Eps15 Is Constitutively Oligomerized Due to Homophilic Interaction of Its Coiled-coil Region*

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Eps15 is a member of an emerging family of proteins containing a novel protein/protein interaction domain, the EH domain, of as yet unknown function. Recent findings of Eps15 association with clathrin adaptor complex AP-2 and its localization in clathrin-coated pits have implicated Eps15 in the regulation of vesicle trafficking. Here we show that Eps15 exists in several multimeric states in vivo. When purified recombinant Eps15 or lysates of NIH 3T3 cells were treated with cross-linking reagents, covalent dimers of Eps15 and larger covalent multimers were detected in high yield. Large Eps15 oligomers co-immunoprecipitated with AP-2 at an efficiency higher than that of Eps15 dimers. Furthermore, cross-linking of the membrane-bound fraction of Eps15 in mildly permeabilized cells was as efficient as that of the cytosolic fraction. Size-exclusion column chromatography of recombinantly produced Eps15 and of total cell lysates was performed to examine the equilibrium ratio of the monomers versus the aggregated forms of Eps15. These experiments showed that essentially all the Eps15 was aggregated, whereas monomers of Eps15 could be obtained only under strong denaturing conditions. To map the region of Eps15 responsible for dimerization, fusion proteins corresponding to the three structural domains of Eps15 were prepared. Cross-linking analysis revealed that the central portion of Eps15, which possesses a coiled-coil region (residues 321–520), serves as the interacting interface. The possibility that hetero-oligomeric complexes of Eps15 dimers and AP-2 function during the recruitment of proteins into coated pits is discussed.

The Eps15 protein was originally discovered as a phosphorylation substrate of the epidermal growth factor receptor kinase (1). Subsequent studies revealed that Eps15 is constitutively associated with the plasma membrane clathrin adaptor protein complex AP-2 (2). Immunomorphological analysis demonstrated that membrane-bound Eps15 is mainly associated with the plasma membrane clathrin-coated pits and vesicles (3). Moreover, Eps15 is distributed asymmetrically within the coat, mostly at the rim of the pits and at the neck of the budding vesicles. All these findings suggested that Eps15 might play a role in clathrin-dependent endocytosis.

Further evidence suggesting a role for Eps15 in protein trafficking comes from the analysis of the primary structure of Eps15 and its homology to other proteins. The predicted amino acid sequence of Eps15 identifies at least three structural domains (1, 4). The amino-terminal region is composed of three relatively conserved repeats of ~70 amino acids referred to as EH domains (for Eps15 homology). The central domain of Eps15 presents the characteristic heptad repeats of coiled-coil proteins. The carboxyl-terminal domain displays several Asp-Pro-Phe (DPF)1 repeats, proline-rich sequences capable of interacting with SH3 domains (5), and the binding site for the α-subunit of AP-2 (6, 7). Importantly, an EH domain is found in End3p, a protein required for endocytosis of α-factor pheromone in yeast cells (8). Moreover, a recent report demonstrated that another EH domain-containing protein, PAN-1, is essential for endocytosis in yeast (9). Finally, a partial sequence has been recently obtained for a mammalian EH domain-containing protein that is associated with the γ-subunit of the Golgi clathrin adaptor complex AP-1 and is located in the trans-Golgi network (10). Thus, EH domains might serve a general role in the functioning of the coats operating along different pathways of protein trafficking.

The major components of the clathrin coat, clathrin and AP complexes, are hetero-oligomers (reviewed in Refs. 11 and 12). Therefore, we examined the multimeric state of Eps15, a novel component of clathrin-coated pits. Using chemical cross-linking and size-fractionation techniques, we show that Eps15 is constitutively oligomerized and that the central domain of this protein, containing a coiled-coil region, is involved in intermolecular homotypic interactions.

EXPERIMENTAL PROCEDURES

Materials—The polyclonal antibodies to Eps15 (Ab577) and to β-subunits of AP-2 (Ab532) have been described elsewhere (1, 13). The water-soluble cross-linking reagent bis(sulfosuccinimidyl) suberate (BS3) was purchased from Pierce. All other chemicals were from Sigma, Fisher, or Pharmacia Biotech Inc.

Glutathione S-transferase (GST)-Eps15 protein was produced as follows. Escherichia coli BL21(DE3) pLysS bacteria cultures expressing pGEX2T-GST-Eps15 cDNA containing the entire open reading frame of Eps15 (residues 2–897) (1) were grown in the presence of 0.1 mM isopropyl-1-thio-β-D-galactopyranosidase for 2 h at 30 °C. Bacteria were lysed by freeze-thaw in lysis buffer (50 mM Tris-HCl, 100 mM NaCl, 25% isopropanol). Aliquots of the lysate were mixed with glutathione, and the bacteria were pelleted by centrifugation. The supernatant was mixed with glutathione-Sepharose 4B beads (Pharmacia Biotech Inc.) and incubated for 1–2 h. The beads were washed with water and with 20 mM Tris-HCl, 100 mM NaCl, 0.5 mM isopropyl-1-thio-β-D-galactopyranosidase (the buffer used for elution contains 150 mM NaCl). The GST-Eps15 fusion protein was eluted with 100 mM reduced glutathione in 20 mM Tris-HCl, 100 mM NaCl, 0.5 mM isopropyl-1-thio-β-D-galactopyranosidase. The eluted GST-Eps15 was dialyzed against 20 mM Tris-HCl, 100 mM NaCl, 0.5 mM isopropyl-1-thio-β-D-galactopyranosidase and was stored at 4 °C until use. When necessary, the GST-Eps15 was treated with PreScission protease (Pharmacia Biotech Inc.) to remove the GST sequence.

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1 The abbreviations used are: DPF, Asp-Pro-Phe; BS3, bis(sulfosuccinimidyl) suberate; GST, glutathione S-transferase; FPLC, fast protein liquid chromatography; PAGE, polyacrylamide gel electrophoresis; CMF-PBS, Ca++-Mg++-free phosphate-buffered saline.
Sucrose, pH 8, 1% Triton X-100, 1% Tween 20, 1 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 554 μM iodoacetamide, and 10 μg/ml aprotinin). The GST-Eps15 fusion protein was immobilized on glutathione-agarose beads and eluted by cleavage with thrombin as described previously (1). However, the efficiency of Eps15 binding to the beads was low. Therefore, to obtain large amounts of Eps15, the GST-Eps15 fusion protein was partially purified by gel filtration on a Superose 6HR FPLC column (Pharmacia) equilibrated with 50 mM Tris-HCl, pH 8.0, containing 100 mM NaCl. Because GST-Eps15 elutes as a molecule with a very high molecular mass (see “Results”), fractions between the 8th and 11th ml from pooling the sample (immediately following the void volume) were collected. GST-Eps15 was the major protein in these fractions as assayed by SDS-polyacrylamide gel electrophoresis (PAGE) and Coomassie Blue gel staining.

Cell Culture—Mouse NIH 3T3 cells expressing -4 × 10^4 human wild-type epidermal growth factor receptors/cell were derived by single-cell cloning of pCD1 cells (16). Cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% newborn calf serum and 50 μg/ml gentamicin. Cells were grown in 100-mm dishes (Costar Corp.) and used for experiments when confluent.

Chemical Cross-linking—The GST-Eps15 fusion protein was diazoylated overnight at 4 °C in Ca^2+-Mg^2+-free phosphate-buffered saline (CMF-PBS) before or after cleavage of the GST domain with thrombin. Thrombin digestion of Eps15 purified by gel filtration was carried out by incubating 50 μg of fusion protein with 0.9 μg of thrombin (660 units/mg) in 100 μM of CMF-PBS containing 2.5 mM CaCl_2 for 90 min at room temperature and was terminated by adding 25 mM EDTA. 5-μg aliquots of dialyzed Eps15 protein (final protein concentration of 50 μg/ml) were incubated with 1.0-17.5 mM freshly dissolved BS3 for 30 min at 4 °C. To stop the cross-linking reaction, 250 mM glycine (final concentration) was added, and the mixture was incubated for an additional 10 min. In control experiments, the unrelated proteins β-amylase, bovine serum albumin, and alcohol dehydrogenase (gel-filtration molecular mass markers, Sigma; final concentration of 50 μg/ml) were incubated with 4.6 μM BS3 for 45 min at 4 °C; quenched with glycine; and processed for SDS-PAGE. The proteins were visualized by Coomassie Blue gel staining.

For cross-linking of cellular Eps15, NIH 3T3 cells were washed three times with ice-cold CMF-PBS and solubilized in TGH buffer (1% Triton X-100, 1% sodium deoxycholate, 10% glycerol, 50 mM NaCl, 50 mM Hepes, pH 7.3, 1 mM dithiothreitol, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovandate, 10 mM sodium fluoride, 1 mM phenylmethylsulfon fluoride, 10 μg/ml leupeptin, 544 μM iodoacetamide, and 10 μg/ml aprotinin) by scraping the cells from the dish with a rubber policeman followed by gentle rotation for 10 min at 4 °C. This procedure allows solubilization of ~90% of the total cellular Eps15 and AP-2. Lysates were then centrifuged at 16,000 × g for 10 min at 4 °C. Supernatants were incubated for 30 min at 4 °C with BS3 (3 μM), followed by quenching with 250 mM glycine for 10 min at 4 °C.

Immunodetection of Eps15—Samples of cell lysates or recombinant Eps15 obtained before or after the cross-linking procedure were separated by SDS-PAGE on 3–10 or 3–7% linear gradient gels. In some experiments, cross-linked lysates of NIH 3T3 cells were incubated with antibodies specific to Eps15 (Ab577) or to β-subunits of AP complexes (Ab32) to immunoprecipitate Eps15 or AP complexes, respectively, as described previously (3). After transfer of the protein to nitrocellulose membrane, Eps15 was immunodetected by enhanced chemiluminescence (Pierce) using a polyclonal serum to Eps15 (Ab577) and protein A (Zymed Laboratories, Inc.) conjugated to horseradish peroxidase. The α- and β-subunits of AP-2 were probed with AC1-M11 (15) and Ab31 and Ab32 (13).

Mild Cell Permeabilization—To perform cross-linking of membrane-bound and cytosolic fractions of Eps15, NIH 3T3 cells were mildly permeabilized by incubation in CMF-PBS containing 0.02% saponin, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovandate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors for 30 min at 4 °C. The saponin extract, containing most of the cytosolic components, was collected, and the permeabilized cells were washed with CMF-PBS to remove residual saponin. To cross-link membrane proteins, permeabilized cells were scraped away from the dish with a rubber policeman followed by gentle rotation for 30 min at 4 °C. The reaction was stopped by the addition of glycine (250 mM), and the permeabilized cells were centrifuged at 16,000 × g for 10 min and solubilized in TGH buffer. In parallel, the saponin extract (cytosol fraction) was incubated with the cross-linker at the same concentration and under the same conditions. The TGH lysates and the saponin fractions were centrifuged at 16,000 × g for 10 min, and the supernatants were electrophoresed as detected above for experiments with whole lysates. Eps15 was detected by Western blotting with Ab577.

Gel Filtration—300 μl of NIH 3T3 cell lysates (~1 mg of protein) or partially purified GST-Eps15 fusion protein cleaved with thrombin (~0.2 mg of protein) were cross-linked with BS3 or mock-treated as described above. Samples were then centrifuged at 100,000 × g for 20 min at 4 °C and applied to a Superose 6 column connected to an FPLC system (Pharmacia). Duplicate samples were run in 8 × 1.5 cm gels, 1% SDS, and 5% β-mercaptoethanol at 95 °C for 5 min prior to gel filtration. Native and denatured samples were run under identical conditions. The column was equilibrated with 1% Triton X-100 and 50 mM Hepes, pH 7.4, and run at 4 °C with a 0.2- or 0.1-ml/min flow rate, and fractions (0.4 or 0.2 ml, correspondingly) were collected every 2 min. The fractions were electrophoresed, and Eps15 was detected by Western blotting (see above). The amount of Eps15 protein was quantitated by densitometry. Molecular mass markers for gel filtration were from Sigma or Pharmacia.

Preparation and Cross-linking of Eps15 Fragments—The GST-Eps15 fusion proteins were obtained by recombinant polynucleotide chain reaction of the appropriate fragments from the Eps15 cDNA, followed by cloning in the pTREX2T expression vector in frame with the GST moiety. GST-EH, GST-COIL, and GST-DPF were engineered to contain the three structural domains of Eps15 encompassing amino acid residues 2–330, 321–520, and 501–874, respectively.2 Purification of the fusion proteins on glutathione-agarose beads and thrombin cleavage of agarose-immobilized GST fusion polyepitides have been previously described (1).

BS3 cross-linking experiments were performed on GST fusion proteins (either before or after thrombin cleavage) in CMF-PBS using 5 μg of fusion proteins (final concentration of 0.1 μg/μl) and BS3 (1 mM) for 10 min at room temperature, followed by quenching with 50 mM glycine for 15 min at room temperature. The samples were electrophoresed, and the gels were stained with Coomassie Blue to visualize cross-linking products.

RESULTS

Cross-linking of Eps15—To test the oligomeric state of Eps15, chemical cross-linking analysis of the bacterially expressed GST-Eps15 fusion protein and of detergent lysates of NIH 3T3 cells was performed. Prior to analysis, the fusion protein was cleaved with thrombin to remove the GST moiety, which is capable of homodimerization (16). Full-length recombinant Eps15 was then incubated with the water-soluble heterobifunctional cross-linker BS3. As seen in Fig. 1, immunode-

\[\text{FIG. 1. Chemical cross-linking of recombinantly produced Eps15. A, GST-Eps15 (50 μg/ml) was cleaved (+) or not (−) with thrombin and incubated or mock-treated with 0.3–10.0 mg/ml BS3. Monomeric and oligomeric forms of Eps15 were resolved by SDS-PAGE on a 3–10% gradient gel and detected by immunoblotting. Arrows show the positions of monomeric (M), dimeric (D), and, presumably, tetrameric (T) forms of Eps15. B, β-amylose (final concentration of 50 μg/ml) was incubated or mock-treated with 2.5 mg/ml BS3 for 45 min at 4 °C and resolved by SDS-PAGE followed by Coomassie Blue gel staining. Note that cross-linking leads to a change in the mobility of β-amylose, but does not yield any oligomeric products.}\]
Oligomerization of Eps15

Fig. 2. Chemical cross-linking of Eps15 in lysates of NIH 3T3 cells and in mildly permeabilized cells. A, GST-Eps15 was cleaved with thrombin and incubated with 3 mM BS3. Monomeric and oligomeric forms of Eps15 were resolved by SDS-PAGE and detected by immunoblotting. TGH lysates of NIH 3T3 cells were subjected to cross-linking with BS3 (3 mM) as described under "Experimental Procedures." Aliquots of cell lysates treated with BS3 were incubated with antibodies to Eps15 (Ab577) and to β-subunits of AP-2 (AbSi2). The proteins present in lysates before and after the cross-linking procedure or the immunoprecipitates (IP) of cross-linked lysates were separated by SDS-PAGE on a 3–7.5% linear gradient gel. The Eps15 protein was detected by Western blotting. The migration positions of the monomeric (M), dimeric (D), and tetrameric (T) forms of Eps15 are indicated by arrows. All lanes are from the same gel. The experiment was repeated four times with similar results. B, cells were mildly permeabilized with saponin, and both the saponin fraction (cytosol) and the permeabilized membranes (membranes) were incubated with BS3 as described under "Experimental Procedures." Permeabilized cells were then lysed in TGH buffer. Both membrane and cytosolic protein fractions were resolved by SDS-PAGE on a 3–10% gradient gel, and the Eps15 protein was detected by immunoblotting.

Cross-linked Eps15 migrated on SDS-PAGE as a smear, presumably Eps15 dimers, whereas the monomeric form (145 kDa) was undetectable. With increased concentration of BS3 (Fig. 1A) or with prolonged reaction times (data not shown), larger covalent aggregates were formed. Under similar conditions, cross-linking of unrelated proteins (β-amylose (Fig. 1B) and bovine serum albumin and alcohol dehydrogenase (data not shown)) did not yield covalent oligomers (Fig. 1B), illustrating the selectivity of Eps15 cross-linking.

Cross-linked Eps15 migrated on SDS-PAGE as a smear, probably due to intramolecular cross-linking. Because no reliable high molecular mass marker is available, it was difficult to determine the precise molecular mass of the large Eps15 oligomers that could represent trimers or tetramers of Eps15. However, in the course of the reaction, all monomers were first trapped in dimerized forms (Fig. 1). Therefore, it is likely that the high molecular mass band (indicated with T in Fig. 1) corresponds to the secondary product of cross-linking of two dimers, i.e. tetramers. We will henceforth refer to the large cross-linked species of Eps15 as tetramers to simplify the description of the results.

The treatment of whole lysates of NIH 3T3 cells with BS3 resulted in the redistribution of all immunoreactive Eps15 from the monomeric to the high molecular mass forms (Fig. 1). From this analysis, it is impossible to establish whether the anti-Eps15 immunoreactive high molecular mass forms represent homodimers/oligomers (i.e. containing only Eps15) or heterocomplexes with other cellular proteins. However, the two major bands (indicated with D and T in cross-linked lysates) comigrated with the cross-linked dimers and tetramers of bacterially expressed Eps15 (Fig. 2), thus suggesting that they are composed of homodimers/oligomers, at least in part. In addition, a cross-linking product of ~260 kDa, and thus smaller than the dimer of recombinant Eps15, was detected in cell lysates. The identity of this anti-Eps15 immunoreactive product is not clear, although it is possible that this band represents a dimer of a 130-kDa species of unclear origin (1) that is specifically recognized by the anti-Eps15 antibody (Fig. 2A). Neither epidermal growth factor, which is capable of triggering the tyrosine phosphorylation of Eps15 in vivo (1), nor Ca2+ ions, which possibly bind to the EH domain (4), were able to alter the efficiency of dimerization as assessed by the formation of cross-linking products (data not shown).

Previous studies showed that a sizable pool of Eps15 is co-immunoprecipitated with AP-2 (2, 3) and that direct interaction occurs between Eps15 and the α-chain of AP-2 (6, 7). However, immunoblotting with AC1-M11 or Ab31 antibodies, specific to the amino- or carboxyl-terminal domains of α-subunits, respectively, could not detect any of Eps15-containing products (data not shown). This observation suggests that the cross-linked products of Eps15 do not contain clathrin adapters. Apparently, there are no free amino groups accessible for intermolecular Eps15/AP-2 cross-linking with BS3 within the interaction domains of the carboxyl termini of Eps15 and the α-subunit. On the other hand, tightly associated subunits of AP-2 are readily intercross-linkable (Ref. 17 and data not shown). Under the conditions of our cross-linking experiments, the bulk of AP-2 complexes form large covalent aggregates that do not enter 3% gels. Because the amount of Eps15 immunoreactivity detected by Western blotting in the BS3-treated and untreated lysates was comparable, it is unlikely that any significant amount of Eps15 was covalently bound to these large AP-2 aggregates. Furthermore, the efficiency of Eps15 cross-linking was not affected by the presence of 1 M NaCl, a condition known to disrupt Eps15/AP-2 interaction (data not shown).

Therefore, oligomerization of Eps15 does not depend on the association of Eps15 with AP-2.

When cross-linked lysates were immunoprecipitated with an antibody specific to AP-2 (AbSi2), Eps15 was detected in immunoprecipitates (Fig. 2A). This result suggested that cross-linking did not destruct the interaction of Eps15 and the α-subunit of AP-2. Interestingly, more Eps15-reactive tetramers than dimers (ratio of immunoreactivity of ~3:1) were coprecipitated. Analysis of immunoprecipitated supernatants showed that all tetramers of Eps15 were coprecipitated with AP-2, whereas a pool of dimers remained in the supernatants (data not shown).

In contrast, the tetramer/dimer ratio was similar (~1:1.5 in Fig. 2A) in cross-linked cell lysates and Eps15 immunoprecipitates. It is therefore possible that very large complexes containing two Eps15 dimers and up to four AP-2 complexes exist in the cell. In addition, these complexes might include clathrin, which can be also detected in Eps15 immunoprecipitates (6). Clathrin does not, however, directly bind to Eps15 and apparently coprecipitates with Eps15 via AP-2 (7).

The localization of Eps15 in coated pits and vesicles and its putative role in endocytosis prompted the question of whether Eps15 is dimerized in coated pits and can be efficiently cross-linked in a membrane-bound state. To test this, cross-linking was performed in cells that were mildly permeabilized with saponin. This permeabilization removes the cytosol while preserving the clathrin coats (3) and allowing the BS3 access to membrane-bound Eps15. Incubation of the permeabilized cells (membrane fraction of Eps15) and the saponin extracts (cytosolic fraction of Eps15) with BS3 produced covalent dimers and tetramers of Eps15 (Fig. 2B), thus establishing the involvement of Eps15 in the homotypical intermolecular interactions in coated pits and in the cytosol.

Size-exclusion Chromatography of Eps15—Effective cross-
linking of Eps15 is an indicator of its oligomerization. However, such a technique does not allow for estimation of the equilibrium ratio of the monomeric and oligomeric forms of Eps15. Therefore, gel-filtration chromatography of recombinant and cellular Eps15 was performed.

When recombinant Eps15 was run through a Superose 6 column, the peak of immunoreactivity eluted at the volume corresponding to the globular proteins with a molecular mass of 1–2 \( \times 10^3 \) kDa, with a maximum at 1.3–1.4 \( \times 10^3 \) kDa (Fig. 3A). The large hydrodynamic radius of Eps15 in solution is indicative of a substantial aggregation of the protein. A similar elution volume was observed for the peak of Eps15 when total lysates of NIH 3T3 cells were fractionated under the same conditions (Fig. 3B). This peak may contain either homo-oligomers of Eps15 or its heteroaggregates with other proteins, for instance, AP-2. The similarities in the elution profiles of recombinant Eps15 and Eps15 from cell lysates argue that most of Eps15 in cell lysates is present as stable homo-oligomers, whereas Eps15/AP-2 complexes are not stable under conditions of high pressure gel filtration on Superose 6. The presence of a pool of these complexes in the fractions containing Eps15 cannot, however, be ruled out because AP-2 immunoreactivity is detectable in these fractions (data not shown). The main peak of AP-2 eluted in the void volume, which is consistent with significant aggregation of AP-2 in the TGH lysates.

To determine the apparent elution volume of monomeric Eps15, recombinant Eps15 or cell lysates were denatured to disrupt protein/protein interactions by heating in the presence of SDS and urea as described under “Experimental Procedures.” The denatured samples were then gel-filtrated under conditions identical to those employed for the native samples. Both recombinant and cellular Eps15, under denaturing condition, eluted as peaks with maximum immunoreactivity at 670 kDa (Fig. 3, A and B). The relative positions of the peaks of native and denatured Eps15 did not change in the presence of 1 M NaCl and 0.2% SDS or in the absence of detergent (data not shown).

Thus, the data of gel-filtration experiments are consistent with the stable aggregation of the entire pool of Eps15 under native conditions. An 2-fold difference in the elution volumes of the monomers and the aggregated forms of Eps15 suggests that most of the aggregates represent dimers of Eps15. However, it is technically difficult to determine the Stokes radius of proteins with an apparent size of \( >1 \times 10^6 \) Da under these conditions because the resolution of the gel-filtration column within this range is poor. As seen in Fig. 3, the left shoulder of the peak of native Eps15 was not as sharp as would be expected for the elution profile of a homogeneous protein. This could be due to the presence of hom- and heteroaggregates of Eps15 larger than Eps15 dimers. We verified the elution position of Eps15 dimers and tetramers by size fractionation of cross-linked Eps15. Samples of the lysates of NIH 3T3 cells containing cross-linked Eps15 (dimers and tetramers) and non-cross-linked Eps15 (monomers) were denatured to produce a mixture of these three forms. Fig. 4 shows that the maximum of the peak of covalent tetramers eluted two fractions earlier than that of dimers. Essentially similar elution patterns were observed when cross-linked recombinant Eps15 was subjected to gel filtration after denaturation (data not shown). Peaks of cross-linked dimers and tetramers (Fig. 4) overlapped with the peak of non-cross-linked Eps15 (Fig. 3), suggesting that Eps15 exists as dimers and tetramers in cell lysates.

Mapping of the Domain of Eps15 Involved in Dimerization—To determine the region of Eps15 responsible for intermolecular interactions, several GST fusion proteins were expressed in E. coli. The first polypeptide corresponded to the region of Eps15 encompassing the three EH domains (EH; Fig. 5); the second fusion protein (COIL) contained the central part of the molecule, which has heptad repeats with predicted coiled-coil structure; and the third polypeptide (DPF) encompassed the carboxyl-terminal region, which bears DPF motifs and the AP-2-binding domain (4, 6). In addition, a GST fusion protein encompassing the entire Eps15 open reading frame (full-length) was used. In an initial set of experiments, we attempted to show interaction of native Eps15, present in cell lysates, with agarose-immobilized GST fusion polypeptides. No specific interactions could be detected, even when full-length Eps15 was used (data not shown), and the reaction was brought to a theoretical saturation, as for example, by performing in
proteolysis of DPF.

Low molecular mass bands of GST-DPF and DPF resulted from the cleavage with BS3 (COIL, and DPF). The purified proteins (5 M) are indicated in kilodaltons. Note that GST-DPF and DPF migrated to positions corresponding to ~95 and ~66 kDa, respectively. The low molecular mass bands of GST-DPF and DPF resulted from the proteolysis of DPF.

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\text{FIG. 5. In vitro cross-linking of Eps15 fragments.}\text{ Fragments of Eps15, corresponding to the N-terminal portion (EH), the central portion (COIL), and the C-terminal portion (DPF) of Eps15 depicted in A, were prepared either as GST fusion proteins (GST-EH, GST-COIL, and GST-DPF) or as pure fragments by thrombin digestion of the agarose-immobilized GST fusion proteins followed by FPLC purification (EH, COIL, and DPF). The purified proteins (5 M) were then cross-linked with BS3 (+ lanes) or mock-treated (− lanes) and analyzed by SDS-PAGE and Coomassie Blue staining (B). Molecular mass markers (lane M) are indicated in kilodaltons. Note that GST-DPF and DPF migrated at the positions corresponding to ~95 and ~66 kDa, respectively. The low molecular mass bands of GST-DPF and DPF resulted from the proteolysis of DPF.}
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\text{vitro binding for 24 h at 4 °C.}
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This result contrasted the readily detectable dimerization/oligomerization of native Eps15 (Figs. 1–4) and raised the possibility that lack of in vitro interaction could be due to the pre-existing dimerized/oligomerized state of Eps15 in vitro. To circumvent this problem, we immunoprecipitated Eps15 from cell lysates and performed far-Western blotting on the specific immunoprecipitates with the various GST fusion fragments of Eps15. Under these conditions, one would expect Eps15 to be present as monomer in a denatured state. Again, no specific interaction of Eps15 fragments with full-length Eps15 immobilized on the nitrocellulose membrane could be detected (data not shown). We reasoned that if a domain of Eps15 is involved in dimerization/oligomerization of the full-length protein, this domain might well be present already as a dimer/oligomer when expressed by itself in bacteria. Detection of such a dimeric/oligomeric state could therefore provide indirect proof of the role of that domain in determining dimerization/oligomerization of full-length Eps15 in vitro. To test this possibility, the purified EH, COIL, and DPF domains were subjected to in vitro cross-linking. We performed cross-linking of the domains either in their GST-fused configuration (as a control) or after purification following cleavage from the GST moiety with thrombin. Fig. 5 shows that all fusion proteins could be cross-linked into dimerized forms if the GST domain was present, due to dimerization of the GST moiety. However, cross-linking of the EH and DPF domains after GST cleavage did not change their migration position on SDS-PAGE, indicating the absence of dimerization. In contrast, the COIL polypeptide was effectively trapped into dimerized forms by the cross-linker in the absence of the GST domain. These results suggest that the COIL domain is responsible for stable association of Eps15 monomers and may serve as an interacting interface for the formation of homodimers in the Eps15 molecules.

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\text{DISCUSSION}
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We have employed two different approaches to demonstrate the constitutive oligomerization of Eps15 in vivo and in vitro. Cross-linking analysis showed that Eps15 monomers were barely detectable following a short incubation of purified Eps15, cell lysates, or permeabilized cells with relatively low concentrations of BS3. This is an indication of an effective cross-linking reaction. It is to be noted that the rate-limiting step in this type of reaction is the interaction of the BS3-linked monomer with another monomer (18). The rate of the reaction is thus directly proportional to the proximity of two monomers and is very slow when determined by random encounters (18). Given the very low concentrations of Eps15 in cell lysates, in permeabilized cells (<10^-7 M), and in the in vitro cross-linking (<10^-6 M), our experiments strongly argue for the presence of pre-existing dimers.

This notion is supported by the data from gel-filtration analysis. The apparent size of native Eps15 was two times bigger than that of the denatured protein, indicative of the absence of monomeric forms and of the constitutive multimeric status of Eps15 in solution. The observation that denatured Eps15 elutes as a molecule with an abnormally high molecular mass, compared with globular proteins of similar molecular mass, suggests that Eps15 is an asymmetric protein. The central domain of Eps15, which serves as a dimerization interface for Eps15 dimerization, displays sequence homology to the rod region of myosin heavy chain, suggesting that Eps15 may be a rod-like protein. Interestingly, Eps15 displays an abnormal mobility on SDS-PAGE as it migrates slower (145–150 kDa) than would be expected from its molecular mass (~100 kDa) (1).

Thus, our data demonstrate that Eps15, a novel component of clathrin-coated pits and vesicles, displays similarity in organization to the principal proteins of the clathrin coat, such as the clathrin triskelion and AP-2, which are hetero-oligomers. Although the importance of Eps15 dimerization and oligomerization as well as the function of Eps15 are unknown, several possibilities can be discussed. Data from co-immunoprecipitation analysis suggest that AP-2 is present in excess to Eps15 in the cell (3). This notion is supported by the data of electron microscopy demonstrating that a limited pool of AP-2 located at the periphery of the coat is associated with Eps15 (3). Given the very stable aggregation of Eps15, it is possible to predict that four AP-2 complexes, which might be associated with two Eps15 dimers, will be in close proximity to each other. This, in turn, may affect the function of AP-2 located at the periphery of the coat. It may, for instance, interfere with the polymerization of clathrin at the edge of the coat and limit the growing clathrin lattice, or it may result in an increased curvature of the coat. Another possibility is that dimerization of Eps15 is necessary to increase the affinity of EH domains for interactions with putative EH domain-binding proteins. More important, other EH domain-containing proteins (Eps15R, PAN-1, and YBL047c) also contain extensive coiled-coil regions (4, 9) that might be involved in dimerization. Therefore, homo- and heterodimerization of EH domain-containing proteins may be a general feature important for the functional activity of this novel family of proteins.

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