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Communication

Eps15 Is a Component of Clathrin-coated Pits and Vesicles and Is Located at the Rim of Coated Pits*

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Eps15, a phosphorylation substrate of the epidermal growth factor (EGF) receptor kinase, has been shown to bind to the α-subunit of the clathrin-associated protein complex AP-2. Here we report that in cells, virtually all Eps15 interacts with the cytosol and membrane-bound forms of AP-2. This association is not affected by the treatment of cells with EGF. Immunofluorescence microscopy reveals nearly absolute co-localization of Eps15 with AP-2 and clathrin, and analysis by immunoelectron microscopy shows that the localization of membrane-associated Eps15 is restricted to the profiles corresponding to endocytic coated pits and vesicles. Unexpectedly, Eps15 was found at the edge of forming coated pits and at the rim of budding coated vesicles. This asymmetric distribution is in sharp contrast to the localization of AP-2 that shows an even distribution along the same types of clathrin-coated structures. These findings suggest several possible regulatory roles of Eps15 during the formation of coated pits.

Clathrin-coated membranes represent specialized organelles involved in the formation of transport vesicles that traffic from the plasma membrane or the *trans*-Golgi network to the endosomal compartment (1–3). At the plasma membrane, clathrin-coated pits serve to selectively concentrate and internalize proteins destined to be retrieved from the surface, in some cases to deliver nutrients to the cell and in others to down-regulate the surface expression of membrane receptors. At the *trans*-Golgi network, this organelle operates in the secretory pathway leading to lysosomal targeting of several lysosome-resident proteins.

The assembly of clathrin into basket-shaped lattices on the cytosolic side of the membrane initiates the formation of the coated pit, and a section of captured membrane becomes a coated vesicle (4). The major proteins that drive clathrin coat formation are the AP[1] complexes or adaptors which also have a number of other functions in vesicle trafficking (1–3). AP-1 and AP-2 complexes are both heterotetramers, consisting of two large chains (γ- and either β1- or β2-adaptin for AP-1 and α- and either β1- or β2-adaptin for AP-2), a medium chain (μ1 or μ2), and a small chain (σ1 or σ2). AP-1 is found in vesicles derived from the *trans*-Golgi network, whereas AP-2 is specific for the plasma membrane-coated vesicles. μ1 and μ2 are the recognition molecules for the tyrosine-sorting signals present in the cytoplasmic tail of proteins that traffic through clathrin-coated vesicles (5, 6). The β-subunits interact with clathrin and *in vitro* they drive the formation of coats that are structurally indistinguishable from clathrin coats obtained from cells (7). The α-subunits also bind to clathrin, but their role in coat formation has not been established (8). There is growing evidence that several other molecules might also interact with the α-subunit of AP-2. They include the membrane protein synaptotagmin (9), GRB2 adapter protein (10), and a group of small molecules containing phosphorylated inositol rings that includes phosphatidylinositide 4,5-diphosphate and inositol 6-phosphate (11, 12). It has been recently shown that another protein, named Eps15, also interacts with the α-subunit (13). This interaction involves regions at the carboxyl terminus of Eps15 and of the α-chain of AP-2, respectively (14).

Eps15 is a 100-kDa protein, currently of unknown function, that was originally discovered as a phosphorylation substrate of the epidermal growth factor (EGF) receptor kinase (15). A prominent feature of Eps15 is the existence of three relatively conserved domains of about 70 amino acid residues referred to as EH domains which are located in its amino terminus (16). EH domains are also found in several other molecules, including the yeast protein End3p, a molecule required for the endocytosis of α-factor pheromone in yeast cells (17).

As a step in understanding the function of Eps15 in mammalian cells, we have investigated the intracellular distribution of Eps15. Here we show that there is a pool of Eps15 that is membrane-bound and that virtually all of these molecules are complexed to AP-2. Furthermore, we have used a combination of immunofluorescence and immunogold electron microscopy techniques to demonstrate that the membrane-bound form of Eps15 localizes to endocytic clathrin-coated pits and coated vesicles. An unexpected result was the strong asymmetric localization of Eps15 in the coated structures, most notably to the edges of forming coated pits and to the rims of budding coated vesicles. Based on these observations, we discuss several possible roles of Eps15 in the clathrin-mediated traffic pathway.

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[1] The abbreviations used are: AP-2, plasma membrane clathrin-associated protein complex also referred to as adaptor; AP-1, Golgi clathrin-associated protein complex; CMF-PBS, Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline; EGF, epidermal growth factor; GTPγS, guanosine 5′-*O*-(thiotriphosphate).

EXPERIMENTAL PROCEDURES

Materials—Polyclonal antibodies to αC (Ab31) and β-subunits (Ab32) and the monoclonal antibody to the β-subunit have been described previously (18).[2] The monoclonal antibody AC1-M11 that recognize α-subunits of AP-2 was a gift from Dr. M. S. Robinson (University of Cambridge, Cambridge, UK). Polyclonal (Ab577) and monoclonal (6G4) antibodies to Eps15 were kindly provided by Dr. P. P. Di Fiore (European Institute of Oncology, Milan, Italy) and Dr. N. Cerf-Bensussan (INSERM, Paris, France), respectively. A monoclonal antibody to the clathrin heavy chain (X-22) and α-subunit (AP-6) were a gift of F. Brodsky (UCSF, San Francisco). Clathrin-coated vesicles were isolated from bovine brain as described (7, 19). EGF was purchased from Collaborative Research Inc.

Cell Culture—Mouse NIH 3T3 cells expressing approximately 4×10^5 human EGF receptors per cell were derived by single-cell cloning of pCO 11 cells (20). Cells were grown in 35–100-mm dishes (Costar) as described (18) and used for experiments when confluent.

Co-immunoprecipitation of APs—Cells treated or not treated with EGF were washed with Ca^{2+},Mg^{2+}-free phosphate-buffered saline (CMF-PBS) and lysed in TGH buffer (1% Triton X-100, 10% glycerol, 50 mM NaCl, 50 mM Na-Hepes, pH 7.3, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 544 μM iodoacetamide, 10 g/ml aprotinin). This procedure allows the complete solubilization of the cytosolic pool and the partial solubilization of membrane-associated pools of AP-2 (18, 21). In other experiments designed to separate the cytosol and membrane fractions, cells were mildly permeabilized by incubation in 0.02% saponin in CMF-PBS, containing 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, protease inhibitors, for 30 min at 4 °C. After removal of the saponin fraction, the permeabilized cells containing the membrane-bound proteins were washed with CMF-PBS and solubilized by scraping the cells away from the dish with a rubber policeman in TGH containing 1% sodium deoxycholate (TGH-deoxycholate) followed by gentle rotation for 10 min at 4 °C.

The TGH lysates, the saponin, and TGH-deoxycholate fractions were centrifuged at 100,000 × *g* for 20 min at 4 °C and incubated with antibodies to APs (Ab31, Ab32) or Eps15 (6G4, Ab577) for 3 h at 4 °C and then 1 h after the addition of Protein A-Sepharose. Unrelated rabbit IgG (Zymed) were used to control for nonspecific immunoprecipitations. Immunoprecipitates were washed twice with cold CMF-PBS or TGH supplemented with 100 mM NaCl and then once without NaCl. The SDS-gel electrophoresis, transfer to nitrocellulose membranes, and Western blot analysis were carried out as described (18, 21). The top (above the 116-kDa molecular mass marker) and bottom portions of the nitrocellulose membrane were blotted with antibody to Eps15 and α-subunits (AC1-M11, Ab31), respectively. Sheep antibodies to mouse IgG (Cappel Inc.) or Protein A (Zymed Inc.) conjugated with horseradish peroxidase were used, with the enhanced chemiluminescence (Amersham, DuPont NEN, or Pierce), to detect primary mouse or rabbit antibodies, respectively. A Bio-Rad PhosphorImager was used for quantitation.

Immunofluorescence Staining—Cells grown on coverslips were fixed with freshly prepared 3.7% formaldehyde for 12 min at room temperature and permeabilized with CMF-PBS containing 0.1% Triton X-100, 1% bovine serum albumin for 5 min. Coverslips were then incubated for 1 h with primary antibody (mouse monoclonal X-22 or AP-6 and rabbit polyclonal Ab577 precleared by centrifugation at 100,000 × *g* for 10 min), washed intensively, and then incubated with secondary anti-mouse IgG and anti-rabbit IgG labeled with Texas Red or fluorescein (Jackson ImmunoResearch) in the same buffer at room temperature. A Nikon Diaphot 300 microscope equipped with 100 × objective lens, and the single fluorochrome filter sets for either Texas Red, fluorescein, or simultaneous Texas Red/fluorescein fluorescence were used for visualization and recording the images.

Immunogold Electron Microscopy—Cells grown in tissue culture dishes were rinsed once with CMF-PBS and then released from the dishes by incubation with CMF-PBS supplemented with 0.5 mM EDTA. 800 μl of the cell suspension was layered in an Eppendorf tube on top of a 200-μl cushion solution containing 8% paraformaldehyde (in 200 mM Hepes, pH 7.4) and centrifuged for 3 min at 3,000 rpm. The supernatant was carefully removed, and fresh 8% paraformaldehyde solution was added. After 1 h fixation at room temperature, the samples were centrifuged for 5 min at 14,000 rpm and left overnight at 4 °C. The pellets were then infiltrated for 15 min with a solution containing 2.1 M sucrose in PBS and then frozen by placing the Eppendorf tube in liquid nitrogen. Frozen samples were sectioned at −120 °C, and the sections were transferred to formvar-carbon-coated copper grids; the grids were then placed on top of a drop of PBS and processed for immunogold labeling as described previously (22). Briefly, stock solutions containing the antibodies and the gold-conjugated protein A were dissolved in 0.5% fish skin gelatin (Sigma) and centrifuged for 1 min at 14,000 rpm prior to labeling. Grids were floated on drops with 0.5% fish skin gelatin for 10 min to prevent nonspecific labeling and then transferred for 3 min to 5-μl drops containing the primary antibodies (rabbit serum specific for Eps15, Ab577, or mouse mAb specific for the β-subunit of AP-1 and AP-2). The grids were rinsed by transfer onto 0.5-ml drops of PBS (15 min) followed by incubation on 5 μl of protein A-gold for 20 min. In the case of the mAb, an additional incubation step with a rabbit-anti-mouse antibody was needed prior to protein A-gold incubation. Two final 15-min washes in 0.5 ml of PBS and in double-distilled water were performed prior to a 10-min contrasting/embedding step in 0.3% uranyl acetate dissolved in 2% methyl cellulose. The grids were examined in a JEOL 1200EX transmission electron microscope and images recorded at a primary magnification of 25,000.

RESULTS AND DISCUSSION

Association of Eps15 with AP-2 in the Cytosol and Membrane Fractions of NIH 3T3 Cells—Triton X-100 lysates of NIH 3T3 cells were incubated with the polyclonal antibodies directed against the αC-subunit (Ab31) or against the β-subunit of AP-2 (Ab32), and the immunoprecipitates were analyzed for the presence of Eps15 by Western blot analysis. As was originally shown by Benmerah and co-workers (13), Eps15 co-immunoprecipitates with AP-2 (Fig. 1*A*). The observed co-precipitation of Eps15 with anti-β antibody was 5- to 6-fold more efficient than with anti-αC antibody. The low efficiency of Ab31, specific for epitopes located in the αC-ear/hinge domain (18) to co-immunoprecipitate Eps15, is probably due to its inability to recognize AP-2 complexes containing αA-subunit, and by steric hindrance presented by Eps15 when bound to the α-ear/hinge (14). In the converse experiment, immunoprecipitation with antibody specific for Eps15 brings down about 5–10% of the total AP-2 present in the TGH lysate as monitored by the appearance of signals for αA and αC chains (Fig. 1*A*).

The Triton X-100 extraction allows the release of the cytosolic and, partially, the membrane pool of AP-2 while 40–50% of total cellular AP-2 remains insoluble (21). To differentiate between the cytosolic and the membrane-bound forms of AP-2, a two-step detergent extraction was used. The cells, still adherent to the tissue culture dish, were permeabilized with 0.02% saponin which permits the release of cytosolic components including APs while preserving the membrane-bound form of AP-2. Retention of membrane-bound forms of AP-2 and clathrin in coated pits was confirmed from the inspection of the images of these cells generated by immunofluorescence labeling of clathrin and AP-2 (not shown). Co-immunoprecipitation analysis shows that the association of Eps15 with AP-2 in the cytosolic fraction is maintained after the saponin treatment (Fig. 1*B*). Interestingly, it was found that these proteins also remain in association in the membrane-bound fraction (Fig. 1*B*). The association is robust enough that it even survives treatment of the membrane-bound fraction with a mixture of Triton X-100 and sodium deoxycholate (TGH-deoxycholate), a condition previously shown to release all AP-2 complexes from membranes (26).

Analysis of the remaining supernatants revealed that the fractions were almost completely depleted of Eps15 by the antibody to β-subunit Ab32 (Fig. 1*C*). Within the limits of sensitivity of the assay, the data are consistent with a situation where most if not all Eps15 is complexed with AP-2 in the cell. In contrast, when the Eps15 antibody is used to bring down the complexes, not more than 10–20% of the total cytosolic and 2–3% of membrane-bound AP-2 is able to co-immunoprecipi-

[2] K. Clairmont, W. Boll, and T. Kirchhausen, submitted for publication.

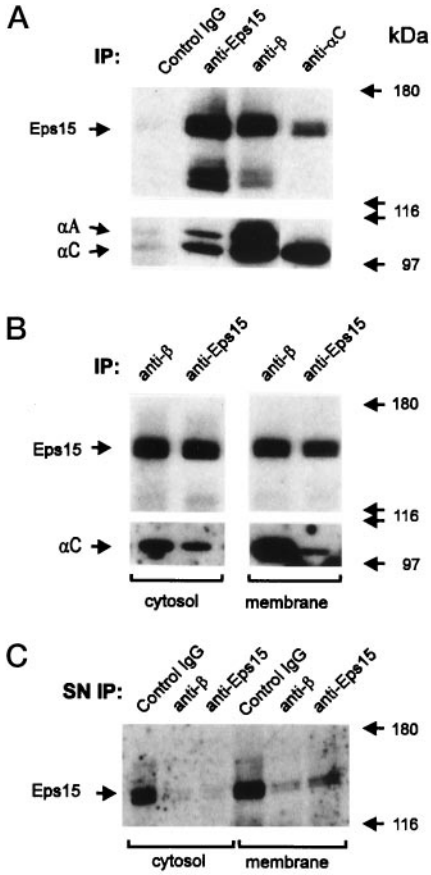

FIG. 1. **Association of AP-2 with Eps15 in NIH 3T3 cells.** *A*, cells were solubilized in TGH, and aliquots of lysates were immunoprecipitated with antibody to Eps15 (Ab577), β-subunits (Ab32), and αC-subunit of AP-2 (Ab31) or control IgG followed by SDS-gel electrophoresis. Proteins were transferred to a nitrocellulose membrane, and Eps15 and α-subunits (A and C) were detected with Ab577 and AC1-M11, respectively. Eps15 migrates on SDS-polyacrylamide gel electrophoresis at the position corresponding to the molecular mass of approximately 145 kDa. The smaller fragments (≈125 kDa) detected by Ab577 are the apparent presumed to be degradation products of Eps15. *B*, cells were permeabilized with saponin and then solubilized in TGH-deoxycholate buffer. Equal aliquots of the cytosolic (saponin eluent) and membrane fraction were incubated with Ab32 (anti-β) and 6G4 (anti-Eps15) to immunoprecipitate APs and Eps15, respectively. Immunoprecipitates were subjected to electrophoresis, and Eps15 and αC subunit were detected by Western blotting with Ab577 and Ab31. *C*, equal aliquots of the cytosolic and membrane fractions obtained as in *B* were incubated with saturative amounts of antibody 6G4 (anti-Eps15) and Ab32 (anti-β) or a corresponding amount of rabbit IgG. Supernatants after immunoprecipitation were electrophoresed and the Eps15 was detected by Western blotting with Ab577.

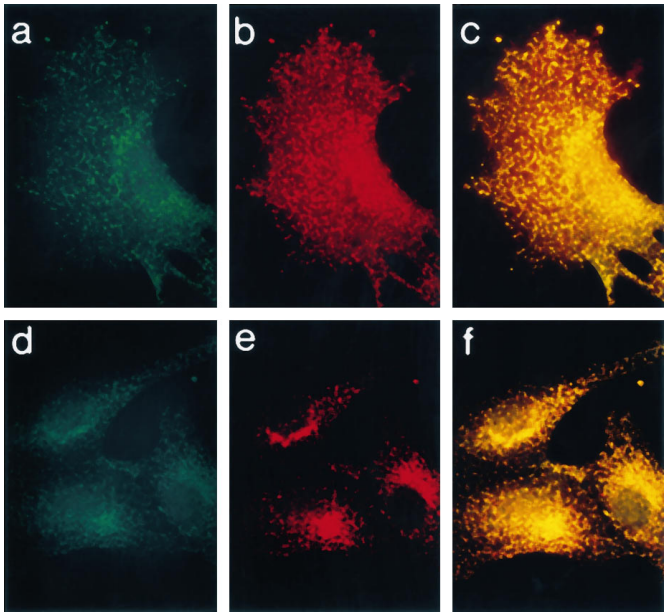

FIG. 2. **Subcellular co-localization of Eps15 with AP-2 and clathrin.** Formaldehyde-fixed NIH 3T3 cells were processed for double-label immunofluorescence microscopy using Ab577 for Eps15 and AP6 (α-subunit of AP-2) (*a–c*) or X-22 (clathrin heavy chain) (*d–f*). Primary antibodies were detected with corresponding secondary IgGs labeled with fluorescein (Eps15) or Texas Red (AP-2 or clathrin). Note the strong co-localization of the patterns of Eps15 (*green*) (*a*, *d*) and AP-2 (*b*) or clathrin (*e*) (*red*) immunoreactivity. Overlapping fluorescence of fluorescein and Texas Red yields *yellow* staining when cells are viewed using a double-fluorochrome filter set (*c*, *f*), whereas *green* or *red* staining is preserved on the *dots* where proteins are not co-localized.

tate with Eps15 (Fig. 1*B*). These results are in agreement with earlier observations indicating that most AP-2 complexes are not bound to Eps15 (13). Thus, it is possible that a limited portion of AP-2 is associated at any given time with Eps15.

Since Eps15 becomes phosphorylated upon cell stimulation with EGF, it has been proposed that Eps15 may play a direct role in the recruitment of AP-2 to the EGF receptor (23). We were unable, however to observe any effect of EGF on Eps15/AP-2 association in NIH 3T3 or in A-431 cells stimulated with 80 nM EGF for 3–60 min (data not shown).

Eps15 Co-localizes with Clathrin and AP-2—To study whether Eps15 and AP-2 are located in the same membrane compartments, double immunofluorescence staining of NIH 3T3 cells with Eps15 and AP-2 antibodies was performed. Fig. 2 shows that Eps15 staining produced a punctate pattern that is typical of clathrin-coated pits. In fact, most dots corresponding to Eps15 co-localize with dots corresponding to AP-2 (Fig. 2, *A–C*). Some labeling was observed with Eps15 which did not co-localize with AP-2, especially in the perinuclear region; this region, corresponding to the *trans*-Golgi network, is identified by immunolocalization of AP-1 (data not shown). That the co-localization of Eps15 with AP-2 is indeed taking place in clathrin-coated areas was established by co-staining the cells with the Eps15 and the clathrin antibody (Fig. 2, *D–F*). The majority but not all clathrin spots contain detectable amounts of Eps15 suggesting that some coats do not contain Eps15, or the amounts of Eps15 are not sufficient to be detected by the immunofluorescence method. A similar pattern of co-localization of Eps15 and clathrin was observed in A-431 cells (data not shown). From these data we conclude that clathrin-coated areas, containing AP-2 complexes, define the cellular region containing most if not all of the membrane-bound Eps15.

Localization of Eps15 at Coated Pits and Coated Vesicles—The intracellular localization of Eps15 in NIH 3T3 cells was further investigated by immunoelectron microscopic visualization of ultrathin sections incubated with the polyclonal antibody specific for Eps15 followed by labeling with protein A-gold (Fig. 3). Analysis of many fields showed the presence of gold particles at the plasma membrane, mostly along the profiles containing the electron-dense appearance at the cytosolic phase that is characteristic of clathrin-coated pits. The gold particles were also found in membrane profiles that are characteristic of clathrin-coated vesicles. The same types of structures were labeled by the monoclonal antibody specific for the β-subunits of AP-1 and AP-2, confirming their assignment as clathrin-coated structures. Closer inspection of the pattern of Eps15 labeling showed, however, a surprising result. In every coated pit analyzed, the gold particles were always found at the edge of the coated area. Likewise, when the pit was deeper and more invaginated, the gold particles were concentrated at the rim of the coat. These results suggest a relative depletion of

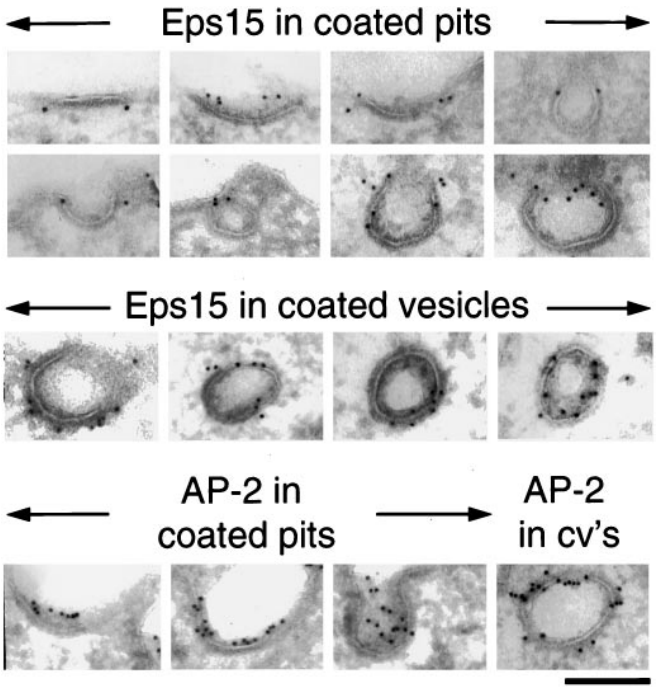

FIG. 3. **Immunoelectron microscopic localization of Eps15 to the rim of coated pits.** The sections of NIH 3T3 cells were labeled with the polyclonal antibody to Eps15 (Ab577) or with the monoclonal antibody to β1/2-chain of AP-2 followed by staining with protein A-gold. The images represent the distribution range of gold particles detected in profiles of coated pits (n = 17 for AP-2 and n = 37 for Eps15) and coated vesicles (n = 15 for AP-2 and n = 12 for Eps15). All images were taken at a primary magnification of 25,000 ×. *Bar* = 20 nm.

Eps15 in the deeper parts of the invaginations. We note the possibility that clathrin/AP coats (or other unknown proteins) might interfere with the accessibility of the Eps15 antibody preventing labeling of Eps15 within the coat. Although this possibility cannot be completely ruled out, it would seem unlikely, since Eps15 labeling was readily detected throughout the outline of profiles corresponding to coated vesicles (Fig. 3) even though these structures are completely surrounded by the same clathrin/AP coat of coated pits. In contrast to the asymmetric distribution of Eps15 in coated pits, labeling for AP-2 was always detected along the complete coated pit profile (Fig. 3). A-431 cells studied in the same way also showed preferential labeling of the edges and rims of coated pits (data not shown). These observations suggest that the unexpected localization of Eps15, presumably to the growing portion of the coat, is a common feature in all clathrin pits and in all cells.

Presence of Eps15 in clathrin-coated vesicles was confirmed biochemically by Western blot analysis of coated vesicles isolated from bovine brains. An immunogenic protein of the same electrophoretic mobility as Eps15 derived from NIH 3T3 cells could be readily detected in purified coated vesicles (data not shown). It is presumed that Eps15 also interacts with AP-2 in the coated vesicles, but this remains to be determined.

The general conclusion from the observations presented here is that endocytic clathrin-coated areas, containing AP-2 complexes, define the cellular region where most if not all of the membrane-bound Eps15 is found. We were surprised by the preferential position of Eps15 to the rims of coated pits and can only make some suggestions of possible functions of this protein. Sequence analysis of Eps15 predicts an extended α-helical region on the middle of the protein; it is possible that Eps15 exists as a dimer that interacts simultaneously with two AP-2 complexes. This could change the local concentration of AP-2 at the edge of an assembling coated pit, promoting local curvature by facilitating the formation of pentagonal facets in the clathrin lattice. A second possibility is that Eps15 is an adaptor that recruits other proteins to the forming clathrin/AP-2 coat. These proteins could be membrane receptors that become trapped in the coated pit. It has been suggested that Eps15 aids in the recruitment of the EGF receptor (23), although there is no evidence to date that a complex is formed *in vivo* between Eps15 and EGF receptors (15, 24).

As a third possibility, Eps15 could recruit molecules involved in the membrane budding process itself perhaps acting at the stage of pit invagination or vesicle scission. It is believed that the latter stage is regulated by dynamin, a protein that also displays a preferential localization to the membrane portion connecting the budding vesicle to the donor membrane (25, 26). An interesting difference between Eps15 and dynamin, is that, as has been shown here, the localization of Eps15 to the rim of pits is detected with wild-type Eps15 and under normal physiological conditions. In contrast, only cells containing mutant forms of dynamin or cell fragments incubated with modified nucleotides like GTPγS show the asymmetric distribution of dynamin (25, 26). A fourth possibility is that Eps15 is a part of the putative docking machinery responsible for the specific targeting of AP-2 to the plasma membrane. Lastly, it has been noted that EH domains bind to several cytosolic proteins, although their identity and function is still unknown (16).

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