Unusual Structural Organization of the Endocytic Proteins AP180 and Epsin 1*

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Epsin and AP180/CALM are important endocytic accessory proteins that are believed to be involved in the formation of clathrin coats. Both proteins associate with phosphorylated membrane inositol lipids through their epsin N-terminal homology domains and with other components of the endocytic machinery through short peptide motifs in their carboxyl-terminal segments. Using hydrodynamic and spectroscopic methods, we demonstrate that the parts of epsin 1 and AP180 that are involved in protein-protein interactions behave as poorly structured flexible polypeptide chains with little or no conventional secondary structure. The predominant cytosolic forms of both proteins are monomers. Furthermore, we show that recombinant epsin 1, like AP180, drives in vitro assembly of clathrin cages. We conclude that the epsin N-terminal homology domains-containing proteins AP180/CALM and epsin 1 have a very similar molecular architecture that is designed for the rapid and efficient recruitment of the principal coat components clathrin and AP-2 at the sites of coated pit assembly.

Clathrin-coated vesicles are involved in a number of membrane transport processes, including receptor-mediated endocytosis, recycling of synaptic vesicles, and sorting of lysosomal enzymes (1). Despite our detailed knowledge of the structural components of the coat and the identity of many endocytic accessory proteins, the molecular events leading to coat formation on the plasma membrane remain elusive. Among the factors considered to be important for coat formation are the neuronal proteins AP180, its ubiquitously expressed homolog CALM (clathrin assembly lymphoid myeloid leukemia protein), and epsin 1 (2, 3). All three proteins have a globular epsin N-terminal homology (ENTH)1 domain in common that is constructed from 8 to 10 α-helical rods (4–6). This domain mediates binding to the rare membrane lipid phosphatidylinositol 4,5-bisphosphate (PI-4,5-P2), which is generally regarded as a major recruiter of components for the endocytic machinery to the plasma membrane (7, 8). AP180 and epsin 1 interact directly with clathrin and the α- and β-appendage domains of the AP-2 adaptor complex (3, 9–14). AP180 binding to clathrin promotes assembly of clathrin triskelia into a population of small cages with a narrow size distribution (15, 16). In neuronal tissue of Drosophila, a knockout of the AP180 ortholog LAP reduces the number of clathrin-coated vesicles; moreover, their size range is much wider than in wild-type flies (17). These studies have led to the suggestion that AP180 and its orthologs might be involved in the control of vesicle size and thus support the notion of playing an important role in vesicle formation. More recently, the potential of AP180 to recruit clathrin to lipid surfaces and to assemble it there was demonstrated with PI-4,5-P2-containing liposomes and lipid monolayers (7). When a combination of AP-2 and AP180 was added to the monolayer, the clathrin lattices became deeply invaginated (7). Whereas the ENTH domains of AP180 and epsin 1 appear to be predominantly important for membrane binding, their carboxyl-terminal segments are designed for protein-protein interactions. Eight tandemly arranged DPW motifs present in epsin 1 mediate its high affinity interaction with the α-appendage domain of AP-2 (9, 12). The association of epsin 1 with the clathrin terminal domain occurs through two clathrin box motifs in the central and carboxyl-terminal parts of the protein (13, 14). The second clathrin box motif is followed by three NPF repeats, which are known to interact with Eps15 homology domains (18). AP180 contains two DPF motifs, which were shown for other endocytic accessory proteins to mediate their interaction with AP-2 (3, 9, 10, 13, 14). In addition, there are three FXDXF motifs present in AP180 that were recently implicated in α-appendage binding (19). The central and carboxyl-terminal segments of AP180 lack typical clathrin box motifs; but instead, five DLL repeats were recently implicated in its clathrin assembly function (11). These findings raise the questions why such multiple tandemly arranged binding motifs are needed and how endocytic proteins utilize them. Are they structurally arranged in a way that they are presented to only one interaction partner at a time, or could they possibly interact with more than one target domain simultaneously? Earlier biophysical studies on AP180 from bovine brain coated vesicles suggested an unusually large Stokes radius (15). Analysis of its circular dichroism spectrum indicated 30% α-helix, 14% β-turn, and 27% β-sheet (20). So far, little is known about the structure of the carboxyl-terminal parts beyond the ENTH domains. Therefore, we have subjected epsin 1 and AP180 as well as recombinant fragments of them to a detailed biophysical analysis using gel filtration, analytical ultracentrifugation, and CD spectroscopy. We conclude that the parts of both proteins that follow their respective ENTH domains are flexible extended polypeptide chains with no relationship to any known conventional secondary structure. Furthermore, we show that epsin 1, like AP180, drives in vitro assembly of clathrin cages and thus might have more functional similarities to other ENTH domain-containing proteins such as AP180 and CALM than previously assumed.

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§ The abbreviations used are: ENTH, epsin N-terminal homology; PI-4,5-P2, phosphatidylinositol 4,5-bisphosphate; GST, glutathione S-transferase.
FIG. 1. Domain structures of epsin 1, AP180, and the expressed recombinant fragments and SDS-PAGE of the proteins used in this study. A, schematic view with binding motifs for AP-2 (DP(F/W) and FXDFX) and clathrin indicated. Known protein-protein interactions, including those with the clathrin amino-terminal domain, involve only the central and carboxyl-terminal regions of the proteins. B, purity of the proteins used for biophysical studies as judged by SDS-PAGE. recomb., recombinant.

EXPERIMENTAL PROCEDURES

Reagents—Restriction enzymes and other reagents for molecular biology were obtained from MBI Fermentas (St. Leon-Roth, Germany) and Roche Molecular Biochemicals (Mannheim, Germany). Calibration standards for gel permeation chromatography and sucrose density gradient centrifugation (MW-GF-1000 kit) were from Sigma (Deisenhofen, Germany). Monoclonal antibody AP180.1 (15) was used to detect AP180. Secondary horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG for immunoblots were purchased from ICN/Cappel (Costa Mesa, CA). The blots were developed with ECL reagent (Amersham Biosciences, Inc., Freiburg, Germany).

Expression of Recombinant Proteins—Epsin 1 DNA in pBluescript was a gift from Hong Chen and Pietro De Camilli (Yale University). The gene for the full-length protein was obtained by cleavage with SstI and ligation into the SsI site of the pET3a expression vector (Novagen, Madison, WI). Epsin 1 was expressed in Escherichia coli BL21(DE3) pLysS (Novagen) with a fusion tag containing thioredoxin and a His6 coding for the carboxyl-terminal end of AP180 was cloned between the SmaI and SsI sites of the pQE32 vector (QIAGEN Inc., Hilden, Germany). The His6-tagged fusion proteins were expressed in E. coli DH5a. The plasmid coding for His6-AP180 was constructed from AP180 clone 36 as described previously (21, 22). A fragment containing the ENTH domain of AP180 was generated from the His6-AP180 plasmid by digestion with Klenow fragment to fill up the recessed 3' termini. The resulting 1250-bp fragment was isolated and cloned into the SalI site of vector pQE31, expressing His6-AP180-(328–745). To obtain a plasmid from which His6-AP180-(328–746) can be expressed, AP180 clone 36 was cut with SsI and HindIII, and the 1907-bp fragment was isolated and cloned between the SsI and HindIII sites of pQE31.

All fusion proteins were purified on an Ni2+-nitrilotriacetic acidagarose affinity matrix (QIAGEN Inc.) according to the manufacturer’s protocol. If not stated otherwise, the bacterial lysates containing the expressed heat-stable His6-epsin 1-(144–575) and His6-AP180-(328–746) fusion proteins were heated in a boiling water bath for 3 min, shock-cooled in blended NaCl and ice water for 5 min, and centrifuged for 15 min at 120,000 g in a Beckman Ti-70 rotor to remove precipitated heat-denatured proteins. The supernatant was incubated with the affinity matrix. To remove the tag from epsin 1, the fusion protein was digested with 8 units/ml thrombin (ICN, Aurora, OH) for 18 h. Epsin 1 and its fragment were further purified by ion exchange chromatography on MonoQ resin. The protein was eluted at pH 8.0 with a 0–0.5 M NaCl gradient buffered with 0.025 M Tris–HCl. Fractions (1 ml) were collected. AP180 from pig brain was obtained as described above (15). All proteins used in this study were finally purified by gel filtration through a Superdex 200 HR10/30 column (Amersham Biosciences, Inc.) equilibrated with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 1.9 mM KH2PO4, and 8.2 mM Na2HPO4). Preparation of Cytosol—Fresh pig brains were obtained from the local slaughterhouse, immediately stored on ice, and processed within 2 h after slaughter. The brains were homogenized in 250 mM sucrose, 25 mM HEPES, 0.5 mM diithiothreitol, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (pH 7.3; 1 ml of buffer/g of tissue) using a Potter S homogenizer (B. Braun Biotech International GmbH, Melsungen, Germany) and centrifuged for 70 min at 125,000 × g in a Beckman Ti-45 rotor. The pellet was discarded, and the supernatant was centrifuged for another 70 min as described above. The clarified cytosol was dialyzed against 50 mM Tris and 100 mM NaCl (pH 7.5) overnight. Finally, the cytosol was centrifuged again for 2 h. When not used immediately, the cytosol was shock-frozen in ethanol and dry ice and stored at −80 °C. Frozen cytosol was rapidly thawed in a water bath at 37 °C and then clarified by ultracentrifugation.

Analytical Gel Filtration Chromatography—Size exclusion chromo-
elution volume of the standard proteins. Plotting Stokes radii against the square roots of the negative decadic logarithms of the distribution coefficients resulted in a straight line (25). This linear relationship was used to translate elution volumes of epsin 1, AP180, and their derivatives into Stokes radii.

Sucrose Density Gradient Centrifugation—To estimate the sedimentation coefficients of proteins in cytosol, we used sucrose gradients (5–20%) made up in 0.5 M Tris-HCl (pH 7.0). The gradients were cast in 5-ml thin-walled ultracentrifuge tubes (13 × 51 mm) using a gradient mixer that was connected to a peristaltic pump. The gradients were overlaid with 200 μl of either pig brain cytosol or a mixture of several standard proteins in 0.5 M Tris-HCl (pH 7.0) at a concentration of 0.14 mg/ml each. We used lysozyme (1.91 S), ovalbumin (3.55 S), bovine serum albumin (4.44 S), lactate dehydrogenase (6.93 S), and catalase (11.20 S) as standards (26). The gradients were centrifuged in a Beckman SW 55 Ti rotor at 40,000 rpm for 18 h and fractionated from the meniscus downwards in 220-μl steps using a micropipette. The fractions were analyzed by SDS-PAGE. The standard proteins were visualized with Coomassie Blue, and the proteins of interest were visualized by Western blotting. The linear relationship between the fraction numbers of the distribution maxima of the standard proteins and their sedimentation coefficients was used to determine the s values of cytosolic epsin 1 and AP180. As an internal control, the sedimentation coefficient of G-actin was determined to be 3.2 S, which is very close to the published value (26).

Heat Denaturation—The susceptibility of epsin 1, AP180, and their fragments to irreversible denaturation was tested by heating them in a boiling water bath for 5 min, followed by shock cooling for 5 min in an NaCl ice water slurry. 10 μg of the respective protein in 100 μl of phosphate-buffered saline and 1 mg/ml glutathione S-transferase (GST) were subjected to the procedure. Precipitated protein was pelleted by centrifugation for 10 min at 90,000 × g in a Beckman Optima TL ultracentrifuge using a Beckman TL-45 rotor. Supernatant and pellet fractions were analyzed by SDS-PAGE and subsequent Coomassie Blue staining.

**Pull-down Experiments and Clathrin Assembly**—Binding experiments with His6-AP180-(328–896) and the immobilized GST-α-appe ndage domain and GST-clathrin terminal domain fusion proteins were performed exactly as described by Scheele et al. (27), as were the clathrin assembly experiments.

**Analytical Ultracentrifugation**—Analytical ultracentrifugation was done in a Beckman/Coulter XL-A analytical ultracentrifuge in either eight- or four-place rotors (An-40 or An-60). Sedimentation rate analysis was done with 12-mm path length double sector centripieces. Sedimentation rate constants were determined by analysis of the boundary movement and were corrected to 20°C and pure water as solvent (s_{20,w}) (28).

**CD Spectroscopy**—CD spectroscopy was performed using a Jobin Yvon Dichrograph III with a bandwidth of 2 nm, a scanning rate of 0.03 nm/s, and a time constant of 2 s. The spectra were evaluated with CDPro software using the expanded reference set of protein spectra as described previously (29).

## RESULTS

### Molecular Dimensions of Epsin 1 and AP180

In the course of purifying recombinant epsin 1, we noted that it migrated anomalously slow upon SDS-PAGE, gave rise to sharp but slightly distorted bands, and eluted from gel filtration columns like a much larger protein than predicted by its sequence. Similar observations were made previously with the endocytic accessory protein AP180 (15, 30). To determine whether both proteins share common structural features in addition to the ENTH domain, we embarked on a detailed structural characterization of epsin 1 and AP180 that also included several functional fragments of both proteins (Fig. 1). We started by fractionating pig brain cytosol on a calibrated gel filtration column to separate the proteins according to their hydrodynamic radii. The square root of the negative decadic logarithm of the distribution coefficients (K_{D}) of macromolecules is linearly related to their Stokes radii (25). Using this relationship, we obtained Stokes radii of 5.3 nm for cytosolic epsin 1 and 7.5 nm for AP180 (Fig. 2 and Table I). These values are characteristic for globular proteins with molecular masses of 230 and 550 kDa, respectively; but according to sequence data, epsin 1 has

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**FIG. 2.** Analytical size exclusion chromatography on calibrated gel filtration columns. A, elution profiles of epsin 1 (C) and AP180 (D) in pig brain cytosol as determined by Western blot analysis of the collected fractions. The elution maxima of the standard proteins are indicated by arrows together with the values for their Stokes radii (R_{S}). B, plots of the Stokes radii (R_{S}) and elution profiles of epsin 1 (B) were calculated from the K_{D} of epsin 1 (B) and AP180 (C) are indicated by arrows.
Hydrodynamic properties of epsin 1, AP180, and their fragments lacking the ENTH domains as determined by different methods

The molecular masses given in the second column were calculated from the compositions based on the amino acid sequences. The last two columns show a comparison of \( s_{20,w} \) values in the presence and absence of the chaotropic denaturant guanidinium chloride. The sedimentation coefficients determined by analytical ultracentrifugation have errors of \( \pm 5\% \). ND, not determined; \( R_g \), Stokes radius.

| Protein                  | Calculated from composition, kDa | Analytical gel filtration, \( R_g \) | Sucrose density gradient centrifugation, \( s_{20,w} \) | Analytical ultracentrifugation |
|--------------------------|----------------------------------|-------------------------------------|-----------------------------------------------------|--------------------------------|
|                          |                                   | \( nm \)                            | \( S \), \( nm \)                                      | Native, \( R_g \) | Denatured, \( s_{20,w} \) |
| Cytosolic epsin 1        | 60.2                             | 5.5 \( \pm 0.6 \)                    | 2.4 \( \pm 0.6 \)                                    | ND               | ND                        |
| Recombinant epsin 1      | 64.6                             | 5.5 \( \pm 0.1 \)                    | ND                                                   | 4.6              | 3.4\( a \)               |
| His\(_6\)-epsin 1-(144–575) | 45.8                        | 4.8 \( \pm 0.1 \)                    | ND                                                   | 4.1              | 2.7 \( b \)               |
| Cytosolic AP180          | 91.4                             | 7.5 \( \pm 0.8 \)                    | 2.5 \( \pm 0.6 \)                                    | ND               | ND                        |
| His\(_6\)-AP180          | 92.3                             | 7.2 \( \pm 0.2 \)                    | ND                                                   | 5.9              | 3.4                       |
| His\(_6\)-AP180-(328–896)| 57.4                             | 6.9 \( \pm 0.1 \)                    | ND                                                   | 5.2              | 2.4\( c \)               |

\( a \) Distribution of \( s_{20,w} \) values between 3.0 and 3.8 \( S \).
\( b \) In 5 \( M \) guanidinium chloride.
\( c \) Distribution of \( s_{20,w} \) values between 2.1 and 2.8 \( S \).
\( d \) In 6 \( M \) guanidinium chloride.

A molecular mass of only \( \sim 60 \) kDa and AP180 of 92 kDa. Assuming that epsin 1 and AP180 are monomers and not associated with any other cytosolic components, these results suggest that epsin 1 and AP180 contain segments that have an extended rod-like structure or that are not compactly folded.

To exclude the possibility that epsin 1 and AP180 form complexes with other cytosolic components, we determined the Stokes radii of purified recombinant epsin 1 and AP180 by analytical gel filtration. We observed that the values for both proteins are very close to the ones determined for their cytosolic forms (Fig. 2). Because the overall structure of the ENTH domain is already known to be globular (4–6), we focused our analysis on the recombinant fragments His\(_6\)-epsin 1-(144–625) and His\(_6\)-AP180-(328–896), which lack the ENTH domain. Both fragments eluted from the gel filtration column only slightly behind the positions of the respective full-length proteins (Fig. 2, B and C). This suggests that the behavior of epsin 1 and AP180 upon gel filtration chromatography is dominated by the structural organization of their carboxyl-terminal segments.

**Sedimentation Properties of Epsin 1 and AP180**—To determine whether the observed large Stokes radii of epsin 1 and AP180 might possibly result from self-association, we analyzed the sedimentation properties of epsin 1 and AP180 in 5–20% sucrose gradients. First, the sedimentation velocities of unfractionated cytosolic epsin 1 and AP180 were examined. After ultracentrifugation, the gradient was fractionated, and each fraction was analyzed by SDS-PAGE and Western blotting with antibodies directed against epsin 1 and AP180. A set of standard proteins with known sedimentation coefficients was used to construct a calibration line from which \( s \) values of 2.4 for epsin 1 and 2.5 for AP180 were obtained (Fig. 3, A and B; and Table I). Considering the molecular masses of both proteins, these \( s \) values are unusually low and suggestive of a very high frictional coefficient. By entering the value for the Stokes radius and that for the \( s \) value into the Svedberg equation, it is possible to estimate the molecular mass of a macromolecule. We did this for cytosolic AP180 and epsin 1 and arrived at molecular masses of 90 and 60 kDa, respectively. These values suggest that both proteins exist in cytosol mainly as monomers.

We next used sedimentation-diffusion equilibrium ultracentrifugation to directly determine the molecular masses of highly purified recombinant epsin 1, AP180, and their fragments lacking the ENTH domains. The data indicate that all examined proteins are predominantly monomers (data not shown). We also analyzed the sedimentation properties of His\(_6\)-epsin 1-(144–625) and His\(_6\)-AP180-(328–896) in strong chaotropic protein denaturants (3–6 \( M \) guanidinium chloride) and observed only very small changes in their frictional coefficients upon denaturation (Table I). The observed hydrodynamic size is consistent with either an extended rod with a thickness of 1.5–2.0 \( nm \) and a length of some 50 \( nm \) or, alternatively, a poorly folded polypeptide chain. Taken together, our results obtained from quantitative gel filtration chromatography and ultracentrifugation strongly suggest that the carboxyl-terminal parts of epsin 1 and AP180 are rather extended, with the consequence that they behave like very large proteins on gel filtration columns and like molecular parachutes during ultracentrifugation.

![Fig. 3. Sucrose gradient centrifugation of pig brain cytosol.](https://example.com/figure3.png)
CD Spectroscopy Reveals Unstructured Polypeptide Chains—

Whereas hydrodynamic methods are suitable for determining the molecular dimensions of macromolecules, they do not, however, tell us whether the molecule is an extended structure such as a rod (e.g., a coiled coil) or an unstructured random coil. To distinguish between these two possibilities, recombinant epsin 1, AP180, and several of their recombinantly expressed fragments were analyzed by CD spectroscopy. The spectra of epsin 1 and AP180 are very similar. Both show typical characteristics of a moderate α-helical content, but are obviously dominated by the signature of random coils. Evaluation of the raw data with CDPro software indicated an α-helical content of 21% for epsin 1 and of 22% for AP180 (Table II). In the spectra of the fragments lacking the ENTH domains (His6-epsin 1-(144–575) and His6-AP180-(328–896)), the α-helical characteristics were almost completely lost, whereas the content of random structures increased from 56 to 66% for the epsin 1 fragment and from 55 to 84% for the AP180 fragment (Fig. 4, A and B). In contrast, in the His6-AP180-(1–329) fragment, which includes the ENTH domain, the α-helical content reached 42%, accounting approximately for the total α-helical content of intact AP180. We also divided the carboxyl-terminal AP180 segment into two fragments and obtained their CD spectra. The first fragment extended from Val329 to Gly745, and the second from Gly745 to Leu896. As expected, the spectra of both fragments revealed little secondary structure; but more important, the sum of the molar ellipticities in the spectra of the three recombinant AP180 fragments (His6-AP180-(1–329), -(328–745), and -(745–896)) was identical to that in the spectrum of recombinant full-length AP180 and similar to that in the spectrum of AP180 isolated from pig brain (Fig. 4D). This observation ruled out the possibility that the lack of secondary structure was an artifact caused by expressing only short fragments that cannot fold properly due to the disruption of intramolecular interactions. The small discrepancies in the β-structure content between recombinant and pig brain AP180 are most likely explained by contaminating proteins present in the AP180 preparation.

Taken together, these results show that besides their respective ENTH domains, epsin 1 and AP180 contain no conventional secondary structure, but are overall unstructured, randomly coiled, and therefore hydrodynamically large polypeptide chains. However, we cannot entirely rule out the possibility that short segments of the polypeptide chains engage in intramolecular interactions, but they seem to lack overall secondary structure and hence also tertiary structure.

**Epsin 1 and AP180 without the ENTH Domains Are**

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**Table II**

Secondary structure contents of recombinant epsin 1, pig brain AP180, recombinant AP180, and recombinantly expressed fragments of both proteins

| Protein                  | Structure | α-Helix | β-Sheet | Turn | Random |
|--------------------------|-----------|---------|---------|------|--------|
| Epsin 1 (recombinant)    | 21        | 10      | 13      | 56   |        |
| His6-epsin 1-(144–575)   | 6         | 16      | 12      | 66   |        |
| AP180 (CCV)              | 18        | 17      | 16      | 47   |        |
| His6-AP180               | 22        | 10      | 13      | 55   |        |
| His6-AP180-(1–329)       | 42        | 9       | 18      | 30   |        |
| His6-AP180-(328–896)     | 5         | 5       | 6       | 84   |        |

**Fig. 4.** Secondary structures of epsin 1 and AP180. A and B, comparison of the CD spectra of full-length AP180 and epsin 1 and their fragments, respectively. The spectra of AP180 are characteristic for proteins with modest α-helical content. In contrast, the spectrum of the ENTH domain of AP180 is dominated by its α-helical content, whereas the spectra of the recombinant carboxyl-terminal portions from both proteins do not match the signatures of known conventional secondary structures. C, comparison of the spectra of recombinant His6-AP180 and AP180 isolated from pig brain clathrin-coated vesicles (CCV). Both spectra are almost identical, suggesting that the unusual CD spectrum is a characteristic not only of the recombinant protein. D, comparison of averaged spectra of full-length AP180 and the sum of the spectra of its recombinant fragments. Both spectra are almost identical, so the observed loss of secondary structure when measuring the carboxyl-terminal parts is not due to disruption of intramolecular interactions. deg, degrees.
Clathrin (1.4 mol) of the clathrin heavy chain (HC) (1.1 mol) and the clathrin amino-terminal domain (GST-TD) of the clathrin heavy chain (HC) (1.1 mol). Immobilized GST beads (1.8 mol) served as a control. The beads were incubated with untreated or boiled 6xHis-AP180-(328–896) in buffer G (25 mM Heps, 125 mM potassium acetate, 5 mM magnesium acetate, pH 7.1) for 30 min. The concentration of the recombinant fragment was 0.73 μM. The beads were washed and recovered by low-speed centrifugation and then analyzed by SDS-PAGE and immunoblotting. Equal amounts of supernatant and pellet fractions were loaded onto the gel. A, pull-down experiments were carried out with the immobilized GST-α-appendage domain (GST-α app.) of the AP-2 adaptor (1.8 μmol) and the GST-amino-terminal domain (GST-TD) of the clathrin heavy chain (HC) (1.1 μmol). Immobilized GST beads (1.8 μmol) served as a control. The beads were incubated with untreated or boiled 6xHis-AP180-(328–896) in buffer G (25 mM Heps, 125 mM potassium acetate, 5 mM magnesium acetate, pH 7.1) for 30 min. The concentration of the recombinant fragment was 0.73 μM. The beads were washed and recovered by low-speed centrifugation and then analyzed by SDS-PAGE and immunoblotting. Equal amounts of supernatant and pellet fractions were loaded onto the gel.

Fig. 5. Carboxyl-terminal segments of epsin 1 and AP180 are heat-stable. A, recombinant epsin 1 and AP180 as well as their fragments lacking the ENTH domains were boiled in the presence of GST and shock-cooled. Denatured precipitated proteins were pelleted by centrifugation. Whereas the full-length proteins were almost completely in the pellet (p), the heat-stable carboxyl-terminal fragments remained in the supernatant (s). B, pull-down experiments were carried out with the immobilized GST-α-appendage domain (GST-α app.) of the AP-2 adaptor (1.8 μmol) and the GST-amino-terminal domain (GST-TD) of the clathrin heavy chain (HC) (1.1 μmol). Immobilized GST beads (1.8 μmol) served as a control. The beads were incubated with untreated or boiled 6xHis-AP180-(328–896) in buffer G (25 mM Heps, 125 mM potassium acetate, 5 mM magnesium acetate, pH 7.1) for 30 min. The concentration of the recombinant fragment was 0.73 μM. The beads were washed and recovered by low-speed centrifugation and then analyzed by SDS-PAGE and immunoblotting. Equal amounts of supernatant and pellet fractions were loaded onto the gel. C, assembly experiments were carried out with heat-treated 6xHis-AP180-(328–896). Clathrin (1.4 × 10^{-10} mol) was incubated on ice in buffer G for 1 h in a final volume of 100 μl with 1.5 × 10^{-10} mol of either untreated or boiled 6xHis-AP180-(328–896). Assembled clathrin was separated from free triskelia by ultracentrifugation. Aliquots of the supernatant and resuspended pellets were subjected to SDS-PAGE. Proteins were stained with Coomassie Blue. Heat-stable—The irreversible denaturation of most proteins upon heating is due to the disruption of native secondary and tertiary structures and concomitant transient exposure of hydrophobic segments. These lead to coagulation of the denatured protein either during heating or upon rapid cooling when the polypeptide chain follows a wrong refolding pathway and becomes trapped in non-native conformations. Therefore, we assumed that where there is no structure, the fragments that lack the ENTH domains ought to remain soluble upon heating in a boiling water bath. To test this hypothesis, we heat-denatured both the amino-terminally truncated fragments of epsin 1 and AP180 and the recombinant full-length proteins, shock-cooled them, and then pelleted any coagulated protein by centrifugation. SDS-PAGE analysis of the supernatant and pellet fractions indeed demonstrated that His6-epsin 1-(144–625) and His6-AP180-(328–896) were both heat-stable, whereas the full-length proteins and GST added as a carrier and internal standard did almost quantitatively precipitate (Fig. 5A). We next tested whether boiling of the recombinant AP180 fragment His6-AP180-(328–896) would compromise its functions. AP180-(328–896) is known to bind the clathrin aminoterminal domain, to associate with the α-appendage domain of the AP-2 adaptor complex, and to induce assembly of clathrin triskelia (11, 31). Binding of the boiled protein fragments to clathrin and the adaptor was assessed by pull-down experiments using the immobilized recombinant α-appendage domain and the clathrin amino-terminal domain as baits. No significant differences between the untreated and heat-treated His6-AP180-(328–896) fragments were observed (Fig. 5B). Similarly, heating did not affect the ability of the fragment to induce clathrin assembly (Fig. 5C).

Epsin 1 and AP180 share little sequence homology. However, we have focused on the accessory proteins epsin 1 and AP180. Both proteins bind to PI-4,5-P2-containing membranes through their respective ENTH domains and to clathrin and AP-2 through short peptide motifs in the carboxyl-terminal segments. Besides the ENTH domain and DP(F/W) peptide motifs, epsin 1 and AP180 share little sequence homology. However, we have shown here that both proteins are monomers with long poorly structured carboxyl-terminal segments that behave like flexible polymers. This manifests itself in unusually large hydrodynamic radii, correspondingly low sedimentation coefficients, and resistance to irreversible heat denaturation. In epsin 1, the
peptide motifs involved in binding the clathrin amino-terminal domain are the type II clathrin box motif 257LMDLADV and the type I motif 480LVDLD (14). It has been suggested that the two chemically distinct motifs recognize different surfaces on the clathrin amino-terminal domain (14). Assuming a fully extended polypeptide chain, the motifs could be as far as 155 nm apart, which corresponds roughly to the circumference of a small clathrin-coated vesicle. The average or effective distance between the clathrin box motifs will probably be considerably shorter than 155 nm, but certainly very much longer than required for an interaction between different surfaces on the same globular clathrin amino-terminal domain, which has a diameter of only ~5 nm (32). This suggests that epsin 1 is designed to engage simultaneously two clathrin amino-terminal domains rather than one. Our observation that epsin 1 can function as an assembly protein supports this conjecture. In between the two clathrin-binding motifs, there are eight copies of the tripeptide DPW, which was shown previously to bind the α-appendage domain of the AP-2 adaptor (12). Their maximal spacing averages 10 ± 2 nm, and they could therefore associate simultaneously with several AP-2 complexes.

AP180 is the most effective clathrin assembly protein (33), and it also associates with the α-appendage domain of the AP-2 adaptor (34, 35). Its flexible carboxyl-terminal segment Val328-Leu896 contains no typical clathrin box motif, but does have five DLL repeats that have been related to the assembly function of AP180 (11). In addition, AP180 contains two DPF peptide motifs, which were shown to be involved in AP-2 binding in amphiphsin, auxlin-1 and -2, and Eps15. Moreover, AP180 contains three FXDXF motifs that were recently demonstrated to mediate binding of the accessory protein HIP1 to AP-2 (19). One of them (640FGDAF) occurs within AP180-(623–680), which binds AP-2 and competes with intact AP180 for AP-2 binding (31).

Several lines of evidences suggest that the interactions between short peptide motifs and their usually stably folded binding partners are of low affinity. For example, despite the apparent high affinity of AP180 for clathrin cages, it proved initially difficult to directly identify the clathrin amino-terminal domain as its contact surface on the heavy chain (36). Only after using recombinantly expressed GST-clathrin amino-terminal domain fusion proteins immobilized at high density on glutathione-Sepharose beads was it possible to prove an interaction between AP180 and the amino-terminal domain (11). This suggests that multimerization of binding sites is required for stable interactions. These might occur either by immobilizing monovalent binding partners on beads or membranes or by their polymerization into supramolecular structures such as a clathrin cage. Accordingly, in most endocytic accessory proteins, short peptide motifs occur in multiple copies. The cooperativity of the interactions provides not only stability, but also specificity. In this scenario, specificity is not born out of a tight key and lock-type fit, but results from many loosely fitting contacts between the interaction partners. Flexibility of the residual structure allows for a random search for additional binding partners without a biased orientation. Recruitment of additional proteins to the ones already present would transform fleeting assemblies into stable structures. This process would be akin to a condensation reaction that consists of a nucleation phase followed by an elongation phase, which, when applied to a clathrin-coated pit, would correspond to the growth of the clathrin lattice. However, the resulting structure could be described as dynamically stable. Upon interfering with individual weak interactions, e.g. by protein phosphorylation at or near the interaction sites or by offering alternative binding partners, the lattice could undergo rapid local and eventually global changes. Thus, by combining multiple weak interactions in a cooperative manner, a dynamic macromolecular structure can be constructed that can also rapidly be remodeled.

Which role do epsin 1 and AP180 play in coat assembly on the plasma membrane? There is already ample evidence that clathrin coat formation on the plasma membrane is linked to lipid metabolism. Current models suggest that locally high concentrations of PI-4,5-P₂ might attract epsin 1, AP180, and AP-2 to select plasma membrane domains (8). With their long flexible polypeptide chains, AP180 and epsin 1 might be more suited to ensnare soluble and membrane-bound coat components than other more structured lipid-binding proteins such as HIP1/HIP1R and the amphiphysins. The affinity of AP-2 for epsin 1 and AP180 might then lead to AP-2 binding and thereby stabilization of a membrane subdomain. Subsequent recruitment of multivalent clathrin triskelia and their polymerization into a lattice will not only fortify the coat, but also extend the coated area (Fig. 7). The efficient cooperation between AP-2 and AP180 in the recruitment of clathrin triskelia to lipid monolayers was recently demonstrated in vitro (7).

Why then are two distinct proteins (epsin 1 and AP180) with similar functions needed? First, our pull-down experiments using the immobilized α-appendage domain and the clathrin amino-terminal domain as baits suggested that epsin 1 binds with somewhat higher affinity to the α-appendage domain compared with AP180 (data not shown), whereas AP180 proved to be slightly more promiscuous with respect to
lipid binding (6, 7). Moreover, both proteins support assembly of clathrin, but only AP180 is also capable of precisely controlling the size of the resulting coat (15, 16). Also, epsin 1 contains the peptide motif NPF, which binds to Eps15 homology domains like that of the accessory protein Eps15, whereas AP180 lacks this capacity, as does its non-neuronal homolog CALM (37). Taken together, this suggests a division of labor between epsin 1 and AP180 in recruiting additional coat components.

Almost all of the known endocytic accessory proteins interact with the clathrin amino-terminal domain and with the α-appendage domain of AP-2. Immunofluorescence studies of fixed cells suggest their collective presence in each of the clathrin-positive fluorescent dots. Because these proteins cannot possibly all be present in stoichiometric amounts at the same time, it is likely that they might act not only at different places in a growing lattice, but possibly also sequentially, as would be expected for a vectorial process. Determining the order of the protein-protein interactions that keep the endocytic machine running will constitute a major challenge for the future.

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