Characterization of antibodies as probes for structural and biochemical studies of tektins from ciliary and flagellar microtubules

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Summary

Rabbit antibodies raised and purified against three tektins, proteins of flagellar doublet microtubules from sea-urchin sperm (*Lytechinus pictus* and *Strongylocentrotus purpuratus*), were used to study tektin biochemistry and their structural localization. Doublet microtubules were fractionated into tektin filaments and separated by SDS–PAGE into three major tektin polypeptide bands ($M_r = 47, 51$ and $55 \times 10^3$), which were used to immunize rabbits. Antibodies against each tektin (anti-tektins) were affinity-purified and then characterized by two-dimensional isoelectric focusing/SDS–PAGE immunoblotting and by immunofluorescence microscopy. In two-dimensional immunoblots of 0-5% Sarkosyl-resistant fractions of flagellar microtubules, the antibody against the $55 \times 10^3 M_r$ tektin (anti-55) stained one major polypeptide of $55 \times 10^3 M_r$ and $pI \approx 6.9$, anti-51 stained two polypeptides of $51 \times 10^3 M_r$ and $pI = 6.15$, and anti-47 stained one major polypeptide of $47 \times 10^3 M_r$ and $pI = 6.15$. The anti-tektins also stained several minor neighbouring polypeptides, which may be isoelectric variants, novel tektins or unrelated proteins. Furthermore, anti-47 crossreacted with the major $55 \times 10^3 M_r$ polypeptide. By immunofluorescence microscopy all three anti-tektins stained methanol-fixed echinoderm sperm flagella and embryonic cilia. In addition, anti-47 and anti-55 stained unfixed, demembranated axonemes. Besides staining axonemes, all anti-tektins labelled the basal body region, and anti-51 labelled the sperm head envelope. These results indicate that the tektins are a complex family of proteins that are components of axonemal microtubules and possibly other cytoplasmic and nuclear structures.

Key words: intermediate filaments, microtubules, tektins.

Introduction

Doublet microtubules of sea-urchin sperm flagella can be fractionated in two ways. First, extraction of doublet tubules with Sarkosyl detergent produces resistant ribbons of three protofilaments composed of tubulin (Meza et al. 1972; Witman, 1970; Witman et al. 1972a,b) and a subset of microtubule proteins that do not comigrate with tubulin (Linck, 1976). Second, doublet microtubules can be fractionated by a combined extraction with Sarkosyl–urea into extended filaments, 2–6 nm in diameter, principally composed of three equimolar polypeptide groups with apparent molecular weights ($M_r$) of 47,000, 51,000 and 55,000 (Linck & Langevin, 1982; Linck et al. 1985). These filament proteins are collectively named tektins. Initial findings indicated the tektins are similar in their properties to mammalian intermediate filament proteins (Linck & Langevin, 1982; Linck & Stephens, 1987).

In previous studies antibodies were prepared against an equimolar mixture of the three major tektins and used to study tektin location in axonemal microtubules. The antibodies (referred to as anti-tektins) stained ciliary and flagellar axonemes fixed with methanol for immunofluorescence microscopy (Amos et al. 1985; Linck et al. 1985). By immunoelectron microscopy the anti-tektins labelled 2–3 nm diameter fibrils at the ends of the Sarkosyl ribbons and similar fibrils remaining after extraction of doublet microtubules with Sarkosyl–urea. The results suggested that these intermediate filament-like proteins exist as filaments or possibly protofilaments in the Sarkosyl-resistant domain of the
A-microtubule wall (Amos et al. 1986; Linck, 1982; Linck & Langevin, 1982).

For our present studies it seemed preferable to have separate, polyclonal antibody preparations against each of the three major tektins. For example, specific anti-tektins might provide information about the identity and relatedness of the tektins and permit the localization of each tektin within the axoneme. This paper reports the preparation of such antibodies against individual tektins from two sea-urchin species and characterizes their crossreactivities with sea-urchin tektins and proteins from other sources by immunoblotting and immunofluorescence microscopy. Preliminary and additional results appear elsewhere (Linck et al. 1986; Steffen & Linck, 1987; unpublished).

Materials and methods

Abbreviations

Ethylene diamine tetraacetate (EDTA), sodium dodecyl sulphate (SDS), polyacrylamide gel electrophoresis (PAGE), isoelectric focussing (IEF), two-dimensional (2-D), phosphate-buffered saline (PBS: 10 mM-phosphate, 0.15 M-NaCl, 2.7 mM-KCl, pH 7.4), PBS-Tween–Na3PO4 (PTN: PBS containing 0.05 % Tween 20 and 0.02 % Na3PO4), bovine serum albumin (BSA), room temperature (RT).

Animals


Biochemical reagents

Biochemical reagents deemed important in this work were obtained from the following sources: SDS, electrophoresis purity reagent from Bio Rad (Richmond, CA, USA); sodium dodecyl sarcosinate (Sarkosyl), Ciba-Geigy Corp. (Greensboro, NC); ampholytes, LKB, pH 5.7, Cat. no. 1809-121, and pH 3.5-10, Cat. no. 1809-101 (Bromma, Sweden); 4-chloro-1-naphthol, Aldrich Chemical Co. (Milwaukee, WI); rabbit anti-(*S. purpuratus* egg)-tubulin, Polysciences, Inc. (Warrington, PA); fluorescein-conjugated goat anti-rabbit IgG, Cooper Biomedical (Malvern, PA); biotin-conjugated goat anti-rabbit IgG, Sigma Chemical Co., Cat. no. B9642 (St Louis, MO); avidin–rhodamine, Sigma, Cat. no. A3026; rabbit anti-chicken desmin, DAKO, Cat. no. A611, Lot no. 096b (Santa Barbara, CA) and Miles Scientific, Cat. no. 65-793-1, Lot no. A200 (Naperville, IL); luciferin, luminol, and 4-methylumbelliferone, Sigma, Cat. nos L6882, A8511 and M1508, respectively; 125I-labelled goat anti-rabbit IgG, New England Nuclear (Boston, MA).

Biochemical procedures: SDS–PAGE, IEF/SDS–PAGE and immunoblotting

The following procedures are described in detail as referenced: SDS–PAGE was performed according to Laemmli (1970). 2-D IEF/SDS–PAGE was modified from that of O'Farrell (1975) as described by Linck & Langevin (1982). IEF pH gradients were measured from duplicate gels focused without protein and cut into 16 mm × 5 mm segments; two matched segments were equilibrated by shaking in 1 ml 0.1 M-NaCl for 1 h at RT; pH was measured with a Radiometer PHM 82 meter with a combined electrode, type GK2321-C or GK2322-C (Copenhagen, Denmark). Immunoblotting was modified from Towbin et al. (1979) as follows: electrophoresis was performed in 10 % methanol, 0.1 % SDS, 0.192 M-glycine, 0.025 M-Tris·HCl, at 25 mA overnight, and then for 1 h under the same conditions minus SDS. Nitrocel lulose strips were stained as indicated with one of the following: (1) Amido Black; (2) an appropriate dilution of rabbit anti-tektin IgG followed by peroxidase-conjugated goat anti-rabbit IgG, followed by addition of substrate, using either 4-chloro-1-naphthol or luciferin/luminol/4-methylumbelliferone (the latter according to Laing, 1986); or (3) 125I-labelled goat anti-rabbit IgG.

Purification and fractionation of cilia, flagella and desmin

The following were purified and fractionated according to previously published procedures: sea-urchin sperm flagellar axonemes (Gibbons & Frunk, 1972); Sarkosyl-resistant protofilament ribbons (Linck, 1976); sea-urchin embryo ciliary axonemes (Stephens, 1977); chicken gizzard desmin (Geisler & Weber, 1980, as modified by Linck & Langevin, 1982). Molluscan gill cilia were a gift from R. E. Stephens and were prepared according to Linck (1973) and Stephens (1983).

Purification of proteins as immunogens

Tektin filaments were prepared, as previously described (Linck & Stephens, 1987), by twice extracting flagellar doublet microtubules of *S. purpuratus* or *L. pictus* sea-urchin sperm flagella with 0.5 % Sarkosyl, 2.0 M-urea, 50 mM-Tris·HCl, 50 mM-lysine, 1 mM-EDTA, pH 8.0 at 4°C. The pellets of tektin filaments were resolved by preparative SDS–PAGE into the three major tektin polypeptide bands (47, 51 and 55 (×103) M). The proteins were visualized by precipitation in the gel with 0.5 M-KCl, cut out, electrodialted, dialysed against deionized water, and extracted with 100 % acetone. Chicken gizzard desmin was similarly purified.

Preparation and purification of antibodies

Preimmune sera were obtained from rabbits by taking 35–50 ml blood from the ear vein, clotting at 37°C for 1 h, removing the clot and centrifuging at 10,000 g for 10 min. Serum from each rabbit was checked by SDS–PAGE immunoblotting and immunofluorescence to determine whether it crossreacted with sea-urchin flagellar axoneme proteins. Rabbits not immune to axonemal proteins were bled weekly, until at least 45 ml of preimmune serum had been collected. Crude IgG was then isolated by two precipitations in 40 % ammonium sulphate; the final crude IgG was dialysed against PBS and stored frozen in samples at –80°C. For immunization, approximately 0.2 mg of a given purified tektin was sonicated into 1.1 ml of 1 mM-Tris·HCl, 0.1 mM-EDTA, pH 7.8; this sample was then emulsified with an equal volume of Freund’s complete adjuvant. Animals were
shaved along the vertebral column and 0.1 ml of adjuvant mixture was injected at each of 10 sites on each side. Animals were boosted after 5 weeks with a similar preparation of antigen in incomplete adjuvant. In the sixth week, and weekly thereafter, the animals were bled and IgG purified in the manner described above.

Affinity purification was carried out as follows: 10 mg of *L. pictus* tektin filaments were solubilized in 2% SDS, 5% 2-mercaptoethanol, 19.2 mM-glycine, 2.5 mM-Tris·HCl, pH 6.8. After boiling for 2 min, the sample was dialysed exhaustively against deionized water, then freeze-dried. The

![Fig. 1. SDS-PAGE/Serva Blue analysis of preparatively purified *S. purpuratus* tektins. Lane a: tektin filaments obtained by two extractions with 0.5% Sarkosyl-2 M-urea (according to Materials and methods). The three major tektin polypeptide bands (Mr, values (×10^3) are indicated) were preparatively separated by SDS-PAGE. Each band was cut out, discarding the zone between bands. Following electroelution, each fraction was analysed. Lane b: purified 47K Mr, polypeptide chains. Lane c: purified 51K Mr, chains. Lane d: purified 55K Mr, chains. The individually purified tektins were used as immunogens. Slightly slower migration of the purified polypeptides may be an electrophoretic artifact related to SDS binding. Note: an ≈53K Mr, polypeptide (arrow) is faintly present in the filament preparations (lane a).](image1)

![Fig. 2. One-dimensional SDS-PAGE immunoblot of *S. purpuratus* flagellar axonemes stained with anti-*S. purpuratus* tektins affinity-purified with *L. pictus* tektin filaments. Lane a: stained with Amido Black for total protein. Lane b: anti-47K Mr, tektin stains the 47K Mr, band and crossreacts significantly with the 55K Mr, band. Lane c: anti-51 stains the 51K Mr, band and weakly an ≈53K Mr, band (not evident in the figure). Lane d: anti-55 stains only the 55K Mr, band.](image2)
SDS-denatured tektin filament preparation was conjugated to cyanogen bromide-activated Sepharose (Pharmacia, Sweden), according to the manufacturer's recommended procedure. After thoroughly washing each affinity column with PBS, immune sera were passed through a 5-ml bed, continuously cycling the IgG for 1 h at RT. The column was then washed extensively with PBS; the wash fraction was recovered and found to contain negligible anti-tektin activity.
by SDS–PAGE immunoblotting procedures. Bound antibody was released by elution with 0.2 M-glycine, pH 2.3, collected in 1-ml fractions and immediately neutralized with 1 M 0.2 M-Tris–base. Antibody was then dialysed against 1:10 (v/v) diluted PBS, divided into samples, freeze-dried and stored at −80°C. The affinity-purified anti-tektins were reconstituted by adding deionized water to yield a normal PBS concentration. A separate affinity column was maintained for each anti-tektin.

**Immunofluorescence microscopy**

Diluted samples of sperm were attached to poly-L-lysine-coated glass coverslips, fixed in methanol at −20°C for 30 min, washed three times (10 min each) in PTN, treated in PTN–1% BSA for 20 min, incubated with primary antibody in PTN–BSA overnight at RT or 2 h at 37°C, washed three times (10 min) in PTN, incubated with secondary antibody in PTN–BSA for 1 h at 37°C, and washed three times (10 min) in PTN. Goat anti-rabbit IgG conjugated to fluorescein or biotin were used as secondary antibodies. Specimens stained with biotin conjugates were washed three times (10 min) with PTN, treated with poly-L-lysine (0.1 mg/ml) in PTN for 10 min to reduce non-specific DNA–avidin binding, incubated in avidin–rhodamine in PTN–BSA for 45 min at 37°C, and washed three times (10 min) in PTN. All specimens were then mounted in paraphenylene diamine (1 mg/ml) in 90% glycerol, 0.1 M-Tris–HCl, pH 9.1.

For unfixed specimens the methanol step was omitted from the protocol described above. Demembranated axonemes were prepared for this purpose by extracting whole sperm in 1% Triton X-100, 10 mM-Tris–HCl, 0.15 M-KCl, 5 mM-MgSO4, 0.5 mM-EDTA, pH 8.0, for 20 min at 4°C, and centrifuging at 1000 g for 5 min to pellet most of the heads. Axonemes in the supernatant fraction were pelleted at 9000 g for 6 min, re-extracted with the Triton solution, and then washed twice with the solution minus Triton.

**Fig. 3.** Two-dimensional SDS–PAGE immunoblot of S. purpuratus filaments stained with affinity-purified anti-S. purpuratus tektins. Tektin filaments were prepared from 0.5% Sarkosyl–2 M-urea (as in Fig. 1) and focused in quadruplicate. The top gel pattern was stained with Serva Blue to reveal the major polypeptides: molecular weights (×10^3) are indicated alongside a 1-D SDS–PAGE profile; pI range is shown along the top. Major spots for a given molecular weight are indicated by letters (and number for the 53K Mr, spot) and isoelectric points are given in Table 1. The anti-55 panel shows strong staining of the 55K Mr, spot a: spot b is also stained, as well as an ~45K Mr, spot x (compare with Fig. 4). The anti-51 panel shows strong specific staining of two 51K Mr, spots (a' and b') and very faint staining of a 53K Mr, spot (not apparent in reproduction). The anti-47 panel shows strong staining of two 47K Mr, spots (c' and d') and also weaker but significant crossreaction with the 55K Mr, spot a. Exact alignment of the spots was made on a fifth identical separated gels using a broad range of molecular weights as the three major tektins. Antibodies to the 55K Mr, tektin (anti-55) specifically stained a 55K Mr, band, the anti-51 stained a 51K Mr, band, and the anti-47 stained a 47K Mr, band but also crossreacted significantly with the 55K Mr, band. The anti-tektins were never seen to stain polypeptides with molecular weights higher than 55K Mr, and rarely did they stain lower molecular weight polypeptides. By 1-D SDS–PAGE immunoblotting the specificities of antibodies raised against L. pictus tektins were found to be qualitatively and quantitatively similar to those from S. purpuratus.

Specimens were examined with a Zeiss UEM microscope equipped with Neofluar 25×/0.8 NA and 63×/1.4 NA objectives. Photographs were taken using either Kodak Tri-X or hypersensitized Technical Pan 2415 35 mm film (Lumicon, Livermore, CA, USA), the latter being developed according to the recommended procedure.

**Results**

**Preparation of tektins and anti-tektins**

Tektin filaments were obtained by twice extracting purified flagellar doublet microtubules from S. purpuratus or L. pictus with 0.5% Sarkosyl–2 M-urea. These conditions were determined, as described by Linek & Stephens (1987), by varying the concentration of both Sarkosyl and urea to optimize the purity and yield of the three major polypeptides (M_r = 47, 51 and 55(×10^3)), referred to in this paper as the 47K M_r, 51K M_r, and 55K M_r, tektins. SDS–PAGE analysis of such tektin filament preparations is illustrated in Fig. 1. The filament proteins were preparatively separated by SDS–PAGE for use as immunogens; each tektin band was cut out according to molecular weight, avoiding the zones between the bands, electroeluted and analysed for purity by SDS–PAGE. As seen in Fig. 1, the three molecular weight fractions were pure.

As described in Materials and methods, preimmune IgG and immune IgG fractions were prepared, adjusted to twice the original serum IgG concentration, and stored at −80°C. Affinity-purified antibodies against S. purpuratus and L. pictus tektins (referred to here as anti-tektins) were obtained using SDS–denatured tektin filaments from L. pictus as the affinity probe in both cases. A different affinity column was maintained for each anti-tektin. The characterization of the affinity-purified antibodies is reported below, primarily for S. purpuratus. The results were essentially identical for L. pictus.

**Specificities of anti-tektins for axoneme proteins**

To a first approximation, the specificities of the anti-tektins were determined by one-dimensional SDS–PAGE immunoblot analysis of axonemal proteins. As shown in Fig. 2, the anti-tektins recognized only proteins with the same apparent molecular weights as the three major tektins. Antibodies to the 55K M_r, tektin (anti-55) specifically stained a 55K M_r, band, the anti-51 stained a 51K M_r, band, and the anti-47 stained a 47K M_r, band but also crossreacted significantly with the 55K M_r, band. The anti-tektins were never seen to stain polypeptides with molecular weights higher than 55K M_r, and rarely did they stain lower molecular weight polypeptides. By 1-D SDS–PAGE immunoblotting the specificities of antibodies raised against L. pictus tektins were found to be qualitatively and quantitatively similar to those from S. purpuratus.
as reported elsewhere (Steffen & Linck, 1987; unpublished).

Specificities of anti-tektins by 2-D immunoblot analysis

The specificities of the anti-tektins were next analysed by 2-D SDS–PAGE immunoblotting against purified tektin filament proteins and are illustrated in Fig. 3 for S. purpuratus. The isoelectric points and nominal molecular weights of the tektin filament proteins are given in Table 1. Anti-55 strongly stained the principal 55K Mr polypeptide a (see Fig. 3) and an adjacent ≈55K Mr spot b; the anti-55 also stained polypeptide spot x at ≈45K Mr, pI 6.4 that occasionally, but rarely, appeared in filament preparations. Anti-51 specifically stained the two main 51K Mr polypeptides a' and b'; although not apparent in the 2-D blot of Fig. 3, anti-51 also stained an ≈53K Mr polypeptide, which is evident in the original tektin filament preparations (Fig. 1). Anti-47 strongly stained the major 47K Mr polypeptide c' and a weaker spot d'; in addition, it crossreacted weakly, but consistently, with the main 55K Mr polypeptide a. These results should be compared with those for the Sarkosyl ribbon proteins presented below.

Specificities for Sarkosyl ribbon proteins

To look more closely at the complex mixture of proteins in the tektin–tubulin region, axonemes were extracted with 0.5% Sarkosyl to reduce the amount of tubulin present. The resulting Sarkosyl-resistant protofilament ribbons were then analysed by 2-D immunoblotting (Fig. 4). The isoelectric points of the Sarkosyl protofilament ribbon proteins are given in Table 1. The relative specificities and crossreactions of the anti-tektins were observed to be essentially identical to those found in the tektin filament preparations. Anti-55 stained only the main 55K Mr spot a and adjacent spots b and c; no other polypeptides stained with anti-55 (in particular, polypeptide x in Fig. 3 was not observed). Anti-51 specifically stained two 51K Mr spots (a' and b'). Anti-47 stained four polypeptides (a", b", c" and d") and crossreacted with the principal 55K Mr spot a. The anti-tektins did not stain the tubulins or the 77K/83K Mr polypeptide pair. Two alpha and one beta tubulin subunits were identified by anti-tubulin staining.

Immunofluoresence localization of tektins

Each of the anti-tektins was examined for its staining distribution in whole, fixed sperm by immunofluorescence microscopy. Representative results are shown in Figs 5 and 6. As with the immunoblot data, the results for L. pictus and S. purpuratus were essentially identical. All three anti-tektins stained the entire length of the sperm flagellum; also, all three antibodies more faintly stained thin-tip regions, ≈4 μm in length, that presumably correspond to the A-tubules extending past the termination of the B-tubules (Figs 5F, 6). In addition to the flagellum, several other structures were specifically stained. All anti-tektins stained the basal body and acrosome regions (Figs 5, 6). Furthermore, anti-47 stained an area coincident with the mitochondrion. Of particular interest was a specific anti-51 staining of the outline of the sperm head or nuclear envelope; we refer to this region microscopically as the sperm head envelope. In the case of L. pictus sperm, anti-L. pictus 51K Mr (affinity-purified with L. pictus tektin filaments) stained the sperm head envelope as brightly as the flagellum (Fig. 5E). In the case of S. purpuratus

<table>
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<tr>
<th>Component*</th>
<th>Isoelectric point†</th>
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<tr>
<td>81K Mr, (†)</td>
<td>6.75</td>
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<tr>
<td>77K Mr, (†)</td>
<td>6.50</td>
</tr>
<tr>
<td>α1-tubulin</td>
<td>5.85</td>
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<tr>
<td>α2-tubulin</td>
<td>5.70</td>
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<td>51K Mr-a'</td>
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<tr>
<td>47K Mr-c'</td>
<td>6.15</td>
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* For identification, see Figs 3, 4.
† Measured from 16 5-mm segments of IEF gels (see Materials and methods); two separate runs were averaged to the nearest 0.05 pI unit. Compare with Linck et al. (1982).

Major components of the tektin filaments are given in bold type in the table.
Fig. 5. Immunofluorescence of whole sperm, fixed and stained with anti-tektins. A–C. *S. purpuratus* sperm stained with antibodies raised against *S. purpuratus* tektins and affinity-purified with *L. pictus* tektin filaments. D–E. *L. pictus* sperm stained with antibodies raised against *L. pictus* tektins affinity-purified with *L. pictus* tektin filaments (A,D, anti-47; B,E, anti-51; and C,F, anti-55). All anti-tektins stained the length of the flagellum and frequently stained a thin, ≈4 μm long distal end piece (*d*). Intermittent staining in E may result from fixed proteins (e.g. tubulin) that sterically block anti-tektin labelling. All anti-tektins also stained the basal body region and the acrosomal region (*a*). Furthermore, anti-47 stained a structure corresponding to the mitochondrion (*m*), and anti-51 stained the sperm head envelope of *L. pictus* (*E*); compare with Fig. 7. The biotin–avidin system and hypersensitized film were employed. Bar, 10 μm.

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Fig. 6. Immunofluorescence of fixed, whole sperm (S. purpuratus), stained with anti-51 IgG. Low-power field and high-power inset (of sperm in lower right) show staining of the flagellum, distal end piece (d), basal body region (b), and sperm head envelope region (arrows). Compare the head envelope staining with Fig. 5E. Fluorescein–goat anti-rabbit IgG system and Tri-X film were used. Bar, 10 μm.

sperm, non-affinity-purified IgG against the S. purpuratus 51K Mr, tektin also stained the head envelope brightly (Fig. 6), whereas no staining was observed with preimmune IgG (not shown). However, following affinity purification of the anti-S. purpuratus tektin with L. pictus tektin filaments, the head envelope staining in S. purpuratus sperm was substantially reduced or eliminated (Fig. 5B).

The anti-tektins were also examined for their ability to stain unfixed axonemes (Fig. 7). Both anti-47 and anti-55 stained the unfixed axonemes brightly, while anti-51 stained them extremely weakly. All three anti-tektins did, however, show bright staining at the ends of the broken flagellar axonemes, 5–30 μm in length. The unfixed axonemes also stained brightly with anti-tubulin (not shown).

Crossreactivity of anti-tektins with cilia

The antibodies to flagellar tektins were examined for their abilities to crosreact with ciliary proteins from echinoderms and molluscs (Fig. 8). Ciliary axonemes were isolated from S. purpuratus blastulae and gastrulae embryos and from molluscan (A. irradians) gill tissue and probed with non-affinity-purified antibodies against S. purpuratus flagellar tektins. In both cases cilia were stained, as observed by immunofluorescence microscopy (not shown). In SDS–PAGE immunoblots of S. purpuratus cilia the anti-tektins crosreacted with polypeptides of molecular weights similar to those in flagella and showed identical specificities; i.e. the anti-55 stained only a 55K Mr band, the anti-51 primarily stained a 51K Mr band (and more weakly 47K, 53K and 55K Mr bands), and the anti-47 prominently stained the 47K Mr band but crosreacted with the 55K Mr band.

In the case of molluscan gill cilia the immunoblot staining was more complex. The anti-55 crosreacted with polypeptide bands with molecular weights of approximately 52K, 54K and 56K Mr; anti-51 crosreacted with 52K and 55K Mr bands; and anti-47 crosreacted with 45K, 49K, 52K and 55K Mr bands.

Other crosreactivities

Anti-tektins were not found to crosreact with purified chicken gizzard desmin by 1-D SDS–PAGE immunoblot; similarly, polyclonal antibodies to desmin (those

Antibody analysis of tektins
Discussion

Echinoderm (sea-urchin) sperm flagellar doublet microtubules contain a set of related polypeptides called tektins that are the major components of the 2–6 nm Sarkosyl–urea-insoluble filaments (Linck et al. 1985; Linck & Langevin, 1982). Three major tektins with apparent molecular weights of 47K, 51K and 55K M_r have been characterized and found to be related, but distinct, gene products; they are different from tubulin but similar to intermediate filament proteins in their structural, electrophoretic and solubility properties (Amos et al. 1986; Beese, 1984; Linck & Langevin, 1982; Linck & Stephens, 1987; cf. Steinert et al. 1985). Previous studies with antibodies raised against undenatured tektins indicated that the antigenic sites of the tektins are masked by their structural organization within the microtubule, except where thin filaments protrude at the ends (Linck et al. 1985). The goal of our present efforts has been to prepare antibodies to each of the tektins for use as specific probes for further molecular studies. We discuss here the specificities, crossreactivities and immunofluorescence staining of these anti-tektins.

Specificity and crossreaction

The specificity of each anti-tektin is largely restricted to the original immunogen. Anti-55 is the most specific, recognizing only the 55K M_r band in 1-D immunoblots of flagellar axoneme proteins. In 2-D immunoblots of tektin filaments or Sarkosyl ribbons, anti-55 recognizes three polypeptides with narrowly defined molecular weights and isoelectric points (spots a, b and c in Figs 3, 4). These three polypeptides comigrate at ~55K M_r on 1-D SDS–PAGE, and all would have been included in the preparation used to raise the anti-55 antibodies. We have not determined whether these polypeptides are distinct or related proteins. One insight into the structure of the 55K M_r tektin may come from the staining by anti-55 of spot x at ~45K M_r and pI 6.4 (Fig. 3). This spot is usually not present in the preparations and is not recognized by anti-47 or anti-51; therefore, it may represent a proteolytic fragment of the 55K M_r tektin. If this is the case, then the pI shift of the major fragment from 7.0 to 6.4 implies that the ~10K M_r cleavage fragment would be a highly basic terminal domain.

Anti-51 is specific in 2-D immunoblots for two polypeptide spots (a’ and b’) with masses of 51K M_r (Figs 3, 4). For the same reasons as with anti-55, we cannot define the nature of these two spots. It is clear, however, that anti-51 does not recognize the 51K M_r polypeptide c’ that is present in Sarkosyl ribbons in an amount equal to the 51K M_r tektin a’ spot. Faint anti-51 staining of 47K, 53K and 55K M_r bands (better seen in 1-D immunoblots, see Fig. 8C) could be attributed

prepared in our laboratory and those commercially available from DAKO and Miles) were not found to crossreact with sea-urchin flagellar tektins, even though sensitive methods of secondary immuno-staining were employed, including the ¹²⁵I radiolabel and the peroxidase–luminol–luciferin methods.
to contamination of the 51K Mr antigen with these neighbouring polypeptides; however, the presence of an ≈53K Mr band consistently in tektin filament preparations (Fig. 1) argues for the existence of at least one other bona fide filament component (Table 1).

In 2-D immunoblots anti-47 stains two to four polypeptides, 47K Mr, spots a", b", c" and d", which vary slightly. Again, it is not known whether these spots are related. Interestingly, anti-47 crossreacts with the 55K Mr tektin spot a. It is unlikely that the 47K Mr antigen preparation was contaminated by 55K Mr antigens, since a substantial area (including the 51K Mr region) was cut out between these two tektins during the electrophoretic purification of the 47K Mr band. This observed crossreaction thus suggests a degree of immunological (i.e. structural) similarity between the 47K and 55K Mr tektins, a conclusion reinforced by the fact that L. pictus and S. purpuratus yield identical results.

Complexity of tektins
Sperm flagellar tektins were originally defined by their solubility properties and apparent molecular weights (Linck et al. 1985; Linck & Langevin, 1982). The
polyclonal antibody studies we have reported here help to establish the identity of the three major molecular weight groups of tektins previously studied, but they also suggest that the tektins may be a more complex family of proteins. Of the polypeptides on 2-D immunoblots that stain with anti-tektins, spots 47K \( M_r \) c", 51K \( M_r \) a' and -b', and 55K \( M_r \) a correspond to the major components of the tektin filaments, but a less abundant 53K \( M_r \) component is also present. As stated earlier, we cannot yet determine the relationship of the other 2-D spots to the main tektins. Clearly, some polypeptides may be isoelectric variants of their parent tektin (e.g. 47K \( M_r \) a", b" and d" may be phosphorylated variants), others may be novel tektins (e.g. spot 51K \( M_r \) c' and the \( \approx 53K \ M_r \) band), and others may not even be tektins. The potential complexity of the tektins is particularly evident in the crossreactions of anti-echinoderm tektins with molluscan ciliary proteins (Fig. 8E-H), in which anti-47 crossreacts with molluscan polypeptides b, c, d and e, anti-51 with bands b and c, and anti-55 with bands a, b and c. These crossreactions with molluscan proteins have been more carefully analysed and are to be reported elsewhere (R. E. Stephens, personal communication). Sea-urchin anti-tektins have also been found to crossreact with tektin-like proteins in *Chlamydomonas* flagella (C. Silflow & K. Joyce, personal communication) and *Drosophila* testes and sperm (E. Raff & J. Hutchens, personal communication).

**Localization of tektins in sperm and cilia**

Immunofluorescence microscopy demonstrates that all three tektins are present throughout the length of flagellar and ciliary axonemes and in the basal body region (Figs 5-7). In a related study we have demonstrated that all three anti-tektins label basal bodies and each of the nine doublet microtubules of sperm flagella, and also label centrioles and/or centrosomes of Chinese hamster ovary cells (Steffen & Linck, 1987; unpublished). At the distal tip of the axoneme (Figs 5F, 6), a thinner segment, \( \approx 4 \mu m \) in length, is also stained by the anti-tektins. In most species it is in this region of cilia and flagella that the B-tubules terminate and the A-tubules extend as singlet microtubules (Gibbons, 1961; Gibbons & Grimstone, 1960; Linck & Langevin, 1981; Satir, 1967; Stephens, 1970); thus, the anti-tektins are presumably staining the A-tubules. In most species it is in this region of cilia and flagella that the B-tubules terminate and the A-tubules extend as singlet microtubules (Gibbons, 1961; Gibbons & Grimstone, 1960; Linck & Langevin, 1981; Satir, 1967; Stephens, 1970); thus, the anti-tektins are presumably staining the A-tubules. These observations are in agreement with our earlier results (Linck et al. 1985) and also with those of Stephens (1977, and personal communication), who showed that 'Component 20' (of Linck, 1976) is the 55K \( M_r \) tektin synthesized during sea-urchin embryo ciliogenesis in an amount consistent with that of a length-determining component of ciliary (and flagellar) A-tubules.

Demembranated, unfixed axonemes were also stained by anti-47 and anti-55, but weakly stained by anti-51. All three anti-tektins did, however, stain brightly the ends of the unfixed, broken axonemes (Fig. 7). In our previous studies (Linck et al. 1985), anti-tektins raised against undenatured tektin filaments did not label flagellar microtubules, unless the tubules were first fixed or disrupted. The positive staining of undisrupted flagellar microtubules reported here may relate to the fact that our individual anti-tektins were raised against SDS-denatured tektins. These anti-tektins may, therefore, recognize a greater number of antigenic sites exposed on the microtubule surface; the weaker staining with anti-51 may imply that the 51K \( M_r \) tektin is less exposed on the surface. The brighter staining of the ends of axonemes with all anti-tektins correlates well with our earlier observation that antibodies do label thin filaments protruding from the frayed ends of microtubules (Amos et al. 1986).

Of interest, also, is the observation that the 51K \( M_r \) tektin antibodies stain a region that we refer to as the sperm head envelope (Figs 5, 6), as intensely as they do the flagellum. Both non-affinity-purified anti-\( S. \ purpuratus \) and anti-L. *pictus* antibodies do label thin filaments protruding from the sperm head envelope (compare Figs SE, 6). However, affinity purification of anti-S. *purpuratus*-51 with L. *pictus* tektin filaments substantially reduces head envelope staining in S. *purpuratus*, conceivably because of differences in the immunological determinants of the envelope components in the two species. Since originally reporting sperm head envelope staining (Linck et al. 1985), noting that it might be artifactual, we have taken precautions to avoid non-specific staining. Treatment of the specimens with polylysine was found to block non-specific DNA-avidin binding. Without polylysine treatment, avidin-rhodamine stains the entire sperm head; after treatment, only the thin outline of the head envelope is stained with anti-51 and not with anti-47 or anti-55. We conclude that the crossreaction of anti-tektins with the envelope region is likely to result from the similarities between tektins and intermediate filament proteins (Amos et al. 1986; Beece, 1984; Linck & Langevin, 1982; Linck & Stephens, 1987) and between intermediate filament proteins and nuclear lamins (Fisher et al. 1986; McKeon et al. 1986). Recently, it has been reported that one monoclonal antibody against sea-urchin tektins crossreacts with nuclear envelopes and lamins from cultured mammalian cells (Chang & Piperno, 1987).

**Relationship of tektins to intermediate filament proteins**

The properties of tektins indicate their similarities to mammalian intermediate filament proteins. Chang & Piperno (1987) have recently reported that a monoclonal anti-tektin crossreacted with desmin and a
polyclonal, non-affinity-purified anti-desmin cross-reacted with a certain tektin. We investigated the possibility of crossreactions between the anti-tektins and chicken gizzard desmin and between anti-desmin and tektins; but no evidence of such crossreactions was observed, using either the peroxidase/4-chloro-1-naphthol method of immunoblot staining or more sensitive methods of detection (i.e. peroxidase/luminol–luciferin or 125I label). Recent studies on cultured mammalian cells, however, have demonstrated that one of our affinity-purified anti-tektins recognizes a keratin in HeLa cells (Steffen & Linck, 1987). Our finding that certain rabbits were immune to sea-urchin tektins and/or mammalian intermediate filament proteins urges prudence in the interpretation of non-purified, polyclonal antibody crossreactivities between specific IF proteins and tektins.

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References


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