Identification of a MAP 2-like ATP-binding Protein Associated with Axoplasmic Vesicles That Translocate on Isolated Microtubules

Susan P. Gilbert and Roger D. Sloboda

Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire 03755, and The Marine Biological Laboratory, Woods Hole, Massachusetts 02543. S. P. Gilbert's current address is Department of Molecular and Cell Biology, Pennsylvania State University, University Park, Pennsylvania 16802.

Abstract. Axoplasmic vesicles were purified and observed to translocate on isolated microtubules in an ATP-dependent, trypsin-sensitive manner, implying that ATP-binding polypeptides essential for force generation were present on the vesicle surface. To identify these proteins [α^{32}P]8-azidoadenosine 5'-triphosphate ([α^{32}P]8-N_3ATP), a photoaffinity analogue of ATP, was used. The results presented here identify and characterize a vesicle-associated polypeptide having a relative molecular mass of 292 kD that bound [α^{32}P]8-N_3ATP. The incorporation of label is ultraviolet light-dependent and ATP-sensitive. Moreover, the 292-kD polypeptide could be isolated in association with vesicles or microtubules, depending on the conditions used, and the data indicate that the 292-kD polypeptide is similar to mammalian brain microtubule-associated protein 2 (MAP 2) for the following reasons: The 292-kD polypeptide isolated from either squid axoplasm or optic lobe cross-reacts with antiserum to porcine brain MAP 2. Furthermore, it purifies with taxol-stabilized microtubules and is released with salt. Based on these characteristics, the 292-kD polypeptide is distinct from the known force-generating molecules myosin and flagellar dynein, as well as the 110–130-kD kinesin-like polypeptides that have recently been described (Brady, S. T., 1985, *Nature (Lond.)*, 317:73–75; Vale, R. D., T. S. Reese, and M. P. Sheetz, 1985*b*, *Cell*, 42:39–50; Scholey, J. M., M. E. Porter, P. M. Grissom, and J. R. McIntosh, 1985, *Nature (Lond.)*, 318:483–486). Because the 292-kD polypeptide binds ATP and is associated with vesicles that translocate on purified MAP-free microtubules in an ATP-dependent fashion, it is therefore believed to be involved in vesicle–microtubule interactions that promote organelle motility.

DIRECTED intracellular organelle movement is a fundamental process characteristic of all cells. This process has been well studied in the neuron because membranous organelles must travel very long distances, up to a meter or more in length, between the cell body and the synaptic terminal. This process, called fast axonal transport, is ATP-dependent, rapid (1–5 μm/s), and selective. Newly synthesized membranous organelles produced in the cell body are transported in the orthograde direction toward the nerve terminal. The organelles that are moving in the retrograde direction, however, are different in nature and are being transported from the synaptic terminal region back to the cell body for degradation (reviewed in Grafstein and Forman, 1980; Brady and Lasek, 1982; Weiss, 1982). The giant axon of the squid *Loligo pealei* has been an important preparation for the study of the molecular basis of such organelle transport. The axon is unmyelinated, up to 1 mm in diameter, and 4–8 μl of axoplasm can be mechanically extruded away from the surrounding plasma membrane and glial sheath (Bear et al., 1937; Lasek, 1974). Moreover, the preparation remains metabolically active for hours, accessible to surrounding solutions for experimentation, and the organelle motility can be observed directly using video-enhanced differential interference contrast microscopy (Brady et al., 1982). These characteristics of the axoplasm from *Loligo pealei* have enhanced the analysis of the relationship between moving organelles and cytoskeletal transport filaments.

Recently, several reconstituted models have been developed to define and characterize the organelle-cytoskeletal interactions that occur during fast axonal transport. Vesicles isolated from the squid giant axon retained their ability to translocate—in an ATP-dependent, trypsin-sensitive fashion—when added back to extruded axoplasm (Gilbert and Sloboda, 1984) or to microtubule-associated protein-free microtubules (Gilbert et al., 1985). The microtubule substrate supported bidirectional movement, and all the organelles moved at the same rate, ~2 μm/s. However, in a related approach, vesicle motility reported by Vale et al. (1985*a*) required the addition of soluble factors from squid axoplasm. In contrast, the motility reported by Gilbert et al. (1985) occurred in the absence of added proteins. These results implied that the motility-promoting molecules could be isolated in association with the vesicles or free in solution, depending upon the buffer conditions employed. In another series of experiments it was noted that single transport filaments and associated organelles were separated from the

bulk of the axoplasm when the ionic conditions and osmolality of the axoplasm were decreased (Allen et al., 1985; Schnapp et al., 1985). These transport filaments were shown to be single microtubules, and these single microtubules were motile (Allen et al., 1985; Vale et al., 1985*a*), that is, they could translocate over a glass surface. Furthermore, this translocation was ATP-dependent and sensitive to heat and trypsin.

Lasek and Brady (1985) demonstrated that the nonhydrolyzable analogue of ATP, adenylyl imidodiphosphate (AMP-PNP), when added to squid axoplasm promoted the attachment of vesicles in solution to microtubules to form a stable complex. This observation allowed Brady (1985) and Vale et al. (1985*b*) to identify proteins from neuronal tissue that bound microtubules with high affinity in the presence of AMP-PNP. The protein purified from squid optic lobe and axoplasm by Vale et al., called kinesin, had an apparent molecular mass of ~600 kD and contained polypeptides of 110–120 and 60–70 kD. Although kinesin promoted the movement of microtubules on glass and the movement of beads along microtubules, an ATPase activity associated with the protein was not demonstrated. In contrast, Brady (1985) correlated an ATPase activity with the presence of a polypeptide from chick brain that bound to microtubules in the presence of AMP-PNP; this molecule may be the vertebrate counterpart to invertebrate kinesin. However, neither line of investigation has demonstrated a direct interaction of kinesin-like molecules with organelles that translocate on microtubules, although the evidence suggests that this protein may be involved. Furthermore, although movement in the axon is bidirectional, motility due to the presence of kinesin is unidirectional in vitro (from the minus to the plus end of microtubules, i.e., in the orthograde direction with respect to the axon). Indeed, kinesin can be immunoprecipitated from an axoplasmic homogenate, and a factor remains soluble that can promote bead movement in the opposite direction (from the plus end to the minus end, i.e., retrograde) (Vale et al., 1985*c*). Thus, more than one molecule may be responsible for the bidirectional transport observed in axoplasm.

Because isolated axoplasmic vesicles were observed to translocate on axonemal microtubules whose dynein arms had been removed (Gilbert et al., 1985), it was assumed that the ATP-binding proteins essential for vesicle motility were present on the vesicle surface. In this paper, we identify the ATP-binding polypeptides associated with purified axoplasmic vesicles using the photoaffinity analogue of ATP, [α^{32}P]8-azidoadenosine 5′-triphosphate ([α^{32}P]8-N_3ATP). The photoreactive azido analogues of nucleotides have been used to identify the active site of ATPases such as the Na^+K^+-ATPase of the erythrocyte membrane (Haley and Hoffman, 1974), the F1-ATPase of mitochondria (Wagenvoord et al., 1977; Scheurich et al., 1978), the Ca^{++}-ATPase of the sarcoplasmic reticulum (Briggs et al., 1980), and the 12 S and 18 S dyneins of *Chlamydomonas* flagella (Pfister et al., 1984; 1985). Furthermore, 8-N_3ATP has been used to identify the ATP-binding polypeptides of type I protein kinase (Hoppe and Freist, 1979) and the polypeptide associated with the ATPase activity in bovine brain microtubule preparations (Murphy et al., 1983). The preceding experiments have all used purified or partially purified enzymes as substrates in a photoaffinity labeling assay. Here, we take the unique approach of using the analogue as a diagnostic tool for the identification of ATP-binding molecules in a complex mixture of proteins; those polypeptides that bind ATP may be potentially important in the process of fast axoplasmic transport.

In the experiments described here, a preparation of axoplasmic vesicles was isolated free of soluble proteins and observed to translocate on isolated axonemal microtubules and squid optic lobe microtubules. By using photoaffinity radioactive labeling techniques, the ATP-binding proteins were identified. The results presented indicate that a high molecular weight polypeptide is present in the vesicle preparation that binds ATP and is recognized by antibodies directed against porcine brain microtubule-associated protein 2 (MAP 2).[1] Furthermore, this polypeptide is distinct from those that comprise the kinesin-like polypeptides isolated from squid optic lobe and axoplasm (Vale et al., 1985*b*), bovine and chick brain (Vale et al., 1985*b*; Brady, 1985), and sea urchin eggs (Scholey et al., 1985). Because the MAP 2-like ATP-binding protein can be isolated in association with vesicles that translocate on isolated microtubules or with in vitro assembled taxol-stabilized microtubules, it is a possible candidate as a mediator of the vesicle–microtubule interactions that occur during vesicle motility.

Materials and Methods

Squid (*Loligo pealei*) and sea urchins (*Arbacia punctulata*) were obtained from the Department of Marine Resources, Marine Biological Laboratory, Woods Hole, MA. Antiserum to squid optic lobe myosin and preimmune serum were the generous gift of Drs. Karl R. Fath and Raymond J. Lasek, Case Western Reserve University, Cleveland, OH. Rabbit antiserum to porcine brain MAP 2 was the generous gift of Dr. Harold Gainer, Laboratory of Neurochemistry and Neuroimmunology, National Institute of Child Health and Human Development, Bethesda, MD. Taxol was provided by Dr. Matthew Suffness, Division of Cancer Treatment, National Institutes of Health, Bethesda, MD. [α^{32}P]8-N_3ATP was purchased at a specific activity of 11–13 Ci/mmol from ICN Biomedicals, Inc., Irvine, CA. Unlabeled 8-N_3ATP was obtained from Sigma Chemical Co., St. Louis, MO. [α-^{32}P] ATP was obtained at a specific activity of 3,000 Ci/mmol from New England Nuclear, Boston, MA or ICN Biomedicals, Inc.

Vesicle and Protein Purification

Vesicles were isolated from extruded axoplasm (5–8 μl per axon) obtained from 12–14 giant axons of the squid *Loligo pealei* using discontinuous sucrose gradient centrifugation as described previously (Gilbert and Sloboda, 1984). The resulting 50 mM, 250 mM, and 300 mM sucrose fractions were pooled and concentrated to 200 μl using a Centricon-10 microconcentrator (Amicon Corp., Danvers, MA). Although the 600 mM fraction contained vesicles, this fraction was enriched with mitochondria (Gilbert and Sloboda, 1984); therefore it was not included in the experiments reported here. The pooled and concentrated vesicle fraction was next loaded onto a Sephacryl S-400 (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) column in a plastic disposable 5-ml pipette containing 4 ml of resin equilibrated at 4°C in vesicle buffer (250 mM sucrose, 100 mM KCl, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 0.1 mg/ml soybean trypsin inhibitor, 20 mM imidazole, pH 7.4). Fractions (200 μl) were collected and protein was determined using a spot assay (Bell et al., 1982) and SDS PAGE. Fractions containing either vesicles or soluble protein were pooled and concentrated using Centricon-10 microconcentrators.

Microtubules were prepared from freshly dissected squid optic lobes using a modification of the taxol procedure of Vallee (1982). Optic lobes were weighed and homogenized in a volume of PM buffer (100 mM Pipes, pH 6.9, 4 mM EGTA, 5 mM $MgSO_4$, 1 mM GTP, 1 mM phenylmethylsulfonyl fluoride) two times the wet weight of the optic lobes. The homogenate

1. *Abbreviations used in this paper*: [α^{32}P]8-N_3ATP, [α^{32}P]8-azidoadenosine 5′-triphosphate; MAP 2, microtubule-associated protein 2; NF, neurofilament.

was spun at 13,000 rpm for 30 min at 4°C in an SS34 rotor (Sorvall Instruments Div., DuPont Co., Newton, CT). The supernatant was removed and adjusted to 20 μM taxol. The preparation was incubated at 19°C for 30 min, followed by centrifugation over a 20% sucrose cushion in PM buffer containing 20 μM taxol (PM-taxol). The pellet was resuspended in PM-taxol, and the microtubules were again centrifuged through a 20% sucrose cushion. The pellet was resuspended in PM-taxol and adjusted to 1 M KCl by adding one-half volume of 3 M KCl (in PM-taxol). The salted microtubule preparation was spun over a sucrose cushion as described above. The pellet was resuspended in PM-taxol, and the centrifugation through sucrose was repeated. The pellet containing MAP-free microtubules was resuspended in PM-taxol to a concentration of ~2 mg/ml. Aliquots of 200 μl were frozen in liquid N_2 and stored at −70°C until use.

Axonemal microtubules were prepared from *Arbacia* spermatozoa and dialyzed against low ionic strength buffer (1 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 0.1% β-mercaptoethanol) for 24–48 h at 4°C to remove the dynein arms (Gibbons and Rowe, 1965). The dialyzed axonemes were adjusted to 50 mM Tris-HCl, pH 8.0 and centrifuged at 17,000 rpm for 15 min at 4°C in a Sorvall SS34 rotor. The axonemes were washed one time with 30 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 5 mM $MgSO_4$. The supernatant was discarded and the pellet was resuspended in 10 mM Hepes, pH 7.4, 100 mM KCl, 5 mM $MgSO_4$, 0.5 mM EDTA, 1 mM dithiothreitol for motility experiments; alternatively the pellet was overlaid with the Hepes buffer and frozen in liquid N_2 to be stored at −70°C until use.

Porcine brain microtubules were prepared according to the procedure of Sloboda and Rosenbaum (1979). Actin was prepared from an acetone powder of rabbit muscle using the technique of Spudich and Watt (1971). Rabbit myosin was prepared from the back and hindleg muscle according to the procedure of Kendrick-Jones et al. (1970) and was the generous gift of Dr. Margaret A. Titus, Brandeis University, Waltham, MA. Protein concentration was determined by the Schacterle and Pollack (1973) modification of the procedure of Lowry et al. (1951).

Photoaffinity Labeling

[α-^{32}P]8-N_3ATP. Axoplasmic vesicles or isolated proteins were labeled radioactively, using a modification of the procedure of Pfister et al. (1984). Briefly, 2–5-μl aliquots of [α-^{32}P]8-N_3ATP in methanol were added to 0.5-ml microfuge tubes (Sarstedt, Inc., Princeton, NJ) that were placed on ice and protected from room light. The methanol was allowed to evaporate by room air or by a stream of N_2 after which the protein or vesicle samples were added as 25–50-μl aliquots containing 0.5–0.9 mg/ml protein. The final concentration of [α-^{32}P]8-N_3ATP was 4–8 μM. The contents of the tube were mixed and either irradiated with 254-nm light (UVG-11 Mineralight lamp, 4 W, UltraViolet Products, San Gabriel, CA) at 4–5 cm from the top of the tube for 90 s or allowed to incubate without ultraviolet (UV) exposure for an equal amount of time. Samples were then reduced for electrophoresis according to Laemmli (1970). In competition experiments, unlabeled ATP (0.01–2 mM) was added to the microfuge tubes at the same time as [α-^{32}P]8-N_3ATP and before the addition of protein.

[α-^{32}P]ATP. For direct photochemical cross-linking experiments (Yue and Schimmel, 1977; Maruta and Korn, 1981), 50-μl reaction mixtures in 0.5-ml microfuge tubes on ice contained protein at 0.5–0.9 mg/ml and ~20 μCi of [α-^{32}P]ATP whose final concentration was 0.2–0.6 μM. The samples were either UV irradiated for 1 h at a distance of 4–6 cm with an R-52G Mineralight Lamp (254 nm, 100 W, UltraViolet Products, Inc.) or incubated on ice with no UV exposure. The contents of the tubes were mixed every 15 min during the irradiation/incubation period, and at the end of the hour, each sample was mixed with the appropriate volume of 5× concentrated electrophoresis sample buffer (Laemmli, 1970).

Video-enhanced Microscopy

Video-enhanced contrast differential interference contrast microscopy (Allen and Allen, 1983) was used to monitor the vesicle isolation procedure and for motility experiments. The optical system included a Nikon Optiphot microscope (Nikon, Inc., Garden City, NY) that was equipped with a 100×/1.35 numerical aperture (NA) planapochromatic objective, an achromatic-aplanatic condenser (1.35 NA), and a 100-W mercury arc for illumination. The image was projected from the microscope by a 10–20× zoom eyepiece (Swift Instruments Inc., San Jose, CA) and focused onto a Hamamatsu C-1000 camera having a Chalnicon video tube (Hamamatsu Corp., Middlesex, NJ) using a Zeiss adaptor. The adaptor contained a projection lens having a focal length of 63 mm with a T2 thread plus a T2 to C-mount adaptor (Carl Zeiss, Inc., Thornwood, NY). The camera was connected to a C1966 image processor (Photonic Microscopy, Inc., Oak Brook, IL), and the video-enhanced images were recorded in real time using a SONY VO-2610 ¾-in. videocassette recorder.

Motility Experiments

The isolated axoplasmic vesicles were tested for their ability to undergo motility before the photoaffinity labeling experiments using the in vitro motility assay previously described (Gilbert et al., 1985). Briefly, axonemal microtubules or squid optic lobe microtubules were allowed to settle onto a No. 0 coverslip (24 × 50 mm, ESCO, Erie Scientific, Portsmouth, NH) and any unattached axonemes or microtubules were removed by washing with vesicle buffer. The preparation was covered with a second coverslip (No. 0, 18 × 18 mm), sealed with valap (vaseline/lanolin/paraffin, 1:1:1) on two opposite sides, and transferred to the microscope for observation. Axoplasmic vesicles were added at the edge of the coverslip "sandwich" and washed across the preparation by absorbing buffer with filter paper at the opposite side. The vesicle addition and the vesicle movement were observed using the microscopy technique described above.

The effect of 8-N_3ATP on endogenous axoplasmic transport was determined. Axoplasm from the squid giant axon was extruded mechanically onto a coverslip (Brady et al., 1982), and 10 μl of 5 mM 8-N_3ATP in buffer X (Brady et al., 1985) was added. The preparation at 16–18°C was irradiated with 254-nm light (UVG-11 Mineralight lamp) from a distance of 8–10 cm for 30 s to 2 min. The axoplasmic preparations were then transferred to the microscope for observation. Control preparations included the following: addition of 8-N_3ATP with no irradiation, addition of buffer instead of 8-N_3ATP with irradiation, and addition of 8-N_3ATP with irradiation, followed by the subsequent addition of 5–10 mM ATP.

PAGE, Immunoblotting, and Autoradiography

Protein samples were analyzed using 5–15% acrylamide/12–48% urea linear gradient slab gels (0.75 mm) with 3% stacking gels according to the buffer formulation of Laemmli (1970) and stained for protein with Coomassie Brilliant Blue R 250 (Fairbanks et al., 1971) or silver nitrate (Morrissey, 1981; Pratt et al., 1984). All gels contained the following molecular mass markers (Sigma Chemical Co.): rabbit muscle myosin (200 kD), β-galactosidase (116 kD), phosphorylase B(97.4 kD), bovine serum albumin (66 kD), ovalbumin (45 kD), and carbonic anhydrase (29 kD).

Electrophoretic transfer of proteins from these gels to nitrocellulose paper (0.45 μm, Schleicher & Schuell, Inc., Keene, NH) was performed in a Bio-Rad Trans Blot Cell (Bio-Rad Laboratories, Richmond, CA) according to the method of Towbin et al. (1979). The transfer was performed at constant current (400 mA) for 90 min in a tank buffer of 25 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.05% SDS. After completion of the transfer, the nitrocellulose sheets were processed for detection of rabbit IgG-antigen immune complexes as described in the procedure provided with the Bio-Rad Kit which uses horseradish peroxidase–labeled secondary antibodies. For blots to the porcine MAP 2 antiserum, the specificity of the serum was tested by immunoprecipitating the MAP 2 antibodies with porcine brain MAP 2 antigen. After centrifugation to remove the precipitated immune complexes, the resulting serum was used for probing the blots.

For autoradiography of the ^{32}P-labeled polypeptides, the silver-stained gels were dried and exposed to Kodak X-Omat AR Film using a Dupont Cronex Lightning Plus intensifying screen. After the appropriate exposure time (3–21 d) at −70°C, the films were developed in Kodak GBX developer.

Results

Vesicle Purification

To determine if key vesicle surface proteins interact with microtubules to effect vesicle movement, it was first necessary to separate isolated vesicles from soluble proteins. To do this, axoplasmic vesicles were first isolated using discontinuous sucrose gradient centrifugation as previously described (Gilbert and Sloboda, 1984; Gilbert et al., 1985). Subsequently, the vesicle-containing fractions from the sucrose gradient were pooled, concentrated, and further fractionated using a Sephacryl S-400 column. Fig. 1 shows the polypeptide composition of the column fractions. Those fractions shown in lanes *d–i* contained vesicles as deter-

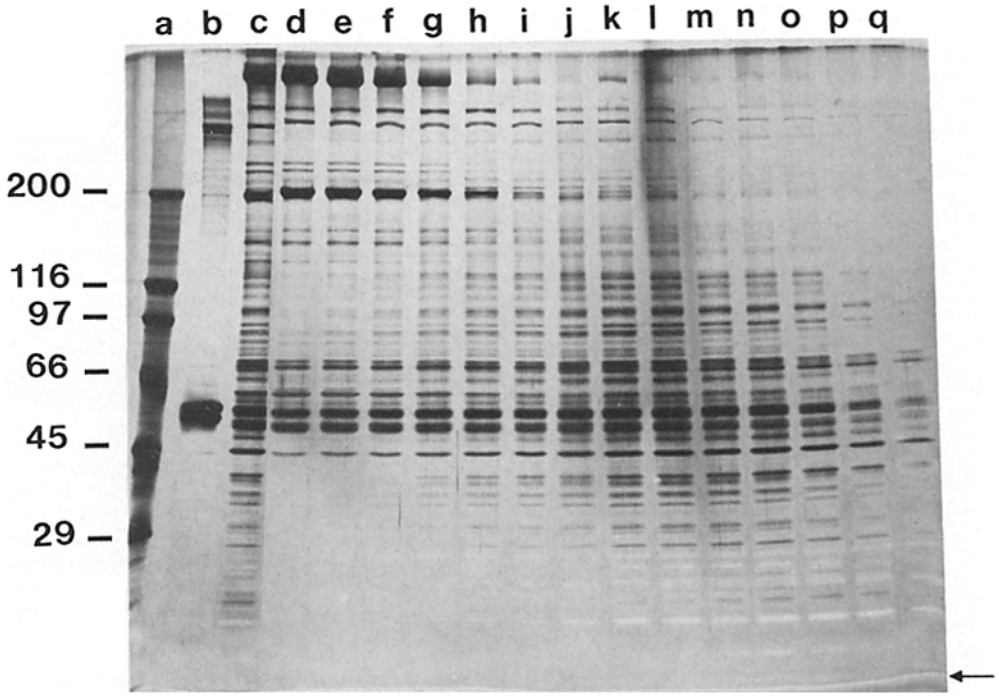

Figure 1. SDS PAGE showing the protein composition of the Sephacryl S-400 column fractions. All lanes are from the same slab gel and are stained with silver nitrate. Lane *a*, relative molecular mass markers ($\times 10^{-3}$), lane *b*, purified porcine brain microtubules. Lane *c*, polypeptide composition of the vesicles before fractionation by the column; lanes *d–q*, column fractions containing protein. Lanes *d–g*, fractions containing vesicles. Lanes *h* and *i*, fractions containing both vesicles and soluble protein; lanes *j–q*, fractions devoid of vesicles, but containing soluble protein. Arrow marks the ion front.

mined by video-enhanced microscopy (data not shown), while those represented by lanes *j–q* were devoid of vesicles. The fractions enriched in vesicles (lanes *d–g*) were pooled, concentrated, and routinely tested to ensure their ability to translocate on isolated microtubules before use in the biochemical experiments described below. The column fractions represented by lanes *j–q*, devoid of vesicles but containing soluble proteins, were also pooled and concentrated for use in later experiments. Because the fractions corresponding to lanes *h* and *i* contained some vesicles as well as soluble proteins, these fractions were not used in the experiments described here.

Inhibition of Endogenous Transport by Unlabeled 8-N_3ATP

To identify ATP-binding proteins of the vesicle preparation, the radioactive photoaffinity analogue of ATP, [α-^{32}P]8-N_3ATP, was used. Before these radioactive experiments, however, the effect on motility of the nonradioactive form of the analogue was determined. Extruded axoplasm was incubated with unlabeled 8-N_3ATP (1–5 mM), and the effect of the analogue on fast axonal transport was assessed using video-enhanced light microscopy. Organelles continued to move at very rapid rates in either the orthograde or the retrograde direction, and no discernable effect on organelle motility was observed for up to 60 min. The length of time for UV irradiation of the photoaffinity analogue was determined by first assessing the effect of irradiation on axoplasmic transport in the absence of 8-N_3ATP. At irradiation times greater than 90 s (i.e., 105 and 120 s), the appearance of the axoplasm changed. Large numbers of organelles were observed in Brownian motion, and those not in Brownian motion demonstrated motility that was spasmodic and intermittent. Therefore, 90 s was the maximum irradiation exposure used to activate the photoaffinity analogue.

When extruded axoplasm was incubated with 5 mM 8-N_3ATP followed by irradiation with 254 nm light for 30–90 s, fast axonal transport ceased. Although an occasional organelle was observed to translocate, the majority of the movements had stopped, and the organelles appeared "frozen" in place. This effect upon transport using 8-N_3ATP was observed at concentrations from 1 to 5 mM. These high concentrations of 8-N_3ATP were required for the analogue to compete effectively with the relatively high endogenous ATP concentration ($\sim$1 mM) that exists in axoplasm (Morris and Lasek, 1982). At 1 mM 8-N_3ATP the effect was not as dramatic as at 5 mM 8-N_3ATP and larger numbers of organelles continued to move. Furthermore, the cessation of motility brought about by irradiation at 254 nm in the presence of 8-N_3ATP was immediate and irreversible. Axoplasm incubated in 5 mM 8-N_3ATP followed by UV irradiation ceased motility, and the organelle movement could not be reactivated by the addition of ATP at concentrations from 5 to 10 mM. This observation indicated that the 8-N_3ATP had interacted with a nucleotide binding site(s) critical for organelle translocation such that when 8-N_3ATP was covalently attached to the site by UV irradiation, ATP turnover and thus transport could not continue.

Radioactive Photoaffinity Labeling

Because the column-purified vesicles devoid of all soluble proteins were competent to translocate on axonemal microtubules in an ATP-dependent, trypsin-sensitive fashion, it was assumed that ATP-binding molecules essential for vesicle motility were present on the vesicle surface. To identify these molecules, experiments were performed using [α^{32}P]8-N_3ATP followed by analysis of the resulting labeled polypeptides by electrophoresis and autoradiography. Control lanes in Fig. 2 illustrate that [α^{32}P]8-N_3ATP can be used to identify known ATP-binding molecules: the ATPase myosin (lanes *c–e*) and the ATP-binding protein actin (lanes *f–h*). Covalent modification of the myosin heavy chain and of actin occurred only in the presence of UV light (lanes *d* and *g*). Lanes *i–n* show results obtained when column-

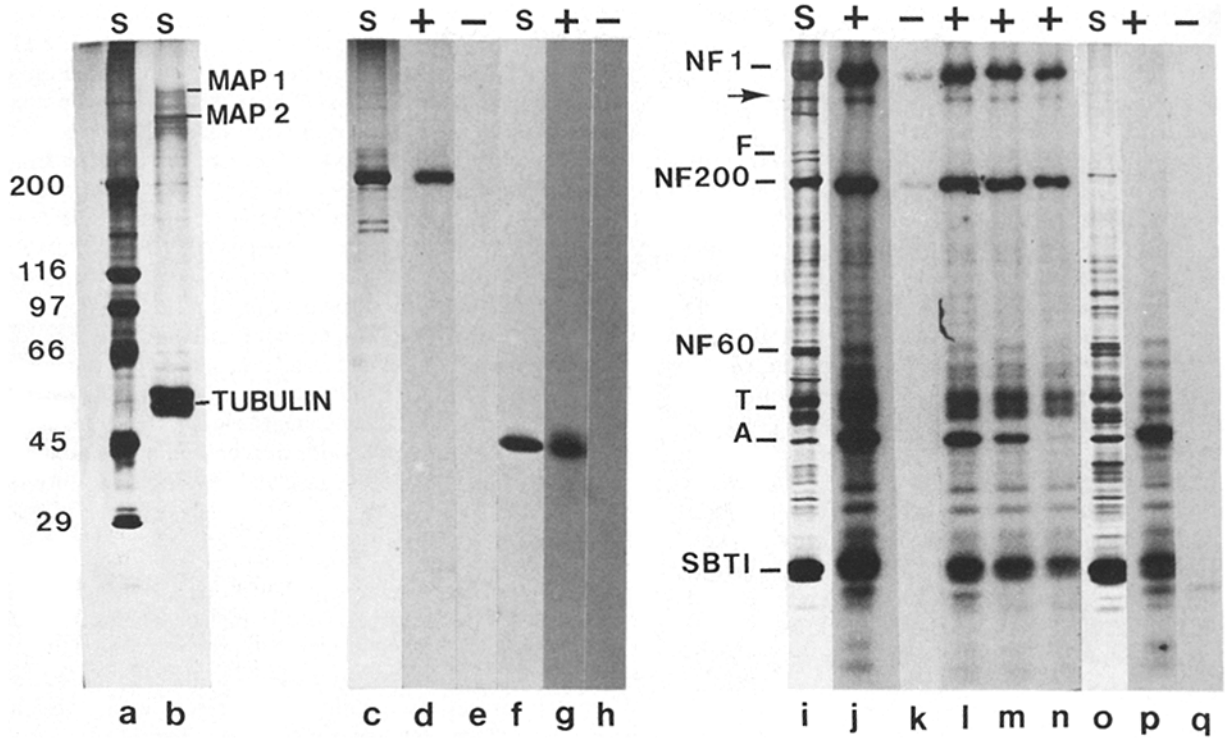

Figure 2. Photoaffinity labeling of ATP-binding proteins. Lane *a*, relative molecular mass markers ($\times 10^{-3}$); lane *b*, purified porcine brain microtubules. Lane *c*, silver stain (*S*) of a rabbit muscle myosin preparation covalently modified by [α-^{32}P]8-N_3ATP in the presence (+, lane *d*) or absence (−, lane *e*) of UV irradiation. Lane *f*, silver stain (*S*) of purified actin, and the corresponding autoradiograph (lanes *g* and *h*) indicates that labeling of the 43-kD polypeptide is UV-dependent. Lane *i*, composition of the column-purified vesicles; known polypeptides are identified to the left of the lane: neurofilament 1 (*NF1*), fodrin (*F*), neurofilament 200 (*NF200*), neurofilament 60 (*NF60*), α- and β-tubulin (*T*), and actin (*A*). Lanes *j–k*, polypeptides of lane *i* that were labeled by [α-^{32}P]8-N_3ATP in the presence (+) and absence (−) of UV irradiation. In addition to some known polypeptides that labeled, a discrete high molecular mass polypeptide of 292 kD (*arrow*, lane *j*) also incorporated label. Lanes *l–n*, a progressive decrease in the incorporation of [α-^{32}P]8-N_3ATP occurred when unlabeled ATP was added to the reaction mixture at 0.01, 0.1, and 1 mM, respectively. Lane *o*, polypeptide composition of the soluble protein from the column; lane *p*, those polypeptides that were labeled by [α-^{32}P]8-N_3ATP in the presence of UV irradiation; lane *q*, labeling in the absence of UV exposure. Soybean trypsin inhibitor (*SBTI*) appears as a predominant band on some of the gel lanes because it is present in the column elution buffer, and its concentration increases when the column fractions are concentrated for the motility and biochemical studies (see Materials and Methods). Lanes *i–q* are from the same gel or its corresponding autoradiograph; the autoradiograph was overexposed to show all labeling that occurred. *S*, silver-stained lanes; + and −, autoradiographs of gel samples generated in the presence (+) or absence (−) of UV exposure.

purified vesicles were incubated with [α^{32}P]8-N_3ATP in the presence and absence of UV irradiation. The vesicle preparation as isolated contained known axoplasmic polypeptides, and these are indicated to the left of lane *i*. In the presence of UV light, the following identifiable polypeptides became radioactively labeled (lane *j*); neurofilament 1 (NF 1, also called high molecular weight neurofilament [Lasek et al., 1979; Pant et al., 1978; Pant et al., 1986]), neurofilament 200 (NF 200), neurofilament 60 (NF 60), α- and β-tubulin, and actin. In addition, a discrete, high molecular weight polypeptide (indicated by the arrow) having a relative molecular mass of ~292 kD also bound [α^{32}P]8-N_3ATP. In the absence of UV irradiation only weak labeling of NF 1 and NF 200 was detected after an identical exposure of the gel (lane *k*). Furthermore, addition of ATP (0.01–1 mM) to the photolysis reaction mixture (lanes *l–n*) resulted in a progressive decrease in the amount of label incorporated, illustrating that [α^{32}P]8-N_3ATP and ATP compete for the same binding sites. Addition of 1 mM GTP to the vesicle preparation resulted in the almost complete elimination of labeling of α- and β-tubulin, yet there was no evident decrease in labeling of the other polypeptides (data not shown).

For comparison to the vesicles, the soluble proteins from the column were also covalently modified by [α^{32}P]8-N_3ATP (lanes *o–q*). The incorporation of the label into soluble polypeptides was UV-dependent (lane *p*) and unlabeled ATP effectively decreased the amount of label incorporated (data not shown). Moreover, the addition of MAP-free squid optic lobe microtubules either to the vesicle preparation or to the soluble proteins did not stimulate [α^{32}P]8-N_3ATP labeling of any additional polypeptides.

As an alternative approach to confirm the specificity of the results described above, direct photochemical cross-linking (Yue and Schimmel, 1977; Maruta and Korn, 1981) of [α^{32}P]ATP to ATP-binding proteins was also used. The results of these experiments (data not shown) indicated that the incorporation of [α^{32}P]ATP into protein was also UV-dependent and the polypeptides labeled were qualitatively the same as those labeled with [α^{32}P]8-N_3ATP.

Photoaffinity Labeling of Purified Kinesin

Vale et al. (1985*b*) have isolated and identified a force-generating protein called kinesin which is involved in micro-

tubule-based motility. Kinesin was isolated from squid optic lobe using published procedures (Vale et al., 1985*b*) and covalently modified using [α^{32}P]8-N_3ATP. Fig. 3, lane *c* shows the Coomassie stain of the purified protein and lane *d*, the silver stain of the same gel. Lanes *e* and *f* are from the autoradiograph and demonstrate that the 116-kD polypeptide, α- and β-tubulin, and actin incorporate label in a UV-dependent manner. Moreover, when increasing concentrations of unlabeled ATP (0.01–1 mM) were added to the reaction mixture, a progressive decrease in incorporation of label was observed (lanes *g–i*). These data indicate that the 116-kD subunit of kinesin is an ATP-binding polypeptide, although ATPase activity associated with this protein has not yet been demonstrated (Vale et al., 1985*b*). To determine if kinesin was present in the vesicle preparation, the polypeptides of kinesin and the column-purified vesicles were resolved side by side on the same gel (Fig. 4). The vesicle preparation (lane *d*) contains several polypeptides in minor amounts that migrate in the 110–120-kD region of the gel. However, distinct incorporation of [α^{32}P]8-N_3ATP in this region of gels of the vesicle preparation was never obtained (compare Figs. 2 and 3). Thus, it appears that kinesin is not a significant component of motility-competent vesicles isolated using the procedures employed here.

Immunoblotting

Fig. 5 illustrates that the 292-kD ATP-binding polypeptide of the vesicle preparation is recognized by antibodies to porcine brain MAP 2 (lanes *f* and *g*). In addition, the 292-kD polypeptide can be isolated in association with squid optic lobe microtubules (lanes *j* and *k*) but is absent from a preparation of kinesin isolated from optic lobe (lanes *h* and *i*). Moreover, the 292-kD polypeptide can be removed from taxol-stabilized microtubules in the presence of salt (lane *l*). These data indicate that the 292-kD polypeptide demonstrates some of the characteristics expected of a microtubule-associated protein as defined by Sloboda et al. (1975; 1976), and has one or more antigenic determinants in common with vertebrate brain MAP 2. Therefore, because the 292-kD polypeptide binds ATP and can be isolated in association with microtubules or with vesicles that translocate on microtubules in an ATP-dependent fashion, these data suggest that the 292-kD polypeptide may be a microtubule–vesicle cross-bridging molecule involved in vesicle motility.

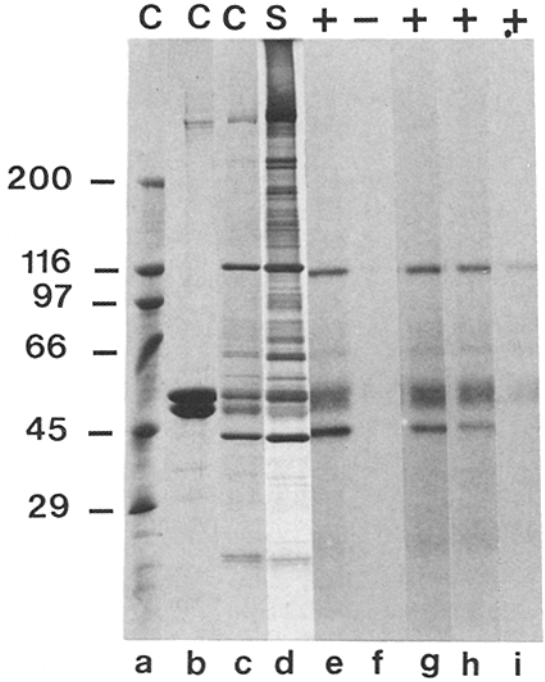

Figure 3. [α-^{32}P]8-N_3ATP labeling of kinesin. Lane *a*, relative molecular mass markers (×10^{-3}), lane *b*, porcine brain microtubules. Lanes *c* and *d*, polypeptide composition of a preparation of kinesin from squid optic lobe after Coomassie staining (*C*) and silver staining (*S*) of the same gel lane. Lane *e*, from the corresponding autoradiograph and shows that in the presence (+) of UV irradiation, the 116-kD polypeptide of kinesin, α- and β-tubulin, and actin were labeled. In the absence (−) of UV exposure (lane *f*), no incorporation of label was observed. Furthermore, the incorporation of [α-^{32}P]8-N_3ATP was effectively decreased by addition of 0.01, 0.1, and 1 mM ATP, respectively (lanes *g–i*).

Discussion

Because it had been previously shown that axoplasmic vesicles from the squid giant axon could translocate on axonemal microtubules whose dynein arms had been removed (Gilbert et al., 1985), these observations implied that the molecules necessary for vesicle motility were present in the vesicle preparation. Thus, the experiments described in this paper were designed to identify those polypeptides important for motility. Purified vesicles devoid of soluble proteins were obtained by fractionation of axoplasmic vesicles on a Sephacryl S-400 column (Fig. 1). These column-purified vesicles were observed to translocate on isolated axonemal microtubules in an ATP-dependent and trypsin-sensitive manner. Furthermore, the axonemal microtubules were never observed to

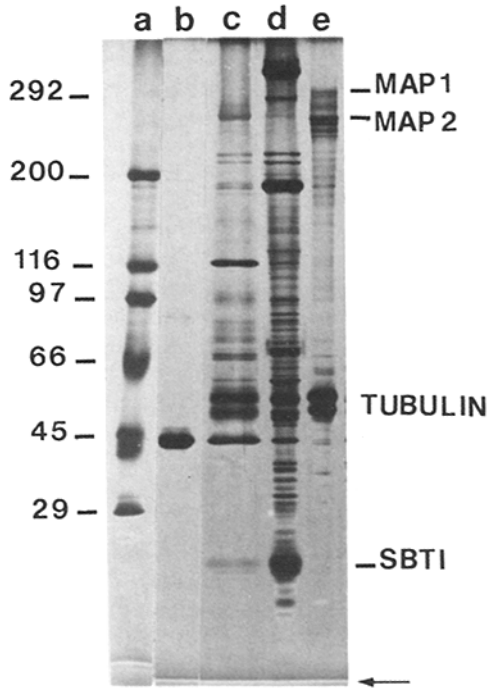

Figure 4. Comparative migration of the polypeptides of the kinesin and vesicle preparations. Lane *a*, relative molecular mass markers (×10^{-3}); lane *b*, actin; lane *c*, squid optic lobe kinesin; lane *d*, axoplasmic vesicles; and lane *e*, porcine brain microtubules. Arrow marks the ion front.

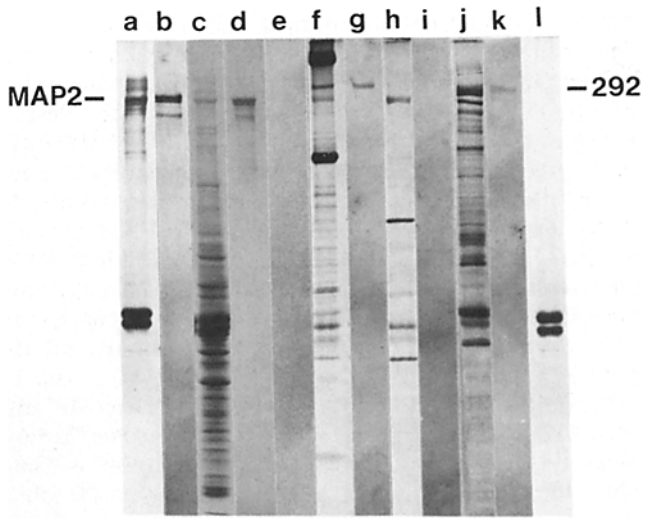

Figure 5. The 292-kD vesicle-associated polypeptide shows immunological cross-reactivity to porcine brain MAP 2. Lane *a*, silver stain of purified porcine brain microtubules; lane *b*, its blot with antiserum to porcine brain MAP 2. Lane *c*, Coomassie stain of the soluble proteins from a porcine brain homogenate; lane *d*, its blot with the antiserum. Lane *e*, blot of purified porcine brain microtubules reacted with antiserum that had been immunoadsorbed with MAP 2 as antigen. No cross-reaction is evident. Lane *f*, silver stain of the vesicle preparation; lane *g*, its blot, showing cross-reaction of the 292-kD polypeptide with MAP 2 antiserum. Lane *h*, silver stain of a kinesin preparation; lane *i*, its blot against the antiserum; no cross-reactivity is evident. Lane *j*, silver stain of squid optic lobe microtubules; lane *k*, its blot showing cross-reactivity between the 292-kD polypeptide and the MAP 2 antiserum. Lane *l*, silver stain of squid optic lobe microtubules treated with 1 M KCl in the presence of taxol to remove the microtubule-associated proteins.

translocate on the glass coverslip. These data indicate that under certain conditions the ATP-binding, force-generating polypeptides essential for motility can be isolated in association with the vesicle surface.

Next, to identify these ATP-binding molecules, the radioactive photoaffinity analogue of ATP, [α^{32}P]8-N_3ATP, was used. In motility experiments, unlabeled 8-N_3ATP in the presence of UV irradiation caused the immediate cessation of axoplasmic transport. Furthermore, organelle translocation could not be reactivated by the addition of up to 10 mM ATP. These results indicated that 8-N_3ATP interacted with nucleotide-binding sites critical for vesicle translocation such that when the analogue was covalently attached to the sites by UV irradiation, ATP turnover and thus transport could not occur. Control experiments (Fig. 2) showed that [α^{32}P]8-N_3ATP could be used to identify known ATP-binding polypeptides; and when the vesicle preparation was covalently modified, a number of identifiable polypeptides were labeled, including NF 1, NF 200, NF 60, α- and β-tubulin, and actin. In addition to these known nucleotide-binding proteins, a discrete high molecular mass polypeptide of 292 kD also bound [α^{32}P]8-N_3ATP. Note also that the photoaffinity analogue is not merely acting as a qualitative protein stain. For example, both the fodrin doublet and the high molecular mass polypeptide that migrates between the 292-kD polypeptide and fodrin show no incorporation of [α^{32}P]8-N_3ATP in an overexposed autoradiograph, yet these polypeptides distinctly stain for protein (Fig. 2, lanes *i* and *j*).

Although a number of vesicle-associated proteins bound [α^{32}P]8-N_3ATP, not all of these ATP-binding polypeptides are important for vesicle translocation for the following reasons. First, NF 1, NF 200, and NF 60 bound [α^{32}P]8-N_3ATP, yet it has been shown that neurofilaments are not involved in organelle translocation (Papasozomenos et al., 1982; Schnapp and Reese, 1982; Allen et al., 1985; Schnapp et al., 1985; Miller and Lasek, 1985). Furthermore, the incorporation of label at 200 kD (Fig. 2, lane *j*) is believed to be due to NF 200 polypeptide only. Although axoplasmic myosin co-migrates at 200 kD, the vesicle preparation showed no cross-reaction with antiserum to squid optic lobe myosin by immunoblotting under conditions in which whole axoplasm demonstrated cross-reactivity with the antiserum at this position on the gel (data not shown). Thus, while the data reported here demonstrate that the neurofilament polypeptides co-purify with the vesicles and bind ATP, several lines of evidence have shown them not to be involved in vesicle motility.

Second, F-actin does not appear to be involved in organelle translocation on isolated microtubules (Allen et al., 1985; Gilbert et al., 1985; Schnapp et al., 1985). In these motility assay systems, isolated microtubules can support bidirectional movement, yet F-actin does not appear to be a significant component of the vesicle–microtubule complex. However, although these in vitro models suggest that actin is not involved, its role in directed organelle movement in vivo is still controversial; for example, agents that disrupt actin microfilaments also inhibit fast axonal transport (Isenberg et al., 1980; and Goldberg, 1982; Brady et al., 1984).

Third, both actin and tubulin bind and hydrolyze nucleoside triphosphates during polymerization. Actin binds 1 mol of ATP per actin monomer, and F-actin has predominantly ADP bound (Straub and Feuer, 1950). Moreover, labeled ATP exchanges freely with G-actin · ADP or G-actin · ATP (Martonosi et al., 1960; Neidl and Engel, 1979). Similarly, the tubulin dimer binds 2 mol of GTP, 1 mol of which binds to an exchangeable site (Jacobs et al., 1974), and tubulin can bind ATP (Zabrecky and Cole, 1980; 1982). Furthermore, the labeling of α- and β-tubulin could be almost completely eliminated by the addition of GTP to the vesicle preparation without decrease of [α^{32}P]8-N_3ATP incorporation into the other vesicle polypeptides (data not shown). These combined results suggest that the binding of [α^{32}P]8-N_3ATP was to the nucleotide-binding sites on actin and tubulin normally occupied by ATP and GTP, respectively.

The 292-kD polypeptide is the only other polypeptide unique to the vesicle preparation that was distinctly labeled by [α^{32}P]8-N_3ATP (Fig. 2). Label incorporation was UV-dependent and competitive with unlabeled ATP. Because the [α^{32}P]8-N_3ATP and unlabeled ATP were added simultaneously to the vesicle preparation, there was some labeling of the 292-kD polypeptide even in the presence of 1 mM ATP. This result was not unexpected because the ATP-binding proteins would have an equal opportunity to bind a labeled or an unlabeled molecule of ATP under the conditions used. Furthermore, unlabeled ATP was competing not only with unbound [α^{32}P]8-N_3ATP but also with bound [α^{32}P]8-N^3ATP and any hydrolyzed products (i.e., [α^{32}P]8-N_3ADP, [α^{32}P]8-N_3AMP). The lack of complete competition by unlabeled ATP therefore may be due to different exchange rates of the various labeled nucleotides before UV irradiation.

Thus, the complete elimination of labeling by unlabeled ATP as is demonstrated by actin (Fig. 2, lanes *l–n*) may reflect both the rate of exchange of nucleotide at the ATP-binding site as well as the specificity of nucleotide binding. Furthermore, because the experiments were performed with ^{32}P at the α position rather than the γ position of the analogue, the label was not transferred to the 292-kD polypeptide by an associated protein kinase reaction (Sloboda et al., 1975). Also, all labeling experiments were performed under conditions in which both protein and analogue concentrations were kept low to minimize nonspecific labeling which increases linearly with concentration (reviewed in Potter and Haley, 1983). Moreover, any [$\alpha^{32}P$]8-N_3ATP molecules not in association with protein at the time of irradiation react with solvent because the highly reactive nitrene is very unstable (Potter and Haley, 1983). Hence, these results suggest that the labeling of the 292-kD polypeptide is specific, i.e., that [$\alpha^{32}P$]8-N_3ATP bound to a distinct ATP-binding site in the presence of UV irradiation. Yet the possibility exists that binding of the analogue may have been due to a nonspecific ionic interaction with cationic groups on the polypeptide rather than due to a specific interaction with an adenine nucleotide binding site. The data presented here or any other similar control experiment cannot exclude this possibility. However, Kerlavage and Taylor (1980) have shown that incorporation of 8-azido analogues can be highly specific. In their experiments, [^{32}P]8-N_3cAMP covalently modified a single tyrosine residue of the cAMP binding site of the regulatory subunit of a type II cAMP-dependent protein kinase from porcine heart.

Furthermore, in experiments in which 0.5% SDS was added to the column buffer, the elution profile of the 292-kD polypeptide changed. In the presence of detergent, the 292-kD polypeptide eluted in the included volume of the Sephacryl S-400 column, corresponding to fractions *k–r* in Fig. 1 (data not shown). This result demonstrates that the 292-kD polypeptide is not present in the vesicle preparation as a soluble monomer. Moreover, the results of Pratt (1986) suggest that the 292-kD polypeptide is not a MAP in association with neurofilaments. She has shown that this polypeptide is present when vesicle–microtubule complexes, essentially free of neurofilaments, are isolated from squid axoplasm. Furthermore, the association of the 292-kD polypeptide with the vesicle–microtubule complex is nucleotide dependent, and the complex contains ATPase activity. These results, combined with the published data summarized above, suggest that the 292-kD polypeptide is one likely candidate for an ATP-binding protein essential for vesicle motility.

Vale et al. (1985*b*) have isolated and identified a force-generating protein called kinesin which induces the movement of microtubules along a glass coverslip and the movement of latex beads along microtubules. Although the data suggest that kinesin is the mechanochemical transducer that promotes organelle movement along microtubules, there is no direct evidence showing an interaction of kinesin with organelles (Vale et al., 1985*c*). The results of Fig. 3, showing the incorporation of [$\alpha^{32}P$]8-N_3ATP into the 116-kD subunit of kinesin, indicate that kinesin is an ATP-binding protein. These data support the hypothesis of Vale et al. (1985*b*; 1985*c*) that orthograde organelle movements are mediated by kinesin; however, ATPase activity associated with the molecule has not yet been demonstrated, and distinct incorporation of [$\alpha^{32}P$]8-N_3ATP in the 116-kD region of gels of the vesicle preparation was never obtained (Fig. 2). Furthermore, no incorporation of label was detected in the 116-kD region of the gels of the soluble proteins (Fig. 2, lane *p*). Hence, the data presented here do not conclusively support or negate a role for kinesin in axoplasmic vesicle motility although they do demonstrate that kinesin is an ATP-binding protein (Fig. 3). Moreover, the results presented here show that axoplasmic vesicles can translocate on isolated microtubules in the presence of ATP, yet kinesin does not appear to be an obvious component of this motility competent preparation.

In addition to ATP binding, another characteristic expected of the force-generating molecule(s) responsible for organelle translocation is that it should purify in association with vesicles or microtubules, depending on the ionic strength, osmolality, and other buffer conditions used. Fig. 2 demonstrates that the 292-kD polypeptide purified with axoplasmic vesicles, while Fig. 5 illustrates that the 292-kD polypeptide also co-purifies with taxol-stabilized microtubules and that it is released from the microtubules in the presence of salt. These data suggest that while the 292-kD polypeptide is vesicle associated, it also demonstrates some of the characteristics of a microtubule-associated protein: it associates with assembled tubulin noncovalently, and it can be removed by salt in the presence of taxol.

Fig. 5 shows that the 292-kD polypeptide present in either a preparation of axoplasmic vesicles or squid optic lobe microtubules cross-reacts with antibodies to porcine brain MAP 2. This observation is the first demonstration that a MAP 2-like protein is present in squid axoplasm and furthermore, the first demonstration of the presence of MAP 2 in an invertebrate. Previously, MAP 2 had been shown to be highly enriched in dendrites and cell bodies relative to axons (Matus et al., 1981; Huber and Matus, 1984; Wiche et al., 1983; De Camilli et al., 1984), yet MAP 2 has clearly been shown to be a distinct component of certain axons (Caceres et al., 1984; Papasozomenos et al., 1985). Although the 292-kD polypeptide is similar to MAP 2 by immunological criteria, it migrates between porcine brain MAP 1 and MAP 2 on 5–15% acrylamide/12–48% urea gradient slab gels (Figs. 2, 4, and 5). Furthermore, the axoplasmic 292-kD polypeptide cannot be phosphorylated under conditions in which NF 1 and NF 200 or porcine brain MAP 2 (Sloboda et al., 1975) become phosphorylated (manuscript in preparation). Finally, because the 292-kD polypeptide is completely released from microtubules by a brief incubation in 1 M salt and it cross-reacts with antiserum to porcine brain MAP 2, it is distinct from the flagellar motive force-generating protein dynein (Gibbons and Rowe, 1965).

In summary, the results presented here suggest that a MAP 2-like protein from squid axoplasm demonstrates characteristics expected of a molecule that generates the motive force for organelle translocation: it binds ATP, is associated with vesicles that translocate on isolated microtubules, and can be isolated in association with vesicles or microtubules. Although ATP binding is one of the most important criteria expected of an ATPase that generates organelle motility, ATP binding alone is certainly not sufficient to identify a protein as a mechanochemical force transducer. However, a novel use of a radioactive azido analogue of ATP has enabled us

to focus on one likely candidate in a complex mixture of polypeptides. Experiments are presently being performed to ascertain the role of the 292-kD polypeptide in those vesicle–microtubule interactions that occur during the important neurological process of fast axoplasmic transport.

We thank Drs. Karl Fath and Raymond J. Lasek (Case Western Reserve Medical School) for myosin preimmune and antisera, Dr. Harold Gainer (National Institutes of Health) for antiserum to MAP 2, and Dr. Margaret A. Titus (Brandeis University) for purified rabbit muscle myosin. We acknowledge Drs. Hiroshi Maruta and Joel L. Rosenbaum, Yale University, for generously loaning us their R-52G Mineralight Lamp to do the direct photochemical cross-linking experiments, and are grateful to the Molecular Genetics Center at Dartmouth College for the use of the LaserWriter used to prepare the manuscript.

This research was supported by grants to R. D. Sloboda (Muscular Dystrophy Association; National Science Foundation [NSF] BNS-85-03597) and S. P. Gilbert (Sigma Xi Grant-in-Aid, Association of Women in Science Judith Pool Award, and R. Melville Cramer Grant-in-Aid). S. P. Gilbert was a graduate student at Dartmouth College supported by NSF BNS-85-03597 to R. D. Sloboda.

Received for publication 12 February 1986, and in revised form 5 May 1986.

References

Allen, R. D., and N. S. Allen. 1983. Video-enhanced microscopy with a computer frame memory. *J. Microsc.* 129:3–17.

Allen, R. D., D. G. Weiss, J. H. Hayden, D. T. Brown, H. Fujiwake, and M. Simpson. 1985. Gliding movement of and bidirectional transport along single native microtubules from squid axoplasm: evidence for an active role of microtubules in cytoplasmic transport. *J. Cell Biol.* 100:1736–1752.

Bear, R. S., F. O. Schmitt, and J. Z. Young. 1937. Investigations on the protein constituents of nerve axoplasm. *Proc. R. Soc. Lond. B Biol. Sci.* 123: 520–529.

Bell, C. W., C. Fraser, W. S. Sale, W.-J. Y. Tang, and I. R. Gibbons. 1982. Preparation and purification of dynein. *Methods Cell Biol.* 24:373–397.

Brady, S. T. 1985. A novel brain ATPase with properties expected for the fast axonal transport motor. *Nature (Lond.).* 317:73–75.

Brady, S. T., and R. J. Lasek. 1982. Axonal transport: a cell-biological method for studying proteins that associate with the cytoskeleton. *Methods Cell Biol.* 25:365–398.

Brady, S. T., R. J. Lasek, and R. D. Allen. 1982. Fast axonal transport in extruded axoplasm from squid giant axon. *Science (Wash. DC.).* 218:1129–1131.

Brady, S. T., R. J. Lasek, and R. D. Allen. 1985. Video microscopy of fast axonal transport in extruded axoplasm: a new model for study of molecular mechanisms. *Cell Motil.* 5:81–101.

Brady, S. T., R. J. Lasek, R. D. Allen, H. L. Yin, and T. P. Stossel. 1984. Gelsolin inhibition of fast axonal transport indicates a requirement for actin microfilaments. *Nature (Lond.).* 310:56–58.

Briggs, F. N., W. Al-Jumaily, and B. E. Haley. 1980. Photoaffinity-labeling of the (Ca+Mg) ATPase of skeletal and cardiac sarcoplasmic-reticulum with (γ-^{32}P)-8-azido-ATP. *Cell Calcium.* 1:205–215.

Caceres, A., L. I. Binder, M. R. Payne, P. Bender, L. Rebhun, and O. Steward. 1984. Differential subcellular localization of tubulin and the microtubule-associated protein MAP2 in brain tissue as revealed by immunocytochemistry with monoclonal hybridoma antibodies. *J. Neurosci.* 4:394–410.

De Camilli, P., P. E. Miller, F. Navone, W. E. Theurkauf, and R. B. Vallee. 1984. Distribution of microtubule-associated protein 2 in the nervous system of the rat studied by immunofluorescence. *Neuroscience.* 11:819–846.

Fairbanks, G., T. L. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry.* 10:2606–2617.

Gibbons, I. R., and A. J. Rowe. 1965. Dynein: a protein with adenosine triphosphatase activity from cilia *Science (Wash. DC).* 149:424–426.

Gilbert, S. P., R. D. Allen, and R. D. Sloboda. 1985. Translocation of vesicles from squid axoplasm on flagellar microtubules. *Nature (Lond.).* 315: 245–248.

Gilbert, S. P., and R. D. Sloboda. 1984. Bidirectional transport of fluorescently labeled vesicles introduced into extruded axoplasm of squid *Loligo pealei*. *J. Cell Biol.* 99:445–452.

Goldberg, D. J. 1982. Microinjection into an identified axon to study the mechanism of fast axonal transport. *Proc. Natl. Acad. Sci. USA.* 79:4818–4822.

Grafstein, B., and D. S. Forman. 1980. Intracellular transport in neurons. *Physiol. Rev.* 60:1167–1283.

Haley, B. E., and J. F. Hoffman. 1974. Interactions of a photo-affinity ATP analog with cation-stimulated adenosine triphosphatases of human red cell membranes. *Proc. Natl. Acad. Sci. USA.* 71:3367–3371.

Hoppe, J., and W. Freist. 1979. Localization of the high-affinity ATP site in adenosine-3′:5′-monophosphate-dependent protein kinase type I. Photoaffinity labeling studies with 8-azidoadenosine 5′-triphosphate. *Eur. J. Biochem.* 93:141–146.

Huber, G., and A. Matus. 1984. Differences in the cellular distributions of two microtubule-associated proteins, MAP1 and MAP2 in rat brain. *J. Neurosci.* 4:151–160.

Isenberg, G., P. Schubert, and G. W. Kreutzberg. 1980. Experimental approach to test the role of actin in axonal transport. *Brain Res.* 194:588–593.

Jacobs, M., H. Smith, and E. W. Taylor. 1974. Tubulin: nucleotide binding and enzymic activity. *J. Mol. Biol.* 89:455–468.

Kendrick-Jones, J., W. Lehman, and A. G. Szent-Györgyi. 1970. Regulation in molluscan muscles. *J. Mol. Biol.* 54:313–326.

Kerlavage, A. R., and S. S. Taylor. 1980. Covalent modification of an adenosine 3′:5′-monophosphate binding site of the regulatory subunit of cAMP-dependent protein kinase II with 8-azidoadenosine 3′:5′-monophosphate. Identification of a single modified tyrosine residue. *J. Biol. Chem.* 255:8483–8488.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Lasek, R. J. 1974. Biochemistry of the squid giant axon. *In* A Guide to the Laboratory Use of the Squid. J. M. Arnold, W. C. Summers, and D. L. Gilbert, editors. Marine Biological Laboratory, Woods Hole, MA. 69–74.

Lasek, R. J., and S. T. Brady. 1985. Attachment of transported vesicles to microtubules in axoplasm is facilitated by AMP-PNP. *Nature (Lond.).* 316: 645–647.

Lasek, R. J., N. Krishnan, and I. R. Kaiserman-Abramof. 1979. Identification of the subunit proteins of 10-nm neurofilaments isolated from axoplasm of squid and *Myxicola* giant axons. *J. Cell Biol.* 82:336–346.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265–275.

Martonosi, A., M. A. Gouvea, and J. Gergely. 1960. Studies on actin. The interaction of C^{14}-labeled adenine nucleotides with actin. *J. Biol. Chem.* 235:1700–1706.

Maruta, H., and E. D. Korn. 1981. Direct photoaffinity labeling by nucleotides of the apparent catalytic site on the heavy chains of smooth muscle and *Acanthamoeba* myosins. *J. Biol. Chem.* 256:499–502.

Matus, A., R. Bernhardt, and T. Hugh-Jones. 1981. High molecular weight microtubule-associated proteins are preferentially associated with dendritic microtubules in brain. *Proc. Natl. Acad. Sci. USA.* 78:3010–3014.

Miller, R. H., and R. J. Lasek. 1985. Cross-bridges mediate anterograde and retrograde vesicle transport along microtubules in squid axoplasm. *J. Cell Biol.* 101:2181–2193.

Morris, J. R., and R. J. Lasek. 1982. Stable polymers of the axonal cytoskeleton: the axoplasmic ghost. *J. Cell Biol.* 92:192–198.

Morrissey, J. H. 1981. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Anal. Biochem.* 117: 307–310.

Murphy, D. B., K. T. Wallis, and R. R. Hiebsch. 1983. Identity and origin of the ATPase activity associated with neuronal microtubules. II. Identification of a 50,000-dalton polypeptide with ATPase activity similar to F-1 ATPase from mitochondria. *J. Cell Biol.* 96:1306–1315.

Neidl, C., and J. Engel. 1979. Exchange of ADP, ATP, and 1:N^6-ethenoadenosine 5′-triphosphate at G-actin. Equilibrium and kinetics. *Eur. J. Biochem.* 101:163–169.

Pant, H. C., P. E. Gallant, and H. Gainer. 1986. Characterization of a cyclic nucleotide- and calcium-independent, neurofilament protein kinase activity in axoplasm from the squid giant axon. *J. Biol. Chem.* 261:2968–2977.

Pant, H. C., G. Shecket, H. Gainer, and R. J. Lasek. 1978. Neurofilament protein is phosphorylated in the squid giant axon. *J. Cell Biol.* 78:R23–R27.

Papasozomenos, S. Ch., L. I. Binder, P. K. Bender, and M. R. Payne. 1985. Microtubule-associated protein 2 within axons of spinal motor neurons: associations with microtubules and neurofilaments in normal and β,β′-iminodipropionitrile-treated axons. *J. Cell Biol.* 100:74–85.

Papasozomenos, S. Ch., M. Yoon, R. Crane, L. Autilio-Gambetti, and P. Gambetti. 1982. Redistribution of proteins of fast axonal transport following administration of β,β′-iminodipropionitrile: a quantitative autoradiographic study. *J. Cell Biol.* 95:672–675.

Pfister, K. K., B. E. Haley, and G. B. Witman. 1984. The photoaffinity probe 8-azidoadenosine 5′-triphosphate selectively labels the heavy chain of *Chlamydomonas* 12 S dynein. *J. Biol. Chem.* 259:8499–8504.

Pfister, K. K., B. E. Haley, and G. B. Witman, 1985. Labeling of *Chlamydomonas* 18 S dynein polypeptides by 8-azidoadenosine 5′-triphosphate, a photoaffinity analog of ATP. *J. Biol. Chem.* 260:12844–12850.

Potter, R. L., and B. E. Haley. 1983. Photoaffinity labeling of nucleotide binding sites with 8-azidopurine analogs: techniques and applications. *Methods Enzymol.* 91:613–633.

Pratt, M. M. 1986. Stable complexes of axoplasmic vesicles and microtubules: protein composition and ATPase activity. *J. Cell Biol.* 103:957–968.

Pratt, M. M., S. Hisanaga, and D. A. Begg. 1984. An improved purification method for cytoplasmic dynein. *J. Cell. Biochem.* 26:19–33.

Schacterle, G. R., and R. L. Pollack. 1973. A simplified method for the quantitative assay of small amounts of protein in biologic material. *Anal. Biochem.* 51:654–655.

Scheurich, P., H. Schafer, and K. Dose. 1978. 8-Azido-adenosine 5′-

triphosphate as a photoaffinity label for bacterial F_1 ATPase. *Eur. J. Biochem.* 88:253–257.

Schnapp, B. J., and T. S. Reese. 1982. Cytoplasmic structure in rapid-frozen axons. *J. Cell Biol.* 94:667–679.

Schnapp, B. J., R. D. Vale, M. P. Sheetz, and T. S. Reese. 1985. Single microtubules from squid axoplasm support bidirectional movement of organelles. *Cell.* 40:455–462.

Scholey, J. M., M. E. Porter, P. M. Grissom, and J. R. McIntosh. 1985. Identification of kinesin in sea urchin eggs, and evidence for its localization in the mitotic spindle. *Nature (Lond.).* 318:483–486.

Sloboda, R. D., W. L. Dentler, and J. L. Rosenbaum. 1976. Microtubule-associated proteins and the stimulation of tubulin assembly in vitro. *Biochemistry.* 15:4497–4505.

Sloboda, R. D., and J. L. Rosenbaum. 1979. Decoration and stabilization of intact, smooth-walled microtubules with microtubule-associated proteins. *Biochemistry.* 18:48–55.

Sloboda, R. D., S. A. Rudolph, J. L. Rosenbaum, and P. Greengard. 1975. Cyclic AMP-dependent endogenous phosphorylation of a microtubule-associated protein. *Proc. Natl. Acad. Sci. USA.* 72:177–181.

Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* 246:4866–4871.

Straub, F. B., and G. Feuer. 1950. Adenosinetriphosphate the functional group of actin. *Biochim. Biophys. Acta.* 4:455–470.

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.

Vale, R. D., B. J. Schnapp, T. S. Reese, and M. P. Sheetz. 1985*a*. Organelle, bead, and microtubule translocations promoted by soluble factors from the squid giant axon. *Cell.* 40:559–569.

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

Vale, R. D., B. J. Schnapp, T. Mitchison, E. Steuer, T. S. Reese, and M. P. Sheetz. 1985*c*. Different axoplasmic proteins generate movement in opposite directions along microtubules in vitro. *Cell.* 43:623–632.

Vallee, R. B. 1982. A taxol-dependent procedure for the isolation of microtubules and microtubule-associated proteins (MAPs). *J. Cell Biol.* 92:435–442.

Wagenvoord, R. J., I. van der Kraan, and A. Kemp. 1977. Specific photolabeling of beef heart mitochondrial ATPase by 8-azido-ATP. *Biochim. Biophys. Acta.* 460:17–24.

Weiss, D. G., editor. 1982. Axoplasmic Transport. Springer-Verlag, New York. 478 pp.

Wiche, G., E. Briones, H. Hirt, R. Krepler, U. Artlieb, and H. Denk. 1983. Differential distribution of microtubule-associated proteins MAP-1 and MAP-2 in neurons of rat brain and association of MAP-1 with microtubules of neuroblastoma cells (clone N_2A). *EMBO (Eur. Mol. Biol. Organ.) J.* 2:1915–1920.

Yue, V. T., and P. R. Schimmel. 1977. Direct and specific photochemical cross-linking of adenosine 5'-triphosphate to an aminoacyl-tRNA synthetase. *Biochemistry.* 16:4678–4684.

Zabrecky, J. R., and R. D. Cole. 1980. ATP-induced aggregates of tubulin rings. *J. Biol. Chem.* 255:11981–11985.

Zabrecky, J. R., and R. D. Cole. 1982. Effect of ATP on the kinetics of microtubule assembly. *J. Biol. Chem.* 257:4633–4638.