Enthoprotin: a novel clathrin-associated protein identified through subcellular proteomics

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Despite numerous advances in the identification of the molecular machinery for clathrin-mediated budding at the plasma membrane, the mechanistic details of this process remain incomplete. Moreover, relatively little is known regarding the regulation of clathrin-mediated budding at other membrane systems. To address these issues, we have utilized the powerful new approach of subcellular proteomics to identify novel proteins present on highly enriched clathrin-coated vesicles (CCVs). Among the ten novel proteins identified is the rat homologue of a predicted gene product from human, mouse, and Drosophila genomics projects, which we named enthoprotin. Enthoprotin is highly enriched on CCVs isolated from rat brain and liver extracts. In cells, enthoprotin demonstrates a punctate staining pattern that is concentrated in a perinuclear compartment where it colocalizes with clathrin and the clathrin adaptor protein (AP1). Enthoprotin interacts with the clathrin adaptors AP1 and with Golgi-localized, γ-ear–containing, Arf-binding protein 2. Through its COOH-terminal domain, enthoprotin binds to the terminal domain of the clathrin heavy chain and stimulates clathrin assembly. These data suggest a role for enthoprotin in clathrin-mediated budding on internal membranes. Our study reveals the utility of proteomics in the identification of novel vesicle trafficking proteins.

Introduction

Clathrin-mediated endocytosis is a major pathway by which proteins and membrane components are internalized into cells. In general terms, this process is initiated by the adaptor protein (AP)*2 complex, which recruits clathrin, leading to clathrin assembly and the formation of clathrin-coated pits (CCPs). Through interactions with AP2, endocytic cargo is recruited to the nascent CCPs, which eventually pinch off to form clathrin-coated vesicles (CCVs) (Schmid, 1997). Clathrin and AP2 also bind directly or indirectly to an array of endocytic regulatory proteins including dynamin, synaptojanin, amphiphysin, endophilin, intersectin, epsins, and huntingtin-interacting proteins (HIPs) (Slepnev and De Camilli, 2000). The trans-Golgi network (TGN) is a second major site of clathrin-mediated membrane budding in cells (Schmid, 1997). Lysosomal hydrolyases, which are bound through mannose 6-phosphate (MP) tags to the cation-dependent and -independent MP receptors (MPRs), are concentrated into clathrin-coated buds from which they enter carrier vesicles for delivery to the endosomal system (Traub and Kornfeld, 1997). The acidic pH of the endosome causes the release of the hydrolyases, after which the MPRs return to the TGN for additional rounds of sorting. The Golgi-localized, γ-ear–containing, Arf-binding proteins (GGAs) and the AP1 complex have both been implicated in sorting of MPRs in this pathway (Traub and Kornfeld, 1997; Le Borgne and Hoflack, 1998; Puertollano et al., 2001; Zhu et al., 2001). However, in contrast to clathrin-mediated budding at the plasma membrane, relatively little is known regarding the regulatory molecular mechanisms for clathrin budding on internal membranes.

The combination of subcellular fractionation and mass spectrometry, known as subcellular proteomics, provides a powerful approach towards the identification of previously unknown components of cellular compartments. Recent examples include proteomics analysis of Golgi membrane proteins (Bell et al., 2001), the nuclear envelope (Dreger et
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al., 2001), the nuclear pore (Rout et al., 2000), and the phagosome (Garin et al., 2001). Each of these investigations led to the identification of known proteins not previously understood to associate with the organelle under study. Moreover, they revealed the presence of novel proteins that had been previously described only as predicted open-reading frames from genome sequencing data, and provided evidence assigning the proteins to the organelle. Therefore, we sought to utilize and extend this approach to the identification of novel functional components of the machinery for clathrin-mediated membrane budding. Here we describe the characterization of a novel clathrin-associated protein identified through proteomics of CCVs.

Results and discussion
Identification of novel CCV-associated proteins through proteomics

Maycox et al. (1992) developed a subcellular fractionation procedure that leads to the isolation of near homogeneous populations of CCVs. We isolated CCVs from adult rat brain using this procedure coupled to an additional sucrose gradient centrifugation step (see Materials and methods). The CCV proteins were separated on one-dimensional SDS-PAGE and detected by Coomassie blue staining (Fig. 1 A). The preparation was highly enriched in clathrin and the subunits of the AP1 and AP2 adaptor complexes (α/β/γ-adaptins), the μ-adaptins, δ2-adaptin, and the clathrin light chains (CLCs).

Figure 1. Identification of enthoprotin as a novel CCV-associated protein. (A) Proteins of CCVs from rat brain were separated on SDS-PAGE and stained with Coomassie blue. The dashes and brackets indicate the migratory positions of major CCV proteins including the clathrin heavy chain (CHC), the large subunits of the AP1 and AP2 adaptor complexes (α/β/γ-adaptins), the μ-adaptins, δ2-adaptin, and the clathrin light chains (CLCs). (B) Homogenates and microsomes from nontransfected (NT) COS-7 cells and homogenates from cells transfected with FLAG-tagged full-length enthoprotin (T) were analyzed by Western blot with antibodies against enthoprotin (3194) or the FLAG epitope tag as indicated. (C) CCVs were prepared from rat brain and liver and the distribution of endogenous enthoprotin in the subcellular fractions was determined by Western blot and compared with that of endogenous clathrin. H, homogenate; P, pellet; S, supernatant; SGp, sucrose gradient pellet; SGs, sucrose gradient supernatant. (D) A crude preparation of CCVs was generated from HEK-293 cells expressing FLAG-tagged full-length enthoprotin, the isolated COOH-terminal domain, or the ENTH domain. The distribution of transfected proteins was determined by anti-FLAG Western blot and compared with that of endogenous clathrin. H, homogenate; P, pellet; S, supernatant. For C and D, equal amounts of protein were loaded in each lane. (E) Identification of enthoprotin as a novel CCV-associated protein. (A) Proteins of CCVs from rat brain were separated on SDS-PAGE and stained with Coomassie blue. The dashes and brackets indicate the migratory positions of major CCV proteins including the clathrin heavy chain (CHC), the large subunits of the AP1 and AP2 adaptor complexes (α/β/γ-adaptins), the μ-adaptins, δ2-adaptin, and the clathrin light chains (CLCs).
united AP2 and is found in CCPs at the leading edge of migrating cells (Conner and Schmid, 2002). KIAA1360 also contains an NH$_2$-terminal serine/threonine kinase domain, two DLL motifs, and an NPF motif. KIAA1360 shows no significant overall homology with KIAA1048. KIAA1576 is homologous to VAT-1, an abundant membrane protein of cholinergic synaptic vesicles in Torpedo (Linial et al., 1989). Numerous synaptic vesicle proteins were identified in the proteomics analysis consistent with the observation that synaptic vesicles are the major cargo for CCVs in adult brain (Maycox et al., 1992). KIAA1576 may represent a novel synaptic vesicle protein in mammals. The proteins with gi 13385744 and gi 13384758 and the protein encoded by the nucleotide sequence gi 13385773, show no significant homologies with known proteins and contain no identifiable domains. However, gi 13384758 and gi 13385773 are homologous to each other and are enriched on CCVs (unpublished data). The protein with gi 12859193 contains a putative GTP-binding domain whereas the protein with gi 12855458 contains a SEC14 domain. The Sec14 domain was originally identified in the phosophatidylinositol transfer protein Sec14p, a protein required for membrane budding at the TGN (Bankaitis et al., 1989). The demonstrated and presumptive functions of these novel proteins to clathrin-mediated membrane trafficking provides strong validation to the suitability of the approach.

Next, we focused on the characterization of the novel protein encoded by KIAA0171 (Table I), identified by the Kanzusa DNA Research Institute (Nagase et al., 1996). The sequence of KIAA0171 is shown in Fig. S1 (available at http://www.jcb.org/cgi/content/full/jcb.200205078/DC1) aligned to a mouse protein predicted from the NCBI contig, NT032476 (gi 20345123) and a hypothetical Drosophila protein encoded by cDNA GH02671. Eleven separate tryptic peptides were identified in the KIAA0171 protein (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200205078/DC1). The protein contains an epsin NH$_2$-terminal homology (ENTH) domain, a module found primarily in endocytic proteins (De Camilli et al., 2002). The ENTH domain is most homologous with that of its Drosophila orthologue (GH02671), a yeast protein encoded by yYSPCC794, and the family of vertebrate epsins (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200205078/DC1). The protein demonstrates no homology to known proteins outside of its ENTH domain. The COOH-terminal domain contains two peptides that match the consensus for the type 2–clathrin box (Drake and Traub, 2001) and a third related sequence. Based on its identification as a novel ENTH domain-containing protein in a proteomics screen, we have named this protein enthoprotin.

To begin the characterization of enthoprotin we generated a polyclonal antibody against a GST fusion protein encoding a fragment from its COOH terminus. After affinity purification, the antibody reacted with FLAG-tagged enthoprotin expressed in COS-7 cells (Fig. 1 B). FLAG-tagged enthoprotin migrated at ~85 kD on SDS-PAGE (Fig. 1 B) although the predicted molecular mass of the protein is 68 kD. Consistent with this observation, peptides corresponding to enthoprotin from the mass spectrometry analysis were found primarily in gel slices ranging from 80 to 84 kD (Table I). The antibody also detected an endogenous protein in homogenates of COS-7 cells that was enriched in microsomes and which migrated at ~80 kD (Fig. 1 B). Thus, in COS-7 cells, the migration of endogenous enthoprotin at 80 kD is consistent with the migration of the FLAG-tagged protein after factoring in the mass of the FLAG-epitope tag.

Endogenous enthoprotin is highly enriched in CCVs prepared from brain and liver extracts (Fig. 1 C). In fact, the profile of enthoprotin reactivity throughout the subcellular fractions parallels that of the clathrin heavy chain (Fig. 1 C). To determine the domain of the protein responsible for CCV targeting, HEK-293 cells were transfected with FLAG-tagged cDNA constructs and CCVs were isolated using a simplified version of the CCV subcellular fractionation procedure (Metzler et al., 2001). Full-length enthoprotin and its COOH terminus (amino acids 136–625) were enriched on CCVs whereas the isolated ENTH domain (amino acids 1–35) was not detected in the CCV fraction (Fig. 1 D).

**Enthoprotin colocalizes with clathrin and AP1 in a perinuclear compartment**

To examine the localization of enthoprotin we transfected COS-7 cells with GFP-tagged constructs. After ~15 h of ex-
expression, GFP-enthropin demonstrates a punctate distribution that is concentrated near the nucleus, but which extends throughout the cell (Fig. 2 A). The isolated COOH terminus demonstrates a nearly identical pattern (unpublished data), consistent with the parallel distributions of the two proteins during the subcellular fractionation of CCVs (Fig. 1 D). The isolated ENTH domain also demonstrates a weak localization to a perinuclear compartment (unpublished data). The perinuclear pool of enthoprotin demonstrates a substantial degree of overlap with markers of the TGN including clathrin and AP1 (Fig. 2 A). In addition, enthoprotin colocalizes in part with endocytosed epidermal growth factor and with the cation-independent MPR, which recycles between the TGN and the endosomal system (unpublished data). Thus, enthoprotin appears to localize primarily to the TGN with an additional pool that is associated with endosomes. Only limited colocalization was seen between enthoprotin and clathrin at peripheral punctae or between enthoprotin and AP2, suggesting that
the protein is not a major component of plasma membrane-derived CCPs and CCVs (Fig 2 A). Essentially identical results were obtained after enthoprotin expression in HeLa cells (unpublished data). Moreover, staining for endogenous enthoprotin in COS-7 cells revealed a punctate staining pattern that was enriched in a perinuclear pool and that colocalized with clathrin (Fig 2 B).

**Enthoprotin binds to coat components of CCVs**

In its COOH-terminal domain, enthoprotin contains two type 2–clathrin boxes. This observation, coupled with the enrichment of enthoprotin on CCVs, suggests that the protein may interact with coat components of CCVs. Interestingly, pulldown analysis reveals that through its COOH terminus, enthoprotin binds specifically to AP1 (as determined by γ-adaptin Western blot), AP2 (as determined by α-adaptin Western blot), and clathrin (Fig. 3 B). No binding was detected to the ENTH domain or to GST alone (Fig. 3 B). To further define the binding sites for these proteins, we generated GST constructs encoding COOH-terminal truncation mutants (Fig. 3 A). The AP1 binding site(s) maps primarily between amino acids 370 and 451, whereas the AP2 binding site(s) maps primarily between amino acids 136 and 281 (Fig. 3 C). Clathrin binding maps to a site(s) between amino acids 281 and 370, which contains a type 2–clathrin box. Thus, enthoprotin binds to major components of CCVs through distinct sites located in its COOH terminus.

**Enthoprotin binds to and colocalizes with GGA2**

Next, we examined for a potential interaction between enthoprotin and GGA proteins. The GGAs have a modular structure consisting of four domains termed: Vps27p, Hrs, STAM; GGA and TOM; hinge; and GAE (Robinson and Bonifacino, 2001). The GAE domain is homologous to the ear domain of γ-adaptin although little is known regarding potential binding partners for this domain. Using a GST pulldown assay, we found that the hinge and GAE domains of GGA2 bound FLAG-tagged enthoprotin, whereas the Vps27p, Hrs, STAM domain of GGA3 and the hinge and ear domains of the β3A subunit of AP-3 did not (Fig. 4 A). We also observed colocalization of FLAG-tagged enthoprotin with GFP-tagged GGA2 at both a perinuclear pool and at peripheral cytoplasmic foci (Fig. 4 B). GGA2 is found at both the TGN and at endosomes consistent with a localization of enthoprotin on these internal membranes.

**Enthoprotin stimulates clathrin assembly**

To better characterize the role of enthoprotin, we further examined its interaction with clathrin. A GST–enthoprotin fusion protein containing the clathrin-binding site between amino acids 281 and 370 bound to clathrin in overlay assays demonstrating that the interaction is direct (unpublished data). Moreover, a GST fusion protein encoding the globular terminal domain of the clathrin heavy chain specifically bound the FLAG-tagged COOH terminus of enthoprotin (Fig. 5 A). Interestingly, epsin, which like enthoprotin contains an ENTH domain, binds to the terminal domain of the clathrin heavy chain and was recently demonstrated to stimulate the assembly of clathrin cages in vitro (Kalthoff et al., 2002). Thus, we sought to explore if enthoprotin is involved in the assembly of clathrin using in vitro clathrin assembly assays (Legendre-Guillemin et al., 2002). Both GST-ΔC1 and GST-ΔC2 (Fig. 3 A) stimulate the assembly of purified clathrin triskelia in a dose-dependent manner (Fig. 5 B). In contrast, no assembly activity is seen with GST or with GST-ΔC3, which does not interact with clathrin (Fig. 5 B).

A key finding of our study is the identification of enthoprotin as a novel ENTH domain–containing protein enriched on CCVs. Thus, enthoprotin joins a growing list of ENTH domain–containing proteins, including AP180, the HIP proteins, and epsins that function in clathrin-mediated vesicle trafficking (De Camilli et al., 2002). Although these proteins share an ENTH domain protein module, they display limited homology outside of the ENTH domain. For example, although epsin 1 is ~42 and 41% identical with epsin...
Figure 4. **Enthoprotin binds to and colocalizes with GGA2.** (A) GST fusion proteins bound to glutathione-Sepharose were used in pulldown assays from COS-7 cells expressing FLAG-tagged full-length enthoprotin. Specifically, bound proteins were analyzed by Western blotting for the FLAG epitope. (B) FLAG-tagged full-length enthoprotin was coexpressed with GFP-tagged GGA2. The enthoprotin and GGA2 fusion proteins were detected by FLAG immunostaining and GFP fluorescence, respectively. Bar, 10 μm.

Figure 5. **Enthoprotin stimulates clathrin assembly.** (A) GST or a GST fusion protein encoding the terminal domain of the clathrin heavy chain (GST-TD) were precoupled to glutathione-Sepharose beads and used in pulldowns from HEK-293 cells expressing FLAG-enthoprotin COOH-terminal or ENTH domains. Specifically bound proteins were analyzed by Western blotting for the FLAG epitope along with an aliquot of the cell lysate (SM, starting material). (B) Clathrin assembly assays were performed with increasing amounts of GST, GST–ΔC1, GST–ΔC2, and GST–ΔC3 as indicated. The GST fusion proteins and the clathrin remaining in the supernatant after high-speed centrifugation were analyzed by SDS-PAGE and Coomassie blue staining. The migratory positions of the clathrin heavy chain (CHC) and the various fusion proteins are indicated. (C) Model of potential enthoprotin function.
Materials and methods

Antibodies and cDNA constructs
A GST fusion protein encoding amino acids 166–370 of enthropin (Figs. S1 and S2, available at http://www.jcb.org/cgi/content/full/jcb.200205078/DC1) was injected into each of two rabbits (3194 and 3197; ~1.5 mg/injection) using Titermax adjuvant (CytRx Corp.) with standard protocols. Antibody production was monitored by Western blots and antibodies were affinity purified using strips of PVDF membrane containing the same fusion protein (Sharp et al., 1993). A monoclonal antibody against the FLAG epitope tag was purchased from Sigma-Aldrich. Monoclonal antibodies against the γ-adaptin subunit of the AP1 complex, the α-adaptin subunit of AP2, and the clathrin heavy chain were purchased from Transduction Laboratories. A monoclonal antibody against clathrin used for immunofluorescence was produced from the hybridoma X22 (American Type Culture Collection). GST–clathrin terminal domain was a gift of Dr. James Keen (Thomas Jefferson University, Philadelphia, PA). GGA2 GST fusion proteins have been previously described (Puertollano et al., 2000). Other enthropin constructs are described in the supplementary materials.

Purification of CCVs and mass spectrometry analysis
Adult rat brains and livers were homogenized in buffer A (10 mM MES, pH 6.8, 0.5 mM EGTA, 1 mM MgCl₂) and CCVs were isolated by differential centrifugation as described (Maycox et al., 1992). The isolated rat brain vesicles (~1 mg) were placed on linear 20–50% sucrose gradients prepared in buffer A and centrifuged in a Sorvall AH629 rotor at 145,000 g for 1.5 h. The gradients were fractionated and the CCVs were identified by Western blots. Three peak fractions were pooled, diluted 10-fold in buffer A, the CCVs were pelleted at 91,500 g for 1 h and then resuspended in gel sample buffer and the proteins were separated by SDS-PAGE and stained with Coomassie blue. The gel lane was then cut horizontally into 62 even-sized gel slices. The slices were dehydrated in acetonitrile and washed by two cycles of 10 min in 100 mM (NH₄)₂CO₃, before the addition of an equal volume of acetonitrile. The completely destained gel slices were then treated for 30 min with 10 mM dithiothreitol to reduce cysteinyl residues and for 20 min with 55 mM iodoacetamide to effect alkylation. After an additional round of (NH₄)₂CO₃ and acetonitrile washes, the slices were extracted with acetonitrile at 37°C. They were then incubated with trypsin (6 mg/ml) in 50 mM (NH₄)₂CO₃ for 5 h at 37°C and the peptides were first extracted in 1% formic acid/2% acetonitrile followed by two further extractions with additions of acetonitrile. All treatments were performed robotically using a MassPrep Workstation (Micromass).

Extracted peptides were applied to a reverse phase guard column and then eluted in-line to a 10 cm by 75 μm PicoFrit column filled with BioBasic C₁₈. The column was eluted at 200 nl/min with a linear gradient of 5–70% acetonitrile/0.1% formic acid. For peptides from gel bands with an apparent molecular mass between 21 and 100 kDa, a 60 min gradient was employed whereas all other peptides were eluted with a 30 min gradient. A 2,000-V charge was applied to the PicoFrit column such that the eluted peptides were electrosprayed into a Cap liquid chromatography quadrupole time-of-flight MS (MicroMass). When doubly or triply charged ions were detected, the quadrupole was used to select the precursor ion, which was passed into a collision cell where fragmentation was induced by collision with argon gas. The collision energies are determined automatically by the instrument based on the m/z values and charge states of each peptide. Fragmentation data were collected in 1-s scans for up to 5 s. The MS/MS data were peaklisted (Masslynx, Micromass) and submitted to Mascot (MatrixScience) software for database search analysis against the NCBI non-redundant database.

Analysis of enthropin in transfected cells
COS-7 cells were plated on polyl-lysine–coated coverslips, and at ~15 h post transfection, cells were fixed with 2% paraformaldehyde/PBS (20 mM NaH₂PO₄, 0.9% NaCl, pH 7.4) for 10 min, permeabilized with 0.2% Triton X-100/PBS for 4 min, and processed for immunofluorescence with appropriate primary and secondary antibodies. For the experiment shown in Fig. 4 B, COS-7 cells were fixed and permeabilized 15 h after transfection using methanol–acetone (50:50) and processed for immunofluorescence with appropriate primary and secondary antibodies. To generate COS-7 cell microsomes, cell lysates were centrifuged for 20 min at 17,000 g. and the supernatant was subsequently centrifuged for 1 h at 56,000 g. CCVs were prepared from HEK-293 cells transfected with various enthropin constructs as previously described (Metzler et al., 2001).

Pulldown assays
Adult rat brain was homogenized in Buffer B (10 mM Hepes-OH, pH 7.4, 0.83 mM benzamidine, 0.23 mM phenylmethylsulphonyl fluoride, 0.5 μg/ml aprotinin and 0.5 μg/ml leupeptin) and centrifuged at 800 g for 5 min. The supernatant was incubated with 1% Triton X-100, 150 mM NaCl for 30 min at 4°C and then centrifuged for 30 min at 205,000 g. At 48 h post-transfection, HEK-293 cells were washed with PBS, scraped in Buffer B, and sonicated. Lysates were centrifuged for 15 min at 220,000 g and Triton X-100 was added to the supernatant fractions to 1% final. Aliquots of the Triton X-100 (1 mg/ml) and the cell lysates (25 μl) were incubated for 3 h or overnight at 4°C with GST fusion proteins precoupled to glutathione-Sepharose beads. Samples were subsequently washed in Buffer B containing 1% Triton X-100, and specifically bound proteins were
processed for Western blotting. In the experiments shown in Fig. 4 A, COS-7 cells were lysed 48 h posttransfection in TNE buffer (25 mM Tris-HCl, 150 mM NaCl, 0.5 mM EDTA) containing 0.5% Triton X-100 and incubated with GST fusion proteins (0.2 mg per sample) bound to glutathione-Sepharose for 4 h at 4°C. The beads subsequently washed with PBS and bound proteins were processed for Western blotting.

Clathrin assembly assays

Clathrin assembly assays were performed as described (Legendre-Guillemin et al., 2002).

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