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Communication

Assembly of Clathrin Coats Disrupts the Association between Eps15 and AP-2 Adaptors*

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Eps15 is a phosphorylation substrate of the epidermal growth factor receptor kinase. *In vivo*, it is largely found in complex with AP-2, the plasma membrane clathrin adaptor protein complex. Although AP-2 is uniformly distributed across the surface of clathrin-coated pits and vesicles, Eps15 is preferentially found in the rims of endocytic clathrin-coated pits (1). This observation suggests that Eps15 may disengage from AP-2 during coat formation. Here we use two new anti-Eps15 antibodies to show that, contrary to our own earlier suggestion, coated vesicles isolated from brain do not contain detectable amounts of Eps15. Furthermore, when AP-2 complexes that are saturated with Eps15 are used for *in vitro* assembly of clathrin-AP-2 coats, normal structures are formed that contain the expected amounts of clathrin and AP-2, but the amount of Eps15 present is dramatically lower than that of AP-2. We propose that during coated pit formation, addition of clathrin to the growing edge at the rim of the pit releases Eps15 from AP-2.

During the past decade, several groups have extensively studied the protein components and the mechanism responsible for the formation of clathrin-coated pits. Clathrin and its tetrameric adaptors AP-1 and AP-2 are among the best characterized structural elements of the coat (for recent reviews, see Refs. 2–4). The APs recruit clathrin, and they facilitate coat formation through its association with clathrin. The interaction involves a short segment located in the hinge region of the β chain of AP-2 (5) and the terminal domain of clathrin.[1] Recently, it was suggested that Eps15 (6), a ~100-kDa molecule that forms complexes with AP-2 (7–9), is involved in the clathrin-mediated internalization pathway. Eps15-AP-2 complexes are found both in the cytosol and at the plasma membrane (1, 9, 10). In solution, the interaction between Eps15 and AP-2 involves a short segment toward the C terminus of Eps15 and the C-terminal ear domain of the α chain of AP-2 (7, 8). Most of the Eps15 in the cell is bound to AP-2, whereas ~80% of AP-2 is free (1). The membrane-bound Eps15/AP-2 complexes are mainly found within clathrin-coated pits (1, 10), usually at the rims and growing edges of coated pits (1).

The formation of coated pits at the plasma membrane requires AP-2 complexes and clathrin to be recruited to the membrane and a poorly understood nucleation event that initiates clathrin coat assembly. After nucleation, new clathrin and AP-2 complexes presumably add to the free edges of the pit, so that an AP-2 complex that is added to the pit early on will move progressively deeper into the pit. How is it, then, that Eps15 is concentrated at the edges of pits, regardless of the state of completion of the pit? We previously examined whether Eps15 is present in coated vesicles (1) and found a band of the approximately correct molecular weight in gels of purified bovine brain vesicle preparations. However, this band was not conclusively identified as Eps15, and in the absence of purified Eps15 quantitation was impossible. We recently used recombinant Eps15 to generate polyclonal anti-Eps15 antibodies (11). Here we readdress the question of the quantity of Eps15 present in complete vesicles. We estimate that less than 1 in 1000 AP-2 complexes in vesicles carry Eps15.

To further examine how Eps15 behaves during the process of coat formation, we made 1:1 complexes between recombinant Eps15 and purified AP-2 and determined their ability to incorporate into clathrin coats assembled *in vitro*. We found that the coats formed by Eps15-AP-2 complexes are indistinguishable from coats formed in the absence of Eps15 but that the AP-2 complexes present in the coats are significantly depleted in Eps15. We suggest that assembly of clathrin into the coat results in the disengagement of Eps15 from AP-2.

EXPERIMENTAL PROCEDURES

Coat Proteins and Antibodies—Clathrin-coated vesicles were isolated from calf brains, and clathrin and AP-2 were purified using previously described procedures (12–14). Recombinant Eps15 was made in *Escherichia coli* and purified by nickel-nitriloacetic acid chromatography (11). Stock solutions of clathrin (3 mg/ml), Eps15 (1 mg/ml), and AP-2 (0.7 mg/ml) were used as starting materials for the experiments described here. Protein amounts were estimated by BCA or by SDS-PAGE[2] and Coomassie Blue staining using purified clathrin and bovine serum albumin as standards. Polyclonal antibodies specific for the N-terminal 538 amino acids or for the C-terminal 368 residues of Eps15 were generated using the corresponding glutathione *S*-transferase fusion proteins as antigens. The monoclonal antibody specific for the $\beta1/\beta2$ subunits of AP-2 was 6A (15).

Gel Filtration Chromatography—Prior to gel filtration, the samples were dialyzed overnight at 4 °C against coat preassembly buffer (33 mM MES, 8 mM Hepes, 167 mM Tris, 100 mM NaCl, 0.67 mM EGTA, 0.02% NaN_3, 0.08% Triton X-100, pH 7.4) or against coat assembly buffer (100 mM MES, 2 mM EDTA, 2 mM DTT, 0.05% Triton X-100, pH 6.6). After centrifugation at 15,000 rpm at 4 °C for 10 min, the supernatants (250 μl) were applied to a preparative grade Superose 6 gel filtration column (H10/30, Pharmacia Biotech Inc.) equilibrated with coat preassembly or with coat assembly buffer. The samples were eluted at a flow of 0.5 ml/min. 0.5-ml fractions were collected and processed for SDS-6% PAGE and Coomassie Blue staining or Western blot analysis.

Coat Assembly—The *in vitro* assembly of coats was performed as described earlier (12) using 1 mg/ml clathrin and 0.25 mg/ml AP-2, in the absence or the presence of 0.3–0.6 mg/ml Eps15. After overnight dialysis at 4 °C against coat assembly buffer, the samples were subjected to a low speed centrifugation (15,000 rpm for 10 min; Eppendorf

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[1] A. Contreras and T. Kirchhausen, manuscript in preparation.

[2] The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; MES, 4-morpholineethanesulfonic acid.

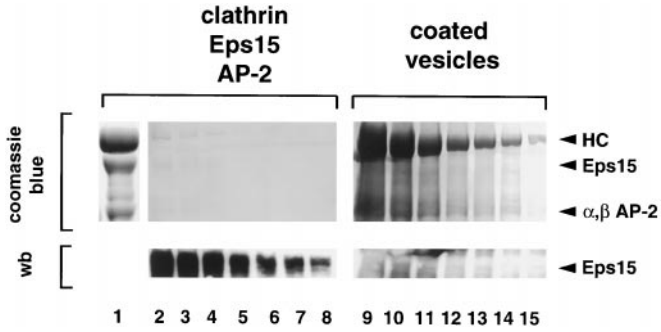

FIG. 1. **Abundance of Eps15 in clathrin-coated vesicles.** Samples corresponding to a control mixture of purified clathrin, recombinant Eps15 and AP-2 (*lanes 1–8*) or to coated vesicles (*lanes 9–15*) were subjected to SDS-6% PAGE fractionation to estimate the amount of Eps15 present in the coated vesicle sample. *Lane 1* contained ~20 μg of clathrin, 5 μg of Eps15, and 5 μg of AP-2. *Lanes 2–8* correspond to 2-fold serial dilutions of the clathrin-Eps15/AP-2 mixture starting with 300 ng of clathrin, 150 ng of Eps15, and 75 ng of AP-2. *Lanes 9–15* correspond to a 2-fold serial dilution of the coated vesicle sample. *Lane 9* has 2.5–5 μg of AP-2. The *top panels* correspond to a gel stained with Coomassie Blue. The *bottom panels* correspond to Western blots (*wb*) from second gels stained with the polyclonal antibody C-Eps15 #2. Similar results were obtained with the antibody N-Eps15 #2 (not shown). The *arrows* indicate the positions for clathrin heavy chain (*HC*) (~180 kDa), Eps15 (~140 kDa), and the α and β subunits (~100 kDa) of AP-2.

centrifuge) followed by a high speed centrifugation step (60,000 rpm for 12 min; TLA 100.4 rotor Beckman). The pellets were resuspended into the same starting volume with coat assembly buffer and processed for SDS-PAGE and Coomassie Blue staining or for negative staining. Assembly of coats was confirmed by direct electron microscopic observation of samples negatively stained with 1.5% uranyl acetate (5). Images were obtained at a primary magnification of 50,000.

RESULTS AND DISCUSSION

The Amount of Eps15 in Coated Vesicles Is Surprisingly Low—Electron microscopic observations of cell sections indicate that, regardless of coat size, the Eps15 present in a coated pit is concentrated at the rim (1). The question of how much Eps15 is present in a coated vesicle was left open, however. A band with a molecular weight appropriate for Eps15 was observed on a Western blot, but no purified Eps15 standards were available to allow firm identification or quantitation of the signal. Because recombinant Eps15 is now available (6, 7, 11), we combined Coomassie Blue staining and Western blot analysis to estimate the content of Eps15 in coated vesicles by comparison with known amounts of Eps15. The results, depicted in Fig. 1, indicate a content of less than 3 ng of Eps15 for ~20 μg of clathrin in the coated vesicle sample (*lane 9*). The amount of Eps15 was estimated by comparison with the signal elicited by Western blot in the Eps15 standard (*lane 8*). The amount of AP-2 in the same coated vesicle sample (*lane 9*) is ~3–5 μg. Because the proportion of AP-2 complexes in the cytoplasm that carry Eps15 is ~10%, it appears that the AP-2 complexes recruited into the vesicle have either been selected to lack Eps15 or have lost Eps15 as part of the assembly process. The ratio of AP-2 complexes to Eps15 is ~1,000:1 in the coated vesicle sample, and because each coated vesicle contains an average of ~30 AP-2 complexes, only one vesicle in 30 contains an Eps15 molecule.

Eps15 Associates Stably with AP-2—To determine whether the association between Eps15 and AP-2 s is perturbed by the assembly process, we first made Eps15-saturated AP-2. Recombinant Eps15 at 0.3 mg/ml was mixed with purified bovine brain AP-2 complexes at 0.25 mg/ml and assayed by gel filtration. After mixing, the AP-2 complexes quantitatively co-elute with Eps15 (*Eps15 + AP-2*, Fig. 2*A*); Eps15 is in excess over AP-2 by Coomassie Blue stain. Thus, all of the AP-2 complexes in this preparation are bound to Eps15 under the initial coat

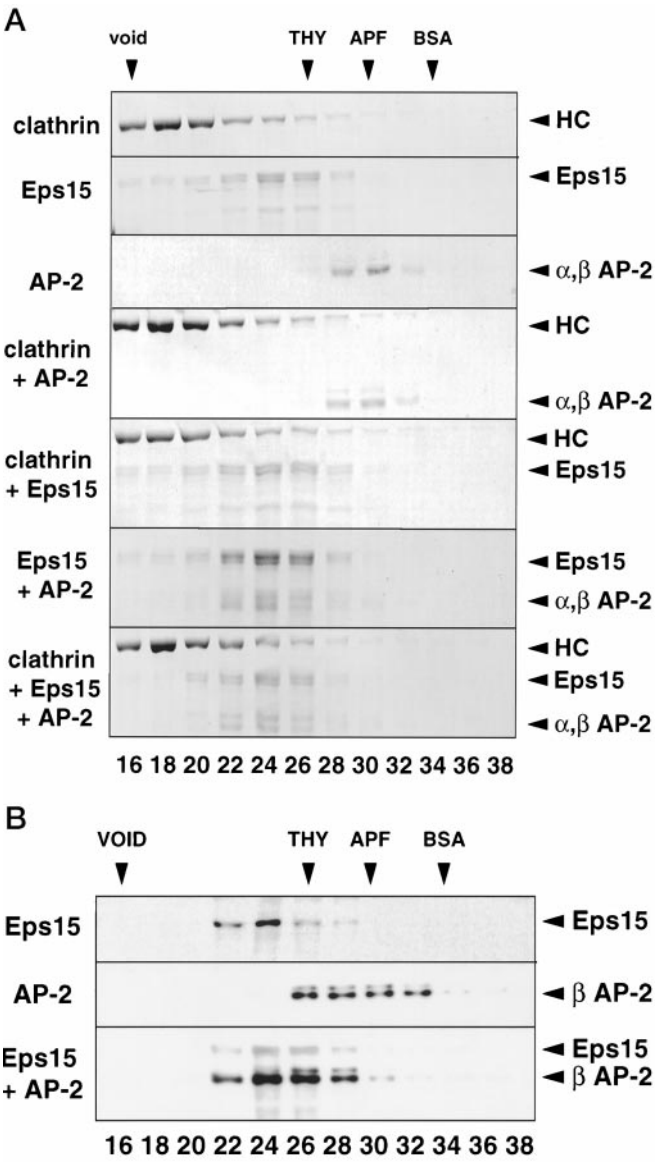

FIG. 2. **The complexes of Eps15 and AP-2 are stable.** Samples of purified clathrin, Eps15, and AP-2, either alone or as mixtures, were subjected to gel filtration chromatography using a Superose 6 column. Aliquots of the eluted fractions were analyzed by SDS-6% PAGE. *A*, the samples were dialyzed overnight against preassembly coat buffer followed by gel filtration chromatography on a Superose 6 column pre-equilibrated with the same buffer. Aliquots of the resulting fractions were subjected to SDS-PAGE followed by Coomassie Blue staining. *B*, the indicated samples were dialyzed overnight against coat assembly buffer and then applied to the Superose 6 column pre-equilibrated in the same buffer. Aliquots from the eluted fractions were subjected to SDS-6% PAGE and Western blot analysis using the polyclonal antibody N-Eps15 #2 specific for Eps15 and the monoclonal antibody 6A specific for the β1/β2 subunits of AP-2. The peak positions for the void and the globular sizing standards thyroglobulin (*THY*, 670 kDa), apoferritin (*APO*, 440 kDa), and bovine serum albumin (*BSA*, 66 kDa) are indicated.

preassembly buffer conditions. The AP-2-Eps15 complexes elute at the same fractions as free Eps15 (Eps15; Fig. 2*A*), indicating that the Stokes radius of the complex is not significantly different from that of Eps15 itself (16). We have recently shown that Eps15 forms parallel dimers and anti-parallel tetramers of ~31 nm in length (11), and so this result is not surprising. Presumably this result indicates that the globular AP-2 complexes (~60-nm diameter) (17) do not bind at the extreme ends of the molecule.

As a control, we also examined whether clathrin could affect

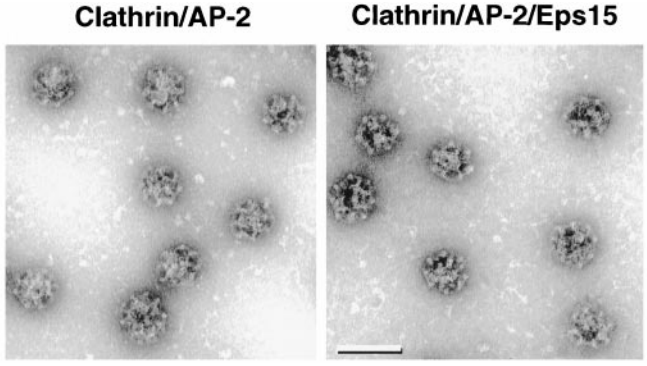

FIG. 3. **Assembly of clathrin-AP-2 coats is not affected by the presence of saturating amounts of Eps15.** Electron microscopic views show negatively stained samples of clathrin-AP-2 coats that were assembled *in vitro* by dialysis against coat assembly buffer. These samples belong to the experiment described in Fig. 4. The *left panel* corresponds to coats from a control sample containing 1 mg/ml of clathrin and 0.25 mg/ml of AP-2 (*lane 8*, Fig. 4). The *right panel* correspond to an equivalent sample supplemented with 0.6 mg/ml of Eps15 before initiation of coat assembly (*lane 12*, Fig. 4). The number of coats formed under these conditions is unaffected, and their geometry is normal. *Bar*, 100 nm.

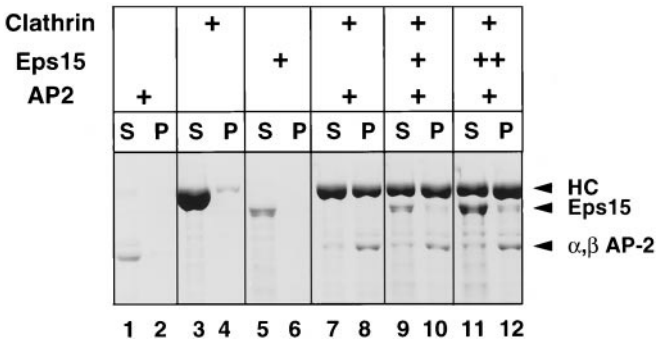

FIG. 4. **Displacement of Eps15 from AP-2 upon *in vitro* formation of clathrin-AP-2 coats.** Samples of clathrin, Eps15, and AP-2, either alone or in combination, were transferred from precoat to coat assembly buffer by overnight dialysis. Clathrin coats were assembled, and after removal of aggregated material by low speed centrifugation, the coats were separated from nonassembled material by high speed centrifugation. Aliquots of the resulting supernatants (*S*) and pellets (*P*) were analyzed by SDS-PAGE and Coomassie Blue staining. Clathrin at 1 mg/ml and AP-2 at 0.25 mg/ml were used in all experiments. Saturating amounts of Eps15 were 0.3 mg/ml (*lanes 5*, *6*, *9*, and *10*) and at 0.6 mg/ml (*lanes 11* and *12*), respectively.

the interaction between Eps15 and AP-2 or associate directly with Eps15 in the same coat preassembly buffer. No perturbation of the Eps15-AP-2 interaction was seen under buffer conditions that prevent coat assembly, and no shift in the elution profile of Eps15 was detected in response to the presence of clathrin (*clathrin + Eps15* and *clathrin + Eps15 + AP-2*, Fig. 2*A*).

We next examined whether the Eps15-AP-2 complex is stable when they are transferred to a solution that facilitates *in vitro* coat formation (12, 14). As expected (18), the AP-2 complexes aggregate under coat assembly conditions in the absence of clathrin, and therefore the amount of material available for gel filtration analysis is reduced. We therefore analyzed the fractions from the gel filtration experiment by Western blot. The gel filtration experiment in Fig. 2*B* shows no apparent perturbation of the AP-2-Eps15 complex.

Eps15 Is Lost during Coat Assembly—We next asked whether the Eps15-saturated AP-2 complexes can participate in the formation of clathrin coats. Under the saturating conditions used above, the presence of Eps15 does not interfere with the efficiency of coat formation. Negative stain electron microscopy (Fig. 3) shows that the normal barrel-shaped geometry of the clathrin-AP-2 coats was maintained in the presence of Eps15. Eps15 also had no effect on the efficiency of coat formation as estimated by counting the number of coats present in four random fields in the absence of Eps15 (91 ± 11 coats/field) or in the presence of Eps15 (86 ± 1 coats/field). We isolated the coats by high speed centrifugation and determined the relative amounts of Eps15, clathrin, and AP-2 by SDS-PAGE and Coomassie staining. The amount of AP-2 in the pellet is unaffected by the presence of excess Eps15 (compare *lane 8* with *lanes 10* and *12* in Fig. 4), but the ratio of Eps15 to AP-2 is very low, far from the stoichiometric ratio expected if Eps15-saturated AP-2 complexes are incorporated into coats without perturbation. Doubling the amount of Eps15 present in the coat-forming reaction may slightly increase the ratio of Eps15 to AP-2 in the pellet, but the ratio is clearly still far from 1:1. We propose, therefore, that the assembly of a clathrin coat interferes with the interaction between Eps15 and AP-2, such that the majority of AP-2 complexes loose their associated Eps15s. Glycerol gradient rate zonal centrifugation analysis confirms that the clathrin coats formed under these conditions have very little Eps15 compared with the amount of AP-2 present in the fractions containing the coats (not shown).

Conclusions—We have shown that the interaction between AP-2 complexes and clathrin is severely disrupted by the coat assembly process. Several interpretations of this observation are possible. For example, it is possible that the coat forming process selects only AP-2 complexes that are free of Eps15 and that this selection drives the dissociation of AP-2 and Eps15. However, such an interpretation would be inconsistent with the electron microscopic observations that show Eps15 located at the rims of growing coated pits (1). A second possibility is that AP-2-Eps15 complexes can bind at the rim of a coated pit, captured (for example) by AP-AP or AP-transmembrane protein cytosolic tail interactions and that clathrin binding then displaces Eps15 from the AP-2 complex. This displacement could be the result of an allosteric change in AP-2 due to clathrin binding (14). Alternatively, it could be due to steric or competitive interactions between clathrin and Eps15 on AP-2 upon binding to each other that results in the disruption of the contact between AP-2 and Eps15. Eps15 and clathrin bind to sites in AP-2 that are very close to each other (17): the α ear, the binding site for Eps15 (7, 8), is only 2–10 nm from the β hinge, the binding site for clathrin (5). Eps15, at 31 nm in length (11), could easily span this distance, and the terminal domain of clathrin is ~7 nm in diameter (19). It is known, however, that clathrin induces a conformational change in the AP-2 complex upon binding and co-assembly to form the coat (14); this conformational change may also be involved in the release of Eps15 from the AP complexes at the growing face of the vesicle.

In studies of the intracellular distribution of Eps15 by immuno-electron microscopy, we observed antibody labeling not only at the rims of coated pits but also in structures we scored as coated vesicles (1). Such profiles are often cross-sections of coated pits (20), and in view of the biochemical data presented here we believe that this ambiguity is likely to have accounted for at least some of the apparent coated vesicle images.

Eps15 appears at the rim of plasma membrane coated pits (1). The results described here suggest a cyclic recruitment and disengagement of Eps15. Eps15 interacts with a variety of other molecules including proteins involved in signal transduction (21) and possibly in lipid modification (see review in Ref. 22). We therefore propose that Eps15 is an antenna to attract these partners to the growing edge of a clathrin-coated structure. An alternative function, that Eps15 participates in the targeting of AP-2 to clathrin lattices, appears less likely, be-

cause chimeric AP-2 adaptors lacking the Eps15 interaction site are still delivered to the plasma membrane. The activities of the Eps15 partners are therefore likely to be important in the regulation of endocytic membrane traffic.

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