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

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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, 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, 1992a).

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) (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 latter 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 pf4 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 KNO₃ replaced the NH₄NO₃.

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 tagged with 2% NP-40 in HMDEK (10 mM Hepes, pH 7.4, 5 mM MgSO₄, 1 mM DTT, 0.5 mM EGTA, 25 mM KCl) for 45 min on ice and the resulting axonemes were sedimented (10 min, 8000 g). Axonemal pellets were washed twice in HMDEK and were resuspended in HMDEK at 5 μg/μl. 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" (Strategene, La Jolla, CA) (Williams et al., 1989). 1. Abbreviations used in this paper: EDC, 1-Ethyl-3-(3-Dimethylaminopropyl)carbodiimide; RSP3, radial spoke protein 3.

RSP3 with carboxy-terminal truncations were synthesized from mRNAs transcribed in vitro after linearizing the RSP3 cDNA with PstI, SacI, or HindIII, which cut just upstream of the 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 Nhel, which cuts just 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 transformed, digested, 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 Kpsl/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 PstI 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 β-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 5' end of the primer, 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 (Strategene) 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 [35S]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 35S-labeled RSP3 with 5 μl of 5 μg/μl 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 labeling 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.

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Transformation of Chlamydomonas

To test the ability of various mutant RSP3 constructs to complement the pf4 mutation, minigene constructs containing mutagenized RSP3 cDNAs were introduced into Chlamydomonas using the glass bead method of transformation (Kindle, 1990). The double mutant pf4 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 (Kozmik and Pearl, 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 pf4 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 of 90-kD form were either mixed with the pf4 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 pf4 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 pf4 and used as a substrate for the binding of radioactive RSP3 synthesized in vitro.

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, lane 0.0, 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
been bound were treated with EDC, two forms of RSP3 were migrating at 150 kD (Fig. 2 a, lane 2). The inability of RSP3 to bind wild-type axonemes in vitro, as in vivo, RSP3 is bound specifically to a specific protein on the axoneme. Furthermore, the results suggest that in vitro, as in vivo, 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.

Cross-linking of RSP3 to pf4 Axonemes

The zero-length cross-linker EDC was used to further test the specificity of in vitro binding; if RSP3 bound to pf4 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 (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 pf4 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 is 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.

Incubation of RSP3 with Brain Microtubules

The above experiments indicate that RSP3 binds specifically to pf4 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 2. Cross-linking of RSP3 to wild-type and pf4 axonemes. (a) This immunoblot of wild-type axonemes probed with anti-RSP3 antisera 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 pf4 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 is 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.
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 pfl4 axonemes (Fig. 3, Δ1/41), 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 pfl4 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).

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 pfl4 axonemes. Each panel is cropped to show only the test protein.

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 pfl4 cells and transformants were assayed for motility. Previous work had shown that transformation of pfl4 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 pfl4 cells (Figs. 4–6). When transformation of the Chlamydomonas double mutant pfl4 nit1-305 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 pfl4 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 nit1+ transformants screened for motility, no motile transformants were obtained with the deleted minigene, whereas ~20% of the nit1+ 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 nit1+ 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...
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. * 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 nit1-305 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 nit1-305 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 nit1-305. 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 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. * 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.

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. 5f). When examined by EM, many sections showed few or no spokes (Fig. 6e); however, some sections were seen to have an apparently normal distribution of radial spokes (Fig. 6f). 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 nit1* 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 pfl4 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 pfl4 axonemes to which RSP3 has been bound in vitro. The fact that RSP3 can bind to pfl4 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 crosslinker EDC. When cross-linked with EDC, wild-type axonemes, as well as pfl4 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 pfl4**

The use of RSP3 cDNA minigenes to complement the paralyzed phenotype of pfl4 by transformation has enabled us to examine functional domains of RSP3 in vivo. Transformation of pfl4 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 pfl4. 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 pfl4 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 pfl4 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 pfl4 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 pfl4 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 pfl4 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 flagellum. 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.

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