The FYVE Domain of Early Endosome Antigen 1 Is Required for Both Phosphatidylinositol 3-Phosphate and Rab5 Binding

CRITICAL ROLE OF THIS DUAL INTERACTION FOR ENDOSONAL LOCALIZATION

(Received for publication, September 8, 1999)

Deidre C. Lawe§§, Varsha Patki§§, Robin Heller-Harrison¶, David Lambright¶, and Silvia Corvera‡

From the Program in Molecular Medicine and the Departments of §Cell Biology and ¶Biochemistry and Molecular Biology, University of Massachusetts Medical School, Worcester, Massachusetts 01605

Early endosome antigen 1 (EEA1) is a 170-kDa polypeptide required for endosome fusion. EEA1 binds to both phosphatidylinositol 3-phosphate (PtdIns3P) and to Rab5-GTP in vitro, but the functional role of this dual interaction at the endosomal membrane is unclear. Here we have determined the structural features in EEA1 required for binding to these ligands. We have found that the FYVE domain is critical for both PtdIns3P and Rab5 binding. Whereas PtdIns3P binding only required the FYVE domain, Rab5 binding additionally required a 30-amino acid region directly adjacent to the FYVE domain. Microinjection of glutathione S-transferase fusion constructs into Cos cells revealed that the FYVE domain alone is insufficient for localization to cellular membranes; the upstream 30-amino acid region required for Rab5 binding must also be present for endosomal binding. The importance of Rab5 in membrane binding of EEA1 is underscored by the finding that the increased expression of wild-type Rab5 increases endosomal binding of EEA1 and decreases its dependence on PtdIns3P. Thus, the levels of Rab5 are rate-limiting for the recruitment of EEA1 to endosome membranes. PtdIns3P may play a role in modulating the Rab5 EEA1 interaction.

Early endosome antigen 1 (EEA1) is a 170-kDa polypeptide required for endosome fusion. EEA1 binds to both phosphatidylinositol 3-phosphate (PtdIns3P) and to Rab5-GTP in vitro, but the functional role of this dual interaction at the endosomal membrane is unclear. Here we have determined the structural features in EEA1 required for binding to these ligands. We have found that the FYVE domain is critical for both PtdIns3P and Rab5 binding. Whereas PtdIns3P binding only required the FYVE domain, Rab5 binding additionally required a 30-amino acid region directly adjacent to the FYVE domain. Microinjection of glutathione S-transferase fusion constructs into Cos cells revealed that the FYVE domain alone is insufficient for localization to cellular membranes; the upstream 30-amino acid region required for Rab5 binding must also be present for endosomal binding. The importance of Rab5 in membrane binding of EEA1 is underscored by the finding that the increased expression of wild-type Rab5 increases endosomal binding of EEA1 and decreases its dependence on PtdIns3P. Thus, the levels of Rab5 are rate-limiting for the recruitment of EEA1 to endosome membranes. PtdIns3P may play a role in modulating the Rab5 EEA1 interaction.

The activity of PI3 kinase has been strongly implicated in the control of endosomal membrane traffic. Treatment of cells with PI3 kinase inhibitors results in a decrease in transferrin receptor recycling, plateau-derived growth factor receptor down-regulation, and transcytosis in epithelial cells (7–12). In addition, wortmannin inhibits endosome fusion in vitro assays (13, 14). These effects coincide with an inhibition of EEA1 binding to early endosomal membranes (3, 15), which accounts for the inhibition of endosome fusion in vitro, and may account for some of the wortmannin-induced phenotypes observed in intact cells. Rab5 has been recognized as a critical regulatory factor in endosome fusion; a mutation in Rab5, which renders it resistant to GTP hydrolysis (Rab5Q79L), results in enlarged endosomes in vivo and enhanced fusion in vitro endosome fusion assays (16, 17). Conversely, mutations in Rab5, which render it resistant to Rab5 by the addition of GDP dissociation inhibitor (19). From these studies it has been proposed that EEA1 is a critical factor required for endosome fusion, where it acts as a docking/tethering protein between vesicles to promote soluble N-ethylmaleimide-sensitive fusion attachment receptor (SNARE)-mediated fusion reactions (20, 21). Although it is clear that EEA1 binds directly to both PtdIns3P and to Rab5-GTP in vitro, the functional role of a dual interaction at the endosomal membrane is not clear. EEA1 co-localizes with Rab5 at early endosomes (2) but, unlike Rab5, does not associate with early endosome precursors such as clathrin-coated or adaptin-coated vesicles. It has been proposed that the combinatorial interaction of EEA1 with both Rab5-GTP and PtdIns3P ensures its precise localization to endosomes containing both signals, as opposed to cellular membranes containing exclusively one or the other (18). Alternatively, PtdIns3P and Rab5 may play a regulatory role in EEA1 function. The half-life of PtdIns3P and the duration of the GTP-bound state of Rab5 may influence the rate and extent of the endosome fusion reaction. In this regard, it is worth noting that introduction of a GFP tag at the COOH terminus of EEA1 results in the gross enlargement of early endosomes in vivo, coinciding with a loss of specificity for PtdIns3P binding (4).
To begin to understand the mechanisms and functional significance of the dual interaction of EEA1 with PtdIns3P and Rab5, we have determined the structural features in EEA1 required to bind these ligands. Our studies indicate that the integrity of the FYVE domain in conjunction with a stretch of 30 amino acids upstream is essential for Rab5 association. Thus, the PtdIns3P binding FYVE domain is nested within the Rab5 binding site, which explains the overlapping influence of both PI3 kinase and Rab5 activities on early endosome formation. Microinjection of diverse constructs of EEA1 into cells revealed that the FYVE domain alone, which binds PtdIns3P3 in vitro with high affinity, is insufficient for membrane localization. The upstream 30 amino acids were absolutely required for membrane binding, which was further enhanced by the presence of the IQ motif. Thus, additional protein-protein interactions may play a critical role in establishing FYVE/PtdIns3P-mediated protein-membrane associations.

**MATERIALS AND METHODS**

*Generation of Constructs and Recombinant Protein—GST1277 was generated as described previously (4). Both GST1306 and GST1336 were reverse chain reaction amplified using primers ending to residues 1306–1411 and 1336–1411 of EEA1, respectively, cloned in frame into the BamHI/Sall sites in pGEX-4T1 (Amersham Pharmacia Biotech), and sequenced for verification. Rab5c cDNA was isolated from a 3T3-F442A murine adipocyte cDNA expression library via EcoRI excision of the 768-base pair Rab5c cDNA insert from Bluescript-Rab5c obtained from cloning procedures and subcloned into EcoRI-digested pCMV5. Correct orientation of Rab5c was determined by restriction analysis and sequencing. The 10×His-Rab5c construct, corresponding to residues 17–185, lacks both hypervariable regions but has normal function with respect to GTP binding/hydrolysis. It interacts with EEA1 in a nucleotide-dependent manner, as does full-length Rab5c expressed as a GST fusion protein.*

*In Vitro Binding of GST Fusion Proteins—GST1277 was immobilized by glutathione-Sepharose affinity purification. GST fusion proteins and a nickel nitrilotriacetic acid-agarose column (Qia-mer's instructions). Purified GST fusion proteins were bound to glutathione-Sepharose beads that had been preblocked in 5% nonfat dry milk in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20). Beads were then washed and incubated with 2 μg of preloaded 10×His-Rab5c for 1 h at 4 °C in 300 μl of cytosol buffer containing 10 mg/ml bovine serum albumin and 0.1% Tween 20. 5 μM N,N′N′N′-tetramis(2-pyridylmethyl)ethylenediamine (TPEN) (Sigma) were included in the incubation where indicated. Following centrifugation, the pellets were washed four times in 1 ml of A buffer, 20 mM MgCl2, 0.1% Tween 20. Bound proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane, which were blocked in 5% dry milk, TBST followed by incubation with anti-His monoclonal antibody (Amersham Pharmacia Biotech). Filters were then incubated with horseradish peroxidase-conjugated goat anti-mouse antibody (Promega), which was detected by Renaissance enhanced lumino reagent (NEN Life Science Products).* 

*Generation of Antibodies—Rab5c antiserum was prepared by injecting the Rab5c carboxyl-terminal peptide QNAAGAPGRGTDQESN coupled to keyhole limpet hemocyanin via an aminos-terminal cysteine residue into New Zealand White rabbits. Affinity purified Rab5c antibodies were purified by chromatography using a glutathione-Sepharose column (Amersham Pharmacia Biotech) for GST fusion proteins and a nickel nitrilotriacetic acid-agarose column (Qiagen) for His fusion proteins and were eluted according to the manufacturer’s instructions.

*Generation of Antibodies—Rab5c antiserum was prepared by injecting the Rab5c carboxyl-terminal peptide QNAAGAPGRGTDQESN coupled to keyhole limpet hemocyanin via an aminos-terminal cysteine residue into New Zealand White rabbits. Affinity purified Rab5c antibodies were purified by chromatography using a glutathione-Sepharose column (Amersham Pharmacia Biotech) for GST fusion proteins and a nickel nitrilotriacetic acid-agarose column (Qiagen) for His fusion proteins and were eluted according to the manufacturer’s instructions.*

*Immunofluorescence—Stable transfectants were grown to 40–50% confluence on coverslips and incubated for 10 min without or with wortmannin as indicated. Coverslips were washed twice with cold phosphate-buffered saline, fixed in 4% formaldehyde/phosphate-buffered saline for 10 min at 4 °C, and permeabilized with 0.2% Triton X-100/phosphate-buffered saline for 10 min at 4 °C. Cells were then blocked with 1% fetal bovine serum/0.5% Triton X-100 in phosphate-buffered saline for 30 min at 4 °C. EEA1 was detected with human antiserum reactive with EEA1 (3) at a 1:10,000 dilution. Rab5c was detected with affinity purified rabbit anti-Rab5c antibody at approximately 10 μg/ml. Microinjected GST fusion proteins were detected with anti-GST monoclonal antibody (Upstate Biotechnology) or fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories Inc.), rhodamine-conjugated goat anti-rabbit antibody, or fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories Inc.).*
RESULTS

The Binding of EEA1 to Rab5 and PtdIns3P Occurs through Overlapping Structural Regions—We investigated the structural features in EEA1 that determine its binding to Rab5 and to PtdIns3P. We have previously shown that the carboxy-terminal 134 amino acids of EEA1, expressed as a GST fusion protein (here referred to as GST1277), will bind specifically to liposomes containing PtdIns3P (4). In addition, this region has been shown in vitro to bind directly to Rab5-GTP (18). This region also contains an IQ motif, a putative calmodulin binding domain composed of extensive coiled-coil, which probably contributes to protein-protein interactions including homodimerization (22). To identify the minimal sequence within this region required to bind Rab5, a series of deletion constructs were generated in frame with GST (Fig. 1A), and their ability to associate with Rab5 was analyzed (Fig. 1B). The constructs were immobilized on glutathione-Sepharose beads and incubated with a 10xHis-tagged Rab5c protein that had been preloaded with either GTP\(\gamma\)S or GDP\(\beta\)S. As expected, GST1277 bound to Rab5 in a GTP-dependent fashion. Interestingly, GST1306, which lacks the IQ motif and contains significantly less of the regions of predicted coiled-coil, also bound Rab5-GTP effectively. A further deletion of 30 amino acids (GST1336) abolished binding (Fig. 1B). Thus, the 30-amino acid region upstream of the FYVE domain is the minimal region necessary for Rab5 binding.

To determine whether this 30-amino acid region was sufficient for Rab5 binding, a GST fusion protein encompassing amino acids 1306–1357 was generated, and its ability to associate with Rab5-GTP was determined. This construct was unable to pull down either Rab5-GTP or Rab5-GDP (data not shown), indicating that additional structural elements are necessary for the interaction.

The ability of GST1277, GST1306, and GST1336 to bind PtdIns3P was also determined using a liposome binding assay (Fig. 1C). All three fusion proteins bound tightly and specifically to liposomes composed of 50% phosphatidylserine, 49% PtdIns, and 1% PtdIns3P (PS/P1), as opposed to liposomes composed of only 50% phosphatidylserine and 50% PtdIns (PS/P1) (Fig. 1C). These results indicate that the FYVE domain in these constructs is completely functional and independent of the structural elements required to bind Rab5-GTP.

To determine whether the three-dimensional structure of the FYVE domain was required for Rab5 binding, we examined the ability of GST1277 to interact with Rab5 after treatment with the zinc chelator, TPEN. The ability of GST1277 to interact with Rab5 in a GTP-dependent manner was abrogated by the removal of Zn\(^{2+}\) (Fig. 2A). As expected, Zn\(^{2+}\) chelation also abrogated the binding of GST1277 to PtdIns3P (Fig. 2B). Thus, these data indicate that an integral FYVE domain, together with amino acids 1306–1357 of EEA1, are necessary for Rab5-GTP binding.

Structural Elements Upstream of the Rab5 and PtdIns3P Binding Domains Enhance Binding to Endosomes—To directly compare the interactions observed in vitro to the interactions required for endosomal localization in intact cells, the GST1277, GST1306, and GST1336 fusion proteins were micro-injected into COS cells, and their subcellular distribution was visualized after fixation by immunofluorescence with an anti-GST antibody (Fig. 3). GST1277 localized to punctate and ring-shaped vesicular structures throughout the cytoplasm (Fig. 3A, top left panel). Most of the injected protein was bound to endo-

![Fig. 1. Delineation of the Rab5 binding site on EEA1. A, a schematic representation of the carboxy terminus of EEA1 and deletion constraints thereof fused to GST at the indicated sites. B, the GST fusion proteins depicted above were immobilized on glutathione-Sepharose and incubated with 10xHis-Rab5c preloaded with GTP\(\gamma\)S or GDP\(\beta\)S as described under “Materials and Methods.” Protein bound to the Sepharose beads was analyzed by Western blotting and detected by PAGE and Coomassie Blue staining.](http://www.jbc.org/)

![Fig. 2. A functional FYVE domain is required for Rab5-GTP binding. A, 10xHis-Rab5c preloaded with GTP\(\gamma\)S or GDP\(\beta\)S was incubated with immobilized GST1277, either in the presence or absence of 5 mM TPEN, and bound 10xHis-Rab5c was detected by Western blotting as in Fig. 1B. B, GST1277 was incubated with either PS/P1 or PI-3P liposomes in the presence or absence of 5 mM TPEN, and the amount of GST protein bound was measured by PAGE and Coomassie Blue staining.)
Fig. 3. The FYVE domain is insufficient for endosome targeting. Cos cells were microinjected with a solution containing 20 μg/ml rhodamine-coupled dextran and (A) 0.15 mg/ml GST1277 or (B) 0.5 mg/ml GST1306 or 0.6 mg/ml GST1336 fusion protein. Following microinjection, coverslips were placed in fresh medium for 2 h, treated or not with 50 nm wortmannin (WTM) for 10 min, fixed, and stained with a monoclonal antibody against GST followed by a fluorescein isothiocyanate-coupled secondary anti-mouse antibody. Microinjected cells were identified via rhodamine-coupled dextran (right panels). The localization of the microinjected GST fusion proteins is depicted in the left panels.

Fig. 4. Endogenous Rab5c levels are rate-limiting for association of EEA1 with cell membranes. PNS from CHO cells stably transfected with either the pCMV5 vector alone (A) or Rab5c (B) were incubated for 15 min with (+) or without (−) 50 nm wortmannin in the absence or presence of 100 μg GTP/μl. The cytosol was then separated from particulate fraction by high speed centrifugation and analyzed by Western blotting with an anti-EEA1 polyclonal antiserum. Plotted are the values expressed as a percentage of total EEA1, which was estimated from the amount present in the homogenate prior to centrifugation.

Wild-type Rab5 Levels Are Rate-limiting for EEA1 Binding to Membranes—The results shown above demonstrate that the simultaneous occurrence of PtdIns3P and Rab5 binding are required for the interaction of the COOH terminus of EEA1 with the endosome. Furthermore, binding of these two ligands occurs through overlapping structural domains. To delineate how endogenous Rab5 and PtdIns3P influence the binding of EEA1 to cellular membranes, we took advantage of our finding that binding of EEA1 to membranes can be observed in broken cells. PNS obtained from pCMV5-transfected CHO cells was placed at 37 °C for 20 min, and the membrane fraction was then removed by high speed centrifugation. Approximately 50% of EEA1 was present in the supernatant (Fig. 4A, lane 1). Inclusion of GTPγS to maximally activate small GTPases markedly enhanced binding to membranes, with only 20% of EEA1 remaining in the supernatant (Fig. 4A, lane 3). Inclusion of wortmannin during the 20-min incubation inhibited binding almost completely, resulting in 90% of EEA1 remaining in the supernatant (Fig. 4A, lane 2). The presence of GTPγS did not overcome the inhibitory effect of wortmannin on EEA1 binding to membranes, indicating that even with maximal activation of Rab5, EEA1 binding was still dependent on PtdIns3P (Fig. 4A, lane 4). Thus, at normal Rab5 levels, PtdIns3P is critical for EEA1 binding.

We next examined whether the levels of expression of Rab5 would influence the interaction of EEA1 with endosomes. CHO cells were stably transfected with Rab5c, and the properties of PNS obtained from Rab5c-overexpressing cells were analyzed (Fig. 4B). Overexpression of Rab5c enhanced binding of EEA1 to the membrane, as only 10–20% of EEA1 could be detected in the supernatant (Fig. 4B, lane 1). GTPγS further enhanced binding, with virtually a complete disappearance of EEA1 from the supernatants (Fig. 4B, lane 3). Inclusion of wortmannin under these conditions had only a modest inhibitory effect on
EEA1 binding (Fig. 4B, lanes 2 and 4). Thus, overexpression of Rab5c not only enhances association of EEA1 with membranes, but renders the interaction insensitive to the levels of PtdIns3P.

The results observed in broken cells are supported by the observation that Rab5c-overexpressing cells display a more intense EEA1 staining pattern localized to peripheral vesicular and ring-shaped tubular structures as compared with cells transfected with vector alone (Fig. 5). In these cells some of the Rab5c co-localized with EEA1 on vesicular structures but most was present in more perinuclear structures (Fig. 5, bottom right panel). Thus, not all Rab5-positive structures contained EEA1; however, overexpression of wild-type Rab5c markedly enhanced EEA1 binding in vivo and led to larger EEA1-containing structures. These results suggest that endosome fusion is directly regulated by the levels of Rab5, which determines the degree of interaction of EEA1 with membranes.

We next examined the effect of PI3 kinase inhibition on EEA1 membrane association in the Rab5c overexpressing cells. Immunofluorescence analysis revealed the majority of EEA1 in association with membranes both in the presence or absence of wortmannin treatment (Fig. 6), in accordance with the fractionation results shown in Fig. 4. Interestingly, whereas in nontreated cells the overexpressed Rab5c was relatively diffuse and only partially co-localized with EEA1 (Fig. 5, bottom, and Fig. 6, top), in wortmannin-treated cells Rab5c was found in a tight perinuclear cluster, where it clearly co-localized completely with EEA1 (Fig. 6, compare bottom left and right panels). Thus, wortmannin treatment appears to cause an alteration in the partitioning of Rab5c among intracellular compartments, suggesting that PI3 kinase activity may influence the GTP cycle on Rab5.

**DISCUSSION**

Recent work from many laboratories has contributed to the identification of the specific molecular elements involved in the process of endosome fusion (20, 23–26). Among these elements are the small GTPase Rab5, PI3 kinase, and the protein EEA1, which interacts functionally with both of these effectors and is thought to provide a tethering system necessary for membrane fusion. Endosome fusion also depends critically on SNARE proteins, an integral part of the fusion system, which directly mediate the formation of the fusion pore (27, 28). How these systems of tethering and fusion, each of which have components that are likely to play regulatory roles, operate in the context of intact cells is currently an unanswered question.

In this manuscript, we have addressed two specific questions related to the mechanisms that regulate endosome fusion in intact cells. First, what is the structural basis for the interaction between Rab5, PtdIns3P, and EEA1, and second, what is the physiological relevance of these interactions in intact cells. Our results indicate that (a) Rab5 binding by EEA1 requires the integrity of the FYVE domain plus a 30-amino acid NH$_2$-terminal extension; (b) the FYVE domain of EEA1 alone, although capable of high affinity binding to PtdIns3P, cannot bind to endosomes nor to any other membrane system in intact cells; both PtdIns3P and Rab5 binding are required for binding of EEA1 to endosomes; and (c) that the rate-limiting factor for EEA1 association with endosomes in vivo is the level of activated Rab5.

Our conclusion that an efficient interaction between EEA1 and Rab5 requires both the integrity of the FYVE domain and a 30-amino acid sequence to the NH$_2$ terminus contrasts with that reached by Simonsen *et al.* (18). In these studies the minimal Rab5 binding region of EEA1 was defined as being comprised of residues 1277–1348, excluding the FYVE domain. It is possible that a weak interaction between this region and a persistently active mutant of Rab5 may be detectable in the two-hybrid system used in these studies but not in a biochemical pull-down assay such as the one used here. However, this weak interaction is clearly functionally insufficient, as mutations in the FYVE domain completely prevent binding of EEA1 to endosomes even in the presence of persistently active Rab5 (2). Overexpression of this mutant form of Rab5 abrogates the normal requirement of PtdIns3P association for EEA1 binding to endosomal membranes (18). Thus the inability of the FYVE domain mutants to localize to endosomes in cells expressing persistently activated Rab5 is likely to be due to the disruption of Rab5 binding.

A dual role of the FYVE domain in mediating the interaction both with PtdIns3P and Rab5 underscores the functional similarity between EEA1 and the yeast protein Vac1p. Point mutations in the FYVE domain of Vac1p abolished its interaction with Vps21p, a Rab GTPase that functions in Golgi to endosome transport (29). Similarly, other Zn$^{2+}$ binding domains are present in Rab effector proteins and contribute directly or indirectly to the interaction with activated Rab GTPases. For example, Rabphilin-3A contains a Zn$^{2+}$ binding domain within its Rab3A binding region, and mutations that disrupt Zn$^{2+}$ binding abolish Rab3A association (30). Comparison of the crystal structure of the FYVE domain of Vps27 with that of the
Rab3A-Rabphilin-3A complex suggests a potential interaction between the COOH terminus of Rab5 and the FYVE domain of EEA1 (31).

What is the physiological role of the dual interaction of EEA1 with PtdIns3P and Rab5? PtdIns3 kinase, Rab5, and EEA1 have been recognized as critical components in the control of endosome fusion, but the mechanisms underlying their interplay are not understood. One current hypothesis is that Rab5 provides a mechanism to ensure targeting of EEA1 to early endosomes and not to other cellular membranes that might contain PtdIns3P (18). However, the finding that PtdIns3P binding by the FYVE domain is not sufficient for EEA1 association with any cellular membrane suggests that Rab5 binding is fundamentally required for the binding event itself. Furthermore, overexpression of wild-type Rab5 enhances EEA1 binding to the point where the FYVE-PtdIns3P interaction becomes unnecessary for endosomal localization. These results suggest that the principal role of Rab5 is to direct the membrane binding of EEA1 and that the FYVE-PtdIns3P interaction is likely to be regulatory.

What type of regulatory role might PtdIns3P play in endosome fusion? Experiments using fusion proteins of the COOH domain of EEA1 indicate that both Rab5 and PtdIns3P are required to achieve the binding of EEA1 to endosomal membranes (Fig. 3). These results suggest that these signals play a synergistic role in mediating EEA1 binding to endosomal membranes and in subsequent endosome fusion. The prediction from this conclusion would be that inhibition of PtdIns3 kinase activity with wortmannin would result in an inhibition of endosome fusion. Although this result is indeed observed in vitro endosome fusion assays (13, 14), results in intact cells differ significantly. For example, wortmannin increases the accumulation of the fluid phase marker Lucifer Yellow and of transferrin in early endosomes and causes only a small decrease in the rate of transferrin recycling (9, 11, 12). Furthermore, a pronounced enlargement and tubulation of endosomes containing these markers is observed after treatment with this toxin (11). This phenotype is not consistent with a block in endosome fusion. In fact, it more closely resembles the phenotype of enhanced endosome fusion produced by expression of persistently activated forms of Rab5 (16).

The discrepancy between the apparent requirement for PtdIns3P to bind EEA1 to membranes and the enhanced fusion of endosomes observed in intact cells after wortmannin treatment suggests more than one specific role for PtdIns3P during the process of endocytosis. For example, PtdIns3P might serve to initiate the recycling of EEA1 and other components of the fusion machinery after endosome fusion, thus preventing the formation of large endosomes while promoting the continual formation of new ones. An intriguing possibility supported by results shown here (Fig. 6) is that PtdIns3 kinase activity may be a critical factor in regulating Rab5 function. Whereas in control cells Rab5 was diffusely distributed, in wortmannin-treated cells it was largely concentrated in enlarged juxtanuclear endosomes that also contained EEA1. These enlarged endosomes might arise from a persistent activation of Rab5 resulting in enhanced EEA1 binding and an uncontrolled increase in the number of endosome fusion events. Further studies aimed at better understanding the interplay among PtdIns3P, EEA1, and Rab5 are necessary to clarify these questions.

Microinjection experiments shown here indicate that whereas the presence of both the FYVE and the upstream sequence required for Rab5 binding are sufficient for endosome localization, the presence of the IQ motif amino-terminal to these sites enhances this interaction. This enhanced interaction might be due to oligomerization of the carboxyl terminus of EEA1 with itself, as amino acids 1277–1411 but not 1307–1411 were able to homodimerize in a yeast two-hybrid assay (22). Oligomerization of the protein would result in multiple Rab5-GTP/PtdIns3P binding sites in one complex, increasing its affinity for membranes containing both signals, thereby stabilizing the protein at the membrane. Alternatively, additional interactions with other endosome components might be involved. In the case of Vac1p, interactions with components of the SNARE fusion machinery have been demonstrated (29). Such interactions might also occur in mammalian cells. Whether these interactions directly involve the IQ domain of EEA1 remains to be determined.

Acknowledgments—We gratefully acknowledge Michelle Morin and John Dumas for producing several cell lines and constructs used here.

REFERENCES
1. Mu, F. T., Callaghan, J. M., Steele-Mortimer, O., Stenmark, H., Parton, R. G., Campbell, P. L., McCluskey, J., Yeo, J. P., Tock, E. P., and Toh, B. H. (1995) J. Biol. Chem. 270, 13503–13511
2. Stenmark, H., Aasland, R., Toh, B. H., and D’Arrigo, G. (1996) J. Biol. Chem. 271, 24048–24054
3. Patki, V., Virbasius, J., Lane, W. S., Toh, W. S., Shpetner, H. S., and Corvera, S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7326–7330
4. Patki, V., Lawe, D. C., Corvera, S., Virbasius, J. V., and Chawla, A. (1998)
FYVE Domain Interaction with Rab5

5. Gaullier, J. M., Simonsen, A., D’Arrigo, A., Bremnes, B., Stenmark, H., and Aasland, R. (1998) Nature 394, 432–433.
6. Burd, C. G., and Emr, S. D. (1998) Mol. Cell 2, 157–162.
7. Hansen, S. H., Olsson, A., and Casanova, J. E. (1995) J. Biol. Chem. 270, 28425–28432.
8. Kundra, R., and Kornfeld, S. (1998) Nature 394, 432–433.
9. Hansen, S. H., Olsson, A., and Casanova, J. E. (1995) J. Biol. Chem. 270, 28425–28432.
10. Reaves, B. J., Bright, N. A., Mullock, B. M., and Luzio, J. P. (1996) J. Cell Sci. 109, 749–762.
11. Shpetner, H., Joly, M., Hartley, D., and Corvera, S. (1996) J. Cell Biol. 132, 585–605.
12. Sprio, D. J., Boll, W., Kirschhausen, T., and Wessling-Resnick, M. (1996) Mol. Biol. Cell 7, 355–367.
13. Jones, A. T., and Clague, M. J. (1995) Biochem. J. 311, 31–34.
14. Li, G., DSouza-Schorey, C., Barbiert, M. A., Roberts, R. L., Klippel, A., Williams, L. T., and Stahl, P. D. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10207–10211.
15. Mills, I. G., Jones, A. T., and Clague, M. J. (1998) Curr. Biol. 8, 881–884.
16. Stenmark, H., Parton, R. G., Steele-Martimer, O., Lutcke, A., Gruenberg, J., and Zerial, M. (1994) EMBO J. 13, 1287–1296.
17. Gorvel, J. P., Chavrier, P., Zerial, M., and Gruenberg, J. (1991) Cell 64, 915–925.
18. Simonsen, A., Lippe, R., Christoforidis, S., Gaullier, J. M., Brech, A., Callaghan, J., Toh, B. H., Murphy, C., Zerial, M., and Stenmark, H. (1998) Nature 394, 494–498.
19. Christoforidis, S., McBride, H. M., Burgoyne, R. D., and Zerial, M. (1999) Nature 397, 621–625.
20. Corvera, S., D’Arrigo, A., and Stenmark, H. (1999) Curr. Opin. Cell Biol. 11, 460–465.
21. Waters, M. G., and Pfeffer, S. R. (1999) Curr. Opin. Cell Biol. 11, 458–459.
22. Callaghan, J., Simonsen, A., Gaullier, J. M., Toh, B. H., and Stenmark, H. (1999) Biochem. J. 338, 539–543.
23. Hay, J. C., and Scheller, R. H. (1997) Curr. Opin. Cell Biol. 9, 505–512.
24. Burd, C. G., Babst, M., and Emr, S. D. (1998) Semin. Cell Dev. Biol. 9, 527–533.
25. Clague, M. J. (1998) Biochem. J. 336, 271–282.
26. Li, G. (1996) Biocell 20, 325–330.
27. Chao, D. S., Hay, J. C., Winnick, S., Prekeris, R., Klumperman, J., and Scheller, R. H. (1999) J. Cell Biol. 144, 869–881.
28. Sollner, T. H., and Rothman, J. E. (1996) Experientia (Basel) 52, 1021–1025.
29. Peterson, M. R., Burd, C. G., and Emr, S. D. (1999) Curr. Biol. 9, 159–162.
30. Ostermeier, C., and Brunger, A. T. (1999) Cell 96, 363–374.
31. Misra, S., and Hurley, J. H. (1999) Cell 97, 657–666.
The FYVE Domain of Early Endosome Antigen 1 Is Required for Both Phosphatidylinositol 3-Phosphate and Rab5 Binding: CRITICAL ROLE OF THIS DUAL INTERACTION FOR ENDOSONAL LOCALIZATION

Deirdre C. Lawe, Varsha Patki, Robin Heller-Harrison, David Lambright and Silvia Corvera

J. Biol. Chem. 2000, 275:3699-3705. doi: 10.1074/jbc.275.5.3699

Access the most updated version of this article at http://www.jbc.org/content/275/5/3699

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 31 references, 14 of which can be accessed free at http://www.jbc.org/content/275/5/3699.full.html#ref-list-1