Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 2643–2647, April 1988
Cell Biology

Evidence for tektins in centrioles and axonemal microtubules

(basal body/cilia/flagella/intermediate filament)

W. STEFFEN AND R. W. LINCK

Department of Cell Biology and Neuroanatomy, University of Minnesota, Minneapolis, MN 55455

Communicated by Aaron Klug, January 14, 1988

ABSTRACT **Affinity purified, polyclonal antibodies were prepared against three tektins (tektins A, B, and C) isolated from sea urchin sperm axonemal microtubules. These antibodies (anti-tektins) were used to localize tektins in axonemes, basal bodies, and centrioles. By immunofluorescence microscopy it could be demonstrated that in sperm tails from *Lytechinus pictus* all three anti-tektins stain all nine axonemal doublet microtubules and A-tubule extensions along their entire length. In addition to staining doublet microtubules, anti-tektin C weakly labeled the central-pair microtubules in sperm tails from *Patiria miniata*. The anti-tektin staining revealed also a clear cross-reaction with basal bodies of sea urchin sperm and with centrioles of cells from hamsters, humans, and pigs. These data provide evidence of tektin or tektin-like proteins in basal bodies and centrioles and suggest that centriole microtubules are constructed according to the same principles as flagellar microtubules.**

Centrioles are important cell organelles in nearly all animal cells and in some plant cells (1, 2). Aside from the clear presence of tubulin in centriolar microtubules (3, 4), their molecular composition is poorly understood. Several antibodies have been reported to cross-react with centriole components (4–7), but the nature and function of these components in relation to the microtubules have not been determined. Perhaps the most basic function of the centriole, in the capacity of a basal body, is to act as a template for the assembly of ciliary and flagellar axonemes (1, 2, 8–10). In particular, the A- and B-subfibers of centriolar triplet microtubules are templates for the assembly of the A- and B-subfibers of ciliary and flagellar doublet microtubules. Because of the relationship between axonemes and centrioles, we have been investigating whether centrioles might share proteins with doublet microtubules, about which considerably more is known.

In related investigations we have found that doublet microtubules from sea urchin sperm flagella can be extracted free of tubulin, leaving filaments, 2–6 nm in diameter, that are composed predominantly of a set of proteins named tektins (11–13). Three distinct, but related, tektins with relative molecular masses between 46 kDa and 57 kDa have so far been characterized. The tektins are different from tubulin yet strikingly similar to intermediate filament proteins in terms of their solubility properties, molecular masses, amino acid composition, fibrous substructure, high α-helical content, and immunological determinants (11–16). Cross-reactivities with polyclonal antibodies to a mixture of tektins indicate the presence of homologous proteins in echinoderm embryonic cilia and molluscan gill cilia (17). Chemical fractionation and immuno electron microscopy studies further suggest that tektins are components of the A-subfibers of doublet microtubules (12, 18), but it has not been determined whether all three tektins are present in each of the flagellar microtubules.

We have prepared and characterized a set of polyclonal antibodies to each of the three tektins (19). In our present report we have used these antibodies to investigate the organization of tektins in the doublet microtubules of sea urchin sperm and to study whether tektins are conserved constituents of centrioles. In light of our findings, we discuss possible functions of tektins for centrioles and microtubules in general. Preliminary reports of this investigation have been presented (16, 20).

MATERIALS AND METHODS

Detailed methods for the purification of proteins and the preparation and characterization of antibodies are described elsewhere (13, 19). Tektin filaments were prepared by extraction of purified *Lytechinus pictus* sperm flagella axonemes with 0.5% sodium dodecyl sarcosinate/2 M urea/50 mM Tris lysine/1 mM EDTA, pH 8, at 4°C. The three major tektin polypeptides were electrophoretically purified by $NaDodSO_4$/PAGE and used to immunize rabbits. Specific antibodies to each tektin were purified by using $NaDodSO_4$-denatured tektin filaments (composed of all three tektins) as the affinity probes. Fab fragments were prepared by using papain as described by Mage (21). Protein A-Sepharose CL-4B was used to separate Fab fragments from uncleaved IgG and Fc fragments. $NaDodSO_4$/PAGE and immunoblotting were carried out as described by Laemmli (22) and Towbin *et al.* (23) with modifications by Linck *et al.* (19). The $NaDodSO_4$ used was electrophoresis purity reagent from Bio-Rad. The polyclonal anti-tubulin was obtained from Polyscience (Warrington, PA).

For immunofluorescence microscopy the staining/detection system is described elsewhere (19); it employed biotin-conjugated goat anti-rabbit IgG, rhodamine-conjugated avidin, and hypersensitized Kodak Tech Pan film. Mammalian cell lines [Chinese hamster ovary cells (CHO), HeLa cells, and pig kidney cells ($LLCPK_1$)] were obtained from R. Kuriyama (University of Minnesota) and grown on cover slips. Splayed axonemes specimens were prepared as follows: a drop of sperm diluted in sea water or Ca^{2+}-free sea water was placed on Parafilm, overlaid with a coverslip, and incubated for 2 min at room temperature. This procedure caused the sperm tails to splay into individual axonemal microtubules that remained connected at the basal body in the sperm head. Mammalian cells and sperm with splayed tails were fixed by immersing the coverslips in methanol at −20°C. All anti-tektins were used at the following concentrations: 50 ng/ml for immunoblot and 10–20 μg/ml for immunofluorescence.

Preabsorption control experiments were conducted in the following two ways: (*i*) Purified tektin filaments (composed of all three tektins) were mixed with a given anti-tektin, to final concentrations of 0.5 mg/ml and 10–20 μg/ml, respectively, incubated at room temperature for 1 hr, and centrifuged at 400,000 × *g* for 30 min. The supernatant was then

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used for immunofluorescence microscopy. (*ii*) Tektins were resolved by $NaDodSO_4$/PAGE and electroblotted onto a nitrocellulose sheet. The sheet was then cut into three horizontal strips corresponding to the various tektins. After blocking with 3% (wt/vol) bovine serum albumin in 10 mM Tris·HCl/0.15 M NaCl, pH 7.5, for 2 hr at room temperature, purified anti-tektins at 10–20 μg/ml were incubated with the appropriate strips. The undiluted, unbound fraction was tested by immunoblotting, and incubation was continued until only a weak immunoblot staining was detectable. The absorbed fraction, affinity-purified anti-tektins eluted from the nitrocellulose sheet, was then used for immunofluorescence microscopy.

RESULTS

We have characterized the flagellar tektins from two species of sea urchins, *L. pictus* and *Stronglyocentrotus purpuratus*. Molecular mass determinations by $NaDodSO_4$/PAGE revealed slight differences between the tektins, as shown in Table 1.

Affinity-purified polyclonal antibodies were prepared against each of the three tektins from *L. pictus* and *S. purpuratus* and characterized, as reported elsewhere (19). Summarizing that investigation, preimmune sera from five of the six rabbits used to raise antibodies showed no detectable staining of axonemal proteins by immunoblotting; a sixth preimmune serum showed only a faint cross-reaction with one ≈50-kDa polypeptide. Immune sera showed strong and relatively monospecific reactions with their respective tektins and did not cross-react with polypeptides above or below the tektin region on the immunoblot (19). To eliminate contamination of antibodies that did not cross-react with the tektins, we purified the specific anti-tektins by using $NaDodSO_4$-denatured tektin filaments as the affinity probe. For the present investigation we have used exclusively the affinity-purified anti-tektins; immunoblot specificities for anti-tektins raised against *L. pictus* proteins are shown in Fig. 1, and the specificities and cross-reactivities of the anti-tektins within each species and between species are summarized in Table 1. On the basis of their molecular masses and immunological relatedness, the tektins can be categorized; for simplicity we refer to these as tektins A, B, and C.

To localize tektins within the axonemes and basal bodies of sea urchin sperm, specimens were examined by immunofluorescence microscopy. We found that sperm of *L. pictus* could be prepared so that the axonemal microtubules were splayed. By immunofluorescence, anti-tubulin antibody staining of sperm prepared in this manner revealed 9 filaments 45 μm long (Fig. 2*A*), which presumably correspond to the nine doublet microtubules. If the central singlet microtubules were also preserved, one would expect to see 11 filaments in such splayed preparations (24, 25); however, the central microtubules were dissolved, most likely by the high salt concentration of sea water.

Table 1. Comparative molecular masses and immunological relatedness of tektins

Tektin	Apparent molecular mass, kDa	
	L. pictus	*S. purpuratus*
A	56–57	55
B	51–52	51
C	46	47

Tektins are arranged in three groups, A, B, and C, in descending order of apparent molecular mass, based on comparative $NaDodSO_4$/PAGE. Anti-tektins are primarily monospecific. Anti-tektins from one species strongly cross-react with only the same tektin type in the other species; thus, the tektins are categorized by similarities in molecular mass and immunological cross-reactivities. Note that anti-tektin C cross-reacts weakly with tektin A within the same species (see Fig. 1 and ref. 19) as well as between species (data not shown).

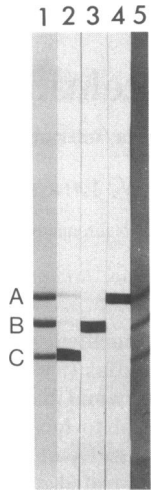

FIG. 1. $NaDodSO_4$/PAGE immunoblot specificities of anti-tektins. Proteins from *L. pictus* tektin filaments are shown on an $NaDodSO_4$/polyacrylamide gel stained with Serva blue (lane 1). The major polypeptide bands (from top to bottom) are tektin A (56–57 kDa), tektin B (51–52 kDa), and tektin C (46 kDa). After transferring tektins to nitrocellulose, strips were stained with affinity-purified anti-tektin C (lane 2), anti-tektin B (lane 3), anti-tektin A (lane 4), and amido black (lane 5). The anti-tektins were primarily monospecific, and only anti-tektin C cross-reacted weakly with tektin A. A full characterization of these anti-tektins has been reported (19).

Staining of splayed flagella with the three anti-tektins (Fig. 2*B*) revealed the same pattern of nine filaments 45 μm long, lengths identical to that of the intact sperm tails. All three anti-tektins were used at the same concentration and yielded identical immunofluorescence patterns of essentially equal staining intensity, suggesting that all three tektins are present along the entire length of each doublet microtubule and A-tubule extension. In addition to staining doublet microtubules, all anti-tektins intensely labeled the basal bodies (Fig. 2*B*).

Because the central-pair microtubules were not preserved in specimens prepared from the sea urchin species used, the presence or absence of tektins in these microtubules was ambiguous. It is known that in some species the central pair microtubules are relatively stable (26). Thus, we examined other species in which the central tubules might be more stable even after splaying. Such was the case with the batstar *Patiria miniata*. In splayed preparations nine brightly stained filaments (i.e., doublet tubules) and two weakly stained filaments (i.e., central-pair tubules) are seen after treatment with anti-tubulin (data not shown) or anti-*L. pictus* tektin C (Fig. 3). These results suggest that the central-pair microtubules may also contain tektin-like polypeptides; the fainter central-pair staining compared to the doublet-tubule staining may reflect a difference in the type or quantity of the central-pair tektin-like antigens.

Since tektin-like proteins are present in basal bodies of sperm flagella, one might also expect to find tektins in centrioles. To study these organelles in higher organisms, we investigated CHO, HeLa, and $LLC\text{-}PK_1$ cells. By immunofluorescence microscopy, affinity-purified anti-tektins stained paired dot-like structures, either juxtanuclear and in the center of microtubule asters or at spindle poles (Fig. 4). The number of the dots was cell-cycle-dependent: i.e., one pair was typically seen in interphase cells and two pairs in mitotic cells. These data indicate that the stained dots

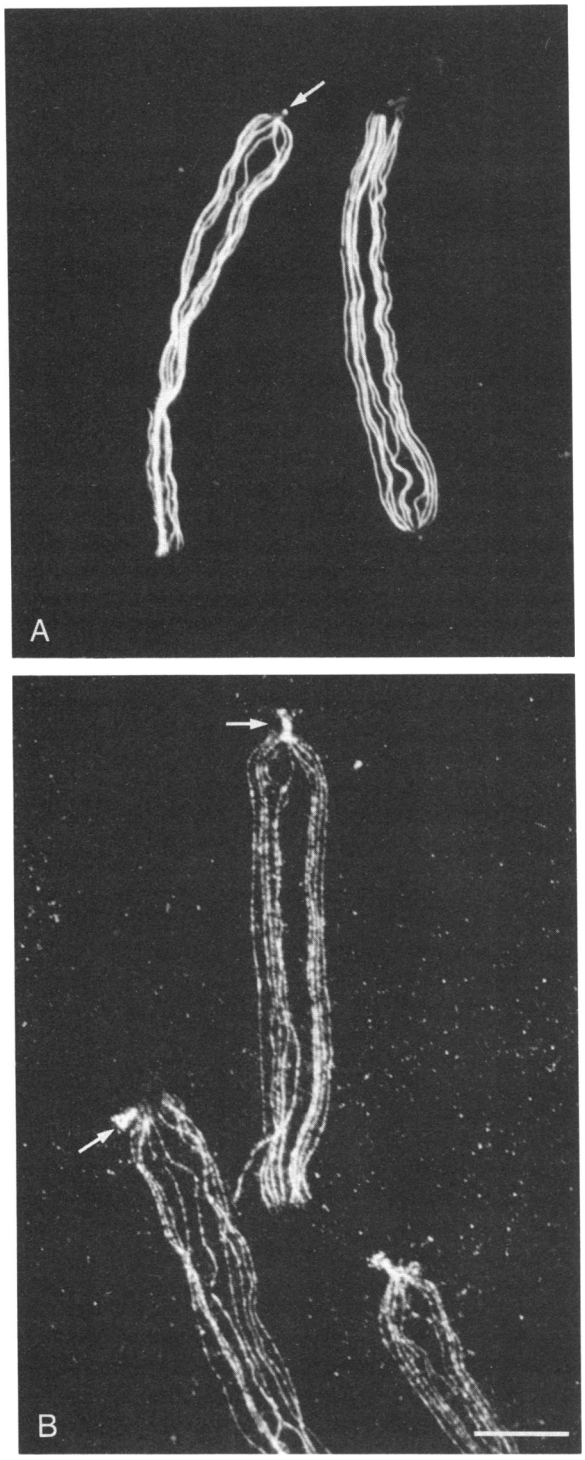

FIG. 2. Immunofluorescence microscopy of splayed sperm tails of *L. pictus* with polyclonal anti-tubulin (*A*) and affinity-purified anti-*L. pictus* tektin B (*B*) is shown. Labeling of the splayed axonemes with anti-tubulin (*A*) revealed a pattern of nine filaments with lengths identical to the length of intact sperm tails. These nine filaments, i.e., the nine outer-doublet microtubules and A-tubule extensions, merge at one end in a dot-like structure (arrow). Based on the location in the sperm and labeling with anti-tubulin, these dots represent basal bodies. All three anti-*L. pictus* tektins also stained the basal bodies and the nine doublet microtubules along their length (*B*). When applied at the same concentrations, anti-tektins A, B, and C (10–20 μg/ml) produced essentially identical staining intensities. The punctuated staining, as seen with anti-tektin B, may result from a partial masking of tektin by tubulin (12, 19). Compared to the staining of basal bodies with anti-tubulin, the labeling with anti-tektin is much more intense. (Bar = 10 μm.)

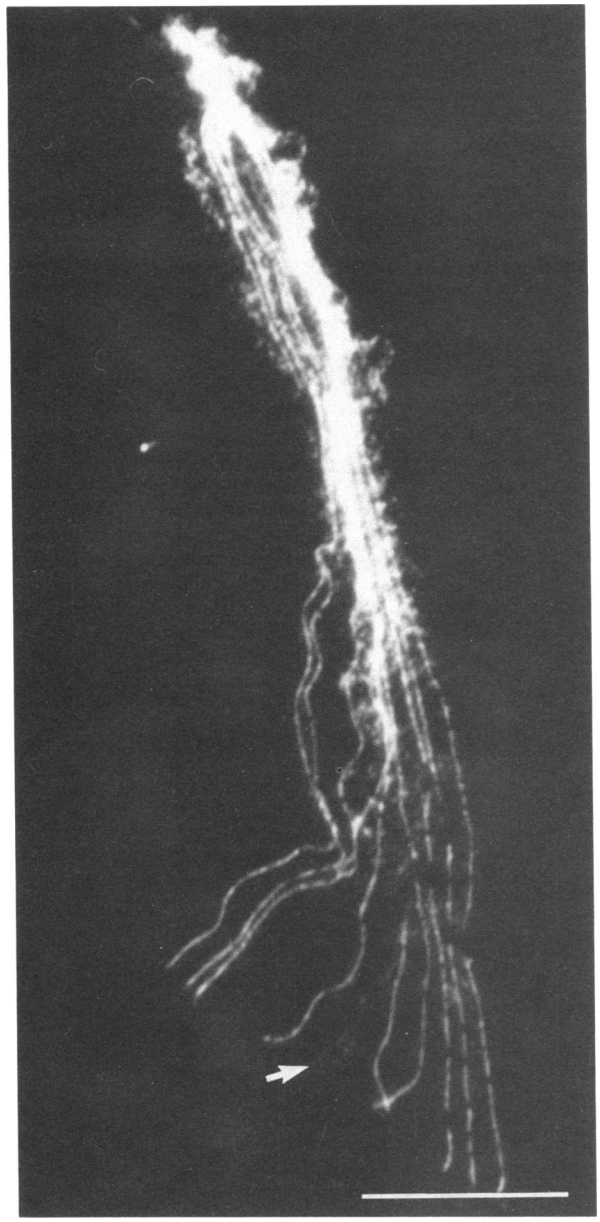

FIG. 3. Immunofluorescence staining of splayed sperm tails from *P. miniata* stained with anti-tektin C. In addition to labeling of the nine outer-doublet microtubules (compare with Fig. 2) anti-tektin C also stained the central-pair microtubules. The fainter staining of the central pair may reflect a difference in type or quantity of the tektin-like antigens in these microtubules. (Bar = 10 μm.)

correspond to centrioles. By using various anti-tektins, centriole staining was observed in all three cell lines: i.e., all anti-tektins (against *L. pictus* and *S. purpuratus* tektins A, B, and C) stained LLC-PK_1 cells, anti-*L. pictus* tektin C stained CHO and HeLa cells, and anti-tektin A (against *L. pictus* and *S. purpuratus* tektin A) stained CHO cells.

To control the specificity of the centriole staining, the anti-tektins were preabsorbed with either whole tektin filaments or the respective purified tektin. Centriole staining was not observed, when preabsorbed anti-tektins were used. Hansson *et al.* (27) described an Fc-specific interaction with vimentin, an intermediate filament protein. Because tektins are similar to intermediate filament proteins (13) and because certain polyclonal anti-tektins cross-react with intermediate filament proteins (16), Fab fragments were used to eliminate the possibility for Fc-specific interactions. Staining with Fab fragments prepared from each anti-tektin yielded images

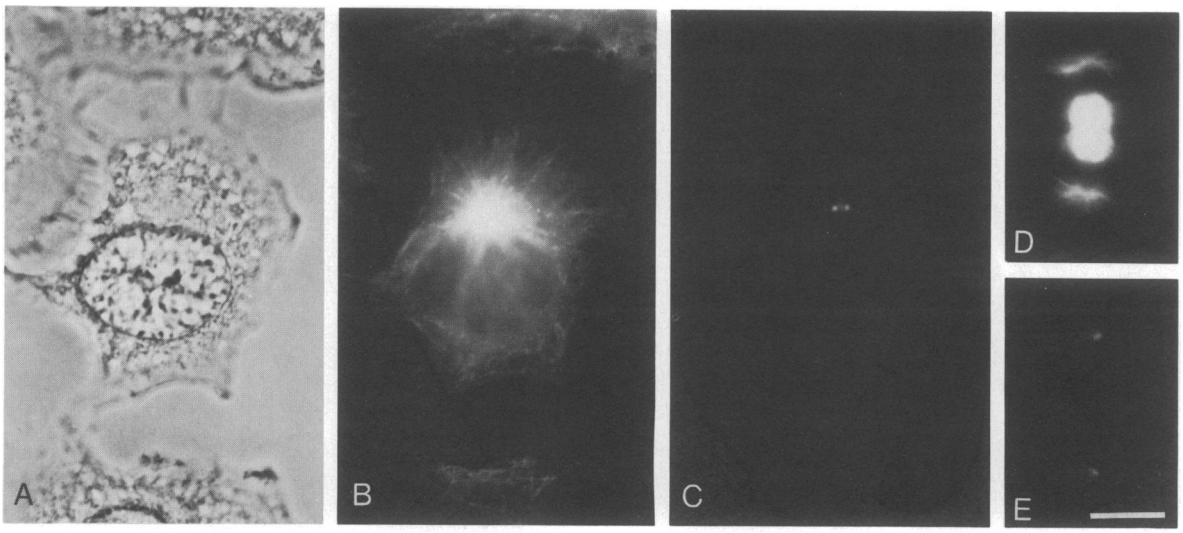

FIG. 4. Immunofluorescence staining of HeLa cells. (*A*) Phase-contrast image of an interphase cell. (*B*) Same cell stained with anti-tubulin, showing a radial pattern of microtubules. (*C*) Same cell stained with anti-tektin C, showing a pair of juxtanuclear dots. (*D*) A late telophase cell stained with anti-tubulin, showing the aster and developing midbody microtubules. (*E*) Same cell stained with anti-tektin C, showing stained dots at the spindle poles. These dots appear as pairs in the centers of aster-like microtubule arrays of interphase cells (*A–C*) and at the spindle poles of mitotic cells (*D–E*), where only one dot of each pair is in the plane of focus. Because of their location, small size, and normally paired appearance, we conclude that these dots correspond to centrioles, not to centrosomes. The concentration of anti-tektins used was the same as for the staining of axonemal microtubules and basal bodies in Fig. 2. Similar staining patterns were obtained with other anti-tektins and with CHO and LLC-PK_1 cells (see text). (Bar = 10 μm.)

(data not shown) similar to those described for uncleaved anti-tektins.

DISCUSSION

The results above demonstrate two major points: (*i*) all three anti-tektins bind to each axonemal doublet microtubule and its A-tubule extension throughout their lengths; (*ii*) all three anti-tektins strongly recognize tektin-like components in sea urchin basal bodies and mammalian centrioles. In addition, anti-tektin C weakly stained the central-pair microtubules of the batstar *P. miniata*. The presence of tektins in flagella and centrioles might correlate with specific features that these microtubule systems have in common: i.e., a high degree of stability and species-specific length (2).

Microtubule stability is controlled by factors that affect the assembly–disassembly kinetics of tubulin (28). As integral proteins in flagella and perhaps centrioles, tektins may participate in regulating microtubule stability. Previous results (12, 18) and those presented here suggest that the tektins exist as filaments, or possibly protofilaments, in the walls of the A-tubule. A 48-nm axial periodicity was measured for tektin filaments by monoclonal antibody labeling (29). In addition, a 96-nm helical repeat was noted in the tektin–tubulin protofilament domain of flagellar microtubules (11). Both of these repeats are fundamental orders in optical and computed Fourier transforms of flagellar microtubules (30, 31); the proposed helical structure of the tektin filament based on these parameters (11, 29) provides for an axial spacing of 4.00–4.08 nm, which matches that of the tubulin subunit lattice (29–33). Thus, whereas a direct molecular interaction between tubulin and tektins has not yet been demonstrated, it is reasonable to suppose that such an interaction could occur and could affect microtubule stability. Tektins, existing either as specific microtubule protofilaments or as smaller fibrils in "seams" between certain tubulin protofilaments (32, 34), would be suitably positioned to stabilize interactions between adjoining tubulin subunits and other microtubule-associated proteins.

Tektins may also be involved in the assembly of ciliary microtubules. In studies of ciliary regeneration in sea urchin embryos (*Strongylocentrotus droebachiensis*), Stephens (35) reported that, following deciliation, regenerated cilia contain one polypeptide in particular (component 20) that is synthesized *de novo* in limited amounts, as determined by pulse–chase experiments; additional synthesis of this protein is required in the second round of regeneration. Component 20 (55 kDa) had been shown to be a major component of the chemically resistant protofilament domain of the A-tubule (18), and it has also been shown to be recognized by our anti-*S. purpuratus* tektin A (36). Furthermore, this anti-tektin A has been used to select a λgt11 cDNA clone from sea urchin embryos that hybridizes with a mRNA of an appropriate length to code fully for tektin A (37). Stephens originally postulated that the quantal *de novo* synthesis of component 20 (tektin A) was consistent with it being a factor involved in the elongation or length determination of ciliary microtubules. Our finding that tektin A is present along the length of each doublet microtubule and A-tubule extension supports such a hypothesis.

Finally, the immunofluorescence staining of basal bodies and centrioles with anti-tektins provides evidence that these microtubule organelles contain tektins. (*i*) The anti-tektins used to stain centrioles were affinity-purified, (*ii*) the immunological specificities resided with the Fab fragments, (*iii*) two types of preabsorption with tektins effectively blocked anti-tektin staining of centrioles, and (*iv*) centriole staining was observed, when various anti-tektins were applied to three mammalian cell lines. On the other hand, an immunoblot identification of the tektin-like components in basal bodies and centrioles appears to be more difficult. We have found that on immunoblots of mammalian cells the anti-tektins recognize several polypeptides in the region between 45 kDa and 70 kDa (ref. 16; unpublished observations). Yet, by immunofluorescence microscopy, certain anti-tektins, besides binding to centrioles, cross-react with other microtubule- and intermediate filament-like structures (16). Thus, it is not yet possible biochemically to determine whether the cross-reacting polypeptides arise solely from centrioles or also from cytoskeletal filaments.

Other reports have described the association of various components with basal bodies and centrioles (4–7), but, with

the possible exception of the localization of a purine nucleoside phosphorylase (5), the functional nature of these components is unexplained. However, it should not be surprising to find flagellar microtubule proteins other than tubulin in centrioles, since A-fibers of flagellar doublet tubules are continuous with the A-fibers of centriolar triplet microtubules (8). Considering the synthesis and possible role of tektin A in ciliogenesis, a similar pattern might be expected in the development of primary cilia, the replication of centrioles and the regulation of their lengths. In light of these results, it will now be interesting to study whether a coassembly of tektin and tubulin influences microtubule stability and length and whether tektins contribute to the formation of centrioles and axonemes.

This work was supported by U.S. Public Health Service Grant GM 35648 (R.W.L.) and by funds generously provided by the Department of Cell Biology and Neuroanatomy, University of Minnesota.

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