The Ear of $\alpha$-Adaptin Interacts with the COOH-terminal Domain of the Eps15 Protein*

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The role of Eps15 in clathrin-mediated endocytosis is supported by two observations. First, it interacts specifically and constitutively with the plasma membrane adaptor AP-2. Second, its NH$_2$ terminus shows significant homology to the NH$_2$ terminus of yeast End3p, necessary for endocytosis of $\alpha$-factor. To gain further insight into the role of Eps15-AP-2 association, we have now delineated their sites of interactions. AP-2 binds to a domain of 72 amino acids (767–739) present in the COOH terminus of Eps15. This domain contains 4 of the 15 DPF repeats characteristic of the COOH-terminal domain of Eps15 and shares no homology with known proteins, including the related Eps15r protein. Precipitation of proteolytic fragments of AP-2 with Eps15-derived fusion proteins containing the binding site for AP-2 showed that Eps15 binds specifically to a 40-kDa fragment corresponding to the ear of $\alpha$-adaptin, a result confirmed by precipitation of Eps15 by $\alpha$-adaptin-derived fusion proteins. Our data indicate that this specific part of AP-2 binds to a cellular component and provide the tools for investigating the function(s) of the association between AP-2 and Eps15.

Eps15 is the prototype of a new family of signal transducers characterized by their ability to interact with a large number of proteins (1). It was initially described as a substrate of the epidermal growth factor (EGF) and platelet-derived growth factors, including the related Eps15r protein. Precipitation of proteolytic fragments of AP-2 with Eps15-derived fusion proteins containing the binding site for AP-2 showed that Eps15 binds specifically to a 40-kDa fragment corresponding to the ear of $\alpha$-adaptin, a result confirmed by precipitation of Eps15 by $\alpha$-adaptin-derived fusion proteins. Our data indicate that this specific part of AP-2 binds to a cellular component and provide the tools for investigating the function(s) of the association between AP-2 and Eps15.

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1 The abbreviations used are: EGF, epidermal growth factor; EGFR, EGFR; EGF receptor; Eps15, EGFR pathway substrate clone 15; GST, glutathione S-transferase; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; EH, Eps15 homology.

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that this antibody recognizes the NH2 terminus of Eps15. The 61kDa and reacted with mAb6G4 (Fig. 1A). Construction of Glutathione S-transferase (GST) Fusion Proteins— Different fusion proteins were derived from eps15 using the GST gene fusion vector (pGEX-1 vector) (Pharmacia-LKB, Les Ulis, France). The cDNA of human eps15 subcloned in pBluescript II KS (Stratagene) was obtained in the laboratory and used as a template to generate different cDNA fragments encoding domains DI, DII, and DIII of eps15 and truncated forms of DIII. A BamHI and a Xhol site were introduced in the upper and lower primers, respectively, to allow subcloning the PCR products in the pGEX-1 vector in frame with the GST moiety. The constructs were checked by nucleotide sequencing (Thermosequenase, Amersham Corp., Les Ulis, France). GST fusion proteins encoding the COOH domain of α-adaptin or parts of this domain were similarly generated by PCR using the cDNA of mouse α-adaptin C, the nonalternatively spliced ubiquitous form of α-adaptin (12), subcloned in pBluescript II SK as a template (a kind gift of Dr. M. Robinson). Sequences of the used primers are available on request. Production of fusion proteins in DH5α bacteria and purification were performed as described elsewhere (6).

Biological Procedures—For biosynthetic labeling, MOLT16 cells were incubated with "S-labeled amino acids (Trans35S-label, Amer sham Corp., Les Ulis, France) for 30 min. After a 3-h chase, the 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 phenylmethylsulfonyl fluoride, 10 μg/ml apro tin, leupeptin, pepstatin, 50 μg/ml trypsin inhibitor (Sigma)). For limited proteolysis of the AP-2 complex, MOLT16 cells were lysed in 50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.5% Triton X-100, in the absence of protease inhibitors. Cells lysates were then digested with trypsin (Life Technologies, Inc., Eragny, France) at a 1:500 protein ratio at 37 °C for various periods of time. Digestion was stopped by addition of a mix of protease inhibitors (see above) and 10% fetal calf serum (Life Technologies). Digested lysates and control undigested lysates were precipitated by GST fusion proteins. For precipitation, cell lysates were cleared with protein A-Sepharose or GST coupled to glutathione-Sepharose 4B beads (Pharmacia Biotech Inc.) and then incubated overnight with mAb 6G4 (10 μg/3 × 106 cells) coupled to protein A-Sepharose (Pharmacia Biotech Inc.) 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-polyacrylamide gel electrophoresis (PAGE) under reducing conditions.

For Western blotting, acrylamide gels were transferred onto nitrocellulose membranes (Schleicher & Schuell) in 10 mM Tris, 0.2 M glycine, and 30% methanol. Nonspecific binding sites were blocked by incubation in Tris-HCl, pH 7.6, containing 5% bovine serum albumin and 0.2% Tween (Sigma). The blots were then sequentially incubated for 1 h, either with the mAb or with the indicated dilutions, followed by peroxidase-labeled sheep anti-mouse antiserum (1:20,000) (Amersham) or with rabbit antiserum Ab32 followed by swine anti-rabbit immunoglobulin antiserum (1:5,000) (Dakopatts, Trappes, France). Labeled bands were revealed using ECL (Amersham).

RESULTS

Eps15 Binds AP-2 via Its Third COOH-terminal Domain—Previous studies have shown that the anti-Eps15 mAb 6G4 as well as a fusion protein encompassing the full-length of Eps15 precipitate the various polypeptides of the AP-2 complex (6). To determine which domain of Eps15 interacts with AP-2, three GST fusion proteins, each one comprising one of the three structural domains of Eps15, GST-DI (amino acids 1–315), GST-DII (amino acids 305–538), and GST-DIII (amino acids 529–896), were prepared (Fig. 1A). Correct translation of the fusion proteins was checked by Coomassie Blue staining and/or immunoblotting. The GST-DI protein had the expected size of 61 kDa and reacted with mAb 6G4 (Fig. 1B, lane 1), indicating that this antibody recognizes the NH2 terminus of Eps15. The epitope was further mapped to the first 97 NH2-terminal amino acids (not shown). The GST-DII fusion protein, visualized by Coomassie Blue staining, had the expected size of 54 kDa (not shown). The GST-DIII protein reacted with a mAb produced against the C terminus of Eps15 (Fig. 1B, lane 3). Its apparent molecular mass of 97 kDa contrasted with its expected size of 67 kDa. However, the delayed migration of GST-DIII is reminiscent of the behavior of the full-length fusion protein which has an expected size of 130 kDa and migrates as a 160–170-kDa polypeptide in SDS-PAGE even in the absence of post-translational modification (2). The delayed migration of Eps15 and its third domain is thus likely due to the amino acid composition of the latter domain and particularly to its high number of prolines, 15 of which are adjacent to an acidic residue in the 15 DPF repeats.

GST-DI-, DII-, and DIII fusion proteins and the anti-Eps15 antibody 6G4 were used to precipitate lysates of 35S-labeled MOLT16 cells (Fig. 2A). As described previously (6), the anti-Eps15 antibody precipitated two major bands of 140 and 102 kDa (lane 2). The 140-kDa band corresponds to Eps15 since it reacts with 6G4 (6) and can be cleaved by endoprotease-Lys C into the same peptides as the in vitro translation product of eps15 cDNA.2 The 102-kDa band contains the α- and β-adaptins, as previously demonstrated by microsequencing and immunoblotting (6). A large band with the same molecular mass of 102 kDa was also precipitated by GST-DIII (lane 5) but not by GST-DI, GST-DII, or the control GST (lanes 3, 4, and 6). The identity of the 102-kDa band precipitated by the adaptins of the AP-2 complex was demonstrated by immunoblotting experiments, which showed that it reacts with anti-α (Fig. 2B, lanes 1 and 2) and anti-β (Fig. 2B, lane 4) but not with an anti-γ adaptin antibody (Fig. 2B, lane 3). In addition, GST-DIII precipitated bands of 50 and 17 kDa (lane 5). Bands of comparable molecular mass were previously observed in 6G4 immunoprecipitates (6), although only the 50-kDa band is visible in the 6G4 immunoprecipitate shown in this experiment (lane 2). These two bands have a molecular mass compatible with that of the two small components of AP-2, μ2 and σ2.

Besides the components of AP-2, GST-DIII coprecipitated polypeptides with molecular masses of 180 and 30–35 kDa (Fig. 2A, lane 5). The molecular mass of these polypeptides was comparable to those of the heavy and light chains of clathrin, which interact with the AP-2 complex (13). The presence of clathrin in the precipitate of GST-DIII was confirmed by immunoblotting with the TD.1 antibody specific for the clathrin heavy chain, which detected a 180-kDa band in the GST-DIII precipitate but not in a control GST precipitate (Fig. 2C, upper panel, lane 2).

Characterization of the Binding Site for AP-2 in the COOH-
mental procedures. In and transferred on a nitrocellulose membrane as indicated in experi-

Precipitated proteins were separated on a 7–15% gradient SDS-PAGE under reducing conditions and autoradiographed. Precipitated proteins were separated on a 7–15% gradient SDS-PAGE under strong denaturing conditions and therefore coimmuno-

A, lysates of biosynthetically labeled MOLT16 cells were first cleared with protein A-Sepharose (lane 1) or with GST coupled to glutathione-Sepharose 4B beads (lane 6) and then precipitated (PP) with mAb 6G4 coupled to protein A-Sepharose (lane 2) or GST-DI (lane 3), GST-DII (lane 4), or GST-DIII (lane 5) coupled to glutathione-Sepharose 4B beads, as described under “Experimental Procedures.” Precipitated proteins were separated on a 7–15% gradient SDS-PAGE under reducing conditions and autoradiographed. Precipitated proteins were separated on a 7–15% gradient SDS-PAGE and transferred on a nitrocellulose membrane as indicated in experimental procedures. In B, the 102-kDa band precipitated by GST-DII was immunoblotted with pan anti-α-adaptin mAb 100/2 (ascitis 1:2,000) (lane 1), anti-α-adaptin A mAb AC2M15 (ascitis 1:2,000) (lane 2), anti-γ-adaptin mAb 100/3 (ascitis 1:1,000) (lane 3), and anti-β-adaptin 100/1 (ascitis 1:5000) (lane 4). In C, precipitates of GST (lane 1) and GST-DII (lane 2) were immunoblotted with anti-clathrin heavy-chain mAb TD 1 (10 μg/ml) (upper panel) or pan anti-α-adaptin mAb 100/2 (lower panel).}

terminal Domain of Eps15—The results described above indicate that the COOH-terminal domain of Eps15 is sufficient for mediating the association between Eps15 and AP-2. To further characterize the region of Eps15 responsible for the association, a series of truncated forms of the GST-DII proteins was used to precipitate cold cell lysates, and the precipitation of AP-2 was detected by immunoblotting using the 100/2 mAb specific for α-adaptin. As summarized in Fig. 3B, a segment comprising amino acids 667–739 and including four of the 15 DPF repeats was required for AP-2 binding. Further trimming of this segment by removing either amino acids 667–675 (Fig. 3A, upper panel), lane 7) or amino acids 712–739 (upper panel, lanes 9 and 16) prevented AP-2 binding. However, the amount of α-adaptin precipitated by the fusion protein comprising only amino acids 667–763 (lanes 6 and 15) was significantly less than the amount of α-adaptins precipitated by fusion proteins containing a larger NH₂-terminal fragment of 43 amino acids (lanes 5 and 14). The NH₂-terminal fragment does not appear to provide a second binding site for AP-2, since GST-529/682 failed to precipitate α-adaptin (lane 3). More likely, the presence of this fragment influences the conformation and/or the accessibility of the binding site present on segment 667–739. However, it was not possible to define more precisely the minimal size of the NH₂-terminal fragment allowing optimal binding of AP-2, since the presence of 6 DPF repeats, very close to each other between amino acids 624–667, precluded the design of specific primers for fusion proteins of intermediate size. Finally, the amount of α-adaptins precipitated by GST-667/739 (lane 17) was consistently less than that precipitated by GST-667/763 (lane 15). Since GST-624/739 precipitated very efficiently α-adaptin (lane 8), the fragment 740–763 is not normally required for binding AP-2. Nonetheless, when the fusion protein does not contain segment 624–667, the presence of segment 740–763 may contribute to the conformation of the binding site present in segment 667–739.

The Eps15-derived Fusion Protein Binds to the Proteolyzed Ear Domain of α-Adaptin—Pretreatment studies did not allow us to identify the component of AP-2 that binds directly to Eps15, since the four components of AP-2 are dissociated only under strong denaturing conditions and therefore communoprecipitate with Eps15 (6, 14, 15). Previous studies have shown that AP-2 consists of a brick correlative structure or head with two small appendages or ears linked to the head by hinges containing sites for proteolytic cleavage. The head and ears can thus be separated by limited proteolysis of AP-2. The head consists of the NH₂-terminal domains of α- and β-adaptins associated with the medium and small chains, whereas the ears correspond to the COOH-terminal domains of the two adaptins. Proteolysis does not affect the interactions between the truncated α and β subunits and the protease resistant 50- and 17-kDa subunits (14, 15). Therefore, to define the AP-2 domain that binds to
Eps15, the head and the ears were prepared by limited proteolysis of AP-2 with trypsin. As shown in Fig. 4, lysates treated with trypsin for 20 min at 37 °C contained fragments of 60–65 kDa that were reactive with AC1-M11 and 100/1 antibodies against the head of α- and β-adaptins, respectively (15) (Fig. 4, b and d, lanes 5), and 40-kDa fragments reactive with 100/2 and Ab32 antibodies against the ears of α- and β-adaptins, respectively (10, 15) (Fig. 4, a and c, lanes 5). When proteolysis was further prolonged, there was a decrease in the amount of immunoreactive 60–65-kDa fragments, indicating that these fragments were proteolyzed to smaller peptides (not shown). Therefore, control undigested lysates or lysates treated with trypsin for 5, 10, or 20 min were precipitated with GST-DIII (lane 1, 2, and 3, respectively). As shown in Fig. 4, a and b, GST-DIII precipitated undigested α-adaptin visible in control precipitates (lanes 1) and in precipitates obtained after a digestion for 5 min (lanes 2) or 10 min (Fig. 4b, lane 3). In addition, GST-DIII precipitated a fragment with a molecular mass of 40 kDa, which was reactive with mAb 100/2 (Fig. 4a, lanes 2–4) and therefore corresponded to the ear of α-adaptin. In contrast, GST-DIIId failed to precipitate the other proteolyzed fragments of AP-2. Thus, it precipitated neither the NH$_2$-terminal domain of α-adaptin recognized by mAb AC1-M11 (Fig. 4b, lanes 2–4), nor that of β-adaptin, recognized by mAb 100/1 (Fig. 4d, lanes 3 and 4). Some intact β-adaptin and a light band of 60 kDa that reacted with the 100/1 antibody were observed in the GST-DIII precipitate after a 5-min digestion (Fig. 4d, lane 2) but were not detectable after longer times of digestion (Fig. 4d, lanes 3 and 4). Since intact α-adaptin remained detectable at 5 and 10 min of digestion (Fig. 4b, lanes 2 and 3), these bands most likely correspond to digested or undigested β-adaptin that is still associated with undigested α-subunit. Finally, GST-DIII did not precipitate the ear of β-adaptin detected by the Ab32 antiserum (Fig. 4c). These results indicate that Eps15 specifically binds the ear of α-adaptin.

GST Fusion Proteins Derived from the COOH-terminal Domain of α-Adaptin Bind Eps15—To confirm the results of the proteolysis studies, a GST-fusion protein encompassing the entire COOH terminus of α-adaptin and including the hinge region was derived from mouse α-adaptin (Fig. 5A). We observed that a large fraction of the α-adaptin ear was cleaved from the GST moiety during purification of the GST fusion protein and released in the bacterial lysate (not shown). This proteolytic cleavage was probably due to the presence of bacterial proteases and could not be prevented by the use of exogenous protease inhibitors. Therefore, to demonstrate the association of Eps15 with the α-adaptin ear, bacterial lysates containing the α-adaptin ear were precipitated with Eps15-derived GST fusion proteins. After three clearing cycles with glutathione-Sepharose 4B beads to remove free GST, the supernatant was precipitated with fusion proteins derived from Eps15 (lane 1), and precipitates by GST-DI (lane 2) or GST-DIIId (lane 3) were analyzed by Western blotting using the α-adaptin ear-specific mAb 100/2. C, two different constructs encoding residues 756–938 (lane 1) and residues 706–938 (lane 2) of mouse α-adaptin C were used to precipitate MOLT16 cell lysates (PP). The presence of Eps15 in the precipitates was revealed by Western blotting (WB) using the 6G4 mAb (upper panel). Coomassie Blue staining of the membranes revealed that similar amounts of fusion proteins were used in the two precipitations (lower panel).
We have recently demonstrated a specific and constitutive interaction between Eps15 and the plasma membrane adaptor, AP-2 (6). In the present study, the binding site of Eps15 for interaction between Eps15 and the plasma membrane adaptor, is indicated and its amino acid sequence is shown. The four DPF repeats are underlined. In addition, we have identified AP-2 (6). In contrast, the GST fusion protein encoding the COOH-terminus of Eps15 was tested for their ability to precipitate AP-2 comprised amino acids 667–739 and included only four of the DPF repeats among the 15 present in human Eps15. Its sequence is shown in Fig. 6A. The binding site of AP-2 was thus close but distinct from the binding site of Crk (residues 765–771) (1), indicating that these proteins bind Eps15 independently. A search of the data banks using the Blast program revealed 90% identity with the corresponding sequence in the murine Eps15 protein, which also interacts with AP-2 (6). In contrast, the sequence was poorly conserved in Eps15, the homology being largely related to the presence of DPF repeats. Furthermore, there was no significant homology with other known proteins, suggesting that the association of Eps15 with AP-2 has a very specific function.

Clathrin-coated pits and vesicles bud from two membrane compartments, the plasma membrane and the trans-Golgi network. Two distinct adaptor complexes link the clathrin lattice to the appropriate membrane: AP-2, associated with the plasma membrane coated vesicles, and AP-1, associated with trans-Golgi coated vesicles (7, 8). Both adaptors are heterotetramers consisting of two 90–110-kDa adaptins (α and β, for the plasma membrane, γ and β, for the trans-Golgi) complexed with two smaller proteins of 48–50 and 16–17 kDa (μ1, μ2 and σ1, σ2 respectively) (7, 8). In agreement with our previous observations (6), Eps15-derived fusion proteins precipitated four proteins with molecular masses consistent with the four components of the adaptor complexes. The α- and β-adaptins, but not γ-adaptin, were found in the GST-Eps15 precipitate, a result that confirms the specific association of Eps15 with AP-2 but does not allow us to determine which subunit of AP-2 interacts with Eps15. Several protein associations with components of the adaptor complexes have already been described. The β1 and β2 subunits, which are 85% identical (14, 16), mediate binding of both AP-1 and AP-2 adaptors to clathrin and promote clathrin coat assembly (17–19). The μ1 and μ2 subunits, which share 40% identity (20, 21), interact with tyrosine-based signals of several integral membrane proteins (22). The α- and γ-adaptins have an overall identity of only 25%, mainly restricted to the NH2 domain (23). This domain contains sequences which simultaneously determine coassembly with the correct μ and σ subunits and targeting to the appropriate membrane (24, 25). In addition, the NH2-terminal domain of α-adaptin binds to clathrin cages (26) and contains a binding site for polyphosphoinositols which, in vitro, inhibit AP-2 self-association, binding of AP-2 to clathrin, and clathrin coat assembly (27, 28). In contrast to the other components of adaptor complexes, the ears of α- and γ-adaptins show no homology, suggesting that they have distinct functions (23). Precipitation of the fragments of AP-2 released by limited proteolysis indicates that the Eps15-derived fusion proteins bound to the ear of α-adaptin. This result was confirmed by precipitation of Eps15 with a fusion protein encompassing the ear of α-adaptin C. Furthermore, the ear of α-adaptin C released in the lysate of transformed bacteria could be precipitated by a GST-Eps15 fusion protein, demonstrating that Eps15 and α-adaptin interact directly. These data are consistent with the specificity of Eps15 for AP-2 and supports the hypothesis that the ear of α-adaptin is endowed with a specific function. A model summarizing the interaction of Eps15 with AP-2 is shown in Fig. 6B.

The association of Eps15 with AP-2 and its homology with End3p strongly suggest that this protein has a function in clathrin-mediated endocytosis. Furthermore, the presence of the clathrin heavy chain in the GST-DIII precipitate indicates that Eps15 may be a component of clathrin-coated pits and vesicles where AP-2 and clathrin may interact, a hypothesis supported by preliminary electron microscopic data.3 Eps15 might thus be related to the unidentified 150-kDa protein observed by Beck and Keen (18) in AP-2 aggregates and in coated vesicles and/or to the 140-kDa protein observed by Lindner and Ungewickel (29) among the components of bovine clathrin-coated vesicles. The putative function of Eps15 in endocytosis remains to be defined. Our study, which delineates

3 G. Raposo, A. Benmerah, B. Bague, A. Dautry-Varsat, and N. Cerf-Bensussan, unpublished results.
the domain of Eps15 involved in AP-2 binding, provides the basis for the design of mutated proteins that could elucidate the in vivo function of Eps15.

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