The endosome fusion regulator early-endosomal autoantigen 1 (EEA1) is a dimer

Judy CALLAGHAN*†[1], Anne SIMONSEN*[1], Jean-Michel GAULLIER*, Ban-Hock TOH† and Harald STENMARK*[2]

*Department of Biochemistry, The Norwegian Radium Hospital, Montebello, N-0310 Oslo, Norway, and †Department of Pathology and Immunology, Monash Medical School, Prahran, Victoria 3181, Australia

EEA1, an early-endosomal protein originally identified as an autoantigen, is essential for endocytic membrane fusion. It interacts with early endosomes via binding to the membrane lipid phosphatidylinositol 3-phosphate (PtdIns3*P*) and the active form of the small GTPase Rab5. Most of the EEA1 sequence contains heptad repeats characteristic of proteins involved in coiled-coil protein–protein interactions. Here we have investigated the ability of EEA1 to self-interact. Crosslinking of cytosolic and recombinant EEA1 resulted in the disappearance of the 180-kDa monomer in SDS/PAGE and the strong appearance of a ~ 350-kDa crosslinked product. Glycerol gradient centrifugation experiments indicated that native EEA1 had the same hydrodynamic properties as the ~ 350-kDa crosslinked complex. Two-hybrid analysis indicated that N- and C-terminal fragments of EEA1 can interact with themselves, but not with each other, suggesting that EEA1 forms parallel coiled-coil dimers. The ability of the C-terminus of EEA1 to dimerize correlates with its ability to bind to Rab5 and early endosomes, whereas its binding to PtdIns3*P* is independent of dimerization. These data enable us to propose a model for the quaternary structure of EEA1.

INTRODUCTION

The mechanism of homotypic fusion between early endosomes, a process that has been characterized *in vitro* by many laboratories [1–8], appears to resemble the heterotypic fusion of endocytic vesicles with early endosomes [1]. Among the molecules that regulate endocytic membrane fusion are the small GTPase Rab5 [1,3,9] and phosphatidylinositol 3-kinase (PI 3-kinase) [4–6]. Recently, the early endosomal autoantigen 1 (EEA1) [10] was shown to be an effector of both Rab5 and the PI 3-kinase product phosphatidylinositol 3-phosphate (PtdIns3*P*) in endosome fusion [8,11]. The 162-kDa EEA1 protein contains two binding sites for the GTP-bound form of Rab5, one comprising an N-terminal zinc finger and one close to the C-terminus [8]. In addition, the very C-terminus of EEA1 contains a FYVE finger [12], which binds to PtdIns3*P* [11,13,14]. An analysis of the amino acid sequence of EEA1 [10] has indicated a high occurrence of α-helical heptad repeats typically found in molecules engaged in coiled-coil protein–protein interactions [15]. In this report we have investigated whether EEA1 is able to self-interact.

MATERIALS AND METHODS

Materials

Crosslinkers and reagents for enhanced chemiluminescence (ECL) detection were from Pierce (Rockford, IL, U.S.A.). Standards for glycerol gradient centrifugation were from Sigma (St. Louis, MO, U.S.A.). Protein-inhibitor tablets (complete) were from Boehringer Mannheim (Mannheim, Germany). Yeast media were from Bio101 (Vista, CA, U.S.A.). Pansorbin was from Calbiochem (La Jolla, CA, U.S.A.). The human autoimmune serum against EEA1 was from a patient with subacute cutaneous systemic lupus erythematosus identified in the Monash Clinical Immunology Laboratory [10]. The rabbit antibody against the N-terminal 16 residues of EEA1 has been described before [8].

Plasmids

The yeast two-hybrid plasmids pLexA $EEA1_{1257–1411}$, pLexA $EEA1_{1277–1411}$, pLexA $EEA1_{1325–1411}$, pGAD $EEA1_{wt}$ and pGAD $EEA1_{1–209}$ have been described previously [8]. pLexA $EEA1_{wt}$, pLexA $EEA1_{1–209}$ and pLexA $EEA1_{1307–1411}$ were obtained by subcloning the respective EEA1 cDNAs [8,12] into the polylinker sites of pLexA/pBTM116 [16]. pGAD $EEA1_{1257–1411}$, pGAD $EEA1_{1277–1411}$, pGAD $EEA1_{1307–1411}$ and pGAD $EEA1_{1325–1411}$ were obtained by subcloning the respective cDNAs [12] into the polylinker sites of pGAD GH (Clontech, Palo Alto, LA, U.S.A.). For expression in *Escherichia coli*, truncated EEA1 constructs were obtained by PCR amplification of full-length cDNA of EEA1 using primers with an *Xho*I site downstream of the termination codon (5′-CCTGCTCGAGTTATCCTTGCAAGTCATTGA-3′), and an *Eco*R1 site upstream of either codon 1257 (5′-CGGAATTCTTGGCAATCTAGTCAACGG-3′) or codon 1325 (5′-CGGAATTCTGCAGTGCAGGAGCTGGGC-3′). The PCR products were subcloned into the pGEX-6P-3 expression vector (Pharmacia, Uppsala, Sweden). The correct sequences were confirmed by dideoxyribose nucleic acid sequencing.

Recombinant proteins

For expression as fusion proteins with glutathione S-transferase (GST) in *E. coli*, BL-21(DE3) or DH5α strains were transformed with the respective pGEX plasmids and protein expression was induced with 0.3–1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C. Recombinant fusion proteins were solubilized with 1 % Triton X-100, sonicated and purified on a glutathione–Sepharose column (Pharmacia) according to the manufacturer's

Abbreviations used: PI 3-kinase, phosphatidylinositol 3-kinase; EEA1, early-endsomal autoantigen 1; PtdIns3*P*, phosphatidylinositol 3-phosphate; GST, glutathione S-transferase; ECL, enhanced chemiluminescence; SDC, synthetic defined complete; BMH, bismaleimidohexane; BS^3, bis(sulphosuccinimidyl)suberate; DSS, disuccinimidyl suberate.

[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed (e-mail stenmark@ulrik.uio.no).

instructions. In order to cleave off the GST tag, the purified proteins were treated with PreScission protease (Pharmacia) according to the manufacturer's instruction. His_6-EEA1 was expressed in insect cells as described [8].

Chemical crosslinking

His_6-EEA1 (4 μg), GST-$EEA1_{1257–1411}$ (4 μg), GST-$EEA1_{1325–1411}$ (4 μg) or HeLa cytosol (20 μg) were incubated with 2.5–10 mM of the covalent crosslinker bis(sulphosuccinimidyl) suberate (BS^3) in PBS for 30 min at 30 °C. Free reactive groups were quenched with 0.2 M Tris, pH 7.0, for 30 min at 4 °C. Rat brain cytosol (30 μg) was incubated with 5 mM of the covalent cross-linker disuccinimidyl suberate (DSS) for 2 h at 4 °C. Free reactive groups were quenched with 0.1 M glycine, pH 7.0, for 30 min at 4 °C. The reactions were analysed by SDS/PAGE followed by immunoblotting with anti-EEA1 antibodies, followed by ECL detection. For a partial crosslinking of EEA1 from cytosol, 100 μl (300 μg) of HeLa cytosol was incubated in the presence of 5 mM bismaleimidohexane (BMH) for 1 h at 4 °C. Free reactive groups were then quenched with 100 mM cysteine for 1 h at 4 °C.

Preparation of cytosol

HeLa cells grown to 8×10^5 cells/ml were pelleted, washed and homogenized in KEHM buffer (50 mM KCl/10 mM EGTA/50 mM Hepes/2 mM $MgCl_2$, pH 7.4). The homogenate was then centrifuged at 80000 ***g*** for 30 min at 4 °C in a Beckman TLA-100.4 rotor in order to pellet the membranes and obtain a cytosolic fraction with a total protein concentration of $\sim$ 5 mg/ml. Rat brain cytosol was prepared by homogenizing rat brains in sucrose buffer (0.32 M sucrose/4 mM Hepes-NaOH, pH 7.3). The homogenate was first centrifuged at 900 ***g*** for 10 min at 4 °C. The resulting supernatant was centrifuged at 100000 ***g*** for 1 h to pellet the membranes and obtain a cytosolic fraction.

Immunoprecipitation of EEA1 from rat brain

A solubilized homogenate of rat brain was prepared by homogenizing rat brains in 50 mM Hepes, pH 7.4/1 mM EDTA/0.1 M NaCl. The homogenate was centrifuged at 900 ***g*** for 10 min. Triton X-100 was added to the resulting supernatant at a final concentration of 0.5 % and incubated on ice for 30 min. The soluble brain proteins were obtained by centrifuging the supernatant at 100000 ***g*** for 30 min. EEA1 was purified by diluting the soluble brain homogenate in lysis buffer (10 mM Tris/HCl, pH 7.0/50 mM NaCl/50 mM EDTA) and incubating with rabbit anti-EEA1 antibody overnight at 4 °C. Pansorbin (10 %) was added for 1 h at 4 °C, the immune complexes were collected by centrifugation and subsequently washed three times in lysis buffer and once in PBS. EEA1 and associated proteins were eluted from the Pansorbin by heating for 4 min at 95 °C in the presence of reducing sample buffer. The eluted proteins were analysed by SDS/PAGE followed by Coomassie Brilliant Blue staining or immunoblotting with human anti-EEA1 antibodies. Protease inhibitors were included in all stages of the preparation of cytosolic and recombinant EEA1. Protein concentrations were estimated using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, U.S.A.).

Yeast two-hybrid methods

The yeast reporter strain L40 [16] [*MATa* his3D200 trp1-901 leu2-3112 ade2 *LYS2*:: (lexA-*HIS3*) (*URA3*::lexA-lacZ) *GAL4*] was co-transformed with the indicated 2μ/*TRP1* pLexA and 2μ/*LEU2* pGAD plasmids using a lithium acetate-based method [16a] and the transformants were selected on synthetic defined complete (SDC) medium lacking tryptophan and leucine. *HIS3* reporter-gene activation analysis was performed by spotting 5 μl of a yeast colony resuspended in 1 ml of TE buffer (10 mM Tris/1 mM EDTA, pH 8.0) on SDC medium lacking tryptophane, leucine and histidine.

Glycerol-gradient centrifugation

BMH-treated HeLa cytosol (300 μg) was added on top of a 12-step (26–60 %) glycerol gradient and centrifuged at 132000 ***g*** (50000 r.p.m.) for 20 h at 4 °C in a Beckman TLS55 rotor. A gradient containing the standards thyroglobulin (670 kDa, 19 S), catalase (220 kDa, 11.2 S) and transferrin (81 kDa, 4.92 S) was included. Eighteen fractions were collected from each gradient and analysed by SDS/PAGE and subsequent immunoblotting using human anti-EEA1 antibodies and ECL as the detection method.

Liposome-binding assay

The lipids and assay conditions were as described [17], except that the liposomes consisted of 63 % phosphatidylcholine (with a trace amount of [^{3}H]phosphatidylcholine), 20 % phosphatidylserine, 15 % phosphatidylethanolamine and 2 % PtdIns3*P*.

RESULTS

Anti-EEA1 antibodies recognize proteins of $\sim$ 180 and $\sim$ 350 kDa from cytosol

Upon immunoprecipitation of cytosol with rabbit anti-EEA1 and subsequent SDS/PAGE and Coomassie Brilliant Blue staining (Figure 1A), a band corresponding to the EEA1 monomer of 162 kDa (apparent molecular mass, 180 kDa) was readily de-

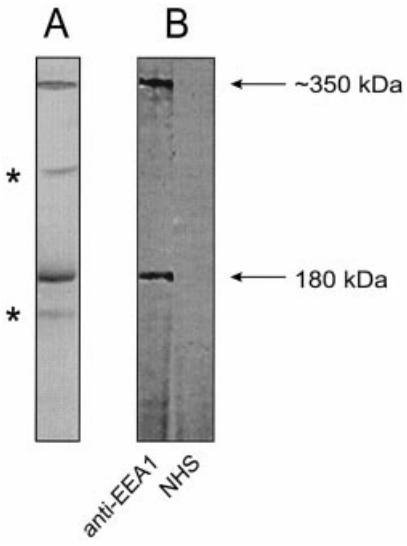

Figure 1 Immunoprecipitation of EEA1 from rat brain

Soluble brain homogenate was immunoprecipitated with rabbit anti-EEA1 antibody followed by either (**A**) Coomassie Brilliant Blue staining or (**B**) immunoblotting with human anti-EEA1 antibodies or normal human serum (NHS). The asterisks indicate proteins co-immunoprecipitated with EEA1. Apparent molecular mass values are indicated.

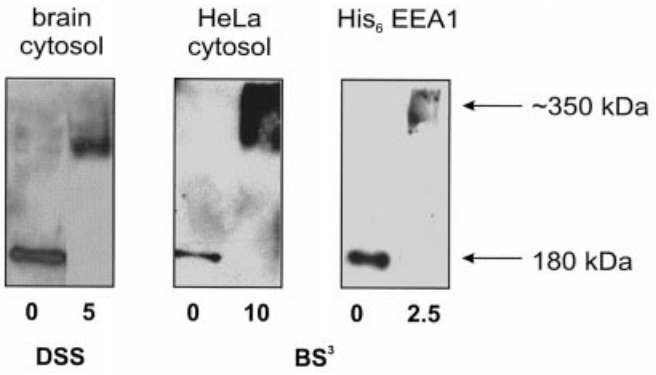

Figure 2 Crosslinking of recombinant and cytosolic EEA1

Rat brain cytosol, HeLa cytosol or recombinant His_6-EEA1 were either untreated or treated with the indicated concentrations (mM) of DSS or BS^3, as described in Materials and methods, and then analysed by SDS/PAGE followed by immunoblotting with anti-EEA1. Apparent molecular mass values are indicated.

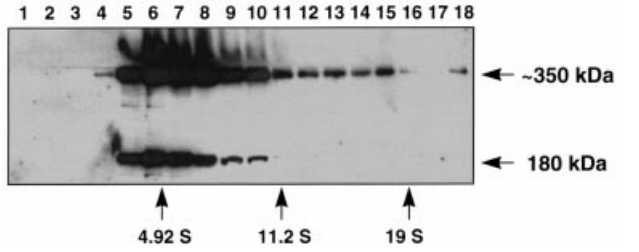

Figure 3 Glycerol-gradient centrifugation of crosslinked and uncrosslinked EEA1

HeLa cell cytosol was incubated with the non-cleavable crosslinker BMH, followed by glycerol-gradient centrifugation. In total, 18 fractions were analysed by SDS/PAGE and anti-EEA1 immunoblotting. The Svedberg-unit standards in the glycerol gradient, the fraction numbers and apparent molecular mass values are indicated.

tected, as expected. However, we also observed a ~ 350 kDa band of variable intensity. This band was also observed after immunoblotting with a human anti-EEA1 autoimmune serum, but not with a control human serum (Figure 1B). Under conditions where EEA1 was not enriched by immunoprecipitation (see below), the ~ 350 kDa band became less visible. The identity of the ~ 350 kDa band is not clear, but one possibility is that it might represent a protein distinct from but immunologically related to EEA1. Another option is that it might represent an SDS-resistant [18] dimer of EEA1.

Chemical crosslinking doubles the apparent molecular mass of EEA1 in SDS/PAGE

To investigate if EEA1 dimerizes, we analysed by anti-EEA1 immunoblotting HeLa and brain extracts that had been treated with chemical crosslinkers. Upon crosslinking with BS^3, the ~ 180 kDa monomer band disappeared, and a smeary band of ~ 350 kDa appeared instead (Figure 2, left and middle panels). The size of this crosslinked product would be consistent with that of an EEA1 homodimer (calculated molecular mass, 324 kDa). To investigate this possibility further, we performed a similar experiment with recombinant EEA1 (Figure 2, right panel). This DSS crosslinking resulted in the appearance of a ~ 350 kDa band similar to that obtained with cytosol. Combined, these results indicate that cytosolic as well as recombinant EEA1 can form a homodimer.

Crosslinked and uncrosslinked EEA1 have similar hydrodynamic properties

In order to study the extent of homodimerization of cytosolic EEA1, we compared the migration rates of native versus crosslinked EEA1 upon centrifugation in a glycerol gradient. For the crosslinking, we used an intermediary concentration of BMH, sufficient to obtain a partial crosslinking of EEA1, Fractions from the glycerol gradient were analysed by SDS/PAGE followed by immunoblotting with anti-EEA1. If the bulk of EEA1 were present as monomers, we would expect to observe uncrosslinked EEA1 in different fractions from those of the crosslinked dimer. However, as shown in Figure 3, the ~ 180-kDa uncrosslinked EEA1 was present in exactly the same fractions as the bulk of the ~ 350-kDa crosslinked product. This indicates that uncrosslinked EEA1 has the same hydrodynamic properties as crosslinked EEA1, and we conclude from these experiments that EEA1 is mainly present in cytosol as a dimer. A small amount of crosslinked EEA1 was present also in the lower fractions of the gradient. This may represent dimeric EEA1 non-covalently associated with either other crosslinked dimers or other high-molecular-mass cytosolic protein complexes. It should be noted that both native and crosslinked EEA1 had sedimentation coefficients of about 5 S. For a globular protein, this would correspond to an apparent molecular mass of ~ 80–100 kDa, which is much lower than that predicted for an EEA1 dimer (324 kDa). Such a big discrepancy between calculated and actual hydrodynamic properties is found with rod-shaped molecules [19], indicating that the EEA1 dimer has an extended rather than a globular conformation.

Evidence that EEA1 forms a parallel coiled-coil dimer

Its high content of α-helical heptad repeats, as well as our interpretation that EEA1 forms an elongated dimer, suggested the possibility that EEA1 may form a coiled-coil dimer [15]. Coiled-coil dimers may be either parallel or antiparallel [15]. To distinguish between these alternatives, we investigated whether the N- and C-terminal parts of EEA1 can interact with themselves or with each other. For this purpose, we made use of the yeast two-hybrid system [20]. EEA1 constructs (Figure 4A) were cloned into the 'bait' vector pLexA, as well as the 'prey' vector pGAD, and then introduced into the two-hybrid reporter yeast strain L40 [16]. Protein–protein interactions were assayed by monitoring activation of the *HIS3* reporter gene. We first checked that dimerization of full-length EEA1 could be detected with the two-hybrid system. As shown in Figure 4(B), full-length EEA1 interacted with itself in the two-hybrid system, as evidenced by the ability of the yeast double transformants to grow on histidine-free medium. Interestingly, the C-terminal part of EEA1 was also able to interact with itself, suggesting that EEA1 may form parallel dimers. This conclusion was supported by the finding that the N-terminal part of EEA1 also self-interacted (Figure 4B), whereas the N-terminal part of EEA1 did not interact with the C-terminus. The very C-terminus of EEA1 contains a

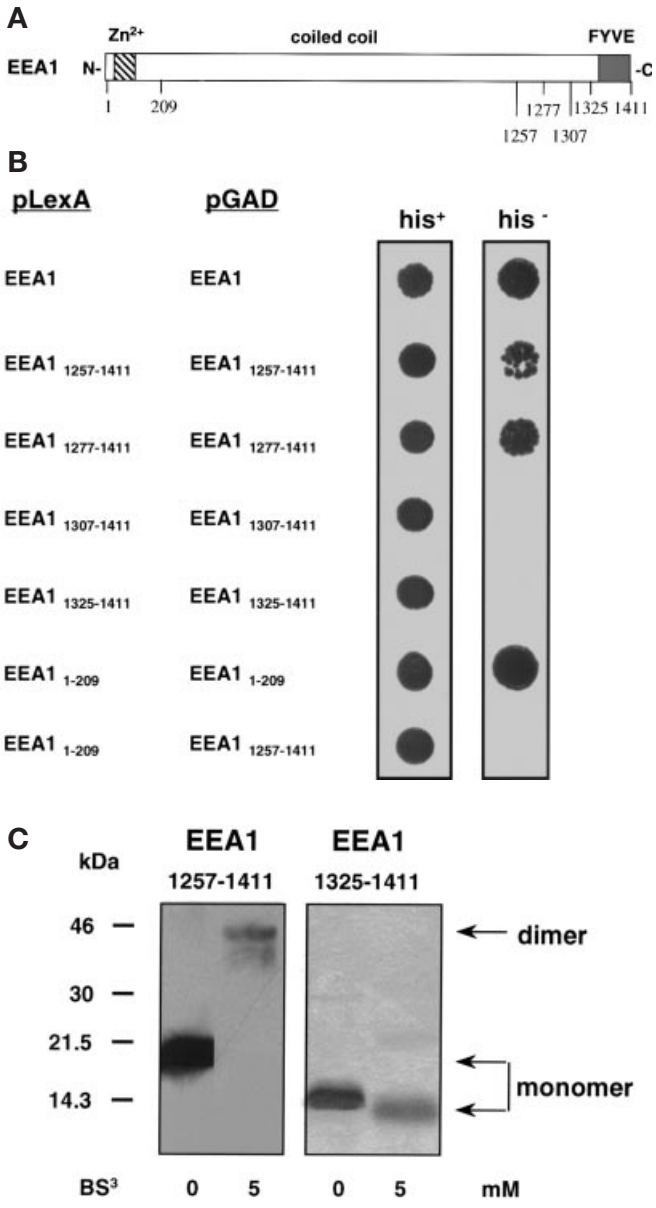

Figure 4 Dimerization of EEA1 constructs as studied with the two-hybrid system and chemical crosslinking

(**A**) Schematic representation of EEA1, with the N-terminal zinc finger, the C-terminal FYVE finger and the putative coiled-coil regions indicated. The positions of relevant residues are indicated (not to scale). (**B**) The indicated EEA1 constructs were prepared as fusions with the Gal4 activation domain in pGAD and the DNA-binding protein LexA in pLexA. L40 reporter yeast cells co-transformed with the pGAD and pLexA constructs as indicated were spotted on to SDC medium lacking tryptophan and leucine (his$^+$) or lacking tryptophan, leucine and histidine (his$^-$). *HIS3* reporter-gene activation, indicative of dimerization, was measured as the ability of the transformants to grow on the his$^-$ medium. (**C**) Chemical crosslinking of recombinant $EEA1_{1257–1411}$ and $EEA1_{1325–1411}$. The proteins were either left untreated or crosslinked with BS^3 as described in Materials and methods and then analysed by SDS/PAGE, followed by immunoblotting ($EEA1_{1257–1411}$) or Coomassie Brilliant Blue staining ($EEA1_{1325–1411}$). ($EEA1_{1325–1411}$ could not be analysed by immunoblotting, as this construct was not recognized by the anti-EEA1 antibody.) The slight shift in migration of $EEA1_{1325–1411}$ upon BS^3 treatment is likely to be the result of BS^3 modification of the monomeric protein. The positions of molecular-mass markers as well as monomers and a putative $EEA1_{1257–1411}$ dimer are indicated.

PtdIns3*P*-binding FYVE finger [11]. A small region of upstream putative coiled-coil sequences was needed for the dimerization of the C-terminus to occur, as $EEA1_{1277–1411}$ but not $EEA1_{1307–1411}$ or $EEA1_{1325–1411}$, dimerized (Figure 4B). A similar result was obtained with chemical crosslinking of the recombinant proteins (Figure 4C). In conclusion, our experiments indicate that EEA1 forms a parallel coiled-coil dimer, and that the C-terminal FYVE finger is not sufficient for the dimerization.

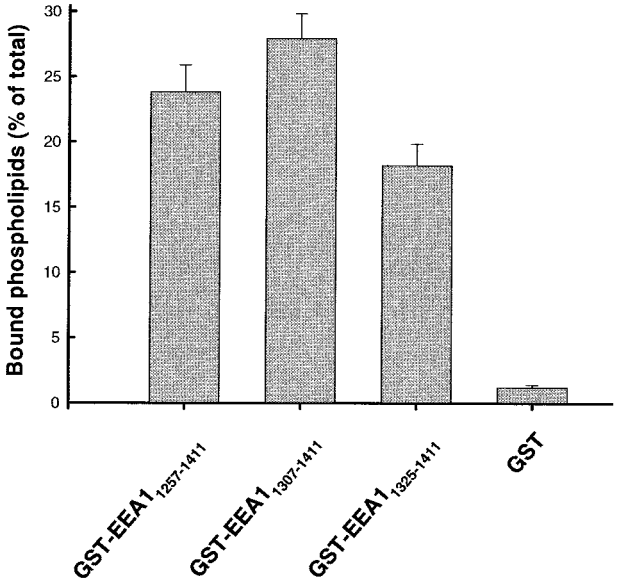

Figure 5 PtdIns3*P* binding of C-terminal EEA1 constructs

GST alone or GST fused to the EEA1 proteins indicated were immobilized on glutathione–Sepharose and incubated with [^{3}H]phosphatidylcholine-containing liposomes containing 2% PtdIns3*P*. The radioactivity associated with the glutathione–Sepharose beads after washing was measured with a scintillation counter. The bars represent the means ± S.E.M. of a typical experiment performed in triplicate.

Dimerization of EEA1 is not required for PtdIns3*P* binding

To study the relationship between PtdIns3*P* binding and dimerization, we investigated the ability of three different C-terminal EEA1 constructs (prepared as fusion proteins with GST) to bind to radiolabelled liposomes [17] containing 2% PtdIns3*P*. According to the two-hybrid experiments shown in Figure 4, one of the constructs, $EEA1_{1257–1411}$, was able to dimerize, whereas the other two, $EEA1_{1307–1411}$ and $EEA1_{1325–1411}$, did not dimerize. However, all three constructs were found to bind strongly to PtdIns3*P*-containing liposomes (Figure 5), whereas GST alone did not bind. This indicates that PtdIns3*P* binding of EEA1 is independent of dimerization.

Table 1 Properties of full-length and C-terminal EEA1 constructs

The table summarizes data from the present study and [12]. ND, not determined.

EEA1 region	Rab5 binding	Endosome binding	PtdIns3*P* binding	Dimerization
1–1411	+	+	ND	+
1257–1411	+	+	+	+
1307–1411	−	−	+	−
1325–1411	−	−	+	−

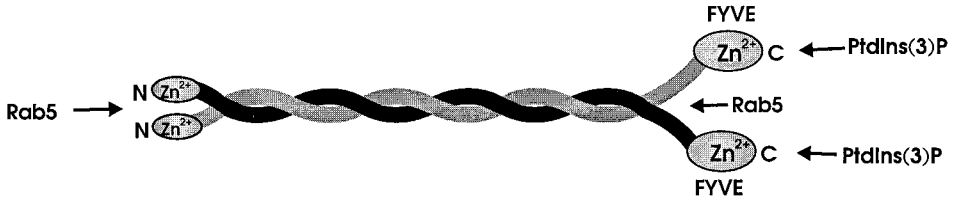

Figure 6 Model of the EEA1 dimer

Schematic view of the EEA1 dimer, as inferred from [8] and the present experiments. The N-terminal C2H2 zinc finger and the C-terminal FYVE zinc finger are indicated. Binding sites for Rab5 [8] and PtdIns3*P* [11] are indicated by arrows.

DISCUSSION

In this report we show evidence that EEA1 exists as a parallel coiled-coil dimer in cytosol. First, EEA1 can be crosslinked, yielding a product with approximately double the apparent molecular mass of the monomer. Second, the crosslinked product has the same sedimentation properties as the uncrosslinked EEA1 in glycerol-gradient centrifugation. Third, two-hybrid experiments indicate that N- and C-terminal EEA1 fragments can homodimerize but not heterodimerize. Fourth, its slow sedimentation upon glycerol-gradient centrifugation suggests that the EEA1 dimer has a rod-like shape.

There is a correlation between the ability of the C-terminus of EEA1 to dimerize and to bind to Rab5 and early endosomes, whereas the C-terminal FYVE finger binds PtdIns3*P* independently of dimerization (Table 1). Based on this information, we propose the following model for the quaternary structure of EEA1 (Figure 6): two EEA1 monomers intertwine through the extensive coiled-coil regions encompassing most of the molecule, whereas the C-terminal FYVE fingers are free to interact individually with PtdIns3*P* molecules. This overall structure of EEA1 would correspond to an extended dimer with a Rab5-binding site at each end, and a duplicate PtdIns3*P*-binding site at the C-terminus. The interesting feature about this model is that it points out EEA1 as a highly asymmetric dimer, with the PtdIns3*P*-binding sites located distant from the N-terminal Rab5 binding C2H2 zinc fingers. Such an asymmetric shape would be consistent with the proposed role of EEA1 in conferring directionality to endocytic membrane fusion [8].

What is the function of EEA1? EEA1 binds Rab5 and PtdIns3*P*, and the immunodepletion of EEA1 from cytosol reversably inhibits homotypic endosome fusion [8]. This indicates that EEA1 acts downstream of Rab5 and PtdIns3*P* in the regulation of membrane docking or fusion. From the model of EEA1 in Figure 6 we speculate that EEA1 could function in tethering one Rab5-positive compartment to another one, as an early step in endocytic membrane docking. The finding that Rab5 is needed on both membranes for endosome–endosome fusion to occur [22] is consistent with this model. The role of EEA1 may be equivalent to that of two other putative coiled-coil proteins, Uso1p and p115, which appear to function in vesicle docking in endoplasmic reticulum–Golgi traffic [23–25]. The initial docking of endoplasmic reticulum vesicles requires Uso1p and the Rab GTPase Ypt1 [25], and EEA1 and Uso1p could thus have in common that they are both regulated by a Rab GTPase and function in membrane docking. The further functional characterization of EEA1 will require the development of assays that measure docking between Rab5-positive compartments.

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