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Generation of Coated Intermediates of Clathrin-Mediated Endocytosis on Protein-Free Liposomes

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Summary

Clathrin-coated buds and dynamin-coated tubules morphologically similar to corresponding structures observed in synaptic membranes can be generated on protein-free liposomes by incubation with cytosol, or with clathrin coat proteins and purified dynamin, respectively. Dynamin- and clathrin-coated intermediates may form independently of each other, despite the coupling between the two processes typically observed in synaptic membranes. Formation of both structures on liposomes can occur in the absence of nucleotides. These findings indicate that interfaces between lipids and cytosolic proteins are fully sufficient to deform lipids bilayers into buds and tubules. They suggest that a main function of membrane proteins is to act as positive and negative regulators of coat assembly, therefore controlling these processes in time and space.

Introduction

In eukaryotic cells, anatomically distinct membranes are functionally connected to each other via vesicular transport. A fundamental basis for these processes is the property of intracellular membranes to generate vesicular buds enriched in selected cargo proteins, which then separate as free vesicles. Vesicle formation starts with the assembly on the donor membrane of a "coat," which acts as a scaffold both to bend the membrane and to select cargo membrane proteins (Pearse and Robinson, 1990; Rothman, 1994; Schekman and Orci, 1996). Several coats have been characterized. They include the COPI coat (Rothman, 1994), the COPII coat (Schekman and Orci, 1996), and the clathrin coats (Pearse and Robinson, 1990).

One of the most thoroughly investigated budding reactions is the formation of clathrin-coated vesicles from the plasmalemma. This process is implicated in a variety of cellular functions, including the internalization of receptors, the uptake of viruses (Mellman, 1996), and the recycling of synaptic vesicle membranes in nerve terminals (Heuser and Reese, 1973; Takei et al., 1996). While some of the protein isoforms that participate in clathrin-mediated endocytosis in nerve terminals are unique, the fundamental aspects of clathrin-mediated endocytosis appear to be highly conserved (De Camilli and Takei, 1996). Thus, clathrin-mediated recycling at the synapse has been a very useful model system to study molecular mechanisms in clathrin-mediated endocytosis.

Nerve terminal clathrin coats comprise, besides clathrin, the clathrin adaptor AP-2 (Robinson, 1994) and the protein AP180 (Ye and Lafer, 1991; Morris et al., 1993). Following formation of a deeply invaginated bud, the generation of a free vesicle is completed when oligomerization of dynamin I at the neck of the coated pit drives the fission reaction (Hinshaw and Schmid, 1995; Takei et al., 1995). A variety of other accessory cytosolic proteins have also been implicated in these processes. These include amphiphysin, synaptojanin I (Wang et al., 1995; David et al., 1996; McPherson et al., 1996; Bauerfeind et al., 1997; Ramjaun et al., 1997; Wigge et al., 1997), Eps15 (Benmerah et al., 1996; Di Fiore et al., 1997), and epsin (Chen et al., 1998). Several potential membrane binding proteins for AP-2–containing clathrin coats have been identified (Kirchhausen et al., 1997). Some of these proteins are nonobligatory passengers of clathrin-coated vesicles. However, a high affinity docking apparatus that plays an essential role in coat formation and contains a trypsin-sensitive site has also been postulated (Kirchhausen et al., 1997). The protein synaptotagmin was proposed to represent this site (Zhang et al., 1994).

In addition to protein–protein interactions, recent studies have suggested an important role of lipids in the recruitment of coats, including the clathrin coat. Both AP-2 and the protein AP180, which has the property of an accessory clathrin adaptor (Ye and Lafer, 1991; Morris et al., 1993), were found to bind phosphoinositides (Ye et al., 1995; Gaidarov et al., 1996). Furthermore, dynamin was shown to interact with membrane acidic phospholipids (Tuma et al., 1993; Liu et al., 1994) and to bind phosphoinositides via its PH domain (Lin et al., 1997). Together with results of other studies implicating lipids in vesicular traffic, these findings have raised the possibility that phospholipids may contribute to the membrane anchoring of "coats" either directly or via allosteric regulation of protein–protein interactions (De Camilli et al., 1996; Kirchhausen et al., 1997; Rapaport et al., 1997).

We have previously established a cell-free system that allows for the visualization of transient endocytic intermediates which participate in the clathrin-dependent reformation of synaptic vesicles (Takei et al., 1995, 1996). These intermediates consist of clathrin-coated pits that are often connected to donor membranes by a narrow tubular stalk decorated with dynamin rings (Takei et al., 1995). The goal of the present study was to determine whether specific synaptic membrane proteins play an essential role in the recruitment of the nerve terminal cytosolic endocytic apparatus. To this aim, we have tested the ability of brain cytosolic proteins to generate endocytic intermediates on a variety of non-neuronal membrane templates and have found that lipids alone can support the formation of clathrin-coated buds and dynamin-coated tubules.

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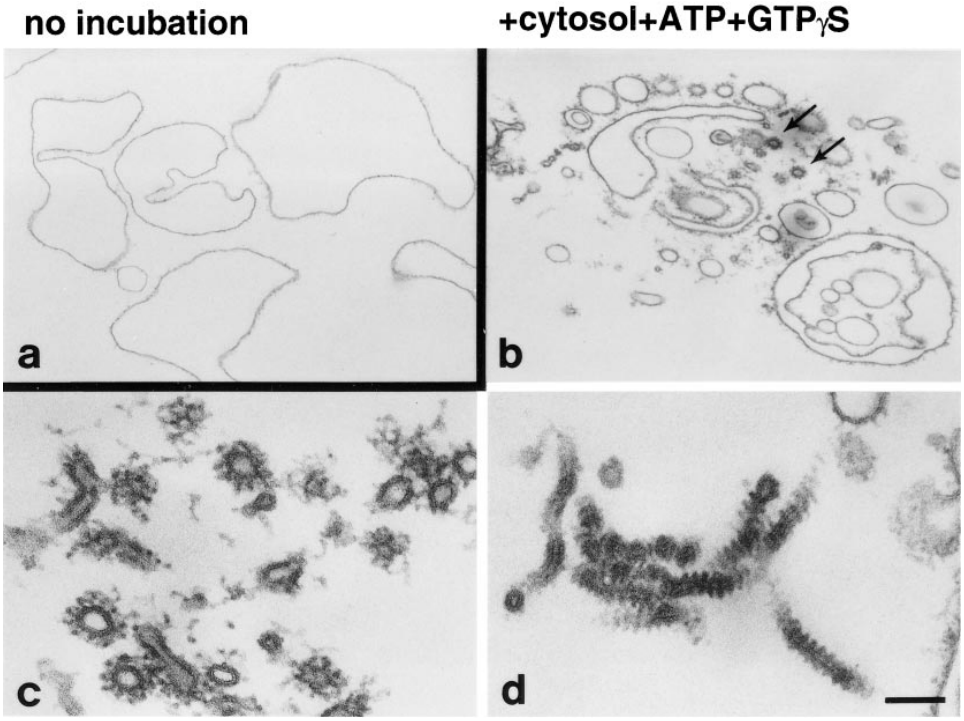

Figure 1. Electron Micrographs Demonstrating the Effects of Various Incubations on the Morphology of Inside-Out Erythrocyte Vesicles

Inside-out resealed vesicles are characterized by the presence of a submembranous cytomatrix at their external surface (a). Incubation of the membranes with rat brain cytosol, ATP, and GTPγS generated clathrin-coated buds ([b] [arrows] and [c]) and dynamin-coated tubules (d). Calibration bar, 400 nm in (a) and (b); 100 nm in (c) and (d).

Results

Brain Cytosol Generates Coated Endocytic Intermediates on Nonneuronal Plasma Membranes

We have previously shown that incubation of synaptic membranes with brain cytosol, ATP, and GTPγS (200 μM) results in the formation of numerous clathrin buds and dynamin-coated tubules (Takei et al., 1995, 1996). These endocytic structures are also positive for other nerve terminal proteins thought to have an accessory role in clathrin-mediated endocytosis, such as amphiphysin and synaptojanin (Bauerfeind et al., 1997; Haffner et al., 1997). When the same cell-free incubations were performed using CHO cell plasma membranes as templates, similar coated intermediates, positive for all of several cytosolic nerve terminal endocytic proteins tested, were generated (data not shown). We next used as templates plasma membranes that do not normally have any endocytic function and that are not expected therefore to contain intrinsic membrane proteins specialized for endocytosis: the plasma membrane of erythrocytes.

Erythrocyte membrane ghosts were processed under conditions that enhance formation of inside-out resealed plasmalemmal vesicles (Sulpice et al., 1994). These vesicles, which were recognizable by EM due to the presence of a residual submembranous cytoskeleton at their external surface, had a smooth profile (Figure 1a). Incubation with brain cytosol plus ATP and GTPγS produced a major morphological change, including the formation of numerous clathrin-coated pits and dynamin-coated tubules (Figures 1b–1d). Consistent with previous studies, ATP alone was sufficient to deform inside-out vesicles (data not shown), probably due to a rearrangement of the submembranous cytoskeleton (Morris et al., 1992). However, the presence of clathrin-coated pits and dynamin-coated tubules did not occur under these conditions.

These findings raised the possibility that specific docking proteins in the membrane may not be essential for the formation of clathrin- and dynamin-coated intermediates. They prompted us to perform similar experiments with protein-free liposomes.

Cytosol Generates Endocytic-like Coated Tubules and Buds on Liposomes

Liposomes prepared from a brain total lipid extract had smooth profiles and large diameters, some exceeding 1 μm (Figure 2a). Incubation with brain cytosol, ATP, and GTPγS resulted in a drastic morphological change with the massive formation of high curvature membrane profiles as seen by both thin sectioning and whole-mount preparations (Figures 2c and 2d). The more prominent change was the formation of dynamin-coated tubules (Figures 2d and 2e), but clathrin-coated buds were also observed (Figures 2d and 2f). At the level of resolution of thin section electron microscopy, these structures were morphologically identical to corresponding

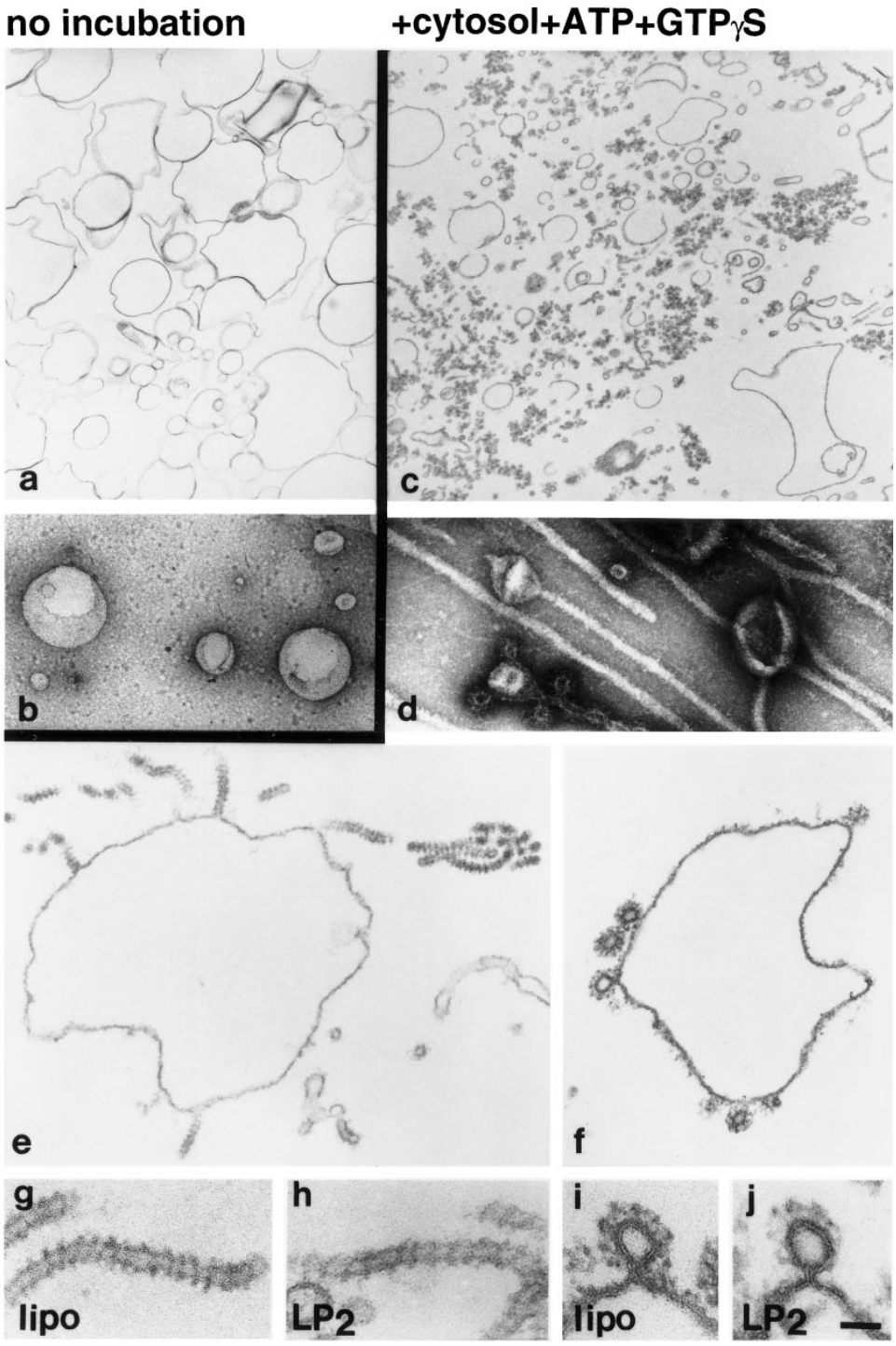

Figure 2. Electron Micrographs Demonstrating the Effect of Brain Cytosol on Liposomes Composed of Total Brain Lipids

Preparations were observed after plastic embedding and thin sectioning with the exception of fields (b) and (d), which show negative staining of whole-mount preparations. (a and b) Control liposomes not incubated with brain cytosol. (c–f) Liposomes incubated with ATP, GTPγS, and brain cytosol. High power observation reveals presence of dynamin-like rings and clathrin coats. Fields (g)–(j) demonstrate the similarity of dynamin-coated tubules and clathrin-coated pits observed on liposomes (lipo) and synaptic membranes (LP2 fraction) incubated with brain cytosol, ATP, and GTPγS. Calibration bar, 500 nm in (a) and (c); 140 nm in (b) and (d); 150 nm in (e) and (f); and 50 nm in (g)–(j).

endocytic intermediates observed in synaptic membranes (LP2 subfractions) incubated under the same conditions (Figures 2g–2j). However, tubules were much more abundant in liposomes, while the number of clathrin-coated pits in these preparations was lower than in synaptic membranes (data not shown). Furthermore, negative staining observations revealed that the majority of the dynamin-coated tubules present in the

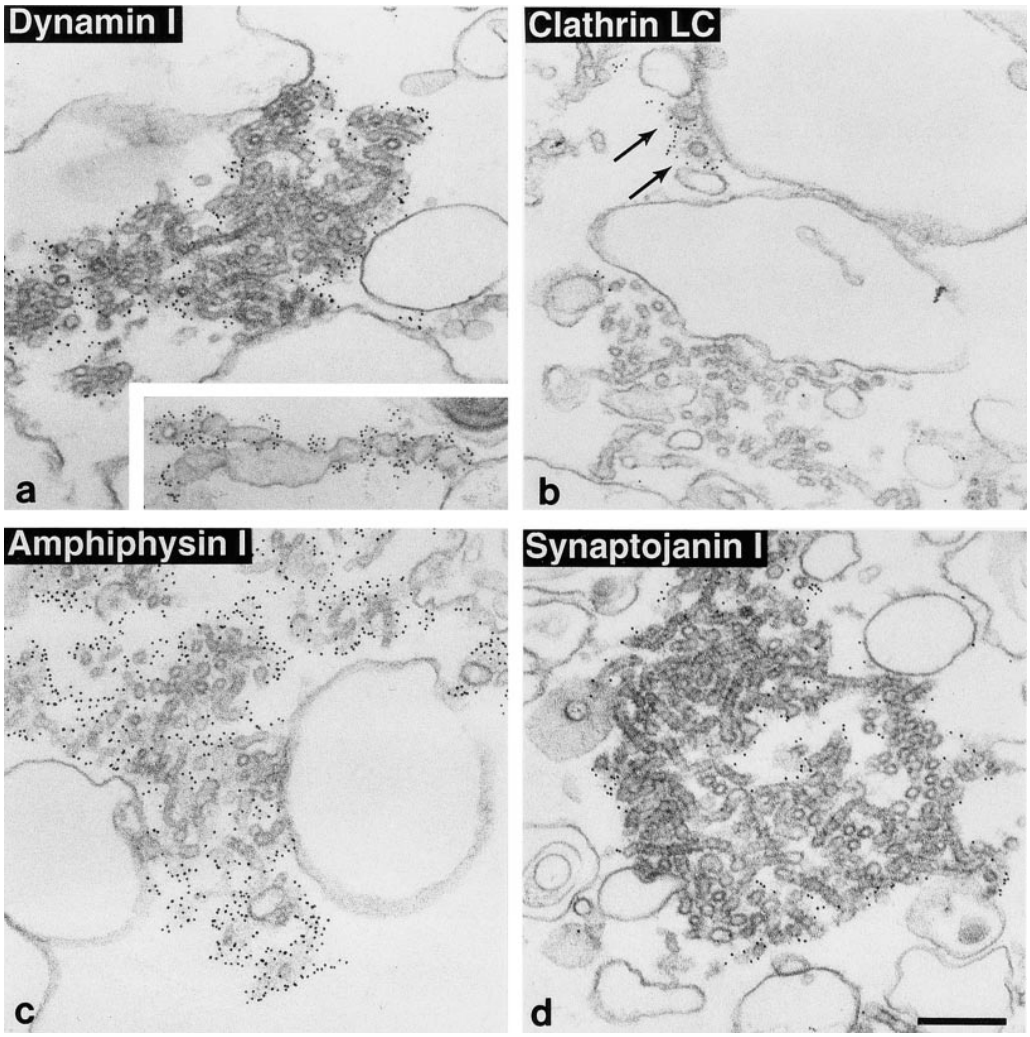

Figure 3. Immunogold Localization of Cytosolic Endocytic Proteins on Liposomes of Total Brain Lipids Incubated with Cytosol, ATP, and GTPγS

Dynamin I and clathrin are concentrated at tubules and buds (arrows in [b]), respectively. Note the concentration of dynamin at sites of liposome constriction in the inset of field (a). Amphiphysin I (c) and synaptojanin I (d) are also concentrated on the coated evaginations. Calibration bar, 200 nm.

liposomes were not capped by a clathrin coat (for example, see Figure 2d). Interestingly, clathrin-coated buds occurred in clusters as though assembly of these buds was a cooperative process.

To confirm that the coats were represented by dynamin and clathrin, we performed electron microscopy immunogold cytochemistry. As shown in Figures 3a and 3b, coated tubules and pits were intensely positive for dynamin I and clathrin immunoreactivity, respectively. Amphiphysin I and synaptojanin I were also concentrated on coated liposomal surfaces (Figures 3c and 3d), consistent with their accessory role in dynamin I action (Bauerfeind et al., 1997; Haffner et al., 1997).

Given the relatively low number of clathrin-coated pits visible in these preparations, we tested the effect of a brain protein extract highly enriched in clathrin coats, which we obtained by stripping purified bovine brain clathrin-coated vesicles (Figure 4a and 4b). Addition of the coat protein fraction to liposomes in the presence of ATP plus GTPγS resulted in a massive formation of clathrin-coated membrane profiles, most of which (and possibly all) were represented by coated pits (Figure 4c, field i). The number of clathrin-coated profiles in these preparations was more than 100-fold higher than on liposomes incubated with total brain cytosol. No dynamin-coated tubules were visible after these incubations, consistent with the very low amount of dynamin present in the coat fraction (see Figure 4b). However, several dynamin-coated tubules capped by clathrin-coated pits were observed when unfractionated brain cytosol, which contains endogenous dynamin, was added to the coat fraction (Figure 4c, field ii). Under these conditions a lower number of clathrin-coated pits was observed, as if dynamin tubulation competed with clathrin-coated pit formation.

To gain some initial insight into the lipid requirement

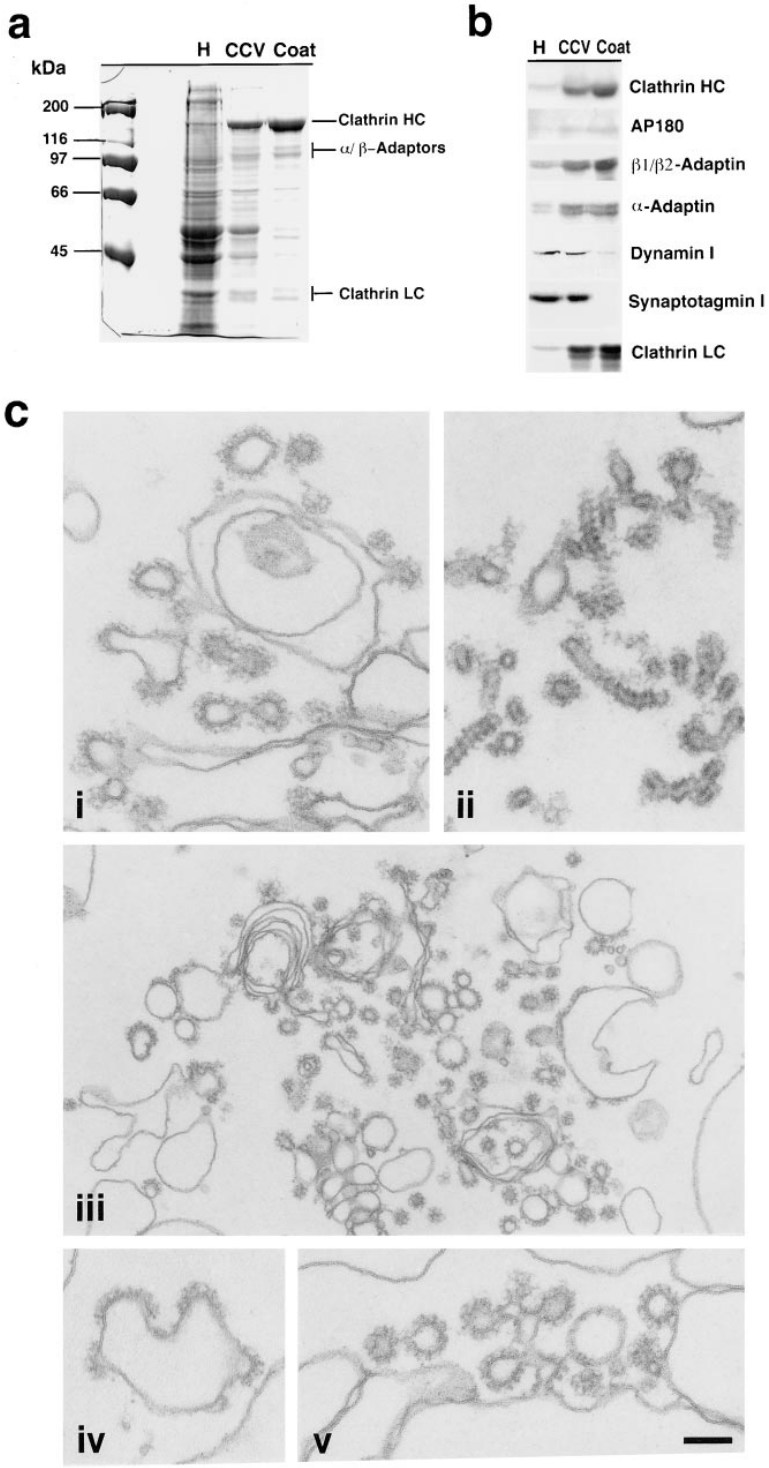

Figure 4. A Clathrin Coat Fraction Induces Massive Formation of Clathrin Coats on Liposomes Irrespective of the Presence of Nucleotides

(a) Preparation of a highly enriched clathrin coat fraction from bovine brain. Total brain homogenate (H; 50 μg protein), clathrin-coated vesicles (CCV), and the final coat protein fraction (coat; 30 μg protein each) were analyzed by SDS-PAGE followed by Coomassie blue staining.

(b) Western blot analysis of the samples shown in (a). Fifty micrograms of proteins were loaded in each lane. Blots were developed by ^{125}I-based autoradiography. Note the absence of synaptotagmin, a membrane marker, in the coat fraction.

(c) Electron micrographs of liposomes of total brain lipids after incubation with the coat fraction. (i) Liposomes incubated in the presence of coat fraction, ATP, and GTPγS. (ii) Liposomes incubated with total brain cytosol supplemented by the coat fraction in the presence of ATP and GTPγS. (iii–v) Liposomes incubated with the coat fraction without added nucleotides and in the presence of an ATP-depleting system. Calibration bar, 100 nm in (i), (ii), (iv), and (v); 180 nm in (iii).

of these coating reactions, total brain cytosol was incubated in the presence of ATP plus GTPγS with liposomes of defined lipid composition. These liposomes contained a basic mixture comprising 20% each of cholesterol, phosphatidylcholine, phosphatidylethanolamine (PE), and phosphatidylserine (PS), and an additional 20% of one of the following phospholipids: phosphatidylinositol (PI), a phosphoinositide mixture (PIs), PE, PS, phosphatidyl glycerol (PG), or phosphatidic acid (PA) (Figure 5a). Dynamin-coated tubule formation, as detected by electron microscopic observation and morphometry, was the highest in PA supplemented liposomes followed, in order of decreasing abundance, by PG, PIs, PI, PS, and PE (Figure 5b). In contrast, the highest number of clathrin-coated pits was observed in PE supplemented liposomes (Figure 5c). Biochemical

a. Compositions of defined liposomes

components \ liposomes	PI	PIs	PE	PS	PG	PA
cholesterol	20 %	20 %	20 %	20 %	20 %	20 %
phosphatidylchorine	20 %	20 %	20 %	20 %	20 %	20 %
Phosphatidylethanolamine	20 %	20 %	40 %	20 %	20 %	20 %
Phosphatidylserine	20 %	20 %	20 %	40 %	20 %	20 %
phosphatidylinositol	20 %					
phosphoinositide mixture		20 %				
Phosphatidylglycerol					20 %	
Phosphatidic acid						20 %

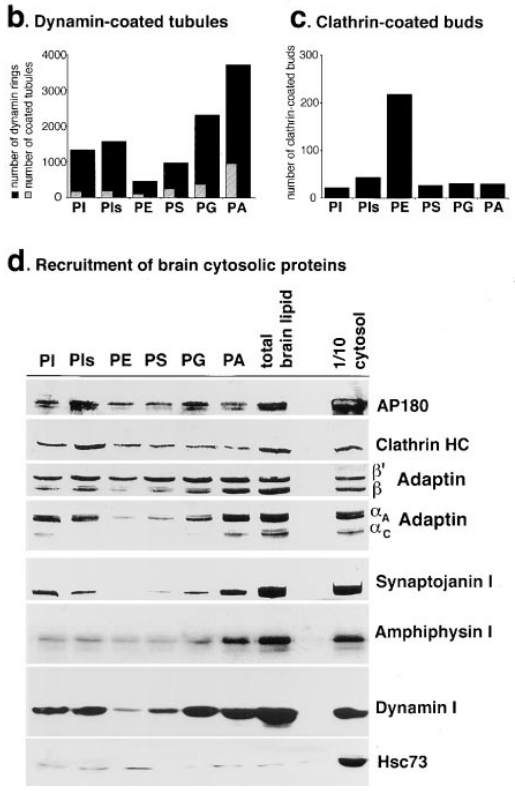

Figure 5. Effect of Phospholipids on Coat Recruitment

(a) Compositions of the lipid mixtures used for the following morphometric (b and c) and biochemical analysis (d).

(b and c) Morphometric analysis of the formation of dynamin-coated tubules and clathrin-coated pits in liposomes of defined compositions incubated with brain cytosol, ATP, and GTPγS. (b) Total number of longitudinal tubular profiles as well as total number of dynamin rings in a total thin section area of 6×10^4 μm^2. The total number of rings represents an estimate derived by multiplying the number of tubules by the average number of rings. (c) Total number of coated pits counted over the same area analyzed for dynamin-coated tubules.

(d) Western blots illustrating the recruitment of endocytic proteins onto liposomes. After incubation of liposomes with brain cytosol, ATP, and GTPγS, bound protein was analyzed. One-tenth of the amount of cytosol added to the liposomes was run in the right lane.

analysis of the property of various liposomes to recruit dynamin was in good agreement with the electron microscopic results (Figure 5d). Dynamin recruitment was the highest with total brain liposomes, lower in PA, PG, PI, and PIs supplemented liposomes, and even lower in PS and PE supplemented liposomes. Qualitatively similar results were observed for synaptojanin I and amphiphysin I. In contrast, no correlation was found between lipid binding of intrinsic components of the clathrin coat (AP180, the AP-2 subunit α-adaptin and clathrin) and the presence of clathrin-coated pits, suggesting that at least a fraction of these proteins bind to membranes (either directly or indirectly) independently and irrespective of coat assembly (Figure 5d).

Coated Tubules and Buds Can Form in the Absence of Nucleotides

Brain cytosol produced a massive tubulation of liposomes even in the absence of nucleotides. Tubules formed under these conditions were somewhat different from those formed in the presence of ATP and GTPγS (Figure 6, compare fields [a] and [d]). They were slightly variable in diameter, and their wall was only sparsely decorated by dynamin rings. A morphology similar to that produced by cytosol alone was observed with liposomes incubated with cytosol plus ATP (data not shown). Surprisingly, tubules with a patchy irregular coat were observed even in the presence of ATP plus GTP (200 μM) (Figure 6c) or in liposomes that had been incubated for 10 min with brain cytosol, ATP, and 200 μM GTPγS, and then supplemented with an excess of GTP (10-fold molar excess to GTPγS) for an additional 10 min to reverse the effect of GTPγS (data not shown). Previous studies of synaptic membranes had shown that tubules did not form if 200 μM GTPγS was replaced with 200 μM GTP (Takei et al., 1996). However, tubules that resemble in morphology the tubules generated by brain cytosol on liposomes in the presence of GTP were previously described at synapses fixed in situ (Heuser and Miledi, 1971; Lovas, 1971; Hama and Saito, 1977), and a few examples of such tubules can be observed in intact synaptosomes (Figure 6e). The connection of these tubules to clathrin had suggested their endocytic nature (Heuser and Miledi, 1971; Lovas, 1971; Hama and Saito, 1977). Thus, tubule formation in the presence of GTP may reflect a phenomenon that occurs physiologically at least under certain conditions.

Despite their different morphology, the tubules observed after all the various incubation conditions were positive for dynamin I, synaptojanin I, and amphiphysin I (Figure 6b and data not shown). Likewise, tubules occasionally observed at synapses in situ were also positive for these proteins (Figures 6f and 6g).

As in the case of dynamin-coated tubules, generation of clathrin-coated buds on liposomes did not require nucleotides. The massive formation of clathrin-coated buds on liposomes incubated with the coat fraction and in the presence of an ATP-depleting system is shown in Figure 4c (fields iii–v).

Dynamin-Coated Tubules Can Form Independently of Clathrin-Coated Buds

It has been speculated that dynamin-coated tubules may result from the progressive polymerization of dynamin at the neck of clathrin-coated buds (De Camilli

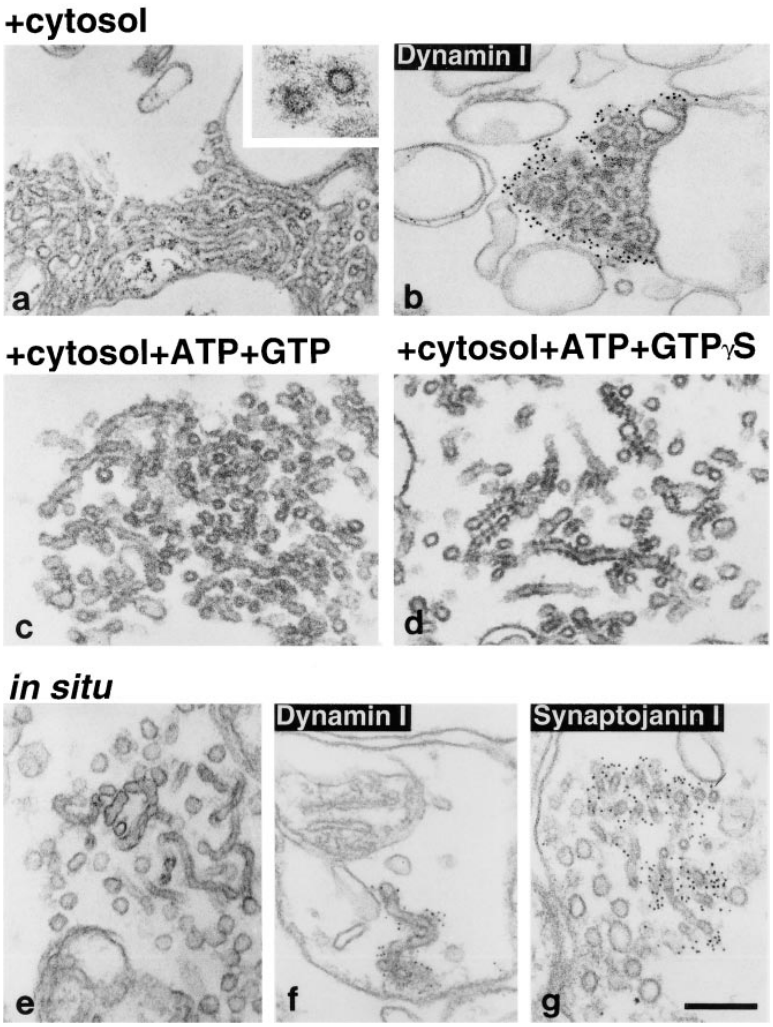

Figure 6. Brain Cytosol Tubulates Liposomes Irrespective of the Presence of Nucleotides

Liposomes of total brain lipids were incubated with cytosol in the absence of nucleotides (fields [a] and [b]), with cytosol plus ATP and GTP (field [c]), or with cytosol plus ATP and GTPγS (field [d]). The inset of field (a) shows clathrin-coated vesicular profiles. Field (b) shows liposomes immunolabeled for dynamin I and demonstrates that the surfaces of the tubules accessible to gold are heavily labeled. Tubules generated in the absence or presence of GTPγS have a similar diameter but regularly spaced dynamin rings are visible only in GTPγS reacted preparations. (e–g) Electron micrographs demonstrating that dynamin-coated tubular structures can occasionally be seen in synaptosomes not subjected to any cell-free incubation. (e) Freshly prepared intact synaptosome. (f and g) Broken synaptosomes immunolabeled for dynamin I (f) and synaptojanin I (g). Calibration bars, 200 nm; 100 nm, inset of (a).

and Takei, 1996). As mentioned above (Figure 2), however, many of the dynamin-coated tubules generated by total brain cytosol on liposomes (irrespective of the nucleotide condition) were not capped by clathrin coats. This finding can be explained either by clathrin-independent tubule formation or by dissociation of the clathrin coat after tubules have started to grow. We therefore tested the effect of purified recombinant dynamin I on liposomes in the absence of other cytosolic proteins and found that purified dynamin alone could tubulate liposomes both in the absence or in the presence of guanylnucleotides (200 μM GTP or GTPγS) (Figure 7). We conclude that the generation of a vesicular bud is not an essential prerequisite for tubule formation. As observed for total brain cytosol (see above), tubules generated in the presence of GTP were shorter, with a less regular diameter. Furthermore, they were often interspersed with small vesicular structures (Figure 7b). These observations suggest the occurrence of both tubule growth and tubule fragmentation under these conditions.

Discussion

Our results demonstrate that the cytosolic endocytic apparatus responsible for clathrin- and dynamin-mediated endocytosis can assemble on liposomes. Thus, the deformation of a lipid membrane by coat proteins does not require the presence of protein anchors within the lipid bilayer. Molecular interfaces between cytosolic proteins and lipids appear to be fully sufficient to generate both clathrin-coated pits and dynamin-coated tubules morphologically identical to corresponding structures observed in situ.

Specific membrane proteins have been implicated in the recruitment of clathrin coats to membranes (Moore et al., 1987; Kirchhausen et al., 1997). Our results imply that protein binding sites do not play an essential role in the formation of endocytic structures but act only as facilitators of coat recruitment. The much higher abundance of clathrin-coated pits generated by total brain cytosol on synaptic membrane fractions (LP2 fractions) (Takei et al., 1995, 1996) than on liposomes is consistent

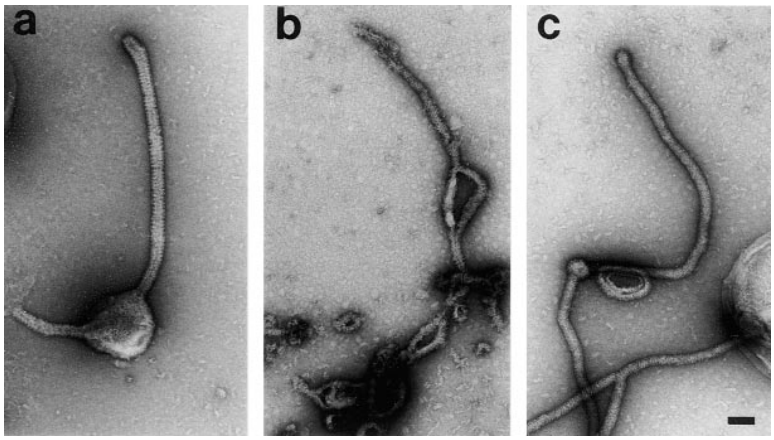

Figure 7. Electron Micrographs Demonstrating Tubulation of Liposomes by Purified Dynamin

Liposomes of total brain lipids were incubated with purified dynamin in the absence of nucleotides (a) or in the presence of either 200 μM GTP (b) or 200 μM GTPγS (c). Tubulation is visible in all conditions. Note in the GTP condition the less regular profile of the tubule and the presence of small liposomal fragments. Calibration bar, 100 nm.

with the possibility that the abundant synaptic protein synaptotagmin (Zhang et al., 1994) acts as a major facilitator in clathrin coat recruitment at the synapse.

Dynamin-mediated tubulation was strikingly more prominent in liposomes than in synaptic membranes. A scaffold of membrane proteins may inhibit dynamin binding or hinder tubulation in biological membranes. Unexpectedly, dynamin-mediated tubulation of lipid bilayers neither requires any cofactor nor the previous formation of a bud neck. Thus, dynamin alone is sufficient to evaginate a low curvature membrane into a tubule. Similar findings were reported by Sweitzer and Hinshaw (1998). Thus, the direct and indirect interactions between dynamin and components of the clathrin coat (Wang et al., 1995; David et al., 1996; McMahon et al., 1997; Ramjaun et al., 1997), which may account for the efficient coupling between clathrin coat formation and dynamin ring oligomerization, do not preclude independent actions of dynamin. Accordingly, it was recently shown that dynamin is implicated in the internalization of caveolae, a clathrin-independent process (Henley et al., 1998; Oh et al., 1998).

Experiments with brain cytosol demonstrated a striking preference of dynamin for acidic phospholipids, in agreement with previously reported biochemical studies of dynamin–phospholipid interactions (Tuma et al., 1993). Furthermore, they showed that phosphoinositides were not required for tubulation despite the presence of a PH domain in dynamin (Lin et al., 1997). Tubule formation appeared to have an antagonistic effect on clathrin-coated pit formation (possibly by sequestering acidic phospholipids or protein factors), and the relative abundance of clathrin-coated pits in PE enriched liposomes compared to other liposomes may reflect this effect.

The formation of clathrin- and dynamin-coated structures on liposomes did not require ATP. ATP independence was previously shown for the self-assembly of clathrin cages, of clathrin-adaptor coats, and of dynamin rings in the absence of membrane templates (Zaremba and Keen, 1983; Keen, 1987; Morris et al., 1989; Pearse and Robinson, 1990). However, studies of the ATP dependence of clathrin-coated pit formation have yielded conflicting results (Moore et al., 1987; Schmid and Carter, 1990; Schmid, 1993). Our findings suggest that oligomerization of clathrin coats and dynamin rings are energy-independent processes, at least under cell-free conditions, irrespective of whether they occur in solution or on a protein-free lipid bilayer. An ATP requirement of clathrin coat assembly on cell membranes could be explained by ATP-dependent reactions that play an accessory role in clathrin coat assembly, such as the release of membrane-associated proteins or the modification of membrane lipids.

Finally, we have shown that liposomes can be tubulated by purified dynamin or cytosol in vitro irrespective of the nucleotide condition. No tubules were previously observed in synaptic membranes reacted with 200 μM GTP (Takei et al., 1996). Tubules, however, could be formed at the same GTP concentration on liposomes. In the presence of 200 μM GTP, tubules were shorter than in other nucleotide conditions, had an irregular profile, and, as shown by experiments with purified dynamin I, coexisted with vesicular fragments, suggesting a coexistence of tubular growth and fragmentation. A direct demonstration that dynamin can act as a GTP-dependent mechanochemical enzyme that cleaves lipid tubules is reported in the independent study by Sweitzer and Hinshaw (1998). These authors show that the tubule-severing activity of dynamin increases in parallel with the concentration of GTP, with a massive effect at 1 mM GTP. The fission reaction is likely to be dependent upon a synchronous conformational change of all dynamin subunits within a ring. Accessory factors present in cell membranes may assist the synchronous disassembly of dynamin rings at physiological concentrations of GTP. At least in some cases, however, dynamin-coated tubules appear to represent physiologically occurring endocytic intermediates (Heuser and Miledi, 1971; Lovas, 1971; Hama and Saito, 1977; Willingham and Pastan, 1983).

The massive predominance of structures coated by clathrin and dynamin in liposomes reacted with total brain cytosol most likely reflects the extremely high concentration in brain of cytosolic components implicated in synaptic vesicle endocytosis. It is possible, however, that some of the clathrin-coated buds visible in our preparations may be generated by Golgi complex– or endosome-derived clathrin coats. Furthermore, some vesicle buds decorated by nonclathrin coats were present in liposomes incubated with total cytosol and nucleotides (data not shown), suggesting that the property of coats

to assemble on liposomes may be general. This is consistent with the report that COPII-mediated budding can be reconstituted in vitro with purified coat proteins, liposomes, and nucleotides (Matsuoka et al., 1998).

Clearly, coat assembly in vivo occurs with a high degree of spatial and temporal specificity. This layer of specificity must be mediated by intrinsic membrane proteins either via protein–protein interactions or via short range modifications of the lipid environment.

Experimental Procedures

Reagents

Polyclonal and monoclonal antibodies against amphiphysin I and synaptojanin I were raised in our laboratory (David et al., 1994; Butler et al., 1997; Haffner et al., 1997). Other monoclonal antibodies were obtained from the following sources: anti-neuronal clathrin light chain (Cl 57.1) and anti-β/β'-adaptin (C420 10A) from Dr. Reinhard Jahn (Göttingen, Germany) and Dr. Thomas Kirchhausen (Boston, MA), respectively; anti-clathrin heavy chain (TD.1) from ATCC (Nathke et al., 1992); anti-dynamin (Hudy-1) from UBI (Lake Placid, NY); anti-α-adaptin (AC1-MC11) from Affinity BioReagents Inc. (Golden, CO); anti-HSC73 (715) from StressGen Biotechnologies Corp. (Victria, BC, Canada); and anti-AP180 from Sigma (St. Louis, MO). Six nanometer protein A-gold conjugates were prepared as described (Slot and Geuze, 1985). Bovine brain lipid extract and phosphoinositides were purchased from Sigma (St. Louis, MO); the remaining lipids were acquired from Avanti Polar Lipids (Alabaster, AL).

Inside-Out Erythrocyte Vesicles

Erythrocyte membrane vesicles were prepared from human blood as described (Sulpice et al., 1994).

Liposomes

Large liposomes were prepared as described (Reeves and Dowben, 1969) with some modifications. Lipids solubilized in a chloroform/methanol (1:2) mixture were dried in a rotary evaporator and then hydrated by a stream of water-saturated nitrogen for 20 min. After gently adding degassed 0.3 M sucrose, the flask was flushed with nitrogen, sealed, and left undisturbed for 2 hr at 37°C to allow spontaneous formation of liposomes. The liposomes were recovered by centrifugation at 12,000 × g for 10 min and resuspended in cytosolic buffer (see below) prior to cell-free incubation.

Brain Cytosol

A fraction containing diluted cytosol was prepared from fresh or frozen rat brain by high speed centrifugation as described (Huttner et al., 1983; Takei et al., 1996). The fraction was desalted and concentrated by amonium sulfate precipitation and dialysis as described (Malhotra et al., 1989).

Coat Proteins

Clathrin-coated vesicles were prepared from four fresh calf brains as described (Campbell et al., 1984; Heilker et al., 1996). Coat proteins were extracted from the coated vesicles with a buffer containing 0.8 M Tris-Cl (pH 7.4), 2 mM EGTA, 0.03% sodium azide, 0.5 mM DTT, 1 mM PMSF for 15 hr at room temperature (modified from Keen et al., 1979). The soluble coat protein containing supernatant was separated from stripped vesicles and other membrane contaminants by centrifugation at 100,000 × g for 1 hr at room temperature. Coat proteins were either used directly or frozen in liquid nitrogen and stored at −70°C.

Preparation of Purified Dynamin

Human dynamin (Urrutia et al., 1997) was produced as a GST fusion protein using a modified Bac-to-Bac baculovirus expression system (GIBCO BRL). A cDNA encoding GST followed by a cleavage site for PreScission protease (Pharmacia) was inserted into pFastBAC1 baculovirus transfer vector (GIBCO BRL). Dynamin 1aa cDNA without the starting methionine was cloned into this vector downstream to the cleavage site. A recombinant virus was produced, amplified, and used to infect insect Tn cells for 48 hr in 1 l spinner cultures. Glutathione-Sepharose 4B affinity-purification of the fusion protein from cell lysates and removal of GST by PreScission protease (Pharmacia) were performed according to manufacturer's protocols. The purity of dynamin in the final material was estimated to be more than 90%, and its GTPase activity was validated using the EnzChek phosphate assay kit (Molecular Probes).

Cell-Free Incubations

Morphological Experiments

Erythrocyte vesicles at a protein concentration of 5 mg/ml or liposomes at a concentration of 1 mg of lipid/ml were incubated in 1 ml of cytosolic buffer (25 mM HEPES-KOH [pH 7.4], 25 mM KCl, 2.5 mM magnesium acetate, 150 mM K-glutamate) with or without "cytosol" (6 mg/ml), coat fraction (0.5 mg/ml), purified dynamin (0.1 mg/ml), various nucleotides as indicated, and either 10 μM Ca^{2+} or 2 mM EGTA (no obvious morphological differences were observed in number or type of coat structures obtained in the absence or presence of Ca^{2+}). Final concentrations of nucleotides were 2 mM ATP, 200 μM GTP, and 200 μM GTPγS. Samples containing ATP were also supplemented with an ATP regenerating system consisting of 16.7 mM creatine phosphate and 16.7 IU/ml creatine phosphokinase. In some of the no-nucleotides samples, an ATP-depleting system consisting of 5 U/ml of hexokinase and 10 mM glucose was used. These mixtures were incubated for 15 min at 37°C, and incubations were stopped by the addition of 1 ml 2× concentrated fixative (see below).

Biochemical Experiments

Liposomes were tagged with the lipophilic dye 1,6 diphenyl-1,3,5-hexatriene (DPH) and incubated under the same conditions described above with the exception that only 1/5 of the material was used for each sample in a total volume of 0.2 ml. At the end of the incubation, liposomes were loaded on 0.5 M sucrose and centrifuged at 120,000 × g in a Beckman TLA100.2 rotor for 30 min at 4°C to separate bound proteins from unbound proteins. Liposomes were recovered in the pellet, and the recovery was assessed using an Hitachi F-3010 fluorescence spectrophotometer by monitoring the emitted fluorescence at 430 nm upon excitation at 360 nm. Equal amounts of the recovered liposomes were analyzed by SDS-PAGE and Western blotting as described (Bauerfeind et al., 1997). Western blots were performed with ^{125}I-protein A for amphiphysin I and dynamin I, and with horseradish peroxidase followed by visualization with 4-chloro-1-naphthol for the other proteins.

Electron Microscopy

For standard electron microscopy, incubation mixtures were fixed in suspension with 3% formaldehyde, 2% glutaraldehyde in 50 mM HEPES-KOH buffer (pH 7.4), pelleted in an Eppendorf centrifuge, and postfixed in OsO_4. In some samples, OsO_4 postfixation was followed by impregnation with 0.2% tannic acid to enhance visualization of membrane coats (Orci et al., 1986). For immunoelectron microscopy, liposomes or synaptic membranes were fixed and labeled as described (Takei et al., 1996). For whole-mount observation, liposomes were first incubated with proteins or cytosol in suspension and then absorbed onto Formvar-coated EM grids pretreated with poly-L-lysine. The samples were fixed with 3% formaldehyde, 2% glutaraldehyde and negatively stained with 2% uranyl acetate.

Morphometry

The number of dynamin- and clathrin-coated profiles on liposomes of defined lipid composition was determined by visually scanning a large area of ultrathin sections (6×10^4 μm^2) at the electron microscope for the presence of longitudinal profiles of dynamin-coated tubules and clathrin-coated pits, respectively. The average number of dynamin rings on the entire length of a continuous tubular profile was calculated by counting the number of rings on 100 tubular profiles for each sample. Considering the regular periodic arrangements of the dynamin rings, this value reflects very closely the length of the tubular profile in the plane of section. The total number of dynamin rings present in the area examined was estimated by multiplying the number of the coated tubules by the average number of dynamin rings.

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