Assembly of Flagellar Radial Spoke Proteins in *Chlamydomonas*: Identification of the Axoneme Binding Domain of Radial Spoke Protein 3

Dennis R. Diener, Lay Hong Ang, and Joel L. Rosenbaum

Department of Biology, Yale University, New Haven, Connecticut 06511

Abstract. Radial spokes of the eukaryotic flagellum extend from the A tubule of each outer doublet microtubule toward the central pair microtubules. In the paralyzed flagella mutant of *Chlamydomonas pf14*, a mutation in the gene for one of 17 polypeptides that comprise the radial spokes results in flagella that lack all 17 spoke components. The defective gene product, radial spoke protein 3 (RSP3), is, therefore, pivotal to the assembly of the entire spoke and may attach the spoke to the axoneme. We have synthesized RSP3 in vitro and assayed its binding to axonemes from *pf14* cells to determine if RSP3 can attach to spokeless axonemes. In vitro, RSP3 binds to *pf14* axonemes, but not to wild-type axonemes or microtubules polymerized from purified chick brain tubulin. The sole axoneme binding domain of RSP3 is located within amino acids 1–85 of the 516 amino acid protein; deletion of these amino acids abolishes binding by RSP3. Fusion of amino acids 1–85 or 42–85 to an unrelated protein confers complete or partial binding activity, respectively, to the fusion protein. Transformation of *pf14* cells with mutagenized RSP3 genes indicates that amino acids 18–87 of RSP3 are important to its function, but that the carboxy-terminal 140 amino acids can be deleted with little effect on radial spoke assembly or flagellar motility.

THE eukaryotic flagellum is a complex, motile organelle comprised of over 200 different polypeptides precisely assembled onto a framework of microtubules. The biflagellate alga *Chlamydomonas* is well suited for the study of the biogenesis and function of flagella due to the availability of a variety of mutants that assemble incomplete, and therefore, immotile flagella. Analysis of these mutants using a combination of genetic, biochemical, and ultrastructural techniques has helped elucidate the polypeptide composition and function of various components of the flagella, including the dynein arms, the radial spokes, and the central pair microtubules (reviewed in Huang, 1986). Molecular approaches are now being used to examine these components in more detail (reviewed in Curry and Rosenbaum, 1993). The genes for several flagellar proteins have been cloned and sequenced (Youngblom et al., 1984; Silflow et al., 1985; Williams et al., 1986, 1989; Mitchell and Kang, 1991; Curry et al., 1992), and some of these genes have been used to restore motility to paralyzed flagella mutants by transformation (Diener et al., 1989; Mitchell and Kang, 1991; Curry et al., 1992).

A second advantage of *Chlamydomonas* for the study of flagellar biogenesis is that the flagella can be experimentally detached from the cell, initiating the regeneration of new flagella (reviewed in Lefebvre and Rosenbaum, 1986; Johnson and Rosenbaum, 1993). Within minutes following deflagellation, new flagellar proteins are synthesized and move to the tip of the incipient flagella, where assembly occurs (Rosenbaum and Child, 1967; Witman, 1975; Johnson and Rosenbaum, 1992). In 90 min the flagella are assembled, each ~10 μm long and capable of beating 60 times per second.

In this paper we address the assembly of one component of the flagellum: the radial spoke. Radial spokes consist of a stalk and a globular head composed of 12 and 5 different polypeptides, respectively (Piperno et al., 1981). In *Chlamydomonas*, pairs of radial spokes are attached every 96 nm to the A subfiber of the outer doublet microtubules (Hopkins, 1970; Warner, 1970; Goodenough and Heuser, 1985) and apparently serve as transient links between the outer doublet microtubules and the central pair projections (Warner and Satir, 1974; Goodenough and Heuser, 1985).

The importance of radial spokes in flagellar motility was demonstrated by the fact that mutants lacking radial spokes or spoke heads have paralyzed flagella (reviewed in Huang, 1986). Analysis of the flagellar waveform of a mutant lacking spoke heads, but which swims (albeit abnormally) due to a bypass suppressor mutation (Huang et al., 1982), suggests that radial spokes are necessary to convert a symmetric flagellar bending pattern into the asymmetric "breaststroke" pattern characteristic of *Chlamydomonas* (Brokaw et al., 1982). One aspect of radial spoke function may be to regulate the activity of dynein. Indeed, mutants that suppress the requirement for radial spokes include a dynein mutant (Huang et al., 1982), as well as strains that are deficient in components of inner dynein arms and a "dynein regulatory

complex" (Huang et al., 1982; Piperno et al., 1992) located near the base of the radial spokes and the inner dynein arms (Mastronarde et al., 1992). An interaction between spokes and dynein was demonstrated directly in experiments showing that dynein isolated from wild-type axonemes was more active than dynein isolated from spokeless axonemes (Smith and Sale, 1992*a*).

Much of the work on radial spokes has relied on the paralyzed flagella mutant of *Chlamydomonas*, *pf14*, the flagella of which lack radial spokes (Witman et al., 1976). The absence of all 17 radial spoke proteins from the flagella of *pf14* is due to a mutation in a single gene: the gene for radial spoke protein 3 (RSP3)[1] (Luck et al., 1977; Piperno et al., 1981). In the absence of RSP3, radial spokes are not assembled, and the flagella are immotile.

The observation that a mutation in RSP3 prevents the assembly of the entire radial spoke suggests that RSP3 attaches radial spokes to the outer doublet microtubules and/or that it maintains the integrity of the entire spoke (Luck et al., 1977). If the former is true, RSP3 may bind directly to *pf14* axonemes where radial spokes are missing. To test this possibility, we synthesized RSP3 in vitro and tested its binding to axonemes. RSP3 binds specifically to *pf14* axonemes and the axoneme binding domain is contained within the amino-terminal 84 amino acids of this 516–amino acid protein. We have also examined the effects of RSP3 mutations in vivo by transforming *pf14* cells with RSP3 cDNA "minigene" constructs and testing for complementation of the mutant phenotype. This approach will be useful for identifying domains that are required to ensure proper targeting and assembly of flagellar proteins during flagellar biogenesis, as well as domains that participate directly in generating flagellar motility.

Materials and Methods

Chlamydomonas Strains and Culture

Chlamydomonas reinhardtii wild-type strain (CC-125) and the paralyzed flagella mutant *pf14* (CC-1032) were obtained from the *Chlamydomonas* Genetics Center (Duke University, Durham, NC). The nitrate reductase mutant *nit1*-305 and the double mutant *pf14 nit1*-305 (Kindle et al., 1989) were obtained from Dr. P. A. Lefebvre (University of Minnesota, Minneapolis, MN). Cells were cultured in R/2 medium (Kindle et al., 1989) or, for nitrate selection of transformants, in media in which KNO_3 replaced the NH_4NO_3.

Isolation of Axonemes

Chlamydomonas were deflagellated by pH shock in 10 mM Hepes, pH 7.4, and flagella were isolated by differential centrifugation (Witman et al., 1972). The isolated flagella were extracted with 2% NP-40 in HMDEK (10 mM Hepes, pH 7.4; 5 mM $MgSO_4$; 1 mM DTT; 0.5 mM EGTA; 25 mM KCl) for 45 min on ice and the resulting axonemes were sedimented (10 min, 8,000 *g*). Axonemal pellets were washed twice in HMDEK and were resuspended in HMDEK at 5 $\mu g/\mu l$. To dissociate the axonemes into outer doublets, axonemes were extracted with HMDEK containing 0.6 M KCl, then dialyzed overnight in a low salt buffer (Piperno et al., 1981). Protein concentrations were estimated by the Bradford method (Bio Rad Laboratories, Hercules, CA) using BSA as a standard.

Construction of Plasmids

A cDNA clone of RSP3 in *Chlamydomonas* had been cloned into the EcoRI site of Bluescript KS^- (Stratagene, La Jolla, CA) (Williams et al., 1989). RSP3 with carboxy-terminal truncations were synthesized from mRNAs transcribed in vitro after linearizing the RSP3 cDNA with PflMI, Sau3AI, or HincII, which cut after the codons for amino acids 237, 146, and 91.

Deletions in the putative axoneme binding domain of RSP3 were constructed by linearizing the RSP3 cDNA with PpuMI at codon 70 followed by digestion for 30, 60, or 120 s at 28°C with Bal 31 (New England Biolabs Inc., Beverly, MA). Digestions contained 7 μg of DNA, 0.25 U of Bal 31, 20 mM Tris-HCl, pH 8, 12 mM $CaCl_2$, 12 mM $MgCl_2$, 0.6 M NaCl, and 1 mM EGTA. The digested DNA was treated with the Klenow fragment of DNA polymerase I, and recircularized by ligation in the presence of ClaI linkers (New England Biolabs Inc.). Deletions of interest were sequenced by the dideoxynucleotide chain termination method. The inserted ClaI sites were used to splice in-frame deletions of the desired size.

A clone in which translation was initiated only at methionine 42 was constructed by removing both the authentic ATG and a downstream out-of-frame ATG. For this the RSP3 cDNA was linearized with NruI, which cuts just upstream of the out-of-frame start codon and digested with Bal 31. The plasmid was then digested with NheI, which cuts 21 bases upstream from the authentic ATG, the ends were repaired, and the plasmid was recircularized. The first AUG in mRNA transcribed from this plasmid encodes methionine 42 of wild-type RSP3.

To produce fusions of RSP3 and RSP6 for in vitro transcription, the RSP6 cDNA (Curry et al., 1992) was digested with NcoI, which cuts at the start codon, treated with S1 nuclease, and digested with HindIII, which cuts in the polylinker, 5′ to the cDNA. A HindIII/NcoI fragment of the RSP3 cDNA, which includes the first 85 codons of RSP3, was then ligated into the RSP6 vector. To identify splices that were in frame, plasmids were transcribed and translated in vitro. Sequencing of a plasmid that produced a translation product of the expected size showed that amino acids 1–85 of RSP3 were fused to amino acids 2–459 of RSP6. Other fragments of the RSP3 cDNA were ligated into the same RSP6 vector in a similar way.

Constructs to be used for transformation were made by exchanging a fragment of an engineered cDNA for the comparable fragment in a plasmid containing the genomic KpnI/EcoRI fragment of RSP3, pEK (Williams et al., 1989). The resulting plasmid contained ~665 bp of genomic sequence upstream and 380 bp downstream of the RSP3 cDNA. To produce truncated RSP3 in vivo, a universal termination linker (Promega, Madison, WI) was inserted into the NotI or the PflMI site following amino acid 376 or 237. These constructs lack introns and so are referred to as "minigenes."

An epitope from influenza hemagglutinin, for which a high affinity mAb is available (12CA5, Field et al., 1988), was inserted into the minigene lacking codons for amino acids 18–87 (Δ18/87) to aid in detecting its expression on immunoblots. The oligonucleotide encoding the epitope had been previously inserted into the α1-tubulin gene of *Chlamydomonas* with no deleterious effects (Kozminski et al., 1993). Likewise, when this oligonucleotide was inserted into the StuI site of the RSP3 gene, 12 amino acids upstream of the carboxy terminus of RSP3, no effect was noted on the ability of the gene to complement the *pf14* phenotype (Karl Johnson, personal communication). The BstEII/MluI fragment from the RSP3 construct containing the epitope sequence was exchanged with the same fragment of Δ18/87 to epitope-tag the mutant RSP3.

Transcription and Translation

Linearized RSP3 and RSP6 cDNAs (Curry et al., 1992) were transcribed with T3 RNA polymerase (Stratagene) following procedures recommended by the supplier. Transcripts were extracted with phenol/chloroform, precipitated with ethanol, and translated in rabbit reticulocyte lysate (Pelham and Jackson, 1976) supplemented with [^{35}S]methionine. Before use in the binding assay, lysate containing the translated protein was treated with RNase A (10 min at 30°C) and centrifuged for 10 min in a microfuge at room temperature.

In Vitro Binding Assay

The binding of RSP3 to axonemes was measured by mixing 5 μl of rabbit reticulocyte lysate containing ^{35}S-labeled RSP3 with 5 μl of 5 $\mu g/\mu l$ flagellar axonemes or outer doublets and incubating at room temperature for 30 min. The axonemes were spun for 10 min in a microfuge and the supernatant, containing the unbound protein, was removed. The axonemal pellets were washed in HMDEK containing 0.2 M KCl and were resuspended in Laemmli sample buffer (Laemmli, 1970). The bound and unbound proteins contained in the pellet and supernatant, respectively, were analyzed by SDS-PAGE (Laemmli, 1970). Gels were stained with Coomassie blue, dried, and exposed to X-OMAT AR film (Eastman Kodak Co., Rochester, NY) to visualize the radioactive proteins.

1. *Abbreviations used in this paper*: EDC, 1-Ethyl-3-(3-Dimenthylaminopropyl) carbodiimide; RSP3, radial spoke protein 3.

Transformation of Chlamydomonas

To test the ability of various mutant RSP3 constructs to complement the *pf14* mutation, minigene constructs containing mutagenized RSP3 cDNAs were introduced into *Chlamydomonas* using the glass bead method of transformation (Kindle, 1990). The double mutant *pf14 nit1*-305, which is both paralyzed and deficient in the gene for nitrate reductase, was cotransformed with the desired RSP3 minigene along with a plasmid that contains the wild-type nitrate reductase gene, pMN24 (Fernández et al., 1989). Transformants were selected on nitrate plates, transferred to microtiter wells, and checked microscopically for motility.

Swim Rate Measurement

Swimming cells were videotaped with a frame rate of eight frames/second. The paths of individual cells during eight frames were traced onto a plastic sheet and measured with a map measurer. Only actively swimming cells were included in calculating mean swim rates; therefore, the swim rates were independent of the percentage of cells that were motile.

Electron Microscopy

For EM, flagella were isolated by the dibucane method (Witman et al., 1978) and fixed on ice with 3% glutaraldehyde containing 1% tannic acid (Mallinckrodt Specialty Chemicals, Chesterfield, MO) in 10 mM phosphate buffer pH 7.4. After postfixation in 1% osmium tetroxide the flagella were dehydrated through acetone and embedded in Araldite (Electron Microscopy Sciences, Fort Washington, PA). Silver to gray sections were stained with 1% uranyl acetate and lead citrate and examined in a Philips 201 electron microscope (Philips Electronic Instruments Co., Mahwah, NJ).

Electrophoresis of Flagellar Proteins and Immunoblotting

Two-dimensional (2D) gel electrophoresis was carried out on isolated axonemes as previously described (L'Hernault and Rosenbaum, 1983). In some experiments the proteins were transferred to nitrocellulose and probed (Kozminski et al., 1993) with a 1:10,000 dilution of anti-RSP3 antiserum (Williams et al., 1989).

Preparation of Chick Brain Tubulin

Tubulin was isolated from chick brains by cycles of polymerization/cold depolymerization as previously described (Snell et al., 1974) except that 4 M glycerol was included in the buffer during microtubule polymerization. After three cycles of polymerization/depolymerization, the tubulin was depleted of microtubule associated proteins by phosphocellulose chromatography (Sloboda and Rosenbaum, 1982).

Cross-linking

The protocol for cross-linking axonemal proteins was similar to that used by King et al. (1992). Radiolabeled RSP3 was bound to axonemes from *pf14* cells, and the axonemes were incubated at room temperature for 1 h in the presence of 0, 1, or 5 mM 1-Ethyl-3-(3-Dimethylaminopropyl)carbodiimide (EDC) (Pierce, Rockford, IL). This compound forms irreversible peptide bonds between closely apposed carboxyl and amino groups. After incubation, the reactions were quenched with 0.1 vol of 2-mercaptoethanol and the samples were prepared for electrophoresis. To act as a marker, wild-type axonemes cross-linked with 5 mM EDC were either mixed with the *pf14* samples before electrophoresis or loaded into adjacent wells. After SDS-PAGE, samples were transferred to nitrocellulose and probed with anti-RSP3 antiserum to locate free and cross-linked RSP3 in wild-type axonemes. Radiolabeled RSP3 on the nitrocellulose was visualized by autoradiography.

Results

RSP3 Binding to Axonemes In Vitro

The paralyzed flagella mutant *pf14* is deficient in RSP3; the flagella lack all 17 radial spoke polypeptides (Piperno et al., 1981) and, ultrastructurally, the radial spokes are missing (Witman et al., 1976). These observations suggest that RSP3 attaches the spoke to the axoneme, perhaps by binding directly to the axonemes. To determine whether or not RSP3 binds directly to the axoneme, axonemes were isolated from *pf14* and used as a substrate for the binding of radioactive RSP3 synthesized in vitro.

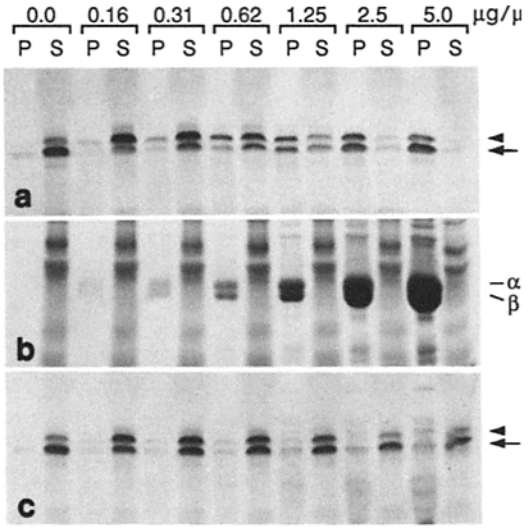

Figure 1. Binding of in vitro synthesized RSP3 to axonemes. Radiolabeled RSP3 was incubated with the indicated μg/μl of axonemes isolated from *pf14* (*a* and *b*) or wild-type cells (*c*). The axonemes were sedimented, washed, and analyzed by SDS-PAGE. In vitro translation of RSP3 mRNA produced two forms of RSP3: an unmodified form (*arrow*, *a* and *c*) and a more slowly migrating modified form (*arrowhead*, *a* and *c*). The autoradiogram (*a*) shows that in the absence of axonemes (0.0 μg/μl) the RSP3 remained in the supernatant (*S*), but with the addition of 0.16–5.0 μg/μl of axonemes from *pf14* cells, increasing amounts of RSP3 sedimented with the axonemal pellet (*P*). The Coomassie blue–stained gel (*b*) from which the autoradiogram shown in *a* was taken illustrates the amount of axonemal proteins, including tubulin (α and β), in the pellets. When incubated with wild-type axonemes, as shown in autoradiogram *c*, RSP3 remained in the supernatant. Only a portion of the autoradiograms and gel are shown.

Translation of RSP3 mRNA synthesized in vitro produces two major proteins of 90 and 96 kD (Fig. 1, lane *0.0*; and Williams et al., 1989). The ratio of the two proteins varied between preparations, but the 90-kD form was always the more abundant immediately after translation; however, occasionally, the 96-kD form became more abundant in the presence of axonemes at the expense of the 90-kD form (Fig. 1 *a*, compare lanes *0.0* and *0.16*). The 96-kD protein comigrates on 2D gels with the predominate flagellar form of RSP3 (Williams et al., 1989 and data not shown), which is phosphorylated (Piperno et al., 1981). The 90-kD protein comigrates with a minor flagellar form of RSP3, which is more abundant in the cell body (Williams et al., 1989 and data not shown). Thus, the 90-kD form is most likely an unmodified cytoplasmic precursor of the phosphorylated flagellar 96-kD form. Production of the 96-kD form in vitro is probably due to phosphorylation of the 90-kD form (Williams et al., 1989) by kinases present in the reticulocyte lysate (Ernst et al., 1987) and by an axonemal kinase previously shown to phosphorylate RSP3 in vitro (Segal and Luck, 1985).

To test for binding, radiolabeled RSP3 synthesized in rabbit reticulocyte lysate was incubated with axonemes isolated

from *pf14* and the axonemes were sedimented by centrifugation. The axonemal pellet and supernatant were subjected to SDS-PAGE, and the amount of RSP3 present was determined by autoradiography. The amount of RSP3 that sedimented (Fig. 1 *a*) varied with the amount of axonemes present (Fig. 1 *b*). If axonemes were omitted from the incubation mixture, all of the RSP3 remained in the supernatant (Fig. 1 *a*, lanes *0.0*) indicating that the protein remained soluble during the incubation. As increasing amounts of axonemes were added, more RSP3 sedimented with the axonemes, until upon addition of 2.5 μg/μl of axonemes, essentially all of the RSP3 sedimented with the axonemes, with little RSP3 remaining in the supernatant (Fig. 1 *a*, lanes *2.5*). Both the 90- and 96-kD forms of RSP3 bound to the axonemes. Other experiments showed that binding was complete in 30 min, was not affected by the presence of ATP or GTP, and, like radial spokes in wild-type flagella, was stable in 0.6 M KCl (data not shown).

To determine whether or not RSP3 has access to binding sites along the entire length of the axoneme in the binding assay, axonemes were dissociated into individual outer doublets before being used as the substrate for RSP3 binding. As with intact axonemes, all the RSP3 was bound by 2.5 μg/μl outer doublets (data not shown) indicating that the added RSP3 has access to its binding site on the A tubule of the outer doublet microtubules of the intact axoneme even though this site is on the inner portion of the outer doublet, facing the central microtubules. Likewise, in vitro reconstitution of inner dynein arms onto axonemes lacking these complexes has shown that dynein arms are able to enter the axoneme and bind along the length of the microtubules (Smith and Sale, 1992*b*).

If RSP3 binds in vitro to a specific spoke binding site on *pf14* axonemes, it should not bind to wild-type axonemes in which these sites are filled by radial spokes. As a test for the specificity of binding the titration shown in Fig. 1 *a* was repeated with dilutions of axonemes from wild-type instead of *pf14* cells. As can be seen in Fig. 1 *c*, RSP3 did not bind to wild-type axonemes within the range of concentrations used. Occasionally, some binding was obtained to 5 μg/μl of wild-type axonemes, but the amount bound was never half the RSP3. The inability of RSP3 to bind wild-type axonemes indicates that, in vitro, RSP3 binds specifically to *pf14* axonemes at sites that are not accessible in wild-type axonemes.

Cross-linking of RSP3 to pf14 Axonemes

The zero-length cross-linker EDC was used to further test the specificity of in vitro binding; if RSP3 bound to *pf14* axonemes in vitro can be cross-linked to the same protein as it is bound to in wild-type axonemes, it would provide further evidence that the binding obtained in vitro is specific. Immunoblot analysis of wild-type axonemes identified the flagellar form of RSP3 at 96 kD (Fig. 2 *a*, lane *1*). After treatment with 1 mM EDC a fraction of the RSP3 formed a complex migrating at ~150 kD (Fig. 2 *a*, lane *2*, *arrowhead*). Likewise, when *pf14* axonemes to which radiolabeled RSP3 had been bound were treated with EDC, two forms of RSP3 were seen: the unbound form, which migrated as the unmodified 90-kD form (Fig. 2 *b*, lanes *1–3*, *arrow*); and a cross-linked form (Fig. 2 *b*, lanes *2* and *3*, *arrowhead*), which migrated slightly faster than the cross-linked RSP3 from wild-type flagella (Fig. 2 *b*, lane *4*). The fact that a discrete band of

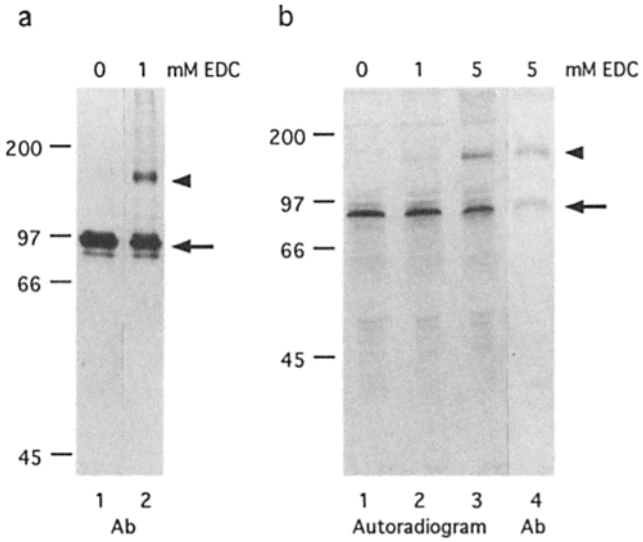

Figure 2. Cross-linking of RSP3 to wild-type and *pf14* axonemes. (*a*) This immunoblot of wild-type axonemes probed with anti-RSP3 antiserum identifies the predominant flagellar form of RSP3 at 96 kD (lane *1*, *arrow*). After treatment with 1 mM EDC (lane *2*) a complex migrating at ~150-kD (*arrowhead*) containing RSP3 is formed. (*b*) The autoradiogram (lanes *1–3*) of *pf14* axonemes to which radiolabeled RSP3 has been bound in vitro shows both the free (90 kD, *arrow*) and cross-linked RSP3 (*arrowhead*) following treatment with 1 or 5 mM EDC (lanes *2* and *3*). An immunoblot of cross-linked wild-type axonemes from an adjacent lane are included for reference (lane *4*); both free and cross-linked RSP3 synthesized in vitro migrate slightly faster than the corresponding forms of RSP3 from wild-type axonemes. Molecular mass markers are in kD.

cross-linked RSP3 was obtained suggests that RSP3 bound a specific protein on the axoneme. Furthermore, the results suggest that in vitro, as in vivo, RSP3 is bound specifically to a 50–60-kD protein. The identity of this protein is unknown, although it appears not to be tubulin (see Discussion). The small difference in the electrophoretic mobility of the cross-linked RSP3 between the wild-type and *pf14* samples reflects the difference between the modified form found in flagella and the unmodified form synthesized in vitro.

Incubation of RSP3 with Brain Microtubules

The above experiments indicate that RSP3 binds specifically to *pf14* axonemes, but they do not address whether RSP3 attaches directly to the microtubules or whether it binds indirectly through some other axonemal protein. To determine if RSP3 can bind directly to microtubules, chick brain tubulin, depleted of microtubule associated proteins, was assembled into microtubules. The microtubules were stabilized with taxol and used in the binding assay at a concentration of 2.0 μg/μl. No RSP3 sedimented with the microtubules (data not shown). The fact that RSP3 does not bind to in vitro polymerized microtubules may mean that RSP3 does not bind directly to axonemal microtubules, but rather to some other axonemal protein.

Identification of the Axoneme Binding Domain of RSP3

Initial experiments to identify the axoneme binding domain

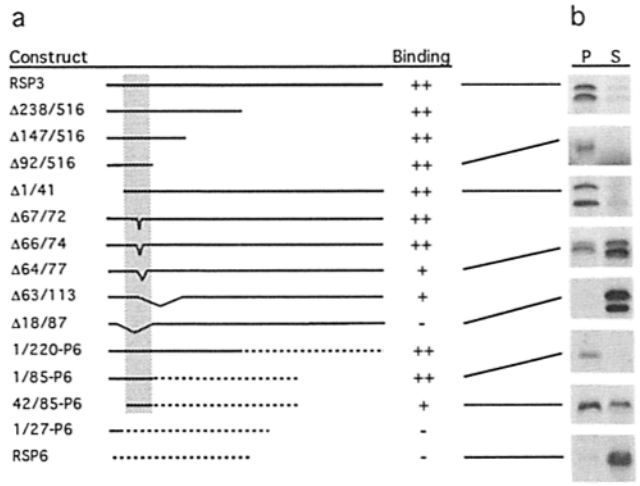

Figure 3. Binding of mutant forms of RSP3 to axonemes in vitro. (*a*) This diagram shows the RSP3 mutations used in the axoneme binding assay and their binding to axonemes. Construct names denote the amino acids of RSP3 that were deleted (Δ) or fused to RSP6 (*P6*, ----). The shaded area delineates amino acids 42–85, the approximate boundaries of the axoneme binding domain. All of the mutant polypeptides remained soluble in reticulocyte lysate in the absence of axonemes. Ranges for binding of test proteins to 2.5 μg/μl of axonemes relative to full length RSP3 are: ++, 80–100%; +, 25–75%; −, 0–20%. (*b*) These autoradiograms show the distribution of the indicated RSP3 deletions or fusion proteins between the pellet (*P*) and supernatant (*S*) after incubation with 2.5 μg/μl of *pf14* axonemes. Each panel is cropped to show only the test protein.

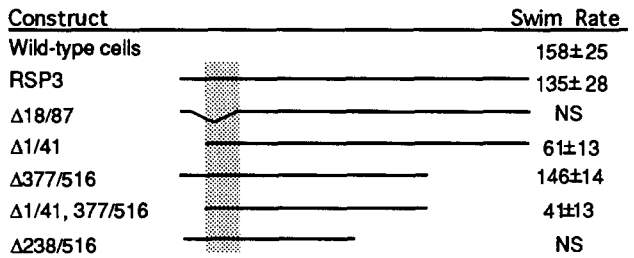

Figure 4. Restoration of motility to *pf14* cells following transformation with various RSP3 constructs. The constructs diagrammed were transformed into *pf14* cells and the swim rate of the transformants was measured. Construct names denote the amino acids deleted from the encoded RSP3. The shaded area delineates amino acids 42–85, the approximate boundaries of the axoneme binding domain. Swim rates listed are the means of 30 cells reported as μm/s ± standard deviation. *NS* indicates that no swimming cells were found.

were done by testing the binding of a series of carboxy-terminal truncations of RSP3 (Fig. 3). Even the smallest of these truncated proteins, consisting of only the amino-terminal 91 (of 516 total) amino acids of RSP3, bound to *pf14* axonemes (Fig. 3, Δ92/516), suggesting that an axoneme binding domain is present within this fragment. To further define the binding domain, RSP3 from which the first 41 amino acids had been deleted, was tested and shown to bind to *pf14* axonemes (Fig. 3, Δ1/41), suggesting that amino acids 42–91 are sufficient for binding. Some small deletions in this domain had no detectable effect on binding (e.g., Fig. 3, Δ67/72), but the deletion of amino acids 64–77 reduced binding activity substantially (Fig. 3, Δ64/77). RSP3 lacking amino acids 18–87 had no binding activity (Fig. 3, Δ18/87). In addition to demonstrating the importance of these amino acids in binding, the latter construct also shows that the amino terminus is the only region of RSP3 with the ability to bind axonemes.

To confirm that the putative axoneme binding domain was sufficient for attachment to the axoneme, a chimeric protein consisting of the amino-terminal 85 amino acids of RSP3 fused to RSP6 was assayed for binding. RSP6, a radial spoke polypeptide located in the spoke head, does not, by itself, bind to axonemes (Fig. 3, *RSP6*); however, the fusion protein binds to axonemes (Fig. 3, *1/85–P6*). The fusion protein consisting of amino acids 42–85 of RSP3 and RSP6 also binds to axonemes (Fig. 3, *42/85–P6*), though not as well as full length RSP3, indicating that these amino acids contain much of the structure necessary for binding. Amino acids 1–27 of RSP3 conferred no binding to RSP6 (Fig. 3, *1/27–P6*).

In Vivo Analysis of RSP3 Deletions

To determine whether the axoneme binding domain identified in vitro was necessary for spoke binding and function in vivo, mutagenized RSP3 clones were used to transform *pf14* cells and transformants were assayed for motility. Previous work had shown that transformation of *pf14* with a 6.6-kb genomic clone including the gene for RSP3 restores wild-type motility to the paralyzed cells (Diener et al., 1989). Likewise, preliminary experiments showed that a "minigene," consisting of the RSP3 cDNA flanked by ~665 and 380 kb of 5′ and 3′ genomic RSP3 sequence, could restore radial spokes and flagellar motility to *pf14* cells (Figs. 4–6). When transformation of the *Chlamydomonas* double mutant *pf14 nitl*-305 was carried out using both the nitrate reductase gene, as a selectable marker, and the RSP3 minigene, cells that had been transformed with both genes swam at about the same rate as wild-type cells (Fig. 4). Furthermore, analysis of the axonemes of these cotransformants by two-dimensional gel electrophoresis showed that radial spoke proteins had been restored to the flagella (Fig. 5 *c*); EM showed that the distribution of spokes in the flagella (Fig. 6 *c*) were indistinguishable from wild-type cells.

If amino acids 42–85 of RSP3 are essential for axoneme binding, an RSP3 construct lacking this domain should not be able to complement the paralysis of the *pf14* mutant. The codons for amino acids 18–87 were deleted from an otherwise intact RSP3 minigene, and this construct was used in cotransformation experiments in parallel with the intact minigene. In over 200 *nitl*$^+$ transformants screened for motility, no motile transformants were obtained with the deleted minigene, whereas ~20% of the *nitl*$^+$ transformants were motile when the intact minigene was used. To ensure that the Δ18/87 construct could be properly expressed, it was necessary to screen the *nitl*$^+$ transformants for the presence of Δ18/87 RSP3. Initial immunoblot analysis of total cell protein with anti-RSP3 antiserum did not identify any positive cotransformants. This may have been because the mutant RSP3 does not accumulate in the flagella of these cells, and it is difficult to detect RSP3 even in the wild-type cell bodies (sans flagella) with anti-RSP3 antiserum. To increase the sensitivity of the screen, an epitope from influenza virus hemagglutinin for which a high affinity mAb is available (Field et al., 1988) was inserted into RSP3 (see Materials

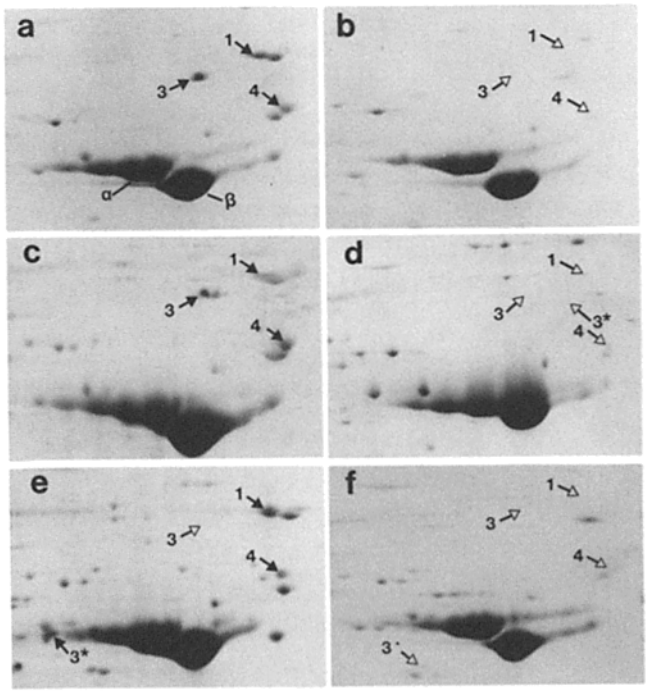

Figure 5. Electrophoretic analysis of axonemes from transformants. Each panel shows the region of a two-dimensional gel including RSP3 from wild-type (*a*), *pf14* (*b*), and various transformants (*c*, complete RSP3 minigene; *d*, Δ1/41; *e*, Δ377/516; *f*, Δ1/41, 377/516). Filled arrows indicate the location of three radial spoke proteins (RSP1, RSP3, and RSP4); open arrows indicate the location where these radial spoke proteins are missing or present in reduced amount. *3** indicates the location of truncated forms of RSP3, identified with anti-RSP3 antiserum on immunoblots of these gels. α and β tubulin are also indicated (α and β) in *a*.

and Methods). Insertion of this epitope into the wild-type *RSP3* gene has no detectable effect on its ability to complement the *pf14* mutation (Karl Johnson, personal communication). After transformation of *pf14 nitl*-305 with the epitope-tagged Δ18/87 minigene and the nitrate reductase gene, two transformants were identified that expressed the mutant RSP3 (data not shown). Still, these cells did not swim. Thus, the Δ18/87 RSP3 can be expressed, yet it cannot restore motility to *pf14* cells. These experiments suggest that the deleted region is important for the formation or function of radial spokes and are consistent with the in vitro data showing that this region includes the axoneme binding domain.

Removal of the amino-terminal 41 amino acids of RSP3 had little effect on in vitro binding. This observation was unexpected because the mutant *pf14* synthesizes a small amount of RSP3, initiating translation at methionine 42, yet it lacks radial spokes. To test for the assembly of the amino-terminal deletion in vivo, a minigene construct encoding RSP3 lacking amino acids 1–41 was transformed into *pf14 nitl*-305 cells. Motile transformants were obtained, but in every clone isolated only a few of the cells were swimming, and those cells that were motile swam at less than half the rate of wild-type cells (Fig. 4). Electrophoretic analysis of flagella isolated from these transformants showed that radial spoke proteins were present, but at a much reduced level relative to wild-type flagella (Fig. 5 *d*).

To test for other domains of RSP3 involved in motility, stop codons were inserted into RSP3 minigenes and the constructs were transformed into *pf14 nitl*-305. When an RSP3 minigene construct containing a stop codon after codon 376, deleting the carboxy-terminal 140 amino acids, was used in cotransformation experiments, motile transformants were obtained that swam at approximately the same speed as wild-type cells (Fig. 4). On the basis of electrophoretic and electron microscopic analyses (Figs. 5 and 6), the axonemes from transformants with this construct were indistinguishable from wild-type except for the altered electrophoretic mobility of the truncated RSP3 (Fig. 5 *e*). These results show that amino acids 377–516 are not essential for either targeting of the protein to the flagellum or for spoke assembly or motility.

A second construct, combining the amino-terminal deletion (Δ1/41) with the deletion of amino acids 377–516, was able to restore partial motility to *pf14* cells, as was found with the construct with just the amino-terminal deletion

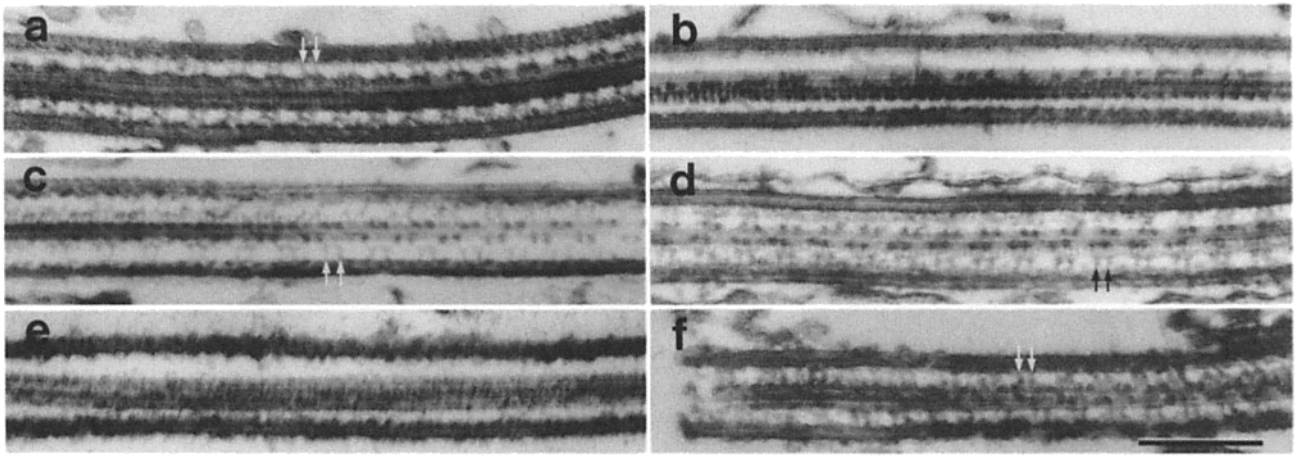

Figure 6. Analysis of axonemes from transformants by EM. These micrographs of longitudinal sections of axonemes from wild-type (*a*), *pf14* (*b*), and transformants (*c*, complete RSP3 minigene; *d*, Δ377/516) illustrate the presence or absence of radial spokes (*arrows*) in these cell lines. Although many sections of flagella from transformants expressing Δ1/41, 377/516 lacked radial spokes (*e*), some sections of these flagella had an apparently normal distribution of radial spokes (*f*). Bar, 0.25 μm.

(Fig. 4). Electrophoretic analysis showed the amount of radial spoke proteins was reduced when compared with wild-type (Fig. 5*f*). When examined by EM, many sections showed few or no spokes (Fig. 6 *e*); however, some sections were seen to have an apparently normal distribution of radial spokes (Fig. 6*f*). This construct, lacking the amino terminal 41 and the carboxy-terminal 140 amino acids, was the shortest construct tested that was able to restore motility to the paralyzed mutant. No motile transformants were obtained from over 200 *nitl*$^+$ transformants examined when a stop codon was placed after amino acid 237 in the minigene used for transformation.

Discussion

In Vitro Binding of RSP3 to Axonemes

Using an in vitro binding assay we have shown that the radial spoke stalk protein RSP3 translated in vitro can bind to spokeless axonemes isolated from *pf14* cells. The specificity of this binding is supported by the observations that (*a*) RSP3 binds poorly, if at all, to wild-type axonemes, that (*b*) amino acids 42–85 of RSP3 are sufficient and necessary for binding, and that (*c*) when cross-linked with EDC, RSP3 forms a complex of ~150 kD in both wild-type axonemes and in *pf14* axonemes to which RSP3 has been bound in vitro. The fact that RSP3 can bind to *pf14* axonemes in the absence of the other 16 radial spoke polypeptides supports the hypothesis that it is involved in anchoring the entire radial spoke onto the flagellar axoneme (Luck et al., 1977).

By examining the ability of in vitro mutagenized RSP3 to bind axonemes we have determined that the amino-terminal 85 amino acids contain the sole axoneme binding site. Deletions made within this domain can abolish binding. Furthermore, fusion of amino acids 1–85 to RSP6, a protein that does not bind axonemes, produces a chimeric protein that binds axonemes as well as full length RSP3. This observation, together with the fact that removal of amino acids 1–41 from RSP3 had little effect on binding, suggest that the binding domain lies between amino acids 42–85. Fusion of these amino acids to RSP6 produced a protein that bound axonemes, but not as well as the fusion of amino acids 1–85, suggesting that amino acids 42–85 do not make up the entire binding domain, or that they cannot function completely in the context of the fusion protein.

Although RSP3 binds axonemes, it may not bind directly to the axonemal microtubules. The axoneme binding domain of RSP3 is basic as is typical for microtubule binding domains, but, other than this, no similarities have been noted (Curry and Rosenbaum, 1993 and Alice Curry, personal communication) between the axoneme binding domain of RSP3 and the microtubule binding domain of MAP2, MAP4, tau (reviewed in Chapin and Bulinski, 1992), kinesin or dynein (reviewed in Bloom, 1992; and Witman, 1992, respectively). Neither does RSP3 contain repeated motifs as are found in some microtubule binding domains (reviewed in Chapin and Bulinski, 1992). Furthermore, unlike flagellar dynein from *Chlamydomonas*, which can bind both axonemal and brain microtubules (Haimo et al., 1979), RSP3 does not bind to chick brain microtubules repolymerized in vitro. Perhaps there is an unidentified axonemal protein that links RSP3 to the microtubule.

As a first step in identifying the protein to which RSP3 binds, axonemes were treated with the zero-length cross-linker EDC. When cross-linked with EDC, wild-type axonemes, as well as *pf14* axonemes to which RSP3 had been bound in vitro, formed a complex (~150 kD) containing RSP3. Although the size of this complex is consistent with it being the fusion of RSP3 (96 kD) and tubulin (55 kD), preliminary results suggest that this is not the case. Anti-α- or β-tubulin antisera do not appear to react with the complex after its precipitation with anti-RSP3 antiserum (D. Diener, unpublished observations). Even though the protein to which RSP3 is cross-linked on the axoneme does not appear to be tubulin, this does not rule out the possibility that RSP3 also binds tubulin.

In Vivo Complementation of Motility in pf14

The use of RSP3 cDNA minigenes to complement the paralyzed phenotype of *pf14* by transformation has enabled us to examine functional domains of RSP3 in vivo. Transformation of *pf14* cells with a construct including the full-length RSP3 cDNA restored radial spokes and motility to the paralyzed flagella; however, when the codons for amino acids 18–87, which include the axoneme binding domain, were deleted, the construct did not complement flagellar paralysis when expressed in *pf14*. In contrast, deletion of 140 amino acids from the carboxy terminus had little effect on motility. These results emphasize the importance of the amino terminus of RSP3 to radial spoke function and are consistent with the in vitro result that this region is necessary to attach RSP3 to the flagellar axoneme. Further analysis of transformants expressing the Δ18/87 construct will be required to determine if the truncated protein is found in the flagella, yet is unable to bind the axoneme.

Unlike paralyzed flagella mutants that have rigid flagella, the flagella of *pf14* cells are able to twitch and bend slowly. This limited movement may depend on the presence of some RSP3. In spite of the fact that the *pf14* mutation produces a premature stop codon at codon 21 of RSP3, these cells still synthesize a small amount of an incomplete form of the protein, apparently by initiation of translation at methionine 42. This protein is found in low amounts both in the cell body (Williams et al., 1989) and in the flagella (L. H. Ang and D. Diener, unpublished observations). When *pf14* cells were transformed with a construct encoding an RSP3 in which the first ATG was methionine 42 (and which either had the complete carboxy terminus or was truncated after amino acid 376) the cells swam at less than half the rate of wild-type cells and their axonemes contained some spokes, but fewer than in wild-type cells. These results indicate that the truncated RSP3 is able to enter the flagella, bind the axoneme, and participate in motility, but the degree to which these functions are compromised has not been determined.

Deletions of the carboxy terminus of RSP3 were also tested for their ability to restore motility to *pf14* cells. Little effect on spoke assembly or flagellar motility was seen after deleting over one fourth of the molecule (amino acids 377–516) suggesting that this portion of the molecule is dispensable for RSP3 assembly and flagellar motility. Constructions in which the next 139 amino acids were also deleted, however, could not complement the *pf14* mutation, suggesting that these amino acids are critical to the function

of RSP3. Further analysis of transformants expressing this construct may be informative as to what aspect of RSP3 function is impaired by this deletion.

Reintroducing mutagenized RSP3 genes into *Chlamydomonas* will be instrumental in elucidating the role of RSP3 in radial spoke assembly and function as well as the function of spokes in flagellar motility. One aspect of flagellar assembly that can now be addressed more directly, for example, is that of targeting proteins to the flagellar compartment; i.e., what part of RSP3 is necessary to direct this protein to the flagella. These techniques can also be applied to study the various functions of any flagellar protein, including other radial spoke proteins and individual components of the dyneins.

The authors would like to thank Mitchell Bernstein and Keith Kozminski for helpful comments on the manuscript and Alice Curry for analysis of sequence comparisons. We are also grateful to Karl Johnson for his observations on *Chlamydomonas* transformants expressing epitope-tagged RSP3 and providing the epitope-tagged RSP3 construct, and to Ian Wilson (Scripps Clinic) for providing the anti-hemagglutinin antibody (12CA5).

This work was supported by National Institutes of Health grant GM 14642 and National Science Foundation grant DCB-8916582 to J. L. Rosenbaum.

Received for publication 23 April 1993 and in revised form 12 July 1993.

References

Bloom, G. S. 1992. Motor proteins for cytoplasmic microtubules. *Curr. Opin. Cell Biol.* 4:66–73.

Brokaw, C. J., D. J. L. Luck, and B. Huang. 1982. Analysis of the movement of *Chlamydomonas* flagella: the function of the radial-spoke system is revealed by comparison of wild-type and mutant flagella. *J. Cell Biol.* 92: 722–732.

Chapin, S. J., and J. C. Bulinski. 1992. Microtubule stabilization by assembly-promoting microtubule-associated proteins: a repeat performance. *Cell Motil. Cytoskeleton.* 23:236–243.

Curry, A. M., and J. L. Rosenbaum. 1993. The flagellar radial spoke: a model molecular genetic system for studying organelle assembly. *Cell Motil. Cytoskeleton.* 24:224–232.

Curry, A. M., B. D. Williams, and J. L. Rosenbaum. 1992. Sequence analysis reveals homology between two proteins of the flagellar radial spoke. *Mol. Cell. Biol.* 12:3967–3977.

Diener, D. R., A. M. Curry, K. A. Johnson, B. D. Williams, P.A. Lefebvre, K. L. Kindle, and J. L. Rosenbaum. 1989. Rescue of a paralyzed flagella mutant of *Chlamydomonas* by transformation. *Proc. Natl. Acad. Sci. USA.* 87:5739–5743.

Ernst, H., R. F. Duncan, and J. W. B. Hershey. 1987. Cloning and sequencing of complementary DNAs encoding the α-subunit of translational initiation factor eIF-2. *J. Biol. Chem.* 262:1206–1212.

Fernández, E., R. Schnell, L. P. W. Ranum, S. C. Hussey, C. D. Silflow, and P. A. Lefebvre. 1989. Isolation and characterization of the nitrate reductase structural gene of *Chlamydomonas reinhardii. Proc. Natl. Acad. Sci. USA.* 86:6449–6453.

Field, J., J. Nikawa, D. Broek, B. MacDonald, L. Rodgers, I. A. Wilson, R. A. Lerner, and M. Wigler. 1988. Purification of a *RAS*-responsive adenylyl cyclase complex from *Saccharomyces cerevisiae* by use of an epitope addition method. *Mol. Cell. Biol.* 8:2159–2165.

Goodenough, U. W., and J. E. Heuser. 1985. Substructure of inner dynein arms, radial spokes, and the central pair/projection complex of cilia and flagella. *J. Cell Biol.* 100:2008–2018.

Haimo, L. T., B. R. Telzer, and J. L. Rosenbaum. 1979. Dynein binds to and crossbridges cytoplasmic microtubules. *Proc. Natl. Acad. Sci. USA.* 76: 5759–5763.

Hopkins, J. M. 1970. Subsidiary components of the flagella of *Chlamydomonas reinhardii. J. Cell Sci.* 7:823–839.

Huang, B. P.-H. 1986. *Chlamydomonas reinhardtii*: a model system for the genetic analysis of flagellar structure and motility. *Int. Rev. Cytol.* 99: 181–215.

Huang, B., Z. Ramanis, and D. J. L. Luck. 1982. Suppressor mutations in Chlamydomonas reveal a regulatory mechanism for flagella function. *Cell.* 28:115–124.

Johnson, K. A., and J. L. Rosenbaum. 1992. Polarity of flagellar assembly in *Chlamydomonas. J. Cell Biol.* 119:1605–1611.

Johnson, K. A., and J. L. Rosenbaum. 1993. Flagellar regeneration in *Chlamydomonas*: a model system for studying organelle assembly. *Trends Cell Biol.* 3:156–161.

Kindle, K. L. 1990. High-frequency nuclear transformation method for *Chlamydomonas reinhardtii. Proc. Natl. Acad. Sci. USA.* 87:1228–1232.

Kindle, K. L., R. A. Schnell, E. Fernandez, and P. A. Lefebvre. 1989. Stable nuclear transformation of *Chlamydomonas* using a gene for nitrate reductase. *J. Cell Biol.* 109:2589–2601.

King, S. M., C. G. Wilkerson, and G. B. Witman. 1991. The M_r 78,000 intermediate chain of *Chlamydomonas* outer arm dynein interacts with α-tubulin *in situ. J. Biol. Chem.* 266:8401–8407.

Kozminski, K. G., D. R. Diener, and J. L. Rosenbaum. 1993. High level expression of nonacetylatable α-tubulin in *Chlamydomonas reinhardtii. Cell Motil. Cytoskeleton.* 25:158–170.

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

Lefebvre, P. A., and J. L. Rosenbaum. 1986. Regulation of the synthesis and assembly of ciliary and flagellar proteins during regeneration. *Annu. Rev. Cell Biol.* 2:517–546.

L'Hernault, S. W., and J. L. Rosenbaum. 1983. *Chlamydomonas* α-tubulin is posttranslationally modified in the flagella during flagellar assembly. *J. Cell Biol.* 97:258–263.

Luck, D., G. Piperno, Z. Ramanis, and B. Huang. 1977. Flagellar mutants of *Chlamydomonas*: studies of radial spoke-defective strains by dikaryon and revertant analysis. *Proc. Natl. Acad. Sci. USA.* 74:3456–3460.

Mastronarde, D. N., E. T. O'Toole, K. L. McDonald, J. R. McIntosh, and M. E. Porter. 1992. Arrangement of inner dynein arms in wild-type and mutant flagella of *Chlamydomonas. J. Cell Biol.* 118:1145–1162.

Mitchell, D. R., and Y. Kang. 1991. Identification of *oda6* as a *Chlamydomonas* dynein mutant by rescue with the wild-type gene. *J. Cell Biol.* 113:835–842.

Pelham, H. R. B., and R. J. Jackson. 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. *Eur. J. Biochem.* 67:247–256.

Piperno, G., B. Huang, Z. Ramanis, and D. J. L. Luck. 1981. Radial spokes of *Chlamydomonas* flagella: polypeptide composition and phosphorylation of stalk components. *J. Cell Biol.* 88:73–79.

Piperno, G., K. Mead, and W. Shestak. 1992. The inner dynein arms I2 interact with a "dynein regulatory complex" in *Chlamydomonas* flagella. *J. Cell Biol.* 118:1455–1463.

Rosenbaum, J. L., and F. M. Child. 1967. Flagellar regeneration in protozoan flagellates. *J. Cell Biol.* 34:345–364.

Segal, R. A., and D. J. Luck. 1985. Phosphorylation in isolated *Chlamydomonas* axonemes: a phosphoprotein may mediate the Ca^{2+}-dependent photophobic response. *J. Cell Biol.* 101:1702–1712.

Silflow, C. D., R. L. Chisholm, T. W. Conner, and L. P. W. Ranum. 1985. The two alpha-tubulin genes of *Chlamydomonas reinhardi* code for slightly different proteins. *Mol. Cell. Biol.* 5:2389–2398.

Sloboda, R. D., and J. L. Rosenbaum. 1982. Purification and assay of microtubule-associated proteins (MAPs). *In* Methods in Enzymology. D. W. Frederiksen and L. W. Cunningham, editors. Academic Press, New York. 409–416.

Smith, E. F., and W. S. Sale. 1992*a*. Regulation of dynein-driven microtubule sliding by the radial spokes in flagella. *Science (Wash. DC).* 257:1557–1559.

Smith, E. F., and W. S. Sale. 1992*b*. Structural and functional reconstitution of inner dynein arms in *Chlamydomonas* flagellar axonemes. *J. Cell Biol.* 117:573–581.

Snell, W. J., W. L. Dentler, L. T. Haimo, L. I. Binder, and J. L. Rosenbaum. 1974. Assembly of chick brain tubulin onto isolated basal bodies of *Chlamydomonas reinhardi. Science (Wash. DC).* 185:357–360.

Warner, F. D. 1970. New observations on flagellar fine structure: the relationship between matrix structure and the microtubule component of the axoneme. *J. Cell Biol.* 47:159–182.

Warner, F. D., and P. Satir. 1974. The structural basis of ciliary bend formation: radial spoke positional changes accompanying microtubule sliding. *J. Cell Biol.* 63:35–63.

Williams, B. D., D. R. Mitchell, and J. L. Rosenbaum. 1986. Molecular cloning and expression of flagellar radial spoke and dynein genes of *Chlamydomonas. J. Cell Biol.* 103:1–11.

Williams, B. D., M. A. Velleca, A. M. Curry, and J. L. Rosenbaum. 1989. Molecular cloning and sequence analysis of the *Chlamydomonas* gene coding for radial spoke protein 3: flagellar mutation *pf14* is an ochre allele. *J. Cell Biol.* 109:235–245.

Witman, G. B. 1975. The site of *in vivo* assembly of flagellar microtubules. *Ann. NY Acad. Sci.* 253:178–191.

Witman, G. B. 1992. Axonemal dyneins. *Curr. Opin. Cell Biol.* 4:74–79.

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

Witman, G. B., R. Fay, and J. Plummer. 1976. *Chlamydomonas* mutants: evidence for the roles of specific axonemal components in flagellar movement. *In* Cell Motility, Book C. Microtubules and Related Proteins. R. Goldman, T. Pollard, and J. Rosenbaum, editors. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. 969–986.

Witman, G. B., J. Plummer, and G. Sander. 1978. *Chlamydomonas* flagellar mutants lacking radial spokes and central tubules. Structure, composition, and function of specific axonemal components. *J. Cell Biol.* 76:729–747.

Youngblom, J., J. A. Schloss, and C. D. Silflow. 1984. The two β-tubulin genes of *Chlamydomonas reinhardtii* code for identical proteins. *Mol. Cell. Biol.* 4:2686–2696.