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Kinesin-related proteins in eukaryotic flagella

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SUMMARY

To identify kinesin-related proteins that are important for ciliary and eukaryotic flagellar functions, we used affinity-purified, polyclonal antibodies to synthetic peptides corresponding to conserved sequences in the motor domain of kinesin (Sawin et al. (1992) *J. Cell Sci.* 101, 303-313). Using immunoblot analysis, two antibodies to distinct sequences (LNLVDLAGSE, 'LAGSE' and, HIPYRESKLT, 'HIPYR') reveal a family of proteins in flagella and axonemes isolated from *Chlamydomonas*. Similar analysis of axonemes from mutant *Chlamydomonas* strains or fractionated axonemes indicates that none of the immunoreactive proteins are associated with dynein arm or spoke structures. In contrast, one protein, ~110 kDa, is reduced in axonemes from mutant strains defective in the central pair apparatus. Immunoreactive proteins with masses of 96 and 97 kDa (the '97 kDa' proteins) are selectively solubilized from isolated axonemes in 10 mM ATP. The 97 kDa proteins co-sediment in sucrose gradients at about 9 S and bind to axonemes or purified microtubules in a nucleotide-dependent fashion characteristic of kinesin. These results reveal that flagella contain kinesin-related proteins, which may be involved in axonemal central pair function and flagellar motility, or directed transport involved in morphogenesis or mating responses in *Chlamydomonas*.

Key words: cell motility, cilium, microtubule, cytoplasmic transport, molecular motor

INTRODUCTION

Although much is known about dynein ATPases and their roles in cilia and flagella (Witman, 1992), the presence of other microtubule-associated molecular motors has not been determined. In this work our goal was to investigate the presence of kinesin or related proteins in cilia and eukaryotic flagella. Kinesin and related proteins form a superfamily defined by common nucleotide and microtubule binding structures in a motor domain (Vale, 1992; Goldstein, 1991; Bloom, 1992), and the members of the kinesin superfamily are versatile and responsible for many diverse microtubule-associated functions such as axonal transport (Vale et al., 1985; Brady et al., 1990), movement and distribution of cytoplasmic membrane elements (Hollenbeck and Swanson, 1990), and chromosome movements during mitosis or meiosis (Gelfand and Scholey, 1992).

The versatility and functional properties of kinesin-related proteins make them attractive candidates for a variety of processes in cilia and flagella. For example, in most cases studied, kinesin-related proteins generate a polarized force such that the kinesin motor, and whatever cargo is attached, move toward the plus end of the microtubules (Vale et al., 1985; Paschal and Vallee, 1987). Thus, kinesin or related proteins are potential candidates for directed transport along axonemal microtubules, possibly characterizing a novel transport mechanism recently described as 'intraflagellar transport' (Kozminski et al., 1993), and presumably responsible for movement of axonemal components to the distal tip for assembly (Johnson and Rosenbaum, 1993). Kinesin-related proteins are also candidates for flagellar 'tipping' responses required for gamete mating in *Chlamydomonas* (Goodenough, 1991) and directional surface movements characteristic of flagella from *Chlamydomonas* (Bloodgood, 1990).

Members of the kinesin superfamily may play a role in the mechanism of ciliary and flagellar motility. In contrast to kinesin, axonemal dynein generates polarized force such that dynein, and the microtubule it is structurally bound to, move toward the minus end of the adjacent microtubule (Sale and Satir, 1977; Fox and Sale, 1987). This observation, along with others, indicates that dynein-driven microtubule sliding must be regulated (Satir, 1985; Brokaw, 1989). One possible mechanism for regulating dynein-driven sliding is via production of an opposing force. Thus, there is ample basis on which to examine the possibility that kinesin or related proteins are located within the axoneme and play structural and functional roles.

Affinity-purified, polyclonal antibodies to conserved sequences in the kinesin motor domain (Sawin et al., 1992a) have proven to be useful reagents in identifying new members of the kinesin superfamily. For example, affinity-purified antibodies led to the discovery of novel kinesin-related proteins in *Xenopus* eggs (Sawin et al., 1992a,b), sea urchin eggs (Cole et al., 1992) and diatom spindles (Hogan et al., 1992). Using this strategy we describe a family of proteins in flagella and axonemes that react with affinity-purified antibodies directed

against two different sequences referred to as LAGSE (LNLVDLAGSE) and HIPYR (HIPYRESKLT). A subset of the immunoreactive proteins is associated with the axonemal central pair apparatus and may be involved in ciliary and flagellar motility or act as a microtubule-associated structural component. In related studies, Johnson and Rosenbaum (1994) have localized kinesin-related proteins to the central pair apparatus in *Chlamydomonas* flagella. A second set of proteins, the 97 kDa proteins, is solubilized from axonemes using ATP and displays nucleotide-dependent microtubule binding properties similar to kinesin. The 97 kDa proteins are candidate molecular motors for intraflagellar transport (Kozminski et al., 1993), flagellar gliding (Bloodgood, 1990), or flagellar tipping responses (Goodenough, 1991).

MATERIALS AND METHODS

Flagella and axoneme isolation

Flagella were isolated from vegetative *Chlamydomonas* as described before (Smith and Sale, 1992) using either the dibucaine method (Witman, 1986) or pH shock (Witman et al., 1972). The method of deflagellation had no effect on the results described below. Axonemes were isolated by suspension of flagella in 0.5% Nonidet P-40 (in 10 mM Hepes, pH 7.4, 5 mM $MgSO_4$, 0.5 mM EDTA, 1 mM dithiothreitol, 30 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, and 0.6 TIU aprotinin; referred to as buffer A), and either 1 mM ATP or 1 mM adenylyl imidodiphosphate (AMP-PNP), and then separated by centrifugation. For most experiments axonemes were isolated in the presence of 1 mM AMP-PNP in buffer A to retain immunoreactive, kinesin-related proteins for subsequent selective extraction with ATP.

ATP solubilization of immunoreactive proteins

Axonemes were washed once in buffer A by centrifugation, suspended at a protein concentration of 8 mg/ml in buffer A supplemented with 10 mM ATP and 10 mM $MgSO_4$, and incubated for 10 minutes on ice. Following extraction, axonemes were pelleted and the supernatant collected. The ATP extract was subsequently fractionated by zonal centrifugation on a 5% to 20% sucrose gradient, made with buffer A as described before (Smith and Sale, 1992). Twenty 0.5 ml fractions were collected from the bottom of the gradient and prepared for gel electrophoresis. The sedimentation values were determined using 12 S and 21 S dynein and catalase (11.3 S) as standards. Gradient fractions were analyzed by gel electrophoresis with Coomassie Blue staining and immunoblots using either control antibodies or affinity-purified, peptide antibodies as described below.

Gel electrophoresis and immunoblots

Flagella, axonemes and extracts were solubilized for electrophoresis in sample buffer and run on mini 7% Laemmli gels. The gels were either stained with Coomassie Blue or prepared for immunoblot analysis. For immunoblots, proteins were transferred to nitrocellulose filters at 1 amp for 2 hours. The nitrocellulose was then blocked for 1 hour at room temperature in 3% gelatin in TBS with a subsequent wash in TBS and 0.05% Tween-20. The blot was incubated in primary antibodies, diluted in 1% gelatin in TBS-0.05% Tween-20, for 1 to 2 hours at room temperature or overnight at 4°C. Following washes in TBS/Tween-20, alkaline phosphatase-conjugated secondary antibodies (Bio-Rad Laboratories Inc., Hercules, California) were diluted 1:3000 in 1% gelatin in TBS-0.05% Tween-20 and incubated with blots for 1 hour at room temperature. The alkaline phosphatase substrate kit (no. 170-6460) from Bio-Rad was used for color development. Preimmune sera or secondary antibodies alone were used as controls and did not show any staining.

Antibody production and purification

Antibodies against the two decapeptides, LNLVDLAGSE (LAGSE) and HIPYRESKLT (HIPYR), were generated and affinity-purified on peptide columns as described by Sawin et al. (1992a).

Microtubule binding assays

Taxol-stabilized microtubules were prepared from purified bovine brain tubulin as previously described (Sale and Fox, 1988). Microtubules were assembled at 37°C for 15 minutes from 5 mg/ml 6 S tubulin in a buffer (PM) containing 0.1 M PIPES, pH 6.9, 2 mM $MgSO_4$, 1 mM EGTA and 1 mM GTP. The microtubules were stabilized by adding an equal volume of PM containing 20 μM taxol (generous gift from Drug Synthesis Branch, Division of Cancer Treatment, NCI), sedimented in an Airfuge (Beckman Instruments, Palo Alto, California) at 100,000 ***g*** for 5 minutes and resuspended at a protein concentration of 1 mg/ml in buffer A containing 20 μM taxol. For rebinding experiments, axonemes were further extracted with buffer A containing 10 mM MgATP and 0.6 M NaCl to remove any residual dynein arms and 97 kDa proteins, sedimented at 12,000 ***g*** for 15 minutes (SS-34 rotor, DuPont, Sorvall) and resuspended at 1 mg/ml protein in buffer A. Microtubules or extracted axonemes were mixed with an equal volume of the peak fractions (~0.7-0.8 mg/ml) from the sucrose gradient, either 10 mM MgATP or 4 mM AMP-PNP was added, and microtubule binding was examined by light microscopy, or by sedimenting in an Airfuge at 100,000 ***g*** for 5 minutes and preparing the resulting pellets and supernatants for gel electrophoresis.

Materials and cell strains

Deionized water was used throughout. ATP was from Boehringer Mannheim Diagnostics Inc. (Houston, TX). All other reagents were from Sigma Chemical Co. (St Louis, MO). Mutant strains of *Chlamydomonas* were obtained from the *Chlamydomonas* Genetics Center (E. H. Harris, Duke University), and the motility and axonemal structural phenotype were verified by light and electron microscopy, respectively.

RESULTS

Immunochemical evidence for kinesin-related proteins in flagella

To identify kinesin-related proteins in flagella, we used the affinity-purified polyclonal antibodies to the LAGSE and HIPYR peptides corresponding to conserved sequences in the motor domain of kinesin as described by Sawin et al. (1992a). As illustrated in Fig. 1, both antibodies reacted with flagellar proteins with masses of about 96, 97, 110 and 125 kDa, a size characteristic of kinesin or kinesin-like protein heavy chains. These proteins were also recognized by affinity-purified antibodies to the kinesin heavy chain motor domain (Rodionov et al., 1991) and affinity-purified antibodies to peptides kin1-3 (Cole et al., 1992) (data not included). In addition, anti-LAGSE antibodies recognized additional proteins of ~45 kDa and >200 kDa (Figs 1 and 2A), and the anti-HIPYR antibody recognized additional proteins of 130 and 150 kDa (Fig. 1), but these proteins were not studied further. On the basis of the coincident recognition of the 96, 97, 110 and 125 kDa proteins with multiple antibodies to different conserved sequences from the kinesin superfamily, we were confident that these immunoreactive proteins included kinesin-related proteins.

Localization of immunoreactive, kinesin-related proteins in flagella

To determine the location of immunoreactive proteins in

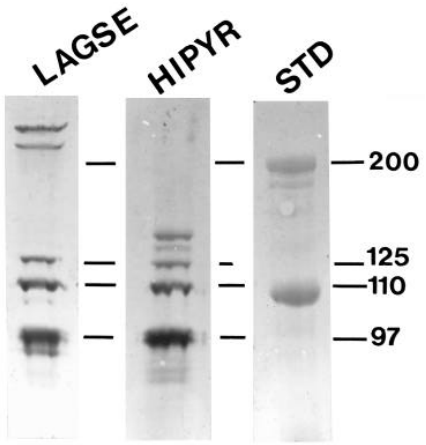

Fig. 1. Immunoblots of proteins from flagella isolated from wild-type *Chlamydomonas* incubated with anti-LAGSE and anti-HIPYR antibodies (see Materials and Methods), each revealing a similar pattern of proteins with masses of 96, 97, 110 and ~125 kDa. Anti-LAGSE antibodies also reacted with higher molecular mass proteins (>200 kDa) and at least one lower molecular mass protein (see Fig. 2A), and anti-HIPYR antibodies reacted with proteins of 130 and 150 kDa.

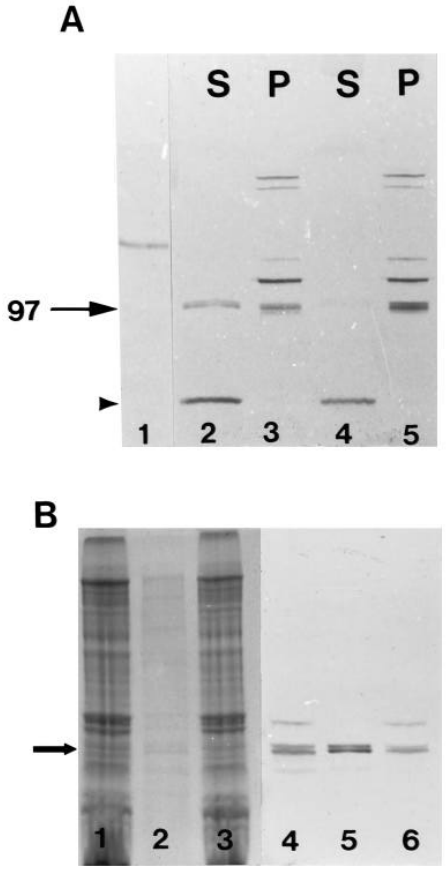

Fig. 2. (A) Immunoblot of flagellar fractions using the anti-LAGSE antibody, illustrating selective solubilization of proteins with a mass of ~97 kDa in buffer containing 1 mM MgATP and 0.5% Nonidet P-40; lane 2, ATP extract; lane 3, resulting axonemal pellet. In contrast, and with the exception of one lower molecular mass protein (arrowhead), all immunoreactive proteins were retained in the axonemes in the presence of detergent and AMP-PNP; lane 4, extract; lane 5, resulting axonemal pellet. Lane 1 is partially purified kinesin heavy chain from sea urchin eggs with a mass of ~130 kDa. (B) Coomassie-stained gel and corresponding immunoblot, using the anti-LAGSE antibody demonstrating selective solubilization of the 97 kDa (arrow) immunoreactive proteins in 10 mM ATP. Lane 1, Coomassie-stained axonemal fraction; and lane 4, the corresponding immunoblot. Lanes 2 and 5, the ATP extract. Lanes 3 and 6, resulting pellet. Protein loads were 20 μl, of approximately 8 mg/ml.

flagella, we used two complementary approaches: analysis of flagellar fractions and analysis of mutant strains of *Chlamydomonas* with defined defects in axonemal structures. First, the distribution of immunoreactive proteins in flagella was examined by fractionation of flagella into axonemal and membrane-matrix components. Fig. 2A, lanes 4 and 5, show that all immunoreactive proteins except the 45 kDa species (arrowhead), remain associated with the axoneme after flagella are extracted with a buffer containing Nonidet P-40 and AMP-PNP (see Brady, 1985; Vale et al., 1985). In contrast, partial but highly selective extraction of the 96 and 97 kDa proteins (the 97 kDa proteins) occurred in the Nonidet buffer containing 1 mM MgATP (Fig. 2A, lanes 2 and 3). The 97 kDa immunoreactive proteins were partially purified by isolating axonemes in the presence of AMP-PNP and subsequent selective and nearly complete solubilization with 10 mM MgATP (Fig. 2B, lanes 2 and 5). Other immunoreactive proteins remain associated with the axoneme in ATP, and using duplicate blots the same results were demonstrated with the anti-HIPYR or kin1-3 antibodies (data not shown). We also discovered that the 97 kDa proteins, as well as a small portion of the 110 kDa protein, could be partially extracted in 600 mM NaCl. Furthermore, partial solubilization of both proteins could be achieved upon extraction in a combination of 1 mM ATP and 600 mM NaCl. However, extraction of axonemes in ATP alone was selective for the 97 kDa proteins. Purification and detailed characterization of the 97 kDa proteins are described below.

We analyzed flagella and axonemes by immunochemical analysis from a selection of mutant strains of *Chlamydomonas* with defects in axonemal structure (Huang, 1986). Using both the anti-LAGSE and anti-HIPYR antibodies, immunoblots did not reveal differences in flagella or axonemes for the 97, 110 or 125 kDa proteins from wild-type cells (*137*) and mutants with defective assembly of dynein arms (*pf28, pf30, pf30pf28*) or radial spokes (*pf14*). In contrast, the immunoreactive band with a mass of ~110 kDa was either missing or greatly reduced in flagella and axonemes from mutant strains with defective central microtubule pair assembly (Fig. 3). The same results were obtained in five separate experiments, and axonemal structure from mutant cells was verified by electron microscopy.

Characterization of microtubule binding of 97 kDa proteins

Solubilization of the 97 kDa proteins permitted study of both their molecular structure and their ability to rebind microtubules in a nucleotide-dependent fashion. Extracts were fractionated by zonal centrifugation on sucrose gradients to partially purify the 97 kDa proteins. The 97 kDa proteins co-sedimented at ~9

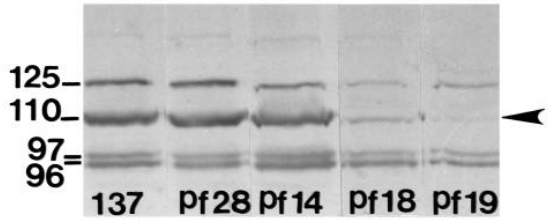

Fig. 3. Immunochemical analysis of axonemes from several mutant strains of *Chlamydomonas.* The strains examined are 137 (wild type), *pf28* (missing the outer dynein arms), *pf14* (missing the radial spokes), and *pf18* and *pf19* (lacking the central pair microtubules in isolated axonemes). The 110 kDa band (arrowhead) is found to be either missing or reduced in the central pair mutants. Equal amounts of protein were loaded for each sample. Equal transfer of axonemal proteins to nitrocellulose was verified by protein staining of blots prior to immunostaining.

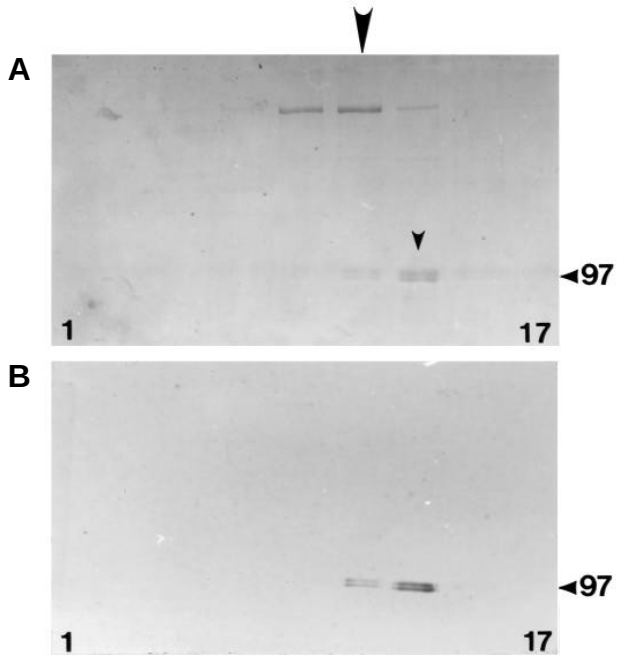

Fig. 4. Coomassie-stained gel (A) and immunoblot (B) of the odd-numbered fractions (1-17) from a 5% to 20% sucrose gradient of the ATP extract, demonstrating co-sedimentation of the 97 kDa proteins at ~9 S (small arrowhead). Inner arm dynein sediments at 11 S (large arrowhead).

S (Fig. 4), a sedimentation rate consistent with that expected for kinesin (Bloom et al., 1988; Hackney et al., 1992).

Reassociation of the 97 kDa fractions with microtubules required the addition of AMP-PNP. This was demonstrated by incubation of gradient fractions with either extracted axonemes or purified microtubules in the presence or absence of nucleotides, as described in Materials and Methods (Fig. 5A,B). In each case the 97 kDa fraction bound to the microtubules only when AMP-PNP was present (Fig. 5A,B, lanes 1 and 2) and in each case 10 mM MgATP induced complete release (Fig. 5A,B, lanes 3 and 4). The presence of apyrase had no effect on the binding of the 97 kDa complex to microtubules, indicating that binding was not due to contaminating

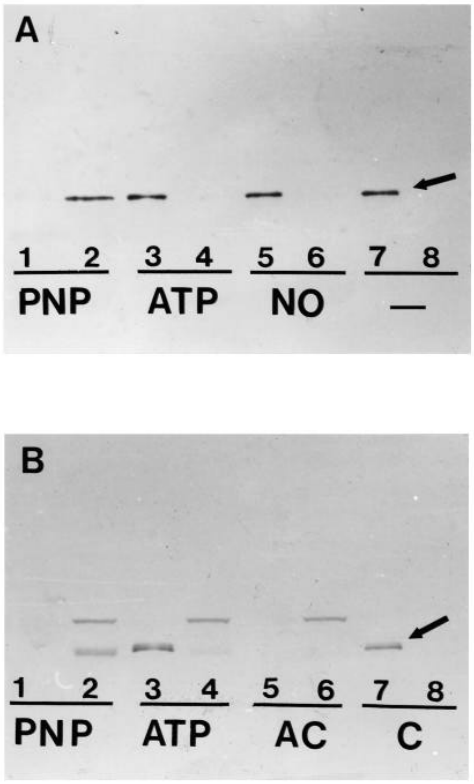

Fig. 5. Immunoblots illustrating that the binding of the 97 kDa protein (arrows) to purified microtubules (A) or extracted axonemes (B) is dependent on the presence of AMP-PNP. Subsequent addition of ATP induced release of 97 kDa proteins from microtubules or axonemes. (A) Samples include: lanes 1 and 2, supernatant and pellet of a mixture of microtubules and sucrose gradient peak fraction (see Fig. 4) in 1 mM AMP-PNP (PNP); lanes 3 and 4, supernatant and pellet following ATP-induced release (ATP); lanes 5 and 6, supernatant and pellet of microtubules and gradient peak fraction with no added nucleotide (NO); lanes 7 and 8, supernatant and pellet of sucrose gradient peak fraction in the absence of microtubules (–). (B) Samples include: lanes 1 and 2, supernatant and pellet of a mixture of extracted axonemes and sucrose gradient peak fraction in the presence of 1 mM AMP-PNP (PNP); lanes 3 and 4, supernatant and pellet following ATP release (ATP); lanes 5 and 6, supernatant and pellet of extracted axoneme control (AC); lanes 7 and 8, supernatant and pellet of sucrose gradient peak fraction /buffer control (C).

ADP or ATP (data not shown). Thus, based upon immunochemical sedimentation and nucleotide-dependent microtubule binding characteristics, the 97 kDa proteins are likely to be kinesin-related microtubule-associated proteins. Light and electron microscopic analysis did not reveal cross-linking or bundling of the microtubules when bound to the 97 kDa proteins (data not shown).

DISCUSSION

We have used an immunochemical approach in the search for kinesin or related proteins in flagella. The strategy was to probe immunoblots of flagella and flagellar fractions (e.g. axonemes and the membrane matrix components) with affinity-purified

antibodies to different, non-overlapping and conserved peptides in the kinesin motor domain. This strategy has been successful for identifying novel kinesin-related proteins in eggs of sea urchin and frogs (Cole et al., 1992; Sawin et al., 1992a,b). In *Chlamydomonas* flagella, proteins with masses of 96, 97, 110 and 125 kDa were found to react with both the anti-LAGSE and anti-HIPYR antibodies, and this same set of proteins also reacted with two other peptide antibodies also raised against the kinesin motor domain. Based upon coincident immunoreactivity with affinity-purified antibodies to distinct but conserved structures, these proteins are excellent candidates for kinesin-related proteins. As previously shown by Wright et al. (1991), none of the flagellar proteins reacted with the monoclonal antibody, SUK4, shown to bind to conventional kinesin (Ingold et al., 1988). Therefore these putative kinesin-related proteins are not likely to be conventional kinesins. Further, the kinesin-related proteins found in *Chlamydomonas* flagella have similar properties to kinesin-related proteins found in sea urchin eggs (Cole et al.,1992). It is possible that some of the sea urchin proteins are precursers to embryonic cilia.

In addition to the four polypeptides described above, anti-LAGSE reacted with proteins with masses of 45 and >200 kDa, and anti-HIPYR reacted with additional proteins of about 130 and 150 kDa. The 45, 130, 150 and >200 kDa proteins were not studied further because they did not react with multiple peptide antibodies, and they were not soluble, were not diminished in mutants with axonemal structural defects, and did not bind microtubules in a nucleotide-dependent fashion. The axoneme contains over 200 peptides (Huang, 1986) and multiple structures that associate with microtubules. However, little is known about the molecular mechanisms responsible for microtubule association. It is attractive to speculate that the microtubule binding domain in kinesin has been adapted to serve as a means to bind diverse structures to axonemal microtubules, independent of motor activities. These kinesin-related domains may retain epitopes recognized by a select group of peptide antibodies.

One provocative result was the observation that the ~110 kDa immunoreactive kinesin-related protein is associated with the central pair. This conclusion is based upon analysis of flagellar and axonemal proteins from a wide selection of mutant *Chlamydomonas* strains with structural defects in the axoneme. As shown in Fig. 3, the 110 kDa component is reduced in *pf18* and *pf19*, two mutants shown to be missing the central pair complex in isolated axonemes (Adams et al., 1981). Johnson and Rosenbaum (1994) have localized this protein to the central pair by immunological methods. Further, as described in results, the 110 kDa protein can be partially solubilized by extraction of wild-type axonemes in 600 mM NaCl, a procedure that partially solubilizes central pair components (Dutcher et al., 1984) and structures (Piperno and Luck, 1979). The simplest interpretation is that the 110 kDa protein is associated with the central pair.

The 97 kDa proteins have several characteristics that suggest they are members of the kinesin superfamily. In addition to their reactivity with multiple peptide antibodies, the 97 kDa proteins bind to microtubules with a nucleotide-dependence characteristic of kinesin (Brady, 1985; Vale et al., 1985); they bind to the axoneme when isolated in the presence of AMP-PNP, but become solubilized when axonemes are isolated in the presence of or extracted in ATP. The solubilized 97 kDa proteins co-sediment in sucrose gradients at about 9 S, a sedimentation rate similar to that for kinesin in the buffer condition employed (Bloom et al., 1988; Hackney et al., 1992). From this result it is predictable that the 96 kDa and 97 kDa proteins form a dimeric complex. The partially purified 97 kDa proteins also re-bind microtubules or axonemes in an AMP-PNP dependent manner, and are released upon addition of ATP (Fig. 5). Thus, in addition to immunochemical evidence, microtubule binding characteristics strongly indicate that the 97 kDa proteins are members of the kinesin superfamily. Although we do not yet know the location in the axoneme, these proteins may be candidate molecular motors for intraflagellar transport (Kozminski et al., 1993).

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