Involvement of the endosomal autoantigen EEA1 in homotypic fusion of early endosomes

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In mammalian cells, fusion between early endocytic vesicles has been shown to require the ubiquitous intracellular fusion factors N-ethylmaleimide-sensitive factor (NSF) and α-SNAP, as well as a factor specific for early endosomes, the small GTPase Rab5 [1–3]. We have previously demonstrated an additional requirement for phosphatidylinositol 3-kinase (PI 3-kinase) activity [4]. The membrane association of early endosomal antigen 1 (EEA1), a specific marker of early endosomes [5,6], has recently been shown to be similarly dependent on PI 3-kinase activity [7], and we therefore postulated that it might be involved in endosome fusion. Here, we present evidence that EEA1 has an important role in determining the efficiency of endosome fusion *in vitro*. Both the carboxy-terminal domain of EEA1 (residues 1098–1411) and specific antibodies against EEA1 inhibited endosome fusion when included in an *in vitro* assay. Furthermore, depletion of EEA1, both from the membrane fraction used in the assay by washing with salt and from the cytosol using an EEA1-specific antibody, resulted in inhibition of endosome fusion. The involvement of EEA1 in endosome fusion accounts for the sensitivity of the endosome fusion assay to inhibitors of PI 3-kinase.

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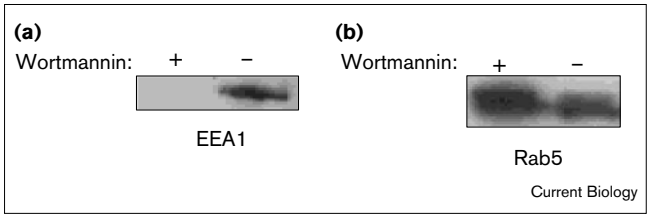
Results and discussion

We checked that EEA1 redistributes from the early endosomes of baby hamster kidney (BHK) cells upon inhibition of PI 3-kinase by wortmannin, as has been shown for other cell types using crude membrane preparations [7]. To generate an enriched early endosomal fraction and separate early endosomes from late endosomes, we used an established protocol involving a sucrose flotation gradient [3]. We observed that EEA1 was absent in an early endosomal fraction from wortmannin-treated cells (Figure 1a), whereas another peripheral marker of early endosomes, the small GTPase Rab5, remained in place (Figure 1b).

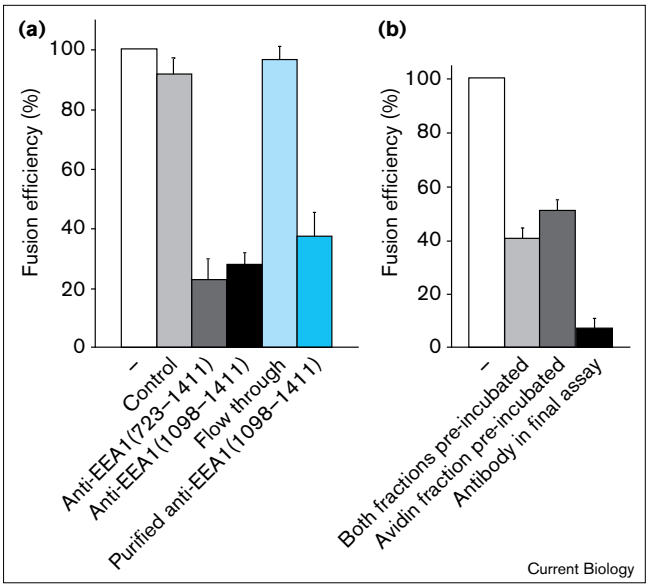
Figure 1



EEA1 dissociates from early endosomes following wortmannin treatment. Post-nuclear supernatants were prepared from untreated cells or cells pre-incubated with 100 nM wortmannin. In each case, a fraction enriched for early endosomes was isolated on a sucrose flotation gradient as described by Gorvel *et al.* [3] and 5 μg was analysed by western blotting for either **(a)** EEA1 or **(b)** Rab5. EEA1 is completely absent from the fraction isolated from wortmannin-treated cells, whereas the level of Rab5 is similar in fractions from treated and untreated cells.

In order to test if EEA1 is necessary for endosome fusion, polyclonal rabbit antibodies raised against the bacterially expressed carboxy-terminal fragments of EEA1 encompassed by residues 723–1411 and 1098–1411 —EEA1(723–1411) and EEA1(1098–1411), respectively — were pre-incubated with postnuclear fractions, which were then combined, with the antibody still present, in an *in vitro* assay for endosome fusion. Both antisera provided a significant inhibition of fusion (Figure 2a). A minimal effect was observed when control serum was used instead of the anti-EEA1 antibodies. The serum raised against EEA1(1098–1411) was affinity purified using antigen, and the flow through from the column was included in a fusion assay (Figure 2a). The inhibitory activity of this residual serum was the same as that of control serum, indicating that the inhibitory effect is specific to components that recognise the antigen. As expected, the affinity purified antibody from this column inhibited fusion (Figure 2a). We also conducted a fusion assay using membranes isolated from postnuclear fractions that had been exposed to the anti-EEA1(723–1411) antibody, facilitating the removal of antibody before fractions were combined. This gave less inhibition than when antibody was included in the final fusion step of the assay (Figure 2b), in accord with there being a significant pool of EEA1 in the rat brain cytosol used to support the fusion of isolated membranes. Interestingly,

Figure 2



Antibodies against EEA1 inhibit endosome fusion. In the endosome fusion assay used, which measures contents mixing, one set of endosomes were labelled with avidin and the other with biotin conjugated to horse radish peroxidase (HRP). **(a)** Both of the postnuclear supernatants combined in the assay were either left untreated (–) or pre-incubated with 5 μl of the indicated rabbit antisera, an equivalent amount of flow through from an affinity column used to purify anti-EEA1(1098–1411) antibody, or 15 μg of this affinity purified antibody. The fusion efficiencies were normalised to the signal obtained using untreated controls. **(b)** Exposure to antiserum of only one of two sets of membrane fractions combined in a fusion assay is sufficient for inhibition. For this experiment, rather than combine postnuclear supernatants, membranes isolated from these supernatants were combined and fusion was supported by rat brain cytosol. This enabled us to allow antibodies to have access to only one of the two sets of membranes used in the fusion assay. We either left both membrane fractions untreated (–), pre-incubated both the avidin-labelled and biotin–HRP-labelled fractions with 5 μl anti-EEA1(723–1411) antiserum, pre-incubated only the avidin fraction with the antiserum or added the antiserum to the complete fusion mix.

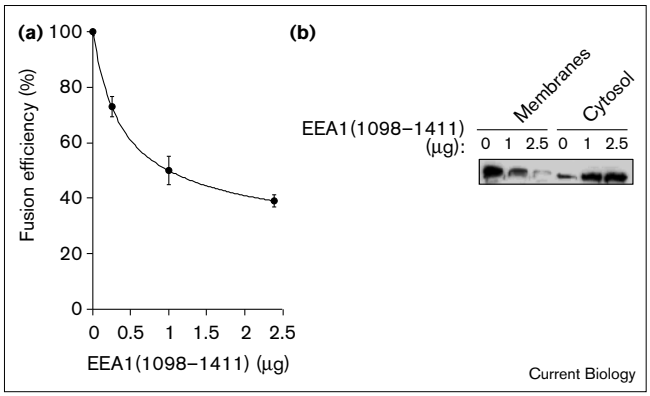
exposure to the antisera of only one of the two membrane fractions combined in this fusion assay was virtually as effective as exposure of both (Figure 2b).

We purified EEA1(1098–1411) from bacteria; this fragment contains a FYVE zinc finger domain and is sufficient for endosomal localisation [6]. Inclusion of this protein also exerted an inhibitory effect on endosome fusion *in vitro* (Figure 3a). This implies that the amino-terminal domain of EEA1, which is missing from the added fragment, has an important role in endosome fusion, as the carboxy-terminal domain associated with endosomes and displaced endogenous wild-type protein over the same concentration range (Figure 3b). The inhibition was insensitive to supplementation of the assay with $ZnCl_2$ indicating that it is not due to zinc chelation by EEA1(1098–1411) [8] (data not shown).

In our hands, the majority of EEA1 in BHK-21 cells was associated with the membrane fraction, although a significant cytosolic fraction was present (Figure 4a). EEA1 association with the membrane fraction could be completely disrupted by incubation with 0.3 M NaCl (Figure 4a). At the concentration of rat brain cytosol we have used in the fusion assay, salt washing resulted in a reduction in endosome fusion (Figure 4c). When we immunodepleted rat brain cytosol using polyclonal antibodies (Figure 4b) in addition to salt washing the membranes, we found that endosome fusion was reduced even further (Figure 4c). Using immunodepleted cytosol and untreated membranes also resulted in a specific reduction in fusion, similar to that obtained by salt treatment alone. Under our standard conditions of endosome fusion supported by rat brain cytosol *in vitro*, we estimate from western blot analysis that roughly equal amounts of EEA1 are supplied by the membrane fractions and the cytosol. The reduction in fusion corresponding to salt washing is consistent with EEA1 removal but cannot be directly attributed to it as other factors might also have been removed. The reduction in fusion due to immunodepletion, however, is clearly specific for the removal of EEA1 and any factors which may be associated with it.

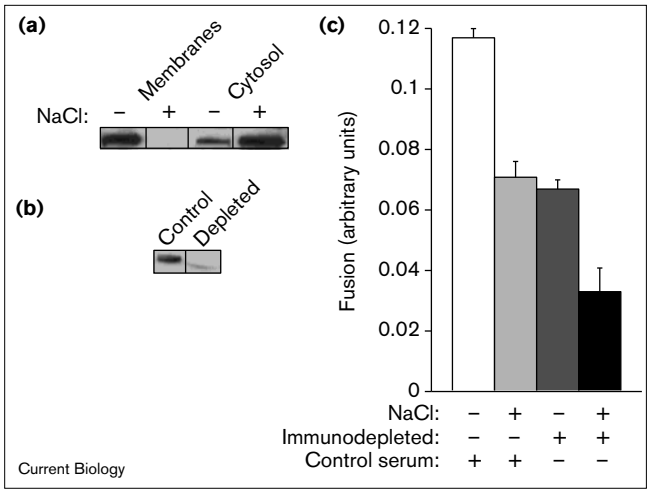
In this report we have provided a body of evidence in support of a role of EEA1 in endosome fusion, a role that we initially postulated on the basis of its redistribution following wortmannin treatment. It has previously been proposed that activation of the small GTPase Rab5 may

Figure 3



Recombinant EEA1(1098–1411) reduces endosomal fusion efficiency and displaces endogenous EEA1 from membranes. **(a)** Varying amounts of His_6-tagged EEA1(1098–1411) were added in a standard fusion assay (volume, 220 μl; protein concentration, 4 mg/ml). Fusion is expressed as a proportion of that obtained in a control incubation in which no fragment was added. **(b)** Membranes incubated with the indicated amounts of the recombinant fragment, under fusion assay conditions, were isolated from the cytosol and each fraction was analysed for endogenous EEA1 by western blot. Displacement of EEA1 from membranes to cytosol was observed over the same concentration range of EEA1(1098–1411) required for inhibition of fusion.

Figure 4



Reduced endosome fusion efficiency correlates with EEA1 depletion. **(a)** Western blot showing removal of EEA1 from BHK membranes by 0.3 M NaCl. Postnuclear supernatants were incubated with or without salt at 4°C and then membrane (30 μg) and cytosol (80 μg) fractions, separated as described in Materials and methods, were analysed for the presence of EEA1. **(b)** Western blot showing immunodepletion of EEA1 from rat brain cytosol using anti-EEA1(1098–1411) antibody; 100 μg protein was loaded per lane. As a control, cytosol was treated with serum from a non-immunised rabbit absorbed onto protein-A–sepharose. **(c)** Early endosome fusion assay as described in Materials and methods. Membranes were isolated following incubation with or without 0.3 M NaCl and recombined with one of the two rat brain cytosol fractions shown in (b).

represent the requirement of PI 3-kinase activity for endosome fusion [9]. Recent data from our laboratory have shown that PI 3-kinase activity is required for an effector action other than activation of Rab5, however, because wortmannin inhibition of endosome fusion is still observed in the presence of a moderate excess of activated Rab5 or GTPγS, which causes constitutive activation of small GTPases [10]. In the presence of a vast excess of activated Rab5, endosome fusion in our assay becomes insensitive to wortmannin. The magnitude of the inhibition of endosome fusion that we observe with each manipulation of EEA1 suggests that wortmannin inhibition of endosome fusion can be fully accounted for by its effect on the distribution of EEA1. In a report of a recent meeting [11], data showing an interaction between Rab5 and EEA1 are discussed. It could be that in the presence of a large excess of Rab5, a relatively low affinity interaction with EEA1 is sufficient to localise it to endosomes. This interaction may be required under all conditions for endosome fusion as it is clear that Rab5 [3] and, from our observations, EEA1 are required for endosome fusion. Under native conditions and at lower levels of Rab5, it is conceivable that the interaction is favoured by the accumulation of EEA1 and Rab5 to early endosomes by virtue of PI 3-phosphate and prenylation-dependent membrane associations, respectively. Rabaptin-5 has also been identified as an effector of Rab5 and a complex of this protein with another protein, Rabex-5, is essential for endosome fusion [12]. The relationship between these proteins and EEA1 on the fusion pathway must now be determined.

Materials and methods

Materials

Polyclonal rabbit antisera raised against EEA1(723–1411) were the kind gift of M. Renz. The cDNA corresponding to EEA1(1098–1411) was inserted into a pTric-HisA vector. Bacterially expressed His_6-tagged protein was purified using Ni^{2+} affinity chromatography followed by dialysis against 50 mM Tris, 1 μM $ZnCl_2$, pH 7.4.

Cell-free fusion assay

Internalisation of avidin and biotin–HRP into BHK-21 cells was carried out essentially as described previously [3,13]. In the standard configuration of the assay, postnuclear fractions were combined in a mixture also containing salts, buffers, biotin–insulin and an ATP-regenerating system as described by Gorvel *et al.* [3]. Alternatively, membranes were prepared by pelleting at 65,000 rpm (using a TLA 100.2 rotor) for 15 min and combined in a fusion assay supplemented with rat brain cytosol or BHK cytosol, a process that allows specific interventions to be made on these components before they are combined. This mixture was then incubated at 37°C for 30 min before lysis on ice with 0.2% Triton X-100. Fusion efficiency was measured by immunoprecipitation of avidin followed by determination of bound HRP.

Immunodepletion

Rat brain cytosol prepared by the method of Waters *et al.* [14] was incubated with end-over-end rotation at 4°C for 2 h together with EEA1 polyclonal serum pre-absorbed onto protein-A–sepharose. The antibody-coated beads were removed and replaced with a fresh batch for a further 2 h incubation before removal and collection of the cytosol.

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