ENTH/ANTH proteins and clathrin-mediated membrane budding

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Summary

The epsin N-terminal homology (ENTH) domain is an evolutionarily conserved protein module found primarily in proteins that participate in clathrin-mediated endocytosis. Structural analyses and ligand-binding studies have shown that a set of proteins previously designated as harboring an ENTH domain in fact contain a highly similar, yet unique module referred to as an AP180 N-terminal homology (ANTH) domain. ENTH and ANTH (E/ANTH) domains bind both inositol phospholipids and proteins and contribute to the nucleation and formation of clathrin coats on membranes. ENTH domains also function in the development of membrane curvature through lipid remodeling during the formation of clathrin-coated vesicles. E/ANTH-bearing proteins have recently been shown to function with adaptor protein-1 and GGA adaptors at the trans-Golgi network, which suggests that E/ANTH domains are universal components of the machinery for clathrin-mediated membrane budding.

Key words: ENTH domain, ANTH domain, Phosphoinositides, Clathrin-coated vesicle, trans-Golgi network

Introduction

Clathrin-mediated membrane budding drives the formation of endocytic vesicles for the internalization of nutrients, receptors and other proteins at the cell surface (McPherson et al., 2001; Conner and Schmid, 2003a). It also operates at the trans-Golgi network (TGN), where it mediates the trafficking of cargo proteins from the TGN to the endosomal/lysosomal system (Brodsky et al., 2001; Hinners and Tooze, 2003). Several studies have revealed the complex molecular machinery underlying the formation and function of clathrin-coated pits (CCPs) and vesicles (CCVs) at the plasma membrane (Slepnev and De Camilli, 2000; Takei and Haucke, 2001). By contrast, the mechanisms of clathrin-mediated events at the TGN remain less clear. Recently, two new and highly related modules, the epsin N-terminal homology (ENTH) and AP180 N-terminal homology (ANTH) domains, have been identified in proteins that participate in clathrin-mediated budding. In this Commentary, we describe recent structural studies that have significantly advanced our understanding of E/ANTH domains. We also discuss how the identification of new E/ANTH proteins at the TGN, notably the ENTH-domain-containing protein enthoprotin, provides evidence that E/ANTH domains are universal elements in the clathrin-budding machinery.

Identification of the ENTH domain

The heterotetrameric clathrin adaptor protein-2 (AP-2) complex plays an important role in the formation of endocytic vesicles. AP-2 contributes to the recruitment of clathrin triskelia to the plasma membrane and the assembly of triskelia into clathrin coats, leading to the formation of CCPs that eventually pinch off from the membrane in a dynamin-dependent manner to form CCVs (Brodsky et al., 2001; Sever, 2002). AP-2 also binds to endocytic cargo, leading to the concentration of cargo in nascent CCPs (Brodsky et al., 2001). Additionally, AP-2 recruits a diverse array of endocytic accessory proteins to sites of CCV formation (Slepnev and De Camilli, 2000; Takei and Haucke, 2001). These proteins bind through short consensus peptide motifs to a globular domain at the C-terminus of the AP-2 α-adaptin subunit termed the α-ear (Owen et al., 1999; Traub et al., 1999; Brett et al., 2002; Ritter et al., 2003).

Members of the epsin family of endocytic regulatory proteins are important binding partners for the α-ear (Chen et al., 1998; Yamabhai et al., 1998; Nakashima et al., 1999; Rosenthal et al., 1999; Spradling et al., 2001) (Fig. 1). The best characterized member of this family, epsin 1, is unusual in that the C-terminal two-thirds of the molecule contains essentially no secondary structure (Kalthoff et al., 2002a). Within this extended, unstructured domain are multiple short peptide motifs that mediate interactions with endocytic proteins (Fig. 1). Included are eight copies of the DPW tripeptide that mediates binding to the α-ear (Owen et al., 1999; Traub et al., 1999) and two distinct clathrin-binding motifs, which mediate interactions with the clathrin heavy chain (Hussain et al., 1999; Rosenthal et al., 1999; Drake and Traub, 2001). Epsin 1 also contains three NPF motifs, which bind to the EH (Eps15 homology) domains of the endocytic proteins Eps15 and intersectin (Chen et al., 1998; Hussain et al., 1999). Epsin 1 is localized to CCPs, and disruption of its interactions with other endocytic proteins blocks clathrin-mediated endocytosis (Chen et al., 1998).

Sequence alignments revealed an ~150 residue region at the epsin 1 N-terminus that is highly conserved in organisms as diverse as yeast, plant, frogs and humans (Rosenthal et al., 1999; Kay et al., 1999). This sequence, first noted in a plant protein (Jones and Hooley, 1997), was called the ENTH

domain (Rosenthal et al., 1999; Kay et al., 1999). X-ray crystallography has shown that the ENTH domain from epsin 1 has a compact globular structure in which eight α-helices are connected by loops of varying length (Hyman et al., 2000; De Camilli et al., 2002). Structurally, the ENTH domain is similar to the VHS (Vps27p, hepatocyte growth-regulated tyrosine kinase substrate, Hrs; signal-transducing adaptor molecule, STAM) domain (Hyman et al., 2000), which is found at the N-terminus of proteins that participate in membrane trafficking (Lohi et al., 2002).

ENTH and ANTH domains bind inositol phospholipids

The module most highly related to the ENTH domain is the ANTH domain (Ford et al., 2001; Itoh et al., 2001; Ford et al., 2002). These two modules are, in fact, so similar that several ANTH-bearing proteins were originally designated as containing ENTH domains. The ANTH domain has been studied primarily in AP180, a brain-specific clathrin-binding protein that stimulates clathrin assembly during the recycling of synaptic vesicles (Fig. 1) (Morgan et al., 2000).

An important breakthrough in understanding the function of E/ANTH domains came with the observation that they bind inositol phospholipids and exhibit a preference for phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] (Ford et al., 2001; Itoh et al., 2001). Nuclear magnetic resonance and X-ray crystallography revealed that PtdIns(4,5)P_2 binds to the epsin 1 ENTH domain through basic residues in α-helix 1, the α1-2 loop, α-helix 3 and α-helix 4. By contrast, in the case of

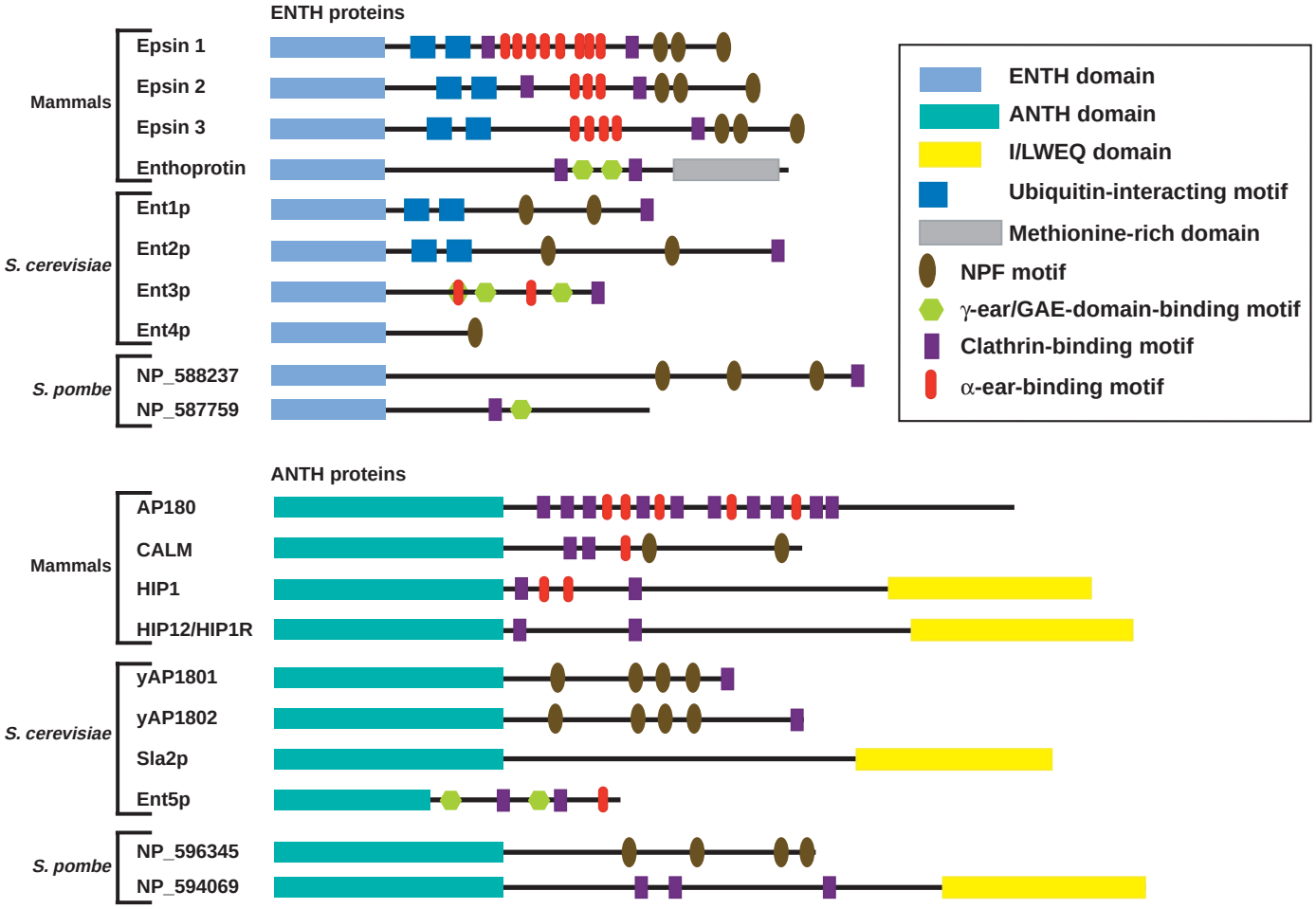

Fig. 1. E/ANTH proteins and their domains. A series of ENTH- and ANTH-bearing proteins from human and yeast are depicted. Within their C-terminal region, these proteins contain short peptide motifs and protein domains that predominantly mediate protein interactions. The I/LWEQ domain, also called the talin-like domain, can bind to actin. The ubiquitin-interacting motif (UIM) is a consensus motif (EDExLxxAxxxSxxE/D) that mediates interactions with ubiquitylated proteins. The methionine-rich domain, found within the C-terminal region of enthoprotin, contains 17% methionine residues but its function is unknown. NPF motifs bind to Eps15 homology (EH) domain-containing proteins. A γ-ear/GAE-domain-binding motif with the consensus sequence [D/E][G/A]$_{(0-1)}$F[G/A][D/E]Φ, where Φ is any hydrophobic amino acid, has been recently identified in enthoprotin and other proteins (Duncan and Payne, 2003). Clathrin-binding motifs encompass multiple sequences that bind to the terminal domain of the clathrin heavy chain including DLL motifs and type I and II clathrin boxes. For HIP1 and HIP12/HIP1R, the C-terminal most clathrin-binding motif represents a putative new binding site for clathrin light chains present within an extended α-helical segment of the proteins (V.L.-G. et al., unpublished). The α-ear-binding motif includes DPW, DPF and FxDxF motifs that bind to the ear of the α-adaptin subunit of AP-2. Hypothetical E/ANTH proteins from *S. pombe* are denoted by their GenBank accession numbers.

the ANTH domain of AP180, PtdIns(4,5)P_2 binding is mediated by lysine residues in α-helix 1 and α-helix 2 and the loop between them. In fact, the sequence around this region (K/G)A(T/I)x_6(P/L/V)KxK(H/Y) is conserved in all ANTH domains and might represent an ANTH-defining consensus sequence (Ford et al., 2002). The corresponding region in ENTH domains is not involved in PtdIns(4,5)P_2 binding and is not conserved between ENTH and ANTH domains (Ford et al., 2002). Intriguingly, on binding of PtdIns(4,5)P_2, the region at the N-terminus of the ENTH domain, which is unstructured when not bound to ligand, forms an α-helix referred to as α0 (Ford et al., 2002). Basic residues on the inner face of α0 stabilize PtdIns(4,5)P_2 binding and their mutation abrogates lipid interactions. α0 is not present or generated in the ANTH domain (Ford et al., 2001; Itoh et al., 2001; Ford et al., 2002). These differences contribute to the classification of ENTH and ANTH domains as distinct modules, despite their overall structural similarities.

Function of E/ANTH domains

E/ANTH-bearing proteins have substantially divergent sequences outside the E/ANTH domain. However, many of them contain sequences that are indicative of functional roles in clathrin-dependent events (Fig. 1). These include motifs for binding to clathrin, AP-2, EH domains and ubiquitylated proteins (Fig. 1). Moreover, mutations in epsin 1 and AP180 that block PtdIns(4,5)P_2 binding inhibit clathrin-mediated endocytosis (Ford et al., 2001; Itoh et al., 2001). Thus, E/ANTH domains appear to be intimately associated with endocytic proteins.

Both epsin 1 and AP180 stimulate the assembly of soluble clathrin triskelia into clathrin cages, the assembly activity being located outside the E/ANTH domain (Ahle and Ungewickell, 1986; Morgan et al., 2000; Kalthoff et al., 2002a). Thus, an attractive model is that E/ANTH proteins are anchored to the membrane by PtdIns(4,5)P_2, leaving their extended C-terminal regions available to recruit coat components and catalyze clathrin assembly (Kalthoff et al., 2002a). Indeed, in vitro, both epsin 1 and AP180 recruit clathrin to PtdIns(4,5)P_2 monolayers and stimulate its polymerization into lattices (Ford et al., 2002). However, clathrin patches formed by epsin 1 are invaginated, whereas AP180-induced lattices are flat (Ford et al., 2002). These data suggest that clathrin assembly may not be sufficient to stimulate membrane invagination and that epsin 1 harbors an activity contributing to membrane curvature that is not found in AP180. One caveat to this interpretation comes from recent studies indicating that Eps15 can bind to AP180 and potently stimulate its clathrin assembly activity (Morgan et al., 2003). Thus, in the absence of Eps15, the studies of Ford et al. may have failed to reveal the full contribution of AP180 to the formation of invaginated CCPs (Ford et al., 2002).

An important distinction between ENTH and ANTH domains is the generation within the ENTH domain of α0 on binding to PtdIns(4,5)P_2. α0 has a series of hydrophobic residues on its outer surface, which has led McMahon and colleagues to speculate that insertion of α0 into the cytosolic leaflet of the bilayer could mechanically facilitate membrane curvature by pushing the lipid head groups apart (Ford et al., 2002) (Fig. 2). To test this hypothesis, Cho and colleagues monitored changes in surface pressure of phospholipid monolayers as a measure of membrane insertion (Stahelin et al., 2003). The presence of PtdIns(4,5)P_2 in the monolayers induced the membrane insertion of the epsin 1 ENTH domain, leading to membrane deformation, and this depended on the presence of the hydrophobic residues on the surface of α0 (Stahelin et al., 2003). No membrane insertion was seen with the ANTH domain of AP180. Thus, the ENTH domain can perform mechanical work on membranes, suggesting two possible models for the formation of membrane curvature. Insertion of the ENTH domain could function synergistically with clathrin assembly to drive membrane invagination. Alternatively, ENTH domains might be sufficient for membrane curvature, with assembled clathrin stabilizing the deformed membrane. The function of the ANTH domain is less clear. The clathrin lattices assembled on PtdIns(4,5)P_2 monolayers in the presence of AP180 are more uniform in size than those assembled by epsin 1 alone, suggesting that ANTH domains may contribute to the regulation of lattice size (Ford et al., 2002). Interestingly, in *Drosophila* AP180-knockout animals, abnormally large and pleiomorphic synaptic vesicles arise during reformation from CCVs (Zhang et al., 1998). Thus, E/ANTH domains have fundamental functions in the formation of CCPs and CCVs.

E/ANTH domain proteins and PtdIns(4,5)P_2 cycling

PtdIns(4,5)P_2 plays a crucial role in the nucleation of clathrin coats (Cremona and De Camilli, 2001). For example, phosphoinositides enhance coat formation on liposomes (Cremona et al., 1999), and masking PtdIns(4,5)P_2 by overexpression of the phospholipase C (PLC) δ pleckstrin homology (PH) domain or downregulating it by expression of lipid phosphatases inhibits the recruitment of coat proteins to the plasma membrane (Jost et al., 1998; Krauss et al., 2003). In brain, PtdIns(4,5)P_2 can be generated from PtdIns by the sequential actions of PtdIns 4-kinase type IIα and PtdIns 4-phosphate 5-kinase type Iγ (PtdIns4*P*-5KIγ) (Fig. 2) (Guo et al., 2003). PtdIns(4,5)P_2 forms on the plasma membrane, and its production is stimulated in part through the activation of PtdIns4*P*-5KIγ by membrane-associated ARF6 (Krauss et al., 2003). Thus, sites for CCP formation could be demarcated by transient increases in the levels of PtdIns(4,5)P_2 on the plasma membrane occurring in localized microdomains (Cremona and De Camilli, 2001). In fact, ultrastructural localization of PtdIns(4,5)P_2 using the PLC δ PH domain reveals that, in neurons, the phospholipid is found primarily on the plasma membrane, where it displays a non-uniform distribution (Micheva et al., 2003). Following electrical stimulation, PtdIns(4,5)P_2 relocalizes to cytoplasmic vesicles, which appear to represent freshly endocytosed membranes (Micheva et al., 2001; Micheva et al., 2003). Through their ability to bind PtdIns(4,5)P_2, E/ANTH proteins could be targeted to PtdIns(4,5)P_2-rich endocytic membranes to establish a scaffold for coat assembly (Fig. 2).

Although E/ANTH proteins are sufficient to bridge coat components to PtdIns(4,5)P_2-containing membranes in vitro (Ford et al., 2002), the situation appears to be more complex in vivo. Interestingly, Fisher and colleagues determined that the *D. melanogaster* epsin homologue, liquid facets, can be

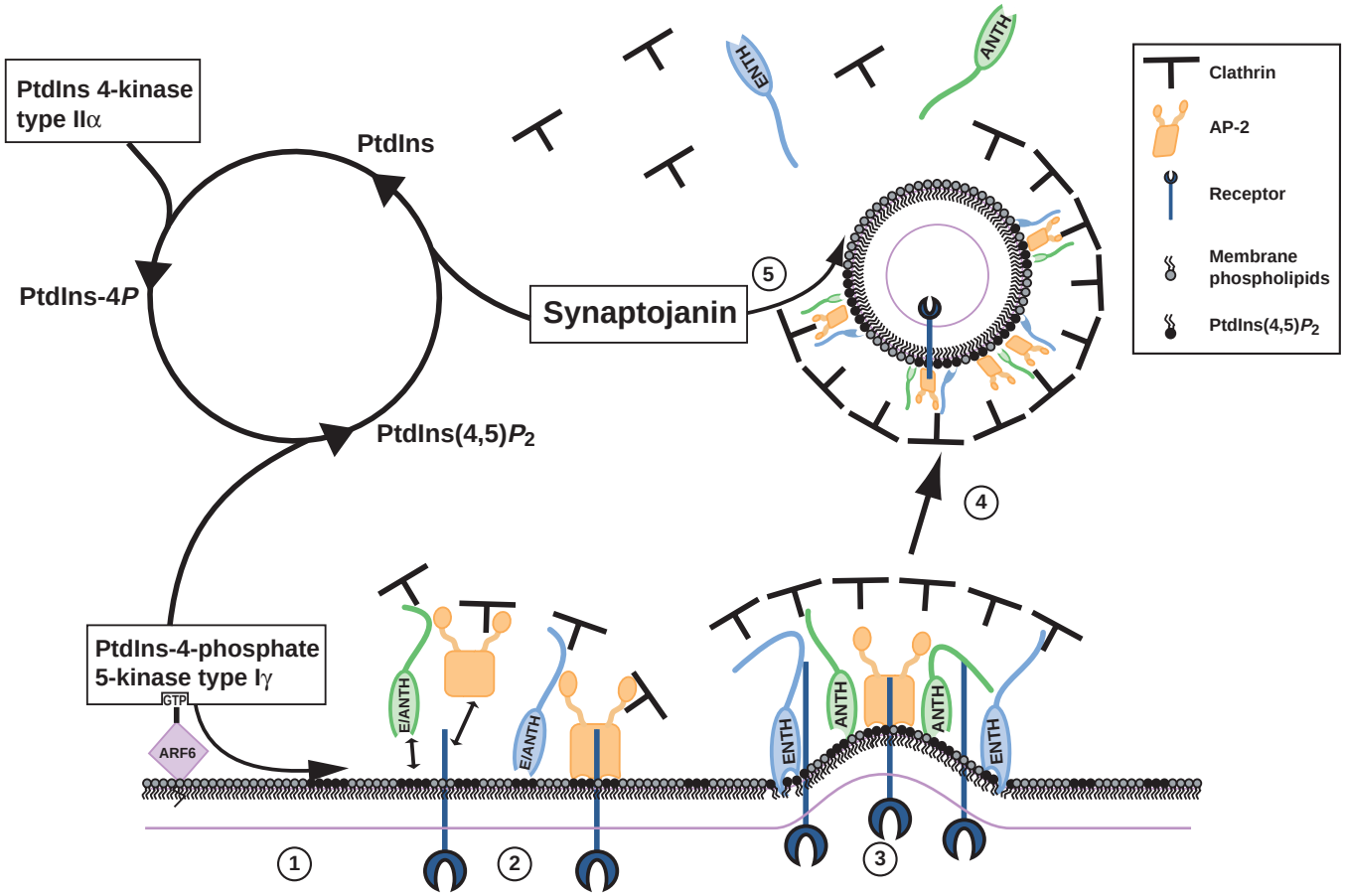

Fig. 2. E/ANTH proteins function in CCP and CCV formation coupled to cycles of PtdIns(4,5)P_2 metabolism. (1) PtdIns(4,5)P_2 (black) is generated from PtdIns by the sequential actions of PtdIns 4-kinase type IIα and PtdIns 4-phosphate 5-kinase type Iγ. ARF6-GTP can bind to and activate PtdIns 4-phosphate 5-kinase type Iγ on the plasma membrane. (2) E/ANTH proteins bind to PtdIns(4,5)P_2 that may form in localized microdomains within the general pool of membrane phospholipids (light gray). The E/ANTH proteins in turn bind additional endocytic proteins. Other coat components including AP-2 can also bind directly to PtdIns(4,5)P_2. Transmembrane receptors, through specific internalization motifs in their cytoplasmic domains, bind to the AP-2 complex. These cooperative interactions lead to the nucleation of clathrin coats on the plasma membrane. (3) Through their C-terminal regions, several E/ANTH proteins stimulate clathrin assembly, leading to the formation of CCPs. α-helix α0, formed at the N-terminus of the ENTH domain in response to phospholipid binding, inserts into the plasma membrane, leading to membrane deformation and curvature. E/ANTH proteins may also function to recruit cargo molecules to nascent CCPs through binding to their cytoplasmic tails. (4) CCVs pinch off from the plasma membrane in a dynamin-dependent mechanism. (5) The lipid phosphatase synaptojanin is recruited to CCVs, where it converts PtdIns(4,5)P_2 to PtdIns. This leads to destabilization of clathrin coats with Hsc70 and auxilin (not pictured) driving coat disassembly.

divided into two pieces, an ENTH domain and an ENTH-less protein, and each part retains partial endocytic function (Overstreet et al., 2003). These observations support the view that the ENTH domain and the remainder of the protein can function separately and that both parts of the protein contribute to epsin targeting and function. Also, Wendland and colleagues recently showed that, in the case of yeast epsin, Ent1p, the ENTH domain interacts with phospholipids, whereas ubiquitin-interaction motifs (UIMs) in the C-terminal region (Fig. 1) bind to ubiquitylated proteins at the membrane (Aguilar et al., 2003). These events in turn promote interactions between NPF motifs in Ent1p and EH-domain-bearing proteins (Aguilar et al., 2003). Moreover, as epsin binds to AP-2 and both proteins interact with PtdIns(4,5)P_2, they are likely to bind to membranes in a cooperative manner (Cremona and De Camilli, 2001). Thus, epsins are recruited to biological membranes by multiple independent interactions instead of being targeted to the membrane by the ENTH domain alone. These results suggest that E/ANTH proteins are unlikely to simply bridge coat components to membranes but instead contribute to the formation of networks of protein-protein and protein-lipid interactions that lead to the stable recruitment of clathrin coats (Fig. 2).

Key to the idea of a PtdIns(4,5)P_2-mediated switch in CCV formation is a mechanism to terminate the signal. Dephosphorylation of PtdIns(4,5)P_2 by lipid phosphatases could weaken the association of E/ANTH proteins and other coat components with membranes (Fig. 2). This would destabilize the clathrin coat and contribute to uncoating along with Hsc70 and auxilin (Lemmon, 2001). Synaptojanin, an endocytic PtdIns(4,5)P_2 phosphatase (McPherson et al., 1996), is a probable candidate to trigger this switch because synaptojanin knockouts have increased endogenous levels of PtdIns(4,5)P_2 and a corresponding increase in the numbers of coated

CCVs (Cremona et al., 1999). Another way of modulating PtdIns(4,5)P_2 levels at the plasma membrane involves the regulation of ARF6 activity by regulatory proteins such as guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). In fact, cycles of activation-inactivation of ARF6 are crucial for trafficking through the plasma-membrane–endosome recycling pathway (Jackson and Casanova, 2000; Donaldson, 2003). Activation of ARF6-specific GAPs could decrease ARF6-GTP levels, decreasing PtdIns(4,5)P_2 production (Donaldson, 2003). Thus, cycles of association of E/ANTH-bearing proteins with membranes coupled to cycles of PtdIns(4,5)P_2 synthesis and dephosphorylation may be a key factor in regulating CCV formation.

Additional roles for E/ANTH proteins in endocytosis

Several E/ANTH proteins, including epsin, AP180, CALM (clathrin assembly lymphoid myeloid leukemia protein), HIP1 (huntingtin-interacting protein 1) and HIP12/HIP1R, stimulate clathrin assembly (Ahle and Ungewickell, 1986; Tebar et al., 1999; Morgan et al., 2000; Engqvist-Goldstein et al., 2001; Mishra et al., 2001; Kalthoff et al., 2002a; Legendre-Guillemin et al., 2002). Thus, in addition to recruiting coat components to membranes, E/ANTH-bearing proteins probably contribute to the formation of ordered clathrin coats. Moreover, a growing body of evidence suggests that E/ANTH proteins function as cargo-specific adaptors that recruit endocytic cargo to sites of CCV formation. For example, genetic disruption of UNC-11, the *C. elegans* orthologue of AP180, disrupts the targeting of synaptobrevin/VAMP (vesicle-associated membrane protein) to recycling synaptic vesicles (Nonet et al., 1999). Moreover, mammalian and yeast epsins carry UIMs. These modules bind to ubiquitylated membrane proteins (Polo et al., 2002; Aguilar et al., 2003) and are thus likely to concentrate ubiquitylated proteins into CCVs. Ubiquitylation is a key signal regulating the endocytosis of a diverse group of cell-surface proteins (Hicke, 1999). Thus, epsins could function as adaptors for a wide range of ubiquitilated endocytic cargo. Hayden and colleagues recently described a more specific adaptor function for the ANTH-bearing protein HIP1 (Metzler et al., 2003). HIP1 binds to clathrin and AP-2 and is a stable component of CCPs and CCVs (Metzler et al., 2001; Mishra et al., 2001; Waelter et al., 2001; Legendre-Guillemin et al., 2002). Interestingly, in neurons, HIP1 is found in the postsynaptic compartment, where it binds to the GluR1 subunit of the AMPA-type glutamate receptor (Metzler et al., 2003). HIP1-knockout mice show a profound deficiency in clathrin-mediated endocytosis of GluR1 following AMPA stimulation, whereas constitutive endocytosis of the transferrin receptor is not affected (Metzler et al., 2003). It is intriguing to speculate that HIP1 couples AMPA receptor complexes to CCPs and as such plays a crucial role in the regulation of synaptic efficacy. It has been shown recently that AP-2 is necessary for the recruitment of only specific classes of cargo to CCPs (Conner and Schmid, 2003b; Motley et al., 2003; Hinrichsen et al., 2003). It is conceivable that E/ANTH proteins could be responsible for the recruitment of AP-2-independent cargo. In addition, as several E/ANTH proteins bind to AP-2, the two classes of adaptors could function co-operatively to recruit cargo to CCPs.

Protein binding by E/ANTH domains

Phospholipid binding allows E/ANTH domains to function at membranes. However, many E/ANTH-bearing proteins have significant pools in the cytosol, which suggests they have additional functions that are not directly related to CCV formation. In addition to VHS domains, E/ANTH domains are structurally related to an *armadillo* repeat segment from β-catenin and to the HEAT repeat unit from karyopherin-β (Hyman et al., 2000). Both *armadillo* and HEAT repeats are protein interaction modules (De Camilli et al., 2002), and the VHS domains can bind to the tails of sorting receptors at the TGN (Lohi et al., 2002). Thus, E/ANTH domains may have additional protein-binding properties. In fact, a two-hybrid screen using the ENTH domain of epsin 1 revealed that it interacts with promyelocytic leukemia Zn^{2+} finger protein (PLZF), a transcription factor (Hyman et al., 2000). The interaction with PLZF occurs in vivo, and PLZF can target epsin 1 to the nucleus (Hyman et al., 2000). Moreover, treatment of cells with leptomycin, an inhibitor of Crm1-dependent nuclear export, leads to an accumulation of epsin 1 and CALM in the nucleus (De Camilli et al., 2002). Thus, epsin 1, through its ENTH domain, may function as a transcriptional regulator.

Recently, we and others have determined that E/ANTH domains isolated from various species are binding partners for tubulin and microtubules (De Camilli et al., 2002; Hussain et al., 2003). A wealth of information has revealed complex links between the endocytic machinery and the actin cytoskeleton (for reviews, see Qualmann et al., 2000; McPherson, 2002), although a role for microtubules in endocytosis is less clear. Interestingly, Simon and colleagues have recently used total internal reflection fluorescence microscopy to show the lateral motion of dsRed-tagged clathrin parallel to the plasma membrane (Rappoport et al., 2003). The dsRed-clathrin spots clearly run on microtubule tracks, depolymerization of microtubules decreases their motility and overexpression of the microtubule-associated protein tau decreases spot run length (Rappoport et al., 2003). Whether these spots represent CCPs or pinched-off CCVs that have failed to uncoat is unclear. Regardless, these data show a clear link between endocytic membranes and microtubules. E/ANTH-bearing proteins could thus link clathrin-coated membranes to microtubules. Microtubule-mediated retrograde transport of epsin 1 from the cell surface to the nucleus could also explain the observation that epsin 1 is present at both cellular localizations. Thus, E/ANTH domains are reminiscent of other modules, such as PH domains, which bind to both inositol phospholipids and proteins (Lemmon et al., 2002). For example, the PH domain of the β-adrenergic receptor kinase binds to both PtdIns(4,5)P_2 and the G protein βγ subunit, and, in fact, simultaneous interaction with both ligands is necessary for the recruitment of the kinase to membranes (Pitcher et al., 1995). Together, these studies suggest multiple roles for E/ANTH domains as lipid- and protein-binding modules.

Enthoprotin (Clint/epsinR): an ENTH-domain-containing protein at the TGN

Until recently, the association of E/ANTH-domain-containing proteins with clathrin had been seen exclusively with CCPs and CCVs at the cell surface (Chen et al., 1998; Rosenthal et al.,

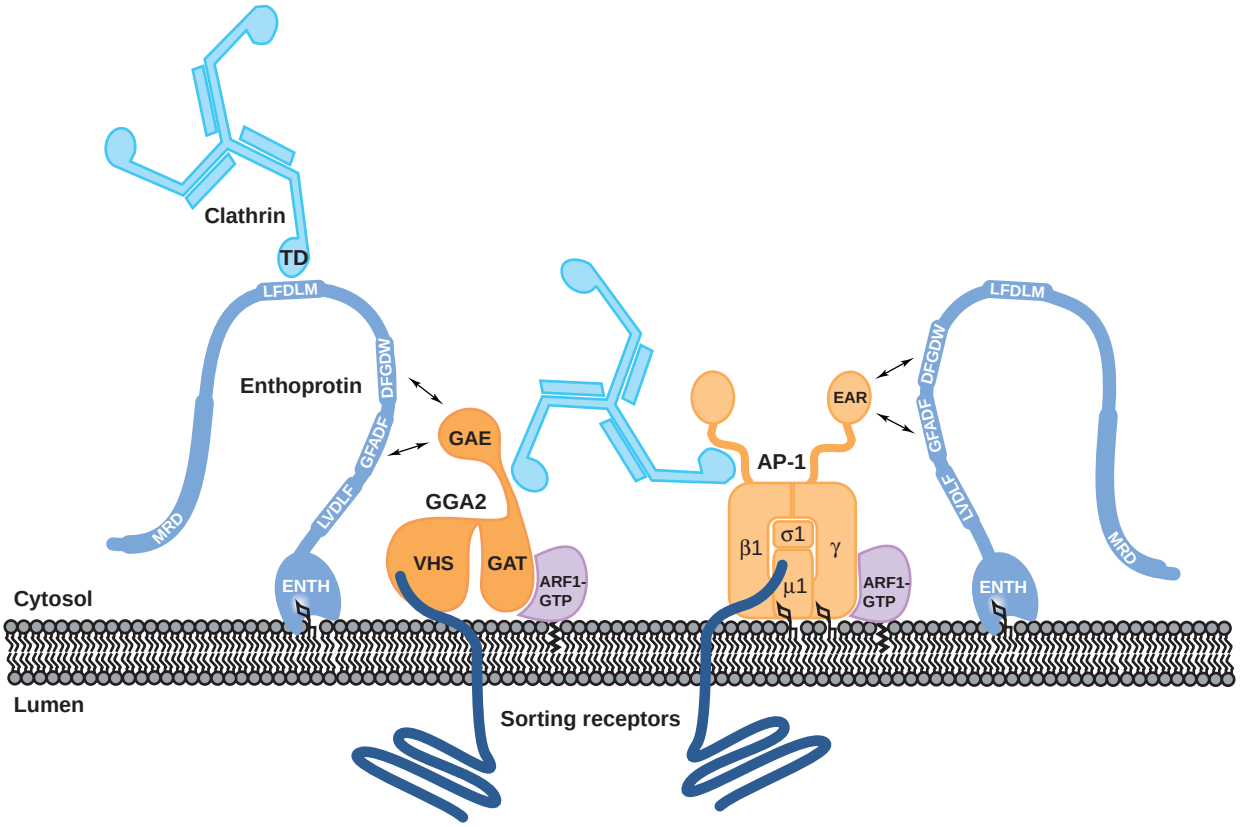

Fig. 3. Enthoprotin interactions at the TGN. TGN clathrin adaptors AP-1 and GGA2 are targeted to the membrane through interactions with ARF1-GTP, where they bind peptide motifs present in the cytoplasmic tails of sorting receptors. AP-1 is composed of four distinct subunits: γ-, β1-, μ1- and σ1-adaptin. γ- and β1-adaptin each contains a globular C-terminal region referred to as an ear domain. PtdIns4*P* binding also contributes to the membrane recruitment of AP-1. GGA2 is a monomeric adaptor containing VHS (Vps27p, Hrs, STAM), GAT (GGA and TOM) and GAE (γ-adaptin ear) domains. The ear domains are linked to the adaptors via flexible hinge domains. Both adaptors recruit clathrin triskelia to the TGN membrane via clathrin-binding motifs present in the hinge. Enthoprotin is targeted to the TGN through a combination of interactions including ENTH domain binding to PtdIns4*P* and additional interactions in the C-terminal region. Its unstructured C-terminal tail is involved in interactions with GGA2 and AP-1 through γ-ear/GAE-domain-binding motifs with the sequence GFADF and DFGDW. Enthoprotin also binds to the terminal domain (TD) of the clathrin heavy chain via clathrin-binding motifs LVDLF and LFDLM and can contribute to clathrin assembly. The ENTH domain could be involved in the production of membrane curvature through membrane insertion.

1999; Tebar et al., 1999; Wendland et al., 1999; Morgan et al., 2000; Engqvist-Goldstein et al., 2001; Metzler et al., 2001). Whether E/ANTH domains have a role at other cellular sites of clathrin-mediated budding was not known. Using a proteomics analysis of CCVs isolated from rat brain, we have recently identified several CCV proteins previously described only as predicted open reading frames from cDNA databases (Wasiak et al., 2002). One is a novel ENTH-domain-containing protein, which we named enthoprotin (Wasiak et al., 2002). Enthoprotin was independently discovered and referred to as Clint (Kalthoff et al., 2002b) and epsinR (Hirst et al., 2003; Mills et al., 2003). Enthoprotin shares little homology with any known protein outside of the ENTH domain. However, it contains two clathrin-binding domains in its C-terminal region (Fig. 1) and binds directly to clathrin (Fig. 3). Interestingly, enthoprotin also binds through its C-terminal domain to adaptor protein-1 (AP-1) and Golgi-localized γ-ear containing ADP-ribosylation factor-binding protein 2 (GGA2), clathrin adaptor proteins functioning predominantly at the TGN (Le Borgne and Hoflack, 1998; Nielsen et al., 2001; Puertollano et al., 2001; Zhu et al., 2001) (Fig. 3). Binding is mediated by the ear domain of the γ-subunit of AP-1 (γ-ear) and the γ-adaptin ear (GAE) domain of GGA2. The γ-ear and GAE domain both bind to two conserved γ-ear/GAE domain-binding motifs in the C-terminal region of enthoprotin (Mills et al., 2003; Miller et al., 2003; Duncan and Payne, 2003; Wasiak et al., 2003) (Fig. 3). Immunofluoresence analysis indicates that enthoprotin localizes primarily to the TGN with a second pool present on membranes of the endosomal system (Wasiak et al., 2002; Kalthoff et al., 2002b; Hirst et al., 2003; Mills et al., 2003). Enthoprotin therefore appears to be a new component of TGN- and endosome-derived CCVs and provides the first evidence of a role for ENTH-bearing proteins on internal membranes.

Further evidence of such a role for E/ANTH proteins has come with the identification in yeast of Ent3p and Ent5p, and ENTH- and ANTH-bearing proteins, respectively (Fig. 1), which Payne and colleagues recently identified though two-hybrid screens using Gga2p and the γ-adaptin subunit of yeast AP-1 (Duncan et al., 2003). Ent3p and Ent5p localize to the TGN and early endosomes. Interestingly, individual deletions of *ENT3* or *ENT5* do not lead to defects in clathrin-mediated protein transport, but double mutants display delayed

maturation of carboxypeptidase S (normally processed to a mature form in the vacuole) and of the α-factor mating pheromone (which depends on clathrin-mediated traffic between Golgi and endosomes) (Duncan et al., 2003). These data strongly support a role for Ent3p and Ent5p in TGN-endosomal traffic. Moreover, they show that E/ANTH protein function on intracellular membranes is conserved throughout eukaryotic evolution.

Interestingly, ENTH domains appear to form two large clusters phylogenetically (Fig. 4). One branch contains mammalian epsins and epsin homologues in *Drosophila melanogaster* and *Caenorhabditis elegans* (liquid facets) (Cadavid et al., 2000). This branch also contains the budding yeast *Saccharomyces cerevisiae* epsin orthologues Ent1p and Ent2p (Wendland et al., 1999) (Fig. 4). The second branch contains enthoprotin, its *D. melanogaster* orthologue, a *C. elegans* protein originally denoted as an epsin, and the *S. cerevisiae* protein Ent3p (Fig. 4). The fission yeast *Schizosaccharomyces pombe* has two genes that encode for ENTH-bearing proteins. One (NP_588237), which has an ENTH domain that clusters with epsins, contains a clathrin-binding domain and multiple copies of the NPF tripeptide, like epsin, Ent1p and Ent2p (Fig. 1). The second (NP_587759) clusters in the enthoprotin branch (Fig. 4) and contains a clathrin-binding domain and a putative γ-ear/GAE domain-binding motif, similar to enthoprotin (Fig. 1). Thus, there appears to be two ENTH domain families: the epsin family, which interacts through NPF motifs with EH domains and functions at the cell surface; and the enthoprotin family, which contains sequences for binding to GGAs and γ-adaptin and functions on internal membranes. Importantly, these families have been maintained in such divergent species as budding yeast, fission yeast and humans. Ent4p, which has not been well characterized, does not fit obviously into either family (Fig. 4).

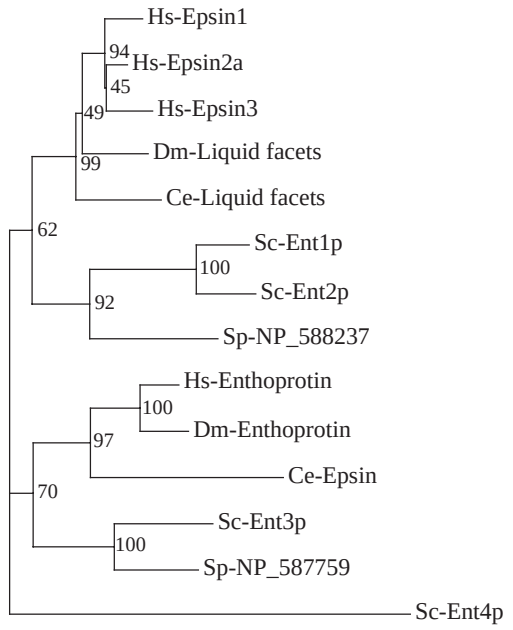

Fig. 4. Phylogenetic analysis of ENTH domains. Amino acid sequences of ENTH-bearing proteins from human, fly, worm and yeast were retrieved from the GenBank database (Hs-Epsin1, NP_037465; Hs-Epsin2a, AAC78608; Hs-Epsin3, AAG45223; Hs-Enthoprotin, DAA00062; Ce-Liquid facets, NP_510458; Ce-Epsin, NP_509973; Dm-Liquid Facets, AAF05113; Dm-Enthoprotin, AY060606; Sc-Ent1p, NP_010120; Sc-Ent2p, NP_013307; Sc-Ent3p, NP_012659; Sc-Ent4p, NP_013062; Sp-NP_587759; Sp-NP_588237). Sequences of the ENTH domains were aligned using ClustalW. The alignment was then used for phylogenetic comparisons using the PHYLIP package [J. Felsenstein, 1993, PHYLIP (Phylogeny Inference Package) 3.6a3; University of Washington, Seattle]. Analysis was performed with a bootstrap procedure that computes the probability of occurrence of the branches for 100 possible trees. Branching order was determined using the Jones-Taylor-Thornton model included in the PHYLIP package. The tree was rooted by the most divergent sequence, Sc-Ent4p, as the outgroup. Bootstrap values are shown at each node. The tree visualization was performed using TreeView 1.6.6 (Page, 1996). Hs, *Homo sapiens*; Dm, *Drosophila melanogaster*; Ce, *Caenorhabditis elegans*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*.

Enthoprotin function

The functional role of the ENTH domain of enthoprotin is unknown. In vitro, the module binds weakly to phospholipids, and modeling studies show that it contains hydrophobic residues on the outer surface of a potential α0 similar to that of epsin 1 (Mills et al., 2003). Thus, the module could contribute to membrane curvature on TGN and endosomal membranes (Fig. 3). Interestingly, constructs of enthoprotin lacking the ENTH domain concentrate on clathrin-enriched membrane fractions (Wasiak et al., 2002) and, when residues responsible for lipid interactions are mutated, full-length enthoprotin remains membrane associated (Mills et al., 2003). Thus, the C-terminal region of the protein appears to contain membrane-targeting sequences. However, ENTH-domain mutants unable to bind phospholipids relocalize from the TGN to large perinuclear puncta (Mills et al., 2003), which suggests that the ENTH domain contributes to the specificity of membrane localization. In in vitro binding assays, enthoprotin shows a slight preference for PtdIns4*P* (Hirst et al., 2003; Mills et al., 2003). Interestingly, Yin and colleagues have recently shown that PtdIns4*P* is enriched in the mammalian Golgi where it binds to AP-1 (Wang et al., 2003). RNA interference (RNAi) of PtdIns 4-kinase type IIα causes a decrease in PtdIns4*P*, decreased Golgi recruitment of AP-1 and disruption in AP-1-mediated trafficking (Wang et al., 2003). The co-recruitment of enthoprotin and AP-1 to PtdIns4*P*-rich Golgi membranes could increase the affinity of the two proteins for each other and for the phospholipid (Fig. 3). Thus, as in the case of Ent1p (Aguilar et al., 2003), a combination of protein and lipid interactions appears to be necessary to target enthoprotin to its proper location.

Fig. 3 shows a model of enthoprotin interactions at the TGN. In this model, binding of the ENTH domain to phospholipids couples with additional membrane-targeting activities in the C-terminal region, including binding to GGA2 and AP-1, to recruit the protein to the TGN. There, through interactions with AP-1, GGA2 and clathrin, enthoprotin contributes to a network of interactions that lead to the formation of CCPs. Enthoprotin has been shown to stimulate clathrin assembly in vitro at acidic pH (Wasiak et al., 2002), although assembly does not occur at physiological pH (Mills et al., 2003). This situation is similar

to that seen for AP180 (Morgan et al., 2003). However, AP180 does assemble clathrin at physiological pH following binding to Eps15 (Morgan et al., 2003). In an analogous manner, enthoprotin could stimulate clathrin assembly following its binding to one of its TGN partners (Fig. 3). In this way, enthoprotin could stimulate the formation of CCPs on the TGN in proximity to GGAs and AP-1, which bind to the cytoplasmic domains of TGN sorting receptors, allowing for the concentration of the receptors in nascent CCPs. Indeed, overexpression of full-length enthoprotin in COS cells leads to abnormal secretion of the lysosomal hydrolase pro-cathepsin D, which is normally transported in CCVs from the TGN to the late endosome and lysosome (Mills et al., 2003). Surprisingly, however, depletion of enthoprotin by RNAi in COS and HeLa cells does not alter the trafficking of cathepsin D (Hirst et al., 2003), which suggests that enthoprotin is not absolutely required for this trafficking pathway. Enthoprotin may therefore function in AP-1 and GGA-dependent pathways that do not involve transport of lysosomal hydrolases. More experiments will be needed to determine how enthoprotin contributes to TGN-endosomal trafficking. Interestingly, disruption of the gene encoding enthoprotin is pupal lethal in *D. melanogaster* (P. A. Leventis, S.W., P.S.M. and G. L. Boulianne, unpublished). Analysis of trafficking defects in mutant larvae should resolve this issue and provide new insights on enthoprotin function.

Concluding remarks

Significant strides have been made in the functional characterization of E/ANTH domains in the four years since their discovery. It is clear that E/ANTH-bearing proteins play a multi-faceted role in the formation of CCPs and CCVs. Through phospholipid binding, they help to bridge components of clathrin coats to membranes and, through their assembly activity, they contribute to the formation of clathrin cages. Moreover, they also appear to function as adaptor proteins, each E/ANTH protein being involved in the recruitment of specific classes of cargo into nascent CCPs. Through alterations in membrane phospholipids, ENTH domains can also contribute to the development of membrane curvature. Finally, E/ANTH domains appear to contribute to the association of endocytic vesicles with the microtubule cytoskeleton. Further analysis of E/ANTH proteins using genetic models in yeast, flies, worms and mammals should greatly increase our understanding of these unique protein modules in the coming years.

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