Localization of Calmodulin and Dynein Light Chain LC8 in Flagellar Radial Spokes

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Abstract. Genetic and in vitro analyses have revealed that radial spokes play a crucial role in regulation of ciliary and flagellar motility, including control of waveform. However, the mechanisms of regulation are not understood. Here, we developed a novel procedure to isolate intact radial spokes as a step toward understanding the mechanism by which these complexes regulate dynein activity. The isolated radial spokes sediment as 20S complexes that are the size and shape of radial spokes. Extracted radial spokes rescue radial spoke structure when reconstituted with isolated axonemes derived from the radial spoke mutant *pf14*. Isolated radial spokes are composed of the 17 previously defined spoke proteins as well as at least five additional proteins including calmodulin and the ubiquitous dynein light chain LC8. Analyses of flagellar mutants and chemical cross-linking studies demonstrated calmodulin and LC8 form a complex located in the radial spoke stalk. We postulate that calmodulin, located in the radial spoke stalk, plays a role in calcium control of flagellar bending.

Key words: calcium • cilia • dynein • flagella • calmodulin

Introduction

Among the least understood and most important problems of ciliary and flagellar motility is the mechanism controlling dynein-driven microtubule sliding (Satir, 1985; Brokaw, 1994). Structural and genetic analyses have revealed that part of the mechanism involves the flagellar radial spoke structures and their interaction with the central pair apparatus (Warner and Satir, 1974; Huang, 1986; Curry and Rosenbaum, 1993; Smith and Lefebvre, 1997; Omoto et al., 1999; Shingyoji et al., 1998; Bannai et al., 2000; Porter and Sale, 2000). For example, in *Chlamydomonas*, or in humans, failure to assemble the radial spokes results in flagellar paralysis (Witman et al., 1978; Sturgess et al., 1979). Analysis of the suppressor mutants, and in vitro functional assays of dynein activity, has revealed a control system that links the radial spokes to control of flagellar dynein activity (Huang et al., 1982; Huang, 1986; Smith and Sale, 1992a; Howard et al., 1994; Habermacher and Sale, 1996; Yoshimura and Shingyoji, 1999; Yang and Sale, 2000). Moreover, phenotypic analysis of suppressor mutants demonstrated the radial spokes play a role, in part, for control of flagellar waveform: motility in suppressors lacking radial spokes is characterized by a failure to generate normal reverse bends (Brokaw et al., 1982). However, little is known about the biochemical mechanism by which the radial spoke structures control dynein-driven motility.

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The radial spokes are T-shaped structures anchored on the A-microtubule of each outer doublet, adjacent to the inner dynein arms (Warner and Satir, 1974; Witman et al., 1978; Goodenough and Heuser, 1985, 1989). In cross sections, the spokes project toward the central pair apparatus, where the spoke heads transiently interact with the central pair projections (Warner and Satir, 1974; Goodenough and Heuser, 1985, 1989; Smith and Lefebvre, 1997; Mitchell and Sale, 1999; Omoto et al., 1999). In the long axis, radial spokes repeat in either pairs or triplet groups every 96 nm along each doublet microtubule, in exact register with the inner dynein arms (Warner and Satir, 1974; Goodenough and Heuser, 1985, 1989; Piperno et al., 1990; Mastronarde et al., 1992; Gardner et al., 1994; Porter and Sale, 2000). Biochemical analysis of *Chlamydomonas* axonemes has demonstrated the radial spokes are composed of ≥17 proteins, 5 located in the spoke head and at least ≥12 located in the spoke stalk (Fig. 1 A; Piperno et al., 1981). Five of the stalk proteins are phosphorylated in vivo (Piperno et al., 1981), and several have been cloned and characterized (for review see Curry and Rosenbaum, 1993). In particular, radial spoke protein 3, RSP3, is located at the proximal end of the spoke stalk in position to target and anchor the spoke to the doublet microtubule (Huang et al., 1981; Diener et al., 1993). Recently, it has also been determined that RSP3 is an A-kinase anchor protein, predicted to anchor protein kinase A (PKA)[1] in position near

[1]*Abbreviations used in this paper:* 2D, two-dimensional; KI, potassium iodide; KP, potassium phosphate; pI, isoelectric point; PKA, protein kinase A.

the inner dynein arms (Gaillard et al., 2001). However, little more is known about the nature of radial spoke proteins or the mechanism for their control of motility.

To further investigate radial spokes, we have taken a direct biochemical approach, isolating intact radial spokes and analyzing the components. Isolated radial spokes sediment at 20S and are the size and shape of radial spoke structures located in the axoneme. Extracted radial spoke complexes, when mixed with isolated axonemes from *pf14* cells, mutants that lack the radial spokes, reconstituted the radial spoke structures. The isolated spokes contain all 17 previously identified protein subunits, plus five additional polypeptides including calmodulin and dynein light chain LC8, perhaps explaining the failure of assembly of radial spokes in the LC8 null mutant cells, *fla14* cells (see Discussion) (Pazour et al., 1998). This is the first localization of calmodulin to a particular structure in the axoneme. This observation is consistent with models in which calcium, known to act directly to alter waveform in reactivated axonemes (Bessen et al., 1980; Kamiya and Witman, 1984; Brokaw and Nagayama, 1985), alters radial spoke function for control of flagellar waveform. Thus, the radial spokes may be one of possibly multiple sites in the axoneme for calcium control of flagellar motility (Wakabayashi et al., 1997) (see Discussion).

Materials and Methods

Chlamydomonas Strains

Wild-type cells (*cc-124*) and paralyzed flagellar mutant cells with defective radial spokes (Huang et al., 1981) were provided by Dr. Elizabeth Harris from the *Chlamydomonas* Stock Center at Duke University. The genetic and biochemical defects of the radial spoke mutants were previously characterized (Huang et al., 1981). *pf14* lacks the entire radial spoke due to the mutation of RSP3 gene (Luck et al., 1977). *pf17* lacks the radial spoke head. *pf24* has a reduced amount of spoke head and stalk and is defective in the gene for RSP2. *pf27* also has a reduced amount of the spoke head and stalk and is defective in phosphorylation of the spoke proteins RSP2, -3, -5, -13, and -17. The double mutant *pf28pf30,* lacking outer dynein arm and inner dynein arm I1, has been described (Piperno et al., 1990; Smith and Sale, 1992b). Cells were grown in the liquid modified medium I under 14–10 light/dark cycles as described previously (Witman, 1986).

Extraction and Fractionation of Axonemal Proteins

Unless stated otherwise, chemicals were purchased from Sigma-Aldrich. Flagella and axonemes were prepared as described previously (Yang and Sale, 1998). In brief, *Chlamydomonas* cells were harvested and deflagellated using dibucaine (Witman, 1986). Flagella were demembranated with 0.5% Nonidet P-40 (Calbiochem) in buffer A (10 mM Hepes, 5 mM $MgSO_4$, 1 mM dithiothreitol, 0.5 mM EDTA, 30 mM NaCl, 0.1 mM PMSF 0.5 TIU/ml aprotinin, pH 7.4), and axonemes were washed once in buffer A. To remove dynein arm complexes, axonemes (8 mg/ml) were resuspended in buffer A with 0.6 M NaCl (15 min on ice). The extraction was repeated to remove residual dynein components. The extracted axonemes were resuspended in 0.5 M potassium iodide (KI) or 0.6 M NaBr in buffer A (10 mg/ml protein, 30 min on ice), followed by microfuge–centrifugation for 10 min. The supernatant was dialyzed in buffer A for 2 h at 4°C and then clarified by centrifugation. 0.6 ml supernatant was loaded on an 11.5-ml 5–20% continuous sucrose gradient in buffer A, sedimented at 35 K rpm (SW41 rotor; Beckman Coulter) for 16 h, and fractionated into 20 equal fractions. Sedimentation coefficients were estimated as described before (Pfister et al., 1982; Mitchell and Sale, 1999) using dynein and central pair apparatus complexes as standards. For SDS-PAGE, aliquots of the fractions were mixed with 5× electrophoresis sample buffer.

For in vitro reconstitution of the radial spokes, increasing amounts of the dialyzed KI extract in buffer A were added to a fixed quantity (50 μl, 1 mg/ml) of *pf14* axonemes or axonemes first extracted with 0.6 M NaCl to remove dynein arms. The final volume was adjusted to 90 μl, and after 30-min incubation on ice, the mixture was centrifuged at 12,000 *g* for 10 min. Pellets and supernatant were processed for Western blot analysis or transmission electron microscopy.

Chemical Cross-linking

The 15S radial spoke stalk fractions derived from *pf17* cells (see Results) were concentrated fivefold using a Microcon centrifugal filter following manufacturer's instructions (Millipore). After extensive dialysis in buffer A lacking dithiothreitol, 60 μl aliquots were incubated for 1 h at room temperature with increasing concentrations of 1,5-difluoro-2,4-dinitrobenzene (DFDNB; Pierce Chemical Co.) as described (Benashski et al., 1997). The reaction was terminated with 10× excess of β-mercaptoethanol, followed by addition of 2× electrophoresis sample buffer.

Immunoprecipitation

KI extract from *pf17* axonemes was dialyzed in PBS. 50 μl of the supernatant was incubated first with 4 μl affinity-purified anti-RSP2 at 4°C overnight. The incubation continued for an additional 2 h after the addition of 20 μl protein A–agarose (Sigma-Aldrich). After extensive washing with PBS, the pellet was suspended with 50 μl sample buffer for Western analysis.

SDS-PAGE

5 μl from axoneme fractions (5 mg/ml) or 15 μl from sucrose gradient fractions were separated by SDS-PAGE, and proteins were revealed by silver or Coomassie blue staining. Molecular weight standard markers were included for all gels and blots. Alternatively, proteins were transferred to nitrocellulose membrane for Western blot analysis as described previously (Yang et al., 2000). Western blot analyses for calmodulin were carried out using a modified procedure (Hulen et al., 1991). In brief, proteins were transferred overnight at 4°C to Immobilon-P (Millipore) using potassium phosphate (KP) buffer, pH 7.0, followed by fixation with 0.2% glutaraldehyde in KP buffer at room temperature for 1 h. After washing with KP buffer, the Immobilon-P membrane was blocked with 2% BSA/TBS and then incubated 1 h at 37°C with the anticalmodulin antibody 1:1,000 in 1% BSA/0.05% Tween 20/TBS, followed by secondary antibody (1:10,000; Bio-Rad Laboratories) in 1% BSA/10% goat serum/0.05% Tween 20/TBS for 1 h 37°C.

Two-dimensional (2D) Gel Electrophoresis

Isoelectric focusing in the first dimension was as described (Piperno, 1995) with minor modifications. Broad-range premixed ampholines (Amersham Pharmacia Biotech), pH 3.5–9.5, were used for the first dimension gel. A 20 × 20–cm two-dimensional (2D) gel apparatus (Bio-Rad Laboratories) was used after the manufacturer's instruction. After isoelectric focusing, the tube gel was ejected into electrophoretic sample buffer and loaded on the second dimension gel for regular SDS-PAGE. The slab gel was run at 100 V until the gel buffer front reached the separating gel and then continued at 85 V overnight. In certain cases, only the acidic half of the isoelectric focusing gel was analyzed in the second dimension using a 1.5-mm thick minigel.

Antibodies

Monoclonal antibodies were produced by the Yale Immunological Services (New Haven, CT), by injecting the axonemes stripped with 0.6 M NaCl into Balb c mice (Cole et al., 1998). Hybridoma supernatants were screened on immunoblots of wild-type and *pf14* axonemes (Fig. 1 A) and confirmed on 2D gel immunoblots and expressed recombinant RSP2 (data not shown). Rabbit polyclonal antibodies against RSP2 and -3 were raised against proteins purified from 2D gels (Williams et al., 1986, 1989). Anti-LC8 polyclonal antibody was provided by Dr. S.M. King (University of Connecticut, Storrs, CT; King and Patel-King, 1995a). Anti–*Dictyostelium* calmodulin antibody (Ab-1) was purchased from Calbiochem. This monoclonal antibody is monospecific and cross-reacts with vertebrate and *Chlamydomonas* calmodulin (Hulen et al., 1991).

Electron Microscopy

Aliquots of sucrose gradient fractions 2 or 3 were directly applied to the glow-discharged carbon- and Formvar-coated copper grids for 2 min followed by staining with 4% uranyl acetate for 30 s. The grid was blotted

dry and observed by electron microscopy at 80 kV. Dialysis of the sample into ammonium acetate or buffer A did not affect appearance of isolated spoke structures. Negative staining of axonemes was performed as before (Witman et al., 1978). For thin section electron microscopy, pelleted axonemes were fixed and embedded as described before (Porter et al., 1992), and sections were stained with uranyl acetate and lead citrate.

Results

Extraction and Purification of Flagellar Radial Spoke Proteins

Our approach was to systematically determine ionic conditions that result in extraction of radial spoke proteins. The results were evaluated by probing for RSP2 and -3. As expected on Western blots, the RSP2 antibody selectively bound to a 118-kD axonemal protein (Fig. 1 A; Luck et al., 1977; Piperno et al., 1981), and the RSP3 antibody selectively bound to a 97-kD axonemal protein (Fig. 1 A; Diener et al., 1993). In control experiments using axonemes from radial spoke mutants, neither RSP2 nor RSP3 were present in axonemes from *pf14*. Both were present in axonemes from *pf17*, RSP2 was greatly reduced in axonemes from *pf24* and both were reduced in axonemes from *pf*27 (Fig. 1 A). As previously reported (Piperno et al., 1981), radial spoke proteins were not soluble in 0.6 M NaCl (Fig. 1 A, lane 1). In contrast, we found that 0.6 M KI efficiently solubilized RSP2 and -3 (Fig. 1 A, lane 7, KIext). The spoke proteins were also effectively solubilized in 0.5 M KI or 0.6 M NaBr.

To determine whether the extracted spoke proteins were retained in large complexes, the KI extract was fractionated by velocity sedimentation on sucrose gradients, and fractions were analyzed by Western blots and protein-stained gels. RSP2 and -3 cosedimented at 20S (Fig. 1 A, fraction 3, right). Similar results were obtained after 0.6 M NaBr extraction. The simplest explanation is that, after extraction and dialysis, the radial spoke proteins were retained in a large complex including most or all radial spoke proteins. Consistent with this interpretation, the 20S peak fraction contained several proteins with masses anticipated for spoke polypeptides (Fig. 1 B, left). To better resolve the proteins of the 20S spoke complex and to distinguish those proteins from the contaminating 20S dynein subunits, axonemes from *pf*28*pf*30, lacking the outer dynein arms and inner dynein I1, were used for extraction. Again, the 20S spoke fraction displayed several protein subunits with masses consistent with known spoke proteins (Fig. 1 C, left). The same proteins were totally lacking in the 20S fraction of extracts derived from *pf14* (Fig. 1 C, right). We further predicted that a smaller radial spoke complex consisting of the spoke stalk, and missing radial spoke head proteins, could be isolated from *pf17* axonemes. As predicted, in the *pf17* extract, a smaller particle containing RSP2 and -3 but missing a subset of polypeptides including RSP1 (Fig. 1 B, gray arrow, right) sedimented at about 15S.

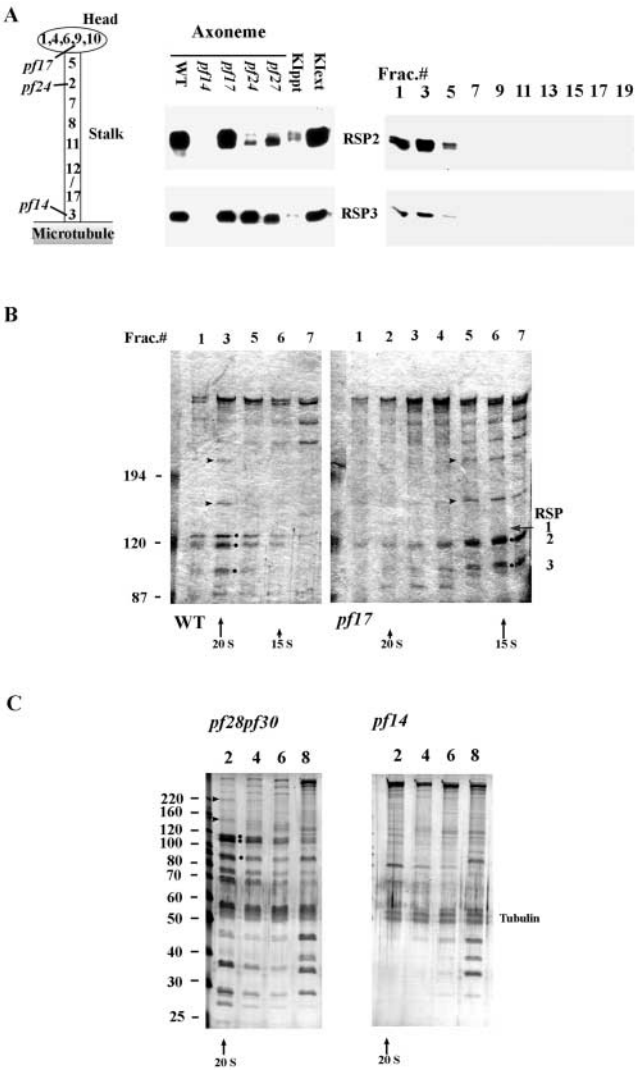

Figure 1. Extraction of flagellar radial spoke proteins and isolation of a 20S radial spoke complex and a 15S radial spoke stalk complex. (A, left) Diagram illustrating the predicted location of radial spoke proteins and the gene products of *pf14*, *pf17*, and *pf24* in the radial spoke stalk and head (Curry and Rosenbaum, 1993). (middle) Western blot analyses of isolated axonemes and axonemal fractions, using antibodies to RSP2 and -3 (lane 1, wild-type axonemes after extraction in 0.6 M NaCl; (lanes 2–5) axonemes from *pf14*, *pf17*, *pf24*, and *pf27*; (lane 6) wild-type axonemes after 0.6 M KI extraction; (lane 7) KI extract from wild-type axonemes. Notably, RSP2 and -3 are extracted with 0.6 M KI (middle, compare lanes 6 and 7) and cosediment at ~20S in the 5–20% sucrose gradient (right, fraction 3). Protein loads and transfers were controlled by Ponceau red staining of blots (not shown). (right) Sucrose gradient fractions of the KI extract from wild-type cells probed with antibodies to RSP2 and -3. (B) Coomassie-stained 5% gels of sucrose gradient fractions reveal cosedimentation of several proteins in addition to RSP2 and -3. RSP1, -2, and -3 are indicated (●; left panel, fraction 3). Extraction of *pf17* axonemes yields a diffuse 15S peak containing a subset of the proteins found in the 20S complex. Arrowheads indicate the 140- and 210-kD proteins that cosediment with both the 20S radial spoke and 15S radial spoke stalk complexes. RSP1 (gray arrow), a spoke head protein, is missing in the 15S complex. (C) Silver-stained 8% gels of sucrose gradient fractions of the axonemal extract from *pf28pf30*, a mutant lacking outer arm dynein and inner arm dynein I1. The 20S radial spoke complex is composed of several proteins (left), that are missing in the 20S fractions from a mutant lacking the radial spokes, *pf14* (right panel). Samples analyzed in B and C were derived from 0.5 M KI (B) or 0.6 M NaBr (C) extract of axonemes first extracted in 0.6 M NaCl. Molecular weights are indicated on the left.

These results suggested that the 20S and 15S complexes were the intact radial spokes and radial spoke stalks, respectively. As a further test, the 20S spoke complex derived from *pf28pf30* cells was analyzed by 2D gel electrophoresis and electron microscopy.

The 20S Radial Spoke Fraction Contains T-shaped Particles Composed of the 17 Previously Defined Radial Spoke Proteins

Genetic and biochemical analyses had revealed that radial spokes are composed of ≥17 proteins with well-defined isoelectric points (pIs) and molecular weights (Piperno et al., 1981). We took advantage of these data to analyze the isolated 20S complex, from *pf28pf30*, by 2D gels. Using precisely defined 2D map positions, all 17 defined radial spoke proteins were identified (Fig. 2). Moreover, the relative intensity of the silver-stained proteins in the 20S complex qualitatively matched the intensity of the ^{35}S-labeled spoke proteins from axonemes (compare to Figure 1 in Piperno et al., 1981). Thus, analysis of isolated radial spokes by 2D gels and silver staining confirms the relative ratios of spoke proteins predicted from 2D gel analysis of axonemes isolated from wild-type and mutant cells (Piperno et al., 1981). For example, radial spoke proteins 1, 2, 3, 4, 5, 6, 7, 9, 10, and 16 are relatively abundant when analyzed by silver staining or ^{35}S labeling.

As a further test, the 20S complexes were directly examined by negative stain electron microscopy. Individual T-shaped particles with the size and shape of radial spokes, as visualized in axonemes, were revealed (Fig. 3). The isolated radial spoke is 40–42 nm long, and a globular structure was often present on the end of the stalk opposite to the T-shaped head (Fig. 3, arrowhead). We conclude that the 20S fraction contains intact radial spokes.

In Vitro Reconstitution of Radial Spoke Structure

We tested whether extracted radial spoke complexes could reconstitute radial spokes when added to isolated axonemes lacking the radial spokes. For these experiments, spokes contained in dialyzed KI extracts were added at approximately the original stoichiometry (Fig. 4, 5-μl extract) to *pf14* axonemes or 0.6 M NaCl extracted *pf*14 axonemes. Based on Western blots using the antibody to RSP2, the radial spokes cosedimented with the axonemes or with extracted axonemes (Fig. 4 A). Binding of spokes was more efficient when *pf14* axonemes were first extracted with NaCl, presumably due to the removal of obstructing dynein arms (Fig. 4 A, compare lanes 1–4 with lanes 5–8). Furthermore, binding of spokes saturated when 20 μl of the spoke fraction (fourfold the predicted original stoichiometry of spokes) were added (Fig. 4 A, lanes 7 and 8).

To determine if spokes were assembled appropriately, axonemes and reconstituted axonemes were examined by electron microscopy. Axonemes or salt-extracted axonemes from *pf*14 do not contain radial spokes (Fig. 4 B, top). As described before (Mitchell and Sale, 1999), 0.6 M NaCl extraction of *pf14* axonemes partially removes dynein structures and disrupts or completely solubilizes the central pair apparatus. Addition of the radial spoke fraction resulted in restoration of T-shaped radial spoke structures

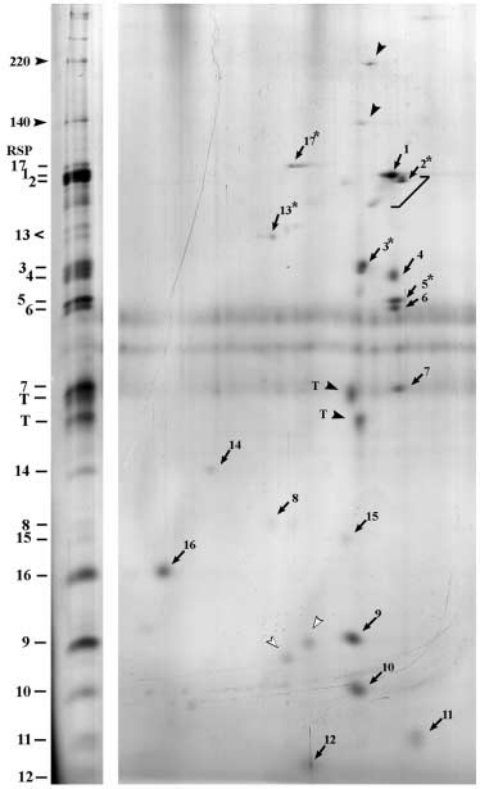

Figure 2. The 20S radial spoke complex contains all 17 previously identified spoke proteins (compare to Piperno et al., 1981). Proteins in one-dimensional (left) and 2D (right) gels of the 20S fraction from *pf28pf30* are revealed by silver staining. The 17 radial spoke proteins are numbered. In addition, present in the 20S fraction are the 140- and 210-kD proteins (arrowheads), as well as two unknown proteins (open arrowheads) and tubulin (T). Phosphorylated proteins are labeled (*), and RSP2 distribution, based on Western blots, are indicated by the bracket. Acidic polypeptides are on the right. No polypeptides are present in the basic half of the gel (not shown). A 9% slab gel was used for the second dimension.

attached to the A-microtubule of doublet cross sections (Fig. 4 B, cross sections, bottom) and often revealed pairs of radial spokes that repeated at 96 nm viewed in long section (Fig. 4 B, bottom). Radial spokes were also restored in regions retaining remnants of the central pair apparatus, but spokes were visualized more clearly in the absence of central pair structure. The simplest interpretation of the reconstitution results is that intact radial spokes were extracted and capable of restoring spoke structure. The relatively high stoichiometry required for saturation in in vitro reconstitution experiments may be a consequence of structural hindrance. We have not yet excluded the possible contribution of additional soluble components in the KI extract.

Together, these results indicate intact radial spokes were isolated, composed in part of the original 17 radial spoke proteins, and capable of restoring spoke structure in vitro. The results confirm the reliability of using flagellar mutants to define structural composition (Huang et al., 1981; Piperno et al., 1981) and provide a new opportunity to analyze the composition, assembly, and function of the radial spokes and radial spoke stalks.

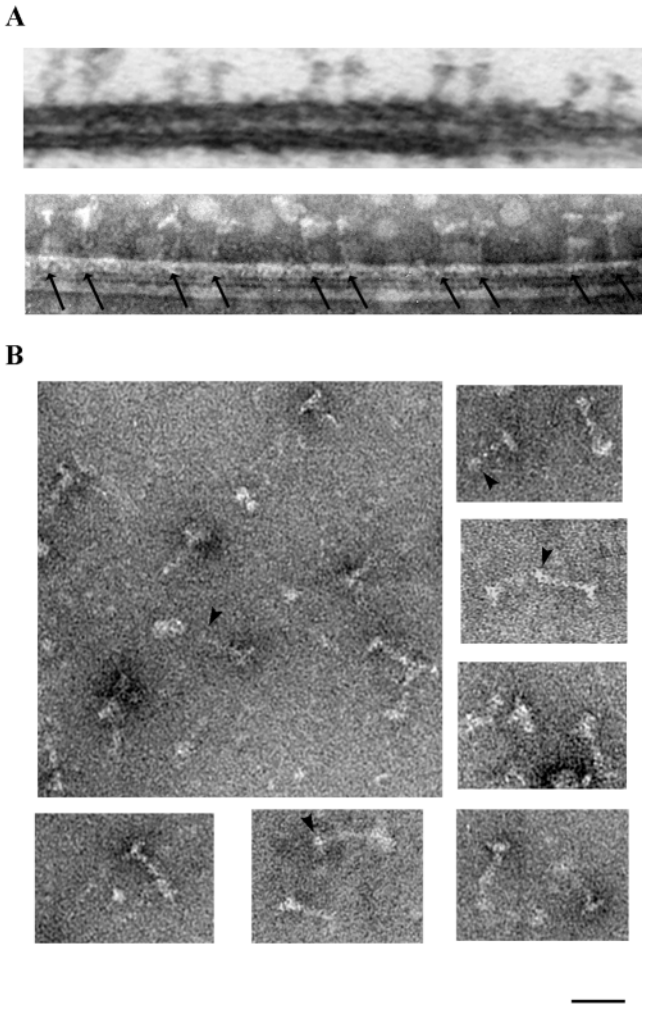

Figure 3. The 20S radial spoke fraction contains T-shaped particles with the dimensions of intact radial spoke in situ. (A) Longitudinal images of thin section (top) and negatively stained (bottom) doublet microtubules, viewed edge-on, reveals paired radial spoke structures (arrows; compare with Witman et al., 1978). (B) Negative-stained images of the 20S radial spoke particles. A globular structure (arrowhead) was often found at the proximal end of the isolated spokes. Bar, 40 nm.

Identification of Novel Spoke Stalk Components Including Calmodulin and Dynein Light Chain LC8

In addition to the 17 known radial spoke proteins, analysis of the isolated radial spokes revealed additional components. Two high–molecular mass polypeptides with apparent masses of 140 and 210 kD always cosedimented with both the 20S radial spoke and the 15S radial spoke stalk fractions (Fig. 1 B, arrowheads). Based on the nonequilibrium 2D gels, the 140- and 210-kD proteins have pIs of 5.5 and 5.4, respectively (Fig. 2). These proteins were also present in the 2D gels of axonemes (Piperno et al., 1981; Dutcher et al., 1984). Based on their presence in the 15S spoke stalk fraction from *pf17*, these proteins are likely to be associated with the spoke stalk located at the proximal end, possibly involved in spoke attachment (see Discussion). Two additional polypeptides with masses of ~26 kD were revealed in 2D gels of the 20S fraction (Fig. 2, open arrowheads). The nature of these two polypeptides is not known.

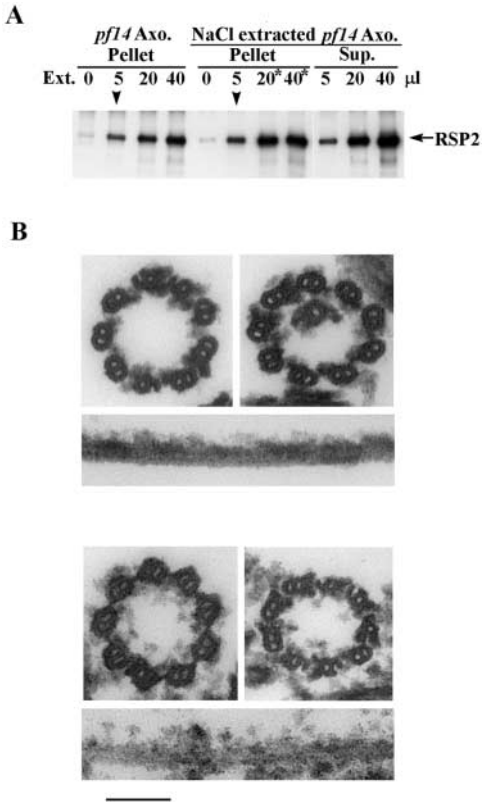

Figure 4. In vitro reconstitution of radial spokes. Western blots (A) and electron microscopy (B) reveal that extracted radial spokes bind to *pf14* axonemes and restore radial spoke structure. Increasing amounts of extract containing radial spokes were incubated with either *pf14* axonemes (lanes 1–4) or *pf14* axonemes preextracted with 0.6 M NaCl (lanes 5–11). Pellets containing axonemes (lanes 1–4 for *pf14* axonemes, and lanes 5–8 for NaCl extracted *pf14* axonemes), and the supernatant for the NaCl-extracted *pf14* axonemes (lane 9–11) were analyzed by Western analysis using RSP2 antibody. A 1:1 stoichiometry of radial spokes to the binding sites was predicted when 5 µl spoke fraction (arrowheads) was added to the axonemes. Rebinding saturated (*) when 20 µl extract was added to NaCl-extracted axonemes. (B) Cross and longitudinal sections of NaCl extracted *pf14* axonemes (top), and the same axonemes reconstituted with extracted radial spokes (bottom). Notably, spoke structures are restored to the A-microtubules (cross section view). Bar, 96 nm.

Three polypeptides smaller than RSP12 with masses of 10, 16, and 18 kD were identified in 14% of the gels. Based on silver staining (Fig. 5 A) and Coomassie staining (not shown), and compared with other spoke proteins including RSP9, 10, 11, and 12, the three proteins were always present in the same relative intensity from a large number of preparations and from several cell strains. Based on the protein staining, the 10-kD protein was equal to or greater in abundance than RSP9 and -10. The 16- and 18-kD proteins, though less abundant than RSP9 and -10, were more abundant than RSP8, -11, and -12. Moreover, proteins with identical molecular masses and pIs were identified in 2D gels of isolated axonemes (Van Eldik et al., 1980; Piperno et al., 1981). Thus, it was unlikely the proteins resulted from degradation.

Based on pI and mass, we postulated that the 18-kD polypeptide is calmodulin (Van Eldik et al., 1980; Lukas et

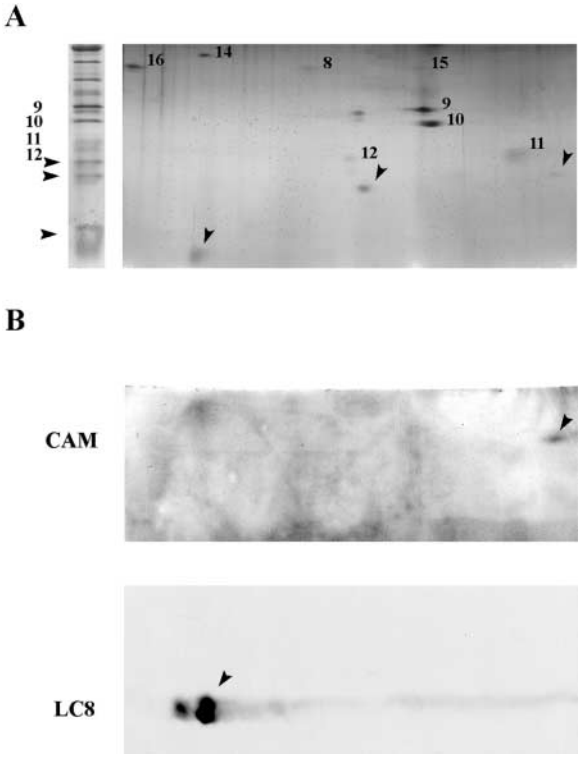

Figure 5. In addition to the radial spoke proteins described above, three small proteins including calmodulin, and dynein light chain LC8 are associated with the isolated radial spoke. (A) The 20S radial spoke fraction was separated by 14% one-dimensional (left) and 2D (right) gels. Silver staining revealed three proteins with masses of ~18, ~16, and ~10 kD (arrowheads). (B) Western blot analyses revealed that the 18-kD protein is recognized by anti–*Dictystelium* calmodulin monoclonal antibody (top). The 10-kD protein is recognized by anti-LC8 antibody (bottom). The identity of the 16-kD protein is not known.

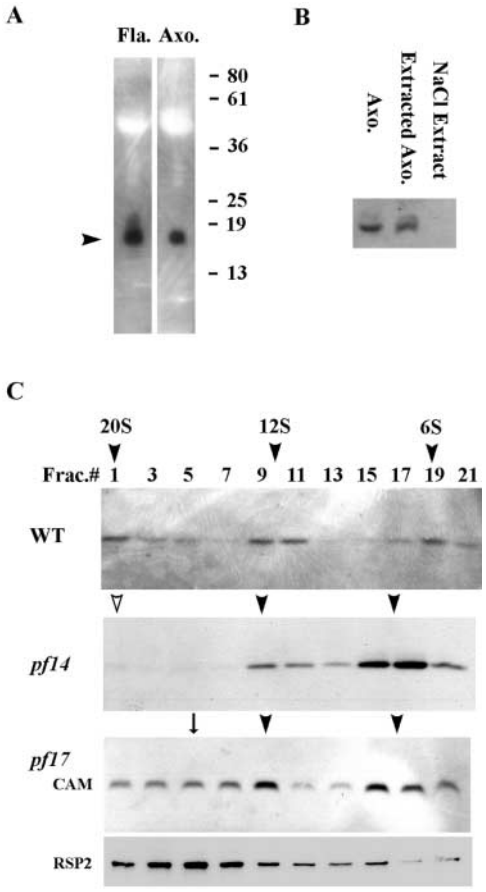

Figure 6. Calmodulin is localized in the radial spoke stalk. (A) Western blot analyses revealed that a fraction of flagellar calmodulin tightly associates with axoneme. (B) The axonemal fraction is not extractable with 0.6 M NaCl. (C) On sucrose gradients, calmodulin in the KI extract sediments as three peaks when derived from wild-type axonemes (top, arrowheads). The 20S calmodulin peak is absent in extracts derived from *pf14* (open arrowhead, middle). In the KI extract from *pf17* axonemes, the discrete 20S calmodulin peak is missing, and rather it sediments as a diffuse peak (arrow, third panel) that overlaps with the second 12S calmodulin peak and cosediments with radial spoke stalk proteins including RSP2 (compare third and fourth panels, C).

al., 1985; Zimmer et al., 1988). Based on pI, mass, and the phenotype of LC8 mutants (see Discussion), we predicted that the 10-kD polypeptide is dynein light chain LC8 (King and Patel-King, 1995a; Pazour et al., 1998). The 16-kD radial spoke protein remains unidentified. Western analysis of 2D gels confirmed our hypotheses: the 18-kD protein reacted with an antibody to calmodulin (Fig. 5 B, CAM, arrowhead), and the 10-kD protein reacted with the anti-LC8 antibody (Fig. 5 B, LC8, arrowhead). Notably, LC8 was resolved into two spots, consistent with the finding that LC8 is a phosphoprotein (Kumar et al., 2000). To further evaluate the presence of calmodulin and LC8 in the radial spokes, axonemal fractions from wild-type and mutant cells were analyzed.

Based on the Western blot analysis of calmodulin, the 18-kD protein is present in flagella and axonemes (Fig. 6 A) and the cell body (not shown), as demonstrated previously (Gitelman and Witman, 1980; Van Eldik et al., 1980). The axonemal fraction is resistant to 0.6 M NaCl extraction (Fig. 6 B), consistent with its presence in the radial spoke. Based on the specificity of the antibody (Hulen et al., 1991) and the resistance to NaCl extraction, it is not likely that the 18-kD protein is centrin (LeDizet and Piperno, 1995). Calmodulin is present in axonemes from a wide range of structural mutants (not shown; Otter, 1989), suggesting that calmodulin is located not only in the radial spoke but also in additional specific axonemal structures. Consistent with this hypothesis, calmodulin appeared in three distinct peaks in sucrose gradient fractions (~20S, ~12S, and ~6S) of the KI extract (Fig. 6 C, arrowheads, WT). The 20S calmodulin peak is absent in the extracts from *pf14* axonemes lacking the radial spokes (Fig. 6 C, open arrowhead). However, the other calmodulin peaks (~12S and ~6S) are retained, consistent with calmodulin's presence in other axonemal structures. In addition, the discrete 20S calmodulin peak is not present in extracts from *pf17* axonemes lacking the radial spoke heads (Fig. 6 C, arrow, third panel). Rather, calmodulin sediments more diffusely, at ~15S, overlapping the 12S fraction and cosedimenting with the radial stalk protein RSP2 (Fig. 6 C, bottom). Thus, the simplest interpretation is that the radial spoke fraction of calmodulin is localized to the radial spoke stalk. Cross-linking experiments described below

were performed to further test this hypothesis. Notably, after extensive analysis of a variety of flagellar structural mutants (*pf3*, *pf6*, *pf16*, *pf18*, *pf28*, *pf30*, *mbo*, etc.; data not shown), the 20S calmodulin peak was only absent in axonemes defective for radial spoke assembly.

To further clarify the location of LC8 within axonemal structures, axonemes from various mutant cells were fractionated and analyzed by Western blots. Previously, LC8 was identified as an abundant axonemal protein and a subunit of outer dynein arm (Piperno and Luck, 1979; Pfister et al., 1982) and inner dynein arm I1 (Harrison et al., 1998). However, a fraction of LC8 remains in the axonemes after dyneins were removed by NaCl extraction, suggesting a third location for LC8 (Pazour et al., 1998). Western blots show that an abundance of LC8 is present in wild type, *pf14* axonemes (lacking radial spokes), and *pf28pf30* axonemes (lacking both outer dynein and I1) (Fig. 7 A). Moreover, similar to RSP3 (Fig. 7 A, bottom), LC8 in *pf28pf30* axonemes is partially, but poorly, extracted by NaCl but becomes soluble in KI, consistent with the location of LC8 in radial spoke (Fig. 7 A, left). In contrast, LC8 in *pf14* spoke-less axonemes is largely soluble in 0.6 M NaCl (Fig. 7 A, right), consistent with the location of this fraction of LC8 in the dynein arms.

As a further test of the localization of LC8 in radial spoke structures, the RSP2 antibody was used to immunoprecipitate the 20S radial spoke complex from KI extracts. Probing an immunoblot of the precipitate for LC8 confirmed that this light chain is a component of the radial spoke (Fig. 7 B, compare lane 3 with 6). Compared with controls without antibody, LC8 in the KI extract was coprecipitated with RSP2 when the antibody to RSP2 was used. As a final test, we predicted that LC8 must be present in both the 20S radial spoke fraction as well as the 15S radial spoke stalk fraction derived from *pf17* cells. Sucrose gradient analysis of KI extracts indicates LC8 is located in the 20S radial spoke fraction (Fig. 7 C, *pf28pf30*) and the 15S radial spoke stalk fraction (Fig. 7 C, *pf17*, arrow). We conclude that LC8, a subunit of the flagellar dynein arms, is also a subunit of the radial spoke and is located in the radial spoke stalk.

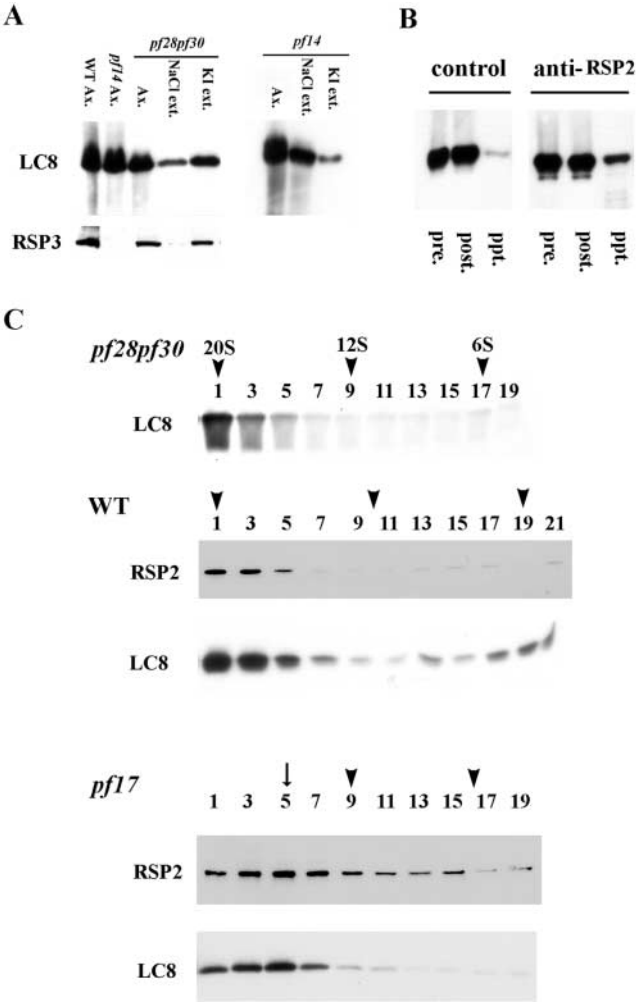

Figure 7. Dynein light chain LC8 is a subunit of the radial spoke stalk, as well as a subunit of dynein structures. (A) Western blot analyses of LC8 and RSP3 in wild-type axonemes (lane 1); *pf14* axonemes (lane 2); *pf28pf30* axonemes (lane 3); 0.6 M NaCl extract (lane 4); and 0.6 M KI extract (lane 5). Notably, LC8 is abundant in *pf14* and *pf28pf30* axonemes, and in *pf28pf30* axonemes, the majority of LC8, and RSP3 (bottom), resists extraction in 0.6 M NaCl, consistent with the localization in radial spokes. In contrast, LC8 in *pf14* is largely extractable in 0.6 M NaCl (right), consistent with this fraction of LC8 being a subunit of dynein. (B) Coprecipitation of LC8 with radial spokes. RSP2 antibody was used to precipitate the 20S radial spoke complex from KI extracts (right). Western blots of LC8 showed that LC8 is present in the immunoprecipitate (lane 3, right). In contrast, little LC8 is precipitated in control experiments performed without addition of RSP2 antibody (left, and compare lane 3 of right and left panels). (lane 1 of each panel) Extract before precipitation (pre). (lane 2 of each panel) Extract after precipitation (post). (C) LC8 is located in the radial spoke stalk. Western analyses, using anti-LC8 and anti-RSP2, of sucrose gradient fractions from 0.5 M KI extracts from *pf28pf30* (top) and wild type (middle). In each case, LC8 cosediments with the 20S radial spoke complex. In contrast, LC8 sediments as a diffuse peak centered at ~15S (arrow, fraction 5, bottom) when derived from *pf17* axonemes.

Calmodulin and LC8 Form a Complex Localized to the Radial Spoke Stalk

Based on cross-linking studies, LC8 is known to form dimers, or higher order structures, in dyneins and myosin V (Benashski et al., 1997). Moreover, based on analysis of *pf17* (Figs. 6 and 7), both LC8 and calmodulin are localized in the spoke stalk. Cross-linking experiments were carried out to (a) determine whether LC8 of the radial spoke fraction forms dimers, (b) confirm the presence of calmodulin in the radial spoke stalk, and (c) test whether LC8 and calmodulin structurally interact in the spoke stalk. Isolated radial spoke stalk fractions from *pf17* were treated with increasing concentration of a short cross-linker, DFDNB. The results of cross-linking were analyzed by Western blots using anticalmodulin (Fig. 8, left) and anti-LC8 antibodies (Fig. 8, right). A 27-kD complex (Fig. 8, arrowhead, left) containing calmodulin appears after treatment with DFDNB, suggesting that calmodulin is cross-linked to a protein of ~10 kD. LC8 is the only 10-kD protein in the spoke stalk fractions. As predicted, LC8 also formed multimeric complexes after the treatment with DFDNB, including bands of 19 and 27 kD (Fig. 8, right). Based on the size and relative intensity, the 19-kD complex must be a LC8 dimer, similar to that found in dynein

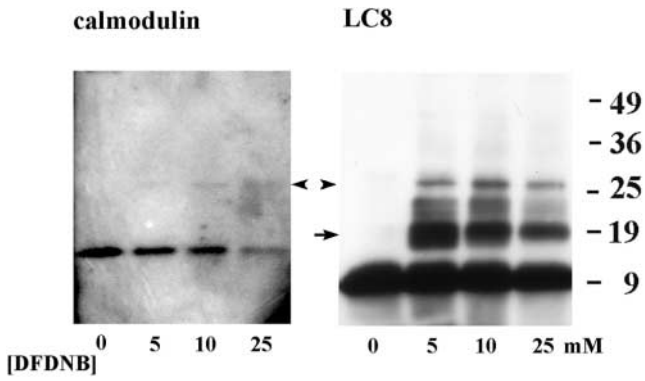

Figure 8. Cross-linking analysis reveals dimerization of LC8 and close interaction of calmodulin and LC8 in the radial spoke stalk. The 15S fraction derived from *pf17*axonemes was treated with DFDNB at concentrations indicated. Samples were then prepared for Western blot analyses using anticalmodulin (left) or anti-LC8 (right) antibodies. A 27-kD cross-linked product reacted with both antibodies (arrowheads). A 20-kD LC8 dimer also results from cross-linking (arrow, right).

and myosin V (Benashski et al., 1997). The 27-kD complex containing LC8 coincides with the 27-kD complex containing calmodulin. The complex formed by the 0.3-nm cross-linker further confirms that both calmodulin and LC8 are in the radial spoke stalk and likely assembled in close proximity in the stalk.

Discussion

A Role for the Radial Spokes in Calcium Signal Transduction

We report the biochemical isolation of flagellar radial spokes and the discovery of novel radial spoke components including calmodulin and dynein light chain LC8. Evidence from several in vitro experimental systems has revealed that calcium acts directly on the 9 + 2 axoneme to alter motility (for reviews see Satir, 1985; Brokaw, 1987). For example, in *Chlamydomonas* axonemes, an increase in calcium from 10^{-7} to 10^{-5} M causes a change in waveform from a ciliary-type bend to a flagellar-type bend in reactivated axonemes (Bessen et al., 1980; Kamiya and Witman, 1984).

Although recognized for many years that the axoneme contains calmodulin, until this study calmodulin had not been localized to a distinct structure (for review see Otter, 1989). Based on our location of one fraction of axonemal calmodulin to the radial spoke, and based on genetic analysis revealing the radial spokes play a role in control of flagellar waveform (Brokaw et al., 1982), we postulate the radial spokes operate, in part, to transduce calcium signals for control of flagellar waveform. As discussed below, this cannot be the only site of calcium action in the axoneme (LeDizet and Piperno, 1995; Frey et al., 1997; Wakabayashi et al., 1997). For example, using defined buffer conditions, calcium can alter waveform in reactivated axonemes lacking radial spoke structures, indicating more than one mechanism for calcium control of axonemal bending (Wakabayashi et al., 1997; Yagi and Kamiya, 2000).

Isolated Radial Spokes Reveal Novel Protein Components

Several lines of evidence indicate we have isolated intact radial spokes and radial spoke stalks. First, radial spoke proteins, solubilized in KI or NaBr, cosediment as large 20S complexes, when derived from wild-type cells, or smaller 15S complexes lacking spoke heads, when derived from *pf17* cells. Second, 2D gels revealed that the 20S complexes contain all 17 previously defined radial spoke proteins in approximately the same relative abundance found in axonemes (Piperno et al., 1981; Table I). The 15S complex lacks a subset of spoke proteins including RSP1 located in the radial spoke head. Third, the 20S radial spoke complexes are the size and shape of native radial spoke structures. Fourth, the extracted radial spoke complexes can rebind to *pf14* axonemes and restore radial spoke structure.

These results confirm the composition of the radial spoke originally defined indirectly by analysis of mutant cells (Piperno et al., 1981) and reveal that the radial spokes are robust structures that, although extractable in KI, resist further disassembly. (The intact 20S radial spokes did not result from reassembly during dialysis: spoke proteins still cosediment in nondialyzed KI extracts.) The mechanism for extraction is not known but may be related to partial solubilization of outer doublet microtubules (Linck, 1976). Biochemical analysis of the isolated radial spokes or spoke stalks revealed at least five new radial spoke proteins that we refer to as RSP18–22 (Table I). The two 26-kD proteins revealed by 2D gel analysis (Fig. 2, open arrowheads) might also be radial spoke components. However, they were not included in Table I since, in contrast to the other 22 proteins, the apparent stoichiometry could not be clearly established by our experiments and they could not easily be identified in previously published 2D gels of axonemes (Piperno et al., 1981). In contrast, RSP18–22 always displayed a consistent stoichiometry compared with other radial spoke proteins. Two of these proteins, calmodulin (RSP20) and LC8 (RSP22), are also components of other axonemal structures (see below). Thus, the localization of calmodulin and LC8 could not have been identified solely by analysis of flagellar mutants.

The 210-kD (RSP18) and 140-kD (RSP19) radial spoke proteins were not previously recognized by analysis of mutants. For example, both proteins are present in axonemes from *pf14*, a radial spoke mutant lacking radial spoke structure (Piperno et al., 1981). Thus, we postulate their assembly is independent of the other spoke proteins but, once assembled, form a tight interaction with the other radial spoke proteins. Although not yet based on direct analysis, we also postulate RSP18 and -19 are located at the proximal end of the radial spoke stalk, contributing to the docking of the radial spoke and possibly interacting with RSP3 (Diener et al., 1993). Consistent with this hypothesis, RSP18 and -19 copurify with both the 20S radial spoke and the 15S radial spoke stalk. Furthermore, based on previously published data, RSP18 and -19 are present in the axoneme at about the same relative abundance as RSP1, -2, and -3 (Figure 1 in Piperno et al., 1981; Figure 3 in Dutcher et al., 1984), consistent with the presence of one copy of RSP18 and -19 for each radial spoke. The apparent reduc-

Table I. Proteins in the Isolated Radial Spoke

RSP no.*	M_r	pI
	kDa	
1	123	5.2
2	118	5.0
3	86‡	5.5
4	76	5.1
5	69	5.1
6	67	5.1
7	58	5.1
8	40	6.5
9	26	5.7
10	24	5.6
11	22	4.8
12	20	6.3
13	98	6.3
14	41	6.8
15	38	5.7
16	34	7.1
17	124	6.2
18	210	5.4
19	140	5.5
20 (calmodulin)	18	4.3
21	16	6.2
22 (LC8)	10	6.8

*RSP1–17 were identified previously (Piperno et al., 1981).
‡The size of rsp3 identified by different laboratories varied slightly.

tion in molar ratio of RSP18 and -19 in the isolated radial spokes or spoke stalks is likely due to dissociation during extraction and isolation.

Dynein Light Chain LC8 Is Located in the Radial Spoke Stalk

LC8 (RSP22) is an extraordinary ubiquitous protein associated with many diverse structures and enzymes (for review see King, 2000). LC8 was originally identified as a light chain of the outer dynein arm (Piperno and Luck, 1979; Pfister et al., 1982), and later shown to be a subunit of cytoplasmic dynein (King et al., 1996), inner arm dynein I1 (Harrison et al., 1998), myosin V (Espindola et al., 2000), and nitric oxide synthase (Jaffrey and Snyder, 1996). Its function is not known, but LC8 is present in each enzyme complex in multiple copies (Benashski et al., 1997) and often present along with calmodulin (for reviews see Stuehr, 1999; Reck-Peterson et al., 2000).

LC8 is also found in multiple copies in the radial spoke stalk, possibly associated with calmodulin. These conclusions are founded on several results. First, based on 2D map position and Western blots, LC8 is found in both the 20S radial spoke and 15S spoke stalk. Second, a fraction of axonemal LC8 is found in mutant axonemes lacking outer arm dynein and inner arm dynein I1. Third, a *Chlamydomonas* LC8 mutant allele fails to assemble radial spokes, as well as dynein structures (Pazour et al., 1998). Based on chemical cross-linking, LC8 forms oligomers, and based on comparison with the abundance of RSP9 and -10, we postulate LC8 may be present in four copies per radial spoke. Further, as discussed below, LC8 can be crossed-linked to calmodulin in radial spoke stalks. Finally, an LC8 binding motif has recently been defined and found in several proteins to which LC8 is postulated to interact (Lo et al., 2001). Interestingly, RSP3 contains three putative LC8 binding motifs (Yang, P., unpublished observation), consistent with a specific interaction of LC8 with the radial spoke stalk and consistent with localization of LC8 at the proximal end of the spoke stalk (see below).

Calmodulin Is Located in Multiple Axonemal Structures Including the Radial Spoke Stalk

For many years it has been recognized that calmodulin is an axonemal component (for review see Otter, 1989). However, until now calmodulin had not been localized to a particular axonemal structure. Our localization of calmodulin depended on the highly acidic nature of calmodulin and its 2D gel map position (Van Eldik et al., 1980; Zimmer et al., 1988), availability of an antibody that reacts with *Chlamydomonas* calmodulin by Western analysis (Hulen et al., 1991), relative insolubility of axonemal calmodulin in 0.6 M NaCl (Otter, 1989), copurification of calmodulin with the 20S radial spoke, and cross-linking of calmodulin and LC8 in radial spoke stalk complexes. Based on the relative insolubility of the axonemal fraction of calmodulin in 0.6 M NaCl, the 18-kD protein is not centrin, which is known to be a calmodulin-like protein and a component of the inner dynein arms (e.g., LeDizet and Piperno, 1995).

Several results indicate calmodulin is a radial spoke stalk component. First, although calmodulin is found in three distinct peaks in sucrose gradients, one fraction cosediments with the 20S radial spokes: the 20S calmodulin fraction is missing in extracts from *pf14* axonemes and is displaced to cosediment with radial spoke stalk proteins in extracts from p*f17* axonemes. Second, the spoke fraction of calmodulin, RSP20, is present in approximately the same abundance as RSP2 and other radial spoke proteins. Third, we recently discovered that the sequence of RSP2, a stalk component, contains two tandemly aligned 1-8-14–type calcium-dependent calmodulin binding motifs (Rhoads and Friedberg, 1997). As predicted, the recombinant RSP2 binds calmodulin (Yang, P., and W.S. Sale, manuscript in preparation). Fourth, calmodulin and LC8 become crossed-linked in radial spoke stalks using a short cross-linker.

These results are consistent with previous observations on axonemal calmodulin and help explain some of the confusion about the localization of calmodulin in the axoneme. A failure to localize calmodulin in axonemes using flagellar mutants is apparently due to location of calmodulin in multiple axonemal structures. In the axoneme, the multiple locations of calmodulin as well as additional EF–hand proteins (LeDizet and Piperno, 1995; King and Patel-King, 1995b) will play distinctive roles in calcium's control of movement. For example, as discussed below, it is likely that the radial spoke plays a role in control of calcium-induced changes in motility (Brokaw et al., 1982). However, it is clear that, in the absence of radial spokes, axonemal motility can also respond to changes in calcium (Wakabayashi et al., 1997), consistent with multiple distinct sites for calcium control.

Role for the Radial Spoke in Calcium Regulation of Flagellar Waveform

Based on our results, LC8, calmodulin, and RSP2 are part of the radial spoke stalk (Fig. 9). Currently, we favor

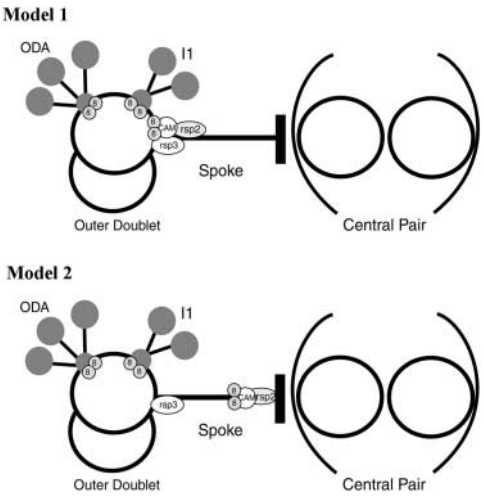

Figure 9. Two models depicting the possible location of the key proteins in the radial spoke stalk including, LC8, calmodulin, RSP2, and RSP3.

model 1 (Fig. 9), localizing the LC8–calmodulin–RSP2 complex of at the proximal end of the spoke stalk. This hypothesis is based on localization of RSP3 at the proximal end of the spoke stalk (Diener et al., 1993), prediction that LC8 binds to RSP3 (see above; Lo et al., 2001), interaction between LC8 and calmodulin in the spoke stalk (Fig. 8), and RSP2 binding to calmodulin (Yang, P., and W.S. Sale, manuscript in preparation). However, distinguishing between model 1 and model 2 will require new structural and biochemical approaches.

Diverse evidence cited above has revealed that calcium and the radial spokes both play a role in regulating flagellar waveform. Calcium binding to calmodulin may change the physical structure of the radial spoke, altering its interaction with the central pair apparatus (Warner and Satir, 1974; Smith and Lefebvre, 1997; Omoto et al., 1999). It is also possible calmodulin or other calcium binding proteins are organized differently in each of *Chlamydomonas*'s two flagella, since each flagellum responds differently to changes in calcium (Kamiya and Witman, 1984). In addition, calcium induced change in radial spoke structure could alter control of axonemal kinases or phosphatases that control flagellar dynein activity (for review see Porter and Sale, 2000). For example, RSP3 is an A-kinase anchor protein predicted to localize PKA to the base of the radial spokes (Gaillard et al., 2001), and PKA is thought to alter dynein activity (Howard et al., 1994). Moreover, an axonemal CK1 is thought to be located on outer doublet microtubules in position to control inner arm dynein activity (Yang and Sale, 2000). It is now possible to test the hypothesis that calcium-induced change in radial spoke structure could locally alter kinase activity and control the pattern of dynein-driven microtubule sliding, resulting in control of the size and shape of flagellar bends.

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