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Inhibition of clathrin-coated pit assembly by an Eps15 mutant

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SUMMARY

Recent data have shown that Eps15, a newly identified component of clathrin-coated pits constitutively associated with the AP-2 complex, is required for receptor-mediated endocytosis. However, its precise function remains unknown. Interestingly, Eps15 contains three EH (Eps15-Homology) domains also found in proteins required for the internalization step of endocytosis in yeast. Results presented here show that EH domains are required for correct coated pit targeting of Eps15. Furthermore, when cells expressed an Eps15 mutant lacking EH domains, the plasma membrane punctate distribution of both AP-2 and clathrin was lost, implying the absence of coated pits. This was further confirmed by the fact that dynamin, a GTPase found in coated pits, was homogeneously redistributed on the plasma membrane and that endocytosis of transferrin, a specific marker of clathrin-dependent endocytosis, was strongly inhibited. Altogether, these results strongly suggest a role for Eps15 in coated pit assembly and more precisely a role for Eps15 in the docking of AP-2 onto the plasma membrane. This hypothesis is supported by the fact that a GFP fusion protein encoding the ear domain of α-adaptin, the AP-2 binding site for Eps15, was efficiently targeted to plasma membrane coated pits.

Key words: Endocytosis, Eps15, Clathrin, AP-2, Dynamin, GFP

INTRODUCTION

The major pathway for receptor mediated endocytosis involves the formation of clathrin-coated vesicles at the plasma membrane. The role of the three major identified coat proteins, AP-2, clathrin and dynamin, is now well characterized. The AP-2 complex plays a central role in both the organization and function of plasma membrane coated pits: it drives clathrin assembly onto the plasma membrane and interacts with tyrosine based signals of membrane receptors. Clathrin gives an organizing framework to the pit. Dynamin is a GTPase that assembles into rings at the neck of invaginated coated pits and its GTPase activity is required for the scission of the vesicle from the plasma membrane (Mellman, 1996; Schmid, 1997; McNiven, 1998).

Eps15 (EGFR pathway substrate clone 15) is a newly identified constituent of plasma membrane clathrin-coated pits that is ubiquitously and constitutively associated with AP-2 (Benmerah et al., 1995; Tebar et al., 1996). This has suggested its role in clathrin-coated pit function and recent results showing that overexpression or microinjection of dominant negative mutants of Eps15 inhibit clathrin-dependent endocytosis have confirmed this hypothesis (Carbone et al., 1997; Benmerah et al., 1998). However, the function of Eps15 within coated pits remains unknown. Sequence analysis has shown that Eps15 is likely to be organized in three distinct structural domains (Fazioli et al., 1993; Wong et al., 1994); an N-terminal domain (DI) composed of three EH domains for Eps15-Homology (see below); a central coiled-coil domain (DII) involved in Eps15 oligomerization (Cupers et al., 1997; Tebar et al., 1997); a COOH-terminal proline rich domain (DIII) which contains all the AP-2 binding sites (Benmerah et al., 1996; Iannolo et al., 1997). Electron microscopic images of rotary shadowed recombinant Eps15, have shown that Eps15 is effectively composed of a globular 'head' that corresponds to the N-terminal EH-domains joined to an extended stalk, the coiled-coil domain linked by a kink to the C-terminal AP-2 binding domain (Cupers et al., 1997).

In the present study, we have focused on the N-terminal EH domains. EH domains were first identified as three repeats of 70 amino acids homologous to each other and to equivalent domains found in proteins from mammals, nematodes, flies and yeasts (Wong et al., 1995). Recently, EH domains were shown to be novel protein-protein interaction modules which specifically interact with NPF based motifs (Salcini et al., 1997) shared by several proteins (Haffner et al., 1997; Salcini et al., 1997; Wendland and Emr, 1998). Results from yeast showing that two EH domains containing proteins are required for endocytosis (Benedetti et al., 1994; Wendland et al., 1996) have suggested their role in intracellular traffic (Di Fiore et al., 1997). These data obtained in yeast are suported by results obtained in mammals showing that both Eps15 (Carbone et al., 1997; Benmerah et al., 1998) and Eps15r (Carbone et al., 1997), an Eps15 related EH domain containing protein

(Schumacher et al., 1995; Wong et al., 1995), are effectively required for clathrin mediated endocytosis. To further study the role of EH domains within the Eps15 protein, we have generated mutant forms of Eps15 lacking EH domains and studied their intracellular distribution and their effect on clathrin-coated pit assembly and function.

MATERIALS AND METHODS

Cells and antibodies

HeLa cells were grown in RPMI supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin and streptomycin. Mouse monoclonal antibody (mAb) 100/1, against β-adaptin, was obtained from Sigma Immunochemicals. Mouse mAb OKT9, against the transferrin receptor, was from ATCC. Mouse mAbs AP-6 (Chin et al., 1989), against AP-2; Hudy-1 (Damke et al., 1994), against dynamin, and the rabbit polyclonal antibody against clathrin (Mangeat and Burridge, 1984) were the kind gifts of Drs F. Brodsky, S. Schmid and P. Mangeat, respectively. Texas Red-conjugated goat anti-mouse immunoglobulins and goat anti-rabbit immunoglobulins were obtained from Molecular Probes (Eu, OR). Cy5-conjugated goat anti-mouse immunoglobulins was obtained from Amersham Pharmacia Biotech.

Generation of GFP constructs

To generate the Eps15 derived constructs, the full length sequence of Eps15 subcloned in the PGEX5.1 vector (Benmerah et al., 1995) was transferred into the EGFP-C2 vector (CLONTECH) allowing fusion with green fluorescent protein (GFP) at the N terminus of Eps15 (Benmerah et al., 1998). To further delete EH domains from the NH2-terminal domain of Eps15, *Hin*dIII sites were introduced by PCR at position 288 to delete the second and the third EH domain (GFP-EΔ95/295). The generated fragment was subcloned using the *Hin*dIII site present at position 825 of the human Eps15 coding sequence. The α703/938 construct, corresponding to the ear domain of α-adaptin C (Robinson, 1993; Page and Robinson, 1995), and the α756/938 construct subcloned in PGEX5.1 (Benmerah et al., 1996), were further transferred into the EGFP-C2 vector to generate the corresponding GFP fusion proteins. All the constructs were checked by nucleotide sequencing (Thermosequenase, Amersham Pharmacia Biotech). Sequences of the used primers are available on request.

Transfections and immunofluorescence

Subconfluent HeLa cells were used for transient expression of the different constructs. Transfections were performed using the CalPhos Maximizer Transfection Kit from CLONTECH. For immunofluorescence studies, transfected HeLa cells were grown on coverslips and used one day after transfection. The cells were washed in phosphate buffer saline (PBS) and fixed in 3.7% paraformaldehyde and 0.03 M sucrose for 30 minutes at 4°C. The cells were then washed once in PBS, and after quenching for 10 minutes in 50 mM NH_4Cl in PBS, washed again in PBS supplemented with 1 mg/ml bovine serum albumin (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 minutes at room temperature. After two washes in the permeabilizing buffer, the presence of antibodies was revealed by incubating the cells for 45 minutes 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 100 mg/ml Mowiol (Calbiochem, La Jolla, CA), 25% glycerol (V/V), 100 mM Tris-HCl, pH 8.5. For surface staining, transfected cells were incubated with mAbs in PBS BSA 1 mg/ml at 4°C. The samples were examined under an epifluorescence microscope (Zeiss) attached to a cooled CCD-camera (Photometrics) or under a confocal microscope (LSM 510, Zeiss).

Endocytosis

Endocytosis of Texas Red-conjugated transferrin (Tf) (Molecular Probes) was performed on subconfluent HeLa cells grown on coverslips one day after transfection. The cells were first incubated for 30 minutes at 37°C in RPMI 20 mM Hepes, pH 7.2, to eliminate receptor-bound 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. For fluid-phase endocytosis, BSA was labelled with Cy5 dye (Amersham Pharmacia Biotech), using the CyDye fluorolink reactive dye kit following the manufacturer's instructions. Cells were incubated in RPMI 20 mM Hepes, pH 7.2, 1 mg/ml BSA containing 0.25 mg/ml Cy5-labelled BSA for 45 minutes at 37°C, washed 5 times with PBS BSA 1 mg/ml, once in PBS and then fixed as described above.

RESULTS

EH domains are required for correct coated pit targeting of Eps15

Recent results showing that glutathione-S-transferase (GST)-EH domains fusion proteins inhibit endocytosis both by microinjection (Carbone et al., 1997) and in a perforated cell assay (C. Lamaze, personnal communication), have suggested their role in the function of Eps15 in endocytosis. To further characterize the function of EH domains, we generated an Eps15 deletion mutant lacking the second and third EH domains (Fig. 1). The mutant was fused to GFP, transiently expressed in

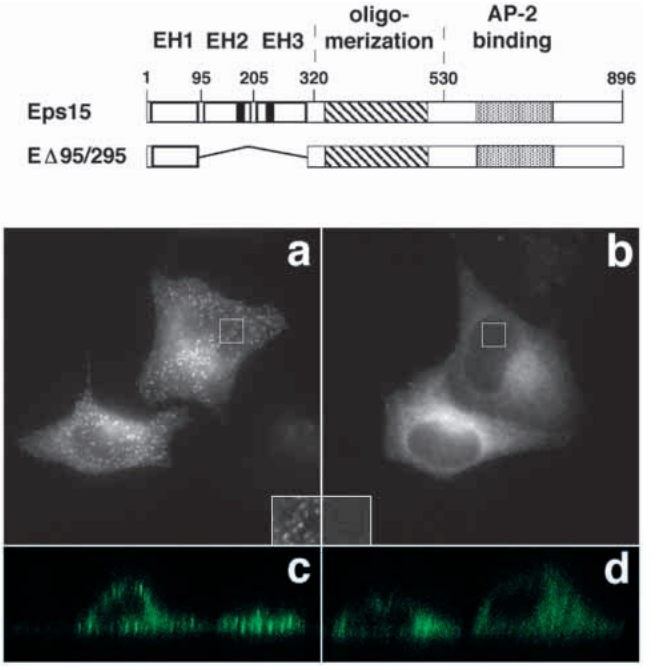

Fig. 1. The NH2-terminal domain of Eps15 is required for Eps15 targeting to the plasma membrane. Structural organization of Eps15 and description of the Eps15 mutant is shown on the top. HeLa cells transiently transfected with GFP-Eps15 (a and c) or GFP-EΔ95/295 (b and d) were fixed and processed for fluorescence microscopy. (a and b) Cells were observed under an epifluorescence microscope attached to a cooled CCD camera; the insets show higher magnifications of representative areas. (c and d) Vertical (z) optical sections obtained by confocal microscopy.

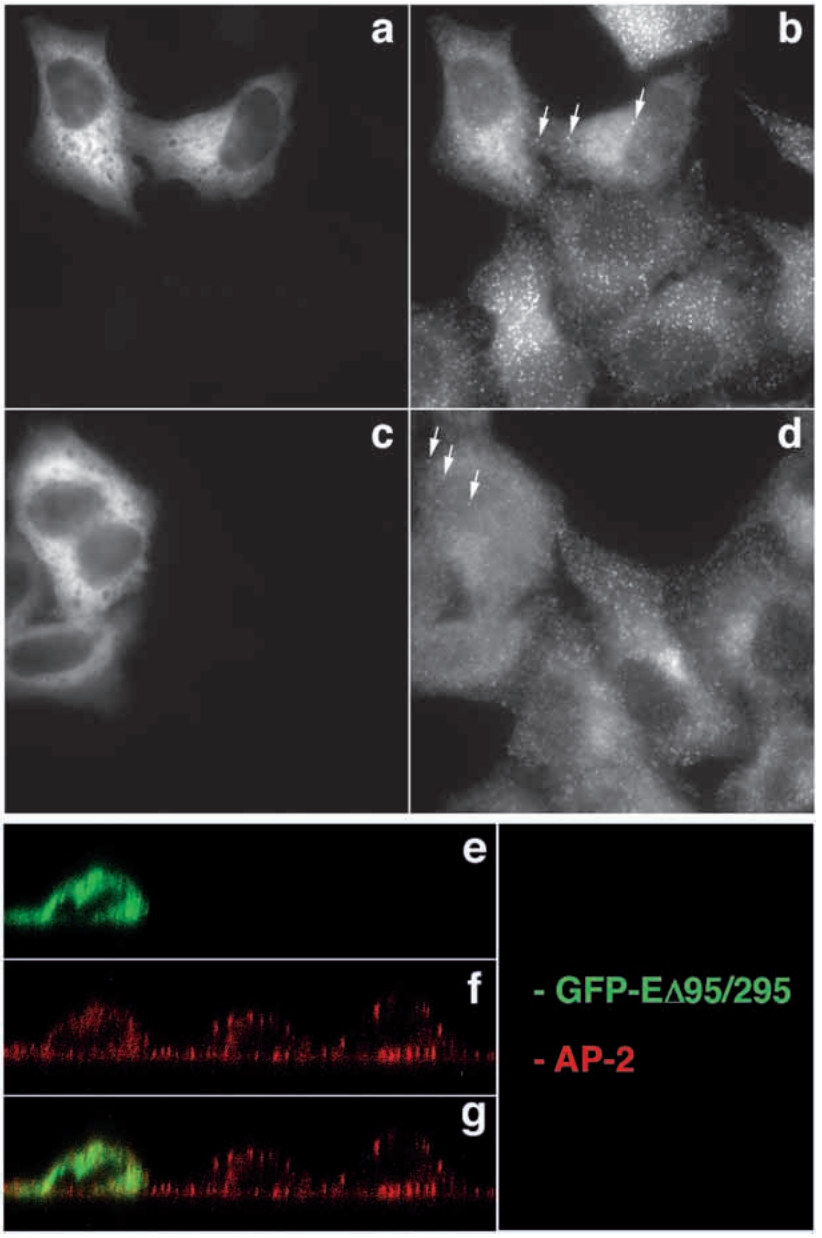

Fig. 2. Disrupted distribution of AP-2 and clathrin in cells expressing the GFP-EΔ95/295 construct. Hela cells transiently transfected with the GFP-EΔ95/295 construct were fixed, permeabilized and processed for fluorescence microscopy using antibodies directed against AP-2 (b and f) or clathrin (d), revealed by Texas Red (b and d) or Cy5-labelled (f and g) secondary antibodies. (a-d) Cells were observed under an epifluorescence microscope attached to a cooled CCD camera. The same fields are shown in a and b, and in c and d. (a and c) Green fluorescence emitted by GFP. Arrows show remaining AP-2 (b) and clathrin (d) spots present at the plasma membrane. (e-g) A vertical (z) optical section obtained by confocal microscopy is shown. (e) Green fluorescence emitted by GFP, (f) red fluorescence corresponding to AP-2 complexes, and (g) combined images.

HeLa cells and its intracellular localization was compared with that of the wild-type protein. Endogenous Eps15 has been observed in coated pits by electron microscopy (Tebar et al., 1996), and colocalized with AP-2 (Tebar et al., 1996; van Delft et al., 1997) as well as the GFP-Eps15 construct (Benmerah et al., 1998), both showing a punctate staining characteristic of plasma membrane coated pits (Fig. 1a and c). The intracellular distribution of the EH deleted mutant was strongly modified compared to the wild-type protein. The clear punctate distribution was completely lost (compare insets in Fig. 1a and b), the staining was mainly cytosolic, as confirmed by confocal microscopy (Fig. 1d), showing that EH domains are required for correct targeting of Eps15 to coated pits.

Expression of an EH-deleted mutant of Eps15 inhibits clathrin-coated pit assembly

As Eps15 is a constitutive component of plasma membrane coated pits, we next analysed the effect of expression of the EH-deleted mutant on the intracellular distribution of clathrin coat components. We first analysed the distribution of the AP-2 complex, the specific marker of plasma membrane clathrin-coated pits, and clathrin. The intracellular distribution of both AP-2 and clathrin was strongly modified in cells expressing GFP-EΔ95/295; the punctate staining characteristic of plasma membrane coated pits was almost completely lost, becoming mostly cytosolic with only a few dots at the plasma membrane (Fig. 2b and d, arrows). The lack of plasma membrane punctate staining for AP-2 was further confirmed using confocal microscopy. As shown in Fig. 2e-g, the AP-2 staining, using a z optical cut, show a characteristic punctate distribution decorating the cell surface. This punctate staining observed in untransfected cells was lost in cells expressing the EH-deleted mutant which showed an increased cytosolic staining (see Fig. 2f, cell on the left). Altogether, these results show that

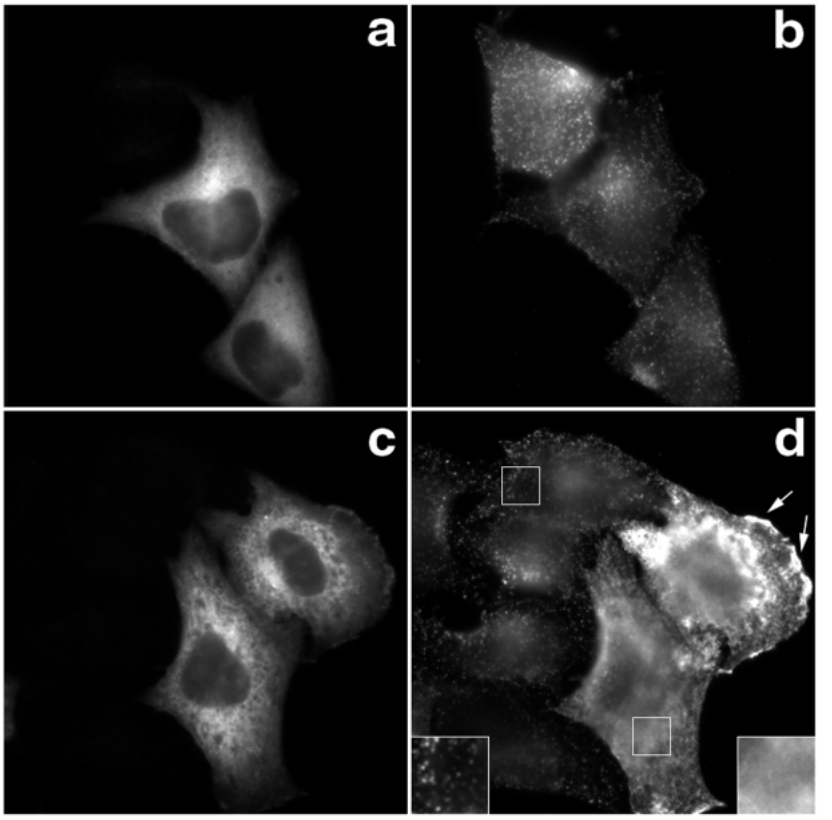

Fig. 3. Dynamin is mislocalized in cells expressing the GFP-EΔ95/295 construct. Hela cells transiently transfected with the GFP-DIIIΔ2 (Benmerah et al., 1998) (a,b) or GFP-EΔ95/295 construct (c,d) were fixed, permeabilized and processed for fluorescence microscopy using the hudy-1 antibody directed against dynamin, revealed by a Texas Red-labelled secondary antibody. (a and c) Green fluorescence emitted by GFP, (b and d) red fluorescence emitted by Texas Red observed under an epifluorescence microscope attached to a cooled CCD camera. The same fields are shown in a and b, and in c and d, respectively. (d) The insets show higher magnifications of representative areas of control (left) and transfected (right) cells.

overexpression of a mutant form of Eps15 lacking EH domains disrupts the punctate distribution of the two structural components of the coat, i.e. AP-2 and clathrin, implying the absence, or a large decrease in the number of plasma membrane coated pits.

Dynamin is mislocalized in cells expressing the EH-deleted mutant of Eps15

Next, we analysed the intracellular distribution of a third protein found in plasma membrane coated pits, i.e. dynamin. Dynamin, a GTPase required for the late steps of coated vesicle formation (Hinshaw and Schmid, 1995; Takei et al., 1995) and more generally for fission events in vesicle formation (Henley et al., 1998; Jones et al., 1998; Llorente et al., 1998; Oh et al., 1998), is found in plasma membrane coated pits and presents an AP-2 like staining (Fig. 3b). In contrast, in cells expressing GFP-EΔ95/295, but not in untransfected cells (Figs 3d and 4a) nor in cells transfected with a control Eps15-derived GFP fusion protein (Fig. 3b), the dynamin staining was brighter and the punctate pattern was lost (Figs 3d and 4a). Using classical fluorescence it was not possible to ascribe a specific localization for dynamin in cells expressing the EH-deleted mutant of Eps15. However, the accumulation of dynamin in ruffling areas (Fig. 3d, arrows) suggested its plasma membrane localization. The redistribution of dynamin to the plasma membrane was confirmed using confocal microscopy. As shown in Fig. 4, both xy (b, c and d) and xz (e, f and g) optical cuts showed that the GFP-EΔ95/295 construct (green) was cytosolic, leaving a dark area corresponding to the nucleus; whereas dynamin staining (red) decorated only the periphery of the cell. Furthermore, little if any yellow staining was observed, showing that dynamin and the GFP-EΔ95/295 constructs were not colocalized. These results showing an homogeneous plasma membrane localization of dynamin in cells expressing the EH-deleted mutant, versus a punctate one in control cells, are in agreement with the lack of clathrin-coated pits in cells expressing the Eps15 mutant (see Discussion).

Expression of the EH-deleted mutant of Eps15 inhibits clathrin dependent endocytosis

In agreement with the abscence of plasma membrane coated pits, expression of the GFP-EΔ95/295 construct inhibited the endocytosis of transferrin, a well known marker of clathrin-coated pit mediated endocytosis. As shown in Fig. 5b, the characteristic perinuclear compartment observed after 15 minutes internalization of transferrin in untransfected cells was not found in cells expressing the EH deleted mutant. Furthermore, the inhibition of transferrin uptake correlated, as expected, with an increase in surface transferrin receptor expression compared to neighboring, untransfected cells (Fig. 5d). In contrast, the internalization of a fluid phase marker was not modified in cells expressing the Eps15 mutant (Fig. 5f). This is in agreement with previous data showing that when clathrin-coated pit mediated endocysosis is inhibited, fluid phase endocytosis is not (Cupers et al., 1994; Damke et al., 1995; Liu et al., 1998).

The ear domain of α-adaptin, the Eps15 binding site on AP-2, is efficiently targeted to plasma membrane coated pits

The results presented above suggest a potential role for Eps15 in clathrin-coated pit formation, i.e. coat protein recruitment and/or assembly at the plasma membrane. Among the known

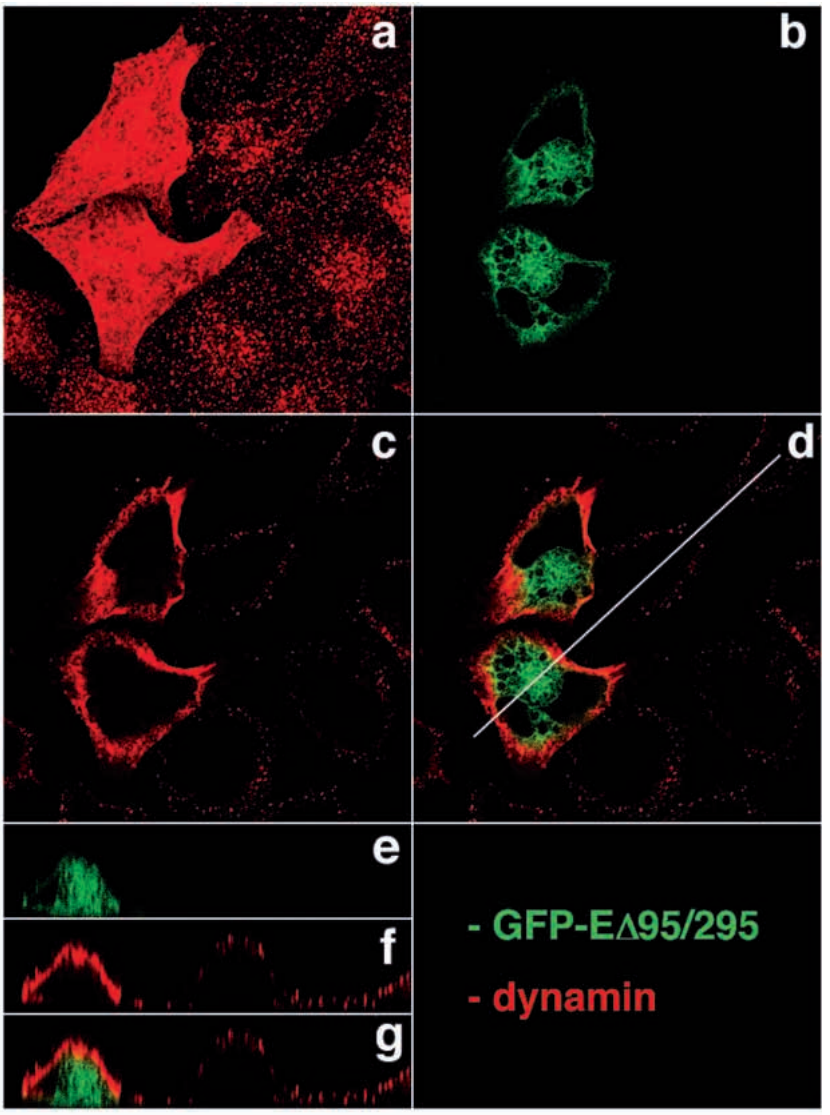

Fig. 4. Dynamin is homogeneously redistributed to the plasma membrane in cells expressing the GFP-EΔ95/295 construct. Hela cells transiently transfected with the GFP-EΔ95/295 construct were fixed, permeabilized and processed for fluorescence microscopy using the hudy-1 antibody directed against dynamin, revealed by a Texas Red-labelled secondary antibody. Cells were analyzed by confocal microscopy, GFP and dynamin staining are shown in green and red, respectively. (a) Superposition of all the xy optical planes for dynamin. Medial xy (b,c,d) and vertical xz (e,f,g) optical sections for GFP, dynamin and combined images respectively. The axis used for the xz vertical cuts is indicated in d by a white line.

coat proteins, AP-2 is believed to play a central role in coated pit formation: in the currently accepted model, AP-2 complexes are first targeted to the plasma membrane by a putative AP-2 docking complex (see Discussion). An attractive hypothesis suggested by our results is that Eps15 might participate in this process. Previous studies carried out to characterize the signals within AP-2 required for its targeting to the plasma membrane lead to conflicting results concerning the role of the C-terminal ear domain of α-adaptin (see Discussion), the AP-2 binding site for Eps15 (Benmerah et al., 1996). To clarify this point, we have examined the intracellular localization of GFP-fusion proteins derived from the ear domain of α-adaptin. As shown in Fig. 6, a GFP-ear construct showed punctate staining at the plasma membrane characteristic of clathrin-coated pits (Fig. 6a and c). Indeed, these dots at the plasma membrane colocalized with both β-adaptin (Fig. 6b) and clathrin (Fig. 6d), showing that the ear domain of α-adaptin is indeed targeted to plasma membrane coated pits. Furthermore, the intracellular localization of a GFP construct encoding residues 756/938 of α-adaptin C was also analysed. This construct, lacking the 50 first amino acids of the ear domain and unable to bind Eps15 (Benmerah et al., 1996; Chen et al., 1998), did not localize to plasma membrane clathrin-coated pits and showed a diffuse cytosolic staining (Fig. 6e). Altogether, these results clearly show that the ear domain of α-adaptin contains the necessary information for its targeting to coated pits. This targeting information is correlated with the capacity to bind Eps15, suggesting a role for Eps15 in sorting AP-2 to plasma membrane coated pits.

DISCUSSION

Data obtained in yeast have shown that proteins containing EH domains are required for endocytosis (Benedetti et al., 1994; Wendland et al., 1996; Tang et al., 1997); and that in the case of End3p, EH domains were required for the function of the protein (Benedetti et al., 1994). These data and the fact that EH domains were also found in Eps15 (Wong et al., 1995), an AP-2 binding protein (Benmerah et al., 1995), have suggested a general role for EH domains in endocytosis. The results presented in this study show that EH domains are required for clathrin-coated pit targeting of Eps15 and suggest a role for Eps15 in clathrin-coated pit assembly.

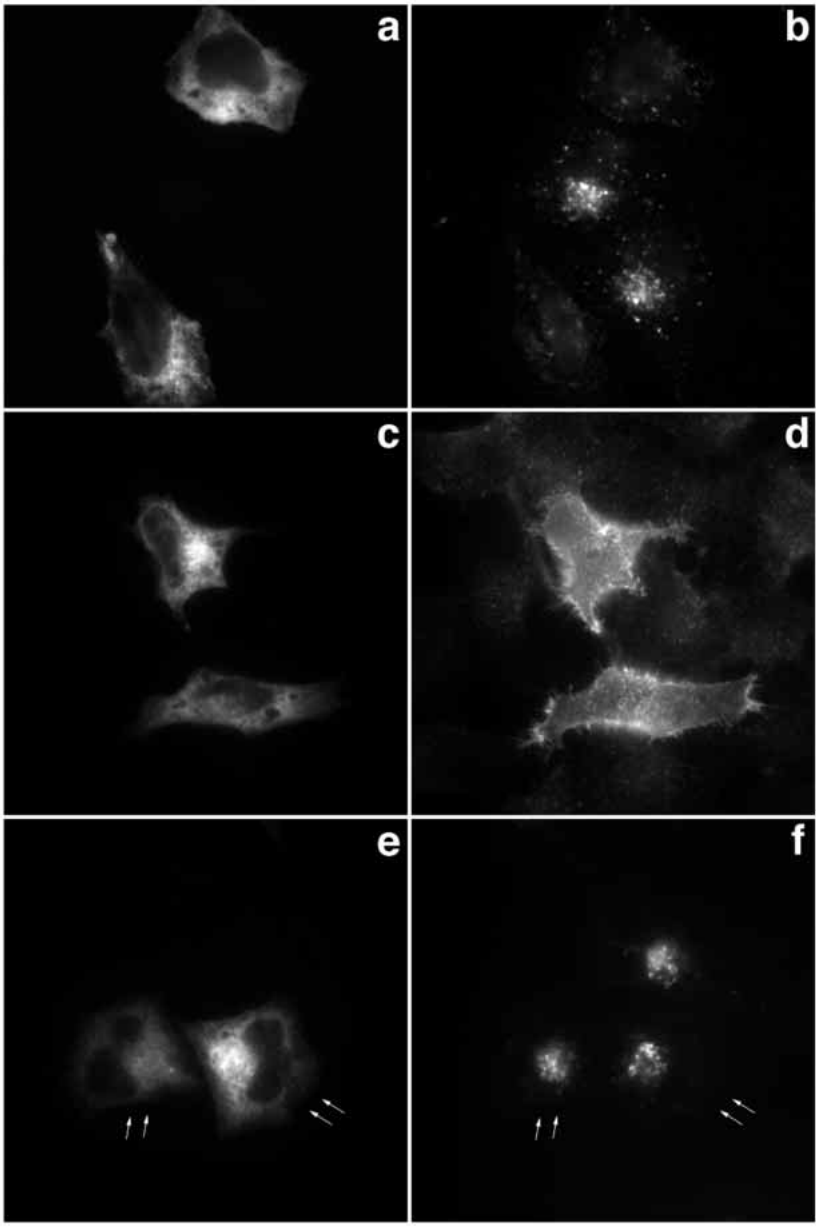

Fig. 5. Expression of the GFP-EΔ95/295 construct inhibits clathrin dependent endocytosis. HeLa cells transfected with GFP-EΔ95/295 were incubated in the presence of Texas Red-conjugated transferrin at 37°C for 10 minutes (b) or with Cy5-conjugated bovine serum albumin for 45 minutes at 37°C (f), then washed and fixed. To detect surface transferrin receptors (d), cells were incubated first with anti transferrin-receptor antibody followed by a Texas Red-conjugated antibody at 4°C. (a,c,e) Green fluorescence emitted by GFP. (b and d) Red fluorescence emitted by Texas Red. (f) Red fluorescence emitted by Cy5. The same fields are shown in a and b, in c and d, and in e and f.

The fact that an EH domain deleted mutant of Eps15 was found to be mostly cytosolic shows that EH domains are required for the correct plasma membrane targeting of Eps15. Recent data obtained by different groups have shown that EH domains from Eps15, Eps15r and pan1p bind to a conserved NPF motif shared by several proteins in yeast and mammals (Haffner et al., 1997; Salcini et al., 1997; Wendland and Emr, 1998). Altogether, these results suggest that an NPF containing protein is involved in plasma membrane targeting of Eps15. Among the NPF containing proteins shown to bind to Eps15 EH-domains, the recently identified protein epsin is a good candidate. Indeed, epsin has been shown to localize in plasma membrane coated pits and to be ubiquitously and constitutively associated with Eps15. Furthermore, epsin was also found on the cytosolic face of the plasma membrane suggesting that it could interact directly or indirectly with the plasma membrane (Chen et al., 1998).

Furthermore, the results presented here also show that expression of an EH-deleted mutant of Eps15 induced a loss of punctate plasma membrane staining for AP-2, clathrin and dynamin, supporting a role for Eps15 in clathrin-coated pit assembly. Formation of a clathrin-coated pit requires first the binding of a few AP-2 complexes onto the plasma membrane. Docked AP-2 complexes then generate a nucleation site for clathrin assembly, leading to the formation of a planar coated pit which then invaginates and pinches off the membrane to give a clathrin-coated vesicle (Schmid, 1997). Eps15 is a constitutive component of coated pits (Tebar et al., 1996) but it is excluded from coated vesicles and from clathrin coats formed in vitro (Cupers et al., 1998). Thus the function(s) of Eps15 should be restricted to coated pits on the membrane, in agreement with our previous results showing that Eps15 is required for the earliest steps of receptor mediated endocytosis (Benmerah et al., 1998). The data presented here are also in keeping with inhibition occuring at a very early step of coated pit assembly, i.e. coat protein recruitment onto the plasma membrane.

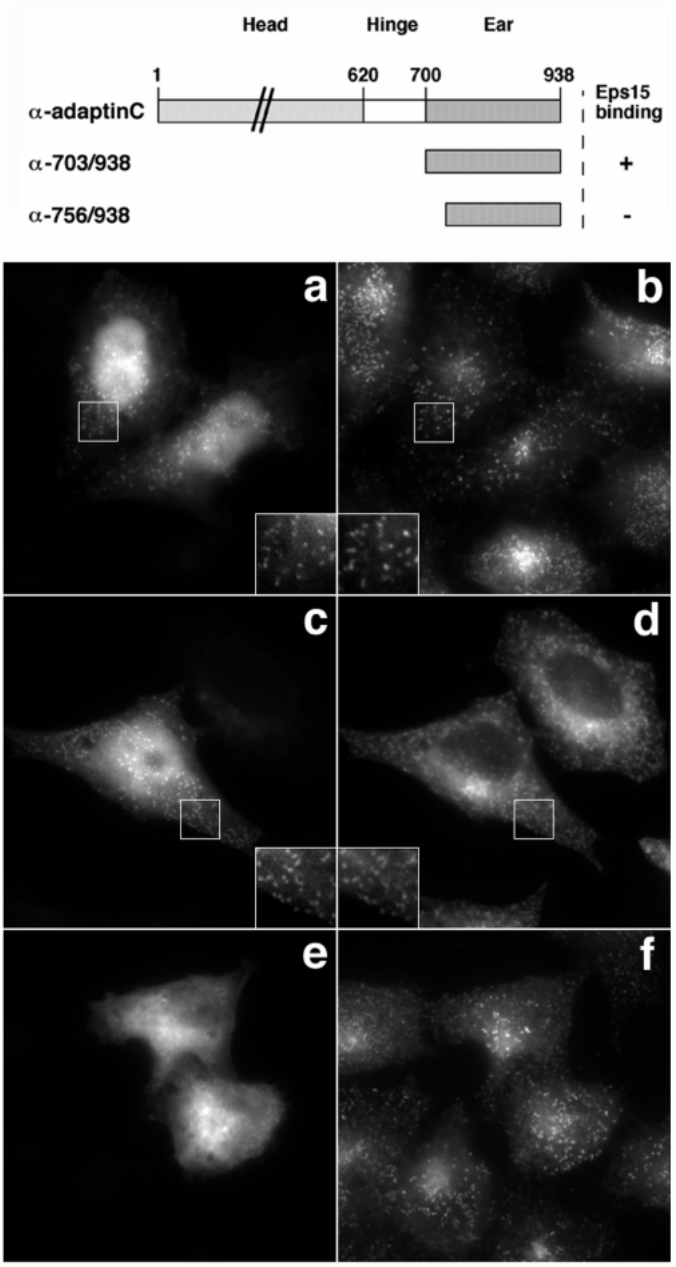

Fig. 6. The ear domain of α-adaptin is efficiently targeted to plasma membrane coated pits. (a) Structural organization of α-adaptin and description of the ear derived GFP constructs. Hela cells transfected with GFP-α706/938 (a-d) and with GFP-α756/938 (e and f) were fixed, permeabilized and processed for immunofluorescence microscopy using the mouse monoclonal antibody 100/1 directed against β-adaptin (b and f) and a polyclonal antibody against clathrin (d), revealed by Texas Red-labelled secondary antibodies. The same fields are shown in a and b, in c and d, and in e and f. In (a and b) and (c and d) the insets show higher magnifications of the same areas. (a,c,e) Green fluorescence emitted by GFP. (b,d,f) red fluorescence emitted by Texas Red.

Furthermore, as Eps15 is constitutively associated with AP-2 (Benmerah et al., 1995), it seems likely that Eps15 function(s) is (are) related to AP-2 function. We do not favor the hypothesis that the Eps15 mutant inhibits AP-2-induced clathrin assembly. If this were the case, AP-2 staining would have been normal and we would have observed a lack of clathrin staining on the plasma membrane, as described when clathrin assembly at the plasma membrane is inhibited by potassium depletion or hypertonic treatment (Hansen et al., 1993). Therefore, the simplest hypothesis to explain our results is that Eps15 plays a role in the recruitment of AP-2 to the plasma membrane.

The mechanisms involved in this process are still poorly understood and the involvement of a specific docking machinery within the plasma membrane has been suggested. This machinery may be different from the one involved in docking AP-1 and AP-3, two AP complexes homologous to AP-2 but with distinct intracellular distributions. Recruitment of both AP-1 to the trans-Golgi network and AP-3 onto endosomes is stimulated by GTPγS and inhibited by brefeldin A and requires the small GTPase ARF-1 (Robinson and Kreis, 1992; Stammes and Rothman, 1993; Traub et al., 1993; Dell'Angelica et al., 1997; Simpson et al., 1997; Faundez et al., 1998). Recruitment of AP-2 to the plasma membrane is inhibited by GTPγS (Seaman et al., 1993) and is not modified by brefeldin A (Robinson and Kreis, 1992), suggesting an ARF independent process. Efforts were made to identify the components of this putative specific AP-2 docking machinery. In vitro studies have shown that binding of AP-2 to salt stripped membranes occurs on saturable, high affinity and protease sensitive sites (Mahaffey et al., 1990; Chang et al., 1993). A 45 kD protein fragment obtained from limited proteolysis of isolated plasma membrane was shown to inhibit AP-2 binding (Mahaffey et al., 1990), suggesting that it could be part of the docking machinery but this fragment has not been further characterized. Synaptotagmins which are plasma membrane AP-2 binding proteins (Ullrich et al., 1994; Zhang et al., 1994) seemed good candidates but direct evidence for their role in AP-2 docking is still lacking.

A second approach was to delineate the domains of AP-2 involved in coated pit targeting and to use them as a bait to catch the different components of the docking complex. APs form heterotetrameric complexes composed of two large subunits (adaptins), α and β2 or β for AP-2 and γ and β1 or β′ for AP-1, a medium chain (μ), and a small one (σ). Rotary shadowing of purified AP-2 complexes show that they consist of a brick-like core or 'head' attached to two smaller appendage 'ears' through protease sensitive hinges. The ears correspond to the COOH-terminal domain of the two adaptins and the head to the NH2-terminal domains of adaptins together with μ2 and σ2 (Keen, 1990; Robinson, 1994). In vitro studies have shown that isolated α-adaptin, but not β-adaptin, binds to purified plasma membrane (Chang et al., 1993), a result in keeping with the hypothesis that β-adaptins which are the most conserved subunits between AP-1 and AP-2 (Keen, 1990; Robinson, 1994), are unlikely to be involved in the specific targeting of AP complexes. The important role of α-adaptin in the targeting of AP-2 was confirmed in vivo using adaptin chimeras. In these experiments, the structurally related domains of α- and γ-adaptins were exchanged and the intracellular targeting of the resulting AP complexes was analysed. Results of these studies show an important role for a N-terminal 100-amino-acid domain (residues 190 to 330) of α-adaptin in the targeting of AP-2 to plasma-membrane coated pits (Page and Robinson, 1995). Furthermore, recent results show that the PIP2 binding site found in the N terminus (residues 21 to 80) of α-adaptin (Gaidarov et al., 1996) is also involved in the recruitment of

AP-2 to the plasma membrane (Gaidarov and Keen, 1997). As α and γ-adaptin COOH terminal domains show little if any sequence similarity (Robinson, 1989, 1990), a specific role for these domains in the function of each AP complex was suggested. Somewhat contracditory results were obtained concerning the role of the COOH-terminal ear domain of α-adaptin in plasma membrane recruitment of AP-2. In vivo studies using ear swapped adaptins have shown that AP-2 complexes lacking the ear of α-adaptin were 92% cytosolic versus 32% for the wild type (Robinson, 1993), suggesting that the ear domain of α-adaptin plays an important role in the incorporation of AP-2 into coated pits and/or its stabilization on the plasma membrane. However, another study using an in vitro system following AP-2 binding to salt stripped membranes has suggested that the ears may not be involved in AP-2 recruitment (Peeler et al., 1993). The fact that a GFP-ear construct is efficiently targeted to plasma membrane coated pits shows that in vivo the ear domain of α-adaptin contains the necessary information to get into coated pits. Altogether, these results suggest that the recruitment of AP-2 to the plasma membrane occurs as a result of interactions between several binding sites on α-adaptin N- and C- terminal domains, with different components forming the putative AP-2 docking complex. Our results showing that an EH-deleted mutant of Eps15 inhibited clathrin-coated pit assembly and that the ear domain of α-adaptin, which directly binds to Eps15 (Benmerah et al., 1996), has a functional coated pit targeting signal, support the hypothesis that Eps15 is part of the AP-2 docking complex.

The results presented in this study also provide new insights concerning the recruitment of dynamin into plasma membrane coated pits. Morphological studies have established that, at equilibrium, dynamin is rarely found in uncoated regions of the plasma membrane (Damke et al., 1994; Warnock et al., 1997), suggesting that dynamin is directly recruited from a cytosolic pool to growing coated pits. The results presented in this study show that in the absence of plasma membrane coated pits, dynamin is redistributed homogeneously to the plasma membrane. If dynamin were recruited directly from the cytosol to growing coated pits, it should have behaved as clathrin and AP-2 and we should have observed a lack of dynamin staining on the plasma membrane. Our results are rather in keeping with a recruitment of dynamin onto the plasma membrane followed by quick incorporation into nascent coated pits. Inhibition of clathrin-coated pit assembly would then stabilize dynamin on the plasma membrane. The possible interaction of dynamin with the plasma membrane is suported by the fact that purified dynamin spontaneously binds to salt stripped membranes (Tuma and Collins, 1995; Sweitzer and Hinshaw, 1998) and to phospholipids containing liposomes (Sweitzer and Hinshaw, 1998; Takei et al., 1998), and by recent results showing that a GFP-dynamin2 construct is found in ruffling areas of the plasma membrane (Cao et al., 1998).

Finally, until very recently, dynamin was thought to be specifically involved in clathrin-coated vesicle budding from the plasma membrane. Thus, mutant forms of dynamin were used to block clathrin dependent endocytosis (Herskovits et al., 1993; van der Bliek et al., 1993; Damke et al., 1994, 1995). Recent studies show that dynamin is instead involved in vesicle budding in distinct trafficking pathways, i.e. vesicle budding from the trans-Golgi network (Jones et al., 1998), transport from endosomes to TGN (Llorente et al., 1998) and internalization through caveolae (Henley et al., 1998; Oh et al., 1998). Therefore, it appears that the use of the EH-deleted mutant of Eps15 is a new powerful tool to specifically study clathrin-coated pit mediated endocytosis. Furthermore, as the three types of mutants, dynamin (Damke et al., 1994), clathrin (Liu et al., 1998) and Eps15 (this study), inhibit different steps of coated pit/vesicle formation, comparative studies should be useful to study both clathrin-coated pit/vesicle formation and clathrin-independent endocytosis.

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