The Epsins Define a Family of Proteins That Interact with Components of the Clathrin Coat and Contain a New Protein Module*  

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Epsin (epsin 1) is an interacting partner for the EH domain-containing region of Eps15 and has been implicated in conjunction with Eps15 in clathrin-mediated endocytosis. We report here the characterization of a similar protein (epsin 2), which we have cloned from human and rat brain libraries. Epsin 1 and 2 are most similar in their NH2-terminal region, which represents a module (epsin NH2 terminal homology domain, ENTH domain) found in a variety of other proteins of the data base. The multiple DPW motifs, typical of the central region of epsin 1, are only partially conserved in epsin 2. Both proteins, however, interact through this central region with the clathrin adaptor AP-2. In addition, we show here that both epsin 1 and 2 interact with clathrin. The three NPF motifs of the COOH-terminal region of epsin 1 are conserved in the corresponding region of epsin 2, consistent with the binding of both proteins to Eps15. Epsin 2, like epsin 1, is enriched in brain, is present in a brain-derived clathrin-coated vesicle fraction, is concentrated in the peri-Golgi region and at the cell periphery of transfected cells, and partially colocalizes with clathrin. High overexpression of green fluorescent protein-epsin 2 mislocalizes components of the clathrin coat and inhibits clathrin-mediated endocytosis. The epsins define a new protein family implicated in membrane dynamics at the cell surface.

Epsin (epsin 1) is a recently characterized protein with a putative role in clathrin-mediated endocytosis. Its COOH-terminal region, which contains three repeats of the EH domain binding consensus NPF, interacts with the EH domains of Eps15 (1–3), whereas its central region binds the clathrin adaptor AP-2 (4). Epsin 1 is enriched in brain and is partially associated in situ with clathrin coats. Disruption of epsin 1 function in fibroblasts by either overexpression, microinjection of antisense RNA, or microinjection of antibody conjugate has a potent inhibitory effect on clathrin-mediated endocytosis (5).

Screening of a prokaryotic expression library from human fibroblasts with a GST fusion protein comprising the EH domains of Eps15 led to the isolation of several clones interacting with these domains. One of them, EHB21 (6), encoded a partial sequence similar (34%) to the COOH-terminal region of rat epsin 1. Based on the EHB21 sequence, we have screened human and rat libraries to obtain full-length open reading frames. The sequences obtained encode a novel protein, epsin 2, which is closely related to but is distinct from epsin 1. We report here a characterization of this protein and demonstrate that epsin 2, like epsin 1, is implicated in clathrin function.

EXPERIMENTAL PROCEDURES

Antibodies—Polyclonal antibodies against epsin 2 were directed against the coding amino acids of the EHB21 clone (6). Rabbit polyclonal antibodies Eps15 and epsin 1 were previously described (2, 5). Mouse monoclonal antibodies directed against clathrin (TD.1 and X.22), a-adaptin, and b-adaptin were obtained from ATCC (Manassas, VA), Affinity Bioreagents (Golden, CO), and Sigma, respectively. Anti-glutamic acid decarboxylase antibodies and anti-synapsin 2 antibodies were described as (7, 8).

Cloning of Epsin 2 cDNA—A 485-base pair fragment of EHB21 (6) comprising its coding region and the first 120 base pairs of the 3′ untranslated region was amplified by PCR from the clone using the following oligonucleotide primers: forward primer, 5′-ctggactaactgtgaccc-3′; reverse primer, 5′-gtgcagcgtgaagccg-3′. This fragment was radiolabelled by [a-32P]dCTP using random primer-labeling (Roche Molecular Biochemicals). The labeled product was used as a probe to screen a human cerebellar cDNA AZAP library (Strategene, La Jolla, CA). A partial clone isolated from the screen (containing a 1 kilobase insert) was used in turn as a probe to screen a human brain agt11 cDNA library (CLONTECH, Palo Alto, CA). Multiple screens of the agt11 human brain library led to several overlapping clones, none of which contained a full-length open reading frame. A full-length clone (17/4) was assembled from clone 4, which contained the termination codon, and from clone 17B, which included the putative start codon ATG (within an apparent Kozak consensus) (8) but lacked the 3′ end. Clone 17/4 was generated by ligation of two gel-purified fragments (1151 and 1086 base pairs, respectively) obtained from digestion of clone 17 and clone 4 within pBlueScript with EcoRI and MluI. The resulting construct was subcloned into pBlueScript (epsin 2a/pBSK). The sequence of several other partial clones was identical to that of clone 17/4, henceforth defined as epsin 2a, with the exception of a 174-base pair deletion (see Fig. 1A), resulting in a 58-amino acid deletion in the open reading frame (epsin 2b). Nucleotide sequences were analyzed using the LaserGene software package (DNASTAR Inc., Madison, WI). The Genetics Computer Group implementation of Paup version 4.0d55 was used to generate a single maximum parsimony tree from the alignment shown in Fig. 2A. The phyllogenetic tree is unrooted with bootstrap values.

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indicated along the branches to assess the confidence of each branch of the topology. All branches are resolved with a high (>50%) confidence (33).

The rat epsin 2 sequence was obtained by PCR cloning from a rat brain cDNA library using primers that were generated based on human epsin 2 sequences. Three pairs of primers (5’-GCAAGGACACGCGACGTACGTO-3’, 5’-TGAGTTCAAGGTTTCTCAATTCG-3’) and (5’-CCCGGCCTGTGCACTAT-3’, 5’-CAGCCACTGGACGAGGTATG-3’), and (5’-ATGACATCTCTGTCTATCAAGCAGG-3’, 5’-CTAGAGAGGAAAGGGTTGGTGTG-3’) were added to a single PCR at a final concentration of 2 mM. The longest PCR product was gel-extracted and subcloned into a TA vector (Invitrogen) and fully sequenced, confirming the generation of a full-length rat epsin 2 clone.

Production of Recombinant Proteins—A portion of the coding region of human epsin 2 containing the three NPF motifs (amino acids 324-642 human epsin 2a), the DPW domains of epsin 2 (amino acids 318–456 of human epsin 2a), and epsin 1 (amino acids 1–401) were amplified by PCR using Taq polymerase. The PCR fragment encoding the NPF motifs was subcloned into the TA vector (Invitrogen, Carlsbad, CA), cleaved with EcoRI, and ligated to pGEX-4T-2 (Amersham Pharmacia Biotech) to obtain a GST-NPF fusion protein. The PCR product of the DPW domain of epsin 2 was digested with EcoRI/HindIII and subcloned into the same vector to obtain a GST-DPW fusion protein. The ENTH/DPW domain fragments were digested with EcoRI/HindIII (epsin 1) and HindIII/Xhol (epsin 2) and subcloned into pcDNA3 (Invitrogen, Carlsbad, CA). GST fusions of the ENTH/DPW of epsin 1 and 2 were prepared by subcloning EcoRI/HindIII inserts into pGEX4 (Amersham Pharmacia Biotech). DH5α or BL21(DE3) host strains were transformed, and both constructs were verified by sequencing (Keck Biotechnology Resource Laboratory, Yale University). Rat epsin 1 constructs and GST fusion proteins of amphiphysin 1 (amino acids 262–405 and 262–375) were previously described (5, 9). Proteins were purified on a glutathione 4B-Sepharose affinity matrix (Amersham Pharmacia Biotech).

Affinity Chromatography—Affinity chromatography of a rat brain extract on the GST-NPF domain of epsin 2 was performed as described previously (9). For the study of AP-2 and clathrin binding to GST fusion proteins, a rat brain homogenate was extracted with 1 x Tris, pH 8.9, 2% Triton X-100, and a protease inhibitor mixture and spun at 100,000 × g for 1 h. The supernatant was desalted in a buffer containing 125 mM KCl, 25 mM Hepes, and 1% Triton X-100. Proteins specifically retained by the affinity matrix were eluted into SDS/PAGE sample buffer, separated by SDS/PAGE, and transferred to nitrocellulose for Western blotting (9).

In Vitro Translations—cDNA clones encoding luciferase (Promega Co.), amphiphysin I (10), rat epsin 1 (5), human epsin 2, and fragments of rat epsin 1 and human epsin 2 comprising both the ENTH and DPW domains (amino acids 1–401 and 1–456, respectively) were in vitro transcribed using five species of S race (Promega Co.) and in vitro translated using a T7-coupled transcription/translation system (Promega Co.) according to manufacturer’s instructions. The radiolabeled products were separated by SDS/PAGE and quantitated so that equivalent amounts of radioactivity could be added in each binding reaction. The products were then incubated for 2 h at 4 °C in phosphate-buffered saline containing 1% Triton X-100, 5 mM EDTA, and 1 mg/ml bovine serum albumin with 50 μl of glutathione-Sepharose with a prebound GST or GST fusion protein of the ENTH/DPW domain of rat epsin 2 (kindly provided by James Keen, Kimmel Cancer Institute). The beads were pelleted by centrifugation and thoroughly washed in the same buffer. The bound proteins were eluted in 40 μl of SDS sample buffer and separated by SDS/PAGE followed by autoradiography.

Subcellular Fractionation—Subcellular fractionation of rat brain to obtain a fraction enriched in clathrin-coated vesicles was performed as described (11).
human epsin 2a). In contrast, epsin 1, whose predicted size is only slightly greater than that of epsin 2, migrates with an apparent molecular mass of about 94 kDa in SDS/PAGE (Fig. 3A). Based on the analysis of the SDS/PAGE mobility of epsin fragments, this difference is primarily due to the different mobilities of the DPW domains of epsin 1 and 2 (not shown). It is most likely due to the higher content of acidic amino acids in the DPW domain of epsin 1 (predicted pI 3.3) than in the DPW domain of epsin 2 (predicted pI 9.08). As shown in Fig. 3B, antibodies directed against epsin 2 do not recognize epsin 1 in either brain or transfected cells.

Western blot analysis of a variety of rat and human tissues demonstrated a widespread tissue distribution of the protein (Fig. 4). Similar results were obtained by Northern blotting (not shown). The highest concentration of the protein was observed in brain.

Epsin 2 Binds Eps15, α-Adaptin, and Clathrin—We next investigated the binding properties of epsin 2. Extracts of rat brain were affinity-purified on GST fusion proteins of epsin 2 fragments, and the bound proteins were revealed by Western blotting. The NPF domain bound specifically to Eps15 (Fig. 5) but not to glutamic acid decarboxylase, another abundant brain cytosolic protein (18) used as a control, in agreement with the isolation of the EHB21 clone as an interactor for its EH domains (6).

The DPW domain of epsin 2 bound AP-2, as revealed by the enrichment in the bound material of the AP-2 subunits, α- and β-adaptins (3) (Fig. 6). Blotting of the material affinity-purified by this construct for a variety of endocytic proteins demonstrated that the DPW domain of epsin 2 also bound clathrin (Fig. 6B), in agreement with the presence of the clathrin binding motif LDL in this domain (14–16). GST, used as a negative control, did not bind either protein, whereas amphiphysin 1, used as a positive control, bound both AP-2 and clathrin as expected (Fig. 6, A and B). A GST fusion protein of the DPW domain of epsin 1 bound clathrin only slightly above background (not shown). We noted, however, that the sequence LMDLAD is present at the very NH2-terminal end of this construct of epsin 1, and unfavorable folding of this fusion protein may interfere with binding to clathrin. We considered the possibility that epsin 1 as well may bind clathrin and that flanking regions may be required for optimal binding of clathrin to epsin. We compared, therefore, binding of GST fusion proteins of longer epsin 1 and epsin 2 constructs (amino acids 1–401 of epsin 1 and amino acids 1–456 of epsin 2). As shown by Fig. 6C, both constructs, but not GST alone, bound similar levels of AP-2 and similar levels of clathrin. Clathrin binding was also observed when the brain extract used for the affinity purification was depleted of AP-2 by preincubation with a GST fusion protein comprising selectively the AP-2 binding domain of amphiphysin 1 (10) (not shown). This finding strongly suggests that not only the interaction between epsin and AP-2 (5), but also the interaction between epsin and clathrin, is direct.

To further demonstrate a direct interaction between clathrin and epsin 1 or 2, in vitro translation experiments were performed. Epsin 1 and epsin 2 fragments corresponding to those used for Fig. 6C, amphiphysin 1 (positive control) and luciferase (negative control), were transcribed and translated in vitro and then incubated with immobilized GST or a GST fusion of the NH2-terminal domain of clathrin. This clathrin domain was previously shown to be the region of clathrin that binds the consensus sequence L/L/I/D/E/N/L/F/D/E (14, 19). As shown by Fig. 7, epsin 1 and epsin 2, their ENTH-DPW domain fragments and amphiphysin 1 bound clathrin, whereas luciferase did not. None of the proteins bound GST alone (not shown). Binding of epsin 1 and 2 to clathrin was similar to that of amphiphysin 1 in this assay.

Epsin 2 Is Partially Associated with Clathrin Coats—Rat brain homogenate was fractionated to generate a highly enriched clathrin-coated vesicle fraction. Epsin 2 was partially recovered in the purified coated vesicles, although it did not co-enrich with clathrin and AP-2, i.e. the intrinsic components of the clathrin coat. Instead, its recovery in the clathrin-coated vesicle fraction was similar to that of Eps15 (Fig. 8), epsin 1, and of other accessory proteins of the clathrin coat (5) (Fig. 8). Synapsin 2, a protein previously found to be absent from clathrin-coated vesicles (8) was not present in the same fraction.

The subcellular localization of epsin 2 was further analyzed in cultured fibroblastic cells. Since endogenous epsin 2 was not detectable by available antibodies, epsin 2 was expressed in...
these cells as a GFP-tagged fusion protein. In cells expressing it at low to moderate levels, GFP-epsin 2 appeared as fine puncta sparse throughout the cell and particularly concentrated in the central region where the Golgi complex is localized. This distribution was very similar to the distribution of clathrin immunoreactivity (Fig. 9, a and b). In highly expressing cells (Fig. 9, c–j), GFP-epsin 2 produced an intense and continuous labeling of the cell surface as well as one or more bright, compact masses in the central region of the cell. In these cells a major redistribution of components of the clathrin coat was observed, as shown by the collapse of clathrin, Eps15, and to a lesser extent, AP-2, into the same central mass(es) positive for GFP-epsin 2 (Fig. 9, c–h). Furthermore, in these cells, an inhibition of the internalization of Texas red-labeled transferrin was observed (Fig. 9, i and j). Similar results were obtained by using epitope-tagged epsin 2 instead of GFP-epsin 2 (data not shown).

**DISCUSSION**

We report here the characterization of a new protein that shares significant primary sequence similarity with epsin (epsin 1) and that we have therefore defined as epsin 2. Epsin 1 and 2 have the same tripartite domain structure, similar protein binding partners, and similar subcellular localization. Furthermore, both proteins are enriched in brain. Despite these similarities, a number of differences have also emerged.

The region of highest similarity between the two proteins is the NH2-terminal domain, most strikingly, the first 150 amino acids. Based on data base searches, this domain defines a new protein module conserved from yeast to man (5) (epsin NH2-terminal homology domain or ENTH domain). Alignment of these sequences reveals several sites with 100% conservation and numerous other sites with conservative amino acid substitutions. It is of interest that about half of these proteins and perhaps more, due to the incomplete sequence of some of these genes, contain one or multiple NPF motifs, even if some of them do not appear to represent true orthologues of either epsin 1 or 2. Since the NPF sequence was found to represent an EH domain binding consensus, this observation suggests that a physiologically important link between ENTH domains and EH domain-mediated interactions. This protein module, first identified in epsin 1 (5), has been recently and independently discussed by Kay et al. (20).

The second most conserved portion in epsin 1 and 2 is the COOH-terminal region, which contains the three NPF motifs.
with the presence of a phosphorylation site for Cdc2 kinase in epsin 1 (5), but not in epsin 2, and with corresponding observation that epsin 1, but not epsin 2, undergoes a phosphorylation-dependent shift in electrophoretic mobility in mitotic cells (5, 17, 21).

Despite these differences, both DPW domains have similar AP-2 binding properties. Thus, the peculiar and distinct organization of the DPW motifs in the two proteins must have additional functions beside AP-2 binding. However, a contribution of at least some DPW repeats to AP-2 binding is plausible. It was shown previously that the unique arrangements of the multiple DPF motifs in the COOH-terminal region of Eps15 is not essential for AP-2 binding, although some of the numerous FIG. 3. Electrophoretic mobility of epsin 1 and 2 in transfected cells and brain. A, Western blot of rat and human brain homogenate using preimmune, anti-epsin 1, or anti-epsin 2 sera. B, Western blots of Chinese hamster ovary cell lysates transfected with the indicated epsin constructs and human and rat brain homogenates. HA, hemagglutinin.

FIG. 4. Tissue distribution of epsin 2. Western blot analysis of the pattern of expression of epsin 2 in human and rat tissues.

FIG. 5. The NPF domain of epsin 2 binds Eps15. A Triton X-100 rat brain extract was affinity-purified on GST or a GST fusion protein comprising the three NPF motifs of epsin 2. The starting material (SM) and the bound material were reacted by Western blotting for Eps15 or, as a control protein, the abundant cytosolic brain protein, glutamic acid decarboxylase.

FIG. 6. The DPW domains of epsin 1 and 2 bind clathrin and AP-2. A and B, a Triton X-100 extract of rat brain was affinity-purified on similar amounts of GST or GST fusion proteins comprising amino acids 262–405 of amphiphysin 1 or the DPW domain of epsin 2 (amino acids 318–456). The bound material was stained by Coomassie Blue (A) or reacted by Western blotting for the heavy chain of clathrin and for the AP-2 subunits α- and β-adaptins (B). Note that lanes corresponding to GST were from the same gel as other samples. C, Western blotting for the heavy chain (HC) of clathrin and for α-adaptin of the material affinity-purified by GST fusion proteins of epsin 1 and 2 fragments comprising both the ENTH and the DPW domains.
DPF motifs were found to participate in such an interaction (1, 22).

Both epsins bind the NH₂-terminal domain of clathrin. Epsin 1 and 2 contain the sequences LMDLAD and LLDLMD, respectively, at the NH₂-terminal side of their DPW domains. Based on the similarity of these sequences to a previously defined consensus for clathrin binding [L(L/I)(D/E/N)(L/F)(D/E)] (14, 15), it is conceivable that such sequences may represent the core of the clathrin binding domain.

All these findings support the hypothesis that epsin 2, like epsin 1, is implicated as an accessory factor in clathrin-mediated endocytosis. This hypothesis is further confirmed by the partial co-localization of GFP-epsin 2 with clathrin in transfected cells and by its property, when greatly overexpressed, to mislocalize clathrin coat components and to block the clathrin-mediated internalization of transferrin. In highly overexpressing cells (c−e), the localization of AP-2, clathrin, and Eps15 is severely disrupted. In these cells the internalization of transferrin is impaired (h and i).

The partial sequences of two proteins, Ibp1 and Ibp2, intersectin-binding proteins 1 and 2, which are likely to represent mouse epsin 1 and 2, respectively, were reported while this study was in progress (22). Intersectins (also referred to as Ese 1 and 2) (23) are the vertebrate homologues of the Drosophila DAP160 protein (24) and contain multiple SH3 domains and EH domains (25, 26). They bind epsin 1 (Ibp1) and 2 (Ibp2) via their NH₂-terminal EH domain and Eps15 via a coiled-coil region (22, 23). Since DAP160 and the intersectins also bind dynamin and synaptojanin (24), proteins of the DAP160/intersectin family may link the function of these proteins to the function of Eps15 and epsin. A picture is emerging in which
formation of a clathrin-coated vesicle is assisted by a variety of accessory factors that interact with each other to form large macromolecular complexes. At least one component of these complexes is a lipid-metabolizing enzyme, the inositol phosphatase synaptotagmin (27). Other proteins of the complex have been linked to actin function from genetic studies in yeast and biochemical studies in mammalian tissues (28–31). It is therefore likely that this network of protein-protein interactions may coordinate formation of a coated bud and then of a free vesicle, with changes in the actin cytoskeleton and in the lipid components of the vesicle membrane.

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Addendum—While this paper was in revision a paper reporting the interaction of Ibp2 (epsin 2) with clathrin was reported (32).

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