Biochemical and Immunochemical Analysis of Rat Brain Dynamin Interaction with Microtubules and Organelles In Vivo and In Vitro

Robin Scaife and Robert L. Margolis

The Fred Hutchinson Cancer Research Center, Seattle, Washington 98104

Abstract. We have purified a 100-kD rat brain protein that has microtubule cross-linking activity in vitro, and have determined that it is dynamin, a putative microtubule-associated motility protein. We find that dynamin appears to be specific to neuronal tissue where it is present in both soluble and particulate tissue fractions. In the cytosol it is abundant, representing as much as 1.5% of the total extractable protein. Dynamin appears to be in particulate material due to association with a distinct subcellular membrane fraction. Surprisingly, by immunofluorescence analysis of PC12 cells we find that dynamin is distributed uniformly throughout the cytoplasm with no apparent microtubule association in either interphase, mitotic, or taxol-treated cells. Upon nerve growth factor (NGF) induction of PC12 cell differentiation into neurons, dynamin levels increase approximately twofold. In the cell body, the distribution of dynamin again remains clearly distinct from that of tubulin, and in axons, where microtubules are numerous and ordered into bundles, dynamin staining is sparse and punctate. On the other hand, in the most distal domain of growth cones, where there are relatively few microtubules, dynamin is particularly abundant. The dynamin staining of neurites is abolished by extraction of the cells with detergent under conditions that preserve microtubules, suggesting that dynamin in neurites is associated with membranes. We conclude that dynamin is a neuronal protein that is specifically associated with as yet unidentified vesicles. It is possible, but unproven, that it may link vesicles to microtubules for transport in differentiated axons.

MICROTUBULES frequently perform functions that require their coalescence into ordered arrays, and their specific association with a variety of subcellular organelles. These linkages most likely result from the specific association of the polymer with proteins of specialized function.

Among the brain-derived microtubule-associated proteins (MAPs),[1] some have been reported to induce linkage between microtubules and intermediate filaments (Bloom and Vallee, 1983) or with F-actin (Griffith and Pollard, 1978). The well-characterized MAPs, tau and MAP2, induce microtubule bundles after cDNA overexpression or microinjection in cultured cells (Kanai et al., 1989; Lewis et al., 1989), and, for MAP2, a putative carboxy terminal domain that could function in dimerization has been suggested as the cause of this bundling effect (Lewis and Cowan, 1990). Other MAPs have also been reported to cross-link between microtubules in vitro (Aamodt et al., 1989; Campbell et al., 1989; Hollenbeck and Chapman, 1986; Turner and Margolis, 1984).

In addition to those MAPs that apparently play structural roles on microtubules by stabilizing and organizing the polymers in the cell, others like kinesin and dynein appear to be involved in microtubule-directed organelle movement (Schroer et al., 1988; Paschal and Vallee, 1987). Biochemical and immunofluorescence studies have identified kinesin as an important mechanochemical enzyme involved in microtubule-directed organelle movement (Vale et al., 1985; Hollenbeck, 1989; Pfister et al., 1989). Additionally, several proteins have been identified in a number of lower eukaryotes, and *Drosophila*, with presumed motility functions and substantial domain homologies to kinesin (Enos and Morris, 1990; Meluh and Rose, 1990; Endow et al., 1990; McDonald and Goldstein, 1990). Cytoplasmic dynein is also a likely candidate for a variety of intracellular microtubule-based motility events, ranging from axonal vesicular transport to mitotic chromosome segregation (Paschal and Vallee, 1987; Pfarr et al., 1990; Steuer et al., 1990). Recently, Shpetner and Vallee (1989) have isolated a microtubule bundling protein dynamin, which they characterized as a third type of putative microtubule-based motor protein.

Several proteins form cross-links between microtubules and appear to induce ATP driven interpolymer sliding in vitro. Both dynein, the motor protein of axonemes, and dynamin have the capacity to link between microtubule polymers in vitro (Goodenough and Heuser, 1984; Shpetner and Vallee, 1989) and to cause one polymer to slide relative to another (Sale and Satir, 1977; Shpetner and Vallee, 1989).

1. *Abbreviations used in this paper*: MAP, microtubule-associated protein; MME buffer, 100 mM Mes, 1 mM $MgSO_4$, 1 mM EGTA, 0.02% sodium azide, pH 6.8; NGF, nerve growth factor.

It is thus evident that microtubule cross-linking may be generated by proteins with other than purely structural roles. In the case of dynein, it is clear that both microtubule cross-linking and motor behavior are interdependent processes with physiological meaning for flagellar motility. Cross-linking by associated proteins can involve apparently specific interactions with microtubules. Both the cytoplasmic homologue of dynein, MAP1C (Paschal et al., 1987), and dynamin (Shpetner and Vallee, 1989) appear to generate ordered microtubule bundles (Amos, 1989; Shpetner and Vallee, 1989).

We have previously demonstrated extensive bundling of rat brain–derived microtubules in the presence of the microtubule stabilizing drug taxol, and we have shown that the activity was ATP sensitive and correlated with the presence of a 100-kD doublet protein (Turner and Margolis, 1984). It is evident that the characteristics of this protein and those of the recently described dynamin are strikingly similar. We have examined this possibility and now confirm that the 100-kD protein we described is a rat brain homologue of dynamin.

In this report, we show that rat brain dynamin may be purified by a rapid procedure, incorporating an N-6–linked ATP affinity column that binds dynamin with substantial discrimination. We confirm the previous report (Shpetner and Vallee, 1989) that dynamin cross-links microtubules in an ATP sensitive manner, and copurifies with microtubules through in vitro assembly cycles.

Additionally, we have generated a specific polyclonal antiserum against rat brain dynamin. We use the antibody to demonstrate that dynamin is a major neuronal protein and is substantially membrane associated. By immunofluorescence assay, it is shown that dynamin has no apparent association with microtubules in the cell body or in mitotic spindles. It further shows no close association with microtubules in axons but rather is present as detergent sensitive foci in axons and accumulates in growth cones. These data together might suggest a neurite specific function for dynamin in the motility of as yet unidentified vesicles upon microtubules in axons but evidence for specific association with microtubules is weak at best. The role of dynamin as a motor protein is still unresolved. Dynamin does not induce ATP dependent sliding of microtubules on coverslips (Shpetner and Vallee, 1989), nor does it exhibit ATPase activity in the absence of crude extract factors (Shpetner and Vallee, 1989). The possibility therefore exists that the in vitro association of dynamin with microtubules may be irrelevant to its true intracellular function.

Materials and Methods

Microtubule Protein Preparation

Fresh rat brains were homogenized in MME buffer (100 mM Mes, 1 mM $MgSO_4$, 1 mM EGTA, 0.02% sodium azide, pH 6.8) at 2 ml/g of tissue. After centrifugation at 4°C for 30 min at 150,000 *g*, microtubule assembly was induced in the clarified supernatant by adding 10% (vol/vol) DMSO and warming to 30°C. The resulting microtubules were collected by centrifugation through 1 ml of MME containing 20% glycerol (vol/vol) for 30 min at 150,000 *g* (30°C).

Pure tubulin was prepared from beef brain microtubule protein that was recycled according to previously published procedures (Margolis et al., 1986). Tubulin was prepared from three cycle-purified microtubule protein by passage through a phosphocellulose chromatography column (Williams and Detrich, 1979) and a subsequent cycle of assembly (in MME buffer plus 20% DMSO and 0.25 mM GTP) at 30°C.

Dynamin Purification

Dynamin was purified by first assembling rat brain microtubules from the crude extract in MME buffer. Pelleted microtubules were disassembled by shearing with a 25 gauge needle and exposure to 4°C for 30 min, and the resulting solution was centrifuged at 12,000 *g* for 10 min. The supernatant was loaded onto a DEAE-cellulose (Whatman Inc., Clifton, NJ) column and the resulting flow-through fraction was then loaded onto an ATP-agarose column with N-6 linkage (No. A9264; Sigma Chemical Co., St. Louis, MO). Dynamin was eluted with 0.2 M NaCl in MME. Peak fractions were drop frozen in liquid nitrogen, and stored at −70°C.

Immunofluorescence Microscopy of Individual Microtubules

Samples containing taxol-stabilized pure tubulin microtubules and dynamin

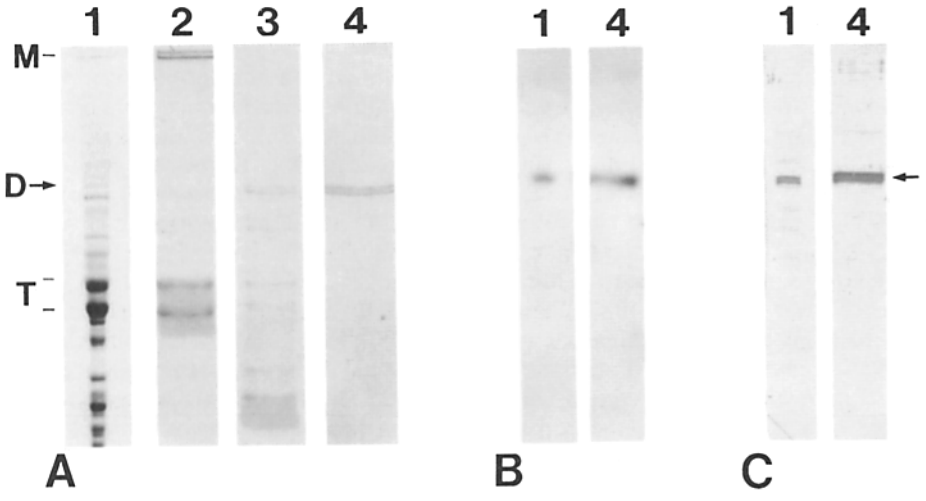

Figure 1. Purification of dynamin from rat brain, analyzed by SDS-PAGE and immunoblotting. (*A*) Clarified rat brain crude extract prepared as described in Materials and Methods (lane *1*) was assembled at 30°C in MME buffer containing 10% DMSO, and microtubules were collected by centrifugation as described in Materials and Methods. The microtubule containing pellet was disassembled at 4°C and clarified by centrifugation (lane *2*). The supernatant fraction was then passed through a DEAE column and the flow-through fraction (lane *3*) applied to an N-6–linked ATP-agarose column, yielding the 100-kD dynamin as a doublet by elution in 0.2 M salt (lane *4*). (*B* and *C*) Immunoblot analysis was performed on samples of the rat brain crude extract (lanes *1*), and of dynamin purified as described above (lanes *4*). (*B*) Western blot using a rabbit antiserum to the 100-kD protein produced in our laboratory and, (*C*), a Western blot of the same protein samples probed with antiserum to dynamin produced in Dr. Vallee's laboratory. The gel positions of tubulin (*T*), HMW-MAPs (*M*), and dynamin (*D*) are indicated.

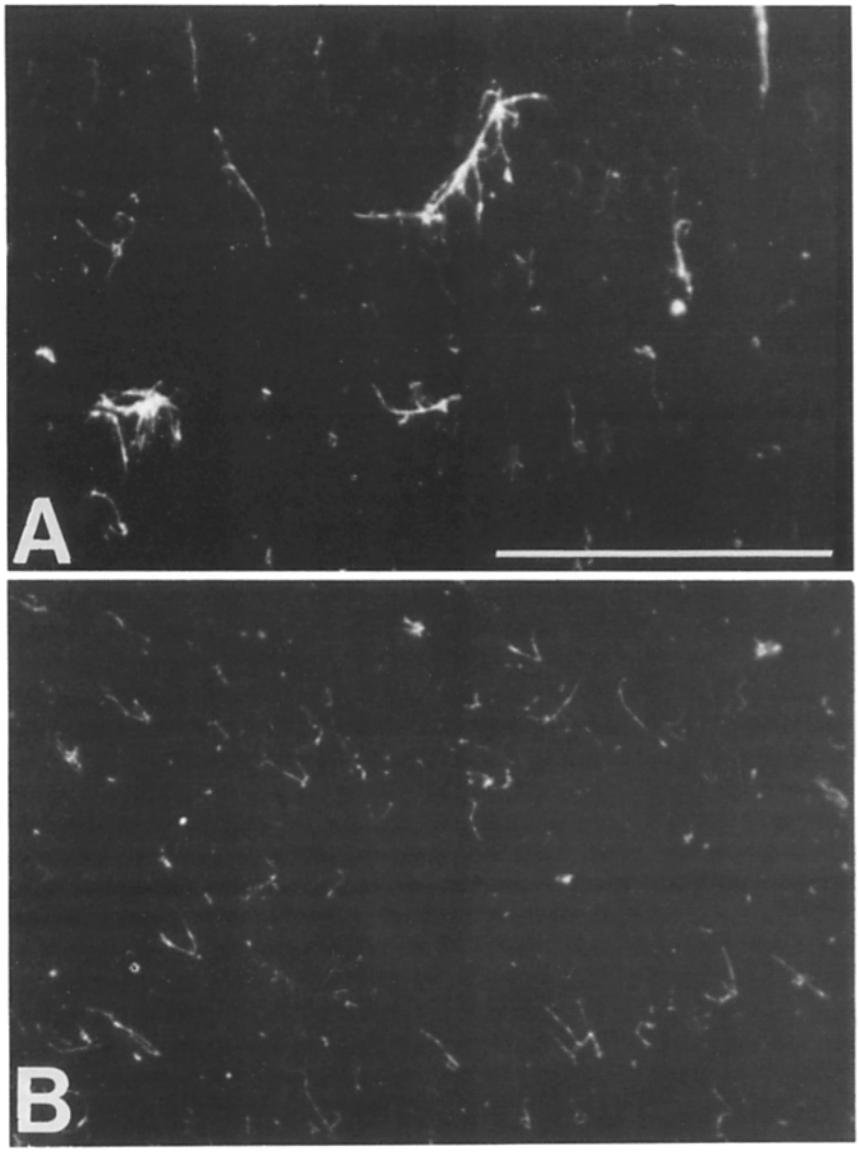

Figure 2. Immunofluorescence assay of microtubule cross-linking by ATP-agarose–purified dynamin. Microtubules were assembled from purified tubulin (0.8 mg/ml) with GTP (0.1 mM) in MME buffer plus 10% DMSO for 20 min at 30°C. After adding taxol (20 μM) to stabilize the microtubules, 1 μl aliquots were incubated for 15 min at 30°C with 9 μl of ATP-agarose–purified dynamin ($\sim$.015 mg/ml final concentration) and with or without Mg^{2+}-ATP (5 mM). Samples were fixed at 30°C in MME buffer containing 20% glycerol and 1% glutaraldehyde. After dilution, fixed microtubules were spun onto glass coverslips and processed for immunofluorescence microscopy with anti-β tubulin serum as described in Materials and Methods. Samples without Mg^{2+}-ATP are shown in *A*; with Mg^{2+}-ATP in *B*. Bar, 2 μm.

(purified as described above) were fixed for 15 min at 30°C in MME buffer containing 20% glycerol and 1% glutaraldehyde (Ted Pella, Redding, CA). Aliquots were then diluted with buffer and centrifuged onto coverslips at 15,000 *g* for 60 min, and subsequently treated successively with 0.5 mg/ml $NaBH_4$, 1:200 mouse anti-β tubulin serum (East Acres Biological, Southbridge, MA) and fluorescein-conjugated goat anti-mouse antibodies (1:200 dilution, TAGO, Inc., Burlingame, CA). Coverslips were mounted on glass slides with PBS containing 90% glycerol and 50 mg/ml *N*-propylgallate, and viewed with a Leitz Ortholux microscope equipped for epifluorescence microscopy.

Cell Culture and Immunofluorescence Microscopy

Rat neuronal PC12 cells were grown on a collagen-treated substrate in DME medium plus 5% FCS and 1% heat-treated horse serum. Cells were induced to differentiate with 100 ng/ml nerve growth factor (NGF) (Sigma Chemical Co.), and after 3–7 d in NGF they were either lysed in SDS containing buffer and immediately heated to 100°C (for subsequent electrophoretic analysis) or prepared for immunofluorescence microscopy by fixation at 37°C for 15 min in MME buffer containing 2.5% paraformaldehyde, 0.1% glutaraldehyde. Taxol (obtained from Dr. Suffness, National Institutes of Health) treatment of cells was at 10 μM for 18 h before fixation. After permeabilization with 0.2% Triton X-100 for 15 min, coverslips were immersed in PBS containing 0.5 mg/ml sodium borohydride, rinsed in PBS containing 0.05% Tween 20, and blocked with 2% FBS before incubation with antibodies. The anti-α and anti-β tubulin sera (diluted 1:250 in PBS plus 1 mg/ml BSA) used were mouse ascites fluids from Amersham Corp. (Arlington Heights, IL). The antidynamin serum was an affinity-purified rabbit serum. Polyclonal antidynamin sera were produced by injection of electrophoretically purified 100-kD antigen as previously described (Margolis et al., 1986) and affinity-purified antiserum to dynamin was obtained by exposure of nitrocellulose strips from blots containing electrophoretically separated dynamin (first purified by DEAE cellulose and ATP-agarose chromatography), to dynamin antiserum, and removal of the dynamin-specific antibodies with 50 mM glycine-HCl, pH 2.2. Control affinity-purified antiserum was obtained from nitrocellulose blots by washing strips that did not contain dynamin. Coverslips were then incubated with 1:250 diluted rhodamine-conjugated goat anti-rabbit (TAGO, Inc.) and then in sequence, rhodamine-conjugated swine anti-goat sera, followed by 2% normal goat serum and 1:250 diluted fluorescein-conjugated goat anti-mouse serum (TAGO, Inc.). Coverslips were mounted on glass slides with PBS containing 90% glycerol and 50 mg/ml *N*-propylgallate, and viewed with a Nikon microscope equipped for confocal microscopy (MRC-500; Bio-Rad Laboratories, Cambridge, MA). Fixation with methanol at −20°C resulted in qualitatively similar images to those presented here, as did use of fluorescein conjugated goat anti-rabbit (TAGO, Inc.) and Texas red-conjugated goat anti-mouse (TAGO, Inc.) secondary sera.

Fractionation and Analysis of Synaptosomal Lysate

Synaptosomal material was obtained from rat brain (Huttner et al., 1983), and after hypo-osmotic lysis, was fractionated by sucrose density gradient

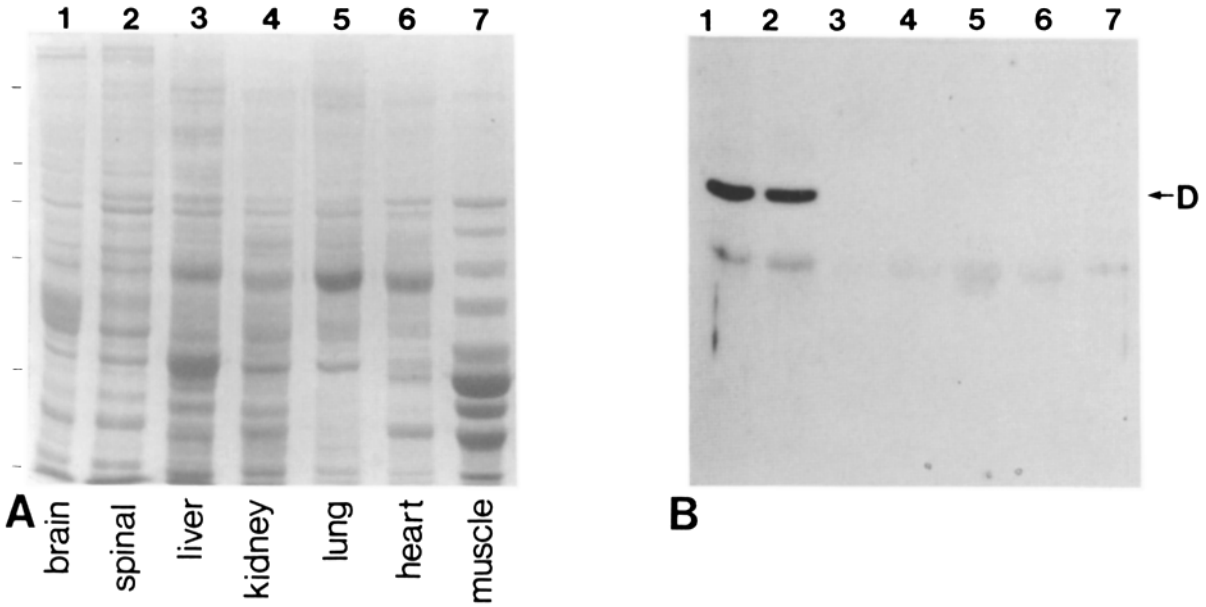

Figure 3. Determination of tissue-specific dynamin distribution by immunoblotting. Crude tissue extracts were obtained from brain (lane *1*), spinal cord (lane *2*), liver (lane *3*), kidney (lane *4*), lung (lane *5*), heart (lane *6*), and muscle (lane *7*) of adult rats and subjected to SDS-PAGE (*A*) and immunoblotted with antiserum to dynamin (*B*). Positions of molecular mass markers (210, 116, 97, 66, 45, and 30 kD) are indicated at the side of the gel and the position of dynamin on the blot is indicated with the letter *D*.

sedimentation and by controlled pore glass bead chromatography, essentially according to published procedures (Huttner et al., 1983). During centrifugation, we employed a linear 12-ml 0.05–1.2 M sucrose gradient for each one milliliter of lysate. The gradient fractions containing dynamin, as determined by immunoblotting procedure, were pooled and loaded onto a 1.5 × 70-cm controlled pore glass bead column for further fractionation. Sucrose gradient and controlled pore glass column fractions were processed for electron microscopy as described (Huttner et al., 1983).

Other Procedures

PAGE was performed using 8% polyacrylamide, 0.1% SDS gels as previously described (Laemmli, 1970). Molecular mass marker positions (205, 116, 97, 66, 45, and 32 kD) are indicated by horizontal bars alongside the gels. Immunoblotting was essentially according to Towbin et al. (1979). After electrotransfer of samples, nitrocellulose sheets were blocked with 1% nonfat milk for 60 min and incubated with 1:2,000 antidynamin (or antisynapsin-I serum; a generous gift from Dr. Greengard, Rockefeller University) for 60 min in PBS with 0.05% Tween-20 and 1% nonfat milk. After this, blots were washed with the same buffer, then exposed to ^{125}I-protein A (New England Nuclear, Boston, MA), and visualized by autoradiography. A Phosphor Imager 400A (Molecular Dynamics, Sunnyvale, CA) was used to quantitate the autoradiographic signal. Protein concentrations were determined by $OD_{560\ nm}$ assay with bicinchoninic acid (Pierce Chemical Co., Rockford IL).

Results

Purification of a 100-kD Rat Brain Antigen and Its Identification as Dynamin

This laboratory has reported that a protein doublet of 100 kD is highly enriched on microtubules assembled in vitro from rat brain tissue in the presence of taxol (Turner and Margolis, 1984). Further, the presence of the 100-kD protein correlates with ATP sensitive bundling of isolated microtubules (Turner and Margolis, 1984). The ATP sensitive bundling led us to suspect the 100-kD protein might bind with high affinity to ATP. We therefore have made use of ATP affinity matrices for the purification of this 100-kD species. We have now devised a simple three step purification procedure that incorporates the ATP affinity column, and that yields a nearly homogeneous 100-kD protein doublet (Fig. 1 *A*). In the first step, soluble 100-kD protein associates and copellets with microtubules assembled from a rat brain crude extract (Fig. 1 *A*, lanes *1* and *2*). The resolubilized pellet is then applied to a DEAE ion exchange column, from which we recover the 100-kD doublet, but no other major MAPs (i.e., MAP1 and MAP2), in the flow-through eluate (Fig. 1 *A*, lane *3*). This eluate is then applied to an N-6 linkage ATP affinity column, and the 100-kD doublet is recovered from this column as a near homogeneous preparation at 0.2 M salt (Fig. 1 *A*, lane *4*). As predicted from the previous study (Turner and Margolis, 1984), the three step purified 100-kD protein cross-links pure tubulin microtubules in vitro in an ATP sensitive manner (Fig. 2, *A* and *B*).

We have developed a rabbit polyclonal antiserum against electrophoretically purified 100-kD protein. On Western blots, this serum recognizes a single doublet antigen in crude rat brain extract (Fig. 1 *B*, lane *1*), of the same apparent molecular mass as the three step purified antigen (Fig. 1 *B*, lane *4*).

The similarity of mass and cross-linking properties of this 100-kD protein to the recently described beef brain microtubule motor protein, dynamin (Shpetner and Vallee, 1989), suggested that the 100-kD protein might be rat dynamin. We have used antisera to probe the relationship between these proteins. For this purpose, a blot of crude rat brain extract (Fig. 1 *C*, lane *1*) and three step purified antigen (Fig. 1 *C*, lane *4*) was sent to the laboratory of Dr. Vallee where it was probed with antiserum raised against dynamin. The major bands detected with this serum correspond to a 100-kD doublet in the crude extract, and the three step purified 100-kD protein (Fig. 1 *C*). Additionally, our antiserum to the 100-kD protein reacts strongly with purified dynamin on ni-

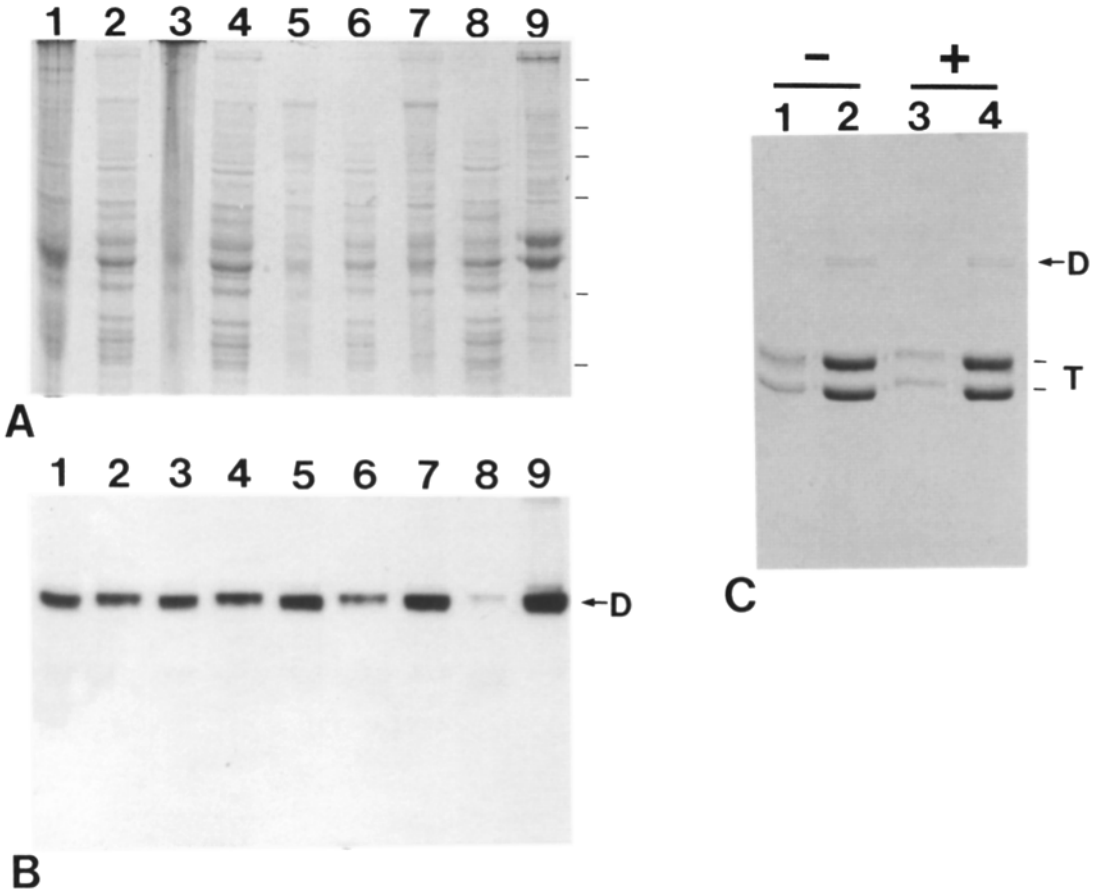

Figure 4. Analysis of dynamin distribution in rat brain soluble, particulate and microtubule fractions. Rat brain homogenate (lane *1*) was centrifuged at low speed (3,000 *g*) for 10 min at 4°C. The resulting pellet was extracted with 2 vol of cold MME buffer before electrophoresis (lane *3*). The low speed supernatant fraction (lane *2*) was centrifuged at medium speed (18,000 *g*) for 20 min at 4°C. The resulting pellet was resuspended in MME buffer and subjected to electrophoresis (lane *5*). The medium speed supernatant (lane *4*) was centrifuged at high speed (150,000 *g*) for 30 min at 4°C. The resulting pellet was resuspended in MME buffer and subjected to electrophoresis (lane *7*). The high speed supernatant (lane *6*) was incubated at 30°C for 30 min with 10% DMSO to assemble microtubules and centrifuged again at high speed for 30 min at 30°C. The resulting supernatant and pellet fractions were subjected to electrophoresis (lanes *8* and *9*, respectively). The Coomassie-stained gel is shown in *A*, and a duplicate immunoblot, probed with our rabbit polyclonal antiserum to dynamin is shown in *B*. Positions of molecular mass markers (210, 116, 97, 66, 45, and 30 kD) are indicated at the side of the gel and the position of dynamin on the blot is indicated with the letter *D* is shown in *B*. In *C*, microtubules were assembled from purified tubulin (0.8 mg/ml) in MME buffer containing 0.1 mM GTP and 10% DMSO, for 20 min at 30°C. After adding 20 μM taxol to stabilize microtubules, 17.5 μl microtubule aliquots were added to 82.5 μl of purified dynamin (~0.015 mg/ml final concentration). Samples were incubated for 20 min at 30°C with or without 5 mM Mg^{2+}-ATP, and centrifuged at 150,000 *g* for 30 min. Supernatants (lanes *1* and *3*) and pellets (lanes *2* and *4*) were subjected to PAGE and stained with Coomassie blue. Lanes *1* and *2*, minus ATP; lanes *3* and *4*, plus ATP. Positions of α and β tubulin (*T*) and dynamin (*D*) are indicated.

trocellulose blots supplied by the laboratory of Dr. Vallee (data not shown). Therefore, the 100-kD protein species that we have identified is clearly the same protein as beef brain dynamin. We hereafter refer to this protein as dynamin. By quantitative analysis of our blots, we have determined that dynamin represents as much as 1.5% of the total extractable protein in rat brain. For this purpose, soluble dynamin content was quantitated by Phosphor Imager analysis of crude rat brain soluble extract and highly purified dynamin Western blot signals, and then corrected for protein content in each of the gel lanes analyzed.

Distribution of Rat Brain Dynamin

To determine the tissue distribution of dynamin, we have analyzed extracts from seven different rat tissues by Western blot assay using the polyclonal serum (Fig. 3). Assaying whole cell extracts that were loaded on an equal protein basis (Fig. 3 *A*), we found high levels of dynamin in both brain and spinal cord, while all other tissues tested negative for the presence of dynamin (Fig. 3 *B*). A ~60-kD antigen is also present in these samples. This antigen is probably human keratin, since its detection is dependent on the presence of mercaptoethanol in the sample buffer (Shapiro, 1987). Affinity-purified dynamin antibody, used below for immunofluorescence assays, does not recognize this 60-kD band. The dynamin epitope(s) recognized by this polyclonal antibody are clearly neuron specific, but it cannot be ruled out that other isotypes of the protein exist in other tissues.

Our polyclonal serum has further allowed us to determine

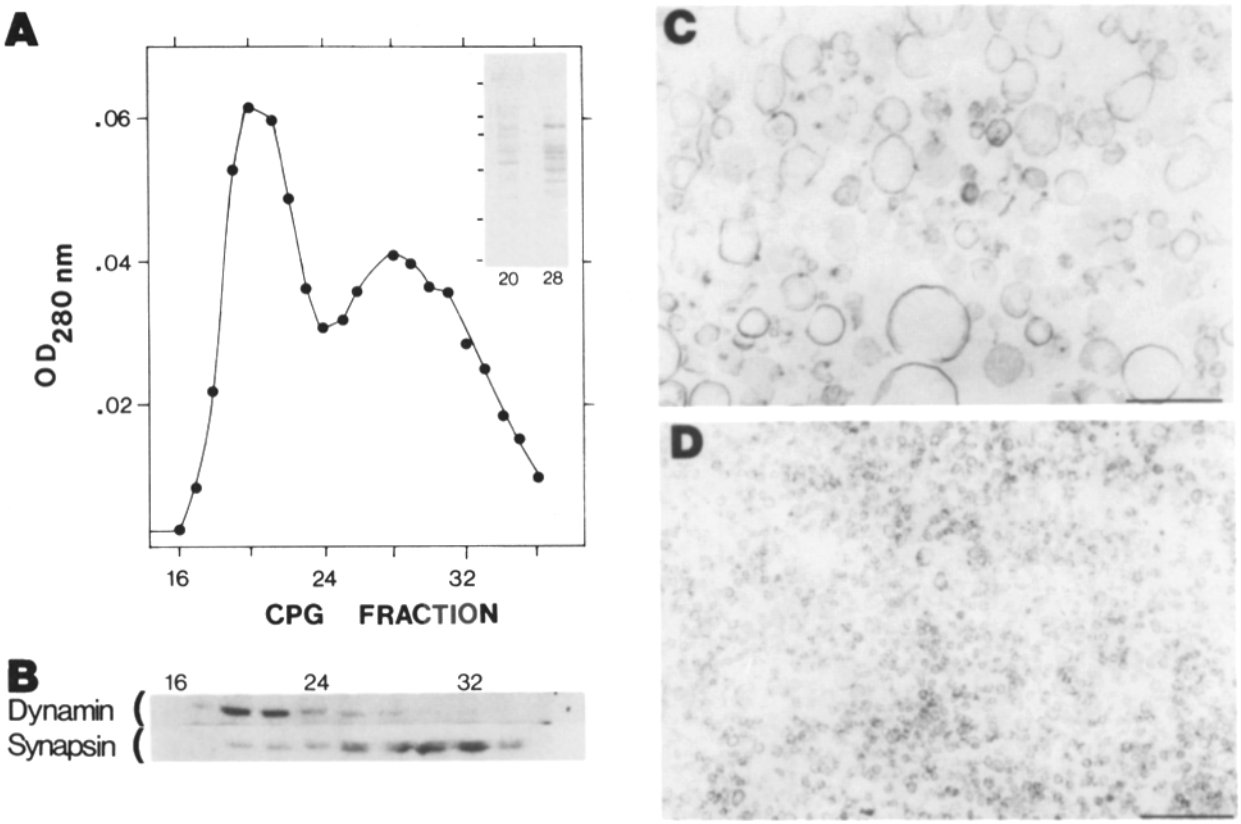

Figure 5. Controlled pore glass bead column separation of dynamin containing membrane fractions from synaptic vesicles. Rat brain synaptosomal lysates (obtained as described in Materials and Methods) were subjected to sucrose density gradient sedimentation (Huttner et al., 1983), and dynamin containing gradient fractions (determined by immunoblot analysis) were loaded onto a controlled pore glass bead column (*A*) The column eluate profile was monitored at 280 nm, and (*A*, *inset*) the two major column peak fractions were analyzed by SDS-PAGE. Positions of molecular mass markers (210, 116, 97, 66, 45, and 32 kD) are indicated. (*B*) Column fractions were also immunoblotted with our polyclonal antidynamin antiserum and with antisynapsin antibodies (supplied by the laboratory of Dr. Greengard). Aliquots of column fractions 20 and 28, the peak fractions containing dynamin or synapsin, were also fixed and processed for electron microscopy (*C* and *D*, respectively). Bar, 0.5 μm.

the distribution of dynamin upon subcellular fractionation of rat brain tissue. By performing sequential centrifugation of whole rat brain tissue homogenate at low, medium, and high speed we have found, surprisingly, that dynamin is relatively abundant in all three particulate fractions (Fig. 4). After a low speed (3,000 *g*) centrifugation of the homogenate, ~70% of the dynamin was recovered in the particulate fraction (Fig. 4, *A* and *B*, lane *3*). The low speed supernatant (Fig. 4, *A* and *B*, lane *2*) was centrifuged at medium speed (18,000 *g*). A substantial proportion of the dynamin was again recovered in the particulate fraction (Fig. 4, *A* and *B*, lane *5*). The medium speed supernatant (Fig. 4, *A* and *B*, lane *4*) was centrifuged at high speed (150,000 *g*). Again, dynamin was recovered in the particulate fraction (Fig. 4, *A* and *B*, lane *7*). The high speed supernatant (Fig. 4, *A* and *B*, lane *6*) was incubated at 30°C for 30 min with 10% DMSO, to assemble microtubules, and centrifuged again at high speed. Nearly all the supernatant dynamin was then recovered along with the microtubules in the pelleted fraction (Fig. 4, *A* and *B*, lane *9*). This soluble dynamin associates quantitatively with microtubules through successive in vitro assembly–disassembly cycles (data not shown). We have also observed quantitative retention of soluble purified dynamin on microtubules in the presence of millimolar ATP (Fig. 4 *C*), in accord with results of Shpetner and Vallee (1989), despite the relaxation of dynamin dependent microtubule bundling at this ATP concentration (Fig. 2 *B*; Shpetner and Vallee, 1989).

A substantial proportion of rat brain dynamin is thus in a particulate form. As dynamin is suggested to be a putative microtubule motor protein (Shpetner and Vallee, 1989), it was of interest to assay for its potential membrane association. We began analysis by isolation of rat brain synaptosomes, which contain high levels of dynamin. Upon hypoosmotic lysis of a synaptosome preparation, we recovered synaptosome associated dynamin in a high speed particulate fraction (data not shown). This material was fractionated further by sucrose density gradient centrifugation (data not shown), and finally by controlled pore glass bead chromatography (Fig. 5). Western blot analysis of the column fraction demonstrates that dynamin is specifically associated with a subfraction of membranous material distinct from the synaptic vesicle peak (Fig. 5 *B*). This conclusion is drawn both on the basis of the absence of synapsin from the dynamin rich fraction (Fig. 5 *B*) and the finding that the dynamin rich fraction consists primarily of large membranous material (Fig. 5 *C*), in contrast to the small vesicles comprising the synapsin rich fraction (Fig. 5 *D*).

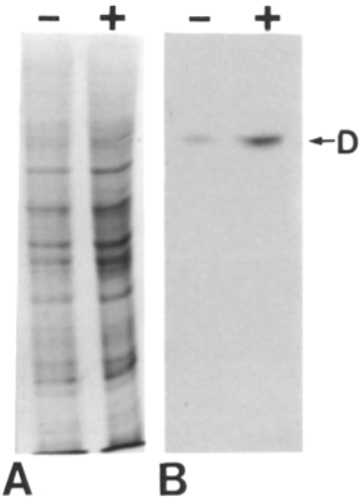

Figure 6. Immunochemical detection of dynamin in PC12 cells. PC12 cells were grown either with or without NGF, as described in Materials and Methods. After 6 d, cells were lysed in Tris-glycine buffer containing 0.5% SDS and, after sonication, aliquots were analyzed by SDS-PAGE (*A*) and immunoblotting with affinity-purified antidynamin serum (*B*). Lane *1*, 35 μg cell lysate, minus NGF; lane *2*, 43 μg cell lysate, plus NGF. The position of dynamin on the blot is indicated by the letter *D*.

Immunofluorescent Localization of Dynamin in PC12 Cells

By Western blot analysis, we have determined that dynamin is expressed in PC12 cells and is present in whole lysates of cells grown both in the presence and absence of NGF (Fig. 6), and that dynamin is the only antigen detectable upon probing the nitrocellulose blot with affinity-purified antiserum to dynamin. Exposure of the cells to NGF induces neurite outgrowth (Greene and Tischler, 1976) and increases the dynamin level approximately twofold (Fig. 6 *B*).

We have applied the antidynamin antibody, affinity purified from nitrocellulose blots, for immunofluorescence analysis of intracellular dynamin distribution (Figs. 7–10). At the earliest stages of neurite extension dynamin was detectable as a nearly uniform punctate stain throughout the cytoplasm (Fig. 7 *A*) in contrast to the distinctly filamentous tubulin staining (Fig. 7 *B*). The staining with antidynamin antibody is, however, specific since substitution with a negative control antiserum (see Materials and Methods) yielded no signal (Fig. 7 *E*). The difference between dynamin and tubulin staining was particularly apparent in the leading ruffles of growth cones where dynamin was very abundant relative to tubulin (Fig. 7, *A* and *B*, *arrows*). We have neither thus far observed a convincing codistribution of dynamin with interphase microtubules in the cell bodies of these cells.

For the cell shown in Fig. 7 (*C* and *D*), differentiation of cells was more advanced. As in earlier stages of differentiation, dynamin staining was present as a punctate pattern throughout the cytoplasm and in the neuritic extensions (Fig. 7 *C*). Dynamin staining was particularly intense relative to the tubulin signal in the distal portions of neuritic extensions (Fig. 7, *C* and *D*, *open arrows*). Additionally, in view of the reports on in vitro microtubule bundling by dynamin, it was surprising to find relatively low levels of dynamin stain in areas of the neurites where tubulin staining was strongest, and where microtubules have a bundled appearance (Fig. 7, *C* and *D*, *solid arrows*). Although the low levels of dynamin stain in these areas could be a result of epitope masking in the dense microtubule bundles, this is not likely to be the case with a polyclonal serum. Also, we have found that microtubule bundling per se does not mask dynamin epitopes, as in vitro formed bundles readily stain with our dynamin antibody (data not shown).

The apparent absence of close association of dynamin with individual microtubules or microtubule bundles in PC12 cells is particularly apparent following taxol treatment (Fig. 8). Extensive rearrangement and bundling of microtubules occurred after addition of taxol to the culture media (Fig. 8 *B*). The dynamin staining, on the other hand, remained uniformly punctate and was clearly not influenced by the dramatic taxol-induced microtubule rearrangements (Fig. 8 *A*).

The punctate nature of the dynamin stain in PC12 cells and the detection of dynamin in particulate fractions from PC12 cells (data not shown) suggest an association of PC12 cell dynamin with cellular membranes. Further, the punctate staining of PC12 growth cones by dynamin antibody (Fig. 7) correlates with evidence that dynamin is present in synaptosome-derived membrane fractions (Fig. 5), suggesting that dynamin is present in synaptosomes as a membrane-associated antigen.

To distinguish whether the bulk of the dynamin staining in axons is associated with membrane surfaces, we have detergent-extracted cells before fixation. Detergent treatment of cells before fixation generally results in the extraction of membrane-associated antigens and concomitant loss of immunofluorescent staining (Brown et al., 1976; Hollenbeck, 1989; Pfister et al., 1989), whereas detergent extraction, performed in a microtubule stabilizing buffer (Osborn and Weber, 1982), has no effect on microtubules or its associated antitubulin staining. Extraction of PC12 cells, after 4 d (Fig. 9, *A* and *B*) or 7 d (data not shown) in NGF, with 0.2% Triton had no significant effect on antibody staining of microtubules (Fig. 9 *B*), but substantially attenuated the dynamin stain (Fig. 9 *A*). After extraction, neuritic extensions were not stained at all (Fig. 9 *A*, *arrows*) while, curiously, cell bodies retained much of their uniform dynamin distribution.

In view of its ability to cross-link and slide on microtubules, dynamin would seem to be an attractive candidate for an anaphase B mitotic motor protein. Since the epitopes recognized by our polyclonal antibodies are restricted to neuronal tissue (Fig. 3), we probed for dynamin in the mitotic spindles of PC12 cells in culture. After release from nocodazole block, PC12 cells in mitosis were fixed and stained with antidynamin (Fig. 10 *A*) and antitubulin (Fig. 10 *B*) antisera. While mitotic spindles are clearly visible with the antitubulin antibody, staining of spindles with antidynamin antiserum was not apparent. Instead, dynamin appears as a uniform punctate background characteristic of its appearance in the cell bodies of interphase cells. Although a dynamin homologue may function in these cells in the mitotic spindle, it is clear that the neuronal dynamin is not spindle associated.

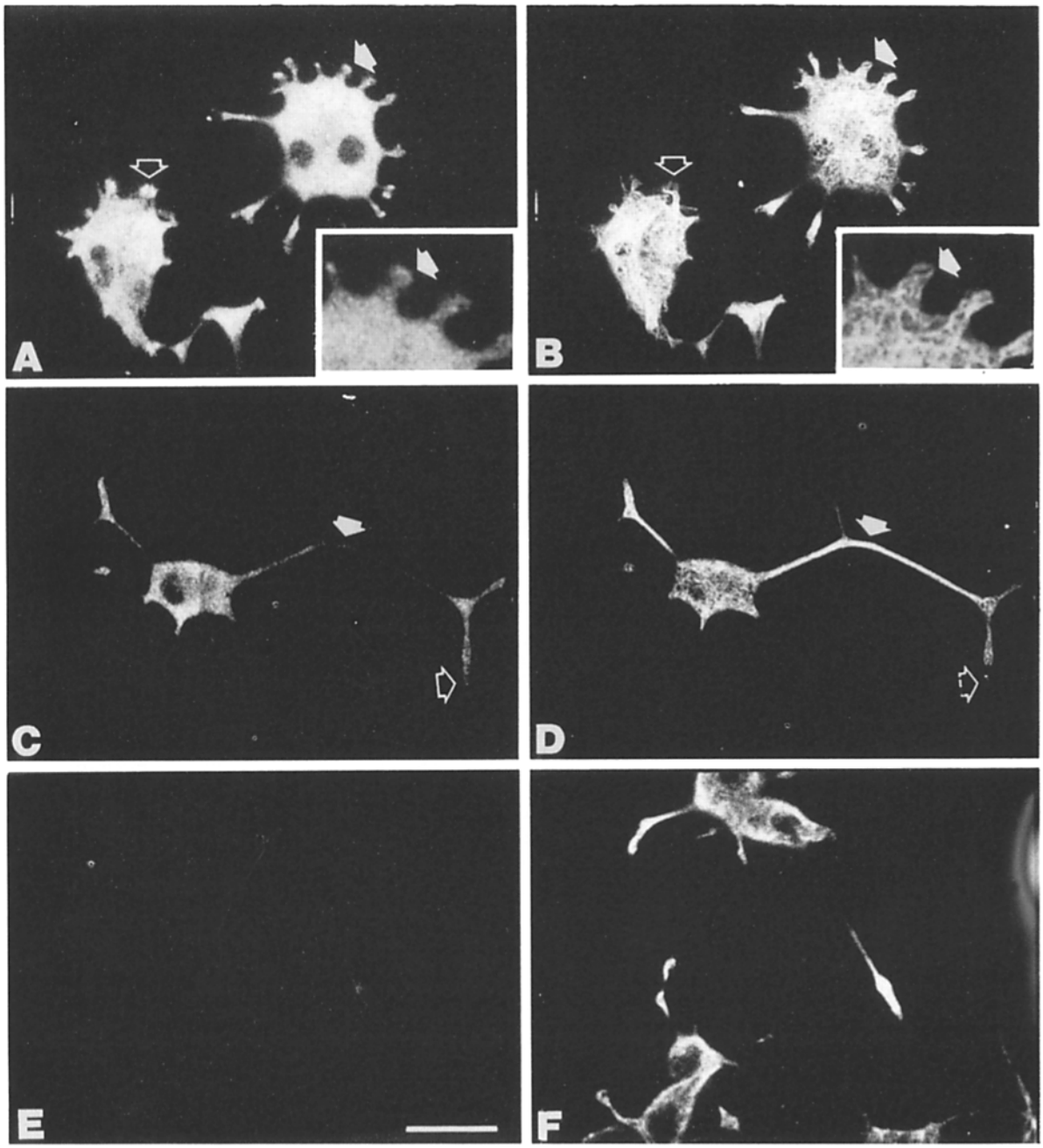

Figure 7. Immunofluorescence analysis of dynamin distribution in PC12 cells. Cells were cultured on coverslips with NGF for 4 d and processed for immunofluorescence microscopy as described in Materials and Methods. All coverslips were stained with anti-α and anti-β tubulin sera (*B*, *D*, and *F*). Staining with affinity-purified antiserum to dynamin is shown in *A* and *C*, while staining with negative control affinity purified antiserum (see Materials and Methods) is shown in *E*. In *A* and *B*, the arrows indicate growth cones of budding neurites. In *C* and *D*, the open arrow indicates a growth cone, the solid arrow indicates a neurite. Bar, 5 μm. The inserts in *A* and *B* represent higher magnification images of the portion of the cell labeled by the solid arrow.

Discussion

We have purified and characterized a rat brain 100-kD doublet protein that associates with microtubules in vitro and induces polymer bundling. This confirms the postulate in our previous work (Turner and Margolis, 1984) that microtubule bundling in vitro is associated with and probably a consequence of the specific presence of a 100-kD protein. We have determined that this protein is dynamin, a 100-kD MAP first isolated from beef brain, which has been reported to induce microtubule cross-linking in vitro (Shpetner and Vallee, 1989). The 100-kD protein described here is dynamin as determined by cross-identification on Western blots and microtubule binding properties. Dynamin is very abundant in neuronal tissue (as much as 1.5% of the total soluble protein content), but it is not apparent as an antigen in non-neuronal tissue. This suggests that dynamin plays a role in processes specific for neuronal cells.

We have developed a simple purification protocol to obtain dynamin from rat brain. As one step in the procedure,

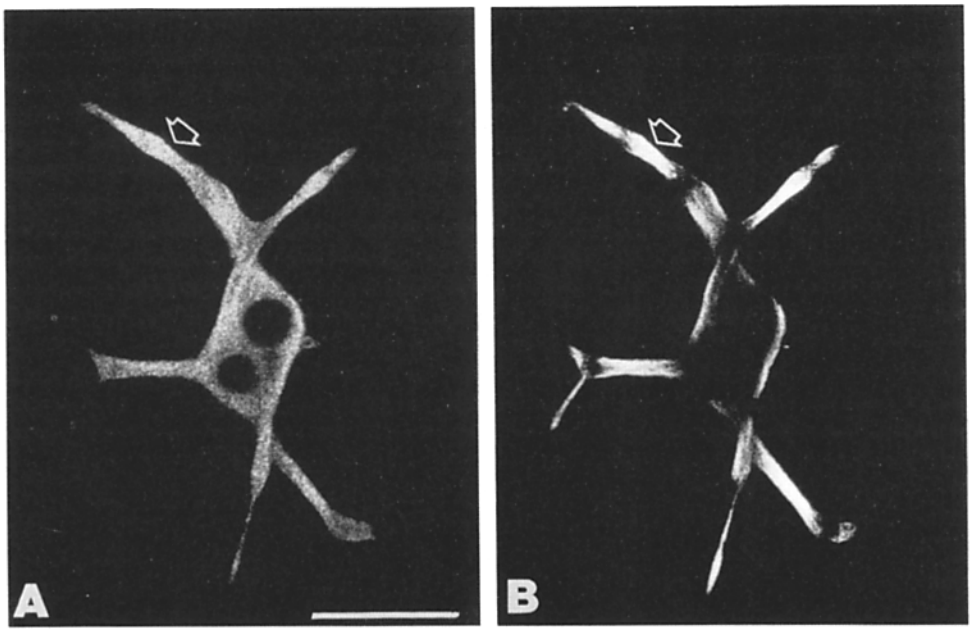

Figure 8. Immunofluorescence analysis of dynamin distribution in taxol-treated PC12 cells. Cells were cultured on coverslips with NGF for 4 d and processed for immunofluorescence microscopy following taxol treatment for 18 h, as described in Materials and Methods. The staining with affinity purified antiserum to dynamin is shown in *A*, while staining with anti-α and anti-β tubulin sera is shown in *B*. The arrow indicates an area of extensive microtubule bundling. Bar, 5 μm.

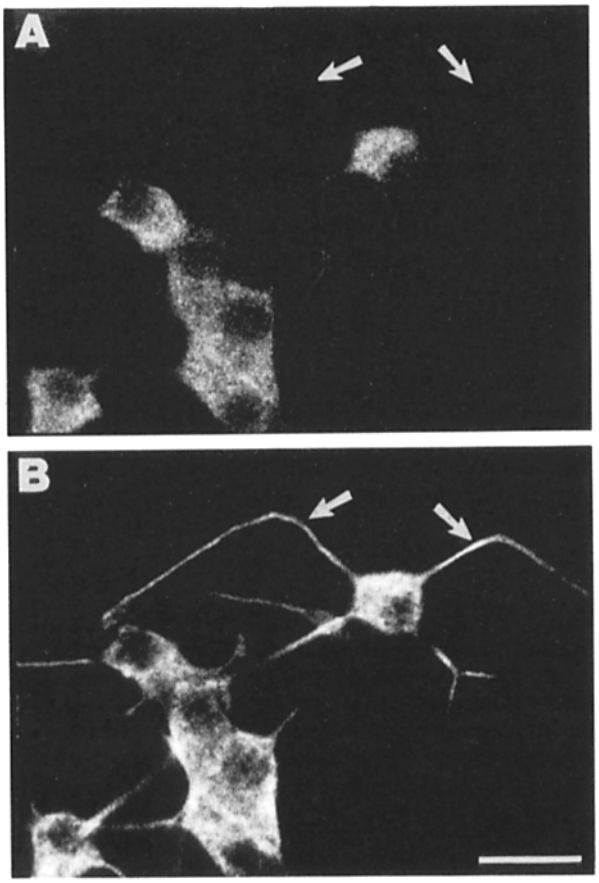

Figure 9. Loss of dynamin immunofluorescence staining by detergent extraction of PC12 cells. After culture in NGF for 4 d, cells were extracted with MME buffer containing 4% PEG and 0.2% Triton X-100 for 3 min at 37°C, and then processed for immunofluorescence microscopy as described in Materials and Methods. The staining with affinity-purified antiserum to dynamin is shown in *A*, while staining with anti-α and anti-β tubulin sera is shown in *B*. Arrows indicate neurites. Bar, 5 μm.

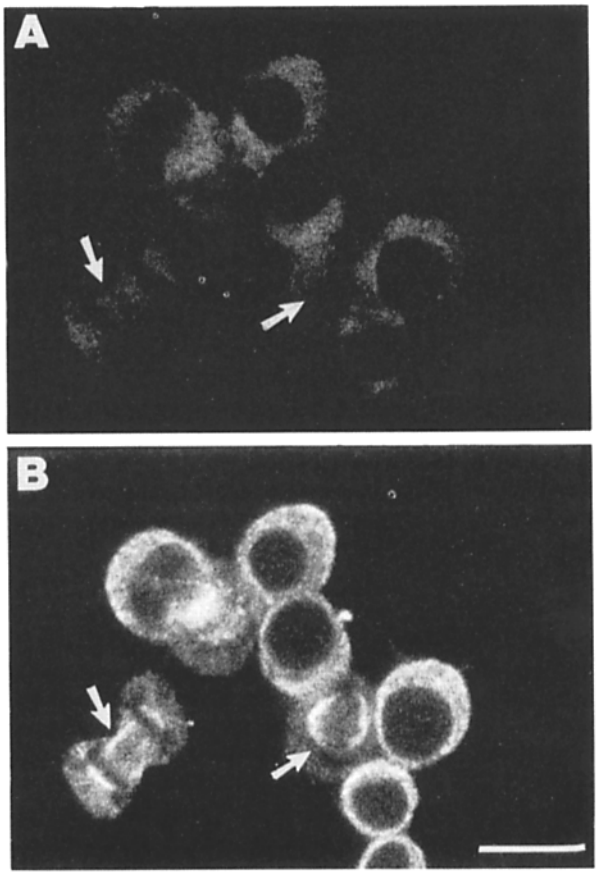

Figure 10. Dynamin and tubulin immunofluorescence staining of mitotic PC12 cells. Cells were grown in media without NGF and incubated overnight with 40 ng/ml nocodazole. Cells were fixed and processed for immunofluorescence microscopy, as described in Materials and Methods following mitotic recovery 45 min after removal of the nocodazole. Staining with the affinity-purified antiserum to dynamin is shown in *A*, while staining with anti-α and anti-β tubulin sera is shown in *B*. Arrows indicate mitotic spindles. Bar, 5 μm.

we use an N-6 linked ATP affinity column that is impressively discriminative for dynamin. On applying a brain crude extract to this column (not shown), we have found that dynamin is by far the most abundant protein eluting in the column specific fraction.

We have also developed a polyclonal antiserum against dynamin and have used it both to study the association of dynamin with subcellular fractions in vitro and to determine the distribution of dynamin in cultured cells by immunofluorescence analysis. Dynamin is abundant in the particulate fraction of neuronal tissue and is apparently associated with membranous material. Immunocytochemistry shows dynamin present in punctate arrays in cells, presumably associated with organelles. The possibility that dynamin may act as a motor protein in mitosis (Shpetner and Vallee, 1989) is not supported by immunofluorescence evidence, which shows that dynamin, like kinesin (Hollenbeck, 1989; Pfister et al., 1989) but unlike cytoplasmic dynein (Pfarr et al., 1990; Steuer et al., 1990) or STOP (Margolis et al., 1990), remains dispersed in the cytoplasm during mitosis. This observation does not of course preclude the possibility that a motor protein substantially homologous to dynamin might perform such a function, but, if so, it does not share epitopes with the neuronal dynamin that we have purified and used for polyclonal antibody production.

Dynamin levels increase approximately twofold after NGF treatment of PC12 cells, in contrast to MAPs such as MAP2 and tau, which increase ~10–12-fold upon NGF treatment (Drubin et al., 1985). These MAPs appear to be structural components of neuronal microtubules, since they are uniformly distributed on polymers (Bloom and Vallee, 1983). These MAPs appear capable of inducing microtubule bundles (Lewis et al., 1989; Kanai et al., 1989), apparently through dimerization (Lewis and Cowan, 1990). This capacity for bundling has been proposed to play a role in the observed bundling of microtubules in axons and dendrites (Lewis et al., 1989; Kanai et al., 1989). Purified dynamin also induces microtubule bundling in vitro, forming bundles that are ATP sensitive. However, it appears unlikely that dynamin is responsible for microtubule bundling in the axon, since it is sparsely represented among the bundled axonal microtubules.

Dynamin appears punctate and randomly dispersed in the cytoplasm, and displays no discernable interaction with the microtubule array of the cell body. Similarly, another presumptive microtubule motor protein, kinesin, appears to be distributed in a dispersed and punctate pattern in the cytoplasm, but kinesin also shows some apparent association along microtubules (Pfister et al., 1989; Hollenbeck, 1989). Similarly, in some instances (particularly in axonal branch points near growth cones), immunofluorescence suggests sporadic colocalization of dynamin with microtubules. Therefore, if dynamin can specifically associate with microtubules in the cell, it would appear to result from a specific activation during PC12 cell differentiation. Even in the most favorable cases, however, colocalization of dynamin with microtubules is very limited and most of the protein is dispersed in a punctate in distribution throughout the cell. Any association of dynamin with microtubules must be transitory, since, upon taxol-induced rearrangement and bundling of the microtubules, the dynamin remains uniformly dispersed and punctate. This contrasts with MAP1A, which remains superimposed with the microtubules upon taxol treatment of PC12 cells (Bloom et al., 1984). In view of the in vitro bundling of microtubules by dynamin, our finding that the neuritic microtubule bundles contain only very little dynamin, if any, is surprising. Of this dynamin only a small fraction can be meaningfully associated with microtubules, since the punctate dynamin evident on neurites is entirely detergent extractable whereas, under these conditions, microtubules and many of their associated proteins remain. Our finding that dynamin is present in rat brain particulate fractions is in agreement with the apparent preferential association of dynamin with membranes in axons.

In distal portions of neurites dynamin is relatively abundant, and it appears in growth cones beyond the region containing microtubules. The abundance of dynamin at these sites may be due to an accumulation of dynamin containing vesicles after their transport along the neurite microtubules. We have not yet determined if dynamin is directly involved in transport of these vesicles. As dynamin does not copurify with classical synaptic vesicles, the nature of the dynamin rich organelles visualized in the cell remains to be determined.

In summary, despite strong evidence for association of dynamin containing vesicles with microtubules in axons, the quantitative association of soluble dynamin with microtubules in vitro and the reported in vitro capacity of dynamin to act as a motor protein on microtubules in the presence of other soluble factors (Shpetner and Vallee, 1989) would argue that it could operate as a motor protein for the transport of specific as yet unidentified vesicles on microtubules toward the axonal synapse where these dynamin containing vesicles seem to accumulate. Any apparent motor function would appear to be specific to neuronal cells and to overlap with kinesin function (Vale et al., 1985; Schroer et al., 1988). It is possible that dynamin and kinesin move different sets of vesicles toward synapses, or that they are distinct for reasons of physiological regulation of vesicle traffic. We would caution against premature judgement, however, with respect to dynamin motor function on microtubules. Unlike dynein or kinesin, dynamin does not move microtubules on glass substrates (Shpetner and Vallee, 1989), nor does it exhibit ATPase activity in the absence of crude factors (Shpetner and Vallee, 1989). Our failure to find it convincingly associated with microtubules in vivo leaves us that much more skeptical as to its potential role in microtubule-based motility, or in microtubule bundling in vivo.

We wish to thank Dr. Richard Vallee and Dr. Robert Obar (Worcester Foundation, MA) for probing our protein preparations with antidynamin serum, Paul Goodwin and Stratton Spyropoulos (Image Analysis Laboratory, Fred Hutchinson Cancer Research Center) for assistance with confocal microscopy, Liz Caldwell (Electron Microscopy Laboratory, Fred Hutchinson Cancer Research Center) for assistance with sample preparation for electron microscopy, Dr. Douglas Palmer (Fred Hutchinson Cancer Research Center) for critical reading of this manuscript, and Pam Noble for secretarial assistance.

Supported by a grant from the Muscular Dystrophy Association to Robert L. Margolis and by fellowship support from the National Institutes of Health to Robin M. Scaife (ESO 7032 National Institute of Environmental Health Sciences; N. K. Mottet, Principal Investigator).

Received for publication 2 February 1990 and in revised form 9 September 1990.

References

Aamodt, E., R. Holmgren, and J. Culotti. 1989. The isolation and in situ location of adligin: the microtubule cross-linking protein from *Caenorhabditis elegans*. *J. Cell Biol.* 108:955–963.

Amos, L. A. 1989. Brain dynein crossbridges microtubules into bundles. *J. Cell Sci.* 93:19–28.

Bloom, G. S., and R. B. Vallee. 1983. Association of microtubule-associated protein 2 (MAP2) with microtubules and intermediate filaments in cultured brain cells. *J. Cell Biol.* 96:1523–1531.

Bloom, G. S., F. C. Luca, and R. B. Vallee. 1984. Widespread cellular distribution of MAP-1A (Microtubule-associated Protein 1A) in the mitotic spindle and on interphase microtubules. *J. Cell Biol.* 98:331–340.

Brown, S., W. Levinson, and J. A. Spudich. 1976. Cytoskeletal elements of chick embryo fibroblasts revealed by detergent extraction. *J. Supramol. Struct.* 5:119–130.

Campbell, E. J., S. A. MacKinlay, and T. H. MacRae. 1989. Cross-linking of microtubules by microtubule-associated proteins (MAPs) from the brine shrimp, Artemia. *J. Cell. Sci.* 93:29–39.

De Brabander, M., G. Geuens, R. Nuydens, R. Willebrords, and J. De May. 1981. Taxol induces the assembly of free microtubules in living cells and blocks the organizing capacity of the centrosomes and kinetochores. *Proc. Natl. Acad. Sci. USA.* 78:5608–5612.

Drubin, D. G., S. C. Feinstein, E. M. Shooter, and M. W. Kirschner. 1985. Nerve growth factor-induced neurite outgrowth in PC12 cells involves the coordinate induction of microtubule assembly and assembly-promoting factors. *J. Cell Biol.* 101:1799–1807.

Endow, S. A., S. Henikoff, and L. Soler-Niedziela. 1990. Mediation of meiotic and early mitotic chromosome segregation in *Drosophila* by a protein related to kinesin. *Nature (Lond.).* 345:81–83.

Enos, A. P., and N. R. Morris. 1990. Mutation of a gene that encodes a kinesin-like protein blocks nuclear division in A. nidulans. *Cell.* 60:1019–1027.

Greene, L. A., and A. S. Tischler. 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA.* 73:2424–2428.

Goodenough, U., and J. Heuser. 1984. Structural comparison of purified dynein proteins with *In Situ* dynein arms. *J. Mol. Biol.* 180:1083–1118.

Griffith, L. M., and T. D. Pollard. 1978. Evidence for actin filament microtubule interaction mediated by microtubule associated proteins. *J. Cell Biol.* 78:958–965.

Hollenbeck, P. J. 1989. Distribution, abundance, and subcellular localization of kinesin. *J. Cell Biol.* 108:2335–2342.

Hollenbeck, P. J., and K. Chapman. 1986. A novel microtubule-associated protein from mammalian nerve shows ATP-sensitive binding to microtubules. *J. Cell Biol.* 103:1539–1545.

Huttner, W. B., W. Schiebler, P. Greengard, and P. De Camilli. 1983. Synapsin I (Protein I), a nerve terminal-specific phosphoprotein. III. Its association with synaptic vesicles studied in a highly purified synaptic vesicle preparation. *J. Cell Biol.* 96:1374–1388.

Kanai, Y., R. Takemura, T. Oshima, H. Mori, Y. Ihara, M. Yanagisawa, T. Masaki, and N. Hirokawa. 1989. Expression of multiple tau isoforms and microtubule bundle formation in fibroblasts transfected with a single tau cDNA. *J. Cell Biol.* 109:1173–1184.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.).* 227:680–685.

Lewis, S. A., and N. Cowan. 1990. Microtubule bundling. *Nature (Lond.).* 345:674.

Lewis, S. A., I. E. Ivanov, G. Lee, and N. J. Cowan. 1989. Organization of microtubules in dendrites and axons is determined by a short hydrophobic zipper in microtubule-associated proteins MAP2 and tau. *Nature (Lond.).* 342:498–505.

Margolis, R. L., C. T. Rauch, and D. Job. 1986. Purification and assay of a 145-kDa protein ($STOP_{145}$) with microtubule-stabilizing and motility behavior. *Proc. Natl. Acad. Sci. USA.* 83:639–643.

Margolis, R. L., C. T. Rauch, F. Pirollet, and D. Job. 1990. Specific association of STOP protein with microtubules in vitro and with stable microtubules in mitotic spindles of cultured cells. *EMBO (Eur. Mol. Biol. Organ.) J.* In press.

McDonald, H. B., and L. S. B. Goldstein. 1990. Identification and characterization of a gene encoding a kinesin-like protein in *Drosophila*. *Cell.* 61:991–1000.

Meluh, P. B., and M. D. Rose. 1990. KAR3, a kinesin-related gene required for yeast nuclear fusion. *Cell.* 60:1029–1041.

Osborn, M., and K. Weber. 1982. Immunofluorescence and immunocytochemical procedures with affinity purified antibodies: tubulin containing structures. *Methods Cell Biol.* 24:98–132.

Paschal, B. M., and R. B. Vallee. 1987. Retrograde transport by the microtubule-associated protein MAP 1C. *Nature (Lond.).* 330:181–183.

Pfarr, C. M., M. Coue, P. M. Grissom, T. S. Hays, M. E. Porter, and J. R. McIntosh. 1990. Cytoplasmic dynein is localized to kinetochores during mitosis. *Nature (Lond.).* 345:263–265.

Pfister, K., M. C. Wagner, D. L. Stenoien, S. T. Brady, and G. S. Bloom. 1989. Monoclonal antibodies to kinesin heavy and light chains stain vesicle-like structures, but not microtubules, in cultured cells. *J. Cell Biol.* 108:1453–1463.

Sale, W. S., and P. Satir. 1977. Direction of active sliding of microtubules in *Tetrahymena* cilia. *Proc. Natl. Acad. Sci. USA.* 74:2045–2049.

Schroer, T. A., B. J. Schnapp, R. S. Reese, and M. P. Sheetz. 1988. The role of kinesin and other soluble factors in organelle movement along microtubules. *J. Cell Biol.* 107:1785–1792.

Shapiro, S. Z. 1987. Elimination of the detection of an artefactual 65 kDa keratin band from immunoblots. *J. Immunol. Methods.* 102:143–146.

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

Steuer, E. R., L. Wordeman, T. A. Schroer, and M. P. Sheetz. 1990. Localization of cytoplasmic dynein to mitotic spindles and kinetochores. *Nature (Lond.).* 345:266–268.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

Turner, P. F., and R. L. Margolis. 1984. Taxol-induced bundling of brain-derived microtubules. *J. Cell Biol.* 99:940–946.

Vale, R. D., T. S. Reese, and M. P. Sheetz. 1985. Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. *Cell.* 42:39–50.

Vallee, R. B. 1986. Purification of brain microtubules and microtubule-associated protein 1 using taxol. *Methods Enzymol.* 134:104–115.

Williams, R. C., Jr., and H. W. Detrich, III. 1979. Separation of tubulin from microtubule-associated proteins on phosphocellulose. Accompanying alterations in concentrations of buffer components. *Biochemistry.* 18:2499–2503.