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 a-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 AP1 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 phosphatidylinositol 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.

* The abbreviations used are: AP-2, plasma membrane clathrin-associated protein complex; CMF-PBS, Ca2+- and Mg2+-free phosphate-buffered saline; EGF, epidermal growth factor; GTPγS, guanosine 5′-O-(thiotriphosphate).
EXPERIMENTAL PROCEDURES

Materials—Polyclonal antibodies to α- (Ab31) and β- subunits (Ab32) and 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 × 10^6 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, 100 mM glycerc, 50 mM NaCl, 50 mM Na-Hepes, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μM iodoacetamide, 10 μg/ml apro- 2

in 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 supernatant of TGH-deoxycholate (TGH-deoxycholate) fraction, 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 and anti-rabbit IgG labeled with Texas Red or fluorescein were used 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 200 microscope equipped with 100 × objective lens, and the single fluorochrome filter sets for either Texas Red, fluorescein, or ProteinA (Zymed Inc.) conjugated with horseradish peroxidase were used, with the enhanced chemiluminescence (Amer-

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tate with Eps15 (Fig. 1B). 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
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 coat 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.

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 a-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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