AP-2/Eps15 Interaction Is Required for Receptor-mediated Endocytosis

Alexandre Benmerah,*‡ Christophe Lamaze,‡ Bernadette Bègue,* Sandra L. Schmid,§ Alice Dautry-Varsat,‡ and Nadine Cerf-Bensussan*

*Développement Normal et Pathologique du Système Immunitaire, Institut National de la Santé et de la Recherche Médicale U 429, Hôpital Necker-Enfants Malades, 75473 Paris Cedex 15, France; ‡Unité de Biologie des Interactions Cellulaires, URA-Centre National de la Recherche Scientifique 1960, Institut Pasteur, 75724 Paris Cedex 15, France; and §Department of Cell Biology, The Scripps Research Institute, La Jolla, California 92037

Abstract. We have previously shown that the protein Eps15 is constitutively associated with the plasma membrane adaptor complex, AP-2, suggesting its possible role in endocytosis. To explore the role of Eps15 and the function of AP-2/Eps15 association in endocytosis, the Eps15 binding domain for AP-2 was precisely delineated. The entire COOH-terminal domain of Eps15 or a mutant form lacking all the AP-2–binding sites was fused to the green fluorescent protein (GFP), and these constructs were transiently transfected in HeLa cells. Overexpression of the fusion protein containing the entire COOH-terminal domain of Eps15 strongly inhibited endocytosis of transferrin, whereas the fusion protein in which the AP-2–binding sites had been deleted had no effect. These results were confirmed in a cell-free assay that uses perforated A431 cells to follow the first steps of coated vesicle formation at the plasma membrane. Addition of Eps15-derived glutathione-S-transferase fusion proteins containing the AP-2–binding site in this assay inhibited not only constitutive endocytosis of transferrin but also ligand-induced endocytosis of epidermal growth factor. This inhibition could be ascribed to a competition between the fusion protein and endogenous Eps15 for AP-2 binding. Altogether, these results show that interaction of Eps15 with AP-2 is required for efficient receptor-mediated endocytosis and thus provide the first evidence that Eps15 is involved in the function of plasma membrane–coated pits.

Receptor-mediated endocytosis plays a major role in the regulation of cellular functions such as uptake of nutrients and downregulation of growth factor receptors (Sorkin and Waters, 1993; Mellman, 1997). Most of the known membrane receptors are internalized from the cell surface through specialized structures of the plasma membrane called coated pits, characterized by the presence of a coat on the cytosolic side of the plasma membrane. The receptors undergoing endocytosis are first recruited and concentrated into coated pits and internalized as a result of the progressive invagination of the coated pits that pinch off from the membrane to give coated vesicles (Smythe and Warren, 1991).

The AP-2 complex, one of the major identified components of the coated along with clathrin, plays a central role in both the organization and the function of plasma membrane coated pits (Kirchhausen, 1993; Robinson, 1994). AP-2 is an heterotetramer composed of two large chains, the α- and β2-adaptins (100 kD), a medium chain μ2 (50 kD), and a small chain ω2 (17 kD). α-Adaptin is necessary for the targeting of AP-2 to the plasma membrane (Robinson, 1993; Page and Robinson, 1995) and seems to modulate the functions of AP-2 through interactions with phosphoinositides (Beck and Keen, 1991; Gaidarov et al., 1996). β2-adaptin binds to clathrin and is involved in the assembly of the clathrin lattice (Gallusser and Kirchhausen, 1993; Shih et al., 1995). The μ2 subunit binds to tyrosine-based endocytic signals present within the cytosolic tail of endocytosed receptors (Boll et al., 1996; Ohno et al., 1995, 1996).

Recently, we have shown that AP-2 is constitutively associated with Eps15 (Benmerah et al., 1995, 1996), a highly conserved protein organized in three distinct structural domains (Fazioli et al., 1993; Wong et al., 1994) (Fig. 1). Its NH2-terminal domain (DI) is composed of three imperfect repeats of 70 amino acids homologous to each other and to domains found in proteins in mammals, yeast, and nematodes. These domains, called EH for Eps15 Homology (Wong et al., 1995), were shown to mediate interaction with other proteins via an NPF motif (Saleini et al., 1997). Moreover, two yeast proteins containing EH domains, End3p and Pan1p, have been implicated in the in-
ternalization step of endocytosis (Benedetti et al., 1994; Wendland et al., 1996). The central domain of Eps15 (DII) is characterized by the presence of heptads required for coiled-coil structures and is involved in the homodimerization of Eps15 (Tebar et al., 1997). The COOH-terminal domain (DIII) is characterized by the presence of repeats of the DPF sequence and contains the AP-2–binding site (Benmerah et al., 1996; Iannolo et al., 1997). The fact that Eps15 is associated with AP-2 and is homologous to yeast proteins involved in endocytosis suggested a possible role for Eps15 in coated pit–mediated endocytosis. This hypothesis is supported by two studies showing a colocalization of Eps15 with both clathrin and AP-2 in vivo, and the presence of Eps15 in plasma membrane coated pits and vesicles (Tebar et al., 1996; van Delft et al., 1997).

However, direct evidence for a role for Eps15 in clathrin-mediated endocytosis has been lacking. To assess the role of Eps15 in the function of plasma membrane coated pits, we have transiently transfected wild-type and mutant constructs of Eps15 and tested their effect on endocytosis. The inhibition obtained in living cells with the mutant constructs was confirmed in a cell-free assay that also allowed us to define at which step along the endocytic pathway the Eps15/AP-2 interaction is required.

**Materials and Methods**

**Cells, Antibodies, and Ligands**

HeLa cells were grown in RPMI supplemented with 10% FCS, 2 mM L-glutamine, penicillin, and streptomycin. A431 cells were cultured in DME containing 10% defined-FCS (Hyclone, Aalst, Belgium). After trypsinization, 4 × 10⁶ cells were seeded onto 15-cm culture dishes 20–24 h before preparing perforated cells, as previously described (Smythe et al., 1992).

The production and characterization of the IgG anti–Eps15 mAb has been previously reported (Benmerah et al., 1995). Mouse mAb PSD4 (IgG1) against the epitope YTDIEMNLGK of the G protein of vesicular stomatitis virus (VSV) was a gift of Dr. T. Kreis (University of Geneva, Geneva, Switzerland; Kreis, 1986). Mouse mAb AC1-M11 (IgG2a) against the NH₂-terminal domain of α-adaptin and mAb AP-6 (IgG2b) against the COOH-terminal domain of α-adaptin were provided by Drs. M.S. Robinson (University of Cambridge, Cambridge, UK) and F. Bordsky (University of California, San Francisco, CA), respectively (Robinson, 1987; Chin et al., 1989). mAb 100/2, against the COOH-terminal domain of α-adaptin, mAb 100/1 (IgG1), against the NH₂ terminus of β-adaptin, and mAb 100/3 (IgG2A), against γ-adaptin (Ahele et al., 1988), were purchased from Sigma Immunochemicals (Sigma, Saint Quentin Fallavier, France). FITC-conjugated goat anti–mouse IgG2a, and anti–mouse IgG2b and anti–mouse IgG anti-sera, were obtained from Southern Biotechnology Associates Inc. (Birmingham, AL).

Biotinylated transferrin (B-Tf) was prepared as previously described (Lamaze et al., 1993) using sulfo-NHS-SS-biotin (6-[N-{[biotinyl] amino} hexanoyl] amino) hexanoic acid, sulfosuccinimidyl ester, sodium salt) obtained from Molecular Probes (Eugene, OR). Biotinylated epidermal growth factor (B-EGF) was purchased from Boehringer-Mannheim S.A. (Meylan, France).

**Plasmids**

The cDNA of human eps15 subcloned in pBluescript II KS (Stratagene, La Jolla, CA) was obtained in the laboratory (Benmerah et al., 1995) and used as a template to generate the different cDNA fragments used in this study.

The glutathione-S-transferase (GST) fusion proteins were derived from eps15 using the GST gene fusion system and the PGEX5.1 vector (Pharmacia-LKB, Les Ulis, France) as previously described (Benmerah et al., 1995, 1996). Briefly, a BamHI and an Xhol site were introduced in the upper and lower primers, respectively, to allow subcloning of the PCR products in the PGEX5.1 vector in frame with the GST moiety. Production of fusion proteins in DH5α bacteria and purification were performed as described (Benmerah et al., 1995, 1996).

The green fluorescent protein (GFP) fusion proteins were derived from the corresponding Eps15-derived GST fusion protein constructs. The different domains of Eps15, or the full-length sequence of Eps15 subcloned in PGEX5.1, were excised using the BamHI/Xhol sites, purified on agarose gel, and then cloned into the BamHI/Xhol sites of the pcDNA3 vector (Invitrogen, Leek, The Netherlands). We then used the XbaI site downstream from the Xhol site to generate BamHI/XbaI fragments that were then cloned into the BamHI and XbaI of the EGFP-C2 vector (CLONTECH, Palo Alto, CA). This vector allowed us to generate Eps15-derived fusion proteins with the GFP at the NH₂ terminus end in the same open reading frame as in the PGEX5.1 vector.

The VSV-tagged form of Eps15 was generated using PCR by introducing a Smal site starting at the stop codon of Eps15 and the VSVG epitope was introduced between the Smal/XbaI sites. The construct was then subcloned into the BamHI/XbaI sites of the pcDNA3 vector (Invitrogen). All the constructs were checked by nucleotide sequencing (Thermosequenase; Amersham, Les Ulis, France). Sequences of the used primers are available on request.

**Biochemical Procedures**

HeLa cells were lysed in 50 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, containing a mix of protease inhibitors (4 mM PMSF, 10 µg/ml aprotinin, leupeptin, pepstatin, 50 µg/ml trypsin inhibitor [Sigma]).

For precipitation, cell lysates were cleared with protein A–Sepharose or GST coupled to glutathione–Sepharose 4B beads (Pharmacia-LKB), and then incubated overnight with mAb 6G4 (10 µg/ml) coupled to protein A–Sepharose (Pharmacia-LKB) or with GST fusion proteins (5–10 µg/10⁶ cells) coupled to glutathione–Sepharose 4B beads (20–30 µl/10⁶ cells). Precipitated proteins were separated by SDS-PAGE under reducing conditions.

For Western blotting, acrylamide gels were transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH) in 10 mM Tris, 0.2% SDS, 10% methanol, 100 µg/ml Mowiol (Calbiochem-Novabiochem, La Jolla, CA), 25% glycerol (vol/vol), 100 µg/ml Dabco (Sigma), and then incubated in 25 mg/ml BSA. The cells were then incubated with the different mAbs at the indicated dilutions, followed by peroxidase-labeled sheep anti–mouse antibody (1:200,000) (Amersham). Labeled bands were revealed using enhanced chemiluminescence (Amersham).

**Transfections and Immunofluorescence**

Subconfluent HeLa cells were used for transient expression of the different constructs. Transfections were performed using the CalPhos Maximizer Transfection Kit (CLONTECH).

For immunofluorescence studies, transfected HeLa cells were grown on coverslips and used 1 or 2 d after transfection. The cells were washed in PBS and fixed in 3.7% paraformaldehyde and 0.03 M sucrose for 15 min at room temperature. The cells were then washed once in PBS, and after quenching for 10 min in 50 mM NH₄Cl in PBS, were washed again in PBS supplemented with 1 mg/ml BSA. The cells were then incubated with the different mAbs in permeabilization buffer (PBS with 1 mg/ml BSA and 0.05% saponin) for 45 min at room temperature. After two washes in the permeabilization buffer, the presence of antibodies was revealed by incubating the cells for 45 min at room temperature in permeabilizing buffer containing labeled secondary antibodies. After two washes in permeabilizing buffer and one wash in PBS, the cells were mounted on microscope slides in 25 mg/ml Dabco (Sigma), 100 mg/ml Mowiol (Calbiochem-Novabiochem, La Jolla, CA), 25% glycerol (vol/vol), 100 mM Tris- HCl, pH 8.5.

The samples were examined under an epifluorescence microscope (Axioptol; Carl Zeiss, Oberkochen, Germany) attached to a cooled CCD camera (Photometrics, Tucson, AZ).

No immunofluorescence staining was ever observed when secondary
antibodies were used without the first antibody or with an irrelevant first antibody.

**Endocytosis**

Endocytosis of Texas red-conjugated Tf (Molecular Probes, Eugene, OR) was performed on subconfluent HeLa cells grown on coverslips 1 or 2 d after transfection. The cells were first incubated for 30 min at 37°C in RPMI 10 mM Hepes, pH 7.2, to eliminate endogenous Tf, and then incubated in RPMI 20 mM Hepes, pH 7.2, 1 mg/ml BSA containing 100 nM Texas red–conjugated Tf. After incubation at 37°C for the indicated times, the cells were rapidly cooled to 4°C, washed twice in cold PBS, and then fixed as described above.

**Cell-free Assays for Receptor-mediated Endocytosis**

Perforated A431 cells were prepared and endocytosis assays were performed as previously described (Carter et al., 1993; Lamaze et al., 1993). Briefly, incubations were performed at 37°C for 30 min in 40-μl reaction volumes containing an ATP-regenerating system, 6 mg/ml cytosol (prepared from K562 human erythroleukemic cells), 2 μg/ml B-Tf, 10 ng/ml B-EGF, 0.2% BSA in KSHM assay buffer (100 mM K-acetate, 85 mM sucrose, 20 mM Hepes, pH 7.4, 1 mM Mg-acetate). Reactions were stopped on ice, cells were pelleted in a refrigerated microfuge, and the supernatants were carefully aspirated.

**Measurement of Avidin Inaccessibility.** The cell pellets were resuspended in avidin (50 μg/ml) and processed as described to mask surface accessible B-Tf or B-EGF (Carter et al., 1993; Lamaze et al., 1993). The avidin reagent was quenched with biocytin, and detergent cell lysates were plated onto ELISA microtiter wells coated with anti-Ti (Boehringer-Mannheim) or anti-EGF antibodies (Upstate Biotechnology, Inc., Lake Placid, NY).

**Measurement of β-Mercaptethane Sulfonate (MesNa) Resistance.** Incubations were performed in the presence of 4 μg/ml disulfite bond–biotinylated transferrin (B-SS-Tf) for 15 min at 37°C. The cell pellets were resuspended in 50 μl of 10 mM MesNa. The tubes were agitated at 4°C. At 30 min, 12 μl of 50 mM MesNa was added to each tube, and at 60 min this was supplemented with 18 μl of 50 mM MesNa. The MesNa solutions were prepared just before each addition in 100 mM NaCl, 1 mM EDTA, 50 mM Tris, 0.2% BSA, pH 8.6. After 90 min, the MesNa was oxidized by the addition of 25 μl of 500 mM iodoacetic acid. After a 10 min agitation, the membranes were solubilized and lysates were plated onto ELISA microtiter wells coated with anti-Ti as described above.

**ELISA-based Detection of Biotinylated Ligand.** Cell-associated B-Tf and B-EGF, or B-SS-Tf, which were inaccessible to avidin or MesNa, respectively were quantitated using streptavidin coupled to HRP (Boehringer-Mannheim). The constitutive presence of Eps15 in plasma membrane–coated pits strongly suggested its role in endocytosis. To test this hypothesis, we first examined whether overexpression of Eps15 could affect the endocytosis of transferrin, which has been extensively used as a marker of endocytosis via clathrin-coated pits. HeLa cells were transiently transfected with GST-Eps15 and transfected cells were allowed to internalize Texas red–conjugated Tf for 15 min at 37°C. As shown in Fig. 2, endocytosis of Tf did not appear to be modified in cells expressing Eps15, and there were no morphological differences between the compartments loaded with Texas red–conjugated Tf in transfected and untransfected cells (compare Fig. 2, c and d). Identical results were obtained using the Eps15-VSV construct (not shown). These results show that the overexpression of wild-type Eps15 does not affect Tf endocytosis.

**Results**

**Effect of the Overexpression of Tagged Eps15 Proteins on the Endocytosis of Transferrin in Intact HeLa Cells**

To define the possible role of Eps15 in endocytosis, we have generated tagged forms of Eps15 by introducing the VSV-G epitope at the COOH-terminal end (Eps15-VSV) or the GFP at the NH2-terminal end (GFP-Eps15) (Fig. 1). The constructs were transiently transfected in HeLa cells and 2 d after transfection, the distribution of the two different constructs was analyzed by immunofluorescence: the Eps15-VSV construct was detected using the PSD4 mAb specific to the VSV-tag and GFP-Eps15 was directly visualized by the green fluorescence emitted by GFP (not shown and Fig. 2 a, respectively). Both fusion proteins displayed a similar punctate staining typical of the distribution of plasma membrane–coated pits and comparable with the staining previously described for endogenous Eps15 (Tebar et al., 1996; van Delft et al., 1997). Moreover, in cells expressing GFP-Eps15 or Eps15-VSV, as reported for the endogenous Eps15 (Tebar et al., 1996; van Delft et al., 1997), the tagged proteins colocalized with AP-2 (Fig. 2, a and b).

The constitutive presence of Eps15 in plasma membrane–coated pits strongly suggested its role in endocytosis. To test this hypothesis, we first examined whether overexpression of Eps15 could affect the endocytosis of transferrin, which has been extensively used as a marker of endocytosis via clathrin-coated pits. HeLa cells were transiently transfected with GST-Eps15 and transfected cells were allowed to internalize Texas red–conjugated Tf for 15 min at 37°C. As shown in Fig. 2, endocytosis of Tf did not appear to be modified in cells expressing Eps15, and there were no morphological differences between the compartments loaded with Texas red–conjugated Tf in transfected and untransfected cells (compare Fig. 2, c and d). Identical results were obtained using the Eps15-VSV construct (not shown). These results show that the overexpression of wild-type Eps15 does not affect Tf endocytosis.

**The Expression of the COOH-terminal Domain of Eps15 Has a Dominant-negative Effect on Tf Endocytosis in Intact HeLa Cells**

We have previously shown that residues 667–739 of the Eps15 COOH-terminal domain bind directly to the ear of α-adaptin. However, the contribution of amino acids 621–667 could not be excluded (Benmerah et al., 1996). To assess their potential contribution to the binding of AP-2, internal deletions were introduced into GST-DII, a GST protein containing the entire COOH-terminal domain of Eps15 (Fig. 1), and the fusion proteins were used to precipitate AP-2. As shown in Fig. 3 a, AP-2 was precipitated by GST-DIII (Fig. 3, lane 1). It was also precipitated, albeit much less efficiently, by GST-DIIIΔ1 (Fig. 3, lane 2), a construct lacking amino acids 667–739. In contrast, GST-
DIIIΔ2, a construct lacking amino acids 621–739, failed to precipitate AP-2 (Fig. 3, lane 3), confirming that amino acids 621–667 present an additional binding site for AP-2, in agreement with results obtained by Iannolo and coworkers (Iannolo et al., 1997).

These results were used to design GFP fusion proteins containing or missing the Eps15 AP-2–binding sites. DIII, the COOH-terminal domain of Eps15, and DIIIΔ2, lacking all the AP-2–binding sites, were transferred from the PGEX5.1 vector into the EGFP-C2 vector, to generate the corresponding GFP fusion proteins. HeLa cells were transiently transfected with these constructs, and transfected cells were tested for their capacity to internalize Texas red–conjugated Tf. As shown in Fig. 3, cells expressing GFP-DIIIΔ2 internalized Tf as well as untransfected cells (Fig. 3, compare a and b). In contrast, HeLa cells transfected with GFP-DIII failed to internalize Tf (Fig. 3, compare b and c), suggesting that the expression of the COOH-terminal domain of Eps15 containing the AP-2–binding site inhibited the internalization of Tf.

**The COOH-terminal Domain of Eps15 Inhibits Receptor-mediated Endocytosis in Perforated A431 Cells**

To confirm the results obtained in vivo and define which step along the endocytic pathway was blocked in cells overexpressing the COOH-terminal domain of Eps15, the fusion proteins were tested in a cell-free assay designed to dissect the formation of coated vesicles from the plasma membrane (Fig. 4 A). This assay uses human A431 cells that are perforated mechanically to wash out the endogenous cytosol. In the presence of cytosol and an ATP-regenerating system, B-Tf or B-EGF are sequestered into deeply invaginated, coated pits and become inaccessible to avidin (Lamaze et al., 1993; Schmid, 1993). Since perforated cells remain fully accessible to exogenously added reagents, Eps15-derived GST fusion proteins could be introduced to test their effect on endocytosis. The data in Fig. 4 B show that the GST-DIII fusion protein containing the AP-2–binding sites significantly inhibited B-Tf sequestration. This inhibition was dose dependent, the half-maximal inhibition occurring at 3 μM GST-DIII, and a plateau was reached at 60% inhibition in the presence of >9 μM GST-DIII. The inhibitory effect of GST-DIII was specific and required the presence of the AP-2–binding site since the GST-DIIIΔ2 that does not bind AP-2 had no effect (Fig. 4 B). To further test the role of Eps15 in receptor-mediated endocytosis, we next studied the effect of DIII on B-EGF sequestration. When GST-DIII was tested on EGF endocytosis in perforated cells, we found that B-EGF endocytosis was inhibited within the same range of concentrations needed for Tf inhibition. Furthermore, no inhibition was observed at these concentrations for GST-DIIIΔ2 that lacks AP-2–binding sites (Fig. 4 C). Altogether, these results show that addition of the COOH-terminal domain of Eps15 in the in vitro assay inhibits both Tf and EGF endocytosis, two prototypes of constitutive and ligand-induced, receptor-mediated endocytosis, respectively. Furthermore, they also suggest that this domain inhibits the formation of clathrin-coated vesicles.

The inaccessibility of B-Tf to avidin, i.e., sequestration, can occur as a result of its inclusion into either constricted

---

**Figure 2.** Subcellular distribution of transfected GFP-Eps15 and effect on Tf endocytosis. (a and b) HeLa cells grown on coverslips were transiently transfected with GFP-Eps15, fixed, and then stained for AP-2 using the AP-6 antibody. The GFP-Eps15 fusion protein was visualized directly by the green fluorescence emitted by the GFP (a). AP-6 antibody was detected with a secondary anti-IgG2b antibody labeled with Texas red (b). The inserts show a higher magnification of the same area. Arrows point to representative spots stained in both green and red (a and b). (c and d) HeLa cells transiently transfected with GFP-Eps15 were incubated in the presence of 100 nM Texas red-conjugated Tf for 15 min at 37°C, washed twice in cold PBS, and then fixed. (c) Green fluorescence emitted by the GFP construct and (d) Texas red-conjugated Tf. a and b, c and d represent the same fields.
coated vesicles that remain plasma membrane associated and/or coated vesicles, representing early and late events in coated vesicle formation, respectively (Fig. 4 A). The data presented in Fig. 4 B show that the inhibition of the avidin signal by GST-DIII is not complete; thus GST-DIII could either partially inhibit both early and late events of coated vesicle formation, or inhibit solely early or late events. To discriminate between early and late events of clathrin-coated vesicle formation, we used a modified version of the in vitro assay for Tf endocytosis. The late events are selectively detected when Tf, biotinylated through a cleavable disulfide bond (B-SS-Tf), becomes inaccessible to the small membrane–impermeable reducing agent, β-mercaptoethane sulfonate (MesNa) (Schmid, 1993). The effect of the GST-DIII construct was studied in the same experiment, both on the avidin and the MesNa signals. As previously described (Carter et al., 1993; Lamaze et al., 1993), the MesNa signal in perforated A431 cells is about half of the avidin signal (Fig. 4 D). This MesNa signal was not inhibited by GST-DIII at concentrations that inhibited the avidin signal (Fig. 4, B and D). Importantly, at the inhibitory concentrations of GST-DIII, the avidin signal was superimposed on the MesNa signal. Altogether, the data obtained using the in vitro assay strongly suggest that adding GST-DIII inhibits early, but not late events of coated vesicle formation.

The COOH-terminal Domain of Eps15 Competes with Endogenous Eps15 for AP-2 Binding

To test whether the inhibitory effect of the GST-DIII construct resulted from a competition between fusion proteins and endogenous Eps15 for AP-2-binding, cell lysates were incubated in the presence of GST fusion proteins before and during precipitation with the anti-Eps15 antibody, 6G4. As previously shown, 6G4 coimmunoprecipitated AP-2 with endogenous Eps15 from untreated lysates and from lysates treated with 10 μM GST-DIIIΔ2 (Fig. 5 A, lanes 1 and 3). In contrast, the amount of AP-2 coimmunoprecipitated with endogenous Eps15 in lysates treated with 10 μM GST-DIII was markedly reduced (Fig. 5 A, lane 2), indicating that GST-DIII at a concentration that inhibits endocytosis in vitro (Fig. 4 B) competed with endogenous Eps15 for AP-2 binding. Further evidence that inhibition resulted from specific competition between GST-DIII and endogenous Eps15 for AP-2 binding is shown in Fig. 5 B. In this experiment, perforated A431 cells were incubated with inhibitory concentrations of GST-DIII in the presence of excess purified AP-2. As can be seen, the addition of purified AP-2 complexes blocked the inhibition of B-Tf and B-EGF endocytosis by GST-DIII. These results provide strong evidence that the DIII construct of Eps15 acts as a dominant-negative inhibitor of constitutive endocytosis by preventing Eps15 from binding to AP-2 complexes.

Discussion

Using two complementary approaches, we provide the first direct functional evidence that Eps15 is involved in constitutive receptor-mediated endocytosis of Tf, as well as ligand-induced endocytosis of the EGF, two receptors endocytosed via clathrin-coated pits. This finding is consistent with our previous discovery that Eps15 is constitutively associated with AP-2 (Benmerah et al., 1995) and the work of others showing that Eps15 is localized in coated pits (Tebar et al., 1996).

The possible role of Eps15 in coated pit–mediated endocytosis was first assessed by testing the effect of overexpressed wild-type constructs on Tf endocytosis; little or no difference in Tf endocytosis was detected between transfected and untransfected cells. The distribution of the transfected tagged forms of the full-length Eps15 was similar to that of the endogenous protein (Tebar et al., 1996; van Delft et al., 1997) and both GFP- and VSV-tagged Eps15 colocalized with AP-2. This suggests both that AP-2–binding sites for Eps15 were not saturated and that AP-2–independent membrane binding sites for Eps15 (van Delft et al., 1997) were also not saturated. The latter finding is consistent with our previous data showing that not all AP-2
complexes are associated with endogenous Eps15 (Benmerah et al., 1995).

Eps15 is associated with AP-2 in the various cell types and species that we have tested (Benmerah et al., 1995), suggesting that the association of Eps15 with AP-2 may play an important role in its function. This led us to generate mutant forms of Eps15 able to interfere with endogenous Eps15/AP-2 association. With this goal in mind, we first more precisely defined the AP-2–binding site of Eps15. Our previous data obtained with GST fusion proteins showed that the 621–739 region of Eps15 bound AP-2 very efficiently. Further trimming of this segment to amino acids 667–739 reduced AP-2 binding. We therefore concluded that amino acids 667–739 contain an AP-2–binding site, whereas amino acids 621–666 are required for the correct presentation and/or conformation of the 667–739 region (Benmerah et al., 1996). However, a GST fusion protein containing the COOH-terminal domain of Eps15 but lacking amino acids 667–739 was still able to precipitate AP-2. This result suggested the presence of an additional binding site in the 621–666 region. Accordingly, deletion of the entire 621–739 region completely abolished AP-2 binding. These results indicate that efficient AP-2 binding is not because of a unique binding site, but instead results from several elementary interacting sites located within the 621–739 region. A similar conclusion was reached by Iannolo and coworkers who identified three distinct AP-2–binding sites located around residues 650–660, 680–690, and 720–730 of the COOH-terminal domain of Eps15 (Iannolo et al., 1997).

To test the effect of the Eps15-binding site for AP-2 in live cells, the Eps15 moieties of the GST-Eps15 constructs used to precipitate AP-2 were fused to the GFP and transiently expressed in HeLa cells. Endocytosis of Tf was strongly inhibited by overexpression of the entire COOH-terminal domain of Eps15 but not by constructs in which all the AP-2–binding sites were deleted. These results strongly suggest that disruption of the Eps15/AP-2 association inhibits the function of plasma membrane–coated pits. However, a possible inhibitory effect on a later step along the Tf receptor trafficking pathway could not be excluded from these experiments.

The ability to reproduce the inhibitory effect of the DIII construct in perforated A431 cells allowed us to confirm the specific role of the Eps15/AP-2 interaction during the formation of clathrin-coated vesicles. In this in vitro assay, endocytosis of both Tf and EGF were strongly inhibited by GST fusion proteins containing intact DIII, but not by fusion proteins lacking the AP-2–binding site. This effect resulted from a competition between fusion proteins and endogenous Eps15 for AP-2 binding. Significantly, inhibition of Tf sequestration reached a plateau at ~60%, suggesting that late events in coated vesicle formation might not be inhibited by GST-DIII. This hypothesis was confirmed by measuring the inaccessibility of B-SS-Tf to MesNa, i.e., its sequestration into coated vesicles (Schmid, 1993). Altogether these results show that Eps15 is involved in the early steps of coated vesicle formation, including coated pit formation and invagination.

A previous study using a kinase-deficient mutant of the EGF-receptor (EGF-R) and a constitutively activated, soluble form of the EGF-R tyrosine kinase has shown that the recruitment of activated EGF-R into coated pits in perforated cells depends on the presence of a cytosolic substrate(s) of the EGF-R kinase other than the receptor itself (Lamaze and Schmid, 1995). The fact that Eps15 was initially cloned as a substrate of the EGF-R kinase makes itself (Lamaze and Schmid, 1995). The fact that Eps15 was

Figure 4. GST-DIII inhibits both EGF and Tf endocytosis in perforated A431 cells. (A) Schematic representation of the perforated cell assay and the different steps measured by the inaccessibility to avidin and to MesNa. (B) The sequestration of B-Tf (B) and B-EGF (C) was measured in perforated A431 cells under standard assay conditions (see Materials and Methods) in the presence of increasing concentrations of GST-DIII (closed symbols) or GST-DIIIΔ2 (open symbols). (D) The sequestration of B-SS-Tf into constricted coated pits and coated vesicles was detected by the inaccessibility to avidin ( ), whereas the sequestration of B-SS-Tf into coated vesicles only is measured by the resistance to MesNa ( ). The experiments were performed as in B and C, except that the incubation at 37°C was shortened to 15 min. The data represent averages (±SD) of three experiments.
Eps15 contains two tyrosine-phosphorylation sites, which may regulate its association with other proteins (van Delft et al., 1997). Indeed, phosphorylation of Eps15 by Fyn, Src, or Yes induces its recruitment to clathrin-coated pits (Sorkin and Carpenter, 1993). Eps15 is involved in the internalization of several ligands, including EGF, FGF, and GM-CSF (Beck et al., 1991). The interaction of Eps15 with AP-2, which is required for the formation of clathrin-coated pits, is important for the endocytosis of these ligands (Ahle et al., 1988).

The interaction of Eps15 with AP-2 is mediated by a conserved domain present in Eps15, called the Ear domain (Beck et al., 1991). The Ear domain interacts with the AP-2 complex, particularly with the β1 adaptin subunit (Beck et al., 1991). This interaction is required for the formation of clathrin-coated pits and the endocytosis of EGF and other ligands (Ahle et al., 1988).

In addition, Eps15 can interact with other proteins that are involved in endocytosis. These proteins include dynamin, amphiphysin, and End3p (Benedetti et al., 1994). The interaction between Eps15 and dynamin is important for the recruitment of dynamin to the neck of the invaginated coated pit (Shupliakov et al., 1997). The interaction with amphiphysin is also important, as amphiphysin is involved in the formation of clathrin-coated pits (David et al., 1996). Furthermore, the interaction with End3p is important for the endocytosis of certain ligands, such as EGF (Ahle et al., 1988).

In conclusion, Eps15 is a key regulator of endocytosis. Its interaction with AP-2, dynamin, amphiphysin, and End3p is important for the formation of clathrin-coated pits and the endocytosis of various ligands. The specific role of Eps15 in endocytosis depends on the ligand and the cell type. Further studies are needed to understand the mechanisms by which Eps15 regulates endocytosis.

References


