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

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Tuba is a novel scaffold protein that functions to bring together dynamin with actin regulatory proteins. 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, 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.

Fission of clathrin-coated and other endocytic vesicles from the plasma membrane involves the cooperation of several membrane-associated proteins, among which the GTPase dynamin plays a key role (1, 2). The participation of dynamin in the fission of endocytic vesicles has been established by a variety of experimental approaches in Drosophila, cultured cells, and cell-free systems, although the precise mechanism of fission and the role of dynamin in this reaction remain unclear (3).

Several dynamin partners thought to participate in dynamin recruitment or function have been identified (3, 4). At the synapse, where endocytosis plays a key role in the recycling of synaptic vesicle membranes, two prominent dynamin partners are amphiphysin and endophilin (5–7). Amphiphysin has a three-domain structure with an evolutionarily conserved N-terminal module of ~250 amino acids called the Bin/amphiphysin/Rvs (BAR) domain, a variable central region, and a C-terminal Src homology-3 (SH3) domain that binds dynamin. Endophilin has a similar domain structure (2). Although the N-terminal domain of endophilin is substantially divergent in amino acid composition from the BAR domain of amphiphysin, it shares some similarity at critical sites, leading to its classification as a BAR domain (8). Accordingly, the BAR domains of amphiphysin and endophilin share functional similarities because they both can bind and deform lipid bilayers and mediate homooligomerization (6, 8–10). Dynamin also can bind and deform lipid bilayers, and it has been proposed that endophilin and amphiphysin might help to recruit and possibly assist dynamin in the generation of membrane curvature at endocytic pits (8, 10).

The closest homologue of amphiphysin and endophilin in Saccharomyces cerevisiae is Rvs167, that forms a stable heterodimer with Rvs161; Rvs167 has a domain structure like amphiphysin, whereas Rvs161 (homologous to the mammalian protein Bin3) possesses only a BAR domain. Mutation of either one or both components of this heterodimer in yeast produces defects in endocytosis and actin function (11). Such a dual phenotype is typical of most mutations in actin regulatory and endocytosis genes in yeast (12). These observations, together with results from a variety of studies in mammalian cells, have suggested a link between endocytosis and actin, although such a link has remained mechanistically elusive (13, 14). Foci of actin can often be seen at endocytic sites (15), and endocytic vesicles with actin tails have also been observed (16). Interest-

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ingly, these actin tails contain both dynamin and dynamin-interacting proteins (17, 18). Several binding partners of dynamin are either physically or functionally linked to actin or actin regulatory proteins. 

Several binding partners of dynamin are either physically or functionally linked to actin or actin regulatory proteins. These include, among others, syndapin/pacsin and intersectin/DAP160 (13, 14). Intersectin, in particular, represents a striking example of a multidomain protein that links dynamin to proteins that directly or indirectly control actin polymerization, such as the phagophidylinositol-4,5-bisphosphate phosphatase, synaptotagmin, N-WASP (neuronal Wiskott-Aldrich syndrome protein), and the Rho family GTPase Cdc42 (19, 20). N-WASP is a member of the WASP superfamily of proteins that includes SCAR/WAVE. All members of this family possess a conserved C terminus that can bind to and activate the Arp2/3 complex, a collection of proteins that catalyzes the nucleation of actin filaments (21). N-WASP exists predominantly in an autoinhibited state. When activated Cdc42 or the adaptor protein Nck binds to N-WASP, this autoinhibition is relieved. Phagophidylinositol-4,5-bisphosphate can further activate N-WASP in combination with Cdc42 and Nck (22–24). Intersectin functions to bring together N-WASP and, through the guanyl nucleotide exchange activity of its Dbl homology (DH) domain, activated GTP-bound Cdc42, thus activating Arp2/3 and promoting actin nucleation (19). The endocytic protein syndapin/pacsin, which, like intersectin, binds both dynamin and N-WASP, can promote N-WASP-dependent actin nucleation (25). Thus, intersectin and syndapin function as molecular links between endocytosis and actin assembly. The Arp2/3 complex plays a key role in generating actin-based protrusive forces that can drive the movement of vesicles and intracellular pathogens such as *Listeria monocytogenes* (26). Members of the Ena (Enabled)/VASP (vasodilator-stimulated phosphoprotein) protein family (Mena (mammalian Ena-VASP-like)) greatly accelerate the actin-based movement of Listeria (27, 28). 

Death of a pleckstrin homology (PH) domain, and the lipid-binding properties of the PH domain are thought to play a critical role in the localization and regulation of DH domain activity (32). We have characterized one of these three proteins, which we have named Tuba. In an independent line of study, we identified the mouse homolog of Tuba in a yeast two-hybrid screen using the Ena/VASP protein EVL as bait. Collectively, our study demonstrates that Tuba is a large scaffold protein that binds dynamin and a variety of actin regulatory proteins and that activates Cdc42. Our results suggest that Tuba functions as an important link between dynamin function, Rho GTPase signaling, and actin dynamics regulated by WASP/WAVE superfamily and Ena/VASP proteins.

**EXPERIMENTAL PROCEDURES**

**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 D1 (our laboratory) and monoclonal antibody Hdy-1 (Upstate Biotechnology, Inc.), anti-ampiphysin-1 monoclonal antibody-3 (33) and anti-N-WASP polyclonal antibody (gifts of P. Aspenstrom (Ludvig Institute for Cancer Research, Uppsala, Sweden) and M. W. Kirschnier (Harvard Medical School)), anti-CR16 polyclonal antibody (gift of H. Y. Ho and M. W. Kirschnier), anti-WAVE1 polyclonal antibody (gift of John Scott, Vollum Institute), anti-Mena monoclonal antibody (our laboratory), 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, Göttingen, Germany), and anti-GM-130 antibody (Graham Warren, Yale University). Anti-Tuba polyclonal antibodies were generated in rabbit. In our system, 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 Cδ 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 previously (10). 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. 1A). 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 EVI was used as bait in the Litwinwochelung-ready 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. 1A) 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 GenBankTM/EBI Data Bank under accession number AY383729. Affinity Chromatography—GST or GST fusion proteins of SH3 domain-containing regions of Tuba were bound to 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 SH3-3 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 (34). 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 μg 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 immunoglobulin labeling was performed on peroxidasedynapsosomes embedded in agarose, followed by Epion embedding and thin sectioning as described (35). Guanine Nucleotide Exchange Assays—Exchange assays using bacterially expressed and purified Rho GTPases were performed essentially as described (36). In particular, 2 μM Rho(A(1908S), Rac1(1888S), or Cdc42(1808S) was added to buffer containing 20 mM Tris (pH 7.5), 0.5 mM dithiotreitol, 5 mM MgCl₂, 1 mM EGTA, 5 mM GTPγS, and 400 nM mant-GTP (Molecular Probes, Inc.) and allowed to equilibrate for 5 min before adding the indicated concentrations of His-tagged Tuba 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 (λEx = 390 nm and λEm = 400 nm) and was corrected to 25°C. Fluorescence was normalized to the initial value at the start of the experiment. The experiment containing the DH and BAR domains of Tuba 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.
Tuba Links Cdc42, Dynamin, and the Actin Cytoskeleton

RESULTS

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. 1A). Although the overall homology to the amphiphysin-1 BAR domain is limited (24% identical and 39% similar) (Fig. 1B), 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-recognition 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 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. 1A). 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. 1A).

A putative ortholog of Tuba, GEI-18 (GEX interactor-18), was identified in Caenorhabditis elegans (Fig. 1A) (40). 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. 1B.

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. 2A) 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 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. 1A). 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. 2B). 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 (Hud-1 antibodies) (Fig. 3A). 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 (41). 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. 3A).

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 presynaptic compartment, where it was primarily concentrated at the periphery of synaptic vesicle clusters (Fig. 3B). These are the regions where clathrin-mediated endocytosis occurs (for example, see a clathrin-coated vesicle in Fig. 3B) and where presynaptic 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.

The N Terminus of Tuba Binds Dynamin—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. 4A). 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. 4B). 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 vitro (Fig. 4C). 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 (data not shown). 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 (42, 43). When expressed in Chinese hamster ovary cells, SH3-1,2,3,4 had a cytosolic distribution and inhibited transferrin internalization (Fig. 4D), 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).

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 in-
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. 5A). Each of the major protein bands was excised, trypsin-digested, and analyzed by MALDI-TOF spectrometry. The identified proteins are listed in Fig. 5A (upper panel). For some proteins, the interaction was validated further by Western blotting (Fig. 5A, 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 (44). 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 (45–48). WAVE1 is a neuron-specific SCAR/WAVE protein and a member of the WASP superfamily (49) that, like N-WASP, regulates actin assembly through binding and activation of the Arp2/3 complex (50). Unlike N-WASP, isolated WAVE1 is constitutively active (50), but is kept in an inhibited state while bound to a protein complex that includes PIR121 and NAP1 (51), two proteins present in the affinity-purified material. Mena, an Ena/VASP protein (30), and Lamellipodin, a novel Ena/VASP-associated protein,3 were also present in the affinity-purified material, as was drebrin, an F-actin-binding protein (52).

Given the large number of proteins present in the affinity-purified complex, 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 coprecipitate (Fig. 5B). Longer exposures revealed that the 140-kDa Mena isoform (30), a neuron-specific isoform of Mena (30), was also coprecipitated.

3 M. Krause and F. B. Gertler, unpublished data.
FIG. 5. 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).

B. 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.
N-WASP as well was found in the precipitated material (data not shown). These results indicate that Tuba interacts with one or more actin regulatory proteins in vivo.

Because the C terminus of mouse Tuba was identified as a binding partner of EVL in a yeast two-hybrid system (Fig. 1A) 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 (34)) 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. 6A). No binding was observed in cells expressing a mutant form of Mena that lacks the proline-rich region (Fig. 6A), 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. 6B, D7 E-M). GST-SH3-6 pull-down experiments with the same lysates were performed, and the affinity-purified material 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. 6B). 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. 6B). 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 the SH3-6 domain could account for the low activity. Because we believe that the BAR domain of Tuba is a functional domain (56), we fused the SH3-6 domain to a mitochondrial anchoring peptide in WAVE1 and localized to synapses, whereas the lower band appears to be an immunoreactive component of the actin cytoskeleton. This study details

**DISCUSSION**

The striking phenotype of *shibire* (dynamin) mutants of *Drosophila* (58) and the many subsequent studies demonstrating a block of endocytosis in cells harboring dynamin mutations have strongly implicated this GTPase in the fission reaction of endocytosis (10, 59, 60). There is also strong evidence from studies in living cells and in cell-free systems of a role for dynamin in the regulation of the actin cytoskeleton. For example, disruption of dynamin function impairs neurite outgrowth (an actin-dependent process) (61) and perturbs actin dynamics in a variety of cellular contexts (14, 62, 63). Dynamin is present in actin tails (17, 18), and many dynamin-binding proteins, including intersectin (19) and syndapin (64, 65), are also regulatory components of the actin cytoskeleton.
the discovery and characterization of a novel protein, Tuba, and its potential role as a molecular link between dynamin and actin.

The N-terminal domain of Tuba binds dynamin via multiple SH3 domains, each of which can bind independently, thus increasing the overall avidity of Tuba for this GTPase. The
interaction between Tuba and dynamin could be important for both dynamin localization and dynamin regulation, as has been shown for other SH3 domain-containing dynamin ligands (2, 4).

The C-terminal half of Tuba is linked to actin assembly via two independent mechanisms. First, the DH domain of Tuba activates Cdc42, a Rho family GTPase that binds and activates N-WASP, thereby triggering Arp2/3-mediated actin nucleation (66). Second, the C-terminal SH3 domain binds a number of key actin regulatory proteins, including Ena/VASP, N-WASP, the N-WASP-interacting protein WIRE, and CR16. Based on the effects of other SH3 domain interactors of N-WASP (19, 23), it is possible that Tuba binding activates N-WASP and that this activation may cooperate synergistically with Tuba-mediated activation of Cdc42 in promoting actin nucleation.

It is interesting to note that a Tuba isoform and Tuba relatives lack the N-terminal dynamin-binding module, suggesting that Tuba family proteins may regulate the actin cytoskeleton in processes other than endocytosis. For example, recent mod-

**Fig. 7. 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.
els for filopodial formation propose that filipodia emerge from clouds of Arp2/3-generated actin nuclei by elongation of filaments that are protected from capping and subsequently bundled (67). The Tuba C-terminal SH3 domain binds to Ena/VASP proteins. Because Ena/VASP proteins function to promote actin filament elongation and have a potential role in filopodial formation (68), we speculate that Ena/VASP proteins are recruited by Tuba to promote actin filament growth after N-WASP-dependent actin nucleation. Therefore, Tuba is well positioned to promote filopodial formation by coordinating the activation of N-WASP (directly and via Cdc42) with the recruitment of Ena/VASP family members to antagonize capping protein, thus permitting filament elongation. The presence of predicted coiled-coil domains in Tuba both in the BAR domain (9, 69) and upstream of the BAR domain (Fig. 1A) suggests a potential multimerization of the molecule as seen for other BAR and SH3 domain-containing proteins (9, 69), thus providing a further mechanism for the functional coordination of multiple binding partners.

The material affinity-purified by this SH3-6 domain also includes the WAVE1-PIR121-NAP125 complex, which is involved in the regulation of Arp2/3-mediated actin nucleation via Rac1 (51). Interestingly GEI-18, the worm TUBA homolog, was identified in a two-hybrid screen for binding partners of GEX3, the worm NAP125 homolog (40, 70), suggesting that interactions between Tuba and the WAVE inhibitory complex are evolutionarily conserved. It will be interesting to see what role, if any, Tuba plays in Rac-mediated signaling.

The SPOTs peptide binding provided further evidence for a direct and specific interaction between SH3-6 and N-WASP and Ena/VASP proteins. Four potential proline-rich SH3-6-binding sites were identified. Interestingly, the first site in

**FIG. 8.** 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.

**FIG. 9.** The DH domain of Tuba specifically catalyzes the activation of Cdc42. The indicated GTPases (2 μM) 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 μM) 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.
N-WASP (RAGPPPSPSAP, peptide 12) is very similar to a site found in the N-WASP-interacting protein WIRE (RGKKP-PPSPRTP), a protein that is also found in the affinity-purified Tuba SH3-6 complex. Far-Western data suggested that SH3-6 binds directly to more than N-WASP and Mena; and given that WIRE is ~50 kDa, we believe that the smallest band in the brain far-Western blot (Fig. 5B) is likely WIRE. Interestingly, the second site in N-WASP and the sites in Mena and EVL all contain PPXLPL.

The presence of binding sites for both N-WASP and dynamin, together with the presence of a Cdc42-specific DH domain, is also a characteristic of intersectin (19). However, in Tuba, the dynamin- and actin cytoskeleton-binding domains are segregated at opposite ends of the protein. In intersectin, an intramolecular regulatory mechanism has been demonstrated showing that the binding of N-WASP stimulates the DH domain to activate Cdc42, thus resulting in a synergistic activation of Arp2/3-mediated actin nucleation (19). The poor solubility of recombinant Tuba has prevented us from testing whether N-WASP binding can stimulate nucleotide exchange activity. The presence of putative consensus sites for SH3 domain binding in Tuba raises the possibility that inhibitory intramolecular SH3 domain-mediated interactions may occur (71). These interactions might be released by the binding of appropriate SH3 domain ligands.

An additional unique feature of the Tuba family is the presence of a BAR domain, rather than a PH domain, downstream of a DH domain. This is an exception to a nearly absolute rule (32, 55). PH domains bind phosphoinositides (72), and their interactions with lipids in the bilayer recruit catalytic DH domain ligands. Thus, BAR domains bind lipids (8, 10). Given poor solubility, we could not reliably test the lipid-binding properties of the BAR domain of Tuba, although preliminary experiments support this possibility. Thus, the BAR domain of Tuba may functionally replace a PH domain. It is worth noting that the critical role of actin in the endocytic reaction.

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The precise role of Tuba in cell and synaptic physiology remains to be defined. The properties of this protein reinforce the hypothesis that the functions of dynamin and actin are strongly interrelated, although we still do not yet understand precisely how. Two major possibilities remain open. The first is that dynamin has a primary function in endocytosis and that its connection to actin may reflect the need to coordinate the endocytic reaction with rearrangements of the actin cytoskeleton surrounding endocytic sites. These rearrangements may be needed to allow budding and fission or to propel the newly formed endocytic vesicles via actin tails. The second is that dynamin functions to regulate the actin cytoskeleton and that the powerful effect of mutant dynamin on endocytosis reflects the critical role of actin in the endocytic reaction.

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REFERENCES

1. Conner, S. D., and Schmid, S. L. (2003) Nature 422, 77–44
2. Slepnev, V. I., and De Camilli, P. (2000) Nat. Rev. Neurosci. 1, 161–172

4 M. A. Salazar and P. De Camilli, unpublished data.
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52. Ishikawa, R., Hayashi, K., Shirao, T., Xue, Y., Takagi, T., Sasaki, Y., and Kohama, K. (1994) J. Biol. Chem. 269, 29928–29933
53. Frank, R. (2002) J. Immunol. Methods 267, 13–26
54. Puster, S., Chakraborty, T., Walter, U., and Wehland, J. (1995) Curr. Biol. 5, 517–525
55. Hoffman, G. R., and Cerione, R. A. (2002) FEBS Lett. 513, 85–91
56. Liu, B. P., and Burridge, K. (2000) Mol. Cell. Biol. 20, 7160–7169
57. Whitehead, I. P., Campbell, S., Rossman, K. L., and Der, C. J. (1997) Biochem. Biophys. Acta 1332, F1–F23
58. Koenig, J. H., and Ikeda, K. (1989) J. Neurosci. 9, 3844–3860
59. Hinshaw, J. E., and Schmidt, S. L. (1995) Nature 374, 190–192
60. Marks, B., Stowell, M. H., Vallis, Y., Mills, I. G., Gibson, A., Hopkins, C. R., and McMahon, H. T. (2001) Nature 410, 231–235
61. Masur, S. K., Kim, Y. T., and Wu, C. P. (1990) J. Neurogenet. 6, 191–206
62. Krueger, E. W., Orth, J. D., Cas, H., and McNiven, M. A. (2003) Mol. Biol. Cell 14, 1085–1096
63. Ochoa, G. C., Slepnev, V. I., Neff, L., Ringstad, N., Takei, K., Danielli, L., Kim, W., Cao, H., McIver, M., Baron, R., and De Camilli, P. (2000) J. Cell Biol. 150, 377–389
64. Qualmann, B., and Kelly, R. B. (2000) J. Cell Biol. 148, 1047–1062
65. Modregger, J., Ritter, B., Witter, B., Paulsson, M., and Ploem, M. (2000) J. Cell Biol. 113, 4511–4521
66. Rohatgi, R., Ma, L., Miki, H., Lopez, M., Kirchhausen, T., Takenawa, T., and Kirschner, M. W. (1999) Cell 97, 221–231
67. Vignjevic, D., Yurur, D., Welch, M. D., Pelquin, J., Svitkina, T., and Borisy, G. G. (2003) J. Cell Biol. 160, 951–962
68. Svitkina, T. M., Bulanova, E. A., Chaga, O. Y., Vignjevic, D. M., Kojima, S., Vasilev, J. M., and Borisy, G. G. (2003) J. Cell Biol. 160, 409–421
69. Ringstad, N., Nemoto, Y., and De Camilli, P. (2001) J. Biol. Chem. 276, 40424–40430
70. Soto, M. C., Qadota, H., Kasuya, K., Inoue, M., Tsuboi, D., Mello, C. C., and Kaibuchi, K. (2002) Genes Dev. 16, 620–632
71. Zamanian, J. L., and Kelly, R. B. (2003) Mol. Biol. Cell 14, 1624–1637
72. Lemmon, M. A., Ferguson, K. M., and Abrams, C. S. (2002) FEBS Lett. 513, 71–76