Parallel Dimers and Anti-parallel Tetramers Formed by Epidermal Growth Factor Receptor Pathway Substrate Clone 15 (EPS15)*

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The recently discovered localization of epidermal growth factor receptor pathway substrate clone 15 (Eps15) to plasma membrane clathrin-coated pits and its constitutive association with the endocytic clathrin adaptor protein complex, AP-2, strongly suggest that Eps15 has an important role in the pathway of clathrin-dependent endocytic traffic. We report here that Eps15 forms dimers and tetramers of distinct shape. The Eps15 dimer is an elongated molecule, 32 nm in length. There is a globular "head" at one end of the molecule and an extended "stalk" of 25 nm which is kinked at about 17 nm away from the head. In the Eps15 dimer, two subunits are arranged parallel to each other, so that the head corresponds to two side by side copies of the N-terminal region I, which contains the three Eps15 homology domains. The proximal part of the stalk is the coiled-coil central region II containing 20 heptad repeats. The kink is at the boundary between region II and the C-terminal region III, which contains the AP-2 binding site, 15 aspartic-proline-phenylalanine repeats, and proline-rich Src homology domain ligand sites. The Eps15 tetramer has a "dumbbell" shape, ~31 nm in length; it is formed by the anti-parallel association of two Eps15 dimers. Formation of these Eps15 tetramers appears to require contacts between regions I of one dimer and regions III of a second apposing dimer. The extended shapes of the Eps15 dimers and tetramers suggest how Eps15 oligomers are located in the clathrin coat. We discuss the implications for accessibility to partners and for proposed functions of Eps15.

In mammalian cells, formation of clathrin-coated pits leads to specialized vesicular traffic for transport of membrane-bound proteins and their ligands from the plasma membrane or the trans-Golgi network to the endosomal compartment (for a recent review see Ref. 1). Coat components such as clathrin and its associated adaptor protein (AP)[1] complexes have been studied extensively, and it is clear that they have important roles in the assembly of the coat as well as in receptor sorting (for a

recent review, see Ref. 2). Eps15 is a newly recognized coat component, which was originally discovered because it is phosphorylated in cells activated by EGF (3). Subsequently it was found to interact *in vivo* with the α-subunit of the endocytic clathrin adaptor AP-2 complex (4–6), and more recently it was discovered that membrane-bound Eps15 colocalizes with endocytic coated pits and vesicles (7, 8). The predicted sequence of Eps15 consists of three distinct regions (3) (see diagram in Fig. 1*A*). Region I, at the N terminus, contains three internal 70–90 amino acid conservative repeats that are homologous to each other, denoted Eps15 homology (EH) domains, whose functions and partners are presently unknown. The central portion, referred to as region II, spans 140 amino acids and contains 20 heptad repeats, characteristic of proteins that form α-helical coiled-coil dimers. Indeed, a recombinant fragment corresponding to this region has recently been shown to form dimers (9). Moreover, in cell extracts of mammalian cells, it has been shown that cytosolic or membrane-bound Eps15 can also form dimers through a homophilic interaction probably mediated by region II, as well as larger oligomers proposed to be tetramers (9). The AP-2 binding site (4, 6), an SH3-ligand site which supports the *in vitro* interaction with the adaptor Crk (10), and 15 aspartic-proline-phenylalanine repeats (3) of unknown function are all located in the C-terminal region III.

Eps15 is believed to have an important role in vesicular traffic, but its function is still unknown. One reason for believing that Eps15's function is important is the conspicuous presence of Eps15 in most, if not all, plasma membrane-coated pits (7, 8). It is prominently located at the rim of forming pits (7), the part of the pit that is thought to be actively engaged in the recruitment of components for coat assembly and for capture of membrane-bound receptors (2, 11, 12). Another reason to suspect that Eps15 is a significant participant in the endocytic pathway comes from genetic evidence suggesting that End3 and Pan-1, two yeast proteins with EH domains, are required for endocytosis in yeast (13–16). A recently described EH domain-containing protein also interacts with the γ-subunit of AP-1, the trans-Golgi network-form of the clathrin adaptor complex suggesting that a homolog of Eps15 may function in the trans-Golgi network (17).

We report here the conformation of dimers and tetramers of Eps15 and describe the relationship between their structural features and the domain organization of the subunits. We have analyzed electron microscopic images of rotary shadowed molecules of recombinant full-length Eps15. These are images resembling "eighth notes" (quavers) and images resembling "dumbbells." From inspection of these images and of fragments corresponding to different combinations of the three regions of Eps15, we derive a physical map of Eps15 and assign structural features of the molecule to specific regions of the polypeptide chain, defined with the help of gel filtration chromatography, chemical cross-linking, and mass spectrometry. The eighth

[1] The abbreviations used are: AP-1 or AP-2, clathrin adaptor protein complexes; BS₃, bis(sulfosuccinimidyl)suberate; EGF, epidermal growth factor; EH, Eps15 homology; Eps15, EGF receptor pathway substrate clone 15; PAGE, polyacrylamide gel electrophoresis; SH, Src homology domain.

notes are Eps15 homodimers containing two parallel subunits. Their total length is ~32 nm. A globular "head" at one end of the molecule is joined to an extended "stalk," often with a kink ~17 nm away from the head. The head contains two copies of the N-terminal region I. The proximal portion of the stalk is a dimeric coiled-coil, formed by region II. The kink joins region II to the C-terminal region III. The dumbbells are Eps15 tetramers, formed by the anti-parallel assembly of two Eps15 homodimers. Each end of the dumbbell is the head of a homodimer. The ends are separated by a 17–18-nm central rod, made of two anti-parallel region II coiled-coils. Thus, each region I head faces region III of the other dimer. We discuss some possible implications of these conformations for the function of Eps15 in clathrin-mediated endocytic traffic.

MATERIALS AND METHODS

Generation of Recombinant Eps15 and Fragments—A human cDNA clone for Eps15 (full) inserted in pCEV29 was kindly provided by Dr. P. P. Di Fiore (Milan, Italy). Four DNA fragments (see Fig. 1*B*) corresponding to (i) the 314 N-terminal residues (region I), (ii) the 538 N-terminal amino acids (regions I + II), (iii) the 566 C-terminal amino acids (region II + III), and (iv) the 412 C-terminal amino acids (region III) were generated by PCR and subcloned downstream of the 6 His-tag into the bacterial pET-28a expression vector (Novagen). The His-tag was added to facilitate the purification of the expressed proteins. All primers sequences are available on request. The construct containing the complete open reading frame of Eps15 (Eps15 (full)) was obtained by insertion of a restriction fragment derived from pET28a/Eps15 (II + III) spanning part of regions II and the complete region III, to the corresponding sites in the vector pET28a/Eps15 (I + II). BL21 cells were transformed with the expression vector constructs to produce Eps15 (full) or its fragments upon induction with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (Sigma) for 4 h at 37 °C (see Fig. 1*C*). Cells were lysed and the His-tagged proteins purified by adsorption to Ni^{2+}-nitrilotriacetic acid-Sepharose beads (18). Bound proteins were eluted with 200 mM imidazole and immediately collected into one-tenth in volume of a solution containing EDTA (30 mM final concentration) to prevent aggregation. Samples were transfer to column buffer (25 mM HEPES, 150 mM NaCl, 0.1 mM EGTA, 0.02%, NaN_3, pH 7.5) by gel filtration using a NAP-5 column (Pharmacia Biotech Inc.) and kept at 4 °C before use.

Electron Microscopy—Single molecule electron microscopy was performed on samples that were glycerol sprayed and rotary shadowed with platinum (19–21). Briefly, 1 μl (~1 mg/ml) of the samples was added to 50 μl of a solution containing 25 mM ammonium bicarbonate and 45% glycerol and then sprayed onto freshly cleaved mica and rotary shadowed with platinum at an angle of ~6–8°. All images were corrected by the average diameter of the platinum grains (~2.5 nm) as described previously (21). Images were obtained with a transmission electron microscope (JEOL 1200EX) operating at 80 kV and primary magnification of 50,000 calibrated with the 4.1 nm repeat pattern of negatively stained T4 phage tails.

Gel Filtration Chromatography—Native Eps15 was obtained from a cell lysate of COS cells grown to confluency in 150-mm Petri dishes. The cells were washed with PBS and then scraped in the presence of 0.4 ml of TGH lysis buffer (50 mM HEPES, pH 7.3, 50 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin) (7). After sonication, the sample was centrifuged at 85,000 rpm for 20 min at 4 °C in a TLA-100.4 rotor (Beckman Optima TLX centrifuge). 0.2 ml of the supernatant (cytosolic fraction; ~3 mg/ml) was then applied to a Superose 6 column (H10/30 Pharmacia), equilibrated in column buffer, and eluted at room temperature at a flow of 0.5 ml/min with 0.5-ml fractions collected per min. The eluted fractions were concentrated by precipitation with 15% (final) trichloroacetic acid followed by SDS-PAGE and Western blot analysis with a rabbit polyclonal antibody raised against a fusion protein of glutathione S-transferase and region III of Eps15.

To analyze the recombinant proteins, 0.2 ml of a solution (at ~1.5 mg/ml) containing purified full-length Eps15 (full) or several of its fragments was loaded on the Superose 6 column, and aliquots from the fractions were analyzed by SDS-PAGE and Coomassie Blue staining.

The elution profile of the column was calibrated with the sizing standards (Sigma) thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa).
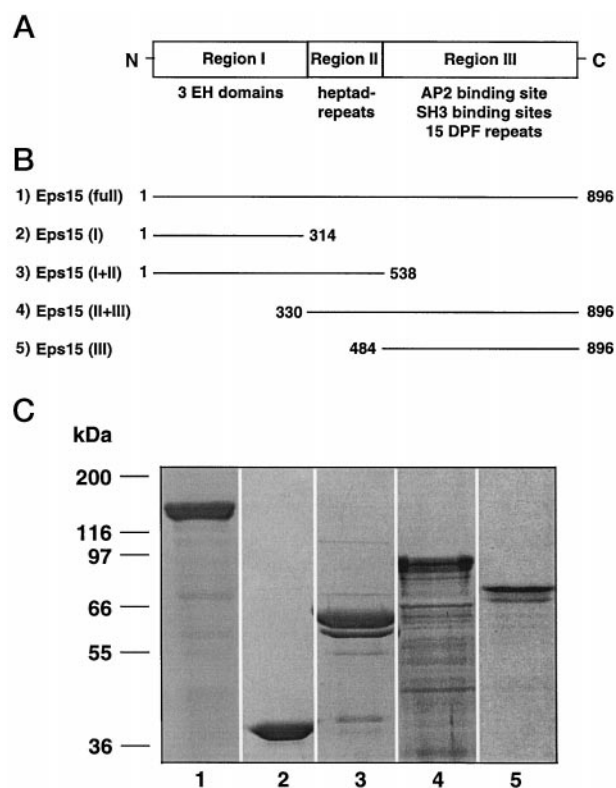

FIG. 1. **Expression of full-length Eps15 and selected fragments.** *A*, schematic representation of the tripartite domain organization of Eps15; the most prominent features within each domain are indicated. *B*, schematic representation of the constructs used in this work; the amino acid residues defining the boundaries of the fragments are indicated. A 6 × histidine-tag was added to the N terminus of all the constructs to facilitate their purification. *C*, SDS-10% PAGE and Coomassie Blue staining analysis of the recombinant forms of Eps15 after their expression in BL21 cells and purification by Ni^{2+}-nitrilotriacetic acid-Sepharose affinity chromatography. *Lane 1*, Eps15 (full); *lane 2*, Eps15 (I); *lane 3*, Eps15 (I + II); *lane 4*, Eps15 (II + III); and *lane 5*, Eps15 (III). Electrophoretic position and size of the markers are indicated.

Chemical Cross-linking—25 μg of a given Eps15 recombinant protein (final concentration of 1.0 mg/ml in 150 mM HEPES, pH 7.0) was incubated with increasing concentrations (between 0.005 and 2.5 mg/ml final) of freshly prepared bis(sulfosuccinimidyl)suberate (BS_3, Sigma) for 2 h at room temperature. The chemical cross-linking reaction was stopped by addition of Tris-HCl (final concentration, 50 mM, pH 7.0), and the cross-linked species were detected by SDS-PAGE and Coomassie Blue staining.

Mass Spectrometry—300 μl (at 1.0 mg/ml) of Eps15 (I + II) free or cross-linked with BS_3 (2.5 mg/ml final) for 2 h at room temperature was transferred into water using a NAP-5 column. The molecular masses of the species present in 2 μl of the samples were determined with a MALDI-TOF mass spectrometer (Perceptive Biosystem Voyager) using sinapinic acid as an acid matrix (Protein Facility, Tufts University).

RESULTS

To assign the structural features of Eps15 to specific regions, we visualized full-length molecules and fragments corresponding to defined regions of Eps15 by electron microscopy. After glycerol spray and platinum rotary shadowing of the samples (Fig. 1), representative results are summarized in the gallery of images shown in Fig. 2. Two classes of views were obtained for recombinant human full-length Eps15 (Eps15 (full), Fig. 2, *a–h*). Images in the first class (*a–d*), representing about 60% of the total, have a globular head, 7 ± 1 nm in diameter, joined to a stalk, 25 ± 3 nm in length (*n* = 15), often with a kink at about 17 nm from the head (*n* = 5). These images resemble musical eighth notes. The remaining 40% of the views (*e--h*) are dumbbells, with two heads of 8 ± 1 nm diameter separated from each

FIG. 2. **Visualization by electron microscopy of rotary shadowed Eps15 and some of its fragments.** The gallery corresponds to images of the complete recombinant Eps15 (Eps15 (full)), selected fragments of Eps15 (Eps15 (I), Eps15 (I + II) or Eps15 (II + III)), and of intact bovine brain clathrin used as a reference. The images were obtained from samples that were glycerol sprayed onto mica followed by rotary shadowing with platinum. The drawings correspond to schematic representations of the images. *Bar*, 20 nm.
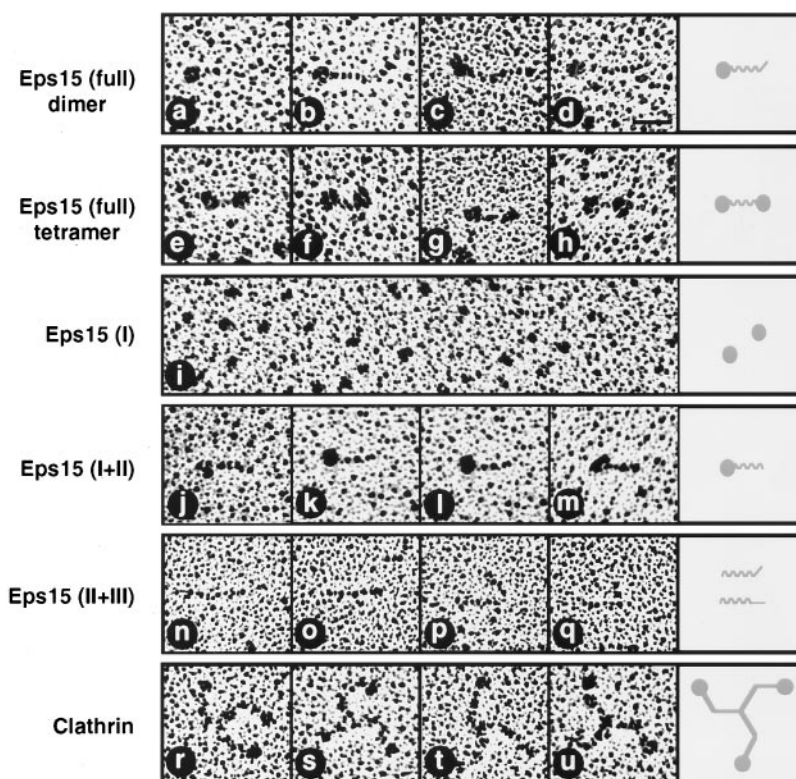
other by a straight rod of 18 ± 3 nm ($n = 7$). As discussed below, the eighth notes and the dumbbells correspond to parallel dimers and anti-parallel tetramers of Eps15, respectively.

The elongated shape of Eps15, deduced from inspection of electron micrographs, was confirmed by determination of its elution behavior when subjected to gel filtration chromatography. The elution profile of recombinant Eps15 (full) (Fig. 3*B*) was the same as the profile of native Eps15 from the cytosol of COS cells (Fig. 3*A*). Eps15 elutes as if it were a molecule larger than the globular ~700-kDa thyroglobulin used as a size marker.

To facilitate assignment of the various regions of Eps15 to the structural features, we examined recombinant fragments corresponding to Eps15 (I), Eps15 (I + II), and Eps15 (II + III). Fig. 2*i* illustrates that Eps15 (I), which contains only region I, has a globular appearance with an average diameter of 7 ± 1 nm ($n = 20$) similar in size to the 52-kDa terminal domain of clathrin (Fig. 2, *r–u*). In solution, Eps15 (I) appears to be a monomer since it elutes at the position expected for a globular protein of about 40 kDa (Fig. 3*C*). Moreover and in agreement with earlier work, chemical cross-linking of Eps15 (I) using BS_3 followed by SDS-PAGE analysis (Fig. 4*B*) yields no new species of higher molecular weight, even after extensive exposure to a relatively high amount of the cross-linking reagent (Fig. 4*B, lane 7*).

Eps15 (I + II) appears as a "head and stalk," where the globular portion has a diameter of 8 ± 1 nm and is linked to a straight stalk of 18 ± 2 nm in length ($n = 20$) (Fig. 2, *j–m*). The elution profile of Eps15 (I + II) is also consistent with an extended structure, since it co-elutes with the ~700-kDa thyroglobulin, rather than with the ~60-kDa bovine serum albumin size marker (Fig. 3*D*). Chemical cross-linking (Fig. 4*C*) of Eps15 (I + II) produced a high molecular weight species in SDS-PAGE, and increasing concentrations of BS_3 chased the monomeric ~60-kDa species into a form running as a relatively sharp band at ~180 kDa. The faint band at ~220 kDa is not present when the protein is purified from a glutathione *S-*
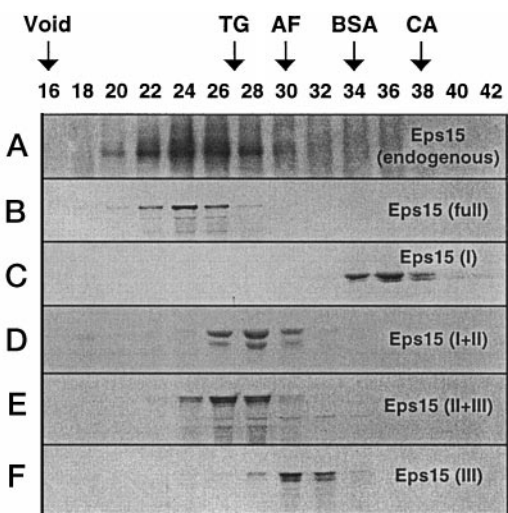

FIG. 3. **Gel filtration chromatography of Eps15 and of selected fragments.** 0.2 ml of samples containing native or recombinant forms of Eps15 were subjected to gel filtration chromatography using a Superose 6 column. Their elution profiles were compared with the behavior of the globular size markers thyroglobulin (*TG*, ~700 kDa), apoferritin (*AF*, ~400 kDa), bovine serum albumin (*BSA*, ~60 kDa), and carbonic anhydrase (*CA*, 29 kDa). *A*, elution profile of native Eps15 present in the cytosol of COS cells and visualized by Western blot analysis. *B–F*, elution profile of recombinant proteins (~1.5 mg/ml) visualized after fractionation by SDS-PAGE and Coomassie Blue staining. *B*, Eps15 (full); *C*, Eps15 (I); *D*, Eps15 (I + II); *E*, Eps15 (II + III); and *F*, Eps15 (III).

transferase-Eps15 fusion by thrombin digestion (not shown) and may be an abnormal aggregate. To resolve whether the 180-kDa oligomer corresponds to a dimer or to a trimer, we determined the true molecular mass of the cross-linked Eps15 (I + II) species by matrix desorption mass spectrometry. Non-cross-linked Eps15 (I + II), used as a control, showed three peaks that corresponded to a protein of 62 kDa with +1, +2,
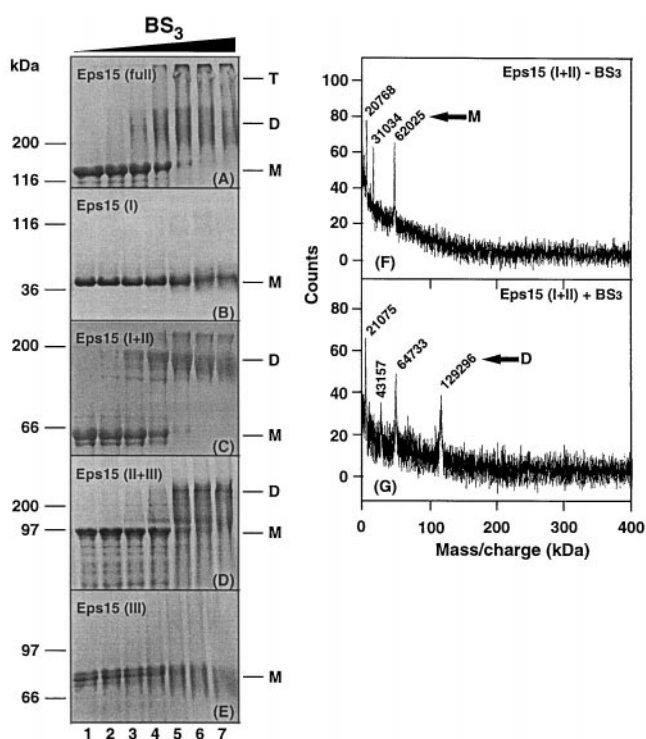
FIG. 4. **Chemical cross-linking and mass spectrometry analysis.** *A–E*, results from cross-linking reactions between increasing amounts of BS$_3$ and 25 μg of Eps15 (full) (*A*); Eps15 (I) (*B*); Eps15 (I + II) (*C*); Eps15 (II + III) (*D*); and Eps15 (III) (*E*) incubated for 2 h at room temperature. The final concentrations of BS$_3$ were 0, 0.005, 0.025, 0.1, 0.5, 1.0, and 2.5 mg/ml (*lanes 1–7*), and the products of the reactions were analyzed by SDS-PAGE followed by Coomassie Blue staining. *F* and *G*, samples of non-cross-linked Eps15 (I + II) (an aliquot from *C, lane 1*) or of Eps15 (I + II) fully cross-linked by treatment with BS$_3$ (an aliquot from *C, lane 7*) were subjected to laser desorption and time of fly-mass spectrometry analysis. *Numbers* above *peaks* indicate the molecular mass (in daltons).

FIG. 5. **Diagrams of Eps15 dimers and tetramers and the possible arrangement of Eps15 at the rim of a clathrin-coated lattice.** *A*, dimers of Eps15 appear as musical eighth notes with two subunits of Eps15 arranged side by side and parallel to each other. *B*, tetramers of Eps15 are shaped as dumbbells and are formed of two dimers aligned side by side and anti-parallel to each other. Region I of one dimer is in contact with region III of the second dimer. *C*, proposal of a model indicating a possible arrangement of Eps15 tetramers at the edge of a clathrin coat.

and +3 charges, which is in excellent agreement with the predicted size of the monomeric fragment (62 kDa) (Fig. 4*F*). In contrast, the sample containing mostly cross-linked Eps15 (I + II) (same as in Fig. 4*C, lane 7*) displays peaks at 129, 65, 43, and 21 kDa and therefore corresponds to different charged forms of the dimer modified with BS$_3$ (Fig. 4*G*). These results indicate that in solution Eps15 (I + II) forms dimers.

We note that the dimensions of the head and the proximal portion of the stalk in the eighth note views of intact Eps15 (full) are similar to those of the corresponding features in dimeric Eps15 (I + II) (Fig. 2, *a–d*). The dimensions of the proximal stalk are consistent with the presence of 20 contiguous heptad repeats in region II forming an extended dimeric α-helical coiled-coil. The length of region II (17–18 nm instead of the expected 21 nm for 20 heptads) might be explained if the head lies to the side of region II rather than at its tip (see diagram in Fig. 5, *A* and *B*). Thus we propose that the heads of the eighth note images contain region I and that the proximal portion of the stalk contains the coiled-coil region II, so that in dimeric Eps15 the chains are arranged side by side and in parallel.

The images of Eps15 (II + III) (Fig. 2, *n–q*) show straight stalks (length of 26 ± 4 nm) often interrupted by a kink at about 16 nm from one end resulting in "V"-shaped views (*n* = 64) (Fig. 2, *p* and *q*). The elution profile of Eps15 (II + III) confirms its extended conformation (Fig. 3*E*), and chemical cross-linking indicates that it is mostly a dimer (Fig. 4*D*). Since the longer portion of the stalk (17 nm) has about the same length (17–18 nm) as the proximal stalk seen in the images of
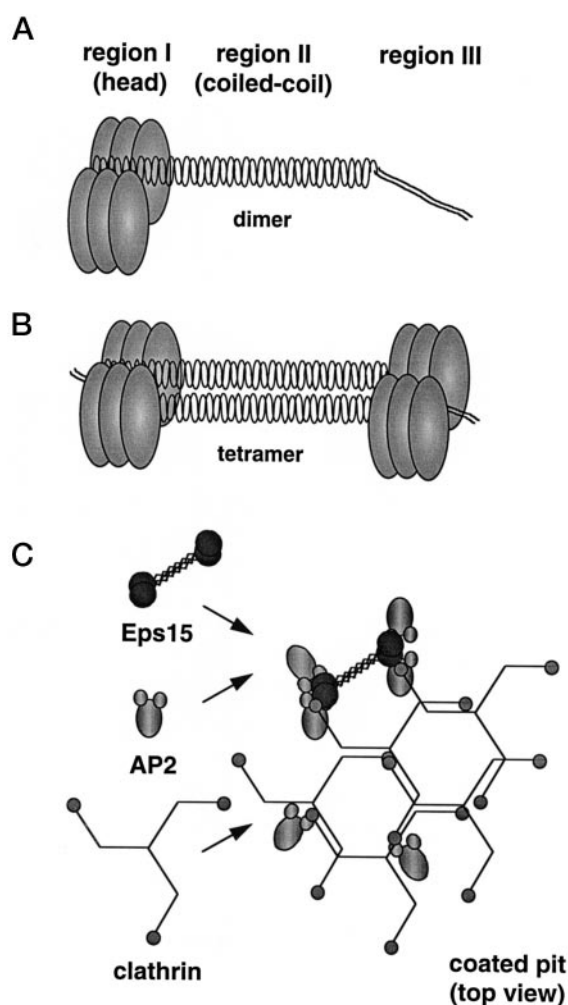
Eps15 (full) and of Eps15 (I + II), we suggest that the kink lies at the boundary between regions II and III. At this point it is not possible to ascertain whether the variability in the angle subtended at the kink reflects flexibility in the joint or whether it simply corresponds to different views of a relatively rigid protein. The extended appearance of the smaller branch of the stalk in Eps15 (II + III) or in full-length eighth note views of Eps15 (full) might indicate that region III has a relatively unfolded conformation. This interpretation is consistent with the hydrodynamic behavior of Eps15 (III) alone (Fig. 3*F*), which co-eluted with apoferritin (a globular protein of ~400 kDa) rather than as a compact protein of ~47 kDa (the true size of Eps15 (III)). Moreover, and as previously shown by a similar *in vitro* experiment, lack of high molecular weight species following extensive cross-linking of Eps15 (III) with BS$_3$ indicates that by itself Eps15 (III) is monomeric (Fig. 4*E*).

Attempts to visualize Eps15 (III) by rotary shadowing were not successful, probably because of the limited contrast afforded by a relatively unfolded monomeric polypeptide chain. As a comparison, clathrin light chains, which are polypeptides of ~25 kDa and also believed to be unfolded in solution, fail to produce enough contrast for visualization using the same plat-

Shape of Eps15

inum-shadowing technique applied here.[2]

Finally, we believe that the dumbbell views of Eps 15 (Fig. 2, *e–h*) correspond to the anti-parallel association of two Eps15 dimers. In these images, each head would correspond to two copies of regions I from one dimer closely apposed to two copies of region III of the second dimer. The straight rod between the two heads therefore corresponds to the side by side anti-parallel arrangement of two coiled coils from region II. The presence of tetramers is consistent with the data obtained by cross-linking of recombinant Eps15 (full) (Fig. 4*A*) and of native Eps15 in the cytosol of NIH 3T3 cells (9). There is a broad band above the position of the monomer, which we interpret as dimer, and a weaker band that barely enters the gel, which might correspond to a tetramer. The extensive side by side contact in the dimers and tetramers probably leads to multiple cross-linking over a significant part of the polypeptide chain and hence to extreme heterogeneity in the migration of cross-linked oligomers.

DISCUSSION

In this study we report the shape of Eps15 and establish the relationships between the structural features of Eps15 and its corresponding domains. We have visualized dimeric Eps15, and we have found that it resembles a musical eighth note. The two subunits are arranged parallel to each other. The head contains two copies of region I; the stalk is a parallel, two strand, α-helical coiled-coil; and the portion beyond the kink contains two copies of region III. We have also visualized the tetrameric form of Eps15. It is dumbbell-shaped, made from two anti-parallel eighth note dimers. Larger oligomers were not detected. Recombinant Eps15, like cytosolic and membrane-bound forms of the molecule, tend to form more dimers than tetramers.

We have found that the three regions of Eps15, defined previously by sequence analysis (3), correspond to the three adjacent structural features visualized by electron microscopy (see schematic diagrams in Fig. 5, *A* and *B*). The N-terminal region I maps to the compact head, the central region II corresponds to the adjacent straight stalk, and the C-terminal region III maps to the extended continuation of the stalk. In the tetramer, four regions II contributed by two dimers overlap to form the central bar of the dumbbell. Since the dumbbell images do not show projections that could correspond to region III, we propose that regions I from one dimer interact with regions III from the other dimer. Thus, contacts between region I and region III seem important to stabilize the tetramer. Contacts between dimers of region II, however, do not seem to be sufficient for tetramer formation. There was no evidence from chemical cross-linking for tetramer formation in samples of either Eps15 (I + II) or Eps15 (II + III), and we could not detect dumbbell images by electron microscopy in samples of Eps15 (I + II), even when they were subjected to chemical cross-linking prior to rotary shadowing (not shown).

The close apposition of regions I and III in the tetramer raises several questions relevant to the function of Eps15. Eps15 becomes phosphorylated in response to stimulation of cells with EGF (3), presumably by the action of the EGF receptor kinase (10). The actual sites of Eps15 phosphorylation are unknown, although a possible tyrosine target in the second EH domain in region I has been suggested (3). Eps15 phosphorylation could therefore modulate the state of tetramerization by

reversibly altering the interaction between regions I and III of two dimers. EH domains are found in many kinds of proteins from yeast to *Drosophila* to man (22). Our results suggest that these domains are involved in oligomerization, since the interaction of the EH domains in region I with region III stabilizes the anti-parallel tetramer. There are 15 aspartic-proline-phenylalanine repeats in region III, and it is possible that these form the EH domain binding site. This is reminiscent of the dimerization of STATS, which occurs via SH2 domain dimerization after phosphorylation (23).

An Eps15 tetramer could interact with up to four AP-2 adaptor complexes, through a contact between the known binding site in region III and the α-ear domain of the AP-2 complex. The dimensions of the tetrameric Eps15 dumbbell put certain constraints on how it could be positioned within a clathrin coat (Fig. 5*C*). Assuming that at least two AP-2 binding sites are satisfied, one at each end of the tetramer, and that region III is close to the heads, then the AP-2 complexes would be separated by at least 17–18 nm. The distance between two vertices in the coat is similar (24, 25), at 15.5 to 16 mm (26). Since Eps15 is located preferentially at the rims of coated pits (7), it is possible that it facilitates the recruitment of APs to the edges of the growing lattice and even that it influences the type of facet (pentagon or hexagon) that forms, defining the local curvature of the coated pit. Other parts of Eps15 might be simultaneously used to recruit molecules that modulate the local properties of the lipid bilayer.

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