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[52] *In Vitro* Reconstitution of Discrete Stages of Dynamin-dependent Endocytosis

By MATTHEW K. HIGGINS and HARVEY T. MCMAHON

Abstract

Many proteins involved in vesicle budding are able to interact with lipids and to deform membranes. In this review we present three *in vitro* assays that can be used to study the roles of these types of proteins and to reconstitute distinct stages of vesicle budding and tubule formation. In the first assay, the dynamic effect of peripheral membrane proteins on liposome morphology is studied, providing insight into the roles of proteins (e.g., the endocytic proteins; dynamins, epsins, amphiphysins, and endophilins) in deforming membranes and in recognizing membrane curvature. In the second assay, preformed lipid nanotubes are used to mimic the neck of a coated vesicle. These nanotubes form a suitable template to study molecules of the dynamin family, allowing visualization of the different conformations of dynamin that occur during vesicle scission. Finally, lipid monolayers have been used as mimics of the internal leaflet of the plasma membrane to investigate early stages of clathrin-coated pit formation. In principle this can equally be used to study budding mechanisms of other coated vesicle types. A combination of these three assays has given considerable insight into the roles of endocytic proteins and is allowing reconstitution and dissection of the different stages of vesicle formation.

Introduction

The formation of vesicles or trafficking intermediates is a complex process, requiring interplay between numerous soluble and membrane-bound proteins and lipids. Together these components work to generate local deformation of a membrane, capture cargo, and promote the formation of a vesicle or tubule intermediate. In cells, these processes can be examined by deleting individual components or by over-expressing

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proteins or their domains. This provides information about trafficking pathways but seldom yields precise information about the individual function of a protein component. Therefore the *in vitro* approach is valuable, allowing more precise roles to be assigned to proteins and allowing vesicle formation to be studied in molecular detail. However, care is needed in interpreting these data as a limited subset of binding partners is used and the lipid composition of model membranes and the concentration of protein components may not reflect those present *in vivo*. Therefore *in vivo* and *in vitro* approaches should be used in tandem, both resulting in different information and with *in vivo* experiments providing crosschecks to confirm *in vitro* data. Despite the pitfalls of studying isolated protein components, tremendous progress has been made in cell biology by such approaches, and only by understanding the molecular details can we begin to intelligently design therapies.

Method 1: Tubulation of Lipid Vesicles by Endocytic Proteins

Introduction

Many endocytic proteins that associate with membranes can deform the lipid bilayer, often causing tubulation. This can be caused by the shape of the lipid binding face of a protein (Peter *et al.*, 2004) or protein oligomer (Praefcke and McMahon, 2004; Sweitzer and Hinshaw, 1998) or can be due to active insertion into the membrane of a helix that stabilizes membrane curvature (Ford *et al.*, 2002). An example of the former is the tubulation of liposomes by the large GTPase, dynamin (see Fig. 1C). Addition of dynamin, in the presence of nonhydrolyzable GTP analogues, causes liposome tubulation, with formation of tubules driven by assembly of an ordered array of dynamin molecules. These dynamin spirals show sufficient order to allow determination of their structure at 20Å resolution by processing of images of frozen tubules (Zhang and Hinshaw, 2001) and are similar in diameter to the necks of endocytic vesicles. In this case tubulation is driven primarily by oligomerization of dynamin into a helix on the membrane template. Indeed, even in the absence of liposomes, dynamin can assemble into ring and tubules-like arrays, forming the conformation preferred by the protein assembly (Hinshaw and Schmid, 1995; Owen *et al.*, 1998).

Dimers of the amphiphysin BAR domain also have intrinsic curvature and therefore bind most favorably to membranes with a curvature complementary to their own (Peter *et al.*, 2004). BAR domains are found in a wide variety of proteins, including amphiphysins (Peter *et al.*, 2004; Razzaq

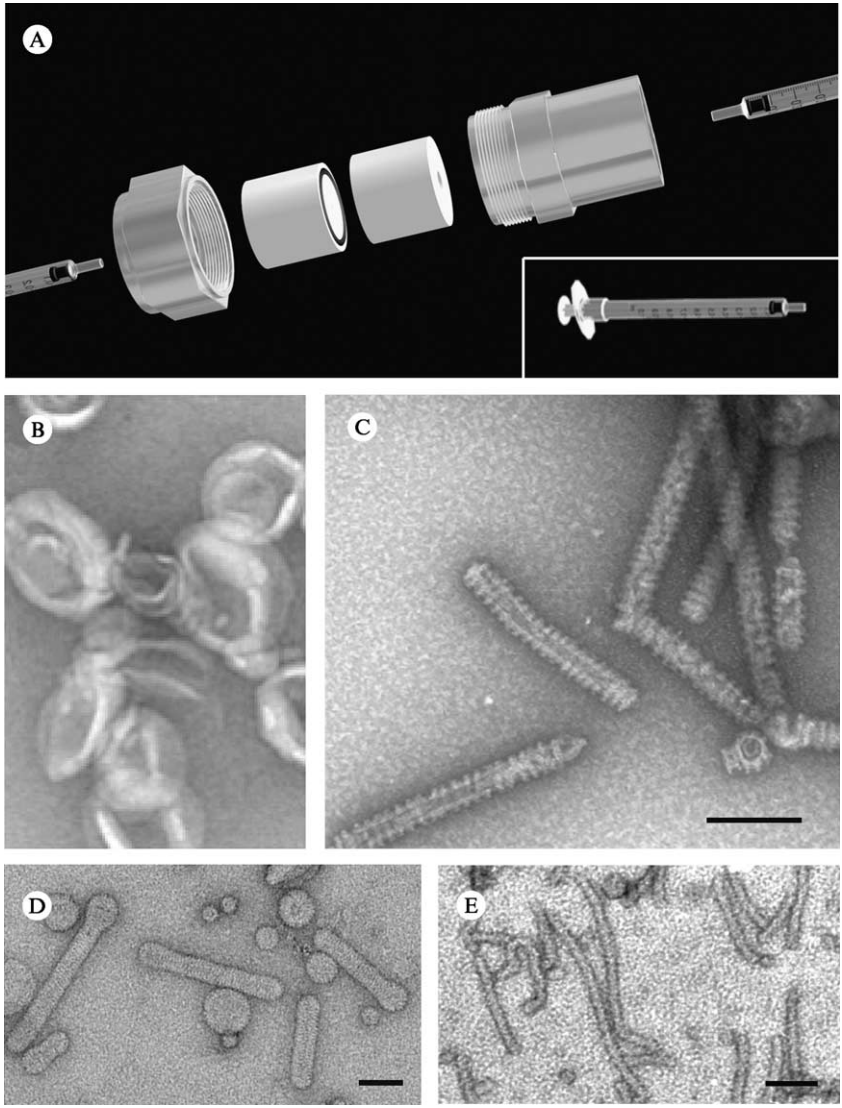

FIG. 1. Studying dynamin, amphiphysin, and epsin mediated liposome tabulation. (A) An ''exploded'' view of the lipid extrusion devise used for the formation of liposomes. (B) Liposomes formed from Folch brain lipid extract. (C) The effect of dynamin on Folch brain lipid liposomes. (D) Tubules formed by incubating liposomes with *Drosophila* amphiphysin. (E) Tubules formed by incubating the ENTH domain of epsin with liposomes. The scale bars are 100 nm.

et al., 2001; Takei *et al.*, 1999), endophilins (Farsad *et al.*, 2001), and many others (Peter *et al.*, 2004), and the presence of the BAR domain can be tested by observing the ability of a protein to tubulate liposomes (Fig. 1D). A variation of the BAR domain is an N-BAR domain where an additional amphipathic helix inserts into the membrane, taking part in driving or stabilization of membrane curvature. The presence of an amphipathic helix that drives tubulation is also seen in the ENTH domain of epsin (Fig. 1E) (Ford *et al.*, 2002). As many other proteins are predicted to contain N-terminal amphipathic helices, the liposome tubulation assay will prove a valuable test for the folding and insertion of these helices into membranes giving rise to increased membrane curvature.

It should be noted that the ability of an isolated protein to form tubules may not reflect an *in vivo* tubule forming capacity. Instead, the formation of a tubule reflects the ability of a protein to increase the curvature of a liposome. Indeed, a tubule is simply a liposome with high curvature around the circular cross-section, but with a relaxed curvature along its length. Therefore, a clearer view of the role of a protein *in vivo* can be obtained by studying proteins in combination. Whereas epsin can tubulate liposomes, in combination with clathrin it forms invaginated clathrin buds (Ford *et al.*, 2002) and *in vitro* the combination does not form tubules. Therefore the ability of a protein to form tubules may not always represent an *in vivo* tubulation activity, as interactions with other proteins may modify the conformation adopted within the cell. However, the ability of a protein to tubulate liposomes does reveal its capacity for altering and stabilizing a particular membrane curvature.

In the method described below, the processes of bilayer deformation can be studied using synthetic liposomes of any defined lipid composition. Liposomes are incubated with the protein of interest and observed in the electron microscope to reveal changes in liposome conformation accompanied with protein binding. In many of our studies where we did not know the precise lipid composition to use, we have instead used Folch brain lipids (Sigma, St. Louis, MO).

Reagents and Equipment

Lipid Stocks. Phosphatidylinositol and phospatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) (Avanti Polar Lipids, Alabaster, AL) weredissolved to 1 mg/ml in 3:1 chloroform:methanol. Cholesterol (Avanti), phosphatidylserine (PtdSer), phosphatidylcholine (PtdChol), and phosphatidylethanolamine (PtdEth) (Sigma) were dissolved to 10 mg/ml in chloroform. Folch extract is a total brain lipid extract containing around 10% of a variety of phosphatidylinositol lipids (Sigma, B1502) and is dissolved to

10 mg/ml in a 19:1 mixture of chloroform:methanol. Concentrated lipid stocks were stored at −80° under argon in glass vials with glass or Teflon lids.

Electron Microscopy Grids. First place a droplet of 2% collodian in amyl acetate (TAAB) onto a bath of filtered milli Q water. Allow the solvent to evaporate, leaving a film of plastic on the water surface. Wash a box of 300 mesh carbon electron microscope grids (TAAB) in acetone, air dry, and place onto the carbon surface with the ''rough'' side in contact with the carbon. Remove the plastic film from the top of the water by laying a piece of parafilm onto the top and pulling it off again. Allow this to dry on the bench. When dry, carbon coat using an E306A carbon evaporator (BOC Edwards, UK), leaving a thin carbon coat on the surface of the grid.

Stain. Prepare 2% uranyl acetate (Biorad, UK) with 0.0025% polyacrylic acid (Sigma) in water. The presence of polyacrylic acid reduces precipitation of the stain. In the absence of polyacrylic acid, stain should be prepared fresh and filtered before use.

Nucleotides. GDP and GTPγS were purchased from Roche. GTPγS should be fresh to avoid breakdown products, including GDP, which will alter the conformation of bound dynamin.

Extrusion of Liposomes. An extrusion device is used to generate liposomes of a defined maximum size (shown in an ''exploded'' view Fig. 1A). The central element of the device consists of two pieces of Teflon with a 0.6 mm hole passing through each block. These holes widen out at one side to 4 mm to allow the insertion of a 1 ml disposable syringe. At the opposite end is a groove to accommodate a 15 mm o-ring. Two of these pieces are placed together with o-rings in contact to form a seal around a filter. These filters are Whatman Cyclopore filters with pore sizes of 0.1, 0.2, or 0.4 μm. A swagelok male pipe weld connector, bored out to hold the Teflon blocks, is used to hold the arrangement in place. 1 ml disposable syringes placed at either end allow the lipid mixture to be extruded through the filter in both directions.

Making Liposomes of a Defined Size

1. Prepare lipid mixtures to a total lipid concentration of 1 mg/ml in a 19:1 mixture of chloroform:methanol in a glass tube. Methanol is necessary to maintain the solubility of some lipids, including PtdIns(4,5)P_2. A common lipid mixture consists of 10% cholesterol, 40% PtdEth, 40% PtdSer, and 10% of the lipid specific for the protein of interest (often PtdIns(4,5)P_2). An alternative is to use a Folch brain lipid extract or a liver lipid extract.

2. Remove solvent from the lipid mixture by evaporation under a gentle stream of Argon. This generates a thin lipid film on the surface of the glass. When this film becomes white, turn up the flow to spread out the last drops and avoid leaving thick lumps of precipitated lipid. Place the dried lipid in a desiccator under vacuum for 10–15 min to complete drying.

3. Rehydrate the dried lipid mixture by adding 1 ml of filtered buffer (50 m*M* HEPES pH 7.4, 120 m*M* NaCl) and leave for 5 min at room temperature. Sonicate in a bath sonicator for 2 min to strip the lipid film off the surface of the glass. Then apply two 2 sec pulses with a small (2 mm) probe sonicator to break lipid particles into liposomes.

4. Filter through 0.1, 0.2, or 0.4 μm polycarbonate filters (Whatman Cyclopore filters, UK) using the extrusion device. The sample should be pushed through the filtration unit between the two 1-ml syringes 11 times. It is important to use an odd number of extrusion steps to ensure that all of the final material has been through the filter and material simply trapped in the extrusion device is not washed back into the sample. A loss of 100–200 μl is usually observed.

Purification of Dynamin and Its Mutants from E. coli

Dynamin can be prepared from a variety of different sources, including rat brain, bacteria, or baculovirus-infected insect cells. The purification protocol relies on the affinity of dynamin for the SH3 domain of amphiphysin 2. The protocol yields approximately 0.5 mg of dynamin from each liter of bacterial culture.

1. To produce dynamin from *E. coli*, the dynamin 1 gene is expressed from pET34b. Grow cells at 37° until log phase, induce with 1 m*M* IPTG, and incubate overnight at 19°. Harvest cells and resuspend in 150 m*M* NaCl, 20 m*M* HEPES pH 7.4, 2 m*M* EDTA, 2 m*M* DTT (incubation buffer). Break by French Press and clarify by centrifugation at 200,000*g*.

2. Lyse 3 l of *E. coli* containing a GST fusion of the Amph2 SH3 domain in incubation buffer. Clarify at 200,000*g* for 30 min and incubate the supernatant with glutathione beads for 40 min. Wash with incubation buffer.

3. Incubate Amph2 SH3 coated glutathione beads with dynamin containing lysate for 40 min and wash with incubation buffer. Elute dynamin with 1.1 *M* NaCl, 20 m*M* PIPES pH6.2, 10 m*M* $CaCl_2$ and dialyze overnight into incubation buffer. Concentrate using a 50 kDa MWCO Vivaspin concentrator. If the preparation is clean, the sample can be concentrated up to and above 0.5 mg/ml without precipitation.

4. Further purify the dynamin by gel filtration. Use an S200 16/60 gel filtration column (Amersham Biosciences) pre-equilibrated with 150 m*M* NaCl, 20 m*M* CHES pH 10, 2 m*M* EDTA, 2 m*M* DTT. Use of a pH 10 buffer prevents dynamin oligomerization, allowing greater concentration and purification by gel filtration. Dialyze into 150 m*M* NaCl, 20 m*M* HEPES pH 7.4, 0.4 m*M* DTT for use.

Analyzing the Effect of Proteins on Liposome Conformation

1. Incubate liposomes (0.1 mg/ml lipid) and protein (0.1–0.5 mg/ml) in buffer for 5 min at room temperature.
2. Place 5 μl liposome mixture onto a glow discharged (hydrophilic) electron microscope grid and leave for 1 min to adhere. Blot excess buffer onto Whatman filter paper and stain by pipetting uranyl acetate onto grids, leave for 15 sec, blot, and then repeat. Blot gently, leaving a thin layer of stain on the grids, leave to air dry, and study in a transmission electron microscope.

Method 2: Analysis of Conformational Changes of Dynamin on Preformed Lipid Nanotubes

Introduction

Different lipid molecules associate together to form membrane structures with very different overall curvatures. When liposomes are made in the presence of sufficient concentrations of nonhydroxylated fatty-acid galactoceramides (NFA-GalCer) they form nanotubes (Wilson-Kubalek *et al.*, 1998). We have used these preformed lipid nanotubes to examine the effect of GTP hydrolysis on the conformation of a dynamin helix (Marks *et al.*, 2001; Stowell *et al.*, 1999). Indeed, in the absence of GTP hydrolysis, purified dynamin aggregates to form helices and, as seen above, can tubulate liposomes. Also, dynamin interacts with lipid nanotubes to form ordered arrays with a diameter similar to the constricted necks of clathrin-coats pits observed *in vivo* (Stowell *et al.*, 1999; Takei *et al.*, 1995), suggesting that the nanotube acts as a good mimic of the neck of a vesicle.

The analysis of dynamin helices formed on lipid tubules provides a picture of what happens to a dynamin assembly upon GTP hydrolysis, as dynamin adopts different conformations in the presence of different nucleotides. While the GTP bound state of dynamin (observed in the presence of GTPγS) forms a tight helix with a spacing of 11 nm, in the GDP bound state the spacing has nearly doubled to 20 nm (Stowell *et al.*, 1999).

This suggests a dramatic increase in the pitch of the helix upon GTP hydrolysis that may drive the scission of the vesicle neck: the ''poppase'' model for dynamin function. Further experiments, with mutants of dynamin, have shown that efficient GTP hydrolysis and the increase in pitch of the dynamin helix are required to allow endocytosis (Marks *et al.*, 2001).

We propose that lipid nanotubes will be important for analysis of the conformational states of other members of the dynamin superfamily. However, it should be noted that these tubules are conformationally stable and may not be as easily ''squeezed'' as liposomes. Therefore conformational changes perpendicular to the membrane plane may be underestimated. Thus (as with the tubulation assay described in Method 1) conclusions must be verified in live cells, where mutants can be expressed and trafficking monitored (Marks *et al.*, 2001).

Reagents and Equipment

Lipid Stocks and Buffers. Lipid nanotube formation uses the same equipment as liposome formation. The major difference is the use of NFA-GalCer (Sigma) as a replacement for phosphatidylethanolamine and phosphatidylserine. NFA-GalCer stocks are prepared to 10 mg/ml in chloroform and stored at -80° under argon. The buffer used for the study of dynamin on lipid nanotubles is 135 m*M* NaCl, 5 m*M* KCl, 20 m*M* HEPES pH 7.4, 1 m*M* $MgCl_2$.

Procedure

Making Lipid Nanotubes

1. The key lipid component required to promote nanotube formation is NFA-GalCer (Sigma C 1516). Tubule formation requires in excess of 20% NFA-GalCer with a typical composition of (w/w) 10% cholesterol, 40% NFA-GalCer, 40% PtdChol, 10% PtdIns(4,5)P_2. This generates lipid tubes with a diameter of around 28 nm, which are filtered to give a uniform length (Fig. 2A).

2. Make nanotubes using the same protocol as for liposomes. After drying the lipid mixture, resuspend in buffer and sonicate as above. To form a homogenous preparation, extrude through a filter with a pore size of 0.2 μm before use.

Studying the Conformational Stage of Dynamin Spirals

1. Incubate nanotubes (0.1 m*M* lipid), dynamin (0.5 μM), and nucleotides (1 m*M*) for 10 min at room temperature. To observe the GTP bound form of dynamin use fresh GTPγS.

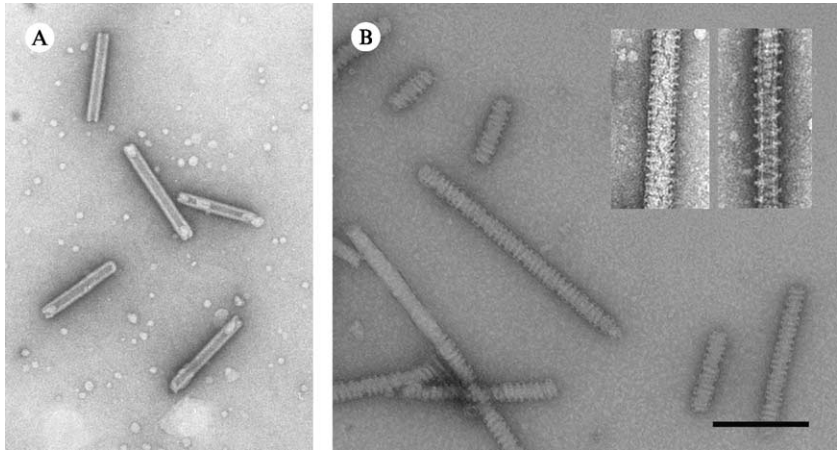

FIG. 2. Studying the conformation of dynamin on a lipid nanotube. (A) Undecorated lipid nanotubes. (B) Lipid nanotubes incubated with dynamin in the presence of GTPγS. The scale bar is 200 nm. The inserts show close-up views of dynamin on lipid tubules in the presence of GTPγS (left hand insert) and GDP (right hand insert) showing that the pitch of the dynamin spiral is greater when GDP bound than when GTPγS bound.

2. Place onto a glow discharged (hydrophilic) electron microscope grid and leave for 1 min to adhere. Blot excess buffer onto Whatman filter paper and stain for two 15 sec incubations with uranyl acetate. Blot gently, leaving a thin layer of stain on the grid surface, air dry, and observe by electron microscopy. Figure 2B shows dynamin spirals observed on lipid nanotubes.

Method 3: Analysis of Early Stages of Endocytosis on a Lipid Monolayer

Introduction

Lipid monolayers have been used for many years as templates for the formation of two-dimensional crystals (Chiu *et al.*, 1997), and for surface tension measurements (Stahelin *et al.*, 2003). We have used them as mimics of the inner leaflet of the plasma membrane to reconstitute the early stages of clathrin-coated vesicle formation (Ford *et al.*, 2001, 2002) and they can equally well be applied to other vesicle and tubule budding mechanisms. A lipid monolayer forms when a droplet of solvent-dissolved lipid is placed onto an aqueous droplet. As the solvent evaporates, lipid molecules become oriented at the air-water interface with head groups in contact

with the aqueous mixture and hydrophobic tails extending into the air. Proteins of interest are injected underneath the monolayer and a hydrophobic electron microscope grid is placed onto its hydrophobic surface. The grid can be removed and stained for study in the electron microscope, revealing the structures that form on the aqueous surface of the monolayer. In addition, the grid can be platinum shadowed, with the height of shadows revealing the degree of invagination (or budding) of the membrane.

This assay is well suited for the study of clathrin coat formation as distinctive clathrin arrays can be readily observed by electron microscopy. It was first used to show the role of AP180 in promoting the formation of flat clathrin lattices on a membrane surface containing PtdIns(3,4)P_2 and the increased degree of invagination of these lattices upon the addition of the AP2 adaptor complex (Ford *et al.*, 2001). Subsequently, it has been used to investigate the nucleation of clathrin lattices by both epsin (Ford *et al.*, 2002) and disabled-2 (Mishra *et al.*, 2002).

Reagents and Equipment

Buffers and Reagents. Nucleation of clathrin lattices by AP180 or AP2 was studied HKM buffer, consisting of 25 m*M* Hepes pH 7.4, 125 m*M* potassium acetate, 5 m*M* magnesium acetate, and 1 m*M* dithiothreitol. Lipid stocks and uranyl acetate stocks were prepared as above.

Purified Proteins. When analyzing clathrin lattice formation, clathrin was purified from pig brain as in Smith *et al.* (1998), dialyzed into HKM buffer, and centrifuged for 20 min at 100,000*g* immediately prior to use to remove aggregates. AP2 was also purified from pig brain as in Smith *et al.* (1998) and rat brain AP180 was expressed in baculovirus-infected Sf9 cells and purified as in Ford *et al.* (2001).

Monolayer Device. This consists of a Teflon block with 60-μl wells (as shown in Fig. 3A and B). Each well has two entrances. The lipid monolayer and electron microscope grid are placed on the large 35 mm diameter top entrance (A) while a smaller side entrance (B) is used for injection of protein samples underneath the monolayer. As traces of lipid contaminants on the Teflon block can result in misleading negative controls, the block should be cleaned thoroughly before and after use by rinsing with hot water, then ethanol, and finally, soaking overnight in a mixture of chloroform/methanol to remove any protein or lipid residue. A humid chamber is used to prevent evaporation during the experiment. A covered container with a wet sponge or paper towel is sufficient.

Carbon Coated Electron Microscopy Grids. These are prepared as described previously; however, if copper grids are used, some copper

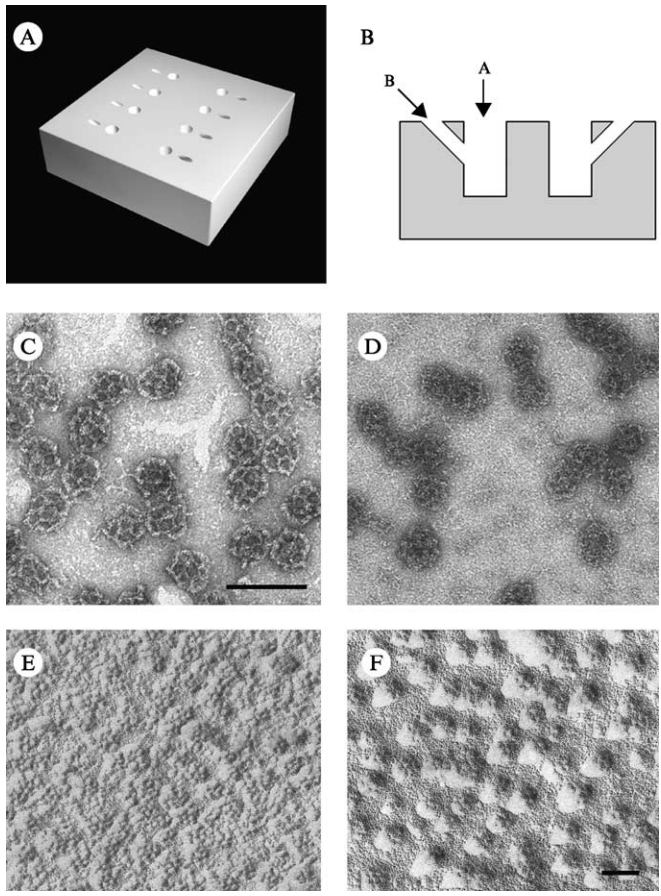

FIG. 3. Studying early stages of vesicle budding on lipid monolayers. (A) A view showing the Teflon block used to investigate the formation of clathrin coats on a lipid monolayer. (B) A cross section through two of the wells. Each well has two entrances. Entrance A is where the lipid monolayer is formed while entrance B is used to inject protein underneath the monolayer. (C) and (D) are images of negatively stained clathrin coats formed in the presence of (C) AP180 or (D) AP180 and AP2. The presence of added AP2 results in formation of more electron dense coats. (E) and (F) are images of platinum shadowed clathrin coats formed in the presence of (E) AP180 or (F) AP180 and AP2. The addition of AP2 leads to invagination of the coats as seen by the increase in the shadow length. Scale bars are 200 nm in length.

leaches from the grid during the incubation with the lipid monolayer. Therefore we use gold electron microscope grids (G204G from Agar Scientific, Stansted, Essex, England) which have been coated using the protocol described above. Forceps are used for handling EM grid, and

self-locking spring forceps are especially useful. Whatman filter paper was used for blotting EM grids.

Platinum Shadowing. An E306A carbon evaporator is required. This is used in conjunction with a 10 cm long 0.2 mm diameter piece of platinum wire (TAAB Laboratories, Aldermaston, Berkshire, England) and a 10 cm long, 1 mm thick tungsten wire (also TAAB). Samples are viewed with a transmission electron microscope.

Procedures

Formation of a Lipid Monolayer

1. Prepare lipid mixtures to a total lipid concentration of 0.1 mg/ml in a 19:1 mixture of chloroform:methanol. Methanol is necessary to maintain the solubility of some lipids, including PtdIns(4,5)P_2. The lipid mixture can be tailored to the protein of interest, with 10% cholesterol, 40% PtdEth, 40% PtdSer, and 10% PtdIns(4,5)P_2 used for the analysis of AP180 and epsin mediated clathrin nucleation. An alternative is to use a brain lipid extract such as Folch extract. Lipid mixtures should be prepared fresh.

2. Arrange the Teflon block in a humid chamber and fill wells with buffer. It is important not to overfill wells, as a concave inner surface will influence monolayer formation.

3. Carefully pipette (or inject with Hamilton syringe) 1 μl of lipid mixture on to the larger entrance of each well (A). As a negative control, pure chloroform or a lipid mixture that will not bind the protein of interest can be injected. These controls are important when studying clathrin coat formation as traces of preformed clathrin cages will otherwise give misleading results. Incubate the block at room temperature for 60 min allowing chloroform to evaporate, leaving a monolayer of lipid on the surface of the buffer.

4. Carefully place one EM grid, carbon side down, onto the top of each buffer droplet. Grids should not be glow discharged before use as a hydrophobic carbon film is required to adhere to the hydrophobic lipid tails of the monolayer.

Analysis of Coat Formation

1. Gently inject proteins into the side injection entrance (B). When analyzing clathrin lattice formation, the final protein concentrations in the well should be 0.5–2 μM for AP180/epsin/adaptor protein, and 30–500 nM for clathrin. Incubate for 60 min at room temperature in the humid chamber.

2. On a piece of parafilm, place 15–20 μl droplets of stain for each grid. Then, to facilitate removal of the grid from the Teflon block, gently inject 30 μl of buffer into the side injection entrance (B in Fig. 3B), thereby raising the grid above the surface of the block. Immediately remove the grid with forceps and lift it vertically off of the droplet. Blot the grid briefly by touching it on a piece of filter paper, then the first stain droplet, and then blot immediately. Touch the grid to the second stain droplet, leave for 30 sec, and blot briefly. This negatively stains the sample by leaving a film of stain on the surface of the grid in which the protein is embedded. However, if the grids is to be platinum shadowed, hold it to the filter paper for several seconds before the stain dries to ensure the removal of excess stain as required for positive staining. Lay the grid on another piece of filter paper to dry.

3. Grids can be examined in a transmission electron microscope immediately, or stored at room temperature. The result of experiments investigating the effect of AP180 and AP2 on clathrin lattice formation in this way is shown in Figure 3C and D.

4. To investigate the degree of invagination of structures formed on the grid surface, a positively stained grid can be platinum shadowed. Set up the vacuum evaporator with a 2 cm long piece of platinum wire coiled tightly around a piece of 1 mm thick tungsten wire. Place the grids to be shadowed on a platform such that the platinum wire maintains an angle of about 10° to the plane of the grid. Place a shield between the grid and the platinum and create a vacuum in the evaporator. Turn on the current and when the platinum wire melts, remove the shield. Often two minutes of shadowing is sufficient, but this depends on the evaporator and should be optimized. The results of single angle platinum shadowing for clathrin lattices formed in the presence of AP180 and AP2 are shown in Fig. 3E and F.

Conclusions and Future Directions

The methods presented here provide snapshots of membrane budding and scission processes. Future developments will make the assays more quantitative rather than qualitative, allowing these studies to address many of the important questions remaining in understanding vesicle formation such as the mechanism of cargo incorporation into a coated vesicle, the energetics of vesicle generation, the use of alternative adaptor molecules, and the regulation of the process. No doubt these techniques will also be applied to many other different membrane budding and scission mechanisms and will help in the assignments of protein functions. The ultimate goal

is reconstitution of complete budding pathways *in vitro* as more and more proteins are purified and added into systems like the ones presented.

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[53] A Continuous, Regenerative Coupled GTPase Assay for Dynamin-Related Proteins

By ELENA INGERMAN and JODI NUNNARI

Abstract

Dynamin-related proteins (DRPs) compose a diverse family of proteins that function, through GTPase stimulated self-assembly, to remodel cellular membranes. The molecular mechanism by which DRPs mediate membrane remodeling events and the specific role of their GTPase cycle is still not fully understood. Although DRPs are members of the GTPase superfamily, they possess unique kinetic properties. In particular, they have relatively low affinity for guanine nucleotides and, under conditions that favor self-assembly, they have high rates of GTP turnover. Established fixed time point assays used for the analysis of assembly stimulated GTPase activity are prone to inaccuracies due to substrate depletion and are also limited by lack of time resolution. We describe a simple, continuous, coupled GTP regenerating assay that tackles the limitations of the fixed time point assays and can be used for the kinetic analysis of DRP GTP hydrolysis under unassembled and assembled conditions.

Introduction

Dynamin-related GTPases (DRPs) have evolved in eukaryotic cells to function in such diverse processes as membrane trafficking, organelle division, and resistance to viral infection (Danino and Hinshaw, 2001; Osteryoung and Nunnari, 2003; Praefcke and McMahon, 2004; Song and

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