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.

**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.

**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 at position 257. To express the last 22 residues of Myc–Hip, Shh-NiIgG was generated by cloning a DNA fragment encoding Shh-N into the pcDSglG1 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 hr after transfection. The supernatant was filtered and concentrated by precipitating with trichloroacetic acid (TCA). To immunoprecipitate Myc–HipΔC22, unconcentrated supernatant from COS-7 cells transfected with Myc–HipΔC22 was incubated with Shh-NiIgG bound to Protein A beads for 4 hr 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 hr 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 hr 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 35S-labelled probes were performed as described.

**Generation of transgenic animals.** To mis-express Hip under the α1(I) 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. Staining of skeleton for bone and cartilage was as described.

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20. Bresciani, E. & Boccaccio, C. The Rab5 effector EEA1 is a core component of endosome docking.

**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 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. However, several observations demonstrate that SNARE function at multiple transport steps and can pair promiscuously, questioning the role of SNAREs in conveying vesicle targeting. Moreover, other proteins have been shown to be important in membrane docking or tethering. 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. In vivo, 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 study.
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 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\(^{18}\).

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)\(^{25,27}\). Of these, most proteins selectively bound the GTP\(^{-}\) 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\(^{-}\)- 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-5\(^{\beta}\), their associated co-factor Rabex-5, and identified EEA1, as described recently\(^{28}\). (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-\(\gamma\)S column efficiently supported endosome fusion (Fig. 1b). Remarkably, 0.4 \(\mu\)g 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-\(\gamma\)S 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 profiles, the sum of fractions 19, 27, 32, 36 and 39 comprises the entire polypeptide composition of the column eluate. These fractions were tested in 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-\(\gamma\)S 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-5\(^{\beta}\), Rabex-5 and Rab5:GST are all indicated, as identified by western blot and mass spectrometry (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-\(\gamma\)S 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 Coomassie 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-5\(^{\beta}\) 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 \(\mu\)l) 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 \(\mu\)l). 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, 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 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.

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 phosphotyidylinositol-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 membrane21. 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-ethylnemithidamide (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 SNAREs25. 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\(^2,25\). 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\(^2,25\), 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\(^1\). 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.

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\(^22,23\), 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\(^9\), 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 Usol\(^9\). 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\(^14\). 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.
NaCl, 10 mM EDTA, 5 mM MgCl2, 1 mM 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 MgCl2, 1 mM 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 30 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 μg GTP-γS, ten column volumes of buffer 2 containing 250 mM NaCl final concentration and 10 μg 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, 1 mM 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 Mg2+ from Rab5 and release the effectors from the column. As EEA1 is a 23-kb binding protein and EDTA chelates Zn2+ (refs 23, 30), our subsequent assays were done in the presence of 1 mM ZnCl2 buffer which contained GTP-γS. Changes: all buffers contained GDP instead of GTP-γS.


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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 45. Here we show that the redox state of plastoquinone also controls the rate of transcription of genes encoding reaction-centre approteins 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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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 45. Here we show that the redox state of plastoquinone also controls the rate of transcription of genes encoding reaction-centre approteins 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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