The ENTH and C-terminal domains of *Dictyostelium* epsin cooperate to regulate the dynamic interaction with clathrin-coated pits

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Accepted 21 July 2008
Journal of Cell Science 121, 3433-3444 Published by The Company of Biologists 2008
doi:10.1242/jcs.032573

Summary

Epsin contains a phospholipid-binding ENTH domain coupled to C-terminal domain motifs that bind coated pit proteins. We examined how these domains interact to influence epsin function and localization in *Dictyostelium*. Although not required for global clathrin function, epsin was essential for constructing oval spores during development. Within the epsin protein, we found that features important for essential function were distinct from features targeting epsin to clathrin-coated pits. On its own, the phospholipid-binding ENTH domain could rescue the epsin-null phenotype. Although necessary and sufficient for function, the isolated ENTH domain was not targeted within clathrin-coated pits. The C-terminal domain containing the coated-pit motif was also insufficient, highlighting a requirement for both domains for targeting to coated pits. Replacement of the ENTH domain by an alternative membrane-binding domain resulted in epsin that sequestered clathrin and AP2 and ablated clathrin function, supporting a modulatory role for the ENTH domain. Within the ENTH domain, residues important for PtdIns(4,5)P_2 binding were essential for both epsin localization and function, whereas residue T107 was essential for function but not coated pit localization. Our results support a model where the ENTH domain coordinates with the clathrin-binding C-terminal domain to allow a dynamic interaction of epsin with coated pits.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/121/??/????/DC1

Key words: ENTH, Membrane traffic, Clathrin, Spore development, Cytokinesis, PtdIns(4,5)P_2, *Dictyostelium discoideum*

Introduction

Clathrin-mediated endocytosis is a highly conserved process in which specific cargo on the plasma membrane is selected and internalized. Clathrin triskelia, key structural proteins of this process, are recruited to the membrane and assemble into coated pits that encompass endocytic cargo. These pits subsequently pinch off to form intracellular clathrin-coated vesicles. A wide variety of adaptors and accessory proteins select appropriate cargo and help recruit clathrin to the membrane. Epsin is one such clathrin adaptor.

First identified as a binding partner of epidermal growth factor receptor substrate 15 (Eps15) (Chen et al., 1998), epsin is thought to contribute to coated vesicle function in eukaryotic cells. Members of the epsin family share a similar structure. At the N-terminus, epsin contains an ENTH domain (epsin N-terminal homology) that binds specifically to the lipid PtdIns(4,5)P_2 (Itoh et al., 2001). At the C-terminus, epsin contains several short binding motifs specific for clathrin and clathrin adaptors such as assembly protein AP2 and Eps15-homology (EH)-domain proteins (Chen et al., 1998; Kay et al., 1999). Accordingly, mammalian epsin coprecipitates with clathrin, AP2 and Eps15 in vitro (Chen et al., 1998; Owen et al., 1999; Traub et al., 1999) and colocalizes with clathrin and various endocytic adaptors in vivo (Chen et al., 1998; Drake et al., 2000; Newpher et al., 2005). Epsin promotes clathrin assembly (Ford et al., 2002; Kalthoff et al., 2002a) and is present, but not necessarily enriched, in purified clathrin-coated vesicles (Chen et al., 1998; Hawryluk et al., 2006). Epsins from different species also contain one or more ubiquitin-interacting motifs (UIMs) that interact with ubiquitylated cargo (Hofmann and Falquet, 2001; Polo et al., 2002; Aguilar et al., 2003; Barriere et al., 2006).

This modular organization suggests a model where the C-terminus of epsin acts as a scaffold for clathrin, clathrin adaptors and specific cargo, whereas the N-terminal ENTH domain tethers and promotes invagination of the coated pit from the plasma membrane. However, how these modules cooperate to facilitate epsin function in living cells remains unclear. Furthermore, domain analysis of fly and yeast epsin has led to the puzzling result that expression of solely the ENTH domain rescues phenotypic deficiencies in these organisms, suggesting that the C-terminus is dispensable (Wendland et al., 1999; Aguilar et al., 2003; Overstreet et al., 2003). The capacity of the isolated ENTH domain to function raises questions about what functional properties the C-terminal domain contributes to epsin.

Dictyostelium discoideum cells offer a model system where clathrin-coated pits associate with the plasma membrane, and clathrin is essential for important biological roles (O'Halloran and Anderson, 1992; Damer and O'Halloran, 2000; Wang et al., 2006). Moreover, *Dictyostelium* cells contain conserved adaptors that associate with clathrin on the plasma membrane (Stavrou and O'Halloran, 2006; Wang et al., 2006; Repass et al., 2007). In this study, we identified the *Dictyostelium* epsin ortholog epnA and found that it plays an essential role in spore development. In addition, our analysis highlighted separate and distinct contributions of the ENTH domain and the C-terminal domain to the localization and to the functional capacity of epsin. We conclude that the ENTH

domain cooperates with the C-terminal domain of epsin to facilitate a dynamic interaction with clathrin-coated pits at the plasma membrane.

Results

Identification of *Dictyostelium* epsin

By searching for genes that shared amino acid sequences similar to the ENTH domain of human Epsin-1 (EPN1), we identified the *Dictyostelium discoideum* ortholog of epsin from the *Dictyostelium* genome database (see Materials and Methods). We identified a single gene, which we named *epnA*, with high amino acid sequence identity (48%) to the Epsin-1 ENTH domain. This was the sole gene that contained an ENTH domain. From this we concluded that *Dictyostelium* contains a single gene for epsin. The predicted amino acid sequence for *epnA* contained multiple short binding motifs for other endocytic adaptors (Fig. 1A), consistent with epsin from other species (Salcini et al., 1997; Chen et al., 1998; Kay et al., 1999; Traub et al., 1999; Cadavid et al., 2000). In addition, *Dictyostelium* epsin also contained two Type I L(L,I)(D,E,N)(L,F)(D,E,S) clathrin-binding motifs (Dell'Angelica et al., 1998; Drake et al., 2000; ter Haar et al., 2000) (Fig. 1A). However, unlike most epsins in other species, the predicted amino acid sequence for *Dictyostelium* epsin did not contain a UIM (Hofmann and Falquet, 2001; Polo et al., 2002; Aguilar et al., 2003; Barriere et al., 2006). In this respect, *Dictyostelium* epsin was similar to *Arabadopsis* epsin, which also lacks a UIM (Holstein and Oliviusson, 2005). To confirm the ability of *Dictyostelium* epsin to bind to clathrin, we performed a pull-down binding assay. Bacterially expressed maltose-binding protein (MBP):epsin fusion protein was bound to amylose resin and incubated with *Dictyostelium* cell lysate. Analysis of the bound and unbound fractions revealed that clathrin sedimented with MBP:epsin, but not MBP alone (Fig. 1B). Under these conditions, we were not able to detect binding between epsin and AP2 (Fig. 1B).

To determine the cellular location of *Dictyostelium* epsin, we cloned a cDNA for *epnA* fused to GFP (green fluorescent protein) and expressed this epsin:GFP fusion construct in a wild-type background. As with mammalian epsins (Chen et al., 1998), *Dictyostelium* epsin showed a punctate distribution largely restricted to the plasma membrane, with some intracellular puncta (Fig. 1C). The epsin puncta colocalized with clathrin on the plasma membrane and also with intracellular clathrin puncta (Fig. 1D; Fig. 4C). *Dictyostelium* epsin puncta also colocalized extensively with AP2 at the plasma membrane (Fig. 1E; Fig. 4B). Thus both the domains and localization of *Dictyostelium* epsin are similar to epsins from other organisms.

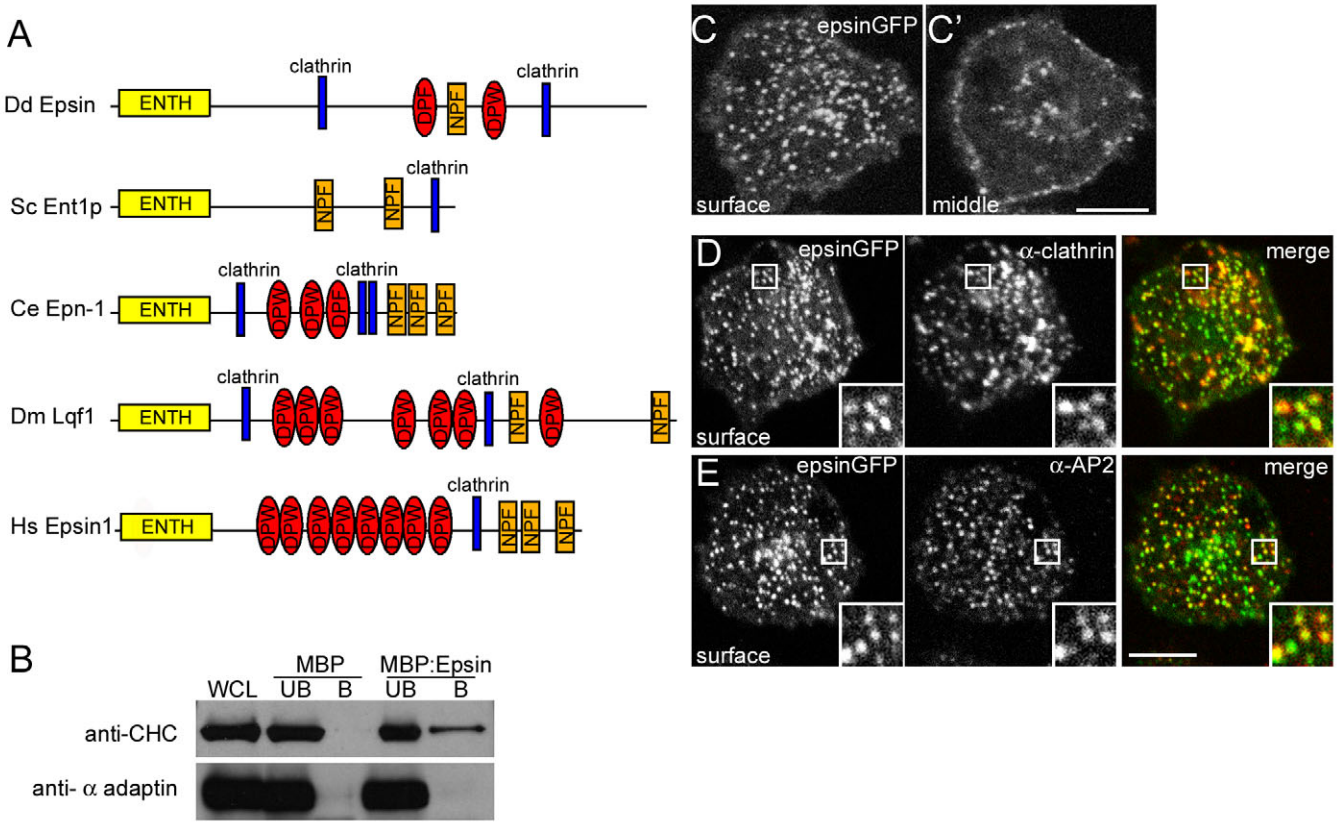

Fig. 1. *Dictyostelium* epsin. (A) Schematic representation of the structural organization of epsin from *Dictyostelium* (Dd), *S. cerevisae* (Sc), *C. elegans* (Ce), *Drosophila* (Dm) and *Homo sapiens* (Hs). Each has a membrane-binding ENTH domain (yellow box) as well as an unstructured C-terminal region containing motifs for binding clathrin (blue boxes), a DPF/DPW motif that binds AP2 (red oval) and an NPF for binding EH-domain-containing proteins. (B) *Dictyostelium* epsin binds clathrin. Amylose resin coupled to maltose binding protein (MBP) or to MBP:epsin was incubated with *Dictyostelium* lysate. Whole cell lysates (WCL) and fractions that did not bind (Unbound; UB) or that did bind (Bound; B) to the resin were immunoblotted for clathrin (anti-CHC) or the α-adaptin subunit of AP2 (α-AP2). (C) Confocal images (surface focal plane) of a wild-type cell expressing epsin:GFP. (C') Confocal image (middle focal plane) from the same cell. (D,E) Epsin colocalizes extensively with clathrin and AP2. Confocal images (surface focal planes) from cells expressing epsin:GFP (green) and immunostained for clathrin (D) or AP2 (E, red). Scale bars: 5 μm.

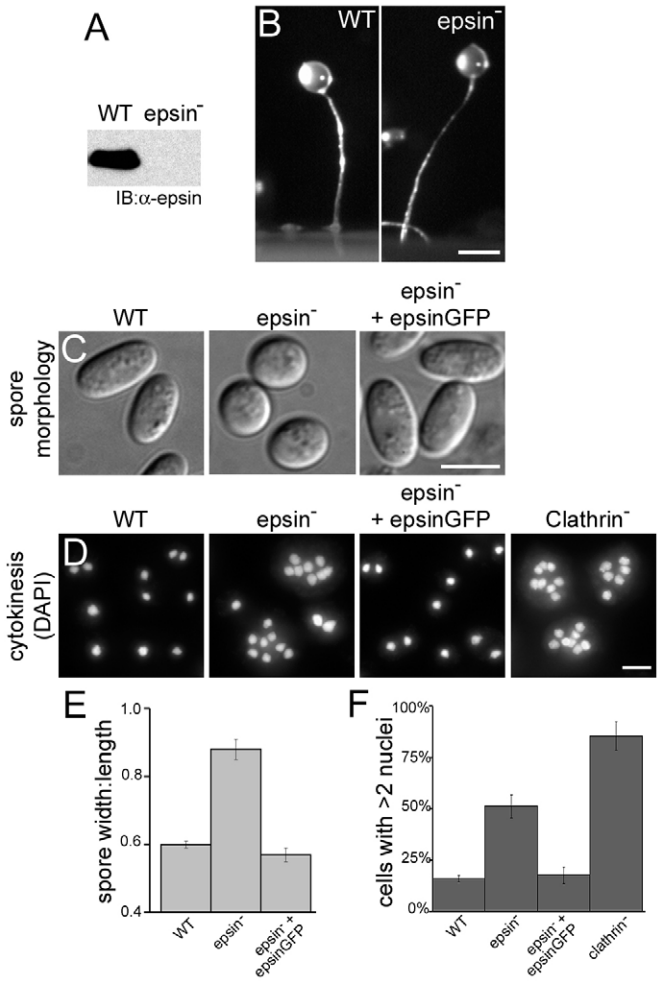

Fig. 2. Epsin-null mutants display defects in cytokinesis and spore morphology. (A) An immunoblot of whole cell lysates of the wild type (WT) and epsin-null mutants (epsin$^-$) stained with anti-epsin antibodies. (B) Wild type (WT) and epsin mutants (epsin$^-$) develop into fruiting bodies. Scale bar: 0.2 mm. (C) DIC images of spores harvested from fruiting bodies of wild-type cells (WT), epsin-null mutants (epsin$^-$), and epsin-null mutants expressing epsin:GFP (epsin$^-$ +epsin:GFP). Expression of epsin:GFP restores wild-type sore morphology. Scale bar: 5 μm. (D) Wild type (WT), epsin-null mutants (epsin$^-$), clathrin-null mutants (clathrin$^-$), and epsin-null mutants expressing epsin:GFP (epsin$^-$ +epsin:GFP) grown in suspension for 72 hours and stained with DAPI to visualize nuclei. Scale bar, 5 μm. (E) Ratio of spore width:length in wild type (WT), epsin-null mutants (epsin$^-$) and epsin-null mutants expressing epsin:GFP (epsin$^-$ +epsinGFP), n=50 for each cell line. (F) Quantification of multinucleated cells in suspension cultures of wild type (WT), epsin mutants (epsin$^-$), epsin mutants expressing epsin:GFP (epsin$^-$ +epsin:GFP) and CHC mutants (clathrin$^-$); n=300 for each cell line. Error bars are s.e.m.

Epsin-null mutants display limited clathrin-associated phenotypes and have abnormal spore morphology

To examine the contribution of *Dictyostelium* epsin to cellular functions, we used targeted gene replacement to generate two epsin-null mutants. The deletion of the *epnA* gene in these mutants was confirmed by PCR of genomic DNA (data not shown), and the absence of epsin protein expression was demonstrated by immunoblotting with anti-epsin antibodies (Fig. 2A). Subsequent experiments revealed no differences in phenotype between the two epsin-null cell lines.

Reconstitution experiments with purified proteins and liposomes suggest that epsin functions to invaginate clathrin-coated pits (Ford et al., 2002). If epsin contributes this essential role to clathrin-coated vesicle formation in living cells, epsin-null cells would be expected to exhibit clathrin-related phenotypic deficits. To test whether clathrin-mediated cellular functions were compromised by the loss of epsin, we assessed the epsin-null mutants for phenotypes displayed by clathrin mutants. These phenotypes include defects in osmoregulation in hypo-osmotic conditions, deficiencies in fluid-phase endocytosis, and abnormal development into fruiting bodies (O'Halloran and Anderson, 1992; Niswonger and O'Halloran, 1997a; Wang et al., 2003). All of these processes were normal in the epsin-null mutants (Fig. 2B and data not shown), suggesting that epsin is not critical for general clathrin function. Both clathrin heavy chain (CHC)-null and clathrin light chain (CLC)-null mutants are known to fail in cytokinesis when grown in suspension cultures (Niswonger and O'Halloran, 1997a; Wang et al., 2003). Similarly to the clathrin mutants, epsin-null cells also accumulated multiple nuclei when grown in suspension cultures (Fig. 2D,F). The absence of many phenotypes characteristic of clathrin mutants suggested that epsin does not supply an essential and global function, such as invagination, to clathrin-coated pit formation. Rather the discrete phenotype suggests that epsin contributes to a subset of clathrin function that includes cytokinesis.

In contrast to clathrin-null cells, epsin-null mutants developed normally into fruiting bodies (Fig. 2B). However, we noted an abnormal phenotype when examining the morphology of spores within mature fruiting bodies. Spores from wild-type fruiting bodies were oblong, but spores from epsin-null fruiting bodies were round (Fig. 2C,E). Measurement of the width:length ratio of wild-type spores was 0.60±0.01 (n=50; mean ± s.e.m.),whereas spores derived from epsin-null mutants had a width:length ratio of 0.88±0.02 (n=50) (Fig. 2D,F). This round spore phenotype was reminiscent of *Dictyostelium* Hip1r, another clathrin accessory protein (Repass et al., 2007). The restricted phenotype during development supported an essential role for epsin in a specialized pathway that controls the correct morphology of spores.

Clathrin and AP2 assemble into puncta on the membrane of epsin-null cells.

Epsins contain domains and motifs that bind plasma membrane lipids as well as clathrin and clathrin adaptors. We therefore tested whether epsin was essential for clathrin pit organization by assessing the ability of clathrin and the clathrin adaptor AP2 to assemble into puncta on the plasma membrane of epsin-null cells. Wild-type cells and epsin-null mutants were transformed with GFP-CLC, a marker known to reflect the endogenous distribution of clathrin (Wang et al., 2006), and then were immunostained with antibodies to AP2. In wild-type cells, clathrin formed puncta on the plasma membrane and in the cytoplasm (Fig. 3A). Clathrin puncta on the plasma membrane of wild-type cells colocalized extensively with AP2 (Fig. 3A, inset). In epsin-null cells, clathrin and AP2 puncta also formed, and the frequency and distribution of the two proteins were indistinguishable from that in wild-type cells (Fig. 3B). Subcellular fractionation of wild-type cells showed that clathrin partitioned into the low-speed and the high-speed membrane fractions. Clathrin showed a similar association with membrane fractions in epsin-null cells (Fig. 3C). Together, these observations suggested that epsin does not play an essential role in organizing clathrin or AP2 in coated pits.

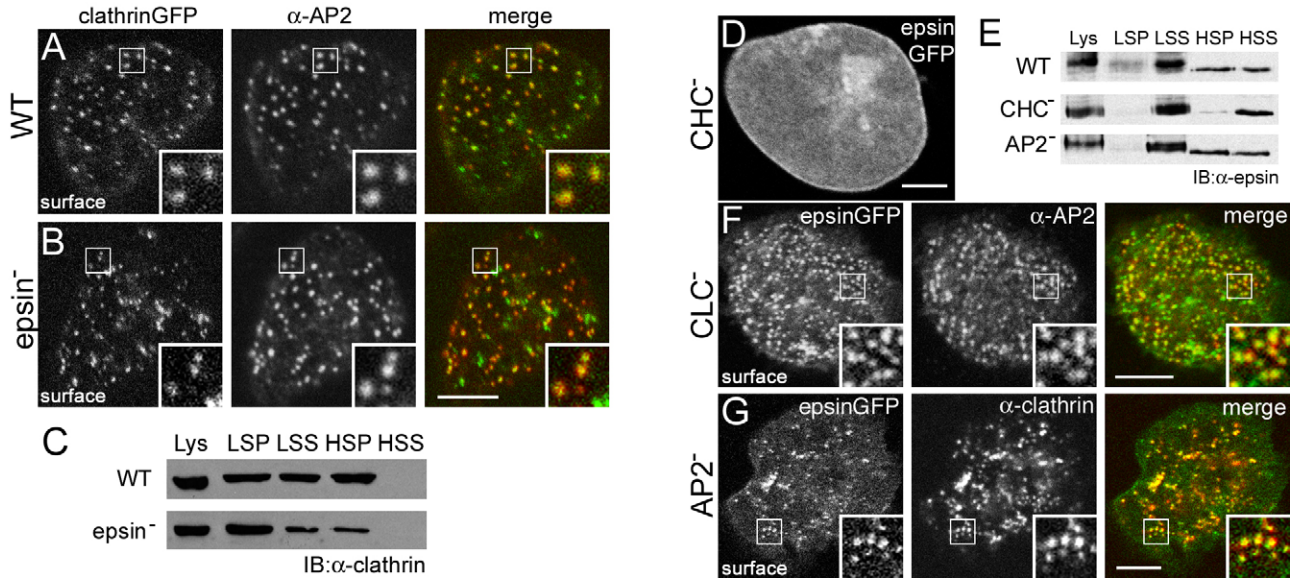

Fig. 3. Epsin requires CHC to form puncta on the membrane. (A,B) Clathrin and AP2 localization in epsin mutants is comparable to that in the wild type. Confocal images (surface focal planes) of (A) wild-type cells (WT) and (B) epsin-null mutants (epsin$^-$) expressing clathrin:GFP (green) and immunostained for the α-adaptin subunit of AP2 (α-AP2) (red). (C) Subcellular fractionation of clathrin is similar in wild-type (WT) and epsin-null cells (epsin$^-$). Immunoblot of samples probed with α-CHC antibodies. Lys, lysate; LSP, low-speed (3000 ***g***) pellet; LSS, low-speed supernatant; HSP, high-speed (100,000 ***g***) pellet; HSS, high-speed supernatant. (D) Epsin localizes to the membrane but does not form puncta in CHC-null cells. Confocal image (middle focal plane) of CHC-null cells (CHC$^-$) expressing epsin:GFP (E) Subcellular fractionation of epsin is altered in CHC mutants, but not in α-adaptin-null mutants. Immunoblot probed with anti-epsin antibodies. (F) Epsin forms puncta that colocalize with AP2 on the membranes of CLC mutants. Confocal images (surface focal plane) of CLC-null mutants expressing epsin:GFP (green) and immunostained for the α-adaptin subunit of AP2 (α-AP2) (red). (G) Epsin forms reduced numbers of puncta at the plasma membrane in α-adaptin-null mutants that colocalize with clathrin. Confocal images (surface focal plane) α-adaptin mutants (AP2$^-$) expressing epsin:GFP (green) and immunostained for clathrin (red). Scale bars, 5 μm.

Epsin localization into puncta on the plasma membrane requires clathrin

To address whether clathrin is required for the association of epsin with the plasma membrane, we examined the distribution of epsin tagged with GFP in clathrin-null and AP2-null mutants. Both the cytokinesis and spore morphology defects of epsin mutants were completely rescued by expression of epsin:GFP (Fig. 2C-F), demonstrating that epsin:GFP was functional, and that the deficiencies displayed by epsin-null cells were specific for the absence of epsin. In clathrin-heavy-chain mutants, clathrin-coated pits are absent (O'Halloran and Anderson, 1992). Likewise, epsin:GFP did not cluster into puncta in clathrin-heavy-chain mutants, but instead uniformly decorated the plasma membrane (Fig. 3D). In *Dictyostelium* cells that lack CLC, clathrin function is diminished, but the heavy chain remains assembled into puncta on the plasma membrane (Wang et al., 2003). In these CLC mutants, epsin:GFP distributed into puncta on the plasma membrane and colocalized with AP2 (Fig. 3F). These observations suggested that the CHC influences the distribution of epsin on the plasma membrane. Subcellular fractionation studies of epsin confirmed this influence. In wild-type cells, epsin fractionated with the membranes of the high-speed pellet. By contrast, epsin was found in the soluble high-speed supernatant in clathrin-heavy-chain mutants, indicating that clathrin-null mutants contained more soluble epsin than wild-type cells did (Fig. 3E).

Epsin does not require AP2 to associate with clathrin at the plasma membrane

The preceding experiments established that clathrin was an important determinant for the association of epsin with membranes and for clustering within puncta on the plasma membrane. In addition to motifs for binding clathrin, the C-terminus of epsin has motifs for binding AP2, the predominant and best characterized clathrin adaptor at the plasma membrane. To examine the contribution of AP2 to the cellular location of epsin, we expressed epsin:GFP in AP2α mutants lacking the large α subunit of AP2. Relative to wild-type cells, AP2α mutants show reduced numbers of clathrin puncta on the plasma membrane (our unpublished results). Nonetheless, epsin continued to colocalize with the remaining clathrin puncta in AP2α-null cells (Fig. 3G). Similarly to the reduced number of clathrin puncta on the membrane of AP2α mutants, epsin formed ~20% fewer puncta on the plasma membrane of AP2α mutants (0.60±0.04 puncta/μm^2, n=1047 puncta; 16 cells) compared with that in wild-type cells (0.77±0.04 puncta/μm^2, n=683 puncta; 12 cells) (Fig. 3G). Subcellular fractionation of epsin in the AP2α mutants revealed that the association of epsin with membrane fractions was similar to that seen in wild-type cells (Fig. 3E). Taken together, these results indicate that, although AP2α is important for building clathrin-coated pits on the plasma membrane, AP2α is not a critical determinant for localizing epsin into coated pits.

The ENTH domain is required but is not sufficient for epsin association with clathrin and AP2 at the plasma membrane

In other organisms, the N-terminal ENTH domain of epsin has been shown to be sufficient for phenotypic rescue (Wendland et al., 1999; Aguilar et al., 2003; Overstreet et al., 2003). To explore the functional properties of *Dictyostelium* epsin in more detail, we generated two expression plasmids for GFP-tagged epsin truncations, epsin$_{1\text{-}333}$ and epsin$_{253\text{-}677}$, which separated the N-terminal ENTH domain from the

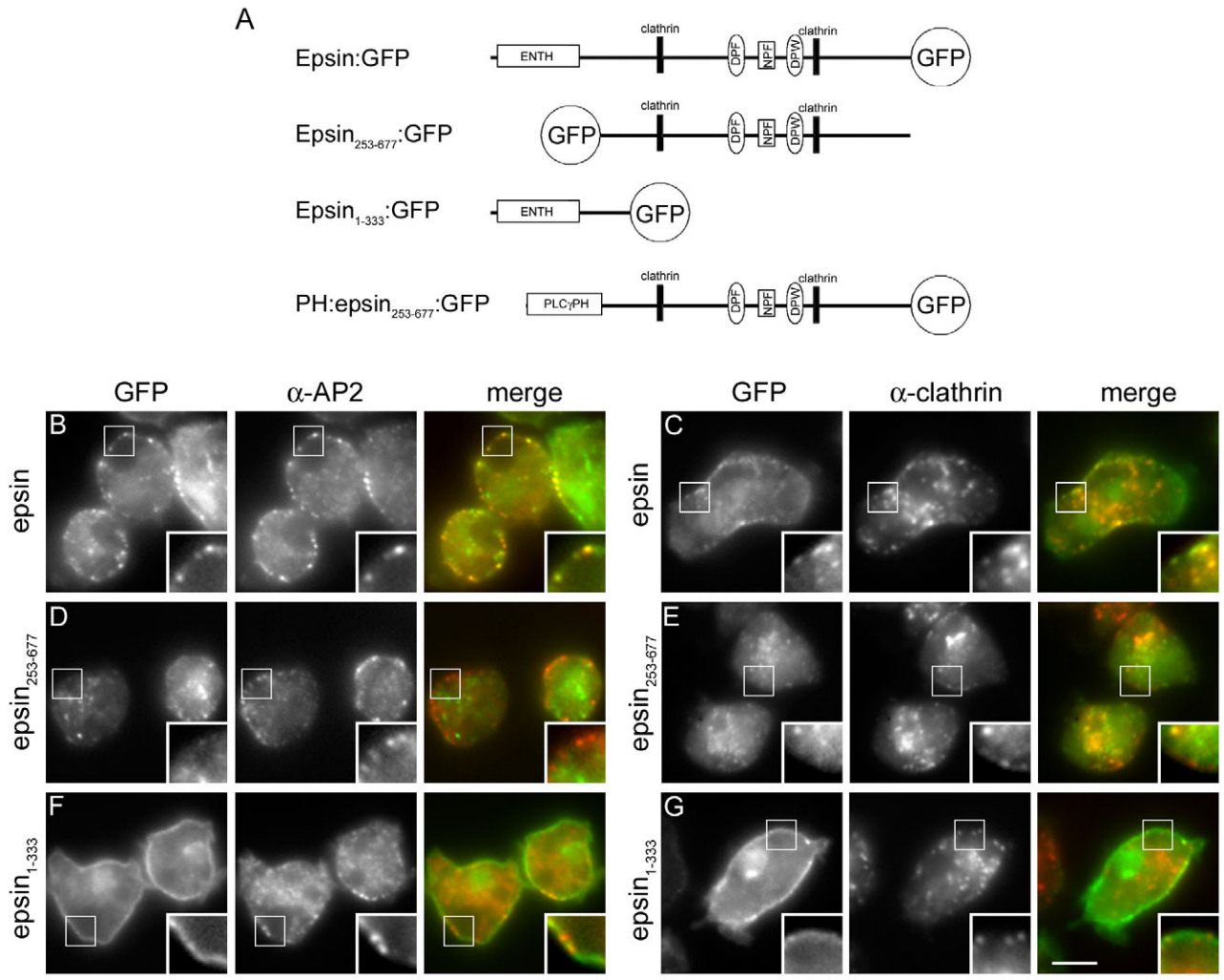

Fig. 4. Both domains of epsin are required for targeting to clathrin-coated pits. (A) Schematic representation of GFP-labeled epsin truncation and chimera constructs. Black bars indicate clathrin-binding motifs, ovals indicate DPF/W AP2 binding motifs and rectangles indicate NPF motifs for binding EH-domain-containing proteins. (B-G) Wide-field fluorescence microscopy (focal planes from the middle of cells). (B,C) Epsin:GFP colocalizes with AP2 in epsin-null cells. Epsin-null cells expressing epsin:GFP (green) were fixed and immunostained with anti-α-adaptin antibody (B) or anti-clathrin antibody (C, red). (D) Epsin$_{253-677}$:GFP does not form puncta at the plasma membrane and does not colocalize with AP2. Epsin-null cells expressing epsin$_{253-677}$:GFP (green) were fixed and immunostained for the α-adaptin subunit of AP2 (α-AP2) (red). (E) Epsin$_{253-677}$:GFP cytoplasmic puncta overlap with clathrin puncta in the cytoplasm but not the plasma membrane. Epsin-null cells expressing epsin$_{253-677}$:GFP (green) were fixed and immunostained with anti-clathrin antibody (red). (F,G) Epsin$_{1-333}$:GFP uniformly decorates the plasma membrane. Epsin-null cells expressing epsin$_{1-333}$:GFP (green) were fixed and immunostained with anti-α-adaptin antibody (F) or anti-clathrin antibody (G, red). Scale bar: 5 μm.

C-terminal domain which contained motifs for binding clathrin, EH-domain proteins and AP2 (Fig. 4A).

We expressed these truncation constructs in an epsin-null background (Fig. 4D-E). Because the C-terminal domain of epsin contains motifs for binding AP2 and clathrin accessory proteins, we expected epsin$_{253-677}$ to associate with the plasma membrane. However, examination by fluorescence microscopy of cells expressing epsin$_{253-677}$ revealed that epsin$_{253-677}$:GFP rarely localized to the plasma membrane, but instead associated with puncta in the cytoplasm (Fig. 4D,E compared with Fig. 4B,C). Thus the C-terminal domain of epsin associated with cytoplasmic puncta of clathrin and was excluded from plasma membrane clathrin puncta, contrary to the normal distribution for full-length epsin.

The complementary N-terminal epsin construct, epsin$_{1-333}$, contained the complete ENTH domain plus a short, unstructured region. Consistent with the capacity of the ENTH domain to bind PtdIns(4,5)P_2, epsin$_{1-333}$:GFP localized uniformly on the plasma membrane and did not form discrete puncta (Fig. 4F,G). Epsin$_{1-333}$:GFP also distributed along the plasma membrane of cells lacking CHC or CLC, as well as AP2α-null cells, suggesting that the ability of epsin$_{1-333}$:GFP to associate with the plasma membrane was independent of clathrin or AP2 (supplementary material Fig. S1). Although the distribution of epsin$_{1-333}$ was uniform, clathrin localized normally into puncta in both epsin-null and wild-type cells expressing epsin$_{1-333}$:GFP (Fig. 4G and data not shown).

In addition to their localization, we also tested whether the N-terminal and the C-terminal truncations of epsin were able to rescue the phenotypic deficiencies of epsin-null cells. We tested the ability of both constructs to rescue cytokinesis by examining whether multinucleated cells accumulated in cultures of epsin mutants

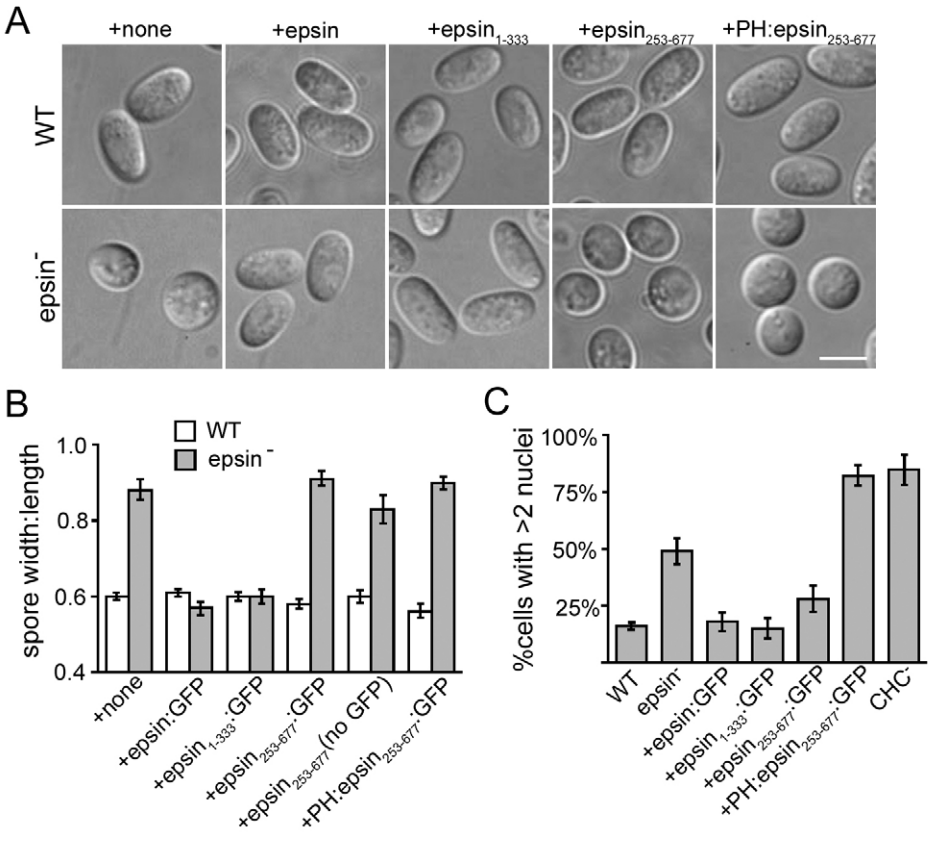

Fig. 5. The ENTH domain rescues phenotypic defects of epsin mutants. (A) DIC images of spores from the wild type (WT) and epsin mutants (epsin$^-$) expressing epsin:GFP, epsin$_{1\text{-}333}$:GFP, epsin$_{253\text{-}677}$:GFP, and PH:epsin$_{253\text{-}677}$:GFP. Scale bar: 5 μm. (B) Ratio of spore width:length in wild type (WT) and epsin-null mutants (epsin$^-$) and epsin-null mutants expressing epsin:GFP, epsin$_{1\text{-}333}$:GFP, epsin$_{253\text{-}677}$:GFP, and PH:epsin$_{253\text{-}677}$:GFP; n=50 for each cell line, error bars represent s.e.m. (C) Quantification of multinucleated cells in suspension cultures of wild-type (WT), epsin mutants (epsin$^-$), clathrin mutants (clathrin$^-$), and epsin mutants expressing epsin:GFP, epsin$_{1\text{-}333}$:GFP, epsin$_{253\text{-}677}$:GFP, and PH:epsin$_{253\text{-}677}$:GFP; n=300 for each cell line. Error bars represent s.e.m.

expressing either of the two constructs. Quantification of multinucleated cells in suspension cultures revealed that both epsin$_{253\text{-}677}$:GFP and epsin$_{1\text{-}333}$:GFP rescued the cytokinesis defect of epsin mutants (Fig. 5C).

We also examined the ability of the two constructs to rescue the spore morphology defect of epsin mutants. Epsin-null cells expressing epsin$_{253\text{-}677}$:GFP developed into fruiting bodies containing round spores that were indistinguishable from the control epsin-null mutants (Fig. 5A,B). The failure of epsin$_{253\text{-}677}$:GFP to restore normal spore morphology was not an artifact of the GFP tag, because epsin-null cells expressing epsin$_{253\text{-}677}$ without GFP also developed into fruiting bodies that contained round spores (Fig. 5B). By contrast, epsin-null mutants expressing the N-terminal construct epsin$_{1\text{-}333}$:GFP developed into fruiting bodies that contained oblong spores indistinguishable from that in the wild type (Fig. 5A,B). Thus epsin$_{253\text{-}677}$:GFP was able to rescue the cytokinesis failure but not the spore morphology defect, whereas epsin$_{1\text{-}333}$ was able to fully rescue both phenotypic defects of epsin-null mutants.

A canonical PtdIns(4,5)P_2-binding domain cannot substitute for the ENTH domain

The analysis of epsin domains suggested that the ENTH domain was both necessary and sufficient to target epsin to the membrane and to rescue the spore morphology and cytokinesis defects of epsin-null mutants. A significant function of the epsin ENTH domain is to bind PtdIns(4,5)P_2 (Itoh et al., 2001; Ford et al., 2002). We therefore asked whether another PtdIns(4,5)P_2-binding domain could functionally replace the ENTH domain of *Dictyostelium* epsin. The PH domain of mammalian PLCδ, a canonical PtdIns(4,5)P_2-binding domain, is of comparable size to the ENTH domain and also binds to PtdIns(4,5)P_2 (Lemmon et al., 1995; Stauffer et al., 1998; Itoh et al., 2001). We generated a construct that tagged the PLCδPH domain with GFP and examined its distribution in epsin-null and wild-type cells. Consistent with membrane-binding properties similar to the ENTH domain of epsin, PLCδPH:GFP displayed a uniform plasma membrane localization comparable with epsin$_{1\text{-}333}$:GFP and did not disrupt clathrin localization or function (supplementary material Fig. S2).

To determine whether this canonical PtdIns(4,5)P_2-binding domain could substitute for the ENTH domain function, we made a chimeric GFP-tagged epsin that replaced the ENTH domain with the PH domain of PLCδ (Fig. 4A). If this alternative PtdIns(4,5)P_2-binding domain was able to substitute for the ENTH domain, the PH-epsin C-terminal domain chimera (PH:epsin$_{253\text{-}677}$:GFP) should distribute similarly to full-length epsin. However, when expressed in wild-type cells, the PH:epsin$_{253\text{-}677}$:GFP chimera localized in a distinct and aberrant pattern. Instead of forming puncta evenly distributed on the plasma membrane and puncta within the cytoplasm, PH:epsin$_{253\text{-}677}$:GFP aggregated into large patches on the plasma membrane (Fig. 6A,B compare with Fig. 4B,C). Moreover, these aberrant patches sequestered both AP2 and clathrin. Staining with anti-AP2 antibody revealed that AP2 puncta frequently clustered within the PH:epsin$_{253\text{-}677}$:GFP patches (Fig. 6A). Staining with anti-clathrin antibody revealed that PH:epsin$_{253\text{-}677}$:GFP caused severe mislocalization of clathrin. In wild-type cells or epsin-null cells rescued with epsin:GFP, clathrin normally forms discrete puncta on the plasma membrane and in the perinuclear region of the cytoplasm (Fig. 4C). However, in wild-type cells expressing PH:epsin$_{253\text{-}677}$:GFP, clathrin aggregated together with PH:epsin$_{253\text{-}677}$:GFP caps at the plasma membrane, and nearly all cytoplasmic and perinuclear clathrin staining was absent (Fig. 6B).

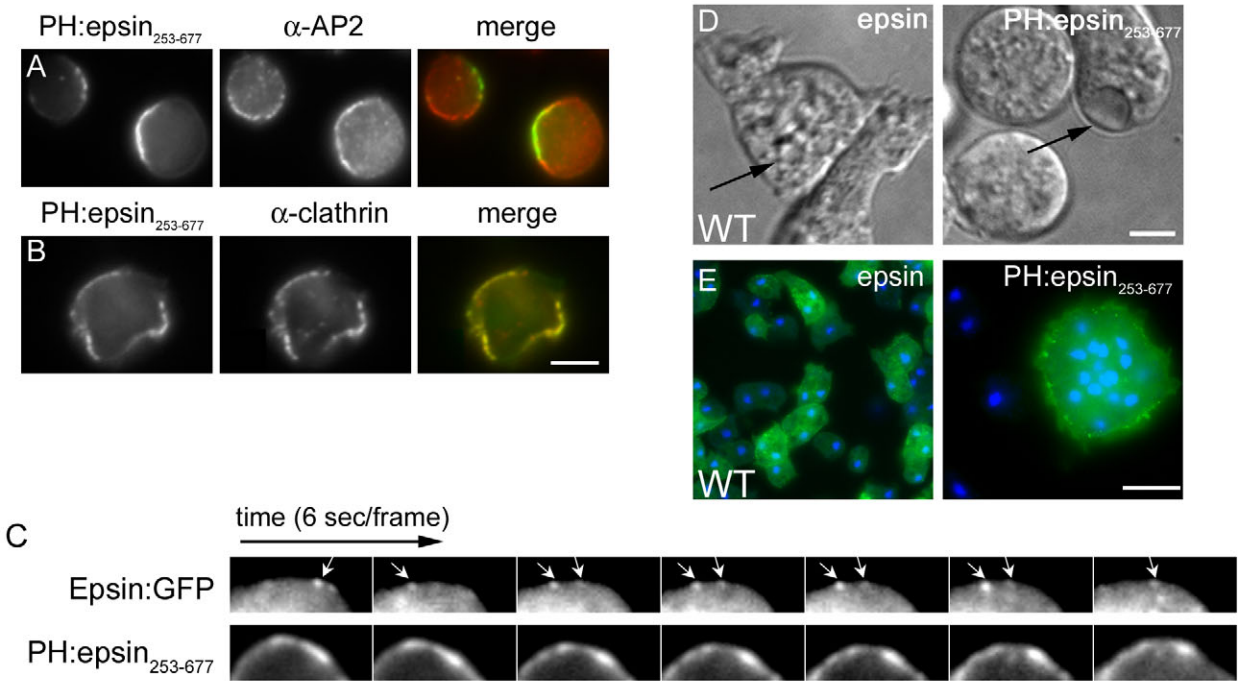

Fig. 6. PH:epsin$_{253\text{-}677}$:GFP forms large patches on the plasma membrane and cells expressing PH:epsin$_{253\text{-}677}$:GFP mislocalize clathrin and AP2 to these patches (A,B). Wild-type cells expressing PH:epsin$_{253\text{-}677}$:GFP (green) were fixed and immunostained with anti-α-adaptin antibody (A) or anti-clathrin antibody (B) (red). Images were acquired under wide-field fluorescence microscopy (focal plane in the middle of the cell). Scale bar: 5 µm. (C) Epsin forms dynamic membrane puncta, but PH:epsin$_{253\text{-}677}$:GFP forms relatively static patches. Plasma membrane images from a time course of wild-type cells expressing either epsin:GFP (top row) or PH:epsin$_{253\text{-}677}$:GFP (bottom row) were imaged under fluorescence microscopy. (D,E) Wild-type cells expressing PH:epsin$_{253\text{-}677}$:GFP display clathrin-associated phenotypic defects, including enlarged contractile vacuoles and cytokinesis defects. (D) Wild-type cells expressing epsin:GFP or PH:epsin$_{253\text{-}677}$:GFP were shifted from medium to water and imaged under DIC optics. Arrows indicate contractile vacuoles. Scale bar: 5 µm. (E) Wild-type cells expressing epsin:GFP or PH:epsin$_{253\text{-}677}$:GFP (green) were cultured in suspension for 3 days, then fixed and stained with DAPI to visualize nuclei (blue). Scale bar: 10 µm.

Epsin-null cells expressing PH:epsin$_{253\text{-}677}$:GFP showed an identical distribution and mislocalization of AP2 and clathrin (data not shown). Thus, substitution of an alternative PH domain for the ENTH domain allowed the chimeric epsin to associate with the plasma membrane, and allowed the C-terminal domain to bind clathrin and AP2. However, the chimeric epsin also sequestered AP2 and clathrin into abnormal patches on the plasma membrane.

Imaging living cells expressing the chimeric epsin revealed that its dynamic association with the plasma membrane was also aberrant (Fig. 6C). Puncta of GFP-epsin associated transiently with the plasma membrane and GFP-epsin could be seen to build up into a discrete spot that subsequently disappeared. By contrast, the patches of PH:epsin$_{253\text{-}677}$:GFP were relatively more static on the membrane and did not appear to form or disassemble. Both the PH:epsin$_{253\text{-}677}$:GFP chimera and epsin GFP were expressed in similar amounts (data not shown). We therefore concluded that substitution of the PH domain for the ENTH domain disrupted the capacity of epsin to form transient puncta on the plasma membrane.

Expression of PH:epsin$_{253\text{-}677}$:GFP impairs clathrin function

To determine whether the sequestration of clathrin on the membrane by PH:epsin$_{253\text{-}677}$ ablated clathrin function, we tested wild-type cells expressing PH:epsin$_{253\text{-}677}$:GFP for phenotypes typical of clathrin mutants: defective osmoregulation, cytokinesis failure and abnormal development.

Clathrin mutants display defects in the size and activity of the contractile vacuole, an osmoregulatory organelle in *Dictyostelium*, and are therefore osmosensitive (O'Halloran and Anderson, 1992; Wang et al., 2003). To test whether expression of PH:epsin$_{253\text{-}677}$:GFP induced osmosensitivity, we shifted cells from medium to water, and examined the contractile vacuole under differential interference contrast (DIC) microscopy. Wild-type cells displayed an increase in contractile vacuole activity, with the contractile vacuole swelling and discharging. Similarly to clathrin-light-chain mutants (Wang et al., 2003), wild-type cells expressing PH:epsin$_{253\text{-}677}$:GFP developed abnormally large contractile vacuoles with prolonged cycles of expansion (Fig. 6D). This effect was due to the expression of PH:epsin$_{253\text{-}677}$:GFP because the contractile vacuole activity was not altered in wild-type cells expressing full-length epsin, either of the two epsin truncations or the PH domain alone (data not shown).

Clathrin is also critical for cytokinesis. Both CLC and CHC mutants are unable to divide in suspension cultures and become large and multinucleated (Niswonger and O'Halloran, 1997a; Wang et al., 2003). When grown in suspension cultures, wild-type cells expressing PH:epsin$_{253\text{-}677}$:GFP also accumulated many nuclei to the same extent as CHC mutant cells, demonstrating a similarly severe defect in cytokinesis (Fig. 5C; Fig. 7E). These defects showed that coupling the C-terminus of epsin to an alternative membrane-binding domain induces dominant-negative phenotypes in growing cells that are characteristic of clathrin mutants.

In contrast to the other clathrin deficiencies induced by expressing PH:epsin$_{253\text{-}677}$:GFP in wild-type cells, expression of PH:epsin$_{253\text{-}677}$:GFP did not impair wild-type cells during development. Clathrin mutants are not able to complete development and aggregate to form stunted structures (Niswonger and

O'Halloran, 1997b; Wang et al., 2003). However, wild-type cells expressing PH:epsin$_{253-677}$:GFP developed into fruiting bodies with a stalk and a sorus that appeared normal in structure (data not shown). Moreover, the fruiting bodies of wild-type cells expressing PH:epsin$_{253-677}$:GFP contained oblong spores identical in shape to wild-type spores, indicating that PH:epsin$_{253-677}$:GFP did not induce the formation of abnormal spores (Fig. 5A,B). Nonetheless, whereas the chimeric PH:epsin$_{253-677}$:GFP protein did not lead to dominant-negative developmental phenotypes, the chimeric epsin also did not rescue the spore defect of epsin-null cells. Examination of the spores housed within the sori of epsin-null cells expressing the chimeric PH:epsin$_{253-677}$:GFP revealed round spores that were identical in morphology to the spores of epsin mutants (Fig. 5A,B).

Identification of residues in the ENTH domain important for localization and function

The inability of the PH domain to substitute for the ENTH domain suggested that the ENTH domain contributed more than PtdIns(4,5)P_2-binding activity to epsin. To determine how the ENTH domain contributed to epsin localization and function, we first asked whether the PtdIns(4,5)P_2-binding ability of the ENTH domain was critical for epsin function. Amino acids R65 and K78 have been shown to be critical for the interaction between the ENTH domain and PtdIns(4,5)P_2 (Itoh et al., 2001). To directly test the importance of this activity, we constructed two plasmids to express mutant versions of either the ENTH domain or full-length epsin with the R65A/K78A mutations. Assessment of the PtdIns(4,5)P_2-binding capacity of ENTH$^{R65A/K78A}$ confirmed that mutating these residues impaired the ability of the ENTH domain to bind PtdIns(4,5)P_2 (Fig. 7A). Examination of the cells expressing the GFP-tagged proteins revealed that ENTH$^{R65A/K78A}$ and epsin$^{R65A/K78A}$ failed to associate with the plasma membrane (Fig. 7B,C), consistent with the insufficiency of the C-terminal domain to associate with clathrin-coated pits. Moreover, ENTH$^{R65A/K78A}$ and epsin$^{R65A/K78A}$ also failed to rescue the round spore phenotype, demonstrating that the ability to bind PtdIns(4,5)P_2 was required for both epsin function and localization (Fig. 7D). We also tested the contribution of another amino acid within the ENTH domain, T107, a residue not predicted to function in PtdIns(4,5)P_2 binding. The analogous amino acid in yeast epsin is essential for viability (Aguilar et al., 2006), demonstrating an important contribution to epsin activity; however, the contribution of this residue to epsin localization has not been examined. We therefore constructed a plasmid that introduced the T107A mutation into the ENTH domain and full-length epsin. In contrast to epsin$^{R65A/K78A}$, the T107A mutation did not affect the localization of epsin. ENTH^{T107A} was distributed uniformly along the plasma membrane and epsin^{T107A} localized within puncta on the plasma membrane (Fig. 7B,C). Similarly to wild-type epsin, these puncta colocalized with plasma membrane clathrin (data not shown). However, despite the wild-type association with the plasma membrane or coated pits, neither ENTH^{T107A} nor epsin^{T107A} were able to rescue the round-spore phenotype of epsin-null cells (Fig. 7D). Thus this mutation separates the contribution of the ENTH domain to epsin localization from its contribution to essential cellular function.

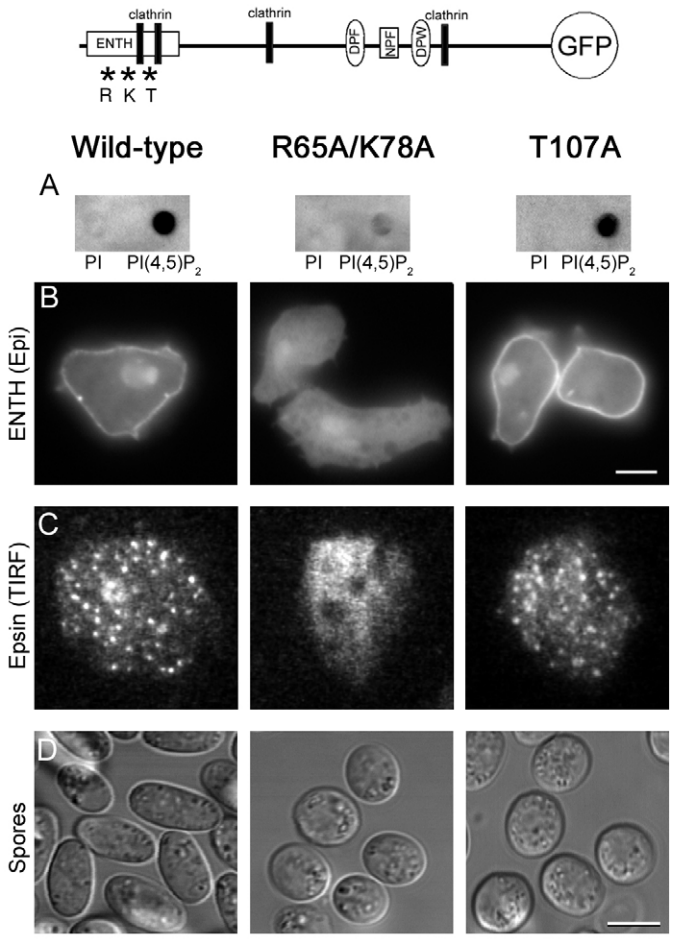

Fig. 7. Epsin^{T107A} localizes to plasma membrane puncta but does not rescue spore morphology. (Top of figure) Schematic representation of epsin, showing the location of point mutations. (A) ENTH$^{R65A/K78A}$, but not ENTH^{T107A}, exhibits impaired binding to PtdIns(4,5)P_2. PtdIns and PtdIns(4,5)P_2 (PI and PI(4,5)P_2, respectively) were spotted onto nitrocellulose membrane and then incubated with lysate from *Dictyostelium* cells expressing either wild-type or mutant ENTH:GFP. Membranes were probed with anti-GFP antibody. (B) Wild-type or mutant ENTH:GFP was expressed in epsin-null cells and imaged by epifluorescence wide-field microscopy (epi); focal planes from the middle of cells. ENTH^{WT} (left) and ENTH^{T107A} (right) localize to the plasma membrane, whereas ENTH$^{R65A/K78A}$ (center) does not. (C) Wild-type or mutant epsin:GFP was expressed in epsin-null cells and imaged by total internal reflection microscopy (TIRF). Epsin^{T107A} (right) forms puncta similar to epsin^{WT} (left). Epsin$^{R65A/K78A}$ (center) does not form puncta on the plasma membrane. (D) Epsin-null cells expressing wild-type or mutant ENTH:GFP were allowed to develop on starvation plates. Spores were harvested and imaged under DIC optics. ENTH$^{R65A/K78A}$ (center) or ENTH^{T107A} (right) cannot rescue spore morphology. Scale bars: 5 μm.

Discussion

Epsin is a phylogenetically conserved clathrin adaptor protein. Our results define determinants essential for targeting epsin into clathrin-coated pits that are distinct from determinants essential for epsin function. In this work, we identified clathrin, but not AP2, as essential for epsin localization within clathrin-coated pits. Our analysis also demonstrated that a cooperative interaction between the two domains of epsin enables this protein to interact dynamically with clathrin pits on the plasma membrane. Our results support a model where the ENTH domain coordinates with the clathrin-binding C-terminal domain to tether epsin to the plasma membrane for a productive and functional interaction with clathrin-coated pits. Independent of targeting to coated pits, the isolated ENTH domain is both necessary and sufficient for rescuing the aberrant spore morphology of epsin-null cells. Thus, determinants for targeting epsin to coated pits are distinct from those that supply function.

Clathrin, but not AP2, is a determinant for localizing epsin within coated pits

By examining the localization of epsin in different mutant backgrounds, we were able to define how other proteins contribute to the localization of epsin within clathrin-coated pits. In wild-type cells, double-label fluorescence microscopy revealed extensive colocalization between clathrin and epsin. A small number of epsin punctae were found without clathrin, but these static experiments could not distinguish whether clathrin was required for a particular phase of a dynamic coated pit or whether a small subset of epsin spots did not require clathrin. We addressed these two possibilities using clathrin mutants, and identified the CHC as necessary for epsin to cluster to plasma membrane puncta. Clathrin-heavy-chain-null mutants distributed epsin uniformly on the plasma membrane, as revealed by microscopy, and contained more soluble epsin than wild-type cells, as revealed by subcellular fractionation (Fig. 3D,E). Among *Dictyostelium* clathrin-associated proteins, this requirement for clathrin to form punctae is unique, because clathrin mutants continue to form puncta of AP180 and AP2 on their membranes (Stavrou and O'Halloran, 2006) (our unpublished results).

By contrast, epsin continued to cluster within clathrin-coated pits in AP2α mutants. Deletion of AP2 in *Dictyostelium* caused a marked decrease in the total number of puncta at the membrane that contain epsin and clathrin (our unpublished results), which is consistent with depletion experiments in vertebrate cell culture (Hinrichsen et al., 2003; Motley et al., 2003). Although the number of epsin puncta is reduced in AP2α mutants, the remaining epsin puncta continue to colocalize with clathrin. Thus, although AP2 increases the number of clathrin puncta on the membrane, the presence of AP2 is not critical for epsin to incorporate into clathrin-coated pits. This is in agreement with our biochemical results, which did not detect a physical interaction between epsin and AP2. Similarly, we have found that deletion of other *Dictyostelium* clathrin accessory proteins, including Hip1r and AP180, does not affect epsin localization to clathrin-coated pits (Stavrou and O'Halloran, 2006) (our unpublished results).

Dictyostelium contains a single epsin gene and did not appear to contain other homologs, including the related epsinR protein, which binds AP1, and is associated with Golgi trafficking events (Kalthoff et al., 2002b; Wasiak et al., 2002; Hirst et al., 2003; Mills et al., 2003). Despite the absence of related genes that could supply redundant function, we found that *Dictyostelium* epsin-null cells manifested only limited clathrin-associated phenotypes. In epsin-null cells, epsin function may be covered by other clathrin adaptors, including AP2 and AP180. Similarly to epsin, AP2 and AP180 bind PtdIns(4,5)P_2 and contain multiple motifs for interacting with other coated pit proteins (Legendre-Guillemin et al., 2004; Edeling et al., 2006). Our findings in epsin-null mutants might indicate that epsin is not essential for initiating clathrin pit assembly, and are consistent with more specialized roles for epsin rather than assembly of the clathrin lattice itself.

Contribution of the C-terminal domain

In addition to examining how other proteins contribute to epsin localization, we also defined determinants within the epsin protein necessary and sufficient for targeting within coated pits. As with other epsins, the *Dictyostelium* epsin C-terminal domain contained motifs for interacting with coated pits. These motifs included clathrin- and AP2-binding motifs, but not a motif for interacting with ubiquitin. The latter motif is also lacking in *Arabidopsis* epsin (Holstein and Oliviusson, 2005). Surprisingly, we found that, although essential, the clathrin-binding C-terminal domain was not sufficient for associating with clathrin pits.

Coupling the C-terminal domain to an alternative membrane-binding domain, a PH domain, created a chimeric molecule capable of associating with clathrin and AP2 on the plasma membrane. Neither the C-terminal domain nor the PH domain on its own associated with clathrin on the membrane. Thus, the C-terminal domain contributes a potent capacity to associate with clathrin-coated pits, but only when targeted to the plasma membrane by a membrane-binding domain.

However, the chimeric PH:epsin$_{253\text{-}677}$ molecule was not functional, and even sequestered clathrin to the extent of abolishing clathrin function. Thus the C-terminal domain is necessary but not sufficient for the functional interaction of epsin with clathrin-coated pits; the ENTH domain is also required. Moreover, the nonproductive and relatively static interaction of the chimeric molecule with clathrin at the plasma membrane suggests that the ENTH domain tempers the clathrin-binding ability of the C-terminal domain, allowing the interaction between epsin and clathrin to be transient, dynamic, and functional.

The ENTH domain is essential for localization and sufficient for function

The insufficiencies of the isolated C-terminal domain highlight a unique contribution of the ENTH domain to both the localization and function of epsin. The ENTH domain binds to PtdIns(4,5)P_2 on the plasma membrane, a phospholipid critical for coated pit assembly (Zoncu et al., 2007). The C-terminal domain of epsin required this membrane-binding function of the ENTH domain in order to cluster within clathrin pits. However, the disruption of clathrin distribution and dominant-negative phenotypes associated with expression of the PH:epsin$_{253\text{-}677}$ chimera suggested a new function for the ENTH domain in modulating the clathrin binding capacity of epsin.

Although the ENTH domain is essential for epsin localization, it is not sufficient. The ENTH domain alone could not cluster to clathrin-coated pits, but instead distributed uniformly over the plasma membrane. By contrast, the ENTH domain was both necessary and sufficient to rescue the spore morphology defects of epsin-null mutants. Thus, the determinants sufficient for clustering epsin within clathrin-coated pits, which require both the C-terminal domain and the ENTH domain, are distinct from the determinants sufficient for supplying function, which are contained solely in the ENTH domain.

Determinants within the ENTH domain that contribute to epsin function

Mutating residues R65 and K78 in the isolated ENTH domain ablated PtdIns(4,5)P_2 binding and also ablated the capacity of the ENTH domain to rescue epsin-null phenotypes. Similarly, mutating R65/K78 in full-length epsin also rendered the protein unable to bind to coated pits and compromised its ability to rescue the round spore phenotype of epsin mutants. This mutant demonstrates that the ability of the ENTH domain to bind PtdIns(4,5)P_2 is required for both epsin localization and essential function. By contrast, mutating the T107 residue rendered epsin non-functional, but still able to localize within clathrin-coated pits. Thus this residue within the ENTH domain contributes to the essential function of epsin, but does not contribute to the ability of epsin to target to and incorporate within clathrin-coated pits.

How does the T107 residue contribute to epsin function? The analogous residue in the yeast epsin homolog is part of a functional

patch that binds to a GTPase-activating protein (GAP) for cell division control protein cdc42 and contributes to regulation of the actin cytoskeleton and cell polarity (Aguilar et al., 2006). The mechanism by which the T107 residue contributes to epsin function may be different than in yeast, because the *Dictyostelium* genome does not contain a gene for cdc42, although it does contain multiple genes that encode Rac GTPases. The *Dictyostelium* ENTH domain could supply a similar function by determining the polar organization of cellular components in the oblong spore. At present, little is known about how *Dictyostelium* spores construct their oblong shape. Interestingly, the clathrin accessory protein Hip1r also forms abnormally round spores in *Dictyostelium* (Repass et al., 2007). Both epsin-null cells and Hip1r-null cells produce round spores with slightly reduced viability, but Hip1r-null spores are more sensitive to heat and detergent treatment, indicating that the spore phenotype of Hip1r-null cells is more severe (Repass et al., 2007). Moreover, the ENTH domain of epsin is required for the phosphorylation and coated pit localization of Hip1r. An important function of the ENTH domain may be to regulate the localization and activity of Hip1r.

Functional contribution of the ENTH domain

The ability of the isolated ENTH domain to rescue epsin-null phenotypes even though it does not localize within coated pits suggests that epsin could have two distinct functions. One function, governed by the ENTH domain, is an essential developmental function that contributes to spore morphology. The capacity of the ENTH domain to function independently of clathrin-coated pits may be universal, as indicated by the ability of the isolated ENTH domains for yeast and *Drosophila* to rescue null phenotypes. Epsin does not require coated-pit localization in order to operate in this capacity, suggesting that this activity might be independent of clathrin. The other function of epsin is within clathrin-coated pits on the plasma membrane.

What is the functional contribution of epsin to clathrin-coated pits? In vitro studies demonstrate that epsin promotes invagination of clathrin assembled on lipid monolayers (Ford et al., 2002). More recently, in vivo studies have suggested that clathrin itself is the driving force in coated-pit invagination from the plasma membrane (Hinrichsen et al., 2006). Consistent with this observation, we found that *Dictyostelium* epsin-null cells manifested only limited clathrin-associated deficits. Our results argue for a more specialized role for epsin during clathrin-mediated endocytosis. Similarly, the *Drosophila* epsin homolog is not required for general clathrin-mediated endocytosis, but is specifically required for the appropriate processing of the Delta ligand during Notch signaling events (Overstreet et al., 2004; Wang and Struhl, 2004; Wang and Struhl, 2005).

It has been suggested that adaptors such as epsin are involved in sorting ligands to distinct endosomal populations (Lakadamyali et al., 2006) and allow precise endocytosis of certain surface receptors that are critical for appropriate cell fate specification (Berdnik et al., 2002; Overstreet et al., 2003; Traub, 2003; Wang and Struhl, 2005). Dissecting how epsin and other adaptors function in eukaryotic cells to tailor clathrin-coated pits amidst large volumes of membrane traffic remains an important challenge.

Materials and Methods

Strains and cell culture

Dictyostelium discoideum strains included Ax2, an axenic wild-type strain, 10G10 and 5B4, epsin-null strains derived from Ax2 (described below), 6A5, an α-adaptin-null line derived from Ax2, 5E2, a CHC-null line derived from Ax2 (Niswonger and O'Halloran, 1997b) and 2A1, a CLC-null strain derived from NC4A2 (Wang et al., 2003). Cells were cultured on tissue culture plates with HL-5 medium (Sussman, 1987) supplemented with 60 U/ml penicillin and 60 μg/ml streptomycin (Invitrogen, Carlsbad, CA) at 18°C. Null cells grown under selection were supplemented with 5 μg/ml blasticidin (ICN Biomedicals, Irvine, CA) and cells carrying expression plasmids were supplemented with 20 μg/ml G418 (geneticin, Gibco-BRL, Invitrogen).

Targeted replacement of *epnA* in *Dictyostelium discoideum*

Genomic sequence upstream of *epnA* was PCR-amplified using 5′-TTAAAAAAG-GTAAAGATGCAGTATTG-3′ and 5′-TTGGAAATTTGGTGTTGCTGGTG-3′. Downstream genomic sequence was PCR-amplified using 5′-AATCAAAGTGGT-GCGAATAGAAATAC-3′ and 5′-AATGATGATAGTAAAACTGATGGTAGAAG-3′. Genomic sequences were cloned on either side of the Bsr cassette in pSP27-BSR (Wang et al., 2002) using *Xho*I/*Hin*dIII (5′) and *Eco*RI (3′), generating the plasmid pSP72-BSR-EpsinKO. 10 μg linearized vector was transformed into Ax2 cells by electroporation. Cells were diluted into 96-well plates and grown under Bsr selection. Clonal transformants lacking the entire *epnA* gene were identified by western blot and PCR analysis, and two of these clones, 10G10 and 5B4, were selected for further study.

cDNA cloning and sequence analysis

The protein sequence of human Epsin-1 (EPN1; accession number NP037465) was used to search the *Dictyostelium* genome database (http://www.dictybase.org) for the best match using BLAST. A single homologous gene product was identified (DDB0183945, accession number XM630177). A complete cDNA clone was obtained from a *Dictyostelium discoideum* cDNA library using polymerase chain reaction (PCR) with primers 5′-TGGAGACTATGATTAAAAGTTATATTAAAAAAGGTAAA-GATGCAGTATTGAATACACCAGAAATTGAAAGAAAGGTTAG-3′ and 5′-GCAGATCCCATGCTATTAGTATTTCTATTCGC-3′ and cloned into pCR2.1 using TA Cloning Kit (Invitrogen) to generate pCR2.1-Epsin. DNA sequences were managed using EditSeq and SeqMan (DNAStar, Madison, WI).

Cloning of expression plasmids

Epsin was cloned into pTX-GFP (Levi et al., 2000) (a kind gift from T. Egelhoff) to generate pTx-EpsinGFP using *Kpn*I and *Eco*RV. The cDNA encoding the epsin$_{1\text{-}333}$ truncation was PCR-amplified from pCR2.1-Epsin with 5′-TGGAG-ACTATGATTAAAAGTTATATTAAAAAAGGTAAAGATGCAGTATTGAATACA CCAGAAATTGAAAGAAAGGTTAG-3′ and 5′-GGTCGACTTCTTCCGCCAG-3′ and ligated into pCR2.1 to generate pCR2.1-epsin$_{1\text{-}333}$. pCR2.1-epsin$_{1\text{-}333}$ was then digested with *Eco*RI, blunt ended and cloned into the *Eco*RV site of pTxGFP, making pTX- pCR2.1-epsin$_{1\text{-}333}$GFP. Epsin$_{253\text{-}677}$ was amplified by PCR from pCR2.1-Epsin using 5′-TATAGTAATAGAGCAGGTGAGGAAACAAGAAG-3′ and 5′-CA-GATCCCATGCTATTAGTATTTCTATTCGC-3′ and ligated into pCR2.1 to make pCR2.1- epsin$_{253\text{-}677}$. Epsin$_{253\text{-}677}$ was cloned from pCR2.1- epsin$_{253\text{-}677}$. into pTx-GFP using *Bam*HI and *Xho*I, making the expression plasmid pTx-GFP- epsin$_{253\text{-}677}$. A cDNA encoding the PH domain of PLCδ (kind gift from Tobias Meyer, Stanford University, Stanford, CA) was cloned into pTxGFP using *Bam*HI and *Not*I to generate pTx-GFP-PH. PH:epsin$_{253\text{-}677}$ was generated by cloning the PH domain into pCR2.1-epsin$_{253\text{-}677}$ with *Hin*dIII and *Sac*I to make pCR2.1-PH:epsin$_{253\text{-}677}$. PH:epsin$_{253\text{-}677}$ was then cloned into pUC18 (Invitrogen) with *Hin*dIII and *Hin*cII to make pUC18-PH:epsin$_{253\text{-}677}$. Finally, PH:epsin$_{253\text{-}677}$ was cloned into pTx-GFP with *Kpn*I, making pTX- PH:epsin$_{253\text{-}677}$GFP. To generate pTX-epsin$_{253\text{-}677}$, an expression plasmid for epsin$_{253\text{-}677}$ without the GFP tag, we amplified a cDNA encoding epsin$_{253\text{-}677}$ with 5′-CAGTGTGCTGGTACCCGGCTTTATAGTAATAG-3′ and 5′-GATGGATAG-GATCCTAATTCGGCTTCAG-3′, ligated into pCR2.1, and cloned into pTxGFP with *Kpn*I and *Bam*HI, effectively replacing GFP with epsin$_{253\text{-}677}$. Plasmid maps were managed using Gene Construction Kit (Textco BioSoftware, West Lebanon, NH).

Dictyostelium transformation

Dictyostelium cell lines were transformed with various expression plasmids by electroporation. 5×10^6 cells in 100 μl buffer H-50 (20 mM HEPES, 50 mM KCl, 10 mM NaCl, 1 mM $MgSO_4$, 5 mM $NaHCO_3$, 1 mM NaH_2PO_4) were mixed with 10 μg plasmid and electroporated using a Bio-Rad Gene Pulser (Bio-Rad, Hercules, CA) at 75 kV and 25 μF.

Fluorescence microscopy

Cells expressing GFP expression plasmids were harvested and allowed to attach to glass coverslips for 10 minutes at 18°C and incubated with low-fluorescence media (Liu et al., 2002) for at least 20 minutes. Cells were fixed with 2% formaldehyde and 0.01% Triton X-100 in PDF (2 mM KCl, 11 mM K_2HPO_4, 13.2 mM KH_2PO_4, 0.1 mM $CaCl_2$, 0.25 mM $MgSO_4$, pH 6.7) at room temperature for 15 minutes and then in 100% methanol at –20°C for 5 minutes, then rinsed with PDF and mounted on glass slides. For immunostaining, cells on coverslips were blocked with 3% BSA (Fisher Scientific, Pittsburgh, PA) in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) for 20 minutes at 37°C, and then incubated with rabbit anti-CLC (Wang et al., 2003) or rabbit anti-AP2 IgG for 1 hour. Coverslips were rinsed with PBS, incubated for 30 minutes with 30 μg/ml goat anti-rabbit IgG conjugated to Texas Red (Molecular Probes, Invitrogen), and rinsed again in PBS. Coverslips were then rinsed in sterile, distilled water and mounted on glass slides. Images were taken using an inverted Nikon Eclipse TE200 microscope (Nikon

Instruments, Dallas, TX) with 100× 1.4 NA PlanFluor objective and a Quantix 57 camera (Roper Scientific, AZ) controlled by Metamorph software (Universal Images, PA). Confocal images were acquired on a Leica TCS-SP2 laser-scanning confocal inverted microscope (Leica Microsystems, Wetzlar, Germany). Images were processed using Metamorph (Molecular Devices, Sunnyvale, CA) and Adobe Photoshop (Adobe Systems, San Jose, CA) software.

Live cell imaging by TIRF microscopy

Cells were imaged by TIRF (total internal reflection fluorescence) microscopy at Washington University School of Medicine, St Louis, Missouri. Cells were harvested, rinsed with PDF, and allowed to attach to acid-washed glass coverslips. Cells were imaged live on an Olympus 1X81 inverted microscope (Olympus America, Center Valley, PA) with an XR/Mega-10 camera (Stanford Photonics, Palo Alto, CA) using a 488 nm laser.

Generation of anti-epsin antibodies

A cDNA for epsin$_{253\text{-}677}$ was cloned from pCR2.1-epsin$_{253\text{-}677}$ into pMAL-C2X (New England Biolabs, Ipswich, MA) with *Bam*HI and *Pst*I so that epsin$_{253\text{-}677}$ was downstream of maltose-binding protein (MBP), resulting in expression of MBP-epsin$_{253\text{-}677}$ fusion protein from the plasmid pMAL-MBP-epsin$_{253\text{-}677}$. The pMAL-MBP-epsin$_{253\text{-}677}$ expression plasmid was transformed into *Eschericha coli* BL21 and the fusion protein was purified according to the manufacturer's protocol. Purified MBP-epsin$_{253\text{-}677}$ was used to generate anti-epsin polyclonal antisera in rabbits (Cocalico Biologicals, Reamstown, PA).

Osmoregulation

Cells were harvested and allowed to attach to glass coverslips for 10 minutes at 18°C. Cells were then shifted to sterile, distilled water and imaged on an inverted Nikon Eclipse TE200 microscope using DIC optics.

Development and spores

To develop fruiting bodies, approximately 5×10^7 cells were harvested, washed with PDF and plated on starvation agar plates [0.2 mM $CaCl_2$, 2.0 mM $MgSO_4$, 20 mM MES pH 6.7, 1% Agar Noble (BD, Sparks, MD)]. Fruiting bodies were allowed to develop for 48 hours and then imaged using a Zeiss STEMI SR stereoscope (Carl Zeiss, Thornwood, NY). Spores were harvested from development plates by sharply striking the inverted plate on a hard surface and resuspending spores from the lid in PDF. Spores were plated on glass coverslips, allowed to settle, and imaged using an inverted Nikon Eclipse TE200 microscope with DIC optics.

Cytokinesis and growth in suspension

Cells were diluted to $1\times10^{4\text{-}5}$ cells/ml and grown in HL-5 on a rotary shaker at 218 r.p.m. at 18°C. Cultures were sampled periodically and counted on a hemocytometer. For DAPI (4′,6-diamidino-2-phenylindole) staining, cells were grown in suspension for 72 hours and then allowed to attach to glass coverslips for 10 minutes. Cells were fixed with 100% methanol at –20°C for 5 minutes and rinsed with PDF. Cells were then stained with 0.05 μg/ml DAPI (Invitrogen) in PDF for 10 minutes, rinsed, mounted on glass slides and imaged on an inverted Nikon Eclipse TE200 microscope.

Subcellular fractionation

Cells were fractionated according to Wang et al. (Wang et al., 2003). Briefly, cells were collected, washed, and resuspended to 4×10^7 cells/ml in MES isolation buffer (10 mM MES (pH 6.5), 50 mM potassium acetate, 0.5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, and 0.02% NaN_3) with protease inhibitors (Fungal Protease Inhibitor cocktail, Sigma-Aldrich, St Louis, MO). Cells were lysed by passing through two pieces of Osmonics (GE Osmonics, Trevose, PA) polycarbonate membrane (pore size: 5 μm) in a Gelman Luer-Lock-style filter (Gelman Sciences, Ann Arbor, MI). Cell lysates were centrifuged at 3000 ***g*** for 10 minutes at 4°C to generate a low speed pellet (LSP) and low speed supernatant (LSS). The LSS was ultracentrifuged at 100,000 ***g*** for 60 minutes at 4°C, resulting in a high-speed supernatant (HSS) and a high-speed pellet (HSP).

Site-directed mutagenesis

Epsin/ENTH$^{R65A/K78A}$ and Epsin/ENTH^{T107A} were generated using the Stratagene Quick Change Site-Directed Mutagensis Kit (Stratagene, La Jolla, CA) with primer pairs 5′-AATTATTATGGGTGTAATTTGGAAAGCTATTAATGATCCAGGCAA-GTTTTGG-3′ and 5′-CCAAAACTTGCCTGGATCATTAATAGCTTTCCAAAT-TACACCCATAATAATT-3′ (for R65A), 5′-GATCCAGGCAAGTTTTGGAGA-CATGTTTATGCATCACTTCTTCTTATCG-3′ and 5′-CGATAAGAAGAAGTGAT-GCATAAACATGTCTCCAAAACTTGCCTGGATC-3′ (for K78A), and 5′-GATTG-TAGACATCATACTATGGAAATTAAAGCATTGGTTGAGTTCCAA-3′ and 5′-TTGGAACTCAACCAATGCTTTAATTTCCATAGTATGATGTCTACAATC-3′ (for T107A).

MBP:epsin binding assay

100 ml *Dictyostelium* suspension culture was harvested, washed in ice-cold binding buffer (20 mM piperazine-N,N′-bis[2-ethanesulfonic acid] pH 6.8, 1.5 mM EDTA, 15 mM $MgCl_2$, 1 mM DTT and fungal protease inhibitor cocktail) (Vithalani et al., 1998) and resuspended to a concentration of 5×10^7 cells/ml. Cells were sonicated 5 times for 15 seconds at 50% power and centrifuged at 14,000 ***g*** for 20 minutes. MBP-epsin$_{253\text{-}677}$ or MBP alone was purified from 1 l bacterial culture according to the manufacturer's protocol (see above), with the exception that the protein was not eluted from the amylose resin. 400 μl of beads were incubated with 1 ml prepared *Dictyostelium* lysate for 2 hours at 4°C with shaking. Beads were washed several times with cold binding buffer, and the bound fraction was eluted with hot RSB (reducing sample buffer). Samples were analyzed using standard immunoblotting protocols.

Lipid binding assay

1 nmol PtdIns or PtdIns(4,5)P_2 (Echelon Biosciences, Salt Lake City, UT) was pipetted onto nitrocellulose membrane and allowed to air dry. The membrane was then blocked in ice-cold binding buffer with 3% dried milk for 30 minutes. *Dictyostelium* lysate was prepared as above and incubated with the membrane for 2 hours at 4°C with gentle shaking. The membrane was washed with 0.1% Tween-20 in PDF and then probed using standard immunoblotting protocols.

We thank members of the O'Halloran lab, Arturo De Lozanne, Janice Fischer and John Heuser for reading early versions of the manuscript. We are also thankful to Tobias Meyer for gift of the PH expression vector, and to John Heuser for advice, help and use of his TIRF microscope. This work is supported by an NSF Graduate Research Fellowship to R.J.B. and NIH RO1 GM048625 to T.J.O.

References

Aguilar, R. C., Watson, H. A. and Wendland, B. (2003). The yeast Epsin Ent1 is recruited to membranes through multiple independent interactions. *J. Biol. Chem.* **278**, 10737-10743.

Aguilar, R. C., Longhi, S. A., Shaw, J. D., Yeh, L. Y., Kim, S., Schon, A., Freire, E., Hsu, A., McCormick, W. K., Watson, H. A. et al. (2006). Epsin N-terminal homology domains perform an essential function regulating Cdc42 through binding Cdc42 GTPase-activating proteins. *Proc. Natl. Acad. Sci. USA* **103**, 4116-4121.

Barriere, H., Nemes, C., Lechardeur, D., Khan-Mohammad, M., Fruh, K. and Lukacs, G. L. (2006). Molecular basis of oligoubiquitin-dependent internalization of membrane proteins in Mammalian cells. *Traffic* **7**, 282-297.

Berdnik, D., Torok, T., Gonzalez-Gaitan, M. and Knoblich, J. A. (2002). The endocytic protein alpha-Adaptin is required for numb-mediated asymmetric cell division in Drosophila. *Dev. Cell* **3**, 221-231.

Cadavid, A. L., Ginzel, A. and Fischer, J. A. (2000). The function of the Drosophila fat facets deubiquitinating enzyme in limiting photoreceptor cell number is intimately associated with endocytosis. *Development* **127**, 1727-1736.

Chen, H., Fre, S., Slepnev, V. I., Capua, M. R., Takei, K., Butler, M. H., Di Fiore, P. P. and De Camilli, P. (1998). Epsin is an EH-domain-binding protein implicated in clathrin-mediated endocytosis. *Nature* **394**, 793-797.

Damer, C. K. and O'Halloran, T. J. (2000). Spatially regulated recruitment of clathrin to the plasma membrane during capping and cell translocation. *Mol. Biol. Cell* **11**, 2151-2159.

Dell'Angelica, E. C., Klumperman, J., Stoorvogel, W. and Bonifacino, J. S. (1998). Association of the AP-3 adaptor complex with clathrin. *Science* **280**, 431-434.

Drake, M. T., Downs, M. A. and Traub, L. M. (2000). Epsin binds to clathrin by associating directly with the clathrin-terminal domain: evidence for cooperative binding through two discrete sites. *J. Biol. Chem.* **275**, 6479-6489.

Edeling, M. A., Smith, C. and Owen, D. (2006). Life of a clathrin coat: insights from clathrin and AP structures. *Nat. Rev. Mol. Cell. Biol.* **7**, 32-44.

Ford, M. G., Mills, I. G., Peter, B. J., Vallis, Y., Praefcke, G. J., Evans, P. R. and McMahon, H. T. (2002). Curvature of clathrin-coated pits driven by epsin. *Nature* **419**, 361-366.

Hawryluk, M. J., Keyel, P. A., Mishra, S. K., Watkins, S. C., Heuser, J. E. and Traub, L. M. (2006). Epsin 1 is a polyubiquitin-selective clathrin-associated sorting protein. *Traffic* **7**, 927.

Hinrichsen, L., Harborth, J., Andrees, L., Weber, K. and Ungewickell, E. J. (2003). Effect of clathrin heavy chain- and alpha-adaptin-specific small inhibitory RNAs on endocytic accessory proteins and receptor trafficking in HeLa cells. *J. Biol. Chem.* **278**, 45160-45170.

Hinrichsen, L., Meyerholz, A., Groos, S. and Ungewickell, E. J. (2006). Bending a membrane: how clathrin affects budding. *Proc. Natl. Acad. Sci. USA* **103**, 8715-8720.

Hirst, J., Motley, A., Harasaki, K., Peak Chew, S. Y. and Robinson, M. S. (2003). EpsinR: an ENTH domain-containing protein that interacts with AP-1. *Mol. Biol. Cell* **14**, 625-641.

Hofmann, K. and Falquet, L. (2001). A ubiquitin-interacting motif conserved in components of the proteasomal and lysosomal protein degradation systems. *Trends Biochem. Sci.* **26**, 347-350.

Holstein, S. E. and Oliviusson, P. (2005). Sequence analysis of Arabidopsis thaliana E/ANTH-domain-containing proteins: membrane tethers of the clathrin-dependent vesicle budding machinery. *Protoplasma* **226**, 13-21.

Itoh, T., Koshiba, S., Kigawa, T., Kikuchi, A., Yokoyama, S. and Takenawa, T. (2001). Role of the ENTH domain in phosphatidylinositol-4,5-bisphosphate binding and endocytosis. *Science* **291**, 1047-1051.

Kalthoff, C., Alves, J., Urbanke, C., Knorr, R. and Ungewickell, E. J. (2002a). Unusual structural organization of the endocytic proteins AP180 and epsin 1. *J. Biol. Chem.* **277**, 8209-8216.

Kalthoff, C., Groos, S., Kohl, R., Mahrhold, S. and Ungewickell, E. J. (2002b). Clint: a novel clathrin-binding ENTH-domain protein at the Golgi. *Mol. Biol. Cell* **13**, 4060-4073.

Kay, B. K., Yamabhai, M., Wendland, B. and Emr, S. D. (1999). Identification of a novel domain shared by putative components of the endocytic and cytoskeletal machinery. *Protein Sci.* **8**, 435-438.

Lakadamyali, M., Rust, M. J. and Zhuang, X. (2006). Ligands for clathrin-mediated endocytosis are differentially sorted into distinct populations of early endosomes. *Cell* **124**, 997-1009.

Legendre-Guillemin, V., Wasiak, S., Hussain, N. K., Angers, A. and McPherson, P. S. (2004). ENTH/ANTH proteins and clathrin-mediated membrane budding. *J. Cell Sci.* **117**, 9-18.

Lemmon, M. A., Ferguson, K. M., O'Brien, R., Sigler, P. B. and Schlessinger, J. (1995). Specific and high-affinity binding of inositol phosphates to an isolated pleckstrin homology domain. *Proc. Natl. Acad. Sci. USA* **92**, 10472-10476.

Levi, S., Polyakov, M. and Egelhoff, T. T. (2000). Green fluorescent protein and epitope tag fusion vectors for Dictyostelium discoideum. *Plasmid* **44**, 231-238.

Liu, T., Mirschberger, C., Chooback, L., Arana, Q., Dal Sacco, Z., MacWilliams, H. and Clarke, M. (2002). Altered expression of the 100 kDa subunit of the Dictyostelium vacuolar proton pump impairs enzyme assembly, endocytic function and cytosolic pH regulation. *J. Cell Sci.* **115**, 1907-1918.

Mills, I. G., Praefcke, G. J., Vallis, Y., Peter, B. J., Olesen, L. E., Gallop, J. L., Butler, P. J., Evans, P. R. and McMahon, H. T. (2003). EpsinR: an AP1/clathrin interacting protein involved in vesicle trafficking. *J. Cell Biol.* **160**, 213-222.

Motley, A., Bright, N. A., Seaman, M. N. and Robinson, M. S. (2003). Clathrin-mediated endocytosis in AP-2-depleted cells. *J. Cell Biol.* **162**, 909-918.

Newpher, T. M., Smith, R. P., Lemmon, V. and Lemmon, S. K. (2005). In vivo dynamics of clathrin and its adaptor-dependent recruitment to the actin-based endocytic machinery in yeast. *Dev. Cell* **9**, 87-98.

Niswonger, M. L. and O'Halloran, T. J. (1997a). A novel role for clathrin in cytokinesis. *Proc. Natl. Acad. Sci. USA* **94**, 8575-8578.

Niswonger, M. L. and O'Halloran, T. J. (1997b). Clathrin heavy chain is required for spore cell but not stalk cell differentiation in Dictyostelium discoideum. *Development* **124**, 443-451.

O'Halloran, T. J. and Anderson, R. G. (1992). Clathrin heavy chain is required for pinocytosis, the presence of large vacuoles, and development in Dictyostelium. *J. Cell Biol.* **118**, 1371-1377.

Overstreet, E., Chen, X., Wendland, B. and Fischer, J. A. (2003). Either part of a Drosophila epsin protein, divided after the ENTH domain, functions in endocytosis of delta in the developing eye. *Curr. Biol.* **13**, 854-860.

Overstreet, E., Fitch, E. and Fischer, J. A. (2004). Fat facets and Liquid facets promote Delta endocytosis and Delta signaling in the signaling cells. *Development* **131**, 5355-5366.

Owen, D. J., Vallis, Y., Noble, M. E., Hunter, J. B., Dafforn, T. R., Evans, P. R. and McMahon, H. T. (1999). A structural explanation for the binding of multiple ligands by the alpha-adaptin appendage domain. *Cell* **97**, 805-815.

Polo, S., Sigismund, S., Faretta, M., Guidi, M., Capua, M. R., Bossi, G., Chen, H., De Camilli, P. and Di Fiore, P. P. (2002). A single motif responsible for ubiquitin recognition and monoubiquitination in endocytic proteins. *Nature* **416**, 451-455.

Repass, S. L., Brady, R. J. and O'Halloran, T. J. (2007). Dictyostelium Hip1r contributes to spore shape and requires epsin for phosphorylation and localization. *J. Cell Sci.* **120**, 3977-3988.

Salcini, A. E., Confalonieri, S., Doria, M., Santolini, E., Tassi, E., Minenkova, O., Cesareni, G., Pelicci, P. G. and Di Fiore, P. P. (1997). Binding specificity and in vivo targets of the EH domain, a novel protein-protein interaction module. *Genes Dev.* **11**, 2239-2249.

Stauffer, T. P., Ahn, S. and Meyer, T. (1998). Receptor-induced transient reduction in plasma membrane PtdIns(4,5)P2 concentration monitored in living cells. *Curr. Biol.* **8**, 343-346.

Stavrou, I. and O'Halloran, T. J. (2006). The monomeric clathrin assembly protein, AP180, regulates contractile vacuole size in Dictyostelium discoideum. *Mol. Biol. Cell* **17**, 5381-5389.

Sussman, M. (1987). Cultivation and synchronous morphogenesis of Dictyostelium under controlled experimental conditions. *Methods Cell Biol.* **28**, 9-29.

ter Haar, E., Harrison, S. C. and Kirchhausen, T. (2000). Peptide-in-groove interactions link target proteins to the beta-propeller of clathrin. *Proc. Natl. Acad. Sci. USA* **97**, 1096-1100.

Traub, L. M. (2003). Sorting it out: AP-2 and alternate clathrin adaptors in endocytic cargo selection. *J. Cell Biol.* **163**, 203-208.

Traub, L. M., Downs, M. A., Westrich, J. L. and Fremont, D. H. (1999). Crystal structure of the alpha appendage of AP-2 reveals a recruitment platform for clathrin-coat assembly. *Proc. Natl. Acad. Sci. USA* **96**, 8907-8912.

Vithalani, K. K., Parent, C. A., Thorn, E. M., Penn, M., Larochelle, D. A., Devreotes, P. N. and De Lozanne, A. (1998). Identification of darlin, a Dictyostelium protein with Armadillo-like repeats that binds to small GTPases and is important for the proper aggregation of developing cells. *Mol. Biol. Cell* **9**, 3095-3106.

Wang, W. and Struhl, G. (2004). Drosophila Epsin mediates a select endocytic pathway that DSL ligands must enter to activate Notch. *Development* **131**, 5367-5380.

Wang, W. and Struhl, G. (2005). Distinct roles for Mind bomb, Neuralized and Epsin in mediating DSL endocytosis and signaling in Drosophila. *Development* **132**, 2883-2894.

Wang, N., Wu, W. I. and De Lozanne, A. (2002). BEACH family of proteins: phylogenetic and functional analysis of six Dictyostelium BEACH proteins. *J. Cell Biochem.* **86**, 561-570.

Wang, J., Virta, V. C., Riddelle-Spencer, K. and O'Halloran, T. J. (2003). Compromise of clathrin function and membrane association by clathrin light chain deletion. *Traffic* **4**, 891-901.

Wang, J., Wang, Y. and O'Halloran, T. J. (2006). Clathrin light chain: importance of the conserved carboxy terminal domain to function in living cells. *Traffic* **7**, 824-832.

Wasiak, S., Legendre-Guillemin, V., Puertollano, R., Blondeau, F., Girard, M., de Heuvel, E., Boismenu, D., Bell, A. W., Bonifacino, J. S. and McPherson, P. S. (2002). Enthoprotin: a novel clathrin-associated protein identified through subcellular proteomics. *J. Cell Biol.* **158**, 855-862.

Wendland, B., Steece, K. E. and Emr, S. D. (1999). Yeast epsins contain an essential N-terminal ENTH domain, bind clathrin and are required for endocytosis. *EMBO J.* **18**, 4383-4393.

Zoncu, R., Perera, R. M., Sebastian, R., Nakatsu, F., Chen, H., Balla, T., Ayala, G., Toomre, D. and De Camilli, P. V. (2007). Loss of endocytic clathrin-coated pits upon acute depletion of phosphatidylinositol 4,5-bisphosphate. *Proc. Natl. Acad. Sci. USA* **104**, 3793-3798.