Yeast epsin-related proteins required for Golgi–endosome traffic define a γ-adaptin ear-binding motif

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Clathrin-coated vesicles (CCVs) are a central component of endocytosis and traffic between the *trans*-Golgi network (TGN) and endosomes. Although endocytic CCV formation is well characterized, much less is known about CCV formation at internal membranes. Here we describe two epsin amino-terminal homology (ENTH) domain-containing proteins, Ent3p and Ent5p, that are intimately involved in clathrin function at the Golgi. Both proteins associate with the clathrin adaptor Gga2p *in vivo*; Ent5p also interacts with the clathrin adaptor complex AP-1 and clathrin. A novel, conserved motif that mediates the interaction of Ent3p and Ent5p with γ-ear domains of Gga2p and AP-1 is defined. Ent3p and Ent5p colocalize with clathrin, and cells lacking both Ent proteins exhibit defects in clathrin localization and traffic between the Golgi and endosomes. The findings suggest that Ent3p and Ent5p constitute a functionally related pair that co-operate with Gga proteins and AP-1 to recruit clathrin and promote formation of clathrin coats at the Golgi/endosomes. On the basis of our results and the established roles of epsin and epsin-related proteins in endocytosis, we propose that ENTH-domain-containing proteins are a universal component of CCV formation.

Clathrin and clathrin adaptors are major structural components of vesicular clathrin coats. In current models, clathrin adaptors are central to the formation of CCVs, nucleating coat assembly by binding to appropriate membranes and recruiting vesicle cargo, clathrin and other coat-associated proteins[1]. A well-characterized example is the clathrin adaptor complex AP-2, which functions in endocytic CCV formation. Many important functions of AP-2 are mediated by the carboxy-terminal 'ear' domain of the α-adaptin subunit, which binds a host of accessory proteins that participate in coat formation, membrane deformation and vesicle release. Among the ear-interacting proteins are two proteins, AP180 and Epsin1, that also bind to clathrin and contain ENTH domains[2–4]. Although structurally related, the ENTH domains of Epsin1 and AP180 bind to phosphoinositides through different regions, perhaps signifying unique functions[5–7]. Indeed, the ENTH domain of Epsin1 stimulates deformation of liposomes and lipid monolayers, whereas the ENTH domain of AP180 does not[7]. Through their ability to bind AP-2, clathrin and phosphoinositides at the plasma membrane, the ENTH domain proteins have been proposed to co-operate with AP-2 in initiating endocytic CCV formation[5,8].

Although CCVs are important transport carriers between the TGN and endosomes, few accessory proteins have been identified for these vesicles[9–13]. Two different types of adaptor — AP-1 and Gga proteins — have been implicated in CCV formation at the TGN and endosomes. AP-1 is composed of subunits that are homologous to AP-2. Gga proteins are monomeric adaptors that contain a C-terminal domain related to the ear domain of AP-1
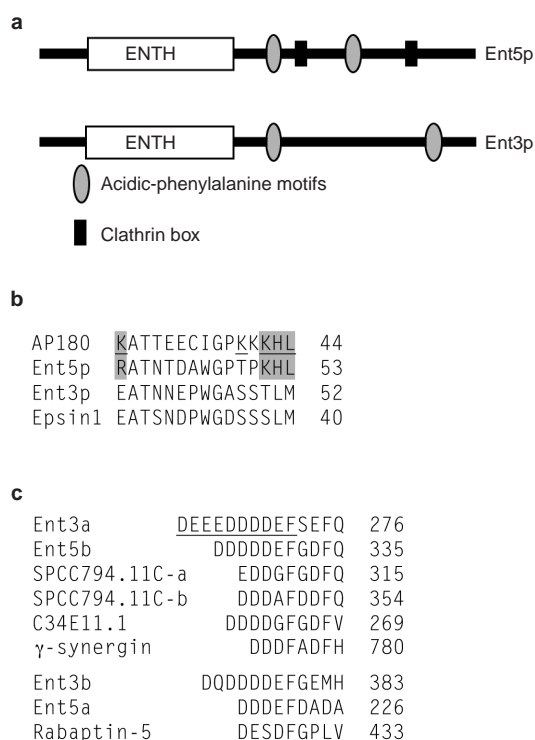

Figure 1 Ent3p and Ent5p represent a conserved family of ENTH-domain-containing proteins. a, Diagrams of Ent3p and Ent5p showing the relative position of ENTH domains, acidic-phenylalanine motifs and clathrin-binding motifs (clathrin box). **b**, Alignment of the AP180 phosphoinositide-binding region with homologous sequences in Ent5p, Ent3p and Epsin1. Residues important for phosphoinositide binding by AP180 are underlined and those residues that are conserved in Ent5p are boxed. **c**, Alignment of acidic-phenylalanine motifs from different species. For Ent3p, Ent5p and SPCC794.11C, the two independent motifs in each protein are labeled a and b, with motif a located closer to the amino terminus. For γ-synergin, one of the eight motifs in the protein is shown; five such motifs occur in the region of γ-synergin shown to bind to Ggas and AP-1. The smallest γ-ear-interacting region of Ent3p is underlined.
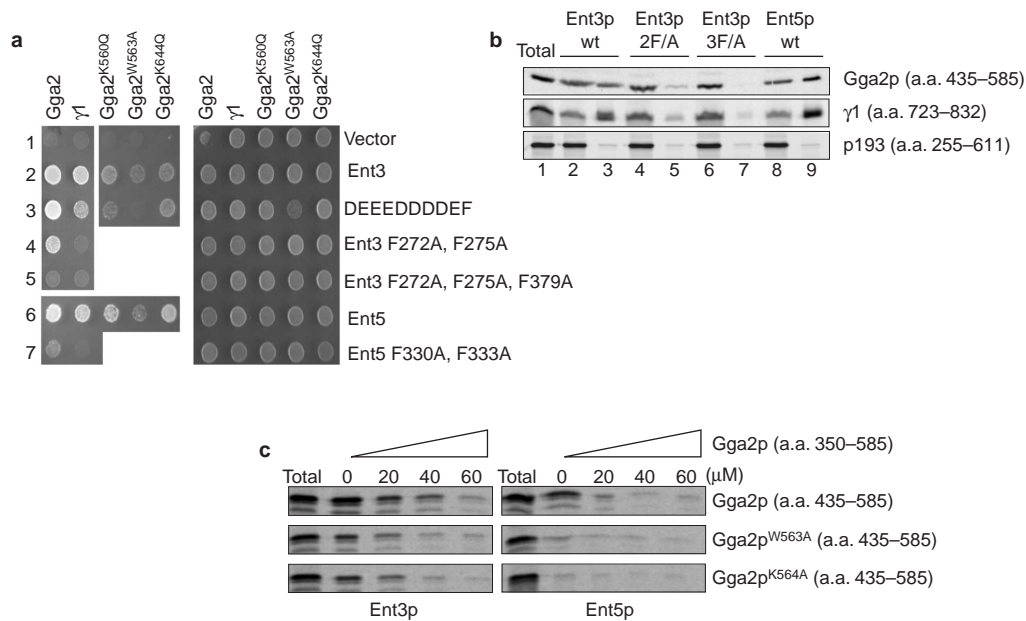
Figure 2 Ent3p and Ent5p interact with the ear domains of Gga2p and AP-1-γ. a, The growth of cells containing the indicated DNA-binding fusions to Gga2p (amino acids 435–585) or γ1 (Apl4p, amino acids 723–832) and activation-domain fusions to full-length Ent3p, Ent5p and the acidic-phenylalanine motif. Equal cell densities were spotted on media that allowed growth with (left) or without (right) interaction. **b**, *In-vitro*-translated T7-Gga2p (amino acids 435–585), T7-γ1 (Apl4p, amino acids 723–832) or T7-p193 (amino acids 255–611) were incubated with glutathione–agarose beads bound to GST–Ent3p, GST–Ent5p or the indicated mutants. Beads were pelleted and supernatant fractions removed. Bound proteins were eluted with SDS sample buffer. Equal aliquots of the total starting sample, supernatant (lanes 2, 4, 6 and 8) and bound fraction (lanes 3, 5, 7 and 9) were analysed by SDS–polyacrylamide gel electrophoresis (PAGE) and autoradiography. **c**, *In-vitro*-translated T7-Gga2p (amino acids 435–585) or the indicated mutants were incubated with glutathione–agarose beads bound to GST–Ent3p or GST–Ent5p and indicated concentrations of unlabeled Gga2p (amino acids 350–585). Total and bound fractions were analysed by SDS–PAGE and autoradiography.

γ-adaptin[9,14,15]. By analogy with α-adaptin, the γ-ear domains of AP-1 and Gga proteins probably recruit accessory proteins. However, key structural differences between γ- and α-ear domains suggest that the spectra of accessory proteins involved in TGN and endocytic CCV formation may be distinct[10,16–19]. In particular, the α-ear contains a major site for binding to AP180, Epsin1 and other accessory molecules in a C-terminal platform that is missing from AP-1 and Gga γ-ears.

To identify molecules that co-operate with clathrin adaptors at the Golgi and endosomes, we performed yeast two-hybrid screens with the γ-ear domains of *Saccharomyces cerevisiae* γ-adaptin (Apl4p) and Gga2p. We identified two uncharacterized yeast ENTH domain proteins, Ydr153cp (renamed Ent5p) and Ent3p, with striking similarity to one another (Fig. 1a). The two-hybrid interactions of Ent3p and Ent5p with the γ-ear domains of Gga2p and γ-adaptin are presented in Fig. 2a. In addition, glutathione *S*-transferase (GST)–Ent3p and GST–Ent5p fusion proteins bound to Gga2p- and γ-adaptin-ears (Fig. 2b), and to Gga2p and AP-1 in yeast cell extracts (data not shown), but not to an unrelated *in vitro* translated protein (Fig. 2b). Notably, the ENTH domain of Ent5p contains several basic residues that are important for binding of phosphoinositides by AP180 (Fig. 1b). However, these basic residues are missing from Ent3p and Epsin1 (refs 5, 6). This relationship may indicate that Ent3p and Ent5p ENTH domains have distinct functions, perhaps analogous to AP180 and Epsin1 ENTH domains. Ent3p and Ent5p lack known ubiquitin-interacting motifs, as well as multiple copies of the AP-2-binding motif, Asp-Pro-Phe/Trp, characteristic of endocytic ENTH domain proteins[3].

Functional epitope-tagged versions of Ent3p and Ent5p (expressed from the native chromosomal loci) associated with Gga2p *in vivo*, as assessed by co-immunoprecipitation (Fig. 3a). However, other yeast AP complexes, AP-2 and AP-3, were not co-immunoprecipitated, suggesting Ent3p and Ent5p interact specifically with γ-ear-containing adaptors. Interestingly, only Ent5p co-immunoprecipitated AP-1, although Ent3p interacted with the γ-adaptin-ear in two-hybrid and *in vitro* binding assays (Fig. 2a, b), and with AP-1 by GST fusion protein affinity chromatography (data not shown). Thus, although Ent3p and AP-1 can bind *in vitro*, *in vivo* they may interact transiently or not at all. Ent5p, but not Ent3p, contains two sequences that match a consensus for clathrin-binding motifs[20]. Consistent with this difference, only Ent5p bound to clathrin in co-immunoprecipitation experiments (Fig. 3a) and GST–Ent5p affinity chromatography analysis (data not shown). This binding is probably direct, as Ent3p does not bind to clathrin, despite binding to Gga2p by co-immunoprecipitation and both Gga2p and AP-1 in affinity chromatography analysis.

In yeast, immunofluorescence microscopy has shown that clathrin localizes primarily to large punctae that are thought to represent the TGN and/or early endosomes[21]. Ent3p and Ent5p were distributed in foci that were coincident with these large clathrin punctae (Fig. 3b), supporting an association with clathrin coats *in vivo*. Diffuse staining of Ent3p and Ent5p throughout the cell probably represents soluble cytoplasmic pools, similar to other coat components. Taken together, our results suggest that Ent3p and Ent5p are novel, γ-ear-interacting proteins associated with TGN/endosome clathrin coats.

Ent3p and Ent5p each contain two highly acidic stretches followed by phenylalanines (Fig. 1a). One such motif from Ent3p was the smallest clone isolated in the two-hybrid screen (Fig. 2a, underlined sequence), suggesting that these residues are sufficient to interact with γ-ears. Such sequences are missing from other yeast ENTH domain proteins, but are present in uncharacterized ENTH domain proteins from *Schizosaccharomyces pombe* and
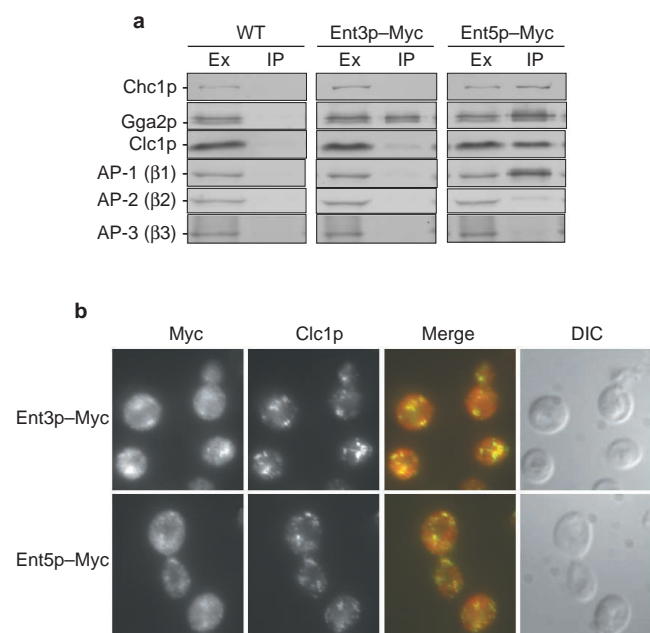
Figure 3 *In vivo* interactions of Ent3p and Ent5p with clathrin and adaptors.
a, Extracts (Ex) from cells expressing untagged Ent proteins (WT), Ent3p-Myc or Ent5p-Myc underwent non-denaturing immunoprecipitations (IP) with anti-Myc tag antibodies. Samples were then analysed by SDS–PAGE and immunoblotting with antibodies against clathrin heavy chain (Chc1p), Gga2p, clathrin light chain (Clc1p) or the β-subunits of AP-1 (β1), AP-2 (β2) or AP-3 (β3). Extract lanes contain 1% of samples used for immunoprecipitation. **b**, Cells expressing Ent3p–Myc or Ent5p–Myc were labelled with antibodies to the Myc tag and to clathrin light chain before visualization. Differential interference microscopy (DIC) images are also shown. In the merged images, Ent–Myc is shown in red and Clc1p is shown in green.

Caenorhabditis elegans, suggesting that Ent3p and Ent5p comprise the founding members of a new sub-family of ENTH proteins (Fig. 1c). Alignment of these sequences reveals a tendency for a second phenylalanine at residue +3 from the invariant phenylalanine. Similar sequences are found in mammalian γ-ear-interacting proteins; once in rabaptin-5 and five times in the region of γ-synergin that interacts with mammalian γ-ears[11] (Fig. 1c). The sequences in γ-synergin were previously suggested as possible sites for interaction with γ-ears[11,19]. The two-hybrid interactions of the Ent3p acidic-phenylalanine sequence with Gga2p and γ-ear domains provides evidence for this possibility.

Ent3p contains two acidic-phenylalanine motifs (Fig. 1c), with either a phenylalanine (motif a) or a methionine (motif b) at position +3 relative to the invariant phenylalanine. Mutation of all three phenylalanines to alanines (F272A, F275A and F379A) in the two motifs reduced binding to background levels, as measured by two-hybrid analysis (Fig. 2a, row 5) and by binding of GST–fusions to *in-vitro*-translated Gga2p γ-ear (Fig. 2b). These results indicate that the motifs are necessary for γ-ear binding. Mutation of both phenylalanines in motif a (F272A, F275A) was sufficient to substantially reduce binding to the AP-1 γ-ear and slightly reduce binding to the Gga2p γ-ear in the two-hybrid assay (Fig. 2a, row 4). The effect of Ent3p F272A, F275A mutations on Gga2p binding was more evident when assayed by *in vitro* binding (Fig. 2b). Mutation of the single phenylalanine of motif b reduced binding to γ-ears only modestly in the two-hybrid assay (data not shown). Ent5p also contains two acidic-phenylalanine motifs (Fig. 1c), one containing a phenylalanine at +3 from the invariant phenylalanine (motif b). Mutations F330A and F333A in motif b markedly reduced binding

to Gga2p and γ-adaptin-ears in the two-hybrid assay (Fig. 2a, row 7). Together, these results suggest that a hydrophobic residue at the +3 position, particularly phenylalanine, favours binding to γ-ears. On the basis of these results and sequence alignments, we propose that the acidic-phenylalanine motif $(Asp/Glu)_{2-3}$-Phe-X-X-Φ (where X is any amino acid and Φ is a hydrophobic residue) represents a conserved γ-ear interaction consensus motif.

Two recent reports proposed different sites on the γ-adaptin-ear as the docking site for γ-synergin[10,19]. One site, corresponding to residues 557–566 of the yeast Gga2p γ-ear, forms a basic surface-exposed strip with two conspicuous hydrophobic pockets. Because of the acidic nature of the interaction motif, we considered this basic surface as a promising candidate site for motif binding. Accordingly, we generated mutations in this region of Gga2p that were analogous to mutations in the γ-adaptin-ear reported to reduce γ-synergin binding[19]. As measured in the two-hybrid assay, the K560Q, W563A and K564Q mutations each affected binding to full-length Ent proteins and the minimal Ent3p binding motif (Fig. 2a, rows 2, 3 and 5). The K560Q and W563A mutants had stronger effects on motif binding than on binding to full-length Ent3p. When assayed by *in vitro* binding, the Gga2p W563A and K564Q mutations markedly reduced binding to Ent5p (Fig. 2c). These mutations also reduced binding to GST–Ent3p, an effect more clearly demonstrated using unlabelled wild-type Gga2p γ-ear as a competitor (Fig. 2c). Our results support the hypothesis that the conserved basic surface (residues 557–566) is a primary interaction site for the acidic-phenylalanine motif, but leave open the possibility of additional interaction surfaces between Ent3p and γ-ears.

Deletion of either *ENT3* or *ENT5* alone resulted in no detectable defect in the subcellular localization of clathrin (Fig. 4a). However, deletion of both genes substantially reduced punctate clathrin staining. Localization of the Golgi membrane protein Gda1p was not affected in double mutants, indicating that the mutations do not have a general effect on Golgi structure. These results suggest a role for Ent3p and Ent5p in clathrin assembly on TGN/endosome membranes.

Consistent with defective clathrin localization, *ent3Δ ent5Δ* cells displayed defects in clathrin-dependent protein transport. In wild-type cells, carboxypeptidase S (CPS) is transported as a precursor from the TGN to the vacuole, where it is processed to a smaller active form[22]. In cells lacking clathrin, or different combinations of Gga proteins and AP-1, maturation of CPS is delayed[23]. Similarly, pulse–chase immunoprecipitation experiments demonstrated that maturation of CPS was substantially delayed in *ent3Δ ent5Δ* cells; the half-time of CPS maturation was greater than 20 min and precursor was still apparent after 60 min (Fig. 4b). In contrast, CPS was fully processed in single mutants or wild-type cells by 20 min. We also assayed maturation of α-factor mating pheromone, which is initiated by the TGN protease Kex2p. The localization of Kex2p is dependent on clathrin-dependent cycling between the TGN and endosomes, and defects in Kex2p localization reduce the efficiency of α-factor maturation[24]. Pheromone maturation was considerably reduced in *ent3Δ ent5Δ* cells, compared with single-mutant or wild-type cells (Fig. 4c). The extent of the defect resembled that in cells lacking Gga2p and AP-1 (ref. 23). Importantly, *ent3Δ ent5Δ* cells exhibited only a marginal delay in the clathrin-independent transport of precursor alkaline phosphatase to the vacuole (Fig. 4d) and no delay in internalization of the lipophilic dye FM4-64 (data not shown). These data support a specific role for both Ent3p and Ent5p in clathrin localization and in traffic between the TGN and endosomes. The fact that deletion of *ENT3* or *ENT5* alone is innocuous suggests that Ent3p and Ent5p are at least partially redundant, consistent with their shared domains and motifs. The role of ENTH domain proteins in TGN/endosome CCV formation is probably evolutionarily conserved, considering the recent identification of a mammalian ENTH domain protein that localizes to the TGN and endosomes[12,13].
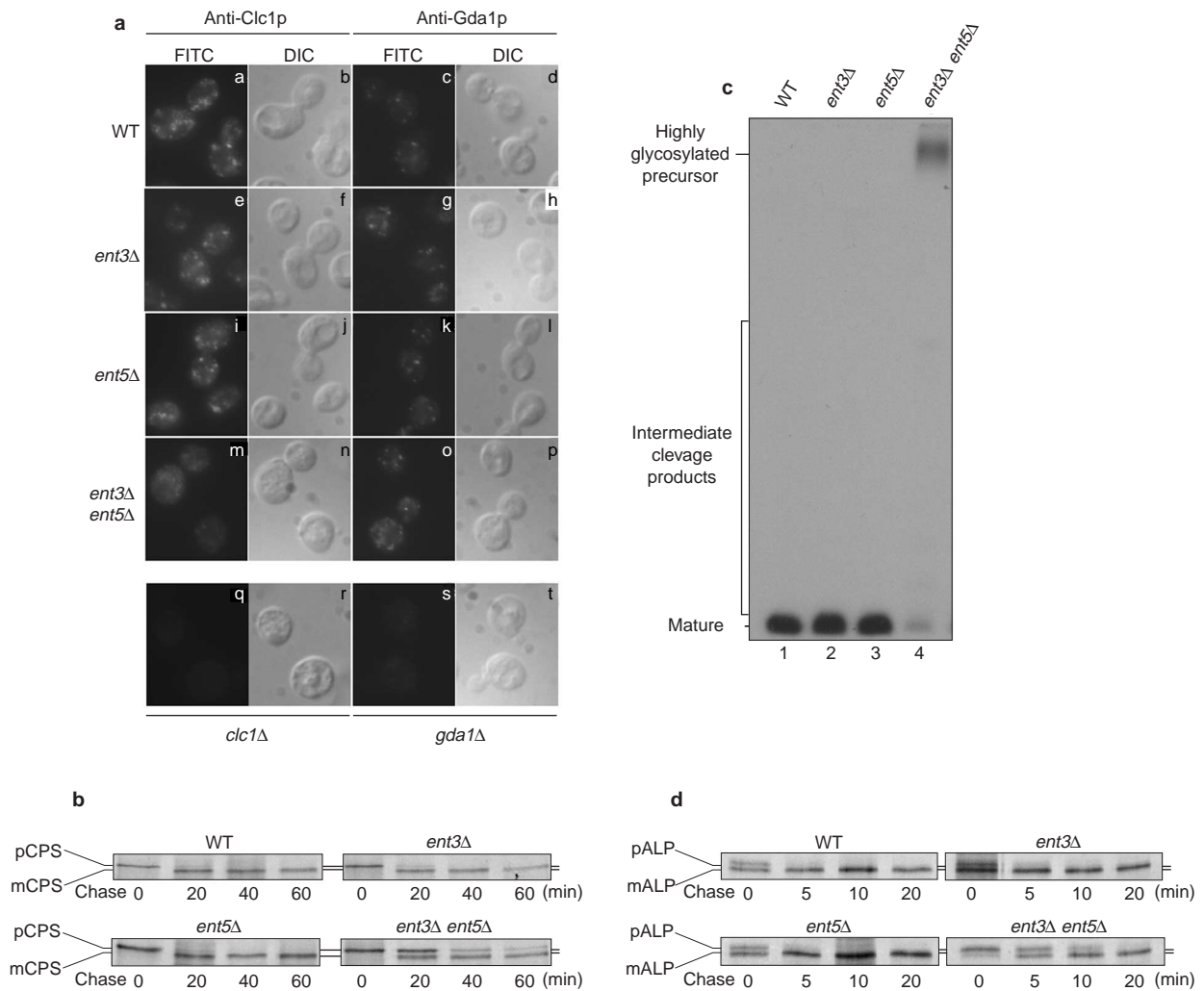
Figure 4 Clathrin defects in *ent3Δ ent5Δ* cells. a, Immunofluorescence microscopy images of wild-type (WT), *ent3Δ*, *ent5Δ*, *ent3Δ ent5Δ*, *clc1Δ* and *gda1Δ* cells (ref. 30) labelled with antibodies to Clc1p or the Golgi membrane protein Gda1p. **b**, Cells with the indicated genotype were metabolically labelled for 10 min at 30 °C and chased for the indicated times. Precursor (pCPS) and mature (mCPS) CPS were immunoprecipitated from cell extracts and analysed by SDS–PAGE. **c**, Cells were metabolically labelled for 30 min at 30 °C, and secreted α-factor was immunoprecipitated and analysed by SDS–PAGE. **d**, Cells were subjected to pulse–chase immunoprecipitation, as in **b**, with antibodies to alkaline phosphatase to detect precursor (pALP) and mature (mALP) forms of ALP.

Our results indicate that the epsin-related proteins Ent3p and Ent5p interact with Gga2p *in vitro* and *in vivo*. Ent5p also associates with clathrin and AP-1 *in vivo*, and both proteins have subcellular distributions that are consistent with localization to clathrin coats at the TGN and/or endosomes. Importantly, we have defined a γ-ear interaction motif. Similar sequences are found in the mammalian γ-ear-interacting proteins γ-synergin and rabaptin-5, making it probable that the structure and function of this motif are conserved from yeast to mammals. Our mutagenesis results also suggest that the motif-binding site on the γ-ear is similarly conserved. We note that several yeast proteins involved in TGN to endosome traffic, including Vps3p and Vps72p, contain such motifs[25,26]. Therefore, these proteins are candidates to interact with AP-1 and/or Ggas. Identification and mutagenesis of acidic-phenylalanine motifs should also promote the discovery and characterization of Gga- and AP-1-interacting proteins in mammalian cells.

Deletion of both *ENT3* and *ENT5* results in defects in clathrin localization and clathrin-dependent trafficking between the TGN and endosomes. Thus, Ent3p and Ent5p seem to be functional accessory factors for CCV formation and function at the TGN/endosomes. Although the phenotypes of mutant cells suggest that there is some redundancy between Ent3p and Ent5p, it may be significant that the two proteins differ in their ability to interact with AP-1 and clathrin, as well as in potential phosphoinositide-binding sites in the ENTH domains. We therefore propose that together, Ent3p and Ent5p supply activities that constitute a universal requirement for ENTH-domain-containing proteins in CCV formation. Our analyses suggest that these functions allow ENTH-domain proteins to co-operate with clathrin adaptors to recruit clathrin and drive the assembly of coated vesicles.

Methods

Strains and Plasmids

Protein purification and protein assays

In-vitro-translated products were produced with the TnT Quick Coupled System (Promega, Madison, WI) according to manufacturer's instructions. GST fusion proteins were purified according to pGEX purification manual (Pharmacia). For competition assays, GST–Gga2p (amino acids 350–585) bound to glutathione–agarose was cleaved using thrombin, which was then inactivated by the addition of phenylmethyl sulphonyl fluoride (Sigma, St Louis, MO). For binding assays, 5 μl of a 50% glutathione–agarose slurry, with or without GST-tagged Ent3p or Ent5p, was incubated with 2.5 μl of *in vitro* translation product in a total of 30 μl HEKG5 buffer (20 mM Hepes at pH 7.5, 1 mM EDTA, 50 mM potassium chloride and 5% glycerol) for 40 min. Beads were washed twice with 150 μl HEKG5 and bound proteins eluted with SDS sample buffer.

Microscopy and protein transport assays

Immunofluorescence microscopy was performed as previously described[30], except that for double-labelling, cells were incubated with anti-Myc monoclonal antibodies (Ab-1, Calbiochem, San Diego, CA) and polyclonal antibodies to clathrin light chain. Secondary antibodies were fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG and Texas Red-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR). Images were captured on a Zeiss Axiovert 200M (Zeiss, Hallbegmoos, Germany) with a Hamamatsu ORCA-ER camera (Hamamatsu, Hamamatsu City, Japan). Co-immunoprecipitations and pulse–chase immunoprecipitations were performed as described[23]

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