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Tuba, a Novel Protein containing Bin/Amphiphysin/Rvs and Dbl Homology Domains, Links Dynamin to Regulation of the Actin Cytoskeleton

A Dissertation
Presented to the Faculty of the Graduate School
of
Yale University
in Candidacy for the Degree of
Doctor of Philosophy

by
Marco Antonio Del Valle Salazar

Dissertation Director: Pietro De Camilli, M.D.

May, 2004

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Abstract

Tuba is a novel scaffold protein that functions to bring together dynamin with components of the actin cytoskeleton. It is concentrated at synapses in brain and binds dynamin selectively through four N-terminal Src homology-3 (SH3) domains. Tuba binds a variety of actin regulatory proteins, including N-WASP, CR16, WAVE1, WIRE, PIR121, NAP1, and Ena/VASP proteins, via a C-terminal SH3 domain. Direct binding partners include N- WASP and Ena/VASP proteins. Forced targeting of the C-terminal SH3 domain to the mitochondrial surface can promote accumulation of F-actin around mitochondria. A Dbl homology domain present in the middle of Tuba upstream of a Bin/amphiphysin/Rvs (BAR) domain activates Cdc42, but not Rac and Rho, and may thus cooperate with the C terminus of the protein in regulating actin assembly. The BAR domain, a lipid-binding module which also generates curvature in lipid membranes, may functionally replace the pleckstrin homology domain that typically follows a Dbl homology domain. The properties of Tuba provide new evidence for a close functional link between dynamin, Rho GTPase signaling, and the actin cytoskeleton.

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Dedication

This thesis is dedicated to my wife, Amanda, who has made incredible sacrifices to allow me to pursue my dreams.

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Acknowledgments

There were many people who contributed to the success of this thesis. First, I am indebted to my mentor, Pietro De Camilli. His dedication to science and his laboratory are inspiring. In addition, he gave me the opportunity to work with a great group of intelligent and insightful individuals who allowed me to grow as a scientist and person. I also thank my committee, Lynn Cooley, Anthony Koleske, and Elke Stein, for their advice and encouragement.

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shared their experience, expertise, and friendship. Special thanks to Gianluca Cestra for his valuable assistance in carrying out experiments when I was no longer in the lab. Laurie Daniell helped teach me electron microscopy and was a good friend—may she rest in peace.

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Vernon Avila and Sanford Bernstein, both at San Diego State University, introduced me to basic science. I would not be here without their assistance and support. Thank you.

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It goes without saying that my success would not have been possible without the encouragement of Evangelina and Antonio Salazar, my mother and father. They taught me early that mediocre work was unacceptable and that success could be obtained only through hard work, and they have supported my endeavors throughout my life. Their sacrifices and love are not unrecognized. Arturo Salazar is a source of inspiration and pride, and his help and encouragement throughout the many facets of my life are greatly appreciated. Rene and Adrian Salazar contribute to the enjoyment of my life.

Most importantly, I am eternally grateful to my wife, Amanda, and my sons, Jakob and Lucas, who have had to endure long periods of my relative and real absence. Their support, encouragement, and patience is incredible, and they have made my life a pleasure even during stressful times. Thank you—I love you.

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Chapter I—Introduction

The function of the neuronal synapse is the subject of intensive investigation and continues to provide an increasing understanding of communication between cells and their environment. Among the many processes which take place at the synapse, clathrin-mediated endocytosis plays a critical role in maintaining normal synaptic physiology. Fundamental to advancing our understanding of clathrin-mediated endocytosis is determining the function of the actin cytoskeleton at the synapse. Accumulating evidence suggests that actin is a participant in clathrin-mediated endocytosis, but it is not clear at which step(s) and to what extent. In this thesis, I will present evidence that Tuba, a novel BAR domain-containing protein, is a link between the endocytic and actin machinery at the synapse. The introduction will provide an overview of synaptic physiology, clathrin-mediated endocytosis, and the relationship between clathrin-mediated endocytosis and the actin cytoskeleton, establishing a framework in which to place Tuba.

THE SYNAPSE

Cell-cell communication is vital to ensuring homeostasis and coordinated action in all organisms. Cells convey information through a variety of means, including the use of chemical mediators. In some cases, cells secrete mediators to be carried by the blood stream to distant targets. Other cases may require cells to secrete regulatory molecules which will convey information to neighboring cells, or to the secreting cell itself. The

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synapse is a special unit of cell-cell communication, where a neural cell transfers information, through chemical mediators in a very specific compartment, to a variety of target cells. Unlike most forms of cell-cell communication, the synapse is designed for speed, allowing cells to convey information on a millisecond time scale. Synaptic communication may involve a distant or neighboring cell, or be autosynaptic, all analogous to non-synaptic information transfer.

Neuronal synapses require at least one member of the synaptic pair, the pre-synaptic member, to be a neuron. Neurons are the fundamental cellular units of the nervous system, and have a distinct morphology closely correlated to their specialized functions. The nucleus is located in the cell body, also called the soma. The soma extends a large number of processes, known as dendrites, which function to receive information from other neurons through synaptic contacts. In addition, synaptic contacts are made directly to the soma itself. One extension from the soma is special, termed the axon, which can be up to a meter in length. It courses to its target to transfer information from the neuronal soma to the postsynaptic cell at the axon terminals. Multiple synapses can be present along the length of the axon. Neurons thus function to integrate information conveyed from other neurons through dendritic and somatic synapses, and generate a signal that conveys the information to other neurons or cells through the axon. Along the way, this signal can be modified by incoming synaptic contacts received by the neuron at either the axon or synaptic terminal.

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The synapse is composed of pre- and post- synaptic compartments (Fig.1). The pre-synaptic compartment (Murthy and De Camilli, 2003) is always neuronal in origin and contains the pre-synaptic vesicle cluster. These vesicles contain neurotransmitters, the chemical entities which transfer information between the two cells of the synapse. The pre-synaptic vesicle cluster is anchored to the membrane at the active zone, the region where synaptic vesicles fuse with the synaptic membrane (Rosenmund et al., 2003). Located at the periphery of the active zone is the endocytic zone, where vesicle membrane is retrieved from the pre-synaptic membrane after synaptic vesicle fusion (Murthy and De Camilli, 2003). The post-synaptic cell can have a variety of post-synaptic specializations, all of which function to enrich the post-synaptic membrane with neurotransmitter receptors as well as to maximize the speed and strength of information transfer between the two cells of the synapse (Sheng, 2001). These components of the synapse play an important role in normal synaptic physiology.

Information transfer at the synapse is either excitatory or inhibitory. Excitatory synapses contain pre-synaptic vesicles filled with excitatory neurotransmitters, such as glutamate (Conti and Weinberg, 1999). There are a variety of post-synaptic receptor types for these neurotransmitters, each with unique properties, which control the response of the post-synaptic cell. In neuron-neuron synapses, excitation leads to downstream propagation to other neurons. At the neuromuscular synapse, the reception of the excitatory signal leads to contraction of the recipient muscle. Inhibitory synapses contain pre-synaptic vesicles filled with inhibitory neurotransmitters, such as GABA and glycine, and the corresponding postsynaptic density contains GABA and glycine receptors, which can

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FIGURE 1. **The synaptic vesicle cycle.** See text for details. Figure courtesy of Helge Gad.

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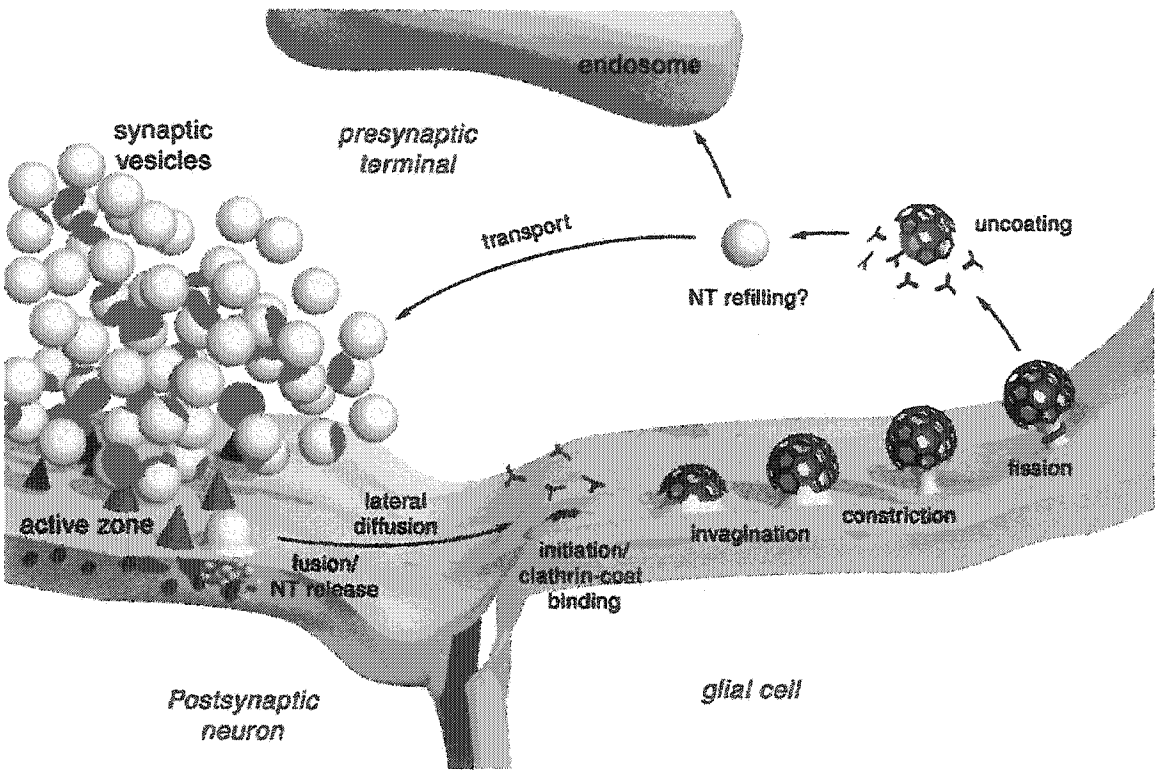

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lead to inhibition of the signal-generating activity of the postsynaptic neuron (Meier, 2003). The convergence of thousands of excitatory and inhibitory synapses onto a single neuron are integrated by the neuronal soma to generate a response.

Overview of Pre-synaptic Compartment Function

In response to an action potential, a neurotransmitter-filled vesicle may or may not fuse with the pre-synaptic plasma membrane, and it is unusual for more than one vesicle to fuse in response to a given stimulus. However, multiple vesicles will fuse with the plasma membrane in response to a train of action potentials, and the pre-synaptic vesicle cluster can be depleted if mechanisms for membrane retrieval and vesicle reformation are not in place. Vesicles exist in three states in the pre-synaptic compartment (Murthy and De Camilli, 2003). The majority of vesicles are not tethered to sites of exocytosis and are instead grouped above the active zone in the pre-synaptic vesicle cluster. These vesicles make up the reserve pool and take the place of vesicles which have fused with the pre-synaptic membrane. A second group of vesicles is tethered at sites of exocytosis, and are in a docked state. However, these vesicles are not competent for fusion. A few vesicles are docked and biochemically "primed" for fusion at the active zone. In response to the reception of excitatory inputs, an action potential is generated at the soma of a neuron and conveyed down the axon to the axon terminal, where specific voltage-gated calcium channels located in the pre-synaptic plasma membrane are activated to allow the influx of calcium, which results in fusion of primed vesicles with the pre-synaptic membrane. Vesicle fusion leads to the release of neurotransmitter into the synaptic cleft, the extracellular space between the two compartments of the synapse. The neurotransmitter

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diffuses across the synaptic cleft and binds to receptors enriched in the post-synaptic membrane (Sheng, 2001).

The vesicle membrane, now located in the pre-synaptic plasmalemma, must be retrieved and recycled to ensure a continuous supply of vesicles, as well as to prevent an increase in dimension of the synapse, which could negatively affect the function of the synapse. This process of membrane retrieval is thought to occur by two mechanisms: kiss-and-run and clathrin-mediated endocytosis. In kiss-and-run, vesicles fuse with the pre-synaptic membrane in a brief and rapidly reversible mechanism, allowing neurotransmitter release and rapid vesicle reformation (Fesce et al., 1994; Palfrey and Artalejo, 2003). Vesicles formed by clathrin-mediated endocytosis require the coat protein clathrin, as well as its adaptor and accessory proteins, to effect *de novo* vesicle production (Murthy and De Camilli, 2003). After its formation, the nascent vesicle may or may not traffic through an endosomal compartment. Endosomes are membrane-bound organelles which function in many cells to sort material internalized by endocytosis (Sachse et al., 2002). While it appears that endosomes are present in the pre-synaptic vesicle compartment, some data suggests that nascent neuronal synaptic vesicles bypass the endosomal compartment. Once vesicles are generated, they must be filled with neurotransmitter. This process is mediated by specific uptake mechanisms and requires a proton ATPase (Gasnier, 2000). Finally, the vesicle is shuttled back to the pre-synaptic vesicle cluster to undergo another round of fusion.

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CLATHRIN AND CLATHRIN-MEDIATED ENDOCYTOSIS

Endocytosis

Endocytosis is the process by which a variety of substances and components of the plasma membrane are retrieved from the cell surface, through invagination of the plasma membrane, with subsequent internalization of the contents in a membrane-bound vesicle (Conner and Schmid, 2003). The cell utilizes endocytosis for a variety of purposes, including retrieval of plasma membrane, nutrient uptake, and in certain cell-types, antigen sampling. In addition, endocytosis is involved in the regulation of signal transduction, as many transmembrane receptors are internalized after activation, leading to signal termination, although in some cases, endosomes have also been shown to have a signaling function (Di Fiore and De Camilli, 2001). Thus, the process of endocytosis is critical to normal cell function.

The cell relies on two broad mechanisms of endocytosis (Conner and Schmid, 2003). In coat-mediated endocytosis, the proteins clathrin and caveolin are utilized to aid in the process of membrane invagination. In contrast, protein coats have not been identified in non-coat-mediated endocytosis. This form of endocytosis includes phagocytosis, macropinocytosis and clathrin and caveolin-independent pinocytosis (also known as micropinocytosis). Phagocytosis is used primarily by a very specific group of cells and does not require a protein coat to aid in invagination of the membrane. Macropinocytosis and clathrin and caveolin-independent pinocytosis also carry out endocytosis without the assistance of a protein coat.

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Coat-Mediated Endocytosis

Cells utilize a variety of mechanisms to regulate endocytosis (Conner and Schmid, 2003). Clathrin-mediated endocytosis is the most extensively characterized mechanism of endocytosis. The protein clathrin is used to mechanically invaginate the plasma membrane and generate vesicles. A variety of adaptor and accessory factors have been identified which participate in clathrin-mediated endocytosis. Clathrin-coated vesicles transport a variety of cargo, including antigen, nutrients, and membrane receptors involved in signal transduction, and it plays a critical role in membrane retrieval at the synapse.

Caveolae-mediated uptake is dependent on the protein caveolin (Pelkmans and Helenius, 2002). Caveolin-mediated endocytosis is not nearly as well understood as clathrin-mediated endocytosis, but is partially distinguished by the high cholesterol content of its vesicles, as well as the insolubility of its lipids to commonly used detergents. Caveolae are thought to participate in internalization of glycosphingolipids and GPI-anchored proteins, nutrients, bacterial toxins, and non-enveloped viruses. In addition, caveolae participate in signal transduction and transcytosis. Thus, one mechanism which cells use to invaginate membrane and carry out the retrieval of specific substances is through the use of proteins which mechanically deform membranes.

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Non-Coat Dependent Endocytosis

Many researchers have observed that endocytic vesicles can be generated independent of a coat to generate a uniform set of vesicles in a process named clathrin and caveolin-independent pinocytosis (Conner and Schmid, 2003). The mechanism responsible for clathrin and caveolin-independent pinocytosis, also called micropinocytosis, is poorly understood. However, many believe that the process may be dependent on the actin cytoskeleton. Certain cell types, such as cells of the thyroid gland, extensively utilize this process (Dunn and Dunn, 2001). It is clear that much work remains to be done in the elucidation of the mechanism of formation and function of micropinosomes.

The endocytic processes described above generate vesicles with diameters no greater than 120 nanometers, and are involved in the concentration and uptake of molecules. The cell also utilizes endocytosis in the internalization of large quantities of extracellular fluid as well as other cells. Macropinocytosis is a form of endocytosis utilized by cells to capture large particles and fluid volumes from the cell exterior (Conner and Schmid, 2003). Macropinosomes have a wide size range, with vesicles being as large as one micron in diameter. The process requires the formation of membrane ruffles, which are often stimulated by growth factors, but the process is not well characterized, and no coat proteins have been identified. It is clear, however, that the process is dependent on the actin cytoskeleton, and bacterial pathogens exploit this actin dependency by injecting actin-regulatory substances into cells which dramatically increase cell ruffling and allow the organism to gain access into the cell. Bulk uptake of extracellular contents of this

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form is important for sampling the environment for antigens, and is thus a mechanism heavily utilized by immature dendritic cells.

Another endocytic mechanism which is designed to capture large particles and cells is phagocytosis (Conner and Schmid, 2003; Underhill and Ozinsky, 2002). Phagocytosis is an actin-mediated form of endocytosis specifically used by cells of the immune system to engulf bacteria, cell debris, and other particles. In a well-characterized example, binding of the Fc receptors on the surface of a neutrophil to a bacterium coated in antibody results in invagination of the plasma membrane around the bacterium, in an actin-dependent process, which results in the ingestion of the bacterium for destruction by the neutrophil (Lee et al., 2003). Taken together, all of the endocytic processes described thus far indicate that cells utilize a variety of mechanisms to effect the uptake of a large variety of substances.

Kiss-and-Run: A Unique Mechanism of Endocytosis

Some cell types have also developed a mechanism where *de novo* vesicle formation is avoided, a process termed kiss-and-run (Fesce et al., 1994; Morgan et al., 2002; Palfrey and Artalejo, 2003). Kiss-and-run is a form of endocytosis occurring in secretory cells and at the neuronal synapse, which involves the brief, rapidly reversible, fusion of the exocytic vesicle with the pre-synaptic membrane, followed by the immediate reformation of the vesicle and transport back into the pre-synaptic compartment. While initially controversial, acceptance of this form of endocytosis is increasing, as evidence for kiss-and-run is accumulating in a variety of cell types which undergo regulated exocytosis. It

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is proposed that the exocytotic pore never dilates, stopping fusion at the stage of a pit; which allows the release of neurotransmitter, but also quick reformation of the vesicle. It is argued that this mechanism is highly advantageous in neurons with intense synaptic activity, where the mechanism of clathrin-mediated endocytosis would be temporally inadequate to replenish the synaptic vesicle pool. The process is poorly understood at the molecular level. Thus, endocytosis does not absolutely require the formation of *de novo* membrane invagination.

The description of the various forms of endocytosis illustrates how cells are heavily dependent on this process for their existence and function. While some forms of endocytosis are universal, such as clathrin-mediated endocytosis, other forms are primarily utilized by highly specialized cells, such as phagocytosis by cells of the immune system.

Clathrin

Clathrin is a highly specialized molecule requiring a variety of adaptor proteins to achieve its function in vesicle formation of clathrin-coated vesicles (Brodsky et al., 2001). Clathrin-coated vesicles obtain their characteristic appearance on electron microscopic images from their clathrin coat. Clathrin exists as a trimer of clathrin heavy chain proteins *in vivo*, termed a triskelion, with the carboxy termini of each molecule interacting to form a hub from which the rest of each molecule radiates (Fig. 2). The remainder of the molecule is divided based on its characteristic morphology into a proximal leg, distal leg, and terminal domain. Each leg is maximally separated from the

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FIGURE 2. **Clathrin coats, a collage of medium and high-resolution views.** The model of a clathrin cage at 21-angstrom resolution was obtained by electron microscopy. The clathrin triskelion is a puckered and relatively rigid molecule. The proximal and distal leg domains of the clathrin heavy chain have similar α-zigzag atomic structures, and the globular terminal domain of the clathrin heavy chain is a β-propeller. From (Kirchhausen, 2000).

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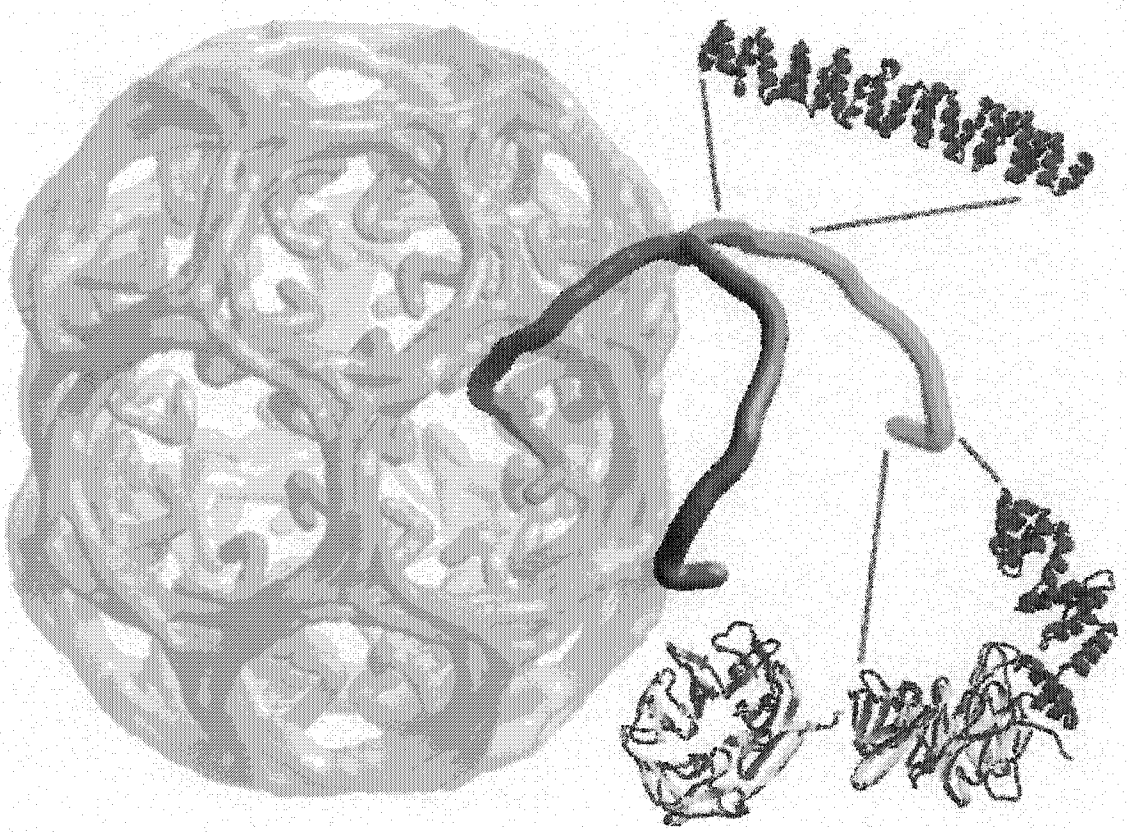

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other legs of the triskelion. The proximal leg of each clathrin heavy chain protein is associated with a clathrin light chain molecule. The proximal and distal legs interact with each other to form pentagons, hexagons, and septagons, with the correct proportion of pentagons and hexagons necessary to correctly invaginate the donor membrane to form a clathrin-coated vesicle, *in vivo*, and clathrin cages *in vitro*. Multiple adaptor and accessory proteins interact with the amino terminus of the clathrin heavy chain molecule, which comprises the terminal domain, or "foot." Adaptor proteins recruit clathrin triskelia to the target membrane and aid in triskelion polymerization, while accessory proteins allow the efficient generation of vesicles. In vitro, clathrin triskelia will form clathrin cages in the absence of lipid or any other accessory factors, demonstrating the triskelion's inherent polymerization and curvature-generating properties. In addition, there are many actin-regulatory proteins which interact with both the clathrin heavy and light chains (Engqvist-Goldstein and Drubin, 2003). This is of significance as there is increasing data suggesting that actin may play a role in endocytosis.

Role of Clathrin in Membrane Deformation

There are two proposed mechanisms to explain how clathrin deforms the donor membrane. In a mechanism advanced by Heuser, preexisting islands of clathrin hexagons located on the donor membrane re-arrange to form the correct proportion of pentagons and hexagons, resulting in membrane invagination and clathrin-coated pit formation (Heuser, 1980). Using quick-freeze, deep-etch, rotary replication of fibroblast plasma membranes, he demonstrated by electron microscopy that flat islands of clathrin exist associated to the plasma membrane, and that the majority of the clathrin was in

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hexagonal form. He noted that with increased membrane curvature, the clathrin lattice contained an increasing proportion of pentagons, and reasoned that membrane curvature developed from the rearrangement of clathrin within the hexagonal flat clathrin lattice, to include clathrin pentagons at the correct location. Other investigators favor the hypothesis that clathrin is polymerized *de novo* in response to a signal initiating clathrin-mediated endocytosis (Kirchhausen, 2000). As the clathrin triskelia assemble into the correct proportion of pentagons and hexagons, the membrane is gradually deformed into a vesicle. In this model, the clathrin coat cannot be changed after polymerization. Among the reasons cited is the geometric, and therefore energetic, challenge of taking a stable structure and effecting its rearrangement. It has been proposed that the observed hexagonal clathrin islands are merely reservoirs for clathrin. However, it is widely accepted that polymerization of the clathrin triskelion is necessary for invagination of the donor membrane and formation of the clathrin-coated vesicle.

Non-Endocytic Coat-Mediated Membrane Budding

Coat-mediated budding has a physiologic role in contexts other than endocytosis. Clathrin-mediated budding occurs from the trans face of the golgi apparatus, as well as from a variety of endosomal compartments (Bonifacino and Glick, 2004). In addition to clathrin, the cell uses other coat proteins to generate vesicles in processes other than endocytosis (Bonifacino and Glick, 2004). COP I vesicles are involved in intra-golgi as well as golgi to endoplasmic reticulum vesicle trafficking, while COP II vesicles are involved in trafficking between the endoplasmic reticulum and the cis face of the golgi apparatus (Barlowe, 2000). Generation of both of these vesicle-types requires distinct

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protein coats which also function to mechanically invaginate the donor membrane. Interestingly, many of the coat proteins of COP I vesicles are believed to form a complex structurally resembling clathrin and the clathrin adaptor AP2 (Bonifacino and Lippincott-Schwartz, 2003). The precise structure of the COP II coat is unknown, although some of its protein components have been crystallized (Bi et al., 2002). Although there are a variety of protein coats which the cell utilizes to invaginate membranes to form vesicles, the structural similarity between many of these coat protein complexes indicates that nature capitalizes on similar principles for the generation of vesicles.

Clathrin Adaptor Proteins

Adaptor proteins involved in clathrin-mediated endocytosis play a critical role in the recruitment of clathrin to the membrane on which the vesicle will be generated (Bonifacino and Traub, 2003; Traub, 2003). The majority of adaptor proteins directly interact with the donor membrane and clathrin, and they are also able to recognize specific motifs on the tails of transmembrane proteins, leading to the differential internalization of protein cargo. Interactions with the lipid bilayer are mediated through a variety of protein domains, which bind to phosphatidylinositol(4,5) bisphosphate at the plasma membrane. In addition to interacting with clathrin, many of the adaptors participating in clathrin-mediated endocytosis also directly interact with each other. The clathrin adaptors thus function to recruit clathrin to sites of endocytosis as well as to concentrate specific proteins for internalization.

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AP2 and AP180 Play Important Roles in Clathrin-Mediated Endocytosis

The most abundant of the endocytic clathrin adaptor proteins is AP2, which is a heterotetramer and a member of a family of adaptor proteins which function throughout the cell in membrane trafficking. AP2 is recruited to the plasma membrane through binding of PtdIns(4,5)P2 and its protein cargo (Fig. 3). Cargo recognized by AP2 consists of transmembrane proteins containing specific dileucine and tyrosine based motifs in their cytoplasmic tails. The cargo-AP2 interaction results in incorporation of those proteins into the nascent vesicle and subsequent internalization into the cell. Clathrin is recruited through binding of the terminal domain of the clathrin heavy chain, and AP2 also interacts with other clathrin adaptor and accessory proteins. Selective disruption of AP2 results in a reduction, but not block, of clathrin-mediated endocytosis, suggesting that although it is the primary clathrin adaptor at the plasma membrane, other adaptors also utilize clathrin to satisfy the cell's needs.

AP180 is the brain-specific isoform of the AP180/CALM adaptor protein family, is enriched in nerve terminals, and like AP2, is recruited to the plasma membrane through its interaction with PtdIns(4,5)P2 (Fig. 3). It binds to the amino terminus of clathrin to aid in its membrane recruitment, and it directly binds to AP2. The coordinated activities of both AP2 and AP180 have been shown to increase the effectiveness with which clathrin is assembled, as compared to either adaptor alone. Unlike AP2, AP180 has been shown to promote the polymerization of clathrin triskelia, as well as regulate vesicle size. Genetic experiments in which either the *Drosophila melanogaster* or *Caenorhabditis elegans* AP180 gene is disrupted show that the organisms have defects in neurologic

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FIGURE 3. **The clathrin adaptor interaction web.** A schematic representation of the protein-protein interactions possible between clathrin, AP-2, and alternate endocytic adaptors. The sorting signal or putative cargo types recognized by the different adaptors are boxed in black. PtdIns(4,5)P2-binding sites are indicated by the spherical gray attachments. AP-2 is modeled on the known molecular architecture of the core and appendages, but the different proteins are not to scale. pGPCR, phosphorylated G protein-coupled receptor; AMPA-R, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor. From (Traub, 2003).

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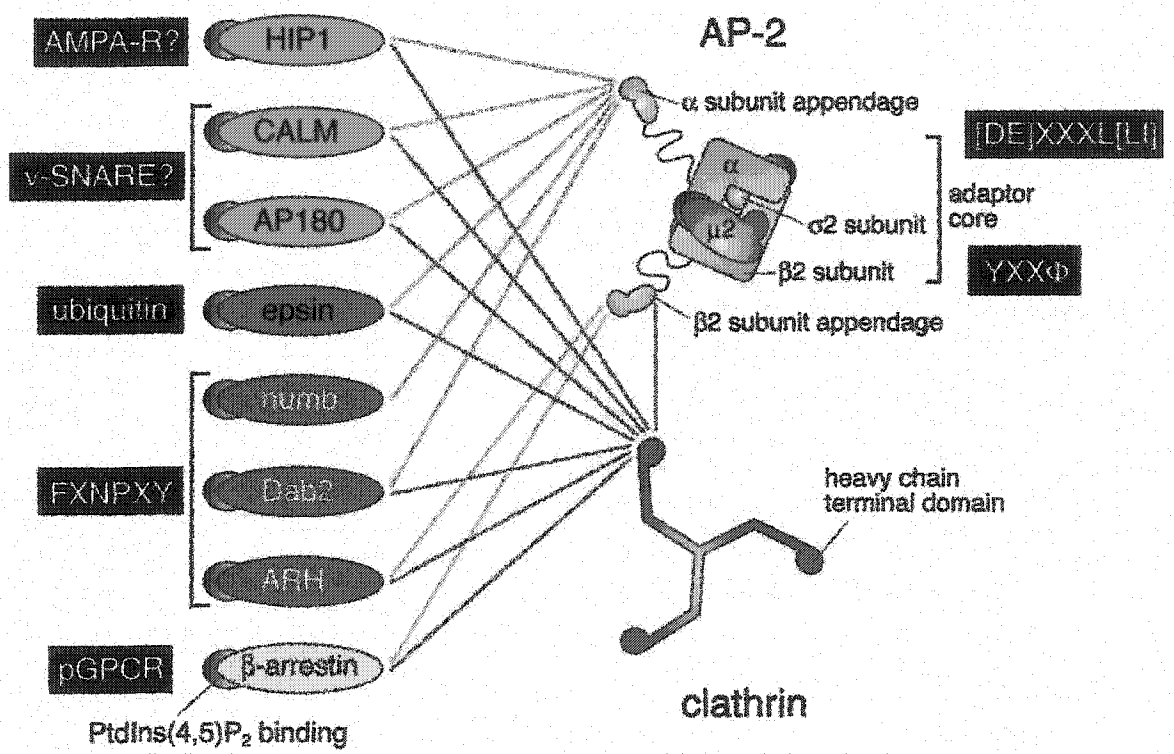

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function, have a decreased number of synaptic vesicles, and synaptic vesicles heterogeneous in their diameter (Nonet et al., 1999; Zhang et al., 1998). AP180 also appears to be necessary for sorting of the SNARE protein synaptobrevin (Nonet et al., 1999). Stonin 2, a recently characterized brain-enriched adaptor protein which binds to AP2 and inhibits clathrin-mediated endocytosis when overexpressed in fibroblasts, also directly interacts with synaptobrevin (Martina et al., 2001; Walther et al., 2004).

The ARH/Dab2/NumbFamily of Clathrin Adaptors

Recently, several other clathrin adaptors have been identified, although their involvement in synaptic vesicle recycling remains unclear. The ARH/Dab2/Numb family of clathrin adaptors rely on an amino terminal phosphotyrosine binding (PTB) domain to interact with both the target membrane and cargo proteins (Bonifacino and Traub, 2003; Traub, 2003). As in other adaptors involved in clathrin-mediated endocytosis from the plasma membrane, the PTB domain directly binds to PtdIns(4,5)P2 (Fig. 3). ARH and Dab2 have been shown to interact with clathrin, while Dab2 and numb have been shown to interact with AP2. ARH was originally identified as the defective gene product in the disease autosomal recessive hypercholesterolemia. It is necessary for the uptake of the low-density lipoprotein receptor, and Dab2 and numb are believed to interact with both the LDL receptor and other members of the LDL receptor family. The PTB domain recognizes the sequence FXNPXY in the cytoplasmic tails of these receptors. Thus, through the recognition of specific protein motifs in the tails of transmembrane proteins, the clathrin adaptors can selectively recruit proteins for internalization through clathrin-coated vesicles.

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Some Clathrin Adaptors Respond to Post-Translational Modifications of their Cargo

In addition to recognizing specific protein sequences, clathrin adaptors also recognize post-translational modifications. The arrestins are a group of proteins which cause the internalization of activated G-protein coupled receptors (Luttrell and Lefkowitz, 2002). Like other clathrin adaptors, arrestins interact with the plasma membrane through PtdIns(4,5)P2-binding, and interact with clathrin and AP2 (Fig. 3). However, they recognize and recruit their cargo based on its state of phosphorylation. As has been well described for adrenergic receptors, arrestins only promote the internalization of receptors which have been phosphorylated due to ligand binding.

Epsin1 provides another example of an adaptor protein responding to specific modifications of its cargo (Bonifacino and Traub, 2003; Traub, 2003). Similar to other clathrin adaptors, epsin is recruited to the plasma membrane through PtdIns(4,5)P2 as well as AP2 binding (Fig. 3). It also directly interacts with the terminal domain of clathrin, providing a mechanism for clathrin recruitment, but is unique in its recognition of protein cargo through its ubiquitin interaction motifs (UIM). In yeast, ubiquitination is a well-described signal for receptor-mediated endocytosis of transmembrane proteins, and the yeast epsin homologs play an important role in that process (Engqvist-Goldstein and Drubin, 2003). In higher eukaryotes, ubiquitination of specific proteins, such as growth factor receptors after ligand binding, are also believed to trigger endocytosis, and epsin may play a role in the recruitment of these receptors to clathrin-coated pits. Eps15, a major interactor of epsin at the synapse, may function cooperatively with epsin to

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promote internalization of ubiquitinated receptors (Bonifacino and Traub, 2003). Eps15 was originally identified as a substrate for the epidermal growth factor receptor tyrosine kinase (Fazioli et al., 1993). It binds to both AP2 and epsin, and also contains UIMs. Unlike other protein adaptors, however, it does not directly interact with clathrin, and it is dependent on epsin and AP2 for its membrane localization.

The previous discussion of the clathrin adaptor proteins indicates that they are critical to the recruitment of clathrin to sites of clathrin-mediated endocytosis. The majority of adaptors can directly interact with the donor membrane through direct interaction with PtdIns(4,5)P2, and they also bind to other adaptor proteins. Each adaptor also contains the ability to recognize specific motifs on the tails of transmembrane proteins, allowing their internalization through recruitment to clathrin-coated vesicles. Thus, the expression of specific clathrin adaptors allows cells to control the molecules they internalize as well as the processes they regulate.

Clathrin Accessory Proteins

Dynamin is Critical to the Process of Clathrin-Mediated Endocytosis

Accessory proteins play an important role in clathrin-mediated endocytosis. The functions of the accessory proteins are varied, with many having the ability to interact with clathrin, the clathrin adaptors, and other accessory proteins. Dynamin is an accessory factor critically important to fission (Hinshaw, 2000). It was originally identified as a tubulin-binding protein (Shpetner and Vallee, 1989), and its structure consists of an amino terminal GTPase domain, a pleckstrin homology domain, a GTPase

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effector domain, and a carboxy terminal proline-rich domain. The pleckstrin homology domain binds specifically to phosphatidylinositol(4,5,)bisphosphate, and may aid in recruitment of dynamin to an appropriate membrane, and/or may play a role in the regulation of GTPase function. The GTPase effector domain is believed to control the activity of the GTPase domain. The proline-rich domain of dynamin is recognized by the SH3 domains of a variety of clathrin-mediated endocytosis accessory proteins (Slepnev and De Camilli, 2000), as well as many proteins involved in the regulation of the actin cytoskeleton (Orth and McNiven, 2003). There is overwhelming evidence that dynamin plays a major role in clathrin-mediated endocytosis; it is also involved in other forms of endocytosis (Schafer, 2002).

The role of dynamin in endocytosis first became apparent with the work of Koenig and Ikeda, which demonstrated that in the *Drosophila melanogaster shibire* mutant, clathrin-mediated endocytosis was arrested at the stage of deeply invaginated pits(Koenig and Ikeda, 1989). The protein mutated in *shibire* mutants is dynamin (van der Bliek and Meyerowitz, 1991). This work, as well as the work of many others, suggests that the function of dynamin is to release the nascent vesicle from the donor membrane. Dynamin forms collars at the neck of clathrin-coated pits through oligomerization of dynamin multimers (Hinshaw, 2000). *In vitro*, stimulation of its GTPase activity leads to vesiculation of liposomes and lipid tubules (Marks et al., 2001; Sweitzer and Hinshaw, 1998). The above work suggests that through activation of its GTPase activity, dynamin undergoes a conformational change, resulting in release of the nascent vesicle from its donor membrane. However, there is no direct evidence that dynamin functions as a

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mechanochemical "pinchase" at the neck of clathrin-coated pits *in vivo*. Alternatively, dynamin may function similar to other GTPases and require interaction with an effector protein to accomplish vesicle fission (Thompson and McNiven, 2001). Unfortunately, other than dynamin itself, no interactors of dynamin's GTPase domain have been identified. It is of interest that there are a variety of proteins which interact with both dynamin and the actin regulatory machinery (Orth and McNiven, 2003). This suggests that dynamin may promote vesicle scission indirectly, through the recruitment of other factors. The relationship between dynamin and actin, and its affect on endocytosis, will be discussed in detail later.

A Multitude of Accessory Factors Play a Variety of Roles in Clathrin-Mediated Endocytosis

In addition to dynamin, there are many other accessory factors involved in clathrin–mediated endocytosis, all with varied roles. Synaptojanin is a protein containing a domain with 3- and 4-phosphatase activity and a separate domain with 5-phosphatase activity, leading to the conversion of PI(4,5)P2 to PI (McPherson et al., 1996; Nemoto et al., 2000). Synaptojanin interacts with clathrin, AP2 (Haffner et al., 2000), and intersectin (see below) (Yamabhai et al., 1998), and has an important role in the regulation of presynaptic PI(4,5)P2 levels (Cremona et al., 1999). Presynaptic PI(4,5)P2 levels are involved in the regulation of dynamin function, clathrin-coat assembly, and the actin cytoskeleton (Cremona and De Camilli, 2001). Intersectin is a large, multi-domain protein which interacts with a variety of proteins involved in clathrin-mediated endocytosis and regulation of the actin cytoskeleton, including epsin, Eps15, dynamin,

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synaptojanin, SNAP25, mSOS, and N-WASP (Hussain et al., 2001; Okamoto et al., 1999; Roos and Kelly, 1998; Sengar et al., 1999; Tong et al., 2000; Yamabhai et al., 1998). Its Dbl homology domain functions as a Cdc42-specific exchange factor (Hussain et al., 2001). It is a component of clathrin-coated vesicles, co-localizes with clathrin in fibroblasts, and inhibits transferrin uptake when its SH3 domains are overexpressed (Hussain et al., 1999; Sengar et al., 1999). Auxilin and Hsp70 function together to release the clathrin coat from the nascent vesicle. Auxilin directly binds to clathrin and AP2 (Scheele et al., 2001). Stimulation of the ATPase activity of Hsc70 by auxilin leads to uncoating of the vesicle (Ungewickell et al., 1995).

The BAR Domains of Amphiphysin and Endophilin Share Similar Properties

The amphiphysin, endophilin, and syndapin (also referred to as pacsin) families form a special superfamily of accessory proteins involved in clathrin-mediated endocytosis. Brain isoforms of the three proteins share a similar domain structure, consisting of an amino terminal BAR domain, a variable middle region, and a carboxy terminal SH3 domain (Slepnev and De Camilli, 2000). The amino terminal BAR (Bin/Amphiphysin/Rvs) domain is a protein module found in a variety of proteins conserved from yeast (Rvs proteins) to humans (amphiphysin and Bin proteins) (Habermann, 2004). Many BAR domain-containing proteins, such as the amphiphysin yeast homolog Rvs167, are involved in the trafficking of membranes as well as the regulation of the actin cytoskeleton (Munn et al., 1995). The BAR domains of amphiphysin and endophilin share similar properties. They are involved in homo- and heterodimerization within their protein family (Peter et al., 2004; Ringstad et al., 2001;

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Wigge et al., 1997a), and they function as lipid-binding and curvature-generating modules *in vitro*, demonstrated by their ability to tubulate liposomes (Farsad et al., 2001; Peter et al., 2004; Takei et al., 1999). The endophilin SH3 domain interacts primarily with synaptojanin, but also binds dynamin (Ringstad et al., 1997). The variable middle region in amphiphysins I and II contains a clathrin/AP2-binding region, directly linking it to the endocytic machinery (Slepnev et al., 2000). The carboxy-terminal SH3 domain of amphiphysin binds primarily to dynamin, but also interacts less avidly to synaptojanin and itself (David et al., 1996; Farsad et al., 2003). Syndapin has only recently been recognized as a member of the amphiphysin superfamily of proteins (Habermann, 2004; Peter et al., 2004). Although its amino terminus has the structure of a BAR domain (Habermann, 2004; Peter et al., 2004), its lipid-binding, dimerization, and curvature-generating properties remain to be characterized. Syndapin directly interacts with multiple proteins involved in clathrin-mediated endocytosis and regulation of the actin cytoskeleton, including dynamin, synaptojanin, N-WASP, mSOS, and synapsin, through its carboxy-terminal SH3 domain (Qualmann et al., 1999). Members of the amphiphysin superfamily can thus function as organizers of the clathrin-coated pit through their multiple interactions with lipids, clathrin and AP2, the clathrin accessory proteins dynamin and synaptojanin, and regulators of the actin cytoskeleton.

Experiments carried out in the reticulo-spinal synapse of the lamprey provide further support that amphiphysin and endophilin are proteins with similar properties (Gad et al., 2000; Shupliakov et al., 1997). Peptides generated to disrupt the SH3-ligand interaction of either amphiphysin I or endophilin I were injected into the synapses. Nerves

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corresponding to the injected synapses were electrically stimulated, and the synapses were then analyzed by electron microscopy to determine the effect of the peptides on endocytosis. The amphiphysin-specific peptide blocked endocytosis at the stage of clathrin-coated pits, indicating that amphiphysin may play a role in recruiting dynamin to the clathrin-coated pit (Shupliakov et al., 1997). Peptides specific for the endophilin SH3 domain also blocked endocytosis at the stage of clathrin-coated pits, but in addition caused the accumulation of actin in regions adjacent to the active zone, sites of endocytosis (Gad et al., 2000). This effect is probably secondary to the effect of inhibiting synaptojanin recruitment. Disruption of endophilin expression in both *D. melanogaster* and *C. elegans* yields similar phenotypes (Schuske et al., 2003; Verstreken et al., 2002). As compared with the neuromuscular junctions of wild type animals, the number of synaptic vesicles is reduced, the amount of pre-synaptic plasma membrane is increased, and the number of coated structures is increased, all indicating that clathrin-mediated endocytosis is impaired. In addition, endophilin/synaptojanin double mutants have an identical phenotype to mutants where expression of only one of the proteins is eliminated, suggesting that they function in the same pathway (Schuske et al., 2003). Interestingly, amphiphysin mutants in *D. melanogaster* were not found to affect endocytosis or clathrin-mediated endocytosis (Razzaq et al., 2001; Zelhof et al., 2001).

The role of syndapin in clathrin-mediated endocytosis has not been examined to the extent of amphiphysin and endophilin. Overexpression of the SH3 domain inhibits transferrin uptake in fibroblasts, consistent with a role in endocytosis (Qualmann and Kelly, 2000). In addition, overexpression of full-length syndapin in fibroblasts leads to

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filopodia formation (Qualmann and Kelly, 2000). Disruption of endophilin function also leads to accumulation of actin (Gad et al., 2000), albeit through mislocalization of synaptojanin as opposed to a direct effect on actin polymerization, as is the case with syndapin (Qualmann and Kelly, 2000).

Together, this data provides further evidence that the BAR domain-containing proteins share similar properties both *in vitro* and *in vivo*, and function as multifunctional adaptors in the process of clathrin-mediated endocytosis.

The BAR Domain

The crystal structure of the *Drosophila melanogaster* amphiphysin BAR domain has been solved, and it begins to provide an explanation for the *in vitro* properties of the module (Peter et al., 2004). The BAR domain is a banana-shaped homodimer, with each subunit composed of three alpha helices (Fig.4). At the dimer interface the three helices come together to form a six-helix bundle. Due to kinking of the helices, the characteristic banana shape is formed. It is at the concave face of the dimer that the BAR domain is believed to interact with membranes. There are multiple positively charged regions of the concave face, facilitating interaction with the negatively charged phospholipid membrane, and mutation of many of these positively charged residues abolish lipid binding. The banana shape of the BAR domain suggests that its curvature generating properties may be due to the BAR domain's concave shape. In addition, the authors also demonstrate that BAR domains preferentially bind to liposomes with the diameter of

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FIGURE 4. **Structure of the *Drosophila* amphiphysin BAR domain.** *A*, Ribbon representation of the antiparallel homodimer (purple and green monomers). The principal side chains that make up the positive patches on the concave surface are marked. *B*, Same view as in *A*, with the surface colored by electrostatic potential (red, -10 kTe-1, blue, +10 kTe-1). *C*, Ribbon diagram viewed along the dyad axis (concave face) perpendicular to *A*. *D*, Electrostatic surface viewed as in *C*. From (Peter et al., 2004).

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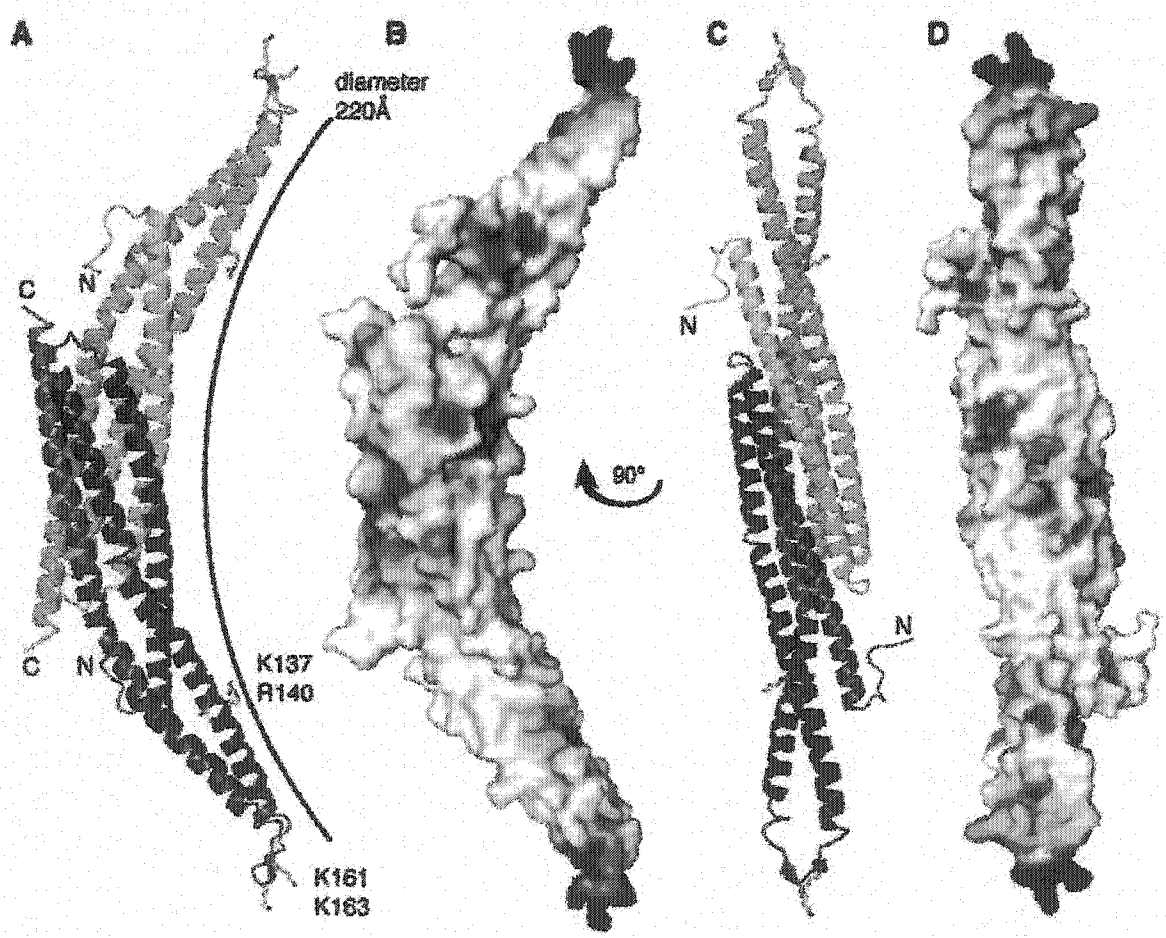

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synaptic vesicles, suggesting a role for the BAR domain as a sensor of curvature. The ability to preferentially interact with membranes containing the correct curvature may regulate when BAR domain-containing proteins are recruited to membranes. This may explain the behavior of amphiphysin I, which is believed to be recruited only to clathrin-coated pits (Heuser and Reese, 1973; Shupliakov et al., 1997). One can also predict that as the BAR domain generates membrane curvature, it increases its avidity for that membrane. Thus, the crystal structure of the BAR domain provides key new insights towards the understanding of its properties.

A Model for Interactions Between Selected Participants of Clathrin-Mediated Endocytosis

Although simplified, figure 5 provides a framework in which to begin to think about the interactions between clathrin and selected clathrin adaptors and accessory factors. The clathrin adaptors AP2 and AP180 are localized to the plasma membrane through interactions with PtdIns(4,5)P2 and the tails of cargo proteins; they then recruit clathrin to sites of endocytosis. Through recognition of the curvature of the membrane, the amphiphysin dimer is recruited to the clathrin-coated pit. Alternatively, amphiphysin recruitment could be mediated via interactions with AP2, clathrin, and/or dynamin. Amphiphysin may also participate in the generation of the neck of the coated-pit through its curvature-generating properties. Dynamin oligomers form collars at the neck of clathrin-coated pits, and through an unrecognized signal and unknown mechanism, initiate the scission reaction, releasing the nascent vesicle from the donor membrane.

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FIGURE 5. **A model for the interaction between selected proteins at the clathrin-coated pit.** See text for details. From (Takei et al., 1999).

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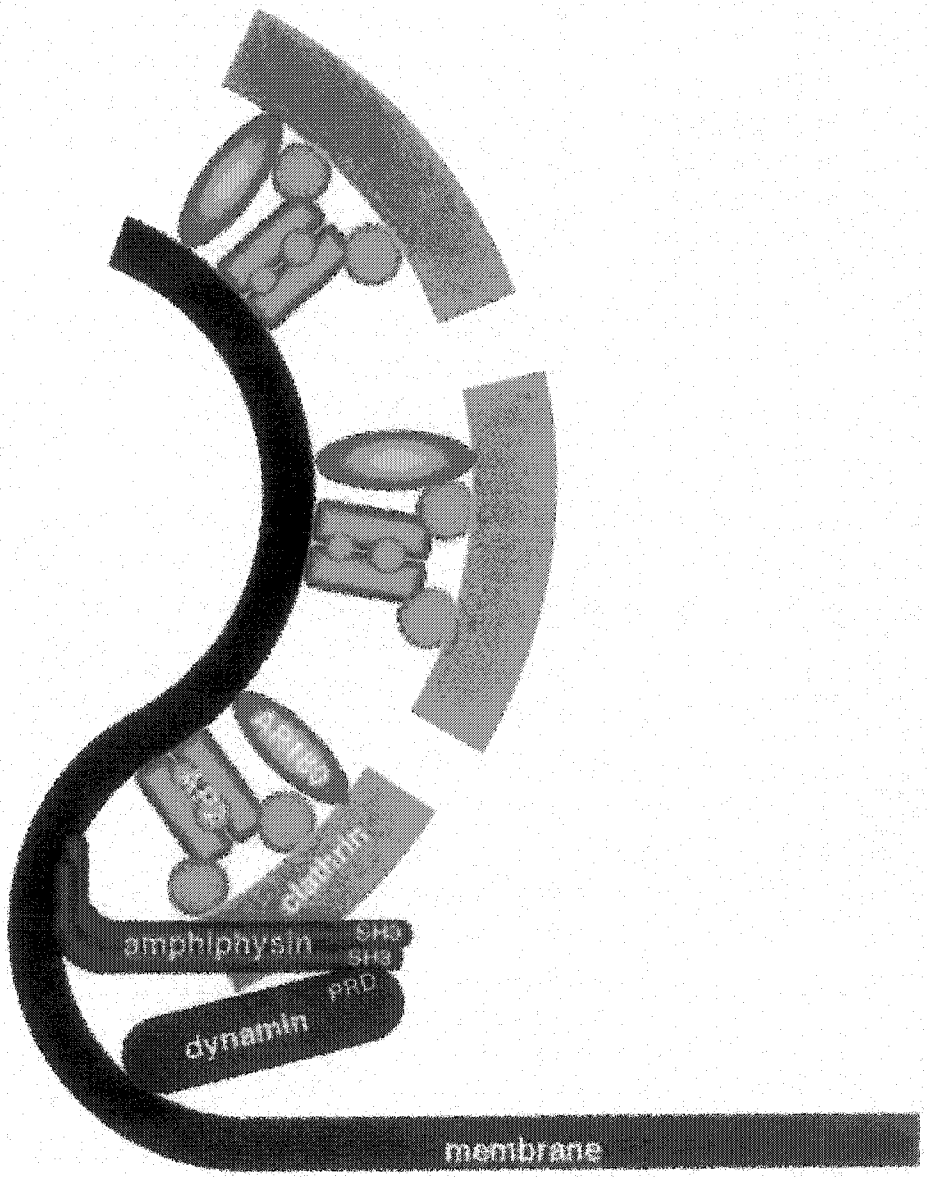

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This model of clathrin-mediated endocytosis provides a framework in which to place the multitude of clathrin adaptors and accessory factors.

Clathrin-Mediated Endocytosis Is Necessary for Normal Synaptic Function

There is abundant evidence pointing to a major role for clathrin-mediated endocytosis at the synapse. As early as the 1970's, Heuser and Reese provided electron microscopic evidence that clathrin-coated structures were the major mechanism by which membrane was retrieved in the pre-synaptic compartment of the neuromuscular junction (Heuser and Reese, 1973). Multiple biochemical experiments subsequently demonstrated that clathrin was enriched at the synapse and that coated vesicles isolated from bovine and rat brain always contained a clathrin coat (Maycox et al., 1992; Pfeffer and Kelly, 1985). Clathrin-coated vesicles are easily purified from brain synaptosomes, subcellular particles isolated by differential centrifugation and composed of only the pre- and postsynaptic compartments (Huttner et al., 1983). Immunogold electron microscopy, carried out on synaptosomes, clearly demonstrates that clathrin-coated structures are present in the pre-synaptic compartment of the synapse, and that they are easily generated with known inducers of endocytosis (De Camilli et al., 1983; Takei et al., 1995; Takei et al., 1996). In addition, these studies also showed that clathrin adaptor and accessory proteins were localized in clathrin-coated structures. Genetic studies in *D. melanogaster* and *C. elegans* which disrupt expression of the clathrin adaptor proteins AP2 (Gonzalez-Gaitan and Jackle, 1997) and AP180 (Nonet et al., 1999; Zhang et al., 1998) and the clathrin accessory protein endophilin (Schuske et al., 2003; Verstreken et al., 2002), as well as electrophysiologic studies in the reticulo-spinal synapse of the lamprey, which interfered

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with the function of the clathrin-accessory factors amphiphysin (Shupliakov et al., 1997) and endophilin (Gad et al., 2000; Ringstad et al., 1999), demonstrated a striking perturbation of clathrin-mediated endocytosis and normal synaptic function. It should be noted, however, that in the case of disruption of endophilin expression in *D. melanogaster* and *C. elegans*, the neuromuscular junction was able to compensate for these change possibly through utilization of the kiss-and-run mechanism of endocytosis (Schuske et al., 2003; Verstreken et al., 2002). Taken together, there is strong evidence that clathrin-mediated endocytosis is the principle mechanism of membrane retrieval at the synapse.

ACTIN AND CLATHRIN-MEDIATED ENDOCYTOSIS

The Actin Cytoskeleton

Actin is an abundant protein present in all eukaryotic cells, and is a major component of the cell's cytoskeleton. It is an ATPase which exists in either a globular (monomeric) or filamentous form, and its properties are affected by whether it is bound to ATP or ADP. Globular actin is polymerized to generate the filamentous form. F-actin is polarized, meaning that the ends of the filament are not equal in form or function. The polarization of the actin filament is utilized by the cell to control where actin polymerization takes place, as polymerization is much more efficient at the barbed or plus end, and the polarized filament is also used by actin-specific motor proteins, the myosins, to effect either cell movement or the transport of objects along the actin filaments, as different myosin family members only move in specific directions along the actin filament.

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N-WASP is a Critical Regulator of the Actin Cytoskeleton

There are many proteins that regulate various aspects of actin's function (Pollard and Borisy, 2003). These include proteins which initiate actin polymerization (Higgs and Pollard, 2001), sever actin filaments (Bamburg and Wiggan, 2002; McGough et al., 2003), cap F-actin (Bear et al., 2002; Sarmiere and Bamburg, 2004), and bind actin monomers (to effect, for example, the state of its bound nucleotide) (dos Remedios et al., 2003). N-WASP is a protein which plays a critical role in the regulation of the actin cytoskeleton (Higgs and Pollard, 2001). It is the ubiquitous homolog of the Wiskott-Aldrich Syndrome protein (WASP), whose disruption causes a disease characterized by recurrent infections, eczema, thrombocytopenia, and cancer of the hematopoetic lineage (Snapper and Rosen, 1999). When activated, N-WASP stimulates a complex of proteins, the Arp2/3 complex, to polymerize actin. The Arp2/3 complex consists of seven proteins that function to nucleate actin polymerization. A variety of proteins, not all of which are members of the WASP superfamily of proteins, directly interact with the Arp2/3 complex to stimulate the polymerization of actin. N-WASP binds directly to the Arp2/3 complex through its carboxy terminal CA (cofilin-like, acidic) region. Adjacent to the CA region, N-WASP contains a verprolin-homology (V) region which directly binds G-actin, concentrating monomeric actin adjacent to the site of actin polymerization.

Full activation of N-WASP requires binding of the activated form of the Rho family small GTPase, Cdc42, to its CRIB (Cdc42 and Rac Interactive Binding) domain. In addition to GTP-Cdc42 binding, N-WASP also requires PtdIns(4,5)P2 binding to a region

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of the protein rich in basic amino acids (the basic region), or SH3 domain binding to its proline-rich region. Phenotypically, N-WASP activation leads to actin-dependent membrane spikes and membrane ruffles, termed filopodia and lamellipodia, emanating from the cell surface (Hall, 1998). N-WASP is a binding partner of multiple proteins and no doubt plays an important role in the regulation of the actin cytoskeleton of most cells.

The cell utilizes differential control of the cytoskeleton to carry out specific tasks. For example, in response to binding a bacteria opsonized with antibody through cell surface Fc receptors, a neutrophil will initiate, among other things, a signal transduction cascade which will result in the polymerization of actin at the correct location and intensity, resulting in the phagocytosis of the bacterium. The cell also utilizes actin for cellular locomotion, muscle contraction, and for generating specific structures, such as microvilli on enterocytes. Actin is a vitally important and versatile cellular component which the cell extensively utilizes to carry out its needs.

Actin in Bulk and Clathrin-Mediated Endocytosis

Actin Participates in Endocytosis in Yeast

A role for actin in clathrin-mediated endocytosis has been postulated for some time. However, until recently, supporting evidence has been scant. The initial evidence implicating actin in the process of endocytosis came from work in yeast (Engqvist-Goldstein and Drubin, 2003). In an effort to identify proteins involved in receptor-mediated endocytosis, Riezman and colleagues developed a screening assay using the internalization of the yeast alpha factor protein. Alpha factor is a secreted pheromone

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which binds to a G-protein-coupled receptor to initiate mating between a and α-mating type yeast. Internalization of the receptor stimulates endocytosis of the receptor-pheromone complex, leading to degradation of the proteins in the lysosome. Many mutants were discovered to perturb both alpha-factor internalization and the actin cytoskeleton. One of these *END* (endocytosis) mutants disrupted the yeast actin gene (Munn et al., 1995); other actin regulatory proteins were also identified. Mutants of the yeast clathrin heavy or light chain proteins also have defects in endocytosis and the actin cytoskeleton (Chu et al., 1996; Tan et al., 1993), and the yeast amphiphysin and endophilin homolog, Rvs167, also displays actin and endocytosis phenotypes when disrupted (Munn et al., 1995). Finally, the yeast genome contains homologs to many of the adaptors and accessory factors known to be involved in clathrin-mediated endocytosis in mammalian synapses, and experiments have demonstrated that these are indeed involved in receptor-mediated endocytosis (Engqvist-Goldstein and Drubin, 2003). Many of these yeast proteins interact genetically and often biochemically with members of both the endocytic and actin machinery.

Supporting a connection between actin and endocytosis in yeast is the work of Kaksonen, *et al.* (Kaksonen et al., 2003), which demonstrates that in budding yeast, actin patches, sites of actin polymerization also believed to function in endocytosis, change their protein composition in a temporally uniform fashion resulting in actin patch motility and endocytic internalization. Using multicolor widefield epifluorescence microscopy, they analyzed the temporal and spatial localization of the Arp2/3 complex, activators of the Arp2/3 complex, an adaptor protein necessary for endocytosis, and Sla2, a protein

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considered a link between the actin and endocytic machinery. Actin polymerization was found to be a late step in endocytosis and necessary for internalization of endocytic components as well as actin patch disassembly. Yeast lacking expression of Sla2 exhibited immobile actin patches capable of nucleating actin tails. This reinforces the idea that actin patches are sites of endocytosis, and that actin polymerization is a requirement of the endocytic process. Thus, it appears that the link between endocytosis and the actin cytoskeleton was established early in evolution.

Actin and Clathrin-Mediated Endocytosis in Vertebrates

There is increasing evidence that actin plays an important role in endocytosis in vertebrates as well. Dunaevsky and Connor demonstrated by immunofluorescence confocal microscopy, in a preparation of frog neuromuscular junction lacking the post-synaptic compartment, that the pre-synaptic compartment has a rich actin network which does not overlap with the pre-synaptic vesicle cluster and sites of exocytosis (Dunaevsky and Connor, 2000). Work by Shupliakov, *et al.* (Shupliakov et al., 2002), in the reticulospinal synapse of the lamprey demonstrated that actin is localized around the active zone and pre-synaptic vesicle cluster of the synapse. Upon stimulation, actin filaments proliferated from the peri-active zonal region, which co-incidentally is the area where endocytosis is greatest. Vesicles, possibly newly formed endocytic vesicles, were found to be tethered to these filaments. When they perturbed the actin cytoskeleton with a variety of toxins, clathrin-mediated endocytosis was impaired and numerous shallow clathrin-coated pits accumulated. In addition, vesicles were found to accumulate and aggregate, suggesting a block in synaptic vesicle transport. Merrifield, *et al.*,

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demonstrated that actin was recruited to sites of endocytosis after the recruitment of first clathrin, and then dynamin, and that actin recruitment was necessary for the disappearance of clathrin from the site of clathrin-coated pit formation (Merrifield et al., 2002). The recruitment of actin to sites of endocytosis indicates that actin plays an important role in endocytosis.

Multiple Proteins Link Clathrin to the Actin Cytoskeleton

There are a variety of proteins in mammals which form a bridge between clathrin and the actin cytoskeleton (Fig. 6). Hip1 was originally identified as an interactor of huntingtin, the causative protein of Huntington disease (Kalchman et al., 1997). Hip1 and its relative, Hip1R, are enriched in clathrin-coated vesicles and co-localize with clathrin (Engqvist-Goldstein et al., 2001; Legendre-Guillemin et al., 2002). Hip1 binds to both clathrin heavy and light chains, as well as the clathrin adaptor AP2 (Legendre-Guillemin et al., 2002; Metzler et al., 2001). Hip1R directly binds F-actin and clathrin light chain (Legendre-Guillemin et al., 2002). Ankyrin is a member of the spectrin-based cytoskeleton, originally described in erythrocytes and which interacts with the actin cytoskeleton, which binds to the clathrin heavy chain protein as well as the spectrin beta subunit (Rubtsov and Lopina, 2000). Overexpression of the ankyrin repeats, a protein module originally described in ankyrin, inhibits the uptake of the low density lipoprotein receptor, which requires clathrin-mediated endocytosis for internalization (Michaely et al., 1999). Myosin VI is a member of the myosin superfamily of actin-dependent motor proteins (Buss et al., 2002). It is enriched in clathrin-coated vesicles and overexpression

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FIGURE 6. **Domain structures of proteins that may function at the interface between actin and the endocytic machinery in clathrin-mediated endocytosis.** *a*, Hip1 and Hip1R, *b*, myosin VI, *c*, Abp1, *d*, cortactin, *e*, intersectin-s and intersectin-l, and *f*, syndapin. The arrows point to interacting proteins. Abbreviations: ANTH, AP180 N-terminal homology domain; CL HC, clathrin heavy chain; CL LC, clathrin light chain; SH3, src-homology 3 domain; A, acidic motif; EH, Eps15-homology domain; DH, Dbl-homology domain; PH, pleckstrin-homology domain. From (Engqvist-Goldstein and Drubin, 2003).

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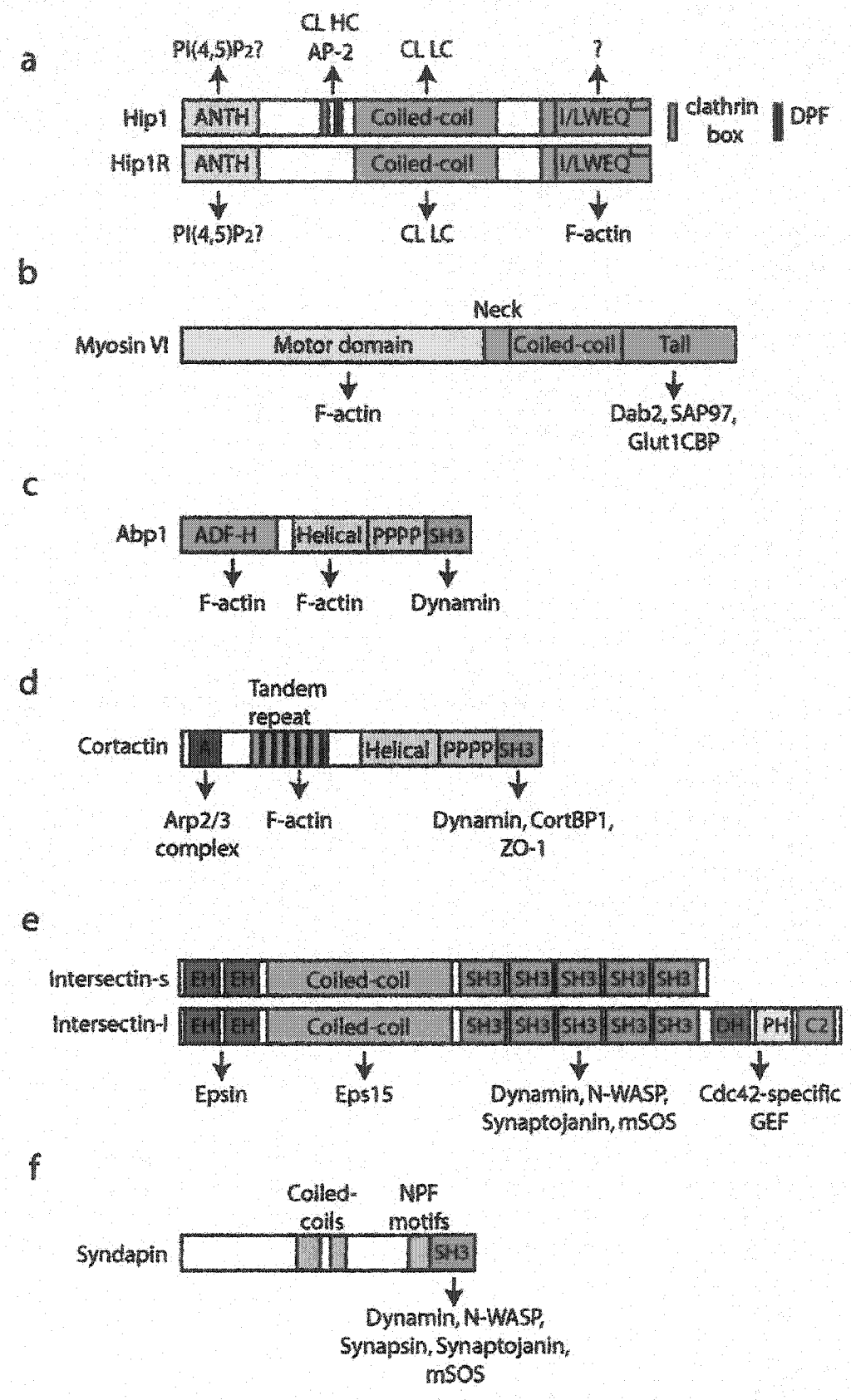

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of the tail domain inhibits transferrin uptake (Buss et al., 2001; Morris et al., 2002). Unlike the other myosin family members, it is a minus end-directed motor (Wells et al., 1999). ACK1 and 2 are tyrosine kinases which interact with the clathrin heavy chain (Teo et al., 2001; Yang et al., 2001b), the sorting nexin, SNX9 (Lin et al., 2002), and are effectors for the small Rho family GTPase Cdc42 (Mott et al., 1999). ACK2 can be co-immunoprecipitated with clathrin (Lin et al., 2002), and overexpression of ACK1 or 2 results in inhibition of transferrin uptake (Teo et al., 2001; Yang et al., 2001b), as well as disruption of stress fibers and focal adhesions (Yang et al., 2001a). The discovery of proteins which interact with clathrin and interact with the actin cytoskeleton under a variety of capacities further suggests that actin plays a role in endocytosis.

Many Clathrin Accessory Proteins are Regulatory Components of the Actin Cytoskeleton

In addition to clathrin, dynamin, a clathrin accessory protein absolutely necessary for clathrin-mediated endocytosis (Hinshaw, 2000), interacts with many proteins that are regulators of the actin cytoskeleton (Fig. 6). Intersectin was introduced earlier as a member of clathrin coated vesicles (Hussain et al., 1999), whose SH3 domains can block transferrin uptake when overexpressed (Sengar et al., 1999). It interacts with epsin, Eps15, dynamin, synaptojanin, mSos, and N-WASP, and contains a Dbl homology domain which functions as a Cdc42-specific exchange factor (Tong et al., 2000; Yamabhai et al., 1998). A variety of its protein interactors are regulators of the actin cytoskeleton, and Cdc42 initiates filopodia formation (Hussain et al., 2001).

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Syndapin/Pacsin contains an SH3 domain that when overexpressed inhibits transferrin uptake (Qualmann and Kelly, 2000). Syndapin interacts with dynamin, synaptojanin, N-WASP, mSos, and synapsin through its SH3 domain (Qualmann et al., 1999). Overexpression of the full-length protein leads to filopodia formation (Qualmann and Kelly, 2000). Abp1 is a Src tyrosine kinase substrate (Larbolette et al., 1999) which directly binds to dynamin and F-actin (Kessels et al., 2001), and the yeast homolog can stimulate actin polymerization through the Arp2/3 complex (Goode et al., 2001). It localizes to lamellipodia (Kessels et al., 2000) and partially localizes with many of the accessory proteins of clathrin-mediated endocytosis (Kessels et al., 2001). Transferrin uptake is inhibited in fibroblasts whose Abp1 expression has been reduced by Abp1-specific RNAi (Mise-Omata et al., 2003). Cortactin is also a Src tyrosine kinase substrate (Wu et al., 1991). It interacts with dynamin (McNiven et al., 2000) and F-actin (Wu and Parsons, 1993), and it is an activator of the Arp2/3 complex, which directly catalyzes polymerization of actin (Uruno et al., 2001). Cortactin localizes to lamellipodia (Kaksonen et al., 2000) and podosomes (Bowden et al., 1999), both of which are actin-rich structures, and it also localizes to endosomes and clathrin-coated pits (Cao et al., 2003), structures which utilize clathrin-mediated budding. The ability of dynamin to play a critical role in clathrin-mediated endocytosis as well as interact with the described actin regulatory proteins indicates that dynamin may provide a link between the actin and endocytic machinery, and that actin may play a role in endocytosis.

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Dynamin May Function as a Regulator of the Actin Cytoskeleton

While dynamin is an essential regulator of endocytosis, there is some data suggesting that dynamin might also function as an actin regulatory protein. Dynamin has been localized to the actin tails of *Listeria monocytogenes* as well as the actin tails of endosomal structures, both sites where endocytosis does not play a role (Lee and De Camilli, 2002; Orth et al., 2002). Further, dynamin mutants were able to either abolish the actin-rich podosome, a structure involved in cell motility in some cell types, or inhibit the dynamics of podosomal actin (Ochoa et al., 2000). Mutants of the GTPase domain of dynamin were also shown to inhibit PIP kinase-induced actin tail number and organelle velocity (Lee and De Camilli, 2002; Orth et al., 2002). Disruption of the interaction between cortactin and dynamin prevented the formation of membrane "waves," actin-rich structures formed in response to stimulation of NIH-3T3 cells with PDGF (Krueger et al., 2003). In addition to inhibition of the wave phenotype, the reorganization of other actin structures, such as stress fibers, was also impaired (Krueger et al., 2003). These observations, albeit preliminary, suggest the possibility that dynamin may itself be a *bona fide* regulator of the actin cytoskeleton. Nevertheless, given the prominence of its role in endocytosis, it is likely that dynamin functions as a bridge between endocytosis and the actin cytoskeleton. Additionally, the association of members of the actin cytoskeleton with clathrin itself indicates that actin plays an important role in clathrin-mediated endocytosis.

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The Role of Actin in Endocytosis

Actin May Specify Sites of Endocytosis

Although it is becoming increasingly apparent that actin plays a role in clathrin-mediated endocytosis, it is unknown at which step(s) it participates. Actin has the ability to play a variety of roles in the endocytic process. Multiple studies indicate that actin may delineate sites of endocytosis. The clathrin adaptor AP2 has been found to co-localize with actin stress fibers (Bennett et al., 2001). Co-localization was disrupted by the actin-specific poison, cytochalasin D (Bennett et al., 2001). This same study also showed that overexpression of the Hub fragment of clathrin disrupted the AP2-stress fiber co-localization and resulted in the dissociation of Hip1R, a protein which binds both clathrin and F-actin that may be responsible for the co-localization of the two proteins. Work in which clathrin dynamics were visualized in live cells also suggests that sites of clathrin-mediated endocytosis may be specified by actin. When cells were treated with the actin-specific drug latrunculin-B, sites of clathrin-coated pit formation were random (Gaidarov et al., 1999). This is in contrast to cells whose actin cytoskeleton was not perturbed, where clathrin-coated pits were continually generated at the same location. The lamprey and *D. melanogaster* neuromuscular junctions also appear to restrict endocytosis to specific sites (Dunaevsky and Connor, 2000; Shupliakov et al., 2002). Thus, actin may play a role in specifying sites of clathrin-mediated endocytosis.

Actin Function at the Clathrin-Coated Pit

While it appears that actin may specify sites of clathrin-mediated endocytosis, actin probably plays a more important role after formation of the clathrin-coated pit.

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Experiments carried out by Lamaze, *et al.*, demonstrated that cells treated with latrunculin-A, endocytosis is blocked at the coated-pit stage (Lamaze et al., 1997). And in the previously described work of Merrifield, et al., actin does not appear at sites of clathrin-mediated endocytosis until after the recruitment of dynamin (Merrifield et al., 2002). This is congruent with work described above which suggests that dynamin forms a significant link between endocytosis and the actin cytoskeleton. What does actin do at the coated pit? Merrifield observed that clathrin-coated structures moved away from the membrane following the appearance of actin (Merrifield et al., 2002). This suggests that nascent clathrin-coated vesicles may contain actin tails which propel them to their destination. Alternatively, actin may be required to release the nascent vesicle from the plasma membrane. A burst in actin polymerization may be able to generate force sufficient to stretch the neck of the clathrin-coated pit, leading to separation of the vesicle from the donor membrane. Polymerization of actin could also be used, with the assistance of myosin family proteins, to constrict the neck of the clathrin-coated pit sufficient to effect the release of the nascent vesicle. Filamentous actin may also be generated to provide tracks on which vesicles can be transported to their destination, using actin-dependent motor proteins such as myosin VI, which directly interacts with clathrin. Taken together, it appears that actin may play an important role in clathrin-mediated endocytosis after the coated pit has formed.

The different potential roles for actin described above are not mutually exclusive. That is, there is no reason why actin cannot simultaneously delineate sites of endocytosis, aid in vesicle fission, and transport the vesicle to its destination, using some or all of the

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delineated mechanisms. Dynamin is likely to play an important role in the recruitment of actin, probably through its interaction with multiple actin-regulatory proteins which when stimulated, lead to the *de novo* polymerization of actin at the appropriate site. PIP2 levels at sites of endocytosis are also likely to have an effect on the polymerization of actin. There are a variety of potential roles for actin filaments in the process of endocytosis, and either alone or working in concert, these functions are likely to increase the efficiency of membrane recycling.

TUBA MAY BE A BRIDGE BETWEEN CLATHRIN-MEDIATED ENDOCYTOSIS AND THE ACTIN CYTOSKELETON

Rationale for the Study of Tuba

The BAR domain was originally identified as a region of homology among the amino termini of the proteins Bin1, amphiphysin 1, and Rvs 167. *In vitro* analysis of the BAR domain carried out in our lab indicates that it functions in lipid-binding, homo- and hetero-dimerization, and curvature generation (Farsad et al., 2001; Ringstad et al., 2001; Takei et al., 1999). When these observations were made, BAR domains were typical of proteins implicated in endocytosis. Database searches using the BAR domain of amphiphysin I identified a novel partial protein, KIAA1010, containing a BAR domain homology region. KIAA1010, like amphiphysin and endophilin, also contained SH3 domains. This raised the possibility that it might be a novel member of the endocytic machinery.

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The putative BAR domain of KIAA1010 was also of interest because of its location within the protein. At the time, the BAR domains of all the described BAR domain-containing proteins were located at the amino terminus. The BAR domain homology region of KIAA1010, however, was located in the middle of the protein. Furthermore, the predicted BAR domain of KIAA1010 was located downstream of a Dbl homology region. Dbl homology domains function as exchange factors for the Rho family of small GTPases, which among other things, regulate the actin cytoskeleton (Hoffman and Cerione, 2002). This observation was exciting, as KIAA1010 would provide a further link between endocytosis and the actin cytoskeleton. Dbl homology domains were invariably followed by pleckstrin homology domains, which function as phosphatidyl-inositol binding modules (Hoffman and Cerione, 2002). The Dbl homology domain of KIAA1010, however, was followed by the putative BAR domain. As described above, one of the functions of the BAR domain is to bind lipids, suggesting that the BAR domain might functionally replace the pleckstrin homology domain.

In summary, KIAA1010 was a protein with a region homologous to the BAR domain of amphiphysin I, as well as multiple SH3 domains, suggesting a structural similarity with amphiphysin and endophilin, proteins shown to be important contributors to clathrin-mediated endocytosis. KIAA1010 also contained a region homologous to Dbl homology domains, which activate Rho family members to effect changes in the actin cytoskeleton. Thus, there was sufficient evidence to investigate whether KIAA1010 was a member of the endocytic machinery, and tantalizing hints that it might also be an actin regulatory protein, resulting in a novel protein which would link endocytosis to the actin

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cytoskeleton. A full-length clone of KIAA1010 was generated and named Tuba. What follows is an initial characterization of the Tuba protein.

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Chapter II--Tuba is a Synaptic Protein

Tuba Is a Novel BAR Domain-Containing Protein

A BLAST search for proteins containing a domain related to the BAR domain of amphiphysin-1 revealed a large number of sequences. In one (KIAA1010), the putative BAR domain is not located at the N-terminus of the protein as in most other sequences, but downstream of a DH domain (Fig. 7*A*). Although the overall homology to the amphiphysin-1 BAR domain is limited (24% identical and 39% similar) (Fig. 7*B*), similarities are concentrated in regions generally conserved among the Bin/amphiphysin family. This region in KIAA1010 is currently identified as a BAR domain by protein module-recognizing algorithms such as those of the Pfam and SMART programs.

We undertook 5'-RACE using human cDNAs from muscle and brain to isolate a full-length protein corresponding to KIAA1010. This protein, which we have named Tuba in line with the tradition of naming large synaptic proteins after musical instruments (Cases-Langhoff et al., 1996), comprises 1577 amino acids with a predicted molecular mass of 178 kDa. The corresponding gene is located on human chromosome 10. The domain structure of Tuba (Fig. 7*A*) includes four N-terminal SH3 domains (referred to as SH3-1, SH3-2, SH3-3, and SH3-4), a predicted coiled-coil domain, a DH domain, the BAR domain, and two additional C-terminal SH3 domains (SH3-5 and SH3-6). In addition, a proline-rich low complexity region containing putative SH3 domain-binding sites is present upstream of the coiled-coil region (Fig. 7*A*).

In an independent line of study, a yeast two-hybrid screen aimed at identifying ligands for the Ena/VASP family protein EVL from an embryonic mouse library led to the isolation of two independent clones highly homologous to the C terminus of KIAA1010

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FIGURE 7. **Domain structure of Tuba and related proteins.** *A*, Tuba, Tuba2, and Tuba3 are shown with domains of interest noted. The *thick lines* below Tuba indicate the portion of Tuba encoded by KIAA1010 and the partial clones isolated in the yeast two-hybrid screen for EVL interactors. Also shown is the shorter isoform of Tuba as well as the *C. elegans* Tuba homolog, GEI-18. *B*, shown is an alignment of the BAR domains of the Tuba family of proteins. The amphiphysin (*Amp*), Tuba, and Tuba3 BAR domains are from human; the Tuba2 sequence is from monkey; and the GEI-18 sequence is from *C. elegans*.

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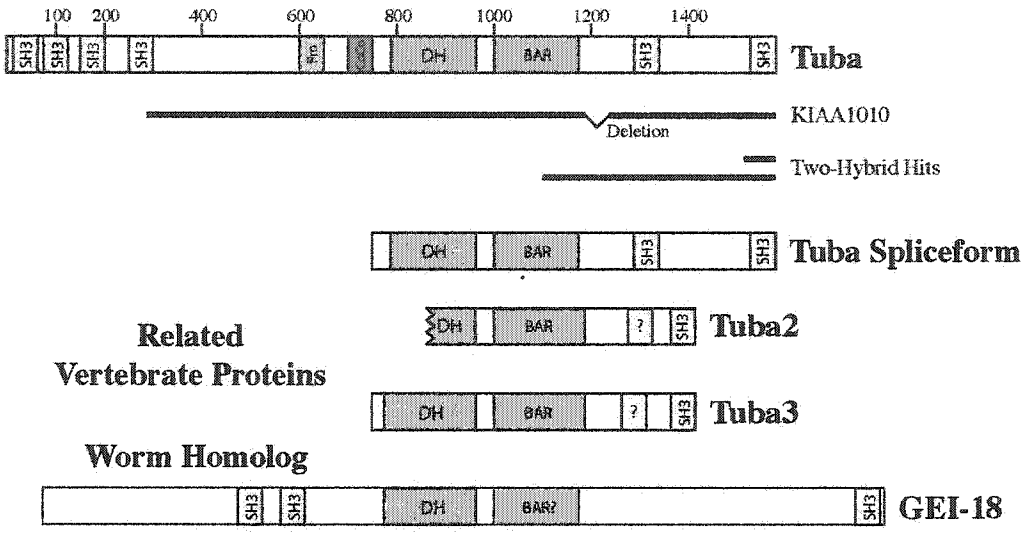

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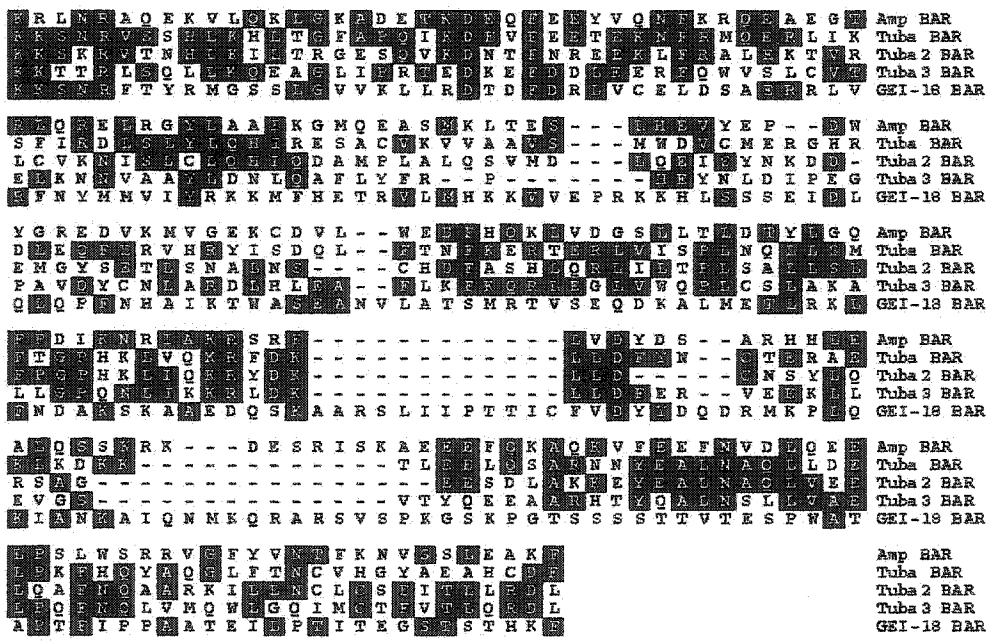

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(Fig. 7*A*) (data not shown). Reverse transcription-PCR using an embryonic mouse cDNA library was carried out to obtain the full-length gene, which turned out to be the mouse ortholog of Tuba. Mouse Tuba is 70% identical and 77% similar to human Tuba, has a similar domain structure, and is encoded by a gene located on mouse chromosome 19.

Searches through genomic and expressed sequence tag data bases revealed numerous expressed sequence tags to two genes that encode proteins homologous to the C-terminal half of Tuba in both human and mouse. We have named these proteins Tuba2 and Tuba3 (Fig. 7*A*). Tuba2 is located on human chromosome 4 and mouse chromosome 3, and Tuba3 is located on human chromosome 5 and mouse chromosome 8. Tuba2 is 41% identical and 60% similar to Tuba, and Tuba3 is 25% identical and 41% similar to Tuba. Tuba2 is 31% identical and 48% similar to Tuba3. Recent searches have also revealed what appears to be an alternately spliced form of Tuba that is similar in structure to Tuba2 and Tuba3 (Fig. 7*A*).

A putative ortholog of Tuba, GEI-18 (GEX interactor-18), was identified in *Caenorhabditis elegans* (Fig. 7*A*) (Tsuboi et al., 2002). Two alternate transcripts of GEI-18 are described that comprise the N- and C-terminal halves of the protein. Although not recognized by Pfam or SMART, the region C-terminal of the DH domain in GEI-18 appears to be very similar to a BAR domain. A comparison of the BAR domains of the Tuba family of proteins with each other and human amphiphysin-1 is shown in Fig. 7*B*.

Tuba Is Ubiquitous

Northern blot analysis of human tissues with a probe corresponding to the C terminus of Tuba (nucleotides 4035–4540) revealed two transcripts of 7.3 and 6 kb (Fig. 8*A*) whose levels varied in different tissues. A probe directed against the N-terminal half of the protein (nucleotides 1246–1696) recognized only the larger transcript (data not shown). A

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FIGURE 8. **Ubiquitous expression of Tuba transcripts and their protein products.** *A*, multiple-tissue Northern blot using a probe directed against the 3' end of Tuba. *B*, multiple-tissue Western blot of rat tissue post-nuclear supernatants with affinity-purified Tuba-specific antibodies. *Sk.*, skeletal.

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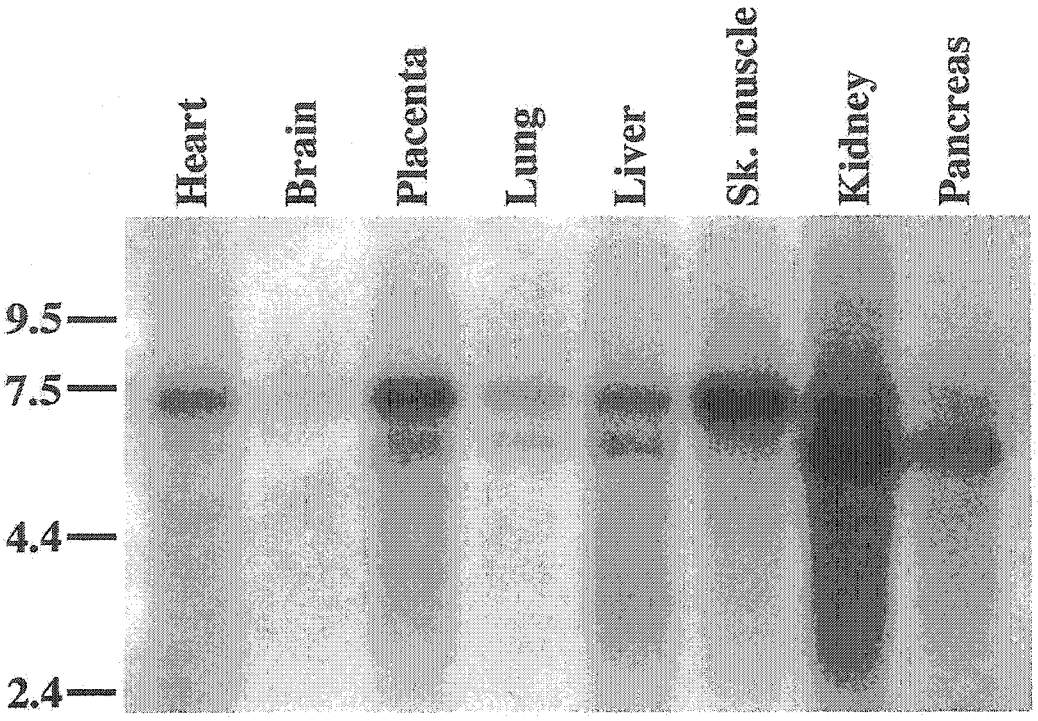

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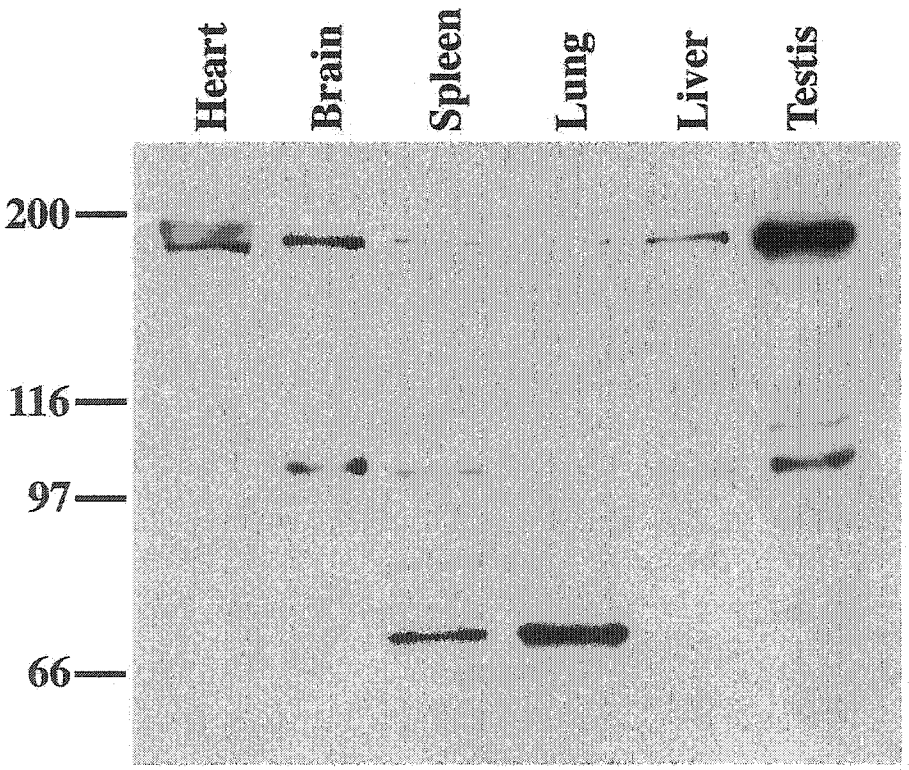

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third transcript of 4.5 kb was observed in a number of mouse tissues with a larger probe corresponding to the C terminus of mouse Tuba (nucleotides 2971–4742) (data not shown). This Tuba mRNA likely encodes only the second half of the protein, *i.e.* a Tuba splice variant similar in domain structure to the homologous proteins Tuba2 and Tuba3 (Fig. 7*A*). When tested by Western blotting against various rat tissues, affinity-purified antibodies generated against the C terminus of Tuba recognized a band at the expected molecular mass of full-length Tuba (180 kDa). The band was the strongest in testis, followed by brain, heart, liver, spleen, and lung. In addition, the same antibodies recognized lower molecular mass bands at 105 and 75 kDa with differential tissue distribution that may represent alternatively spliced forms of Tuba or proteolytic C-terminal fragments (Fig. 8*B*). The 75-kDa band may also represent cross-reactivity of the antibodies against either Tuba2 or Tuba3, whose molecular masses are predicted to be in this range. Collectively, these data indicate that Tuba has a broad tissue distribution and may exist in multiple isoforms.

Tuba Is Found at the Synapse

To determine the localization of Tuba in brain, where dynamin participates in the clathrin-mediated endocytosis of synaptic vesicles, rat brain cryosections were stained for Tuba by immunofluorescence. Tuba immunostaining yielded a punctate pattern outlining the surface of neuronal perikarya and dendrites that co-localized with immunoreactivity for the synaptic markers amphiphysin-1 and dynamin-1 (Hudy-1 antibodies) (Fig. 9*A*). In addition, Tuba immunoreactivity was observed within neuronal cell bodies at locations that corresponded to the Golgi complex, as shown by counterstaining for the Golgi marker GM-130 (Nakamura et al., 1995). However, high magnification observation indicated that GM-130 and Tuba did not have an overlapping distribution, suggesting that the two antigens localized to distinct Golgi complex subcompartments (Fig. 9*A*).

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FIGURE 9. **Tuba is concentrated at the synapse in brain.** *A*, double immunofluorescence of rat brainstem frozen sections with antibodies directed against Tuba (*red*) and other antigens (*green*) as indicated. Tuba co-localizes with the synaptically enriched proteins amphiphysin-1 and dynamin-1. Synapses appear as bright fluorescent puncta that outline the surface of perikarya and dendrites. In addition, Tuba immunoreactivity is present in the Golgi complex area, as shown in the section counterstained for the Golgi complex marker GM-130. Within the Golgi complex, however, Tuba and GM-130 do not have an overlapping distribution. *B*, immunogold labeling of a lysed synaptosome demonstrating the concentration of Tuba at the periphery of the synaptic vesicle cluster in the pre-synaptic compartment. The *arrow* points to a clathrin-coated vesicle.

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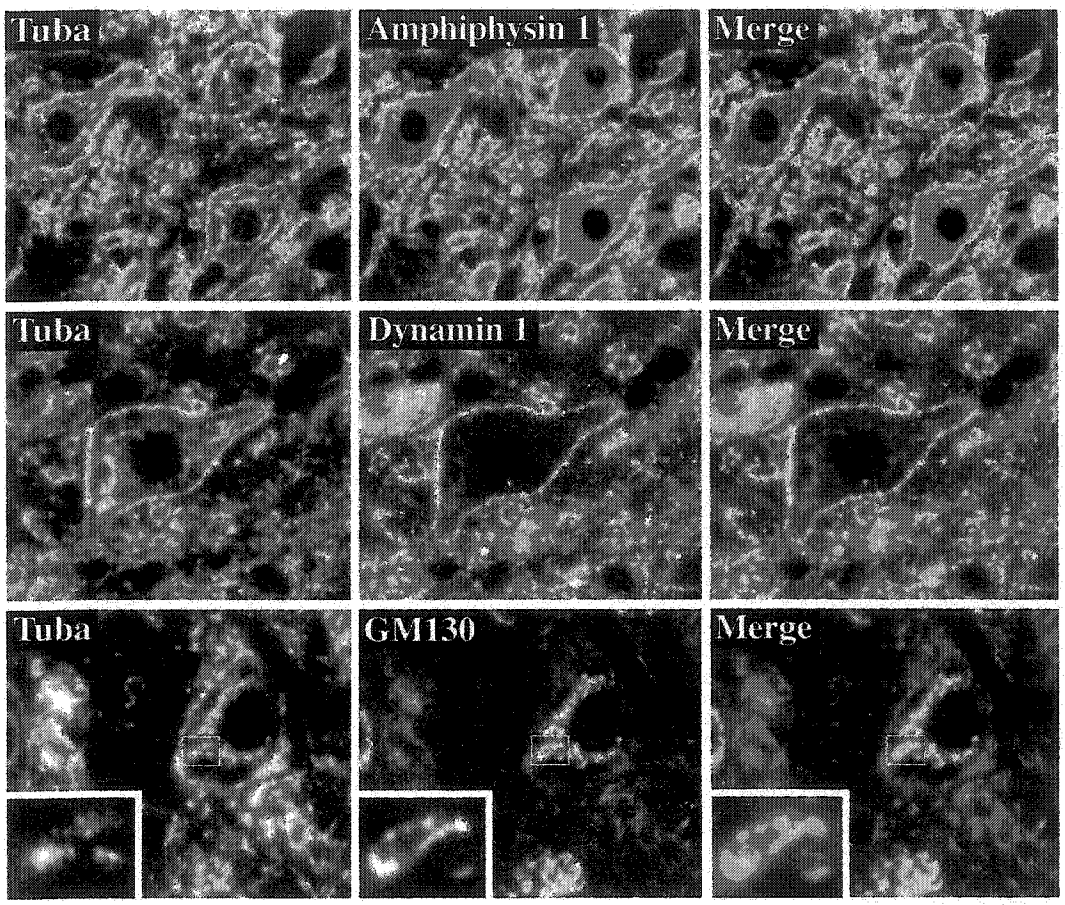

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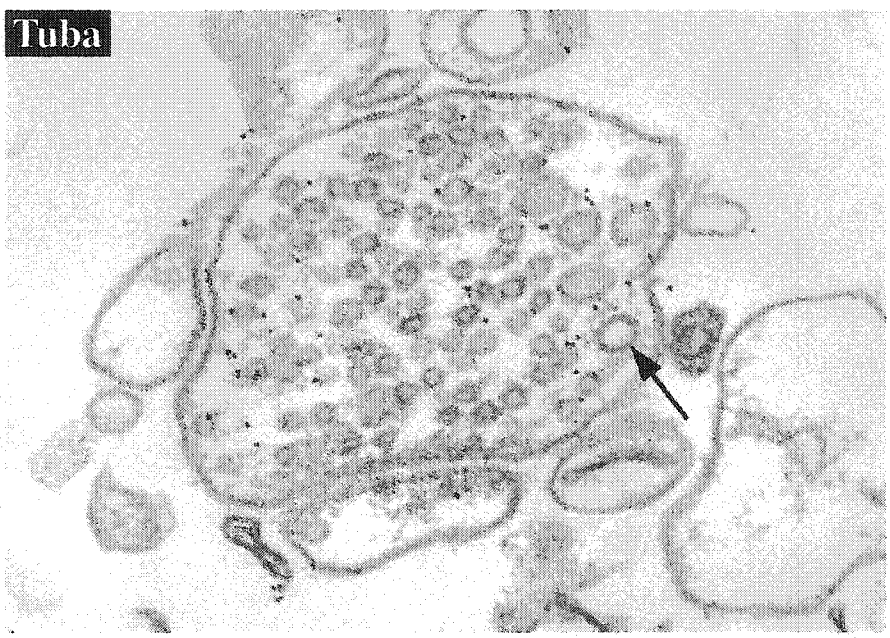

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To analyze the synaptic localization of Tuba in more detail, lysed synaptosomes were processed for anti-Tuba immunogold electron microscopy using a pre-plastic embedding procedure. Gold immunolabeling was detected in the pre-synaptic compartment, where it was primarily concentrated at the periphery of synaptic vesicle clusters (Fig. 9*B*). These are the regions where clathrin-mediated endocytosis occurs (for example, see a clathrin-coated vesicle in Fig. 9*B*) and where pre-synaptic actin is concentrated. This localization is consistent with a role of Tuba in endocytosis and actin function, as proposed for other BAR domain-containing proteins. To begin elucidating the physiological role of Tuba, the binding partners of its SH3 domains were investigated.

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Chapter III—Tuba is a Dynamin-Binding Protein

To identify binding partners of the N-terminal SH3 domains of Tuba, a GST fusion protein comprising the four N-terminal SH3 domains (SH3-1,2,3,4) was generated and incubated with Triton X-100-solubilized rat brain extracts in affinity chromatography experiments. As shown by Coomassie Blue staining of SDS gels of the material retained by the beads, the fusion protein, but not GST alone, specifically and efficiently retained a protein of 100 kDa (Fig. 10*A*). This protein was identified as dynamin-1 by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) spectrometry (data not shown) and immunoblotting (Fig. 10*B*). The interaction between the SH3 domains of Tuba and dynamin-1 is direct because it could be confirmed by far-Western blotting using HA tagged SH3-1,2,3,4 as a probe (data not shown). Furthermore, dynamin (but not synaptotagmin) could be coprecipitated with Tuba from Triton X-100-solubilized rat brain extracts, demonstrating that the interaction can occur *in vivo* (Fig. 12*A*). The separate analysis of each of the four SH3 domains in affinity purification experiments revealed that all of them bound dynamin and that SH3-4 had the highest affinity for dynamin, followed by, in order of decreasing affinity, SH3-1, SH3-3, and SH3-2 (Fig. 11). We conclude that dynamin is the main ligand of the Tuba N-terminal region.

To better determine whether the interaction between dynamin and the N-terminus of Tuba is relevant *in vivo*, we expressed HA-tagged SH3-1,2,3,4 in Chinese hamster ovary cells and determined its effect on transferrin uptake, a dynamin-dependent endocytic reaction. It was shown previously that SH3 domains that bind dynamin can function as potent inhibitors of this process, probably by titrating out dynamin (Shupliakov et al., 1997; Wigge et al., 1997b). When expressed in Chinese hamster ovary cells, SH3-1,2,3,4 had a cytosolic distribution and inhibited transferrin internalization (Fig. 12*B*),

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FIGURE 10. **The N-terminal SH3 domains of Tuba bind dynamin-1.** *A*, bead-immobilized GST and a GST fusion protein of the N-terminal region of Tuba (SH3-1,2,3,4) were incubated with a Triton X-100-solubilized rat brain extract, and the bound material was analyzed by SDS-PAGE and Coomassie Blue staining. A band of 100 kDa (dynamin) was selectively affinity-purified. The greater abundance of dynamin than of the fusion protein used as bait is likely to reflect the oligomerization state of dynamin. *B*, shown is an anti-dynamin Western blot of the material affinity-purified by GST or the GST fusion protein.

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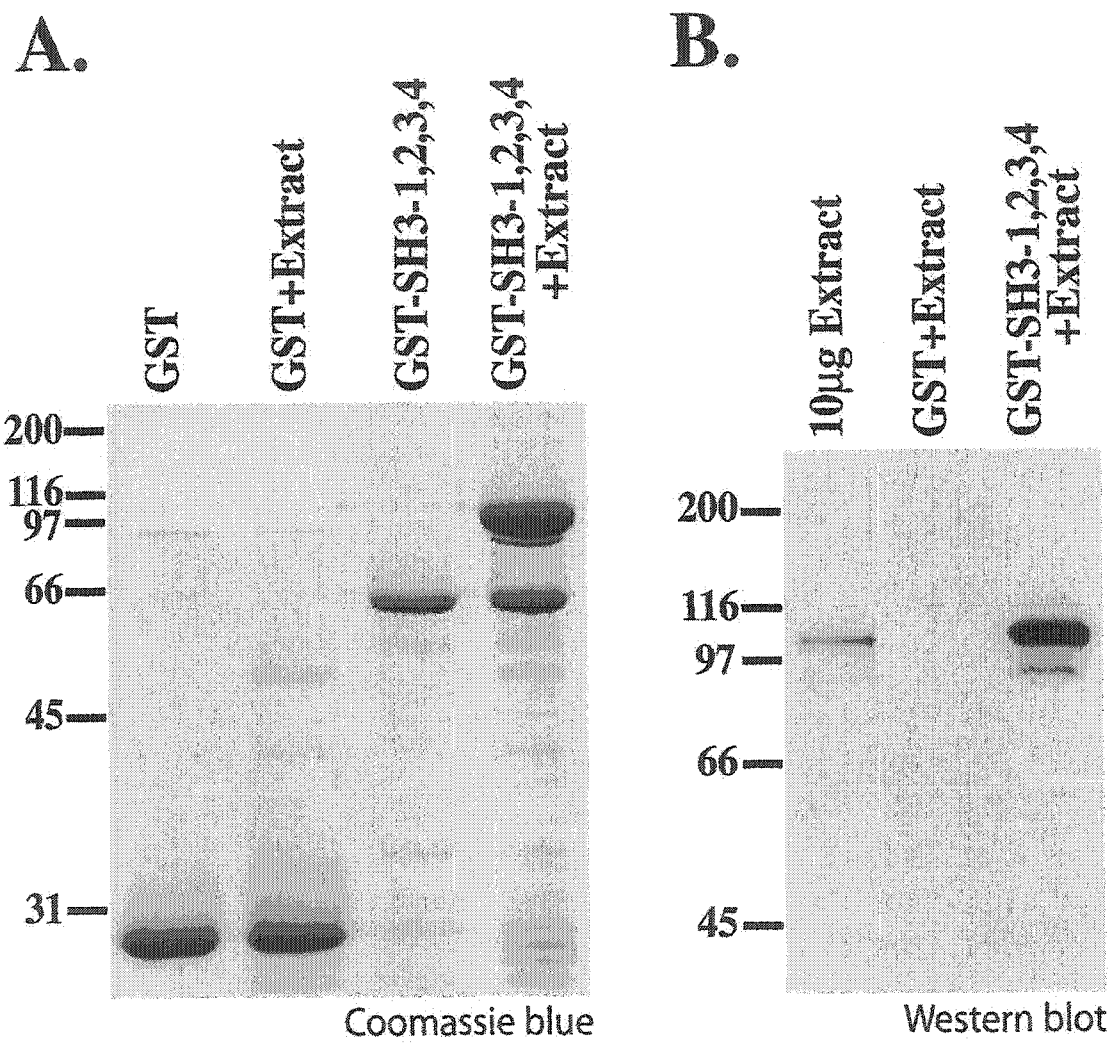

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FIGURE 11. **Tuba SH3 domains 1, 3, and 4 interact with dynamin.** Bead immobilized GST and GST fusion proteins encompassing different combinations of the N-terminal SH3 domains of Tuba were incubated with a Triton X-100-solubilized rat brain extract, and the bound material was analyzed by SDS-PAGE and Coomassie Blue staining. A band of 100 kDa (dynamin) was selectively affinity-purified by SH3 domains 1, 3, and 4, but not SH3-6. SH3-2 interacts with dynamin, but only weakly. Bands below the 100 kDa dynamin band represent fusion proteins. Note the intensity of the dynamin band is greater with increasing numbers of SH3 domains.

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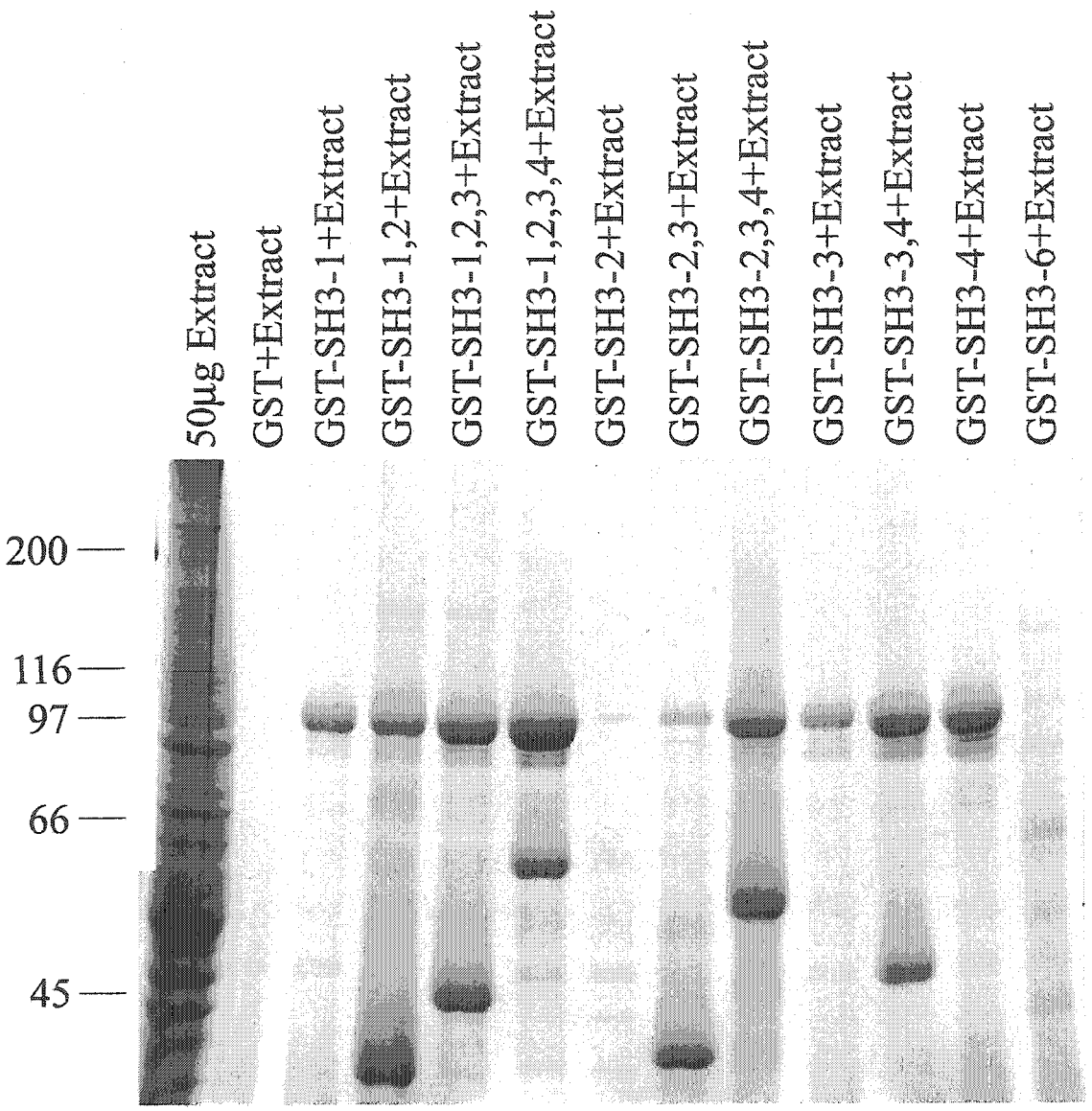

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FIGURE 12. **Tuba and dynamin may interact *in vivo*.** *A*, control or anti-Tuba antibodies were used to generate immunoprecipitates from a Triton X-100-solubilized rat brain extract. Western blots for Tuba, dynamin, and synaptotagmin of the immunoprecipitates are shown. *B*, Chinese hamster ovary cells were transfected with HA-tagged Tuba SH3-1,2,3,4 and then incubated for 7 min with Alexa-transferrin prior to fixation in 4% formaldehyde, followed by anti-HA immunofluorescence. Overexpression of Tuba SH3-1,2,3,4 inhibited transferrin uptake, as demonstrated by the lack of intracellular transferrin fluorescence in the transfected cell.

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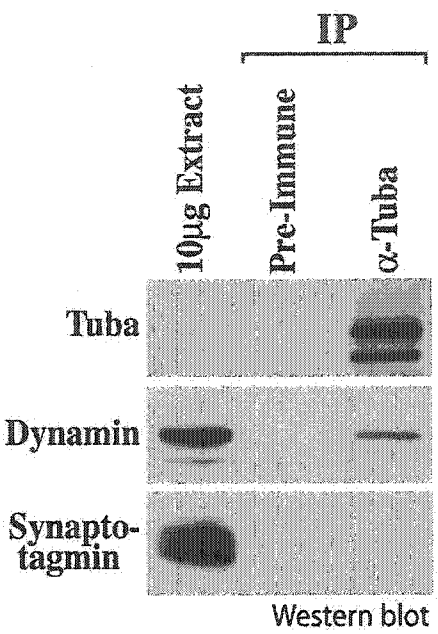

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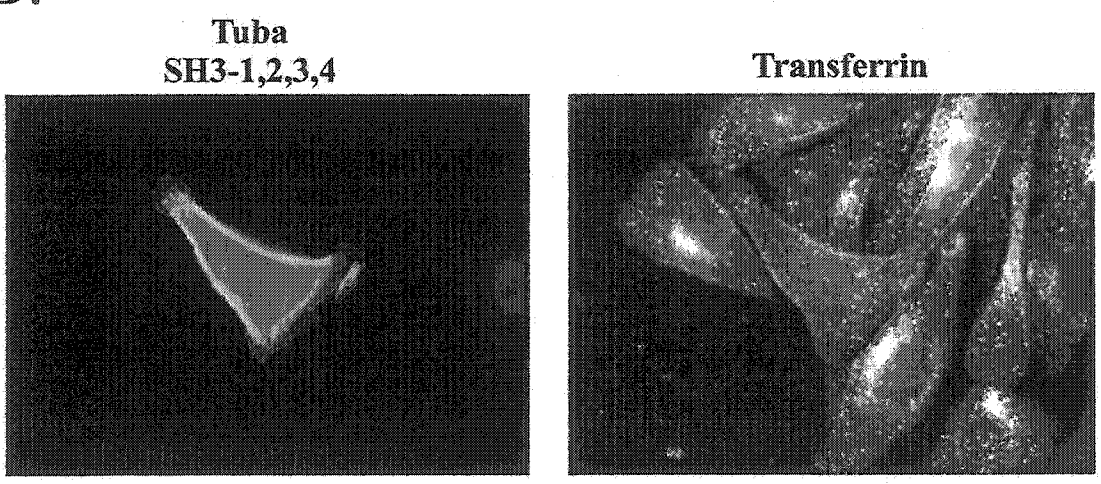

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suggesting that the N terminus of Tuba can interact with dynamin *in vivo*. Cytosolic expression of the C terminal SH3 domain (which does not bind dynamin; see below) had no effect on transferrin uptake (data not shown).

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Chapter IV—Tuba Interacts with Components of the Actin Cytoskeleton

The C-terminal SH3 Domain of Tuba Binds to an Actin Regulatory Complex

We next searched for interactors of the C-terminal SH3 domains of Tuba. GST fusion proteins of SH3-5 and SH3-6 were generated and used in pull-down experiments with Triton X-100-solubilized rat brain extracts. No major interactors were found for SH3-5 (data not shown). However, SH3-6 (but not GST alone) pulled down a variety of proteins as revealed by the Coomassie Blue-stained SDS gel of the affinity-purified material (Fig. 13). Each of the major protein bands was excised, trypsin-digested, and analyzed by MALDI-TOF spectrometry. The identified proteins are listed in Fig. 13 (*upper panel*). For some proteins, the interaction was validated further by Western blotting (Fig. 13, *lower panels*) (data not shown). Two of the major bands were actin and tubulin. Another was Hsp70, which is often found in eluates of pull-down experiments, possibly reflecting the promiscuous role of this ATPase in protein folding reactions. All other bands represent proteins that are either directly or indirectly linked to the regulation of actin dynamics.

The most abundant protein was N-WASP, the ubiquitous and brain enriched homolog of the Wiskott-Aldrich syndrome protein WASP (Miki et al., 1996). CR16 and WIRE (also known as WICH) are both related to WIP, a proline-rich protein that binds to actin and interacts with the N-terminal WASP-homology 1 (WH1) domain of N-WASP (Aspenstrom, 2002; Ho et al., 2001; Kato et al., 2002; Martinez-Quiles et al., 2001). WAVE1 is a neuron-specific SCAR/WAVE protein and a member of the WASP superfamily (Miki et al., 1998) that, like N-WASP, regulates actin assembly through binding and activation of the Arp2/3 complex (Machesky et al., 1999). Unlike N-WASP,

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FIGURE 13. **The C-terminal SH3 domain (SH3-6) of Tuba binds actin regulatory proteins.** *A*, bead-immobilized GST and a GST fusion protein of SH3-6 were incubated with a Triton X-100-solubilized rat brain extract, and the bound material was analyzed by SDS-PAGE and Coomassie Blue staining (*upper panel*). The identities of the proteins were determined by MALDI-TOF and Q-TOF mass spectrometric analysis. Binding of WAVE1 and N-WASP was confirmed by Western blot analysis (*lower panels*).

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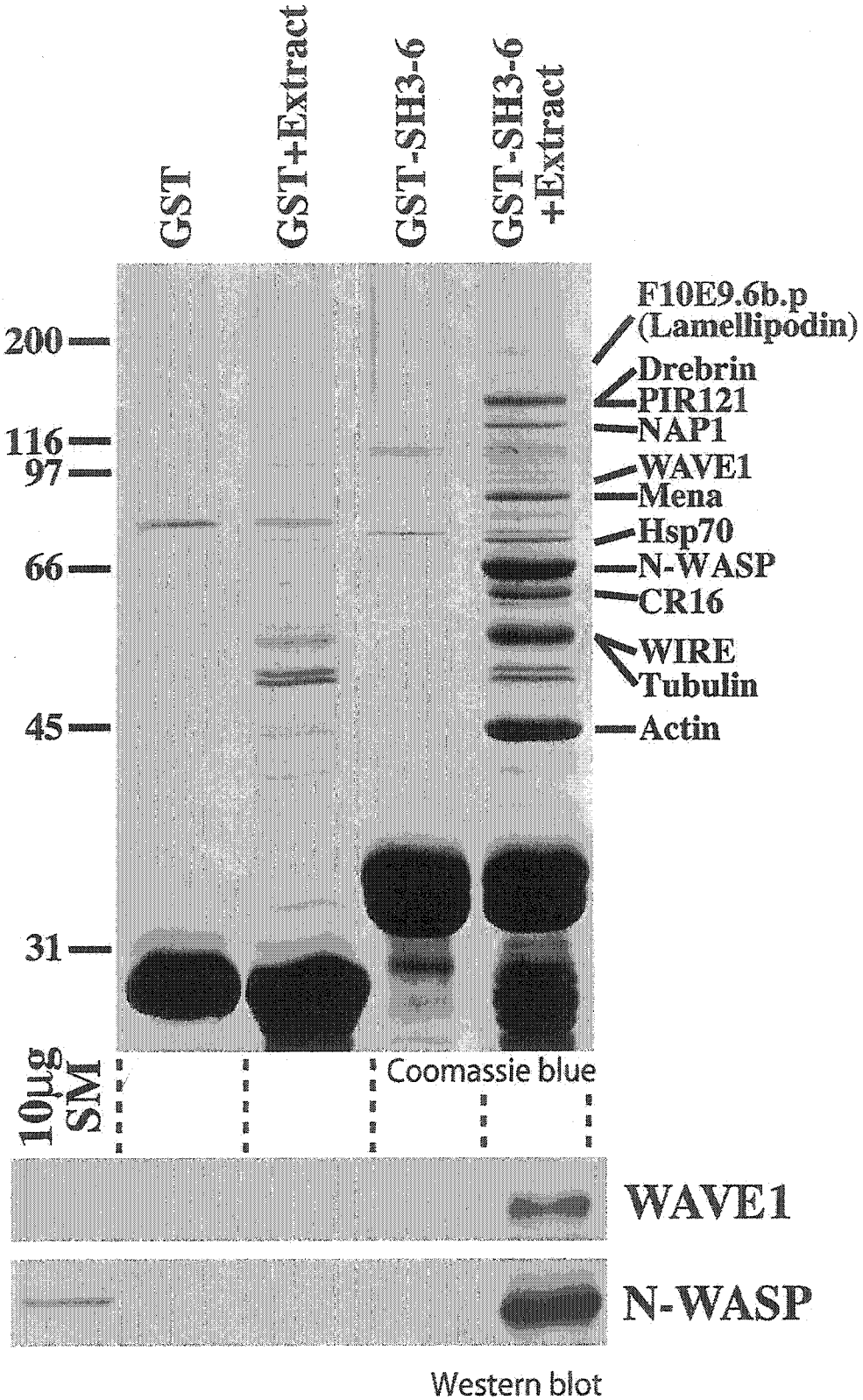

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isolated WAVE1 is constitutively active (Machesky et al., 1999), but is kept in an inhibited state while bound to a protein complex that includes PIR121 and NAP1 (Eden et al., 2002), two proteins present in the affinity-purified material. Mena, an Ena/VASP protein (Gertler et al., 1996), and Lamellipodin, a novel Ena/VASP-associated protein (M. Krause and F. B. Gertler, unpublished data), were also present in the affinity-purified material, as was drebrin, an F-actin-binding protein (Ishikawa et al., 1994).

A gel overlay with 32P-labeled GST-SH3-6 was performed on the material affinity purified by SH3-6 from mouse brain lysate. Two prominent bands bound by SH3-6 were adjacent to the 66-kDa marker (Fig. 14). Western blotting demonstrated that the upper band precisely co-migrated with N-WASP (data not shown), whereas the lower band appears to be CR16 (Fig. 14). The overlay experiment also revealed a prominent band at 50 kDa and a somewhat weaker signal at 140 kDa. Western blotting indicated that the band at 140 kDa corresponds to Mena (Fig. 14). The identity of the 50-kDa band remains unknown. Immunoprecipitation experiments demonstrate that N-WASP and Tuba are co-immunoprecipitated from rat brain extracts with Tuba specific antibodies (data not shown).

The C-terminal SH3 Domain of Tuba Can Promote F-Actin Recruitment

Because the Tuba SH3-6 domain can bind to a number of actin regulatory proteins, we wondered whether concentrating this domain on a surface within the cell would result in recruitment or nucleation of F-actin. To accomplish this, we fused the SH3-6 domain to a mitochondrial anchoring sequence (SH3-6-mito), a method that has been used successfully to map protein domains that induce actin recruitment within living cells (Kessels and Qualmann, 2002). Because Tuba is expressed in brain and localizes to synapses, we used CAD cells, a neuron-like cell line, to express SH3-6-mito. Interestingly, in 15–20% of transfected cells expressing high levels of SH3-6-mito, F-

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FIGURE 14. **Tuba directly interacts with N-WASP.** The material from mouse brain lysates affinity-purified by GST or GST-SH3-6 was separated by SDS-PAGE and overlaid with 32P-labeled SH3-6 (*left panel*). The blot was stripped and reprobed with antibodies against potential ligands (*right panel*).

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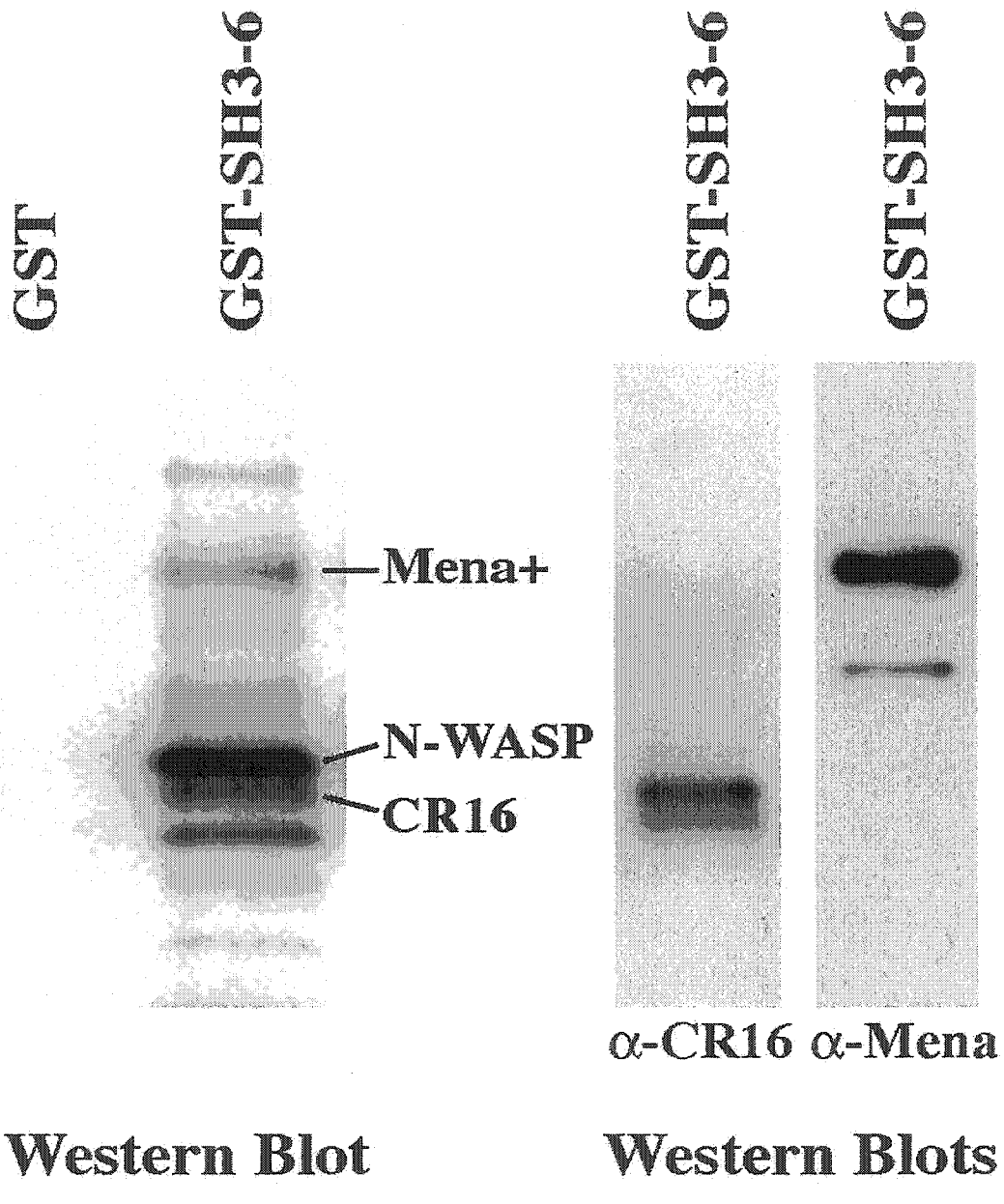

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actin was found to co-localize with the SH3-6-mito fusion (Fig. 15, *D–F*). Therefore, overexpression of the SH3-6 domain can promote F-actin nucleation and/or recruitment within cells, presumably via one or more of the actin regulatory proteins it is known to bind *in vitro*.

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FIGURE 15. **The C-terminal SH3 domain of Tuba recruits F-actin.** SH3-6-mito, a DsRed2 fusion protein of SH3-6 with a mitochondrial targeting sequence at the C terminus, was transiently transfected into CAD cells. *A–C* show control cells stained for F-actin (*A*) that lacks any notable DsRed2 signal (*B* and merge in *C*). *D–F* demonstrate the co-localization of F-actin (*D*) with SH3-6-mito (*E* and merge in *F*) in two highly expressing cells.

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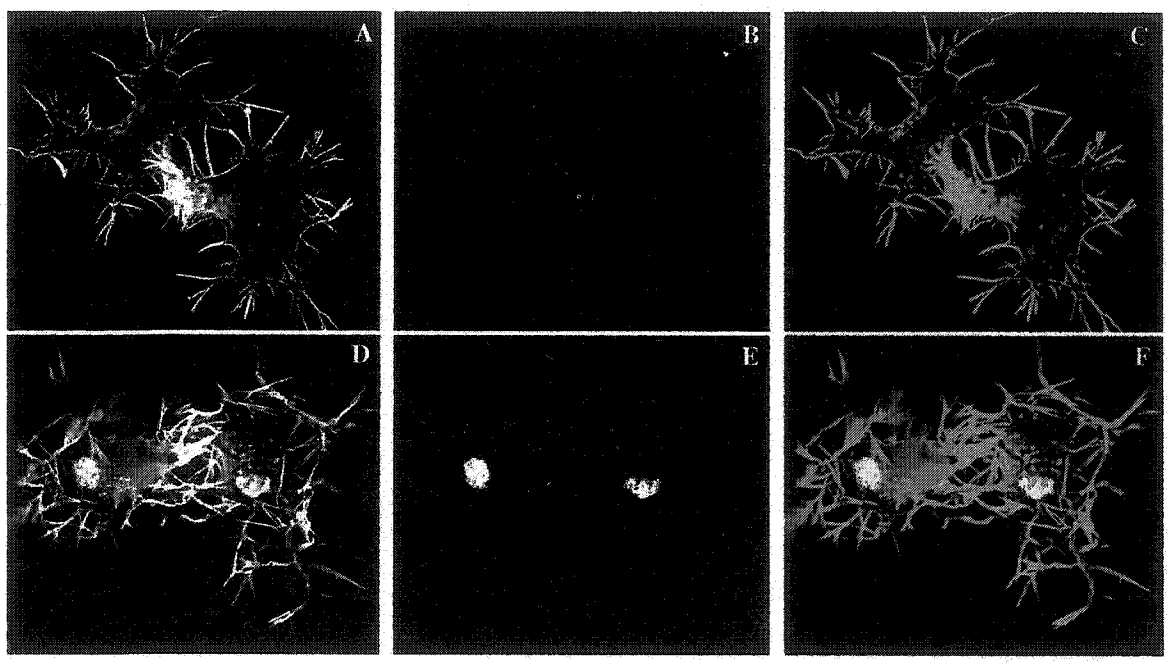

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Chapter V—Tuba is a Cdc42-Specific Exchange Factor

The DH-BAR Region of Tuba Catalyzes the Formation of Active Cdc42

The results described above indicate that the C-terminal SH3 domain of Tuba can promote actin nucleation and/or recruitment. The presence of a DH domain in Tuba, a protein module involved in the activation of Rho family GTPases, suggests a further link to the actin cytoskeleton. The function of this domain was investigated.

Like other GTPases, Rho family GTPases cycle between a GTP-bound active state and a GDP bound inactive state. DH domains form the core catalytic domains of enzymes that activate Rho family GTPases by catalyzing the exchange of GDP for GTP (Hoffman and Cerione, 2002). We studied the substrate specificity of the DH domain of Tuba by testing its guanyl nucleotide exchange activity on three major representative members of the Rho family: RhoA, Rac1, and Cdc42. A His-tagged Tuba DH domain was used in a mant-GTP-based assay, which allows the guanyl nucleotide exchange reaction to be monitored fluorometrically (Snyder et al., 2002). Unfortunately, the insolubility of the BAR domain prevented us from testing the DH-BAR fragment in this assay. The DH domain of Tuba specifically catalyzed, in a concentration dependent manner, the activation of Cdc42, but not Rho or Rac (Fig. 16). The His-tagged DH-PH domain of Vav2, a DH domain known to have a promiscuous exchange activity on Rho, Rac, and Cdc42 (Liu and Burridge, 2000), was used as a control to demonstrate that all three GTPases used were competent for exchange (Fig. 16). The DH domain of Tuba, although specific for Cdc42, displayed relatively low activity compared with the DH-PH domain of Vav2: 10 M Tuba DH domain was comparable to 200 nM Vav2 DH-PH domain. DH domains without associated PH domains are generally less active than DH-PH fragments. Because we believe that the BAR domain of Tuba is a functional replacement for the PH domain, this

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FIGURE 16. **The DH domain of Tuba specifically catalyzes the activation of Cdc42.** The indicated GTPases (2 M) were incubated with 400 nM mant-GTP for 200 s prior to addition of the indicated concentrations of His-tagged Tuba DH domain. Exchange activity was followed by the increase in fluorescence (*fluor.*), normalized to its starting value, and reflects the binding of mant-GTP to the GTPases. To verify the integrity of the GTPases, a fragment of Vav2 (0.2 M) containing the DH and PH domains and previously shown to be active on Rho, Rac, and Cdc42 was used to load mant-GTP onto the GTPases under identical conditions.

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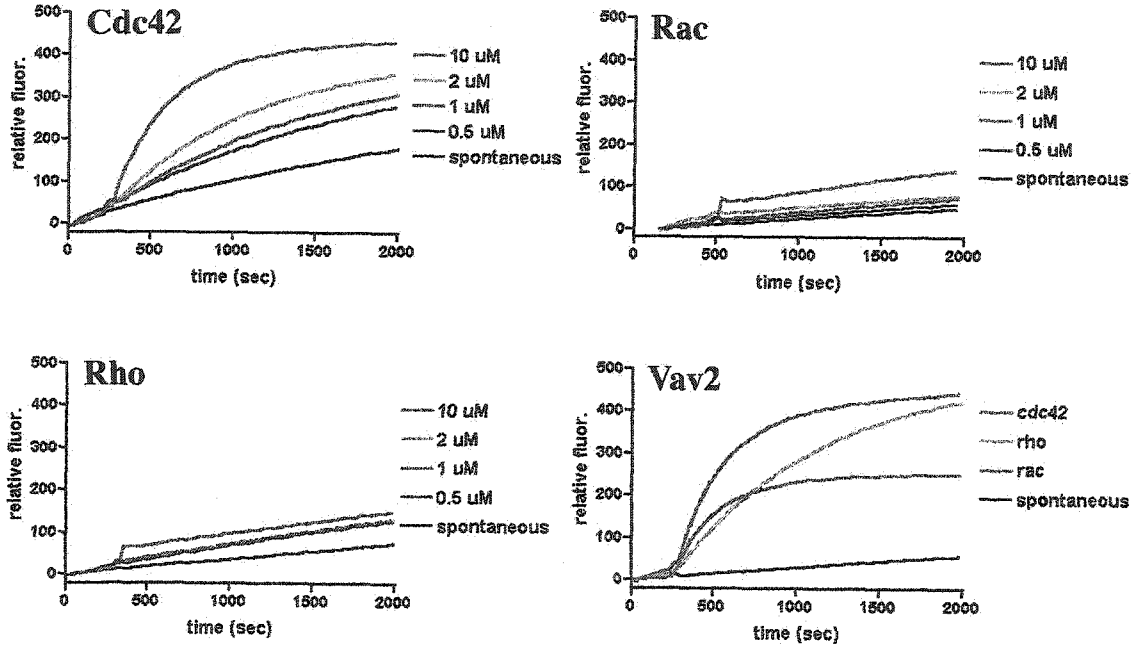

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absence of the BAR domain could account for the low activity.

The BAR Domain of Tuba May Mediate Binding to Lipids

All characterized Dbl homology domain-containing proteins contain a pleckstrin homology domain immediately downstream of the DH domain (Hoffman and Cerione, 2002). This is not the case in Tuba. PH domains, through their ability to interact with phosphatidyl inositides, are believed to locate the DH domain to the correct membrane environment. Some PH domains also stimulate the catalytic efficiency of their associated DH domain upon PI binding (Rossman et al., 2003). Analysis of multiple sequence alignments of BAR domain-containing proteins indicate that the BAR domain of Tuba is most closely related to the BAR domain of the amphiphysin family of proteins, proteins whose BAR domains have been shown to bind lipids (Habermann, 2004; Peter et al., 2004; Takei et al., 1999). This allowed us to predict that the BAR domain of Tuba may be acting to functionally replace the PH domain. We therefore attempted to carry out experiments to demonstrate that this was indeed the case. Unfortunately, the BAR domain of Tuba is insoluble, as demonstrated by clumping of BAR domain-containing constructs transfected into fibroblasts, as well as the complete insolubility of bacterially-expressed BAR domain-containing fusion proteins (data not shown). In an effort to circumvent these limitations, DH and DH-BAR Tuba constructs were *in vitro* translated in the presence of 35S-labeled methionine, and used in a co-sedimentation liposome-binding assay (Fig. 17).

The BAR domain of endophilin was previously shown to be necessary for endophilin-lipid interactions in liposome co-sedimentation assays (Farsad et al., 2001), allowing the use of full-length endophilin as a control. In the presence of liposomes (right half of Figure 17), *in vitro* translated endophilin was located in the pellet after sedimentation, as demonstrated by the presence of radioactive material in the pellet fraction, but was not

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FIGURE 17. **The BAR domain of Tuba may bind to lipids.** HA-tagged constructs consisting of either full-length endophilin, endophilinΔ34, which lacks the amino terminal 34 amino acids of the endophilin BAR domain, Tuba DH, or Tuba DH-BAR, were *in vitro* translated in the presence of ^{35}S-methionine. 10% of each reaction was used in co-sedimentation assays, which differed for each construct by the presence or absence of liposomes generated from a purified brain lipid fraction. The left half of the figure demonstrates sedimentation in the absence of liposomes (- Liposomes), whereas sedimentation in the presence of liposomes is shown on the right half (+ Liposomes). S, supernatant. P, pellet.

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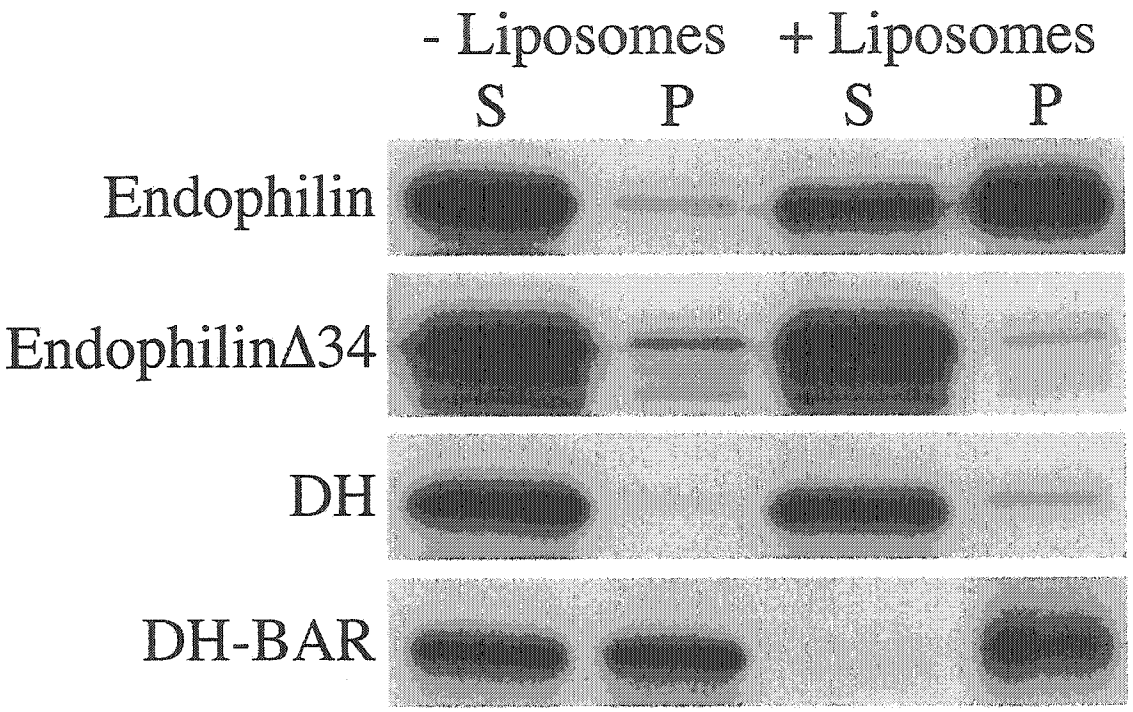

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found in the supernatant, indicating that the majority of the endophilin was bound to lipid. Note that only trace amounts of endophilin were present in the pellet fraction when liposomes were not included in the co-sedimentation assay (left half of Figure 17), providing further evidence that sedimentation of endophilin is dependent on lipid binding. A mutant form of endophilin lacking the amino terminal 34 amino acids of the BAR domain was unable to interact with liposomes, as had been previously demonstrated (Fig. 17) (Farsad et al., 2001).

The DH domain of Tuba was unable to interact with liposomes (Fig.17). This was not unexpected as no Dbl homology domain has been shown to exhibit lipid-binding properties. The Tuba DH-BAR construct was able to co-sediment with liposomes, suggesting that the BAR domain of Tuba is able to interact with lipids (Fig. 17). The control sedimentation, where liposomes were not added, provides a demonstration of the insolubility of the Tuba BAR domain (left half of Figure 17). Significant amounts of the DH-BAR construct were found in the pellet fraction of this control reaction. Although this casts some doubt on the results of the liposome-DH-BAR co-sedimentation reaction, note that there is almost no radioactive material in the soluble fraction (right half of Figure 17), which was not seen in the fraction lacking liposomes. Thus, albeit preliminary, these results suggest that the BAR domain of Tuba shares the lipid-binding properties of other BAR domains and may be acting to functionally replace the pleckstrin homology domain.

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Chapter VI—Independent Identification of Tuba

As we were completing our initial characterization of Tuba, we discovered that another group had independently identified Tuba. Adam Kwiatkowski, a graduate student in the laboratory of Frank Gerteler at the Massachusetts Institute of Technology, identified Tuba in a two-hybrid screen. Their focus, however, was on the interaction of Tuba with the Ena/VASP family of proteins. We subsequently initiated a collaboration to develop a more complete understanding of Tuba.

Because the C terminus of mouse Tuba was identified as a binding partner of EVL in a yeast two-hybrid system (Fig. 7*A*) and the co-immunoprecipitation results demonstrated that Tuba and Mena interact *in vivo*, the Ena/VASP interaction was further characterized. Pull-down experiments with lysates prepared from Ena/VASP-deficient cells (referred to as D7 cells (Bear et al., 2000)) stably infected and sorted for equal expression of enhanced green fluorescent protein (EGFP) fusions of Mena, EVL, or VASP showed that the SH3-6 domain of Tuba bound to each of the three proteins, but more robustly to EVL and Mena (Fig. 18*A*). No binding was observed in cells expressing a mutant form of Mena that lacks the proline-rich region (Fig. 18*A*), indicating that this region, present in all Ena/VASP proteins, is essential for binding.

The two-hybrid results suggest that EVL and, by analogy, all Ena/VASP proteins bind to the SH3-6 domain of Tuba directly. To test this further, lysates from D7 cells and D7 cells expressing EGFP-Mena were blotted and overlaid with 32P-labeled GST-SH3-6. A band at 115 kDa (the expected size of EGFP-Mena) was observed only in the lysate from D7 cells expressing EGFP-Mena (Fig. 18*B*, *D7 E-M*). GST-SH3-6 pull-down experiments with the same lysates were performed, and the affinity purified material

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from these pull down experiments was blotted and overlaid with 32P-labeled GST-SH3-6. Again, SH3-6 bound directly to a band with the predicted molecular mass of EGFP-Mena only in cell lysates expressing EGFP-Mena (Fig. 18*B*). Thus, Tuba binds directly to Ena/VASP proteins, consistent with the initial two-hybrid results. A direct interaction between SH3-6 and other proteins present in the affinity-purified material was also observed (Fig. 18*B*). One prominent band had the predicted electrophoretic mobility of N-WASP, and Western blotting suggested that this band was indeed N-WASP (data not shown). The identity of a major band just above N-WASP remains unknown, but we speculate that it might be a member of the WIP family of proteins. Interestingly, the intensity of this band decreased in cells expressing EGFP-Mena, possibly due to competition between Mena and this protein binding to SH3-6.

Given the large number of proteins present in the Tuba SH3-6 affinity purified complex (Fig. 13), we investigated whether the occurrence of these interactions in cells was supported by immunoprecipitation experiments. When Tuba was immunoprecipitated from embryonic day 15 lysates, Mena was found to co-precipitate (Fig. 19). Longer exposures revealed that the 140-kDa Mena , a neuron-specific isoform of Mena (Gertler et al., 1996), was also co-precipitated. These results indicate that Tuba interacts with one or more actin regulatory proteins *in vivo*.

Because both Mena and N-WASP appear to bind directly to the SH3-6 domain of Tuba, we sought to map the binding site(s) within these proteins. A series of overlapping peptides corresponding to the proline-rich regions of N-WASP, WAVE1, EVL, and Mena were synthesized by the SPOTs method (Frank, 2002). This peptide blot was overlaid with 32P-labeled GST-SH3-6 (Fig. 20*A*), and the resulting signal intensity at each peptide spot was measured and compared with the background level (Fig. 20*B*). N-

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FIGURE 18. **Interaction of the SH3-6 domain of Tuba with Ena/VASP proteins.** *A*, D7 cells, which contain no Ena/VASP protein family members, were infected with EGFP fusions of each member of the Ena/VASP family or of a Mena construct lacking the proline-rich domain (Δ*PRO*). Lysates from cells expressing equal levels of the fusion proteins were affinity-purified by GST or GST-SH3-6. The starting lysates, as well as the supernatant (*Sup*) and pellets of the affinity-purified material, were then processed by Western blotting with anti-EGFP antibodies. *B*, lysates of D7 cells or of D7 cells stably infected with EGFP-Mena (*D7 E-M*) were incubated with GST-SH3-6 in affinity chromatography experiments. The starting lysates and the pull-down (*PD*) products were separated by SDS-PAGE, blotted onto nitrocellulose, and overlaid with 32P-labeled SH3-6.

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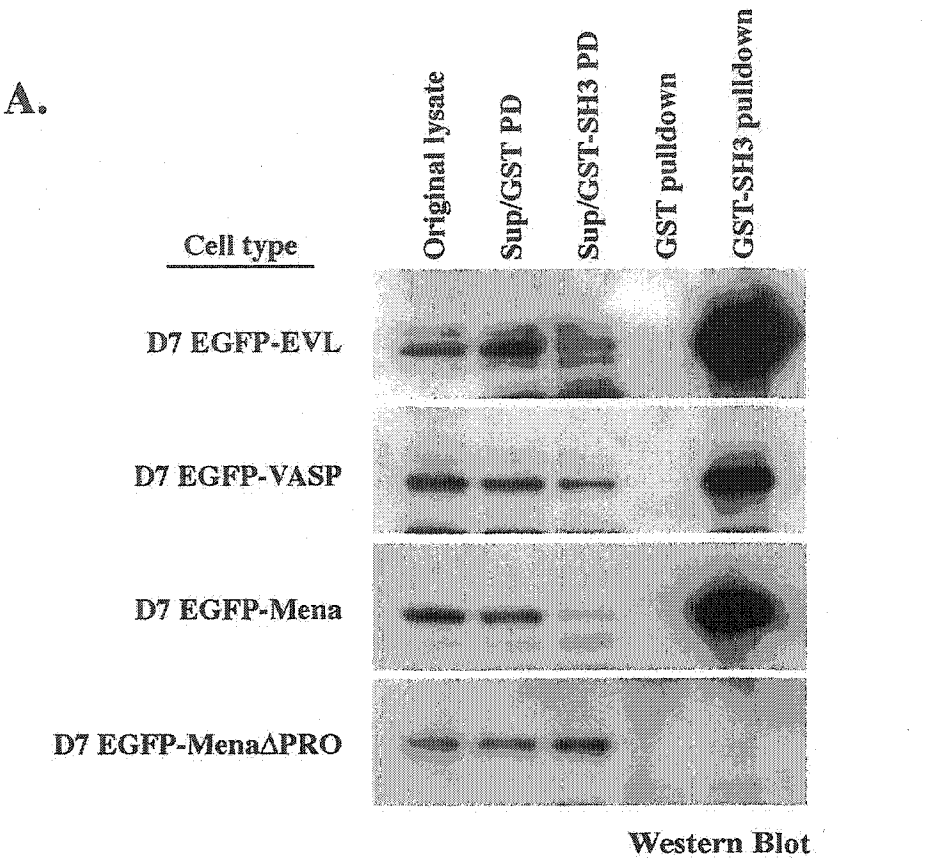

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FIGURE 19. **Mena co-immunoprecipitates with Tuba.** Control or affinity-purified anti-Tuba antibodies were used for immunoprecipitation (*IP*) from embryonic day 16 mouse lysates. The *upper panel* is a Western blot for Tuba; the *lower panel* is a Western blot for Mena. *Sup*, supernatant. *SM*, starting material.

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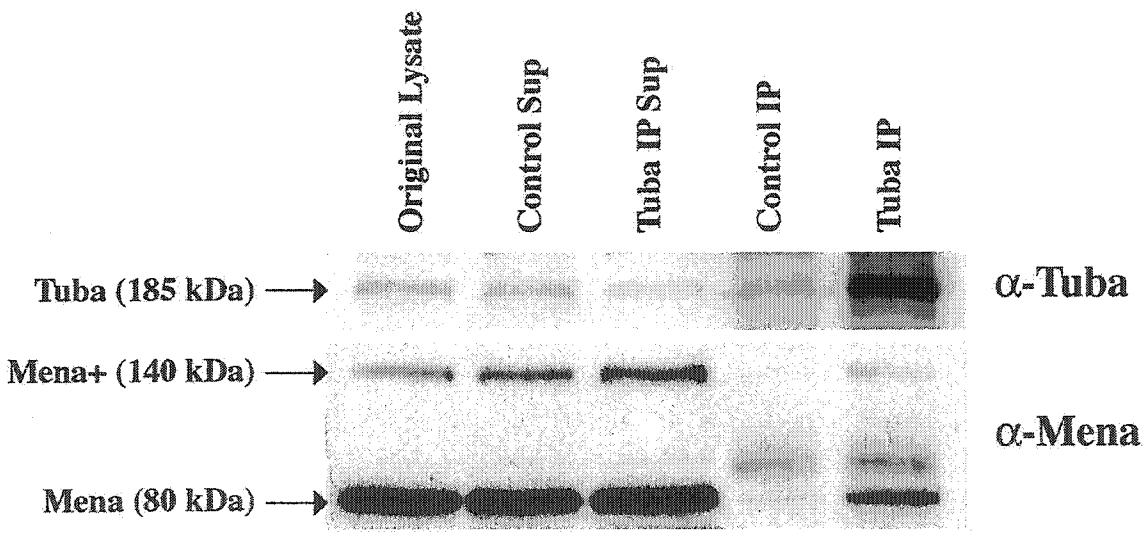

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FIGURE 20. **Mapping of SH3-6-binding sites.** *A*, shown is a SPOTs membrane containing an array of overlapping peptides corresponding to the proline-rich regions of N-WASP, WAVE1, EVL, and Mena overlaid with 32P-labeled GST-SH3-6. *B*, individual peptide spots are listed with intensity value demonstrated graphically as value/background. Peptide 118 is a negative control proline-rich sequence that binds Ena/VASP proteins, but not SH3 domains. Peptides in which the intensity/background is 6 are in *boldface*. *C*, potential binding sites for SH3-6 in N-WASP, EVL, and Mena are listed with their corresponding peptide spot number.

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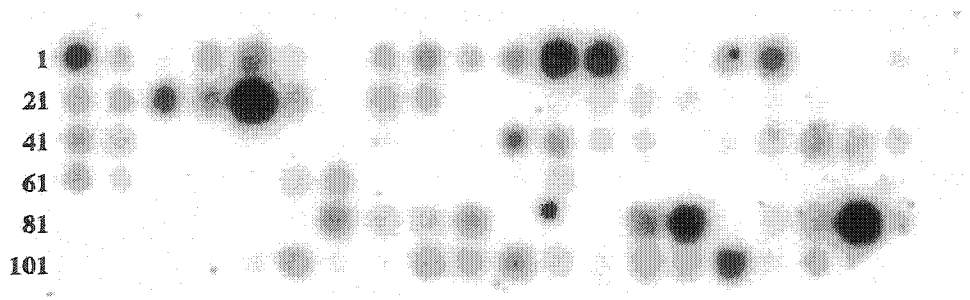
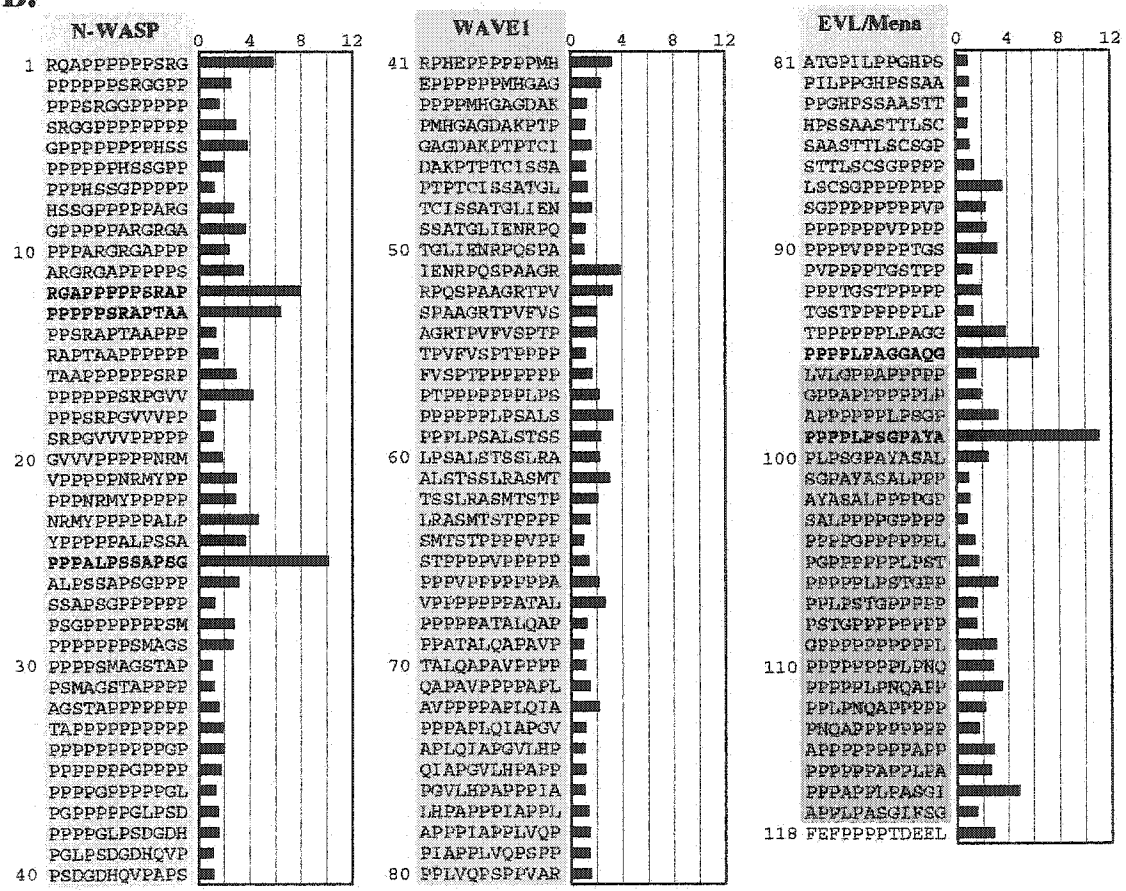
A.



B.



C.

```
N-WASP:   RGAPPPPPSRAP (#12)
          PPPALPSSAPSG (#25)
EVL:      PPPPLPAGGAQG (#95)
Mena:     PPPPLPSGPAYA (#99)
```

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WASP has two potential binding sites for SH3-6 (where the signal level was at least 6-fold over the background level), whereas EVL and Mena each have one. Strong binding to any peptide in WAVE1 was not observed. The four peptides demonstrating the strongest binding are listed in Fig. 20*C*. These results provide further evidence for direct and specific binding between SH3-6 and N-WASP and Ena/VASP proteins.

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Chapter VII—Materials and Methods

A variety of materials and techniques were utilized to make the observations described in this thesis. The protocols employed are well established and will be described only briefly.

Antibodies and Reagents

The following antibodies were used in this study: Rat anti-hemagglutinin (HA) monoclonal epitope (clone 3F10, Roche Applied Science), anti-dynamin polyclonal antibody DG1 (our laboratory) and monoclonal antibody Hudy-1 (Upstate Biotechnology, Inc.), anti-amphiphysin-1 monoclonal antibody-3 (Floyd et al., 2001) and anti-N-WASP polyclonal antibody (gifts of P. Aspenstrom (Ludvig Institute for Cancer Research, Uppsala, Sweden) and M. W. Kirschner (Harvard Medical School), anti-CR16 polyclonal antibody (gift of H. Y. Ho and M. W. Kirschner), anti-WAVE1 polyclonal antibody (gift of John Scott, Vollum Institute), anti-Mena monoclonal antibody (gift of F. B. Gertler), anti-green fluorescent protein polyclonal antibody (Clontech), anti-actin monoclonal antibody (Sigma), anti-synaptotagmin monoclonal antibody (gift of Reinhard Jahn, Max-Planck Institute for Biological Chemistry, Gottingen, Germany), and anti-GM-130 antibody (Graham Warren, Yale University).

Anti-Tuba polyclonal antibodies were generated by injecting rabbits with a glutathione *S*-transferase (GST) fusion protein encompassing the final 292 amino acids of human Tuba. The antibodies were affinity purified on the antigen coupled to SulfoLink beads (Pierce) according to the manufacturer's instructions. A GST fusion protein of the PH domain of phospholipase C was kind gift of Antonella De Matteis (Consorzio Mario Negri Sud, Italy). A GST fusion protein of the BAR domain of amphiphysin-1 was described

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previously (Takei et al., 1999). The KIAA1010 clone was obtained from the Kazusa Institute.

5'-Rapid Amplification of cDNA Ends (RACE)

To obtain the full-length sequence of Tuba from the KIAA1010 clone, human skeletal muscle Marathon-Ready cDNAs (Clontech) were utilized for 5'-RACE using KIAA1010-specific primers and the Advantage 2 PCR enzyme system (Clontech). Based on this sequence, a full-length clone was generated by PCR using probes corresponding to the 5'- and 3'-ends of the Tuba sequence and human brain Marathon-Ready cDNAs (Clontech) as a template. Nucleotide sequencing confirmed the sequences of KIAA1010 and of the N-terminal region of the protein obtained by 5'-RACE with the exception of the absence in KIAA1010 of 40 amino acids in the second half of the BAR domain (see Fig. 1*A*). Multiple clones generated by PCR in different amplification cycles yielded only sequences including the 40 amino acids. The nucleotide sequence of human Tuba has been deposited in the GenBankTM/EBI Data Bank under accession number AY196211.

Yeast Two-hybrid Screen

Full-length EVL was used as bait in the LexA two-hybrid system (Clontech) to probe an embryonic day 19 mouse library. Two independent clones of Tuba comprising amino acids 1502–1577 and 1092–1577 (see Fig. 1*A*) were identified as strong interactors. Full-length murine Tuba was constructed by ligating the larger of the two clones with fragments generated by reverse transcription-PCR using Tuba specific primers and a mouse cDNA library. The nucleotide sequence of mouse Tuba has been deposited in the Gen-BankTM/EBI Data Bank under accession number AY383729.

Affinity Chromatography

GST or GST fusion proteins of SH3 domain-containing regions of Tuba were bound to

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glutathione beads (Amersham Biosciences) and incubated with a Triton X-100-solubilized rat brain extract. Bound material was recovered by centrifugation, followed by elution with SDS and separation by SDS-PAGE. For biochemical analysis of the interaction of the SH3-6 domain of Tuba with actin regulatory proteins in non-neuronal cell extracts, D7 fibroblastic cells, which lack endogenous expression of all Ena/VASP proteins, were used (Bear et al., 2000). Uninfected or infected D7 cells were grown to confluency and extracted in Nonidet P-40 lysis buffer. Cell extracts were clarified by centrifugation and used for affinity chromatography experiments as described above. 10–15 ug of GST or GST fusion protein was incubated with 1 mg of lysate.

Immunocytochemistry of Brain Tissue

Immunofluorescence of frozen rat brain sections was performed by standard procedures on formaldehyde-perfused brains. Anti-Tuba immunogold labeling was performed on lysed synaptosomes embedded in agarose, followed by Epon embedding and thin sectioning as described (De Camilli et al., 1983).

Guanine Nucleotide Exchange Assays

Exchange assays using bacterially expressed and purified Rho GTPases were performed essentially as described (Snyder et al., 2002). In particular, 2 M RhoA(C190S), Rac1(C188S), or Cdc42(C188S) was added to buffer containing 20 mM Tris (pH 7.5), 100 mM NaCl, 5 mM MgCl2, 1 mM dithiothreitol, 10% glycerol, and 400 nM mant-GTP (Molecular Probes, Inc.) and allowed to equilibrate for 5 minutes before adding the indicated concentrations of His-tagged Tuba DH domain or 200 nM His tagged Vav2 DH-PH fragment. Increased fluorescence indicative of mant-GTP binding to GTPases was monitored using a PerkinElmer LS-55 spectrophotometer (ex 360 nm and em 440 nm, slits 5/5 nm) thermostatted to 25 °C. Fluorescence was normalized to the initial value at the start of the experiment. A fragment containing the DH and BAR domains of Tuba

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is insoluble upon expression in *Escherichia coli*, preventing a comparison of exchange rates between this larger portion of Tuba and the isolated DH domain of Tuba.

Transferrin Uptake

Chinese hamster ovary cells were transfected with pcHA (pcDNA3-based vector)-full-length Tuba or pcHA-Tuba SH3-1,2,3,4 using FuGENE 6 transfection reagent (Roche Applied Science) and incubated overnight. They were then washed with serum-free medium and incubated in serum-free medium for 24 h in the presence of Alexa-transferrin (Molecular Probes, Inc.) during the last 7 min before a brief wash with phosphate-buffered saline, followed by fixation with 4% formaldehyde. Cells were stained by immunofluorescence according to standard procedures.

Peptide Binding

Overlapping SPOTs peptides corresponding to the proline-rich regions of mouse N-WASP (amino acids 217–399), SCAR/WAVE1 (amino acids 274–402), EVL (amino acids 157–210), and Mena (amino acids 277–351) were custom-synthesized by Sigma. All peptides are 12-mers offset by three amino acids, and each spot carries an equivalent amount of peptide (5 nmol) covalently attached to the membrane. A negative control peptide was added (no. 118 on the blot) that contains a known proline-rich binding site for Ena/VASP proteins that does not bind SH3 domains. The Tuba SH3-6 domain in pGEX-2TK was labeled by phosphorylating with protein kinase A and [gamma-32P]ATP. The relative intensity of each spot was calculated by converting the PhosphorImager scan into an 8-bit image and measuring the average pixel intensity in a 74-pixel circle centered over each spot in Scion Image. This value was then divided by the background (the average intensity value of the 10 lowest spots) for comparison.

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Mitochondrial Targeting of the Tuba SH3-6 Domain

The mouse Tuba SH3-6 domain was fused in-frame to a modified retroviral expression vector downstream of an ATG start codon and upstream of a fusion between DsRed2 and the mitochondrial targeting sequence from the *L. monocytogenes* protein ActA (excluding Ena/VASP-binding regions) (Pistor et al., 1994). This construct, referred to as SH3-6-mito, was used to transiently transfect CAD cells. Cells were plated onto poly-D-lysine-coated coverslips 24 h post-transfection. The medium was replaced with serum-free medium to induce differentiation 3–4 h after plating. Cells were incubated for an additional 24–36 h before fixation with 4% formaldehyde. Previously established procedures were used to visualize cells by immunofluorescence.

Lipid-Binding of the Tuba BAR Domain

Liposome sedimentation was performed using 10 μg sucrose-loaded liposomes incubated with 10% of ^{35}S-labeled *in vitro* translated reaction product (TnT Coupled Reticulocyte Lysate System, Promega) in 50 μl 25 mM Hepes-KOH, pH 7.4, 25 mM KCl, 2.5 mM Mg^{2+} acetate, and 150 mM K-glutamate for 20 minutes at 37°C. Liposomes were sedimented at 100,000 *g* in a Beckman Coulter TLA 100.3 rotor for 20 minutes. The supernatant was thoroughly removed and resuspended in 2% SDS, while the sedimented liposomes were gently washed in buffer and resuspended in 2% SDS. Samples were subjected to SDS-PAGE and analyzed by autoradiography at –20°C for 18 hours.

Miscellaneous

We carried out Western and Northern blotting following standard procedures. For Northern blotting, human multiple tissue RNA blots were obtained from Clontech. For multiple-tissue Western blotting, tissues were isolated and prepared as described previously (McPherson et al., 1996). Far-Western assays and gel overlays were carried out using standard procedures. Immunoprecipitations were carried out as described

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previously (McPherson et al., 1996).

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Chapter VIII—Discussion

Multiple lines of evidence suggest that actin plays a role in the process of clathrin-mediated endocytosis. Although proteins have been identified which interact with both clathrin and actin or the actin regulatory machinery, dynamin appears to function as a key bridge between endocytosis and the actin cytoskeleton (Engqvist-Goldstein and Drubin, 2003). The *shibire* mutant of *Drosophila melanogaster* (Koenig and Ikeda, 1989) gave the first indication that dynamin, which is the product of the gene disrupted in *shibire* flies (van der Bliek and Meyerowitz, 1991), plays a critical role in endocytosis. *shibire* mutants also display defects in growth cone dynamics (Masur et al., 1990), suggesting a role for dynamin in the regulation of actin, as growth cones are heavily dependent on their actin cytoskeleton for normal function. Subsequent work by a multitude of groups has confirmed the predictions made from the analysis of the *shibire* phenotype, mainly that the block in endocytosis is a result of the inhibition of the fission activity of dynamin (Hinshaw, 2000). There is also an increasing body of evidence indicating that dynamin is involved in the regulation of the actin cytoskeleton (Orth and McNiven, 2003). In addition to directly interacting with a variety of actin-regulatory proteins, dynamin mutants have a variety of effects on the actin cytoskeleton in a variety of cellular contexts, including the disruption of podosomes and alteration of podosome actin dynamics (Ochoa et al., 2000). Tuba, a novel protein whose discovery and characterization has been the focus of this thesis, further strengthens the connection between the endocytic and actin regulatory machinery.

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Tuba was discovered in database searches for novel proteins with regions homologous to the BAR domain of the synaptic and endocytic protein amphiphysin I. Originally identified as the partial open reading frame KIAA1010, a full-length clone was generated using 5'-RACE. Full-length Tuba has a molecular weight of 177 kiloDaltons. Database analysis indicates that it is one of several proteins in a novel family of proteins. The Tuba transcript and protein are ubiquitous. When examined by immunofluorescence in brain, Tuba was found to co-localize with the synaptic and endocytic markers amphiphysin 1 and dynamin 1, and electron microscopic studies localize Tuba to regions of endocytosis in the rat brain synapse. Thus, Tuba is a novel protein localizing to the endocytic compartment of the synapse.

The amino terminus of Tuba contains four tandem SH3 domains. GST pull-down assays utilizing constructs containing these four domains isolated dynamin 1. Dynamin is a protein absolutely necessary for clathrin-mediated and other forms of endocytosis, and functions in the release of the nascent vesicle from the donor membrane. It also interacts with a variety of actin-regulatory proteins, possibly providing a link between actin and the endocytic machinery. The amino terminus of Tuba, through a high avidity interaction, specifically interacts with the endocytic protein, dynamin.

Tuba contains two carboxy-terminal SH3 domains. While no interactor was identified for the penultimate SH3 domain, the terminal SH3 domain was found to isolate a multiplicity of proteins by GST affinity chromatography experiments from rat brain extracts. The majority of these proteins were identified by western blotting and MALDI-

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TOF mass spectrometry as regulators of the actin cytoskeleton. Far-western assays indicate that only N-WASP, CR16, and Mena interact directly with the terminal SH3 domain of Tuba. The isolation of proteins which are involved in regulation of the actin cytoskeleton, but are not binding partners of the terminal SH3 domain of Tuba, suggests that the proteins are constituents of a complex.

N-WASP, in response to activation by Cdc42 and PtdIns(4,5)P2 or SH3 domain binding, stimulates actin polymerization activity via the binding of the Arp2/3 complex (Higgs and Pollard, 2001). WIRE (Aspenstrom, 2002) and CR16 (Ho et al., 2001; Kato et al., 2002) are two N-WASP-interacting proteins whose functions are unknown. Both proteins are proline-rich, indicating a function in the recruitment and coordination of actin regulatory proteins, through SH3 domain binding, to ensure efficient polymerization of actin. Mena is a member of the Ena/VASP family of proteins , and is the mammalian homolog of *enabled*, a *D. melanogaster* mutant which functions to suppress the lethality caused by absence of the *abelson* tyrosine kinase, which itself causes neural defects (Gertler et al., 1990). Mice lacking Mena expression fail to form a corpus callosum, which is a brain structure composed of axons involved in interhemispheric cortico-cortical communication (Lanier et al., 1999). In addition, mice heterozygous for profilin I, a Mena binding partner and G-actin binding protein, which also lack Mena expression, are inviable and have neurulation defects (Lanier et al., 1999). As neurulation and axon migration are actin-dependent processes, it is likely that Mena plays a role in the regulation of the actin cytoskeleton. Recent work indicates that Mena functions as an F-actin capping protein antagonist, allowing greater actin filament length (Bear et al.,

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2002). Thus, Mena is a critical component of the actin regulatory machinery. Lamellipodin, a novel member of the Ena/VASP family of proteins, may also function as a capping protein, and is currently being characterized by Frank Gertler and colleagues.

NAP1 is a member of a protein complex isolated from brain extracts by GTP-Rac1 (Kitamura et al., 1997), and directly binds to the SH3 domains of the adaptor Nck (Kitamura et al., 1996), a protein which interacts with tyrosine kinases through its SH2 domain, and N-WASP, Sos, PAK, and dynamin through its three SH3 domains. In addition, the NAP1 *D. melanogaster* mutant, *kette*, exhibits axonal pathfinding and actin cytoskeletal defects (Hummel et al., 2000), and the *C. elegans* NAP1 mutant, *gex-3*, has multiple defects in morphogenesis resulting in embryonic lethality (Soto et al., 2002). Recent work in *D. melanogaster* indicates that Kette inhibits WAVE1 function, but also activates the *Drosophila* WASP homolog (Bogdan and Klambt, 2003). In this study, a membrane-tethered form of Kette was found to cause a large accumulation of F-actin bundles. These bundles were suppressed by inhibiting WASP expression in a dose-dependent fashion, suggesting that WASP and NAP1 genetically interact, and that NAP1 stimulates WASP's actin polymerizing property. WAVE1 is a member of the WASP superfamily of proteins (Takenawa and Miki, 2001). Unlike N-WASP, it is constitutively active. N-WASP and WAVE1 share a similar carboxy-terminal domain structure, and thus the ability to bind and activate the Arp2/3 complex. The amino termini of WAVE1 and N-WASP, however, differ in the absence in WAVE1 of the CRIB regulatory region that in N-WASP binds GTP-Cdc42 and regulates the activation of N-WASP. GTP-Rac is necessary to localize WAVE to sites of membrane ruffling and stimulates WAVE

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activity. In fibroblasts, WAVE2 (an isoform of WAVE1), IRSp53, a protein which functions to link Rac to WAVE, and active Rac exist in a complex which is necessary to induce membrane ruffling (Miki et al., 2000). PIR121 (also known as Sra1, p140, and CYFIP) is involved in the regulation of WAVE function. Disruption of PIR121 in *Dictyostelium discoideum* results in cells which are severely limited in their locomotion (Blagg et al., 2003). In addition, PIR121/SCAR (the *Dictyostelium* WAVE homolog) double mutants have a phenotype identical to SCAR mutants, which the authors argue suggests that PIR121 is downstream of SCAR *in vivo*(Blagg et al., 2003). The *C. elegans* PIR121 mutant, *gex-2*, is phenotypically very similar to the NAP1 mutant, *gex-3*, giving rise to defects in morphogenesis and resultant embryonic lethality (Soto et al., 2002). As discussed below, PIR121 is a member of a complex which regulates WAVE1 activity.

NAP1, PIR121, and WAVE1 exist in a complex whose function is to regulate the activity of WAVE1 (Eden et al., 2002). When bound to NAP1 and PIR121, WAVE1 is inactive, unable to catalyze the polymerization of actin through its bound Arp2/3 complex. Upon binding of either activated Rac1 or the adaptor Nck, however, WAVE1 is released from the complex to catalyze actin polymerization. It is unknown whether activation of N-WASP leads to activation of WAVE1. However, it is easy to imagine how subsequent activation of WAVE1 would allow a greater filament-generating capacity. It is also possible that WAVE1 is a member of an actin regulatory that is utilized in many contexts and thus plays no role in endocytosis. The actin complex isolated by the terminal SH3 domain of Tuba is likely to not only maximize the efficiency of actin polymerization, but also ensure polymerization in the correct tempero-spatial context.

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Interestingly, the *Caenorhabditis elegans* Tuba homolog, GEI-18, was isolated in a two-hybrid screen using the nematode NAP1 homolog, GEX-3, as bait (Tsuboi et al., 2002). In *C. elegans*, GEX-3 plays an important role in morphogenesis, with homozygous null mutants exhibiting embryonic lethality (Soto et al., 2002). The GEX-3-GEI18 interaction provides further evidence that Tuba and NAP1 function in the same pathway in mammals. Confirmation of similar properties between the nematode and human Tuba orothologs would allow the study of Tuba in a well-established genetic system.

Dbl homology domains function as exchange factors for the Rho family of small GTPases (Hoffman and Cerione, 2002). Exchange factors are necessary to generate GTP-bound small GTPases, which can then bind to effectors to effect changes in cell function. We found that the DH domain of Tuba specifically catalyzes the activation of Cdc42. This result is very exciting given that the terminal SH3 domain of Tuba isolates a complex of actin regulatory proteins containing the Cdc42 effector, N-WASP. In addition to Cdc42 binding, N-WASP also requires co-activation with either PtdIns(4,5)P2 or an SH3 domain-containing protein (Higgs and Pollard, 2001). The terminal SH3 domain of Tuba directly interacts with the proline-rich region of N-WASP, providing the co-stimulation needed for N-WASP activation. Thus, Tuba has the ability to, by itself, activate N-WASP, resulting in the polymerization of actin.

The DH domain of Tuba is interesting due to its association with a BAR domain. With the exception of proteins of the Tuba family, all known DH domain-containing proteins

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contain a pleckstrin homology domain immediately downstream of the DH domain (Rossman et al., 2003). Pleckstrin homology domains interact with PtdIns(4,5)P2 at the plasma membrane, and are believed to help localize the DH domain to its correct environment. As BAR domains have the ability to bind lipids (Farsad et al., 2001; Peter et al., 2004; Takei et al., 1999), the BAR domain of Tuba may be functionally replacing the PH domain. Preliminary experiments indicate that the BAR domain of Tuba indeed functions as lipid-binding module. In the case of some, but not all DH domains, PtdIns(4,5)P2 binding to the PH domain also increases the catalytic activity of the DH domain (Rossman et al., 2003). Unfortunately, due to the insolubility of the Tuba BAR domain, we were unable to test whether liposome-BAR domain interactions increase Tuba DH domain catalysis. The significance of this experiment is indicated by the poor catalytic efficiency of the Tuba DH domain when compared with other DH domains.

The recently solved crystal structure of the *Drosophila melanogaster* BAR domain (Peter et al., 2004) suggests many interesting potential roles for the BAR domain of Tuba. The authors demonstrated that BAR domains preferentially recognize liposomes with smaller diameters, obtaining much more BAR domain recruitment with 50 nm liposomes, as opposed to 300 nm liposomes. This suggests that BAR domains function as sensors of membrane curvature. This has important functional implications for the BAR domain of Tuba. With the exception of muscle amphiphysin, which contains a lysine-rich insertion at the carboxy terminus of its BAR domain and allows the BAR domain to specifically interact with PtdIns(4,5)P2 (Lee et al., 2002), BAR domains are not known to bind to specific classes of lipids. If Tuba does indeed function in endocytosis, it may be

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recruited to the neck of clathrin-coated pits or vesicles only when the curvature of the membrane reaches its optimum for BAR domain binding. Note that the diameter of synaptic and clathrin-coated vesicles is approximately 60 nm. Also remember that the catalytic efficiency of the BAR domain of Tuba is poor. As mentioned above, membrane binding of the corresponding PH domain of selected DH domains increases the catalytic efficiency of that DH domain (Rossman et al., 2003). This suggests that the catalytic efficiency of the DH domain of Tuba may be enhanced through the recognition and binding of the Tuba BAR domain to a membrane with the correct curvature.

The BAR domain forms dimers (Peter et al., 2004). This suggests that Tuba may exist as a homodimer *in vivo* through intermolecular BAR domain interactions. There is no evidence, however, to support this hypothesis. The dimerization property of the BAR domain may also allow the heterodimerization of Tuba with other Tuba family members, as well as other BAR domain-containing proteins. It is difficult to predict the effect of Tuba-Tuba2 or -Tuba3 dimerization as these proteins have not been characterized. However, dimerization with amphiphysin I would give Tuba another mechanism for interacting with the endocytic machinery, through amphiphysin's clathrin/AP2 binding region (Slepnev et al., 2000), and provide another means for appropriate tempero-spatial recruitment of Tuba to sites of endocytosis. Heterodimerization with endophilin would provide additional regulation of both endocytosis and the actin cytoskeleton, as recruitment of synaptojanin would lead to a decrease in PtdIns(4,5)P2 levels (Cremona et al., 1999). Thus, the ability of Tuba to heterodimerize with other BAR domain-

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containing proteins would strengthen the functional connection of Tuba to both endocytosis and regulation of the actin cytoskeleton.

The crystal structure and bioinformatics studies of the BAR domain indicate that it is present in a variety of proteins in spite of substantial amino acid dissimilarity (Habermann, 2004; Peter et al., 2004). Most of these proteins appear to be involved in vesicle trafficking and/or regulation of the actin cytoskeleton. In particular, it was quickly appreciated that the structure of the BAR domain of *Drosophila* is almost identical to the Rac/Arf-binding motif of arfaptin (Tarricone et al., 2001), although Rac interacts with arfaptin in either its GTP or GDP bound state, while only GTP-Arf can bind arfaptin. Racs and Arfs are members of the Rho and Arf families of small GTPases. The function of Rac is to regulate the activity of the actin cytoskeleton (Burridge and Wennerberg, 2004), while Arfs direct vesicle trafficking and actin function (Randazzo et al., 2000). Arfaptin is thought to mediate the cross-talk between Rac and Arfs (Tarricone et al., 2001). The fact that the Rac binding region of arfaptin is a BAR domain raises the possibility that the BAR domain of Tuba may also have the ability to directly bind small GTPases. If this were the case, the BAR domain of Tuba could recruit GDP-Cdc42, the substrate of its associated DH domain. Alternatively, in a manner analogous to arfaptin, the BAR domain of Tuba might mediate the cross-talk between Rac and Cdc42.

The ability of the Tuba BAR domain to bind Cdc42 provides a possible explanation for the relatively poor catalytic efficiency of the DH domain. There is no indication that the BAR domain of any protein recognizes specific lipid-types. If the BAR domain of Tuba

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were to possess the ability to interact with GDP-Cdc42, it might function to, in effect, concentrate Cdc42 in the vicinity of its exchange factor, potentially increasing the catalytic efficiency of the DH domain. Alternatively, the BAR domain may recognize GTP-Cdc42-rich membranes, allowing Tuba to function in a positive feed-back mechanism, with the BAR domain recognizing both the high concentration of Cdc42 as well as the curvature of the membrane as indicators that Tuba should bind that particular membrane. One should also consider that binding of Cdc42 or another small GTPase to the Tuba BAR domain would mask the BAR domain's hydrophobic face, resulting in increased solubility of the BAR domain *in vitro*, and of Tuba *in vivo*. While small GTPase-BAR domain interactions predict interesting functions, it is also possible that the Tuba BAR domain is incapable of binding to this class of proteins.

The structure of the BAR domain suggests that it is incapable of simultaneously binding to both Cdc42 and lipid membranes. If this is indeed the case, the positive feed-back model proposed above for the BAR domain of Tuba would not be possible, as Cdc42 and lipid membranes would compete for BAR domain binding. Recall that the functional BAR domain is a dimer of BAR domain subunits, and that each BAR domain monomer forms half of the functional structure (Peter et al., 2004). This may preclude BAR domain subunits acting in different capacities. Also, if Cdc42 binding were to increase Tuba solubility, this might adversely affect Tuba recruitment to clathrin-coated pits. A role for the Tuba BAR domain in increasing catalytic efficiency of the DH domain is attractive. BAR domain-binding to its membrane target may be sufficient to stimulate DH domain efficiency. However, formation of active Cdc42 by the Tuba DH domain

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through BAR domain recruitment of GDP-Cdc42 could occur as well. This latter possibility is less likely if membrane and Cdc42 binding are mutually exclusive. It is not unreasonable to predict that multiple molecules of Tuba are present in the protein network surrounding clathrin-coated pits in different states. The function of the BAR domain of Tuba might differ depending on its lipid-binding state, as well as the molecules which are interacting with other areas of the Tuba protein. Determining the properties of the Tuba BAR domain will be critical to understanding the mechanism of Tuba function *in vivo*.

The insolubility of the Tuba BAR domain makes the testing of most of these hypotheses difficult. It is clear, however, that the combination of the data presented by Peter, *et al.* (Peter et al., 2004), with the location of the Tuba BAR domain, as well as the binding partners of the terminal SH3 domain of Tuba, provide a tantalizing glimpse at the many possible roles that the BAR domain may play in Tuba.

How might Tuba function *in vivo*? It is likely that Tuba normally exists in an inactivated state. While there is no evidence to support this statement, the cell is unlikely to allow the constitutive production of active Cdc42 and subsequent polymerization of actin, as this would exhaust valuable cellular resources and potentially harm the cell. Intersectin, a dynamin-binding protein sharing many properties with Tuba, provides a model for a switch of an actin regulatory protein from an inactive to an active state. Recent work (Zamanian and Kelly, 2003) demonstrates that intersectin normally exists in an inactivated state through an intramolecular interaction, where one of its SH3 domains

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interacts with its downstream DH domain, resulting in inhibition of exchange activity. The mechanism of inhibition release was not identified, and it did not involve binding of the DH domain-interacting SH3 domain to its ligand, suggesting that the SH3-DH domain interaction does not occur by a conventional mechanism. Tuba contains six SH3 domains through which inhibition could be accomplished. However, we did not address the mechanism of Tuba inhibiton in our studies.

We have presented evidence suggesting that Tuba localizes to synaptic sites of endocytosis. Based on its strong interaction with dynamin, we have also proposed that it is involved in the process of endocytosis. And due to the actin regulatory properties of Tuba, Tuba is likely to be recruited to sites of clathrin-mediated endocytosis no earlier than the formation of the coated pit. There are multiple potential mechanisms by which Tuba may be brought to these sites. One possibility is that dynamin is responsible for the recruitment of Tuba to the neck of clathrin-coated pits. Dynamin contains a PH domain with which it interacts with membranes (Hinshaw, 2000), and may itself be enriched at the neck of coated pits by one of its major binding partners, amphiphysin I (David et al., 1996). Amphiphysin I recruitment to coated pits is likely to precede dynamin, through the curvature-sensing property of the BAR domain as well as its interactions with clathrin and AP2 (Peter et al., 2004; Slepnev et al., 2000). Supporting this idea is the work of Merrifield, *et al.* (Merrifield et al., 2002), which suggests that the formation of actin is not initiated until after dynamin is localized to sites of clathrin-mediated endocytosis. Recall that one of the major functions of Tuba may be the stimulation of the *de novo* polymerization of actin.

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Alternatively, Tuba may not require external factors for its recruitment to the neck of coated pits, utilizing the curvature sensing activity of its BAR domain as well as the postulated BAR-Cdc42 interaction to recognize its membrane destination. The ability of Tuba to independently recognize its site of action suggests a substantial responsibility in dynamin recruitment, as the amino terminus of Tuba provides a mechanism for the concentration of dynamin. Finally, it is also possible that Tuba is recruited to the neck of coated pits through interactions with a member of the endocytic machinery. Tuba could also be localized to clathrin-coated pits through SH3-ligand interactions, as Tuba contains multiple SH3 domains and consensus SH3 domain-binding sites. Tuba SH3 domains 2 and 5 are candidates for this type of interaction, as binding partners for either of these SH3 domains have yet to be identified. This may be due to poor solubilization of its interactors with the methods used to create the brain extract, or a low concentration of the protein at the synapse. While affinity chromatography experiments were attempted with regions of Tuba rich in consensus SH3 domain binding sites, these constructs were unstable and subject to extensive proteolysis. Heterodimerization between the BAR domain-containing proteins Tuba and amphiphysin could also directly recruit Tuba to its site of action. Thus, multiple mechanisms are predicted, either alone or in concert with each other to recruit Tuba to sites of endocytosis.

Localization to its appropriate sub-cellular site leads to BAR domain-membrane interactions through the curvature sensing properties of the BAR domain. This leads to the stimulation of the catalytic activity of the Tuba DH domain and subsequent

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generation of active Cdc42. GTP-Cdc42 is in close approximation to its effector, N-WASP, which is bound to the Tuba carboxy-terminal SH3 domain. The activated Cdc42-N-WASP interaction leads to the polymerization of actin through the actions of the Arp2/3 complex, which is bound to the carboxy terminus of N-WASP. The remaining actin regulatory proteins isolated from rat brain by the terminal SH3 domain of Tuba then ensure the efficiency of actin polymerization.

The following will briefly summarize the envisioned *in vivo* function of Tuba (fig. 21). Tuba is first released from its intramolecular inhibition through an unknown mechanism, possibly phosphorylation. Via the curvature-sensing property of the BAR domain, as well as the dynamin-binding capability of the SH3 domains of the amino terminus, Tuba is recruited to the neck of the clathrin-coated pit (fig. 21A). The BAR domain, upon recognizing the curvature of the pit neck, stimulates activation of the DH domain and formation of GTP-Cdc42 (fig. 21B). The newly-generated active Cdc42 is then bound by its effector, N-WASP, which is tethered to the terminal SH3 domain of Tuba (fig. 21B). GTP-Cdc42 and SH3 domain binding to N-WASP is sufficient to activate the Arp2/3 complex, leading to the polymerization of actin (fig. 21C). The remaining members of the actin regulatory complex then function to ensure adequate and efficient formation of filamentous actin. Tuba is thus an integral component of the endocytic machinery and links it to the actin cytoskeleton.

What role is actin playing in clathrin-mediated endocytosis at the synapse? Actin may function, albeit not directly, as a dynamin effector. Dynamin interacts with many

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FIGURE 21. **Model for the *in vivo* mechanism of Tuba function.** See text for details. Dyn, dynamin.

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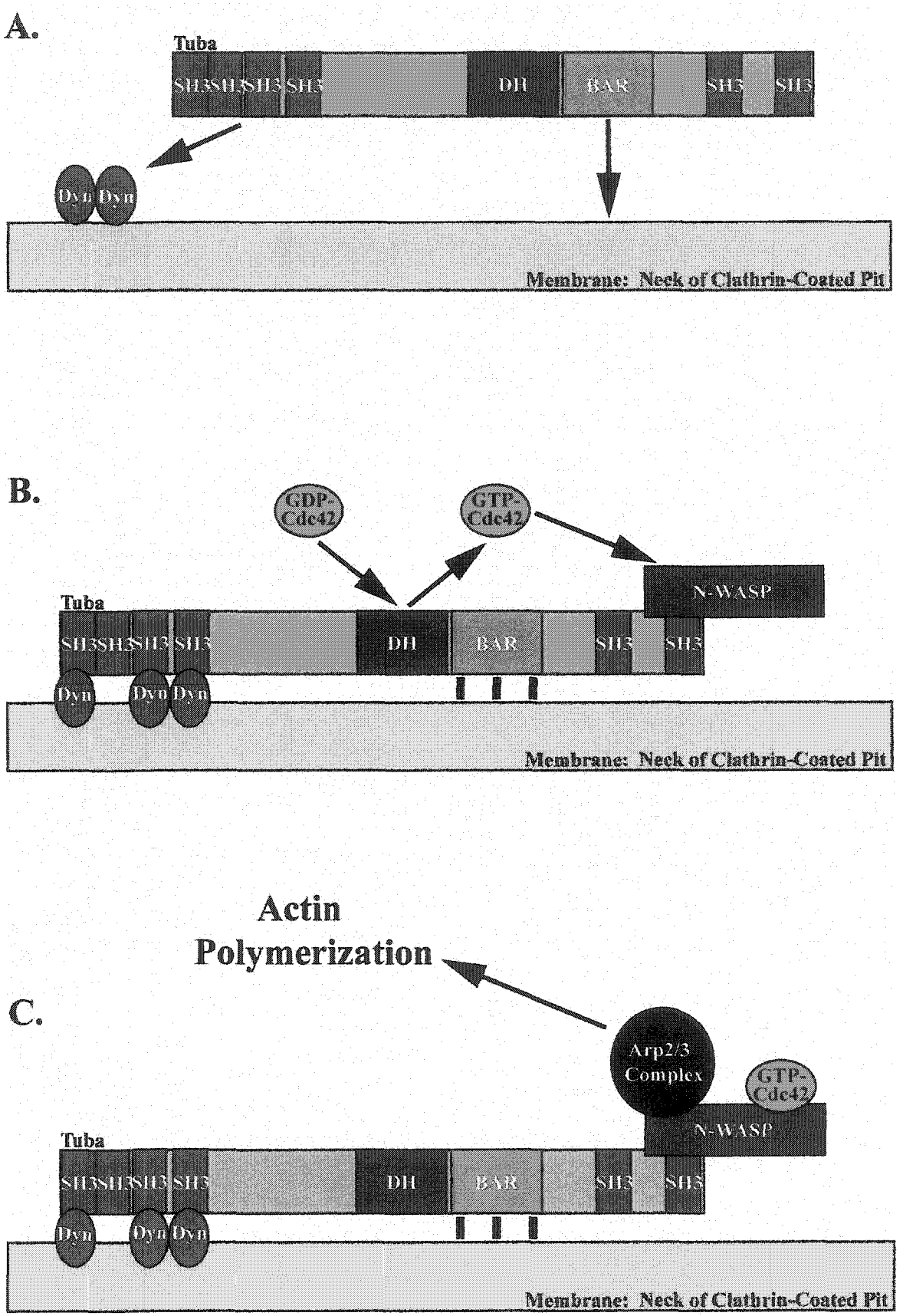

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proteins which have been shown to play a role in both endocytosis and regulation of the actin cytoskeleton (Engqvist-Goldstein and Drubin, 2003). Polymerization of actin has two potential roles in the scission reaction. First, actin may form a meshwork around the neck of the clathrin-coated pit that with the aid of myosin family proteins will mechanically cinch the neck, freeing the nascent vesicle from the donor membrane. Alternatively, the polymerization of actin my occur parallel to the neck of the coated pit, between the donor membrane and vesicle. As actin polymerization progresses, one can envision the neck of the clathrin coated pit stretching to the point of vesicle release. There is very little evidence that actin plays a role in the fission reaction, suggesting that actin is not involved in vesicle scission.

In addition, or instead of, a role in scission, polymerization of actin may be used by the synapse to aid in the locomotion of newly formed vesicles to their destinations. At least two mechanisms are possible. In one scenario, actin tails may be formed upon release of the vesicle from the membrane. The actin tail would then be used to propel the newly formed vesicle away from the membrane or shuttle it to a specific destination. Alternatively, the creation of actin filaments might allow the transport of new vesicles using myosin family motors, such as myosin VI (Buss et al., 2002). It is important to note that most of these roles of actin are not mutually exclusive, and in fact probably work in concert to ensure efficient membrane retrieval and reformation of functional synaptic vesicles. Multiple studies have demonstrated actin filaments emanating from sites of endocytosis (Dunaevsky and Connor, 2000; Shupliakov et al., 2002), and some suggest that perturbation of the actin cytoskeleton disturbs clathrin-mediated endocytosis

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(Bennett et al., 2001; Gaidarov et al., 1999). It will be important to determine what role actin plays in clathrin-mediated endocytosis, and whether dynamin is responsible for the recruitment and initiation of actin function.

Although the properties of Tuba are congruent with the postulated mechanisms described above, it is also possible that Tuba functions independently of endocytosis. However preliminary, there is some data suggesting that dynamin may function as a *bona fide* regulator of the actin cytoskeleton (Lee and De Camilli, 2002; Ochoa et al., 2000). In addition, there is a predicted splice isoform of Tuba which has its amino terminus at the Dbl homology domain, thus encompassing only the actin regulatory portion of the molecule. This implies that Tuba has two functional forms. The short, carboxy-terminal, isoform of Tuba contains the majority of the elements which are involved in the regulation of the actin cytoskeleton. Full-length Tuba was expressed in all tissues tested, and many of those tissues also displayed immunoreactivity for a protein at 100 kD, the approximate size of the short isoform of Tuba. It may be that the short form of Tuba is a component of the actin regulatory machinery in various cell types. As this isoform of Tuba contains all of the necessary components to activate N-WASP and actin polymerization, it would be advantageous for the cell to utilize this form of the protein. It is also possible that the amino terminal extension of Tuba is the defining characteristic allowing Tuba to participate in endocytosis. We have already described the dynamin-binding ability of the amino terminal SH3 domains, but the region spanning Tuba from the SH3 domains to the DH domain contains multiple consensus SH3 domain-binding sites as well as two predicted coiled-coil regions through which it could interact with

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other components of the endocytic machinery as well as an endocytosis-specific mechanism of regulation. Tuba family members Tuba2 and Tuba3, similar to the short isoform of Tuba, only show homology to the regions of Tuba involved in regulation of the actin cytoskeleton. As neither the short Tuba splice isoform, Tuba2, or Tuba3 have been characterized, we can only predict that they may regulate the actin cytoskeleton independent of the process of endocytosis.

In regards to Tuba itself, it cannot be stated with certainty that it is involved in clathrin-mediated endocytosis. However, the characterization of Tuba, when integrated with the work of others described throughout the chapters of this thesis, strongly suggests that Tuba is a link between the endocytic and actin machinery.

There are three fundamental questions whose answers will greatly increase our understanding of Tuba. First, is Tuba a regulator of the actin cytoskeleton *in vivo*? We have demonstrated that the DH domain of Tuba is an exchange factor for Cdc42, and that the carboxy terminal SH3 domain of Tuba interacts with N-WASP and a complex of actin regulatory proteins, strongly suggesting that Tuba is a regulator of the actin cytoskeleton. However, the insolubility of full-length Tuba prevented us from showing an effect of Tuba overexpression in fibroblasts, and overexpression of SH3-6 in fibroblasts also failed to perturb the actin cytoskeleton (data not shown). The effect of Tuba on N-WASP can initially be tested *in vitro*, using established actin polymerization assays (Machesky et al., 1999). In the presence of G-actin, N-WASP, GTP-Cdc42 or liposomes containing PtdIns(4,5)P2, and the Arp2/3 complex, addition of Tuba SH3-6

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should initiate actin polymerization. The best way to confirm Tuba's effect on the actin cytoskeleton *in vivo* is through disruption of Tuba expression. This can be done in mice through Tuba gene-knockout experiments, which would be expected to demonstrate defects in actin-dependent processes such as body morphogenesis, signal-dependent actin cytoskeletal rearrangement in individual cells, and axon migration. Recall that the short isoform of Tuba is not believed to participate in endocytosis, and its absence might account for the majority of the predicted phenotypes. RNA interference is another tool which could be utilized to test the effects of the absence of Tuba on cells in culture, again assaying for defects in the rearrangement of the actin cytoskeleton in response to external signals. Finally, the *C. elegans* Tuba homolog, GEI-18, provides another modality in which to study the effect of Tuba on actin *in vivo*.

Understanding the role of the BAR domain of Tuba would also provide significant insight into the mechanistic details of Tuba function. As discussed above, the BAR domain of Tuba has the potential to regulate Tuba localization as well as DH domain function. *In vitro* studies would initially be needed to refine our knowledge of the properties of the Tuba BAR domain. Preliminary experiments indicate that the BAR domain of Tuba has the ability to bind lipids. This needs to be confirmed. If the BAR domain contained the ability to localize Tuba independent of dynamin or SH3-6 binding, it would suggest that Tuba might be responsible for recruiting and concentrating dynamin and N-WASP at clathrin-coated pits. The relationship between Cdc42 and the Tuba BAR domain must also be investigated. Can the BAR domain directly interact with Cdc42, and if so, does this alter DH domain function or lipid-binding? Is binding of the BAR

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domain to lipids sufficient to increase the catalytic efficiency of the DH domain of Tuba? The crystal structure of the BAR domain is a homodimer, suggesting that Tuba is a homodimer *in vivo*. Does Tuba dimerize with amphiphysin or other BAR domain-containing proteins? This would have significant implications for Tuba localization and function. Answers to these questions would give us a greater understanding of the BAR domain in general, and of Tuba specifically. Unfortunately, the insolubility of the BAR domain will make addressing many of these inquiries technically challenging. *In vivo* examination of many of these issues needs to be based on the results from *in vitro* investigation due to the many hypotheses now available regarding the function of the Tuba BAR domain.

Of greatest interest is whether or not Tuba is indeed necessary for clathrin-mediated endocytosis. While most investigators believe that actin plays a role in endocytosis, it is unknown if actin function is essential, or at what stage it participates. Experiments similar to those done to help elucidate the role of amphiphysin and endophilin in the lamprey spinal cord could be employed (Gad et al., 2000; Shupliakov et al., 1997). Peptides inhibiting the Tuba SH3 interactions with dynamin, N-WASP, or both proteins could help suggest the step at which Tuba is recruited to the endocytic process, and if Tuba is at all necessary for normal synaptic physiology. The function of Tuba in endocytosis could also be probed with gene knockout experiments in mice. Electrophysiology combined with morphologic analysis of the synapse as well as morphometry of indicators of endocytosis, such as clathrin-coated structures, would also imply a role for Tuba in endocytosis. A potential problem with these experiments is that

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proteins such as intersectin, which also simultaneously interact with dynamin, N-WASP, and catalyze the formation of Cdc42, might play a functionally redundant roles in the synapse, masking the effect of Tuba disruption.

While much work remains, the study of Tuba will not only shed light on the properties of a very interesting protein, but may also help elucidate a fundamental unknown of clathrin-mediated endocytosis.

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Bibliography

Aspenstrom, P. 2002. The WASP-binding protein WIRE has a role in the regulation of the actin filament system downstream of the platelet-derived growth factor receptor. *Exp Cell Res.* 279:21-33.

Bamburg, J.R., and O.P. Wiggan. 2002. ADF/cofilin and actin dynamics in disease. *Trends Cell Biol.* 12:598-605.

Barlowe, C. 2000. Traffic COPs of the early secretory pathway. *Traffic.* 1:371-7.

Bear, J.E., J.J. Loureiro, I. Libova, R. Fassler, J. Wehland, and F.B. Gertler. 2000. Negative regulation of fibroblast motility by Ena/VASP proteins. *Cell.* 101:717-28.

Bear, J.E., T.M. Svitkina, M. Krause, D.A. Schafer, J.J. Loureiro, G.A. Strasser, I.V. Maly, O.Y. Chaga, J.A. Cooper, G.G. Borisy, and F.B. Gertler. 2002. Antagonism between Ena/VASP proteins and actin filament capping regulates fibroblast motility. *Cell.* 109:509-21.

Bennett, E.M., C.Y. Chen, A.E. Engqvist-Goldstein, D.G. Drubin, and F.M. Brodsky. 2001. Clathrin hub expression dissociates the actin-binding protein Hip1R from coated pits and disrupts their alignment with the actin cytoskeleton. *Traffic.* 2:851-8.

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.

Blagg, S.L., M. Stewart, C. Sambles, and R.H. Insall. 2003. PIR121 regulates pseudopod dynamics and SCAR activity in Dictyostelium. *Curr Biol.* 13:1480-7.

Bogdan, S., and C. Klambt. 2003. Kette regulates actin dynamics and genetically interacts with Wave and Wasp. *Development.* 130:4427-37.

Bonifacino, J.S., and B.S. Glick. 2004. The mechanisms of vesicle budding and fusion. *Cell.* 116:153-66.

Bonifacino, J.S., and J. Lippincott-Schwartz. 2003. Coat proteins: shaping membrane transport. *Nat Rev Mol Cell Biol.* 4:409-14.

Bonifacino, J.S., and L.M. Traub. 2003. Signals for sorting of transmembrane proteins to endosomes and lysosomes. *Annu Rev Biochem.* 72:395-447.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Bowden, E.T., M. Barth, D. Thomas, R.I. Glazer, and S.C. Mueller. 1999. An invasion-related complex of cortactin, paxillin and PKCmu associates with invadopodia at sites of extracellular matrix degradation. *Oncogene*. 18:4440-9.

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.

Burridge, K., and K. Wennerberg. 2004. Rho and Rac take center stage. *Cell*. 116:167-79.

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.

Buss, F., J.P. Luzio, and J. Kendrick-Jones. 2002. Myosin VI, an actin motor for membrane traffic and cell migration. *Traffic*. 3:851-8.

Cao, H., J.D. Orth, J. Chen, S.G. Weller, J.E. Heuser, and M.A. McNiven. 2003. Cortactin is a component of clathrin-coated pits and participates in receptor-mediated endocytosis. *Mol Cell Biol*. 23:2162-70.

Cases-Langhoff, C., B. Voss, A.M. Garner, U. Appeltauer, K. Takei, S. Kindler, R.W. Veh, P. De Camilli, E.D. Gundelfinger, and C.C. Garner. 1996. Piccolo, a novel 420 kDa protein associated with the presynaptic cytomatrix. *European Journal of Cell Biology*. 69:214-223.

Chu, D.S., B. Pishvaee, and G.S. Payne. 1996. The light chain subunit is required for clathrin function in Saccharomyces cerevisiae. *Journal of Biological Chemistry*. 271:33123-33130.

Conner, S.D., and S.L. Schmid. 2003. Regulated portals of entry into the cell. *Nature*. 422:37-44.

Conti, F., and R.J. Weinberg. 1999. Shaping excitation at glutamatergic synapses. *Trends Neurosci*. 22:451-8.

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.

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

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

terminals. *Proceedings of the National Academy of Sciences of the United States of America*. 93:331-335.

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

Di Fiore, P.P., and P. De Camilli. 2001. Endocytosis and signaling. *Cell*. 106:1-4.

dos Remedios, C.G., D. Chhabra, M. Kekic, I.V. Dedova, M. Tsubakihara, D.A. Berry, and N.J. Nosworthy. 2003. Actin binding proteins: regulation of cytoskeletal microfilaments. *Physiol Rev*. 83:433-73.

Dunaevsky, A., and E.A. Connor. 2000. F-actin is concentrated in nonrelease domains at frog neuromuscular junctions. *J Neurosci*. 20:6007-12.

Dunn, J.T., and A.D. Dunn. 2001. Update on intrathyroidal iodine metabolism. *Thyroid*. 11:407-14.

Eden, S., R. Rohatgi, A.V. Podtelejnikov, M. Mann, and M.W. Kirschner. 2002. Mechanism of regulation of WAVE1-induced actin nucleation by Rac1 and Nck. *Nature*. 418:790-3.

Engqvist-Goldstein, A.E., and D.G. Drubin. 2003. Actin assembly and endocytosis: from yeast to mammals. *Annu Rev Cell Dev Biol*. 19:287-332.

Engqvist-Goldstein, A.E., R.A. Warren, M.M. Kessels, J.H. Keen, J. Heuser, and D.G. Drubin. 2001. The actin-binding protein Hip1R associates with clathrin during early stages of endocytosis and promotes clathrin assembly in vitro. *J Cell Biol*. 154:1209-23.

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.

Farsad, K., V. Slepnev, G. Ochoa, L. Daniell, V. Hauke, and P. De Camilli. 2003. A putative role for intramolecular regulatory mechanisms in the adaptor function of amphiphysin in endocytosis. *Neuropharmacology*. 45:787-96.

Fazioli, F., L. Minichiello, B. Matoskova, W.T. Wong, and P.P. Di Fiore. 1993. eps15, a novel tyrosine kinase substrate, exhibits transforming activity. *Molecular & Cellular Biology*. 13:5814-5828.

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

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

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.

Frank, R. 2002. The SPOT-synthesis technique. Synthetic peptide arrays on membrane supports--principles and applications. *J Immunol Methods.* 267:13-26.

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.

Gaidarov, I., F. Santini, R.A. Warren, and J.H. Keen. 1999. Spatial control of coated-pit dynamics in living cells. *Nat Cell Biol.* 1:1-7.

Gasnier, B. 2000. The loading of neurotransmitters into synaptic vesicles. *Biochimie.* 82:327-37.

Gertler, F.B., J.S. Doctor, and F.M. Hoffmann. 1990. Genetic suppression of mutations in the Drosophila abl proto-oncogene homolog. *Science.* 248:857-60.

Gertler, F.B., K. Niebuhr, M. Reinhard, J. Wehland, and P. Soriano. 1996. Mena, a relative of VASP and Drosophila Enabled, is implicated in the control of microfilament dynamics. *Cell.* 87:227-39.

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

Goode, B.L., A.A. Rodal, G. Barnes, and D.G. Drubin. 2001. Activation of the Arp2/3 complex by the actin filament binding protein Abp1p. *J Cell Biol.* 153:627-34.

Habermann, B. 2004. The BAR-domain family of proteins: a case of bending and binding? *EMBO Rep.* 5:250-5.

Haffner, C., G. Di Paolo, J.A. Rosenthal, and P. de Camilli. 2000. Direct interaction of the 170 kDa isoform of synaptojanin 1 with clathrin and with the clathrin adaptor AP-2. *Curr Biol.* 10:471-4.

Hall, A. 1998. Rho GTPases and the actin cytoskeleton. *Science.* 279:509-14.

Heuser, J. 1980. Three-dimensional visualization of coated vesicle formation in fibroblasts. *J Cell Biol.* 84:560-83.

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.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Higgs, H.N., and T.D. Pollard. 2001. Regulation of actin filament network formation through ARP2/3 complex: activation by a diverse array of proteins. *Annu Rev Biochem.* 70:649-76.

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

Ho, H.Y., R. Rohatgi, L. Ma, and M.W. Kirschner. 2001. CR16 forms a complex with N-WASP in brain and is a novel member of a conserved proline-rich actin-binding protein family. *Proc Natl Acad Sci U S A.* 98:11306-11.

Hoffman, G.R., and R.A. Cerione. 2002. Signaling to the Rho GTPases: networking with the DH domain. *FEBS Lett.* 513:85-91.

Hummel, T., K. Leifker, and C. Klambt. 2000. The Drosophila HEM-2/NAP1 homolog KETTE controls axonal pathfinding and cytoskeletal organization. *Genes Dev.* 14:863-73.

Hussain, N.K., S. Jenna, M. Glogauer, C.C. Quinn, S. Wasiak, M. Guipponi, S.E. Antonarakis, B.K. Kay, T.P. Stossel, N. Lamarche-Vane, and P.S. McPherson. 2001. Endocytic protein intersectin-l regulates actin assembly via Cdc42 and N-WASP. *Nat Cell Biol.* 3:927-32.

Hussain, N.K., M. Yamabhai, A.R. Ramjaun, A.M. Guy, D. Baranes, J.P. O'Bryan, C.J. Der, B.K. Kay, and P.S. McPherson. 1999. Splice variants of intersectin are components of the endocytic machinery in neurons and nonneuronal cells. *J Biol Chem.* 274:15671-7.

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.

Ishikawa, R., K. Hayashi, T. Shirao, Y. Xue, T. Takagi, Y. Sasaki, and K. Kohama. 1994. Drebrin, a development-associated brain protein from rat embryo, causes the dissociation of tropomyosin from actin filaments. *J Biol Chem.* 269:29928-33.

Kaksonen, M., H.B. Peng, and H. Rauvala. 2000. Association of cortactin with dynamic actin in lamellipodia and on endosomal vesicles. *J Cell Sci.* 113 Pt 24:4421-6.

Kaksonen, M., Y. Sun, and D.G. Drubin. 2003. A pathway for association of receptors, adaptors, and actin during endocytic internalization. *Cell.* 115:475-87.

Kalchman, M.A., H.B. Koide, K. McCutcheon, R.K. Graham, K. Nichol, K. Nishiyama, P. Kazemi-Esfarjani, F.C. Lynn, C. Wellington, M. Metzler, Y.P. Goldberg, I. Kanazawa, R.D. Gietz, and M.R. Hayden. 1997. HIP1, a human homologue of S. cerevisiae Sla2p, interacts with membrane-associated huntingtin in the brain. *Nat Genet.* 16:44-53.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Kato, M., H. Miki, S. Kurita, T. Endo, H. Nakagawa, S. Miyamoto, and T. Takenawa. 2002. WICH, a novel verprolin homology domain-containing protein that functions cooperatively with N-WASP in actin-microspike formation. *Biochem Biophys Res Commun.* 291:41-7.

Kessels, M.M., A.E. Engqvist-Goldstein, and D.G. Drubin. 2000. Association of mouse actin-binding protein 1 (mAbp1/SH3P7), an Src kinase target, with dynamic regions of the cortical actin cytoskeleton in response to Rac1 activation. *Mol Biol Cell.* 11:393-412.

Kessels, M.M., A.E. Engqvist-Goldstein, D.G. Drubin, and B. Qualmann. 2001. Mammalian Abp1, a signal-responsive F-actin-binding protein, links the actin cytoskeleton to endocytosis via the GTPase dynamin. *J Cell Biol.* 153:351-66.

Kessels, M.M., and B. Qualmann. 2002. Syndapins integrate N-WASP in receptor-mediated endocytosis. *Embo J.* 21:6083-94.

Kirchhausen, T. 2000. Three ways to make a vesicle. *Nat Rev Mol Cell Biol.* 1:187-98.

Kitamura, T., Y. Kitamura, K. Yonezawa, N.F. Totty, I. Gout, K. Hara, M.D. Waterfield, M. Sakaue, W. Ogawa, and M. Kasuga. 1996. Molecular cloning of p125Nap1, a protein that associates with an SH3 domain of Nck. *Biochem Biophys Res Commun.* 219:509-14.

Kitamura, Y., T. Kitamura, H. Sakaue, T. Maeda, H. Ueno, S. Nishio, S. Ohno, S. Osada, M. Sakaue, W. Ogawa, and M. Kasuga. 1997. Interaction of Nck-associated protein 1 with activated GTP-binding protein Rac. *Biochem J.* 322:873-8.

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.

Krueger, E.W., J.D. Orth, H. Cao, and M.A. McNiven. 2003. A Dynamin-Cortactin-Arp2/3 Complex Mediates Actin Reorganization in Growth Factor-stimulated Cells. *Mol Biol Cell.* 14:1085-96.

Lamaze, C., L.M. Fujimoto, H.L. Yin, and S.L. Schmid. 1997. The actin cytoskeleton is required for receptor-mediated endocytosis in mammalian cells. *J Biol Chem.* 272:20332-5.

Lanier, L.M., M.A. Gates, W. Witke, A.S. Menzies, A.M. Wehman, J.D. Macklis, D. Kwiatkowski, P. Soriano, and F.B. Gertler. 1999. Mena is required for neurulation and commissure formation. *Neuron.* 22:313-25.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Larbolette, O., B. Wollscheid, J. Schweikert, P.J. Nielsen, and J. Wienands. 1999. SH3P7 is a cytoskeleton adapter protein and is coupled to signal transduction from lymphocyte antigen receptors. *Mol Cell Biol.* 19:1539-46.

Lee, E., and P. De Camilli. 2002. From the Cover: Dynamin at actin tails. *Proc Natl Acad Sci U S A.* 99:161-6.

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, W.L., R.E. Harrison, and S. Grinstein. 2003. Phagocytosis by neutrophils. *Microbes Infect.* 5:1299-306.

Legendre-Guillemin, V., M. Metzler, M. Charbonneau, L. Gan, V. Chopra, J. Philie, M.R. Hayden, and P.S. McPherson. 2002. HIP1 and HIP12 display differential binding to F-actin, AP2, and clathrin. Identification of a novel interaction with clathrin light chain. *J Biol Chem.* 277:19897-904.

Lin, Q., C.G. Lo, R.A. Cerione, and W. Yang. 2002. The Cdc42 target ACK2 interacts with sorting nexin 9 (SH3PX1) to regulate epidermal growth factor receptor degradation. *J Biol Chem.* 277:10134-8.

Liu, B.P., and K. Burridge. 2000. Vav2 activates Rac1, Cdc42, and RhoA downstream from growth factor receptors but not beta1 integrins. *Mol Cell Biol.* 20:7160-9.

Luttrell, L.M., and R.J. Lefkowitz. 2002. The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. *J Cell Sci.* 115:455-65.

Machesky, L.M., R.D. Mullins, H.N. Higgs, D.A. Kaiser, L. Blanchoin, R.C. May, M.E. Hall, and T.D. Pollard. 1999. Scar, a WASp-related protein, activates nucleation of actin filaments by the Arp2/3 complex. *Proc Natl Acad Sci U S A.* 96:3739-44.

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-Quiles, N., R. Rohatgi, I.M. Anton, M. Medina, S.P. Saville, H. Miki, H. Yamaguchi, T. Takenawa, J.H. Hartwig, R.S. Geha, and N. Ramesh. 2001. WIP regulates N-WASP-mediated actin polymerization and filopodium formation. *Nat Cell Biol.* 3:484-91.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Masur, S.K., Y.T. Kim, and C.F. Wu. 1990. Reversible inhibition of endocytosis in cultured neurons from the Drosophila temperature-sensitive mutant shibirets1. *J Neurogenet.* 6:191-206.

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.

McGough, A.M., C.J. Staiger, J.K. Min, and K.D. Simonetti. 2003. The gelsolin family of actin regulatory proteins: modular structures, versatile functions. *FEBS Lett.* 552:75-81.

McNiven, M.A., L. Kim, E.W. Krueger, J.D. Orth, H. Cao, and T.W. Wong. 2000. Regulated interactions between dynamin and the actin-binding protein cortactin modulate cell shape. *J Cell Biol.* 151:187-98.

McPherson, P.S., E.P. Garcia, V.I. Slepnev, C. David, X. Zhang, D. Grabs, W.S. Sossin, R. Bauerfeind, Y. Nemoto, and P. De Camilli. 1996. A presynaptic inositol-5-phosphatase. *Nature.* 379:353-7.

Meier, J. 2003. The enigma of transmitter-selective receptor accumulation at developing inhibitory synapses. *Cell Tissue Res.* 311:271-6.

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.

Metzler, M., V. Legendre-Guillemin, L. Gan, V. Chopra, A. Kwok, P.S. McPherson, and M.R. Hayden. 2001. HIP1 functions in clathrin-mediated endocytosis through binding to clathrin and adaptor protein 2. *J Biol Chem.* 276:39271-6.

Michaely, P., A. Kamal, R.G. Anderson, and V. Bennett. 1999. A requirement for ankyrin binding to clathrin during coated pit budding. *J Biol Chem.* 274:35908-13.

Miki, H., K. Miura, and T. Takenawa. 1996. N-WASP, a novel actin-depolymerizing protein, regulates the cortical cytoskeletal rearrangement in a PIP2-dependent manner downstream of tyrosine kinases. *EMBO Journal.* 15:5326-5335.

Miki, H., S. Suetsugu, and T. Takenawa. 1998. WAVE, a novel WASP-family protein involved in actin reorganization induced by Rac. *Embo J.* 17:6932-41.

Miki, H., H. Yamaguchi, S. Suetsugu, and T. Takenawa. 2000. IRSp53 is an essential intermediate between Rac and WAVE in the regulation of membrane ruffling. *Nature.* 408:732-5.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Mise-Omata, S., B. Montagne, M. Deckert, J. Wienands, and O. Acuto. 2003. Mammalian actin binding protein 1 is essential for endocytosis but not lamellipodia formation: functional analysis by RNA interference. *Biochem Biophys Res Commun.* 301:704-10.

Morgan, J.R., G.J. Augustine, and E.M. Lafer. 2002. Synaptic vesicle endocytosis: the races, places, and molecular faces. *Neuromolecular Med.* 2:101-14.

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.

Mott, H.R., D. Owen, D. Nietlispach, P.N. Lowe, E. Manser, L. Lim, and E.D. Laue. 1999. Structure of the small G protein Cdc42 bound to the GTPase-binding domain of ACK. *Nature.* 399:384-8.

Munn, A.L., B.J. Stevenson, M.I. Geli, and H. Riezman. 1995. end5, end6, and end7: Mutations that cause actin delocalization and block the internalization step of endocytosis in Saccharomyces cerevisiae. *Molecular Biology of the Cell.* 6:1721-1742.

Murthy, V.N., and P. De Camilli. 2003. Cell biology of the presynaptic terminal. *Annu Rev Neurosci.* 26:701-28.

Nakamura, N., C. Rabouille, R. Watson, T. Nilsson, N. Hui, P. Slusarewicz, T.E. Kreis, and G. Warren. 1995. Characterization of a cis-Golgi matrix protein, GM130. *J Cell Biol.* 131:1715-26.

Nemoto, Y., B.G. Kearns, M.R. Wenk, H. Chen, K. Mori, J.G. Alb, Jr., P. De Camilli, and V.A. Bankaitis. 2000. Functional characterization of a mammalian Sac1 and mutants exhibiting substrate-specific defects in phosphoinositide phosphatase activity. *J Biol Chem.* 275:34293-305.

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.* 10:2343-2360.

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.

Okamoto, M., S. Schoch, and T.C. Sudhof. 1999. EHSH1/intersectin, a protein that contains EH and SH3 domains and binds to dynamin and SNAP-25. *J Biol Chem.* 274:18446-54.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Orth, J.D., E.W. Krueger, H. Cao, and M.A. McNiven. 2002. From the Cover: The large GTPase dynamin regulates actin comet formation and movement in living cells. *Proc Natl Acad Sci U S A.* 99:167-72.

Orth, J.D., and M.A. McNiven. 2003. Dynamin at the actin-membrane interface. *Curr Opin Cell Biol.* 15:31-9.

Palfrey, H.C., and C.R. Artalejo. 2003. Secretion: kiss and run caught on film. *Curr Biol.* 13:R397-9.

Pelkmans, L., and A. Helenius. 2002. Endocytosis via caveolae. *Traffic.* 3:311-20.

Peter, B.J., H.M. Kent, I.G. Mills, Y. Vallis, P.J. Butler, P.R. Evans, and H.T. McMahon. 2004. BAR domains as sensors of membrane curvature: the amphiphysin BAR structure. *Science.* 303:495-9.

Pfeffer, S.R., and R.B. Kelly. 1985. The subpopulation of brain coated vesicles that carries synaptic vesicle proteins contains two unique polypeptides. *Cell.* 40:949-57.

Pistor, S., T. Chakraborty, K. Niebuhr, E. Domann, and J. Wehland. 1994. The ActA protein of Listeria monocytogenes acts as a nucleator inducing reorganization of the actin cytoskeleton. *Embo J.* 13:758-63.

Pollard, T.D., and G.G. Borisy. 2003. Cellular motility driven by assembly and disassembly of actin filaments. *Cell.* 112:453-65.

Qualmann, B., and R.B. Kelly. 2000. Syndapin isoforms participate in receptor-mediated endocytosis and actin organization. *J Cell Biol.* 148:1047-62.

Qualmann, B., J. Roos, P.J. DiGregorio, and R.B. Kelly. 1999. Syndapin I, a synaptic dynamin-binding protein that associates with the neural Wiskott-Aldrich syndrome protein. *Mol Biol Cell.* 10:501-13.

Randazzo, P.A., Z. Nie, K. Miura, and V.W. Hsu. 2000. Molecular aspects of the cellular activities of ADP-ribosylation factors. *Sci STKE.* 2000:RE1.

Razzaq, A., I.M. Robinson, H.T. McMahon, J.N. Skepper, Y. Su, A.C. Zelhof, A.P. Jackson, N.J. Gay, and C.J. O'Kane. 2001. Amphiphysin is necessary for organization of the excitation-contraction coupling machinery of muscles, but not for synaptic vesicle endocytosis in Drosophila. *Genes Dev.* 15:2967-79.

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.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

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.

Roos, J., and R.B. Kelly. 1998. Dap160, a neural-specific Eps15 homology and multiple SH3 domain- containing protein that interacts with Drosophila dynamin. *J Biol Chem.* 273:19108-19.

Rosenmund, C., J. Rettig, and N. Brose. 2003. Molecular mechanisms of active zone function. *Curr Opin Neurobiol.* 13:509-19.

Rossman, K.L., L. Cheng, G.M. Mahon, R.J. Rojas, J.T. Snyder, I.P. Whitehead, and J. Sondek. 2003. Multifunctional roles for the PH domain of Dbs in regulating Rho GTPase activation. *J Biol Chem.* 13:13.

Rubtsov, A.M., and O.D. Lopina. 2000. Ankyrins. *FEBS Lett.* 482:1-5.

Sachse, M., G. Ramm, G. Strous, and J. Klumperman. 2002. Endosomes: multipurpose designs for integrating housekeeping and specialized tasks. *Histochem Cell Biol.* 117:91-104.

Sarmiere, P.D., and J.R. Bamburg. 2004. Regulation of the neuronal actin cytoskeleton by ADF/cofilin. *J Neurobiol.* 58:103-17.

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

Scheele, U., C. Kalthoff, and E. Ungewickell. 2001. Multiple interactions of auxilin 1 with clathrin and the AP-2 adaptor complex. *J Biol Chem.* 276:36131-8.

Schuske, K.R., J.E. Richmond, D.S. Matthies, W.S. Davis, S. Runz, D.A. Rube, A.M. van der Bliek, and E.M. Jorgensen. 2003. Endophilin is required for synaptic vesicle endocytosis by localizing synaptojanin. *Neuron.* 40:749-62.

Sengar, A.S., W. Wang, J. Bishay, S. Cohen, and S.E. Egan. 1999. The EH and SH3 domain Ese proteins regulate endocytosis by linking to dynamin and Eps15. *Embo J.* 18:1159-71.

Sheng, M. 2001. Molecular organization of the postsynaptic specialization. *Proc Natl Acad Sci U S A.* 98:7058-61.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

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., O. Bloom, J.S. Gustafsson, O. Kjaerulff, P. Low, N. Tomilin, V.A. Pieribone, P. Greengard, and L. Brodin. 2002. Impaired recycling of synaptic vesicles after acute perturbation of the presynaptic actin cytoskeleton. *Proc Natl Acad Sci U S A.* 99:14476-81.

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 by amphiphysin fragments comprising these sites. *J Biol Chem.* 275:17583-9.

Snapper, S.B., and F.S. Rosen. 1999. The Wiskott-Aldrich syndrome protein (WASP): roles in signaling and cytoskeletal organization. *Annu Rev Immunol.* 17:905-29.

Snyder, J.T., D.K. Worthylake, K.L. Rossman, L. Betts, W.M. Pruitt, D.P. Siderovski, C.J. Der, and J. Sondek. 2002. Structural basis for the selective activation of Rho GTPases by Dbl exchange factors. *Nat Struct Biol.* 9:468-75.

Soto, M.C., H. Qadota, K. Kasuya, M. Inoue, D. Tsuboi, C.C. Mello, and K. Kaibuchi. 2002. The GEX-2 and GEX-3 proteins are required for tissue morphogenesis and cell migrations in C. elegans. *Genes Dev.* 16:620-32.

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

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. *Nature Cell Biology.* 1:33-39.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Takenawa, T., and H. Miki. 2001. WASP and WAVE family proteins: key molecules for rapid rearrangement of cortical actin filaments and cell movement. *J Cell Sci.* 114:1801-9.

Tan, P.K., N.G. Davis, G.F. Sprague, and G.S. Payne. 1993. Clathrin facilitates the internalization of seven transmembrane segment receptors for mating pheromones in yeast. *Journal of Cell Biology.* 123:1707-16.

Tarricone, C., B. Xiao, N. Justin, P.A. Walker, K. Rittinger, S.J. Gamblin, and S.J. Smerdon. 2001. The structural basis of Arfaptin-mediated cross-talk between Rac and Arf signalling pathways. *Nature.* 411:215-9.

Teo, M., L. Tan, L. Lim, and E. Manser. 2001. The tyrosine kinase ACK1 associates with clathrin-coated vesicles through a binding motif shared by arrestin and other adaptors. *J Biol Chem.* 276:18392-8.

Thompson, H.M., and M.A. McNiven. 2001. Dynamin: switch or pinchase? *Curr Biol.* 11:R850.

Tong, X.K., N.K. Hussain, E. de Heuvel, A. Kurakin, E. Abi-Jaoude, C.C. Quinn, M.F. Olson, R. Marais, D. Baranes, B.K. Kay, and P.S. McPherson. 2000. The endocytic protein intersectin is a major binding partner for the Ras exchange factor mSos1 in rat brain. *Embo J.* 19:1263-71.

Traub, L.M. 2003. Sorting it out: AP-2 and alternate clathrin adaptors in endocytic cargo selection. *J Cell Biol.* 163:203-8.

Tsuboi, D., H. Qadota, K. Kasuya, M. Amano, and K. Kaibuchi. 2002. Isolation of the interacting molecules with GEX-3 by a novel functional screening. *Biochem Biophys Res Commun.* 292:697-701.

Underhill, D.M., and A. Ozinsky. 2002. Phagocytosis of microbes: complexity in action. *Annu Rev Immunol.* 20:825-52.

Ungewickell, E., H. Ungewickell, S.E. Holstein, R. Lindner, K. Prasad, W. Barouch, B. Martin, L.E. Greene, and E. Eisenberg. 1995. Role of auxilin in uncoating clathrin-coated vesicles. *Nature.* 378:632-5.

Uruno, T., J. Liu, P. Zhang, Y. Fan, C. Egile, R. Li, S.C. Mueller, and X. Zhan. 2001. Activation of Arp2/3 complex-mediated actin polymerization by cortactin. *Nat Cell Biol.* 3:259-66.

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.

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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.K. Diril, N. Jung, and V. Haucke. 2004. Functional dissection of the interactions of stonin 2 with the adaptor complex AP-2 and synaptotagmin. *Proc Natl Acad Sci U S A.* 101:964-9.

Wells, A.L., A.W. Lin, L.Q. Chen, D. Safer, S.M. Cain, T. Hasson, B.O. Carragher, R.A. Milligan, and H.L. Sweeney. 1999. Myosin VI is an actin-based motor that moves backwards. *Nature.* 401:505-8.

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. *Mol Biol Cell.* 8:2003-15.

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

Wu, H., and J.T. Parsons. 1993. Cortactin, an 80/85-kilodalton pp60src substrate, is a filamentous actin-binding protein enriched in the cell cortex. *J Cell Biol.* 120:1417-26.

Wu, H., A.B. Reynolds, S.B. Kanner, R.R. Vines, and J.T. Parsons. 1991. Identification and characterization of a novel cytoskeleton-associated pp60src substrate. *Mol Cell Biol.* 11:5113-24.

Yamabhai, M., N.G. Hoffman, N.L. Hardison, P.S. McPherson, L. Castagnoli, G. Cesareni, and B.K. Kay. 1998. Intersectin, a novel adaptor protein with two Eps15 homology and five Src homology 3 domains. *J Biol Chem.* 273:31401-7.

Yang, W., Q. Lin, J. Zhao, J.L. Guan, and R.A. Cerione. 2001a. The nonreceptor tyrosine kinase ACK2, a specific target for Cdc42 and a negative regulator of cell growth and focal adhesion complexes. *J Biol Chem.* 276:43987-93.

Yang, W., C.G. Lo, T. Dispenza, and R.A. Cerione. 2001b. The Cdc42 target ACK2 directly interacts with clathrin and influences clathrin assembly. *J Biol Chem.* 276:17468-73.

Zamanian, J.L., and R.B. Kelly. 2003. Intersectin 1L Guanine Nucleotide Exchange Activity Is Regulated by Adjacent src Homology 3 Domains That Are Also Involved in Endocytosis. *Mol Biol Cell.* 14:1624-37.

Zelhof, A.C., H. Bao, R.W. Hardy, A. Razzaq, B. Zhang, and C.Q. Doe. 2001. Drosophila Amphiphysin is implicated in protein localization and membrane

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morphogenesis but not in synaptic vesicle endocytosis. *Development*. 128:5005-5015.

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.

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