Tektins Are Heterodimeric Polymers in Flagellar Microtubules with Axial Periodicities Matching the Tubulin Lattice*

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Tektins are proteins that copartition with tubulin in a stable ribbon of three protofilaments from ciliary and flagellar microtubules. After purification, tektins A, B, and C from sea urchin sperm flagellar microtubules appear as extended relatively insoluble filaments, <5 nm in diameter. We used cross-linking reagents to investigate the associations and structural organization of subunits within tektin polymers isolated from stable protofilament ribbons of Stronglylocentrotus purpuratus. We show by SDS-polyacrylamide gel electrophoresis, immunoblots, and transmission electron microscopy that tektins are continuous heteropolymers in the stable protofilament ribbons, and thus flagellar microtubules. Our results also provide evidence for the arrangement of different tektin polypeptides within “core” filaments containing equimolar tektins A and B. Treatment of these core filaments with bis(sulfosuccinimidyl)suberate and with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide yielded a predominant cross-linked ~106-kDa heterodimer of tektins A and B; similar results were obtained by glutaraldehyde cross-linking of tektins solubilized under mild conditions. Finally, cross-linking with 5,5’-dithiobis(sulfosuccinimidylpropionate) revealed a 16 nm periodicity in isolated tektin AB filaments that can be related to the 8 nm tubulin dimer lattice and to periodically associated microtubule components.

Cilia, flagella, basal bodies, and centrioles are evolutionarily conserved from protists to mammals in terms of their basic structure and the stability of their microtubules. Another apparently conserved feature of axonemal microtubules from sources as diverse as echinoderms, molluscs, and the freshwater alga Chlamydomonas is a stable subset of three to four protofilaments arising from the outer doublet and possibly central pair singlet microtubules (1-5). In doublet microtubules these stable protofilament ribbons (pf-ribbons) are derived from the A-tubule wall, near the inner A-B-tubule junction. Sea urchin pf-ribbons isolated from 0.5% Sarkosyl detergent containing 0.5% Sarkosyl detergent contain prominent non-tubulin polypeptides that have been proposed to confer stability to the pf-ribbon and axonemal microtubules (6). Further extraction of the Sarkosyl-resistant pf-ribbons with increasing concentrations of urea produced an insoluble filamentous material composed of proteins named tektins (7-9).

Tektins A (~55 kDa), B (~51 kDa), and C (~47 kDa) are biochemically defined as the 0.5% Sarkosyl + 2 M urea-insoluble, filamentous fraction from sea urchin sperm flagellar axonemes (6). Antibodies specific for all three tektins stain intermittently along the length of all nine outer doublet microtubules fixed for immunofluorescence microscopy (10) and by immuno-EM anti-tektin antibodies label filaments protruding from the ends of pf-ribbons (8, 11). Since pf-ribbons contain a significant amount of tektins, these immunolabeling results were interpreted to suggest that tektins were longitudinal polymers associated with the stable tubulin protofilaments; however, direct evidence for this model has been lacking. Of more general interest, immunological studies have suggested the presence of tektin-like proteins in other stable microtubule organelles, including basal bodies, centrioles, cilia, and byproducts from species, including echinoderms, molluscs (Spisula), and cultured mammalian cells (10, 12-14).

Tektins have biochemical, immunological, and structural similarities to intermediate filament proteins but little sequence identity (15-17), and thus they are a distinct class of coiled-coil proteins. Tektin filaments exhibit strong α-type x-ray diffraction patterns (18). cDNA sequence analysis of tektins A and B indicates that each polypeptide chain has a central rod of approximately 310 residues composed of four major regions strongly predicted to form coiled coils (17, 19).

In our present study we used cross-linking reagents to analyze Sarkosyl-insoluble protofilament ribbons and isolated tektin filaments. Our results begin to elucidate the molecular arrangement of tektin AB filaments within the microtubule and are consistent with an important role for tektins in the organization of cilia and flagella.

EXPERIMENTAL PROCEDURES

Protein Purification—All steps were conducted at 0–4 °C, unless otherwise stated. Sperm flagellar axonemes were prepared from the sea urchin Stronglylocentrotus purpuratus, as modified from Gibbons and Fronk (20). Protein determinations were made according to Lowry et al. (21). Pf-ribbons were prepared by extracting axonemes with a solution of 0.5% Sarkosyl (W. R. Grace), 10 mM Tris, 1 mM EDTA, pH 8.0, for 2 h, and centrifuging at 50,000 g for 90 min; the pellet was reextracted with this same solution and recentrifuged to collect the pellet of purified pf-ribbons. Pf-ribbons that were not used immediately were stored in 50% glycerol, 50 mM Tris, 1 mM EDTA, 1 mM DTT, pH 8.0, at –20 °C or –80 °C. Tektin filaments were isolated from fresh or glycinated pf-ribbons by double extractions with 0.5% Sarkosyl, 2 or 4 M urea (as specified), 50 mM Tris, 1 mM EDTA, 1 mM DTT, pH 8.0, for 1–2 h, followed by centrifugation as before. Experiments using fresh or glycinated ribbons gave identical results. Soluble tektin protein was obtained by incubating tektin filaments in 1 mM HEPES, 0.1 mM EDTA, 10 mM DTT, pH 8.0, for 30 min, followed by centrifugation at 300,000 g for 10 min.

Chemical Cross-linking—Microtubule fractions were resuspended after centrifugation to a final concentration of 1 mg/ml in 10 mM HEPES, 1 mM EDTA, pH 8.0 (for BS3 and DTSSP) or in 100 mM MES, pH 6.0 (for EDC). The cross-linkers BS3, DTSSP, and EDC were obtained from...
FIG. 1. SDS-PAGE analysis showing the fractionation of \textit{S. purpuratus} sperm flagellar axonemes by Sarkosyl and Sarkosyl/urea extractions. Axonemes were extracted twice with 0.5% Sarkosyl ± 2 or 4 mM urea and centrifuged at 100,000 \times g for 90 min. Shown is the sedimentable material from whole axonemes (lane 1), control) and from axonemes extracted with Sarkosyl (lane 2), with Sarkosyl + 2 mM urea (lane 3), and with Sarkosyl + 4 mM urea (lane 4). Proteins were stained with Serva Blue. Lanes represent the polypeptide composition of axonemes (lane 1), pf-ribbons (lane 2), tektin ABC filaments (lane 3), and tektin AB filaments (lane 4).

FIG. 2. SDS-PAGE of pf-ribbons cross-linked by DTSSP. Lane 1, non-cross-linked (control) pf-ribbons. Lane 2, cross-linked pf-ribbons. Lane 3, the Sarkosyl + 2 mM urea-insoluble fraction from cross-linked pf-ribbons. Lane 4, the Sarkosyl/urea-insoluble fraction from cross-linked pf-ribbons after DTSSP cleavage. Samples in lanes 1 and 4 were reduced with 2-mercaptoethanol prior to electrophoresis; samples in lanes 2 and 3 were not reduced to prevent DTSSP cleavage. Lane 1 shows the completely resolved pf-ribbon polypeptides. In lane 2 the staining at the top is due to protein complexes that did not enter the gel; however, most of the α- and β-tubulin did not appear to be cross-linked into this complex. In lane 3 the Sarkosyl/urea-insoluble cross-linked proteins did not enter the gel. In lane 4 this same material is resolved into tektins A, B, and C after cleavage of the cross-linker.

Pierce. BS² and DTSSP were dissolved to 30 mg/ml in dimethyl sulfoxide; EDC was prepared in water at 50 mM. The cross-linkers were diluted to appropriate concentrations into protein solutions of 1 mg/ml. Reactions were incubated for 1–2 h at room temperature and stopped by the addition of 1 M Tris, pH 8.0, to a final concentration of 50 mM. Cross-linked specimens were centrifuged as above for SDS-PAGE or applied to carbon grids for EM.

**SDS-PAGE and Immunoblotting**—SDS-PAGE was performed according to Laemmli (22) under reducing or nonreducing conditions. Proteins were stained with Serva Blue R (Serva, Heidelberg, Federal Republic of Germany) or transferred to nitrocellulose. Blotting procedures were modified from Towbin et al. (23), using 20% methanol, 10 mM CAPS, pH 11. Nitrocellulose strips were stained appropriately with colloidal gold (24), or monospecific, affinity-purified polyclonal antibodies to \textit{Lytechnius pictus} tektins A, B, or C (25) or monoclonal antibodies to the \textit{S. purpuratus} 83-kDa polypeptide, the \textit{S. purpuratus} 77-kDa polypeptide, or \textit{S. purpuratus}-acetylated α-tubulin (14). Primary antibodies were visualized by alkaline phosphatase-conjugated goat anti-rabbit or goat anti-mouse antibodies, incubated in 0.2 mg/ml nitro blue tetrazolium, 0.2 mg/ml 5-bromo-4-chloro-3-indol phosphate (Sigma), 0.1 M Tris, 5 mM MgCl₂, pH 9.5.

**EM**—In most cases samples of pf-ribbons or tektin filaments were prepared in tubes and then applied to carbon-coated grids. Grids were rinsed with 10 mM Tris, 1 mM EDTA, pH 8.0, and stained for 30 s with 1% uranyl acetate. In some cases specimens were prepared in situ on grids: pf-ribbons or filaments were applied to grids and the excess material washed off. The ribbons and filaments that remained sparsely attached to the carbon film were rigorously extracted by washing rapidly with five drops of Sarkosyl/urea solution, incubated on a sixth drop for 1 h at 4 °C, followed by two more drops of Sarkosyl/urea and seven drops of TriS-EDTA buffer. The grid sample was then negatively stained directly or processed for cross-linking.

Grids with samples to be cross-linked in situ were washed several times with 10 mM HEPES, 1 mM EDTA, pH 8.0. After removing excess buffer, each grid was placed on a drop of HEPES-EDTA buffer containing 1 mg/ml DTSSP (diluted from 30 mg/ml in dimethyl sulfoxide) and incubated at room temperature for 1–2 h. Grids were then washed with Tris-EDTA buffer and negatively stained. Specimens were examined in a JEOL 100CX electron microscope operated at an acceleration voltage of 80 kV. Dimensions and spacings in negative stain images were determined by computed Fourier transforms, using tobacco mosaic virus as a standard.

**RESULTS**

Flagellar Axonemes Are Differentially Extracted by Sarkosyl/Urea—\textit{S. purpuratus} sperm flagellar axonemes can be fractionated by 0.5% Sarkosyl to yield stable ribbons of three to four protofilaments (6). Since this is the starting material for our investigations, the fractionation and SDS-PAGE profiles are shown in Fig. 1; the principal polypeptides include α- and β-tubulin, tektins A, B and C, and 77 and 83-kDa polypeptides.
31802  
Tektins Are Heterodimeric Polymers

The non-tektin proteins can be selectively solubilized from the pf-ribbons by inclusion of urea in the Sarkosyl solution. Extraction of pf-ribbons with Sarkosyl + 2 M urea yields filamentous preparations composed exclusively of tektins A, B, and C in equimolar amounts (9). For the purposes of our current studies, it was necessary to further fractionate tektin filaments; we found that Sarkosyl + 4 M urea was optimally effective at solubilizing tektin C (Fig. 1, lane 4), yielding filaments composed exclusively of tektins A and B in equimolar amounts. The negative stain EM appearance of pf-ribbons and tektin filaments is discussed later.

Tektins A, B, and C Are Selectively Cross-linked within Pf-ribbons by DTSSP—DTSSP is a homobifunctional N-hydroxysuccinimidyl ester conjugation reagent that has been used to cross-link keratins (26). It is cleaved by reducing agents. Using SDS-PAGE and EM, we examined the effects of DTSSP on pf-ribbons and on cross-linked pf-ribbons that were subsequently extracted with Sarkosyl + 2 M urea (Fig. 2). Following treatment with DTSSP, most of the non-tubulin proteins were cross-linked into complexes that were too large to be resolved by SDS-PAGE (lane 2); however, most of the tubulin appeared not to be cross-linked into this complex. After extraction of the
cross-linked ribbons with Sarkosyl + 2 M urea (lane 3), the insoluble fraction was collected by centrifugation and found to contain only high molecular weight material, which could not be further separated by SDS-PAGE under nonreducing conditions. Incubation of the cross-linked Sarkosyl/urea-extracted material with 2-mercaptoethanol and subsequent analysis by SDS-PAGE demonstrated that the insoluble high molecular weight material was composed of tektins A, B, and C (lane 4). Thus, tektins A, B, and C were selectively cross-linked in the pf-ribbon by DTSSP.

Immunoblots confirmed that cross-linked tektins A, B, and C remained insoluble essentially in the absence of other pf-ribbon proteins (Fig. 3). Before cross-linking, the major protein components of control pf-ribbons could be recognized by well characterized monospecific antibodies (10, 14, 25). Pf-ribbons were cross-linked and extracted with Sarkosyl (following hydrolysis of the DTSSP cross-linker prior to electrophoresis). Lanes 1–7 show that the insoluble material is composed principally of tektins A, B, and C and a small amount of cosedimenting tubulin. This amount of residual tubulin was not detected by SDS-PAGE with protein staining (see Fig. 1, lanes 3–4, and Fig. 2, lane 4).

EM Analysis—The structure of pf-ribbons, tektin filaments, and the cross-linked products were examined by negative stain EM. The polypeptide composition of these specimens can be correlated to the SDS-PAGE analysis in Figs. 1 and 2. As a control, non-cross-linked S. purpuratus flagellar axonemal microtubules are fractionated by Sarkosyl into characteristic ribbons of three protofilaments (Fig. 4a). Sarkosyl + 2 M urea extraction further fractionates pf-ribbons into <5-nm diameter filaments and bundles of filaments (Fig. 4b) composed exclusively of tektins A, B, and C in equimolar amounts (cf. Fig. 1, lane 3); we refer to these as tektin ABC filaments. The same approach was used to examine the effects of DTSSP. Fig. 4c shows that cross-linked pf-ribbons were similar to control ribbons; however, perhaps due to the cross-linking the ribbons were frequently paired. When cross-linked pf-ribbons were subsequently extracted with Sarkosyl + 2 M urea, <5-nm diameter filaments appeared that were associated with globular material (Fig. 4d). We initially attributed the globular material to the presence of cross-linked non-tektin proteins. This hypothesis was disproved when tektin ABC filaments were first isolated and then cross-linked with DTSSP. While untreated tektin ABC filaments appeared as bundles of smooth <5-nm filaments (Fig. 4b), DTSSP-cross-linked tektin ABC filaments again displayed, only more clearly, a series of axially repeating globules (Fig. 4e). The globules disappeared after incubation of the cross-linked tektin filaments with 50 m~ DTT to cleave the DTSSP (Fig. 4f). Taken together with SDS-PAGE and immunoblot data (cf. Figs. 2 and 3), these results demonstrate directly that tektins preexist as axially continuous filaments in pf-ribbons (and thus flagellar microtubules) and were stabilized by cross-linking.

Tektin AB Filaments—To determine whether or not tektin C gave rise to the associated globules along cross-linked filaments, we took advantage of the fact that tektins C can be selectively solubilized, leaving stable tektin AB filaments (Fig. 1, lane 4, and Fig. 5). Prior to cross-linking, tektin AB filaments (Fig. 5a) had a smooth appearance similar to tektin ABC filaments (cf. Fig. 4b). After cross-linking tektin AB filaments with DTSSP, structural repeats were once again seen only more clearly (Fig. 5b), indicating that tektins A and/or B form the basis of the globular repeats. The presence of tektin C seemed to occlude or disrupt this high degree of order. The globules have a uniform diameter of ~10 nm and an axial repeat of 16 nm, as determined from computed Fourier transforms of single straight filaments with globules, similar to those in b. Samples were prepared in tubes and then applied to grids. Bar, 100 nm (a, b).

Fig. 5. Negative stain EM of non-cross-linked and cross-linked tektin AB filaments. a, tektin AB filaments appeared similar to control tektin ABC filaments (cf. Fig. 4b). b, after DTSSP-cross-linking, tektin AB filaments again displayed axially repeating globules, with diameters of ~10 nm and a periodicity of 16 nm. Filaments frequently appeared double or intertwining. c, the 16 nm periodicity was determined from computed Fourier transforms of single straight filaments with globules, similar to those in b. Samples were prepared in tubes and then applied to grids. Bar, 100 nm (a, b).
Tektins Are Heterodimeric Polymers

FIG. 6. SDS-PAGE and immunoblot analysis of tektin AB filaments cross-linked by DTSSP. Non-cross-linked and cross-linked tektin AB filament samples were subjected to SDS-PAGE in identical gels and stained with Serva Blue (a) or transferred to nitrocellulose (b–d). Nitrocellulose replicas were stained with colloidal gold (b) or probed with affinity-purified polyclonal anti-L. pictus tektin A (c) or anti-L. pictus tektin B (d). In all panels the samples in lanes 1 and 2 were reduced with mercaptoethanol prior to electrophoresis; samples in lanes 3–6 were not reduced. Lane 1, molecular weight standards. Lanes 2 and 3, non-cross-linked tektin AB filaments. Lanes 4–6, tektin AB filaments cross-linked with 0.01, 0.1, and 1.0 mg/ml DTSSP, respectively. Asterisks indicate ~120 and ~200 kDa oligomers, both of which are stained with anti-tektins A and B. In lane 3 the filaments were not DTSSP-cross-linked but still show the presence of the 120- and 200-kDa bands, presumably due to pre-existing disulfide bonds; with greater DTSSP cross-linking these bands disappear as high molecular weight material accumulates at the top of the lanes.

~120-kDa band would appear to represent a tektin AB heterodimer (predicted mass of 106 kDa). Incubation with DTSSP initially increased the appearance of the putative dimer while decreasing the amount of tektin A and B monomers. Again, tektins A and B were present in the presumed dimeric complex by immunoblot analysis (c and d), indicating that tektin AB heterodimers could be resolved from cross-linked and non-cross-linked tektin AB filaments.

In the absence of a reducing agent we were unable to make accurate molecular mass determinations of tektin oligomers by SDS-PAGE. BS$^7$ is a nonhydrolyzable N-hydroxysuccinimidyl ester cross-linking reagent that has a similar spacer arm length to DTSSP (11.4 and 12 A, respectively). Low concentrations of BS$^7$ cross-linked tektin AB filaments into prominent ~107-kDa complexes, which by immunoblot analysis contained both tektins A and B (Fig. 7). The predicted mass of tektin AB heterodimers is 106 kDa. Oligomers corresponding to predicted tektin AA or BB homodimers (110 or 102 kDa, respectively) were not observed with BS$^7$. The higher molecular mass complexes presumably represented tektin trimers, tetramers, and higher oligomers. Repeating globular structures were not present on tektin filaments cross-linked by BS$^7$ (data not shown).

FIG. 7. SDS-PAGE immunoblot analysis of tektin AB filaments cross-linked by BS$^7$. a, SDS-PAGE of non-cross-linked tektin AB filaments (lane 1) and filaments cross-linked with 0.01, 0.1, and 1.0 mg/ml BS$^7$ (lanes 2, 3, and 4, respectively). Proteins were stained with Serva Blue. Masses of standards and tektin oligomers are indicated (kilodaltons). With increased cross-linking, oligomers of 107 kDa and higher appear. b, immunoblots of tektin AB filaments cross-linked with 0.01 mg/ml BS$^7$ stained with colloidal gold (lane 1) or probed with affinity-purified polyclonal anti-L. pictus tektin A (lanes 2 and 3) or anti-L. pictus tektin B (lanes 4 and 5). Lanes 2 and 3 and lanes 4 and 5 differ by a factor of two in the titer of affinity-purified primary antibody used. Tektins A and B and approximate oligomer masses are labeled (kilodaltons). The 107-kDa dimer (and higher oligomers) are composed of both tektins A and B.

tektin filaments were aggregation artifacts, following the extraction of flagellar microtubules with Sarkosyl/urea. In our current investigation we used cross-linking reagents to specifically cross-link tektins in pf-ribbons before Sarkosyl/urea extraction, eliminating potential extraction artifacts. The presence of individual filaments (rather than bundles) composed of tektins A, B, and C, following the cross-linking and extraction of pf-ribbons, demonstrates that tektins naturally exist as axially continuous filaments in the pf-ribbons and thus flagellar microtubules. These filaments might either be associated with
certain tubulin protofilaments or they might exist as a non-tubulin protofilament in the A-tubule wall (7, 8, 11).

Tektin Filaments Are Composed of Tektin AB Heterodimers—Cross-linking and solubilization experiments would be expected to identify homo- and/or heterodimers as adjacent neighbors, if they are present in the tektin filament structure. The apparent molecular masses of tektins A and B by SDS-PAGE are 55 and 51 kDa, respectively. After cross-linking with BS3, DTSSP, and EDC, a new predominant band of 106–107 kDa appears that is stained with antibodies against both tektins A and B, whereas a faint band of 102 kDa is stained only with anti-tektin B.

oligomers are also seen in cross-linked samples (Figs. 6–8), as would be expected from a polymer (28–30).

Tektin Filaments Have a 16 nm Axial Periodicity—The evidence indicates that the globular structures repeating along DTSSP-cross-linked tektin AB filaments arise from tektin A, B, or both (Fig. 5), but it is not entirely clear whether the globules are native structures that are preserved, or whether they are induced by the DTSSP. Such globules were not observed on non-cross-linked AB filaments stained with phoshpotungstic acid instead of uranyl acetate or after rotary shadowing; the globules were also not observed on negatively stained AB filaments cross-linked with glutaraldehyde, BS3, or EDC. Thus, our data would suggest that the globules are not present on tektin AB filaments isolated from Sarkosyl/urea but are induced by cross-linking with DTSSP. Whether these structures are real or induced, we think that their appearance reflects an inherent structural periodicity of the tektin filament. The size of the globules (~10 nm in diameter) and their axial repeat (16 nm) are reproducible and regular (Fig. 5). This periodicity is of particular interest since it is precisely twice the 8 nm repeat of the tubulin dimer in the microtubule lattice (31–35). The 16 nm periodicity also matches the observed spacings of various microtubule-associate components (36–40) and correlates to a 48 nm spacing along tektin filaments (11). Thus, while we have not yet been able to obtain direct evidence for the interaction of tektin and tubulin, the 16 nm spacing along tektin filaments may provide a structural basis for this interaction and/or for the attachment of microtubule-associated components.

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Fig. 8. SDS-PAGE immunoblot analysis of tektin AB filaments cross-linked with EDC. a, SDS-PAGE of non-cross-linked tektin AB filaments (lane 1) and filaments cross-linked with 2, 4, and 10 mM EDC (lanes 2, 3, and 4, respectively). Masses of standards and tektin oligomers are indicated (kilodaltons). b, immunoblots of tektin AB filaments cross-linked with 2 mM EDC, stained with affinity-purified polyclonal anti-L. pictus tektin A (lane 1) or anti-L. pictus tektin B (lane 2). Tektins A and B and approximate oligomer masses are labeled (kilodaltons). After cross-linking a predominant band of 106 kDa appears that is stained by antibodies to both tektins A and B, whereas a faint band of 102 kDa is stained only with anti-tektin B.

Fig. 9. Immunoblot analysis of soluble tektins cross-linked by glutaraldehyde. Tektin AB filaments were solubilized in low salt buffer plus DTT. The solubilized tektins at 2 mg/ml were cleared by ultracentrifugation and cross-linked with 0.1% glutaraldehyde for 30 min at 4°C. Adjacent nitrocellulose strips were probed with polyclonal anti-L. pictus tektin A (lane 1) and with polyclonal anti-L. pictus tektin B (lane 2). Tektins A and B and molecular mass standards are indicated (kilodaltons). The monospecific antibodies against tektins A and B both labeled the 106-kDa oligomer as indicated.
Tektins Are Heterodimeric Polymers

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