which upregulation of *Hip* by Hedgehog signals attenuates further Hedgehog signalling. Attenuation of signalling by physical binding of ligand may then allow differential responses to be generated within a field of competent cells. In *Drosophila*, a single *Hedgehog* gene mediates all Hedgehog signalling whereas in mammals three *Hedgehog* genes have largely non-overlapping activities. We have failed to identify a *Hip* orthologue in *Drosophila*. This suggests that in addition to diversification of Hedgehog signals, vertebrates may have evolved novel mechanisms of Hedgehog signal modulation that could facilitate distinct aspects of vertebrate development. □

Methods

Standard molecular biology and protein biochemistry techniques were performed as described[26].

Database searches. The Hip protein sequence was used to search the National Center for Biotechnology Information database using the BLAST program. Motif analysis of Hip and sequence alignments from Hip, Gene 5, 345 and pCZA361.11 were done with the GeneWorks program (IntelliGenetics). Transmembrane prediction was performed by SOSUI algorithm[27].

Epitope tagging, co-immunoprecipitation, endoglycosidase and PI-PLC treatment. To generate Myc–Hip, *Hip* cDNA was cloned into pcDNA3 (Invitrogen) and a Myc epitope was inserted between amino-acid residues 23 and 24. To generate Myc–HipΔC22, a stop codon was introduced 5′ to the last 22 residues in Myc–Hip. Shh-N::IgG was generated by cloning a DNA fragment encoding Shh-N into the pCD5IgG1 vector. To determine the localization of Myc–Hip and Myc-HipΔC22, COS-7 cells transfected with Myc–Hip or Myc–HipΔC22 using LipofectAMINE were collected and lysed 48 h after transfection. The supernatant was filtered and concentrated by precipitation with trichloroacetic acid (TCA). To immunoprecipitate Myc-HipΔC22, unconcentrated supernatant from COS-7 cells transfected with Myc–HipΔC22 was incubated with Shh-N::IgG bound to Protein A beads for 4 h at 4 °C. After extensive washing with buffer (25 mM HEPES (pH 7.6), 0.1 mM EDTA, 100 mM NaCl and 0.1% NP40), the beads were resuspended in sample buffer. Protein was run on SDS–PAGE and western blotting was done using anti-Myc antibody. To examine glycosylation of Hip, COS-7 cells transfected with Myc–Hip were lysed in RIPA buffer 48 h after transfection. The supernatant was mixed with an equal volume of buffer (20 mM sodium phosphate (pH 6.8), 40 mM EDTA, 0.1% SDS and 20 mM β-mercaptoethanol), boiled for 10 min and 0.4 U of endoglycosidase F (Boehringer Mannheim) was then added. The mixture was incubated at 37 °C for 24 h and analysed by western blotting using an anti-Myc antibody.

***In situ* hybridization.** Whole-mount *in situ* hybridization using digoxigenin-labelled probes and section *in situ* hybridization using ^{35}S-labelled probes were performed as described[28].

Generation of transgenic animals. To mis-express *Hip* under the α1(II) collagen promoter/enhancer, the full-length *Hip* cDNA was cloned into the α1(II) collagen expression vector. Transgenic animals were generated by pronuclear injection as described[29]. Staining of skeleton for bone and cartilage was as described[29].

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The Rab5 effector EEA1 is a core component of endosome docking

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Intracellular membrane docking and fusion requires the interplay between soluble factors and SNAREs. The SNARE hypothesis[1] postulates that pairing between a vesicular v-SNARE and a target membrane z-SNARE is the primary molecular interaction underlying the specificity of vesicle targeting as well as lipid bilayer fusion. This proposal is supported by recent studies using a minimal artificial system[2]. However, several observations demonstrate that SNAREs function at multiple transport steps and can pair promiscuously, questioning the role of SNAREs in conveying vesicle targeting[3–6]. Moreover, other proteins have been shown to be important in membrane docking or tethering[7–9]. Therefore, if the minimal machinery is defined as the set of proteins sufficient to reproduce *in vitro* the fidelity of vesicle targeting, docking and fusion as *in vivo*, then SNAREs are not sufficient to specify vesicle targeting. Endosome fusion also requires cytosolic factors and is regulated by the small GTPase Rab5 (refs 10–20). Here we show that Rab5-interacting soluble proteins can completely substitute for cytosol in an *in vivo*

endosome-fusion assay, and that the Rab5 effector EEA1 is the only factor necessary to confer minimal fusion activity. Rab5 and other associated proteins seem to act upstream of EEA1, implying that Rab5 effectors comprise both regulatory molecules and mechanical components of the membrane transport machinery. We further show that EEA1 mediates endosome docking and, together with SNAREs, leads to membrane fusion.

Rab proteins and their downstream effectors contribute to vesicle targeting specificity [10] and their delivery to the membrane has been proposed to regulate the assembly of SNARE complexes[11–14]. Early-endosome fusion requires cytosol, Rab5 (ref. 15), its effector Rabaptin-5 (refs 16, 17) and several cytosolic and peripheral membrane proteins[18–20], which modulate the membrane fusion activity. However, it is not clear to what extent these molecules alone can support endosome fusion. For example, the Rabaptin-5/Rabex-5 complex is necessary but not sufficient to replace cytosol in the fusion reaction[16].

To determine the minimal factors required for this reaction, we sought to purify all possible Rab5 effectors and determine their contribution to endosome docking and fusion. For this we used an affinity-chromatography approach. We found that 22 proteins from bovine brain cytosol bound specifically to immobilized glutathione *S*-transferase (GST)–Rab5 (Fig. 1a). This specificity is apparent from five lines of evidence. (1) Most proteins selectively bound the GTP-γS form of Rab5. Only two proteins were enriched in the Rab5:GDP eluate and, owing to incomplete nucleotide exchange, these were also present in the Rab5:GTP-γS column. (2) Immobilized GST alone (Fig. 1a) or GST–Rab4 (not shown) yielded a distinct protein pattern. (3) As expected, we purified the previously described Rab5 effectors, Rabaptin-5 and Rabaptin-5β, their associated co-factor Rabex-5, and identified EEA1, as described recently[18]. (4) Abundant cytosolic proteins such as actin or XMAP215 were not detected in the eluate (not shown). (5) These proteins displayed activity in endosome fusion, as we show later.

We next tested whether the eluate of the Rab5 affinity column could functionally substitute for cytosol in the *in vitro* assay of homotypic endosome fusion. Basal fusion reactions were performed with cytosol at a final concentration of 3 mg ml^{-1}, which corresponds to 80% of the maximal fusion efficiency obtained at saturating cytosol concentration (5 mg ml^{-1}). Whereas the GST–Rab5:GDP-column eluate or a control protein (GST, not shown) yielded only background fusion, the eluate of the GST–Rab5:GTP-γS column efficiently supported endosome fusion (Fig. 1b). Remarkably, 0.4 μg of the eluate, in which the known Rab5 effectors (EEA1, Rabaptin-5 and Rabex-5) were present at a concentration similar to cytosol (data not shown), was able to support fusion to cytosolic levels. Moreover, the eluate stimulated fusion in a concentration-dependent manner, reaching 50% of the maximal fusion signal as measured by solubilization of the membranes with detergent. It should be noted that *N*-ethylmaleimide-sensitive fusion protein (NSF), which is necessary for endosome fusion[21,22] was present on the membrane (not shown). These results indicate that the Rab5-interacting proteins can account for the cytosolic requirement in endosome docking and fusion.

Given the multitude of polypeptides eluted from the Rab5:GTP-γS column, we investigated their relative contribution in the *in vitro* assay. We subjected the eluted proteins to further separation by Superose-6 gel-filtration chromatography (Fig. 2a). Judging from the protein elution profile, the sum of fractions 19, 27, 32, 36 and 39 comprises the entire polypeptide composition of the column eluate. These fractions were tested in early-endosome fusion either

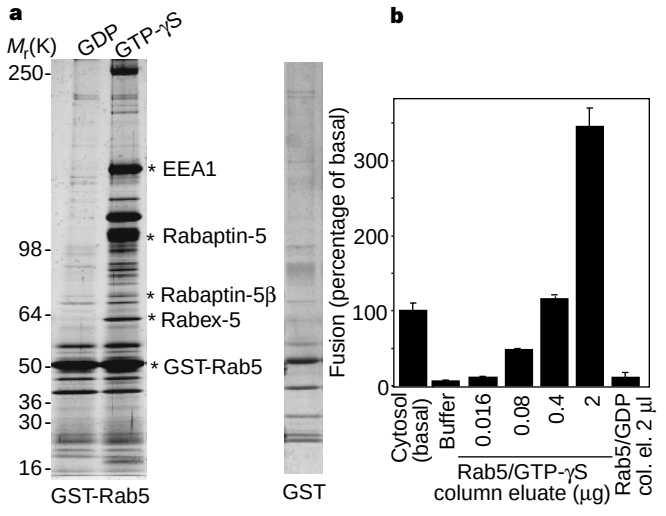

Figure 1 Twenty-two cytosolic proteins bind to Rab5 and can substitute cytosol in endosome fusion. **a**, Left panel: GST–Rab5:GDP and GST–Rab5:GTP-γS affinity columns were incubated with bovine brain cytosol and bound proteins were eluted and analysed by gradient SDS–polyacrylamide gel electrophoresis (PAGE) (6–17%) followed by silver staining. Molecular mass standards are indicated on the left side of the gel. EEA1, Rabaptin-5, Rabaptin-5β, Rabex-5 and Rab5:GST are all indicated, as identified by western blot and microsequencing (not shown). Right panel: GST column was treated as described above for GST–Rab5. **b**, The endosome homotypic fusion assay was done with 3 mg ml^{-1} cytosol (basal), buffer, increasing concentration of Rab5:GTP-γS column eluate or Rab5:GDP column eluate.

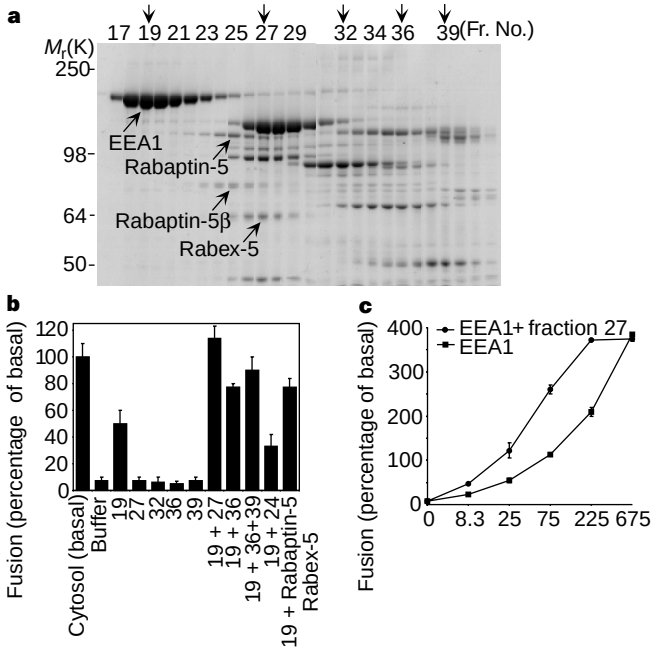

Figure 2 Fractionated eluate reveals a core activity of EEA1, regulated by the other effectors. **a**, The mixture of the Rab5 effectors was separated on a Superose 6 gel filtration column. Fractions eluted from the column were analysed by SDS–PAGE followed by Coommasie staining. Fractions 19, 27, 32, 36 and 39 (top arrows) were tested in the endosome fusion assay (see below). Molecular mass standards are indicated on the left. Arrows mark the position of EEA1, Rabaptin-5, Rabaptin-5β and Rabex-5 identified by western blotting (not shown). **b**, Early-endosome fusion assay done in the presence of cytosol (basal) or the reagents indicated. Fraction 19 was added to 25 nM final concentration of EEA1 in the fusion reaction. Fraction 27 was added in amounts (3 μl) that give a final concentration of Rabaptin-5 equal to that obtained by the addition of cytosol. The other fractions were added at equal volumes to fraction 27. Rabaptin-5/Rabex-5 complex was added at a concentration similar to that of fraction 27 (20 nM). **c**, *In vitro* fusion between early endosomes was done in the presence or absence of fraction 27 with increasing amounts of EEA1. Fraction 27 was added at a final concentration of Rabaptin-5 equal to that found in cytosol (3 μl). Fusion obtained with 3 mg ml^{-1} cytosol is normalized to 100 %.

individually or in combination. Fractions 27, 32, 36 and 39 could not support endosome fusion (Fig. 2b). Unexpectedly, fraction 19, which contained almost exclusively a single protein of relative molecular mass 170,000 (M_r 170K), yielded significant fusion activity. We identified this protein as EEA1 by western blotting and mass spectrometry analysis (not shown).

When the endosomes were supplied with EEA1 at endogenous concentrations, the efficiency of membrane fusion was lower than that obtained in the presence of cytosol. However, a combination of EEA1 and fraction 27 (containing Rabaptin-5, Rabaptin-5β, Rabex-5 and other proteins) resulted in full recovery of the fusion efficiency. Addition of recombinant Rabaptin-5/Rabex-5 complex also stimulated the fusion activity although less potently than fraction 27, suggesting that other unidentified proteins present in the fraction cooperate with the Rabaptin-5 complex. Fractions 36 and 39 could also stimulate the activity of EEA1, albeit less efficiently than fraction 27. These data argue strongly that different Rab5-interacting proteins can exert a regulatory function on EEA1-mediated early-endosome fusion.

As EEA1 alone significantly supported endosome fusion in the absence of cytosol, we investigated the role of this protein further. If EEA1 is a bona-fide core component of the docking and fusion machinery, and if other Rab5 effectors modulate the nucleotide binding of Rab5 (for example, the Rabaptin-5/Rabex-5 complex) and the membrane recruitment and activity of EEA1, this regulation may be bypassed by increasing levels of EEA1. Figure 2c shows that 75 nM EEA1 (threefold concentration compared with cytosol) was able to drive fusion between early endosomes with similar efficiency to cytosol. Higher concentrations of EEA1 markedly enhanced fusion efficiency. Addition of fraction 27 enhanced this activity but fusion became insensitive to fraction 27 when excess EEA1 was supplied (Fig. 2c). The rate of fusion obtained with excess EEA1 was tenfold higher than the cytosol-driven reaction (not shown). These results indicate strongly that EEA1 is an integral component of the docking and fusion machinery.

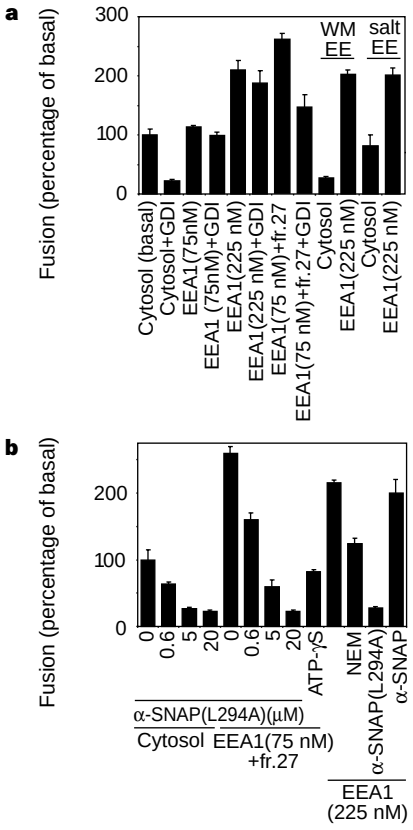

Figure 3 EEA1-mediated fusion can bypass Rab5 and PtdIns(3)P, but is inhibited by α-SNAP(L294A), ATP-γS and NEM. **a**, Endosome fusion was performed in the presence of the indicated reagents. GDI was added to a final concentration of 18 μM. Wortmannin treatment of the early endosomes (WM EE) was done by incubating post-nuclear supernatant (PNS) fraction with 1 μM wortmannin for 20 min at room temperature. Subsequently, endosomes were isolated on a sucrose flotation gradient as with normal endosomes. Similarly, salt-washed endosomes (salt EE) were obtained by incubating PNS with 1 M final concentration of NaCl on ice for 30 min, followed by isolation of the endosomes on a sucrose flotation gradient. **b**, Fusion assay was done in the presence of cytosol (basal) or the indicated reagents. ATP-γS was used at concentration 1 mM in the absence of an ATP regenerating system. The NEM concentration was 3 mM.

Under native conditions of endosome fusion (that is, in the presence of cytosol) the recruitment of EEA1 to the endosome is dependent on Rab5 and phosphatidylinositol-3-phosphate (PtdIns(3)P)[18,23]. Although GDP dissociation inhibitor (GDI) inhibited the cytosol-dependent fusion between early endosomes, it had no effect on fusion induced by 75 nM or 225 nM EEA1 (Fig. 3a). However, fusion under stimulatory conditions provided by fraction 27 demonstrates a partial GDI sensitivity. Therefore, affinity-purified EEA1 bypasses the requirement for Rab5 but the enhancement of its activity by fraction 27 is Rab5-dependent. In addition, the requirement for PtdIns(3)P on EEA1-mediated fusion was tested by pre-treating endosomes with wortmannin to inhibit phosphotidylinositol-3-OH kinase (PI(3)K)[18,24]. Although unable to fuse in the presence of cytosol, wortmannin-treated endosomes fused efficiently in the presence of excess EEA1 (Fig. 3a). Therefore, as for Rab5, when EEA1 is supplied as the only cytosolic protein factor it can bypass the requirement for PtdIns(3)P. This indicates Rab5 and PtdIns(3)P as upstream regulators of EEA1.

Peripheral endosomal membrane proteins did not seem to be required (or rate-limiting) for the EEA1-mediated fusion as salt-washed endosomes fused with equal efficiency to the control endosomes. Under these conditions, NSF was only partially removed by the salt wash (not shown), consistent with its tight association with the membrane[21]. Therefore, there are presumably sufficient amounts of NSF to ensure priming of SNAREs. Altogether, these results indicate that EEA1 is a component of the minimal endocytic machinery for membrane docking and fusion.

To determine the hierarchical order of function between EEA1 and SNAREs, we examined the effect of various reagents known to block SNARE priming. First, we found that EEA1-mediated fusion was inhibited by 60% and 40% by ATP-γS and *N*-ethylmaleimide (NEM) treatment, respectively (Fig. 3b). Similar inhibition was observed by ATP depletion (not shown). Second, we took advantage of a more specific inhibitor. We used a mutant of α-SNAP, α-SNAP(L294A), which, by inhibiting the stimulation of NSF ATPase activity, fails to prime SNAREs[25]. When added to a fusion reaction conducted either with cytosol or in the presence of EEA1 with fraction 27, α-SNAP(L294A) caused a strong, dose-dependent inhibition of fusion. At high concentrations of α-SNAP(L294A) (20 μM), fusion was completely abolished. The reaction was also inhibited to a similar extent when excess EEA1 alone was used. In contrast, wild-type α-SNAP did not inhibit endosome fusion. These data indicate that the NSF- and SNAP-dependent priming of SNAREs is at least partially required for EEA1-mediated fusion of early endosomes.

At which stage does EEA1 become important in the docking and fusion reaction? As the assay of homotypic endosome fusion cannot distinguish between docking and fusion, we developed a morphological docking assay. For this purpose, rhodamine-labelled transferrin was internalized in HeLa cells and endosomes were purified. These membranes were incubated at 37 °C for 25 min with various reagents (Fig. 4a) and subsequently observed under a fluorescence microscope. The combination of the fusion and fluorescence assays enabled us to determine the docked state of the membranes. Under experimental conditions that do not produce fusion, that is, in the absence of cytosol, only small scattered fluorescent structures were

seen (not shown). Larger structures formed in the presence of cytosol, resulting from the basal docking and fusion activity. EEA1 alone markedly stimulated the enlargement of endosomes and its effect was enhanced by the concomitant addition of fraction 27. The same morphological effect was obtained in the presence of α-SNAP(L294A), despite the fact that under these conditions membrane fusion is inhibited. Therefore, these clustered structures must be due predominantly to docking.

Consistent with these results, ATP-γS also had only a marginal effect on docking, although fusion was inhibited. We quantified the docked clusters and compared the values with the data from the fusion assay (Fig. 4b). Both α-SNAP(L294A) and ATP-γS are thought to prevent SNARE priming[1,25,26]. Here, they have no effect on EEA1-mediated endosome docking. Only at higher concentrations of α-SNAP(L294A) (20 μM), beyond the levels required to prevent SNARE priming[25], did we observe an inhibition of endosome docking. Golgi membranes failed to dock in the presence of EEA1 (not shown), demonstrating that the effect of EEA1 is specific for endosomes. After the initial incubation in the presence of EEA1 and α-SNAP(L294A) to block fusion, membranes were diluted. No dispersion of the membranes was observed, which indicates that the membranes were tightly associated and resistant to dilution (not shown).

We have shown that Rab5 is at the centre of a complex regulatory system involving multimeric interactions. Surprisingly, the Rab5 effector EEA1 alone can support fusion between early endosomes. We propose that EEA1 is a core component of the minimal machinery that docks and fuses endosomes. Our results suggest the following sequence of events. The activation of Rab5 on the endosome is ensured by the Rabaptin-5/Rabex-5 complex[16]. EEA1 recruitment and activity is regulated by active Rab5 and PtdIns(3)P[18]. In a minimal system, however, it is possible to bypass these regulatory mechanisms and proceed to a further downstream step that leads directly to docking and fusion. In fact, in the absence of cytosol and with EEA1 as the only soluble factor, endosome fusion becomes uncoupled from the Rab5- and PtdIns(3)P-dependent regulation. This observation has two implications. First, membrane factors other than Rab5 and PtdIns(3)P must participate in the recruitment and activation of EEA1 and, second, the activity of EEA1 may normally be counteracted by cytosolic inhibitory factors. Rab5 and its effectors would then be necessary to override this negative regulation. Again, in our minimal system this negative regulation is either lost or, alternatively, purified EEA1 may have become activated as a result of the interaction with Rab5 on the column. In addition, given the stimulatory and Rab5-dependent effect of the column-eluate fractions, other Rab5 effectors can modulate EEA1 recruitment and/or function.

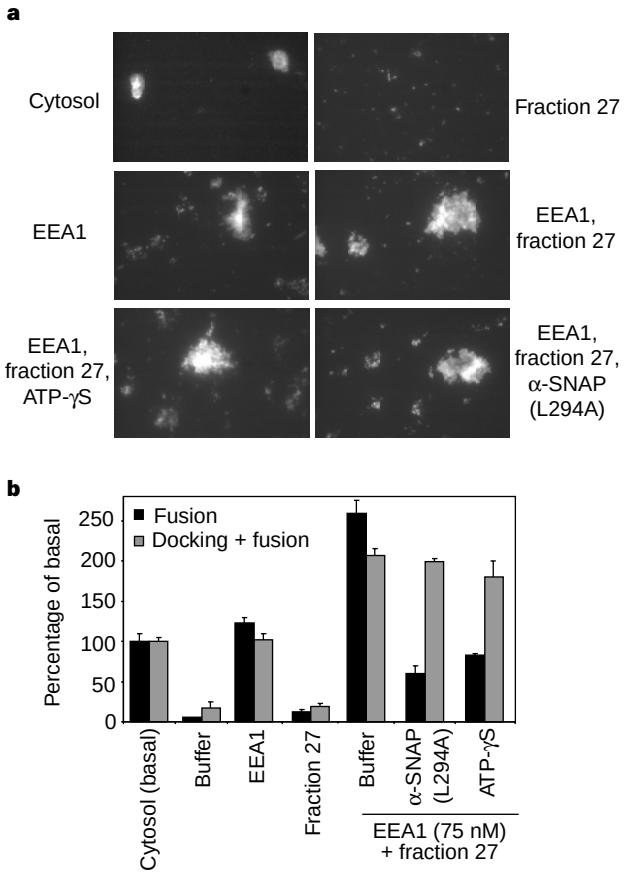

Figure 4 Endosome clustering requires EEA1 but not primed SNAREs. **a**, Visual docking of endosomes. Early endosomes, labelled with rhodamine-labelled transferrin, were incubated with cytosol or the indicated reagents; after 25 min the reaction was visualized with a Zeiss Axiophot fluorescence microscope. Concentrations used were: EEA1, 75 nM; fraction 27, 1.5 μl per 10 μl reaction; α-SNAP(L294A), 5 μM; ATP-γS, 1 mM. **b**, Comparison between fusion and docking plus fusion. Fusion (solid bars) is measured as a percentage of basal (in the presence of cytosol). Quantitation of docking (shaded bars) was done as described (see Methods). Reagents were used at the concentration described in **a**.

By exploiting a combination of morphological and biochemical assays, we now propose a role for EEA1 in endosome membrane docking (referred to also as tethering[8,27]). Although tethering molecules have been identified[27,28], this is the first direct evidence for such a role by a Rab effector. The function of EEA1 in docking, together with its restricted localization to early endosomes[29], implies that this molecule is crucial in the targeting of vesicles to this organelle. EEA1-mediated docking does not seem to require v- and t-SNARE priming as it was not affected by ATP-γS and α-SNAP(L294A). The docking of endoplasmic reticulum vesicles to Golgi has also been shown to occur in the absence of SNARE priming through the tethering protein Uso1p[8]. These results are therefore incompatible with a primary role of SNAREs in vesicle targeting. We consider it more likely that the specificity of vesicle targeting results from the combinatorial interaction between different cytosolic and membrane proteins (Rab effectors, SNAREs and perhaps others).

Although not required for docking, SNARE priming seems to be necessary for fusion. It is not inconceivable, however, that EEA1 may also participate in the fusion event itself. Three lines of evidence support this hypothesis. First, overexpression of a truncated mutant of EEA1 arrests the membranes in a docked stage and inhibits endosome fusion *in vivo*[18]. Second, we could not distinguish kinetically between EEA1-mediated docking and fusion (data not shown), indicating that the two stages are tightly coupled in the early-endosome system. Third, EEA1-mediated fusion is only partially ATP- and NEM-sensitive. One interpretation is that EEA1 may, to a certain extent, fuse endosomes in a SNARE-independent fashion. However, the observation that fusion is strongly inhibited by α-SNAP(L294A) indicates that this mutant could transcend the inhibition of NSF ATPase activity, raising interesting possibilities for a new role of α-SNAP (and NSF) in endocytic membrane fusion. Finally, the multiplicity of putative Rab5 effectors encourages us to consider the role of Rab5 in regulating other aspects of endosome function beyond docking and fusion. □

Methods

Purification of Rab5 effectors. Bacterial DH5α cells (120 l) were grown to express GST–Rab5 and the protein was purified according to manufacturer instructions (Pharmacia). This procedure resulted in 1 g of GST–Rab5 bound to 20 ml of glutathione Sepharose 4B beads. This material was then incubated with nucleotide-exchange buffer (buffer 1) containing 20 mM HEPES, 100 mM

NaCl, 10 mM EDTA, 5 mM $MgCl_2$, 1mM DTT, 1 mM GTP-γS, pH 7.5, for 90 min at room temperature under rotation. Afterwards, buffer 1 was removed and the GTP-γS form of GST–Rab5 was stabilized with buffer (buffer 2) containing 20 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, 1mM DTT, pH 7.5, in the presence of 1 mM GTP-γS for 20 min at room temperature under rotation. Beads were then incubated for 120 min at 4 °C with bovine brain cytosol obtained as follows: 14 bovine brains were homogenized in a blender with buffer 2 and the homogenate was centrifuged at 4,200*g* at 4 °C for 50 min. The resulting postnuclear supernatant was then centrifuged at 100,000*g* at 4 °C for 60 min. The high-speed supernatant was dialyzed against buffer 2 (without nucleotide) before incubation with the affinity column. After incubation with cytosol, beads were washed with ten column volumes of buffer 2 containing 10 μM GTP-γS, ten column volumes of buffer 2 containing 250 mM NaCl final concentration and 10 μM GTP-γS, and one column volume of 20 mM HEPES, 250 mM NaCl, 1 mM DTT, pH 7.5.

Bound proteins were eluted with 1.5 column volumes of a buffer containing 20 mM HEPES, 1.5 M NaCl, 20 mM EDTA, 1mM DTT, 5 mM GDP, pH 7.5, incubated with the beads for 20 min at room temperature under rotation. EDTA was used at this step to remove Mg^{2+} from Rab5 and release the effectors from the column. As EEA1 is a Zn^{2+}-binding protein and EDTA chelates Zn^{2+} (refs 23, 30), our subsequent assays were done in the presence of 1 mM $ZnCl_2$ filtered (0.22 μM) before its use. The GDP form of GST–Rab5 (1 ml) was made as described above for the GTP-γS form of GST–Rab5 with the following changes: all buffers contained GDP instead of GTP-γS, except for the elution buffer which contained GTP-γS (1 mM) instead of GDP. In the case of GST column (100 μl), there was no nucleotide in the buffers.

Fractionation of Rab5 effectors. The eluate (30 ml) from the 20-ml affinity column containing the mixture of Rab5-interacting proteins was first treated twice for 1 h at 4 °C with 1.5 ml glutathione Sepharose beads to remove GST–Rab5 which leaked from the affinity column during the elution step. The sample was then desalted using PD10 columns from Pharmacia in a buffer containing 20 mM HEPES, 150 mM NaCl, 1mM DTT, pH 7.5, and diluted three times with 20 mM HEPES, 1 mM DTT, pH 7.5, resulting in a final volume of 135 ml. The diluted sample was loaded on a 1-ml MQ FPLC column (Pharmacia) and bound proteins were step-eluted with 20 mM HEPES, 1 M NaCl, 1mM DTT, pH 7.5, in a total volume of 1 ml (concentration step). This eluate was fractionated on a 24-ml Superose 6 FPLC gel filtration column (Pharmacia). Fractions of 0.4 ml were collected, aliquoted and frozen at −80 °C.

Endosome docking assay. HeLa cells grown in suspension were harvested, washed with PBS and incubated for 5 min with 20 μg ml^{-1} rhodamine-labelled transferrin at 37 °C for 5 min. Then endosomes were isolated[15] and incubated in a 10 μl reaction for 25 min at 37 °C with reagents mentioned in Fig. 4. At the end of the reaction, samples were put on ice and visualized with a Zeiss Axiophot fluorescence microscope. Images were taken using a Cohu camera. Quantitation of docking was done by taking random pictures, followed by counting of the occupied squares (an indication of organelle area) on a reference grid.

Other preparations. GST–Rab5 and GST proteins were expressed in *Escherichia coli* using the P-GEX vector (Pharmacia). his-Rab GDI[16], his-α-SNAP and his-α-SNAP(L294A)[25] were produced as described. The preparation of recombinant Rabaptin-5/Rabex-5 complex will be described elsewhere (R. Lippe and M. Zerial, manuscript in preparation). Early endosome fusion assay was done as described[16].

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Photosynthetic control of chloroplast gene expression

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Redox chemistry—the transfer of electrons or hydrogen atoms—is central to energy conversion in respiration and photosynthesis. In photosynthesis in chloroplasts, two separate, light-driven reactions, termed photosystem I and photosystem II, are connected in series by a chain of electron carriers[1–3]. The redox state of one connecting electron carrier, plastoquinone, governs the distribution of absorbed light energy between photosystems I and II by controlling the phosphorylation of a mobile, light-harvesting, pigment–protein complex[4,5]. Here we show that the redox state of plastoquinone also controls the rate of transcription of genes encoding reaction-centre apoproteins of photosystem I and photosystem II. As a result of this control, the stoichiometry between the two photosystems changes in a way that counteracts the inefficiency produced when either photosystem limits the rate of the other. In eukaryotes, these reaction-centre proteins are encoded universally within the chloroplast. Photosynthetic control

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