Curvature of clathrin-coated pits driven by epsin

Marijn G. J. Ford*, Ian G. Mills*, Brian J. Peter*, Yvonne Vallis, Gerrit J. K. Praefcke, Philip R. Evans & Harvey T. McMahon

MRC Laboratory of Molecular Biology, Hills Road, Cambridge, CB2 2QH, UK
** These authors contributed equally to this work*

Clathrin-mediated endocytosis involves cargo selection and membrane budding into vesicles with the aid of a protein coat. Formation of invaginated pits on the plasma membrane and subsequent budding of vesicles is an energetically demanding process that involves the cooperation of clathrin with many different proteins. Here we investigate the role of the brain-enriched protein epsin 1 in this process. Epsin is targeted to areas of endocytosis by binding the membrane lipid phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2). We show here that epsin 1 directly modifies membrane curvature on binding to PtdIns(4,5)P_2 in conjunction with clathrin polymerization. We have discovered that formation of an amphipathic α-helix in epsin is coupled to PtdIns(4,5)P_2 binding. Mutation of residues on the hydrophobic region of this helix abolishes the ability to curve membranes. We propose that this helix is inserted into one leaflet of the lipid bilayer, inducing curvature. On lipid monolayers epsin alone is sufficient to facilitate the formation of clathrin-coated invaginations.

Clathrin-mediated endocytosis is essential for many cellular processes, including synaptic vesicle recycling, nutrient uptake and removal of receptors and ion channels from the cell surface[1–5]. The formation of a new vesicle is thought to be driven by both the polymerization of the coat protein clathrin into a cage-like structure and the selection/modification of membrane lipids[5,6]. Epsin 1 is implicated in clathrin-mediated endocytosis by binding to the coat components, clathrin, Eps15 and the AP2 complex (Fig. 1a)[7–9]. There are now at least four known mammalian epsins with different expression patterns (epsins 1–3 and epsin R; see http://www.ncbi.nlm.nih.gov or http://www.kazusa.or.jp/en/). The epsin 1 homologue in *Drosophila* (*liquid facets*) is an essential gene and as with yeast epsins it has been implicated in endocytosis[10,11]. The most conserved feature of the epsin family is the epsin N-terminal homology (ENTH) domain. This domain is structurally homologous but distinct from the AP180 N-terminal homology (ANTH) domain found at the N termini of AP180, CALM and HIP. Both domains bind to PtdIns(4,5)P_2 (refs 12, 13).

Tubulation of liposomes

Although both the ANTH domain of AP180 and the ENTH domain of epsin bind to lipids they have distinct effects on liposome shape. Epsin or its ENTH domain converted liposomes made from total brain lipids (Folch extract containing 10% phosphoinositides) into tubules (Fig. 1b). In contrast, the ANTH domain of either AP180 or CALM had no effect on liposome morphology. The outer diameter of the tubules that formed owing to the action of the ENTH domain on liposomes was approximately one-third that formed by the action of either dynamin (not shown) or the N-terminus of amphiphysin (Fig. 1b). Addition of the ENTH domain to synthetic liposomes containing 10% PtdIns(4,5)P_2 (but not liposomes substituted with 10% phosphatidylserine (PtdSer)) also induced tubulation, but the tubules were frequently irregular and many were fragmented into small vesicles (not shown). To begin to understand these effects of epsin we investigated the structural basis of epsin binding to phosphoinositol head groups.

PtdIns(4,5)P_2 binding by epsin

The epsin ENTH domain was crystallized in the presence of inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3) and the structure was solved at 1.7 Å resolution (Fig. 2a; See also Supplementary Information Table 1). Compared with an earlier structure of this domain solved in the absence of a lipid head group[14] (Fig. 2a, middle structure), a new helix became ordered at the N-terminus, which we have named 'helix zero'. There is also a small rotation in α8 due to different crystal packing. Helix 0 (α0, residues 3–15) folds back across the face of α1 and the loop between α1 and α2. This creates a deep basic groove that forms the binding pocket for the Ins(1,4,5)P_3 ligand (Fig. 2a, c). The ligand is coordinated by residues from α0, α1, the α1–α2 loop, α3 and α4, and all three phosphates are multiply coordinated (Fig. 2b). This coordination is consistent with NMR data, although α0 was undefined in the structure[12]. We obtained an additional crystal form in the presence of Ins(1,4,5)P_3 (solved to 1.57 Å and identical to that solved by ref. 14 in the absence of ligand), which contained no density for bound ligand or α0. These data indicate that binding of Ins(1,4,5)P_3 and α0 formation are coupled. The increase in α-helicity induced by the inositol head group was confirmed by circular dichroism spectroscopy (data not shown). α0 is amphipathic, displaying a series of hydrophobic residues (L6, M10, I13) on its outer surface (Fig. 2c; see also Supplementary Information Fig. 1), while the head group is coordinated by its inner surface. This helix is orientated such that it has the potential to interact with the membrane.

The coordination of Ins(1,4,5)P_3 in the crystal structure suggested that epsin is specific for particular head groups, and we confirmed this by affinity measurement using isothermal titration calorimetry (Fig. 3a). We found head-group binding only if the inositol bears a phosphate on both positions 4 and 5, as binding of Ins(1,4)P_2 and Ins(1,5)P_2 was not detected. Ins(1,4,5)P_3 bound with an affinity of 3.6 ± 0.4 μM. In the crystal structure residues R8, H73 and the main chain N of N30 coordinate phosphate 4, and K11, R25 and R63 coordinate phosphate 5 (Fig. 2b, c). This coordination of the ligand by residues on the inner surface of helix 0 yields a large binding enthalpy that compensates for the entropic penalty of the formation of helix 0 (Supplementary Information Table 2). Epsin bound $InsP_6$ with a higher affinity than Ins(1,4,5)P_3 (0.55 μM compared with 3.6 μM) and with a 1:1 stoichiometry. The short chain lipid di-*O*-octanoglyceryl-phosphatidylinositol-4,5-biphosphate (diC_8PtdIns(4,5)P_2) also had a higher affinity than its corresponding head group, implying small additional contributions by the glycerol backbone, although the head group is still the main determinant of the strength of the interaction. Head-group binding measured by calorimetry, and lipid binding measured by pelleting with liposomes, showed the same specificities (ref. 12, Fig. 3b and

data not shown). For comparison, the ANTH domain of AP180 had an affinity of 30 μM for Ins(1,4,5)P_3 and 5.1 μM for InsP_6. In contrast to epsin, two ANTH domains bound to each InsP_6 molecule, indicative of a binding site exposed on the surface of the protein (see Fig. 2a), in agreement with our previous observations[13] and the much smaller entropic loss (Supplementary Information Table 2). For this reason and on the basis of consensus sequences surrounding the AP180 lipid-binding site we propose the subdivision of the epsin/AP180 families. The family of ANTH domain proteins can be defined by the consensus sequence (K/G)A(T/I)x_6(P/L/V)KxK(H/Y). The mammalian ENTH domain family can also be defined by a consensus over the same region, (D/E)ATx_2(D/E)PWGP. This takes into account that not all epsins necessarily bind PtdIns(4,5)P_2, indeed we predict that *Drosophila* epsin-like protein and its mammalian homologue, which we call epsinR (epsin-related protein, KIAA0171), bind to a less phosphorylated head group (Fig. 2d).

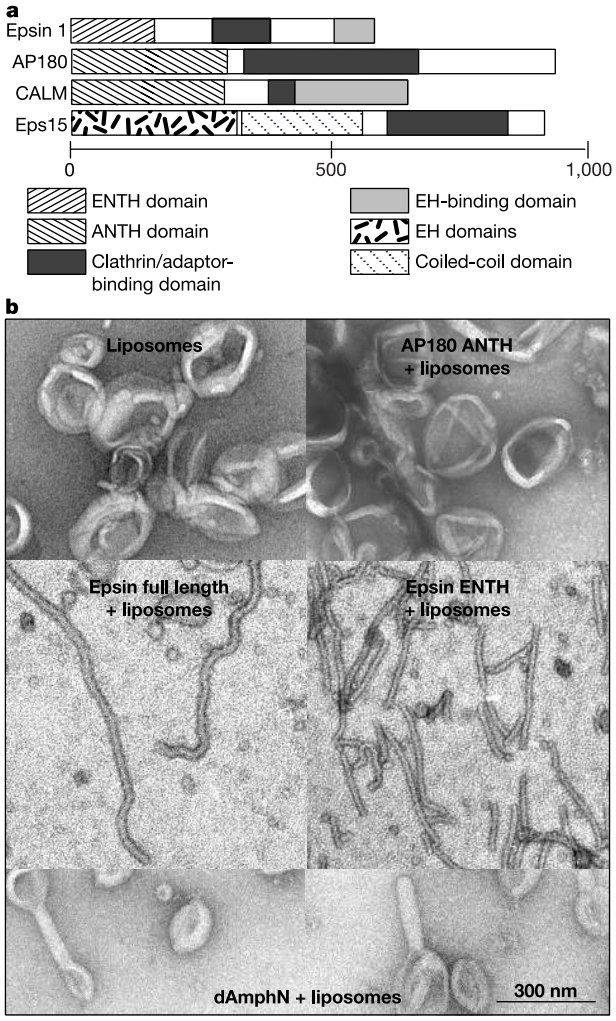

Figure 1 Epsin 1 ENTH domain tubulates liposomes. **a**, Modular arrangement of endocytic proteins. The epsin family can be recognized by the presence of an N-terminal lipid-binding ENTH domain and a clathrin/adaptor-binding domain. The ENTH domain is homologous over the first 150 residues to the ANTH domains of AP180, CALM and HIP1, although the lipid-binding residues are very different (see Fig. 2d). The ENTH domain is followed by ubiquitin-interacting motifs. The adaptor-binding motifs in epsin 1 (eight DPW motifs) and DPF-like motifs in other endocytic proteins bind to the appendage domains of the AP2 complex[25]. NPF motifs at the C terminus of epsin 1 interact with the Eps15 homology (EH) domain of Eps15 (refs 7–9). The scale bar is in amino acid residues. **b**, Electron microscopy of liposomes in the presence of the domains indicated. dAmphN, *Drosophila* amphiphysin N-terminus[26]. The outer diameter of tubules formed with epsin ENTH domain was 15 ± 1 nm, that of tubules formed with full-length epsin was 19 ± 3 nm, and that in the presence of dAmphN was 46 ± 2 nm.

Cell expression studies of epsin 1

In vivo, expression of the epsin ENTH domain in COS cells gave many intensely stained rod-like profiles (not seen with PtdIns(4,5)P_2 binding mutants), which may be indicative of folding in the plane of the membrane or tubulation (Fig. 4). Full-length epsin showed either a general cytoplasmic distribution or accumulation in puncta on the plasma membrane[9] (Fig. 4). The puncta co-labelled with antibodies for the AP2 complex (see enlargements), clathrin, Eps15 and dynamin, but not for the AP1 complex, the early endosome marker EEA1, or the Golgi marker GM130 (Supplementary Information Fig. 2 and see http://www2.mrc-lmb.cam.ac.uk/groups/hmm/epsin/IF). These puncta probably represent endocytically incompetent coated-pits, because dynamin is not present in mature vesicles and the puncta were found on the plasma membrane (confocal sectioning not shown). An inhibition of coated vesicle formation by epsin was confirmed using the suspension cell line RPMI 1788 cells (Fig. 3d). Cells transfected with epsin or with the AP180 carboxy terminus[13] had reduced numbers of coated vesicles, whereas vesicles accumulate with the uncoating mutant auxilin N844 (ref. 15).

To probe the function of PtdIns(4,5)P_2 binding we made a double mutant, R63L H73L, that no longer bound to liposomes containing PtdIns(4,5)P_2 or to Ins(1,4,5)P_3 (Fig. 3a, b). The expression pattern of the full-length mutant in COS cells was now always cytoplasmic and the AP2 complex tended to aggregate (Fig. 4, third row) whereas clathrin remained largely unaltered with a punctate distribution (not shown). Transferrin endocytosis was inhibited in more than 90% of these cells, even in cells with a low level of protein expression. Thus targeting of epsin to the plasma membrane by phosphoinositide binding is linked to correct localization of the cargo recruitment complex AP2. The retention of clathrin puncta in cells expressing R63L H73L shows that other proteins can maintain clathrin recruitment.

In contrast to R63L H73L epsin, we reported previously that overexpression of the AP180 C-terminus caused a redistribution of both the perinuclear and plasma membrane pools of clathrin[13]. This correlates with the greater binding capacity of AP180 C-terminus for clathrin whereas epsin binds better to the AP2 complex (Fig. 3c; see also ref. 16). The epsin used in our experiments (epsin 1) had no effect on AP1 staining (and did not bind to γ-adaptin; Fig. 3c). Thus epsin 1 mutants may be more specific inhibitors of AP2-mediated, coated-vesicle transport, whereas AP180 mutants would be predicted to affect all clathrin-mediated budding events.

Role of helix 0 in membrane curvature

Tubulation of liposomes has been observed with a number of proteins[17–19]. In the cases of dynamin, amphiphysin and endophilin the tubulation can be attributed at least in part to self-oligomerization. Electron microscopy observations revealed that amphiphysin (Fig. 1b) and dynamin can both form tubular extensions from liposomes. With epsin, these intermediates were not seen, implying that it does not tubulate by means of oligomerization. In the case of epsin ENTH domain, we were not able to detect oligomerization by crosslinking on liposomes or by gel-filtration or ultracentrifugation, in the absence or presence of saturating concentrations of head group, and at protein concentrations up to 0.5 mM (not shown). The protein clearly behaved as a monomer, so we conclude that monomeric interactions cause changes in membrane curvature.

We have noted the formation of an amphipathic helix 0 on binding of Ins(1,4,5)P_3 to the ENTH domain (Fig. 2). The hydrophobic outer surface of helix 0 was targeted for mutagenesis. Mutants of residue L6 to E, Q, H and W showed less binding to liposomes, implying a role for helix 0 in membrane interactions

(Fig. 3b). Furthermore, the ability of these mutants to tubulate liposomes was related to hydrophobicity of the substituted residue (Fig. 5). Thus L6Q with its hydrophobic and polar component bound to liposomes but failed to tubulate them. L6H tubulated liposomes (although less markedly than wild type), whereas L6W with its larger hydrophobic surface led to extensive tubulation and vesiculation. Although the L6E mutant still bound to short chain lipids in solution (Fig. 3a), the negative charge on the outer surface of this helix probably repels the membrane. From this we conclude that the hydrophobic outer surface of helix 0 is crucial for the induction of membrane curvature.

We tested these mutants in full-length epsin for their distribution in COS cells (Fig. 4, fourth row and data not shown) and found that all the L6 mutants still localize in puncta just like wild-type epsin. Consistent with epsin targeting being predominantly due to binding of PtdIns(4,5)P_2 head groups, we found that the L6E and L6Q mutants can indeed bind to short chain lipid diC_8PtdIns(4,5)P_2 and Ins(1,4,5)P_3 with affinities close to wild type (Fig. 3a).

Membrane invagination coupled to clathrin polymerization

To study the effects of epsin on clathrin recruitment in conjunction with lipid binding we used lipid monolayers containing PtdIns(4,5)P_2 (Fig. 6). We previously introduced this methodology for visualization and dissection of clathrin assembly by electron microscopy[13], and we found here that AP180 recruited clathrin to the monolayer where it polymerized into patches of defined size (see also Fig. 6f). These patches are approximately the diameter of coated vesicles in the brain, consistent with the use of brain isoforms of proteins and brain-derived clathrin. This points to a function of AP180 in limiting vesicle size (see also refs 13, 20, 21). We showed

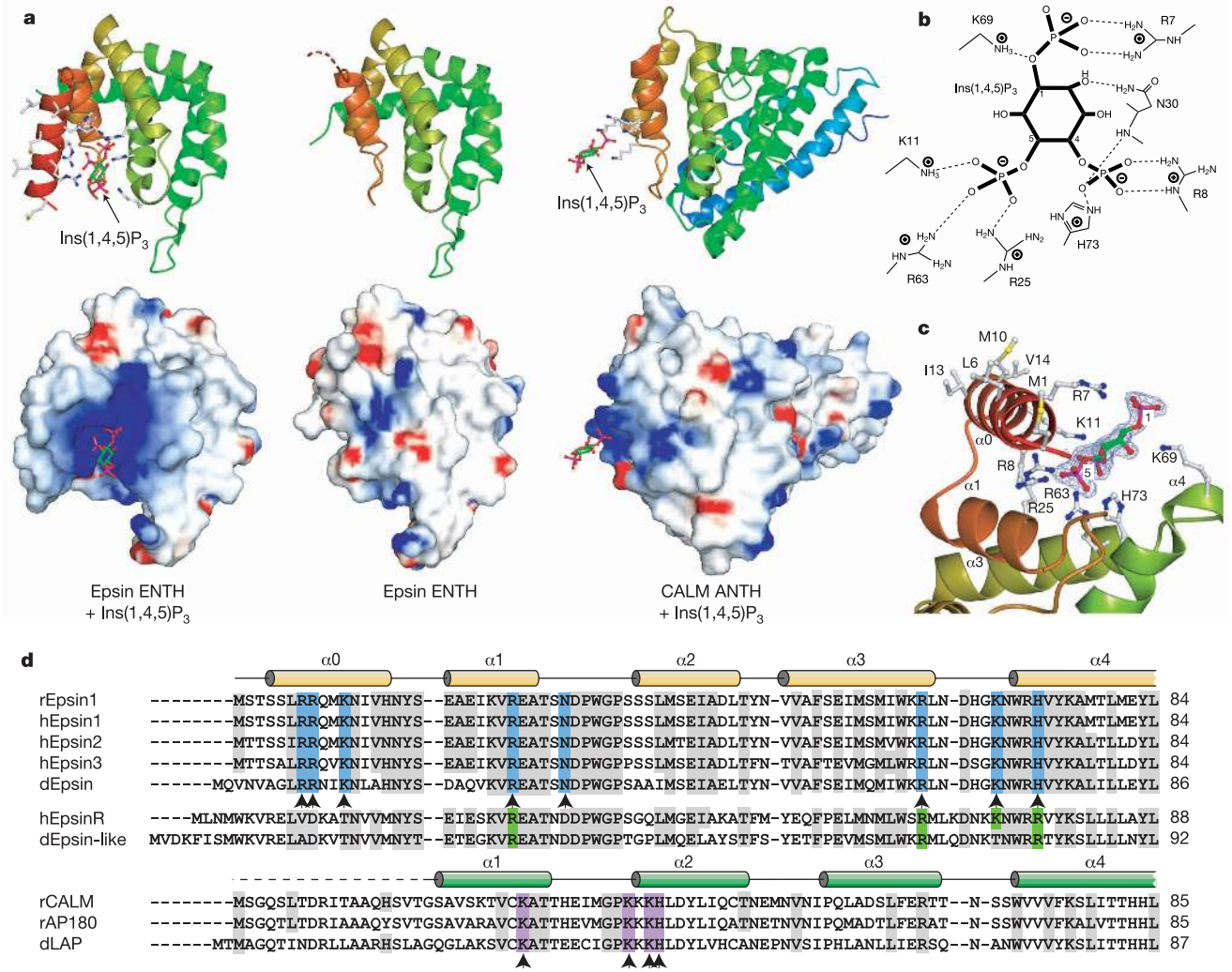

Figure 2 Structure of epsin ENTH bound to Ins(1,4,5)P_3. **a**, Ribbon diagrams of epsin ENTH bound to Ins(1,4,5)P_3 (Protein DataBank (PDB) accession number 1H0A), and for comparison the previous structures of epsin ENTH solved in the absence of Ins(1,4,5)P_3 (PDB 1EDU[14]) and CALM ANTH bound to diC_8PtdIns(4,5)P_2 (PDB 1HFA[13]). The structures are coloured red to blue from N- to C-termini, with corresponding helices having the same colour. Surface electrostatic potentials (red $-$ 10 kT e^{-1}; blue $+$ 10 kT e^{-1}) of each structure are shown below. Ins(1,4,5)P_3 binds to CALM on a positively charged surface not present in epsin. **b**, Schematic diagram of the interactions responsible for binding the Ins(1,4,5)P_3 molecule. **c**, Close-up view of the Ins(1,4,5)P_3 binding site, showing the residues responsible for interaction with the ligand. The hydrophobic residues L6, M10 and I13 exposed on formation of helix 0 are also marked. The structure shows that lipid binding and an interaction of helix 0 with the bilayer could happen simultaneously. The electron density for the ligand is shown, contoured at 0.168 e Å^{-3}. **d**, Sequence alignments comparing the lipid-binding residues of all the epsin family members with corresponding residues from the ANTH domains of AP180, CALM and LAP (the *Drosophila* AP180 homologue). Critical residues for Ins(1,4,5)P_3 binding to epsin 1 are coloured in blue and are conserved in epsins 1, 2 and 3 and in *Drosophila* epsin (*liquid facets*). The lipid-binding residues are not well conserved in epsinR/*Drosophila* epsin-like (see residues coloured green), suggesting a different lipid specificity of this epsin. The epsin Ins(1,4,5)P_3-binding residues are not conserved in AP180 and CALM, where a different set of residues have been identified as being involved in Ins(1,4,5)P_3 binding, coloured in purple[13]. h, human; d, *Drosophila*; r, rat.

that these patches are flat lattices, whereas the presence of the AP2 complex gave limited invagination[13]. Epsin, similar to AP180, was able to recruit and promote clathrin polymerization on the monolayer (Fig. 6a, d, e; see also http://www2.mrc-lmb.cam.ac.uk/groups/hmm/epsin/EM), but the clathrin was less uniformly polymerized than in the presence of AP180, as seen from the high background of clathrin triskelia (Fig. 6d, compare with b and c) and the less efficient recruitment of clathrin to liposomes by epsin (data not shown). Where there was clathrin polymerization by epsin, it was generally more extensive than with AP180, and showed clear signs of invagination. Pentagons can be resolved in the clathrin patches (implying curvature) and the three-dimensional shape of the clathrin-coated buds can be visualized using stereo images (Fig. 6d). Shadowing the clathrin patches with platinum also showed that they are invaginated; however, this process destroyed most of these structures (not shown). Even without shadowing we frequently found footprints (both large and small) where the invaginations have been washed off (see left of panel c). We also found this process at an intermediate stage of partial detachment (see right of panel b). Neither the R63L H73L nor the L6E mutants formed clathrin-coated invaginations (data not shown). These observations provide clear evidence of a role for epsin in membrane invagination together with the polymerization of clathrin.

Epsin was originally identified as a principal binding partner of

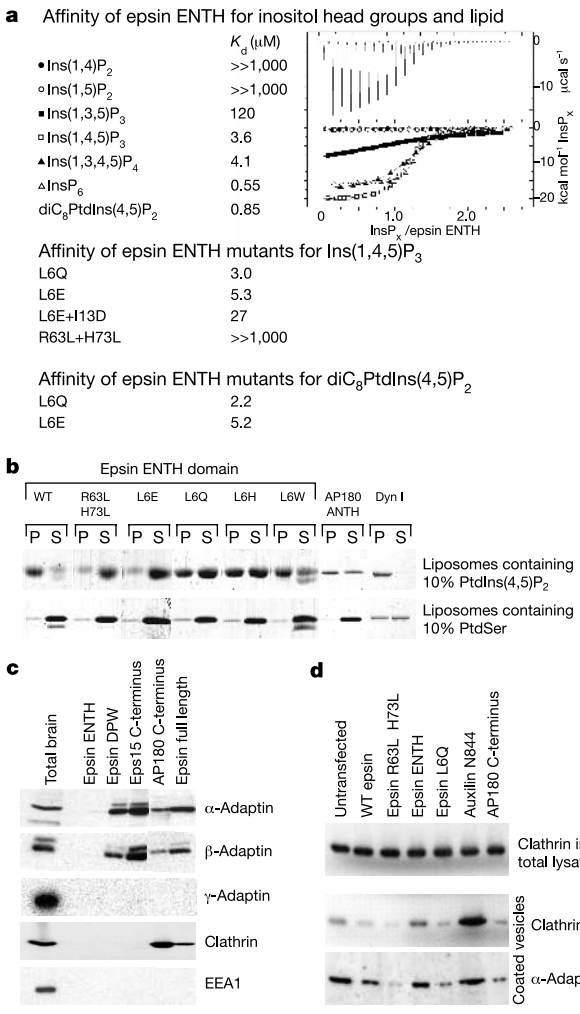

Figure 3 Binding of epsin ENTH and mutants to phosphoinositides. **a**, Dissociation constants (K_d) for wild-type and mutant epsin ENTH domains with inositol phosphates and the short chain lipid diC$_8$PtdIns(4,5)P_2 determined by calorimetric titration at 10 °C. **b**, Liposome pelleting assays. Only liposomes containing 10% PtdIns(4,5)P_2 efficiently sedimented epsin ENTH, whereas the Ins(1,4,5)P_3 binding site mutant (R63L H73L) was not pelleted. Dyn 1, dynamin 1; P, pellet; S, supernatant. **c**, Binding partners in rat brain extract for the indicated proteins. **d**, Purified clathrin-coated vesicles from RPMI1788 B cells transiently transfected with the indicated proteins. Clathrin in the total lysates is shown as a control for cell numbers used. Reduced numbers of clathrin-coated vesicles are present in cells overexpressing full-length epsins.

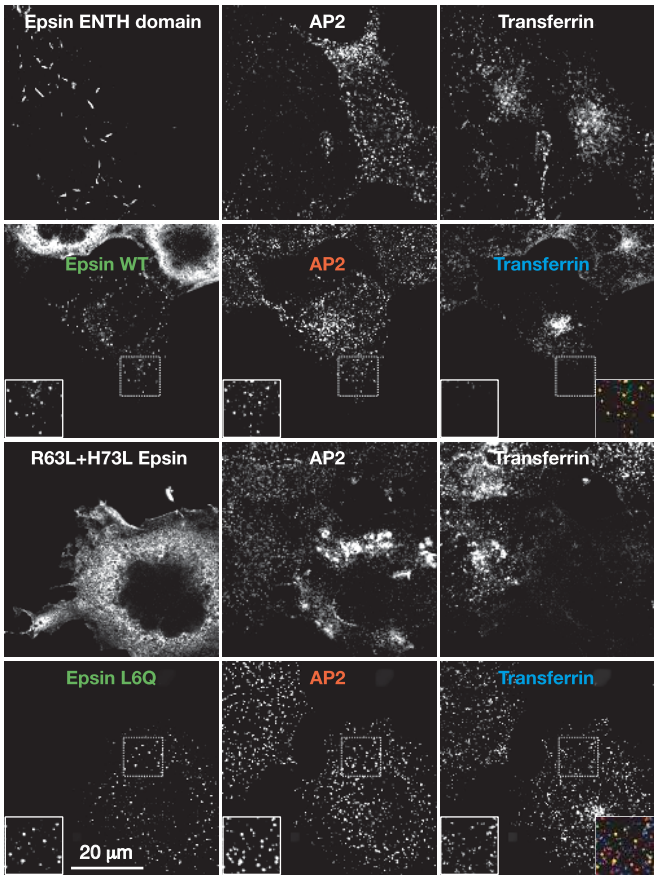

Figure 4 Effect of full-length epsin and mutants on the distribution of the AP2 complex in COS-7 cells visualized by sequential imaging with confocal microscopy. The three columns show the distribution of transfected epsin proteins (left, anti-Myc tag), endogenous AP2 (middle, AP6 antibody) and transferrin uptake (right). A strong perinuclear staining of transferrin is indicative of wild-type endocytosis. Selected enlargements of cells are shown (bottom left corner) to illustrate the co-localization of epsin with AP2, with a merged image in the right of the transferrin column. For co-localization with other endocytic proteins see http://www2.mrc-lmb.cam.ac.uk/groups/hmm/epsin/IF

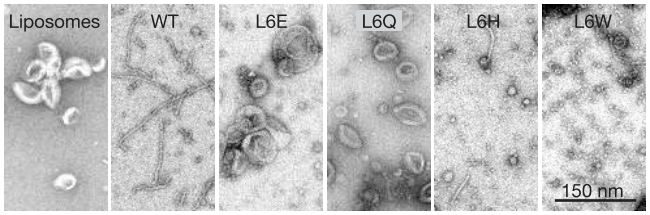

Figure 5 Mutations of L6 on helix 0 affect the ability of epsin to tubulate liposomes. Total bovine brain liposomes (Folch extract) were incubated with 40 μM mutant and wild type (WT) epsin ENTH domains, negatively stained and visualized by electron microscopy. Hydrophobicity of the mutant residue and relative levels of liposome binding (taken from Fig. 3b) are shown.

Eps15, which has been localized to the edge of a forming coated pit[22]. Therefore epsin may also be targeted to this region where the need for curvature-forming molecules will be greatest. This implies that epsin will not be needed to maintain curvature once the clathrin lattice is laid down and indeed we have previously shown that AP2 facilitates curvature of the lattice on membranes[23]. Our results also point to the cooperativity between AP2 and epsin recruitment to membranes, as we found that the localization of the R63L H73L mutant is cytoplasmic, and AP2 staining is no longer punctate (leading to an inhibition of transferrin endocytosis). Thus membrane invagination and cargo recruitment may go hand-in-hand. Epsin 1 is not enriched in mature coated vesicles of the brain (I.G.M. and H.T.McM., unpublished observations; see also ref. 9), and on liposomes it forms narrower tubules than those formed by amphiphysin or a dynamin. Thus if epsin were finally concentrated at the neck of the budding vesicle, then the narrowing of the neck may provide a weak point for the separation of the vesicle from the parent membrane on GTP hydrolysis of dynamin.

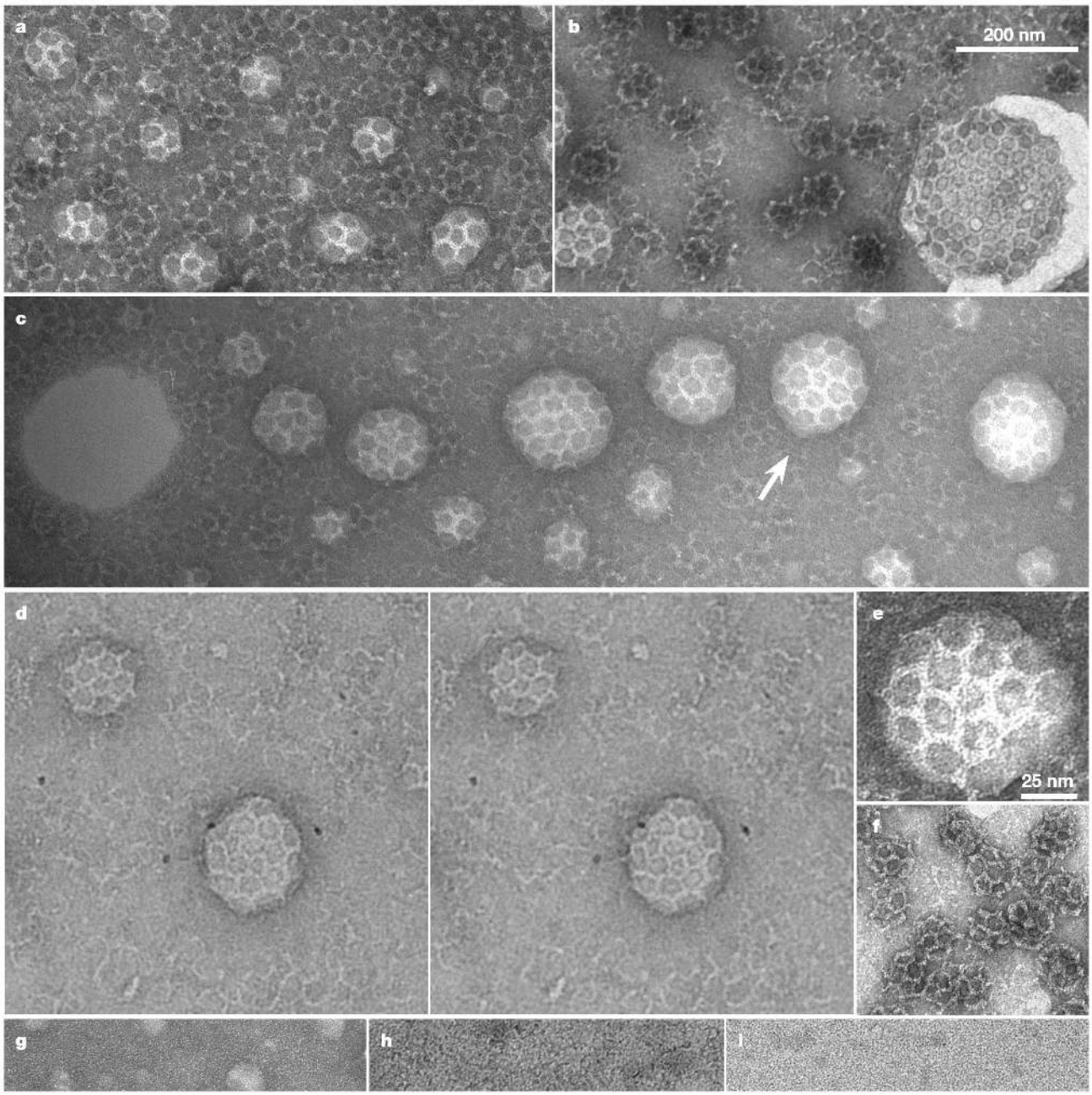

Figure 6 Clathrin recruitment to lipid monolayers by epsin. **a**, Epsin recruits clathrin to lipid monolayers containing 10% PtdIns(4,5)P_2 and stimulates clathrin lattice assembly. The irregular lattices contain both pentagons and hexagons and therefore are slightly raised from the surface of the monolayer. The background lattices are irregular and variable in size and shape. **b**, **c**, In the presence of epsin and AP180, invaginated assemblies are still formed, but the background lattices show a more regular structure reminiscent of the lattices formed when AP180 and clathrin alone are incubated with the monolayers (see **f**). The assemblies contain pentagons and occasionally heptagons (see arrow). We presume that empty circular profiles in the monolayer result from assemblies that have been sheared off. **d**, Stereo image of a section of monolayer incubated with epsin and clathrin. The assemblies clearly protrude from the surface of the monolayer. **e**, Close-up view of an epsin and clathrin assembly. **g**–**i**, Control experiments with monolayers. **g**, epsin ENTH and clathrin; **h**, epsin alone; **i**, clathrin alone. The scale bar in **b** applies to all panels except **e**. A gallery of high-resolution images is available at http://www2.mrc-lmb.cam.ac.uk/groups/hmm/epsin/EM.

So what is the role of epsin in endocytosis? We have shown that epsin causes a strong degree of membrane curvature and tubulation, even fragmentation of membranes with a high PtdIns(4,5)P_2 content. We envisage that epsin binding to membranes facilitates their deformation by insertion of helix 0 into the outer leaflet of the bilayer, pushing the head groups apart. This would reduce the energy needed to curve the membrane into a vesicle, making it easier for the clathrin cage to fix and stabilize the curved membrane (see also ref. 24). Thus our experiments point to a pioneering role for epsin in vesicle budding as it provides both a driving force and a link between membrane invagination, clathrin polymerization and AP2 complex recruitment. □

Methods

See Supplementary Information for the complete description of the Methods (Protein expression and purification; Transfections; Crystallisation and structure determination; Isothermal titration calorimetry).

Liposome tubulation, sedimentation and electron microscopy

Synthetic liposomes contained 10% cholesterol, 40% phosphatidylethanolamine, 40% phosphatidylcholine, and 10% of the test lipid (in most cases phosphatidylinositol(4,5)bisphosphate). Bovine brain lipids (Folch Fraction 1, which contains approximately 10% phosphatidylinositol lipids) were purchased from Sigma (B1502). Liposomes were resuspended at 1 mg m^{-1} in 50 mM HEPES, pH 7.4, 120 mM NaCl and extruded through a 0.4-μm cyclopore filter. For electron microscopy (EM), proteins (4 μM) were incubated with liposomes (0.1 mg ml^{-1}) for 1 min and absorbed onto a glow-discharged carbon-coated EM grid and stained with uranyl acetate before visualisation. For tubulation and sedimentation assays GST tags have been cleaved from the proteins but the presence of an N-terminal GST tag did not prevent either the tubulation of Folch liposomes by epsin or sedimentation. Hydrophobicity of the L6 mutants was determined using the Hopp–Woods scale[27]. For spin assays liposomes and protein (final volume of 40 μl) were incubated for 10 min at room temperature and then spun at 30,000 r.p.m. for 10 min in a Beckman TLA 100 rotor.

Lipid monolayers (same composition as for synthetic liposomes) were formed on the surface of a buffer droplet in a Teflon block and protein(s) of interest were introduced into the buffer[28]. A carbon-coated gold EM grid was placed on the monolayer, incubated at room temperature for 60 min, and then removed and stained with uranyl acetate.

Received 25 April; accepted 17 July 2002; doi:10.1038/nature01020.

1. Cremona, O. & De Camilli, P. Synaptic vesicle endocytosis. *Curr. Opin. Neurobiol.* **7,** 323–330 (1997).
2. Schmid, S. L. Clathrin-coated vesicle formation and protein sorting: an integrated process. *Annu. Rev. Biochem.* **66,** 511–548 (1997).
3. Hirst, J. & Robinson, M. S. Clathrin and adaptors. *Biochim. Biophys. Acta* **1404,** 173–193 (1998).
4. Marsh, M. & McMahon, H. T. The structural era of endocytosis. *Science* **285,** 215–220 (1999).
5. Brodsky, F. M., Chen, C. Y., Knuehl, C., Towler, M. C. & Wakeham, D. E. Biological basket weaving: formation and function of clathrin-coated vesicles. *Annu. Rev. Cell Dev. Biol.* **17,** 517–568 (2001).
6. Schmidt, A. *et al.* Endophilin I mediates synaptic vesicle formation by transfer of arachidonate to lysophosphatidic acid. *Nature* **401,** 133–141 (1999).
7. Salcini, A. E., Chen, H., Iannolo, G., De Camilli, P. & Di Fiore, P. P. Epidermal growth factor pathway substrate 15, Eps15. *Int. J. Biochem. Cell Biol.* **31,** 805–809 (1999).
8. McPherson, P. S. *et al.* EH domain-dependent interactions between Eps15 and clathrin-coated vesicle protein p95. *Biochem. Biophys. Res. Commun.* **244,** 701–705 (1998).
9. Chen, H. *et al.* Epsin is an EH-domain-binding protein implicated in clathrin-mediated endocytosis. *Nature* **394,** 793–797 (1998).
10. Cadavid, A. L., Ginzel, A. & Fischer, J. A. The function of the *Drosophila* fat facets deubiquitinating enzyme in limiting photoreceptor cell number is intimately associated with endocytosis. *Development* **127,** 1727–1736 (2000).
11. Wendland, B., Steece, K. E. & Emr, S. D. Yeast epsins contain an essential N-terminal ENTH domain, bind clathrin and are required for endocytosis. *EMBO J.* **18,** 4383–4393 (1999).
12. Itoh, T. *et al.* Role of the ENTH domain in phosphatidylinositol-4,5-bisphosphate binding and endocytosis. *Science* **291,** 1047–1051 (2001).
13. Ford, M. G. J. *et al.* Simultaneous binding of PtdIns(4,5)P2 and clathrin by AP180 in the nucleation of clathrin lattices on membranes. *Science* **291,** 1051–1055 (2001).
14. Hyman, J., Chen, H., Di Fiore, P. P., De Camilli, P. & Brunger, A. T. Epsin 1 undergoes nucleocytosolic shuttling and its eps15 interactor NH(2)-terminal homology (ENTH) domain, structurally similar to Armadillo and HEAT repeats, interacts with the transcription factor promyelocytic leukemia Zn(2) + finger protein (PLZF). *J. Cell Biol.* **149,** 537–546 (2000).
15. Morgan, J. R., Prasad, K., Jin, S., Augustine, G. J. & Lafer, E. M. Uncoating of clathrin-coated vesicles in presynaptic terminals: roles for Hsc70 and auxilin. *Neuron* **32,** 289–300 (2001).
16. Drake, M. T., Downs, M. A. & Traub, L. M. 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 (2000).
17. Sweitzer, S. M. & Hinshaw, J. E. Dynamin undergoes a GTP-dependent conformational change causing vesiculation. *Cell* **93,** 1021–1029 (1998).
18. Takei, K., Slepnev, V. I., Haucke, V. & De Camilli, P. Functional partnership between amphiphysin and dynamin in clathrin-mediated endocytosis. *Nature Cell Biol.* **1,** 33–39 (1999).
19. Farsad, K. *et al.* Generation of high curvature membranes mediated by direct endophilin bilayer interactions. *J. Cell Biol.* **155,** 193–200 (2001).
20. Zhang, B. *et al.* Synaptic vesicle size and number are regulated by a clathrin adaptor protein required for endocytosis. *Neuron* **21,** 1465–1475 (1998).
21. Kalthoff, C., Alves, J., Urbanke, C., Knorr, R. & Ungewickell, E. J. Unusual structural organization of the endocytic proteins AP180 and epsin 1. *J. Biol. Chem.* **277,** 8209–8216 (2001).
22. Tebar, F., Sorkina, T., Sorkin, A., Ericsson, M. & Kirchhausen, T. Eps15 is a component of clathrin-coated pits and vesicles and is located at the rim of coated pits. *J. Biol. Chem.* **271,** 28727–28730 (1996).
23. Owen, D. J., Vallis, Y., Pearse, B. M., McMahon, H. T. & Evans, P. R. The structure and function of the beta 2-adaptin appendage domain. *EMBO J.* **19,** 4216–4227 (2000).
24. Nossal, R. Energetics of clathrin basket assembly. *Traffic* **2,** 138–147 (2001).
25. Owen, D. J. *et al.* A structural explanation for the binding of multiple ligands by the α-adaptin appendage domain. *Cell* **97,** 805–815 (1999).
26. Razzaq, A. *et al.* Amphiphysin is necessary for organization of the excitation-contraction coupling machinery of muscles, but not for synaptic vesicle endocytosis in *Drosophila*. *Genes Dev.* **15,** 2967–2979 (2001).
27. Ford, M. G. J. *et al.* Simultaneous binding of PtdIns(4,5)P2 and clathrin by AP180 in the nucleation of clathrin lattices on membranes. *Science* **291,** 1051–1055 (2001).
28. Hopp, T. P. & Woods, K. R. Prediction of protein antigenic determinants from amino acid sequences. *Proc. Natl Acad. Sci. USA* **78,** 3824–3828 (1981).

Supplementary Information accompanies the paper on *Nature*'s website (http://www.nature.com/nature) and from http://www2.mrc-lmb.cam.ac.uk/groups/hmm/epsin/.

Acknowledgements

We thank O. Perisic and R. Williams for advice on protein–membrane interactions; B. Collins and staff at the ESRF for assistance with data collection; J. Butler for assistance with analytical ultracentrifugation; and J. Berriman for assistance with electron microscopy. We also thank B. Pearse, N. Unwin, M. Stowell, M. Goedert, S. Munro, P. Rosenthal and members of our laboratories for extensive discussions, and J. Gallop for a supply of dynamin. I.G.M. was supported by an MRC Postdoctoral Fellowship, B.J.P was supported by an EMBO Long Term Fellowship, and G.J.K.P. was supported by a Marie Curie Fellowship.

Competing interests statement

The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to H.T.McM. (e-mail: hmm@mrc-lmb.cam.ac.uk) or P.R.E. (e-mail: pre@mrc-lmb.cam.ac.uk).