Membrane Fission Is Promoted by Insertion of Amphipathic Helices and Is Restricted by Crescent BAR Domains

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SUMMARY

Shallow hydrophobic insertions and crescent-shaped BAR scaffolds promote membrane curvature. Here, we investigate membrane fission by shallow hydrophobic insertions quantitatively and mechanistically. We provide evidence that membrane insertion of the ENTH domain of epsin leads to liposome vesiculation, and that epsin is required for clathrin-coated vesicle budding in cells. We also show that BAR-domain scaffolds from endophilin, amphiphysin, GRAF, and β2-centaurin limit membrane fission driven by hydrophobic insertions. A quantitative assay for vesiculation reveals an antagonistic relationship between amphipathic helices and scaffolds of N-BAR domains in fission. The extent of vesiculation by these proteins and vesicle size depend on the number and length of amphipathic helices per BAR domain, in accord with theoretical considerations. This fission mechanism gives a new framework for understanding membrane scission in the absence of mechanoenzymes such as dynamin and suggests how Arf and Sar proteins work in vesicle scission.

INTRODUCTION

All eukaryotic cells rely on intracellular compartmentalization of vital processes within membrane organelles, whose shapes and dynamic interplay are tightly regulated to support their functions (Antonny, 2006; McMahon and Gallop, 2005; Shibata et al., 2009). Basic cellular compartments, including the endoplasmic reticulum (ER), the Golgi complex (GC), mitochondria, and intracellular transport intermediates (such as endocytic vesicles), contain in their structures highly curved tubular and spherical membrane elements undergoing persistent transformations and mutual conversion (McMahon and Gallop, 2005; Shibata et al., 2009). To form these intracellular membrane shapes, there are two essentially different types of membrane-sculpting events: generation of membrane curvature without disturbing membrane integrity and membrane remodeling by fission and fusion. A lipid bilayer, constituting the structural basis of all cell membranes, resists both bending and remodeling (fission) (Chernomordik and Kozlov, 2003). Therefore, forces have to be applied and energy supplied to intracellular membranes in order to drive membrane curvature and fission. Several unrelated mechanisms have been suggested for protein-mediated membrane sculpting (Farsad and De Camilli, 2003; Antonny, 2006; McMahon and Gallop, 2005; Shibata et al., 2009) and scission (Chernomordik and Kozlov, 2003; Corda et al., 2006; Hurley and Hanson, 2010; Liu et al., 2009; Schmid and Frolov, 2011). The mechanisms of curvature generation by peripheral membrane proteins may be classified into two groups: (1) hydrophobic insertion mechanisms, based on penetration of hydrophobic or amphipathic protein domains into the lipid bilayer matrix, and (2) scaffolding mechanisms, where intrinsically curved and sufficiently rigid hydrophilic protein domains (or assemblies thereof) adhere to the lipid bilayer surface and impress their shapes on the membrane (McMahon and Gallop, 2005; Shibata et al., 2009). This has enabled a quantitative and unifying understanding of the action of practically all peripheral membrane proteins proven to date to generate membrane curvature. The state of the current understanding of membrane fission is less advanced. So far, several hypothetical models of membrane division have been suggested for Arf1 and dynamin (Beck et al., 2011; Roux et al., 2006; Schmid and Frolov, 2011) and for ESCRTIII (Hurley and Hanson, 2010), but these do not provide a quantitative basis on the forces driving membrane scission.

The present work establishes that shallow hydrophobic insertions, previously shown to generate membrane curvature, are sufficient to drive membrane fission resulting in the transformation of continuous membranes into separate vesicles. Previous work showed that the ENTH domain-containing protein epsin and N-BAR domain-containing proteins endophilin and amphiphysin could generate membrane vesicles in addition to the reported tubules with diameters from 20 to 50 nm (Ford et al., 2002; Gallop et al., 2006; Peter et al., 2004). This suggested...
that, in addition to promoting membrane curvature during endocytic vesicle formation (McMahon and Boucrot, 2011), ENTH and N-BAR domains could also promote membrane scission. As the common feature of these domains is the presence of membrane-inserting amphipathic helices at their N termini, we hypothesize that this structural module might be the key factor necessary and, likely, sufficient for membrane fission.

A theoretical analysis was conducted of the elastic energy of small vesicles and membrane tubules, using a coarse-grained model, accounting effectively for the molecular features of lipids and proteins. This analysis predicted that proteins containing shallow insertion domains promote membrane scission, whereas a protein whose membrane interaction face is crescent-like, such as crescent BAR domains (without insertions or twists), which bend membranes by the scaffolding mechanism, prevent membrane fission, hence, counteracting membrane insertions. We validated these predictions using a new in vitro quantitative vesiculation assay and found a crucial role for epsin during clathrin-coated vesicle (CCV) budding in cells.

RESULTS

Predictions from a Biophysical Model

Membrane fission involves rearrangements of membrane continuity requiring specialized protein modules. To foresee the effect of shallow hydrophobic insertions and/or crescent-like protein scaffolds on membrane fission, we undertook a comparative analysis of system energies in tubular and vesicular states based on a coarse-grained semiquantitative physical model (see Extended Experimental Procedures). The results can be presented in the form of phase diagrams (Figures 1A and 1B; Figure S1 available online) predicting formation of the vesicular state, tubular state, and coexistence between them for different protein-to-lipid ratios, $x$, and different ratios between the scaffold and lipid bilayer bending moduli, $k_p/k_m$. The latter parameter characterizes the ability of scaffolds to generate membrane curvature. Vanishing values of this parameter, $k_p/k_m = 0$, describe proteins that do not produce any scaffolding effect and bend membranes solely by shallow insertion of amphipathic helices. The larger $k_p/k_m$, the stronger the scaffolding effect. Qualitatively an increase of $k_p/k_m$ is equivalent to a decrease in the number of amphipathic helices per scaffold for a given protein rigidity $k_p$ (Figure 1A). The extended phase diagram in Figure 1B corresponds to an N-BAR with an extra amphipathic domain in the middle (such as endophilin). The points corresponding to $k_p/k_m = 0$ describe scaffold-less proteins such as epsin ENTH domains. Complete modeling is presented in the Extended Experimental Procedures.

The major conclusion illustrated by the phase diagrams (Figures 1A, 1B, and S1D–S1F) is that shallow hydrophobic insertions are predicted to be sufficient for vesicle formation, driving membrane fission, whereas crescent-like protein scaffolds are predicted to support formation of continuous membrane tubules, hence disfavoring fission (Figures 1A, 1B,
Figure 2. Epsin Is Required for CCV Scission

(A) Effect of RNAi (siRNA pool1) of epsin proteins on transferrin (Tf) uptake measured by flow cytometry. Clathrin (CHC), AP2, and FCHo proteins depletion were used as positive controls (black bars). The values were normalized to the mean of the control cells (gray bars). The background (cells without Tf) for each cell line is shown (white bars). The number of cells analyzed is displayed on each bar. ***p < 0.0001. Data are the mean ± SD.

(B) Effects of 5 independent pools of siRNA against Epsin1+2+3 (red bars) on Tf uptake and of the rescue of pools 1 and 2 (but not CHC and AP2 RNAi) by coexpression of rat epsin1-RFP (green bars). Experiments were done as in (A). Data are the mean ± SD.

(C) Effect of epsin1+2+3 RNAi on the dynamics of clathrin-coated structures (CCS) and rescue by coexpression of rat epsin1-RFP. CCS labeled by s2-EGFP.

Bar, 5 μm.

(D) Scatter plots of individual lifetimes of CCS from three different cells, measured on data set similar to (C). Median with interquartile range is shown on graph and mean ± SD is written at the bottom, n is the number of events analyzed. ***p < 0.0001.

(E) Fraction of CCS with longer duration than the time series.
and S1). The mechanistic background for these predictions comes from a qualitative consideration of the mechanical stability of a funnel-like membrane neck, an unavoidable intermediate stage of the fission process (Figure 1C). Fission occurs if this neck is unstable, i.e., possesses extra energy, which can be released as a result of membrane scission. Geometrical considerations show that the saddle-like shape of the neck membrane is characterized by having a larger midplane area than outer and inner leaflet surface areas (occupied by lipid head groups). Expansion of the head group region with respect to the bilayer midplane would stress and destabilize the neck, hence, favoring its fission. This reasoning can be expressed in exact terms of the insertion contribution to the membrane modulus of Gaussian curvature and the role of the latter in determining membrane conformations (Huse and Leibler, 1991; Schwarz and Gompper, 1999).

Shallow hydrophobic insertions, such as amphipathic helices from proteins like epsin, span mainly the polar head regions of membrane monolayers and do not penetrate deeply into the monolayer hydrocarbon region (Kweon et al., 2006). As a result, these insertions expand the bilayer surface(s) with respect to the bilayer midplane and, hence, are predicted to destabilize the neck and favor membrane fission. Although this effect is strongest if insertions are introduced into both membrane monolayers (expanding the head groups region on both sides), estimations show that amphipathic helices inserted only in the outer monolayer at biologically reasonable concentrations can be sufficient to drive fission on their own. Moreover, insertions are predicted not only to make fission energetically favorable but also to accelerate this reaction by reducing its energy barrier. The fission rate was previously proposed to be limited by the energy of the membrane stalk intermediate (Bashikrov et al., 2008; Kozlovsky and Kozlov, 2003), and computations show that a positive contribution to outer monolayer spontaneous curvature generated by the insertions (Campelo et al., 2008) decreases the stalk energy (Kozlovsky and Kozlov, 2003). Taken together, shallow hydrophobic insertions are expected to support membrane fission into small vesicles both in terms of the overall energy balance and kinetically.

Crescent-like scaffolds, such as BAR and F-BAR domains that do not penetrate lipid monolayers, do not change the area balance between membrane surfaces and the midplane. Instead, they mold, locally, the membrane into a cylindrical shape, which is curved only along the line of the scaffold-membrane interface (Figure S1H). The energetically most favorable situation for multiple crescent-like scaffolds is where they are oriented parallel to each other on a tubular surface. Hence, pure crescent-like scaffolds are not expected to support membrane fission but rather are predicted to generate tubular shapes (Figure S1).

“Hybrid” proteins, such as N-BAR domains, with both insertion and scaffolding effects, are predicted to generate coexisting vesicles and tubules with the degree of preference for the former or latter depending on the amount of the amphipathic helices per scaffold and on the effective rigidity of the scaffold, which includes the strength of the scaffold binding to the membrane surface (Figures 1A, 1B, and S1). Hence, scaffolding by BAR domains is predicted to restrain membrane fission mediated by hydrophobic insertions, and BAR domains with an increasing number of amphipathic helices are predicted to support increasing membrane vesiculation (Figures S1D–S1I) and so potentially in vivo will be on the pathway to membrane fission.

**Epsin Is Required for CCV Scission**

Epsin proteins were initially chosen as a paradigm for insertion activity in the absence of scaffolding. Epsin proteins play a role in cargo selection and membrane sculpting of CCV (Ford et al., 2002; Wang et al., 2011) but have not been linked so far with membrane fission. The lack of endocytic defects in epsin1 and 2 double knockout-derived cells (Chen et al., 2009) and in cells depleted of epsin1 by RNA interference (RNAi) (Chen and Zhuang, 2008; Kazazic et al., 2009) is likely due to protein redundancy, as there are at least four epsin proteins in humans: epsin1, -2, and -3 and epsinR (Clint/enthoprotein). EpsinR is involved in CCV formation from intracellular compartments (Mills et al., 2003), whereas the remaining epsins are believed to function from the plasma membrane. We measured the effects of individual or combinatorial depletion of epsin1, -2, and -3 by RNAi on clathrin-mediated endocytic activity as measured by transferrin (Tf) uptake (Figure 2A). We found that only simultaneous depletion of epsin1, -2, and -3 (1+2+3 RNAi) led to a significant decrease in Tf uptake, giving a similar effect to depletion of clathrin, FCHO proteins (Henne et al., 2010), or AP2 (Figures 2A, S2A, and S2B). This phenotype was specific as it was confirmed using up to five different 1+2+3 siRNA pools (comprising 24 different siRNAs) in three different cell lines and could be specifically rescued by coexpression of rat epsin1-RFP, which was resistant to the epsin1 siRNA in pools 1 and 2 (Figures 2B and S2A). Rat epsin1-RFP did not rescue, as expected, clathrin or AP2 RNAi. The perturbation was specific to clathrin-mediated endocytosis, as the uptake of the fluid-phase marker dextran was not affected (Figure S2C). Because epsin3 is known to be upregulated in some tumors (Coon et al., 2011), the phenotype was confirmed in a normal diploid cell line (hTERT-RPE1) where we have shown by mass spectrometry that epsins 1, 2, and 3 are all expressed (Figures 2A and S2B). The block of Tf uptake upon co depletion of epsins was largely due to a defect in scission of clathrin-coated structures (CCS) as epsin 1+2+3 RNAi cells had most (63.3%) of their AP2- and clathrin punctae arrested **(F)**. Scatter plots of individual maximum fluorescence intensities of CCS from three different cells. Data are presented as in (D), excepted for the Log10 vertical axis. **∗∗∗** p < 0.0001.

**G** Morphological analysis of CCS in BSC1 cells treated or not with epsin1+2+3 RNAi. Representative electron microscopy images for various categories quantified (top). Bars, 100 nm. Coated structures were classified as 1, shallow; 2, invaginated; 3, constricted; and 3*, multilobed. Repartition between the various categories of 70 structures from control (white bars) and 1+2+3 RNAi (black bars) cells is shown. Large image on left and ×3 image are from RNAi-treated cells.

**H** Effect of epsin1+2+3 RNAi on recruitment of endogenous dynamin 2 (DNM2, green) and clathrin (CLTA, red). Bar, 5 μm.

**I** Scatter plots of individual lifetimes (top) and individual maximum fluorescence intensities (bottom) of endogenous dynamin2. Data are presented as in (D) and **(F)**, respectively.

See also Figure S2.
and enlarged (“1+2+3,” Figures 2C–2F). These defects were rescued by the re-expression of rat epsin1-RFP (“rescue,” Figures 2C–2F). By electron microscopy (EM), the number of CCS per \( \mu \text{m} \) cell perimeter was not significantly different in the control and epsin 1+2+3 RNAi cells (control: 0.065 ± 0.042 CCS/\( \mu \text{m} \), n = 70; 1+2+3 RNAi: 0.070 ± 0.036 CCS/\( \mu \text{m} \), n = 70; \( p > 0.05 \)). The relative abundance of different stages of CCS—shallow, invaginated, and constricted—were similar in the two samples. However, a marked increase in the number of multiheaded CCS were observed in the epsin 1+2+3 RNAi sample, representing 23% of the total number of pits versus 4% in the control (Figure 2G). Large patches of flat clathrin-coated plasma membrane were also observed, reminiscent of what Brodin and colleagues (Jakobsson et al., 2008) observed when interactions of epsin with clathrin and AP2 were perturbed acutely in the giant lamprey synapse. The diameter of CCVs still attached to the membrane was not significantly different in RNAi-treated cells (\( p > 0.05 \), Student’s t test; control 106 nm, 1+2+3 RNAi 102 nm; \( n = 50 \)), but the neck diameter of constricted coated pits (stage 3) was significantly greater (\( p > 0.001 \), Student’s t test; control 25.7 nm, 1+2+3 RNAi 35.0 nm; \( n = 50 \)). The defect in scission in epsin 1+2+3 RNAi cells was not due to a lack of recruitment of dynamin as both dynamin 1 and 2 were detected for significantly longer times and at higher intensities at the arrested CCS (Figures 2H, 2I, and S2E), although from live cell imaging we do not know if it is present on the neck. Altogether, these studies reveal that epsin is required for CCV scission.

**Epsin Can Mediate CCV Scission in Dynamin-Depleted Cells**

To test whether epsin could support CCV budding independently of dynamin, we tested the potential rescue by slight over-expression of epsin in two situations where dynamin function was impaired: when dynamin was locked at the neck (using the small-molecule dynamin inhibitor dynasore) and when dynamin was depleted (dynamin1 and 2 \([\text{DNM1+2}]\) RNAi). Mild over-expression of epsin did not significantly rescue the CCV budding defect induced by dynasore, but did rescue DNM1+2 RNAi, as judged by Tf uptake (Figures 3A and 3B) and the rescue in clathrin-AP2 dynamics (Figures 3C and 3D). This suggested that epsin can support the scission of the neck of CCS when dynamin expression is reduced (DNM1+2 RNAi) but not when dynamin is locked at the neck (dynasore). The ability of epsin to promote CCV scission with dynamin RNAi was sensitive to its amphipathic helix insertion (Figures 3B and S3). Mutation of a charged residue on the hydrophobic face of the amphipathic helix L6E (reducing membrane binding and curvature induction...
[Ford et al., 2002]) did not rescue scission to the same extent (despite being recruited to CCS) as wild-type (WT) epsin or a mutant with increased membrane binding, L6W (Ford et al., 2002). Thus, we concluded that epsin supports CCV scission and works alongside dynamin.

Epsin ENTH Domain Causes Membrane Vesiculation

The membrane-binding face of epsin ENTH domain has an intense positively charged patch (Figure S4A), which allows the domain to be recruited to negatively charged membranes, where it binds to PtdIns(4,5)P₂ (PIP₂), inducing the folding of an N-terminal sequence into an amphipathic helix forming a pocket for the head group of this lipid (Ford et al., 2002). The folding of this helix is relatively specific for PIP₂, where three residues of the helix are involved in hydrogen bonding with the lipid. This exposes a hydrophobic surface, which along with surrounding hydrophobic residues is proposed to sit in the hydrophobic phase of the membrane (Figures S4A and S4B).

When incubated with liposomes, epsin ENTH domain forms many small nanovesicles and extremely narrow tubules of ~20 nm diameter (Figure 4A). An assay was needed to quantify the nanovesicle formation. In a standard lipid cosedimentation assay, proteins that bind to liposomes generally pellet (P) with the liposomes whereas soluble proteins that do not bind remain in the supernatant (S). When we performed this assay with epsin ENTH domain, it was puzzling that the protein appeared to remain in the supernatant even in the presence of PIP₂-containing Folch liposomes (Figure 4A, Samples 2 and 4). This indicated either that the protein did not bind or that the membranes were now in the supernatant fraction. To monitor the membrane distribution after velocity sedimentation, we exploited our observation that Coomassie dye stains both proteins and lipids on the same SDS-PAGE gel (where stain and fix have no alcohol so as not to dissolve the lipids). Liposomes (filtered to 200 nm) were found in the pellet fraction as visualized by Coomassie staining of the lipids close to the dye front of the gel (Figure 4A, Sample 1). However, on addition of epsin ENTH domain to liposomes, the lipid signal moved to the supernatant fraction (Figure 4A, Sample 2). Thus the protein must have interacted with the liposomes and changed the apparent density. A partial shift...
could also be achieved by sonication of the liposomes (Figure 4A, Sample 3). This observation forms the basis of an unbiased biochemical assay for membrane vesiculation, where small vesicles were found to resist pelleting. EM of the starting material compared to liposomes in the presence of epsin showed dramatic membrane vesiculation of the starting material and possible micelle formation (Figure 4A, lower EM panel). The increased number of nanovesicles in the presence of epsin was consistent with the vesiculation of 200 nm liposomes to 20 nm giving at least a 100-fold increase in vesicle number. After sedimentation only small liposomes were found in the supernatants of any of these samples (Figure 4A).

To assess the dynamic range of the assay, we filtered liposomes using polycarbonate membrane filters with defined pore sizes. Liposomes filtered to a diameter of 200 nm sedimented efficiently, whereas liposomes filtered to 30 nm did not (Figure 4B). Electron microscopy confirmed that the liposomes were indeed filtered to approximately the defined size (with some heterogeneity) and that vesicles with diameters smaller than 100 nm tended to resist pelleting. (The broad transition between flotation and pelleting also partially reflects the range of diameters achieved with the filtration process; Figure S4C).

It is likely that more highly curved liposomes have a greater contribution from lipids to their apparent density (on centrifugation) than larger liposomes, leading to a difference in pelleting, consistent with previous observations (Goormaghtigh and Scarborough, 1986). We concluded that the relative distribution of lipids between pellet and supernatant in these experiments is an unbiased biochemical measure of the extent of vesiculation and that this bulk assay agrees with the EM observation of extensive vesiculation of liposomes by epsin ENTH domain.

Vesiculation of liposomes by the epsin ENTH domain was concentration dependent with maximal vesiculation around 2.5 μM protein (Figure S4D, but see comment later). If all protein was bound to the membrane surface and an individual epsin occupies an area equivalent to 20 lipids (Figure S4B) then at 0.125 mg/ml lipid the membrane would be 70% saturated. As can be seen in the saturation curve, vesiculation occurred at much lower concentrations but did not go to completion in the time given (Figure S4D).

To test whether the observed flotation of small vesicles is limited to the particular Folch extract mix used in these experiments, we made a synthetic mixture containing 10% cholesterol, 5% PIP2, 55% PC, and 30% PS (used to achieve a strong electrostatic attraction for epsin, as would be expected in the plasma membrane inner leaflet where the protein binds in vivo). The addition of epsin resulted in robust vesiculation as determined by the sedimentation assay (Figure S4E).

### Epsin-Mediated Vesiculation Is due to Amphipathic Helix Insertion

To understand the nature of epsin-dependent vesiculation, we next tested epsin mutants. Epsin L6W resulted in a slight increase in vesiculation compared to WT after 1 hr at 37°C (Figure 5A). Samples taken for electron microscopy after 5 min incubations showed that L6W resulted in uniform small vesicle production, whereas WT protein gave tubules and vesicles with a wide distribution of sizes (Figures 5A–5C). Experiments at 4°C showed a large increase in vesiculation with L6W over WT protein (Figure S5A). After 1 hr incubation, vesiculation by L6E was less efficient than WT ENTH domain at 37°C (Figure 5A), and no vesiculation was observed either at 4°C (Figure S5A) or in the 5 min time point processes for electron microscopy (Figure 5B). Vesicles produced by L6W for 5 min at 37°C had diameters centered around 20 ± 4 nm, whereas WT protein had a much broader distribution (Figures 5C and S5B). This would indicate that epsin works in a stochastic manner to bud vesicles off larger structures, and that stable tubule intermediates are not required on the way to vesiculation (as predicted by our theoretical model).

Altered vesiculation that accompanies mutations of epsin’s amphipathic helix points to the importance of this module in vesicle generation. However, vesiculation may not be a direct property of the helix but simply a reflection of the amount of protein bound to membrane, where the helix can be considered as an anchor. This is particularly plausible because at 4°C we observed that only L6W, which binds membranes much better than WT protein, led to vesiculation (Figure S5A). An alternative strategy to determine the importance of the amphipathic helix was to exploit the PIP2 dependence of helix folding (Ford et al., 2002). One PIP2 binds per one epsin molecule, and thus if one epsin covers approximately 20 lipids (Figure S4B) then 5% PIP2 should allow complete saturation of the membrane. With 10 μM epsin ENTH domain, there was complete vesiculation of this lipid mix (Figure 5D). As expected, this resulted in a dramatic shift of lipids from the pellet to the supernatant after a high-speed spin, which correlated with vesiculation as judged by electron microscopy. To reduce the amphipathic helix concentration in the membrane 10-fold, we lowered the PIP2 content to 0.5%. This also resulted in maximal vesiculation (Figure 5D). As a control, there was no vesiculation when PIP2 was removed altogether, despite significant association of the protein by electrostatic attraction to the PS-containing membranes. Thus, our experiments show that a relatively low density of amphipathic helices (1 helix to 200 lipids) is required to achieve maximal vesiculation (as determined by movement of membranes into the supernatant in the sedimentation assay). We do not observe epsin dimer formation on membranes and the low concentration of protein required for vesiculation argues against a molecular crowding model for membrane vesiculation. From these measurements, we can calculate that formation of a 20 nm vesicle with 10% coverage of the membrane by epsin ENTH footprints will require at least 30 molecules. Calculations based on the spontaneous curvature of epsin (Campeolo et al., 2008) and bilayer curvature of a 20 nm vesicle show that about 100 molecules are required (in very close agreement with the biochemical measurement).

To further address effects of epsin ENTH domain amphipathic helix insertion into membranes, we tested for trypsin sensitivity of this sequence, which has multiple lysine and arginine residues and is proposed to be unfolded in solution (Ford et al., 2002). Limited proteolysis gave a distinct cleavage product of 1–2 kDa and a corresponding decrease in molecular mass of the parent protein (Figure 5E). By mass spectrometry, we identified the cleaved peptide as a fragment of the amphipathic helix (Figure S5C). This cleavage was protected by liposomes, showing that it is inserted (unlike a soluble synaptobrevin...
The stability of epsin for up to 30 min in the presence of trypsin+membranes showed that epsin did not dissociate at a significant rate. Cleaved ENTH domain no longer bound at a significant level to membranes (Figure 5E). As a proof of principle of helix insertion, we showed that reduced PIP2 levels led to reduced helix protection/insertion and with 0% PIP2 there was no protection (Figure S5E). We could also show that there was a strong correlation between the protection of epsin from trypsin cleavage and vesiculation (Figures 5F and S5F). The assay also allowed us to look at membrane “binding/insertion” of our different epsin mutants where we could show that the L6W mutant bound more tightly than WT protein (Figure S5G).

**Figure 5. Membrane Vesiculation Is due to Amphipathic Helix Insertion**

(A) Membrane vesiculation due to epsin ENTH domain and mutants was assessed by the biochemical vesiculation assay and by electron microscopy. Protein (10 μM) was incubated with 0.125 mg/ml Folch + 5% PIP2 liposomes for 1 hr at 37°C. AP180 ANTH domain, which also binds to PIP2 (Ford et al., 2001), was used as a control. Data are mean of three experiments ± SD with a sample gel shown on the right. *p < 0.001.

(B) Electron microscopy samples taken for samples in (A) after 5 min.

(C) Quantitation of membrane vesiculation after 5 min incubation with WT and L6W epsin ENTH domain. The WT protein gives a broader distribution of vesicle sizes with many vesicles of larger diameters. Data in each case are from 169 objects in at least three different fields. One 200 nm vesicle is estimated to give 141 vesicles of 20 nm.

(D) PIP2 dependence of epsin vesiculation. Epsin ENTH (10 μM) was incubated for 1 hr with either 200 nm or 30 nm-filtered synthetic liposomes (30% PS, 10% cholesterol, 55%–60% PC plus indicated amount of PIP2, final concentration of liposomes: 0.125 mg/ml). We see no effect of protein addition on the 30 nm-filtered liposomes. Vesiculation is dependent on PIP2, but binding can still be observed.

(E) Limited trypsin proteolysis (20 min at 37°C) of epsin ENTH domain was inhibited by Soybean trypsin inhibitor (Inh.) or by liposomes (left). For cleaved peptide sequence, see Figure S4C. The amphiphatic helix was either pretrypsinized or not before addition of liposomes (right).

(F) The amount of vesiculation shows a strong correlation with the amount of epsin protected, as assessed by a trypsin assay in (E). Thus it is not so important to know the amount of epsin added or membrane bound but the amount of helix insertion. See also Figure S5.

**Positive Correlation of the Number of Hydrophobic Insertions and Membrane Fission by N-BAR-Domain Proteins**

To examine the prediction that BAR domains restrain amphipatic helix-induced membrane fission, we tested the effects of various BAR and N-BAR proteins on liposome morphology. Endophilin A1 (EndoA1) has previously been observed to give
a mixture of vesicles and tubules formed from larger liposomes (Gallop et al., 2006; Figure 6A). Here we used endophilin A3, a form of the protein expressed in nonneuronal tissue and localizing to membranes in fibroblasts (Hughes et al., 2004), thus allowing us to also test the phenotypes in vivo (see below). EndoA1 and EndoA3 generated a mixture of vesicles and tubules (Figures 6A and S6) from 200 nm liposomes. In the biochemical vesiculation assay almost 60% of the starting material was vesiculated in 1 hr (Figure 6B). By comparison, amphiphysin2 (Amph) had higher than background vesiculation but was significantly less active than EndoA3. GRAF, which does not have any amphipathic helices (Lundmark et al., 2008), was inactive in vesiculation (Figure 6B). Epsin ENTH domain showed over 80% vesiculation in the same incubation. EM observation of the samples confirmed these results (Figures 6C and S6A). The degree of vesiculation correlated strongly with the number of amphipathic helices. Thus endophilin with four amphipathic helices (N terminus and middle of the BAR domain) had higher activity compared to amphiphysin which only has two N-terminal amphipathic helices, which was better than GRAF with no amphipathic helices (Figure 6D).

Given that vesicles were observed for epsin, endophilin, and amphiphysin, we wondered what effect the BAR domains have on the final product of vesiculation. We thus measured the size distribution of vesicles generated by the various BAR proteins. We already noted above that epsin makes 20 nm vesicles (outer diameter). Endophilin vesicles have a wider distribution with an endophilin mutant showing an average diameter of 27 nm (Figure S6J), whereas amphiphysin vesicles are around 47 nm (Figure S6B). Vesicles of diameter 30 and 45 nm were previously observed for EndoA1 and Amph N-BAR domains, respectively (Peter et al., 2004; Gallop et al., 2006) (Figure S6C). Thus, an increased number of amphipathic helices on a BAR domain correlates with increased vesiculation and smaller vesicle size, as predicted by the quantitative assessment based on membrane physics.

Scaffolding by BAR Domains Restrains Membrane Fission

To test the balance between scaffolding and hydrophobic insertion, we altered the hydrophobicity of the N-terminal amphipathic helix of endophilin A3 (Endo-WT). To shift the
membrane bending capacity toward predominant scaffolding, we replaced the N-terminal amphipathic helix with stretches of four or eight lysines (K4A4 and K8, Figure 6E) to compensate for the reduced membrane binding in the absence of the N-terminal amphipathic helix (Gallop et al., 2006). To shift the protein toward the other extreme of a more pronounced hydrophobic insertion, we doubled the N-terminal amphipathic helix (double amphipathic helix [DAH]). Membrane binding of purified proteins showed that Endo-K8 had similar binding to Endo-WT (Figure 6F). In contrast, Endo-K4A4 bound less well and Endo-DAH bound membranes slightly better than the WT protein (Figure 6F). All four endophilin constructs were recruited as expected to plasma membrane puncta in cells (Figure S6D), suggesting proper folding and functionality. The majority of cells expressing Endo-WT had many internal tubules and/or vesicles labeled with protein (Figures 6G and S6D). Individual tubules were very dynamic and often vesiculated during observations (Figures S6E–S6G). In contrast, cells expressing Endo-DAH had more internal vesicles (85% ± 13%) and less tubules (17% ± 10%) than cells expressing Endo-WT, with most of these tubules being very short (Figures 6G and S6D). Virtually all (91% ± 2%) Endo-DAH tubules observed vesiculated (Figure S6F). Compared to Endo-WT, Endo-DAH vesiculated sooner after formation (Figure S6G). These Endo-positive intracellular punctae were indeed endocytic membrane vesicles, as they labeled positive after a pulse with FM4-64 (Figure S6H). The majority of the cells expressing Endo-K8 had tubules (Figures 6G and S6D), which were very stable with only a minority of them vesiculating within the time of observation (Figure S6F). Virtually no cells expressing Endo-K4A4 had tubules or vesicles (Figures 6G and S6D), consistent with its impaired membrane binding ability. Thus, increasing the number of amphipathic helices on a BAR domain increases its ability to induce membrane fission and vesiculation.

Endophilin has a C-terminal SH3 domain (Figure 6E) that can bind to dynamin, which could contribute to the membrane scission observed in vivo. Thus, we assessed the impact of the endophilin mutations on membrane fission in vitro using our biochemical vesiculation assay. We already noted that EndoA3-WT led to approximately 50% vesiculation (Figure 6B). Doubling the length of the N-terminal amphipathic helix led to 80% vesiculation (Figure 6H), consistent with the nanovesicles observed by electron microscopy (Figures 6I and S6I). This correlated very well with an increase in vesicle production in cells (Figure 6G). Vesiculation was decreased for Endo-K8 (Figure 6G). Given that Endo-WT, -K8, and -DAH all bound to membranes to similar extents (Figure 6F), the major consequences on membrane curvature/vesiculation must be a result of the differences in the area occupied by amphipathic helices per scaffold. In conclusion, mutants of endophilin designed to shift it toward the scaffolding or hydrophobic insertion extremes, appear to shift the protein behavior in vitro and in vivo to tubules or vesicles, respectively (Figure 6J). Thus, our experiments on endophilin N-terminal helix mutants show that hydrophobic insertions can not only drive an increase in positive membrane curvature but also help drive membrane scission, likely through destabilizing the membrane neck.

Amphipathic Helix Addition to a BAR Domain Is Sufficient to Mediate Membrane Fission

Finally, to test further whether amphipathic helices could counteract the scaffolding activity of BAR domains, we tested a BAR-domain protein with no known amphipathic helix. Expression of WT β2-centaurin BAR+PH domain (centaurin-WT, Figure 7A) induced extensive tubulation when expressed in cells (Peter et al., 2004) (Figure 7B) and some tubulation of liposomes (Figure 7D). Initially we observed that GRAF and centaurin competes with epsin for lipid vesiculation (Figures 7E and S7), but this effect may be due to competition for binding sites on the membrane. To circumvent this, we added one or two copies of the N-terminal amphipathic helix from endophilin onto centaurin (Figure 7A) and tested the ability of the mutants to induce membrane fission both in vivo and in vitro. Expression of centaurin containing a double amphipathic helix (centaurin-DAH) caused remarkable vesiculation in vitro (Figure 7D) and in cells (Figure 7B), whereas addition of a single amphipathic helix (centaurin-SA) gave an intermediate phenotype. This further confirmed the prediction of the model that amphipathic helices support membrane scission and that this activity is counteracted by BAR-domain scaffolding.

Experimental Evidence Agrees Quantitatively with the Model

We found a strong positive correlation for vesicle production in vivo and in vitro for the different numbers of amphipathic helices per BAR domain (Figure 6J). Additionally, the experimental data reflected the predictions of our model qualitatively. Epsin ENTH domains having no scaffolding effect, and endophilin-DAH possessing elongated amphipathic helices, are predicted to transform flat membranes directly to the vesicular phase for all system compositions without intermediate generation of a thermodynamically equilibrium tubular phase, although kinetically trapped but nonequilibrium tubules might be observed (Figure S11 for $k_p = 0$). Indeed epsin ENTH domains generated small vesicles without formation of equilibrium membrane tubules (Figure 4), and endophilin-DAH converted the membranes, predominantly, into small spherical vesicles (Figures 6H, 6I, and S6J) with rare tubules. Further, proteins such as centaurin that lack the membrane-inserting modules but have crescent-like scaffolding domains will, according to the model, bend membrane into tubular shapes, but no membrane fission will occur (Figures 1 and S1), which agrees with our experimental observations (Figure 7).

In addition to the qualitative agreement, the model predicted quantitatively the percentage of membrane vesiculation by endophilin mutants with a varying overall area, $A_{\text{basal}}$, occupied in the membrane plane by the amphipathic helices belonging to one BAR domain (Figure 7F). The area $A_{\text{basal}}$ is very small for K8 mutant (taken as $A_{\text{basal}} = 8 \text{ nm}^2$) and was estimated as $A_{\text{basal}} = 20 \text{ nm}^2$ for endophilin WT and $A_{\text{basal}} = 32 \text{ nm}^2$ for endophilin-DAH. The theoretical curve (assuming 50% coverage of the membrane surface area by the proteins) along with the results of measurements in the liposome system and in cells are presented in Figure 7F, which shows a good agreement between the model predictions and the experimental results taking account the considerable variations in the latter. Finally, we computed the average radii of vesicles generated by the epsin
ENTH domains, amphiphysin, and endophilin DAH (again for 50% membrane area occupied by the proteins). There is good agreement (Figure 7G), further validating the model and substantiating our experimental results.

DISCUSSION

Epsin Supports CCV Budding

Epsin proteins are associated with CCS (Chen et al., 1998) and accumulate gradually with peak accrual coinciding with CCV budding (Taylor et al., 2011). In our in vitro assays, epsin ENTH domain was sufficient to drive membrane fission. In cells, clathrin will act as a scaffold on curvature and may even limit the extent of curvature under the cage. It is likely that as clathrin coats mature, epsin molecules get pushed to the edge of the cage, consistent with its nonenrichment in mature CCV (Mills et al., 2003), and with its proposed localization at the neck of the nascent vesicle (Saffarian et al., 2009) where it will largely be unrestrained by clathrin (Figure 7H). Scission of CCV is believed to be primarily carried out by dynamin in higher eukaryotes. We now show

Figure 7. Amphipathic Helix Addition to a BAR Scaffold Is Sufficient to Mediate Membrane Scission

(A) Schematic representation of mutant centaurin proteins. |2-Centaurin WT BAR+PH domain had no amphipathic helices. Centaurin-SAH and centaurin-DAH had respectively a Single Amphipathic Helix or a Double Amphipathic Helix from EndoA3 at their N terminus. All constructs had a Myc-tag at the N terminus. (B) Confocal images of COS-7 cells expressing the BAR+PH domains of centaurin-WT, centaurin-SAH, or centaurin-DAH. The first row represents the maximal projection of a 3D stack of images acquired at 0.25 μm apart. The second row displays the insets of the boxed regions. Note the tubules (white arrows) and the internal vesicles (red arrows). Bar, 10 μm.

(C) Histogram showing the percentage of transfected cells displaying internal tubules (white) and internal vesicles (red). Cells could present both. Data are the mean ± SD of >300 cells for each construct from three independent experiments. **p < 0.001.

(D) EM of liposomes with 9 μM of the indicated proteins.

(E) Competition between epsin ENTH domain and |2-centaurin for vesiculation/tubulation of Folch liposomes. Mean ± SD for three independent experiments. Red bar: p < 0.001.

(F) Predicted percentage of vesiculated membrane by N-BAR domains covering 50% of the total membrane area as a function of the total area of inclusions per scaffold Ains. Points represent the measured values in vitro and in vivo (Figure 6) for Endo-K8 (Ains = 7 nm²), Endo-WT (Ains = 20 nm²), and Endo-DAH (Ains = 32 nm²).

(G) Predicted and measured diameters of vesicles generated as a result of membrane fission by Amph (Ains = 12 nm²), Endo-DAH (Ains = 32 nm²), and epsin ENTH domain (Ains = 6 nm²). In the computations 50% membrane coverage was used.

(H) Model of the concentration of epsin to the region of membrane scission during CCV maturation.

See also Figure S7.
that dynamin-mediated fission of CCVs is severely compromised in the absence of epsin (Figure 2) and that, in certain conditions, epsin can palliate the depletion of dynamin and support CCV budding (Figure 3). However, epsin cannot support budding when dynamin activity is blocked by dynasore and dynamin accumulates at the neck of CCS. The failure of fission may be due to the stabilization of the neck by an oligomeric dynamin scaffold, rather like BAR scaffolds. Altogether, this suggests that epsin might provide the required force to destabilize the neck of nascent vesicles and that scaffolding generated by dynamin oligomers might act as a “tether” with membrane fission promoted upon cooperative GTP hydrolysis-mediated depolymerization.

Shallow Hydrophobic Insertions Promote and BAR-Domain Scaffolds Restrict Membrane Fission

Compared to BAR scaffolds alone, N-BAR modules (e.g., endophilin, amphipysis) contain additional amphipathic helices that insert into membranes. Amphipysis and endophilin both are recruited to endocytic spots with dynamin (Taylor et al., 2011). Hydrophobic insertions are likely to enable these BAR proteins to create the neck and may further position them on the pathway to membrane scission. For dynamin-independent pathways we speculate hydrophobic insertions will be a major driving force for membrane scission. At sufficient concentrations many different proteins with insertions may contribute to membrane fission, or curvature may be limited by associated scaffolds. For example, Arf and Sar proteins contribute to the formation of COP coated vesicles (Beck et al., 2011; Lee et al., 2005). Because both Arf and Sar have an amphipathic helix that extends upon GTP binding, it is likely that these proteins contribute to the scission reaction by the hydrophobic insertion mechanism. For these proteins, the effects of amphipathic helix insertion may initially be controlled by the COP coat, just as the effects of epsin will be limited by the clathrin coat (Figure 7H), perhaps controlling the timing of membrane fission. Arf proteins are also known to bind to the BAR-domain protein, arfaptin (Williger et al., 1999), suggesting a potential regulation of the extent of curvature produced, which could well lead to vesicle budding. Finally, budding of some viruses also relies on amphipathic helix insertion, such as the M2 protein of influenza virus (Rossman et al., 2010). We will likely discover many more examples as the importance of hydrophobic insertions in membrane fission is recognized.

EXPERIMENTAL PROCEDURES

A full description of the methods is in the Extended Experimental Procedures.

Cell Culture, RNAi, Live-Cell Imaging, and Ligand Uptake Measurement by Flow Cytometry

HeLa, BSC1, NTERT-RPE1, COS-7, and BSC1 stably expressing α2-EGFP, SK-MEL-2 DNM2<sup>−/−</sup>-EGFP, and DNM2<sup>−/−</sup>-EGFP CLTA<sup>−/−</sup>-RFP genome-edited (Doyon et al., 2011) cells were grown on 35 mm glass-bottom dishes (MatTek, imaging) or 100 mm dishes (ligand uptake). RNAi was carried out by double transfection (on days 1 and 2) with oligofectamine (Invitrogen) and 80 pmol of each indicated siRNA (see Extended Experimental Procedures) and analyzed on day 3. It is important to note that efficient knockdown of CHC, AP2, FCHO1<sup>−2</sup>, DNM1<sup>−2</sup>, and Epsin1<sup>−2</sup> induce extensive cell mortality and that effectively knocked down cells are often in a minority (see Figure S2D). AlexaFluor 488-labeled human transferrin (20 μg/ml) and FITC-labeled Dextran 3000 kDa (1 mg/ml) uptake was carried at 37°C for 7 and 15 min, respectively, and analyzed using LSR II flow cytometer (Becton Dickinson). Live-cell imaging was performed as in Henne et al. (2010).

Liposome Preparation, Binding, and Vesiculation

Purified untagged proteins and Folch liposomes spiked with 5% PIP2 were used in the experiments, unless otherwise indicated. Folch liposomes (50:50 mix of Sigma Aldrich[B-1502]; Avanti Polar Lipids[131101P] with 0%-5% PIP2[Avanti Polar Lipids, 840046F] in 100 mM NaCl, 20 mM HEPES (pH 7.4) were extruded seven times through 200 nm polycarbonate membranes (Nuclepore). For tubulation/vesiculation assays 5 or 10 μl of 1 mg/ml liposomes were used in 40 μl reactions. Samples were spread on glow-discharged electron microscopy grids (Agar Scientific) and stained using 2% uranyl acetate.

Biochemical Membrane Fission Assay

Liposomes as above were incubated with protein for 1 hr at room temperature (although much shorter times can be used) and were spun at 250,000 × g for 15 min in a Beckman TLA100 rotor. Resuspended pellets and supernatants were analyzed by SDS-PAGE. We monitored the distribution of both proteins and lipids by SDS-PAGE using Bis-Tris gels run in MES buffer (to avoid excess counternions at the gel front that interfere with lipid staining). Gels were stained with 0.1% Coomassie in 10% acetic acid for 5 min and then destained in water. Alcohol was avoided in order to not solubilize the lipids in the gels. Loading dye (Bromophenol Blue) can interfere with the quantitation and so at least 30 min was given for this dye to leach from the gel. The extent of vesiculation was measured as the percentage of lipid found in the supernatant. This is a slight underestimation as empty lanes have a background that has not been subtracted as this can vary across the gel. Gels were quantitated using ImageJ.

Statistical Analysis

Results are mean ± standard deviation (SD) or median with interquartile range, as indicated. Significance was calculated using the Student’s t test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and seven figures and can be found with this article online at doi:10.1016/j.cell.2012.01.047.

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