Rib72, a Conserved Protein Associated with the Ribbon Compartment of Flagellar A-microtubules and Potentially Involved in the Linkage between Outer Doublet Microtubules*

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Ciliary and flagellar axonemes are basically composed of nine outer doublet microtubules and several functional components, e.g. dynein arms, radial spokes, and interdoublet links. Each A-tubule of the doublet contains a specialized “ribbon” of three protofilaments composed of tubulin and other proteins postulated to specify the three-dimensional arrangement of the various axonemal components. The interdoublet links hold the doublet microtubules together and limit their sliding during the flagellar beat. In this study on Chlamydomonas reinhardtii, we cloned a cDNA encoding a 71,985-Da polypeptide with three DM10 repeats, two C-terminal EF-hand motifs, and homologs extending to humans. This polypeptide, designated as Rib72, is a novel component of the ribbon compartment of flagellar microtubules. It remained associated with 9-fold arrays of doublet tubules following extraction under high and low ionic conditions, and anti-Rib72 antibodies revealed an ~96-nm periodicity along axonemes, consistent with Rib72 associating with interdoublet links. Following proteolysis- and ATP-dependent disintegration of axonemes, the rate of cleavage of Rib72 correlated closely with the rate of sliding disintegration. These observations identify a ribbon-associated protein that may function in the structural assembly of the axoneme and in the mechanism and regulation of ciliary and flagellar motility.

In organisms ranging from protists to mammals, cilia and flagella act as propulsive and sensory organelles (1). In mice and humans, mutations affecting cilia lead to a variety of phenotypes, including abnormalities in left-right axis development (e.g. situs inversus), blindness, male sterility, polycystic kidney disease, polydactyly, and respiratory and liver diseases (2–5). This study describes a flagellar microtubule-associated protein that has been contemporaneously discovered and studied by three laboratories. The timely and detailed studies of this previously unknown protein now shed light on its role in the structural assembly of the ciliary axoneme and in the mechanism and regulation of motility.

Cilia and flagella are composed of ~250 different polypeptides, whose assembly is nucleated by a modified centriole, the basal body, to form a membrane-bound axoneme (6). The three-dimensionally precise and complex structure of the axoneme is essential to its function, e.g. the propagation of regular bending waves. In most eukaryotes, the axoneme is composed of nine outer doublet microtubules, two central singlet microtubules, and numerous axoneme-associated components. Outer dynein arms, inner dynein arms, radial spokes, and interdoublet links are bound at precise points both around and along the A-microtubule, with axial spacings characteristic of each of the different components and species. In Chlamydomonas reinhardtii, outer dynein arms are attached to docking sites with axial repeats of 24-nm intervals (7, 8). Radial spokes and inner dynein arms have more complex subspacings, but have an overall axial repeat of 96 nm (9, 10). These spacings are multiples of the 8-nm tubulin dimer repeat; however, it is unlikely in Chlamydomonas, with only one isofrom each of α- and β-tubulin (11, 12), that the tubulin lattice alone provides the three-dimensional spatial information for the binding of all the different axonemal components. How the evolutionarily conserved and precise structure of the axoneme is formed remains an intriguing and important question.

This question has been partially addressed by evidence implicating a unique feature of the A-microtubule as a scaffold for the three-dimensional architecture of the axoneme. In organisms as divergent as protists (Chlamydomonas), echinoderms, and mollusks, extraction of doublet microtubules with the detergent Sarkosyl causes the breakdown of A-microtubules into “ribbons” of three adjoining protofilaments, as seen by electron microscopy (13–18). In parallel with molecular cloning studies in sea urchin and mouse (19–22), chemical cross-linking studies demonstrated that three polypeptides, tektins A, B, and C, remain as extended ~5-nm diameter filaments after extraction of the ribbons with 2 M urea (23). Structural studies indicated that a tektin polymer forms one of the protofilaments of the ribbons and A-microtubules, approximately in the section of the wall to which the radial spokes and inner B-tubule wall are attached (24). Other studies indicated that tektin A, which has
a dramatic steady-state turnover rate in ciliated epithelia, may assemble into the ribbon of pre-existing doublet microtubules (25). Tektins and ribbons have observed and/or predicted axial periodicities of 16, 24, 32, 40, and 48 nm (17, 21, 24, 26). Collectively, these observations led to the hypothesis that tektin filaments act as molecular rulers that relay spatial information for the binding of axonemal components within the axoneme. Associated with the ribbons of *Chlamydomonas* flagella are polypeptides of 43 and 70 kDa. The Rib43a protein and RB43a gene have been characterized (18). Rib43a has homologs in species ranging from *Caenorhabditis elegans* and *Drosophila* to human; and although it does have a coiled-coil structure similar to half a tektin subunit, it has no primary sequence homology to tektins. Further studies of ribbons and their components will likely provide important insights into the assembly and function of cilia and flagella.

Ciliary and flagellar beating is based on localized sliding between adjacent outer doublet microtubules that oscillate back and forth within a certain distance (27). Therefore, the outer doublets, while remaining connected to each other, must have the freedom to undergo shearing motion over a limited length. The classic experiment by Summers and Gibbons (28) showed that, when ATP is added to fragmented axonemes of sea urchin sperm after brief trypsin treatment, axonemes disintegrate via sliding of the outer doublet microtubules. Subsequent work by Sale and Satir (29) demonstrated that the dynein-mediated sliding occurs in a polar minus-end direction. Summers and Gibbons (28) suggested that trypsin disrupts the structure connecting adjacent outer doublet microtubules while leaving the force generator dynein functionally intact. An interdoublet microtubule connection was thought to have the function of limiting the sliding distance and thus converting sliding to bending; proteolytic disruption of this structure presumably allows limitless sliding. In fact, electron microscopy studies indicated that interdoublet links and radial spokes are rapidly lost due to the proteolysis, whereas dynein persists for some time (30). The interdoublet links, also called nexin links, seem to be more fundamental than radial spokes for axoneme function because later studies with *Chlamydomonas* mutants indicated that axonemes can beat without radial spokes under certain conditions (31, 32). We may thus expect that the interdoublet links are essential both for maintaining the axonemal structure and for producing regular bending waves. However, despite the expected functional importance of interdoublet links, little is known about their molecular identity and properties. Some studies suggest that the link is highly elastic and stretchable (33, 34), whereas other studies suggest that it can reversibly detach from the outer doublets and thus is not necessarily elastic (35, 36). Additional studies indicated that adjacent outer doublets are indeed connected by an elastic component (37, 38), and yet the connection can reversibly detach from the outer doublets (38). Clearly, more information must be obtained regarding the molecules that constitute the interdoublet link.

The study reported here started independently in our two laboratories, one aiming at characterizing the ~70-kDa ribbon component and the other aiming at a protein constituting or associated with the interdoublet links. To our surprise, our laboratories arrived at the same protein. We report here the characterization of a 72-kDa ribbon-associated protein that potentially functions in the structural assembly of the axoneme and in maintaining the linkage of the nine outer doublet microtubules during dynein-mediated sliding and flagellar beating. Our results extend those of Patel-King et al. (39), who also reported the predicted structure of the 72-kDa protein and postulated it to be a regulatory subunit of a nucleoside-diphosphate kinase.

### EXPERIMENTAL PROCEDURES

**Strains and Culture**—The cell strains used in this study were Chlamydomonas reinhardtii wild-type 157c, od1 lacking the outer arm with 200 nM, and p/g14 lacking the radial spokes (41). Cells were grown in light/dark cycle or in rich medium containing sodium acetate and additional potassium phosphate under continuous light (43, 44).

**Isolation of Axonemes**—Flagellar axonemes were isolated by the diatom method of Wittman et al. (15, 45) or by the pH shock method of Wittman et al. (14, 15). Flagella were demembranated to yield axonemes by extraction with Nonidet P-40 in HMDEK solution (30 mM HEPES, 5 mM MgSO₄, 1 mM DTT, 1 mM EGTA, and 50 mM potassium acetate, pH 7.4) or in HSSD solution (10 mM HEPES, 1 mM SrCl₂, 4% sucrose, and 1 mM DTT, pH 7.4). Axonemes were finally resuspended in HMDEK solution or purified by sucrose gradient centrifugation (14, 43, 44) and resuspended in HSSD solution.

**High and Low Salt Extraction of Axonemes**—Flagellar axonemes were extracted with high ionic strength solution (HMDEK solution plus 0.6 M KCl) for 30 min at 4°C. The suspension was then dialyzed overnight against low ionic strength solution (2 mM HEPES, 0.2 mM EDTA, and 0.5 mM DTT, pH 7.4). The integrity of the extracted axonemes was observed by dark-field microscopy. The extracted axonemes were centrifuged at 10,000 × g for 10 min to obtain the pellet and supernatant for SDS-PAGE analysis.

**Isolation and Purification of Ribbons**—Axonemes were fractionated into protofilament ribbons as previously described (18) with the modifications described under “Materials and Methods.”

**Sliding Disintegration**—Sliding disintegration of axonemes was induced by a method based on the method of Summers and Gibbons (28). Protease (trypsin, elastase, or nagarse) and ATP (final concentration of 0.1 mM) were added to an axonemal suspension at room temperature. These proteases have been shown to be effective in inducing disintegration of *Chlamydomonas* axonemes (46). The protein ratio of protease to axonemes was varied between 1:2000 and 1:50. In some experiments, the progression of disintegration was monitored using a dark-field microscope equipped with a 100-watt mercury arc lamp and SIT camera (Hamamatsu Photonics, Hamamatsu, Japan). The progress of sliding disintegration was quantified by measuring the number of intact axonemes versus those that underwent disintegration. Because the axonemes were not first fragmented, the outer doublet microtubules remained held together at the base even after disintegration took place. This condition facilitated the measurement of the numbers of disintegrated axonemes. In other experiments, the progression of disintegration was monitored spectrophotometrically by measuring the turbidity of the samples at 350 nm after addition of ATP and protease. For protein analysis of the disintegrating axonemes, aliquots of samples were transferred at regular intervals to SDS-PAGE sample buffer and immediately boiled for 3 min.

**Sequencing of the 72-kDa Ribbon-associated Protein by Tandem Mass Spectrometry**—This procedure was modified from that of Kinter and Sherman (47) and conducted at room temperature except when stated otherwise. The relevant protein band was cut from the gel and cut into pieces of ~1 nm². Gel pieces were extracted with 200 μl of 50% methanol and 5% acetic acid overnight and again for 3 h. A 5-min incubation with 200 μl of acetonitrile was carried out to dehydrate the gel pieces. The acetonitrile was discarded, and dehydration was completed by centrifugation under vacuum for 3 min. Gel pieces were rehydrated by incubation for 30 min in 30 μl of 10 mM DTT in 100 mM ammonium bicarbonate. The DTT solution was discarded, and 30 μl of 100 mM iodoacetamide in 100 mM ammonium bicarbonate was added with incubation for 30 min. The iodoacetamide solution was discarded, and the gel pieces were dehydrated with acetonitrile as described above. After dehydrating the acetonitrile, gel pieces were rehydrated with 200 μl of 100 mM ammonium bicarbonate for 10 min. The ammonium bicarbonate was discarded, and the gel pieces were dehydrated with acetonitrile as described above. After desiccating the acetonitrile, dehydration of the gel pieces was completed by centrifugation under vacuum for 3 min. Gel pieces were transferred to 200 μl of trypsin reagent (20 μg/ml of ice-cold 50 mM ammonium bicarbonate) was added to the sample, and the gel pieces were allowed to rehydrate on ice for 10 min with occasional vortexing. The gel pieces were centrifuged for 30 s, and excess trypsin solution was removed. 5 μl of 50 mM ammonium bicarbonate was added to the sample, and the
sample was vortexed. Digestion was carried out overnight at 37 °C. 50 μL of 50 mM ammonium bicarbonate was added to the digest, and the sample was extracted for 10 min with occasional gentle vortexing. The gel pieces were centrifuged for 30 s, and the peptide-containing supernatant was transferred to a plastic microcentrifuge tube. This extract was repeated two more times, and the supernatants were combined. The volume of the extract was reduced to <20 μL in a vacuum centrifuge. 1 μL of peptide extract was manually injected onto a Ther-moHypersil BetaBasic18 C_{18} microbore column (50 × 0.18 mm, 150-A pore size) running at 2 μL/min. A linear gradient from 100% solution A (95% acetonitrile/water with 1% formic acid) to 40% solution B (5% acetonitrile/water with 1% formic acid) was run for 40 min with direct nanoelectrospray into a Finnigan LCQ ion trap. Data-dependent tandem mass spectra were acquired by automatic switching between mass spectrometry and tandem mass spectrometry mode by the instrument (described in the instrument documentation) and were searched using Sequest software (48) against the latest version of the expressed sequence tag data bases filtered for Clamydomonas.

Clones AY398026 and AY628903 were sequenced by the DNA Sequencing and Synthesis Facility of Iowa State University (Ames, IA). Sequence assembly and analysis were performed using the Wisconsin Package Version 10.2 of Genetics Computer Group (Madison, WI). Molecular mass and pI predictions were performed using the Compute pI/MW Program of ExPasy. Pattern and profile searches were performed using ScanProsite, ExPasy, and BLAST through the NCBI Protein Database.

Peptide Sequencing of the 72-kDa Protease-sensitive Axonemal Protein—For determination of a partial sequence of the 72-kDa protease-sensitive axonemal protein, axonemes obtained from a 5-liter culture of E. coli were extracted with high ionic strength solution, followed by dialysis against low ionic strength solution. The extracted axonemes were centrifuged and separated by SDS-PAGE. The relevant band was excised and subjected to in-gel digestion with modified trypsin (Promega, Madison, WI) according to the method of Rosenfeld et al. (50). The reaction was stopped by addition of 10% trifluoroacetic acid. The polypeptides were eluted from the gel by soaking twice with 0.1% trifluoroacetic acid in 60% acetonitrile for 40 min at room temperature and fractionated by reverse-phase chromatography on an RPC C218 column (Amersham Biosciences, Uppsala, Sweden). Fractions of four discrete peaks were collected and subjected to sequencing on an ABI 494 instrument (Applied Biosystems, Foster City, CA) at the National Institute for Basic Biology Center for Analytical Instrumentation (Okazaki, Japan).

Cloning and Sequencing of cDNA—Two sets of degenerate primers were designed to amplify two peptide sequences (EGGGLPPDPDVPDYYR and TFPELEADEYTLTYMENYK) and used to PCR-amplify fragments of genomic DNA. A cDNA library (a gift from Dr. P. A. Lefebvre, University of Minnesota, St, Paul, MN) was screened with the genomic fragments. Clones that hybridized with both probes were sequenced using an ABI PRISM 310 genetic analyzer (Applied Biosystems). The cDNA sequence was completed using 5'- and 3'-RACE (5'-RACE system, Invitrogen). To obtain a clone covering the entire coding region, reverse transcription-PCR was carried out on total RNA using primers designed from the end sequences determined by RACE (Superscript II reverse transcriptase, Invitrogen).

Northern Blot Analysis—Total RNA was prepared from wild-type vegetative cells by the TRIzol regent method (Invitrogen) every 10 min after deflagellation induced by pH shock (43). The RNA samples were separated on a formaldehyde-containing 1.5% agarose gel (51) and transferred to a Biodyne B membrane (Nihon Pall, Ltd., Tokyo, Japan). An ~700-bp DNA fragment was labeled with [α-32P]dCTP using the Klenow fragment of DNA polymerase I and used as a probe. Hybridization was performed according to standard methods (51). RSP14 (formerly CRY-1) is a clone coding for the SI4 ribosomal protein that is not involved in flagellar assembly (52), was used as a loading control in certain experiments.

Bacterial Expression of Rib72—The coding region of the cDNA was amplified with primers BE1 (CATCCATGCGTCGGCGGCG- GCCTCCG) and BE2 (CGGGATCCGAGCCAGCAGGCGGAGCAG) and ligated into the NcoI and BamHI sites of the bacterial expression vector pQE60 (QIAGEN, Hilden, Germany), resulting in a fusion protein containing a His tag sequence at its N terminus (described in the instrument documentation) and used to in-gel digestion with modified trypsin (Promega, Madison, WI) according to the method of Grunig et al. (53). The expression of the fusion protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a logarithmically growing culture of E. coli to a final concentration of 0.1 mM. The cells were suspended in Buffer A (50 mM sodium phosphate and 0.6 M NaCl, pH 8.0) containing 1 mg/ml lysozyme, incubated on ice for 30 min, and lysed by sonication. The lysate was centrifuged at 400,000 × g for 30 min at 4 °C, and the resulting supernatant was applied to a HiTrap chromatography column (Amersham Biosciences). After the column was washed with Buffer A containing 75 mM imidazole, the recombinant protein was eluted with Buffer A containing 500 mM imidazole. The eluted protein was dialyzed against 500 volumes of HMEDEK solution containing 0.6 M KCl for 3 h at 4 °C. The dialysis solution was then exchanged three times with HMEDEK solution, each time with a lower KCl concentration (0.3, 0.1, and 0 M).

Polysaccharide Production—Bacterially expressed Rib72 was used as the antigen for production of anti-Rib72 antibody. Recombinant Rib72 was dialyzed against PBS and emulsified with complete adjuvant (Naralai Tesque, Inc., Kyoto, Japan). Two rabbits were immunized hypodermically twice with a 3-week interval. Serum was obtained 1 week after the second injection. Antibody was blot-purified (53) using Rib72 from pf14 axonemes.

SDS-PAGE and Immunoblotting—Proteins were analyzed by SDS-PAGE with a 7 or 7.5% acrylamide gel or 5–20% acrylamide gradient gels by the method of Laemmli (54). Gels were stained with Serva Blue R or silver (55). For quantitation, gels were loaded with protein and stained with Serva Blue in the linear region of protein-dye binding, scanned on a GS-700 imaging densitometer (Bio-Rad), and analyzed by Molecular Analyst software (Bio-Rad). Immunoblot procedures were modified from those of Towbin et al. (56). In the first method, proteins transferred to polyvinyldene difluoride membranes (Millipore Corp., Bedford, MA), incubated with blocking buffer (0.05% Tween 20 and 3% skimmed milk in PBS, pH 7.4), and probed with primary antibodies diluted 1:200 in blocking buffer. Immunoreactive bands were detected using alkaline phosphatase-conjugated secondary antibody (Cappel Research Products, Durham, NC) and a 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium phosphatase substrate system (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). In the second method, proteins were transferred to nitrocellulose (Bio-Rad) in 10 mM CAPS, pH 11, and 10% methanol at 24 mA for 30 min using a Genie Blotter (Idea Scientific, Minneapolis, MN). Transferred proteins were stained with 0.02% Ponceau S (Sigma) in 3% trichloroacetic acid. Blots were blocked with SuperBlock® (Pierce) in PBS and 0.1% Tween 20 overnight at 4 °C. Antibodies were diluted 1:10 in SuperBlock, washed with PBS and 0.1% Tween 20, and detected using SuperSignal® WestPico® chemiluminescent substrate according to the manufacturer (Pierce).

Immunofluorescence Microscopy—Immunofluorescence microscopy was performed according to Sanders and Salisbury (57) with minor modifications. Whole cell nucleodacilagellar apparatus was isolated by the method of Wright et al. (58) were fixed with 3% formaldehyde for 5 min at room temperature, followed by treatment with cold methanol (~20 °C). Specimens were incubated with either preimmune serum or antiserum (anti-Rib72 antibody) diluted 1:200 in IF blocking buffer (5% bovine serum albumin in PBS or 0.125% sodium cholate, pH 7.2). After washing with blocking buffer, specimens were incubated with fluorescein isothiocyanate-labeled anti-rabbit IgG antibody (Zymed Laboratories Inc., South San Francisco, CA) diluted 1:500 in blocking buffer. Samples were observed with an Axioplan microscope (Zeiss, Oberkochen, Germany).

EM—All procedures were performed at room temperature. Immunoreactive whole-mount axonemal material was perforated according to rock et al. (59) or Johnson (60) with some modifications. Isolated axonemes (wild-type, ada1, and pf14) or isolated ribbons were absorbed onto carbon-coated grids. Grids were first rinsed with blocking solution (1% bovine serum albumin in PBS or 0.125%–0.5% bovine serum albumin plus 0.125–0.5% fish gelatin in TEB buffer (10 mM Tris, 0.1 mM EDTA, 1 mM DTT, pH 8.0)) and then incubated for 1 h in anti-Rib72 antibody diluted 1:20 to 1:500 in blocking solution. The grids were washed with blocking solution. In some experiments, specimens were treated with primary antibody, washed, and then negatively stained with 1% uranyl acetate. For most other experiments, after primary antibody treatment and washing, specimens were layered with secondary antibody for 1 h, i.e. anti-rabbit IgG conjugated with 5- or 10-nm colloidal gold at 1:40 (4.4 × 10^10 gold particles/μl Zymed Laboratories Inc. or 200 × 10^10 gold particles/μl, Ted Pella, Redding, CA) in TEB buffer. Grids were finally washed with PBS, followed by distilled water, or with TEB buffer and negatively stained with 1% uranyl acetate. For some experiments, prior to immunostaining, axonemes were extracted on carbon film with Sarkosyl solution for 30 min. For quantitation, gold particles

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RESULTS

Identification and Cloning of Rib72, a Ribbon-associated Polypeptide—Extensive extraction of Chlamydomonas flagellar axonemal microtubules with 0.7% Sarkosyl yielded a pure preparation of three-protofilament ribbons consisting of tubulin and associated proteins (Fig. 1a), as previously reported (14, 15, 18). Upon SDS-PAGE, such preparations were composed of three major and several minor polypeptides (Fig. 2). Based on densitometry of SDS gels from four independent preparations (e.g. Fig. 2, lane c), the major ribbon polypeptides were present in the following approximate amounts of total ribbon protein: αβ-tubulin, 72 ± 15%; Rib43a, 3 ± 2%; and an 70-kDa polypeptide, 9 ± 1%. The molar ratio of these proteins would thus be 1 mol of αβ-tubulin (100 kDa)/0.12 ± 0.11 mol of Rib43a (43 kDa)/0.18 ± 0.07 mol of ~70-kDa polypeptide; however, the actual molar ratios of the ribbon proteins cannot yet be determined because the dye-binding ratios for the different proteins are not known. In addition, variations in the relative amounts of ribbon proteins may result from the degree of Sarkosyl extraction and washing to which the preparations were subjected. Rigorous homogenization of axonemes produced the most homogeneous preparations of three-protofilament ribbons (Fig. 1a), but may also result in the fractionation or selective solubilization of certain ribbon proteins. Gentler homogenization yielded ribbons with supernumerary protofilaments and some contaminating A-tubules.

Tandem mass spectrometry of tryptic digests of the ~70-kDa polypeptide (excised from stained polyacrylamide gels) yielded the peptide sequences FYGYFK and LPGYTVCLPQSLSDK. A computer search of the Chlamydomonas expressed sequence tag data base indicated positive matches with clones AV396026 and AV626093. These two clones were obtained from the Kusua DNA Research Institute4 and were used to construct an overlapping sequence of a full-length cDNA (to be described below).

Identification of a Protein Whose Loss Correlates with Axonemal Disintegration—Axonemal proteins engaged in holding the nine outer doublets together must remain attached to the axoneme as long as the nine outer doublets are connected, but may be lost when the axonemal structure is dissociated. To identify proteins that display such properties, we exposed Chlamydomonas axonemes to high and low salt conditions while examining the degree of dissociation by light microscopy and the pattern of proteins remaining in the axonemes by SDS-PAGE. In these experiments, both wild-type cells and radial spoke-deficient pf14 mutant cells were used because radial spokes might also function to maintain the axonemal structure and thus interfere with the identification of interdoublet links. Extraction of axonemes with 0.6M KCl, followed by dialysis against 2 mM HEPES, did not separate the nine outer doublets (data not shown). The proteins responsible for maintaining the interdoublet connections must therefore be contained in the sedimentable fraction following centrifugation (Fig. 3).

Next, wild-type axonemes were induced to disintegrate by addition of trypsin and ATP (28). To monitor the progress of disintegration, aliquots were withdrawn from the reaction mixture at regular intervals, mixed with trypsin inhibitor, and observed by dark-field microscopy. Under these conditions, axonemal fragments underwent sliding disintegration. However,

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Rib72 a Flagellar Microtubule Protein

because axonemal samples were not first fragmented, most axonemes disintegrated byfraying apart attheir distal ends, but remaining intact at their proximal ends. From videotaped images, the number of apparently intact axonemes versus disintegrated axonemes was determined and used to quantitate the disintegration process as a function of time (Fig. 4). At the same time points, aliquots of samples were taken for SDS-PAGE analysis (Fig. 4, b and c). In the case shown in Fig. 4, disintegration took place with a half-time of ~3 min. Upon SDS-PAGE analysis designed to resolve proteins <200 kDa, three polypeptide bands appeared to decrease in intensity at a rate similar to that of the disintegration process (Fig. 4, b and c). Of these three polypeptide bands, only one with an apparent molecular mass of ~70 kDa was also present in the extracted/dialyzed pf14 axonemes (Fig. 3). To study this polypeptide further, it was cloned and sequenced as follows.

Cloning of the cDNA of the Protease-sensitive Polypeptide—pf14 axonemes were extracted with high and low salt solutions and resolved by SDS-PAGE. The ~70-kDa polypeptide band was cut out, digested with trypsin, and separated by reverse-phase chromatography. Of the ~40 major peaks that appeared, five were selected, and their amino acid sequences were determined. PCR was carried out to amplify cDNA fragments corresponding to two of these sequences, yielding probes A and B. Screening of the cDNA library yielded four clones that hybridized with probe A and five clones that hybridized with probe B. Finally, extension of each cDNA by 5’- and 3’-RACE confirmed that these partial cDNAs were derived from a single transcript.

Sequence of the cDNA and Predicted Structure of Rib72—The cDNAs corresponding to the ~70-kDa ribbon-associated polypeptide and the ~70-kDa protease-sensitive polypeptide were sequenced and found to be identical (GenBankTM/EBI accession number AAM44303). The cDNA is predicted to encode a polypeptide of 71,985 Da with a pI of 7.15 and is referred to here as Rib72 due to its association with the ribbon (see below). In agreement with Patel-King et al. (39), sequence analysis indicated that near its C terminus, Rib72 has two EF-hand motifs, characteristic of calcium-binding proteins (Fig. 5). In addition, there are three ~100-residue DM10 domains, which occur in related and/or unrelated proteins predicted from cDNA sequences from C. elegans, Drosophila, and mammals. Based on several structure prediction programs, Rib72 could contain only highly interrupted short stretches of β-sheet and α-helix of less than 17 and 48 residues, respectively, and only one potential region of coiled coil of ~30 residues. BLAST searches indicated that Rib72 homologs of unknown function are present in C. elegans, Drosophila, echinoderm, tunicate, mouse, and human.

Genetic Mapping—To obtain a Rib72 genomic clone, the Chlamydomonas bacterial artificial chromosome library was screened using a 2.8-kb XhoI fragment from the expressed sequence tag AV626093. Three overlapping bacterial artificial chromosome clones were isolated (27p11, 24g19, and 17k3). These bacterial artificial chromosome clones map to linkage group II, overlapping with the S6175 molecular marker (62) and in agreement with Patel-King et al. (39). No known flagellar mutants are located in this region.

Up-regulation of Rib72 Message after Deflagellation—When Chlamydomonas cells are deflagellated, the levels of mRNAs encoding proteins involved in flagellar assembly rapidly increase, and flagella are regenerated within 2 h (62). We investigated the cellular level of RIB72 mRNA after deflagellation. Cells were deflagellated by pH shock, and total RNA was isolated at 10-min intervals. Northern blot analysis showed that the level of RIB72 mRNA increased ~5-fold by 40 min after deflagellation (Fig. 6), indicating that Rib72 is involved in flagellar regeneration.

Polyclonal Anti-Rib72 Antibodies—Polyclonal antiserum was raised against bacterially produced Rib72 protein. Im-

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P. Kathir, M. LaVoie, W. J. Brazelton, N. A. Haas, P. A. Lefebvre, and C. D. Silflow, Eukaryotic Cell, in press.
munoblot analysis of axonemes and ribbons demonstrated that the antiserum and affinity-purified anti-Rib72 antibody (53) specifically recognized a single band corresponding to Rib72 (Fig. 7).

Proteolytic Cleavage of Rib72 Correlates with Sliding Disintegration—Anti-Rib72 antibody was used to more closely examine the relationship between the proteolytic cleavage of Rib72 and the protease/ATP-induced sliding disintegration of axonemes. Previous studies indicated that trypsin, elastase, and nagarse are effective in inducing sliding disintegration of Chlamydomonas axonemes; the efficiency of disintegration is dependent upon the specific protease (46). Axonemal disintegration was monitored by the turbidity of the axonemal sus-
Fig. 8. Immunoblot analysis of Rib72 degradation following protease-induced sliding disintegration. a–c, shown are changes in the turbidity of suspensions of wild-type axonemes after addition of 0.1 mM ATP with (●) or without (○) trypsin (a), elastase (b), or nagarse (c). ATP and proteases were simultaneously added at time 0 (arrowheads). a’–c’, aliquots of the suspensions used in the above experiments were withdrawn at the indicated time points (arrowhead; 0, 30, 60, 90, 150, and 330 s), resolved on 7.5% gels, and probed by Western blotting with anti-Rib72 antibody to show the proteolytic degradation of Rib72. The axoneme/protease protein ratio was 100:1 in all three suspensions.

pension (Fig. 8, a–c) (30, 45, 63), and aliquots of the suspension were removed at regular intervals for SDS-PAGE/immunoblot analysis. As shown in Fig. 8 (a’–c’), Rib72 was cleaved into lower molecular mass species with each of the three proteases; and in each case, the time course of digestion paralleled that of the change in turbidity.

Subcellular Localization of Rib72—Indirect immunofluorescence microscopy using anti-Rib72 antibody demonstrated that this protein was localized along the length of the axonemes (Fig. 9). The staining intensity was greater in the outer dynein arm-less mutant oda1 than in the wild-type axonemes, perhaps because the bulky dynein arms in fixed samples blocked antibody access to the axoneme interior. In nucleoflagellar apparatuses from both oda1 (Fig. 9) and wild-type (data not shown) axonemes, no staining was detected in the basal bodies.

Biochemical Localization of Rib72 in Microtubules—Rib72 was localized to the ribbon compartment by two independent methods: immunoblotting of the Sarkosyl-insoluble fraction (described here) and immuno-EM (following paragraphs). Two identical samples of axonemes were gently resuspended in equal volumes of either TED buffer or TED buffer + 0.7% Sarkosyl, incubated for 16 h at 4 °C, centrifuged at 100,000 × g, gently resuspended in wash buffer (TED buffer), and recen trifuged. Equivalent volumes of the pellets were then analyzed by SDS-PAGE/immunoblotting (Fig. 7); anti-Rib72 antibody staining was detected by chemiluminescence, and the resulting film was analyzed by densitometry. In this experiment, 87% of the anti-Rib72 antibody staining intensity was retained in the ribbon fraction compared with the axoneme control.

Immuno-EM Localization of Rib72 in Microtubules—Rib72 was also localized to protofilament ribbons in a series of immuno-EM experiments (Figs. 1, 10, and 11) and quantitative determinations (Table I). Anti-Rib72 antibodies, as detected by gold-conjugated secondary antibodies, specifically labeled ribbons prepared by extensive Sarkosyl extraction of wild-type axonemes (Fig. 1, a and b), with background and control label-

Fig. 9. Differential interference contrast (a) and fluorescence (b) images of a nucleoflagellar apparatus separated from oda1 cells. The sample was incubated with anti-Rib72 antibody, followed by labeling with fluorescein isothiocyanate-labeled anti-rabbit IgG antibody. The fluorescein isothiocyanate fluorescence was present along the length of the axonemes and only faintly at the proximal portion. Staining at the regions corresponding to basal bodies and transition zones was particularly weak. Bar = 5 μm.
axonemes, as suggested by the immunofluorescence observations. In *oda1* axonemes treated with trypsin and ATP, labeling occurred along separated doublet microtubules with an apparent axial periodicity of ~100 nm (Fig. 11a). Similarly, an ~100-nm axial periodicity was evident when samples were stained with a relatively high concentration of anti-Rib72 antibody alone (no secondary antibody). Such periodic staining was observed with *oda1* axonemes (data not shown) and *pf14* axonemes lacking spokes (Fig. 11b). Attempts to localize the Rib72 antigen by thin-section electron microscopy have so far been unsuccessful.

Examination of Potential Calcium Effects on Sliding Disintegration and on Ribbons—Given the demonstration that the tryptic digestion of Rib72 in axonemes is calcium-dependent (39), we examined the rates of sliding disintegration in 1 mM CaCl$_2$ versus 1 mM EGTA (between pCa 3 and >8) in the presence of 5 mM MgSO$_4$, but found no observable difference.
We also examined the tryptic cleavage of Rib72 in isolated ribbons in CaCl₂ and EGTA. In 0.1 mM CaCl₂ versus 1 mM EGTA, no difference in Rib72 degradation was observed when ribbons were incubated with 0.2 µg of trypsin for 5–60 min at 22 °C. Similarly, when ribbons were incubated in 1 mM CaCl₂ versus 1 mM EGTA for 30 min at 22 °C, no differences were observed within trypsin concentrations from 0.01 to 1 µg.

**DISCUSSION**

Rib72 is a novel *Chlamydomonas* flagellar microtubule-associated protein recently described by Patel-King et al. (39) and our laboratories (this report; GenBankTM/EMBL accession AAM44303). Rib72 (71,985 Da) contains two C-terminal EF-hand motifs (39), but no extended stretches of β-sheet, α-helix, or coiled coil; thus, it would seem unlikely to form fibrous polymers, unlike tektins from sea urchin ciliary and flagellar A-tubule ribbons (21, 23, 59) or possibly Rib43a, another component of *Chlamydomonas* ribbons (18). Rib72 also contains three ~100-residue repeats, which Patel-King et al. (39) attributed to forming a regulatory subunit of flagellar nucleoside-diphosphate kinase. More recently, the Simple Modular Architecture Research Tool® categorized these repeat sequences as DM10 domains of unknown function; only one DM10 domain is present in some (but not all) nucleoside-diphosphate kinases. Thus, it is possible that three such DM10 domains provide Rib72 with functions in addition to (or instead of) regulating nucleoside-diphosphate kinase.

**Structural Organization of Rib72 in Flagellar Microtubules**—Our investigations here shed additional light on the structural organization and potential function of Rib72 in axonemes. Upon immunofluorescence microscopy of fixed material (in which all antigenic sites should be labeled), Rib72 was uniformly distributed along axonemes, but apparently absent from basal bodies (Fig. 9) (39). Upon immunoblot analysis, ~87% of Rib72 remained associated with the ribbon fraction (Fig. 7). The remaining Rib72 may arise from the fragmentation and solubilization of the ribbons during extraction or, alternatively, from a second, more soluble locus or subset of more labile microtubules (e.g. newly assembled, regenerated microtubules). The molar ratio of the three major polypeptides of ribbons is 1 mol of αβ-tubulin dimer/0.12 ± 0.11 mol of Rib43a/0.08 ± 0.07 mol of Rib72. Upon immuno-EM, anti-Rib72 antibodies labeled intact A-tubules infrequently (occasionally appearing at ~100-nm intervals) (Fig. 11), but labeled with greater frequency as the tubules lost structural integrity and finally heavily labeled the emerging ribbons (Fig. 10 and Table I) and purified ribbons (Fig. 1). Occasionally, a ~5-nm wide fibril extended beyond the three-protofilament ribbon and was labeled with anti-Rib72 antibody (Fig. 10c). Attempts to subfractionate the ribbons and enrich for the fibrils led only to their complete dissolution. Finally, in the protease digestion experiments, all three enzymes (trypsin, elastase, and nagarse) initially cleaved an ~10-kDa fragment from Rib72 (Fig. 8).

Taken together, these data and the properties of Rib72 suggest, but do not distinguish, two models: 1) in which mostly linear Rib72 polypeptide chains lie along the protofilaments of the ribbon with an axial repeat of 96 nm and with DM10 domains binding at regular intervals to tubulin, Rib43a, or other proteins and 2) in which globular molecules of Rib72 are periodically arranged along the protofilaments of the ribbon with axial spacings of ~16 or 24 nm. In either case, the results indicate that the majority of the Rib72 polypeptide chain is located on the inner microtubule surface, or it is located on the outer microtubule surface but masked by Sarkosyl-extractable proteins. Furthermore, the results suggest that an ~10-kDa terminal piece (potentially the C-terminal EF-hand) resides on the outer microtubule surface, accessible to protease and antibody.

**Association of Rib72 with Interdoublet Links**—The other aspect of this study suggests that Rib72 interacts functionally with the system of linkages that hold the doublet microtubules in their 9-fold configuration and limit microtubule sliding. The cleavage of Rib72 by trypsin is concomitant with the sliding disintegration of the nine outer doublet microtubules. Of the three polypeptides whose disappearance correlated with axonemal disintegration (Fig. 4), only Rib72 remained present in pf14 axonemes that retained their 9-fold configurations of doublet tubules after high and low ionic strength extractions (Fig. 3). In addition, our immuno-EM suggests that certain Rib72 antigenic sites are exposed at ~100-nm intervals along outer doublet A-tubules (Fig. 11), which is close to the 96-nm axial repeat of interdoublet links in species ranging from protists to mammals (34–36, 64, 65). Relevant to the apparent association of interdoublet links with the ribbon compartment of A-tubules, molluscan ciliary axonemes have been shown to decompose (upon extraction at low and high ionic strength and heating) into a set of nine ribbons held together by interdoublet links containing nexin (16). Thus, Rib72, a ribbon component, would appear to be involved in the system of interdoublet links, whose destruction triggers axonemal disintegration (28, 66).

Both the biochemical composition and functional properties of interdoublet links have been controversial. Although early ultrastructural studies (33, 34, 49) and theoretical considerations (61) suggest that the links are elastic and highly extendable, later studies suggest they are not (35, 36, 65). Warner (35) examined *Tetrahymena* cilia after extraction with high and low salt solutions and showed that when doublet microtubules undergo sliding, one end of the links binds to variable positions along the doublet microtubules. This behavior of the interdoublet link is similar to what we might expect for inner arm dyneins. Bozkurt and Woolley (36) found that interdoublet links never appear to become stretched under conditions in which axonemes are strongly bent, i.e. when the interdoublet link is expected to be extensively stretched. On the other hand, our physiological study directly measuring the longitudinal elasticity in the axoneme has suggested that the axoneme does have a longitudinal elasticity, but that the elastic component is capable of dislocating along the microtubule (38). Therefore, it may be that the link is both detachable and stretchable, whereas its stretched state is too unstable to be observed by electron microscopy.

This investigation extends our understanding of Rib72 initially described by Patel-King et al. (39) and raises a number of questions concerning this protein and its functions in microtubule assembly and ciliary/flagellar motility. What is the molecular structure of Rib72 within the ribbon? What are the functions of the DM10 and EF-hand domains in flagellar microtubule assembly and/or the regulation of motility? What are the important details concerning the interactions between Rib72 and the interdoublet links? These questions pose testable hypotheses that will be addressed in our ongoing investigations.

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Rib72 a Flagellar Microtubule Protein
