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Two proteins isolated from sea urchin sperm flagella: structural components common to the stable microtubules of axonemes and centrioles

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SUMMARY

Biochemical fractionation of axonemal microtubules yields the protofilament ribbon (pf-ribbon), an insoluble structure of 3-4 longitudinal protofilaments composed primarily of α/β tubulin, tektins A, B and C, and two previously uncharacterized polypeptides of 77 kDa and 83 kDa. We have isolated the 77/83 kDa polypeptides (termed Sp77 and Sp83) from sperm flagella of the sea urchin *Stronglyocentrotus purpuratus* and raised polyclonal antibodies against them. Sp77 and Sp83 copurify exclusively with the pf-ribbon. Both the anti-Sp77 and anti-Sp83 antibodies detected the nine outer doublets and the basal bodies of sea urchin sperm by immunofluorescence microscopy. In addition, the anti-Sp83 antibody, but not the anti-Sp77 antibody, detected a single 83 kDa polypeptide on immunoblots of unfertilized sea urchin egg cytoplasm, and a single polypeptide of 80 kDa on blots of isolated mitotic spindles from Chinese hamster ovary (CHO) cells. Previous studies have shown that tektins are present in the basal bodies and centrosomes/centrioles of cells ranging from clam to human. We found that anti-Sp83 decorates the spindle poles in sea urchin zygotes, and the interphase centrosome and spindle poles in CHO cells. In CHO cells arrested in S phase with aphidicolin, anti-Sp83 detects multiple centrosomes. The staining of the centrosome was not disrupted by prolonged nocodazole treatment, suggesting that the 80 kDa polypeptide is associated with the centrioles themselves. Our observations demonstrate that, like tektins, Sp77 and Sp83 are structural proteins associated with stable doublet microtubules, and may be components of basal bodies and centrioles of sea urchins and mammalian cells.

Key words: Basal Body, Centrosome, Microtubule Associated Protein, Spindle pole, Tektin

INTRODUCTION

In animal cells, the microtubule network is organized by the centrosome, an organelle consisting of a pair of centrioles surrounded by a network of pericentriolar material (PCM), which directly nucleates the microtubules (Gould and Borisy, 1977; reviewed in Brinkley, 1985). A homologous organelle to the centriole is the basal body, which nucleates the axoneme of cilia and flagella (Gall, 1961; Wheatley, 1982, 1997). Isolated basal bodies will assemble functional centrosomes when injected into cells (Heideman and Kirschner, 1975; Hamaguchi and Kuriyama, 1982) and during development, with a few notable exceptions (Schatten et al., 1986), the functional centriole is originally supplied at fertilization by the basal body of the sperm, which duplicates to form the poles of the mitotic spindle (Wilson and Matthews, 1895; Sluder and Rieder, 1985; reviewed in Schatten, 1994).

The structure of the centriole microtubules and the molecular mechanisms underlying their assembly are poorly understood, in part because of the difficulty in obtaining pure preparations of basal bodies/centrioles for biochemical analysis (see Kimble and Kuriyama, 1992; Palazzo et al., 1992; Kellogg et al., 1994). Importantly, the triplet microtubules of the basal body are contiguous with the nine outer doublets of the axoneme, and the structural similarities observed between doublet and triplet microtubules suggest that they assemble using similar protein subunits (Gibbons and Grimstone, 1960; Gall, 1961; Behnke and Forer, 1967; Allen and Borisy, 1974; Linck, 1990). Because the axonemes of cilia and flagella can be readily isolated in sufficient quantity to allow biochemical analysis, the isolation and characterization of proteins of the axonemal doublet microtubules offers an opportunity to characterize proteins of the centriole as well (Steffen and Linck, 1988; Norrander et al., 1996).

Subfractionation of ciliary/flagellar axonemes with the detergent sarkosyl solubilizes the majority of the outer doublet microtubule proteins, but leaves intact a structure known as the protofilament ribbon (pf-ribbon) (Witman et al., 1972; Meza et al., 1972; Linck, 1976; Linck and Langevin, 1982). Pf-ribbons, consisting of 3-4 longitudinal protofilaments, are composed of α/β tubulin, tektins A (approx. 55 kDa), B (approx. 51 kDa) and C (approx. 47 kDa), and two previously uncharacterized polypeptides with molecular masses of 77 and 83 kDa (Linck, 1990). Although previous studies have demonstrated that the axonemal tektins possess biochemical and immunological properties similar to intermediate filament proteins (IFs)

(Chang and Piperno, 1987; Steffen and Linck, 1989), sequence analysis of cDNAs encoding the tektins from sea urchin and mammals has revealed that they represent an independent class of filamentous proteins, distinct from the IFs (Norrander et al., 1992, 1996; Chen et al., 1993; Yuan et al., 1995). Ultrastructural and biochemical analysis of isolated pf-ribbons have shown that the tektins assemble as a heteropolymeric filament within the wall of the axonemal A-tubule (Linck et al., 1985; Pirner and Linck, 1994; Nojima et al., 1995; Norrander et al., 1996). While the exact functions of the tektin filament have not been demonstrated, their biochemical properties and localization within the axoneme strongly suggest that they contribute to the stability and three-dimensional form of doublet microtubules (Nojima et al., 1995; Norrander et al., 1996).

In addition to their association with doublet microtubules, the tektins have been localized to the basal body, the interphase centrosomes/centrioles and the mitotic/meiotic spindle poles of species ranging from clam to human (Steffen and Linck, 1988; Steffen et al., 1994). These findings suggest that pf-ribbons contribute to the stability and structure of both doublet and triplet microtubules. However, before we can assess the role played by the pf-ribbon structure within triplet microtubules, it is essential that we characterize the 77 and 83 kDa proteins.

In this study we have isolated the 77 and 83 kDa polypeptides from the sperm flagella of the sea urchin *Stronglyocentrotus purpuratus* (termed Sp77 and Sp83), and using affinity-purified polyclonal antibodies prepared against each of these polypeptides, determined if they were present in basal bodies and centrioles. First we localized Sp77 and Sp83 within the sea urchin sperm flagella and asked whether they localized to the flagellar basal body. Next, we assayed isolated sea urchin sperm axonemes to determine whether Sp77 and Sp83 are components specific to the pf-ribbon or are randomly distributed throughout the axoneme. Finally we tested whether anti-Sp77 or anti-Sp83 detected homologous polypeptides in either sea urchin zygotes or mammalian somatic cells, and determined whether or not Sp77 or Sp83 localized to centrosomes and centrioles.

MATERIALS AND METHODS

Unless otherwise stated, all reagents used in this study were obtained from Sigma Chemical Co. (St Louis, MO).

Abbreviations and buffers

The following were used: artificial sea water (ASW: 423 mM NaCl, 9 mM KCl, 23 mM $MgCl_2$, 25 mM $MgSO_4$, 2 mM $NaHCO_3$, 9 mM $CaCl_2$, 5 mM Tris-HCl, pH 8-8.3), bovine serum albumin (BSA), foetal calf serum (FCS), para-amino butyric acid (PABA), phosphate-buffered saline (PBS: 10 mM Na phosphate, 150 mM NaCl, 2.7 mM KCl, pH 7.4), Pipes buffer (PEM: 100 mM Pipes, pH 6.96, 5 mM EGTA, 0.5 mM EDTA, 1 mM $MgSO_4$, 1 mM DTT), Pipes/glycerol buffer (PEMG: 100 mM Pipes, pH 6.96, 5 mM EGTA, 0.5 mM EDTA, 1 mM $MgSO_4$, 1 mM DTT, 900 mM glycerol), sodium dodecyl sarcosinate (sarkosyl), sodium dodecyl sulphate (SDS).

Fractionation of protofilament ribbons

Protofilament ribbons (pf-ribbons) were prepared from isolated sea urchin flagellar axonemes (*L. pictus* or *S. purpuratus*) by extraction with 0.5% sarkosyl as described (Pirner and Linck, 1995). Further fractionation with varying concentrations of urea was also done as described (Linck and Langevin, 1982).

SDS PAGE and immunoblotting

Protein samples were separated by SDS polyacrylamide gel electrophoresis according to Laemmli (1970), using BioRad electrophoresis grade SDS (Richmond, CA). Protein gels were stained with Coomassie R250, or transferred directly onto nitrocellulose (S&S, Keene, NH) under transblot conditions as originally described for peptide sequencing: 10 mM CAPS buffer, pH 11.0, 20% methanol, at 100 V for 2 hours (Matsudaira, 1987). Blots were stained with Ponceau S and then immunostained. Quantitation of scanned SDS gels and immunoblots was done on a Macintosh Powerbook 520C using NIH Image software (developed at the US National Institutes of Health, and available on the Internet at: http://rsb.info.gov/nih-image/).

Preparation of antibodies

Sp77 and Sp83 were purified from isolated pf-ribbons by SDS-PAGE, transferred to nitrocellulose and used to raise polyclonal antibodies in rabbits using the method of Knudsen (1985). The immune serum was tested by blot of isolated pf-ribbons for reactivity. Antisera was affinity purified against SDS-purified Sp77 or Sp83 by the 'Strip-dip' method (Talian et al., 1983). A pre-immune IgG fraction for each antibody was used as a control.

Mammalian cell culture

Chinese hamster ovary (CHO) cells were cultured in Ham's F-10 medium (Gibco, Grand Island, NY), 10% FCS at 37°C and 5% CO_2. Cells were grown on sterile poly-L-lysine coated coverslips, then fixed in methanol-50 mM EGTA, pH 7.65.

To arrest CHO cells in S phase, cells were cultured on poly-L-lysine coverslips in complete F-10 medium containing 10 μg/ml aphidicolin as described (Sluder and Lewis, 1987; Balczon et al., 1995), and then processed for immunofluorescence microscopy as above.

To depolymerize cytoplasmic microtubules in CHO cells, cells were cultured for 20 hours in medium containing 0.5 mg/ml nocodazole.

Sea urchin zygotes, mitotic spindles and cytoplasmic extracts

Sea urchin eggs were isolated by intercoelmic injections of 0.56 M KCl as described by Fuseler (1973). Eggs were stored in artificial sea water (ASW) at 18°C as a monolayer in glass Petri dishes. Sperm was stored dry on ice until use. Sperm was diluted in ASW-10 mM Paba (pH 8.0), and added to diluted eggs (2.5 ml eggs/50 ml ASW-10 mM Paba). After 2 minutes, the fertilized eggs were briefly centrifuged, resuspended in fresh ASW-Paba, and the formation of the fertilization membrane (FM) was observed by light microscopy. At 15 minutes post-fertilization (PF), the eggs were passed three times through a Nitex screen to strip the FM, pelleted in a hand crank centrifuge and resuspended in fresh aerated ASW. They were then allowed to develop at 18°C.

Biochemical fractionation of the eggs was carried out by a modification of the method of Porter et al. (1988). Briefly, eggs were washed three times with ASW, then suspended in two volumes of PEMG plus protease inhibitors (10 mg/ml aprotinin, leupeptin, pepstatin, soybean trypsin inhibitor), and homogenized in a Dounce homogenizer with a loose fitting piston. Egg homogenate was separated by ultracentrifugation in a Beckman TI 50.2 or TI 70.2 rotor (Beckman Instruments: Palo Alto, CA). Egg homogenate was used fresh or drop-frozen in liquid N_2 and stored at −80°C. Before use, frozen samples were thawed and given a high speed clearing spin (100,000 ***g***, 15 minutes).

Taxol-stabilized microtubules were polymerized from sea urchin egg cytoplasmic extract diluted in PEMG by the addition of 20 mM taxol-1 mM GTP, and sedimented by centrifugation (Vallee and

Bloom, 1983). Salt-labile microtubule associated proteins were extracted with PEMG-taxol-GTP-750 mM KCl for 1 hour on ice (Suprenant and Marsh, 1987).

Immunomicroscopy

Splayed sea urchin flagella were prepared according to Steffen and Linck (1988), and fixed in −20°C methanol. Dual immunostaining of flagella or mitotic spindles was performed as previously described (Steffen et al., 1994) using goat anti-mouse FITC and goat anti-rabbit Texas Red secondary antibodies (Molecular Probes, Eugene, OR). Images were recorded onto hypersensitived Kodak Tech Pan film as described by Silva (1981a,b) (Kodak, Rochester, NY). Immunocytochemical staining of sea urchin zygotes with anti-Sp 83 was done by the method of Wright et al. (1991).

Immunoelectron microscopy of isolated pf-ribbons was performed by the method of Linck (1985) as modified here. Diluted samples of pf-ribbons were allowed to settle onto carbon-coated grids. The following steps were carried out on 100 ml drops placed on parafilm in a humidified chamber at room temperature: 3× 10 minutes in blocking buffer (50 mM Tris, pH 8, 1 mM EDTA, 1 mM DTT-10% w/v cytochrome *c*), a brief wash in wash buffer I (50 mM Tris, pH 8.0, 1 mM EDTA, 0.5% tween), 2 hours incubation with primary antibody in dilution buffer (50 mM Tris, pH 8.0, 1 mM EDTA, 1% cytochrome *c*, 0.1% tween), 3× 10 minute washes in wash buffer II (50 mM Tris, pH 8.0, 1 mM EDTA, 1% cytochrome *c*, 0.5% tween), 2 hours incubation with goat anti-rabbit/5 nm gold in dilution buffer (BioCell-Cardiff, Wales UK), 3× 10 minute washes in wash buffer II. Immunogold-labeled samples were negatively stained with uranyl acetate and viewed with a JEOL 100 CX (JEOL USA) at 80 kV.

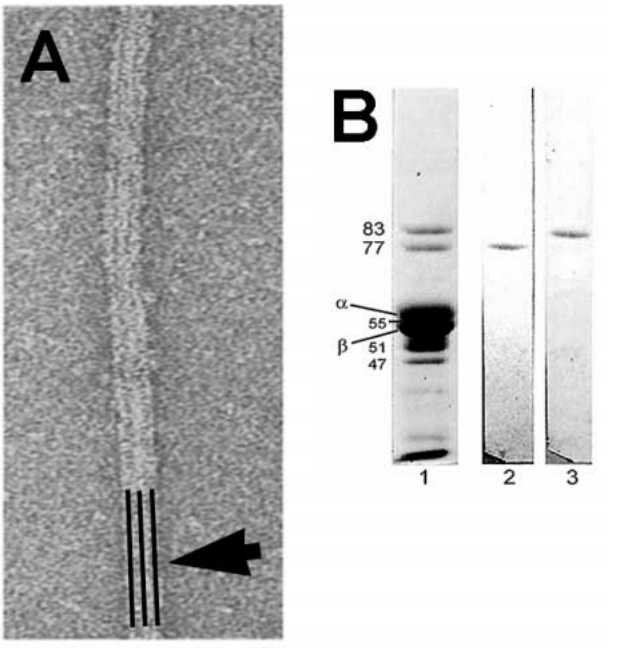

Fig. 1. (A) Isolated protofilament ribbon (pf-ribbon) seen in negative stain TEM. Note the three longitudinal protofilaments indicated by the black bars (arrow). Bars, 50 nm. (B) SDS-PAGE/immunoblot of isolated *S. purpuratus* pf-ribbons. Lane 1: Coomassie blue-stained proteins. The positions of α/β tubulin, tektins A (55), B (51) and C (47) and Sp77/Sp83 are indicated. Lanes 2 and 3 are identical immunoblots stained with either affinity-purified anti-Sp77 (lane 2), or affinity-purified anti-Sp83 (lane 3).

RESULTS

Sp77 and Sp83 are localized to the pf-ribbons of sea urchin sperm flagella

Stable pf-ribbons were isolated from sperm flagellar doublet microtubules of the sea urchin *Stronglyocentrotus purpuratus* by extraction with sarkosyl detergent (Fig. 1A), as previously described (Pirner and Linck, 1995). From these isolated pf-ribbons, the 77 and 83 kDa polypeptides were individually purified by SDS-PAGE. The two polypeptides are referred to as Sp77 and Sp83 (for ***S**tronglyocentrotus **p**urpuratus* polypeptides of 77 and 83 kDa, respectively). The isolated polypeptides were used to raise polyclonal antibodies specific for each protein and the immune serum was further affinity purified against the original purified proteins.

Affinity-purified anti-Sp77 and anti-Sp83 antibodies gave strong, monospecific reactions to their respective antigens on immunoblots of isolated pf-ribbons (Fig. 1B). No crossreaction was seen between either antibody and neither recognized tubulin or the tektins. Identical results were obtained using blots of isolated *Lytechinus pictus* pf-ribbons, which indicates crossreactivity between these two sea urchin species (not shown).

Using immunofluorescence microscopy, Sp77 and Sp83 were localized to *L. pictus* sperm tails splayed onto coverslips (Fig. 2). Both anti-Sp77 (Fig. 2A) and anti-Sp83 (Fig. 2B) recognize all nine doublet microtubules along their length, and the basal body region of the flagellum (Fig. 2A,B, large arrows). In addition, the anti-Sp83 antibody brightly stains a structure overlaying the nine doublets, which is presumably the central pair microtubules (Fig. 2B, series of small arrows). The immunofluorescence patterns are similar to those seen using antibodies raised against the flagellar tektins (Steffen and Linck, 1988). While we did not observe anti-Sp77 staining of the central pair, it is important to point out that because of their relative lability during fixation we were unable consistently to preserve the central pair microtubules for our immunofluorescence analysis (as judged by anti-β-tubulin staining, not shown). Thus, we cannot rule out the possibility that the central pair microtubules also contain Sp77.

In addition to immunofluorescence techniques, we localized Sp77 and Sp83 biochemically within sperm flagellar axonemes. Intact axonemes were fractionated with sarkosyl, and the soluble and insoluble fractions were separated by ultracentrifugation. Sp77 and Sp83 were localized in the resulting fractions using immunoblot analysis, and we found that both Sp77 and Sp83 cofractionated exclusively with the pf-ribbon fraction (Fig. 3B,C, lane 2). Neither polypeptide was solubilized by detergent treatment, or by buffer extraction alone (Fig. 3B,C, lanes 3 and 5). The SDS PAGE/immunoblots were further analysed by NIH Image gel densitometry, and we found that >93% of the Sp77 and Sp83 cofractionate with the pf-ribbon fraction (data not shown). From this we conclude that both Sp77 and Sp83 are axonemal proteins specific to the pf-ribbon of the A-tubule.

The tektin filament was originally identified by its resistance to progressive biochemical extraction (Linck and Langevin, 1982). To study the stability of the association of Sp77/83 with the pf-ribbon under progressively harsher extraction conditions, we followed the Sp77 and Sp83 polypeptides during pf-ribbon extraction using a fixed concentration of

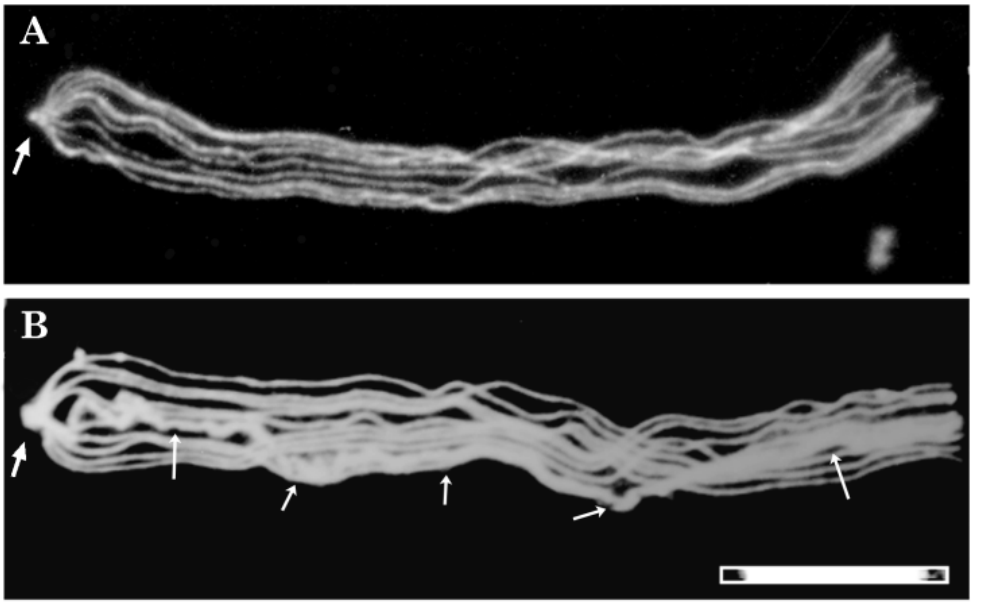

Fig. 2. Localization of Sp77 and Sp83 to splayed sperm tails of *L. pictus*. (A) anti-Sp77. (B) anti-Sp83. Each of the antibodies recognize all nine outer doublet fibers, as well as the basal body region (large arrows). In addition, anti-Sp83 decorates the central pair microtubules, seen as a bright irregular line overtop of the nine outer filaments in B (series of small arrows). Immunofluorescence microscopy. Bar, 10 μm.

detergent (0.5%) and increasing concentrations of urea (from 0.5 M to 4.0 M). After extraction, the insoluble material was pelleted by ultracentrifugation, and analysed by immunoblot. Replica blots were immunostained with either anti-Sp77, anti-Sp83 or anti-β-tubulin antibodies (Fig. 4). From these blots it was determined that Sp77, Sp83 and β-tubulin began to become solubilized at urea concentrations around 1.25 M, and all three components were completely removed from the tektin filament by treatment with 2.0 M urea (Fig. 4B-D). At 4.0 M urea, tektin C also became solubilized (Fig. 4A), which left a pure tektin A/B filament, as has previously been shown (Pirner and Linck, 1994).

Finally, we used immunoelectron microscopy to confirm that the cofractionation of Sp77 and Sp83 with pf-ribbons was due to a specific structural interaction between Sp77/Sp83 and the ribbon structure. Both anti-Sp77 and anti-Sp83 specifically decorated isolated pf-ribbons along their length (Fig. 5A-C). Pf-ribbons labelled with pre-immune IgG fraction showed no gold particle decoration (Fig. 5D). Bound and unbound gold particles were counted directly off micrographs to determine the specificity of labelling; more than 80% of the gold label present was found to be attached to the pf-ribbons (not shown).

Anti-Sp83 identifies an 83 kDa polypeptide in sea urchin zygotes and localizes to the spindle poles

To determine whether Sp77 or Sp83 were present as soluble,

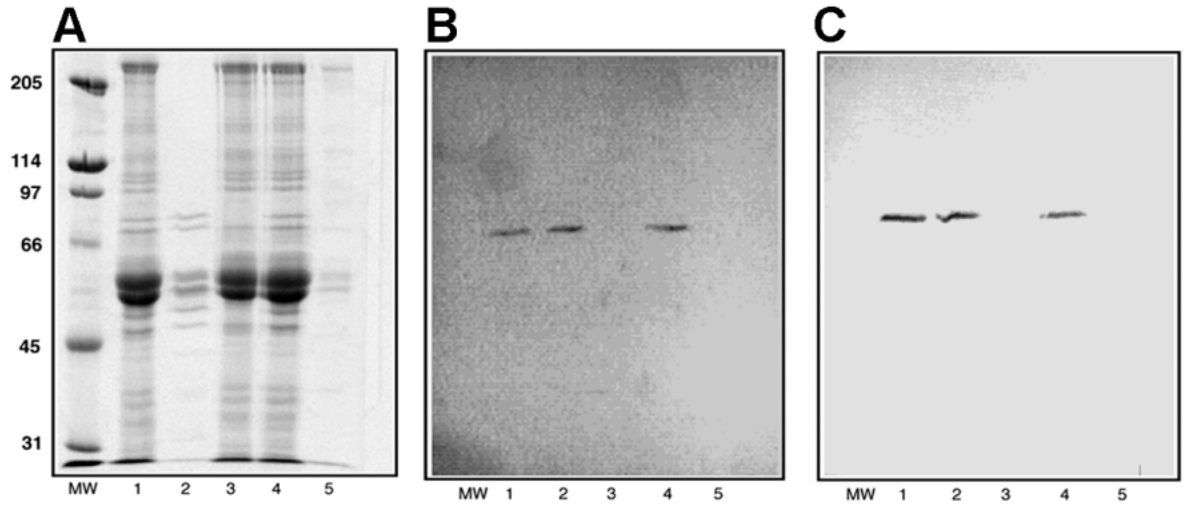

Fig. 3. Distribution of Sp77 and Sp83 following detergent-fractionation of sperm flagellar axonemes. (A) SDS-PAGE of the fractionation procedure. (B and C) Replica nitrocellulose immunoblots stained with either anti-Sp77 (B) or anti-Sp83 (C). Lanes for all three panels are as follows: 1, intact axonemes; 2, pf-ribbons (sarkosyl-insoluble pellet); 3, axoneme minus pf-ribbons (sarkosyl-soluble supernatant); 4, axonemes treated with buffer alone (control pellet); 5, soluble polypeptides from axonemes treated with buffer alone (control supernatant). Sp77 and Sp83 are not extracted from the pf-ribbon by treatment with sarkosyl (lane 3), or by treatment with buffer alone (lane 5). Positions of molecular mass markers are indicated (MW lane).

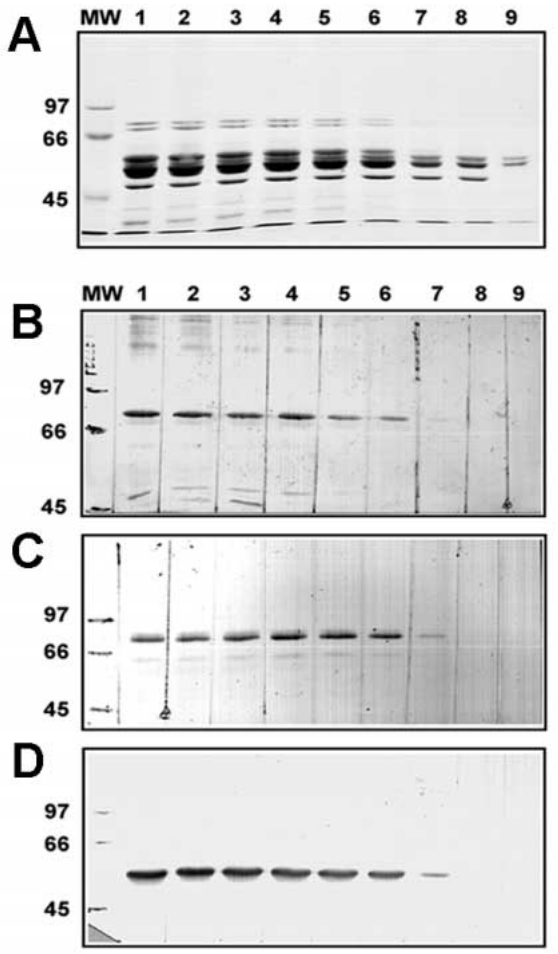

Fig. 4. SDS-PAGE/immunoblot analysis of pf-ribbon extraction using detergent-urea. Isolated pf-ribbons from *L. pictus* were incubated in 0.5% sarkosyl with increasing concentrations of urea from 0 to 4 M, and insoluble material was separated by SDS-PAGE onto four identical gels. (A) Total protein shown by Coomassie blue stain. (B-C) Immunoblots of gels identical to that shown in A. These are immunostained with: (B) anti-Sp83; (C) anti-Sp77; (D) anti-β-tubulin. Lanes: 1, no urea; 2, 0.5 M urea; 3, 0.75 M urea; 4, 1.0 M urea; 5, 1.25 M urea; 6, 1.5 M urea; 7, 1.75 M urea; 8, 2.0 M urea; 9, 4.0 M urea. Positions of molecular mass markers are indicated (MW lane).

cytoplasmic polypeptides or as microtubule associated proteins, we made cytoplasmic extracts from unfertilized *L. pictus* eggs and assembled microtubules from these extracts by addition of taxol/GTP, as previously described (Vallee and Bloom, 1983). The assembled microtubules were then salt-extracted with 0.75 M KCl to remove some of the associated proteins, as described by Suprenant and Marsh (1987). The individual fractions of this isolation procedure were analysed by replica immunoblot stained with either anti-Sp83 or anti-β-tubulin (Fig. 6). Anti-Sp77 gave non-specific crossreactions with multiple protein bands on immunoblots of egg cytoplasmic extracts (not shown), and we therefore chose to focus on the Sp83-like polypeptide for the remainder of this study. Immunoblots revealed that the anti-Sp83 antibody recognises a single polypeptide in sea urchin egg cytoplasmic extracts (Fig. 6A). This polypeptide has the same M_r as Sp83 from control ribbons (Fig. 6A, lanes 1 and 2). Virtually all of the cytoplasmic 83 kDa polypeptide copelleted with the taxol-assembled microtubules as the reactive band was absent in the microtubule-depleted extract (Fig. 6A). The 83 kDa polypeptide was not solublized by high-salt treatment and the salt-extracted taxol-microtubules retain the 83 kDa band (Fig. 6A). A replica immunoblot was stained with anti-β-tubulin and used as a control to follow the presence of tubulin during this biochemical extraction (Fig. 6B).

Our finding of an 83 kDa microtubule associated protein (MAP) in sea urchin egg cytoplasm raises the question of whether Sp83 has homology to the major *Echinoderm* 75-80 kDa MAP, identified as EMAP (Suprenant et al., 1992; Li and Suprenant, 1994). We tested this possibility using a polyclonal anti-EMAP (kindly provided by Kathy Suprenant, University of Kansas) and compared the behaviour of EMAP and Sp83 by immunofluorescence and immunoblotting. Anti-EMAP did not crossreact with any polypeptides on immunoblots of purified pf-ribbons (data not shown). By immunofluorescence microscopy, Suprenant et al. (1992) found that anti-EMAP does not decorate isolated sperm tails. We confirmed this result (data not shown). In addition we prepared an immunoblot of sea urchin egg cytoplasm, and cut a single gel lane into two strips. Blotting of each half-strip with anti-Sp83 and anti-EMAP, we were able to determine that the polypeptide recognized by anti-Sp83 had a slightly larger M_r compared to the polypeptide recognized by anti-EMAP (data not shown).

The localization of this 83 kDa polypeptide in fixed zygotes of the sea urchin *L. pictus* was investigated using immunoperoxidase light microscopy. Fig. 7A shows a zygote just after syngamy, in which two perinuclear spots are detected by the anti-Sp83 antibody (arrows), and these represent the duplicated sperm aster. As these zygotes enter mitosis, anti-Sp83 detected an expanded region at the spindle poles, but does not stain the spindle fibers (Fig. 7B,C). Interestingly, the antibody appears to detect a broad region around the spindle poles, and some of the astral microtubules. However, when we compared anti-β-tubulin staining of similar zygotes, we saw that the bulk of the astral microtubules are not detected by anti-Sp83 (Fig. 7F-J). After the first division, anti-Sp83 recognised the mitotic spindle poles in both blastomeres, and while it did not label the spindle fibers, we observed an increase in the labelling of the astral microtubules (Fig. 7D). Zygotes labelled with pre-immune IgG, or secondary antibody alone showed no staining (data not shown).

Anti-Sp83 recognizes the centrosome and spindle poles of cultured mammalian cells

We screened cultured mammalian cells with anti-Sp83 for the presence of polypeptides immunologically related to Sp83. While no anti-Sp83 immunoreactivity was detected on immunoblots of whole CHO cell extract (not shown), the antibody recognized a single polypeptide of 80 kDa on immunoblots of isolated CHO mitotic spindles (Fig. 8). Anti-Sp77 failed to recognize any polypeptides on blots of CHO cells (not shown).

Immunofluorescence microscopy revealed that anti-Sp83 (Fig. 9B, arrows) labelled the centrosome region coincident with the microtubule organizing center (MTOC), which was

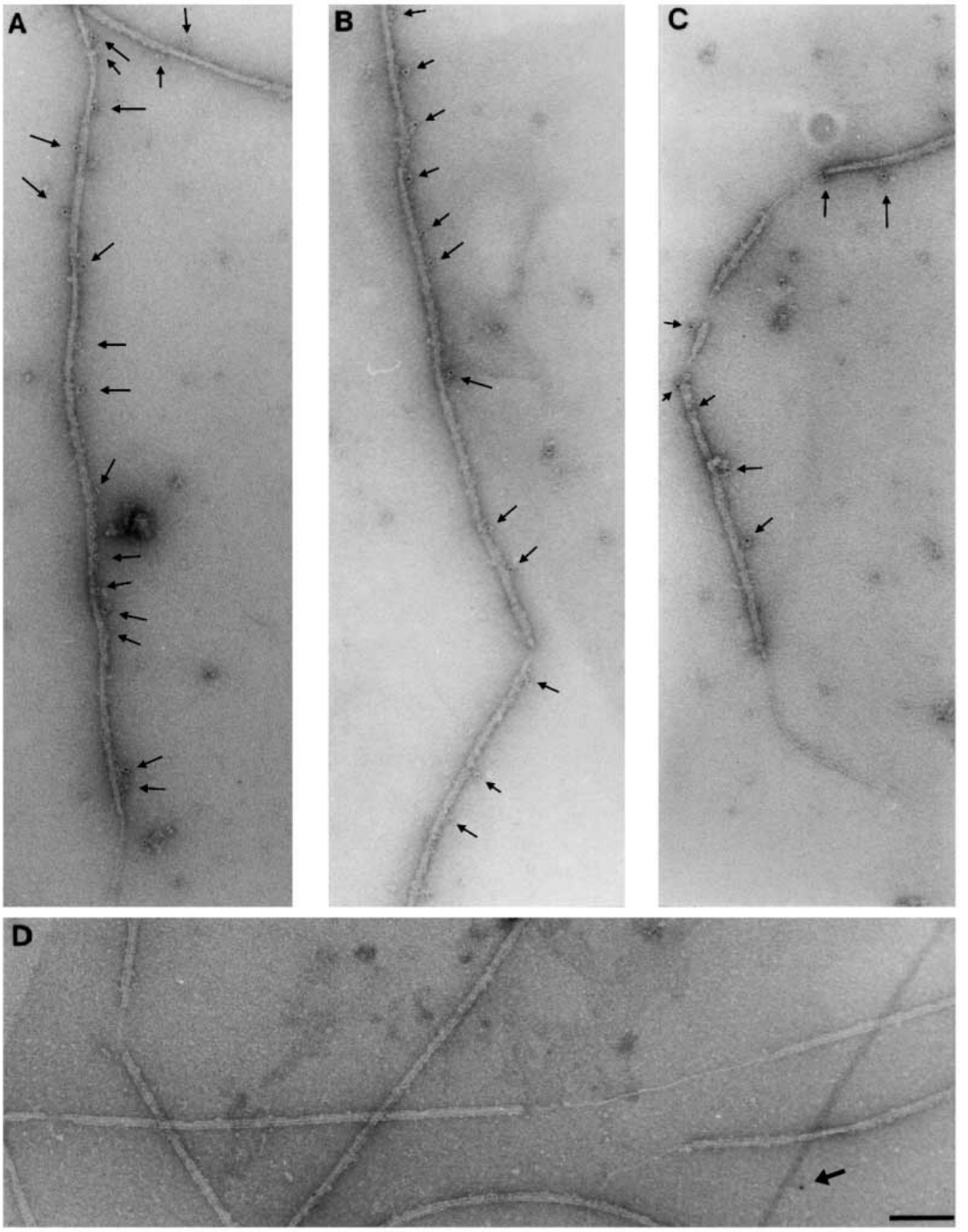

Fig. 5. Immuno-EM labelling of isolated pf-ribbons with: anti-Sp83 (A and B), anti-Sp77 (C) or pre-immune IgG (D). The lengths of the isolated pf-ribbons were decorated with either anti-Sp83 or anti-Sp77. In A-D the gold particles are indicated by arrows along the length of the ribbons. In D the pre-immune IgG fraction does not decorate isolated ribbons. Note the single gold particle (arrow) is unattached to any ribbon structures. Bar, 100 nm.

detected with anti-β-tubulin (Fig. 9A, arrows). In mitotic CHO cells, the anti-Sp83 labelled two small structures at the spindle poles, presumably the centrioles (Fig. 9D), but did not detect the astral or spindle fibers. Anti-Sp83 also detected the centrosome and mitotic spindle poles in mouse (NIH 3T3 fibroblasts) and human (HeLa) cells (data not shown).

To further confirm that the region recognized by anti-Sp83 in mammalian cells was the centrosome, we blocked DNA synthesis in CHO cells by treating them with aphidicolin, a specific inhibitor of the α-DNA polymerase. Blocking DNA synthesis arrests cells in a phase of the cell cycle which allows multiple copies of centriole-containing centrosomes to accumulate without successive mitotic divisions (Sluder and Lewis, 1987; Raff and Glover, 1988; Balczon et al., 1995). Aphidicolin-arrested CHO cells were double-labelled with anti-Sp83 and anti-β-tubulin antibodies. As can be seen in Fig. 10, in cells arrested with aphidicolin, anti-Sp83 detected multiple spots (centrosomes) (Fig. 10B, arrows), which were coincident with the enlarged MTOC that was stained by anti-tubulin (Fig. 10A).

Finally, we tested whether the anti-Sp83 antibody staining pattern followed the behaviour of the centriole microtubules. Prolonged nocodazole treatment depolymerizes cytoplasmic

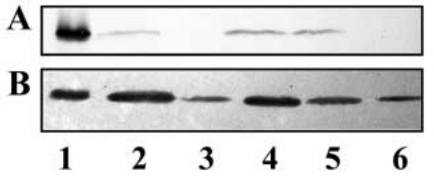

Fig. 6. Immunoblot analysis of unfertilized sea urchin egg cytoplasm and taxol-isolated microtubules. Identical nitrocellulose blots of this fraction were stained with either anti-Sp83 (A) or anti-β-tubulin (B). Lanes are: 1, isolated pf-ribbons; 2, total soluble egg cytoplasmic protein; 3, microtubule-depleted cytoplasmic supernatant; 4, taxol-stabilized microtubule pellet. The assembled microtubules were then suspended in taxol-containing buffer with 0.75 M KCl and resedimented, giving the salt-extracted microtubule pellet (lane 5) and the salt-labile MAP-containing supernatant (lane 6). Anti-Sp83 detects a single 83 kDa polypeptide in the cytoplasm (lane 2), which cosediments with the taxol microtubules (lane 4) and is not extracted by 0.75 M KCl (lane 5).

microtubules, and affects the structure of the centrosome by dispersing the PCM, yet this treatment does not disrupt the structure of the stable triplet microtubules of the centrioles (Kuriyama, 1982; Sellitto and Kuriyama, 1988; Kochanski and Borisy, 1990). To determine if anti-Sp83 staining was dispersed by microtubule depolymerization, CHO cells in culture were treated with nocodazole for 24 hours to depolymerize their microtubule network. Nocodazole-treated CHO cells were double-immunolabelled with anti-β-tubulin and anti-Sp83 (Fig. 10). Anti-β-tubulin recognises the two centrioles, associated with the nucleus (Fig. 10C). Anti-Sp83 antibody also detected these structures (Fig. 10D). Thus, the polypeptide recognized by the anti-Sp83 antibody in Fig. 10 is not dispersed by nocodazole treatment, and its continued presence at the centrosome suggests that it may be associated with the centrioles themselves.

DISCUSSION

Localization of Sp77 and Sp83 within flagellar axonemes

The first goal of this study was to isolate Sp77 and Sp83 from sea urchin sperm flagella, and to determine to what extent these polypeptides were associated with the stable pf-ribbon. While the 77 and 83 kDa polypeptides had previously been observed in preparations of isolated pf-ribbons, it remained unclear as to their distribution throughout the axoneme and whether or not they were directly associated with the pf-ribbon structure (Linck, 1990). We purified both polypeptides and produced affinity-purified polyclonal antibodies specific to each (Fig. 1). By immunofluorescence microscopy we demonstrated that both Sp77 and Sp83 are present along the entire length of the nine outer doublet microtubules, and importantly, both polypeptides localized to the basal body (Fig. 2). In addition, we found that anti-Sp83 decorated the central pair (CP) microtubules. While we did not detect anti-Sp77 staining of CP microtubules, it is important to point out that it is difficult to preserve the CP for immunomicroscopy (see Steffen and Linck, 1988). Thus the lack of central pair staining by anti-Sp77 may be due to the CP microtubules depolymerizing prior to fixation. The anti-Sp83 staining of the central pair in the present work (Fig. 2) compares with the previous immunolocalization of tektin C to the central pair microtubules of batstar sperm flagella (Steffen and Linck, 1988), and provides further evidence that pf-ribbons may be components of CP microtubules (see Steffen and Linck, 1988; Stephens et al., 1989; Linck, 1990).

Biochemical fractionation of sea urchin flagellar axonemes followed by immunoblot analysis revealed that Sp77 and Sp83 are not randomly distributed throughout the axoneme, but are specific to the stable region of the A tubule: >92% of the Sp77 and Sp83 present within these preparations copurified specifically with the pf-ribbon fraction (Figs 3

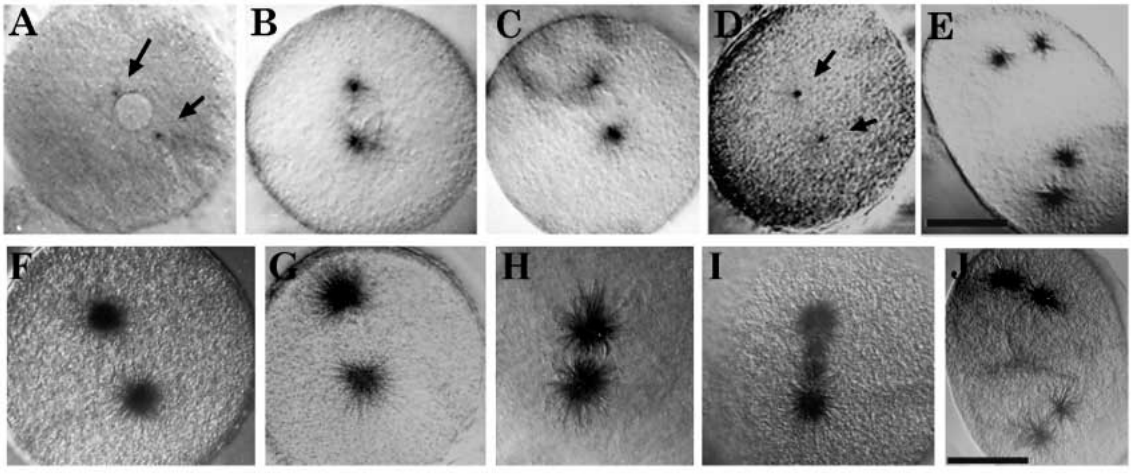

Fig. 7. Anti-Sp83 immunocytochemistry of sea urchin zygotes fixed during the first division cycle. Anti-Sp83 (A-E) and anti-β-tubulin (F-J). (A) At the time of syngamy, anti-Sp83 detects two spots near the nucleus (arrows). (B) Another zygote, fixed after pronuclear fusion, in which the antibody clearly detects the duplicated poles. (C) During prometaphase, the nuclear envelope is stretched between the two poles, which are detected by anti-Sp83. (D) A zygote fixed during first mitosis. Anti-Sp83 recognises only the poles (arrows) but not the fibers of the spindle. (E) Prometaphase of the second embryonic cell cycle. Anti-Sp83 detects the duplicated spindle poles in each blastomere, and short microtubules at the aster but not the spindle fibers themselves. Anti-tubulin staining of zygotes reveals the large astral arrays of microtubules, as well as the spindle fibers (F-J). Bright field optics. Bars, 10 μm.

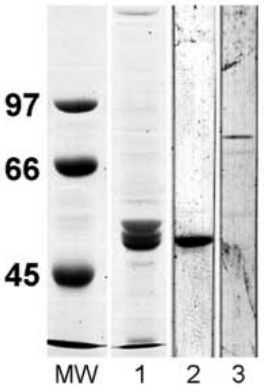

Fig. 8. Immunoblot of isolated mitotic spindles from CHO cells. Lane 1, SDS-PAGE stained with Coomassie blue to show total protein, and replica immunoblots stained with either anti-β-tubulin (lane 2) or anti-Sp83 (lane 3). MW lane, molecular mass markers.

and 4). Ultrastructural analysis using immunoelectron microscopy confirmed that both Sp77 and Sp83 associated directly with the isolated pf-ribbons (Fig. 5). Thus Sp77 and Sp83 can be considered structurally defined proteins within the axoneme, exclusive to the stable region of the flagellar A tubules.

Progressive biochemical extraction of isolated ribbons revealed that Sp77/83 are completely solubilized from the tektin filament by treatment with 0.5% sarkosyl and 2.0 M urea (Fig. 4). Thus on the basis of solubility, Sp77/83 have biochemical properties distinct from the tektin proteins. Interestingly, tubulin found associated with isolated pf-ribbons was also solubilized from the tektin filament by treatment with 0.5% sarkosyl and 2.0 M urea. This observation suggests the possibility that Sp77 and/or Sp83 may interact directly with α/β tubulin in their association with the tektin filament. Given that these polypeptides only disassociate in the presence of 0.5% detergent and 2.0 M urea these interactions would be dependent upon both hydrophobic and interchain hydrogen bonds. While we have no direct evidence for such an interaction within the pf-ribbon, a requirement for coassembly between Sp77/83, tubulin and the tektin filament could explain why purified tubulin fails to associate with isolated tektin filaments in vitro, even though the tektin backbone has potentially interactive domains which match those of the microtubule lattice (Pirner and Linck, 1994; Nojima et al., 1995; Norrander et al., 1996).

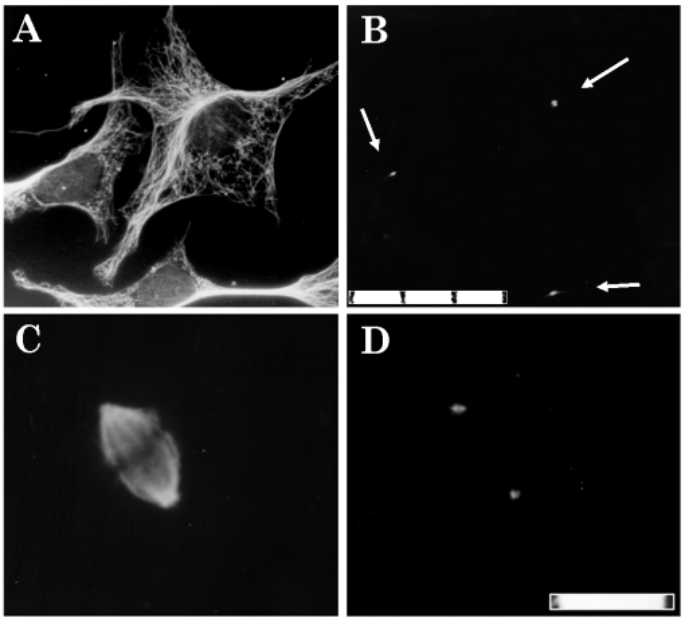

Fig. 9. Localization of the Sp83-like polypeptide in CHO cells double-labelled with monoclonal anti-β-tubulin (A and C) and polyclonal anti-Sp83 (B and D), and visualized by immunofluorescence microscopy. During interphase, anti-tubulin (A) decorates the entire microtubule network, while anti-Sp83 (B) decorates only the area coincident with the microtubule organizing center (MTOC, arrows). During mitosis, the anti-tubulin (C) recognises both the spindle fibers and spindle poles while anti-Sp83 (D) labelled only the spindle poles. Each scale division, 10 μm (A,B); bar, 10 μm (C,D).

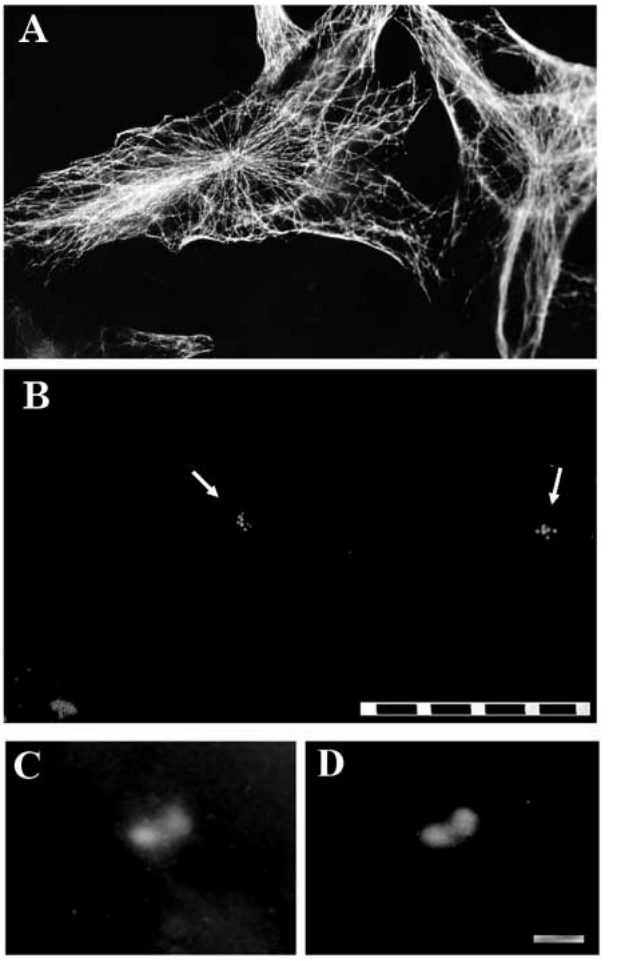

Fig. 10. Localization of the Sp83-like polypeptide in CHO cells arrested in S phase with aphidicolin or treated with nocodazole and visualized by fluorescence optics. CHO cells were arrested for 60 hours in S phase using aphidicolin and double-labelled with anti-β-tubulin (A) and anti-Sp83 (B). During this arrest, the centrosomes continued to duplicate; each cell contains multiple closely spaced centrosomes (B, arrows) present within the enlarged MTOC region (A). (C,D) Highly magnified views of a CHO cell treated for 12 hours with nocodazole and then doubled-immunolabelled with a monoclonal anti-β-tubulin antibody (C) and polyclonal anti-Sp83 (D). The anti-β-tubulin decorates two centrioles (C), which are also recognized by the anti-Sp83 (D). Each scale division, 10 μm (A,B); bar, 1 μm (C,D).

Sp 83 is a microtubule associated protein in sea urchin zygotes and localizes to the spindle poles

We assayed sea urchin egg cytoplasmic extracts by immunoblot and found that the affinity-purified anti-Sp83 antibody recognized a single polypeptide of 83 kDa (Fig. 6). Unfortunately the anti-Sp77 antibody detected multiple high molecular mass bands in a non-specific manner on these blots, and as it failed to detect any polypeptides on blots of CHO cell proteins, we chose to focus on Sp83 for the remainder of the study.

When microtubules were assembled from egg cytoplasmic extracts using taxol, we found that virtually all of the 83 kDa polypeptide present in the cytoplasm cofractionated with the microtubules. Further extraction of these assembled microtubules with 0.75 M KCl to remove the MAPs did not solubilize the 83 kDa polypeptide, further suggesting that Sp83 homologue is associated with microtubules in a salt-stable fashion. This is consistent with our finding in flagella that Sp83 and tubulin may associate using both hydrophobic and hydrogen bonds.

Given the molecular masses of Sp77 and Sp83, we asked whether these polypeptides were related to the 75-80 kDa MAPs previously observed in echinoderms (Kellor and Rebhun, 1982; Vallee and Bloom, 1983; Scholey et al., 1984; Bloom et al., 1985; Hirokawa and Hisanga, 1987; Hosoya et al., 1990). It has been demonstrated that the various 75-80 kDa echinoderm MAPs reported actually represent a single polypeptide, which has been identified as a protein called EMAP (Suprenant et al., 1993; Li and Suprenant, 1994), and is thought to function in anchoring cargo proteins to the microtubules of the mitotic apparatus in echinoderm embryos (Brisch et al., 1996). Using anti-EMAP antibodies (supplied by Kathy Suprenant, University of Kansas) we determined that Sp77 and Sp83 are both polypeptides distinct from EMAP: (1) anti-EMAP does not recognize sea urchin flagella by immunofluorescence microscopy; (2) anti-EMAP does not detect any polypeptides on immunoblots of isolated pf-ribbons from *L. pictus* or *S. purpuratus* flagella; (3) the M_r of the sea urchin egg cytoplasmic 83 kDa polypeptide detected by anti-Sp83 is slightly larger than that detected by anti-EMAP (data not shown).

In early sea urchin zygotes, we found that the 83 kDa polypeptide was localized specifically to the duplicated sperm aster and spindle poles throughout the first embryonic division cycle, but did not decorate the spindle fibers (Fig. 7). Interestingly, anti-Sp83 also decorates a broad region at the spindle poles and a few short microtubules at the aster. However, when we compare the staining patterns of Sp83 and β-tubulin, it is clear that Sp83 does not detect the entire aster (Fig. 7F-J). It may be that Sp83 associates with some of the astral microtubules, which have increased stability (see Shaw et al., 1997). Another possibility is that this staining represents the transport of centrosomal components to the cell center along cytoplasmic microtubules, as has recently been suggested (see Balczon, 1997). Our finding that anti-Sp83 decorates the centrosomes of the sea urchin zygote is consistent with the localization of Sp83 to the flagellar basal body, which in sea urchin zygotes functions to assemble the centrosome following fertilization (Wilson and Matthews, 1895; Sluder et al., 1990; Holy and Schatten, 1991).

Anti-Sp83 recognizes an 80 kDa polypeptide in somatic cultured cells

Anti-Sp83 detected a single polypeptide of approx. 80 kDa on immunoblots of mitotic spindles isolated from CHO cells (Fig. 8). In CHO cells, the approx. 80 kDa polypeptide was found to be associated with the interphase centrosome and the mitotic spindle poles by immunofluorescence microscopy (Fig. 9). Similar staining patterns were found in mouse (NIH 3T3 fibroblasts) and human (HeLa) cells (not shown). As has previously been demonstrated by Balczon et al. (1995), CHO cells arrested in prolonged S phase assemble multiple centriole-containing centrosomes. We repeated this experiment using aphidicolin, a specific inhibitor of DNA polymerase-α, to inhibit DNA synthesis and found that these cells assembled multiple, closely spaced centrosomes within the enlarged MTOC region (Fig. 10A,B). Within this enlarged MTOC anti-Sp83 detected up to eight centrosomes (Fig. 10B).

We also examined the distribution of the approx. 80 kDa polypeptide in CHO cells continuously treated with nocodazole for 24 hours, which has been shown both to depolymerize the microtubule network and to disperse the PCM from the centrosome, but importantly does not disrupt the structure of the triplet microtubules of the centriole (Kuriyama, 1982; Sellito and Kuriyama, 1988; Kochanski and Borisy, 1990). We found that the approx. 80 kDa polypeptide remained associated with a pair of perinuclear structures, which were detected by anti-tubulin antibodies in double-label immunofluorescence studies (Fig. 10). The location, appearance and size of these structures (0.25 μm) suggests that they are the centriole pair.

Conclusions

The localisation of Sp77 and Sp83 proteins, which is exclusively to the stable region of the axonemal A microtubule, and their resistance to biochemical extraction, suggest that they perform a structural function, possibly by mediating the interaction between tubulin and the tektin protofilament. Our findings also suggest that these polypeptides appear to be components of the centriole microtubules. Both polypeptides localize to the flagellar basal body, and in zygotes and somatic cells there appears to be a homologous 80 kDa polypeptide that colocalizes with the centriole. Thus these proteins, found in the walls of flagellar microtubules, may represent conserved components of the stable doublet and triplet microtubules in axonemes, basal bodies and centrioles.

In future it will be of interest to determine the interactions between Sp77, Sp83, tubulin and the tektin filament, and the signals that regulate how these components assemble into stable microtubules.

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REFERENCES

Allen, C. and Borisy, G. (1974). Structural polarity and directional growth of microtubules of *Chlamydomonas* flagella. *J. Mol. Biol.* **90**, 381-402.

Balczon, R., Bao, L., Zimmer, W. E., Brown, K., Zinkowski, R. P. and Brinkley, B. R. (1995). Dissociation of centrosome replication events from cycles of DNA synthesis and mitotic division in hydroxyurea-arrested Chinese hamster ovary cells. *J. Cell Biol.* **130**, 105-115.

Balczon, R. (1997). A role for microtubules in centrosome replication events. *Mol. Biol. Cell* **8**, 55a.

Behnke, O. and Forer, A. (1967). Evidence for four classes of microtubules in individual cells. *J. Cell Sci.* **2**, 169-192.

Bloom, G. S., Luca, F. C., Collins, C. A. and Vallee, R. B. (1985). Use of multiple monoclonal antibodies to characterize the major microtubule-associated proteins in sea urchin eggs. *Cell Motil.* **5**, 431-446.

Brisch, E., Daggett, M. A. F. and Suprenant, K. A. (1996). Cell cycle-dependant phosphorylation of the 77 kDa echinoderm microtubule-associated protein (EMAP) in vivo and association with the $p34^{cdc2}$ kinase. *J. Cell Sci.* **109**, 2885-2893.

Brinkley, B. R. (1985). Microtubule organizing centers. *Ann. Rev. Cell Biol.* **1**, 145-172.

Chang, X. J. and Piperno, G. (1987). Cross-reactivity of antibodies specific for flagellar tektin and intermediate filament subunits. *J. Cell Biol.* **104**, 1563-1568.

Chen, R., Perrone, C. A., Amos, L. and Linck, R. W. (1993). Tektin B1 from ciliary microtubules: primary structure as deduced from the cDNA sequence and comparison with tektin A1. *J. Cell Sci.* **106**, 909-918.

Fuseler, J. F. (1973). Repetitive procurement of mature gametes from individual sea stars and sea urchins. *J. Cell Biol.* **57**, 879-881.

Gall, J. G. (1961). Centriole replication. *J. Biophys. Biochem. Cytol.* **10**, 163-193.

Gibbons, I. R. and Grimstone, A. V. (1960). On flagellar structure of certain flagellates. *J. Biophys. Biochem. Cytol.* **7**, 697-716.

Gould, R. R. and Borisy, G. G. (1977). The pericentriolar material in Chinese hamster ovary cells nucleates microtubule formation. *J. Cell Biol.* **73**, 601-615.

Hamaguchi, Y. and Kuriyama, R. (1982). Aster formation in sand dollar eggs by microinjection of calcium buffers and centriolar complexes isolated from starfish sperm. *Exp. Cell Res.* **141**, 450-454.

Heideman, S. R. and Kirschner, M. W. (1975). Aster formation in eggs of *Xenopus laevis*. Induction by isolated basal bodies. *J. Cell Biol.* **67**, 105-117.

Hirokawa, N. and Hisanaga, S. L. (1987). 'Buttonin', a unique button-shaped microtubule-associated protein (75 kDa) that decorates spindle microtubule surface hexagonally. *J. Cell Biol.* **104**, 1553-1561.

Holy, J. and Schatten, G. (1991). Spindle pole centrosomes of sea urchin embryos are partially composed of material recruited from maternal stores. *Dev. Biol.* **147**, 343-353.

Hosoyo, N., Hosoyo, H., Mohri, T. and Mohri, H. (1990). A 70 kD microtubule-binding protein from starfish egg, purification, characterization, and localization during meiosis and mitosis. *Cell Motil. Cyto.* **15**, 168-180.

Kellogg, D. R., Moritz, M. and Alberts, B. M. (1994). The centrosome and cellular organization. *Annu. Rev. Biochem.* **63**, 639-674.

Kellor, T. C. S. I. and Rebhun, L. I. (1982). *Stronglyocentrotus purpuratus* spindle tubulin I. Characterization of its polymerization and depolymerization in vitro. *J. Cell Biol.* **93**, 788-796.

Kimble, M. and R. Kuriyama (1992). Functional components of microtubule-organizing centers. *Int. Rev. Cytol.* **136**, 1-50.

Knudsen, K. A. (1985). Proteins transferred to nitrocellulose for use as immunogens. *Anal. Biochem.* **147**, 285-288.

Kochanski, R. S. and Borisy, G. G. (1990). Mode of centriole duplication and distribution. *J. Cell Biol.* **110**, 1599-1605.

Kuriyama, R. (1982). Effect of colcemid on the centriole cycle in Chinese hamster ovary cells. *J. Cell Sci.* **53**, 155-177.

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

Li, Q. and Suprenant, K. A. (1994). Molecular characterization of the 77 kDa echinoderm microtubule-associated protein. *J. Biol. Chem.* **269**, 31777-31784.

Linck, R. W. (1976). Flagellar doublet microtubules: fractionation of minor components and α-tubulin from specific regions of the A-tubule. *J. Cell Sci.* **20**, 405-439.

Linck, R. W. (1990). Tektins and microtubules. *Adv. Cell Biol.* **3**, 35-63.

Linck, R. W., Amos, L. A. and Amos, W. B. (1985). Localization of tektin filaments in microtubules of sea urchin sperm flagella by immunoelectron microscopy. *J. Cell Biol.* **100**, 126-135.

Linck, R. W. and Langevin, G. L. (1982). Structure and chemical composition of insoluble filamentous components of sperm flagellar microtubules. *J. Cell Sci.* **58**, 1-22.

Matsudaira, P. (1987). Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* **262**, 10035-10038.

Meza, I., Huang, B. and Bryan, J. (1972). Chemical heterogeneity of protofilaments forming the outer doublets from sea urchin flagella. *Exp. Cell Res.* **74**, 535-540.

Nojima, D., Linck, R. W. and Egelman, E. H. (1995). At least one of the protofilaments in flagellar microtubules is not composed of tubulin. *Curr. Biol.* **5**, 158-167.

Norrander, J. M., Amos, L. A. and Linck, R. W. (1992). Primary structure of tektin A1: Comparison with intermediate-filament proteins and a model for its association with tubulin. *Proc. Nat. Acad. Sci. USA* **89**, 8567-8571.

Norrander, J. M., Perrone, C. A., Amos, L. A. and Linck, R. W. (1996). Structural comparison of tektins and evidence for their determination of complex spacings in flagellar microtubules. *J. Mol. Biol.* **257**, 385-397.

Palazzo, R. E., Vaisberg, E., Cole, R. W. and Rieder, C. L. (1992). Centriole duplication in lysates or Spisula solidissima oocytes. *Science* **256**, 219-221.

Pirner, M. A. and Linck, R. W. (1994). Tektins are heterodimeric polymers in flagellar microtubules with axial periodicities matching the tubulin lattice. *J. Biol. Chem.* **269**, 31800-31806.

Pirner, M. A. and Linck, R. W. (1995). Methods for the isolation of tektins and sarkosyl-insoluble protofilament ribbons. *Meth. Cell Biol.* **47**, 373-380.

Porter, M. E., Grissom, P. M., Scholey, J. M., Salmon, E. D. and McIntosh, J. R. (1988). Dynein isoforms in sea urchin eggs. *J. Biol. Chem.* **263**, 6759-6771.

Raff, J. W. and Glover, D. M. (1988). Nuclear and cytoplasmic mitotic cycles continue in Drosophila embryos in which DNA synthesis is inhibited by aphidicolin. *J. Cell Biol.* **107**, 2009-2019.

Schatten, G. (1994). The centrosome and its mode of inheritance: the reduction of the centrosome during gametogenesis and its restoration during fertilization. *Dev. Biol.* **165**, 299-335.

Schatten, H., Schatten, G., Mazia, D., Balczon, R. and Simerly, C. (1986). Behavior of centrosomes during fertilization and cell division in mouse oocytes and in sea urchin eggs. *Proc. Nat. Acad. Sci. USA* **83**, 105-109.

Scholey, J. M., Neighbors, B., McIntosh, J. R. and Salmon, E. D. (1984). Isolation of microtubules and a dynein-like MgATPase from unfertilized sea urchin eggs. *J. Biol. Chem.* **259**, 6516-6525.

Sellitto, C. and Kuriyama, R. (1988). Distribution of pericentriolar material in multipolar spindles induced by colcemid treatment in Chinese hamster ovary cells. *J. Cell Sci.* **89**, 57-65.

Shaw, S. L., Yeh, E., Maddox, P., Salmon, E. D. and K. Bloom (1997). Astral microtubule dynamics in yeast: a microtubule-based searching mechanism for spindle orientation and nuclear migration into the bud. *J. Cell Biol.* **139**, 985-994.

Silva, R. (1981a). Hypersensitizing, part 1. *Astronomy* **April,** 39-42.

Silva, R. (1981b). Hypersensitizing, part 2. *Astronomy* **May,** 48-50

Sluder, G. and Lewis, K. (1987). Relationship between nuclear DNA synthesis and centrosome reproduction in sea urchin eggs. *J. Exp. Zool.* **244**, 89-100.

Sluder, G., F. J. Miller, F. J., Cole, R. and Rieder, C. L. (1990). Protein synthesis and the cell cycle: centrosome reproduction in sea urchin eggs is not under translational control. *J. Cell Biol.* **110**, 2025-2032.

Sluder, G. and Rieder, C. L. (1985). Experimental separation of pronuclei in fertilized sea urchin eggs: chromosomes do not organize a spindle in the absence of centrosomes. *J. Cell Biol.* **100**, 897-903.

Steffen, W., Fajer, E. A. and Linck, R. W. (1994). Centrosomal components immunologically related to tektins from ciliary and flagellar microtubules. *J. Cell Sci.* **107**, 2095-2105.

Steffen, W. and Linck, R. W. (1988). Evidence for tektins in centrioles and axonemal microtubules. *Proc. Nat. Acad. Sci. USA* **85**, 2643-2647.

Steffen, W. and Linck, R. W. (1989). Relationship between tektins and intermediate filament proteins: an immunological study. *Cell Motl. Cyto.* **14**, 359-371.

Stephens, R. E., Oleszko-Szuts, S. and Linck, R. W. (1989). Retention of

cilliary ninefold structure after removal of microtubule. *J. Cell Sci.* **92**, 391-402.

Suprenant, K. A., Dean, K., McKee, J. and Hake, S. (1993). EMAP, an echinoderm microtubule-associated protein found in microtubule-ribosome complexes. *J. Cell Sci.* **104**, 445-456.

Suprenant, K. A. and Marsh, J. C. (1987). Temperature and pH govern the assembly of microtubules from unfertilized sea urchin egg extracts. *J. Cell Sci.* **87**, 71-84.

Talian, J. C., Olmsted, J. B. and Goldman, R. D. (1983). A rapid procedure for preparing fluorescein-labeled specific antibodies from whole antiserum: its use in analysing cytoskeletal architecture. *J. Cell Biol.* **97**, 1277-1282.

Towbin, H., Staehin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitro-cellulose sheets: procedure and some applications. *Proc. Nat. Acad. Sci. USA* **76**, 4350-4354.

Vallee, R. B. and Bloom, G. S. (1983). Isolation of sea urchin egg microtubules with taxol and identification of mitotic spindle microtubule-associated proteins with monoclonal antibodies. *Proc. Nat. Acad. Sci. USA* **80**, 6259-6263.

Wheatley, D. N. (1982). *The Centriole: A Central Enigma of Cell Biology.* Amsterdam, Elsevier Biomedical Press.

Wheatley, D. N. (1997). 'Essential function'(s) of the centriole: Questions. *Cell Biol. Int.* **21**, 191-192

Wilson, E. B. and Matthews, A. P. (1895). Maturation, fertilization, and polarity in the echinoderm egg. New light on the 'Quadrille of the Centers'. *J. Morphol.* **10**, 319-342.

Witman, G. B., Carlson, K., Berliner, J. and Rosenbaum, J. L. (1972). Chlamydomonas flagella. I. Isolation and electrophoretic analysis of microtubules, matrix, membranes and mastigonemes. *J. Cell Biol.* **54**, 507-539.

Wright, B. D., Henson, J. H., Wedaman, K. P., Willy, P. J., Morand, J. N. and Scholey, J. M. (1991). Subcellular localization and sequence of sea urchin kinesin heavy chain: evidence for its association with membranes in the mitotic apparatus and interphase cytoplasm. *J. Cell Biol.* **113**, 817-833.

Yuan, L., Liu, J. G. and Höög, C. (1995). Rapid cDNA sequencing in combination with RNA expression studies in mice identifies a large number of male germ cell-specific sequence tags. *Biol. Reproduct.* **52**, 131-138.