

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 (AP)1. 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 hydrolases, 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 hydrolases, 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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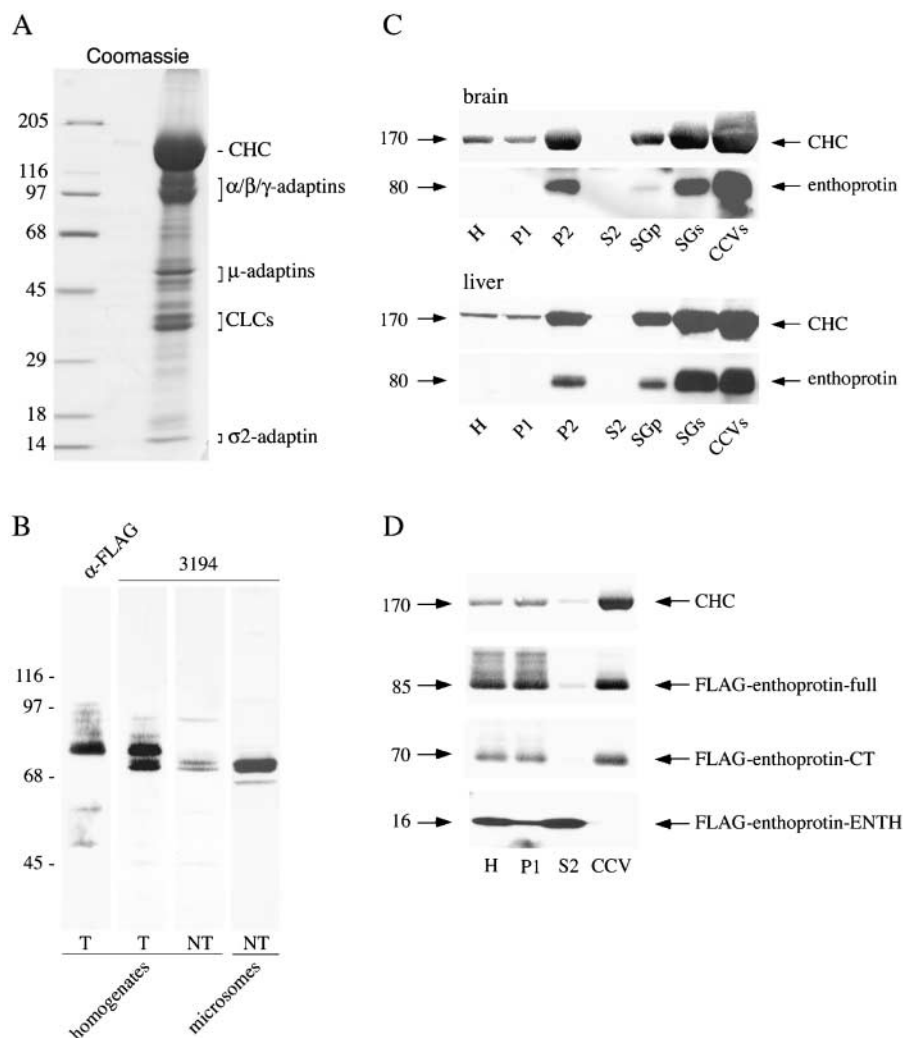
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*Abbreviations used in this paper: AP, adaptor protein; CCP, clathrin-coated pit; CCV, clathrin-coated vesicle; ENTH, epsin NH₂-terminal homology; GGA, Golgi-localized, γ -ear-containing, Arf-binding protein; HIP, huntingtin-interacting protein; MPR, mannose-6-phosphate receptor; TGN, trans-Golgi network.

Key words: clathrin adaptors; endosome; ENTH domain; GGAs; TGN

Figure 1. Identification of enthoproten 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 ($\alpha/\beta/\gamma$ -adaptilins), the μ -adaptilins, $\sigma 2$ -adaptilin, 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 enthoproten (T) were analyzed by Western blot with antibodies against enthoproten (3194) or the FLAG epitope tag as indicated. (C) CCVs were prepared from rat brain and liver and the distribution of endogenous enthoproten 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 enthoproten, 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.



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 prepara-

tion was highly enriched in clathrin and the subunits of the AP1 and AP2 adaptor complexes (Fig. 1 A). The gel lane was cut into 62 equivalent-sized slices and each slice was subjected to in-gel trypsin digest and liquid chromatography quadrupole time-of-flight mass spectrometry. The procedure was repeated two times with independently isolated preparations.

Approximately 250 proteins were identified (unpublished data). Ten of the proteins were hypothetical gene products that had not been previously detected at the protein level (Table I). The protein encoded by KIAA1435 contains seven WD-40 repeats and a FYVE domain. During the course of our study, this protein was detected in a targeted proteomics approach aimed at the identification of phosphatidylinositol(3)P-binding proteins and has been named FENS-1 (Ridley et al., 2001). FENS-1 is implicated in vesicular trafficking based on its FYVE domain-dependent localization to early endosomes (Ridley et al., 2001). KIAA1048 has a serine/threonine kinase domain at its NH₂ and COOH terminus contains multiple DPF, NPF, and DLL motifs implicated in binding to AP2, Eps15 homology domains, and clathrin, respectively. During the course of this study, KIAA1048 was identified through its association with the ear domain of α -adaptilin and has been named AAK1 (Conner and Schmid, 2002; Ricotta et al., 2002). AAK1 phosphorylates the $\mu 2$ -sub-

Table I. Summary of novel proteins identified in the CCV preparation

Protein/gi	Domains/similarities	Mascot score	gel mass	predicted mass
			kD	kD
KIAA1435 ^a	7 WD-40 repeats and a FYVE domain	301	43	46.3
KIAA1048 ^a	Serine/threonine kinase, contains NPF/DPF/DLL repeats	579	116	93.6
KIAA1360	Serine/threonine kinase, contains NPF/DLL repeats (start codon not identified)	36	88	88.6
KIAA1576	Homologous to Torpedo VAT-1	69	52	45.9
13385744	No conserved domains or homologies	82	17	18.5
13385773 ^{a,b}	Homologous to gi13384758, no other homologies or known domains	181	40	29.6
13384758 ^a	Homologous to gi13385773, no other homologies or known domains	56	31	28.6
12859193	GTP-binding protein	76	48	30.4
12855458 ^a	SEC14 domain	73	36	40.8
KIAA0171 ^a	ENTH domain, two type II clathrin boxes, clathrin box-like sequence	355	80	68.2

^aProteins identified in two independent preparations.

^bDNA sequence.

Detailed descriptions of each protein are provided in the text. The gel mass indicates the relative migratory position on the gel (estimated in kD) from where the best peptide matches were obtained as indicated by the listed Mascot scores. The predicted mass is determined from translation of the longest open reading frame.

unit of AP2 and is found in CCPs at the leading edge of migrating cells (Conner and Schmid, 2002). KIAA1360 also contains an NH₂-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 phosphatidylinositol transfer protein Sec14p, a protein required for membrane budding at the TGN (Bankaitis et al., 1989). The demonstrated and presumptive links 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 Kazusa 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 NH₂-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 YSPCC794, 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-

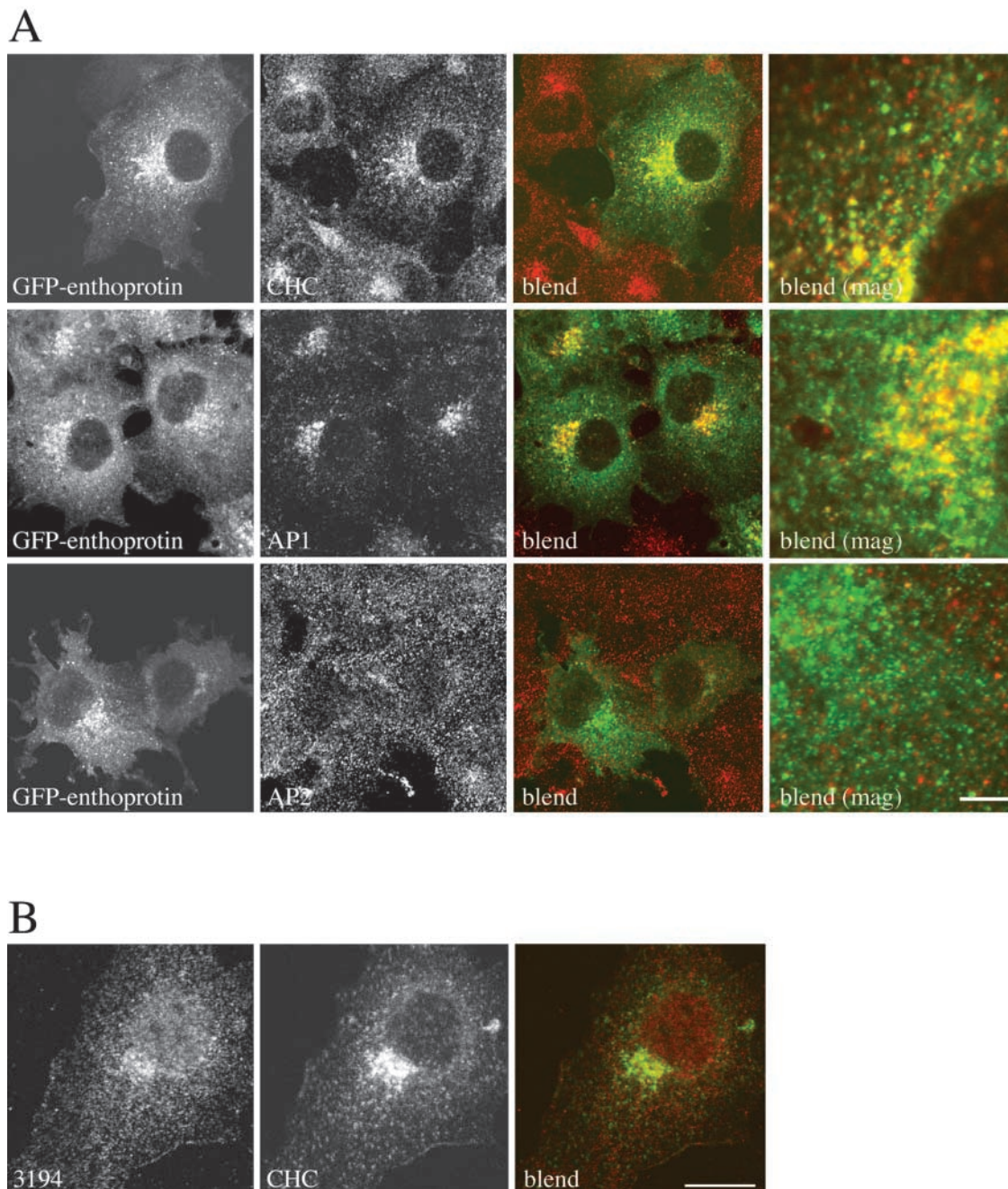


Figure 2. Localization of enthoprotin in COS-7 cells. (A) GFP-tagged full-length enthoprotin was transfected into COS-7 cells and its localization was compared with that of endogenous clathrin heavy chain (CHC), AP1, and AP2 determined by indirect immunofluorescence. The colocalization of enthoprotin (green) with the various markers (red) is revealed in the blended images (blend) and with higher magnification (blend mag). (B) The localization of endogenous COS-7 cell enthoprotin (red) and the clathrin heavy chain (CHC) (green) were determined by indirect immunofluorescence and the overlap of the two signals was revealed in the blended image (blend). Bar, 4 μm in the magnified panels, 20 μm in all other panels.

pression, GFP-enthoprotin 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

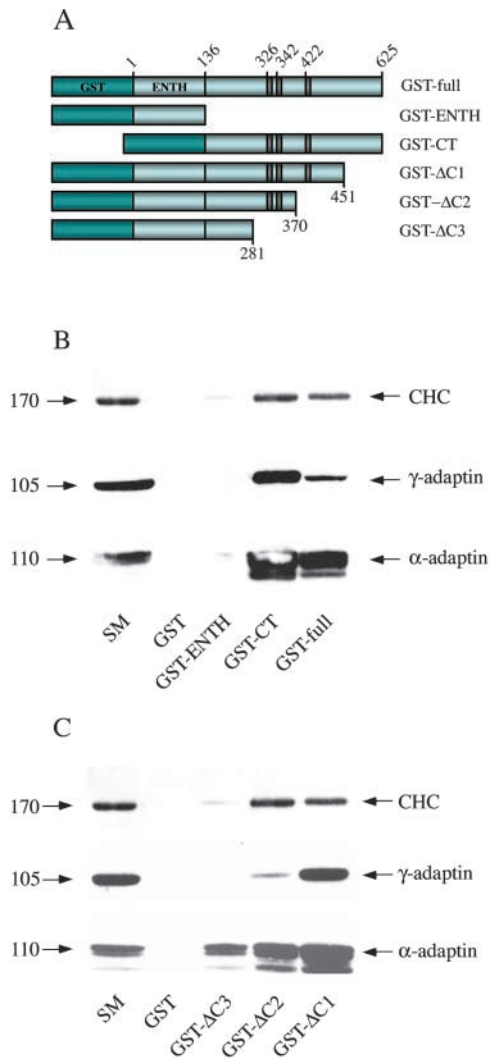


Figure 3. Enthoprotin binds to coat components of CCVs.

(A) Domain models of enthoprotin GST fusion proteins. The gray bars represent the type 2-clathrin boxes (starting at amino acids 326 and 422) and the clathrin box–like sequence (starting at amino acid 342). (B and C) Various enthoprotin GST fusion proteins were coupled to glutathione-Sepharose and used in pull-down assays from brain extracts. Specifically, bound proteins were analyzed by Western blots, along with an aliquot of the brain extract (SM, starting material) with antibodies against the clathrin heavy chain (CHC), γ -adaptin, and α -adaptin.

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, pull-down 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 pull-down 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 β 3A 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-Guillemain et al., 2002). Both GST- Δ C1 and GST- Δ C2 (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- Δ C3, 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 \sim 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 pull-down 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 μ m.

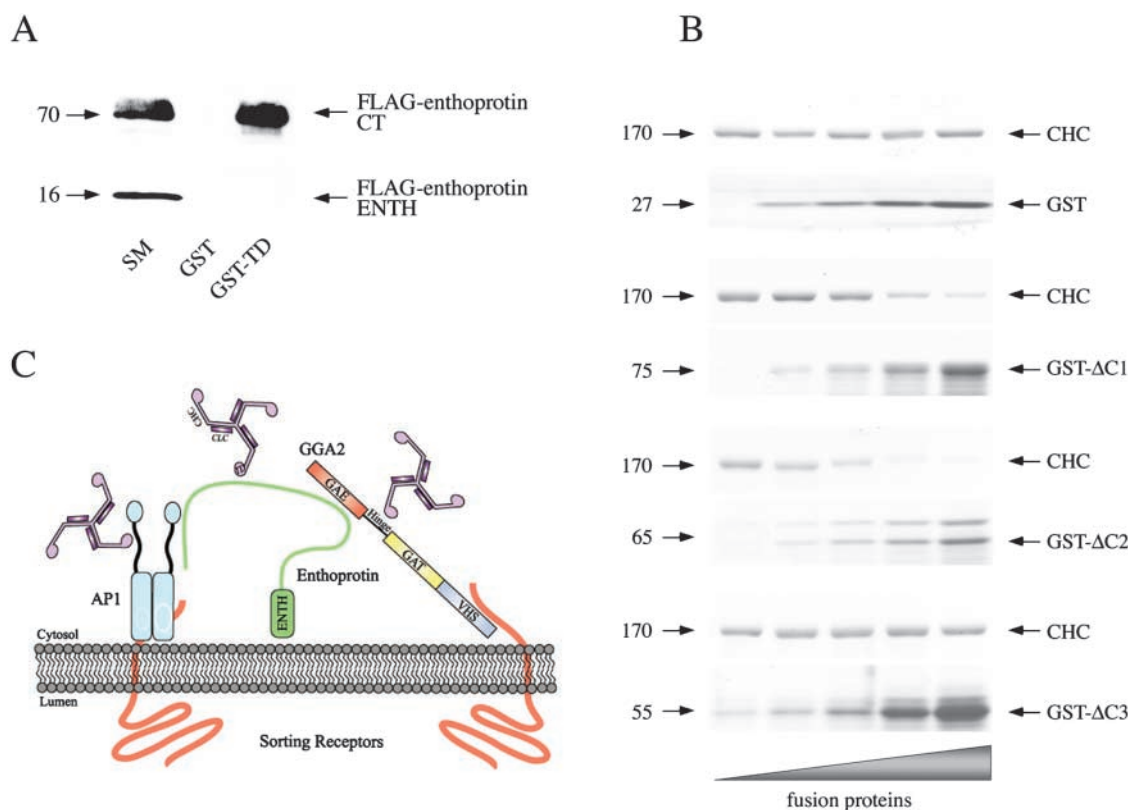
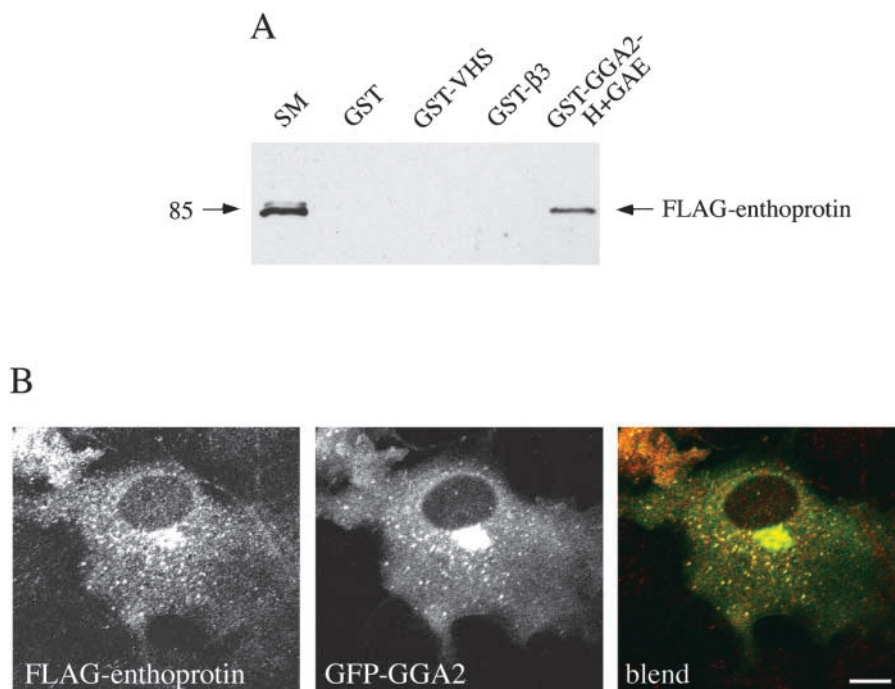


Figure 5. Enthoprotin stimulates clathrin assembly. (A) GST or a GST fusion protein encoding the terminal domain of the clathrin heavy chain (GST-TD) were precoupled to glutathione-Sepharose beads and used in pull-downs from HEK-293 cells expressing FLAG-enthoprotin COOH-terminal or ENTH domains. Specifically 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- Δ C1, GST- Δ C2, and GST- Δ C3 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.

2a and 3, respectively, outside of the ENTH domain, it is only 15% identical with enthoprotin over the same region. However, intriguingly, AP180, HIP1, HIP12, and epsin 1 each bind to clathrin and stimulate clathrin assembly (Ahle and Ungewickell, 1986; Engqvist-Goldstein et al., 2001; Legendre-Guillemain et al., 2002; Kalthoff et al., 2002). In this respect, these proteins appear to be members of a functionally related protein family regulating clathrin-mediated trafficking events. AP180 functions in the endocytic recycling of synaptic vesicles (Morgan et al., 2000) and the HIPs and the epsins are each localized primarily to the plasma membrane where they function in endocytosis (Rosenthal et al., 1999; Engqvist-Goldstein et al., 2001; Metzler et al., 2001). In contrast, enthoprotin is localized primarily to membranes of the TGN and the endosomal system. Enthoprotin is thus the first member of the ENTH domain-containing group of proteins that appears to function primarily on internal membranes. However, it is worth noting that enthoprotin binds to AP2. This binding is unlikely to be indirect through clathrin as clathrin and AP2 display distinct binding sites on enthoprotin. The interaction could result from the presence of AP2 on endosomes (Sorkina et al., 1999), although it is not possible to rule out a role for enthoprotin in the formation of CCVs at the plasma membrane.

In addition to its interaction with AP1, enthoprotin binds to the hinge and GAE domain of GGA2. Both of these adaptor proteins, which are colocalized with enthoprotin, function in the trafficking of the MPRs between the TGN and the endosomal–lysosomal system (Fig. 5 C). Multiple lines of evidence demonstrate functional interactions between clathrin and the GGAs in vivo (for review see Robinson and Bonifacino, 2001). The GGAs also bind to the cytoplasmic tails of the MPRs and recruit them to clathrin-coated areas on the TGN from which they enter carrier vesicles for delivery to endosomes (Puertollano et al., 2001; Zhu et al., 2001). AP1 is also thought to function in MPR exit from the TGN (Traub and Kornfeld, 1997; Le Borgne and Hoflack, 1998) although it may also function as an adaptor in the recycling of the MPRs from endosome back to the TGN (Meyer et al., 2000). Enthoprotin may thus cooperate with these adaptors in clathrin assembly at either of these compartments (Fig. 5 C).

In conclusion, the use of subcellular proteomics has led us to the identification of enthoprotin as a novel component of the machinery for clathrin coat assembly on internal membranes. Previous subcellular proteomics studies identifying novel proteins have focused primarily on their localization to the organelle under study. We have now extended the proteomics approach to the identification of novel functional components of vesicular trafficking pathways.

Materials and methods

Antibodies and cDNA constructs

A GST fusion protein encoding amino acids 166–370 of enthoprotin (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 enthoprotin 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 cystinyl 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 ng/ μ l 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 μ m 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 kD, 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 are 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 was peaklisted (MassLynx; MicroMass) and submitted to Mascot (MatrixScience) software for database search analysis against the NCBI non-redundant database.

Analysis of enthoprotin in transfected cells

COS-7 cells were plated on poly-L-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, cells were washed with PBS, scraped in Buffer A and lysed with a teflon homogenizer. 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 enthoprotin 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 μ g/ml aprotinin and 0.5 μ g/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-soluble brain extract (2 mg) and the cell lysates (0.2 mg) 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-Guillemain et al., 2002).

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