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)* 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 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. 1A). 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

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The abbreviations used are: AP-1 or AP-2, clathrin adaptor protein complex; BS, 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 func-
tion 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. 1B) corre-
sponding 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. 1C). Cells were lysed and the His-tagged proteins purified by adsorption to Ni2+-
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 transferred to column buffer (25 mM
HEPES, 150 mM NaCl, 0.1 mM EGTA, 0.02%, NaN3, 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 per-
formed 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 60°. All images were cor-
rected 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
a–h

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 Na3VO4, 0.5 mM phenylmethylsulfonyl fluoride,
and 0.1 mM leupeptin) (7). After sonication, the sample was centrifuged at
50,000 × g (at 1.0 mg/ml) of Eps15 (I + II) free or
cross-linked with BS3 (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 correspond-
ing 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
eight notes. The remaining 40% of the views (e–h) are dumb-
bells, 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. 3B) was the same as the profile of native Eps15 from the cytosol of COS cells (Fig. 3A). 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. 2i 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. 3C). Moreover and in agreement with earlier work, chemical cross-linking of Eps15 (I) using BS3 followed by SDS-PAGE analysis (Fig. 4B) yields no new species of higher molecular weight, even after extensive exposure to a relatively high amount of the cross-linking reagent (Fig. 4B, 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. 3D). Chemical cross-linking (Fig. 4C) of Eps15 (I + II) produced a high molecular weight species in SDS-PAGE, and increasing concentrations of BS3 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-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,
and +3 charges, which is in excellent agreement with the predicted size of the monomeric fragment (62 kDa) (Fig. 4F). In contrast, the sample containing mostly cross-linked Eps15 (I + II) (same as in Fig. 4C, lane 7) displays peaks at 129, 65, 43, and 21 kDa and therefore corresponds to different charged forms of the dimer modified with BS3 (Fig. 4G). 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 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. 3F), 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 BS3 indicates that by itself Eps15 (III) is monomeric (Fig. 4E).

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.
Finally, we believe that the dumbbell views of Eps15 (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. 4A) 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 (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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