-His expression vector (Invitrogen, Carlsbad, CA, USA). To analyze binding to Rab5, [^{35}S]-labeled proteins were translated *in vitro* using the Quick-coupled TNT kit (Promega, Madison, WI, USA). An equal amount of each

construct (in 10 μl) was incubated with 30 μl of packed GST-Rab5 beads in 300 μl of Buffer A (20 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, pH 7.5 and 1 mM either GDP or GTPγS). After a 2-h incubation at 4 °C, the beads were washed twice each in Buffer A containing 10 μM GDP or GTPγS, and Buffer A containing 250 mM NaCl plus 10 mM GDP or GTPγS. After a final wash in 20 mM HEPES, 250 mM NaCl, 1 mM DTT, pH 7.5, specifically bound proteins were eluted with 45 μl of elution buffer (20 mM HEPES, 1.5 M NaCl, 20 mM EDTA, 1 mM DTT, pH 7.5) containing 1 mM GTPγS to elute from Rab5-GDP and 5 mM GDP to elute from Rab5-GTPγS. Eluted proteins were solubilized in sample buffer and analyzed by SDS-PAGE and autoradiography.

Transfections and metabolic labeling

HeLa cells were cultured on 12-mm round coverslips and transfected with Lipofectamine Plus as per manufacturer's protocol (Life Technologies). HeLa cells were metabolically labeled by incubation overnight in complete medium containing 0.125 mCi/ml Easy-tag [35S]methionine/cysteine (New England Nuclear, Boston, USA) and lysed at 4 °C in 10 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% TX 100, 10% glycerol containing 0.35 mg/ml PMSF, 5 μM leupeptin, 100 μg/ml aprotinin, 100 mM sodium fluoride and 100 mM sodium orthovanadate. The lysates were cleared by centrifugation at 16 000 × *g* for 20 min, pre-absorbed with 5 μg rabbit IgG/protein-A Sepharose to reduce the background, and then immunoprecipitated with 5 μg affinity purified anti-hVps34 or anti-p150 antibodies as indicated.

Microinjections

Microinjections were conducted using an Eppendorf 5171/5242 automated injection system mounted on a Nikon Diaphot inverted microscope, using needles pulled on a Sutter P-87 micropipette puller. Recombinant proteins (2–4 mg/ml) or expression plasmid (2–5 μg/ml) were mixed with FITC-labeled Dextran (3 mg/ml) in PBS (pH 7.4). Cells for microinjection were plated on coverslips and incubated for 24 h in culture medium. The cells were transferred to medium containing 20 mM HEPES (pH 7.3) prior to injection. After injection of recombinant protein and cDNA the cells were allowed to recover for 2 h and 24 h, respectively, before processing.

Cellular fractionation of HeLa cells

HeLa cells were grown to 80% confluency and then trypsinized, counted, and centrifuged at 800 × *g* for 8 min at 4 °C. The pellets were processed by: (a) direct lysis in 2X SDS-sample buffer; (b) lysis in 10 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, followed by centrifugation at 14 000 × g for 15 min; (c) swelling in 1 mM $MgCl_2$, 1 mM DTT and 1 mM EGTA, pH 7.4, followed by homogenization in 0.25 M sucrose, 3 mM imidazole pH 7.4, 0.35 mg/ml PMSF, 1 mM benzamidine, 100 μg/ml aprotinin and 1 μg/ml leupeptin using 10 strokes of a Dounce homogenizer with a loose-fitting pestle, and centrifugation at 125 000 × *g* for 1 h at 4 °C; (d) lysis in Triton X-100 as above, at 37 °C, followed by centrifugation at 14 000 × *g* for 15 min, or (e) lysis in 10 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA, 1% Saponin, followed by centrifugation at 14 000 × *g* for 15 min. In each case, the supernatants and pellets were then solubilized in Laemmli sample buffer such that they reflected 2.5×10^6 cells/ml, and 50 μl was loaded per lane of an SDS-PAGE gel. Where indicated, the cells were transfected with either V5- or EE-p150, Myc-hVps34, or Rab5 12 h prior to fractionation. Endosomal fractions from HeLa cells were purified as previously described (14).

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