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Selective Membrane Recruitment of EEA1 Suggests a Role in Directional Transport of Clathrin-coated Vesicles to Early Endosomes*

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The molecular mechanisms ensuring directionality of endocytic membrane trafficking between transport vesicles and target organelles still remain poorly characterized. We have been investigating the function of the small GTPase Rab5 in early endocytic transport. *In vitro* studies have demonstrated a role of Rab5 in two membrane fusion events: the heterotypic fusion between plasma membrane-derived clathrin-coated vesicles (CCVs) and early endosomes and in the homotypic fusion between early endosomes. Several Rab5 effectors are required in homotypic endosome fusion, including EEA1, which mediates endosome membrane docking, as well as Rabaptin-5·Rabex-5 complex and phosphatidylinositol 3-kinase hVPS34. In this study we have examined the localization and function of Rab5 and its effectors in heterotypic fusion *in vitro*. We report that the presence of active Rab5 is necessary on both CCVs and early endosomes for a heterotypic fusion event to occur. This process requires EEA1 in addition to the Rabaptin-5 complex. However, whereas Rab5 and Rabaptin-5 are symmetrically distributed between CCVs and early endosomes, EEA1 is recruited selectively onto the membrane of early endosomes. Our results suggest that EEA1 is a tethering molecule that provides directionality to vesicular transport from the plasma membrane to the early endosomes.

Vesicular transport between membrane compartments requires high specificity and tight regulation to deliver cargo molecules to the correct acceptor organelle and maintain the integrity of distinct compartments. To ensure the proper directionality of membrane flow, the target organelle must possess appropriate molecular machinery allowing for specific recognition and docking of the incoming vesicle. The binding of membrane proteins of the transport vesicle, v-SNAREs,[1] with membrane proteins of the target organelle, t-SNAREs, was initially proposed to be the central principle underlying vesicle targeting as well as membrane fusion (1–3). It has become apparent, however, that v- and t-SNAREs (referred also as R- and Q-SNARES; Ref. 4) do not display a tight segregation between vesicle and target membranes because both can be incorporated in transport vesicles and cycle between organelles (5, 6). Moreover, SNARE proteins can pair promiscuously and mediate multiple transport events (7–9). Thus, SNARE pairing must be subjected to yet another layer of regulation to maintain the fidelity and vectoriality of vesicular transport. This function appears to be provided by the Rab family of small GTPases through their downstream effectors (10–14).

We have been investigating the function of Rab5 in early endocytic trafficking. Rab5 plays a role in the formation of clathrin-coated vesicles (CCVs) at the plasma membrane (15), their subsequent fusion with early endosomes (EE), in the homotypic fusion between EE (16, 17), and in the interaction of EE with microtubules (18). In accordance with this functional diversity, Rab5 lies at the center of a complex machinery comprising several effector proteins (13). Among them, the Rabaptin-5·Rabex-5 complex activates and stabilizes Rab5 in the GTP-bound conformation (19, 20). Rab5 effectors can efficiently replace cytosol in homotypic EE fusion *in vitro* (13). Of these proteins, EEA1 was identified as a core component of the homotypic endosome docking and fusion machinery and was shown to play a role in the docking/tethering of endosome membranes (13). The membrane recruitment of EEA1 depends on Rab5-GTP as well as phosphatidylinositol 3-phosphate (PI(3)P) (21–23). hVPS34, the phosphatidylinositol 3-kinase (PI3-K) that specifically produces PI(3)P is also a Rab5 effector, suggesting that this interaction ensures the spatial and temporal coordination of the two binding sites for EEA1 on the endosome membrane (24). EEA1 is predominantly localized to the early endosomes and is regarded as a specific marker of this compartment (25). Because of this localization and given its function in endosome membrane docking (13), it has been proposed that EEA1 may confer directionality to Rab5-dependent vesicular transport to the early endosomes. It has not yet been determined, however, whether the same molecular machinery that regulates homotypic EE fusion, and most notably EEA1, also controls the heterotypic CCV-EE fusion. It also remains to be established whether the presence of Rab5 and its effectors is necessary on both membranes undergoing heterotypic fusion or, alternatively, whether these components are asymmetrically distributed. Clearly, in homotypic EE fusion Rab proteins, their effectors, and SNAREs are evenly distributed between the fusion partners, rendering the study of directionality of membrane transport difficult. To address these questions, we have therefore examined the requirement for Rab5 and the

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[1] The abbreviations used are: SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; CCV, clathrin-coated vesicle; EE, early endosome; PI(3)P, phosphatidylinositol-3-phosphate; PI3-K, phosphatidylinositol 3-kinase; RabGDI, Rab GDP dissociation inhibitor; XTPγS, xanthosine 5′-*O*-(γ-3-thiotriphosphate); REP, Rab escort protein; NSF, *N*-ethylmaleimide-sensitive factor; MES, 4-morpholineethanesulfonic acid.

membrane recruitment of the Rab5 effectors Rabaptin-5 and EEA1 in heterotypic CCV-EE fusion *in vitro*.

EXPERIMENTAL PROCEDURES

Membrane Preparations, Fusion, and Recruitment Assays—CCVs labeled with biotinylated transferrin were prepared from HeLa cells by a modification of the procedure described by Woodman and Warren (26). Briefly, 2–4 × 10^{10} HeLa cells in suspension were concentrated about 20 times, washed twice in phosphate-buffered saline at room temperature and incubated in a serum-free medium containing 50 μg/ml biotinylated transferrin for 3 min at 37 °C. The internalization was stopped by washing the cells twice in ice-cold vesicle buffer (140 mM sucrose, 70 mM potassium acetate, 20 mM MES-KOH, pH 6.6, 1 mM EGTA, 0.5 mM magnesium acetate). The cell pellet was then resuspended in two cell volumes of hypotonic buffer (10 mM MES-KOH, pH 6.6, 1 mM EGTA, 0.5 mM magnesium acetate, 1 mM dithiothreitol) and homogenized with a potter homogenizer in the presence of protease inhibitors (1 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride). A postnuclear supernatant was prepared by centrifugation at 500 × *g* for 10 min and incubated with 0.1 mg/ml ribonuclease type A for 20 min at room temperature. Subsequent purification steps were performed according to the original protocol.

EE labeled with anti-transferrin antibody were prepared as described (16), and standard *in vitro* fusion assays were performed as described (20). Preloading of EE or CCV with Rab5-XTPγS was done by incubating the membranes with 180 nM REP-1·His-Rab5D136N complex and 1 mM XTPγS for 5 min at 37 °C in the absence of ATP-regenerating system. Membrane recruitment assays were performed essentially as described (24) using EE from HeLa cells and CCVs from human placenta purified according to Steel *et al*. (27) except for the recovery of membranes by ultracentrifugation which was done using a 10% sucrose cushion.

Others—The following procedures were previously described: purification of RabGDI from bovine brain cytosol (20); purification of His-RabGDI from *Escherichia coli* and preparation of Rab5-RabGDI complex (28); preparation of REP-1·His-Rab5D136N complex (29); synthesis of XTPγS (30); and the immunodepletion of EEA1 (23) or Rabaptin5·Rabex5 complex (20) from HeLa cytosol. The rabbit affinity-purified anti-EEA1 antibodies (23) used in the fusion assays were a kind gift of Dr. H. Stenmark.

RESULTS AND DISCUSSION

To investigate the recruitment of Rab5 effectors onto CCVs and EE, we first tested whether the presence of active Rab5 is necessary on both membrane partners undergoing heterotypic fusion. For this purpose, we took advantage of an *in vitro* assay measuring fusion between CCVs containing internalized biotinylated transferrin (donor membranes) and EE labeled with anti-transferrin antibody (acceptor membranes) (20). Fusion signals were quantified as percentage of the basal reaction (Fig. 1, *lane 2*) performed in the presence of 3 mg/ml cytosol and ATP-regenerating system. Omission of cytosol (Fig. 1, *lane 1*) or ATP-regenerating system (*lane 3*) markedly reduced fusion, demonstrating that this process depends on the supply of soluble proteins and energy. To test for Rab5 requirement, we employed the Rab5D136N mutant that binds xanthosine nucleotides with high affinity (31). Because Rab GDP dissociation inhibitor (RabGDI) extracts endogenous Rab proteins from the membrane in the GDP-bound form, this particular mutant can be rendered resistant to extraction by RabGDI in the presence of XTPγS (29). Therefore this system allowed us to test the activity of Rab5D136N in endocytic membrane fusion in the absence of endogenous functional Rab5. Donor CCVs and acceptor EE were separately pre-loaded with Rab5D136N upon delivery through its chaperone Rab escort protein-1 (REP-1; Ref. 32) in the presence of XTPγS. This pre-incubation step was performed in the absence of ATP-regenerating system to prevent membrane fusion. Subsequently, donor and acceptor membranes were combined and allowed to fuse under standard conditions, in the absence (Fig. 1, *lane 4*) or presence of 0.5 μM RabGDI to remove endogenous Rab5 (Fig. 1, *lanes 5–8*) as well as other Rab proteins (33, 34). Preincubation of both fusing partners with Rab5D136N·XTPγS in the absence of RabGDI (Fig. 1, *lane 4*) caused a more than 3-fold increase in the fusion efficiency, thus indicating that the mutant protein is functionally active. Consistently, removal of Rab proteins by RabGDI completely inhibited the fusion of CCVs and EE (Fig. 1, *lane 5*). The pretreatment of one set of membranes (either donor or acceptor) with Rab5D136N·XTPγS failed to restore membrane fusion in the presence of RabGDI (Fig. 1, *lanes 6* and *7*, respectively). Only when both membranes were preloaded with Rab5D136N·XTPγS, was the RabGDI-mediated inhibition of CCV-EE fusion rescued (Fig. 1, *lane 8*). The level of rescued fusion did not reach the efficiency observed upon Rab5D136N stimulation in the absence of RabGDI (Fig. 1, *lane 4*) because of the removal of endogenous Rab5. These results indicate that active Rab5 is necessary on both membrane partners in the heterotypic CCV-EE fusion, as previously determined for the homotypic fusion between early endosomes (35).[2]

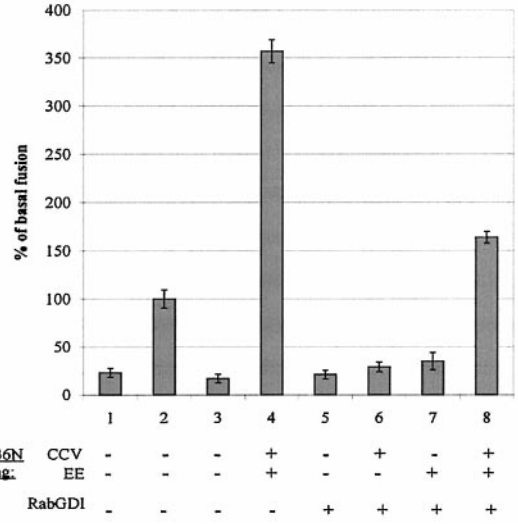

FIG. 1. **Requirement for Rab5 on both membrane sides in the heterotypic CCV-EE fusion.** Donor CCV and acceptor EE were separately preloaded with Rab5D136N-XTPγS, as indicated on the graph and subsequently allowed to fuse in the presence of 3 mg/ml cytosol with (*lanes 5–8*) or without (*lane 4*) addition of 0.5 μM bovine brain RabGDI. The fusion values are expressed as a percentage of the basal reaction (*lane 2*) performed with untreated membranes, 3 mg/ml cytosol, and ATP-regenerating system in the absence of RabGDI. *Lanes 1* and *3* show the extent of fusion of untreated membranes in the absence of cytosol and ATP-regenerating system, respectively.

The requirement for Rab5 on both CCV and EE raises the question of whether the Rab5 effectors which function in homotypic EE fusion (13, 20, 24) are also symmetrically distributed between the donor CCV and acceptor EE membranes. We first examined the requirement for EEA1 in the heterotypic CCV-EE fusion. When the fusion reaction was conducted in the presence of anti-EEA1 antibodies a 60% reduction of the fusion signal was observed compared with the basal reaction (Fig. 2). This inhibition was specific as preimmune serum did not significantly affect the reaction. Do the antibodies inhibit the membrane-bound pool of EEA1 or the cytosolic fraction that is recruited on the endosome membrane? This question was addressed in a second set of experiments where EEA1 was depleted from cytosol using anti-EEA1 antibodies or preimmune serum prior to adding it to the assay. As shown in Fig. 2, cytosol depleted for EEA1 did not support CCV-EE fusion, in contrast to the cytosol treated with preimmune serum. These data establish the requirement for EEA1 in the heterotypic fusion

[2] R. Lippé and M. Zerial, unpublished data.

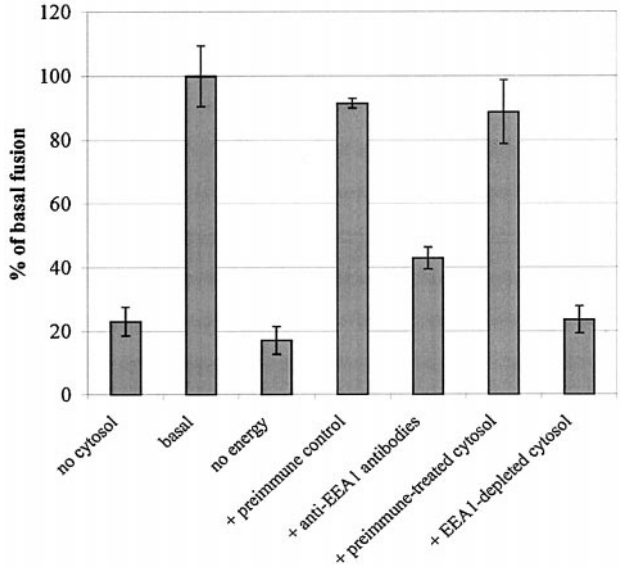

FIG. 2. **Requirement for EEA1 in the CCV-EE fusion.** Fusion assay was performed under standard conditions either with an addition of 1 μl of affinity purified anti-EEA1 antibodies or 1 μl of preimmune serum (in the presence of 3 mg/ml of untreated cytosol) or in the presence of EEA1-depleted cytosol or control cytosol treated with preimmune serum at final concentration of 3 mg/ml, as indicated on the graph. Fusion values are expressed as a percentage of the basal reaction done in the presence of untreated cytosol and ATP-regenerating system.

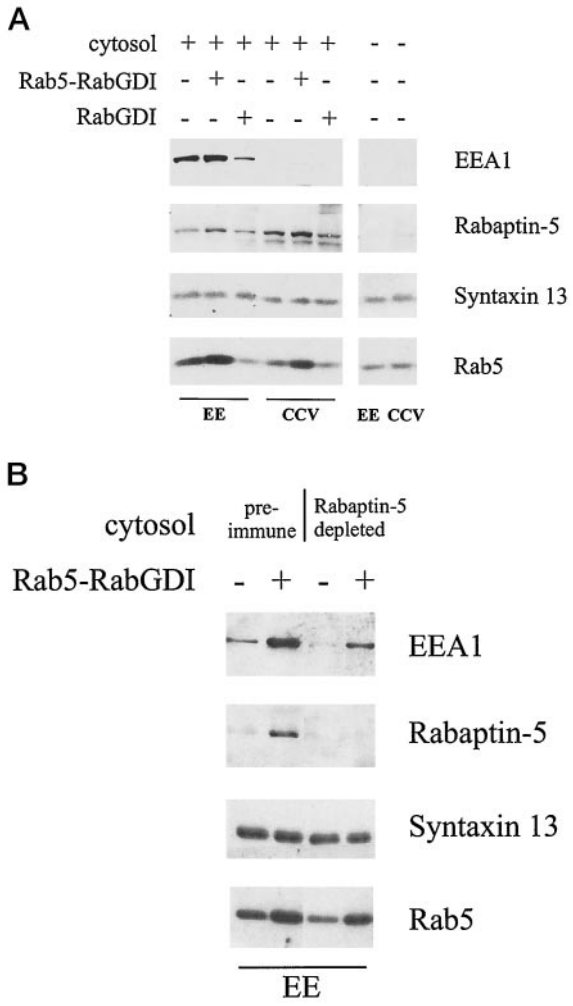

FIG. 3. **Recruitment of Rabaptin-5 and EEA1 on EE and CCVs.** *A*, aliquots of EE and CCVs were incubated for 30 min at 37 °C with 3 mg/ml of cytosol and ATP-regenerating system either without further additions or in the presence of 100 nM Rab5-RabGDI complex or with addition of 5 μM His-RabGDI, as indicated. To control for the endogenous levels of Rabaptin and EEA1, membranes were incubated under the assay condition without cytosol (*right panels*). *B*, aliquots of EE were incubated with 3 mg/ml Rabaptin-5·Rabex-5 depleted cytosol or cytosol treated with preimmune serum in the presence or absence of 100 nM Rab5-RabGDI complex, as indicated. The recruitment of Rab5 effectors was analyzed by Western blotting using anti-Rab5, anti-Rabaptin-5, and anti-EEA1 antibodies. The recovery of membranes in each lane was monitored using syntaxin 13 as a membrane marker.

between EE and CCVs and, more importantly, demonstrate that the cytosolic pool of EEA1 is required for the fusion reaction.

Given that the association of EEA1 with the membrane is necessary for the homotypic fusion between EE (23, 36), the data presented so far argue that the heterotypic fusion reaction requires the translocation of EEA1 from cytosol to the membrane. Does the distribution of EEA1 between EE and CCV reflect the symmetrical localization of Rab5? Given the dynamic equilibrium between cytosolic- and membrane-bound pools, it is necessary to examine the membrane recruitment of Rab5 effectors rather than their steady-state distribution in sub-cellular fractions. For this purpose, we tested the ability of CCV and EE to recruit cytosolic EEA1 and Rabaptin-5 *in vitro*. Aliquots of EE or CCVs were incubated with cytosol under standard fusion assay conditions. Subsequently, the membranes were recovered by centrifugation through a sucrose cushion and the presence of bound proteins was detected by immunoblotting. The recovery of membranes was controlled by monitoring the presence of an endocytic t-SNARE, syntaxin 13 (14, 37, 38). As a further control, EE and CCV membranes were incubated under the assay conditions without cytosol or any reagent, and reisolated by ultracentrifugation. Under the experimental conditions used, EEA1 and Rabaptin-5 were hardly detectable in the pelleted membranes (Fig. 3*A*, *right panels*), indicating that these proteins are not stably associated with, but translocate to, the membranes during the incubation with cytosol. As shown in Fig. 3*A*, the binding pattern of the two Rab5 effectors was clearly different. Rabaptin-5 was efficiently recruited from cytosol onto both CCV and EE membranes, whereas EEA1 was found associated preferentially with EE with only background amounts detected on CCVs. The membrane association was modulated by Rab5 as recruitment of Rabaptin-5 and EEA1 on the endosome membranes was inhibited by addition of RabGDI and stimulated by Rab5-RabGDI complex. However, even upon loading CCVs with exogenous Rab5, EEA1 failed to be efficiently recruited onto these membranes. Moreover, the recruitment of Rabaptin-5 and EEA1 onto EE seemed to be functionally coupled. When Rabaptin-5 and Rabex-5 were immunodepleted from cytosol, the efficiency of EEA1 recruitment onto EE was decreased (Fig. 3*B*). Removal of Rabaptin-5·Rabex-5 complex from cytosol also did not enable the EEA1 binding to CCVs (data not shown), ruling out the possibility that the Rabaptin-5·Rabex-5 complex competes with EEA1 for Rab5 binding on the vesicles. These results therefore establish that EEA1 is primarily, if not exclusively, recruited on the acceptor organelle in the heterotypic CCV-EE fusion.

EEA1 is a core component of the early endocytic transport machinery. It is required for docking and, subsequently, for fusion between EE. Morphological studies have shown that this protein is absent from the plasma membrane (25), and our results demonstrate that it is unable to associate with CCVs *in vitro*. This is in contrast to the broad distribution of SNAREs, and in particular of syntaxin 13, which functions in endosome fusion (14) and is also present on CCVs (Fig. 3*A*). Based on previous data and the results reported here, we propose that EEA1 specifies the target compartment in the heterotypic CCV-EE fusion and thus confers directionality to vesicular

transport from the plasma membrane to the early endosome. In this respect the function of EEA1 would resemble that of other proposed docking factors such as the Exocyst and TRAPP complexes that specify the target compartments in the secretory vesicle to plasma membrane and ER to Golgi transport pathways in yeast, respectively (39–41). It is also possible that other Rab5 effectors may participate with EEA1 in the vesicle targeting process. What determines the asymmetrical distribution of EEA1? EEA1 has two Rab5-binding sites. The first is located in the N terminus and involves the C_2H_2 zinc-finger motif, and the second is in the C terminus, immediately adjacent to the FYVE finger (21, 23, 25). Rab5 alone is not sufficient for the membrane recruitment of EEA1, given that even an excess of Rab5 on CCVs cannot recruit EEA1 to this membrane. Thus, neither the N-terminal nor C-terminal binding site alone can mediate a stable association of EEA1 with Rab5 on the membrane. Instead, what is critical for the association of EEA1 with the endosome membrane is the concomitant presence of both Rab5-GTP and PI(3)P (21, 23), which appear to cooperatively bind to the C terminus of EEA1 on the endosomal membrane. The recent finding that the PI3-K hVPS34 is a Rab5 effector explains how PI(3)P production can be coupled to Rab5 localization (24). However, hVPS34 seems also to be absent from CCVs (24). Lack of sufficient amounts of PI(3)P would explain why EEA1 cannot be recruited on the vesicle membrane. The mechanism responsible for the selective targeting of hVPS34 to the early endosome is unclear at present and most likely involves the accessory protein Vps15p/p150 (42, 43). Another possibility is that the clathrin coat could represent an additional mechanical barrier preventing EEA1 from binding to the vesicles. However, this is not very likely considering that, firstly, incubation of CCVs with cytosol and ATP-regenerating system results in the removal of coat, presumably because of the action of hsc70, the uncoating ATPase (44). Secondly, we show that another coiled-coil protein of a relatively large size such as Rabaptin-5 (19) can be efficiently recruited to the vesicles under the same conditions.

We have established that Rab5 must be present on both CCVs and EE membranes in heterotypic fusion. In the light of these findings, an attractive possibility is that while EEA1 is anchored to the endosome via its C terminus, the N terminus binds to Rab5 on the CCV, thus tethering the vesicle to the early endosome. Clearly, a single EEA1 dimer present in cytosol (45) cannot be recruited by Rab5 on the CCV (Fig. 3*A*). However, EEA1 is found in large oligomers together with Rabaptin-5 and NSF on the endosome membrane (14), and it is likely that PI(3)P binding is essential for this process. Upon incorporation into the oligomers, the high density of EEA1 molecules in the membrane may result in a sufficient number of low affinity binding sites which are then capable of stabilizing the interaction of the N terminus of EEA1 with Rab5 on the CCV membrane. This model also predicts that, because of the lack of recruitment of EEA1, CCVs should in principle be unable to undergo homotypic fusion, a process previously proposed to account for the biogenesis of the early endosomes (46). Finally, it will be interesting to examine the role of EEA1 and other Rab5 effectors in vesicle targeting in polarized cells such as epithelial cells and neurons. In these cells the endocytic circuits in each plasma membrane domain are operated by distinct machineries (47) while being nevertheless subjected to regulation by Rab5 GTPase (48, 49).

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