Sequential Roles for Phosphatidylinositol 3-Phosphate and Rab5 in Tethering and Fusion of Early Endosomes via Their Interaction with EEA1*§

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Early endosome antigen 1 (EEA1) is a 170-kDa polypeptide required for endosome fusion in mammalian cells. The COOH terminus of EEA1 contains a FYVE domain that interacts specifically with phosphatidylinositol 3-phosphate (PtdIns-3-P) and a Rab5 GTPase binding region adjacent to the FYVE domain. The functional interaction of EEA1 with both PtdIns-3-P and Rab5 has been well characterized to provide specificity required to target EEA1 to early endosomes. To test this hypothesis, we generated constructs containing point mutations in the COOH terminus that impair Rab5 but not PtdIns-3-P binding. These constructs localized to endosomes in intact cells as efficiently as their wild-type counterparts. Furthermore, overexpression of the truncated constructs, both wild-type and mutated, impaired the function of endogenous EEA1 resulting in the accumulation of small, unattached endosomes. These results suggest that association with Rab5 is not necessary for the initial binding and tethering functions of EEA1. A role for Rab5 binding was revealed, however, upon comparison of endosomes in cells expressing full-length wild-type or mutated EEA1. The mutant full-length EEA1 caused the accumulation of endosome clusters and suppressed the enlargement of endosomes caused by a persistently active form of Rab5 (Rab5Q79L). In contrast, expression of wild-type EEA1 with Rab5Q79L enhanced this enlargement. Thus, endosome tethering depends on the interaction of EEA1 with PtdIns-3-P, and its interaction with Rab5 appears to regulate subsequent fusion.

Protein trafficking in the secretory and endocytic pathways requires the coordinated participation of distinct protein classes, among which are tethering proteins and Rab GDPases. In the endocytic pathway the small GTPase Rab5 has been postulated to mediate the obligatory docking or tethering steps required prior to homotypic endosome fusion, as well as to participate in the fusion process itself (3, 4).

EEA1 is a large ~170-kDa polypeptide that contains extensive regions of coiled-coil as well as a conserved Zn2+ finger motif at its extreme COOH terminus, called the FYVE domain (5). The FYVE domain interacts specifically with PtdIns-3-P, the most abundant product of PI3-kinase activity in eukaryotic cells (6, 7), and is crucial for the interaction of EEA1 with endosomal membranes (8). The dual interaction of EEA1 with PtdIns-3-P and Rab5 has been hypothesized to confer increased specificity and stability to the association of EEA1 with endosomal membranes (3, 4). To directly test this hypothesis we have identified critical residues required for the interaction of the EEA1 COOH-terminal domain with Rab5, and documented the effect of mutation of these residues on EEA1 localization and endosome fusion dynamics. The results shown here suggest a temporal order of events in which endosome tethering is mediated by the interaction of EEA1 with PtdIns-3-P and subsequent endosome fusion is dependent on the interaction of EEA1 with Rab5.

MATERIALS AND METHODS

Plasmids and Recombinant Proteins—Gst1277 was generated as described previously (6). Site-directed mutations within this plasmid were generated by polymerase chain reaction using Vent Polymerase (New England Biolabs), and the resulting mutants were sequenced for verification. 10XHis-Rab5c and Gst-Rab5c plasmids and purified recombinant protein production have been described previously (9). Both G FP-Gab1 and GFP-Rab5Q79L was generated by polymerase chain reaction from either wild-type or double-mutated Gst1277 and cloned in frame into the SalI-BamHI sites in pEGFP-C1 (CLONTECH). GFP-Rab5Q79L was generated by polymerase chain reaction from the full-length cDNA (a gift from D. Lambright) and cloned in frame into the SalI-BamHI sites in pEGFP-C1 (CLONTECH). Rab5c was generated by subcloning into the EcoRI-SalI sites in pDsRed1-C1 (CLONTECH). The Rab5c cDNA was generated as described elsewhere (6). The cDNA encoding GFP was subcloned into the EcoRI-XhoI sites 5' to full-length EEA1 cDNA to generate GFP-EEA1. Both EEA1dm and GFP-EEA1dm were constructed by insertion of nucleotides 3975–4233 PCRN.
amplified from GST1277dm into the Pet-BamHI sites of EEAIwt or GFP-EEAIwt, respectively.

**Cell Culture and Preparation of Cytosolic Extracts**—COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Life Technologies, Inc.). The cells were grown in 100-mm dishes and transfected using LipofectAMINE (Life Technologies, Inc.). To prepare cytosolic extracts from cells transfected with either GFP-1277wt or GFP-1277dm, 5 μg of GST-tagged fusion protein was bound to glutathione-Sepharose beads that had been pre-blocked in 5% nonfat dry milk in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20). The beads were then incubated with 100 μM GTPγS or GDPβS in buffer A (20 mM HEPES, pH 7.2, 100 mM NaCl, 0.5 mM MgCl2, 2 mM EDTA, and 1 mM dithiothreitol) at 30 °C for 30 min followed by the addition of MgCl2 to a final concentration of 20 mM. Beads were then washed and incubated with 2.5 μg of cytosolic extract for 1 h at 4 °C in a final volume of 300 μl of cytosol buffer containing 10 mg/ml bovine serum albumin and 0.1% Tween 20. Following centrifugation, the pellets were washed four times in 1 ml of buffer A, 20 mM MgCl2, 0.1% Tween 20. Bound proteins were resolved by SDS-PAGE and transferred to nitrocellulose membrane, which was blocked in 5% dry milk, TBST followed by incubation with anti-GFP monoclonal antibody (Zymed Laboratories Inc.). Filters were then incubated with horseradish peroxidase-conjugated goat anti-mouse antibody (Promega), which was detected by Renaissance enhanced luminal reagent (PerkinElmer Life Sciences).

Liposome binding assays were performed as described previously (6) with minor modification. PS/PI liposomes were composed of 50% phosphatidylserine and 50% phosphatidylinositol (Avanti), whereas PI3P liposomes contained 1% PtdIns-3-P (Matreya), 50% phosphatidylserine, and 50% phosphatidylinositol (Avanti), whereas PI3P (PerkinElmer Life Sciences). Liposome pellets were analyzed by SDS-PAGE and immunoblotted with an anti-GFP monoclonal antibody as described above.

**Fluorescence Microscopy**—COS-7 cells were grown to 40–50% confluence on coverslips and transfected using the calcium phosphate precipitation method. 24 h post-transfection, cells were either untreated oruntreated with 50 μM wortmannin for 10 min as indicated or incubated with Texas red-labeled transferrin (Molecular Probes) at a concentration of 25 μg/ml at 37 °C for the indicated time. Coverslips were then washed twice with cold phosphate-buffered saline (PBS), fixed in 4% formaldehyde/PBS for 10 min at 4 °C, and imaged using a conventional wide-field microscope fitted with a 60× or 100× Nikon plan-apo objective. Images were acquired with a cooled CCD camera (Hamamatsu) and MetaMorph software. Coverslips labeled with antibody were fixed as described above followed by permeabilization with 0.2% Triton X-100/PBS for 10 min at 4 °C. Cells were then blocked with 1% fetal bovine serum in PBS for 30 min at 4 °C and stained in the same buffer with a monoclonal antibody directed to the NH2-terminus of EEAI (Transduction Laboratories). Anti-mouse antibody coupled to fluorescein isothiocyanate was used to detect the primary antibody.

To capture the information contained within the whole three-dimensional volume of the cell, 18 optical sections at a resolution of 200 nm/pixel and spaced at 250 nm intervals were taken. To remove out-of-focus information, image restoration was performed using a iterative constrained deconvolution algorithm described previously (10). Morphometric analysis of endosomes was done on three-dimensional images. Prior to analysis of each three-dimensional image, a threshold intensity was set so that 33% of the light in the image would be above this threshold (this yielded a different threshold value for each three-dimensional image). All voxels (voxel = pixel3) below the threshold intensity were set to 0 (background). The light above threshold was considered the signal in the vesicles. A “vesicle” was defined as each group of contiguous, above threshold voxels.

**Live Cell Imaging**—For live cell imaging COS-7 cells were transfected with EGFFP-tagged constructs and after 24 h were imaged using high speed, three-dimensional microscopy (10–13). The microscope was configured to 133 nm/pixels using a 60× objective, and the laser illumination was configured to provide a 488 nm excitation wavelength with a flux on specimen of ~12 watts/cm2. Exposure times of 5 ms were used to acquire each of 18 optical sections, spaced by 250 nm. Each set of 18 optical sections was acquired in less than 1 s, allowing 20 ms for each 250-nm shift in focus. Stacks were acquired every 10 s for 20 continuous minutes. The haze originating from light sources outside the in-focus plane of the cell was reduced by image restoration. Stacks were projected into single two-dimensional images, which were concatenated into a QuickTime video format.

**RESULTS**

To dissect the specific structural requirements for the interaction of EEAI with Rab5, we built upon previous studies demonstrating that, whereas the FYVE domain of EEAI is necessary and sufficient for its interaction with PtdIns-3-P, the FYVE domain and an additional stretch of 30 amino acids NH2-terminal to the FYVE domain are necessary for Rab5 binding (9). The structure of this Rab5 binding region, a parallel coiled-coil homodimer depicted in Fig. 1A1, was derived from the crystal structure of the EEAI COOH-terminal region bound to inositol 1,3-diphosphate (28). This region contains three glutamine residues positioned on the outer surface of a helix, which may potentially contribute to the interaction of the COOH terminus of EEAI with Rab5. To test this, we intro-

**FIG. 1. Point mutation of the Rab5 binding region within the EEAI COOH terminus.** A, structure of amino acids 1320–1340 of EEAI based on the crystal structure of the homodimeric EEAI COOH-terminal region bound to Ina-1,3-P2, as described by Dumas et al. (28). B, GST fusion proteins of EEAI containing the sequence indicated by the amino acid residues were immobilized on glutathione-Sepharose beads. Beads were then incubated with 10XHis-Rab5 previously loaded with either GTPγS or GDPβS. Bound Rab5 was detected by Western blotting with an anti-His antibody.
bound to Sepharose beads and previously loaded with either GFP-1277wt or GFP-1277dm were incubated with GST-Rab5. GDP

Treatment with 50 nM wortmannin for 10 min. Cells were fixed in 4% formaldehyde and imaged using conventional wide-field fluorescence microscopy using a Nikon 100× objective. B, cytosolic extracts from cells transfected with either GFP-1277wt or GFP-1277dm were incubated with GST-Rab5 bound to Sepharose beads and previously loaded with either GTP·S or GTP·P (top) or with liposomes containing phosphatidylinositol (PI) or PtdIns-3-P (PI3P) (bottom); C, no liposome control. Bound GFP protein was detected by Western blotting with an anti-GFP antibody.

duced point mutations converting these glutamine residues, singly or in combination, to alanine and then examined the ability of the resulting mutants to interact with Rab5 in vitro. Mutation of glutamine 1328 to alanine substantially reduced, whereas mutation of glutamine 1338 virtually abolished, the interaction between the COOH terminus of EEA1 and Rab5. The combination of these two point mutations completely ablates Rab5 binding. Interestingly, mutation of glutamine 1335 significantly enhanced this interaction (Fig. 1B). None of the point mutations altered the function of the FYVE domain as assessed by liposome binding assays (not shown). Thus, these mutations result in a selective impairment of the interaction of EEA1 with Rab5.

To examine how the inability of EEA1 to bind Rab5 would influence its membrane localization, the subcellular localization of GFP-tagged constructs encoding the wild-type COOH terminus of EEA1 (GFP-1277wt) and the same construct harboring mutations of glutamines 1328 and 1338 to alanine (GFP-1277dm) were transfected into COS-7 cells and after 24 h were fixed before (left panels) or after (right panels) treatment with 50 nM wortmannin for 10 min. Cells were fixed in 4% formaldehyde and imaged using conventional wide-field fluorescence microscopy using a Nikon 100× objective. B, cytosolic extracts from cells transfected with either GFP-1277wt or GFP-1277dm were incubated with GST-Rab5 bound to Sepharose beads and previously loaded with either GTP·S or GTP·P (top) or with liposomes containing phosphatidylinositol (PI) or PtdIns-3-P (PI3P) (bottom); C, no liposome control. Bound GFP protein was detected by Western blotting with an anti-GFP antibody.

body to GFP. Although there was no difference in the ability of these constructs to associate with PtdIns-3-P liposomes in vitro, the mutant was deficient in Rab5 binding (Fig. 2B), indicating that the endosomal localization of the COOH terminus of EEA1 is mediated through its interaction with PtdIns-3-P and is independent of its interaction with Rab5. It is important to note, however, that the FYVE domain of EEA1 alone (amino acids 1336–1411) is not sufficient for endosome localization in vivo (9). This is most likely due to the inability of the FYVE domain to oligomerize rather than to specific loss of Rab5 binding. In support of this, sedimentation equilibrium experiments demonstrate that the FYVE domain alone (amino acids 1336–1411) is monomeric in solution, whereas the longer COOH-terminal construct, which contains a region of coiled-coil, forms dimers crucial for high affinity membrane binding (not illustrated).

It has been shown that overexpression of the EEA1 COOH terminus results in the formation of unusually small early endosomes and blocks endosome enlargement induced by the GTPase-deficient Rab5, Rab5Q79L (4). Membrane localization is required for this effect, as point mutation of the FYVE domain within this construct restores the Rab5Q79L-induced endosome enlargement (14). It has been suggested that EEA1 functions downstream of Rab5 in endosome fusion because of this effect. To examine the requirement for the EEA1-Rab5 association in this assay, RFP-tagged Rab5Q79L was co-transfected into COS-7 cells with either GFP-1277wt or GFP-1277dm. Both GFP proteins co-localized with Rab5Q79L on endosomal membranes (Fig. 3, left) and when overexpressed blocked the enlargement induced by Rab5Q79L (Fig. 3, right). This would indicate that the inhibitory effect of the EEA1 COOH terminus on Rab5-induced endosome enlargement oc-
Rab5 previously loaded with either GTP-Cytosolic extracts from COS-7 cells transfected with either GFP-EEA1wt or GFP-EEA1dm were incubated with GST-Rab5 previously loaded with either GTP-S or GDPβS bound to Sepharose beads. Bound GFP protein was detected by Western blotting with an anti-GFP antibody. The gray level of each band was determined after scanning using the histogram function in Adobe Photoshop 6.0.

curs independently of its direct interaction with Rab5 and is most likely due to the inability of these deletion constructs to allow endosome tethering to occur. Thus, although EEA1-mediated tethering is crucial for endosome fusion, this function of EEA1 is independent of its interaction with Rab5.

If both membrane localization and EEA1-mediated tethering are independent of Rab5 association, the physiological role of the interaction between EEA1 and Rab5 remains unknown. To attempt to elucidate this role, we examined the effects of overexpressing full-length wild-type (GFP-EEA1wt) or mutated (GFP-EEA1dm) EEA1 on endosome function in COS-7 cells. To confirm that the expressed proteins indeed differentially interact with Rab5, cytosolic extracts of cells expressing either construct were incubated with immobilized GST-Rab5. This experiment was necessary, as the NH2-terminal domain of EEA1 has been reported to be critical for binding Rab5 in vitro (3). However, our experiments indicate that the Q1328A and Q1338A mutation greatly impaired the binding of full-length EEA1 to Rab5 (Fig. 4). The small residual binding observed is likely to be because of the oligomerization of some of the mutant EEA1 with the wild-type endogenous molecule (15). Thus, the contribution of the NH2-terminus of EEA1 to Rab5 binding appears to be minimal in these experiments.

GFP-EEA1wt was localized mainly in single vesicular structures distributed throughout the cytoplasm, with an area of concentration toward the perinuclear region where larger, ring-like endosomes could be seen (Fig. 5, left panels). In contrast, GFP-EEA1dm was found in large clusters distributed throughout the cytoplasm (Fig. 5, right panels). These clusters represent tightly packed individual endosomes, clearly visible in the enlarged inset region. Similar endosome clustering has been observed in vitro under conditions in which SNARE priming is impaired (3). Thus, the accumulation of nonfused endosome clusters in cells overexpressing GFP-EEA1dm suggests a role for the interaction between EEA1 and Rab5 in the regulation of endosome fusion following tethering.

We next examined the dynamic behavior of endosomes in living cells expressing GFP fusion proteins of wild-type or mutant full-length EEA1. GFP-EEA1wt was present on endosomes of varying diameter, many of which had a characteristic ring-like appearance, probably because of their relatively large, spherical morphology combined with the optical properties of the wide-field microscope. These ring-like endosomes have been observed by immunofluorescence of endogenous EEA1 in several different cell types (16). The dynamic behavior of a group of such endosomes in a cell expressing GFP-EEA1wt is illustrated in Fig. 6A. In this sequence, three endosomes appear to come into close proximity with each other, form a tight cluster, and fuse into a single, larger structure. Comparison of the radius of the smaller and larger final endosomes indicates an increase consistent with the fusion of three smaller endosomes into one. In cells expressing GFP-EEA1dm, these fusion events were not observed. Instead, clusters of four or more large endosomes were abundant, and these failed to fuse within the imaging time frame (Fig. 6B). These results suggest that the inability of Rab5 to bind the mutant EEA1 protein results in a decrease in the efficiency of homotypic endosome fusion.

To further test this hypothesis, we analyzed the behavior of endosomes in cells co-expressing RFP-Rab5Q79L and either GFP-EEA1wt or GFP-EEA1dm. As expected, expression of RFP-Rab5Q79L resulted in the formation of enlarged endosomes, an effect that was significantly enhanced by co-expression of GFP-EEA1wt (Fig. 7, upper panels). In contrast, coexpression of GFP-EEA1dm blocked the endosome enlargement induced by RFP-Rab5Q79L (Fig. 7, lower panels). These results suggest that the direct binding of Rab5 to the COOH terminus of EEA1 is required for endosome fusion at a step subsequent to tethering.

To assess the functional consequences of the altered endosome fusion dynamics induced by overexpression of EEA1 constructs, the trafficking of Texas red-transferrin was analyzed (Fig. 8). When nontransfected cells or cells expressing GFP-EEA1wt were incubated with Texas red-transferrin for 40 min, intense transferrin fluorescence was detected in a tight pericentriolar region, which represents the recycling endosome. When the intensity scale was set so as not to saturate the signal in the pericentriolar region, the transferrin signal was virtually undetectable in the GFP-containing endosomes. In contrast, after a similar uptake period, the transferrin signal detected in the pericentriolar region of cells expressing GFP-EEA1dm was comparable with that detected in GFP-containing endosomes, indicating a delay in traffic to the pericentriolar recycling endosome. In cells expressing GFP-1277wt, in which endosome tethering is impaired, little transferrin signal was detected in the recycling endosome after a 40-min uptake, and extensive co-localization of Texas red-transferrin and GFP-1277wt was observed. These results suggest that the transit of transferrin through the early endosome to the recycling endosome is facilitated by EEA1-mediated endosome tethering and by fusion events that require the direct interaction between EEA1 and Rab5.
and upper middle corners of the cell can be seen spaced by 10 s and comprising a larger segment of the cell can be seen as QuickTime videos (Videos 1 and 2 in Supplemental Material). Rather, endosomes seemed to remain tethered for long periods in seven movies obtained from independent coverslips, in which no single, large lumen was observed. Thus, the introduction of the GFP tag on the EEA1 NH2-terminus appears not to alter its function measurably. Furthermore, these results support the hypothesis that the accumulation of vesicle clusters is due to the impairment of EEA1 binding to Rab5.

**DISCUSSION**

The process of endocytosis in eukaryotic cells plays a fundamental role in the control of cellular homeostasis. Through endocytosis, cells accumulate critical nutrients and regulate the surface complement of receptors. Thus, understanding the molecular basis of the endocytic process has been an important goal for cell biologists for many years. In vitro assays that measure the ability of endosomes containing exogenously added labels to fuse with each other have been used extensively to identify and characterize key elements involved in endosome fusion. Using these assays, the activities of PI3-kinase and of the GTPase Rab5 were found to be essential for endosome fusion in vitro (17–19). The discovery that EEA1 can interact with both PtdIns-3-P, a product of PI3-kinase, and activated Rab5 suggested that it may be an effector protein for these regulatory molecules. A model consistent with available results has been proposed in which PtdIns-3-P and Rab5 function cooperatively to recruit EEA1 to early endosomes (3, 4), where EEA1 then interacts with SNARE proteins to mediate endosome fusion (20, 21). The goal of the present work was to test this model and to more precisely define the respective roles of the interactions between EEA1 and PtdIns-3-P and EEA1 and Rab5 in endosome function. The results shown here indicate that the interaction of EEA1 with PtdIns-3-P is necessary and sufficient for binding to endosomal membranes. Once bound, EEA1 may serve to link Rab5 activity to the machinery that regulates homotypic endosome fusion.

**FIG. 8. Transferrin uptake in cells expressing GFP constructs of wild-type and mutant EEA1.** After 24 h of transfection with the indicated constructs, cells were incubated with Texas red-labeled transferrin for 40 min, washed three times in PBS, fixed, and imaged as described in the legend for Fig. 5. Shown are superimposed images obtained in the red and green channels.

To rule out that any of the observed phenotypes were due to the insertion of a GFP-tag, cells expressing nontagged full-length EEA1wt and EEA1dm were analyzed. Transfected cells could be identified by the increase in fluorescence intensity after staining with anti-EEA1 antibody. Neither the number nor the size distribution of endosomal structures in cells expressing EEA1wt at up to 15 times over the endogenous level (Fig. 9) differed appreciably from nontransfected cells. Cells overexpressing EEA1dm at similar levels (5–15 times over endogenous level) differed from those overexpressing EEA1wt in that enlarged particles were observed throughout the cytoplasm (Fig. 9B). These particles represent endosome clusters, as no single, large lumen was observed. Thus, the introduction of the GFP tag on the EEA1 NH2-terminus appears not to alter its function measurably. Furthermore, these results support the hypothesis that the accumulation of vesicle clusters is due to the impairment of EEA1 binding to Rab5.

**FIG. 7. Rab5Q79L-induced endosome enlargement is enhanced by wild-type but suppressed by mutant full-length GFP-EEA1 constructs.** COS-7 cells were co-transfected with RFP-Rab5Q79L and either GFP-EEA1wt or GFP-EEA1dm and fixed after 24 h. The fluorescence contained within the whole three-dimensional volume of the cell was acquired as described under “Materials and Methods.” Enlarged endosomes were seen in cells expressing RFP-Rab5Q79L alone (left upper and lower panels) and in cells co-expressing RFP-Rab5Q79L and GFP-EEA1wt at any level of expression (upper middle and right panels). In contrast, enlarged endosomes were not seen in cells co-expressing RFP-Rab5Q79L and GFP-EEA1dm, despite the presence of sufficient RFP-Rab5Q79L to cause endosome enlargement (lower middle and right panels). Fluorescence intensity values for each image in the red and green channels are depicted by the numbers in the bottom left and right corners of each image, respectively.

**FIG. 6. Endosome dynamics in live cells expressing GFP-tagged full-length wild-type and mutant EEA1.** COS-7 cells expressing GFP-EEA1wt (A) or GFP-EEA1dm (B) were imaged using high speed, three-dimensional microscopy (See “Materials and Methods”). Shown are projections of all 18 optical sections projected into a single two-dimensional image plane depicting an area containing clusters of ring-like endosomes. The asterisks were placed in the lumen of contiguous endosomes. In cells expressing GFP-EEA1wt, contiguous endosomes were seen to fuse, in this case, within 1 min after tethering, resulting in a single, larger endosome. These homotypic fusion events were observed at a rate of approximately two events within a 5-min period in seven movies obtained from independent coverslips, in which ~20% of the cell area was visualized. In contrast, fusion events were never observed within similar time periods in cells expressing GFP-EEA1dm. Rather, endosomes seemed to remain tethered for long periods of time and subsequently separated. A full set of 100 projections spaced by 10 s and comprising a larger segment of the cell can be seen as QuickTime videos (Videos 1 and 2 in Supplemental Material). Scale bar = 1 μm.

**FIG. 5.** Shown are superimposed images obtained in the red and green channels.
EEA1 bound immobilized Rab5, EEA1 constructs containing two point mutations adjacent to the FYVE domain did not (Fig. 4). Thus, the physiological significance of the in vitro interaction of the NH$_2$ terminus of EEA1 with Rab5 remains to be determined.

Despite the failure of mutant EEA1 constructs to interact with Rab5, these constructs bound to endosomes to an extent indistinguishable from the wild-type constructs and in a wortmannin-sensitive manner. The most logical interpretation of these data is that the interaction of EEA1 with Rab5 is not required for the initial binding of EEA1 to early endosome membranes. What then is the role for the interaction between EEA1 and Rab5? Results shown here suggest that this interaction is required to regulate homotypic endosome fusion. Two lines of evidence support this hypothesis. First, endosomes in cells expressing the full-length mutant are found frequently in clusters, reminiscent of the clustered, unfused structures seen in yeast upon disruption of Vps9p (22, 23), a guanine-nucleotide exchange factor for Vps21p, which is a homologue of Rab5 (24). Second, the potent effect of persistently active mutants of Rab5 to accumulate large endosomes is impaired in cells expressing the full-length EEA1 mutant that cannot bind Rab5. These experiments suggest that Rab5 cooperates with EEA1 in the regulation of endosome fusion after EEA1 has bound. This model is consistent with genetic evidence in yeast for the molecular organization of an analogous pathway; Vac1p, the yeast orthologue of EEA1/Rabenosyn5, associates with the Rab5 homologue Vps21p and with Vps45p, a Sec1 homologue that binds and regulates the activity of the yeast SNARE Pep12p. In this system, the requirement for the GTPase is subsequent to membrane tethering.

The results presented here also raise a number of important issues. One in particular is that endosomes from cells expressing wild-type, mutant, or truncated EEA1, although morphologically distinct, all accumulate transferrin. Thus, the traffic of transferrin receptor from the plasma membrane to early endosomes that contain EEA1 does not appear to depend on the function of EEA1. The only consequence of disrupted EEA1 function on transferrin traffic appears to be a delay in the movement of transferrin from early endosomes into the perinuclear recycling compartment. The finding that transferrin traffic into endosomes occurs independently of EEA1 function is consistent with the inability of wortmannin, a potent PI3-kinase inhibitor, to inhibit transferrin uptake (25, 26). Thus, much more work is required to elucidate the mechanisms that control the vectorial delivery of plasma membrane-derived vesicles to endosomes.

In conclusion, the results presented here, derived from experiments in intact cells, suggest a model for the spatial and temporal order of key biochemical events in early endosome fusion. In this model, tethering and fusion are regulated by the sequential interaction of EEA1 with PtdIns-3-P, to mediate tethering, and with Rab5, to mediate fusion. The requirement for tethering factors acting in conjunction with Rab GTPases in other membrane transport steps (27) suggests that interactions similar to those presented here may temporally and spatially control multiple intracellular trafficking pathways.

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