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The EH and SH3 domain Ese proteins regulate endocytosis by linking to dynamin and Eps15

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Clathrin-mediated endocytosis is a multistep process which requires interaction between a number of conserved proteins. We have cloned two mammalian genes which code for a number of endocytic adaptor proteins. Two of these proteins, termed Ese1 and Ese2, contain two N-terminal EH domains, a central coiled-coil domain and five C-terminal SH3 domains. Ese1 is constitutively associated with Eps15 proteins to form a complex with at least 14 protein–protein interaction surfaces. Yeast two-hybrid assays have revealed that Ese1 EH and SH3 domains bind epsin family proteins and dynamin, respectively. Overexpression of Ese1 is sufficient to block clathrin-mediated endocytosis in cultured cells, presumably through disruption of higher order protein complexes, which are assembled on the endogenous Ese1–Eps15 scaffold. The Ese1–Eps15 scaffold therefore links dynamin, epsin and other endocytic pathway components.

Keywords: EH domains/endocytosis/Eps15/Ese/SH3 domains

Introduction

Endocytosis via clathrin-coated pits is a multistep process which requires several conserved proteins (Riezman *et al*., 1997; Schmid, 1997). Clathrin heavy and light chains are brought to the plasma membrane through association with a heterotetrameric complex known as clathrin adaptor complex 2, or AP-2 (Robinson, 1997). At coated pits, membrane is bent through the assembly of the clathrin triskelion into a caged lattice. The GTPase dynamin is then recruited to the neck of coated pits where it assembles into a collar for vesicle fission (Warnock and Schmid, 1996; Urrutia *et al*., 1997). From genetic, cell biological and biochemical analyses, it is clear that additional components such as GTPases, kinases, phosphatases, ubiquitin-conjugating enzymes, lipid-modifying enzymes and the actin cytoskeleton are all required for clathrin coat and vesicle formation (De Camilli *et al*., 1996; Riezman *et al*., 1997; Robinson, 1997; Schmid, 1997). The process by which these numerous molecular interactions and activities are coordinated temporally and spacially to induce endocytosis remains unknown.

One essential component of the early endocytic pathway in mammals is the Eps15 homology (EH) domain protein, Eps15. This protein was discovered in a search for substrates of the epidermal growth factor receptor (Fazioli *et al*., 1993). In 1995, Benmerah *et al*. reported that Eps15 is constitutively associated with α-adaptin of the AP-2 complex (Benmerah *et al*., 1995). The Eps15 protein has also been localized to the neck of clathrin-coated pits by immunoelectron microscopy (Tebar *et al*., 1996, 1997). Recently, two groups have used dominant inhibitory mutants of Eps15, or antibodies against Eps15 (and its relative Eps15R), to demonstrate that these proteins are required for clathrin-mediated endocytosis (Carbone *et al*., 1997; Benmerah *et al*., 1998). Eps15 contains three large structural domains (Fazioli *et al*., 1993; Wong *et al*., 1995). The N-terminal third contains three copies of an EH domain (Wong *et al*., 1995; Di Fiore *et al*., 1997). The central region of Eps15 forms an extended coiled-coil, which is followed by a C-terminus containing a large number of aspartic acid–proline–phenylalanine repeats, α-adaptin-binding sequences (Benmerah *et al*., 1996, 1998; Iannolo *et al*., 1997) and proline-rich stretches which can bind to SH3 domains (Schumacher *et al*., 1995). The full-length Eps15R protein has a similar organization (Schumacher *et al*., 1995), and both Eps15 and Eps15R can be alternatively spliced to generate a number of protein isoforms (Coda *et al*., 1998).

Two EH domain proteins have been identified in *Saccharomyces cerevisiae*, named Pan1p and End3p, which are required for endocytosis and organization of the actin cytoskeleton (Raths *et al*., 1993; Benedetti *et al*., 1994; Tang and Cai, 1996; Wendland *et al*., 1996). Pan1p and End3p form a complex *in vivo* (Tang *et al*., 1997). Indeed, overexpression of End3p can suppress the phenotype of *pan1-4* hypomorphic mutants, and Pan1p is mislocalized in *end3* mutants, indicating that these proteins function together (Tang *et al*., 1997). Additional studies have revealed that the EH domains of Pan1p bind to yeast homologues of mammalian clathrin-binding proteins, AP180 and CALM (yAP180A and yAP180B), through NPF motifs (asparagine–proline–phenylalanine) in the yAP180A/B C-termini (Salcini *et al*., 1997; Wendland and Emr, 1998). These data have led to the proposal that the Pan1p–End3p complex functions as a multivalent adaptor to coordinate protein–protein interactions during endocytosis (Tang *et al*., 1997; Wendland and Emr, 1998). Specifically, the Pan1p–End3p complex may coordinate ubiquitination, inositol lipid modification, actin polymerization/depolymerization and clathrin cage formation during the early stages of endocytosis (Wendland *et al*., 1998). It has not been determined whether analogous EH heteromeric protein complexes exist in multicellular animals.

Purified dynamin forms tubules from protein-free liposome preparations *in vitro* (Sweitzer and Hinshaw, 1998; Takei *et al*., 1998). These tubules are vesiculated on hydrolysis of GTP (Sweitzer and Hinshaw, 1998). The localization and function of the dynamin GTPases must, therefore, be tightly regulated *in vivo*, in order to restrict the conversion of cellular membranes into coated tubules and vesicles (McNiven, 1998). The dynamin pleckstrin homology (PH) and proline-rich domains are believed to function as intramolecular regulators of its localization and self-assembly (Schmid *et al*., 1998). These domains are responsive to inositol phospholipids and SH3 domains, respectively (Schmid *et al*., 1998).

Here we report the identification of two novel mammalian genes, *Ese1* and *Ese2*. *Ese* genes are subject to alternative splicing reactions to produce several transcripts which code for complex adaptor proteins. The Ese1 protein contains two N-terminal EH domains, a central coiled-coil domain and five SH3 domains. This protein forms a complex *in vivo* with Eps15 which is analogous to the Pan1p–End3p complex in yeast. The Ese1 SH3 domains bind to dynamin, and its EH domains bind to epsin family proteins. The Ese1–Eps15 complex therefore regulates endocytosis in multicellular animals by linking dynamin and other components of the endocytic machinery.

Results

Ese1 and Ese2 encode multidomain proteins

Numerous SH3 domain-containing proteins have been implicated in the endocytic process (Gout *et al*., 1993; Munn *et al*., 1995; Wang and Moran, 1996; de Heuvel *et al*., 1997; Ringstad *et al*., 1997; Shupliakov *et al*., 1997; Wesp *et al*., 1997; Wigge *et al*., 1997b). Kay and coworkers have reported the isolation of several novel SH3-encoding sequences (Sparks *et al*., 1996). To test for endocytic functions of the corresponding proteins, we have isolated mouse cDNAs corresponding to the human SH3P17 and SH3P18 partial cDNAs from this reference. Sequence and functional analyses of SH3P17 and SH3P18 open reading frames have implicated these proteins in endocytosis via clathrin-coated pits, and therefore we have named them Ese1 and Ese2, respectively (Ese for EH domain and SH3 domain regulator of endocytosis; see below). The Ese1 and Ese2 proteins are predicted to encode two N-terminal EH domains followed by a coiled-coil domain and five SH3 domains (Figure 1). Interestingly, EH domains have also been identified in Eps15, Eps15R and the yeast endocytic partner proteins, Pan1p and End3p. Coiled-coil domains typically are associated with protein–protein dimerization, or tetramerization, as noted for Eps15 (Cupers *et al*., 1997; Tebar *et al*., 1997). The Ese proteins are 53% identical over the full length of Ese1 (645 of 1213 residues in Ese1 line up with identical residues in Ese2) and are highly related to the Ese proteins from *Xenopus* (DDBJ/EMBL/GenBank accession No. AF032118) and *Drosophila* (Roos and Kelly, 1998) (Figure 1). *Xenopus* intersectin is 81% identical to mouse Ese1 and 54% identical to mouse Ese2, suggesting that intersectin is an Ese1 orthologue (980/1213 residues of mouse Ese1 and 645/1198 residues of mouse Ese2 line up with identical residues in the *Xenopus* protein). *Drosophila* dynamin-associated protein, Dap160-1 (see below) is 32% identical to both mouse Ese proteins (393/1213 residues of mouse Ese1 and 387/1198 residues of mouse Ese2 line up with identical residues in the *Drosophila* protein). These homologies extend over the entire length of Dap160-1, except that Dap160-1 has only four SH3 domains corresponding to the first, second, fourth and fifth SH3 domains of the mouse Ese proteins. Additional sequence analysis reveals the presence of a very large number of potential phosphorylation sites and at least one SH3-binding consensus (Ren *et al*., 1993) in the N-terminus of each Ese protein.

In order to determine where the *Ese* genes are expressed, we have performed Northern analysis on mRNA derived from several adult mouse tissues (Figure 2A). These genes are both widely expressed. The highest levels of Ese1 mRNA were noted in brain, heart and skeletal muscle. Interestingly, brain expresses high levels of many proteins involved in synaptic vesicle endocytosis and recycling.

Our Northern blot reveals the presence of many alternative mRNA transcripts for both Ese1 and Ese2. For example, brain and skeletal muscle mRNA contain unusually large Ese1 transcripts of ~15 kb in length. In addition, numerous Ese1 expressed sequence tags (ESTs) have been identified which skip sequences encoding individual EH domains or regions of the coiled-coil domain, indicating that this gene has the potential to code for many distinct proteins. Indeed, the $SH3_C$ domain was spliced out of the human SH3P17 partial cDNA described by Sparks *et al*. (1996). The predicted Ese2 protein on the other hand encodes a C-terminal extension of 45 amino acids in comparison with the human SH3P18 partial cDNA, revealing that this gene is also alternatively spliced. Lung, liver and kidney express high levels of a 2.4 kb Ese2 transcript, which is significantly smaller than the 3591 bp sequence required to code for our full Ese2 protein (Figure 1), indicating that a major isoform of Ese2 has only a subset of the domains described above.

We have begun to characterize additional transcripts from both *Ese1* and *Ese2* genes. One of our Ese1 clones isolated from a mouse brain cDNA library contained an extended reading frame. We have used PCR from mouse brain cDNA libraries to identify the remaining coding sequences from this transcript. The splicing event which produces this extended protein adds an additional 501 amino acids to Ese1, and codes for a DBL homology domain, a PH domain and a C2 domain (Figure 2B). DBL and PH domains are found together in guanine nucleotide exchange factors for the Rho family of small GTPases. This long form of Ese1 is, therefore, expected to function as an activator of Rho-GTPases, which in turn regulate the actin cytoskeleton and numerous signal transduction pathways (Hall, 1998). C2 domains on the other hand are Ca^{2+}-activated membrane-binding domains and protein–protein interaction domains (Nalefski and Falke, 1996). We have identified a number of ESTs which code for a novel C2 domain fused to the sequence coding for the last 11 amino acids of Ese2. We have therefore used PCR to isolate sequences coding for the alternatively spliced exon(s) which can be included C-terminal to the SH3 domains but before the stop codon. The alternatively spliced exon(s) of Ese2 also codes for a DBL/PH + C2 domain cassette which can be included within the Ese2 transcript (Figure 2B).

Fig. 1. Alignment of protein sequences of the Ese family in mouse, *Xenopus* and *Drosophila*. EH and SH3 domains areas are indicated with overlining. Amino acid identities (in bold) and similarity are boxed.

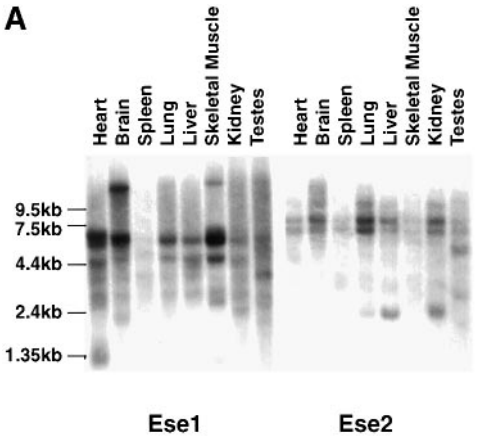
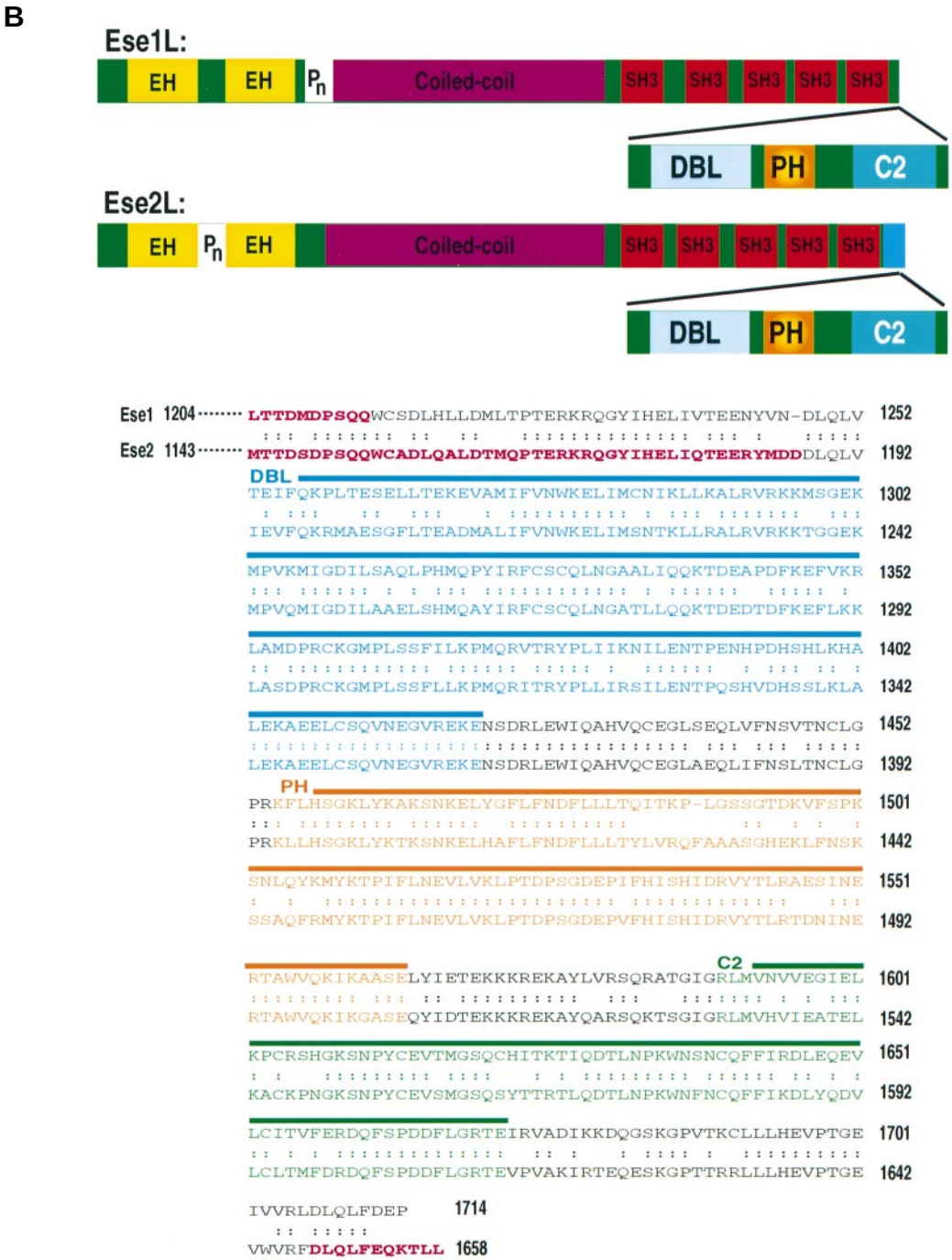


Fig. 2. (**A**) Expression of *Ese1* and *Ese2* genes in adult mouse tissues. Multiple tissue Northern blots of the indicated adult mouse tissues were probed to detect expression of *Ese1* or *Ese2*. (**B**) Alternative transcripts from the *Ese1* and *Ese2* genes code for Ese1L and Ese2L proteins, respectively, with C-terminal DBL/PH and C2 domains. Ese1 and Ese2 sequence junctions are indicated in bold red letters.



As the *Ese* genes are subject to complex alternative splicing to produce proteins with novel predicted functions (e.g. regulation of the cytoskeleton and membrane binding), we feel that it is important to establish a naming system for these and other protein products from the complex *Ese* genes. We suggest that the proteins with two EH domains, a central coiled-coil domain and five SH3 domains be named Ese, and that alternative proteins are named as modifications of this protein. In other words, the Ese1 and Ese2 long forms described in Figure 2B will be known as Ese1L and Ese2L, respectively. In the event that specific domains are spliced out from the transcripts coding for Ese1 and Ese2 proteins, as shown in Figure 1, then the name should be listed as an Eseδ variant. We suggest, therefore, that the protein encoded by the spliced variant identified by Kay and co-workers in the original SH3P17 clone be designated as Ese1δS_C to indicate removal of $SH3_C$. In this study, we have characterized the properties and interactions of the Ese1 protein described in Figure 1.

Ese and Eps15 proteins interact in vivo

In order to define the function of Ese proteins, we screened for partners using the yeast two-hybrid system. Our first screen used the central coiled-coil domain of Ese1, from amino acid 330 to 732, fused to the GAL4 DNA-binding domain. This bait was transformed into the Y190 reporter strain together with plasmid cDNA libraries from several mouse tissue sources. Yeast colonies were selected for growth on histidine minus plates in the presence of 40 mM 3-aminotriazole (3AT) to select for interaction between library-encoded GAL4 activation domain fusions and the GAL4 DNA-binding domain Ese1 coiled-coil bait. Yeast colonies which survived selection for expression of the *His3* gene were also tested for induction of the integrated *LacZ* gene which is GAL4 responsive in Y190. Prey plasmids were recovered from 37 positive yeast colonies which were selected for expression of both *His3* and *LacZ*. One class of cDNAs recovered in this screen included Eps15, which was isolated twice and Eps15R which was isolated from four yeast colonies. Other positive clones are currently being analysed to test whether they encode real Ese1-binding partners *in vivo*. Interestingly, the Eps15 and Eps15R clones were all partial cDNA fusions which minimally included central and C-terminal sequences from the region coding for the coiled-coil domain to the 3′ untranslated region (UTR) (Figure 3A). In the case of Eps15, the positive clones start from amino acid 306 and 376, whereas in Eps15R, the N-terminal boundary of clones were amino acids 4, 10, 222 and 386. These data indicate that minimal sequences required for interaction between the Ese1 coiled-coil domain and Eps15(R) include either or both of the central coiled-coil and/or the C-terminal third of these proteins.

We next wanted to analyse the Ese proteins *in vivo* and to test for their association with Eps15 or Eps15R. Polyclonal antiserum against the N-terminal 21 amino acids of Ese1 was generated in rabbits and peptide affinity purified. In addition, polyclonal serum was raised in chickens against a GST fusion containing the C-terminus of Ese1 from amino acid 665 to the stop codon. This region of Ese1 contains all five of the SH3 domains. We next prepared cell lysates from A431, PC12, MDCK and HeLa cells which represent cell lines from several tissue types and species. The rabbit anti-peptide antiserum was used to precipitate Ese1 from each lysate, and precipitates were analysed by Western blotting using the chicken anti-Ese serum. In each cell line, we observed the presence of several specific bands in the range of 150 kDa which were precipitated in the absence but not in the presence of the peptide to which our serum was generated (Figure 3B). Thus, the Ese1 protein is expressed in many cell lines, and runs on gels in a range consistent with the predicted mol. wt of 137 kDa. The presence of several Ese protein bands suggests that either multiple isoforms of Ese1 can be co-expressed in a single cell, that Ese2 is co-precipitated with Ese1 (note that the chicken antiserum raised against Ese1 is expected to cross-react with Ese2) or that Ese1 is covalently modified.

Ese1 immunoprecipitation samples were also analysed for co-immunoprecipitation of Eps15. In each case, we detected multiple Eps15 proteins which co-purify with Ese1 (Figure 3B). The anti-Ese1 peptide antisera and commercially available anti-Eps15 antisera which we have used were raised against epitopes which are not shared by Ese and Eps15 proteins, ruling out the possibility of cross-reactivity. Ese1 contains multiple C-terminal SH3 domains and Eps15 contains a C-terminal SH3-binding motif. Thus, while Eps15 proteins bound to the coiled-coil domain of Ese1 in our yeast two-hybrid screen, association between these proteins may also require interaction between their respective C-termini *in vivo*. To test whether the C-terminus of Eps15 is required for its association with Ese1, we replaced the entire Eps15 C-terminus with a Flag epitope tag (Eps15δC). Full-length Ese1 was transfected into Cos-1 cells either alone or together with Eps15 or C-terminally truncated Eps15 (Eps15δC). Cell lysates were precipitated with rabbit anti-Ese1 antisera, mouse anti-Flag monoclonal antibodies or with rabbit anti-Eps15 antisera, and immunoprecipitates were Western blotted with chicken anti-Ese antisera. The anti-Flag antibody efficiently precipitated Ese1 from cells expressing Flag-tagged Eps15δC, indicating that the C-terminally truncated Eps15 protein can bind to Ese1 *in vivo* (Figure 3C). The Ese1 and Eps15 proteins are, therefore, associated *in vivo* through interaction of their central coiled-coil regions and do not require the presence of SH3 and SH3-binding motifs in their respective C-termini.

Localization of the Ese1–Eps15 complex

The association of Ese1 and Eps15 is reminiscent of the complex between two EH domain-containing proteins in yeast (Pan1p and End3p). To determine the subcellular localization of Ese1, we first tested our rabbit and chicken antibodies in immunofluoresence experiments. Unfortunately, these sera did not recognize endogenous or transfected Ese1 in this assay. We therefore engineered a myc epitope-tagged version of this protein (mycEse1). Tagged Ese1 was expressed in Cos cells and detected by confocal immunofluorescence microscopy using the mouse anti-myc monoclonal antibody 9E10. Interestingly, the transfected Ese1 protein is highly concentrated on circular structures in most transfected cells (Figure 4A and A′) (compare single optical section in Figure 4A with the projection of all sections through the same cell shown in

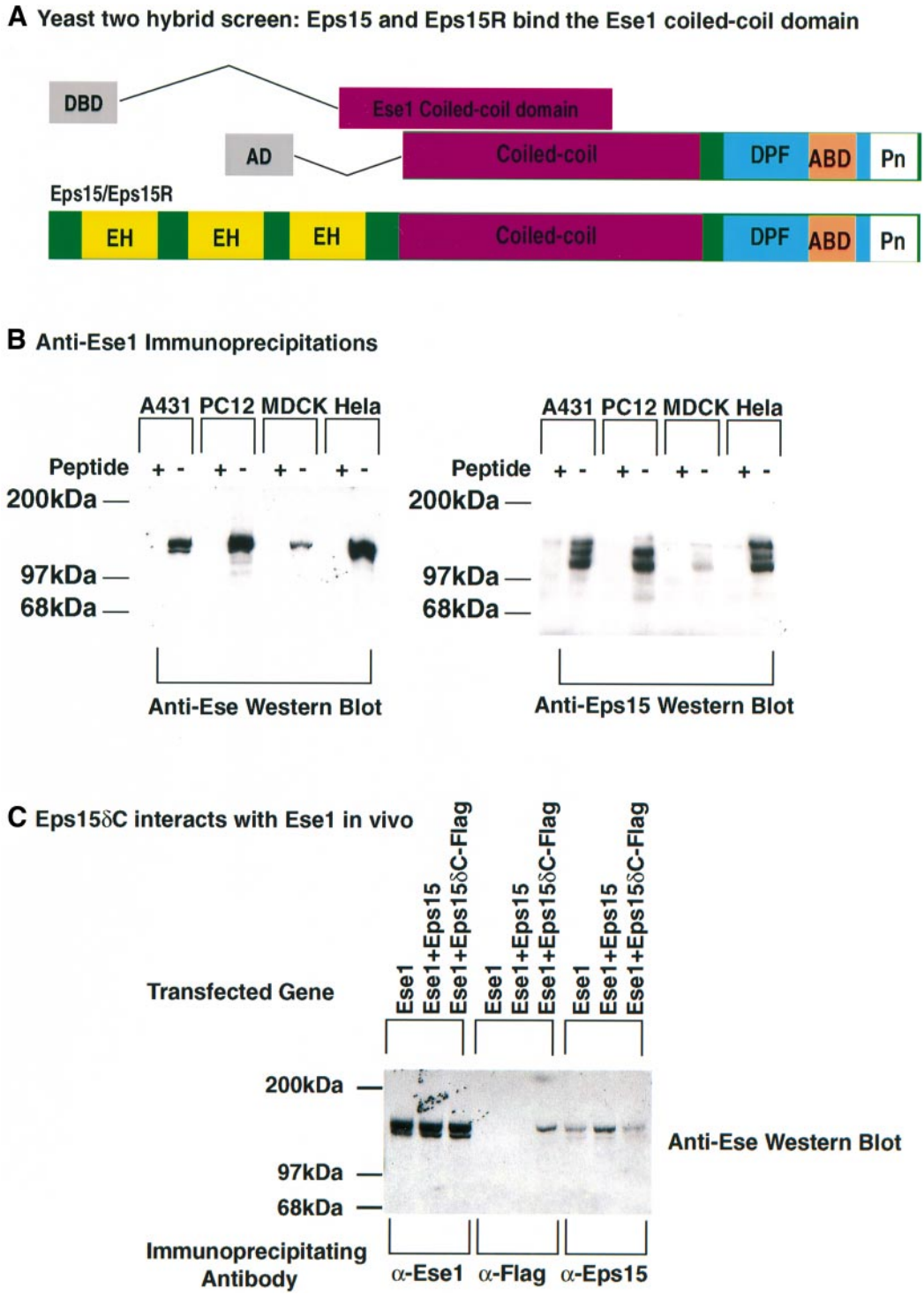


Fig. 3. Ese and Eps15 associate *in vivo*. (**A**) Schematic representation of association of Eps15/Eps15R with Ese1 in the yeast two-hybrid screen. The Ese1 coiled-coil domain fused to the Gal4 DNA-binding domain (DBD) interacted with Eps15/Eps15R Gal4 activation domain (AD) fusions. Note that the AD–Eps15/Eps15R diagram represents the shortest interacting coding region isolated. (**B**) Endogenous Ese1 proteins were precipitated with rabbit anti-peptide antisera against the N-terminus of Ese1. Immunoprecipitates were then analysed by Western blots for the presence of Ese1/2 with chicken anti-Ese antisera or for co-precipitation of Eps15 with rabbit antisera raised against the C-terminus of Eps15. (**C**) Cos-1 cells were transfected with pcDNA3Ese1, pcDNA3Eps15 or the Eps15 C-terminal deletion mutant expression plasmid pcDNA3Eps15δC-Flag as indicated. Cell lysates were immunoprecipitated with rabbit anti-Ese1, mouse anti-Flag or rabbit anti-Eps15. Proteins were transferred to nitrocellulose and probed to detect the presence of Ese1 in each immunoprecipitation. Note the precipitation of Ese1 with anti-Flag monoclonal antibody in the sixth lane, indicating that Ese1 forms a complex with the C-terminally truncated Eps15δC protein *in vivo*.

4A′). In some optical sections, we have observed rings of fluorescent staining surrounding a non-staining area, suggesting that the Ese1-induced structures are vesicles rather than inclusion bodies (data not shown and Figure 6 below). Overexpression of Ese1 in 10T1/2, BHK and HeLa cells using the vaccinia virus T7 expression system (Stenmark *et al*., 1995) also leads to its localization on large circular structures (data not shown). This concentration of

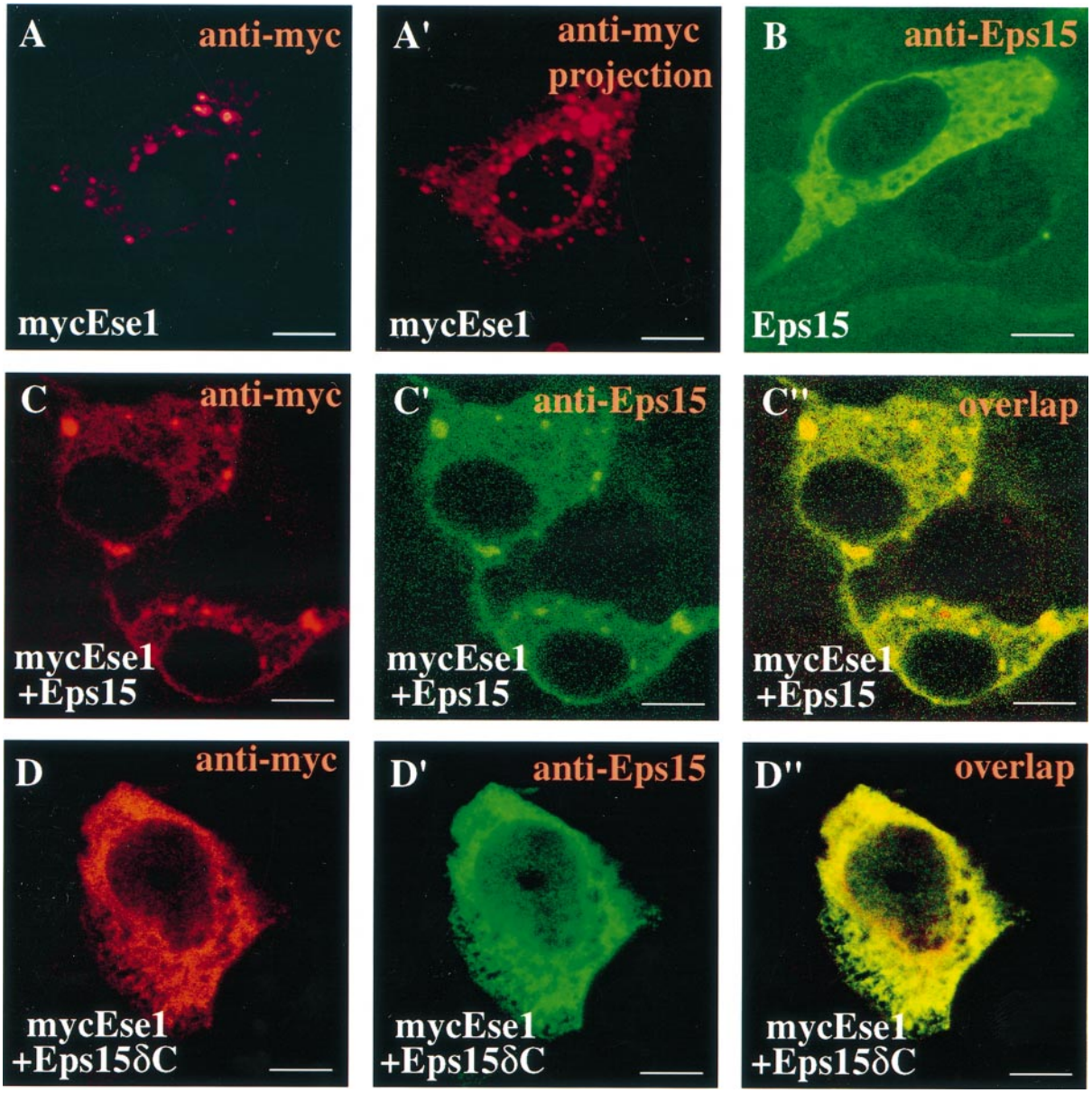


Fig. 4. Ese1 and Eps15 proteins function together. Confocal immunofluorescent microscopy was used to detect Ese1 or Eps15 proteins in Cos cells transfected with mycEse1 (**A** and **A**′), Eps15 (**B**), mycEse1 + Eps15 (**C**, **C**′ and **C**″) or mycEse1 + Eps15δC (**D**, **D**′ and **D**″). (C″) and (D″) represent the overlapping images from (C/C′) and (D/D′), respectively. Overlap in (C″) and (D″) is indicated in yellow. Scale bar, 10 μm.

ectopically expressed Ese1 contrasts with the localization of Eps15 in transiently transfected cells, since Eps15 is dispersed throughout the cytoplasm (Figure 4B). Ese1 and Eps15 proteins form a complex *in vivo*, and yet our data suggested that these proteins were localized to distinct subcellular compartments in transfected Cos cells. Given these data and the fact that End3p controls the localization of Pan1p in yeast, we considered the possibility that Ese1 may function to control Eps15 localization. We therefore determined the subcellular distribution of mycEse1 and Eps15 in Cos cells co-transfected with both genes. In co-transfected cells, mycEse1 is still found in circular structures (Figure 4C). However, in contrast to cells expressing Eps15 alone, in co-transfected cells Eps15 was now partially co-localized with Ese1 in the same structures (Figure 4C′ and C″). Thus, Ese can regulate the localization of Eps15.

The C-terminal third of Eps15 contains several regions which are required for association with α-adaptin of the AP-2 clathrin adaptor complex (Benmerah *et al*., 1998). We found that this region of Eps15 is not required for its association with Ese1 (Figure 3C). In order to test whether Eps15 function is required for Ese localization, we co-transfected mycEse1 and Eps15δC. Interestingly, the mycEse1 protein is still partially co-localized with Eps15δC (Figure 4D, D′ and D″), but is no longer found to be concentrated within circular structures, indicating that Ese1 requires Eps15 function for its distinctive subcellular localization in transfected cells. In addition, these data suggest that the Ese1–Eps15 complex may require association with AP-2, which binds to the Eps15 C-terminus, in order to form large circles or vesicles in Ese1-transfected cells. Taken together, these results demonstrate that Ese1 and Eps15 function cooperatively to regulate subcellular localization of the Ese1–Eps15 complex.

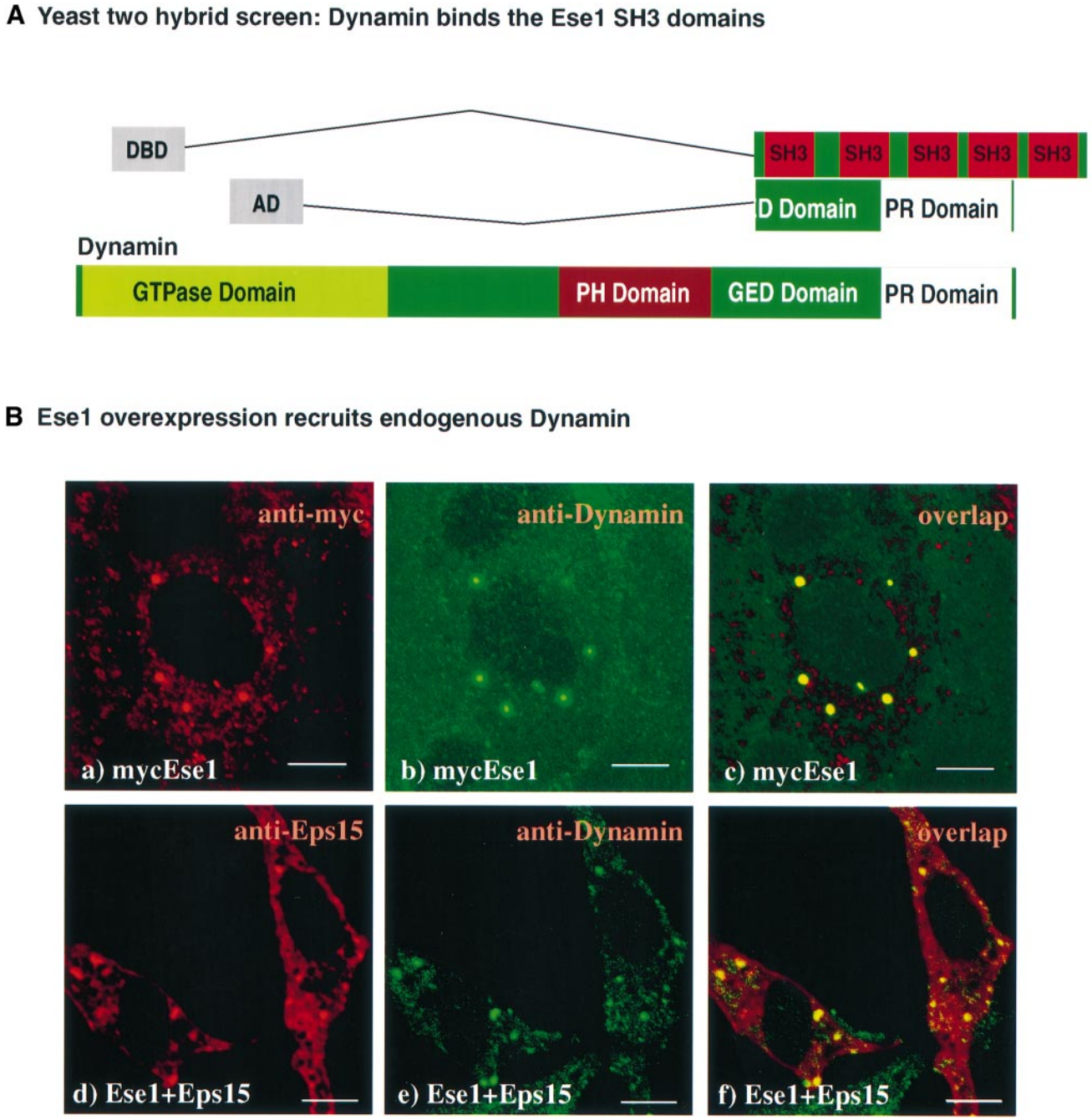


Fig. 5. Ese1 links to dynamin and Eps15. (**A**) Schematic representation of association between dynamin and Ese1 in the yeast two-hybrid screen. Ese1 SH3 domains fused with the Gal4 DNA-binding domain (DBD) interacted with dynamin–Gal4 activation domain (AD) fusions. Note that the AD–dynamin diagram represents the shortest interacting coding region isolated. (**B**) Confocal immunofluorescent microscopy was used to detect transfected mycEse1 (a), transfected Eps15 (d) or endogenous dynamin (b and e) in transfected Cos cells. (c) and (f) represent the overlapping images from (a/b) and (d/e), respectively. Overlap in (c) and (f) is indicated in yellow. Scale bar, 10 μm.

Ese and dynamin

To identify Ese partners which bind to the SH3 domains of this multidomain protein, we performed yeast two-hybrid screens using baits composed of the GAL4 DNA-binding domain fused to individual SH3 domains of Ese1. Initial screens with $SH3_B$ and $SH3_C$ domain fusions resulted in the artifactual isolation of many proline-rich fragments which did not represent real Ese partners. We then focused our effort on screens with GAL4 fusions containing all five SH3 domains (amino acids 665–1213). We selected 41 $His3^+/LacZ^+$ colonies from such screens for further analysis. Six colonies were found to encode fragments of the dynamin II gene and two encoded fragments of dynamin I (Figure 5A). The other 33 clones currently are being analysed to determine the *in vivo* relevance of their affinity for Ese1. All dynamin II clones had 5′ start sites between amino acids 252 and 278, and terminated within the 3′ UTR. The two dynamin I clones were identical and contained a small coding region from amino acid 673 to the C-terminus. Dynamin sequences contained within each Ese1-interacting clone therefore minimally code for the proline-rich motifs (Figure 5A),

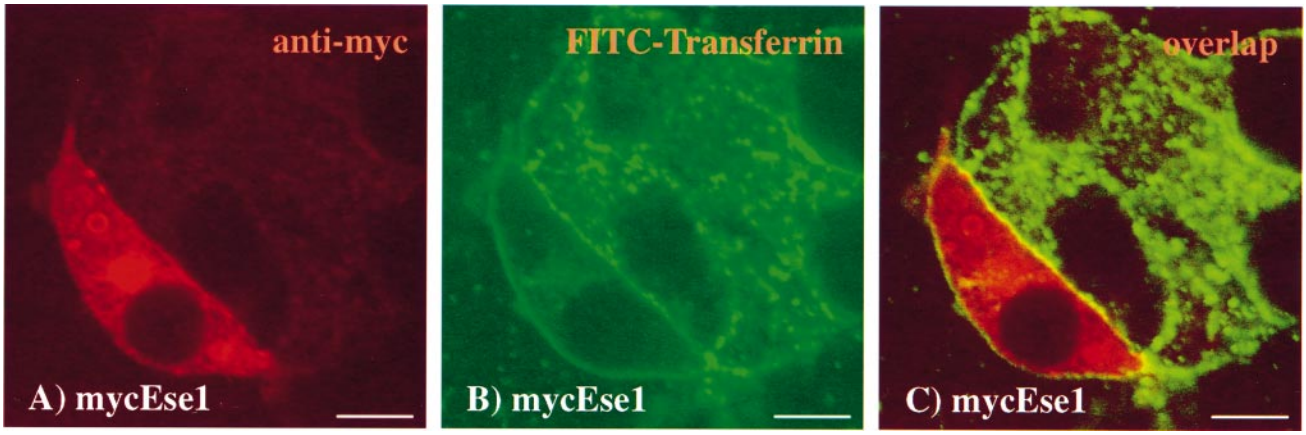


Fig. 6. Ese1 overexpression blocks endocytosis of transferrin in Cos-1 cells. Confocal immunofluorescent microscopy was used to detect transfected mycEse1 (**A**) and internalized FITC-labelled transferrin (**B**). Overlapping images are shown in (**C**), revealing that Ese1 overexpression blocks clathrin-mediated endocytosis of transferrin. Mononuclear morphologically normal cells from both transfected and untransfected groups were assessed for internalization of transferrin. Cell counts from each group are discussed in the text. The scale bar is equal to 10 μm.

which are known to bind SH3 domains *in vitro* (Gout *et al*., 1993). This result is consistent with the interaction between Dap160 and dynamin that has been described recently in *Drosophila* (Roos and Kelly, 1998). To determine the significance of Ese1–dynamin binding, we tested for functional interactions between these proteins. MycEse1 was again transfected into Cos cells. The subcellular localization of dynamin in Ese1-overexpressing cells was analysed by staining for endogenous dynamin and for the myc tag on Ese1. We were unable to detect endogenous dynamin in untransfected cells, probably due to its lack of subcellular concentration. In many Ese1 transfectants, however, endogenous dynamin was recruited to the Ese1 staining vesicles, and both proteins were co-localized (Figure 5B, panels a, b and c). Taken together, our results and the recently reported co-localization of endogenous dynamin and Dap160 in *Drosophila* (Roos and Kelly, 1998) demonstrate that Ese proteins bind dynamin and can regulate its subcellular localization. Given that Ese1 functions in a complex with Eps15 as discussed above, and Ese can regulate the subcellular distribution of dynamin, we asked whether Eps15 also co-localized with dynamin. Eps15 transfected into Cos cells was localized diffusely as shown above (Figure 4B). Endogenous dynamin was not concentrated and was therefore not visible in Eps15-transfected cells (data not shown). When Ese1 and Eps15 were co-transfected, however, we note that Eps15 and endogenous dynamin are co-localized on the Ese1-induced circles (Figure 5B, panels d, e and f), indicating that all three proteins co-localize at these structures.

Ese and Epsin family proteins

In order to identify additional Ese partners, we generated a fusion between the GAL4 DNA-binding domain and the N-terminal EH domain region of Ese1 (amino acids 1–393). This fusion protein was also used in yeast two-hybrid screens with several GAL4 activation domain cDNA libraries. A total of 11 $His3^+/LacZ^+$ colonies were identified as containing library-encoded fusion proteins which interact with the Ese1 EH domain bait. Two of these clones coded for mouse homologues of the epsin protein which recently was identified on the basis of its affinity for Eps15 and α-adaptin, and is required for clathrin-mediated endocytosis (Chen *et al*., 1998). The two epsin clones code for C-terminal fragments from amino acids 403 and 470, respectively. One of the Ese1 EH domain-interacting clones which we obtained coded for a C-terminal fragment of a novel epsin family protein (Stukenberg *et al*., 1997; Chen *et al*., 1998). This cDNA has been isolated independently by M.Yamabhai and B.K.Kay on the basis of affinity between the encoded protein and the EH domains of an Ese protein (the protein has been named Ibp-2, DDBJ/EMBL/GenBank accession No. AF057286). The Ibp2 clone which we have identified in our screen includes amino acids 326–509 of the partial coding sequence in DDBJ/EMBL/GenBank. Each epsin family protein which we have isolated contains a C-terminal region which codes for three copies of an NPF motif. This is also the region of epsin which binds to Eps15 (Salcini *et al*., 1997). It has been shown previously that AP-2 can bind both epsin and Eps15 independently (Chen *et al*., 1998). Here we have shown that the C-terminus of epsin family proteins can bind not only to the EH domains of Eps15 (Salcini *et al*., 1997; Chen *et al*., 1998), but also to the EH domains of Ese1. These data suggest that either multiple epsin proteins exist in the Ese–Eps15 complex or the interaction between individual proteins in various AP-2–epsin–Eps15–Ese–dynamin complexes may be subject to dynamic rearrangement during clathrin-coated pit formation, invagination and scission.

Ese1 overexpression blocks endocytosis

Overexpression of Pan1p in yeast induces phenotypes which are identical to those observed in *pan1* loss-of-function mutants (Tang and Cai, 1996), suggesting that by altering the relative ratios of Pan1p to its numerous partners, the function of higher order Pan1p complexes may be blocked. As the Ese–Eps15 complex contains many protein–protein interaction domains which bind partners including dynamin, epsin and AP-2, we reasoned that overexpressed Ese1 may disrupt the formation of higher order complexes between Ese proteins and partners. We therefore tested whether the clathrin-mediated endocytosis pathway was functional in mycEse1-overexpres-

sing cells. Cos-1 cells were once again transfected with myc-tagged Ese1 and, 48 h post transfection, we added fluorescein isothiocyanate (FITC)-labelled transferrin to cultures for 30 min. Cells were then fixed and analysed for expression of mycEse1 and for internalization of transferrin. As shown in Figure 6, transfected cells do not internalize transferrin, in contrast to their untransfected neighbours. Cell counts in a representative experiment indicate that 96% of Ese1-overexpressing cells do not internalize labelled transferrin ($n = 46$), whereas 100% of untransfected cells were capable of clathrin-mediated endocytosis ($n = 100$). In preliminary experiments, it appears that overexpression of dynamin II may override the Ese1-induced endocytic block. These data indicate that overexpression of Ese1 blocks endocytosis, perhaps through sequestration of dynamin or other Ese1 partners into non-productive binary complexes during endocytosis or recycling of the transferrin receptor.

Discussion

It recently has been demonstrated that two EH domain-containing proteins in *S.cerevisiae*, Pan1p and End3p, function together to regulate endocytosis (Tang *et al*., 1997). In this study, we report the identification of novel EH domain proteins, Ese1 and 2, which together with Eps15(R) form an analogous complex in multicellular animals. Eps15 is constitutively associated with α-adaptin (Benmerah *et al*., 1995). Ese1 proteins bind dynamin and regulate its subcellular localization. Therefore, these mammalian EH proteins, like the yeast proteins discussed above, regulate endocytosis by forming a multivalent complex which links several critical endocytic proteins. Eps15 contains a minimum of six protein–protein interaction surfaces, including three EH domains, a central coiled-coil domain, an α-adaptin-binding domain and a proline-rich motif. The Ese1/2 proteins have a minimum of eight protein–protein interaction surfaces, including two EH domains, a central coiled-coil domain and up to five SH3 domains. In addition, the Eps15 protein forms dimers and tetramers (Cupers *et al*., 1997; Tebar *et al*., 1997) through its central coiled-coil, suggesting that the Ese–Eps15 complex may contain multimers of both proteins. This complex contains binding sites for dynamin, α-adaptin and epsin family proteins.

It is interesting to note that synaptojanin, a mammalian inositol 5′ phosphatase implicated in endocytosis, binds to the *Drosophila* Dap160 protein *in vitro* (McPherson *et al*., 1996; Roos and Kelly, 1998). One form of synaptojanin even has NPF motifs which can bind to EH domains (Haffner *et al*., 1997; McPherson *et al*., 1998). The AP180 homologue CALM also has NPF motifs and, on the basis of studies in yeast described above, this protein is also expected to associate with the Ese–Eps15 complex. It will be important to determine whether these and other endocytic proteins interact with the Ese–Eps15 complex *in vivo*. The fact that numerous endocytic proteins can bind to Ese and/or Eps15 does not suggest that these proteins are constitutively associated with the Ese–Eps15 complex. Indeed, there may be many distinct Ese–Eps15 complexes, with different partners, during sequential steps in the endocytic process. In addition, alternative splicing reactions generate a diverse array of Ese transcripts which code for larger and smaller Ese proteins. The EseL proteins, for example, are predicted to bind directly to cellular membranes and regulate the actin cytoskeleton. Further study is required to define the role of other Ese proteins and their complexes in control of membrane trafficking. During review of this manuscript, Yamabhai and colleagues described the cloning and characterization of *Xenopus* intersectin which binds to dynamin, synaptojanin and epsin *in vitro* (Yamabhai *et al*., 1998). In addition, Guipponi *et al*. (1998) have published the sequence and expression analysis of human Ese1/intersectin and Ese1L/intersectin long form.

Many of the endocytic proteins bind to two partners through a single overlapping site which precludes simultaneous binding to both. For example, epsin and Eps15 compete for α-adaptin despite the fact that epsin and Eps15 also bind to each other. Here we have shown that the epsin C-terminus can bind to the EH domain region of Ese1, and it is known to bind the EH domain region of Eps15 (Chen *et al*., 1998). In addition, it is clear that at least three distinct classes of early endocytic SH3 proteins (amphiphysin, endophilin and Ese) bind to the proline-rich C-terminus of dynamin (Schmid *et al*., 1998). Given the dramatically different structure of amphiphysin, endophilin and Ese, it seems likely that these three proteins interact with dynamin for unique reasons. Perhaps one of these SH3-containing proteins recruits dynamin, whereas the other two SH3 proteins regulate its self-assembly and GTPase functions at the coated pit.

We have shown that overexpression of Ese1 blocks endocytosis of transferrin, probably through alteration of Ese/partners ratios. Overexpressed Ese1 would, therefore, be functioning as a dominant inhibitory protein through recruitment of partners into non-productive complexes which do not contain all of the necessary components for endocytosis to proceed. With the identification of Ese proteins, the Ese–Eps15 complex and Ese–Eps15 partners, future studies must define the molecular mechanism by which numerous protein interactions are controlled and coordinated in Ese–Eps15 complexes to regulate clathrin-mediated endocytosis.

Materials and methods

Ese cloning, plasmids and yeast two-hybrid screens

High stringency screening was used to isolate the two mouse *Ese* genes as per standard methodology (Sambrook *et al*., 1989). *Ese1* was cloned from an adult mouse brain cDNA library using a PCR product from nucleotides 1707–2197 of the coding sequence as probe. *Ese2* was cloned from a mixed tissue adult mouse cDNA library using a mixture of three probes [EST #583881 (Research Genetics Inc.), EST #652549 (Research Genetics Inc.) and a PCR product from nucleotide 2712 to 3456 of the *Ese2* coding sequence]. The *Ese1* sequence was obtained from a single clone, whereas the *Ese2* reading frame was predicted from the overlap of two cDNA clones. The DBL/PH/C2 region of Ese1L was obtained using PCR with an upstream primer designed from sequences within the DBL/PH domain region: GAAGGAGAACTCAGACCGGCT-GGAGTGGAT (this sequence was obtained from one partial Ese1L clone which we had isolated from a mouse brain cDNA library). This upstream primer was paired with downstream primers for the vector. The DBL/PH/C2 region of Ese2L was obtained using PCR with upstream and downstream primers flanking the site in Ese2 where sequence divergence had been noted within an EST clone (upstream Ese2 sequence: GACAGAGGAGCGGTACATGGA and downstream Ese2 sequence: AGCTCCCCTGGTTCTGGCTTC). The mouse Eps15 cDNA was generated through a combination of high stringency library screening with

EST sequences from the *Eps15* gene and RT–PCR according to established methods.

pcDNA3Ese1. Full-length Ese1 was cloned into the *Not*I site of pcDNA3 (Invitrogen Inc.). The *Ese1* cDNA includes 53 nucleotides of 5′ UTR plus a natural *Not*I site and 288 nucleotides of 3′ UTR plus a small region of polylinker including a *Not*I site.

pcDNA3mycEse1. The 5′ end of pcDNA3Ese1 from the *Eco*RI site in the pcDNA3 polylinker to the start codon was replaced with the DNA sequence GAATTCAGAACC**ATG**GAACAAAAGCTTATTTCTGAAGAAGACTTGGGGCCCATG, where the first underlined sequence corresponds to an *Eco*RI site which was fused into the pcDNA3 *Eco*RI site and the extended underlined sequence codes for a myc epitope tag. This is followed by nine nucleotides which code for glycine, proline and the natural *Ese1* start codon. This sequence was joined to the seqences coding for amino acids 2–1213 (the remainder of Ese1) The new start codon in this tagged *Ese1* construct is in bold. The 3′ end of *Ese1* in this vector is the same as in pcDNA3Ese1 above.

pcDNA3Eps15. This plasmid was constructed from four pieces. It contains the full-length Eps15. The 5′ UTR of this plasmid has been constructed to be GGATCCACC**ATG**, where a *Bam*HI site is underlined and the start codon is in bold. This *Bam*HI site was fused to the *Bam*HI site in pcDNA3. The 3′ UTR in this vector is 204 nucleotides of the mouse natural 3′ UTR fused to a short cloning linker ending in the sequence AAGCTTGGGCCC, where an *Apa*I site is underlined; this *Apa*I site was fused to the *Apa*I site in pcDNA3.

pcDNA3Eps15δC. This vector is the same as pcDNA3Eps15 except that sequences downstream from and including mouse Eps15-coding nucleotide 1500 have been replaced with CCTGGATTACAAGGATGATGATGACAAA**TGA**CTCGAG, where the first underlined sequence codes for the Flag epitope, an in-frame stop codon is in bold and an *Xho*I site is underlined. This *Xho*I site was fused to the polylinker in pcDNA3. The resulting plasmid encodes amino acids 1–501 of mouse Eps15 fused to a C-terminal Flag epitope. The 5′ end of Eps15 in this construct is as indicated above for pcDNA3Eps15.

pGBT9Ese1cc. The *Ese1* sequence coding for amino acids 330–732 was fused directly to GAATTC (*Eco*RI site) on the 5′ end and to **TAG**GATCC (stop codon followed by a *Bam*HI site) on the 3′ end. This fragment was cloned into *Eco*RI–*Bam*HI-digested pGBT9 in-frame with the GAL4 DNA-binding domain.

pGBT9Ese1A3.3. This plasmid encodes the bait for our SH3 screen. It encodes all five SH3 domains from amino acid 665 to 1213 and was subcloned into pGBT9 on an *Eco*RI fragment which fuses the Ese1 SH3 region in-frame with the GAL4 DNA-binding domain.

PGBT9Ese1Nterm. This plasmid codes for the N-terminal 393 amino acids of Ese1, including both EH domains. It was subcloned into pGBT9 on an *Eco*RI–*Sal*I fragment.

Yeast two-hybrid screens were performed as per standard protocols with each bait transformed into *S.cerevisiae* strain Y190. Yeast clones were selected for expression of the bait and each screen was performed in the presence of 20–40 mM 3AT.

Northern blot analysis

A multiple tissue Northern blot (Clontech) was pre-hybridized in 5 ml of ExpressHyb Solution (Clontech) at 68°C for 30 min. Probe was added at 1×10^6 c.p.m./ml for 1 h. The blot was washed twice (2× SSC, 0.05% SDS) at room temperature, twice (0.1× SSC, 0.1% SDS) at 50°C and then exposed to film overnight.

Antibodies and Western blot analysis

Western blot analysis was performed according to standard protocols. Briefly, cultured cell lines or 48 h post-tranfection Cos-1 cells were washed with phosphate-buffered saline (PBS) and lysed in 1 ml of cold lysis buffer [50 mM HEPES pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EGTA, 1.5 mM $MgCl_2$, 10 mM NaF, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 1 mM Na_3VO_4]. Supernatants were clarified by centrifugation and immunoprecipitated with specific antiserum as indicated. Antigen–antibody complexes were purified on anti-rabbit agarose or anti-mouse agarose (Sigma Chemical Co.). Samples were run on 7.5% PAGE gels and transferred to nitrocellulose membranes. Filters were blocked in 5% dry milk powder/0.05% Tween-20/PBS, washed in 1% dry milk powder/0.05% Tween-20/PBS and probed with the appropriate antisera at 1 μg/ml in wash buffer (in the case of chicken anti-Ese1, we used 10 μg/ml to probe Western blots). Probed filters were washed further, probed again with 1/5000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG (Amersham) or anti-chicken-IgY (Zymed), washed and signal detected using the enhanced chemiluminescence detection system as per the manufacturer's instructions (Amersham).

Rabbit anti-Ese1 antiserum was raised against a peptide of the following sequence: MAQFPTPFGGSLDVWAITVEE. The antiserum was affinity purified over the same peptide (Research Genetics). This peptide was also used at 5 μg to compete for the 5 μg of antibody per immunoprecipitation reaction. Rabbit anti-Eps15 antisera #C20 (1 μg: Santa Cruz Biotech) and mouse anti-Flag M2 mAb (3 μg: Kodak) were also used for immunoprecipitation experiments. Chicken anti-Ese1 antiserum which was used for Western analysis was raised against a fusion protein between GST and amino acids 665–1213 of mouse Ese1. This serum was cleared of antibodies reacting against GST by incubation with GST on glutathione–agarose beads.

Immunofluoresence and endocytosis assays

For immunofluorescent staining, Cos-1 cells typically were plated at a density of 2×10^5 per 22×50 mm coverglass and transfected with 2.5 μg of plasmid using Ca/phosphate co-precipitation. After 12–16 h, the cells were washed twice with PBS ($-Mg^{2+}-Ca^{2+}$) and fed with fresh 10% fetal bovine serum (FBS) in Iscove's medium. In some experiments, we used lipofection reagents and methods for transfection of cDNAs. Two days post-transfection, cultures were fixed with cold methanol for 30 min at room temperature. Cultures were washed three times for 10 min with PBS, blocked for 1 h at room temperature with 1% bovine serum albumin (BSA) in PBS and then incubated with primary antibody in blocking solution for 1 h at room temperature. Slides were then washed three times for 10 min with PBS and incubated with secondary antibody/1% BSA/PBS in the dark for 1 h at room temperature. Finally, slides were washed three times for 10 min in the dark at room temperature and mounted using Dabco anti-fade solution (Sigma Chemical Co.). Slides were analysed on the confocal microscope using the 63× objective and optical filters to separate signals on each channel.

For primary antibodies, we used mouse anti-myc monoclonal 9E10 (10 μg/ml: Santa Cruz Biotech.), rabbit anti-Eps15 antiserum #C20 (1 μg/ml: Santa Cruz Biotech.) or H896 (1 μg/ml: Santa Cruz Biotech.) and mouse anti-dynamin I #D25520, which recognizes dynamin in Cos-1 cells by both immunoprecipitation and Western blotting (data not shown) (20 μg/ml: Transduction Labs, Inc.). As secondary antibodies, we used Texas red-labelled goat anti-mouse (1:100 dilution), FITC-labelled goat anti-mouse (1:80 dilution) and Texas Red-labelled goat anti-rabbit antibodies (1:150 dilution) (all three from Jackson ImmunoResearch Laboratories Inc.). We were unable to detect endogenous Eps15 staining above background in non-transfected cells. Prominent cytoplasmic Eps15 staining in transfected cells obscured the detection of clathrin-coated pit-associated Eps15 at the membrane (Figure 4B). In Figure 5B, panels a and b, dynamin staining with both primary and secondary antibodies was performed first. We then stained for mycEse1 using biotinylated 9E10 followed by streptavidin conjugated to Texas Red. Following application of the secondary antibody to stain for dynamin, all further incubations and washes were performed under dark conditions. Endocytosis assays were performed as previously described (Wigge *et al*., 1997a).

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