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Direct evidence for a membrane deforming motif in endophilin: implications beyond synaptic vesicle recycling

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Direct Evidence for a Membrane Deforming Motif in Endophilin: Implications Beyond Synaptic Vesicle Recycling

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Abstract

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Effective neurotransmission is dependent on fast, reproduceable synaptic vesicle recycling. The synaptic vesicle recycling process is a complex event involving both protein-protein, as well as protein-lipid interactions. A central part of the retrieval process of synaptic vesicles lies in the ability of soluble proteins to deform the plasma membrane into a nascent bud which will eventually reform a fully competent synaptic vesicle upon fission. This process involves clathrin-coat proteins, which form a protein scaffold around the vesicle bud, as well as proteins which have more recently been thought to be involved in generating the high curvature membranes present at the tubular neck of the nascent vesicle bud.

Endophilin 1, a protein highly enriched in the pre-synaptic neuronal subcompartment has been implicated in many stages of synaptic vesicle retrieval. The following work represents evidence of a direct role for endophilin in tubular membrane

deformation, which may play an important part in the regeneration of synaptic vesicles. This behavior of endophilin complements that of one of its major pre-synaptic binding partners, dynamin, a large GTPase strongly implicated in the fission process of endocytosis. Endophilin forms a coordinated complex with dynamin along membrane tubules, and stabilizes these membrane tubules against the biomechanical changes imparted by dynamin to the membrane in a GTP-dependent manner.

The membrane deforming motif in endophilin comprises a putative amphipathic helical region. This motif is conserved in amphiphysin, another major pre-synaptic dynamin binding partner implicated in synaptic vesicle recycling, and in another endophilin-related protein localized to the Golgi complex. Membrane deforming amphipathic helices have since been found in other proteins localized to additional cellular subcompartments, implicating this type of membrane interaction in diverse functions within the cell.

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Neurotransmission and the Synaptic Vesicle Cycle

The Neuron Doctrine

The vertebrate central nervous system has evolved into a specialized organ responsible for higher cognitive and functional behaviors. Fast, reliable, stereotyped relays of messages from one neuron to the next is responsible for both intrinsic homeostatic functions such as breathing, to much of the responses necessary for adaptation and higher neuronal functioning. The concept of neurotransmission was revolutionized in the second half of the nineteenth century by the seminal work of the neuroanatomist, Santiago Ramon Y Cajal. Using new stains for microscopy and impeccable observational skills, Cajal was the first neurobiologist expanding the cell theory, introduced by Schleiden and Schwann in the 1830s, to suggest that the nervous system was a collection of diverse cellular entities which communicated with each other through non-continuous connections. These connections were termed synapses by Charles Sherrington in 1897, after the Greek word for "clasp" (Cowan and Kandel, 2001). Thus the neuron doctrine, which holds that the nervous system is comprised of individual cells, termed neurons, gained fervor in the scientific community. Cajal's belief was in contrast to the one popularized by his famous contemporary, Camillo Golgi, whose

reticularist model contended that the nervous system was one big syncycium of interconnected parts with a directly communicating protoplasm (Cowan and Kandel, 2001).

In fact, the neuron doctrine has been unequivocally supported by rigorous experimentation, with the ultimate proof provided by electron microscopic studies of the synapse by George Palade and others in the mid 1950s (Cowan and Kandel, 2001; De Robertis and Bennett, 1955; Palay and Palade, 1955). Interestingly, it has been determined that certain few neurons directly communicate through specialized intercytoplasmic junctions, termed gap junctions, which allow for bi-directional flow of ions and small signaling molecules (Bennett, 1997; Cowan and Kandel, 2001; Falk, 2000; Unger et al., 1999). The function of these electrical synapses, however, is mainly thought to be the synchronization of activity within certain groups of neurons (Bennett, 1966; Bennett, 1972; Cowan and Kandel, 2001).

Since the establishment of the neuron doctrine, it has been determined that the central nervous system is comprised of roughly 10^{11} cells of more various types than any other organ known (Kandel, 2000). These cells participate in up to 10^{15} synaptic connections. Furthermore, more of the primate genome is believed to be active in the central nervous system compared with any other organ (Gilliam et al., 2000). This, in part, reflects the complex underpinnings of neuronal function.

The Synapse

The synapse is the principal computational unit of the nervous system. It has long been known that neurotransmission relies on the propagation of electrical impulses. In fact, there are four principal ways that neurons may communicate: a chemical interaction mediated by small neurotransmitter molecules secreted into the synaptic cleft between two neurons; an electrical interaction mediated by diffusion of an electrical impulse between two neurons; ephaptic interactions mediated by electrical field effects of closely apposed neurons; and regional interactions mediated by release of chemical and gaseous signaling molecules. This discussion will primarily deal with the chemical synaptic interactions which are by far the most dominant in the mammalian nervous system.

The chemical synapse is comprised of a presynaptic nerve terminal, a 20-40 nm synaptic cleft maintained by intercellular adhesive contacts, and a postsynaptic nerve terminal (De Camilli et al., 2001a; Heuser and Reese, 1977). Synapses are most often asymmetric intercellular junctions between two communicating excitable cells, typically neurons, or neurons and muscle cells (De Camilli et al., 2001a). Synaptic connections arise during development through a complex and poorly understood mechanism of axonal pathfinding and target recognition underlying the intricate circuitry of the nervous system (Benson et al., 2001). The synapse is so structurally robust, that "synaptosomes" containing only the presynaptic and postsynaptic terminals connected via the synaptic

cleft can be biochemically purified (Gray and Whittaker, 1962; Hannah et al., 1999; Takei et al., 1995; Takei et al., 1996).

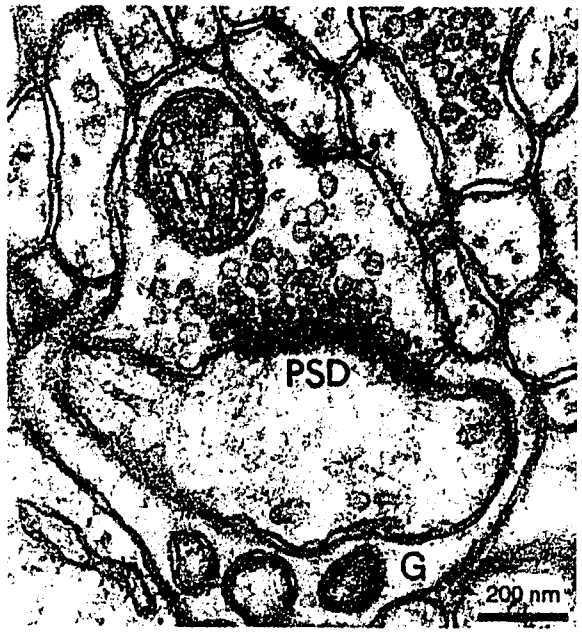
The morphological hallmark of a presynaptic terminal, or bouton, is the presence of a dense sub-plasmalemmal cytomatrix around which exist tens to hundreds and, in some instances, up to thousands of small 35-50nm membrane bound vesicles loaded with neurotransmitter molecules (Figure 1.1) (De Camilli et al., 2001a; Jahn et al., 1990). The active zone, a term coined by Couteaux and Pecot-Dechavassine, defines the release site of the neurotransmitter containing vesicles (Couteaux and Pecot-Dechavassine, 1970). Occasionally, a nerve terminal may contain more than one active zone with distinct postsynaptic contacts (De Camilli et al., 2001a). The terminal bouton also contains cytoskeletal elements which are believed to be involved in the generation and organization of this specialized compartment. The postsynaptic terminal also contains a dense sub-plasmalemmal cytomatrix, termed the postsynaptic density, in which the cognate receptors of the neurotransmitter molecules are clustered (De Camilli et al., 2001a).

Chemical synaptic transmission begins with the propagation of an electrical depolarizing impulse, termed the action potential, down the axon to the nerve terminal. At the nerve terminal, the depolarizing impulse opens voltage gated calcium channels localized at or near the active zone, which then allow an increase in the local calcium concentration by up to four orders of magnitude. The increase in cytosolic calcium triggers fusion of the synaptic vesicle with the synaptic plasma membrane surrounding the active zone

Figure 1.1: Electron micrograph of a mammalian central synapse

Ultrastructure of the synapse between a parallel fiber axon terminal and the dendritic spine of a Purkinje cell in the cerebellar cortex of a rat. Note the numerous synaptic vesicles adjacent to the cell membrane. PSD = post-synaptic density, G = glial cell

Reproduced from (De Camilli et al., 2001a)



in a highly coupled process linking excitation with neurotransmitter release.

Subsequent to the release of neurotransmitter molecules into the synaptic cleft, neurotransmitters bind to their cognate receptors, which either directly or indirectly gate ion channels. The subsequent influx of ions through the postsynaptic ion channels generates a postsynaptic potential that then may or may not generate another action potential to continue the propagation of the signal. A given neuron may integrate the potentials generated by several presynaptic contacts enabling complex computational processing. Thus, an electrical impulse is converted into a chemical signal, which is then reconverted into an electrical impulse. By virtue of its many different regulated steps, the synapse is also the principle effector of plasticity within the nervous system, which underscores the need to functionally understand this important physiologic structure.

The Quantal Nature of Neurotransmitter Release: Synaptic Vesicles

The concept that neurotransmitters were released in discrete packets, or quanta, was postulated before physical evidence of synaptic vesicles was known. Based on physiological recordings of the frog neuromuscular junction in the early 1950s, Bernard Katz and colleagues determined that there existed spontaneous stimulation independent potential changes of uniform 0.5 millivolt amplitudes (Fatt and Katz, 1951; Fatt and Katz, 1952). They postulated that these mini-end plate potentials (MEPPs), as they

were called, must define the minimal unit, or quantum, of neurotransmitter release to generate such a change in potential (Del Castillo and Katz, 1954; Fatt and Katz, 1952).

In 1955, electron microscopic studies by Palay and Palade, and de Robertis and Bennett gave quantal theory a morphological correlate (De Robertis and Bennett, 1955; Palay and Palade, 1955). The presence of numerous small membrane-bound vesicles present at the nerve terminal led to the suggestion that these organelles must be the determinants of quantal neurotransmitter release by fusing with the plasma membrane and releasing their stored quantity of neurotransmitter (Figure 1) (Katz and Miledi, 1965). It has since been found that the quantum of neurotransmitter which generates the MEPP corresponds to the amount which is loaded in synaptic vesicles of the frog neuromuscular junction, roughly five to ten thousand molecules of acetylcholine (Kuffler and Yoshikami, 1975). The most compelling evidence that quantal release translates to synaptic vesicle exocytosis is a study correlating amounts of released quanta measured electrophysiologically in the frog neuromuscular junction with electron micrographic images of synaptic vesicles appearing to be in intermediate stages of fusion with the plasma membrane (Ceccarelli et al., 1973; Haimann et al., 1985; Heuser, 1989; Heuser et al., 1979). Although there is little doubt, it should be mentioned, for the sake of completeness, that since no absolute physical proof for the causal relationship between vesicle exocytosis and synaptic trasmission has yet been found, the vesicle theory of quantal release remains a hypothesis.

Neurotransmitter molecules released from synaptic vesicle exocytosis cross the synaptic cleft within two microseconds, and expose the postsynaptic receptors to a concentration of 1 mM neurotransmitter, sufficient to open up to two thousand postsynaptic ion channels. This would theoretically generate a postsynaptic potential on the order of that seen with the MEPP. It has been estimated that at each release site, or active zone, an action potential has a probability of 0.3 for causing release of a synaptic vesicle (although this is likely variable and regulated at different synapses). It follows, then, that in a large neuromuscular junction containing about one thousand active zones, an action potential results in the release of roughly three hundred vesicles within 1.5 milliseconds, generating the large postsynaptic end-plate potential capable of stimulating muscle fiber excitation.

Biogenesis of Synaptic Vesicles

The identification of synaptic vesicles begged the next question of how and when these organelles are created. The generation of mature synaptic vesicles is thought to occur within the nerve terminal itself, compared with the direct generation from the Golgi complex typical for vesicles of regulated exocytosis. Precursor proteins and membranes, however, are formed in the endoplasmic reticulum (ER) and Golgi complex. These precursors are transported down the axon via membrane-bound tubulo-vesicular organelles through fast axonal transport. These macromolecules include the integral

membrane synaptic vesicle proteins, such as synaptophysin and SV2, as well as lipid molecules (Hannah et al., 1999).

A fair amount of molecular sorting occurs to generate a mature synaptic vesicle, given its compositional difference with the plasma membrane. Most evident is the virtual lack of gangliosides and a relatively large enrichment of cholesterol in synaptic vesicles when compared with the plasma membrane (Hannah et al., 1999). Cholesterol is known to be important for the structure of high curvature membranes, and depletion of cholesterol from the plasma membrane prevents efficient formation of vesicles, resulting in shallow buds which appear stunted in their ability to form high curvature (Rodal et al., 1999; Subtil et al., 1999). In addition, synaptophysin has been shown to directly bind cholesterol, potentially serving as its biochemical sorter in synaptic vesicles (Huttner and Schmidt, 2000; Thiele et al., 2000).

Different lines of evidence point to the generation of a synaptic vesicle occurring at the nerve terminal (Hannah et al., 1999). First, the pleiomorphic, membranous organelles which contain the synaptic vesicle precursor proteins do not share the same size or morphology with synaptic vesicles (Tsukita and Ishikawa, 1980). Second, it is known that different synaptic vesicle proteins are transported on different vesicular carriers (Okada et al., 1995), with the full complement of synaptic vesicle proteins co-existing on the same organelle only in the mature synaptic vesicle at the nerve terminal. These axonal carriers also contain other non-synaptic vesicle cargo destined for traffic to the distal axon/nerve terminal (Okada et al., 1995). Third, neurotransmitter molecules are

loaded into vesicles at the nerve terminal. Finally, it is believed that nascent transport vesicles carrying the synaptic vesicle precursors need to undergo some exo-endocytic recycling before they can load neurotransmitter molecules, implying a degree of sorting at this stage requisite for the generation of a mature synaptic vesicle. It is unclear whether some axonal endosomal sorting compartment may also be important in this process (Heuser and Reese, 1973). Thus, it can be thought that a synaptic vesicle is ultimately formed upon endocytosis from the plasma membrane, a fact which has important ramifications for the recycling nature of synaptic vesicles.

Synaptic Vesicles Exist in Distinct Pools

Two pools of synaptic vesicles exist at the nerve terminal: the readily releasable pool, and the reserve pool (Hannah et al., 1999; Regehr and Stevens, 2001). Electrophysiological evidence exists for two pools of synaptic vesicles. Upon a high-frequency train of action potentials, there ensues a burst of fast synaptic vesicle exocytosis, followed by a lower, steady-state level of release (Elmqvist and Quastel, 1965; Liu and Tsien, 1995; Regehr and Stevens, 2001; Rosenmund and Stevens, 1996). The burst defines the readily releasable pool of synaptic vesicles, while the steady-state is thought to correspond to a reserve pool of synaptic vesicles. This reserve pool is not immediately available for release, but is able to generate continued neurotransmission at a lower rate upon continuous stimulation, therefore highlighting the importance of this pool of vesicles during high-frequency synaptic firing. Once depleted, the readily releasable

pool of vesicles is regenerated only upon a discontinuation of the excitatory train (Stevens and Tsujimoto, 1995). Given continuous high frequency stimulation, neurotransmission will grind to a halt as both pools of synaptic vesicles are exhausted.

Morphologically, the readily releasable pool of synaptic vesicles is thought to be those vesicles docked with the active zone in close proximity to the presynaptic plasma membrane (Rosenmund and Stevens, 1996; Zucker, 1973). These vesicles are the ones postulated to be immediately available to fuse with the plasma membrane and release their neurotransmitter into the synaptic cleft upon stimulation (Schikorski and Stevens, 1997; Stevens and Tsujimoto, 1995). Alteration of the functional size of the readily releasable pool can be an important modulator of synaptic transmission and plasticity (Rosenmund and Stevens, 1996). Around these docked vesicles exist many more synaptic vesicles in a cluster thought to comprise the reserve pool of vesicles (Schikorski and Stevens, 1997). While not immediately available to fuse with the plasma membrane and release their stored neurotransmitters, these vesicles are believed to be gradually recruited to the release sites at the plasma membrane where they may then dock and fuse. It is believed that the inherent delay in this recruitment process is what differentiates the readily releasable pool from the reserve pool, electrophysiologically.

There are several proteins thought to be important in the dynamics of these two pools of synaptic vesicles. The synapsins are a highly conserved protein family having activity dependent association with synaptic vesicles (Bähler et al., 1990; De Camilli et al., 1990; De Camilli et al., 1983a; De Camilli et al., 1983b; Huttner et al., 1983). At rest,

the synapsins are clustered around the synaptic vesicles. Upon stimulation, the synapsins become phosphorylated and no longer bind to the synaptic vesicles, diffusing along the axon (Chi et al., 2001). It is thought that this release from synapsin binding is what frees the vesicle in the reserve pool to progress to docking at the presynaptic plasma membrane (Chi et al., 2001). Binding may then proceed to large multi-domain proteins, such as RIM, piccolo, and bassoon, which are closely associated with the active zone cytomatrix, through other synaptic vesicle proteins such as rab 3A (Lin and Scheller, 2000). From this "docked" site, vesicles are ready to fuse with the plasma membrane in a calcium dependent manner to release neurotransmitter molecules into the synaptic cleft.

Synaptic Vesicle Exocytosis

Due to the ability to biochemically purify a homogeneous population of synaptic vesicles, these organelles have been catalysts for the study of regulated exocytosis (Huttner et al., 1983; Lin and Scheller, 2000). One postulated mechanism for fusion of the synaptic vesicle membrane with the presynaptic plasma membrane occurs via the SNARE proteins (Lin and Scheller, 2000; Rothman, 1994; Schiavo et al., 1997). The SNAREs comprise a diverse family of membrane associated molecules which have the ability to bind to one another to form a robust complex having high melting temperatures and resistance to SDS denaturation (Brunger, 2001; Lin and Scheller, 2000). SNAREs have been classified into those associated with the vesicle (vSNAREs), and those associated with the target membrane (tSNAREs) (Parlati et al., 2000). In general, a

SNARE complex consists of one vSNARE and two different tSNAREs (Brunger, 2001), with the specificity of the vesicle-target membrane interaction thought to be inherent in the specific interactions of the various SNARE proteins (McNew et al., 2000). The prototypical vSNARE is the synaptic vesicle integral membrane protein, synaptobrevin. The prototypical tSNAREs are the integral membrane protein, syntaxin, and the lipid modified protein SNAP-25. It is generally believed that complex formation between the respective SNARE proteins of the vesicle and target membrane contains enough energy to begin a process which pulls the apposing membranes together . The energy contained within the SNARE complex is thought to thermodynamically drive the approximation of the two adjacent membranes until they mix, allowing for fusion. SNARE mediated membrane fusion has been implicated in many forms of membrane trafficking events (McNew et al., 2000).

Two major lines of evidence support the SNARE hypothesis of membrane fusion. The first involves use of bacterial toxins from Clostridia which selectively target various sites in the different neuronal SNARE proteins for proteolytic cleavage. These toxins potently block neurotransmission, and are fatal if left untreated (Pellizzari et al., 1999). The block in neurotransmission lies at the level of synaptic vesicle exocytosis, with no effect on the total number of synaptic vesicles and an increase in the numbers of vesicles docked at the active zone (Hunt et al., 1994). This implicates the SNAREs to be necessary in a late stage of fusion with the plasma membrane, after docking of the vesicle to the active zone. The other line of evidence directly tests the ability of the SNARE proteins to mediate lipid bilayer fusion. Using artificial proteoliposomes containing

various populations of vSNAREs and tSNAREs, Rothman and colleagues showed that the SNARE proteins are the minimal machinery sufficient to produce coordinated membrane fusion between proteoliposomes containing complementary SNARE proteins (Weber et al., 1998).

The probability of synaptic vesicle exocytosis increases five to six orders of magnitude with the influx of calcium at the nerve terminal; however, the nature of the calcium sensor in this process has remained elusive (Lin and Scheller, 2000). The calcium channels at the nerve terminal exist at the active zone, and generate a stimulation dependent increase in local calcium concentration from roughly 100 nM to 100 μM . The time from influx of calcium to vesicle release can occur within 100-200 microseconds, indicating that the calcium sensor must reside in an area at least within 100 nm from the calcium channels, close to the active zone (Lin and Scheller, 2000).

Several synaptic proteins are known to bind calcium. The integral synaptic vesicle protein, synaptotagmin, has been the candidate most intensively studied. The calcium binding sites of synaptotagmin reside in its two C2 domains, homologous to the calcium binding region in protein kinase C (PKC). The low affinity binding of this domain for calcium is consistent with the concentrations of calcium needed to stimulate membrane fusion. Furthermore, various perturbation studies of synaptotagmin function, including protein overexpression and microinjection of antibodies and peptides, has revealed a role for this protein in synaptic transmission (Bommert et al., 1993; Mikoshiba et al., 1995). Moreover, synaptotagmin is known to be able to oligomerize, bind to the

tSNARE proteins, syntaxin and SNAP-25, as well as bind to phospholipids in a calcium dependent fashion (Chapman et al., 1995; Li et al., 1995; Schiavo et al., 1997). These data are consistent with a model whereby calcium binding by synaptotagmin at the active zone is involved in the facilitation of SNARE complex formation and fusion. However, some of the many mammalian isoforms of synaptotagmin do not bind calcium, and gene knock-out studies in C.elegans, D. melanogaster, and mice have produced ambiguous results concerning the role for synaptotagmin in calcium dependent synaptic vesicle exocytosis (Broadie et al., 1994; Chapman, 2002; Geppert et al., 1994; Littleton et al., 1994; Littleton et al., 1993; Nonet et al., 1993). Therefore, the true identity of the synaptic calcium sensor remains obscure.

Synaptic Vesicle Recycling

Even at low levels of stimulation, the complement of synaptic vesicles would rapidly become depleted if there were no compensatory mechanism for their replenishment. Given the presence of variable-frequency stimulation, and the ability of the nervous system to quickly alter the flux of neuronal firing, the rapid regeneration of synaptic vesicles is crucial for the maintenance of productive neurotransmission. In addition, the other consequence of unopposed synaptic vesicle exocytosis would be the large accumulation of membrane at the synapse, with the resulting drastic disruption of synaptic architecture. This problem is elegantly solved through the local recycling of synaptic vesicles at the nerve terminal (Ceccarelli et al., 1973; Heuser and Reese, 1973).

Synaptic vesicle recycling at the nerve terminal can regenerate and maintain the synaptic vesicle pool, while at the same time, balance the total surface area of the presynaptic membrane through a cycle of exo- and endocytosis (De Camilli et al., 2001b).

Indeed, exocytosis is tightly coupled to endocytosis in the synaptic vesicle cycle. The nature of the tight coupling is most likely through a combination of sensitivities to the same biochemical trigger, calcium, as well as through the detection, by the endocytic machinery, of synaptic vesicle components delivered to the presynaptic plasma membrane (De Camilli et al., 2001b). Experimental evidence supports both of these potential coupling mechanisms. Formation of endocytic complexes is facilitated through calcium-dependent dephosphorylation of several proteins in the endocytic pathway by the phosphatase, calcineurin (Robinson et al., 1994; Slepnev et al., 1998). Furthermore, the putative exocytic calcium sensor, synaptotagmin, binds to the alpha-adaptin subunit of the clathrin adaptor AP-2, serving to potentially nucleate the formation of a clathrin-coated pit at sites where synaptotagmin has been delivered to the plasma membrane (Haucke and De Camilli, 1999). In addition, in the vertebrate fish, lamprey, artificial uncoupling of exocytosis from endocytosis resulted in the endocytosis of synaptic vesicles only until the vesicle pool had replenished, arguing for a biochemical stimulus present at the plasma membrane which was triggering the endocytic process, namely, the presence of synaptic vesicle proteins delivered to the plasma membrane by exocytosis (Gad et al., 1998). There is evidence showing that the rate of endocytosis directly correlates with the rate of exocytosis, further demonstrating the intimate coupling of these two processes (Klingauf et al., 1998; Marks and McMahon, 1998). Moreover, recent

data have implicated phosphoinositide metabolism in the exo-endocytic cycle of vesicles recycling from the plasma membrane, suggesting lipid-mediated coupling of these two processes (Cremona and De Camilli, 2001; Cremona et al., 1999; Czech, 2003; Kim et al., 2002; Wenk et al., 2001).

Using styryl dyes which fluoresce upon embedding into lipid bilayers, the kinetics of evoked synaptic vesicle recycling have provided new data demonstrating robust vesicle turnover (Betz et al., 1992; Betz et al., 1996; Ryan and Smith, 1995). These experiments reveal kinetics which are dependent on the intensity and duration of stimulation. Following a brief 10 Hz stimulation, the half-life of synaptic vesicle endocytosis is roughly twenty seconds in central synapses as measured by these methods (Ryan and Smith, 1995; Ryan et al., 1996; Wu and Betz, 1996). However, different kinetics of uptake have been found under different stimulation protocols and in different synapses, reflecting a potential functional role for various recycling dynamics within the diversity of the nervous system (De Camilli et al., 2001b; Klingauf et al., 1998). Based on the kinetics of styryl dye unloading after internalization, it has been determined that roughly another 30 seconds is required after endocytosis for subsequent availability of the nascent vesicle for another round of exocytosis, placing the total time of recycling at just under one minute (Betz and Bewick, 1992; Ryan et al., 1993). Thus, the recycling pathway appears most critically important for maintenance of the reserve pool of synaptic vesicles.

Interestingly, there is segregation at the nerve terminal with respect to regions of exocytosis and endocytosis of synaptic vesicles. While exocytosis is thought to occur principally at the active zone proper, where synaptic vesicles are seen to be closely apposed to the presynaptic plasma membrane by electron microscopy, the major endocytic activity primarily occurs at the outer boundaries of the active zone, in a loose halo around the synaptic vesicle cluster (De Camilli et al., 2001b; Gad et al., 1998; Jarousse and Kelly, 2001; Teng and Wilkinson, 2000). In fact, the periphery of the active zone is enriched in proteins involved in endocytosis (Jarousse and Kelly, 2001; Roos and Kelly, 1999). The mechanism for this functional segregation of exo- and endocytosis is not understood, although clearly there must be a role for protein-protein and protein-membrane interactions which favor this distribution. Conceptually, a separation of these two processes in space would in some ways prevent a functional competition unproductive for neurotransmission, and there may also be steric requirements for the separation of the different machineries required for exo- and endocytosis.

Two Putative Pathways for Synaptic Vesicle Retrieval

In what way does the local recycling of synaptic vesicles occur? In the early 1970s, electron microscopic studies of stimulated nerve terminals revealed clathrin-mediated endocytosis to be a major pathway of synaptic vesicle retrieval after exocytosis (Heuser and Reese, 1973). This highly specialized version of a general endocytic pathway is necessary for the maintenance of the synaptic vesicle pool, and perturbation

of this system results in rapid depletion of synaptic vesicles at the nerve terminal (Gad et al., 2000; Koenig and Ikeda, 1989; Shupliakov et al., 1997).

The clathrin-mediated pathway for endocytosis of synaptic vesicles has been rigorously supported through experimentation using a variety of methods. Biochemically, the brain is an abundant source of clathrin-coat proteins, derived from a coated vesicle fraction enriched in synaptic vesicle proteins (Maycox et al., 1992). Furthermore, by immunofluorescence, clathrin-coat proteins are highly concentrated in the nerve terminal, indicative of a major role for these proteins in a synaptic process (De Camilli et al., 2001b). Moreover, cellular manipulation experiments, through which the clathrin-mediated endocytic pathway was perturbed by either peptide or antibody microinjection, have clearly shown a major role for this pathway in synaptic vesicle recycling (Gad et al., 2000; Ringstad et al., 1999; Shupliakov et al., 1997). Some of the most striking examples of the importance of the clathrin-mediated recycling pathway are seen in the microinjection studies using the living giant reticulospinal synapse of the vertebrate fish, lamprey. In this preparation, disrupting proteins involved in clathrin-mediated synaptic vesicle recycling demonstrate a crucial role for this pathway in the maintenance of the synaptic vesicle pool, at either low or high levels of stimulation (Gad et al., 2000; Ringstad et al., 1999; Shupliakov et al., 1997). Such a disruption of clathrin-mediated endocytosis in the lamprey reticulospinal synapse results in near total depletion of the synaptic vesicle cluster, with many "trapped" intermediates of the clathrin-mediated endocytic pathway seen at the periphery of the active zone (Gad et al., 2000; Ringstad et al., 1999; Shupliakov et al., 1997). Furthermore, genetic studies in D. melanogaster and

mice have further supported a major role for clathrin-mediated synaptic vesicle recycling, demonstrating both lethality and severe neurological dysfunction (Cremona et al., 1999; Di Paolo et al., 2002; Gonzalez-Gaitan and Jackle, 1997; Guichet et al., 2002; Verstreken et al., 2002; Zhang et al., 1998).

An alternative pathway for synaptic vesicle recycling has also been proposed, independent of clathrin-mediated endocytosis. This hypothesis stemmed from electrophysiological studies of membrane capacitance, as well as electron micrographs showing synaptic vesicles in a state of partial fusion with the plasma membrane (Koenig and Ikeda, 1996). Known as 'kiss and run,' this hypothesis posits that the vesicle may, rather than completely collapsing into the presynaptic plasma membrane, only form a transient fusion pore which may then be resealed quickly to reform the synaptic vesicle (Ceccarelli et al., 1973; Fesce et al., 1994). Opening of the fusion pore would be sufficient for release of neurotransmitter, without compromising the overall integrity of the vesicle. Advantages for this pathway would be potentially faster recycling kinetics derived from reversal of a partial fusion event, as well as circumventing the need to sort vesicle constituents from the pool of proteins and lipids present in the plasma membrane.

The most tenable evidence that the 'kiss and run' mode of vesicle recycling occurs has been from capacitance studies of large secretory granule secretion (Breckenridge and Almers, 1987a; Breckenridge and Almers, 1987b; Spruce et al., 1990). The addition of membrane upon fusion of a large vesicle granule with the plasma membrane of a secretory cell is sufficient to detect an increase in the capacitance of the plasma membrane. Using

these techniques, coupled with biochemical detectors of granule content release, it has been shown that a given granule may undergo a 'flicker' state where the capacitance of the plasma membrane quickly changes in a reversible fashion in conjunction with secretion (Almers and Tse, 1990; Fesce et al., 1994; Neher, 1993). The amplitude of the capacitance change is similar to what would be seen with a single total fusion event. This led to the notion that the vesicle was reversibly fusing with the plasma membrane and releasing its contents in brief spurts.

Although the 'flicker' or 'kiss and run' mechanism of fusion has been demonstrated for secretion of these large vesicles, evidence for the same phenomenon occurring with synaptic vesicles has not been nearly as robust, particularly since the small size of synaptic vesicles is below the reliable detection limit for capacitance studies. Furthermore, the large secretory granules studied for capacitance changes are more akin to the large dense-core vesicles containing neuropeptides present in some neurons, rather than the small neurotransmitter-containing synaptic vesicles. These neuropeptide-containing large dense-core vesicles most likely do not undergo exocytosis at the active zone, and thus may have a qualitatively different mechanism of fusion distinct from that of synaptic vesicles (De Camilli et al., 2001a). This fact limits the ability to generalize a process involved with large granule secretion to synaptic vesicle exocytosis. The 'kiss and run' model is still a viable one, however, and more experimentation may reveal a potential role for this pathway in synaptic vesicle recycling (Klingauf et al., 1998).

The Cast of Players in Synaptic Vesicle Retrieval

Clathrin-mediated synaptic vesicle retrieval involves an intricate sequence of protein and membrane dynamics. A central question in this process is the how the nascent vesicle bud is generated from the plasma membrane, and then severed to form an independent organelle. Many proteins have been implicated in this process, from clathrin, to the adaptins, to a whole group of accessory proteins the functions of which have become increasingly studied in recent years (De Camilli et al., 2001b; Schmid, 1997; Slepnev and De Camilli, 2000).

Clathrin-coat proteins purified from brain are comprised principally of clathrin heavy and light chains, the hetero-tetrameric adaptor, AP2, and the large monomeric adaptor, AP180 (Brodsky et al., 2001). Each of these proteins has independently been implicated as important components for clathrin-coat formation. The triskelion is the principal structural unit of clathrin assemblies (Brodsky, 1985). The clathrin triskelion is comprised of three clathrin heavy chains (Brodsky, 1988; Nathke et al., 1992). The central hub of the triskelion is where the light chains are localized (Brodsky et al., 1991; Liu et al., 1995), and the terminal domains represent the NH2-terminal regions of the clathrin heavy chains which are known to be involved in protein-protein interactions with adaptors (ter Haar et al., 2000). Rather than forming a major structural part of the triskelion, the clathrin light chains are mainly thought to exert regulatory effects on coat formation (Brodsky et al., 2001; Winkler and Stanley, 1983; Ybe et al., 1998). The presence of clathrin light chains inhibits clathrin cage formation at physiological pH, and

phosphorylation of light chains may play a role in regulating the interaction of the light chains with the heavy chains (Brodsky et al., 1991; Chu et al., 1999).

Clathrin triskelia can organize to form hexagonal and pentagonal lattices (Brodsky et al., 2001). Geometric models of these shapes indicate that while triskelial hexagons can form flat sheets, triskelial pentagons introduce curvature in the lattice due to stearic considerations of such a pentagonal assembly (Kirchhausen, 2000). With the appropriate ratio of hexagons to pentagons, clathrin is able to form structures resembling geodesic domes, or 'buckminster fullerenes' (Musacchio et al., 1999; Smith et al., 1998). Under acidic conditions below pH 6.5, clathrin itself can oligomerize into lattices and cages in solution comprised solely of triskelial units (Brodsky, 1988); however, at physiologic pH, clathrin adaptor proteins are required for cage formation (Ahle and Ungewickell, 1986).

Both AP2 and AP180 can independently and synergistically stimulate free clathrin cage formation (Ford et al., 2001). AP180 has also been shown, both in vitro and in vivo in C. elegans and D. melanogaster, to be necessary for the stringent size determination of the clathrin-coat for synaptic vesicles (McMahon, 1999; Nonet et al., 1999; Zhang et al., 1998). AP2 and AP180 are also able to bind to phospholipids (De Camilli and Takei, 1996; Ford et al., 2001; Mao et al., 2001), and therefore provide an additional structural link to the site of action for the clathrin-coat. In fact, purified clathrin-coat proteins can form coated buds on protein-free artificial liposomes, demonstrating that soluble components alone are sufficient to generate the structural

intermediates in endocytosis (Takei et al., 1998). In addition, the μ2-subunit of AP2 has been shown to bind to a tyrosine based sorting motif present in synaptotagmin, and therefore, provides a link for the sorting of synaptic vesicle components implicit in clathrin-mediated synaptic vesicle retrieval (Haucke and De Camilli, 1999; Haucke et al., 2000). Consistent with this idea, AP180 C. elegans mutants were shown to mislocalize the vSNARE, synaptobrevin, indicating an important role for AP180 in the sorting of this important exocytic protein (Nonet et al., 1999).

Recently discovered in Drosophila, the novel synaptic protein, stoned B, has been shown to associate with clathrin-coat proteins and to be important in synaptic vesicle recycling (Andrews et al., 1996; Martina et al., 2001; Walther et al., 2001). Stoned B has structural homology to the μ2-subunit of AP2, and concordantly, interacts directly with synaptotagmin (Phillips et al., 2000). Deletion of the *Stoned* B locus results in mislocalization of synaptotagmin and defects in neurotransmission attributed to a dysfunction of synaptic vesicle recyling (Fergestad and Broadie, 2001; Fergestad et al., 1999; Stimson et al., 2001). This further demonstrates how proteins associated with the clathrin-coat can be intimately involved in sorting of synaptic vesicle proteins and in the efficiency of synaptic vesicle recycling.

Ring Around the Collar

In addition to the proteins comprising the clathrin-coat, several other proteins have been implicated in this endocytic pathway (De Camilli et al., 2001b). While

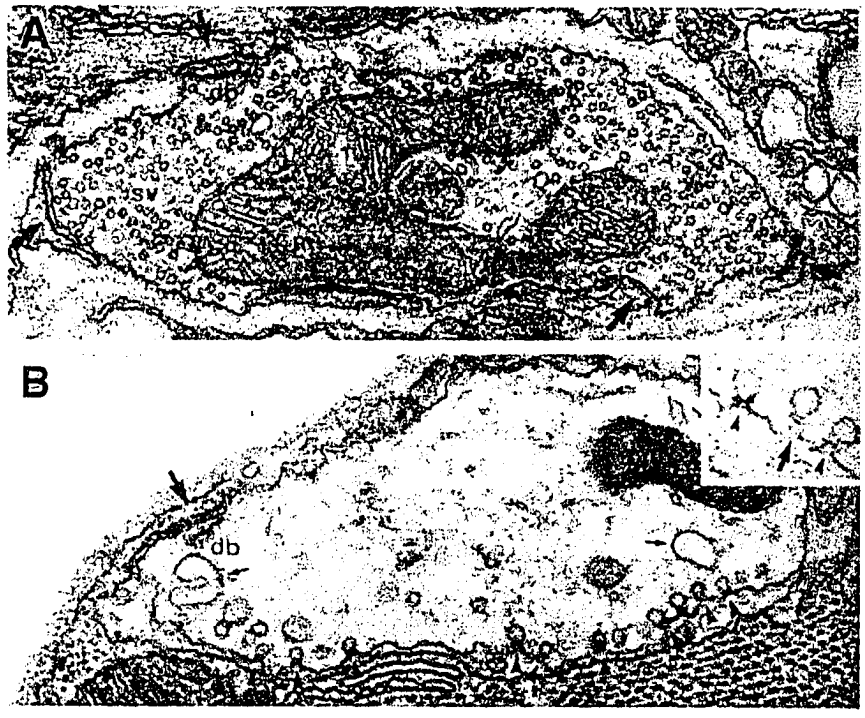
clathrin-coat proteins are involved in budding from the plasma membrane, they are not sufficient to cause fission of the bud into a vesicle. The first evidence that factors other than the clathrin-coat proteins were involved in clathrin-mediated synaptic vesicle retrieval came from the study of a Drosophila temperature-sensitive mutant severely defective in neurotransmission (Koenig and Ikeda, 1989). Morphological analysis of the *shibire* temperature sensitive mutation in D. *melanogaster* showed that, at the restrictive temperature, paralysis coincided with depletion of the synaptic vesicle cluster and the arrest of clathrin-mediated endocytosis at deeply invaginated buds (Figure 1.2) (Koenig and Ikeda, 1989). Interestingly, electron dense collars were found at the necks of these clathrin-coated buds (Koenig and Ikeda, 1989). The mutant gene was subsequently found to encode the Drosophila ortholog of dynamin, a 100kDa GTPase initially cloned as a microtubule associated protein (Shpetner and Vallee, 1989; van der Bliek and Meyerowitz, 1991). Dynamin has three different isoforms totalling up to twenty-seven different splice variants (McNiven et al., 2000). Dynamin 1 is specific to the brain and is highly enriched at the nerve terminal. Dynamin 2 is ubiquitously expressed, while dynamin 3 is predominantly in testes, with some brain and lung expression.

Figure 1.2: The shibire mutant in D. melanogaster displays a temperature sensitive arrest of synaptic vesicle recycling due to failure of vesicle fission as a result of a defect in the gene encoding the GTPase, dynamin

(A) At the permissive temperature, the pre-synaptic compartment is replete with synaptic vesicles and the fly behaves normally. **(B)** At the restrictive temperature, paralysis coincides with a depletion of synaptic vesicles and an accumulation of clathrin-coated profiles arrested at a deeply invaginated state. Note the electron-dense ring-like structures evident at the neck of many of the coated buds.

Reproduced from the following reference: (Koenig and Ikeda, 1989)

(C) The domain structure of dynamin, the 100 kDa GTPase product of the shibire gene. The GTPase activity of the NH2-terminal GTPase domain is required for fission of the nascent clathrin-coated bud into a vesicle. The COOH-terminal proline- and arginine-rich domain is responsible for dynamin binding to various SH3 domain-containing proteins. CC = coiled coils; GED = GTPase effector domain



Dynamin is comprised of five major domains: an NH2-terminal GTPase domain; a middle domain containing a region of coiled-coils potentially involved in dynamin-dynamin interactions; a pleckstrin-homology (PH) domain with affinities for phosphoinositides, and which may also participate in protein-protein interactions; a GTPase effector domain (GED) thought to stimulate dynamin GTPase activity as well as to regulate dynamin-dynamin oligomerization through two predicted coiled-coil segments; and a COOH-terminal region rich in prolines and arginines (PRD) known to bind several proteins containing src-homology 3 (SH3) domains (Figure 1.2C) (Hinshaw, 2000). The large NH2-terminal GTPase domain, the most highly conserved amongst the dynamins, is characterized by very high GTPase activity and low affinity for nucleotides. Indeed, these traits of a large GTPase with high hydrolytic activity and low nucleotide affinity have defined a family of GTPases, including members ranging in function from the immune system to mitochondrial dynamics (McNiven et al., 2000) (Danino and Hinshaw, 2001).

Two important lines of evidence initially implicated dynamin as an important endocytic protein in mammals. Overexpression, in mammalian cells, of a dynamin construct unable to bind GTP acted in a dominant negative fashion to potently block endocytosis (Damke et al., 1994; van der Bliek et al., 1993). Furthermore, treatment of isolated synaptic membranes with brain cytosol and GTPγS, a non-hydrolyzable analog of GTP, could mimic the fission arrest phenomenon seen in the *shibire* mutant, with multiple electron dense rings constricting the membrane below a clathrin-coated bud (Figure 1.3A) (Takei et al., 1995). These electron dense collars were immuno-gold

positive for dynamin. Moreover, dynamin was shown to form stacks of rings in solution by electron microscopy (Hinshaw and Schmid, 1995), and purified dynamin alone deformed both natural membranes and artificial lipid bilayers into tubules with the approximate diameter of the neck of a clathrin-coated bud (Takei et al., 1998). Upon addition of GTP, some tubules generated by dynamin on liposomes were seen to constrict in diameter, and many tubules fragmented to small vesicular structures (Sweitzer and Hinshaw, 1998). Thus, fission of a nascent clathrin-coated bud from the plasma membrane requires dynamin, and specifically, a GTP-dependent function of dynamin. Given the in vitro and in vivo dynamics observed with dynamin and lipid bilayers, the GTP-dependent function is likely a mechanochemical transduction of energy sufficient to mediate scission of the tubular neck of the clathrin-coated bud (Marks et al., 2001). An alternative mechanism has also been proposed whereby dynamin may stimulate a downstream fission machinery in a GTP-dependent fashion (Sever et al., 1999), and it is possible that both mechanisms may work in tandem.

Dynamin exists as dimers and tetramers in solution and is able to form high order oligomers under conditions of low salt, or on the surface of lipid bilayers. Recently, a 20 Ångstrom structure of dynamin oligomerized in a tubule has been solved by electron cryo-microscopy (Klockow et al., 2002; Zhang and Hinshaw, 2001). This structure shows the unit of dynamin assembly to be a dimer, and a dynamin ring to be composed of dimeric oligomers with an eleven-fold axis of symmetry (Klockow et al., 2002; Zhang and Hinshaw, 2001). Dynamin is assembled as a continuous stack of rings which is poised in such a way as to mediate constriction with conformational change (Klockow et al., 2002;

Zhang and Hinshaw, 2001). This model supports a mechanochemical function for dynamin, although downstream effectors may still play a role in the fission reaction.

Protein Complexes in the Endocytic Fission Ring

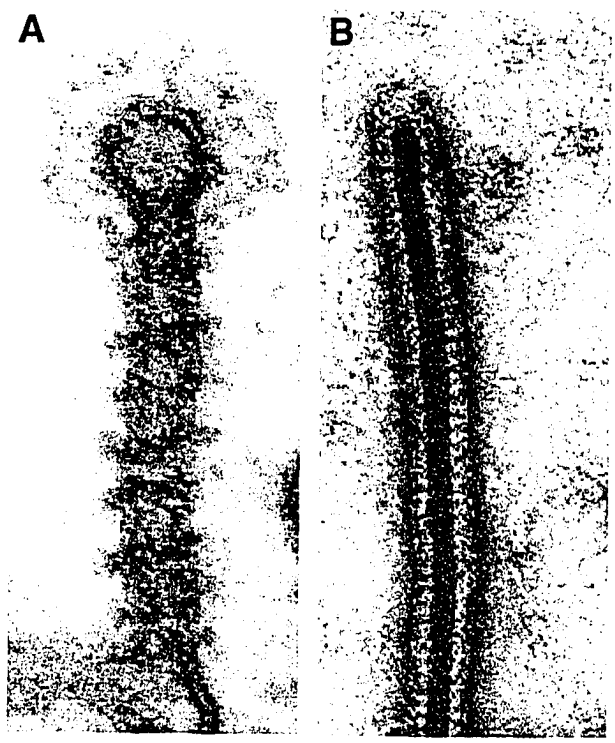
Thin-section electron microscopic specimens of membrane tubules generated by purified dynamin do not exhibit the dense ring pattern observed when membranes are treated with brain cytosol and GTPγS. The rings generated by purified dynamin are only seen by negative stain electron microscopy, and they are thinner and spaced more closely together than the rings formed with brain cytosol (Figure 1.3) (Takei et al., 1999). The rings seen with brain cytosol, therefore, most likely represent a complex of proteins involved in membrane deformation during clathrin-mediated endocytosis, which together are able to generate the electron density observed by thin sectioning.

The most likely candidates for the proteins involved in the endocytic fission ring include major dynamin interacting partners at the nerve terminal. One such protein is amphiphysin, a 128 kDa protein comprising a highly conserved NH2-terminal coiled-coil region and a COOH-terminal SH3 domain (Figure 1.4) (David et al., 1996). The coiled-coil region is involved in protein-membrane interactions as well as dimerization/multimerization (Ramjaun et al., 1997; Wigge et al., 1997a), while the SH3 domain binds dynamin and the synaptic polyphosphoinositide phosphatase, synaptojanin (David et al., 1996; de Heuvel et al., 1997). Furthermore, upstream of its

Figure 1.3 Membrane tubules generated by purified dynamin have a different protein coat compared with tubules generated by brain cytosol

(A) Thin section electron micrograph of epon-embedded membrane tubules generated in the presence of synaptic membranes, brain cytosol, and GTPγS. Note the presence of electron-dense rings similar to those seen in the shibire nerve terminals at the restrictive temperature. These tubules are highly immunoreactive against anti-dynamin antibodies. Reproduced from (Takei et al., 1995)

(B) Negative stain electron micrograph of membrane tubules generated in the presence of liposomes and purified rat brain dynamin. Note that although the dimensions of the membrane tubule are the same as compared with cytosol in A (roughly 25-100 nm diameter), the morphology of the protein coat on the tubule is different, with purified dynamin being represented by thinner rings spaced more closely together. This suggests that a protein complex is likely responsible for the thick electron-dense structure seen with brain cytosol.



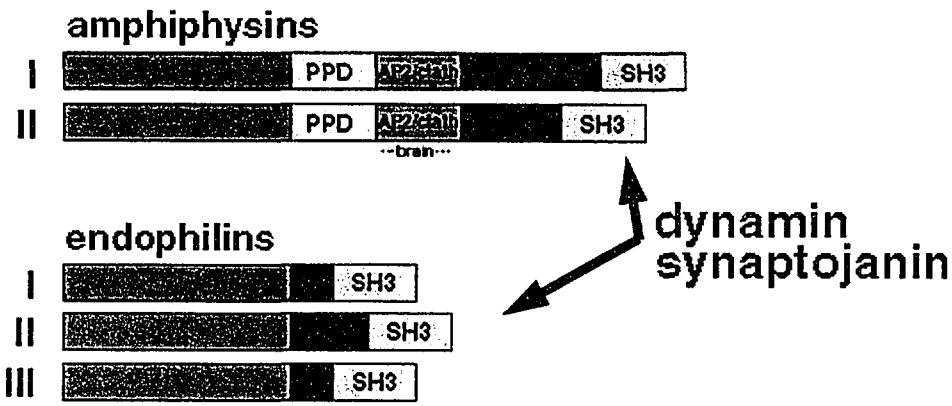
SH3 domain, neuronal isoforms of amphiphysin contain partially overlapping sequences known to bind clathrin and AP2 (Slepnev et al., 2000), thus providing for a putative link between interactions with dynamin and the clathrin-coat (Takei et al., 1999).

The role of amphiphysin in endocytosis has been shown by the strong inhibition of endocytosis found using its SH3 domain for acute perturbation studies. Cells transiently expressing the amphiphysin SH3 domain exhibit a profound block in clathrin-mediated endocytosis (Wigge et al., 1997b). Moreover, microinjection of the amphiphysin SH3 domain in the stimulated nerve terminal of the lamprey reticulospinal synapse results in depletion of synaptic vesicles secondary to a block in clathrin-mediated synaptic vesicle retrieval (Shupliakov et al., 1997). The block in synaptic vesicle recycling is at the late fission step, as numerous deeply invaginated clathrin-coated buds were seen accumulated at the periphery of the active zone (Shupliakov et al., 1997). Chronic perturbation of amphiphysin function mediated by targeted disruption of the amphiphysin gene, in a subset of mutant mice, leads to severe cognitive deficits correlated with dysfunctional synaptic vesicle recycling (Di Paolo et al., 2002). Amphiphsyin knockout mice also suffer premature death due to an enhanced susceptibility to seizures (Di Paolo et al., 2002). These data point to a role for amphiphysin in the high-efficiency synaptic vesicle recycling required for higher order brain functioning.

In vitro, amphiphysin forms a complex with dynamin, which, by comparison, forms more widely spaced electron dense rings visible also by thin sectioning (Takei et al., 1999). These rings more closely resemble the morphology of the rings seen using total brain

Figure 1.4: Major pre-synaptic dynamin binding partners

Amphiphysin and endophilin are proteins which are highly enriched at the synapse, and which interact with dynamin, and the polyphosotidlyinositol phosphatase, synaptojanin, via their COOH-terminal SH3 domains. Both proteins have a highly conserved NH2-terminal domain predicted to form alpha helices and coiled-coils. Brain isoforms of amphiphysin have a central domain with binding sites for clathrin and the clathrin adaptor protein, AP2.



cytosol, rather than the rings seen with dynamin alone (Takei et al., 1999), supporting the notion that a complex of proteins is present at the thick electron-dense collar present at the tubular neck of the clathrin-coated bud. Moreover, recombinant amphiphysin alone was able to tubulate liposomes to roughly the same diameter as the tubules generated by dynamin (Takei et al., 1999). Thus, the role of amphiphysin in the synaptic vesicle cycle may relate to its structural interaction with dynamin at the tubular neck of the clathrin-coated bud.

Lipid Metabolism in Synaptic Vesicle Recycling

As mentioned, the other major binding partner of amphiphysin at the nerve terminal is the polyphosphoinositide phosphatase, synaptojanin (Figure 1.4) (de Heuvel et al., 1997). Synaptojanin is concentrated at the nerve terminal, and is found on coated endocytic intermediates in an incubation using synaptic membranes with brain cytosol and GTP_S (Haffner et al., 1996; Haffner et al., 1997). This led to the speculation that phosphoinositide metabolism is an important part of the synaptic vesicle cycle (Cremona et al., 1999).

Synaptojanin has two phosphoinositide phosphatase domains which mediate the dephosphorylation of the phosphoinositides, $PI_{4,5}P$ (PIP_2) and $PI_{3,4,5}P$ (PIP_3) (McPherson et al., 1996). The synaptojanin COOH-terminal proline-rich domain mediates protein-protein interactions. Both chronic and acute perturbations have

implicated synaptojanin in synaptic vesicle recycling. Targeted disruption of the synaptojanin locus in mice leads to severe neurological deficits, failure to thrive, and a concomitant deficiency in neurotransmission (Cremona et al., 1999). An increase in brain PIP_2 levels compared with wild type mice was noted, indicating a possible causal link between the phenotype and the defect in phosphoinositide metabolism (Cremona et al., 1999). Furthermore, microinjection studies in the lamprey synapse have demonstrated a block in endocytosis with the ensuing accumulation of clathrin-coated vesicles and the proliferation of an actin cytomatrix (Gad et al., 2000).

The importance of lipids in synaptic vesicle recycling has been underscored by several observations. Dynamin shows a preference for acidic phospholipids, with its PH domain having an affinity for PIP_2 (Klein et al., 1998; Salim et al., 1996). PIP_2 is a biologically active lipid, and the observed effects of synaptojanin perturbation may reflect the affinity of the clathrin-coat adaptors to PIP_2, as well as the ability of PIP_2 to nucleate actin dynamics (Cremona and De Camilli, 2001; Takenawa and Itoh, 2001). Indeed, a brain-enriched isoform of a PI(4)P 5-kinase, which generates PIP_2 by the addition of a phosphate to position 5' of the inositol ring in the precursor, PI(4)P, has recently been identified (Wenk et al., 2001). This kinase is enriched at the nerve terminal, and localizes to clathrin-coated intermediates in the cell free incubation previously mentioned with brain cytosol (Wenk et al., 2001).

Interestingly, amphiphysin has been reported to act as an inhibitor of phospholipase D, an enzyme mediating the conversion of phosphatidyl-choline to

phosphatydic acid by cleavage of the choline moiety of the head group (Lee et al., 2000). The action of phospholipase D is a part of an enzymatic cascade leading to PIP_2 generation, and therefore, inhibition of this enzyme would theoretically block this cascade, preventing additional formation of PIP_2. Along with its binding partner, synaptojanin, amphiphysin may work to decrease PIP_2 levels in a function which may have importance for synaptic vesicle dynamics. Thus, a putative cycle of phosphoinositide metabolism may occur in tandem with the synaptic vesicle cycle, whereby generation of PIP_2 after exocytosis leads to coated bud formation in addition to undefined actin dynamics, and removal of PIP_2 leads to vesicle uncoating and competence to enter the recycling pool of synaptic vesicles (Cremona and De Camilli, 2001).

Endophilin and Synaptic Vesicle Recycling

Another major binding partner of dynamin and synaptojanin is endophilin 1, a 40kDa SH3 domain-containing protein enriched in the synapse (Figure 1.4) (de Heuvel et al., 1997; Ringstad et al., 1997). Microinjection studies in the living giant reticulospinal synapse of lamprey have implicated endophilin in many stages of clathrin-mediated synaptic vesicle endocytosis, from early events generating deep membrane curvature in the developing clathrin-coated bud, to later stages such as fission and uncoating of the nascent vesicle(Gad et al., 2000; Ringstad et al., 1999). Furthermore, endophilin was found to be necessary for generation of small synaptic-like microvesicles (SLMVs) in a PC12 cell based assay (Schmidt et al., 1999).

In Drosophila, the endophilin 1 ortholog is essential, with mutants containing P-element insertions in the endophilin 1 gene leading to death at the third instar larval stage (Guichet et al., 2002; Verstreken et al., 2002). Drosophila endophilin is a crucial part of synaptic function, and analysis of the nerve terminals in these mutants prior to death revealed depletion of synaptic vesicles and the presence of large vacuoles (Guichet et al., 2002; Rikhy et al., 2002; Verstreken et al., 2002). In some nerve terminals which were only mildly affected, numerous clathrin-coated intermediates were seen which remarkably resembled the shallow pits seen in the lamprey synapse upon microinjection of endophilin antibodies (Guichet et al., 2002; Ringstad et al., 1999).

In a cell free assay with rat brain cytosol and synaptic membranes incubated with GTP_S, endophilin co-localized with dynamin on membrane tubules, and selective depletion of endophilin from the cytosol resulted in diminished numbers of dynamin-coated tubules (Ringstad et al., 1999). These data suggest that endophilin plays a major role in synaptic vesicle recycling, and that the generation or stabilization of the dynamin-coated membrane tubule at the neck of the clathrin-coated bud may be a part of its function at the synapse. Interestingly, both endophilin and amphiphysin, the two major presynaptic binding partners of dynamin, co-localize to the membrane tubules generated with brain cytosol (Ringstad et al., 1999; Takei et al., 1999). However, it is unclear whether these two SH3 domain containing proteins are functioning in a similar manner with dynamin at the neck of the clathrin-coated bud, or whether they serve differential roles in space and/or time.

Recently, endophilin was biochemically identified in a cytosolic extract containing lysophosphatidic acid acyl transferase (LPA-AT) activity, making endophilin the first cytosolic protein reported to have such an activity (Schmidt et al., 1999). This acyl transferase activity, which generates phosphatidic acid (PA) by the transfer of arachidonoyl-CoA to lysophosphatidic acid (LPA), was thought to be crucial for the generation of membrane curvature by the conversion of an "inverse cone" lipid (LPA) to a "cone-shaped" shaped lipid (PA) in the cytoplasmic leaflet of the membrane (Schmidt et al., 1999). The authors of this study speculated that this activity was necessary for the transition from the positive membrane curvature of the budding vesicle, to the negative membrane curvature of the neck of the clathrin-coated bud (Schmidt et al., 1999), thereby being involved in fission. No direct evidence for a membrane deforming activity of endophilin was shown, however. Whether endophilin is involved in generating membrane curvature through this enzymatic activity or through some unknown mechanism potentially related to its role in the generation of dynamin tubules remains to be answered. To this end, the goal of the following study is to investigate a direct role for endophilin in the generation of membrane curvature.

Concluding Remarks

Fast point-to-point neurotransmission underlies the complex physiology of the nervous system. Elucidating the molecular mechanisms of this action is crucial to our

understanding of nervous system function. The cell biology of the nerve terminal is an important piece in this puzzle, and within this context, the efficient recycling of synaptic vesicles serves to maintain the high transfer of information characteristic of neuronal activity. Undoubtedly, we have only scratched the surface of the complex molecular interactions that are a part of the synaptic vesicle recycling process, and much is left to be studied. Clearly, clathrin-mediated endocytosis is an important part of synaptic vesicle retrieval and remains the most well characterized pathway known to function in this process. It will be interesting to see whether parallel pathways exist which may define a unique biology within synaptic vesicle recycling. Studying the detailed function of the various macromolecules shown to play a role in clathrin-mediated synaptic vesicle endocytosis will provide us with more tools to model our understading of the dynamic function of the nervous system.

Chapter 2

Generation of High Curvature Membranes Mediated by Direct Endophilin-Bilayer Interactions

Abstract

Endophilin 1 is a presynaptically enriched protein which binds the GTPase dynamin and the polyphosphoinositide phosphatase synptojanin. Perturbation of endophilin function in cell free systems and in living synapses has implicated endophilin in endocytic vesicle budding (Gad et al., 2000; Guichet et al., 2002; Rikhy et al., 2002; Ringstad et al., 1999; Schmidt et al., 1999; Verstreken et al., 2002). Here we show that purified endophilin can directly bind and evaginate lipid bilayers into narrow tubules similar in diameter to the neck of a clathrin-coated bud, providing new insight into the mechanisms through which endophilin may participate in membrane deformation and vesicle budding. This property of endophilin is independent of its putative lysophosphatydic acid acyl transferase (LPA-AT) activity, is mediated by its NH2-terminal region, and requires an aminoacid stretch homologous to a corresponding region in amphiphysin, a protein previously shown to have similar effects on lipid bilayers (Takei et al., 1999). Endophilin co-oligomerizes with dynamin rings on lipid tubules and inhibits dynamin's GTP-dependent vesiculating activity. Endophilin B, a protein with homology to endophilin 1, partially localizes to the Golgi complex and also deforms lipid

bilayers into tubules, underscoring a potential role of endophilin family members in diverse tubulo-vesicular membrane trafficking events in the cell.

Introduction

A major pathway of synaptic vesicle retrieval after exocytosis is clathrin-mediated endocytosis (Heuser and Reese, 1973). This specialized version of a general endocytic pathway is necessary for the maintenance of the synaptic vesicle pool, and perturbation of this process results in rapid depletion of synaptic vesicles (Gad et al., 2000; Koenig and Ikeda, 1989; Ringstad et al., 1999; Shupliakov et al., 1997). The 100kDa GTPase, dynamin, has been implicated in the "fission" stage of clathrin-mediated endocytosis (Koenig and Ikeda, 1989), and disruption of dynamin function results in an accumulation of electron-dense "collars" at the tubular neck of deeply invaginated clathrin-coated buds (Koenig and Ikeda, 1989; Takei et al., 1995). These collars are enriched with dynamin, as well as two of the major SH3 domain-containing dynamin binding partners at the synapse: endophilin 1 (endophilin) and amphiphysin 1 (amphiphysin) (David et al., 1996; de Heuvel et al., 1997; Ringstad et al., 1997). Recombinant dynamin 1 and amphiphysin have been shown to deform artificial lipid bilayers into narrow tubules, both independently as well as cooperatively in a complex, likely reflecting a role for these proteins in membrane binding and deformation at the neck of clathrin-coated pits (Takei et al., 1998; Takei et al., 1999)

Endophilin has been implicated in many stages of clathrin-mediated synaptic vesicle endocytosis, from early events generating membrane curvature, to later stages such as vesicle fission and uncoating (Gad et al., 2000; Ringstad et al., 1999). Selective depletion of endophilin from rat brain cytosol inhibited the generation of synaptic-like

microvesicles (SLMVs) in broken PC12 cells, and markedly reduced the formation of dynamin-coated tubules on synaptic membranes in the presence of ATP/ GTPγS (Ringstad et al., 1999; Schmidt et al., 1999). We therefore tested whether endophilin plays a direct role in the generation of membrane curvature in a similar fashion to dynamin and amphiphysin.

Materials and Methods

Liposomes

Liposomes composed of brain lipid extract (type 1, Folch fraction 1, Sigma) were made as described (Takei et al., 1999). Briefly, a lipid mixture solubilized in chloroform was added to a 2:1 chloroform:methanol mixture and thoroughly mixed in a glass tube. Lipids were adhered along the sides of the glass tube under a stream of nitrogen gas by gently rotating the tube until the chloroform:methanol solvent had evaporated. This procedure generates layers of lipids dried as multiple bilayers along the glass surface. 300mM filtered sucrose was then gently added to the tube with the dried lipid bilayers, and the tube was allowed to incubate, covered with parafilm, at 37 °C for 15-60 minutes. During this incubation, the dried bilayers gradually begin to peel off of the glass surface and swell. Following the incubation, vigorous vortexing shears the bilayers into vesicles of heterogeneous size. For liposomes of specific size, this mixture was passed through a filter with the requisite pore size. Liposomes loaded with sucrose could thus be spun down due to the increased density of sucrose compared with the incubation buffer of the subsequent reactions. Synthetic liposomes were made in a similar fashion, using HPLC purified DOPC (40%), DOPS (40%), and DOPE (20%) from Avanti Polar Lipids.

Purified Proteins

Dynamin was affinity purified from rat brain cytosol using amphiphysin 1-SH3 domain as described (Owen et al., 1998). Briefly, three to five rat brains were homogenized and spun in a single step at high speed to remove nuclei, particulates, and heavy membranes. This supernatant was then incubated for two hours at 4 °C with three to five milligrams of a recombinant GST-tagged amphiphysin 1 construct, comprising a COOH-terminal fragment including the SH3 domain, coupled to glutathione sepharose beads. The beads were then washed extensively in the homogenization buffer, and the bound material was eluted in elution buffer (Pipes, pH 6.2, 1.2M NaCl, 10mM Ca^{2+}, 1mM dithiothreitol (DTT)). The eluate was dialyzed overnight into 20mM Hepes, pH 7.4, 100mM NaCl, 1mM DTT. This material, as assessed by SDS PAGE, yielded roughly 90-95% dynamin, and 5-10% synaptojanin. All data shown use this material. Synaptojanin was immunodepleted from this affinity purified material using a monoclonal antibody to yield a more highly purified dynamin preparation, and similar results were obtained.

Recombinant rat endophilin A1, human amphiphysin 1, human endophilin B1, and PLCδ-PH were cloned in pGEX (Pharmacia), and purified as Glutathione-S Transferase (GST) fusion proteins according to standard methods (Pharmacia). For endophilin and amphiphysin, the GST tag was subsequently cleaved by PreScission Protease (Pharmacia).

Endophilin deletion constructs were prepared by PCR to yield the following fragments: amino acids 1-125, 1-261,126-352, 34-352. The constructs were subcloned and purified

as above. Point mutants were generated using mutant primers and plasmid extension by PCR.

Clathrin-coat fraction was purified from bovine calf brain as described (Takei et al., 1999).

Electron Microscopy

Liposomes (0.1mg/ml final) were incubated at 37 °C for 10-20 minutes in buffer A (25mM Hepes-KOH, pH 7.4, 25mM KCl, 2.5mM Mg^{2+} acetate, 150mM K-glutamate) with various proteins and nucleotides at the following final concentrations: dynamin 0.1mg/ml, amphiphsyin 0.1mg/ml, endophilin 0.1mg/ml, endophilin deletion constructs 0.1-0.2 mg/ml, coat proteins 0.5mg/ml, PLCδ-PH 0.1mg/ml, GTP 1mM, GTPγS 0.5mM (Takei et al., 1999). At the end of the incubation, aliquots were adsorbed onto 200-400 μM formvar- and carbon-coated copper EM grids for 3-5 minutes at room temperature, washed in 0.1M Hepes pH 7.4, stained in 1-2% uranyl acetate, blotted and allowed to air dry. For incubations at 4 °C, all of the above was performed on ice.

Average ring spacing was determined by counting the number of rings in ten 350nm tubules, and then dividing by the tubule length. To quantify the number of tubules seen under various conditions, six 13μm^2 areas of the grid were scored. Each independent tubule and each branch of a tubule was counted as a tubule.

Liposome Binding

Liposome sedimentation was performed using 100μg sucrose-loaded liposomes incubated with 5-10μg protein (1-25μg for saturable binding) in 400-500μl buffer A or in Hepes-KCl pH 7.4 (to determine salt sensitivity) for 10-20 minutes at 37 °C. Liposomes were sedimented at 100,000g in a Beckman TLA 100.3 rotor for 20 minutes, the supernatant was thoroughly removed, and sedimented liposomes were solubilized in 2% SDS. To monitor recovery, liposomes were labeled with 0.5% NBD-phosphatidyl choline and absorbance was measured at 460 nm. In some cases, proteins in the pellet and supernatant were concentrated by chloroform precipitation and methanol extraction. Samples were subjected to SDS-PAGE and analyzed by either Coomassie staining, or by slot blot analysis using affinity purified polyclonal endophilin antibodies and ^{125}I. Band intensities were quantified by optic densitometry or phosphorimaging.

For the crosslinking assay, 4μg of the endophilin 125 amino acid fragment (with or without 8μg liposomes) was pre-incubated in Hepes pH 7.4, 100mM KCl for 20 minutes at 37 °C, then an equal volume of 2X buffer with the hetero-bifunctional chemical crosslinker bis(sulfosuccinimidyl) suberate (BS^3) was added to achieve final concentrations of 0, 1, 5, and 10mM crosslinker. The mixture was incubated for an additional 30 minutes at 37 °C, and the samples were then prepared for SDS-PAGE analysis.

Miscellaneous Procedures

Immunogold labeling of endophilin was performed by standard agarose embedding/labeling protocols as described (Ringstad et al., 1999) followed by thin sectioning. The GTPase activity of dynamin was determined by phosphate release as described (Takei et al., 1999). Western blotting and immunofluoresence was performed using standard procedures as described (Ringstad et al., 1997).

Results

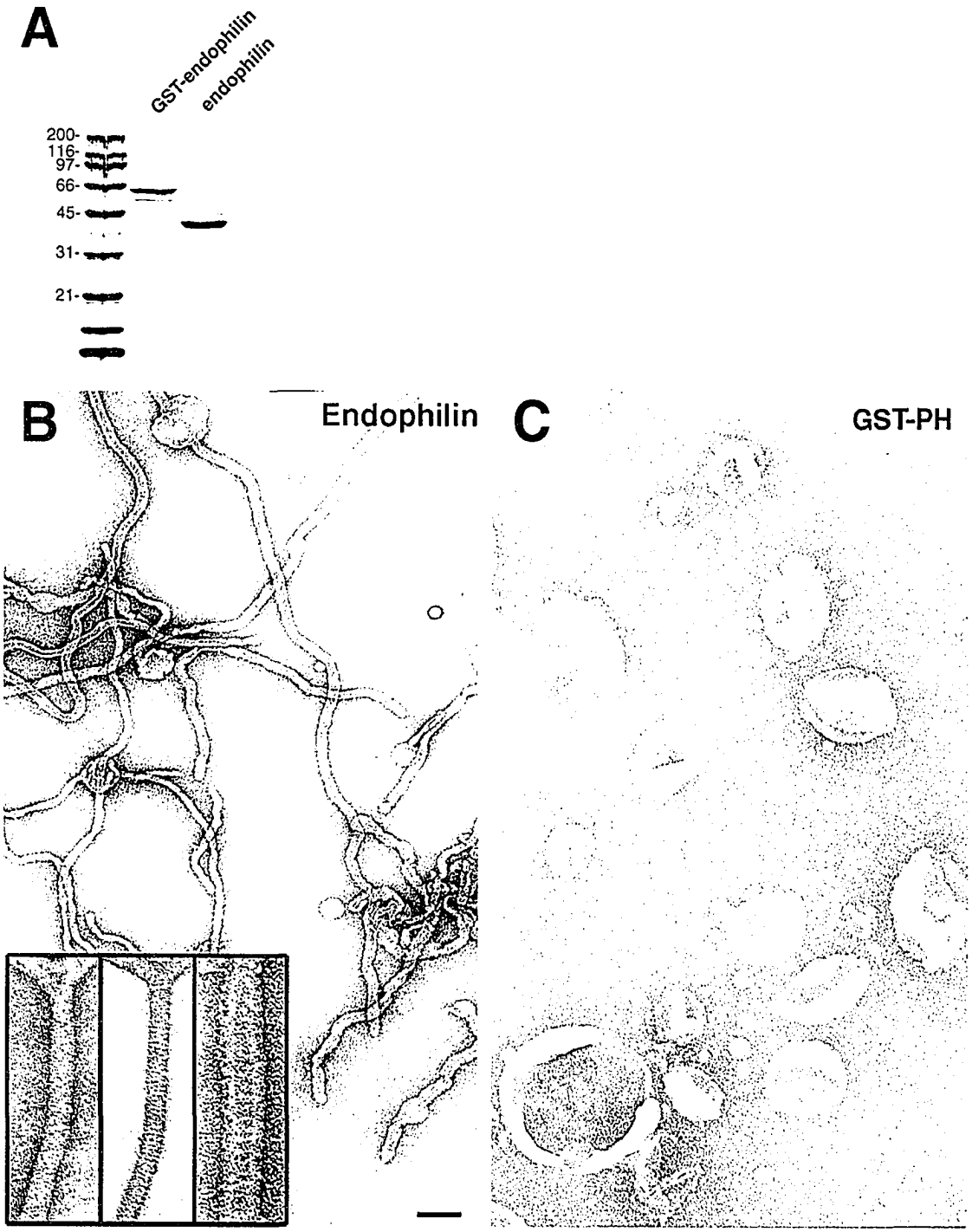
Endophilin Directly Binds and Deforms Lipid Bilayers

To analyze the direct effects of endophilin on lipid bilayers, we took advantage of a liposome-based assay previously shown to support generation of coated intermediates of clathrin-mediated endocytosis (Takei et al., 1998; Takei et al., 1999). This liposome assay has demonstrated that soluble components of clathrin-mediated endocytosis are sufficient for reproducing the complement of morphological intermediates seen in this process, and rules out a significant contributory structural role for membrane proteins in the budding process. Using this assay allows for the study of purified proteins in isolation and in combination, as well as the study of the different lipid components which may be required.

Upon a brief fifteen minute incubation with liposomes at 37 °C, purified recombinant endophilin (Figure 2.1A) efficiently and robustly deformed lipid bilayers into tubules with outer diameters ranging between 20-100nm (Figure 2.1B). The tubule surfaces were decorated by tightly packed thin transverse striations, reminiscent of the coat observed on tubules generated by recombinant amphiphysin (Figure 2.1B, see inset for a comparison of the endophilin, amphiphysin, and dynamin coats on tubules) (Takei et al., 1999). The recombinant pleckstrin homology (PH) domain of phospholipase C delta (PLCδ), a known phopholipid binding domain, did not deform the lipid bilayers into tubes, thereby distinguishing this activity from non-specific changes due to protein-

Figure 2.1: Endophilin directly binds and deforms lipid bilayers.

(A) Recombinant endophilin 1 was subcloned into pGEX6P1 (Pharmacia), expressed and purified on glutathione-S sepharose beads. The GST-tag was subsequently cleaved using PreScission Protease (Pharmacia), yielding the full length protein in a highly purified fraction. This material was used for the subsequent assays. **(B)** Negative stain EM of liposomes composed of a brain lipid extract after addition of recombinant endophilin 1 or **(C)** of the PH domain of PLCδ (GST-tagged) as a control, respectively. Liposomes were incubated for 15' at 37 °C with the purified proteins. Endophilin deformed the spherical liposomes into long tubules with 20-100nm diameters. Inset shows a comparison of the protein coats observed on the tubules generated by endophilin (left), amphiphysin (center), and dynamin (right). Bar, 100nm; 70nm for inset.



membrane interactions (Figure 2.1C). Endophilin binding to liposomes appeared to saturate at a molar ratio of under 1:200 (protein to lipid) in a liposome sedimentation assay (Figure 2.2A), and displayed a non-linear loss of binding with increasing salt (Figure 2.2B). By electron microscopy, it is evident that not all liposomes are tubulated by endophilin, reflecting a cooperativity in the process which is likely reflected in an underestimation of the molar ratio saturation noted above.

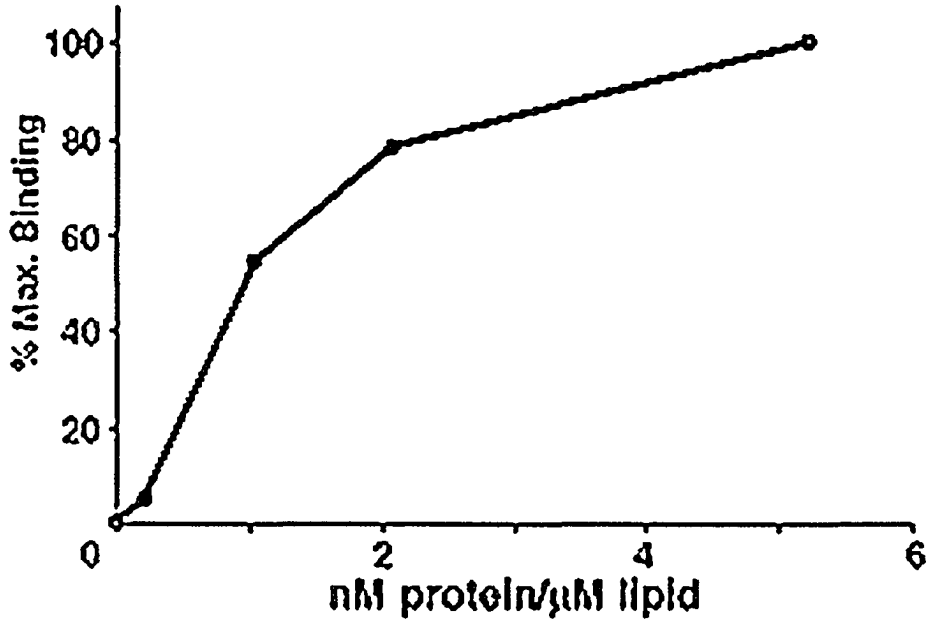
The NH2-terminus of Endophilin is Necessary and Sufficient for Tubulation and Lipid Binding

Deletion constructs of endophilin defined amino acids 1-125 as both necessary and sufficient for tubulation (Figure 2.3A). Correspondingly, this 125 amino acid fragment was also seen to co-sediment with liposomes, while a construct that lacked this fragment stayed in the supernatant (Figure 2.3A). In the presence of a chemical crosslinker, bis(sulfosuccinimidyl) suberate (BS^3), the endophilin N-terminal fragment crosslinked into higher order oligomers in a liposome stimulated manner, whereas in the absence of liposomes, the fragment crosslinked only to a dimer (Figure 2.3B). Similar results were obtained using a second crosslinker, EDC, and with the full length protein (unpublished results). The presence of coated striations on the tubules and the cross-linking data together suggest that endophilin generates bilayer curvature by oligomerizing on the surface of liposomes.

Figure 2.2: Binding characteristics of endophilin to liposomes.

(A) Saturable binding of endophilin to liposomes with increasing concentrations of endophilin. This is likely an underestimation of endophilin saturation on the membrane, since by electron microscopy, not all liposomes are tubulated under these conditions, suggesting a cooperativity which favors tubule formation at areas of higher protein concentrations **(B)** Salt sensitivity of endophilin binding to liposomes with increasing KCl concentration.

A



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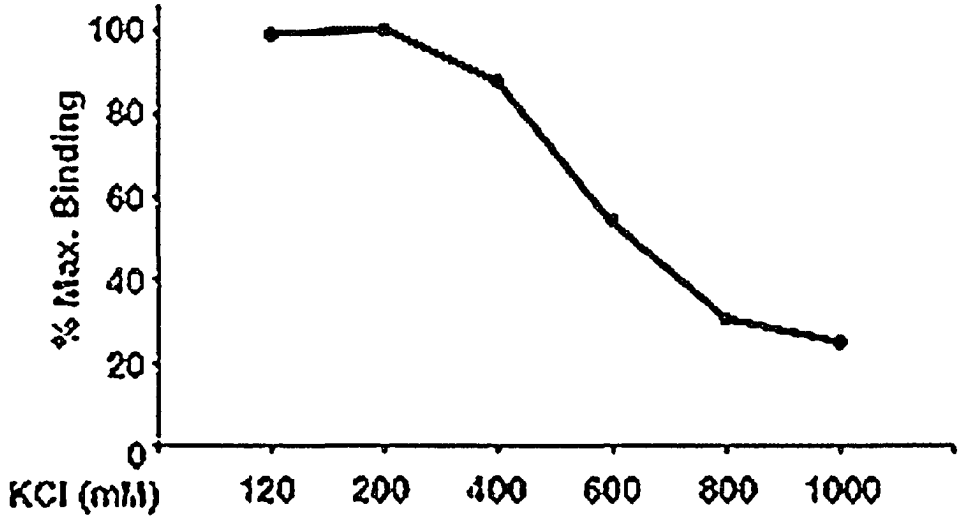
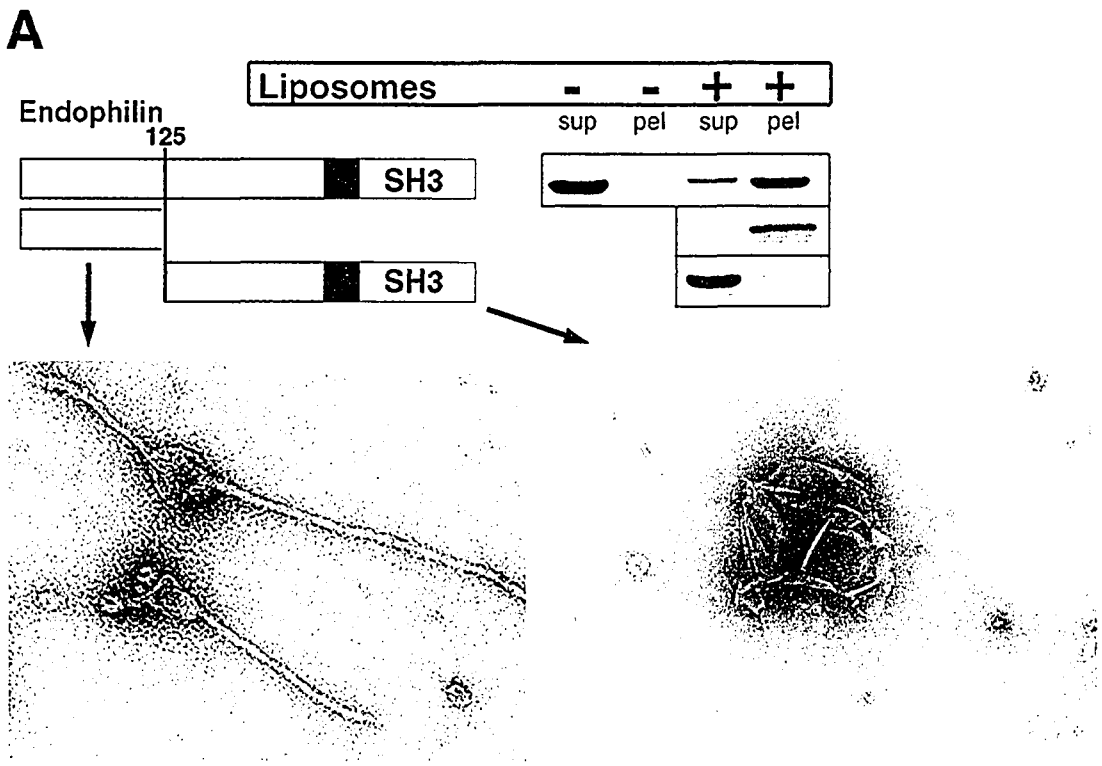


Figure 2.3: The NH2-terminal region of endophilin is necessary and sufficient for lipid binding and tubulation.

(A) Deletion constructs of endophilin were incubated with or without liposomes as shown, and co-sedimentation with liposomes was determined. In the absence of liposomes, full length endophilin remains in the supernatant. The grey area in the cartoon represents the moiety conserved amongst the endophilins. Amino acids 1-125 within this region bind and tubulate liposomes. **(B)** The NH2-terminal 125 amino acid fragment was incubated with or without liposomes as shown, with increasing concentrations of the chemical crosslinker BS^3 for 20' at 37 °C . Samples were then prepared for SDS-PAGE analysis on a 4-12% gradient gel. In the absence of liposomes, the endophilin construct crosslinks into a dimer, while in the presence of liposomes higher molecular weight products are formed suggestive of oligomerization (see arrowheads).

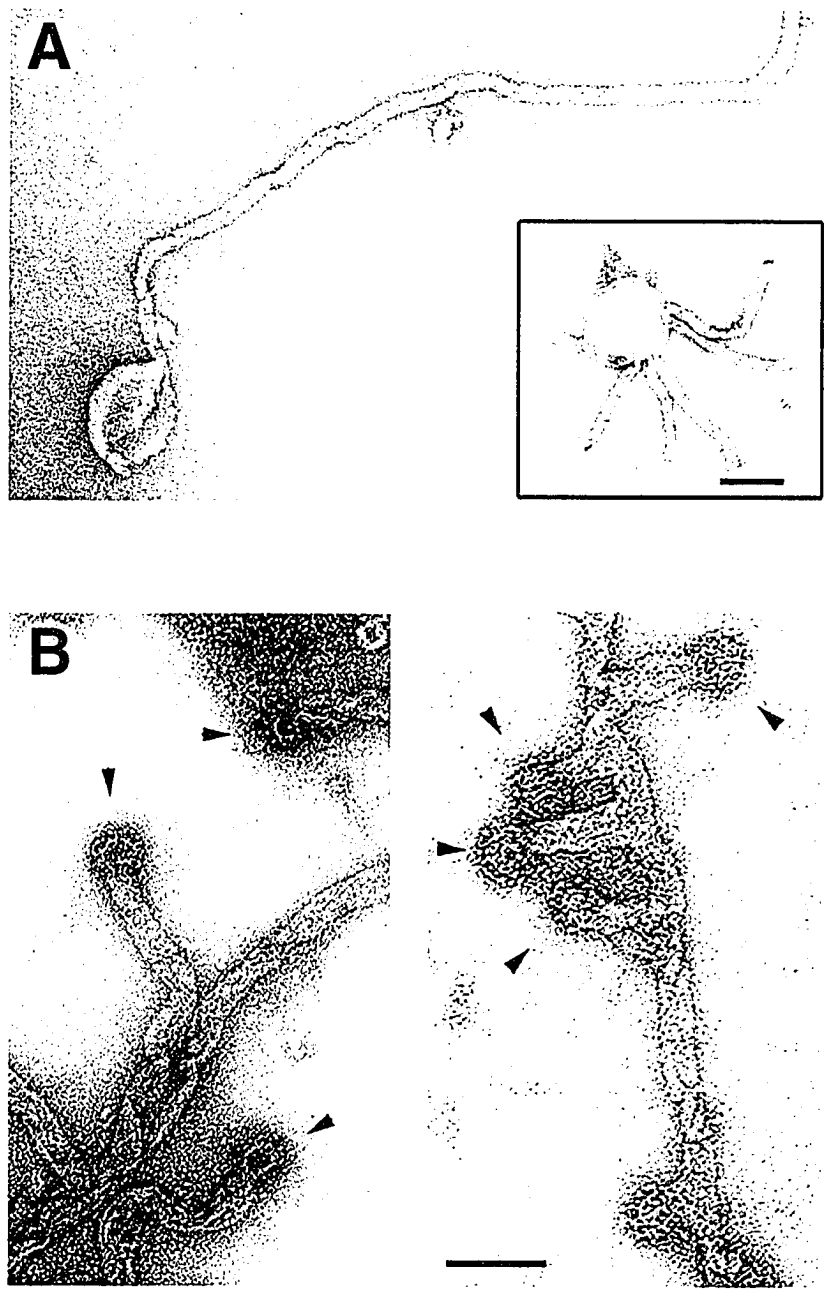


Membrane Tubulation by Endophilin is Independent From LPA-AT Activity

The property of endophilin to deform the lipid bilayer is independent from its reported lysophosphatidic acid acyl transferase (LPA-AT) activity (Schmidt et al., 1999). Endophilin tubulated synthetic liposomes devoid of the putative substrates for the LPA-AT reaction, arachidonoyl-CoA and lysophosphatidic acid (LPA) (Schmidt et al., 1999) (Figure 2.4A). Furthermore, endophilin tubulated liposomes in a reaction incubated on ice, minimizing any potential enzymatic contribution (Figure 2.4A, inset). Thus, bilayer deformation may represent a thermodynamically favorable transition mediated by endophilin polymerization at the liposome surface, rather than by the active enzymatic generation of specific membrane micro-domains. We did observe that endophilin binds and tubulates liposomes made from a brain lipid extract more efficiently than synthetic liposomes, thus indicating some role for the composition of the bilayer. In addition, using purified lipid components, endophilin was found to have a very mild affinity for the presence of LPA in the synthetic liposomes when compared to several other lipids. Addition to the liposomes of PA, as a head group control, or LPC, as an acyl chain control, did not have the same effect as addition of LPA, suggesting that the slight increase in binding due to the presence of LPA in the liposomes is apparently specific. What role this mild lipid preference may have in this process is hard to determine and requires further experimentation.

Figure 2.4: The lipid tubulation activity of endophilin does not require lysophosphatidic acid (LPA)-acyl transferase (LPA-AT) activity.

(A) Endophilin was incubated with synthetic liposomes lacking both LPA and arachidonoyl Co-A, the two substrates for its reported LPA-AT activity. The inset shows a liposome composed of brain lipids after incubation with endophilin for 20' at 4 °C. Bar, 100nm. **(B)** Clathrin-coated buds are observed on endophilin tubules when liposomes are incubated with a mixture of purified clathrin-coat proteins and recombinant endophilin 1. Bar, 200nm.



Incubation of liposomes, purified clathrin-coat proteins, and endophilin generated tubules associated with clathrin-coated buds, consistent with a potential role for this protein in generating tubular membrane curvature at the neck of the nascent clathrin-coated pit (Figure 2.4B). Similar observations were made for amphiphysin (Takei et al., 1999). Of note, amphiphysin has binding sites for clathrin and the endocytic clathrin adaptor, AP2 (Slepnev et al., 2000), but we have never observed binding to clathrin or to clathrin adaptors by endophilin.

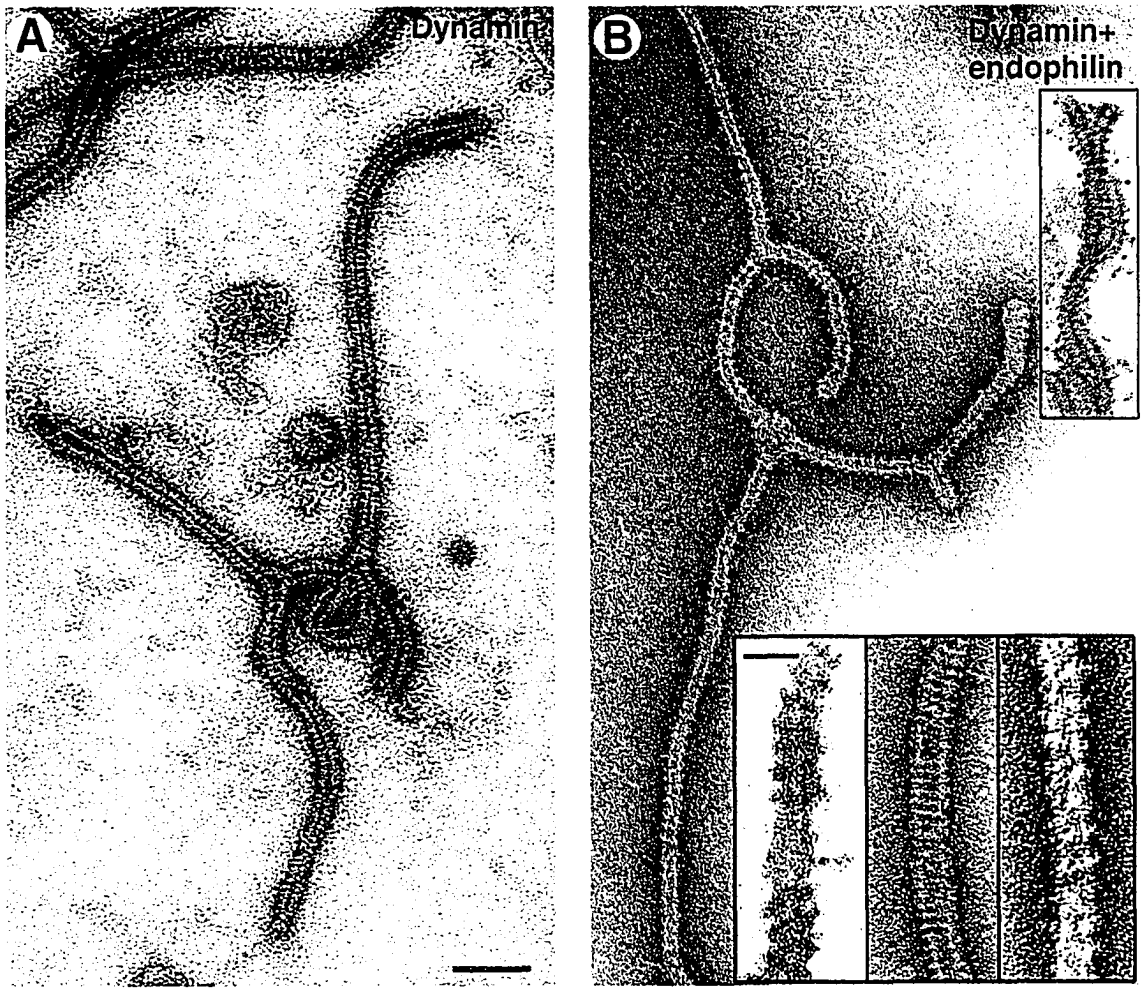
Endophilin and Dynamin Form a Protein Complex on Bilayer Tubules

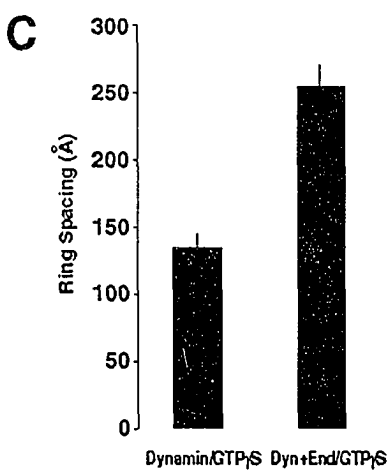
Since endophilin, like amphiphysin, interacts with dynamin via its SH3 domain, we investigated whether dynamin and endophilin could form a complex on liposomes similarly to what has been described for dynamin and amphiphysin (Takei et al., 1999). The coat generated by affinity purified dynamin 1 on bilayer tubules was represented by rings with an average spacing of 132 +/- 5 Ångstroms (Figure 2.5A), in agreement with the previously reported ring spacing determined by electron diffraction (Sweitzer and Hinshaw, 1998). Liposomes incubated with both recombinant endophilin and purified dynamin created a different ring morphology (Figure 2.5B, and lower inset). The rings were thicker, and had an average spacing of 202 +/- 15 Ångstroms, a difference of over 50% compared to the rings formed by dynamin alone (Figures 2.5A and 2.5B).

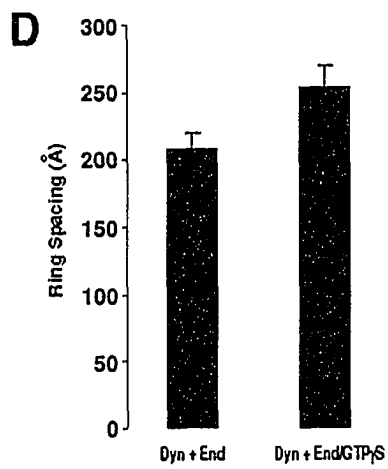
In the presence of GTPγS, the difference in ring spacing between dynamin alone and the dynamin-endophilin complex became more pronounced mainly due to an increase

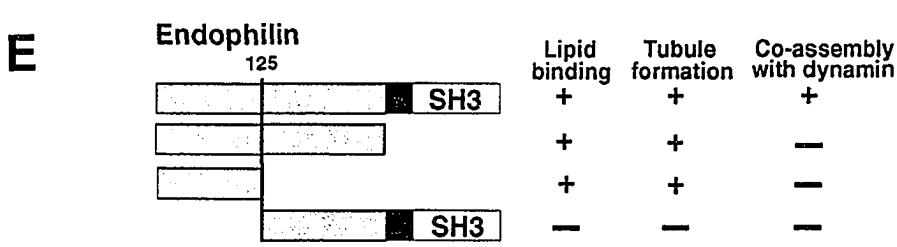
in the spacing of the dynamin-endophilin complex on the liposomes (Figure 2.5C). In the presence of GTPγS, the spacing between the endophilin-dynamin ring complex increased 25% to 255 +/- 15 Ångstroms compared with conditions in which no nucleotides were used (Figure 2.5D). This increase in ring spacing resulted in an almost two-fold difference between the endophilin-dynamin complex and dynamin alone under equivalent conditions (Figure 2.5C). Thus, due to the regular assembly of endophilin and dynamin into rings around lipid tubules, we noted a distinct change in ring conformation attributable to the presence of the GTP analog, GTPγS. This nucleotide dependent conformational change may underlie some of the dynamics observed with dynamin and lipid bilayers, and suggests a mechanochemical basis for dynamin function. Both the NH2-terminal lipid binding domain of endophilin and its COOH-terminal dynamin binding SH3 domain were necessary for the formation of the complex with dynamin on the membranes, since neither domain alone could reproduce the phenomenon (Figure 2.5E). The ring morphology seen with endophilin and dynamin is similar to the ring morphology generated by the dynamin-amphiphysin complex (Takei et al., 1999) and by brain cytosol with ATP/ GTPγS (Takei et al., 1998; Takei et al., 1995). This finding supports the presence of a multi-protein complex in the thick electron-dense rings observed at the neck of clathrin-coated pits on synaptic membranes, and explains the co-localization of endophilin with dynamin on coated tubules formed by incubating synaptic membranes with brain cytosol and ATP/ GTPγS (Figure 2.5B, upper inset) (Ringstad et al., 1999; Ringstad et al., 1997).

Figure 2.5: Complex of endophilin with dynamin along membrane tubules. **(A)** and **(B)** The tubular coat produced by dynamin is modified by the presence of endophilin. Liposomes were incubated for 15' at 37 °C with affinity purified dynamin **(A)** or with dynamin plus recombinant endophilin **(B)** Bar, 200nm. The upper inset of **(B)** shows the immunogold localization of endophilin on dynamin-coated tubules formed by incubating synaptic membranes with brain cytosol, ATP and GTPγS. See also Ref. (Ringstad et al., 1999). The lower inset of **(B)** shows a comparison of the tubules generated on liposomes by brain cytosol, ATP and GTPγS (left, thin section), with those generated by purified dynamin (center, negative stain) and dynamin plus recombinant endophilin (right, negative stain). Note the similarity of the electron-dense rings produced by brain cytosol with those formed by the dynamin-endophilin complex. Bar, 50nm. **(C)** Spacing between protein rings along the lipid tubules in the presence of GTPγS increases from 132 +/- 5 Ångstroms with dynamin alone to 202 +/- 15 Ångstroms with the complex of endophilin and dynamin. **(D)** In the presence of GTPγS, the ring spacing of the endophilin-dynamin complex increases by roughly 25%, indicating a structural change occuring upon binding the nucleotide. **(E)** Both the NH2-terminal lipid binding region and the SH3 domain of endophilin (which binds dynamin) are required to form a complex of endophilin and dynamin on the lipid bilayer.





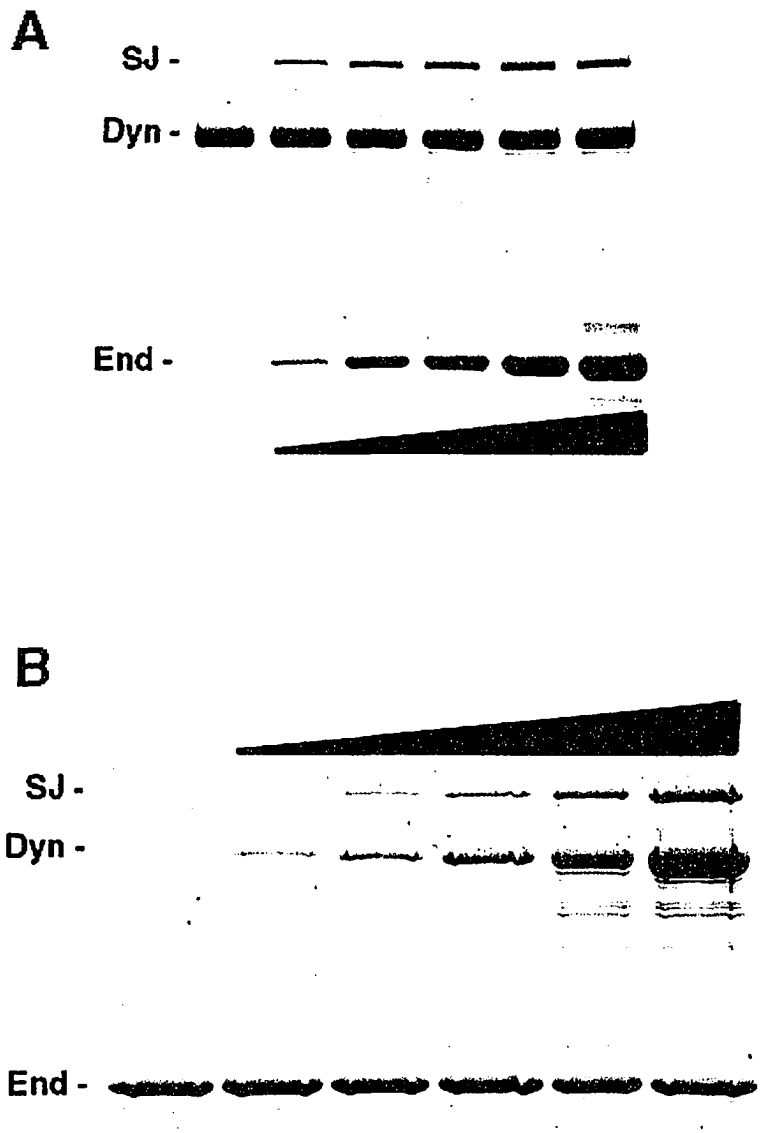




Since endophilin and dynamin were forming a complex on liposomes, we were interested in what role adding endophilin to dynamin had on the recruitment of these two proteins onto lipsomes. Increasing the amount of endophilin in the reaction mixture had very minimal if any effect on the recruitment of dynamin onto liposomes (Figure 2.6A). Correspondingly, increasing the amount of the purified dynamin/synaptojanin fraction also did not stimulate the recruitment of endophilin onto the liposomes (Figure 2.6B). Both endophilin and dynamin appeared to be quantitatively binding to the liposomes under these conditions. Interestingly, increasing the endophilin concentration in the reaction mixture did have a selective stimulatory effect on the recruitment of synaptojanin onto the liposomes (Figure 2.6A). This is consistent with the observations demonstrating a phenotype similar to what has been found with perturbation of synaptojanin function as a part of the phenotype seen upon perturbation of endophilin function at the lamprey synapse. This suggests that one of the major roles of endophilin in synaptic vesicle recycling may be to help recruit synaptojanin to its site of action at the clathrin-coated vesicle. This recruitment would serve to localize the lipid phosphatase activity of synaptojanin to the PIP_2 substrates on the clathrin-coated vesicle to aid the uncoating process.

Figure 2.6: Recruitment of synaptojanin to liposomes is specifically enhanced by endophilin.

(A) Using a fixed concentration of an affinity purified mixture of dynamin and synaptojanin from rat brain cytosol, increasing amounts of endophilin were added and incubated with liposomes. Note that with increasing endophilin concentrations, there is a selective enhancement of synaptojanin recruitment to liposomes, with no effect on dynamin recruitment. **(B)** The converse experiment, where the endophilin concentration is fixed and the amount of the dynamin/synaptojanin material is increased shows that there is no stimulation of further recruitment of endophilin under these conditions.

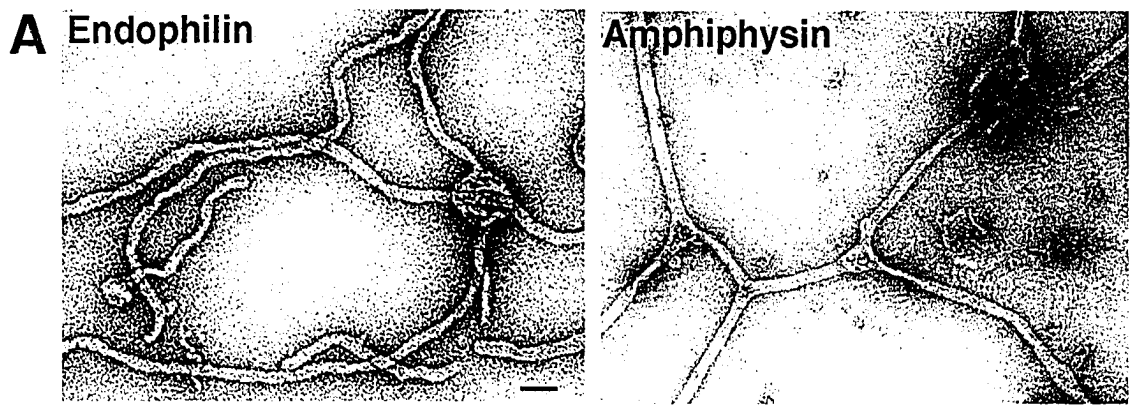


Lipid Binding and Tubulation by Endophilin Requires an Amphipathic Stretch Conserved in Amphiphysin

We were intrigued by the similarities in the membrane deformation properties of endophilin and amphiphysin (Figure 2.7A), and in the tubular coats which they form (Figure 2.1A, inset). The two proteins have an overall similar domain structure, each with a phylogenetically highly conserved NH2-terminal region comprising predicted alpha helices and coiled-coils, and a COOH-terminal SH3 domain which binds dynamin and synaptojanin (Figure 2.7B). Furthermore, the membrane tubulating properties of both proteins map to their respective NH2-termini (Takei et al., 1999). We explored whether endophilin and amphiphysin share any primary sequence similarity within this region. A BLAST alignment of full-length rat endophilin 1 and human amphiphysin 1 identified the highest scoring region to be a 29 amino acid stretch (41% identity and 75% similarity) within the first 35 amino acids of endophilin and the first 41 amino acids of amphiphysin (Figure 2.8A). Secondary structure algorithms (nnpredict and PSIPRED) for this 29 amino acid stretch predict alpha helices and random coils. When plotted on a helical wheel, a similar amphipathic pattern emerged for both endophilin and amphiphysin within this homologous region, creating a putative hydrophobic patch and an opposite hydrophilic face consisting of several basic residues (Figure 2.8A). This feature may allow this domain to interact with phospholipid headgroups via the hydrophilic face, and then to partially embed into the bilayer via the hydrophobic patch in a manner which favors membrane deformation. Accordingly, deleting most of this region of homology abolished binding to liposomes (Figure 2.8B) and tubulation (unpublished

Figure 2.7: Endophilin and amphiphysin share similar membrane tubulation properties

(A) Incubation of liposomes with endophilin or amphiphysin generates membrane tubules with similar morphology (Scale bar = 100nm). **(B)** Schematic cartoon of the basic domain structure of endophilin and amphiphysin. The grey area corresponds to a conserved region of predicted alpha helices and coiled-coils in each protein, respectively, along the NH2-terminus. This region is known to be involved in both lipid binding and in dimerization. The respective SH3 domains of endophilin and amphiphysin both bind dynamin and synaptojanin.



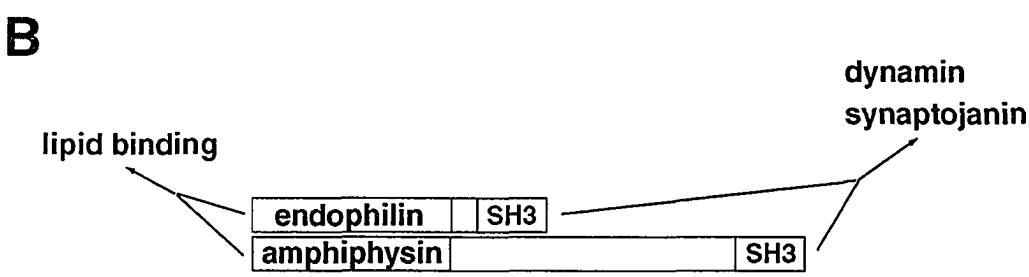
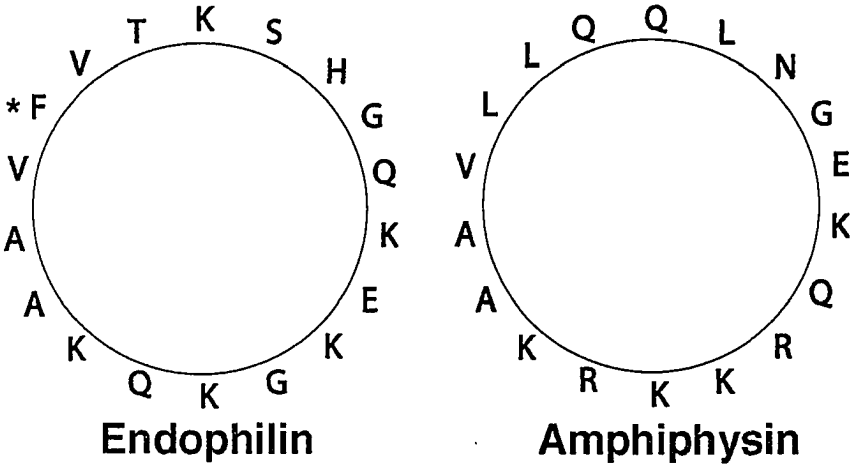


Figure 2.8: An endophilin amino acid stretch homologous to a corresponding region in amphiphysin is required for lipid binding and tubulation.

(A) BLAST alignment of rat endophilin 1 and human amphiphysin 1 reveals a region of homology within the NH2-terminus of both proteins. Plotting this region, which is predicted to form alpha helices and random coils, along a helical wheel reveals a similar amphipathic pattern. (Hydrophobic residues are highlighted in grey. *Phenylalanine targeted for point mutations) **(B)** Endophilin and amphiphysin deletion constructs missing most of this region of homology no longer co-sediment with liposomes (s= supernatant; p = pellet), and also fail to tubulate liposomes (not shown).

A

```
Endophilin      7  KKQFHKATQKVSEKVGGAEGTKLDDDFKE 35
                   +K+  +A +KV +K G A+ TK D+ F E
Amphiphysin    14  QKRLNRAQEKVLQKLGKADETK-DEQFEE 41
```



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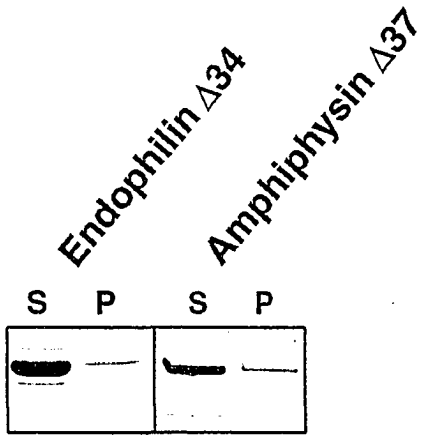


results) for both endophilin and amphiphysin.

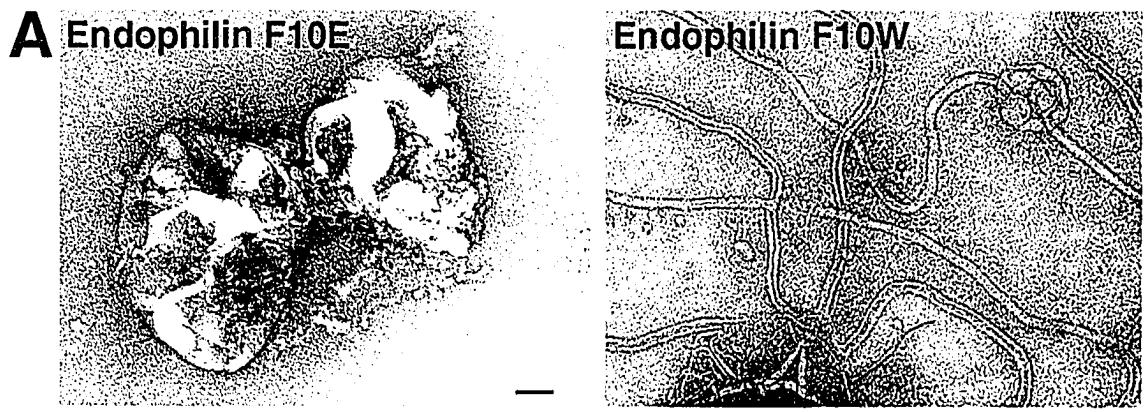
We further examined the role of putative amphipathic chemistry in the lipid binding dynamics of endophilin. We knew from earlier studies that there was a hydrophilic, salt sensitive component to liposome binding, as increasing salt concentrations prevented endophilin-liposome association (see Figure 2.2B). To test for a potential role for hydrophobic interactions in endophilin lipid binding, a point mutation of a conserved hydrophobic residue to an acidic residue (phenylalanine to glutamate) within this putative hydrophobic patch in endophilin was generated via site directed mutagenesis (Figure 2.9 A and B). As predicted, this mutation within the endophilin hydrophobic patch abolished both lipid binding and tubulation (Figure 2.9 A and B). As a control, a conservative hydrophobic to hydrophobic point mutation (phenylalanine to tryptophan) of the same residue preserved both lipid binding and tubulation, highlighting the importance of hydrophobicity within this region of endophilin for lipid binding and tubulation (Figure 2.9 A and B).

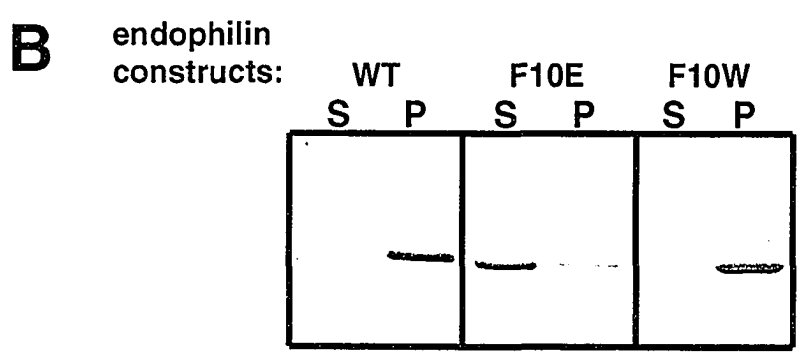
Endophilin and Amphiphysin have Different Effects on Dynamin Vesiculation of Bilayer Tubules

Given the similar morphology of amphiphysin and endophilin coated tubules both in the presence and absence of dynamin, we compared their individual effects on dynamin-mediated membrane fragmentation. Dynamin coated membrane tubules readily

Figure 2.9: Hydrophobic interactions are involved in endophilin lipid binding and tubulation.

(A) and **(B)** A point mutation (F10E) in endophilin of a hydrophobic residue to an acidic residue within the putative hydrophobic patch in the endophilin-amphiphysin homology region, prevents liposome binding and tubulation, whereas a conservative hydrophobic to hydrophobic mutation (F10W) preserves liposome binding and tubulation (WT, F10E, F10W = wild type and mutant endophilins, respectively; p and s = pellet and supernatant, respectively).





fragment into small vesicles in the presence of GTP (Sweitzer and Hinshaw, 1998; Takei et al., 1999). As previously reported, amphiphysin supports this fragmentation (Takei et al., 1999). Accordingly, following an incubation of liposomes with dynamin + GTP, or dynamin with amphiphysin + GTP, a large number of small vesicles were seen as evidenced by electron microscopy (Figure 2.10A and B). Any tubules which remained were generally much shorter. In contrast, when liposomes were incubated with dynamin and endophilin + GTP, fragmentation was inhibited, with a large number of long tubules persisting (Figure 2.10A and B).

The differential effects on liposome vesiculation by dynamin observed between endophilin and amphiphysin were confirmed using a dynamic light scattering assay (Figure 2.11). In this assay, larger complex structures, such as long tubules, scatter more light than small spherical vesicles and liposomes. The light scattering is measured by a fluorimeter, and there is the advantage of looking at the population of the reaction as opposed to isolated areas on an electron microscope grid. Using this assay, addition of dynamin to liposomes significantly increased the amount of light scattered (Figure 2.11A). Subsequent addition of GTP caused a rapid decrease in the amount of light scattered, corresponding with the robust vesiculation occurring under these conditions as seen by electron microscopy (Figure 2.11A). Addition of amphiphysin to dynamin caused an immediate decline in light scattering, even in the absence of GTP (Figure 2.11B). After addition of GTP, the degree of light scattered decreased to below baseline levels, reflecting the reported enhanced vesiculation of liposomes under these conditions (Figure 2.11B). By contrast, addition of endophilin to dynamin caused an even greater

Figure 2.10: Different effects of endophilin and amphiphysin on dynamin properties in vitro.

(A) Liposomes incubated with buffer, GTP, and either purified dynamin (center), or dynamin plus amphiphysin (left) vesiculate liposomes. In contrast, addition of endophilin to dynamin (right) inhibits this phenomenon. Bar, 100nm. **(B)** Morphometric analysis of the reactions shown in **(A)**. **(C)** Phosphate release from $\gamma^{32}P$ labeled GTP incubated with dynamin and dynamin interactors, or GST as a control, in the absence (hatched bars) or presence (solid bars) of liposomes. Note the decrease in phosphate release by dynamin in the presence of endophilin and amphiphysin.

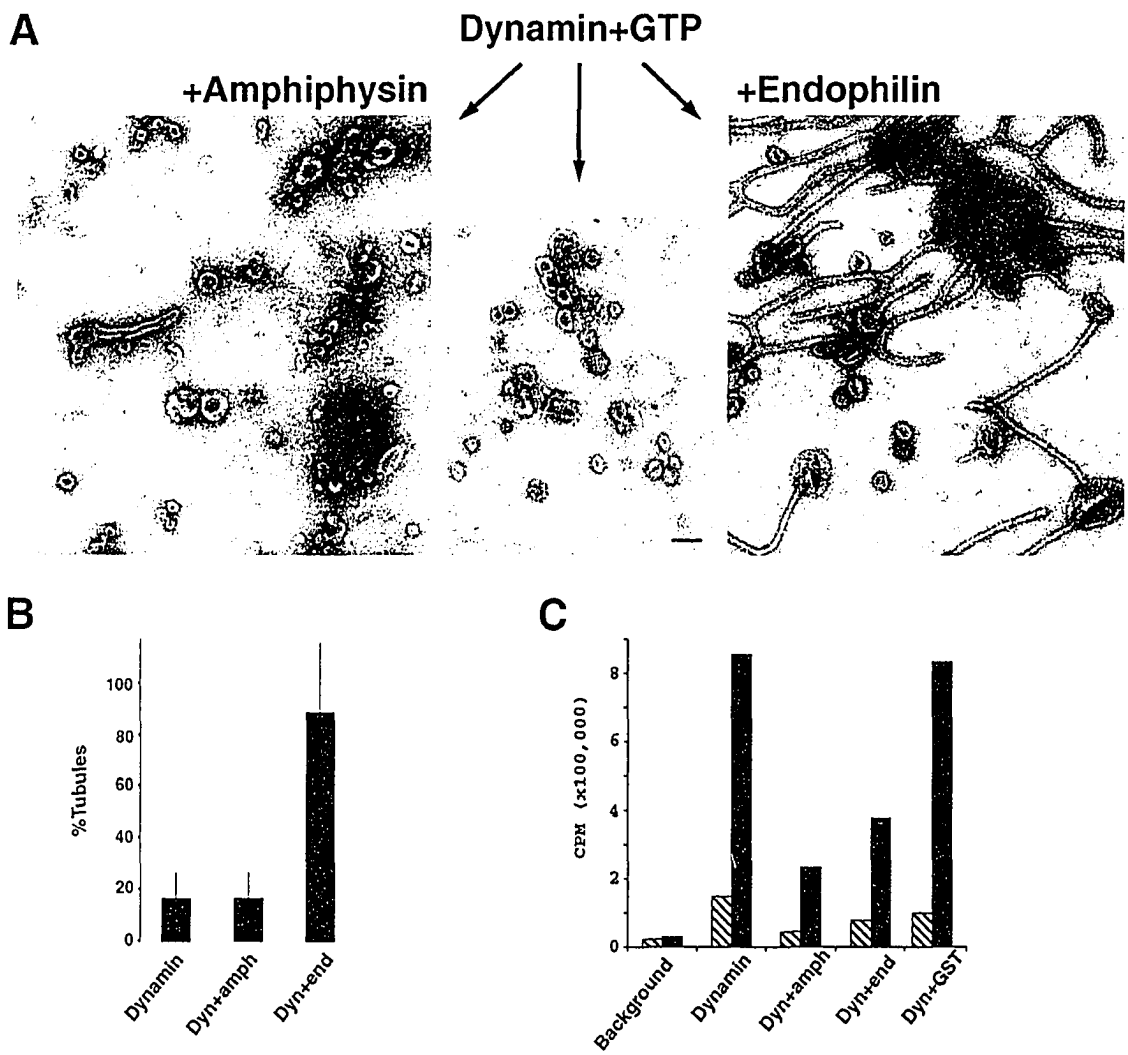
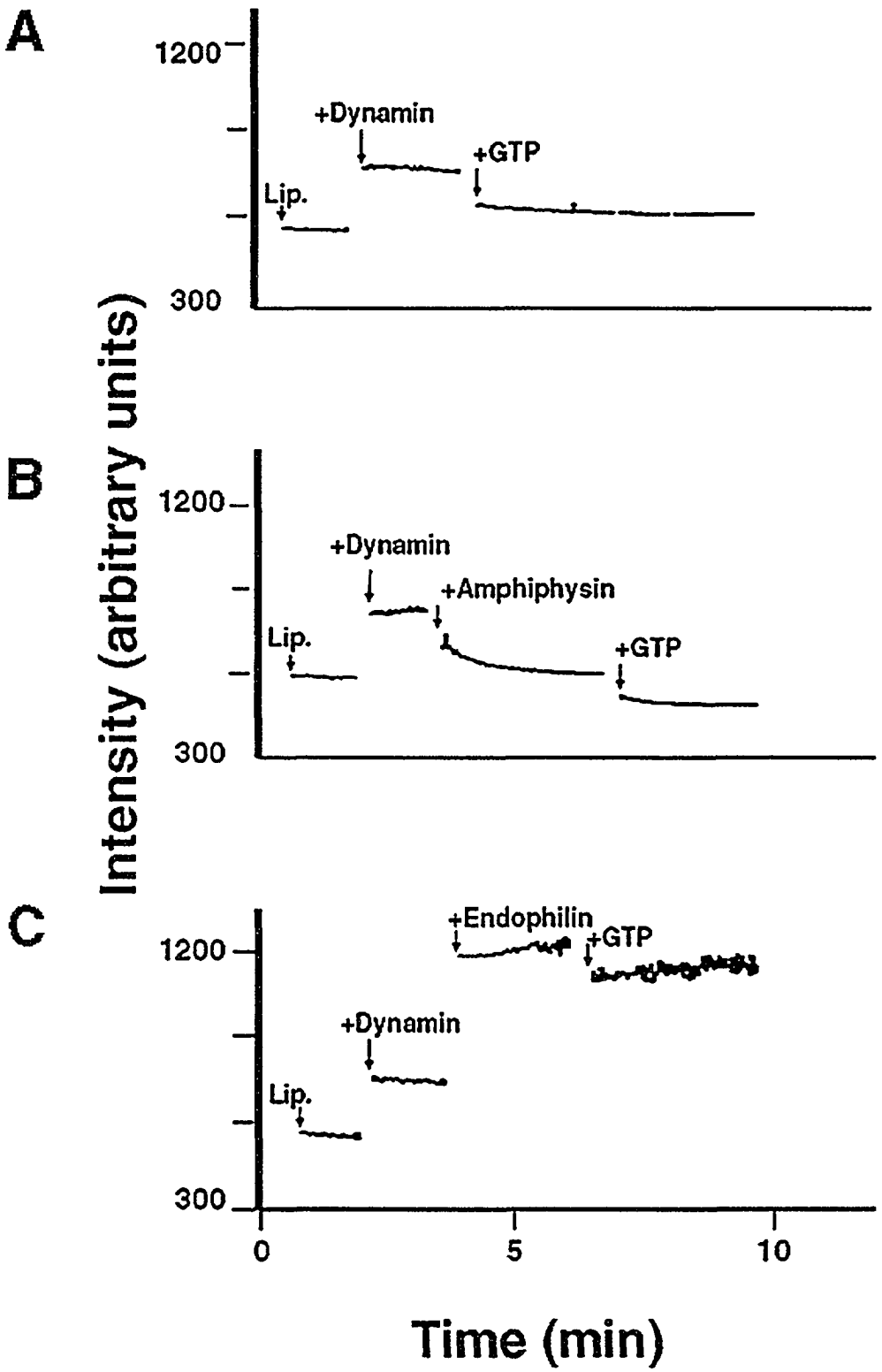


Figure 2.11: Dynamic light scattering analysis of the differential effects on dynamin vesiculation with endophilin and amphiphysin.

(A) Using a Hitachi fluorimeter, we assayed for real-time changes in light scattering based on changes in liposome morphology upon incubation with tubulating proteins. Incubation of liposomes with dynamin increased the level of scattered light relative to background. Addition of GTP, known to cause rapid vesiculation under these conditions, diminished the level of light scattering, corresponding to smaller structures. **(B)** Addition of amphiphysin to dynamin causes an immediate fall in scattered light, followed by an even further drop upon addition of GTP. **(C)** Addition of endophilin to dynamin causes a synergistic increase in the level of light scattered which persists even after the addition of GTP, consistent with the presence of long tubules observed by electron microscopy under these conditions.



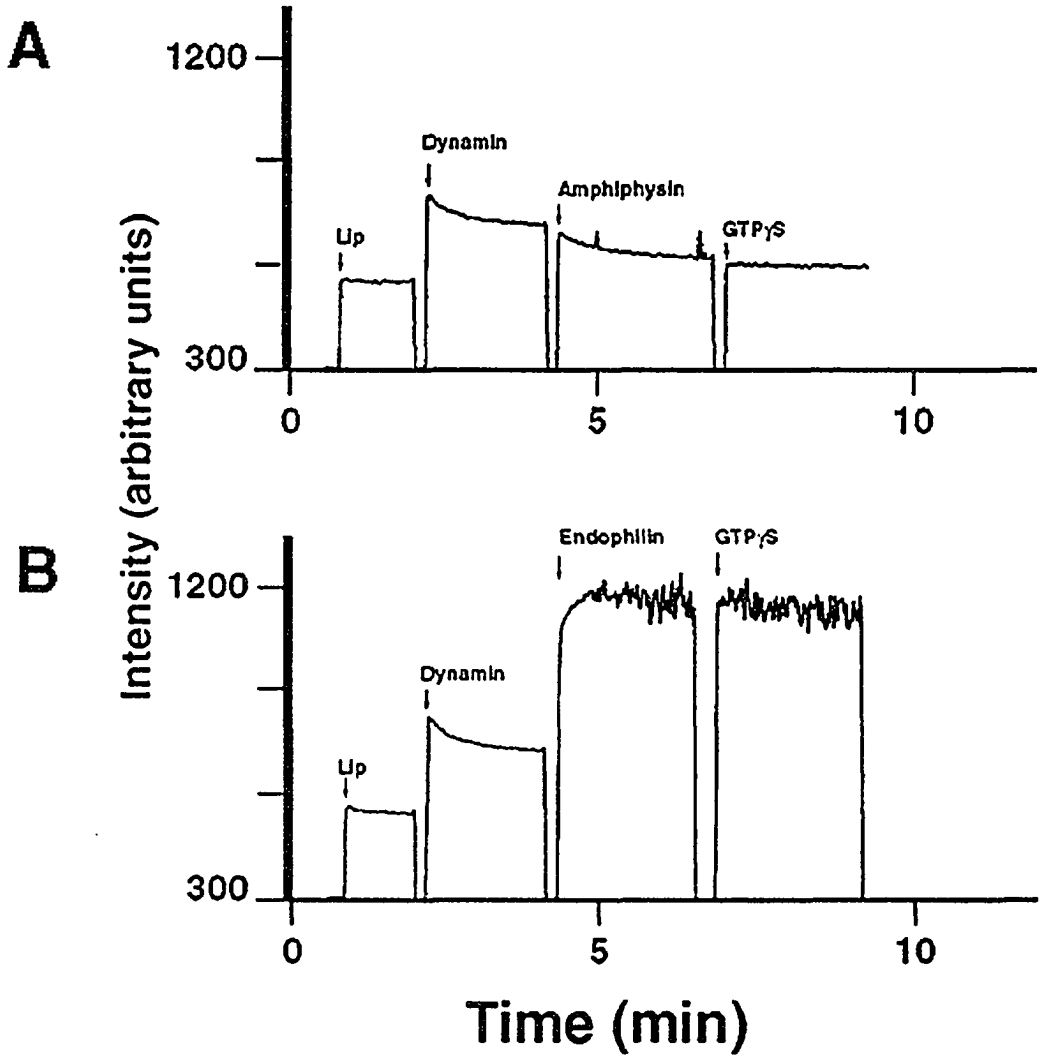
increase in light scattering compared to what was seen with dynamin alone (Figure 2.11C). This high level of light scattering persisted even after the addition of GTP (Figure 2.11C). An endophilin deletion construct defective in liposome binding and tubulation was unable to generate an increase in light scattering, indicating that the light scattering observed was a reflection of changes in liposome morphology (not shown).

For amphiphysin, addition of GTPγS maintained the level of light scattering to that which persisted after addition of amphiphysin to dynamin, consistent with the observation of tubules comprising a dynamin-amphiphysin complex under these conditions (Figure 2.12A). Not surprisingly, addition of GTPγS to the reaction including dynamin and endophilin maintained the high degree of light scattering seen without addition of nucleotides (Figure 2.12B). An aliquot of this reaction including dynamin, endophilin, and GTPγS was taken for observation under the electron microscope, and, indeed, these conditions of highly scattered light corresponded to a large degree of liposome tubulation (Figure 2.12C).

What could be the possible source of difference between these two conditions? Some clues can be found in the crystal structure of the amphiphysin 2 SH3 domain, which bears high identity with the amphiphysin 1 SH3 domain (Owen et al., 1998). When compared with other known SH3 domains, the amphiphysin SH3 domain contains two additional insertions which are highly acidic (Owen et al., 1998). These insertions appear to destabilize dynamin rings in solution, and a chimeric Grb2 (growth hormone receptor bound 2) SH3 domain onto which these loops were grafted conferred a degree of

Figure 2.12: Effect of GTPγS on dynamin light scattering with endophilin and amphiphysin.

(A) Addition of GTPγS to amphiphysin and dynamin maintains the level of light scattering seen at equilibrium after addition of amphiphysin to dynamin. This reflects the tubules seen by electron microscopy under these conditions. **(B)** Addition of GTPγS to dynamin and endophilin makes no appreciable change from that of GTP addition. **(C)** An aliquot of this reaction was analyzed by electron microscopy to confirm that the high level of light scattering corresponded with tubule formation.



C

instability to the formation of dynamin rings in solution compared with the wild-type Grb2 SH3 domain (Owen et al., 1998). When tested in the liposome tubulation assay, the isolated recombinant amphiphysin I SH3 domain completely blocked tubulation by dynamin as assayed by both electron microscopy and light scattering (Figure 2.13B). A mutant amphiphysin SH3 domain which no longer binds dynamin had no effect (Figure 2.13C). Moreover, the endophilin SH3 domain also did not inhibit tubulation by dynamin (Figure 2.13D). Furthermore, in pull-down assays using purified proteins, the amphiphysin-dynamin interaction was less robust in the presence of GTP as compared to either no nucleotides or GTPγS (Figure 2.14A), whereas for endophilin, the interaction with dynamin was not modulated by the presence of nucleotides (Figure 2.14B).

These results suggest that the presence of endophilin stabilizes the lipid tubule against the structural changes in dynamin which lead to vesiculation in the presence of GTP. Furthermore, the interaction of amphiphysin with dynamin is inherently unstable on liposomes and in solution, particularly in the presence of GTP. This may be due to unique features of the amphiphysin SH3 domain and may have an important function in the observed interactions with dynamin. Interestingly, both endophilin and amphiphysin inhibited phosphate release by dynamin-GTP in the presence and absence of liposomes (Figure 2.10C). These results may reflect unique dynamics of dynamin oligomers in co-assembly with endophilin vs. amphiphysin: one which allows for a mechano-chemical transduction productive for vesiculation, and another which hinders this. Therefore, despite important similarities, the dynamin-endophilin interaction is qualitatively different from the the dynamin-amphiphysin interaction on lipid bilayers. This difference

Figure 2.13: Effects of the isolated SH3 domains of endophilin and amphiphysin on dynamin tubule formation.

(A) Tubule generated by incubation of purified dynamin with liposomes. **(B)** Dynamin tubules fail to form when dynamin is incubated in the presence of the isolated amphiphysin SH3 domain. Inset shows the light scattering data for this reaction showing very little increase in light scattering upon addition of dynamin. **(C)** Tubules are seen when dynamin is incuabated with a mutant amphiphysin SH3 domain which no longer binds dynamin. Inset shows that the change in light scattering occurs upon addition of dynamin to this reaction. **(D)** Incubation of dynamin with the isolated SH3 domain of endophilin has no negative effect on dynamin tubule formation. Inset shows the light scattering data for this reaction showing the typical increase in light scattering observed upon incubation of dynamin with liposomes.

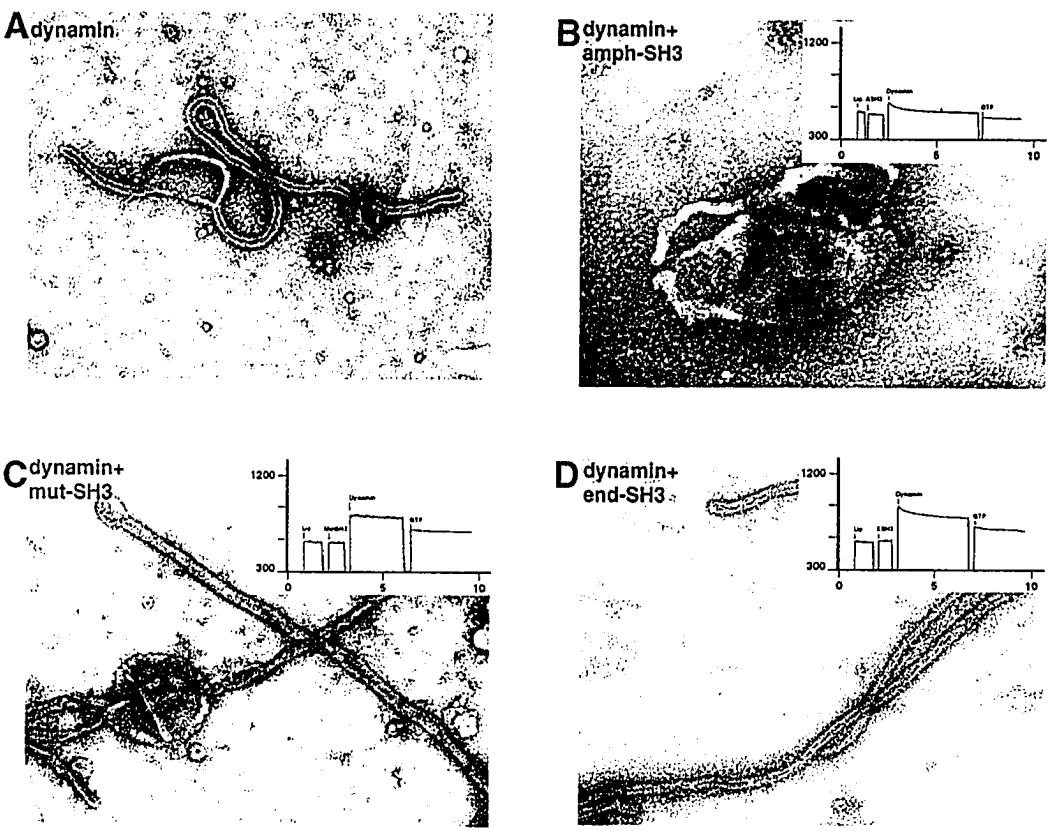
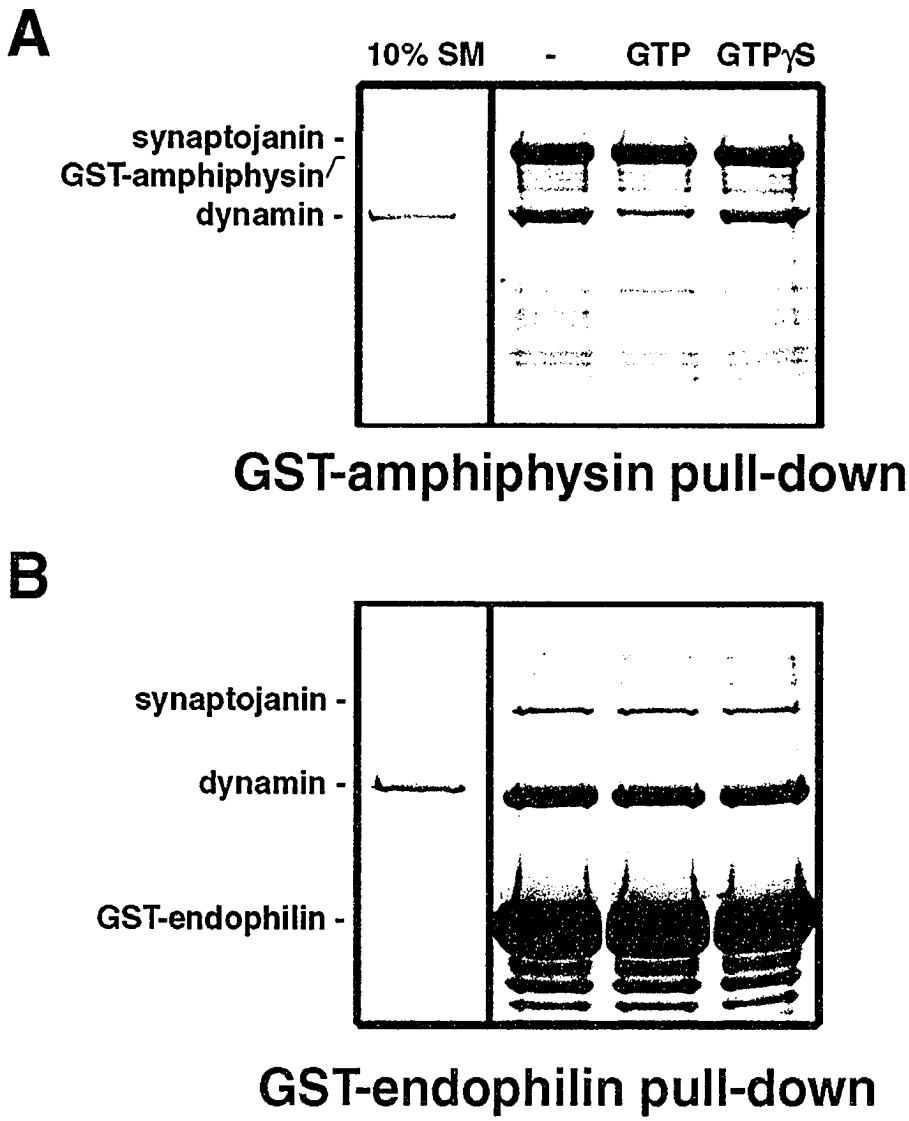


Figure 2.14: Effects of GTP on dynamin binding for endophilin and amphiphysin. **(A)** Solution binding of dynamin to amphiphysin in affinity chromatography diminishes in the presence of GTP, likely reflecting diminished amounts of oligomerized dynamin under these conditions. **(B)** No change in dynamin binding by endophilin is seen regardless of the presence of GTP, indicative of the relative preservation of dynamin oligomers seen under these conditions.



may have significant ramifications for the *in vivo* dynamics of these complexes at the neck of clathrin-coated pits.

Discussion: Tubular Dynamics in Synaptic Vesicle Recycling

Collectively, our results provide evidence that endophilin directly binds and deforms lipid bilayers into narrow tubules within the size range of the tubular neck of clathrin-coated pits. This activity of endophilin is a protein mediated phenomenon, which likely occurs by oligomerization at the surface of the bilayer. Endophilin tubulates membranes at 4°C, and also tubulates synthetic bilayers devoid of the putative substrates of its reported LPA-AT activity, showing that formation of high curvature membranes by endophilin can be independent of lipid-modifying enzymatic activity. We hypothesize that the tubulating activity of endophilin reflects one possible function for this protein in clathrin-mediated endocytosis. Along with dynamin and amphiphysin, endophilin may be part of a protein complex governing aspects of membrane deformation and fission.

Our findings provide a rationale for the observed requirement for endophilin in the tubulation of synaptic membranes by brain cytosol in the presence of GTPγS (Ringstad et al., 1999). These observations may also partially explain the arrest of clathrin-mediated synaptic vesicle retrieval at shallow stages of invagination upon perturbation of endophilin function in lamprey (Ringstad et al., 1999). Endophilin membrane binding and protein-protein interactions may serve to localize and functionally orient the protein at

the clathrin-coated pit. Importantly, the selective recruitment of synaptojanin to lipid bilayers by endophilin may have a function in the spatial localization of this lipid phosphatase at the clathrin-coated vesicle, and provides a suggestion for the observed phenotype of increased clathrin-coated vesicles upon perturbation of endophilin function at the lamprey synapse (Gad et al., 2000). Amphiphysin and endophilin tubulate lipid bilayers both independently and as a complex with dynamin; however, their distinct effects on dynamin *in vitro* suggest a potential differential modulatory role on dynamin function *in vivo* which needs to be explored further.

Acknowledgments

Much of the material in this chapter has been published in the following reference: (Farsad et al., 2001)

Chapter 3

An Endophilin-like Protein Localizes to the Golgi Complex and Also Tubulates Lipid Bilayers: Implications for Membrane Traffic

Abstract

Tubular membranous structures have been increasingly implicated in vesicular trafficking events. We have shown that dynamin, endophilin, and amphiphysin are three proteins involved in synaptic vesicle recycling which display independent and cooperative abilities to deform spherical/planar membranes into long membranous tubules decorated by a protein coat (Farsad et al., 2001; Takei et al., 1998; Takei et al., 1999). For endophilin and amphiphysin, specifically, we have found a conserved putative amphipathic amino acid stretch at the NH2-terminal region of the proteins which is necessary for the lipid binding and liposome tubulation activity shared by these proteins (Farsad et al., 2001). We were interested whether other proteins in the database could be found with similarity to the endophilin protein in an effort to identify more proteins capable of this membrane deformation. A BLAST search found a recently discovered protein family, the endophilin B family (in contrast to the above mentioned endophilin A family), which shares strong homology to the endophilin NH2-terminus, including the

putative amphipathic amino acid region implicated for endophilin liposome tubulation. Endophilin B is expressed in many tissues, with several putative isoforms present in brain. Immunocytochemistry reveals both a synaptic staining pattern, as well as internal membranous staining which partially co-localizes with the Golgi compartment. Electron microscopy of purified Golgi membranes shows extensive localization of endophilin B along the Golgi stacks and vesicles. The purified, recombinant endophilin B protein binds and tubulates liposomes in a manner similar to endophilin A. For endophilin B, although deletion of the NH2-terminal putative amphipathic amino acid region blocks liposome tubulation, binding to liposomes is preserved, suggesting an alternate lipid binding site present in the protein. Implications for a membrane deforming domain involved in diverse membrane trafficking events are discussed.

Introduction

Membrane trafficking is characterized by the selective transport of cargo along membrane bound intracellular carriers. Membrane traffic is part of the compartmentalization of the cell which allowed for the evolution of eukaryotic biology, with its associated specialization of diverse cellular functions within distinct intracellular organelles (Shorter and Warren, 2002). Pleiomorphic tubulo-vesicular intracellular membranes have been noted in virtually every process involving membrane defined compartments and events: the plasma membrane (Henley et al., 1999; Schnitzer et al., 1996); axonal and dendritic process outgrowth (Martinez-Arca et al., 2001); fast axonal transport (Nakata et al., 1998); endosomes and lysosomes (Gruenberg and Maxfield, 1995; Lippincott-Schwartz et al., 1991; Prekeris et al., 1999); the endoplasmic reticulum (ER) (Bannykh et al., 1996; Dabora and Sheetz, 1988; Klumperman, 2000; McIlvain et al., 1993); cytokinesis (Verma, 2001); and the dynamics of the Golgi apparatus (Lippincott-Schwartz et al., 2000; Lippincott-Schwartz et al., 1991; Shorter and Warren, 2002).

The simplest of intracellular organelles is the vesicle, used by the cell primarily as a structure for transport and storage of various macromolecules involved in homeostasis as well as regulated cell function. Synaptic vesicles are some of the best characterized of vesicular organelles by virtue of their ability to be purified to high biochemical homogeneity (Hannah et al., 1999; Huttner et al., 1983). Studies of how synaptic vesicles

are formed and recycle from the plasma membrane has revealed that, in addition to vesicular based membrane traffic, tubular membrane invaginations and evaginations also are involved in the dynamics of these and other membrane-bound structures. The large GTPase, dynamin 1, and its two major synaptic binding partners, endophilin 1, and amphiphysin 1, have all been shown to be involved in the tubular invagination of the clathrin-coated bud during the process of synaptic vesicle retrieval (De Camilli et al., 2001b). Perturbing the function of any of these proteins through microinjection studies in the living giant reticulospinal synapse of the lamprey results in depletion of the presynaptic vesicle cluster due to a potent inhibition of clathrin-mediated endocytosis (Gad et al., 2000; Ringstad et al., 1999; Shupliakov et al., 1997).

Endophilin 1 (hereafter called endophilin A), a 40 kilo-Dalton (kDa) presynaptically enriched protein that binds the GTPase dynamin and the polyphosphoinositide phosphatase synptojanin, has been implicated in many stages of clathrin-mediated synaptic vesicle retrieval (Ringstad et al., 1999) (Gad et al., 2000). Depletion of endophilin 1 from brain cytosol has been shown to inhibit tubular synaptic membrane formation by dynamin in the presence of GTPγS (Ringstad et al., 1999). Moreover, purified recombinant endophilin A has been shown to rapidly deform artificial liposomes into long tubular structures of various diameter through a process of lipid stimulated oligomerization and mechanochemical membrane deformation (Farsad et al., 2001). This membrane deforming activity of endophilin A may be suggestive of its putative role in the process of synaptic vesicle retrieval.

The basic domain structure of endophilin A includes a highly conserved NH2-terminal domain consisting primarily of alpha helices and coiled coils by secondary structure prediction (Farsad et al., 2001), and a COOH-terminal SH3 domain which interacts primarily with dynamin, synaptojanin, and amphiphysin (Micheva et al., 1997; Ringstad et al., 1997). The lipid binding and membrane tubulation activity of endophilin A has been mapped to a putative amphipathic region within the conserved NH2-terminus of the protein (Farsad et al., 2001). In an effort to explore whether this membrane deformation function of endophilin A is conserved in proteins functioning in other compartments within the cell, we searched the database for proteins with sequence homology to the NH2-terminus of endophilin A (Farsad et al., 2001).

Materials and Methods

Antibodies

Rabbit polyclonal antibodies were raised against the COOH-terminal portion of the endophilin B protein including the SH3 domain. The antibodies were affinity purified to 1 mg/mL concentration. Affinity purified antibodies were tested by Western blotting, immunofluorescence, and electron microscopy. The Golgi-complex specific antibodies to giantin and TGN-38 were kindly provided by the laboratory of Graham Warren.

Cell Culture and Transfection

Chinese Hamster Ovary fibroblasts (CHO) and Normal Rat Kidney fibroblasts (NRK) were grown according to standard protocols in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, glutamine, and penecillin/streptomycin. For transient transfection studies, cells were grown on flamed 22 millimeter square coverslips in six-well plates containing 3 milliliters of medium. Constructs were subcloned into pCDNA 3.1 with an HA-tag or in pEGFPC1 vectors, and 5 μg of plasmid DNA was transfected with Lipofectamine 2000 reagent for 16 hours. Cells were rinsed in phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde in 120 mM sodium phosphate buffer warmed to 37 °C for 30 minutes. Cells were rinsed in PBS and blocked in goat serum dilution buffer. Immunocytochemistry was performed with polyclonal antibodies to endophilin B, a monoclonal antibody against the HA-tag, and antibodies to the Golgi-complex (anti-giantin, polyclonal antibody; anti-GM130, monoclonal antibody) using standard protocols. Primary antibodies were visualized with Texas Red and Oregon Green conjugated secondary antibodies.

Liposomes

Liposomes composed of brain lipid extract (type 1, Folch fraction 1, Sigma) were made as described (Takei et al., 1999). Briefly, a lipid mixture solubilized in chloroform was added to a 2:1 chloroform:methanol mixture and thoroughly mixed in a glass tube. Lipids were adhered along the sides of the glass tube under a stream of nitrogen gas by gently rotating the tube until the chloroform:methanol solvent had evaporated. This procedure generates layers of lipids dried as multiple bilayers along the glass surface. 300 mM

filtered sucrose was then gently added to the tube with the dried lipid bilayers, and the tube was allowed to incubate, covered with parafilm, at 37 °C for 15-60 minutes. During this incubation, the dried bilayers gradually begin to peel off of the glass surface and swell. Following the incubation, vigorous vortexing shears the bilayers into vesicles of heterogeneous size. For liposomes of specific size, this mixture was passed through a filter with the requisite pore size. Liposomes loaded with sucrose could thus be spun down due to the increased density of sucrose compared with the incubation buffer of the subsequent reactions. Synthetic liposomes were made in a similar fashion, using HPLC purified DOPC (40%), DOPS (40%), and DOPE (20%) from Avanti Polar Lipids.

Purified Proteins

Dynamin was affinity purified from rat brain cytosol using amphiphysin 1-SH3 domain as described (Owen et al., 1998).

Recombinant human endophilin B1 was cloned in pGEX (Pharmacia), and purified as Glutathione-S Transferase (GST) fusion proteins according to standard methods (Pharmacia). The GST-tag was subsequently cleaved by PreScission Protease (Pharmacia).

Endophilin B1 deletion constructs were prepared by PCR to yield the following fragments: amino acids 46-365, the isolated SH3 domain, the NH2-terminal portion of the protein without the SH3 domain. The constructs were subcloned and purified as above.

Electron Microscopy

Negative Staining: Liposomes (0.1 mg/ml final) were incubated at 37 °C for 10-20 minutes in buffer A (25 mM Hepes-KOH, pH 7.4, 25 mM KCl, 2.5mM Mg^{2+} acetate, 150 mM K-glutamate) with purified, recombinant endophilin B1 with the GST-tag cleaved off (Takei et al., 1999). At the end of the incubation, aliquots were adsorbed onto 200-400 μM formvar- and carbon-coated copper EM grids for 3-5 minutes at room temperature, washed in 0.1M Hepes pH 7.4, stained in 1-2% uranyl acetate, blotted and allowed to air dry. For incubations at 4 °C, all of the above was performed on ice.

EPON embedding: CHO cells were grown to 60-80% confluence in 10cm tissue culture dishes and transiently transfected overnight with pEGFPC1 vector containing either no insert, full-length endophilin B1, or an endophilin B1 construct lacking the NH2-terminal region responsible for tubulation. Transfection efficiency was observed at 80% based on inspection under a fluorescence microscope prior to fixation. Cells were rinsed with 37 °C PBS, and fixed in the dish with 10 mL 4% glutaraldehyde warmed to 37 °C for one hour at room temperature. After 15 minutes of fixation, the glutaraldehyde was removed and 1 mL of fresh 4% glutaraldehyde was added. The cells were gently scraped in this fixative solution, and were then spun for 10 minutes at 14,000 rpm in a table top centrifuge.

Cryo-Sections: Incubations with purified Golgi membranes were spun down and infiltrated for 48 hours in 2.3 M sucrose after fixation. Pellets were frozen and sectioned

according to standard methods, followed by post-section immunostaining using affinity purified rabbit polyclonal antibodies to endophilin B1.

In Vitro Budding Assay

An in vitro Golgi budding assay was performed in an incubation using purified rat liver Golgi membranes, purified, recombinant, lipidated ARF 1, and purified coatomer proteins. Briefly, 1 mg/mL Golgi membranes, 20 μg coatomer, and 4 μg ARF 1 were incubated with 1.5 mM GTP and an ATP regenerating system for two minutes at 37 °C in assay buffer (25 mM Hepes, pH 7.3, 70mM KCl, 2 mM Magnesium-Acetate, 5% sucrose, protease inhibitor tablets). A 200 μl pipette tip was filled with 10% porcine gelatin in 20mM Hepes buffer, the tip sealed with melted plastic from another pipette tip, and overlayed with fixative (8% paraformaldehyde in 250 mM Hepes, pH 7.3, 200 mM sucrose) and 12% sucrose in 25 mM Hepes, 100 mM KCl. The membranes were layered on top of this fixative/sucrose mixture and recovered by spinning in a Beckman TLS55 rotor, with adaptor tubes made to fit the pipette tips using an epon mold, at 12,000 rpm (6000 X g) for 10 minutes. The supernatant was removed and replaced with fixative without sucrose, and the pellets were left at 4 °C for two days. The pellets were then infiltrated with 2.3 M sucrose in 25 mM Hepes buffer and prepared for cryo-sectioning and electron microscopic analysis.

Liposome Binding

Liposome sedimentation was performed using 100 μg sucrose-loaded liposomes incubated with 5-10 μg protein in 400-500 μl buffer A or in Hepes-KCl pH 7.4 (to determine salt

sensitivity) for 10-20 minutes at 37 °C. Liposomes were sedimented at 100,000g in a Beckman TLA 100.3 rotor for 20 minutes, the supernatant was thoroughly removed, and sedimented liposomes were solubilized in 2% SDS. In some cases, proteins in the pellet and supernatant were concentrated by chloroform precipitation and methanol extraction. Samples were subjected to SDS-PAGE and analyzed by either Coomassie staining.

Results

A BLAST search of the Swissprot database was performed for full-length endophilin 1. The search revealed a family of endophilin-like proteins which share a common domain structure with the endophilin family, including a conserved NH2-terminal domain comprising of alpha helices and coils, as well as a COOH-terminal SH3 domain (Farsad et al., 2001; Huttner and Schmidt, 2000; Modregger et al., 2003)(GenBank accession number AF263364; GenBank accession number AF263293) (Figure 3.1A and B). These endophilin-like proteins have been recently named endophilin B family of proteins (Huttner and Schmidt, 2000). The endophilins will be called the endophilin A family hereafter. Phylogenetic analysis of the endophilin B genes reveals that, although displaying broad homology to the endophilin A genes, they indeed cluster as a separate gene group (Figure 3.1C). The cDNA for endophilin B was cloned into vector pGEX 6P1, and antibodies were raised to the recombinant endophilin B1 protein. By Western blot analysis, this antibody recognized a 41 kDa protein mainly in heart, brain, spleen and lung, with lower expression in liver and testis (Figure 3.1D). Additional higher molecular weight bands were also detected specifically in brain, possibly

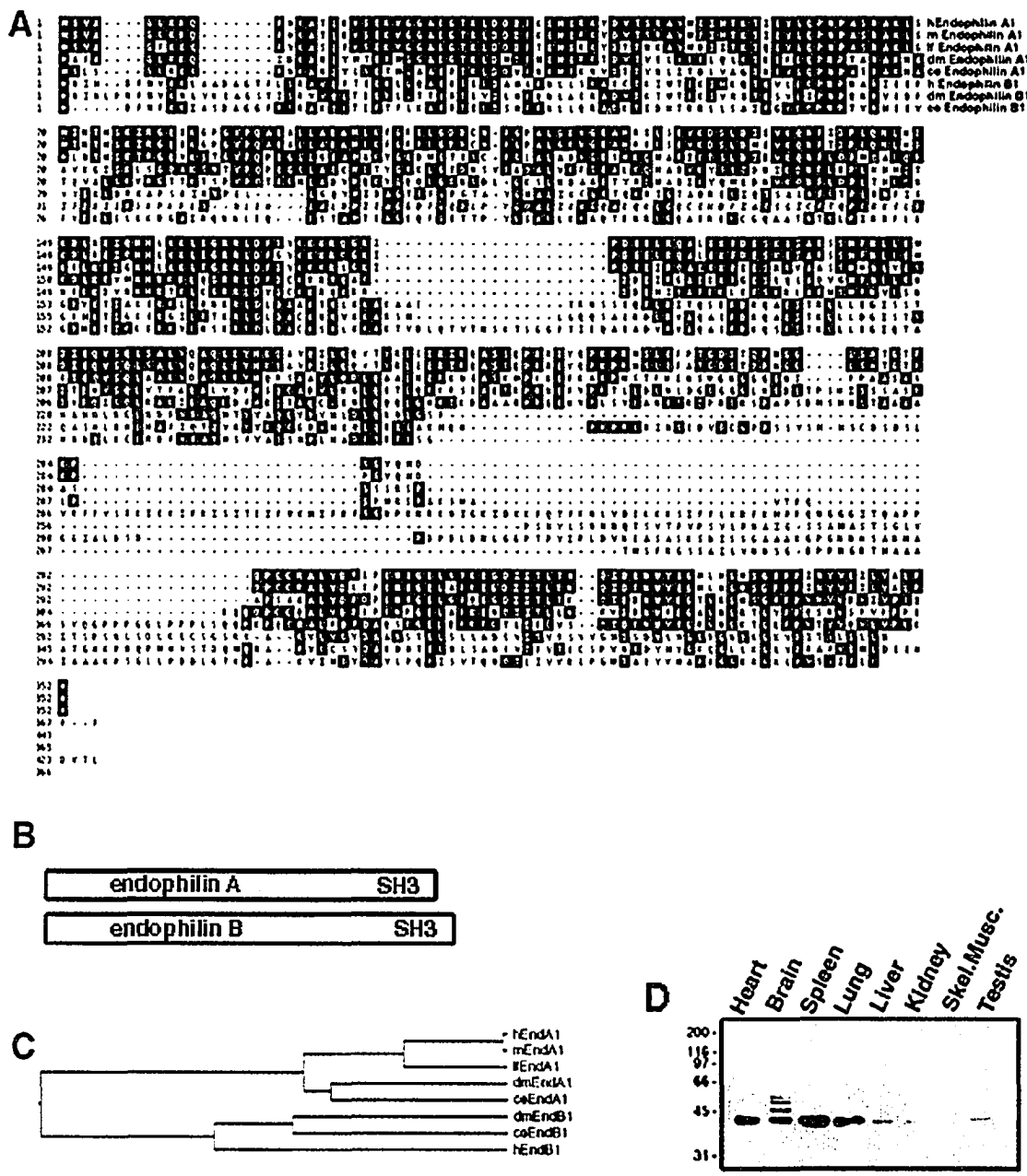
representing other splice isoforms or post-translational modifications (GenBank accession number AF263364) (Figure 3.1D).

By immunofluoresence of rat brainstem frozen sections, endophilin B was detected in a synaptic-like pattern along the neuro-somal periphery, as well as on intracellular membranes (Figure 3.2A)(Farsad et al., 2001). The synaptic-like staining colocalized with syaptic markers such as amphiphysin (which shows additionally diffuse cytosolic staining) as shown (Figure 3.2A). Further analysis of the intraneuronal staining pattern of endophilin B showed partial co-localization with the Golgi-complex marker, GM-130 (Figure 3.2B). In CHO cells, endogenous endophilin B localized to a perinuclear membrane compartment strongly positive for GM-130, in addition to a more diffuse reticular staining pattern throughout the cell (Figure 3.2C). Endophilin B staining was also seen to partially co-localize with the Golgi-specific marker, giantin, as well (not shown). Moreover, there was also partial co-localization with a trans-Golgi network specific marker, TGN-38, indicating the presence of endophilin B throughout both the cis- and trans-Golgi compartments (Figure 3.2D). Brefeldin A caused dispersion of the perinuclear staining by endophilin B, consistent with localization to the Golgi complex (data not shown). There was no co-localization to endosomal compartments labeled with transferrin (data not shown).

In electron microscopic analyses of cryosections of purified rat liver Golgi complex, endophilin B immunogold staining was seen throughout the Golgi stacks (Figure 3.3A). In particular, some vesicular structures were densely stained as well

Figure 3.1: The endophilin B protein family is homologous to endophilin A.

(A) Cross-species sequence alignment (Clustal method) of the endophilin A1 and endophilin B1 class of proteins showing broad homology along the NH2-terminal regions as well as along the COOH-terminal SH3 domains. h = human, m = mouse, lf = lamprey (L. fluviatilis), dm = D. melanogaster, ce = C. elegans. **(B)** Cartoon schematic for endophilin A and endophilin B illustrating the homology observed with the sequence alignments. **(C)** Phylogenetic tree of the alignment above showing that although the two protein families are very similar, the endophilin B family clusters as a separate grouping across species. **(D)** Multiple tissue Western blot using a polyclonal rabbit antibody raised against full-length endophilin B1 shows expression primarily in heart, brain, spleen, and lung, with lower expression evident in liver and testis. Note the presence of brain specific higher molecular weight bands.



(Figure 3.3A, upper and lower right insets). By contrast, a control antibody raised against endophilin A1 showed no specific Golgi-complex staining (Figure 3.3B).

To observe the localization of endophilin B during active Golgi dynamics, an in vitro Golgi budding assay, using only purified rat liver Golgi, ARF, coatomer, and an ATP regenerating system was used. This assay is able to cause virtually complete Golgi-complex fragmentation into vesicles if taken to completion. The in vitro budding reaction was carried out for only five minutes in order to preserve and observe budding intermediates as they are formed from the Golgi stacks. After a five minute incubation at 37 °C, endophilin B staining could be seen throughout both stacks and vesicles, with an apparent concentration of stain around the periphery of the stacks, around vesicular structures (Figure 3.4). This pattern of staining around the stack periphery was also noted in the absence of obvious vesicle formation (Figure 3.4). Immunogold staining was often present around clusters of small vesicular structures as well (Figure 3.4).

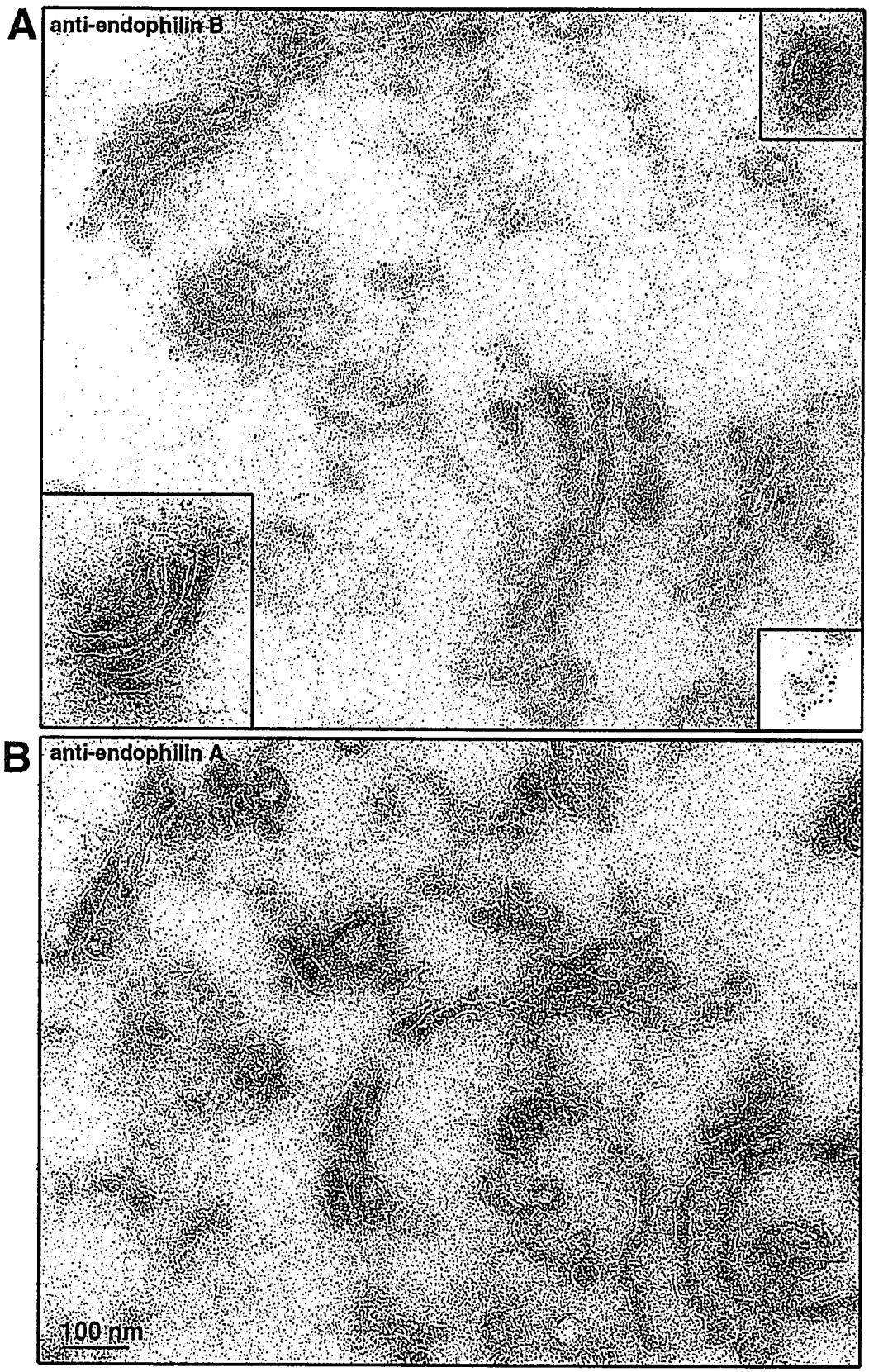
In order to study the in vitro dynamics of endophilin B with isolated lipid bilayers, the recombinant endophilin B protein was expressed in bacteria. The amphipathic region of homology between endophilin A1 and amphiphysin is also conserved in endophilin B1, therefore, we tested the purified recombinant endophilin B1 constructs for its ability to bind and tubulate liposomes (Figure 3.5A). Incubation of the purified recombinant human endophilin B1 protein (GST-tag cleaved) with liposomes generated bilayer tubules in a manner morphologically indistinguishable from endophilin A1 and amphiphysin (Figure 3.5B). Deletion of the homologous amphipathic region in endophilin B1 also blocks

Figure 3.2: Immunocytochemical localization of endophilin B

(A) Immunofluoresence of a rat brainstem frozen section using antibodies directed against recombinant endophilin B1 (upper panel) and amphiphysin 1 (lower panel). Note the localization of endophilin B in a synaptic-like pattern at the cell periphery similar to the localization of amphiphysin. In addition to this synaptic staining, endophilin B localizes to intracellular particles, while amphiphysin shows a diffuse cytosolic staining pattern. **(B)** Endophilin B (upper panel) partially co-localizes with a Golgi complex-specific marker, GM-130 (lower panel) in a large brainstem neuron. **(C)** Endophilin B1 (upper panel) partially co-localizes with a Golgi-specific marker, GM-130 (lower panel) in cultured CHO cells. **(D)** NRK cells were fixed and prepared for immunocytochemistry. Endophilin B, on the left, partially co-localizes with TGN-38, a marker for the trans-Golgi network, seen on the right. Bar, 10 μm.

Figure 3.3: Endophilin B localizes to Golgi stacks and vesicles by electron microscopy.

(A) Affinity purified rabbit polyclonal antibodies to endophilin B were used to localize the protein in preparations of isolated Golgi membranes from rat liver. Immunogold staining was seen throughout the Golgi stacks, with some heavy staining seen around vesicles found in the preparation (see insets on upper and lower right). **(B)** The same rat liver Golgi membrane preparation does not have any reactivity to affinity purified rabbit polyclonal antibodies raised against the similar endophilin A protein.



tubulation, consistent with the importance of this motif for liposome tubulation as seen for both endophilin A1 and amphiphysin as well (not shown). Interestingly, although deletion of this region involved in tubulation for both endophilin and amphiphysin also blocks binding to the liposomes, in endophilin B1, this deletion, despite blocking membrane tubulation, does not prevent membrane association (Figure 3.5C). The endophilin B deletion mutant lacking the tubulation motif still co-sedimented with liposomes, indicating the presence of an alternate lipid binding site downstream of the region necessary for liposome tubulation. This alternate lipid binding site may help localize endophilin B to a specific membrane subcompartment, or to the Golgi-complex itself.

Discussion: A Lipid-Interacting/Deforming Domain for Tubulo-vesicular Dynamics in Membrane Trafficking

Endophilin B1, a member of a newly discovered family of proteins which we find localizes to both synaptic and Golgi/intracellular membrane compartments, displays a similar membrane tubulating activity as that of endophilin A1 and amphiphysin. All three proteins share a short NH2-terminal amphipathic region which we show to be required for membrane tubulation. This may have implications for a shared function for this motif in tubulo-vesicular dynamics in cellular trafficking events.

Figure 3.4: Localization of endophilin B in an in vitro Golgi budding assay

Isolated rat liver Golgi membranes were used for an in vitro budding assay using purified coatomer proteins, recombinant ARF, and an ATP regenerating system. The incubation was performed for five minutes at 37 °C before being spun down and prepared for cryosectioning. Cryosections of the reaction were then incubated with endophilin B antibodies according to standard protocols. Endophilin B staining is seen throughout the Golgi, with an apparent concentration of stain around the periphery of the stacks and around vesicular clusters. Panels represent examples of similar reaction conditions.

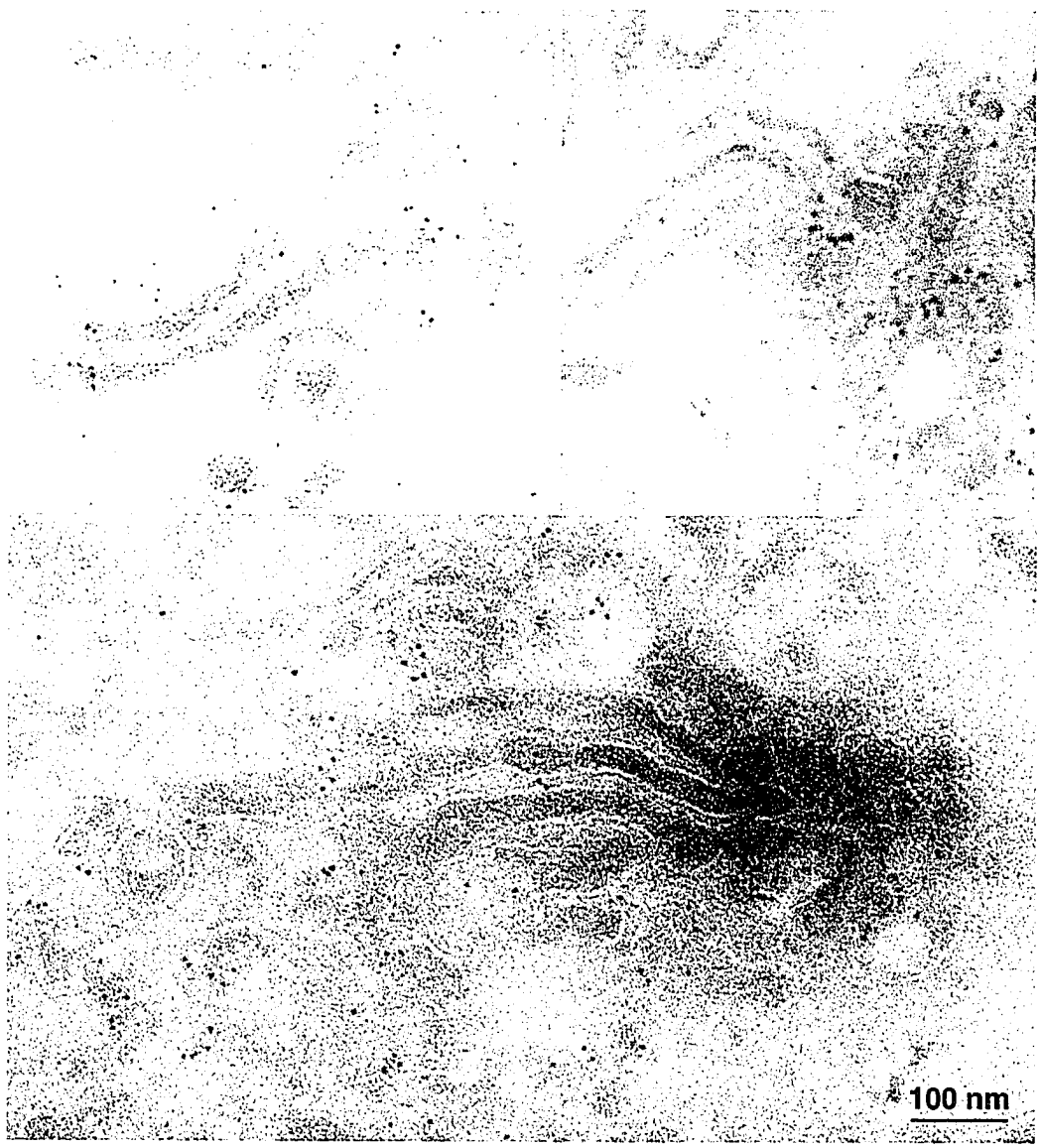
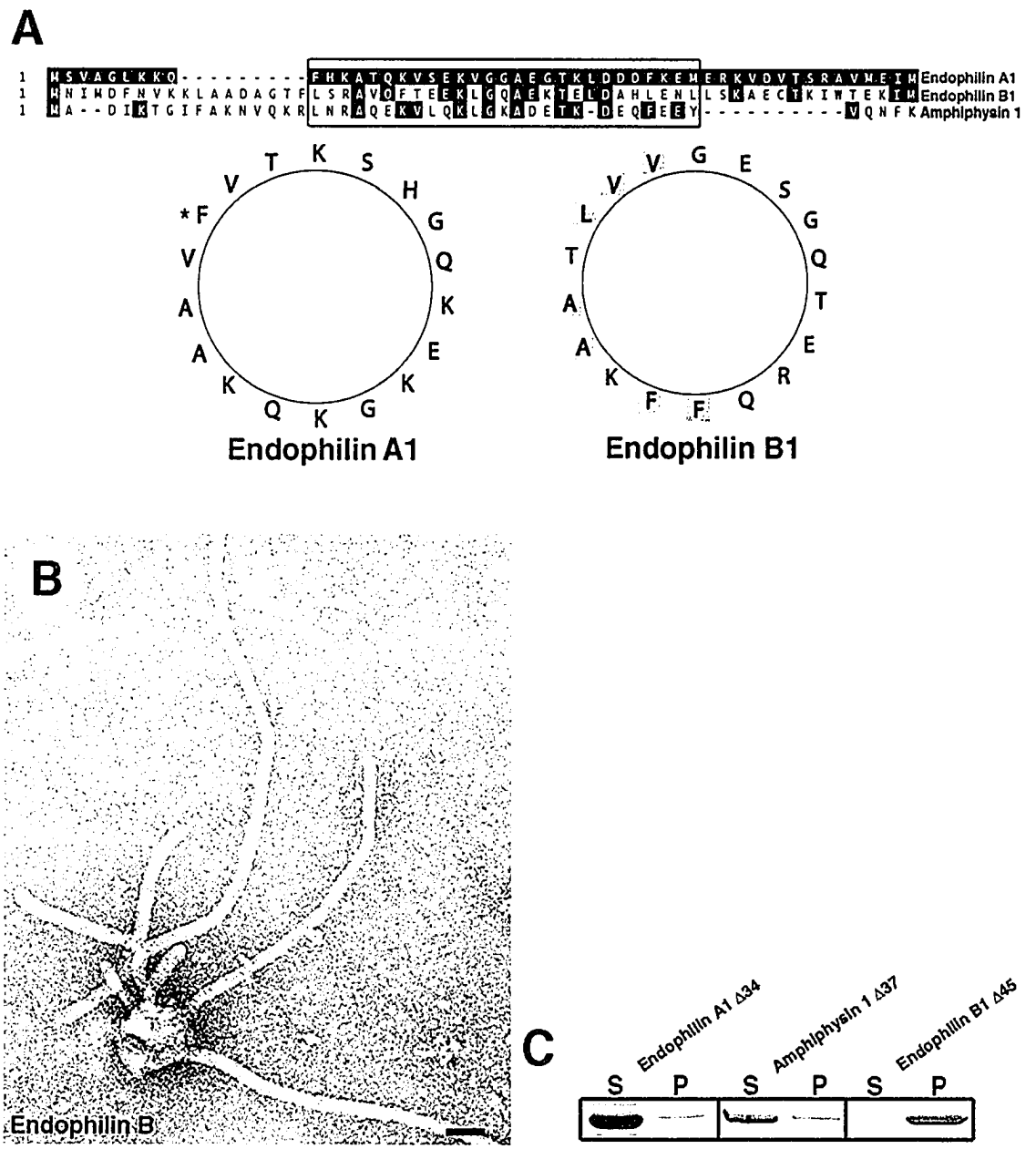


Figure 3.5: Endophilin B tubulates liposomes.

(A) Alignment of the NH2-terminal regions of endophilin A1, endophilin B1, and amphiphysin 1 shows homology for all three proteins along the putative amphipathic coil region identified previously for endophilin A1 and amphiphysin 1. Helical wheel alignment of this homologous region of endophilin B shows an amphipathic distribution of the amino acid residues if they were to fall along a helical axis. **(B)** Purified recombinant endophilin B1 deforms liposomes into tubules similar to those seen with endophilin and amphiphysin. Bar, 100nm. **(C)** Deletion of the region of homology between endophilin A1, endophilin B1, and amphiphysin 1 abolishes tubulation for all three proteins (not shown). While this deletion also blocks the lipid binding properties of endophilin A1 and amphiphysin 1, endophilin B1 is still able to associate with liposomes, suggesting the presence of an additional lipid binding site(s).



Endophilin B is seen to localize both to synaptic regions in brain stem sections, as well as to intracellular membranes positive for Golgi complex markers. By western blot, the endophilin B antibodies recognize several bands in the brain, possibly indicating the presence of different splice isoforms. It would be intriguing if the different splice isoforms differentially localized within the cell. This may explain the peripheral and intracellular membrane staining observed in neurons, while mainly intracellular membrane staining is seen in fibroblasts. A neuron-specific isoform of endophilin B may be involved in dynamics at the plasma membrane, in addition to other isoforms active at the Golgi complex.

By immunofluorescence, endophilin B partially co-localizes with both cis- and trans-Golgi markers, indicating a diffuse presence throughout the Golgi complex. Ultrastructural immuno-gold electron microscopy reveals relatively dense staining throughout the Golgi stacks as well as on some vesicles. In an in vitro Golgi budding assay, there was some evidence that staining appeared to concentrate along the periphery of the Golgi stacks, where vesicles were also seen in relatively higher proportions by inspection. More rigorous quantitative experimentation will need to be performed to determine whether this differential distribution is significant.

The possibility exists that endophilin B may be involved in vesicle budding and tubulation at the Golgi complex, in an analogous fashion to the known role of endophilin A1 at the plasma membrane. The fact that endophilin B shares the same membrane

bilayer tubulation dynamics as other proteins involved in vesicular budding is further suggestive of a possible role for this protein in Golgi complex vesicle dynamics. In contrast to endophilin A1 and amphiphysin, deletion of the membrane tubulation region in endophilin B does not abrogate its association with lipid bilayers. This suggests that there is an alternate lipid binding domain within endophilin B, likely within the stretch between the tubulation domain and the COOH-terminal SH3-domain, and further experiments should help to map this additional membrane binding site. For example, muscle amphiphysin 2, which localizes to the PIP2-rich T-tubule system, is an example of a tubulating protein which has a second lipid binding motif, in addition to its tubulation domain, that selectively recruits and localizes this protein to PIP2-rich regions of the membrane (Lee et al., 2002). Elucidation of the biochemical properties of this internal membrane binding site may help to determine whether there are particular lipid affinities which may aid in the localization or partitioning of endophilin B to the Golgi complex. Furthermore, understanding what role endophilin B may play in the Golgi complex will be facilitated by experiments which perturb the function of endophilin B, such as antibody microinjections or overexpression experiments. In addition, finding putative binding partners for endophilin B may also help delineate the pathways in which this protein participates.

Conceptually, it is conceivable how tubular membrane deformation would be an appropriate intermediate in the constriction and ultimate scission of a nascent bud from a donor membrane. Thus, along with membrane coat proteins such as clathrin and the COPs, other proteins primarily active at the tubular neck of the buds generated by these

coat proteins would need to be present and conserved. In addition, tubular membrane carriers may independently be a significant part of membrane traffic. Dynamin has been shown to be intimately involved in the vesiculation process in various cellular endocytic events (Hinshaw, 2000; McNiven et al., 2000). Indeed, dynamin-dependent endocytosis has developed into its own class of endocytic process. The ability of dynamin to tubulate membranes, and its postulated role in fission have been major avenues of debate and research within the field of endocytosis.

It is likely more than just coincidence that two major dynamin binding partners in the brain, endophilin and amphiphysin, also tubulate membranes. As noted, endophilin and amphiphysin form a complex with dynamin along membrane tubules, further underscoring a potential functional role for this membrane-deforming capacity in vivo (Ringstad et al., 1999; Takei et al., 1999). The role of endophilin and amphiphysin in endocytosis for non-synaptic events is less clear. Perhaps the requirement for rapid, efficient, and highly reproduceable synaptic vesicle retrieval at the synapse presents a unique function for these proteins with dynamin in this process. The elucidation of a temporal sequence for the recruitment of the soluble proteins responsible for synaptic vesicle recycling will be invaluable toward understanding the role for these proteins in this highly specialized form of clathrin-mediated endocytosis.

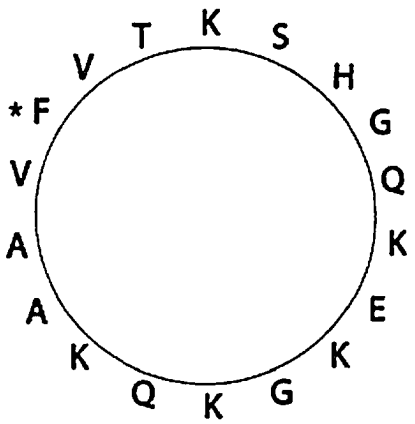
It is becoming apparent that tubulo-vesicular dynamics represent an intrinsic part of membrane traffic. Virtually every trafficking event involving membranous subcompartments has, in addition to budding and fusion, elements of tubulo-vesicular

dynamics. Trafficking through the ER, Golgi, lysosomes, mitochondria, endosomes, the plasma membrane, and organelle transport all have documented tubulo-vesicular dynamics (Lippincott-Schwartz et al., 2000; Lippincott-Schwartz et al., 1991; Sciaky et al., 1997; White et al., 1999). This may represent a highly conserved functionality which has evolved with cellular compartmentalization. Recent advances in microscopy have allowed time-lapse visualization of these trafficking events, and what has become apparent is that the budding process often involves tubular intermediates (Prekeris et al., 1999; Sciaky et al., 1997). Furthermore, organelles which were previously thought of primarily as vesicular in nature, such as axoplasmic transport organelles, are now seen as pleiomorphic tubular structures (Nakata et al., 1998).

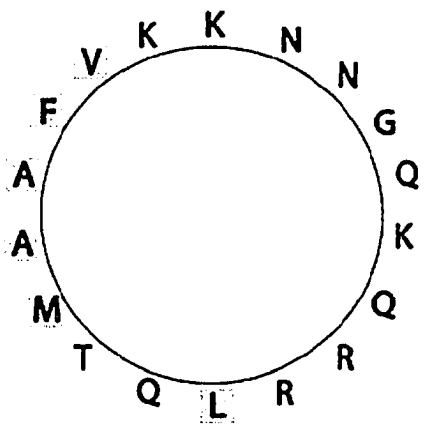
Perhaps the growing list of proteins with the intrinsic ability to tubulate lipid bilayers, most of which have demonstrated importance in membrane dynamics, is a reflection of the fact that we are only at the beginning of uncovering other proteins which share this characteristic potentially involved in diverse membrane trafficking events. The few proteins currently known to perform or putatively perform this tubular membrane deformation are found in various locations within the cell, presumeably participating in different cellular processes. This is a suggestive hint that proteins sharing this function may be active in various events important to eukaryotic cell dynamics.

Figure 3.6: More proteins with homology to endophilin A

A recently identified protein, RICH 1, has been found to have homology to the endophilin NH2-terminal domain. An alignment of endophilin A1, endophilin B1, amphiphysin 1, and RICH 1 shows that the proteins have homology along the same putative amphipathic region. An alignment of the RICH1 amino acid residues in this homologous region along a helical wheel shows a pattern very similar to endophilin A1, making it likely that this protein is also involved in lipid binding and/or tubulation.



Endophilin A1



RICH 1

Recently, RICH (RhoGAP Interacting with CIP4 Homologues), a novel RhoGAP containing protein was found in a yeast two-hybrid screen looking for CIP 4 (CDC-42 Interacting Protein 4) interacting proteins (Richnau and Aspenstrom, 2001). RICH has an NH2-terminal domain with homology to endophilin A1, and the amphipathic motif of endophilin A1 is also conserved (Figure 3.6). A splice isoform of RICH which only contains the endophilin homology domain (RICH 1B) localizes to intracellular punctate compartments (Richnau and Aspenstrom, 2001). Preliminary data suggest that RICH also has a membrane interacting/tubulating function, further expanding the diversity of proteins with this motif in membrane dynamics of the cell (Richnau and Aspenstrom, unpublished results). The application of proteomic and cell biological tools to this field should help determine the breadth and depth of tubulo-vesicular events in the cell.

Acknowledgements

The following work was performed by Niels Ringstad, with the help of Kristin Rose: the cloning of endophilin B; the raising of polyclonal antibodies to endophilin B; the multiple tissue western blot; the GM-130 colocalization in rat brainstem sections and CHO cells. We wish to thank the following people in the laboratory of Dr. Graham Warren: James Shorter for rat liver Golgi preparations, Joerg Malsam for his help with the Golgi in vitro budding assay, and Laurence Pelletier for various help and discussions.
Cryo-sections of rat liver golgi preparation and immunostaining were performed by Marc Pypaert.

Chapter 4

Mechanisms of Membrane Deformation

Abstract

Membrane traffic requires the generation of high curvature lipid-bound transport carriers represented by tubules and vesicles. The mechanisms through which membranes are deformed has gained a lot of recent attention. A major advance has been the demonstration that direct interactions between cytosolic proteins and lipid bilayers are important in the acquisition of membrane curvature. Rather than being driven only by the formation of membrane associated structural scaffolds, membrane deformation requires physical perturbation of the lipid bilayer. A variety of proteins have been identified which directly bind and deform membranes. An emerging theme in this process is the importance of amphipathic peptides that partially penetrate the lipid bilayer.

Introduction

Cellular compartmentalization requires membrane-bound structures. Traffic between membranous organelles occurs via tubular and vesicular membrane carriers which bud and fuse, effectively maintaining the compartmentalized state while allowing for dynamic flux. Over the past several years, we have garnered greater understanding of the molecular processes by which the trafficking organelles – the tubules and vesicles – form and behave. Generation of these structures can be driven by a cooperation of mechanisms both extrinsic and intrinsic to the membrane. Mechanical forces applied to the membrane by the cytoskeleton can induce membrane tubule formation. Proteinaceous coats selectively associated with the surface of membrane buds are key mediators of vesicle formation in the endocytic and secretory pathways. Accessory factors to the main constituents of coat proteins have also recently been found to be an integral part of both vesicle formation as well as cargo selection within the bud. Proteins that can deform the membrane into tubules have been identified and characterized. In addition, lipid components of the membrane, either directly or via interaction with proteins, have been suggested to facilitate the structural changes necessary to deform membranes.

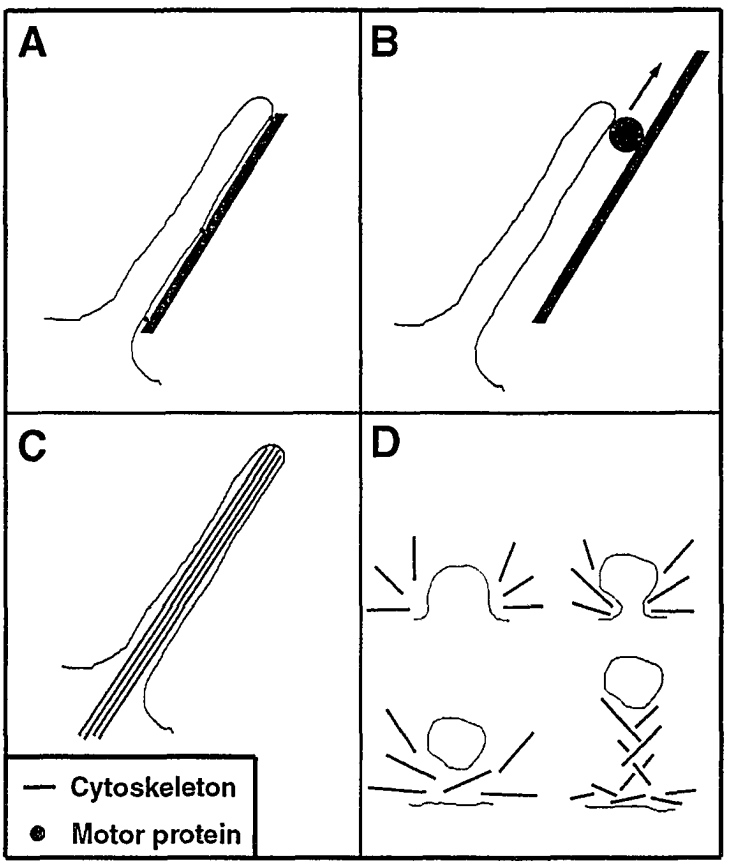
Extrinsic Forces on the Membrane

Cytoskeletal elements have long been known to play some role in membrane traffic, not only by forming the structural scaffold and network over which membrane

traffic flows, but also by directly deforming membranes (Dabora and Sheetz, 1988; Lippincott-Schwartz et al., 2000; Vale and Hotani, 1988). A characteristic property of membrane bilayers is that the application of an external focal force results in bilayer tubule formation rather than a broad 'tenting' of the membrane (Figure 4.1). Many intracellular membrane tubules are generated in this fashion (Allan and Schroer, 1999; Hirokawa, 1998; Robertson and Allan, 2000; Roux et al., 2002; Tooze and Hollinshead, 1992). For example, microtubule motors can pull a developing tubule along a preformed microtubule track in vitro (Dabora and Sheetz, 1988; Robertson and Allan, 2000; Roux et al., 2002). Microtubule-dependent mechanisms, possibly in cooperation with other cytosolic factors (Dreier and Rapoport, 2000), have also been shown to play a role in vitro and in vivo for the tubular dynamics of the endoplasmic reticulum (ER) (Dabora and Sheetz, 1988; Vale and Hotani, 1988) (Klopfenstein et al., 1998; Waterman-Storer and Salmon, 1998), as well as for the Golgi and endosome tubulation events following treatment with the fungal metabolite, brefeldin A (BFA) (Lippincott-Schwartz et al., 1991).

Other cytoskeletal elements, such as actin filaments and membrane-tethered myosin motors, may similarly participate in membrane deformation (Buss et al., 2001; Geli and Riezman, 1996; Hasson and Mooseker, 1995; Morris et al., 2002; Schafer, 2002). One obvious example of actin-dependent membrane deformation is the formation of cell surface tubular microvilli, formed by the extension of actin filaments against the plasma membrane. Actin dynamics have also been suggested to play a role in endocytosis, particularly because of the reproduceable association between abnormalities

Figure 4.1: Cytoskeletal mechanisms for membrane deformation. Cytoskeletal elements may have multiple roles in membrane deformation: **(A)** cytoskeleton-dependent formation and maintenance of tubular organelle structures; **(B)** formation of membrane tubules pulled by a cytoskeletal motor protein; **(C)** external cytoskeletal forces abutting the membrane and causing deformation; **(D)** cytoskeletal elements constricting the membrane and propelling a vesicle along a polymerizing 'comet'



in endocytosis and actin dynamics in yeast mutants (Schafer, 2002). In mammalian cells, the data have not been as conclusive; however, several proteins have been found which likely serve to couple the endocytic machinery with actin dynamics (Qualmann et al., 2000; Schafer, 2002).

Recent data have illustrated a qualitative temporal relationship between endocytosis and actin polymerization (Merrifield et al., 2002; Pelkmans et al., 2002). Using techniques of video microscopy, actin and dynamin were found to be recruited to sites of clathrin-mediated endocytosis just prior to (for dynamin) and immediately after (for actin) the movement of a nascent clathrin-coated vesicle away from the plasma membrane (Merrifield et al., 2002). In another study observing caveolar internalization of SV40 virus, brief bursts of dynamin 2 signal at the plasma membrane, as well as actin dynamics involving a reorganization of the actin cytoskeleton and formation of actin tails, were found to be associated with caveolae loaded with SV40 virus (Pelkmans et al., 2002). While confirming the role of dynamin in endocytosis prior to fission, the data further highlight the importance of actin filaments in this process. The roles actin could play in endocytosis are diverse. Actin filaments may be directly involved in membrane deformation, may help to sever the highly curved neck of the developing bud in fission, and may be part of a machinery propelling the nascent vesicle along an actin comet (Figure 4.1D) (Qualmann et al., 2000; Schafer, 2002). Furthermore, actin dynamics may influence local remodeling of the cortical cytoskeleton to facilitate endocytosis, and also may serve as a potential scaffold for the endocytic apparatus (Qualmann et al., 2000; Schafer, 2002).

Myosin motors may also play a role with actin in membrane traffic events (Hasson and Mooseker, 1995). A type I myosin is required for receptor-mediated endocytosis in yeast (Geli and Riezman, 1996). In mammalian cells, myosin VI has been implicated in endocytic events (Buss et al., 2001; Morris et al., 2002). Myosin VI interacts with clathrin and AP2 through its COOH-terminal domain, and overexpression of this domain blocks transferrin uptake (Buss et al., 2001). Myosin VI, one of only two known minus-end directed motors, may provide a mechanism for movement of the nascent vesicle away from the plasma membrane, in the direction of the minus end of an actin filament oriented along the long axis of microvilli (Buss et al., 2001). Working with actin filaments, myosin-dependent forces could provide many mechanical functions to generate membrane deformation in this process. However, outside of the results seen in yeast genetics, a requirement for actin in general endocytic events has not been unequivocally shown. There is most probably some significance to the findings implicating actin in vesicular dynamics, and membrane-dependent activities are one of the many potential events in which actin dynamics are involved. Thus, the cytoskeleton may potentially affect membrane traffic by both structural and dynamic forces acting on the membrane.

Intrinsic Forces on the Membrane: Protein-Mediated Effects

Over the last several years, emerging data have implicated cytosolic proteins in bilayer deformation upon recruitment to the membrane. Oligomerization of these

proteins into a coat scaffold on the membrane has traditionally been thought to promote budding by imposing curvature on the membrane (Figure 4.2A) (Heuser and Keen, 1988; Kadota and Kadota, 1973; Rothman and Warren, 1994). This view, first developed for the clathrin coat, was then extended to other protein coats observed on vesicles, such as COPI (Rothman and Warren, 1994) and COPII (Kuehn and Schekman, 1997), and has since been supported by data revealing an intrinsic curvature in the structure of coat protein scaffolds (Bi et al., 2002; Musacchio et al., 1999). Importantly, the observation that coat assembly, bilayer invagination, and, in some cases, even fission could occur on protein-free liposomes demonstrated that no integral membrane proteins were required for this process (Bremser et al., 1999; Drake et al., 2000; Matsuoka et al., 1998; Spang et al., 1998; Takei et al., 1998; Zhu et al., 1999).

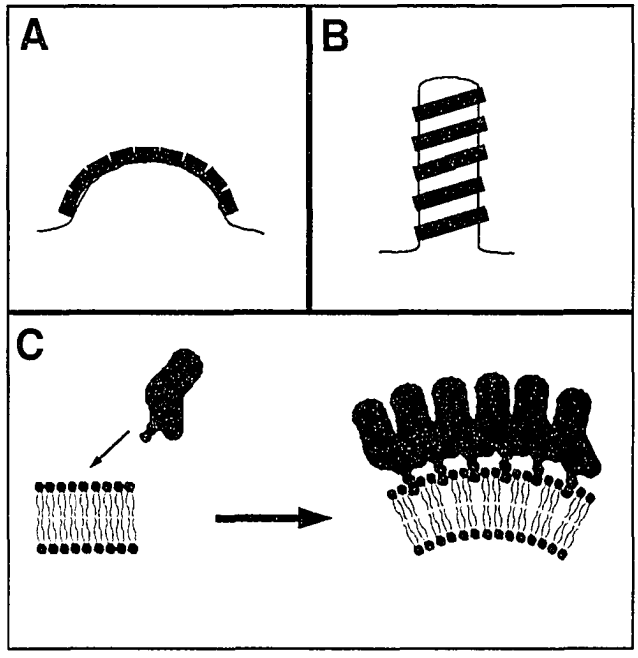
As with the soluble proteins which constitute the coat on a developing bud, cytosolic proteins have also been found to drive bilayer tubulation following recruitment to the membrane (Figure 4.2B). The first evidence that cytosolic proteins play a physiological role in the generation of membrane tubules came from studies of dynamin, a GTPase critically implicated in the fission reaction of clathrin-coated vesicles and other membrane trafficking events (Damke et al., 1994; Hinshaw, 2000; McNiven et al., 2000). Purified dynamin has the property to self-assemble into rings and spirals both in solution, as well as at the narrow tubular stalks of endocytic vesicles (Hinshaw and Schmid, 1995; Takei et al., 1998). Both in vitro and in vivo, dynamin can deform lipid bilayers into narrow tubules coated by dynamin spirals (Marks et al., 2001; Sweitzer and Hinshaw, 1998; Takei et al., 1998). A predominant theory emerged: coat proteins were involved in

budding from the donor membrane, and dynamin rings were involved in forming the tubular neck of the clathrin-coated bud (Hinshaw and Schmid, 1995; Takei et al., 1995). Upon GTP hydrolysis, constriction of the dynamin ring would mediate fission (Hinshaw, 2000; Klockow et al., 2002; Marks et al., 2001; Zhang and Hinshaw, 2001) (but see (Sever et al., 1999; Stowell et al., 1999) for alternative interpretations).

The identification and characterization of proteins associated with clathrin and dynamin, in conjunction with recent theoretical considerations of membrane biophysics, have since expanded and revised this view. With respect to clathrin for example, to effectively drive membrane curvature, the rigidity of the coat protein polymer has to supercede the resistance of mechanically bending the membrane, described as the membrane bending elastic modulus (Nossal, 2001). This notion has recently been challenged for clathrin, due to estimations that the rigidity of clathrin triskelia is similar to the membrane bending elastic modulus (Nossal, 2001). If true, clathrin could at best serve to maintain an already curved membrane, thereby preventing its collapse back into an effectively planar form (Nossal, 2001). Thus, mechanisms in addition to coat protein lattice formation that may help in deforming the bilayer are likely to come into play.

With respect to dynamin, the identification of other endocytic proteins which tubulate lipid bilayers has implicated these proteins as well in physiological membrane deformation. Amphiphysin and endophilin, two major interactors of dynamin, were found to robustly deform liposomes into narrow membrane tubules (Farsad et al., 2001; Takei et al., 1999). Epsin, an interactor of clathrin and of the clathrin adaptor AP-2 (Chen et al., 1998; Rosenthal et al., 1999; Traub et al., 1999), was also shown shown to

Figure 4.2: Protein-mediated membrane deformation. (A) Polymerizing vesicle coat proteins could potentially drive budding. **(B)** Polymerizing membrane tubulating proteins such as dynamin, endophilin, and amphiphysin are potential affectors of membrane deformation. **(C)** Membrane deformation according to the bilayer couple hypothesis. By penetration of an amphipathic helix into the interfacial section of the bilayer, proteins could possibly drive membrane deformation due to bilayer surface area discrepancy.



induce membrane tubulation (Ford et al., 2002). There is no evidence as of yet that these proteins self-assemble into rings, like dynamin, in the absence of a lipid bilayer. The property of these proteins to deform membranes is likely to reflect a unique interaction with the membrane bilayer (see below), since not all proteins which bind lipid bilayers are able to induce deformation.

There is evidence for a function of these endocytic proteins in early stages of clathrin-mediated budding, prior to the generation of a tubular neck, suggesting that the physiological role for these proteins may not be restricted to the formation of tubular membranes. For example, dynamin can be found on the dome of clathrin-coated buds, and antibody disruption of dynamin function (lamprey synapse) leads to markedly impaired clathrin-coated bud formation (H Gad, O Bloom, P Löw, P De Camilli, V Slepnev, O Shupliakov, L Brodin, *Molecular Biology of the Cell, Suppl.*, 11:218a, 2000). Furthermore, impairment of endophilin function at the synapse by antibody injection (lamprey synapse) or by genetic disruption (Drosophila) results in synaptic vesicle depletion and the accumulation of shallow clathrin-coated pits (Gad et al., 2000; Guichet et al., 2002; Ringstad et al., 1999; Verstreken et al., 2002). Amphiphysin also binds clathrin and AP-2 in addition to dynamin (Brett et al., 2002; Slepnev et al., 2000), and clathrin-coated buds generated in the presence of amphiphysin have a more homogenous, smaller size than buds generated in the presence of clathrin-coat fractions alone (unpublished data). Finally, epsin is able to recruit clathrin onto a lipid monolayer and induces 'puckered' clathrin-coated structures (Ford et al., 2002). Thus, via their ability to

deform planar membranes, these proteins may assist clathrin in early stages of bud formation.

While the above considerations apply to clathrin coats, it is still unknown whether similar mechanisms, i.e. cooperation of scaffold proteins and membrane-deforming proteins, may function also in the case of coats comprising the COP proteins. In addition, caveolin polymerization and membrane interactions are thought to play a role in caveolar budding (Drab et al., 2001; Fernandez et al., 2002; Fra et al., 1995; Rothberg et al., 1992). As a final note, one possibility is that various properties of integral membrane proteins may also contribute to membrane deformation. For example, this has been proposed for peripherin, a transmembrane protein concentrated at areas of high curvature in the outer segment discs of retinal photoreceptors, and which induces flattened microsomal vesicles when expressed in vitro (Arikawa et al., 1992; Molday et al., 1987; Wrigley et al., 2000).

Intrinsic Forces on the Membrane: Lipid-Mediated Effects

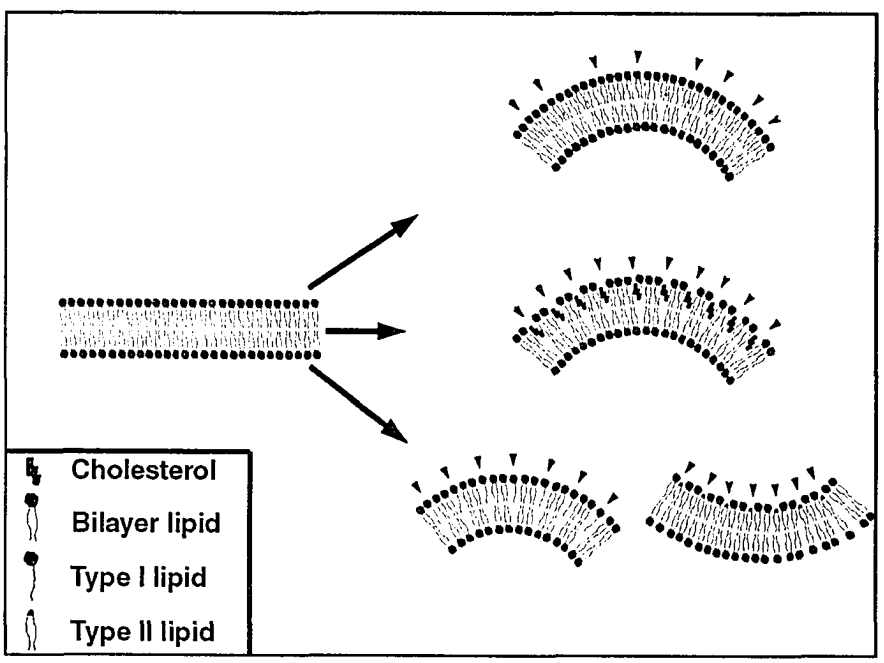
The role of lipid-specific dynamics in enabling or generating membrane curvature has also been an area of provocative research (Evans, 1974; Farge and Devaux, 1992; Lipowsky, 1991). For example, selective transfer of lipids between bilayer leaflets has been proposed as a means by which surface area asymmetries could influence budding and endocytosis (see below) (Farge and Devaux, 1992; Farge et al., 1999). In addition, certain

lipid species are postulated to favor bilayer curvature due to their physico-chemical properties and/or their relative geometries (Burger, 2000; Chernomordik, 1996).

For example, cholesterol is required for the generation of high curvature clathrin-coated buds in vivo, since cholesterol depleting compounds prevent maturation of a bud past a shallow level of curvature (Rodal et al., 1999; Subtil et al., 1999). One possible function of cholesterol is to selectively intercalate into the budding-side leaflet of the bilayer in order to enable bud formation without producing unfavorable hydrophobic-hydrophilic interactions as the bilayer is distorted. Two proteins enriched in endocytic vesicle carriers, synaptophysin and caveolin, bind cholesterol (Murata et al., 1995; Thiele et al., 2000). These proteins may function to selectively concentrate this lipid in the budding portion of the bilayer, thus allowing more favorable phase interactions as the nascent bud forms (Murata et al., 1995; Thiele et al., 2000). In this way, the influence of cholesterol on membrane structure would encompass two non-exclusive possibilities. First, selective enrichment of cholesterol into one leaflet of the membrane may alter the relative bilayer surface areas to favor budding. Second, through differential partitioning, cholesterol may minimize the energy needed for budding by both decreasing local membrane stiffness, and by preserving hydrophobic and van der Waals forces between the leaflets as the bilayer deforms (Baba et al., 2001; Brown et al., 2001; Brown et al., 2002).

In addition, enzymatic alteration of lipids has been suggested to facilitate membrane deformation by generating particular lipid geometries. Formation of type I

Figure 4.3: Lipid driven membrane deformation. (Above) Transfer of lipids to one leaflet could promote deformation by creating surface area discrepancy between the leaflets. (Middle) Selective accumulation of cholesterol could decrease membrane rigidity, create bilayer surface area discrepancy, and facilitate budding. (Below) Type I and type II lipids, based on their relative geometries, have been postulated to play a role in membrane deformation.



lipids, such as lysophosphatidyl choline (LPC), may favor positive curvature by adopting a wedge-like geometry, and formation of type II lipids, such as phosphatidyl ethanolamine (PE), may favor negative curvature by effectively creating the reverse geometry (Burger, 2000; Chernomordik, 1996). For example, sphingomyelinase, an enzyme which cleaves phosphorylcholine from sphingomyelin to generate the type II lipid, ceramide, promotes the formation of bilayer invaginations independently from a protein-mediated effect (Holopainen et al., 2000). Furthermore, phospholipase A2 activity was shown to be required for the 60-80 nm Golgi tubules formed upon treatment with BFA, as well as for the formation of tubular endosomal recycling organelles (de Figueiredo et al., 2001; de Figueiredo et al., 1998). While the effect of phospholipase A2 on membrane deformation is not well understood mechanistically, one suggestion is that formation of type I lysolipids in the membrane may have a role. Putative roles for phospholipase C and phospholipase D in influencing membrane structure through lipid modification have also been described (Basanez et al., 1997; Ktistakis et al., 1996; Liscovitch, 1996). Of note, lipid metabolism may alter bilayer structure not only directly, by affecting lipid geometries, but also indirectly, via the regulated recruitment of membrane deforming proteins (Burger et al., 2000; Ford et al., 2002).

Endophilin has been reported to have lysophosphatidic acid acyl transferase (LPAAT) activity, mediating the transfer of a fatty acyl-CoA to lysophosphatidic acid (LPA), a type I lipid (Modregger et al., 2003; Schmidt et al., 1999). Similarly, BARS, a protein involved in Golgi tubulation and fission, was also reported to have LPAAT

activity (Weigert et al., 1999). It remains unclear what role LPAAT activity plays in induction of membrane curvature, especially since endophilin has been directly shown to deform lipid bilayers independently from this enzymatic activity (Farsad et al., 2001). Furthermore, LPA is a relatively soluble lipid, and like most Type I lipids, it is likely not significantly present in biological membranes (Burger, 2000). It has been suggested that perhaps endophilin undergoes the LPAAT reaction in the cytosol, with soluble LPA, and then partitions into the bilayer upon the creation of the insoluble reaction product, phosphatidic acid (PA) (Huttner and Schmidt, 2000). However, this would serve to promote positive rather than negative curvature. Moreover, at neutral biological pH, PA likely acts as a bilayer promoting lipid, rather than a Type II negative curvature promoting lipid like PE (Burger, 2000). In addition, it is difficult to reconcile the apparent opposing activities of an acyl transferase and a phospholipase (see above for PLA2) both creating the same membrane deformation. However, although the importance of the LPAAT activity of these proteins is as yet undefined, the ability of these proteins to bind certain lipids may prove important for their biological function.

Amphipathic Peptides and the Bilayer-Couple Hypothesis

The bilayer-couple hypothesis, initially popularized by Sheetz and Singer in 1974, postulates that the two halves of a closed lipid bilayer, by virtue of asymmetries between the bilayer leaflets, may have differential responses to various perturbations (Sheetz et al., 1976; Sheetz and Singer, 1974). Thus, a relative increase in surface area of

one leaflet of a closed bilayer, as discussed above, is predicted to increase the spontaneous curvature of the bilayer. In order to minimize its energy state and maintain hydrophobic and van der Waals interactions between the leaflets, an unopposed bilayer will conform to its spontaneous curvature (Nossal, 2001). Specifically, the leaflet to which additional surface area is added will be the side to which the bilayer will deform in compensation. Sheetz and Singer observed that compounds with amphipathic qualities, presumably by intercalating into a particular leaflet of the membrane bilayer, were able to deform erythrocyte membranes according to the predictions of the bilayer-couple theory (Sheetz et al., 1976; Sheetz and Singer, 1974).

The bilayer-couple theory may explain the mechanism through which certain proteins affect morphological changes in planar membranes. By physically penetrating into one face of the bilayer, amphipathic peptides may cause membrane deformation (Figure 4.2C). The NH2-termini of the tubulogenic proteins endophilin and amphiphysin contain an amino acid stretch predicted to form an amphipathic helix necessary for lipid bilayer tubulation (Farsad et al., 2001). The ENTH domain of epsin also forms an amphipathic helix, upon binding phosphatidyl inositol 4,5-bisphosphate (PIP2), which is necessary for bilayer tubulation (Ford et al., 2002). In addition, the ARF family GTPases, involved in recruitment of coat proteins for vesicular trafficking along the secretory and endocytic pathways, have an NH2-terminal amphipathic helix critical for membrane binding(Amor et al., 1994; Antonny et al., 1997; Goldberg, 1998; Pasqualato et al., 2001; Pasqualato et al., 2002), which potentially could play a role in budding. An intriguing possibility exists that all of these proteins are sharing a common mechanism for

enabling membrane deformation through the interactions of an amphipathic peptide with the lipid bilayer. Identifying the mechanisms for how these protein-bilayer interactions are regulated will be paramount to understanding the dynamics of this process.

Recent biophysical studies using the prototypical amphipathic helical peptide, melittin, have shown that an amphipathic helix oriented parallel to a lipid bilayer surface would be ideally poised to reside at an interfacial location between the head groups and the hydrophobic core (Hristova et al., 2001; Lam et al., 2001). The steep gradient from the polar head groups to the non-polar tails within a lipid monolayer is estimated to be on the order of the diameter of an alpha helix, rendering an amphipathic helix an appropriate structural solution to a protein binding the monolayer in this fashion (White et al., 2001). Thus, perhaps many of these proteins involved in membrane deformation may function by orienting an amphipathic helix parallel to the membrane surface in such a way as to affect changes in membrane structure. A striking illustration of the power of an amphipathic peptide in affecting such a process was shown in the ability of a designed amphipathic 18-mer peptide to form extensive tubules 40-50 nm in diameter and up to 600 nm in length from liposomes comprised of various lipid combinations (Lee et al., 2001).

There are likely some key factors which are essential for these peptides to affect membrane deformation. For example, penetration into the bilayer is necessary for this process, since proteins which bind superficially to the bilayer without penetration, such as the PH domain of PLC delta or the ANTH domain of AP180 (similar to the epsin

ENTH domain, but without the ligand-induced amphipathic helix), do not cause deformation (Farsad et al., 2001; Ford et al., 2002; Ford et al., 2001; Hurley and Misra, 2000). Another likely part of this process is clustering of these proteins such that sufficient concentrations are achieved to enable a significant membrane deforming effect. Proteins such as endophilin and amphiphysin may cluster by polymerization, whereas proteins such as epsin may cluster due to the presence of localized interacting proteins. In vitro, and with overexpression, clustering may be a product of high protein concentrations, whereas the situation in vivo is likely more subtle and regulated. It remains to be seen whether different mechanisms for clustering have different effects on membrane structure.

Is there a role for the interaction of amphipathic peptides with lipid bilayers in both monomeric and polymeric forms? Studies with melittin have shown that the monomeric amphipathic helix had only modest effects on bilayer structure at lower concentrations, causing only slight increases in area per lipid. In contrast, melittin monomers cystein-linked to create dimers, affected a significant change in bilayer structure and perturbation at the same monomer/lipid concentration used for monomeric melittin (Hristova et al., 2001). Thus, the self-association of an amphipathic helix is thought to have a qualitatively different effect, compared with non-associating monomers, on the structural perturbation of a lipid bilayer (Hristova et al., 2001). As such, it is possible that biological membrane dynamics may use the effects of both monomeric and polymeric proteins to create variations on bilayer perturbation and deformation. The ability of these proteins to concentrate in the membrane, either alone or in various

combinations, may affect the degree to which membrane structure is perturbed. Indeed, at high concentrations, both monomeric and dimeric melittin significantly perturbed bilayer structure, and were ultimately membrane-lytic (Hristova et al., 2001).

Membrane-deforming proteins likely work in conjunction with clathrin-coat proteins to promote budding. In vitro data with epsin, amphiphysin, and endophilin illustrate this point. As mentioned above, co-incubation of epsin with clathrin induced puckered clathrin polymers on a lipid monolayer (Ford et al., 2002). Clathrin-coat proteins incubated with amphiphysin resulted in coated buds associated with amphiphysin tubules (Takei et al., 1999). Both epsin and amphiphysin are well known to interact biochemically with clathrin, and as such, it makes sense that these proteins could couple clathrin bud formation with other membrane deforming activities. Interestingly, when incubated with coat proteins, many endophilin generated tubules were also capped by clathrin-coated buds, despite the fact that endophilin has no known binding properties to clathrin-coat proteins (Farsad et al., 2001). This suggests that endophilin tubules and clathrin coats may serve as structural, rather than biochemical, substrates under conditions favorable for budding (Farsad et al., 2001). Thus, proteins which alone are able to drive membrane curvature may facilitate clathrin-mediated bud formation by altering bilayer structure to favor this process.

Future Directions and Closing Remarks

Our understanding of the mechanisms generating membrane deformation will no doubt increase our awareness of how this process affects various aspects of cell biology. Roles for membrane budding and tubulation have been described in both immunity and disease. For example, 'reverse' budding, budding away from the cytosol, is a mechanism for the formation of the multi-vesicular bodies (MVB) in the late endosomal pathway (Katzmann et al., 2002; Piper and Luzio, 2001; Stahl and Barbieri, 2002). Recent work in this field has demonstrated a role for three protein complexes, ESCRT I, II, III (Babst et al., 2002a; Babst et al., 2002b; Katzmann et al., 2001), in addition to the mono-ubiquitin pathway (Dupre et al., 2001; Hicke, 2001) and phosphoinositide metabolism (Odorizzi et al., 1998; Odorizzi et al., 2000), in the generation of the lumenal vesicles of the MVBs. Budding into the MVBs is an efficient way to target membrane proteins/receptors to lysosomes for degradation (Katzmann et al., 2002). In addition, the lumenal vesicles of the MVBs are also involved in the immune response by both the loading of the major histocompatibility complex (MHC) class II molecules with antigen, as well as by the formation of exosomes, secreted lumenal vesicles containing MHC class II molecules and T-cell costimulatory factors which are potent immune stimulators and potential anti-tumor agents (Denzer et al., 2000; Katzmann et al., 2002; Schartz et al., 2002; Thery et al., 2002; Wolfers et al., 2001). Furthermore, in the case of enveloped viruses such as HIV-1, viral budding from the plasma membrane has apparently usurped the MVB

machinery in an analogous process of budding away from the cytosol into the extracellular space (Garrus et al., 2001; Hicke, 2001; Katzmann et al., 2002; Pornillos et al., 2002). How this budding process occurs will likely represent a new mechanism in membrane deformation.

The parasite Toxoplasma gondii represents a new example of the role for tubulating proteins in disease. Once the parasite enters the cell into the parasitic vacuole, parasite secreted proteins have been shown to be involved in the generation of a 60-90 nm tubular network emanating into the vacuole from the vacuolar membrane (Mercier et al., 2002). The parasite secreted protein, gra2, is involved in the formation of these tubules, and impaired tubule formation results in diminished parasite virility (Mercier et al., 2002). Gra2 contains two amphipathic alpha helical regions which are critical for tubulation (Mercier et al., 2002). Thus, Toxoplasma gondii uses a secreted tubulogenic protein, with requisite amphipathic helices, in its infectious biology.

Membrane-deforming proteins involved in diverse cellular processes besides intracellular membrane traffic have also been described. A member of the amphiphysin protein family has been localized to the muscle T-tubule system, where its membrane-deforming properties may likely play a role in the biology of these structures (Lee et al., 2002). Furthermore, dynamin and endophilin isoforms have been localized to the tubular plasma membrane invaginations often observed at podosomes, dynamic actin/membrane structures involved in motility and adhesion (Ochoa et al., 2000).

The number of factors involved in generating membrane curvature has increased and has underscored our appreciation of the complexity of the process. Many issues remain to be resolved, and it is likely that the process is driven by a cooperation of both proteins and lipids. A major contribution to bilayer deformation is from the reversible recruitment of cytosolic proteins, which have the advantage of being recycled, to the membrane. The interaction of amphipathic peptides with the membrane, as identified in proteins such as endophilin, amphiphysin, and epsin, is an emerging theme that may describe one mechanism for membrane deformation. Ultimately, more membrane-deforming factors will be identified, and we will likely find that nature has created more than one solution to this problem.

Chapter 5

Membrane Tubulating Proteins and a Putative Regulatory Role in the Recruitment of Clathrin-Coat Proteins to Membranes: A Study with Amphiphysin

Abstract

Amphiphysin 1 is a brain-specific protein enriched at the synapse and a major binding partner for the large GTPase, dynamin (David et al., 1996). Amphiphysin has been shown to be involved in synaptic vesicle recycling by both acute and chronic perturbation studies (Di Paolo et al., 2002; Shupliakov et al., 1997). One potential role for amphiphysin function in the synaptic vesicle recycling process involves its binding affinities for both clathrin-coat proteins and dynamin, as well as for lipid bilayers and biological membranes (Slepnev et al., 2000; Takei et al., 1999). Here we show that amphiphysin directly stimulates clathrin recruitment onto liposomes in an in vitro assay. Amphiphysin-dependent clathrin-coat recruitment is enhanced by the interactions of amphiphysin with dynamin (through the dynamin PRD domain) and endophilin (through the endophilin SH3 domain). We show that the amphiphysin SH3 domain is also able to bind full-length amphiphysin, likely via an internal poly-proline region, and clathrin recruitment onto liposomes by amphiphysin is enhanced in the presence of the amphiphysin SH3 domain. Thus, amphiphysin stimulated clathrin recruitment onto

liposomes may be regulated via intramolecular binding between the amphiphysin COOH-terminal SH3 domain and its internal poly-proline region.

Introduction

Clathrin-mediated endocytosis remains the best characterized pathway of synaptic vesicle retrieval (De Camilli et al., 2001b). In addition to the proteins which comprise the clathrin-coat, several other proteins have been implicated in this process. These accessory proteins are thought to function in various processes related to clathrin-mediated budding, including playing a role at the tubular neck of the clathrin-coated bud, where they may possibly regulate the fission reaction (Chen et al., 1991; Koenig and Ikeda, 1989; Ringstad et al., 1999; Takei et al., 1995; Takei et al., 1999). Dynamin, amphiphysin, and endophilin are some of the best characterized proteins in this process. Amphiphysin and endophilin are major dynamin binding partners at the synapse, and all three proteins localize to the tubular neck of the clathrin-coated pit (David et al., 1996; Gad et al., 2000; Ringstad et al., 1999; Ringstad et al., 1997; Takei et al., 1998; Takei et al., 1995).

A role for these proteins in synaptic vesicle recycling has been shown by both in vitro and in vivo experimentation. Perturbation of either dynamin, amphiphysin, or endophilin function at the synapse demonstrates, among other things, an arrest of the clathrin-mediated endocytic process at various stages of acquisition of deep membrane invagination and fission (Gad, 2000; Gad et al., 2000; Guichet et al., 2002; Hill et al., 2001; Koenig and Ikeda, 1989; Ringstad et al., 1999; Shupliakov et al., 1997; Verstreken et al., 2002). These findings suggest that these proteins, all of which are known to

independently and coordinately form lipid bilayer tubules, may participate in membrane deformation during clathrin-mediated budding (Farsad et al., 2001; Takei et al., 1999).

A role for dynamin and SH3-mediated interactions at this late stage in clathrin-mediated synaptic vesicle recycling of deep membrane invagination and fission has been suggested by microinjection experiments in the living reticulospinal synapses of lamprey (Shupliakov et al., 1997). Microinjection of the amphiphysin SH3 domain in this preparation results in a near-total depletion of synaptic vesicles corresponding to a potent block of late stages in clathrin-mediated synaptic vesicle retrieval (Shupliakov et al., 1997). Under these conditions, numerous deeply invaginated clathrin-coated pits were accumulated at the peri-active zone (Shupliakov et al., 1997). Furthermore, targeted disruption of the amphiphysin 1 gene in mice revealed, in a subset of mice, a phenotype displaying severe cognitive deficits and a lowered seizure threshold correlated with deficient synaptic vesicle recycling (Di Paolo et al., 2002). Moreover, biochemical analysis of brain cytosol in these mice revealed a selective impairment in the recruitment of the clathrin-coat proteins, clathrin and AP2 (clathrin adaptor protein-2), onto lipid bilayers (Di Paolo et al., 2002), suggesting a possible etiology for the decreased efficiency of synaptic vesicle recycling.

The brain-specific isoforms of amphiphysin contain internal binding sites for clathrin and AP2, and it has been postulated that one possible function of amphiphysin in synaptic vesicle recycling is to help coordinate endocytic protein complexes involving clathrin and AP2 (Slepnev et al., 2000; Takei et al., 1999). Consistent with this,

dephosphorylation of amphiphysin by the calcium-dependent phosphatase, calcineurin, stimulated the formation of endocytic protein complexes including amphiphysin, clathrin, AP2, and dynamin, while phosphorylated amphiphysin was less competent for the formation of these protein complexes (Slepnev et al., 1998). Moreover, it has been shown that amphiphysin helps to coordinate clathrin-coated buds on tubules with dynamin in vitro (Takei et al., 1999). In this study we expand on these observations by using purified components to explore the role of amphiphysin in the recruitment of clathrin-coat proteins onto lipid bilayers, and investigate a potential role for an endocytic protein complex in this recruitment process.

Materials and Methods

Electron microscopy

Liposomes (0.1mg/ml final) were incubated at 37 °C for 10-20 minutes in buffer A (25mM Hepes-KOH, pH 7.4, 25mM KCl, 2.5mM Mg^{2+} acetate, 150mM K-glutamate) with various proteins and nucleotides at the following final concentrations: dynamin 0.1 mg/ml, amphiphsyin 0.1 mg/ml, endophilin 0.1 mg/ml, coat proteins 0.5 mg/ml, clathrin 6μg, AP2 16 μg, GTP 1mM, GTPγS 0.5mM (Takei et al., 1999). At the end of the incubation, aliquots were adsorbed onto 200-400 μM formvar- and carbon-coated copper EM grids for 3-5 minutes at room temperature, washed in 0.1M Hepes pH 7.4, stained in

1-2% uranyl acetate, blotted and allowed to air dry. For incubations at 4 °C, all of the above was performed on ice.

Purified Proteins and Peptides

Dynamin was affinity purified from rat brain cytosol using amphiphysin 1 SH3-domain as described above (Owen et al., 1998).

Recombinant rat endophilin A1 and human amphiphysin 1 were cloned in pGEX (Pharmacia), and purified as Glutathione-S Transferase (GST) fusion proteins according to standard methods (Pharmacia). The GST tag was subsequently cleaved by PreScission Protease (Pharmacia).

Amphiphysin SH3-domain G-P R-L mutant (GPRL) was generated by primer mediated mutagenesis and subcloned into pGEX and pCDNA vectors.

Amphiphysin partial constructs were prepared by PCR to yield the following fragments: amino acids, the poly-proline domain (PPD), amino acids, the protein with the deleted COOH-terminal SH-domain. The constructs were subcloned and purified as above.

Clathrin-coat fraction was purified from bovine calf brain as described (Takei et al., 1999). Briefly, clathrin-coated vesicles were purified from bovine brains and the clathrin-coat

proteins were extracted in 0.8M Tris-HCl, pH 7.4, 2mM EGTA, 0.03% sodium azide, 0.5mM dithiothreitol (DTT) and 1mM phenylmethylsulphonyl fluoride (PMSF) for 15 hours at room temperature. Stripped coat proteins were isolated by centrifugation at 100,000 g in a TLA 100.1 rotor for 1 hour at room temperature and stored at –70 °C. For experiments studying purified clathrin, the coat-protein fraction was separated by gel filtration.

A peptide corresponding to the clathrin binding region of amphiphysin was synthesized and purified as observed by FPLC (Slepnev et al., 2000).

Liposomes

Liposomes composed of brain lipid extract (type 1, Folch fraction 1, Sigma) were made as described (Takei et al., 1999). Briefly, a lipid mixture solubilized in chloroform was added to a 2:1 chloroform:methanol mixture and thoroughly mixed in a glass tube. Lipids were adhered along the sides of the glass tube under a stream of nitrogen gas by gently rotating the tube until the chloroform:methanol solvent had evaporated. This procedure generates layers of lipids dried as multiple bilayers along the glass surface. 300mM filtered sucrose was then gently added to the tube with the dried lipid bilayers, and the tube was allowed to incubate, covered with parafilm, at 37 °C for 15-60 minutes. During this incubation, the dried bilayers gradually begin to peel off of the glass surface and

swell. Following the incubation, vigorous vortexing shears the bilayers into vesicles of heterogeneous size.

Liposome Recruitment

Liposome sedimentation was performed using 100μg sucrose-loaded liposomes incubated with 5-10μg protein (1-25μg for saturable binding) in 400-500μl buffer A or in Hepes-KCl pH 7.4 (to determine salt sensitivity) for 10-20 minutes at 37 °C. Liposomes were sedimented at 100,000g in a Beckman TLA 100.3 rotor for 20 minutes, the supernatant was thoroughly removed, and sedimented liposomes were solubilized in 2% SDS. To monitor recovery, liposomes were labeled with 0.5% NBD-phosphatidyl choline and absorbance was measured at 460 nm. Samples were subjected to SDS-PAGE and analyzed by Coomassie staining or by Western blotting with the respective antibodies (amphiphysin polyclonal antibody, CD5; clathrin heavy-chain monoclonal antibody TD-1; AP2 monoclonal antibody to alpha-adaptin subunit, Mab061; dynamin polyclonal antibody DG1; endophilin polyclonal antibodies).

The effect of clathrin recruitment by amphiphysin was studied by the addition of various proteins and peptides, including a peptide corresponding to the amphiphysin binding region within the dynamin proline-arginine domain.

Light Scattering

Dynamic light scattering induced by liposomes was used to monitor a gross indicator of change in morphology for the pool of liposomes in the reaction mixture. Light scattering at a wavelength of 350 nm was measured in a Hitachi F-3010 fluorescence spectrophotometer with a heated cuvette holder. Excitation and emission slit widths were set at 3 nm. Liposomes at a concentration of 100 μg/ml were incubated with proteins at a final concentration of 20-40 μg/ml and 1 mM nucleotide for the times indicated in a reaction cuvette containing a stir bar. The recordings were traced in real time.

Cell Culture and Transfection

Chinese Hamster Ovary fibroblasts were grown according to standard protocols in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, glutamine, and penecillin/streptomycin. For transient transfection studies, cells were grown on flamed 22 millimeter square coverslips in six-well plates containing 3 milliliters of medium. Constructs were subcloned into pCDNA 3.1 with an HA-tag, and 5 μg of plasmid DNA was transfected with Lipofectamine 2000 reagent for 16 hours. Cells were rinsed in phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde in 120 mM

sodium phosphate buffer warmed to 37 °C for 30 minutes. Cells were rinsed in PBS and blocked in goat serum dilution buffer. Immunocytochemistry was performed with polyclonal antibodies to amphiphysin (CD 5), and monoclonal antibodies to clathrin (X-22, Upstate Biochem.) and AP2 (Mab0166, Santa Cruz) using standard protocols. Primary antibodies were visualized with Texas Red and Oregon Green conjugated secondary antibodies.

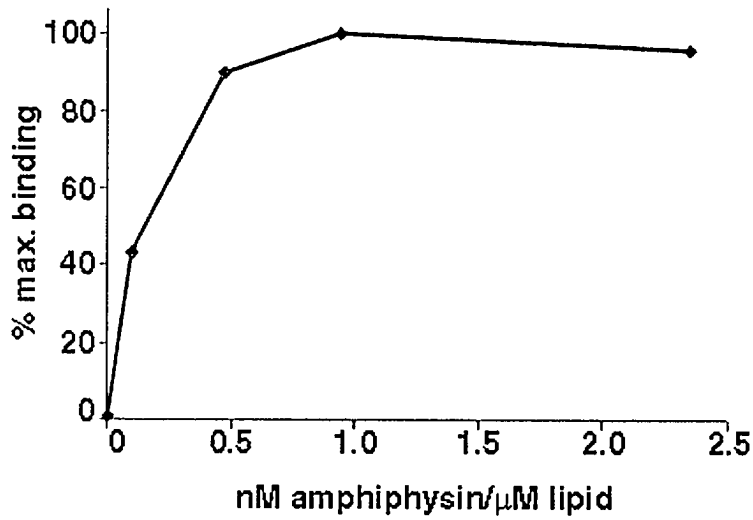
Results

Amphiphysin Binds Liposomes in a Saturable Reaction

To examine the effect of amphiphysin on recruitment of clathrin-coat proteins to liposomes, we used a liposome sedimentation assay to test for liposome associating proteins (Farsad et al., 2001). As mentioned, this assay takes advantage of the property of sucrose loaded lipomes to readily sediment, thereby allowing assessment of liposome binding through centrifugation. Purified recombinant amphiphysin, with the GST-tag cleaved off, readily binds to liposomes in a saturable fashion upon a 15 minute incubation at 37 °C (Figure 5.1). The liposome binding region of amphiphysin has been mapped to its NH2-terminus, with the first 37 amino acids being critical for lipid interactions as defined by deletional mutagenesis (See Figure 2.8B, and (Farsad et al., 2001)).

Figure 5.1: Amphiphysin binding to liposomes is saturable.

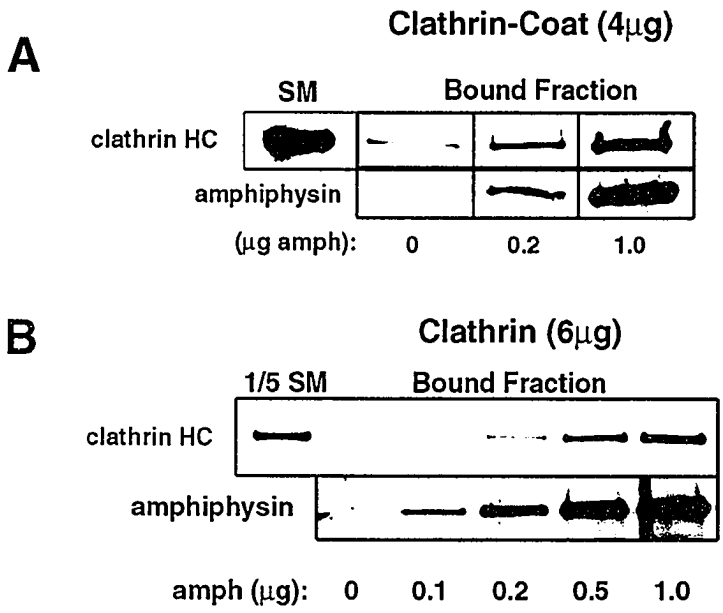
Recombinant human amphiphysin 1 in pGEX6P1 vector was purified and the GST-tag was subsequently cleaved using PreScission Protease (Pharmacia). Liposomes made from a total brain lipid extract were incubated with increasing concentrations of purified recombinant amphiphysin 1 (GST-tag cleaved) for 15 minutes at 37 °C. Protein bound to liposomes was analyzed by centrifugation at 100,000g in a Beckman TLA 100.3 rotor, SDS-PAGE of the pelleted material, and densitometric analysis of the band intensities. Amphiphysin has an apparent saturation of 1:1000 molar ratio protein:lipid, although see chapter 2 for discussion on why this is likely an underestimation due to a cooperative process of membrane binding.



Amphiphysin Stimulates Recruitment of Clathrin-coat Proteins onto Liposomes

The brain isoforms of amphiphysin contain internal binding sites for clathrin and the clathrin adaptor protein, AP2 (Slepnev et al., 2000). Given the strong affinity of amphiphysin for lipid bilayers, we examined what effect these known protein-protein interactions would have on the potential recruitment of clathrin-coat proteins onto liposomes. Incubation of amphiphysin with clathrin-coat proteins, purified from bovine brain, stimulated the recruitment of coat proteins onto liposomes in a dose-dependent fashion (Figure 5.2A). Clathrin was then purified from the coat-protein fraction by gel filtration. Recruitment of purified clathrin onto liposomes was directly stimulated by amphiphysin (Figure 5.2B). Recruitment of the AP2 complex onto liposomes was only mildly stimulated in the presence of amphiphysin (not shown). In our experience, recruitment was consistently more robust when the full clathrin-coat protein fraction was used, making it likely that other macromolecular interactions work cooperatively in this process. Separation of the coat complex into clathrin and AP2 may somehow render the individual components less 'coatogenic' than when present together with potentially other proteins in the coat complex. Indeed, reformation of clathrin-coated buds on liposomes by adding separated clathrin and AP2 is much less efficient than when clathrin-coat proteins are added directly without separation (not shown). Thus, it appears as though amphiphysin has a specific ability to stimulate clathrin recruitment onto

Figure 5.2: Amphiphysin stimulates recruitment of clathrin onto liposomes. **(A)** Liposomes were incubated with a fixed concentration of clathrin-coat proteins (4 μg), with increasing concentrations of amphiphysin. Binding to liposomes was deterimined by co-sedimentation with centrifugation and western blot analysis using antibodies to the clathrin heavy chain (HC) and amphiphysin. Increasing amphiphysin concentrations leads to increasing amounts of coat proteins recruited to the liposomes as determined by the presence of the clathrin heavy chain. **(B)** Amphiphysin directly stimulates clathrin recruitment onto liposomes. Clathrin-coat proteins were separated into clathrin and adaptin fractions through gel filtration and used in the liposome recruitment assay.



liposomes, and there may be other interactions between and amongst clathrin-coat proteins which enhance this recruitment.

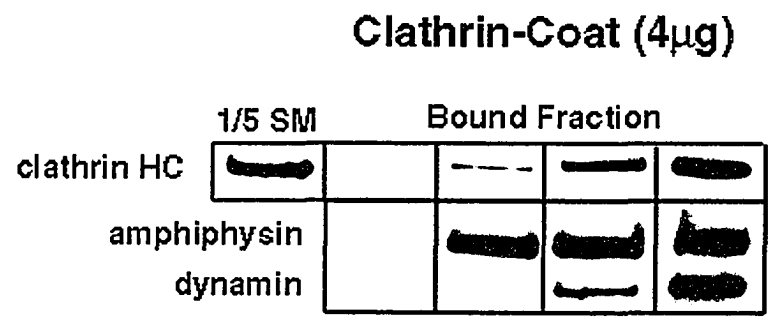
Dynamin Increases Amphiphysin-mediated Clathrin-coat Recruitment

Since amphiphysin has been postulated to potentially serve as a multi-protein adaptor, linking the clathrin-coat with dynamin on the membrane (Takei et al., 1999), we examined what role addition of purified dynamin has on the recruitment of clathrin by amphiphysin. Co-incubation of dynamin with amphiphysin enhanced the recruitment of clathrin-coat proteins onto liposomes (Figure 5.3A). This enhanced recruitment was seen with purified clathrin as well (Figure 5.3B). To further examine the effect of the dynamin-amphiphysin interaction on clathrin-coat recruitment onto liposomes, a peptide corresponding to the proline- and arginine-rich COOH-terminal domain (PRD) of dynamin, known to bind amphiphysin (Grabs et al., 1997), was incubated with amphiphysin and clathrin-coat proteins. The dynamin PRD also enhanced clathrin-coat recruitment onto liposomes mediated by amphiphysin (Figure 3.3C). These data suggest that an interaction with the amphiphysin COOH-terminal SH3 domain is potentially involved with its ability to bind and/or recruit the clathrin-coat proteins onto liposomes.

Figure 5.3: Dynamin increases amphiphysin-mediated clathrin recruitment onto liposomes.

(A) Liposomes were incubated with clathrin-coat proteins, or with clathrin-coat proteins and amphiphysin. With the concentrations of clathrin-coat proteins and amphiphysin held constant, increasing the concentration of dynamin stimulated further recruitment of clathrin-coat proteins above that seen with amphiphysin. **(B)** The stimulatory effect of dynamin on amphiphysin-mediated clathrin-coat recruitment was seen with gel purified clathrin as well. **(C)** The stimulatory effect of dynamin on amphiphysin-mediated clathrin-coat recruitment was seen with increasing concentrations of the dynamin PRD peptide, which interacts with the amphiphysin SH3 domain. This suggests that there is an intrinsic effect on amphiphysin incurred through an SH3-domain interaction with the dynamin PRD-domain which increases the ability of amphiphysin to recruit clathrin onto liposomes.

A



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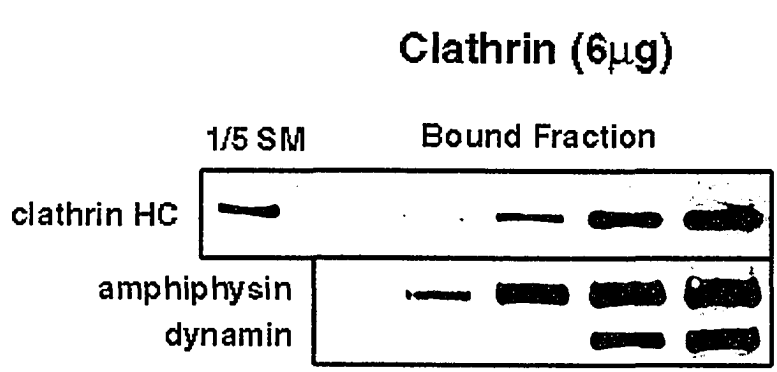

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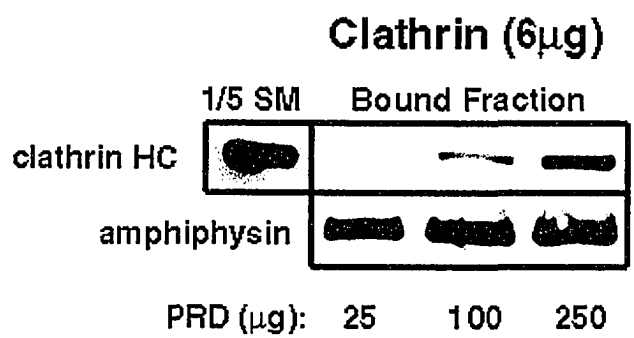


In order to explore a potential contribution of dynamin itself on clathrin-coat recruitment, we incubated dynamin and purified clathrin with liposomes. Upon this incubation, we detected a dose-dependent increase in the amount of clathrin heavy chain recruited onto liposomes in the presence of dynamin (Fig. 5.4A). This interaction between dynamin and clathrin-coat proteins was examined further by investigating what effect coat proteins would have on the GTPase activity of dynamin. As measured in a phosphate release assay, clathrin-coat proteins strongly inhibited dynamin GTPase activity in a dose-dependent fashion (Figure 5.4B). This finding independently suggests a direct interaction between dynamin and clathrin-coat proteins. Thus, dynamin itself interacts with clathrin, and presents an independent stimulus for clathrin recruitment onto liposomes.

Amphiphysin-Endophilin Interaction Stimulates Amphiphysin-mediated Clathrin-Coat Recruitment

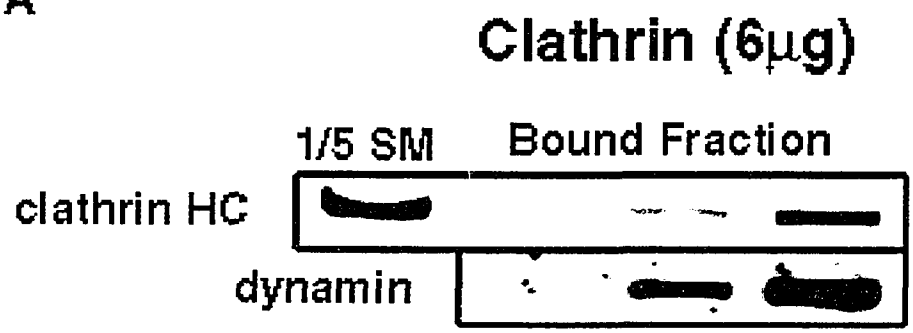
Endophilin has been reported to interact, via its SH3 domain, with a putative poly-proline binding site in amphiphysin. Under our experimental conditions, the endophilin SH3 domain directly bound amphiphysin (Figure 5.5A). A construct which lacked the endophilin SH3 domain was unable to bind amphiphysin (Figure 5.5A).

We next tested the effect of the purified endophilin SH3 domain on the amphiphysin-mediated recruitment of clathrin-coat proteins onto liposomes. Co-

Figure 5.4: Interaction between clathrin-coat proteins and dynamin

(A) Liposomes incubated with purified clathrin and increasing dynamin concentrations showed that dynamin is able to stimulate clathrin recruitment onto liposomes in a dose-dependent fashion. **(B)** Dynamin was incubated with or without increasing concentrations of brain purified clathrin-coat proteins, and the effect on dynamin GTPase activity was determined through release of radioactive phosphate. Increasing clathrin-coat concentrations had a potent inhibitory effect on dynamin GTPase activity, likely reflecting a modulatory interaction.

A



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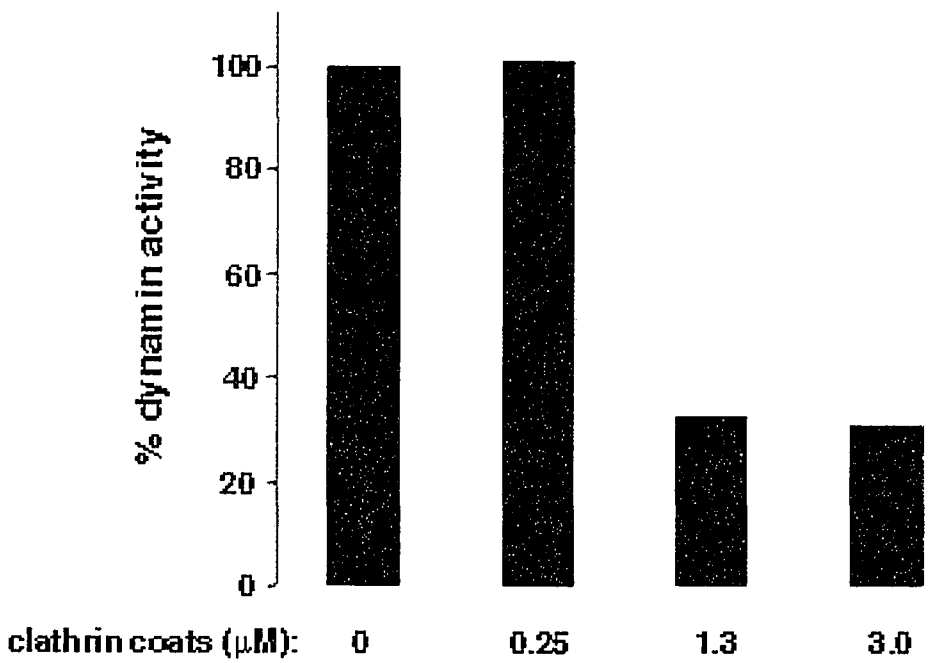


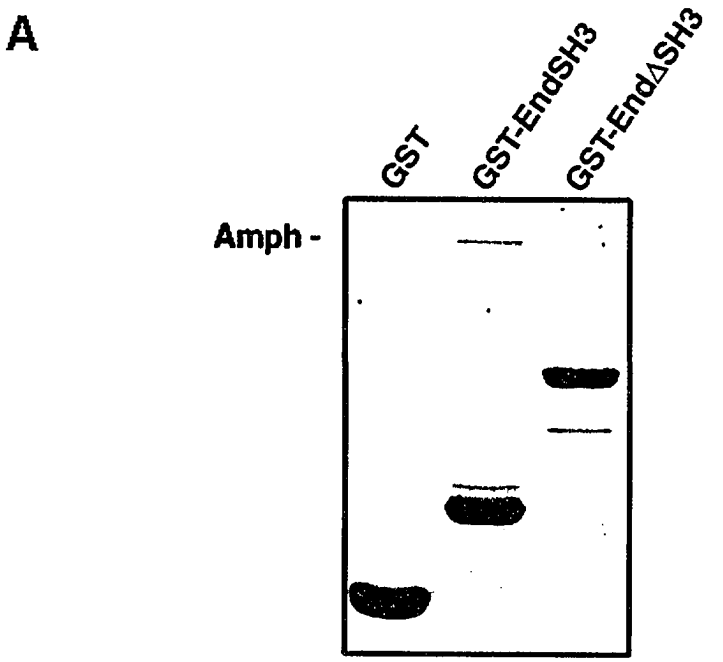
incubation of the endophilin SH3 domain with amphiphysin enhanced amphiphysin-mediated clathrin-coat recruitment onto liposomes (Figure 5.5B). There was also an apparent increase in the amount of amphiphysin recruitment onto liposomes in the presence of the endophilin SH3 domain (Figure 5.5B). Endophilin does not interact with clathrin-coat proteins (not shown), therefore, the enhanced coat recruitment in this reaction must somehow be due to an effect on the property of amphiphysin to either bind or recruit clathrin-coat proteins.

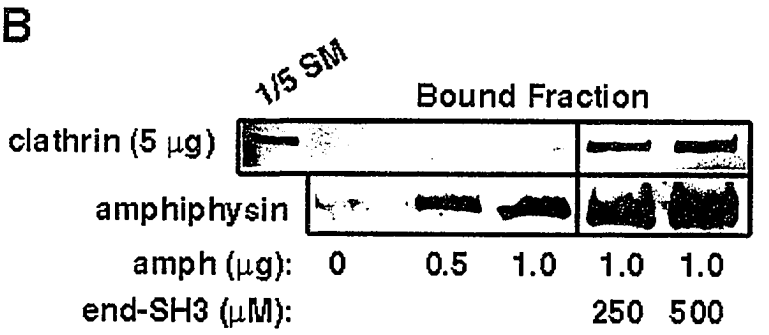
The Amphiphysin SH3 Domain Binds Full-length Amphiphysin and Stimulates Amphiphysin-mediated Clathrin-Coat Recruitment onto Liposomes

Upon analysis of the putative endophilin binding site in amphiphysin, the poly-proline stretch in amphiphysin reported to bind endophilin was found to be located upstream of the amphiphysin binding sites for clathrin and AP2 (Figure 5.6A and (Micheva et al., 1997; Slepnev et al., 2000; Wechsler-Reya et al., 1997)). This poly-proline stretch spans roughly one hundred amino acids, and contains several serine phosphorylation sites (Floyd et al., 2001). Studying the various amphiphysin isoforms, we noted that some splice variants of amphiphysin which did not have the clathrin and AP2 binding sites, such as the muscle specific isoforms, were also missing this poly-proline stretch (Butler et al., 1997). We became interested in whether this poly-proline stretch potentially could regulate amphiphysin interactions with clathrin-coat proteins.

Figure 5.5: Amphiphysin-Endophilin interaction stimulates amphiphysin-mediated clathrin recruitment onto liposomes

(A) Recombinant full-length amphiphysin was incubated in affinity chromatography experiments with endophilin constructs, or GST alone. Amphiphysin was able to directly and specifically bind to the endophilin SH3 domain. **(B)** The presence of the endophilin SH3 domain, shown to bind amphiphysin, was able to increase the amount of clathrin recruitment to the liposomes above that seen with amphiphysin alone.





For example, interactions with amphiphysin binding partners could stimulate recruitment of clathrin-coat proteins by somehow making the clathrin and AP2 binding sites more available.

The fact that the isolated PRD of dynamin, as well as the isolated SH3 domain of endophilin, stimulated clathrin recruitment to liposomes by amphiphysin, points to perhaps a more subtle dynamic intrinsic to amphiphysin itself. Given the results obtained from the interaction of these isolated domains with amphiphysin in clathrin-coat recruitment, we investigated whether there could be a potential intramolecular interaction within amphiphysin itself. The src kinase has been shown to regulate its activity by intramolecular interactions involving its SH2 and SH3 domains (Xu et al., 1999). We tested, therefore, whether the amphiphysin SH3 domain could interact with the full-length protein, presumably via the poly-proline domain upstream of the clathrin and AP2 binding site. Indeed, incubation of purified amphiphysin with its isolated GST (glutathione S-transferase)-tagged SH3 domain, immobilized on glutathione sepharose beads, revealed an interaction above that seen with GST alone (Figure 5.6B).

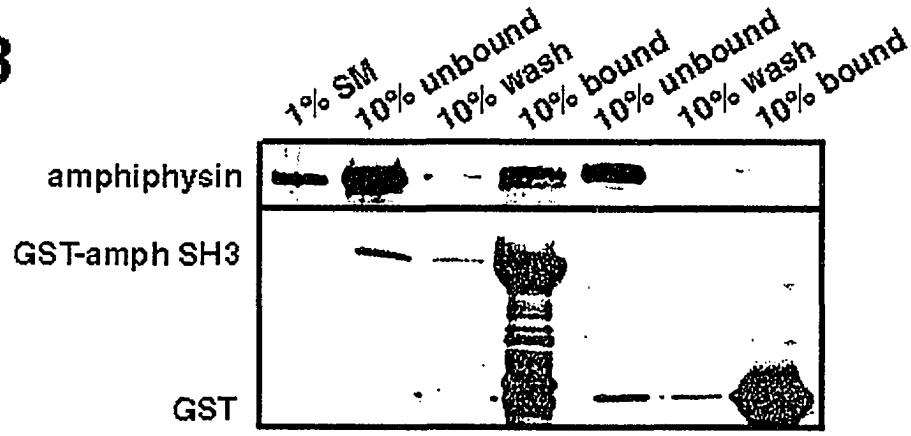
Having observed an interaction between amphiphysin and the amphiphysin SH3 domain, we next tested what the consequence of this interaction would be on amphiphysin-mediated clathrin-coat recruitment. Addition of the amphiphysin SH3 domain to the reaction with amphiphysin and clathrin-coat proteins increased recruitment of clathrin-coat proteins onto liposomes above that observed with amphiphysin alone (Figure 5.6C). This result suggests that a putative SH3-mediated interaction with the

Figure 5.6: A putative amphiphysin intramolecular interaction regulating amphiphysin-mediated clathrin recruitment onto liposomes

(A) Schematic cartoon of the amphiphysin primary protein sequence showing the highly conserved NH2-terminal domain known to bind and tubulate lipid bilayers, an internal poly-proline rich region just upstream of the clathrin and AP2 binding sites, and a COOH-terminal SH3 domain. **(B)** Interaction of full-length amphiphysin with the amphiphysin SH3 domain. Affinity chromatography using purified reagents shows a specific binding of full-legth amphiphysin to the immobilized GST-amphiphysin SH3 domain above that of GST alone. **(C)** Effect of the amphiphysin SH3 domain on amphiphysin-mediated clathrin recruitment onto liposomes. Increasing concentrations of the amphiphysin SH3 domain stimulates further amphiphysin-mediated clathrin recruitment than that seen with amphiphysin alone.

A

B



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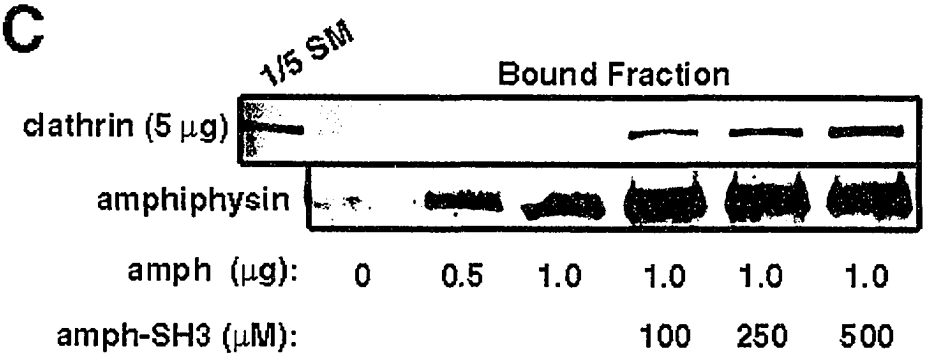


central poly-proline domain of amphiphysin upstream of its clathrin and AP2 binding sites has a stimulatory role on the recruitment of clathrin-coat proteins to liposomes.

Evidence for a putative intramolecular interaction within amphiphysin regulating clathrin-coat protein recruitment is seen in transfection studies of fibroblasts. Transfection of the amphiphysin SH3 domain in fibroblasts blocks receptor mediated endocytosis, while transfection of a mutant amphiphysin SH3 domain, which has a G-P and R-L mutation (GPRL) within its SH3 domain, a mutation which renders the SH3 domain non-functional with respect to binding capacity (Grabs et al., 1997), has no effect. By contrast, this mutation in the amphiphysin SH3 domain has a potent inhibitory effect on the endocytosis of the transferrin receptor when transfected into fibroblasts in the context of the full-length protein, while a cell line stably transfected with wild type amphiphysin 1 does inhibit transferrin uptake (V. Slepnev and G. Ochoa, unpublished results). Although one possible interpretation of these results is that the mutant amphiphysin no longer interacts with dynamin, thereby blocking the fission reaction, it does not fully explain why the two SH3 domains produce opposite results depending on whether they are being expressed alone or part of the full-length protein.

Immunofluorescence studies of these transfected cells show a gradual accumulation of dysfunctional aggregates composed of amphiphysin, clathrin, and AP2 (V. Slepnev and G. Ochoa, unpublished results). At low levels of amphiphysin GPRL expression, the aggregations first appear at the cell membrane, and then with further overexpression, the aggregates form large inclusion bodies within the cell (V. Slepnev and

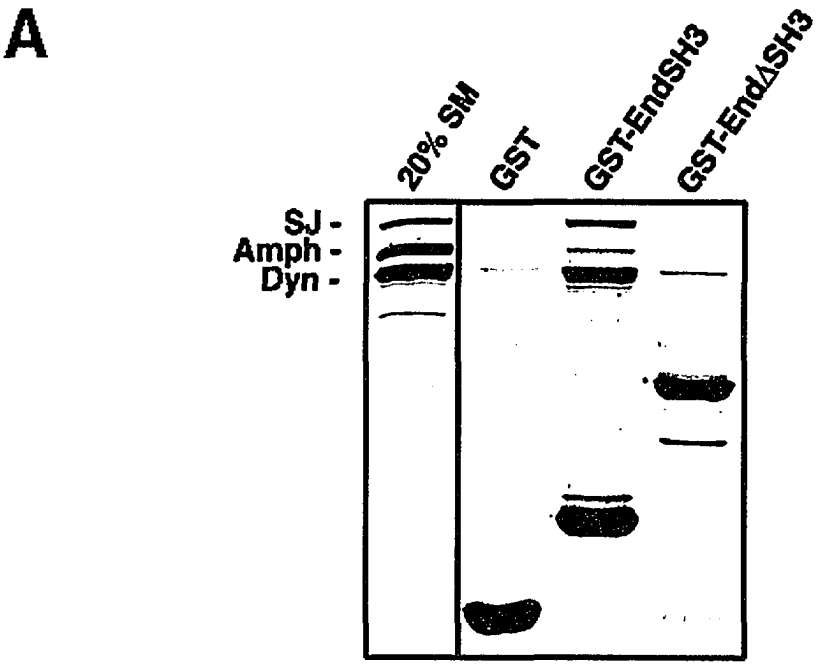
G. Ochoa, unpublished results). The wild type expressing cell line does not form such aggregates. Furthermore, caveolin internalization was not perturbed in amphiphysin GPRL transfected cells, thus indicating that clathrin-independent, dynamin-dependent endocytosis was still functional (V. Slepnev and G. Ochoa, unpublished results). Thus, transfection of an amphiphysin construct with a binding-deficient SH3 domain resulted in a potent block of endocytosis corresponding to the massive accumulation of protein aggregates comprising clathrin and AP2. This effect may be secondary to unregulated interactions between amphiphysin and clathrin-coat proteins due to a mutant amphiphysin SH3 domain.

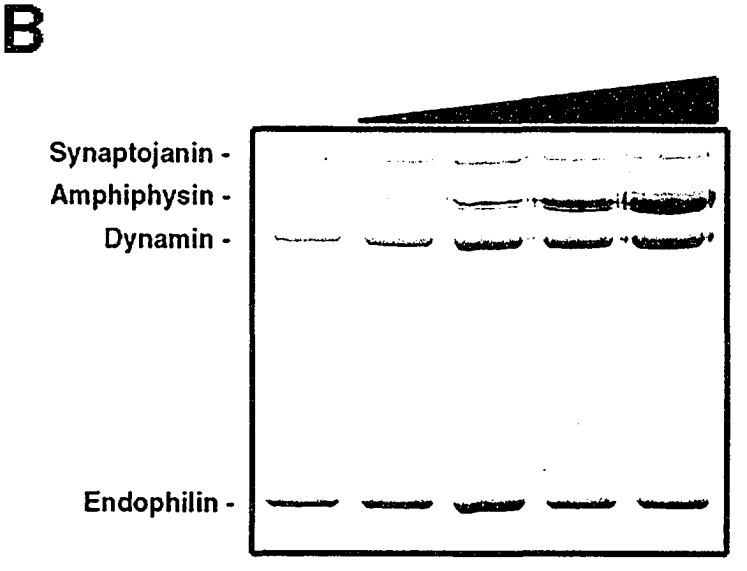
Ternary Protein Complexes with Amphiphysin, Endophilin, and Dynamin: A Role in Facilitating Clathrin Recruitment onto Liposomes

Given that interactions with dynamin and endophilin enhanced amphiphysin-mediated clathrin-coat recruitment onto liposomes, we were interested in seeing whether these proteins could interact in a ternary complex. Using GST fusion proteins of endophilin constructs, a protein complex consisting of dynamin, synaptojanin, and amphiphysin was specifically seen only in the presence of the endophilin SH3 domain (Figure 5.7A). A construct which lacked the endophilin SH3 domain was unable to bind the protein complex above background levels (Figure 5.7A). In addition, we observed simultaneous binding of dynamin, synaptojanin, endophilin, and amphiphysin on

Figure 5.7: A ternary complex including amphiphysin, endophilin, dynamin, and synaptojanin observed in solution and on liposomes

(A) A complex between amphiphysin, endophilin, dynamin, and synaptojanin forms in solution. The endophilin SH3 domain was used for affinity chromatography with the brain purified dynamin/synaptojanin fraction and the recombinant amphiphysin protein. A complex including all of the proteins was selectively observed only in the presence of the endophilin SH3 domain. **(B)** Amphiphysin, endophilin, dynamin, and synaptojanin co-sediment in the presence of liposomes. Increasing concentrations of amphiphysin does not compete away the presence of a fixed concentration of either endophilin or dynamin, suggesting that there are potential non-competitive binding sites for all three proteins.





liposomes (Figure 5.7B). Furthermore, increasing amphiphysin concentrations recruited slightly more dynamin to the membrane (Figure 5.7B).

Both amphiphysin and endophilin have been shown to co-localize with dynamin along coated tubules at the neck of clathrin-coated buds (Ringstad et al., 1999; Takei et al., 1998), and both proteins have been shown to form an in vitro tubular ring complex with dynamin along liposomes (Farsad et al., 2001; Takei et al., 1999). As seen with the light scattering assay above, addition of amphiphysin to dynamin enhances the fragmentation of liposomes by dynamin in a GTP dependent fashion, while addition of endophilin to dynamin prevents this process (see Figure 2.11). However, when endophilin and dynamin are incubated with liposomes and GTP, the presence of amphiphysin does not diminish the high light scattering above that created upon addition of GTP (Figure 5.8). This likely reflects the presence of light scattering structures, such as tubules, upon incubation of liposomes with these proteins.

This was observed by negative stain electron microscopy. Co-incubation of amphiphysin, endophilin, and dynamin with liposomes produces a phenotype consistent with an endophilin-dynamin dominant complex. In the presence of GTPγS, regular protein rings are seen decorating lipid tubules characteristic of the morphology generated by a complex of dynamin and endophilin, and/or amphiphysin (Figure 5.9A). In the presence of GTP, tubules are still visualized in addition to smaller vesicular structures (not shown). This is similar to the effect produced when liposomes are incubated with

Figure 5.8: Incubation of liposomes with amphiphysin, dynamin, and endophilin shows persistence of high light scattering structures.

Liposomes incubated with dynamin and endophilin show a cooperative increase in the amount of light scattering observed. The addition of amphiphysin under these conditions does not reverse the light scattering effect. This reflects the persistence of tubular structures seen by electron microscopy under these conditions, suggesting that the presence of endophilin is able to prevent the loss of light scattering normally seen upon incubation of dynamin with both GTP and amphiphysin.

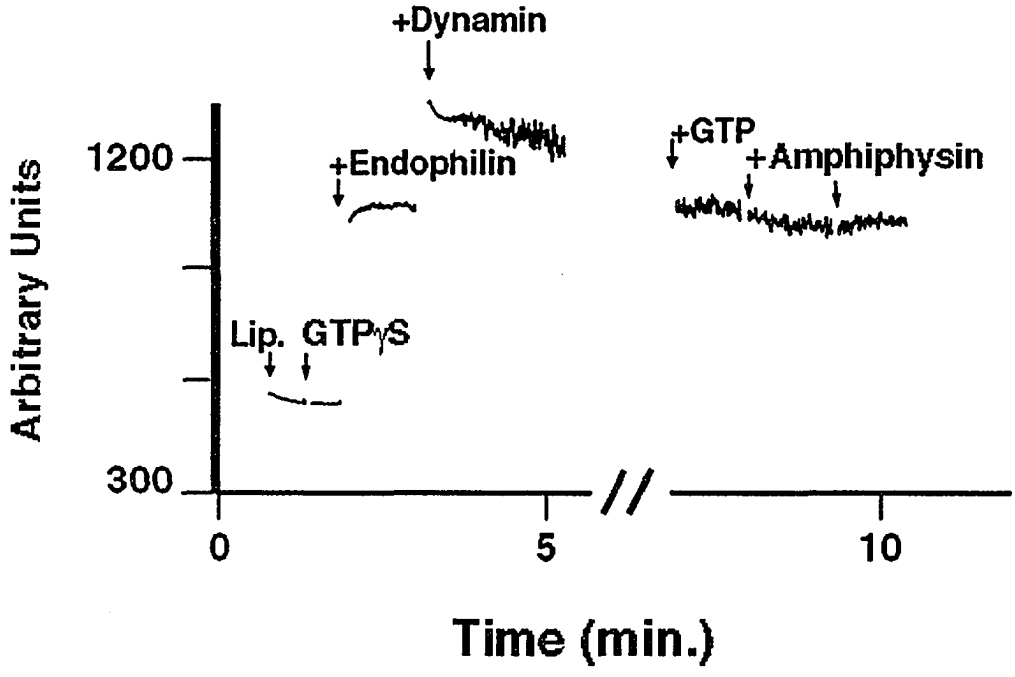
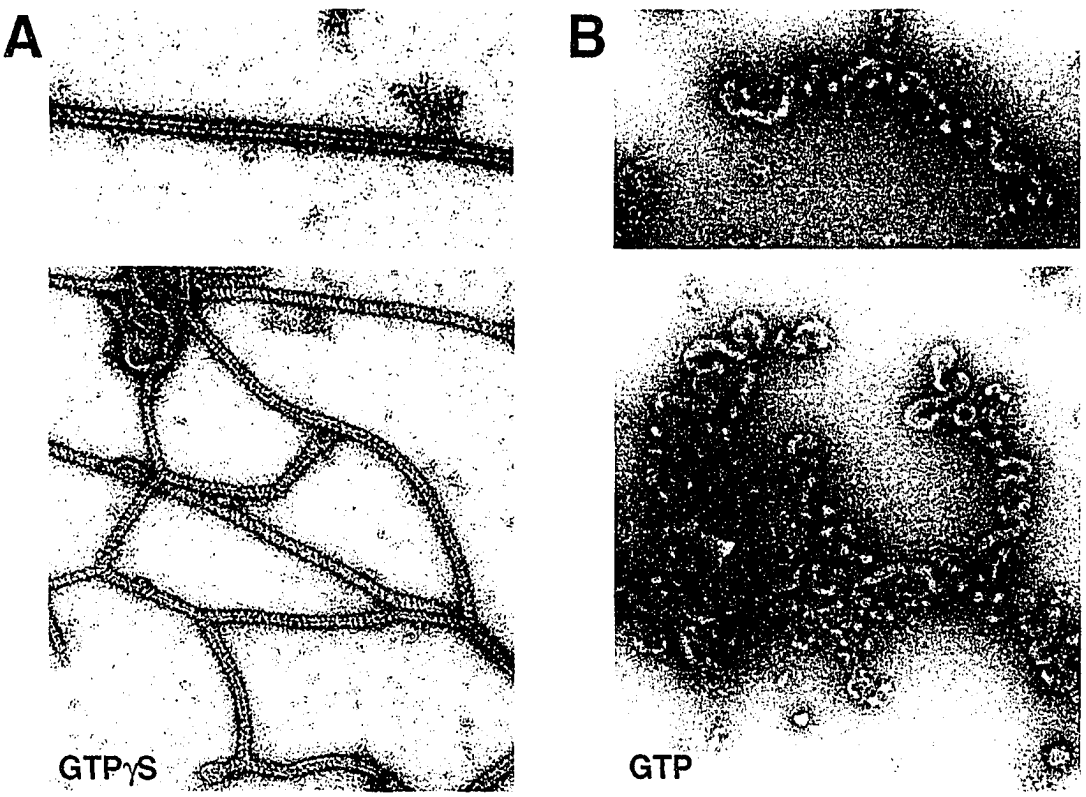


Figure 5.9: Effect of liposome incubation with amphiphysin, endophilin, and dynamin seen by electron microscopy

(A) Liposomes incubated with amphiphysin, endophilin, and dynamin in the presence of GTPγS show long, straight tubules with a morphology characteristic of dynamin with either of its two binding partners under these conditions as visualized by negative stain electron microscopy. **(B)** In the presence of GTP, a phenotype more similar to the dynamin and endophilin complex is seen; however, occasionally we observed tubulated structures which were twisted and contorted as though in the process of vesiculation. This may represent an intermediate to vesiculation seen in the presence of amphiphysin, endophilin, and dynamin.



dynamin and endophilin in the presence of GTP. Occasionally in this preparation, however, the tubules assumed a contorted, twisted appearance, as though a rotational force is being applied (Figure 5.9B). This appeared to be an intermediate structure in the process of vesiculation, and suggests a potential in vitro interaction between dynamin, endophilin, and amphiphysin in creating this phenotype. More definitive experiments will be needed to demonstrate a direct interaction amongst these three proteins on membranes.

Discussion

In this study, we have expanded on previous results showing an interaction between clathrin, AP2, and amphiphysin to examine the solution-membrane dynamics of this interaction in an in vitro system with purified components. We have shown amphiphysin to have a specific interaction with lipid bilayers composed of a total brain lipid extract, as evidenced by saturation binding in a liposome sedimentation assay. Amphiphysin has been shown to interact with clathrin-coat proteins in solution (Slepnev et al., 2000). In our assay, addition of the lipid binding amphiphysin to purified clathrin-coat proteins enables the recruitment of clathrin-coat proteins onto the liposomes. This indicates that amphiphysin binds both liposomes and clathrin-coat proteins in a bivalent manner, consistent with the separation of these two binding sites in the amphiphysin molecule. The lipid binding region of amphiphysin maps within the NH2-terminal 37

amino acids (Farsad et al., 2001), while the clathrin and AP2 binding sites are further downstream in the primary sequence structure of amphiphysin (Slepnev et al., 2000).

In this in vitro assay, amphiphysin recruited clathrin more robustly than the clathrin adaptor, AP2 (not shown). The fact that intact coat-proteins were more efficiently recruited to liposomes than the separated components argues for a more complex interaction both within the coat complex itself, as well as between amphiphysin and the coat complex. Indeed, targeted disruption of the amphiphysin 1 locus in mice resulted in a diminished steady-state level of membrane association for both clathrin and AP2, indicative of the intimate association of these proteins in vivo (Di Paolo et al., 2002). Multivalent binding between amphiphysin, clathrin, and AP2 may help concentrate these proteins in order to facilitate high efficiency synaptic vesicle recycling dynamics. Consistent with this, amphiphysin knock-out mice exhibited reduced synaptic vesicle recycling efficiency associated with severe cognitive deficits in fear conditioning and memory (Di Paolo et al., 2002).

Amphiphysin-stimulated clathrin-coat recruitment onto liposomes was more robust in the presence of proteins involved in synaptic vesicle recycling known to interact with amphiphysin, namely, dynamin and endophilin (Grabs et al., 1997; Micheva et al., 1997). Addition of either the dynamin PRD or the endophilin SH3 domains increased the recruitment by amphiphysin of clathrin-coat proteins more than what was observed with amphiphysin alone. The fact that these isolated amphiphysin interacting domains increased the recruitment ability of amphiphysin for clathrin-coat proteins suggests that

these interactions somehow affect clathrin-coat recruitment by an intrinsic effect on amphiphysin itself. Using a GST-amphiphysin 2 construct, dynamin has been shown to displace clathrin from binding amphiphysin in solution, suggesting that binding of dynamin and clathrin to amphiphysin is competitive (McMahon et al., 1997). Our results clearly show an enhancement of amphiphysin-mediated clathrin recruitment to liposomes in the presence of dynamin, as opposed to a competitive interaction. Although these differences may be a reflection of solution versus membrane dynamics, a complex of amphiphysin, dynamin, and clathrin has also been isolated in solution upon dephosphorylation of endocytic proteins after nerve terminal stimulation (Slepnev et al., 1998).

Intriguingly, despite the fact that both the dynamin PRD as well as the endophilin SH3 domain increase clathrin-coat recruitment mediated by amphiphysin, these two domains interact with different sites within the amphiphysin protein. One possible suggestion for how binding to different sites within amphiphysin produces the same effect with respect to clathrin-coat recruitment could be that these two binding sites somehow interact with each other in the native amphiphysin protein. The fact that these two binding sites are an SH3 domain and a poly-proline stretch, respectively, makes this possibility all the more plausible. Accordingly, we were able to detect a specific interaction between the full-length amphiphysin molecule and its SH3 domain by using purified components. Moreover, addition of the amphiphysin SH3 domain to the reaction including full-length amphiphysin and clathrin-coat proteins increased the recruitment of the coat proteins relative to amphiphysin alone. If present, amphiphysin

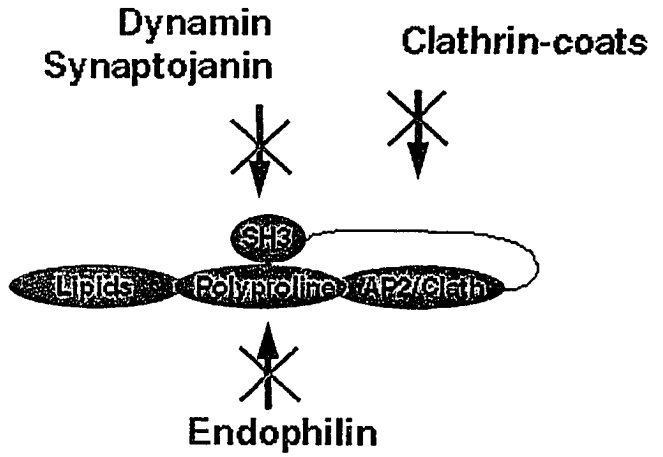
intramolecular binding must be of a low affinity nature, since amphiphysin is not detected by Coomassie staining in affinity chromatography experiments with whole brain extract, using the amphiphysin SH3 domain as bait. However, within the molecule itself, there is such a high local concentration of ligand, that such an interaction may still be present.

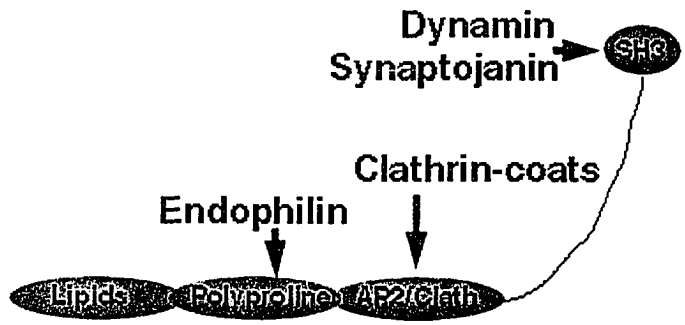
Since the poly-proline region of amphiphysin lies upstream of the binding sites for clathrin and AP2, it is conceivable that putative intramolecular binding to this region, whereby the COOH-terminal amphiphysin SH3 domain folds back over itself to bind the upstream poly-proline stretch, regulates binding to clathrin-coat proteins. Of note, analysis of the exon-intron structure of the amphiphysin genes has determined the presence of an "endocytic exon," exon 12, comprising four sub-exonal components found in amphiphysin isoforms which are thought to participate in endocytosis (Wechsler-Reya et al., 1997). In addition to the sequences mediating binding to clathrin and AP2, the poly-proline stretch is part of this "endocytic exon" (Wechsler-Reya et al., 1997), suggesting a functional link between these regions of amphiphysin.

An intramolecular interaction between the amphiphysin SH3 domain and the upstream poly-proline region is appealing because it could potentially serve as a single switch to generate a non-reactive amphiphysin molecule (Figure 5.10). Through such an interaction, amphiphysin ostensibly would be unable to bind any of its major binding partners – dynamin, synaptojanin, endophilin, clathrin, or AP2. Perhaps only through proper localization, amphiphysin could then be activated by exposing its binding sites. One potential function of amphiphysin interactions with dynamin and endophilin at

Figure 5.10: Model showing how an amphiphysin intramolecular interaction could regulate association with amphiphysin binding partners

Amphiphysin SH3-domain mediated intramolecular interaction with the polyproline region upstream of the clathrin/AP2 binding site could conceivably block the association of amphiphysin with its major binding partners. Binding to either the SH3-domain or the polyproline region by other proteins would expose the remaining binding sites, and may explain the enhanced recruitment of clathrin-coat proteins onto liposomes in the presence of domains interacting with these regions in amphiphysin.





highly concentrated foci would be to create an equilibrium favoring the open amphiphysin state only at areas primed for endocytosis. Consistent with this hypothesis, transfection of an amphiphysin construct which has a mutated SH3 domain resulted in a potent inhibition of endocytosis and an accumulation of protein aggregates comprising the mutant amphiphysin construct, clathrin, and AP2. The mutant amphiphysin construct may no longer be able to maintain intramolecular interactions with its SH3 domain, and therefore may produce unregulated and dysfunctional binding to clathrin and AP2. Thus, a single intramolecular interaction within amphiphysin may serve to regulate the dynamics of this protein for its role in facilitating efficient synaptic vesicle recycling.

A complex consisting of endophilin, amphiphysin, dynamin was detected in solution and, putatively, on liposomes, suggesting that multimeric ternary interactions between these proteins may enhance clathrin-coat recruitment. Endophilin and amphiphysin have distinct binding sites for dynamin and synaptojanin (Cestra et al., 1999; Grabs et al., 1997; Ringstad et al., 1999), making it possible that a large macromolecular endocytic complex may exist at the nerve terminal. Moreover, endophilin, amphiphysin, and dynamin have all been shown to be dimers in solution and/or on the membrane, suggesting the potential for multivalent interactions (Farsad et al., 2001; Klockow et al., 2002; Ramjaun et al., 1999; Ringstad et al., 2001; Zhang and Hinshaw, 2001).

A protein complex such as this would be a way to maintain the high specificity required for synaptic vesicle recycling, as well as to maintain the efficiency of recycling by concentrating a pool of proteins involved in the endocytic reaction. Endocytic proteins have been shown to be highly concentrated in a localized area surrounding the active zone, where most synaptic vesicle recycling is believed to occur (De Camilli et al., 2001b; Gad et al., 1998). Clustering of proteins may facilitate protein polymerization phenomena such as clathrin-cage formation and dynamin oligomerization with endophilin and amphiphysin. As seen with microtubules and microfilaments, polymerization of proteins is directly related to protein concentration, and maintaining a highly concentrated pool of proteins would serve to increase the efficiency of producing a rapid polymerization event such as clathrin-coat formation (McKinley, 1983).

It is noteworthy that dynamin was found to have an independent stimulatory effect on clathrin recruitment to liposomes. This interaction with the clathrin-coat proteins was also reflected in the potent inhibition of dynamin GTPase activity upon incubation with clathrin-coat proteins. Dynamin has been shown to have affinity for the appendage domain of the alpha-adaptin subunit of AP2 (Wang et al., 1995b). In addition, the dynamin PH domain is believed to have binding affinity for WD40/beta-transducin repeats, found in the beta-gamma subunit of hetero-trimeric G-proteins known to interact with dynamin (Wang et al., 1995a). The amino-terminal domain of the clathrin heavy chain has a beta-propeller structure which is related to those of WD40 repeats, and therefore may serve as a potential binding substrate for dynamin (ter Haar et al., 2000). Although the direct inhibition of dynamin GTPase activity by clathrin-coat proteins

needs to be confirmed, it may suggest a mechanism whereby the fission activity of dynamin is regulated by the clathrin-coat so that unproductive and potentially destructive fission would not prematurely occur before the coat has assembled. This implies that another factor may relieve this inhibition to promote GTP hydrolysis and fission.

References

Ahle, S., and E. Ungewickell. 1986. Purification and properties of a new clathrin assembly protein. *EMBO Journal.* 5:3143-9.

Allan, V.J., and T.A. Schroer. 1999. Membrane motors. *Curr Opin Cell Biol.* 11:476-82.

Almers, W., and F.W. Tse. 1990. Transmitter release from synapses: does a preassembled fusion pore initiate exocytosis? *Neuron.* 4:813-8.

Amor, J.C., D.H. Harrison, R.A. Kahn, and D. Ringe. 1994. Structure of the human ADP-ribosylation factor 1 complexed with GDP. *Nature.* 372:704-8.

Andrews, J., M. Smith, J. Merakovsky, M. Coulson, F. Hannan, and L.E. Kelly. 1996. The stoned locus of Drosophila melanogaster produces a dicistronic transcript and encodes two distinct polypeptides. *Genetics.* 143:1699-711.

Antonny, B., S. Beraud-Dufour, P. Chardin, and M. Chabre. 1997. N-terminal hydrophobic residues of the G-protein ADP-ribosylation factor-1 insert into membrane phospholipids upon GDP to GTP exchange. *Biochemistry.* 36:4675-84.

Arikawa, K., L.L. Molday, R.S. Molday, and D.S. Williams. 1992. Localization of peripherin/rds in the disk membranes of cone and rod photoreceptors: relationship to disk membrane morphogenesis and retinal degeneration. *J Cell Biol.* 116:659-67.

Baba, T., C. Rauch, M. Xue, N. Terada, Y. Fujii, H. Ueda, I. Takayama, S. Ohno, E. Farge, and S.B. Sato. 2001. Clathrin-dependent and clathrin-independent endocytosis are differentially sensitive to insertion of poly (ethylene glycol)-derivatized cholesterol in the plasma membrane. *Traffic.* 2:501-12.

Babst, M., D.J. Katzmann, E.J. Estepa-Sabal, T. Meerloo, and S.D. Emr. 2002a. Escrt-III: an endosome-associated heterooligomeric protein complex required for mvb sorting. *Dev Cell.* 3:271-82.

Babst, M., D.J. Katzmann, W.B. Snyder, B. Wendland, and S.D. Emr. 2002b. Endosome-associated complex, ESCRT-II, recruits transport machinery for protein sorting at the multivesicular body. *Dev Cell.* 3:283-9.

Bähler, M., F. Benfenati, F. Valtorta, and P. Greengard. 1990. The synapsins and the regulation of synaptic function. *Bioessays.* 12:259-263.

Bannykh, S.I., T. Rowe, and W.E. Balch. 1996. The organization of endoplasmic reticulum export complexes. *Journal of Cell Biology.* 135:19-35.

Basanez, G., M.B. Ruiz-Arguello, A. Alonso, F.M. Goni, G. Karlsson, and K. Edwards. 1997. Morphological changes induced by phospholipase C and by sphingomyelinase on large unilamellar vesicles: a cryo-transmission electron microscopy study of liposome fusion. *Biophys J.* 72:2630-7.

Bennett, M.V. 1966. Physiology of electrotonic junctions. *Ann N Y Acad Sci.* 137:509-39.

Bennett, M.V. 1972. Electrical versus chemical neurotransmission. *Res Publ Assoc Res Nerv Ment Dis.* 50:58-90.

Bennett, M.V. 1997. Gap junctions as electrical synapses. *J. Neurocytology.* 26:349-366.

Benson, D.L., D.R. Colman, and G.W. Huntley. 2001. Molecules, maps and synapse specificity. *Nat Rev Neurosci.* 2:899-909.

Betz, W.J., and G.S. Bewick. 1992. Optical analysis of synaptic vesicle recycling at the frog neuromuscular junction. *Science.* 255:200-3.

Betz, W.J., F. Mao, and G.S. Bewick. 1992. Activity-dependent fluorescent staining and destaining of living vertebrate motor nerve terminals. *Journal of Neuroscience.* 12:363-375.

Betz, W.J., F. Mao, and C.B. Smith. 1996. Imaging exocytosis and endocytosis. *Current Opinion in Neurobiology.* 6:365-371.

Bi, X., R.A. Corpina, and J. Goldberg. 2002. Structure of the Sec23/24-Sar1 pre-budding complex of the COPII vesicle coat. *Nature.* 419:271-7.

Bommert, K., M.P. Charlton, W.M. DeBello, G.J. Chin, H. Betz, and G.J. Augustine. 1993. Inhibition of neurotransmitter release by C2-domain peptides implicates synaptotagmin in exocytosis. *Nature.* 363:163-165.

Breckenridge, L.J., and W. Almers. 1987a. Currents through the fusion pore that forms during exocytosis of a secretory vesicle. *Nature.* 328:814-7.

Breckenridge, L.J., and W. Almers. 1987b. Final steps in exocytosis observed in a cell with giant secretory granules. *Proceedings of the National Academy of Sciences of the United States of America.* 84:1945-9.

Bremser, M., W. Nickel, M. Schweikert, M. Ravazzola, M. Amherdt, C.A. Hughes, T.H. Sollner, J.E. Rothman, and F.T. Wieland. 1999. Coupling of coat assembly and vesicle budding to packaging of putative cargo receptors. *Cell.* 96:495-506.

Brett, T.J., L.M. Traub, and D.H. Fremont. 2002. Accessory protein recruitment motifs in clathrin-mediated endocytosis. *Structure (Camb).* 10:797-809.

Broadie, K., H.J. Bellen, A. DiAntonio, J.T. Littleton, and T.L. Schwarz. 1994. Absence of synaptotagmin disrupts excitation-secretion coupling during synaptic transmission. *Proceedings of the National Academy of Sciences of the United States of America.* 91:10727-10731.

Brodsky, F.M. 1985. Clathrin structure characterized with monoclonal antibodies. II. Identification of in vivo forms of clathrin. *Journal of Cell Biology.* 101:2055-2062.

Brodsky, F.M. 1988. Living with clathrin: its role in intracellular membrane traffic. *Science.* 242:1396-402.

Brodsky, F.M., C.Y. Chen, C. Knuehl, M.C. Towler, and D.E. Wakeham. 2001. Biological basket weaving: formation and function of clathrin-coated vesicles. *Annu Rev Cell Dev Biol.* 17:517-68.

Brodsky, F.M., B.L. Hill, S.L. Acton, I. Nathke, D.H. Wong, S. Ponnambalam, and P. Parham. 1991. Clathrin light chains: arrays of protein motifs that regulate coated-vesicle dynamics. *Trends Biochem Sci.* 16:208-13.

Brown, M.F., R.L. Thurmond, S.W. Dodd, D. Otten, and K. Beyer. 2001. Composite membrane deformation on the mesoscopic length scale. *Phys Rev E Stat Nonlin Soft Matter Phys.* 64:010901.

Brown, M.F., R.L. Thurmond, S.W. Dodd, D. Otten, and K. Beyer. 2002. Elastic deformation of membrane bilayers probed by deuterium NMR relaxation. *J Am Chem Soc.* 124:8471-84.

Brunger, A.T. 2001. Structure of proteins involved in synaptic vesicle fusion in neurons. *Annu Rev Biophys Biomol Struct.* 30:157-71.

Burger, K.N. 2000. Greasing membrane fusion and fission machineries. *Traffic.* 1:605-13.

Burger, K.N., R.A. Demel, S.L. Schmid, and B. de Kruijff. 2000. Dynamin is membrane-active: lipid insertion is induced by phosphoinositides and phosphatidic acid. *Biochemistry.* 39:12485-93.

Buss, F., S.D. Arden, M. Lindsay, J.P. Luzio, and J. Kendrick-Jones. 2001. Myosin VI isoform localized to clathrin-coated vesicles with a role in clathrin-mediated endocytosis. *Embo J.* 20:3676-84.

Butler, M.H., C. David, G.-C. Ochoa, Z. Freyberg, L. Daniell, D. Grabs, O. Cremona, and P. De Camilli. 1997. Amphiphysin II (SH3P9; BIN1), a member of the amphiphysin/RVS family, is localized in the cortical cytomatrix of axon initial segments and nodes of Ranvier in brain and around T-tubules in skeletal muscle. *Journal of Cell Biology.* 137:1355-1367.

Ceccarelli, B., W.P. Hurlbut, and A. Mauro. 1973. Turnover of transmitter and synaptic vesicles at the frog neuromuscular junction. *Journal of Cell Biology.* 57:499-524.

Cestra, G., L. Castagnoli, L. Dente, O. Minenkova, A. Petrelli, N. Migone, U. Hoffmuller, J. Schneider-Mergener, and G. Cesareni. 1999. The SH3 domains of endophilin and amphiphysin bind to the proline-rich region of synaptojanin 1 at distinct sites that display an unconventional binding specificity. *J Biol Chem.* 274:32001-7.

Chapman, E.R. 2002. Synaptotagmin: a Ca(2+) sensor that triggers exocytosis? *Nat Rev Mol Cell Biol.* 3:498-508.

Chapman, E.R., P.I. Hanson, S. An, and R. Jahn. 1995. Ca2+ regulates the interaction between synaptotagmin and syntaxin 1. *Journal of Biological Chemistry.* 270:23667-71.

Chen, H., S. Fre, V.I. Slepnev, M.R. Capua, K. Takei, M.H. Butler, P.P. Di Fiore, and P. De Camilli. 1998. Epsin is an EH-domain-binding protein implicated in clathrin-mediated endocytosis [In Process Citation]. *Nature.* 394:793-7.

Chen, M.S., R.A. Obar, C.C. Schroeder, T.W. Austin, C.A. Poodry, S.C. Wadsworth, and R.B. Vallee. 1991. Multiple forms of dynamin are encoded by shibire, a Drosophila gene involved in endocytosis. *Nature.* 351:583-586.

Chernomordik, L. 1996. Non-bilayer lipids and biological fusion intermediates. *Chem Phys Lipids.* 81:203-13.

Chi, P., P. Greengard, and T.A. Ryan. 2001. Synapsin dispersion and reclustering during synaptic activity. *Nat Neurosci.* 4:1187-93.

Chu, D.S., B. Pishvaee, and G.S. Payne. 1999. A modulatory role for clathrin light chain phosphorylation in Golgi membrane protein localization during vegetative growth and during the mating response of Saccharomyces cerevisiae. *Mol Biol Cell.* 10:713-26.

Couteaux, R., and M. Pecot-Dechavassine. 1970. Synaptic vesicles and pouches at the level of "active zones" of the neuromuscular junction. *C.R. Hebd. Seances Acad. Sci. D. Sci. Nat.* 217:2346-2349.

Cowan, W.M., and E.R. Kandel. 2001. Brief History of Synapses and Synaptic Transmission. *In* Synapses. W.M. Cowan, T.C. Sudhof, and C.F. Stevens, editors. Johns Hopkins University Press, Baltimore. 1-87.

Cremona, O., and P. De Camilli. 2001. Phosphoinositides in membrane traffic at the synapse. *J Cell Sci.* 114:1041-52.

Cremona, O., G. Di Paolo, M.R. Wenk, A. Luthi, W.T. Kim, K. Takei, L. Daniell, Y. Nemoto, S.B. Shears, R.A. Flavell, D.A. McCormick, and P. De Camilli. 1999. Essential role of phosphoinositide metabolism in synaptic vesicle recycling. *Cell.* 99:179-88.

Czech, M.P. 2003. Dynamics of phosphoinositides in membrane retrieval and insertion. *Annu Rev Physiol.* 65:791-815.

Dabora, S.L., and M.P. Sheetz. 1988. The microtubule-dependent formation of a tubulovesicular network with characteristics of the ER from cultured cell extracts. *Cell.* 54:27-35.

Damke, H., T. Baba, D.E. Warnock, and S.L. Schmid. 1994. Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. *Journal of Cell Biology.* 127:915-934.

Danino, D., and J.E. Hinshaw. 2001. Dynamin family of mechanoenzymes. *Curr Opin Cell Biol.* 13:454-60.

David, C., P.S. McPherson, O. Mundigl, and P. De Camilli. 1996. A role of amphiphysin in synaptic vesicle endocytosis suggested by its binding to dynamin in nerve terminals. *Proc Natl Acad Sci U S A.* 93:331-335.

De Camilli, P., F. Benfenati, F. Valtorta, and P. Greengard. 1990. The synapsins. *Annual Review of Cell Biology.* 6:433-460.

De Camilli, P., R. Cameron, and P. Greengard. 1983a. Synapsin I(protein I) a nerve terminal specific phosphoprotein. Its general distribution in synapses of the central and peripheral nervous system demonstrated by immunofluorescence in froxen and plastic sections. *Journal of Cell Biology.* 96:1337-1354.

De Camilli, P., S.M. Harris, Jr., W.B. Huttner, and P. Greengard. 1983b. Synapsin I (Protein I), a nerve terminal-specific phosphoprotein. *Journal of Cell Biology.* 96:1355-73.

De Camilli, P., V. Haucke, K. Takei, and E. Mugnaini. 2001a. The structure of synapses. *In* Synapses. W.M. Cowan, T.C. Sudhof, and C.F. Stevents, editors. Johns Hopkins University Press, Baltimore. 89-133.

De Camilli, P., V.I. Slepnev, O. Shupliakov, and L. Brodin. 2001b. Synaptic vesicle endocytosis. *In* Synapses. W.M. Cowan, T.C. Sudhof, and C.F. Stevens, editors. Johns Hopkins University Press, Baltimore. 217-274.

De Camilli, P., and K. Takei. 1996. Molecular mechanisms in synaptic vesicle endocytosis and recycling. *Neuron.* 16:481-486.

de Figueiredo, P., A. Doody, R.S. Polizotto, D. Drecktrah, S. Wood, M. Banta, M.S. Strang, and W.J. Brown. 2001. Inhibition of transferrin recycling and endosome tubulation by phospholipase A2 antagonists. *J Biol Chem.* 276:47361-70.

de Figueiredo, P., D. Drecktrah, J.A. Katzenellenbogen, M. Strang, and W.J. Brown. 1998. Evidence that phospholipase A2 activity is required for Golgi complex and trans Golgi network membrane tubulation. *Proc Natl Acad Sci U S A.* 95:8642-7.

de Heuvel, E., A.W. Bell, A.R. Ramjaun, K. Wong, W.S. Sossin, and P.S. McPherson. 1997. Identification of the major synaptojanin-binding proteins in brain. *Journal of Biological Chemistry.* 272:8710-6.

De Robertis, E., and H.S. Bennett. 1955. Some features of the submicroscopic morpphology of synapses in frog and earthworm. *J. Biophys. Biochem. Cytol.* 1:47-58.

Del Castillo, J., and B. Katz. 1954. Quantal components of the end-plate potential. *J. Physiol.* 124:560-573.

Denzer, K., M.J. Kleijmeer, H.F. Heijnen, W. Stoorvogel, and H.J. Geuze. 2000. Exosome: from internal vesicle of the multivesicular body to intercellular signaling device. *J Cell Sci.* 113 Pt 19:3365-74.

Di Paolo, G., S. Sankaranarayanan, M.R. Wenk, L. Daniell, E. Perucco, B.J. Caldarone, R. Flavell, M.R. Picciotto, T.A. Ryan, O. Cremona, and P. De Camilli. 2002. Decreased Synaptic Vesicle Recycling Efficiency and Cognitive Deficits in Amphiphysin 1 Knockout Mice. *Neuron.* 33:789-804.

Drab, M., P. Verkade, M. Elger, M. Kasper, M. Lohn, B. Lauterbach, J. Menne, C. Lindschau, F. Mende, F.C. Luft, A. Schedl, H. Haller, and T.V. Kurzchalia. 2001. Loss of caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice. *Science.* 293:2449-52.

Drake, M.T., Y. Zhu, and S. Kornfeld. 2000. The assembly of AP-3 adaptor complex-containing clathrin-coated vesicles on synthetic liposomes. *Mol Biol Cell.* 11:3723-36.

Dreier, L., and T.A. Rapoport. 2000. In vitro formation of the endoplasmic reticulum occurs independently of microtubules by a controlled fusion reaction. *J Cell Biol.* 148:883-98.

Dupre, S., C. Volland, and R. Haguenauer-Tsapis. 2001. Membrane transport: ubiquitylation in endosomal sorting. *Curr Biol.* 11:R932-4.

Elmqvist, D., and D.M. Quastel. 1965. A quantitative study of end-plate potentials in isolated human muscle. *J Physiol.* 178:505-29.

Evans, E.A. 1974. Bending resistance and chemically induced moments in membrane bilayers. *Biophys J.* 14:923-31.

Falk, M.M. 2000. Biosynthesis and structural composition of gap junction intercellular membrane channels. *Eur J Cell Biol.* 79:564-74.

Farge, E., and P.F. Devaux. 1992. Shape changes of giant liposomes induced by an asymmetric transmembrane distribution of phospholipids. *Biophys J.* 61:347-57.

Farge, E., D.M. Ojcius, A. Subtil, and A. Dautry-Varsat. 1999. Enhancement of endocytosis due to aminophospholipid transport across the plasma membrane of living cells. *Am J Physiol.* 276:C725-33.

Farsad, K., N. Ringstad, K. Takei, S.R. Floyd, K. Rose, and P. De Camilli. 2001. Generation of high curvature membranes mediated by direct endophilin bilayer interactions. *J Cell Biol.* 155:193-200.

Fatt, P., and B. Katz. 1951. An analysis of the end-plate potential recorded with an intra-cellular electrode. *J. Physiol.* 115:320-370.

Fatt, P., and B. Katz. 1952. Spontaneous subthreshold activity at motor nerve endings. *J. Physiol.* 117:109-128.

Fergestad, T., and K. Broadie. 2001. Interaction of stoned and synaptotagmin in synaptic vesicle endocytosis. *J Neurosci.* 21:1218-27.

Fergestad, T., W.S. Davis, and K. Broadie. 1999. The stoned proteins regulate synaptic vesicle recycling in the presynaptic terminal. *J Neurosci.* 19:5847-60.

Fernandez, I., Y. Ying, J. Albanesi, and R.G. Anderson. 2002. Mechanism of caveolin filament assembly. *Proc Natl Acad Sci U S A.* 99:11193-8.

Fesce, R., F. Grohovaz, F. Valtorta, and J. Meldolesi. 1994. Neurotransmitter release: Fusion or `kiss-and-run'? *Trends in Cell Biology.* 4:1-4.

Floyd, S.R., E.B. Porro, V.I. Slepnev, G.C. Ochoa, L.H. Tsai, and P. De Camilli. 2001. Amphiphysin 1 binds the cyclin-dependent kinase (cdk) 5 regulatory subunit p35 and is phosphorylated by cdk5 and cdc2. *J Biol Chem.* 276:8104-10.

Ford, M.G., I.G. Mills, B.J. Peter, Y. Vallis, G.J. Praefcke, P.R. Evans, and H.T. McMahon. 2002. Curvature of clathrin-coated pits driven by epsin. *Nature.* 419:361-6.

Ford, M.G., B.M. Pearse, M.K. Higgins, Y. Vallis, D.J. Owen, A. Gibson, C.R. Hopkins, P.R. Evans, and H.T. McMahon. 2001. Simultaneous binding of PtdIns(4,5)P2

and clathrin by AP180 in the nucleation of clathrin lattices on membranes. *Science*. 291:1051-5.

Fra, A.M., E. Williamson, K. Simons, and R.G. Parton. 1995. De novo formation of caveolae in lymphocytes by expression of VIP21-caveolin. *Proc Natl Acad Sci U S A*. 92:8655-9.

Gad, H., Bloom, O., Low, P., De Camilli, P., Slepnev, V., Shupliakov, O., Brodin, L. 2000. Dynamin Plays a Role in Early Stages of Clathrin-mediated Synaptic Vesicle Endocytosis. *Mol Biol Cell, Suppl.* 11:216a.

Gad, H., P. Low, E. Zotova, L. Brodin, and O. Shupliakov. 1998. Dissociation between Ca2+-triggered synaptic vesicle exocytosis and clathrin-mediated endocytosis at a central synapse. *Neuron*. 21:607-16.

Gad, H., N. Ringstad, P. Low, O. Kjaerulff, J. Gustafsson, M. Wenk, G. Di Paolo, Y. Nemoto, J. Crun, M.H. Ellisman, P. De Camilli, O. Shupliakov, and L. Brodin. 2000. Fission and uncoating of synaptic clathrin-coated vesicles are perturbed by disruption of interactions with the SH3 domain of endophilin. *Neuron*. 27:301-12.

Garrus, J.E., U.K. von Schwedler, O.W. Pornillos, S.G. Morham, K.H. Zavitz, H.E. Wang, D.A. Wettstein, K.M. Stray, M. Cote, R.L. Rich, D.G. Myszka, and W.I. Sundquist. 2001. Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. *Cell*. 107:55-65.

Geli, M.I., and H. Riezman. 1996. Role of type I myosins in receptor-mediated endocytosis in yeast. *Science*. 272:533-535.

Geppert, M., Y. Goda, R.E. Hammer, C. Li, T.W. Rosahl, C.F. Stevens, and T.C. Südhof. 1994. Synaptotagmin I: A major Ca2+ sensor for transmitter release at a central synapse. *Cell*. 79:717-727.

Gilliam, T.C., E.R. Kandel, and T.M. Jessell. 2000. Genes and Behavior. *In* Principles of Neural Science. E.R. Kandel, J.H. Schwartz, and T.M. Jessell, editors. McGraw-Hill, New York. 1414.

Goldberg, J. 1998. Structural basis for activation of ARF GTPase: mechanisms of guanine nucleotide exchange and GTP-myristoyl switching. *Cell*. 95:237-48.

Gonzalez-Gaitan, M., and H. Jackle. 1997. Role of Drosophila alpha-adaptin in presynaptic vesicle recycling. *Cell*. 88:767-76.

Grabs, D., V.I. Slepnev, Z. Songyang, C. David, M. Lynch, L.C. Cantley, and P. De Camilli. 1997. The SH3 domain of amphiphysin binds the proline-rich domain of dynamin at a single site that defines a new SH3 binding consensus sequence. *J Biol Chem*. 272:13419-25.

Gray, E.G., and V.P. Whittaker. 1962. The isolation of nerve endings from brain: an elctron microscopic study of cell fragments derived by homogenization and centrifugation. *J. Anat.* 96:79-88.

Gruenberg, J., and F.R. Maxfield. 1995. Membrane transport in the endocytic pathway. *Current Opinion in Cell Biology.* 7:552-63.

Guichet, A., T. Wucherpfennig, V. Dudu, S. Etter, M. Wilsch-Brauniger, A. Hellwig, M. Gonzalez-Gaitan, W.B. Huttner, and A.A. Schmidt. 2002. Essential role of endophilin A in synaptic vesicle budding at the Drosophila neuromuscular junction. *Embo J.* 21:1661-1672.

Haffner, C., K. Takei, M. Butler, C. David, A. Hudson, D. Grabs, and P. De Camilli. 1996. Evolutionary conservation of synaptojanin, an inositol 5-phopshatase highly concentrated at clathrin coats in nerve terminals. *Molecular Biology of the Cell.* 7 Suppl.:78a.

Haffner, C., K. Takei, H. Chen, N. Ringstad, A. Hudson, M.H. Butler, A.E. Salcini, P.P. Di Fiore, and P. De Camilli. 1997. Synaptojanin 1: localization on coated endocytic intermediates in nerve terminals and interaction of its 170 kDa isoform with Eps15. *FEBS Lett.* 419:175-80.

Haimann, C., F. Torri Tarelli, R. Fesce, and B. Ceccarelli. 1985. Measurement of quantal secretion induced by ouabain and its correlation with depletion of synaptic vesicles. *Journal of Cell Biology.* 101:1953-1965.

Hannah, M.J., A.A. Schmidt, and W.B. Huttner. 1999. Synaptic vesicle biogenesis. *Annu Rev Cell Dev Biol.* 15:733-98.

Hasson, T., and M.S. Mooseker. 1995. Molecular motors, membrane movements and physiology: emerging roles for myosins. *Curr Opin Cell Biol.* 7:587-94.

Haucke, V., and P. De Camilli. 1999. AP-2 recruitment to synaptotagmin stimulated by tyrosine-based endocytic motifs. *Science.* 285:1268-71.

Haucke, V., M.R. Wenk, E.R. Chapman, K. Farsad, and P. De Camilli. 2000. Dual interaction of synaptotagmin with &mgr;2- and alpha-adaptin facilitates clathrin-coated pit nucleation. *Embo J.* 19:6011-9.

Henley, J.R., H. Cao, and M.A. McNiven. 1999. Participation of dynamin in the biogenesis of cytoplasmic vesicles. *Faseb J.* 13 Suppl 2:S243-7.

Heuser, J. 1989. The role of coated vesicles in recycling of synaptic vesicle membrane. *Cell Biology International Reports.* 13:1063-76.

Heuser, J.E., and J. Keen. 1988. Deep-etch visualization of proteins involved in clathrin assembly. *Journal of Cell Biology*. 107:877-86.

Heuser, J.E., and T.S. Reese. 1973. Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. *Journal of Cell Biology*. 57:315-344.

Heuser, J.E., and T.S. Reese. 1977. Structure of the synapse. American Physiological Society, Bethesda. 261-294 pp.

Heuser, J.E., T.S. Reese, M.J. Dennis, Y. Jan, L. Jan, and L. Evans. 1979. Synaptic vesicle exocytosis captured by quick freezing and correlated with quantal transmitter release. *J Cell Biol*. 81:275-300.

Hicke, L. 2001. A new ticket for entry into budding vesicles-ubiquitin. *Cell*. 106:527-30.

Hill, E., J. van Der Kaay, C.P. Downes, and E. Smythe. 2001. The role of dynamin and its binding partners in coated pit invagination and scission. *J Cell Biol*. 152:309-23.

Hinshaw, J.E. 2000. Dynamin and its role in membrane fission. *Annu Rev Cell Dev Biol*. 16:483-519.

Hinshaw, J.E., and S.L. Schmid. 1995. Dynamin self-assembles into rings suggesting a mechanism for coated vesicle budding. *Nature*. 374:190-2.

Hirokawa, N. 1998. Kinesin and dynein superfamily proteins and the mechanism of organelle transport. *Science*. 279:519-26.

Holopainen, J.M., M.I. Angelova, and P.K. Kinnunen. 2000. Vectorial budding of vesicles by asymmetrical enzymatic formation of ceramide in giant liposomes. *Biophys J*. 78:830-8.

Hristova, K., C.E. Dempsey, and S.H. White. 2001. Structure, location, and lipid perturbations of melittin at the membrane interface. *Biophys J*. 80:801-11.

Hunt, J.M., K. Bommert, M.P. Charlton, A. Kistner, E. Habermann, G.J. Augustine, and H. Betz. 1994. A post-docking role for synaptobrevin in synaptic vesicle fusion. *Neuron*. 12:1269-1279.

Hurley, J.H., and S. Misra. 2000. Signaling and subcellular targeting by membrane-binding domains. *Annu Rev Biophys Biomol Struct*. 29:49-79.

Huttner, W.B., W. Schiebler, P. Greengard, and P. De Camilli. 1983. Synapsin I (protein I), a nerve terminal-specific phosphoprotein. *Journal of Cell Biology*. 96:1374-88.

Huttner, W.B., and A. Schmidt. 2000. Lipids, lipid modification and lipid-protein interaction in membrane budding and fission--insights from the roles of endophilin A1 and synaptophysin in synaptic vesicle endocytosis. *Curr Opin Neurobiol.* 10:543-51.

Jahn, R., J. Hell, and P.R.w. Maycox. 1990. Synaptic vesicles: key organelles involved in neurotransmission. *Journal de Physiologie.* 84:128-133.

Jarousse, N., and R.B. Kelly. 2001. Endocytotic mechanisms in synapses. *Curr Opin Cell Biol.* 13:461-9.

Kadota, K., and T. Kadota. 1973. Isolation of coated vesicles, plain synaptic vesicles, and flocculent material from a crude synaptosome fraction of guinea pig whole brain. *J Cell Biol.* 58:135-51.

Kandel, E.R. 2000. Nerve Cells and Behavior. *In* Principles of Neural Science. E.R. Kandel, J.H. Schwarz, and T.M. Jessel, editors. McGraw-Hill, New York. 19-35.

Katz, B., and R. Miledi. 1965. The qauntal release of transmitter substances. Springer-Verlag, New York.

Katzmann, D.J., M. Babst, and S.D. Emr. 2001. Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I. *Cell.* 106:145-55.

Katzmann, D.J., G. Odorizzi, and S.D. Emr. 2002. Receptor downregulation and multivesicular-body sorting. *Nat Rev Mol Cell Biol.* 3:893-905.

Kim, W.T., S. Chang, L. Daniell, O. Cremona, G. Di Paolo, and P. De Camilli. 2002. Delayed reentry of recycling vesicles into the fusion-competent synaptic vesicle pool in synaptojanin 1 knockout mice. *Proc Natl Acad Sci U S A.* 99:17143-8.

Kirchhausen, T. 2000. Clathrin. *Annu Rev Biochem.* 69:699-727.

Klein, D.E., A. Lee, D.W. Frank, M.S. Marks, and M.A. Lemmon. 1998. The pleckstrin homology domains of dynamin isoforms require oligomerization for high affinity phosphoinositide binding. *J Biol Chem.* 273:27725-33.

Klingauf, J., E.T. Kavalali, and R.W. Tsien. 1998. Kinetics and regulation of fast endocytosis at hippocampal synapses. *Nature.* 394:581-5.

Klockow, B., W. Tichelaar, D.R. Madden, H.H. Niemann, T. Akiba, K. Hirose, and D.J. Manstein. 2002. The dynamin A ring complex: molecular organization and nucleotide-dependent conformational changes. *Embo J.* 21:240-50.

Klopfenstein, D.R., F. Kappeler, and H.P. Hauri. 1998. A novel direct interaction of endoplasmic reticulum with microtubules. *Embo J.* 17:6168-77.

Klumperman, J. 2000. Transport between ER and Golgi. *Curr Opin Cell Biol.* 12:445-9.

Koenig, J.H., and K. Ikeda. 1989. Disappearance and reformation of synaptic vesicle membrane upon transmitter release observed under reversible blockage of membrane retrieval. *Journal of Neuroscience.* 9:3844-3860.

Koenig, J.H., and K. Ikeda. 1996. Synaptic vesicles have two distinct recycling pathways. *Journal of Cell Biology.* 135:797-808.

Ktistakis, N.T., H.A. Brown, M.G. Waters, P.C. Sternweis, and M.G. Roth. 1996. Evidence that phospholipase D mediates ADP ribosylation factor-dependent formation golgi coated vesicles. *Journal of Cell Biology.* 134:295-306.

Kuehn, M.J., and R. Schekman. 1997. COPII and secretory cargo capture into transport vesicles. *Curr Opin Cell Biol.* 9:477-83.

Kuffler, S.W., and D. Yoshikami. 1975. The number of transmitter molecules in a quantum: an estimate from iontophoretic application of acetylcholine at the neuromuscular synapse. *J Physiol.* 251:465-82.

Lam, Y.H., S.R. Wassall, C.J. Morton, R. Smith, and F. Separovic. 2001. Solid-state NMR structure determination of melittin in a lipid environment. *Biophys J.* 81:2752-61.

Lee, C., S.R. Kim, J.K. Chung, M.A. Frohman, M.W. Kilimann, and S.G. Rhee. 2000. Inhibition of phospholipase D by amphiphysins. *J Biol Chem.* 275:18751-8.

Lee, E., M. Marcucci, L. Daniell, M. Pypaert, O.A. Weisz, G.C. Ochoa, K. Farsad, M.R. Wenk, and P. De Camilli. 2002. Amphiphysin 2 (Bin1) and T-tubule biogenesis in muscle. *Science.* 297:1193-6.

Lee, S., T. Furuya, T. Kiyota, N. Takami, K. Murata, Y. Niidome, D.E. Bredesen, H.M. Ellerby, and G. Sugihara. 2001. De novo-designed peptide transforms Golgi-specific lipids into Golgi-like nanotubules. *J Biol Chem.* 276:41224-8.

Li, C., B. Ullrich, J.Z. Zhang, R.G.W. Anderson, N. Brose, and T.C. Südhof. 1995. Ca2+-dependent and -independent activities of neural and non-neural synaptotagmins. *Nature.* 375:594-599.

Lin, R.C., and R.H. Scheller. 2000. Mechanisms of synaptic vesicle exocytosis. *Annu Rev Cell Dev Biol.* 16:19-49.

Lipowsky, R. 1991. The conformation of membranes. *Nature.* 349:475-81.

Lippincott-Schwartz, J., T.H. Roberts, and K. Hirschberg. 2000. Secretory protein trafficking and organelle dynamics in living cells. *Annu Rev Cell Dev Biol.* 16:557-89.

Lippincott-Schwartz, J., L. Yuan, C. Tipper, M. Amherdt, L. Orci, and R.D. Klausner. 1991. Brefeldin A's effects on endosomes, lysosomes, and the TGN suggest a general mechanism for regulating organelle structure and membrane traffic. *Cell.* 67:601-16.

Liscovitch, M. 1996. Phospholipase D: role in signal transduction and membrane traffic. *J Lipid Mediat Cell Signal.* 14:215-21.

Littleton, J.T., M. Stern, M. Perin, and H.J. Bellen. 1994. Calcium dependence of neurotransmitter release and rate of spontaneous vesicle fusions are altered in Drosophila synaptotagmin mutants. *Proceedings of the National Academy of Sciences of the United States of America.* 91:10888-10892.

Littleton, J.T., M. Stern, K. Schulze, M. Perin, and H.J. Bellen. 1993. Mutational analysis of Drosophila synaptotagmin demonstrates its essential role in Ca(2+)-activated neurotransmitter release. *Cell.* 74:1125-34.

Liu, G., and R.W. Tsien. 1995. Properties of synaptic transmission at single hippocampal synaptic boutons. *Nature.* 375:404-8.

Liu, S.H., M.L. Wong, C.S. Craik, and F.M. Brodsky. 1995. Regulation of clathrin assembly and trimerization defined using recombinant triskelion hubs. *Cell.* 83:257-67.

Mao, Y., J. Chen, J.A. Maynard, B. Zhang, and F.A. Quiocho. 2001. A novel all helix fold of the AP180 amino-terminal domain for phosphoinositide binding and clathrin assembly in synaptic vesicle endocytosis. *Cell.* 104:433-40.

Marks, B., and H.T. McMahon. 1998. Calcium triggers calcineurin-dependent synaptic vesicle recycling in mammalian nerve terminals. *Curr Biol.* 8:740-9.

Marks, B., M.H. Stowell, Y. Vallis, I.G. Mills, A. Gibson, C.R. Hopkins, and H.T. McMahon. 2001. GTPase activity of dynamin and resulting conformation change are essential for endocytosis. *Nature.* 410:231-5.

Martina, J.A., C.J. Bonangelino, R.C. Aguilar, and J.S. Bonifacino. 2001. Stonin 2: an adaptor-like protein that interacts with components of the endocytic machinery. *J Cell Biol.* 153:1111-20.

Martinez-Arca, S., S. Coco, G. Mainguy, U. Schenk, P. Alberts, P. Bouille, M. Mezzina, A. Prochiantz, M. Matteoli, D. Louvard, and T. Galli. 2001. A common exocytotic mechanism mediates axonal and dendritic outgrowth. *J Neurosci.* 21:3830-8.

Matsuoka, K., L. Orci, M. Amherdt, S.Y. Bednarek, S. Hamamoto, R. Schekman, and T. Yeung. 1998. COPII-coated vesicle formation reconstituted with purified coat proteins and chemically defined liposomes. *Cell.* 93:263-75.

Maycox, P.R., E. Link, A. Reetz, S.A. Morris, and R. Jahn. 1992. Clathrin-coated vesicles in nervous tissue are involved primarily in synaptic vesicle recycling. *Journal of Cell Biology.* 118:1379-1388.

McIlvain, J.M., Jr., C. Lamb, S. Dabora, and M.P. Sheetz. 1993. Microtubule motor-dependent formation of tubulovesicular networks from endoplasmic reticulum and Golgi membranes. *Methods Cell Biol.* 39:227-36.

McKinley, D.N. 1983. Model for transformations of the clathrin lattice in the coated vesicle pathway. *J Theor Biol.* 103:405-19.

McMahon, H.T. 1999. Endocytosis: an assembly protein for clathrin cages. *Curr Biol.* 9:R332-5.

McMahon, H.T., P. Wigge, and C. Smith. 1997. Clathrin interacts specifically with amphiphysin and is displaced by dynamin. *FEBS Lett.* 413:319-22.

McNew, J.A., F. Parlati, R. Fukuda, R.J. Johnston, K. Paz, F. Paumet, T.H. Sollner, and J.E. Rothman. 2000. Compartmental specificity of cellular membrane fusion encoded in SNARE proteins. *Nature.* 407:153-9.

McNiven, M.A., H. Cao, K.R. Pitts, and Y. Yoon. 2000. The dynamin family of mechanoenzymes: pinching in new places. *Trends Biochem Sci.* 25:115-20. 3_00001538 3_00001538.

McPherson, P.S., E.P. Garcia, V.I. Slepnev, C. David, X.M. Zhang, D. Grabs, W.S. Sossin, R. Bauerfeind, Y. Nemoto, and P. De Camilli. 1996. A presynaptic inositol-5-phosphatase. *Nature.* 379:353-357.

Mercier, C., J.F. Dubremetz, B. Rauscher, L. Lecordier, L.D. Sibley, and M.F. Cesbron-Delauw. 2002. Biogenesis of nanotubular network in Toxoplasma parasitophorous vacuole induced by parasite proteins. *Mol Biol Cell.* 13:2397-409.

Merrifield, C.J., M.E. Feldman, L. Wan, and W. Almers. 2002. Imaging actin and dynamin recruitment during invagination of single clathrin-coated pits. *Nat Cell Biol.* 4:691-8.

Micheva, K.D., A.R. Ramjaun, B.K. Kay, and P.S. McPherson. 1997. SH3 domain-dependent interactions of endophilin with amphiphysin. *FEBS Lett.* 414:308-12.

Mikoshiba, K., M. Fukuda, J.E. Moreira, F.M. Lewis, M. Sugimori, M. Niinobe, and R. Llinas. 1995. Role of the C2A domain of synaptotagmin in transmitter release as determined by specific antibody injection into the squid giant synapse preterminal. *Proc Natl Acad Sci U S A.* 92:10703-7.

Modregger, J., A.A. Schmidt, B. Ritter, W.B. Huttner, and M. Plomann. 2003. Characterization of Endophilin B1b, a brain-specific membrane-associated lysophosphatidic acid acyl transferase with properties distinct from endophilin A1. *J Biol Chem.* 278:4160-7.

Molday, R.S., D. Hicks, and L. Molday. 1987. Peripherin. A rim-specific membrane protein of rod outer segment discs. *Invest Ophthalmol Vis Sci.* 28:50-61.

Morris, S.M., S.D. Arden, R.C. Roberts, J. Kendrick-Jones, J.A. Cooper, J.P. Luzio, and F. Buss. 2002. Myosin VI binds to and localises with Dab2, potentially linking receptor-mediated endocytosis and the actin cytoskeleton. *Traffic.* 3:331-41.

Murata, M., J. Peranen, R. Schreiner, F. Wieland, T.V. Kurzchalia, and K. Simons. 1995. VIP21/caveolin is a cholesterol-binding protein. *Proc Natl Acad Sci U S A.* 92:10339-43.

Musacchio, A., C.J. Smith, A.M. Roseman, S.C. Harrison, T. Kirchhausen, and B.M. Pearse. 1999. Functional organization of clathrin in coats: combining electron cryomicroscopy and X-ray crystallography. *Mol Cell.* 3:761-70.

Nakata, T., S. Terada, and N. Hirokawa. 1998. Visualization of the dynamics of synaptic vesicle and plasma membrane proteins in living axons. *J Cell Biol.* 140:659-74.

Nathke, I.S., J. Heuser, A. Lupas, J. Stock, C.W. Turck, and F.M. Brodsky. 1992. Folding and trimerization of clathrin subunits at the triskelion hub. *Cell.* 68:899-910.

Neher, E. 1993. Secretion without full fusion. *Nature.* 363:497-8.

Nonet, M.L., K. Grundahl, B.J. Meyer, and J.B. Rand. 1993. Synaptic function is impaired but not eliminated in C. elegans mutants lacking synaptotagmin. *Cell.* 73:1291-1305.

Nonet, M.L., A.M. Holgado, F. Brewer, C.J. Serpe, B.A. Norbeck, J. Holleran, L. Wei, E. Hartwieg, E.M. Jorgensen, and A. Alfonso. 1999. UNC-11, a Caenorhabditis elegans AP180 homologue, regulates the size and protein composition of synaptic vesicles. *Mol Biol Cell.* 10:2343-60.

Nossal, R. 2001. Energetics of clathrin basket assembly. *Traffic*. 2:138-47.

Ochoa, G.C., V.I. Slepnev, L. Neff, N. Ringstad, K. Takei, L. Daniell, W. Kim, H. Cao, M. McNiven, R. Baron, and P. De Camilli. 2000. A functional link between dynamin and the actin cytoskeleton at podosomes. *J Cell Biol.* 150:377-89.

Odorizzi, G., M. Babst, and S.D. Emr. 1998. Fab1p PtdIns(3)P 5-kinase function essential for protein sorting in the multivesicular body. *Cell.* 95:847-58.

Odorizzi, G., M. Babst, and S.D. Emr. 2000. Phosphoinositide signaling and the regulation of membrane trafficking in yeast. *Trends Biochem Sci.* 25:229-35.

Okada, Y., H. Yamazaki, Y. Sekine-Aizawa, and N. Hirokawa. 1995. The neuron-specific kinesin superfamily protein KIF1A is a unique monomeric motor for anterograde axonal transport of synaptic vesicle precursors. *Cell.* 81:769-80.

Owen, D.J., P. Wigge, Y. Vallis, J.D. Moore, P.R. Evans, and H.T. McMahon. 1998. Crystal structure of the amphiphysin-2 SH3 domain and its role in the prevention of dynamin ring formation. *Embo J.* 17:5273-85.

Palay, S.L., and G. Palade. 1955. The fine structure of neurons. *J. Biophys. Biochem. Cytol.* 1:69-88.

Parlati, F., J.A. McNew, R. Fukuda, R. Miller, T.H. Sollner, and J.E. Rothman. 2000. Topological restriction of SNARE-dependent membrane fusion. *Nature*. 407:194-8.

Pasqualato, S., J. Menetrey, M. Franco, and J. Cherfils. 2001. The structural GDP/GTP cycle of human Arf6. *EMBO Rep.* 2:234-8.

Pasqualato, S., L. Renault, and J. Cherfils. 2002. Arf, Arl, Arp and Sar proteins: a family of GTP-binding proteins with a structural device for 'front-back' communication. *EMBO Rep.* 3:1035-41.

Pelkmans, L., D. Puntener, and A. Helenius. 2002. Local actin polymerization and dynamin recruitment in SV40-induced internalization of caveolae. *Science.* 296:535-9.

Pellizzari, R., O. Rossetto, G. Schiavo, and C. Montecucco. 1999. Tetanus and botulinum neurotoxins: mechanism of action and therapeutic uses. *Philos Trans R Soc Lond B Biol Sci.* 354:259-68.

Phillips, A.M., M. Smith, M. Ramaswami, and L.E. Kelly. 2000. The products of the Drosophila stoned locus interact with synaptic vesicles via synaptotagmin. *J Neurosci.* 20:8254-61.

Piper, R.C., and J.P. Luzio. 2001. Late endosomes: sorting and partitioning in multivesicular bodies. *Traffic*. 2:612-21.

Pornillos, O., J.E. Garrus, and W.I. Sundquist. 2002. Mechanisms of enveloped RNA virus budding. *Trends Cell Biol.* 12:569-79.

Prekeris, R., D.L. Foletti, and R.H. Scheller. 1999. Dynamics of tubulovesicular recycling endosomes in hippocampal neurons. *J Neurosci.* 19:10324-37.

Qualmann, B., M.M. Kessels, and R.B. Kelly. 2000. Molecular Links between Endocytosis and the Actin Cytoskeleton. *J Cell Biol.* 150:F111-F116.

Ramjaun, A.R., K.D. Micheva, I. Bouchelet, and P.S. McPherson. 1997. Identification and characterization of a nerve terminal-enriched amphiphysin isoform. *J Biol Chem.* 272:16700-6.

Ramjaun, A.R., J. Philie, E. de Heuvel, and P.S. McPherson. 1999. The N terminus of amphiphysin II mediates dimerization and plasma membrane targeting. *J Biol Chem.* 274:19785-91.

Regehr, W.G., and C.F. Stevens. 2001. Physiology of synaptic transmission and short-term plasticity. *In* Synapses. W.M. Cowan, T.C. Sudhof, and C.F. Stevens, editors. Johns Hopkins University Press, Baltimore. 135-175.

Richnau, N., and P. Aspenstrom. 2001. RICH-a RhoGAP domain-containing protein involved in signalling by Cdc42 and Rac1. *J Biol Chem.* 28:28.

Rikhy, R., V. Kumar, R. Mittal, and K.S. Krishnan. 2002. Endophilin is critically required for synapse formation and function in Drosophila melanogaster. *J Neurosci.* 22:7478-84.

Ringstad, N., H. Gad, P. Low, G. Di Paolo, L. Brodin, O. Shupliakov, and P. De Camilli. 1999. Endophilin/SH3p4 is required for the transition from early to late stages in clathrin-mediated synaptic vesicle endocytosis. *Neuron.* 24:143-54.

Ringstad, N., Y. Nemoto, and P. De Camilli. 1997. The SH3p4/Sh3p8/SH3p13 protein family: binding partners for synaptojanin and dynamin via a Grb2-like Src homology 3 domain. *Proc Natl Acad Sci U S A.* 94:8569-74.

Ringstad, N., Y. Nemoto, and P. De Camilli. 2001. Differential expression of endophilin 1 and 2 dimers at central nervous system synapses. *J Biol Chem.* 276:40424-30.

Robertson, A.M., and V.J. Allan. 2000. Brefeldin A-dependent membrane tubule formation reconstituted in vitro is driven by a cell cycle-regulated microtubule motor. *Mol Biol Cell.* 11:941-55.

Robinson, P.J., J.P. Liu, K.A. Powell, E.M. Fykse, and T.C. Südhof. 1994. Phosphorylation of dynamin I and synaptic-vesicle recycling. *Trends in Neurosciences.* 17:348-53.

Rodal, S.K., G. Skretting, O. Garred, F. Vilhardt, B. van Deurs, and K. Sandvig. 1999. Extraction of cholesterol with methyl-beta-cyclodextrin perturbs formation of clathrin-coated endocytic vesicles. *Mol Biol Cell.* 10:961-74.

Roos, J., and R.B. Kelly. 1999. The endocytic machinery in nerve terminals surrounds sites of exocytosis. *Curr Biol.* 9:1411-4.

Rosenmund, C., and C.F. Stevens. 1996. Definition of the readily releasable pool of vesicles at hippocampal synapses. *Neuron.* 16:1197-207.

Rosenthal, J.A., H. Chen, V.I. Slepnev, L. Pellegrini, A.E. Salcini, P.P. Di Fiore, and P. De Camilli. 1999. The epsins define a family of proteins that interact with components of the clathrin coat and contain a new protein module. *J Biol Chem.* 274:33959-65.

Rothberg, K.G., J.E. Heuser, W.C. Donzell, Y.S. Ying, J.R. Glenney, and R.G. Anderson. 1992. Caveolin, a protein component of caveolae membrane coats. *Cell.* 68:673-82.

Rothman, J.E. 1994. Mechanisms of intracellular protein transport. *Nature.* 372:55-63.

Rothman, J.E., and G. Warren. 1994. Implications of the SNARE hypothesis for intracellular membrane topology and dynamics. *Current Biology.* 4:220-33.

Roux, A., G. Cappello, J. Cartaud, J. Prost, B. Goud, and P. Bassereau. 2002. A minimal system allowing tubulation with molecular motors pulling on giant liposomes. *Proc Natl Acad Sci U S A.* 99:5394-9.

Ryan, T.A., H. Reuter, B. Wendland, F.E. Schweizer, R.W. Tsien, and S.J. Smith. 1993. The kinetics of synaptic vesicle recycling measured at single presynaptic boutons. *Neuron.* 11:713-724.

Ryan, T.A., and S.J. Smith. 1995. Vesicle pool mobilization during action potential firing at hippocampal synapses. *Neuron.* 14:983-9.

Ryan, T.A., S.J. Smith, and H. Reuter. 1996. The timing of synaptic vesicle endocytosis. *Proceedings of the National Academy of Sciences of the United States of America.* 93:5567-5571.

Salim, K., M.J. Bottomley, E. Querfurth, M.J. Zvelebil, I. Gout, R. Scaife, R.L. Margolis, R. Gigg, C.I.E. Smith, P.C. Driscoll, M.D. Waterfield, and G. Panayotou. 1996. Distinct specificity in the recognition of phosphoinositides by the pleckstrin

homology domains of dynamin and Bruton's tyrosine kinase. *EMBO Journal.* 15:6241-6250.

Schafer, D.A. 2002. Coupling actin dynamics and membrane dynamics during endocytosis. *Curr Opin Cell Biol.* 14:76-81.

Schartz, N.E., N. Chaput, F. Andre, and L. Zitvogel. 2002. From the antigen-presenting cell to the antigen-presenting vesicle: the exosomes. *Curr Opin Mol Ther.* 4:372-81.

Schiavo, G., G. Stenbeck, J.E. Rothman, and T.H. Söllner. 1997. Binding of the synaptic vesicle v-SNARE, synaptotagmin, to the plasma membrane t-SNARE, SNAP-25, can explain docked vesicles at neurotoxin- treated synapses. *Proceedings of the National Academy of Sciences of the United States of America.* 94:997-1001.

Schikorski, T., and C.F. Stevens. 1997. Quantitative ultrastructural analysis of hippocampal excitatory synapses. *J Neurosci.* 17:5858-67.

Schmid, S.L. 1997. Clathrin-coated vesicle formation and protein sorting: an integrated process. *Annu Rev Biochem.* 66:511-48.

Schmidt, A., M. Wolde, C. Thiele, W. Fest, H. Kratzin, A.V. Podtelejnikov, W. Witke, W.B. Huttner, and H.D. Soling. 1999. Endophilin I mediates synaptic vesicle formation by transfer of arachidonate to lysophosphatidic acid. *Nature.* 401:133-41.

Schnitzer, J.E., P. Oh, and D.P. McIntosh. 1996. Role of GTP hydrolysis in fission of caveolae directly from plasma membranes. *Science.* 274:239-42.

Sciaky, N., J. Presley, C. Smith, K.J. Zaal, N. Cole, J.E. Moreira, M. Terasaki, E. Siggia, and J. Lippincott-Schwartz. 1997. Golgi tubule traffic and the effects of brefeldin A visualized in living cells. *J Cell Biol.* 139:1137-55.

Sever, S., A.B. Muhlberg, and S.L. Schmid. 1999. Impairment of dynamin's GAP domain stimulates receptor-mediated endocytosis. *Nature.* 398:481-6.

Sheetz, M.P., R.G. Painter, and S.J. Singer. 1976. Biological membranes as bilayer couples. III. Compensatory shape changes induced in membranes. *J Cell Biol.* 70:193-203.

Sheetz, M.P., and S.J. Singer. 1974. Biological membranes as bilayer couples. A molecular mechanism of drug- erythrocyte interactions. *Proc Natl Acad Sci U S A.* 71:4457-61.

Shorter, J., and G. Warren. 2002. Golgi architecture and inheritance. *Annu Rev Cell Dev Biol.* 18:379-420.

Shpetner, H.S., and R.B. Vallee. 1989. Identification of dynamin, a novel mechanochemical enzyme that mediates interactions between microtubules. *Cell.* 59:421-432.

Shupliakov, O., P. Low, D. Grabs, H. Gad, H. Chen, C. David, K. Takei, P. De Camilli, and L. Brodin. 1997. Synaptic vesicle endocytosis impaired by disruption of dynamin-SH3 domain interactions. *Science.* 276:259-63.

Slepnev, V.I., and P. De Camilli. 2000. Accessory factors in clathrin-dependent synaptic vesicle endocytosis. *Nat Rev Neurosci.* 1:161-72.

Slepnev, V.I., G.C. Ochoa, M.H. Butler, and P. De Camilli. 2000. Tandem arrangement of the clathrin and AP-2 binding domains in amphiphysin 1, and disruption of clathrin coat function mediated by amphiphysin fragments comprising these sites. *J Biol Chem.*

Slepnev, V.I., G.C. Ochoa, M.H. Butler, D. Grabs, and P.D. Camilli. 1998. Role of phosphorylation in regulation of the assembly of endocytic coat complexes. *Science.* 281:821-4.

Smith, C.J., N. Grigorieff, and B.M. Pearse. 1998. Clathrin coats at 21 A resolution: a cellular assembly designed to recycle multiple membrane receptors. *Embo J.* 17:4943-53.

Spang, A., K. Matsuoka, S. Hamamoto, R. Schekman, and L. Orci. 1998. Coatomer, Arf1p, and nucleotide are required to bud coat protein complex I-coated vesicles from large synthetic liposomes. *Proc Natl Acad Sci U S A.* 95:11199-204.

Spruce, A.E., L.J. Breckenridge, A.K. Lee, and W. Almers. 1990. Properties of the fusion pore that forms during exocytosis of a mast cell secretory vesicle. *Neuron.* 4:643-54.

Stahl, P.D., and M.A. Barbieri. 2002. Multivesicular bodies and multivesicular endosomes: the "ins and outs" of endosomal traffic. *Sci STKE.* 2002:PE32.

Stevens, C.F., and T. Tsujimoto. 1995. Estimates for the pool size of releasable quanta at a single central synapse and for the time required to refill the pool. *Proc Natl Acad Sci U S A.* 92:846-9.

Stimson, D.T., P.S. Estes, S. Rao, K.S. Krishnan, L.E. Kelly, and M. Ramaswami. 2001. Drosophila stoned proteins regulate the rate and fidelity of synaptic vesicle internalization. *J Neurosci.* 21:3034-44.

Stowell, M.H., B. Marks, P. Wigge, and H.T. McMahon. 1999. Nucleotide-dependent conformational changes in dynamin: evidence for a mechanochemical molecular spring. *Nat Cell Biol.* 1:27-32.

Subtil, A., I. Gaidarov, K. Kobylarz, M.A. Lampson, J.H. Keen, and T.E. McGraw. 1999. Acute cholesterol depletion inhibits clathrin-coated pit budding. *Proc Natl Acad Sci U S A.* 96:6775-80.

Sweitzer, S.M., and J.E. Hinshaw. 1998. Dynamin undergoes a GTP-dependent conformational change causing vesiculation. *Cell.* 93:1021-9.

Takei, K., V. Haucke, V. Slepnev, K. Farsad, M. Salazar, H. Chen, and P. De Camilli. 1998. Generation of coated intermediates of clathrin-mediated endocytosis on protein-free liposomes. *Cell.* 94:131-41.

Takei, K., P.S. McPherson, S.L. Schmid, and P. De Camilli. 1995. Tubular membrane invaginations coated by dynamin rings are induced by GTPγS in nerve terminals. *Nature.* 374:186-90.

Takei, K., O. Mundigl, L. Daniell, and P. De Camilli. 1996. The synaptic vesicle cycle: A single vesicle budding step involving clathrin and dynamin. *Journal of Cell Biology.* 133:1237-1250.

Takei, K., V.I. Slepnev, V. Haucke, and P. De Camilli. 1999. Functional partnership between amphiphysin and dynamin in clathrin- mediated endocytosis. *Nat Cell Biol.* 1:33-9.

Takenawa, T., and T. Itoh. 2001. Phosphoinositides, key molecules for regulation of actin cytoskeletal organization and membrane traffic from the plasma membrane. *Biochim Biophys Acta.* 1533:190-206.

Teng, H., and R.S. Wilkinson. 2000. Clathrin-mediated endocytosis near active zones in snake motor boutons. *J Neurosci.* 20:7986-93.

ter Haar, E., S.C. Harrison, and T. Kirchhausen. 2000. Peptide-in-groove interactions link target proteins to the beta- propeller of clathrin. *Proc Natl Acad Sci U S A.* 97:1096-100.

Thery, C., L. Zitvogel, and S. Amigorena. 2002. Exosomes: composition, biogenesis and function. *Nat Rev Immunol.* 2:569-79.

Thiele, C., M.J. Hannah, F. Fahrenholz, and W.B. Huttner. 2000. Cholesterol binds to synaptophysin and is required for biogenesis of synaptic vesicles. *Nat Cell Biol.* 2:42-9. java/Propub/cellbio/ncb0100_42.fulltext java/Propub/cellbio/ncb0100_42.abstract.

Tooze, J., and M. Hollinshead. 1992. In AtT20 and HeLa cells brefeldin A induces the fusion of tubular endosomes and changes their distribution and some of their endocytic properties. *J Cell Biol.* 118:813-30.

Traub, L.M., M.A. Downs, J.L. Westrich, and D.H. Fremont. 1999. Crystal structure of the alpha appendage of AP-2 reveals a recruitment platform for clathrin-coat assembly [see comments]. *Proc Natl Acad Sci U S A.* 96:8907-12.

Tsukita, S., and H. Ishikawa. 1980. The movement of membranous organelles in axons. Electron microscopic identification of anterogradely and retrogradely transported organelles. *J Cell Biol.* 84:513-30.

Unger, V.M., N.M. Kumar, N.B. Gilula, and M. Yeager. 1999. Three-dimensional structure of a recombinant gap junction membrane channel. *Science.* 283:1176-80.

Vale, R.D., and H. Hotani. 1988. Formation of membrane networks in vitro by kinesin-driven microtubule movement. *J Cell Biol.* 107:2233-41.

van der Bliek, A.M., and E.M. Meyerowitz. 1991. Dynamin-like protein encoded by the Drosophila shibire gene associated with vesicular traffic. *Nature.* 351:411-414.

van der Bliek, A.M., T.E. Redelmeier, H. Damke, E.J. Tisdale, E.M. Meyerowitz, and S.L. Schmid. 1993. Mutations in human dynamin block an intermediate stage in coated vesicle formation. *Journal of Cell Biology.* 122:553-63.

Verma, D.P. 2001. Cytokinesis and Building of the Cell Plate in Plants. *Annu Rev Plant Physiol Plant Mol Biol.* 52:751-784.

Verstreken, P., O. Kjaerulff, T.E. Lloyd, R. Atkinson, Y. Zhou, I.A. Meinertzhagen, and H.J. Bellen. 2002. Endophilin mutations block clathrin-mediated endocytosis but not neurotransmitter release. *Cell.* 109:101-12.

Walther, K., M. Krauss, M.K. Diril, S. Lemke, D. Ricotta, S. Honing, S. Kaiser, and V. Haucke. 2001. Human stoned B interacts with AP-2 and synaptotagmin and facilitates clathrin-coated vesicle uncoating. *EMBO Rep.* 2:634-40.

Wang, D.S., R. Shaw, M. Hattori, H. Arai, K. Inoue, and G. Shaw. 1995a. Binding of pleckstrin homology domains to WD40/beta-transducin repeat containing segments of the protein product of the Lis-1 gene. *Biochem Biophys Res Commun.* 209:622-9.

Wang, L.H., T.C. Sudhof, and R.G. Anderson. 1995b. The appendage domain of alpha-adaptin is a high affinity binding site for dynamin. *J Biol Chem.* 270:10079-83.

Waterman-Storer, C.M., and E.D. Salmon. 1998. Endoplasmic reticulum membrane tubules are distributed by microtubules in living cells using three distinct mechanisms. *Curr Biol.* 8:798-806.

Weber, T., B.V. Zemelman, J.A. McNew, B. Westermann, M. Gmachl, F. Parlati, T.H. Sollner, and J.E. Rothman. 1998. SNAREpins: minimal machinery for membrane fusion. *Cell.* 92:759-72.

Wechsler-Reya, R., K. Elliott, M. Herlyn, and G.C. Prendergast. 1997. The putative tumor suppressor BIN1 is a short-lived nuclear phosphoprotein, the localization of which is altered in malignant cells. *Cancer Res.* 57:3258-63.

Weigert, R., M.G. Silletta, S. Spano, G. Turacchio, C. Cericola, A. Colanzi, S. Senatore, R. Mancini, E.V. Polishchuk, M. Salmona, F. Facchiano, K.N. Burger, A. Mironov, A. Luini, and D. Corda. 1999. CtBP/BARS induces fission of Golgi membranes by acylating lysophosphatidic acid. *Nature.* 402:429-33.

Wenk, M.R., L. Pellegrini, V.A. Klenchin, G. Di Paolo, S. Chang, L. Daniell, M. Arioka, T.F. Martin, and P. De Camilli. 2001. PIP kinase Igamma is the major PI(4,5)P(2) synthesizing enzyme at the synapse. *Neuron.* 32:79-88.

White, J., L. Johannes, F. Mallard, A. Girod, S. Grill, S. Reinsch, P. Keller, B. Tzschaschel, A. Echard, B. Goud, and E.H. Stelzer. 1999. Rab6 coordinates a novel Golgi to ER retrograde transport pathway in live cells. *J Cell Biol.* 147:743-60.

White, S.H., A.S. Ladokhin, S. Jayasinghe, and K. Hristova. 2001. How membranes shape protein structure. *J Biol Chem.* 276:32395-8.

Wigge, P., K. Kohler, Y. Vallis, C.A. Doyle, D. Owen, S.P. Hunt, and H.T. McMahon. 1997a. Amphiphysin heterodimers: potential role in clathrin-mediated endocytosis. *Molecular Biology of the Cell.* 8:2003-2015.

Wigge, P., Y. Vallis, and H.T. McMahon. 1997b. Inhibition of receptor-mediated endocytosis by the amphiphysin SH3 domain. *Curr Biol.* 7:554-60.

Winkler, F.K., and K.K. Stanley. 1983. Clathrin heavy chain, light chain interactions. *Embo J.* 2:1393-400.

Wolfers, J., A. Lozier, G. Raposo, A. Regnault, C. Thery, C. Masurier, C. Flament, S. Pouzieux, F. Faure, T. Tursz, E. Angevin, S. Amigorena, and L. Zitvogel. 2001. Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. *Nat Med.* 7:297-303.

Wrigley, J.D., T. Ahmed, C.L. Nevett, and J.B. Findlay. 2000. Peripherin/rds influences membrane vesicle morphology. Implications for retinopathies. *J Biol Chem.* 275:13191-4.

Wu, L.G., and W.J. Betz. 1996. Nerve activity but not intracellular calcium determines the time course of endocytosis at the frog neuromuscular junction. *Neuron.* 17:769-779.

Xu, W., A. Doshi, M. Lei, M.J. Eck, and S.C. Harrison. 1999. Crystal structures of c-Src reveal features of its autoinhibitory mechanism. *Mol Cell.* 3:629-38.

Ybe, J.A., B. Greene, S.H. Liu, U. Pley, P. Parham, and F.M. Brodsky. 1998. Clathrin self-assembly is regulated by three light-chain residues controlling the formation of critical salt bridges. *Embo J.* 17:1297-303.

Zhang, B., Y.H. Koh, R.B. Beckstead, V. Budnik, B. Ganetzky, and H.J. Bellen. 1998. Synaptic vesicle size and number are regulated by a clathrin adaptor protein required for endocytosis. *Neuron.* 21:1465-75.

Zhang, P., and J.E. Hinshaw. 2001. Three-dimensional reconstruction of dynamin in the constricted state. *Nat Cell Biol.* 3:922-6.

Zhu, Y., M.T. Drake, and S. Kornfeld. 1999. ADP-ribosylation factor 1 dependent clathrin-coat assembly on synthetic liposomes. *Proc Natl Acad Sci U S A.* 96:5013-8.

Zucker, R.S. 1973. Changes in the statistics of transmitter release during facilitation. *J Physiol.* 229:787-810.