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Subunit interactions within the *Chlamydomonas* flagellar spokehead

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Abstract

The radial spoke (RS)/central pair (CP) system in cilia and flagella plays an essential role in the regulation of force generation by dynein, the motor protein that drives cilia/flagella movements. Mechanical and mechanochemical interactions between the CP and the distal part of the RS, the spokehead, should be crucial for this control; however, the details of interaction are totally unknown. As an initial step toward an understanding of the RS-CP interaction, we examined the protein-protein interactions between the five spokehead proteins (radial spoke protein (RSP)1, RSP4, RSP6, RSP9, and RSP10) and three spoke stalk proteins (RSP2, RSP5 and RSP23), all expressed as recombinant proteins. Three of them were shown to have physiological activities by electroporation-mediated protein delivery into mutants deficient in the respective proteins. Glutathione S-transferase pulldown assays in vitro detected interactions in 10 out of 64 pairs of recombinants. In addition, chemical crosslinking of axonemes using five reagents detected seven kinds of interactions between the RS subunits in situ. Finally, in the mixture of the recombinant spokehead subunits, RSP1, RSP4, RSP6, and RSP9 formed a 7-10S complex as detected by sucrose density gradient centrifugation. It may represent a partial assembly of the spokehead. From these results, we propose a model of interactions taking place between the spokehead subunits.

Keywords

cilia and flagella; radial spokes; pulldown assays; chemical crosslink; protein electroporation

Introduction

One of the crucial problems in the study of cilia and flagella motility is how dynein-driven microtubule sliding is controlled. Genetic and biochemical analyses have indicated that the radial spoke (RS) and central pair (CP) are important for this control. The RS is a T-shaped complex anchored to the outer doublet microtubule, consisting of a thin stalk and a bulbous head, the spokehead. The spokehead is the site where the RS-CP interaction takes place [Goodenough and Heuser, 1985; Yang et al., 2001; Warner and Satir, 1974]. In *Chlamydomonas* mutants that fail to assemble the RS or the CP, flagella are paralyzed and the apparent motor activity of inner arm dynein is reduced [Witman et al., 1978; Smith and

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Sale, 1992; Smith, 2002]. Though it is not completely clear how the CP and RS control the activity of dynein, several lines of evidence suggest that one of the mechanisms is through the modulation of phosphorylation state of an inner arm dynein subunit [Howard et al., 1994; Habermacher and Sale, 1996; King and Dutcher, 1997; Habermacher and Sale, 1997; Yang and Sale, 2000; Smith, 2002]. The RS and CP may well sense the flagellar mechanical state, and relay this information to the outer-doublets and dynein arms [Warner and Satir, 1974; Lindemann, 2003; Smith et al., 2004]. In *Chlamydomonas* flagella and *Paramecium* cilia, the CP rotates within the nine doublet microtubules [Omoto and Kung, 1979; Kamiya et al., 1982; Omoto et al., 1999; Mitchell and Nakatsugawa, 2004]. As the CP rotates, the RS interacting with particular projections of the CP sequentially changes, which may result in a successive change in the location of active dyneins on the nine doublet microtubules [Omoto et al., 1999; Wargo and Smith, 2003]. The CP-RS interaction also must be important for the control of dynein activity in the cilia and flagella of multicellular organisms [Yoshimura and Shingyoji, 1999], in which the CP does not rotate [Tamm and Tamm, 1981]. Human patients deficient in the spokehead have been identified and shown to suffer from primary ciliary dyskinesia (nonmotile cilia syndrome) (Castleman et al., 2009).

In the RS/CP signal transduction system, the distal end of the RS, the spokehead, is thought to interact with one of the several projections of CP to transmit the signal to the outer doublet microtubules. However, neither the nature of interactions between the spokehead and the CP nor the manner of subunit assembly in the spokehead is known. In *Chlamydomonas*, the RS is composed of at least 23 proteins (termed radial spoke proteins; RSPs) [Piperno et al., 1981; Yang et al., 2006] of which five proteins, RSP1, RSP4, RSP6, RSP9, and RSP10, have been thought to constitute the spokehead [Piperno et al., 1981, Huang et al., 1981]. Axonemes of *pf1*, a mutant lacking the spokehead, specifically lack these five proteins. Extraction of wild-type axonemes under low ionic conditions releases the five proteins and a stalk component, RSP5 [Piperno et al., 1981]. Electron microscopy has revealed that the extracted axonemes retain radial spokes that lack the spokeheads. From this solubilization pattern, RSP5 has been thought to constitute the link between the spokehead and the spoke stalk [Piperno et al., 1981]. However, in the axoneme of *pf24*, a mutant deficient in a stalk subunit, RSP2, the amounts of all five spokehead subunits and three stalk proteins, RSP2, RSP16, and RSP23 are reduced while the amount of RSP5 remains unchanged [Huang et al., 1981; Patel-King et al., 2004]. Thus, the protein subunits mediating the spokehead-stalk attachment remain to be determined.

Although the genes of all RS proteins contained in the spokehead or in the distal portion of the stalk have been cloned and sequenced [Curry et al., 1992; Patel-King et al., 2004; Yang et al., 2004; Yang et al., 2005; Yang et al., 2006], how these proteins interact with each other is unknown. In this study, to help understand the subunit organization of the spokehead, we investigated the protein-protein interactions between the spokehead subunits and also between spokehead and stalk subunits that may be localized near the spokehead. Toward this end, we expressed all five spokehead proteins and three stalk proteins as recombinant proteins and examined pairwise interactions by pulldown assays. The physiological activities of the RSP4, RSP6 and RSP9 recombinant proteins were checked using electroporation-mediated protein delivery into mutants that lack them. In addition, we performed chemical crosslinking of axonemes and subsequent immunoblot assays using various antibodies specific to radial spoke proteins. These assays detected various protein-protein interactions, allowing us to draw a rough topological map of the subunit arrangement within the spokehead and the distal region of the spoke stalk.

Materials and Methods

Chlamydomonas Strains and Culture

Chlamydomonas reinhardtii wild type (137c), and spokehead-deficient mutants, *pf1*, *pf17*, and *pf26* were used. The mutants *pf1* and *pf17* are deficient in RSP4 and RSP9, respectively, and both lack the entire spokehead [Huang et al., 1981]. They are non-motile. The mutant *pf26* has a temperature-sensitive mutation in RSP6; cells are motile when grown at permissive temperature (25°C), but most cells become non-motile when grown at restrictive temperature (32°C). At both temperatures, the *pf26* axoneme retains the morphologically normal radial spokehead and stalks [Huang et al., 1981]. In addition, a recombinant *Chlamydomonas* strain, *pf1R-HA*, which expresses HA-tagged RSP4, was constructed as follows: *pf1* was transformed with a plasmid that included the 4.4 kb EcoRI/SalI fragment containing the RSP4 gene (Curry et al., 1992) using the glass bead method [Kindle, 1990]. The RSP4 gene was modified to encode an HA epitope [Field et al., 1988] 16 amino acids from C-terminus. Motile transformants were shown to express the tagged gene on immunoblots using an anti-HA antibody. These cells were cultured in liquid Tris-Acetate-Phosphate (TAP) medium [Gorman and Levine, 1965] with aeration on a 12h/12h light/dark cycle.

Expression and Purification of His-tagged Recombinant RS subunits

Recombinant RS subunits, except RSP6, were expressed in *E. coli* carrying each coding sequence in an expression vector, pProExHTa (Invitrogen). Because RSP6 was not expressed efficiently in *E. coli*, it was expressed in insect culture cells using a baculovirus expression system. Each recombinant protein contained a 6×His tag sequence at its N-terminal. Expression of the fusion proteins was induced by addition of isopropyl-β-D-thiogalactopyranoside to a logarithmically growing culture of *E. coli* (DH5α or BL21 strain) to a final concentration of 0.5-1 mM, and the cultures were grown for additional 2-5 h at 30°C or 37°C. The cells were incubated in Buffer A (50 mM sodium phosphate, 300 mM NaCl, pH 8.0) supplemented with 2 mg/ml lysozyme on ice for 1 h, lysed by sonication, and centrifuged at 400,000 × g at 4°C for 30 min. Ni-NTA agarose (QIAGEN) was added to the resulting supernatants and gently mixed at 4°C for 30 min. After the Ni-NTA agarose was washed with Buffer A containing 20 mM imidazole, the recombinant proteins were eluted with Buffer A containing 250 mM imidazole.

Expression and Purification of His-tagged Recombinant RSP6

The coding sequence of RSP6 was amplified by PCR from a full-length cDNA clone of RSP6 using the primers 5'-
GCGTATACAATATG**CACCACCACCACCACGCCGCGGA** TGTGGGCCAGGCT
and 5'-GCTCTAGACTACTCGTCCTCCTCCTCCG. These primers introduce a Bst1107I site (GTATAC, underlined) at the 5' end and an XbaI site (TCTAGA, underlined) at the 3' end of the coding sequence, and a 6×His-tag sequence (italicized) after the start codon ATG (boldfaced) of the RSP6 coding sequence. This modified coding sequence of RSP6 was inserted between the XbaI and Bst1107I sites of the transfer vector p2Bac (Invitrogen). Recombinant baculovirus was obtained using the BaculoGold transfection kit (PharMingen) following the manufacturer's protocol (Invitrogen). Recombinant protein was obtained from Sf21 insect culture cells, and purified using Ni-NTA agarose following the same above-mentioned protein purification protocol.

Expression and Purification of Glutathione S-Transferase (GST)-tagged Recombinant RS subunits

The recombinant RS subunits were each expressed in *E. coli* carrying their respective coding sequences in the expression vector pGEX-BS (pGEX-BS was made by replacing the multiple cloning site (MCS) of pGEX-6P-2 between the *Bam*HI and *Sal*I recognition sites with a similar fragment from the MCS of pBluescriptII). The resulting fusion proteins contained a GST tag sequence at their N-terminus. Expression of the fusion proteins GST-RSP and intact GST protein encoded by the blank vector were induced by addition of isopropyl- β -D-thiogalactopyranoside to a logarithmically growing culture of *E. coli* (BL21 or DH5 α strain) to a final concentration of 1 mM, and the cultures were grown for additional 2-5 h at 30°C. The cells were suspended in Buffer B (50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 8.0) with 2 mg/ml lysozyme, incubated on ice for 1 h, lysed by sonication, and centrifuged at 400,000 $\times$ g for 30 min at 4°C. Glutathione Sepharose (GE Healthcare Bio-Science AB) was added to the resulting supernatants, and gently mixed on a rotator at 4°C for 30 min. After the Glutathione Sepharose was washed with Buffer B, the recombinant proteins were eluted with Buffer B containing 16 mM glutathione.

Antibodies

Antibodies against RSP1, RSP9 and RSP10 were raised using recombinant His-tagged proteins. For each protein, two rabbits were immunized, and antisera were obtained using standard procedures. These antibodies were blot-purified using the respective recombinant RSPs on PVDF membranes. The purified antibodies detected single proteins in western blot analysis of axonemes (Fig. 3). Polyclonal antibody against RSP23 was raised by injecting rats with bacterially expressed polypeptides. To avoid cross-reaction with an axonemal protein NDK7 [Patel-King et al., 2004], which has a similar domain to the N-terminal half of RSP23, a cDNA fragment corresponding to the C-terminal 237 amino acid sequence of RSP23 (composed of 586 amino acids) was amplified by PCR using an EST clone AV387709 (obtained from Kazusa DNA Research Institute, Japan) as a template. Resulting cDNA was cloned in a vector pMal-c2x (New England Biolabs) and expressed as a maltose-binding-protein (MBP) fusion protein in *E. coli*. MBP-RSP23C was purified following the manufacturer's recommendations. The rabbit anti-RSP4/6 and anti-RSP2 sera, raised against the axonemal proteins purified from 2D gels, were previously described [Williams et al., 1986; Yang et al., 2001]. Anti penta-His antibody was purchased from QIAGEN. Anti-HA tag antibody (12CA5) was from Roche.

Immunoblot

Immunoblot procedures were modified from those of Towbin et al. (1979). Proteins were transferred to PVDF membranes (Millipore Corp., Bedford, MA), and probed with primary antibodies. Immunoreactive bands were detected using horseradish peroxidase-conjugated second antibody and an ECL Advance western blotting detection kit (GE Healthcare). For chemical crosslinking experiments, blotted membranes were used repeatedly after the bound antibodies were removed with a stripping buffer (100 mM β mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.5)[Kaufmann et al., 1987].

Protein Electroporation

Electroporation for introduction of recombinant proteins was carried out after Hayashi et al. [2002]. Briefly, mutant cells from which cell walls had been removed by autolysin treatment were kept in an electroporation cuvette, recombinant protein was added to the final concentration of 0.3-0.5 mg/ml, and an electric impulse was applied with an ECM600 apparatus (BTX). Motility recovery (%) was defined as the ratio of the number of motile cells to the total number of control cells. Since a small fraction of *pf26* cells were motile

even without electroporation, motility recovery (%) of *pf26* was corrected by subtracting the number of motile control cells from the number of motile cells following electroporation. Average swimming velocities and standard errors were measured by tracing tracks of about 30 swimming cells recorded on videotapes.

GST pulldown assay

The expressed and purified recombinant proteins were dialyzed against 1000-2000 volumes of Buffer C (50 mM Tris-HCl, 5 mM MgCl₂, 100 mM NaCl, 10% glycerol, 1 mM DTT, pH 7.5) at 4°C. GST-fusion proteins or intact GST (2 µM) were incubated with 10 µl glutathione beads and 10 µM BSA for 30 min at 4°C. After being washed with buffer C containing 10 µM BSA, the beads were incubated with His-tagged proteins (2 µM) and 10 µM BSA for 2 h at 4°C. The beads were then washed with buffer C three times, incubated with buffer C containing 16 mM glutathione for 30 min at 4°C, and spun down. The supernatants were collected and subjected to SDS-PAGE. The protein bands were electroblotted onto PVDF filters, and reacted with anti-His-tag antibodies. Immunoreactive bands were detected with a chemiluminescence detection system (GE Healthcare).

Chemical crosslinking

Flagellar axonemes were isolated by the method of Witman et al. [1978]. As crosslinkers, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), disuccinimidyl suberate (DSS), dimethyl pimelimidate 2 hydrochloride (DMP), m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), and bismaleimidohexane (BMH) (all are from Pierce Chemical, Rockford, IL), were used. Axonemes were suspended in HME buffer (30 mM HEPES, 5mM MgSO₄, 1mM EGTA, pH 7.4) at a final concentration of 2 mg/ml, and treated with the crosslinkers for 0.5-1 h at room temperature [King et al., 1991]. Reactions were terminated by the addition of SDS-PAGE sample solution containing 2-mercaptoethanol and Tris. The resultant samples were analyzed by immunoblotting.

Sedimentation assays of recombinant protein mixtures

The five His-tagged recombinant spokehead subunits were mixed together and incubated for 6 h at 4°C, and the sample was examined using sucrose-density centrifugation for the formation of multi-subunit assemblies. The sample was loaded on a 5-20% sucrose gradient made in 3 ml of Buffer C, centrifuged at 44,000 rpm for 5 h 30 min at 4°C and fractionated. Sedimentation coefficients were estimated using ribonuclease A (~2 S), BSA (~4 S), aldolase (~7 S), catalase (~11 S) and three-headed outer arm dynein in the axonemal extract (~23 S) (Takada et al., 1992), as standards. For comparison with the native structure, spokeheads were extracted from wild-type axonemes under low ionic strength conditions (0.2 mM EDTA, 0.1% β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride and 5 mM Tris/HCl, pH 8.3) [Piperno, 1981]. The isolated radial spokeheads, as well as the mixture of His-tagged recombinant radial spoke proteins, were dialyzed against 1000-2000 volumes of Buffer C before centrifugation.

Results

Expression of functional spokehead proteins

To detect interactions between spokehead proteins by pulldown assays, we expressed all of the five spokehead subunits (RSP1, RSP4, RSP6, RSP9, and RSP10) and three spoke stalk proteins, RSP2, RSP5 and RSP23, that may participate in the spokehead-stalk interaction [Piperno et al., 1981; Huang et al., 1981] as recombinant proteins with a His- or a GST-tag at the N-terminus of each protein. We did not produce recombinant RSP16 because, although important for flagellar motility, it is dispensable for spokehead-stalk attachment

[Yang et al., 2008]. These recombinant proteins were obtained in a soluble state. After purification with Ni-NTA agarose beads or glutathione beads, most RSPs were obtained as major components as examined by SDS-PAGE and Coomassie Brilliant Blue staining (Fig. 1). Recombinant RSP10 with either tag displayed two closely separated bands. The reason for the appearance of two bands is not understood as the native RSP10 in isolated RS displayed only a single band, at a position close to that of the lower band of the recombinant. Some samples appeared to have undergone significant degradation during the process of protein solubilization from bacteria. However, we used these proteins without further purification.

Rescue of spokehead-deficient mutants by electroporation-mediated protein delivery

Three recombinant RSPs were assayed for functional activity using electroporation-mediated protein delivery. Currently three mutants that are deficient in spokehead subunits are available: *pf1*, which lacks RSP4; *pf17*, which lacks RSP9; and *pf26*, which has temperature-sensitive defects in RSP6. Of these, *pf1* and *pf17* are always non-motile, while *pf26* is motile when grown at 25°C but non-motile when grown at 32°C. We subjected these cells to electroporation in the presence of recombinant His-tagged RSP4, RSP6, and RSP9. The mutant *pf26* was grown at 32°C. After electroporation, 7.1%, 11.8%, and 10.9% of the originally non-motile cells recovered motility in *pf1*, *pf17* and *pf26*, respectively (Supporting Information Fig. S1). These recovery rates are similar to those observed in previous protein electroporation experiments using subunits of inner arm dyneins [Hayashi et al., 2001; 2002]. The motility in the rescued cells was almost the same as in the wild type. For example, the swimming velocity of the rescued *pf17* cells at 25°C was $160.9 \pm 22.2 \mu\text{m}\cdot\text{s}^{-1}$, close to the velocity of wild type cells ($187.2 \pm 18.2 \mu\text{m}\cdot\text{s}^{-1}$). Therefore, at least these three recombinant proteins were functional in vivo.

GST-pulldown assays

We used GST-pulldown assays to detect interactions between eight recombinant RS proteins (five spoke head proteins RSP1, RSP4, RSP6, RSP9, and RSP10; and three spoke stalk proteins, RPS2, RSP5, and RSP23). The GST-tagged RSPs were coupled to glutathione beads, mixed with the His-tagged RSPs in all possible combinations, incubated at 4°C for 2 h, and centrifuged. As a negative control, we also incubated His-tagged proteins with GST protein conjugated with glutathione beads.

Co-precipitation was detected between various combinations of RSPs (Fig. 2). His-RSP4 co-precipitated with GST-RSP1 and, conversely, His-RSP1 with GST-RSP4. Similarly, His-RSP4 co-precipitated with GST-RSP9 and, conversely, His-RSP9 with GST-RSP4. In addition, His-RSP4 co-precipitated with GST-RSP10. However, in this case, co-precipitation was not observed in the reverse combination. We surmise that the GST-tag attached to the RSP4 sequence might have interfered with its interaction with RSP10.

Like RSP4, RSP6 was found to interact with RSP9 and RSP10, although GST-tagged RSP6 and His-tagged RSP9 did not interact. In this case also, we speculate that the GST tag attached to RSP6 hindered the interaction with His-RSP9. The overall similarity in the interaction pattern of RSP4 and RSP6 was as expected since these proteins are paralogs (67% similarity) (Curry et al., 1992). However, unlike RSP4, RSP6 did not show interaction with RSP1. These results suggest that protein interactions in RSP4 and RSP6 are not symmetrical.

In the three stalk subunits examined, we found that RSP2 interacts with RSP23. This observation is consistent with a previous report that indicated a functional interaction between these proteins [Patel-King et al., 2004] (see Discussion).

His-RSP5 was found to co-precipitate with all of the GST-RSP fusion proteins. Apparently this was due to its non-specific association with GST; in fact, it also co-precipitated with GST alone. In contrast, GST-RSP5 did not coprecipitate with any of the His-tagged RSPs. Thus, although RSP5 has been proposed to localize at the spokehead-stalk boundary [Piperno et al., 1981], our pulldown assays did not detect interactions between RSP5 and other recombinant RSPs. Nor did they detect any other interactions between spokehead subunits and stalk subunits.

RS subunits interactions detected by chemical crosslinking

For assays using chemical crosslinking of spokehead proteins in situ, wild-type axonemes were treated with five kinds of crosslinkers, EDC, DSS, DMP, MBS, and BMH, and the resultant crosslinked products were probed on immunoblots using antibodies against RSP1, RSP2, RSP4/RSP6, RSP5, RSP9, RSP10, and RSP23. In a series of analyses, we used the same membrane repeatedly, after removing the bound antibody [Kaufmann et al., 1987]. When a band of the same electrophoretic mobility was detected with antibodies against two different RSPs, and its apparent molecular weight was close to the sum of the molecular weights of the two respective RSPs, the band was regarded as representing their crosslinked product. In such a case, the two proteins are likely to be interacting with each other.

Such crosslinking experiments detected several inter-RSP interactions (Fig. 3). RSP10 is likely to interact with both RSP4 and RSP6, as detected by crosslinking with BMH (Fig. 3) or MBS (not shown). Although the antibody does not distinguish between RSP4 and RSP6, the crosslinked sample showed two closely separated bands; judging from the molecular weight difference, the upper band is likely to represent the RSP4-RSP10 product and the lower band the RSP6-RSP10 product. Interactions between these proteins were also detected by the pulldown assays (Fig. 2). Some crosslinked samples probed with the anti-RSP4/6 antibody (Fig. 3) or with anti-HA antibody (see below; Supporting Information Fig. S2) showed bands at 130-140 kD and ~250 kD. They may reflect some homomeric or heteromeric interactions between RSP4 and RSP6, although these proteins did not interact with each other in pulldown assays (Fig. 2). No other subunit interactions within the spokehead were detected by crosslink experiments.

Chemical crosslinking detected possible interactions between the spokehead and stalk proteins (Fig. 3). First, a crosslinked product between RSP1 and RSP23 was detected in samples treated with BMH (Fig. 3) or MBS (not shown). Second, a product between RSP2 and RSP10 was detected in samples treated with MBS. Third, a band that might represent RSP2-RSP4 or RSP2-RSP6 was detected in DSS-treated axonemes using the anti-RSP4/6 antibody. In a separate experiment, we crosslinked the axonemes from a recombinant strain that expresses an HA-tagged RSP4. A band that apparently corresponded to the crosslinked product RSP2-RSP4-HA was detected by both anti-RSP2 antibody and anti-HA antibody (Supporting Information Fig. 2S). Hence the band detected by both anti-RSP2 antibody and anti-RSP4/6 antibody is likely to represent a product between RSP2 and RSP4. Crosslinking with EDC yielded a 310 kD product that was detected by anti RSP1 antibody (not shown); however, we were unable to identify the crosslinked counterpart. Apart from this band, EDC or DMP did not yield clear crosslinked products detectable with any antibodies used. When the axonemes of *pfl*, a spokehead-less mutant, was examined in place of wild type axonemes, none of the bands that we assigned as crosslinked products appeared (data not shown). Thus, the inter-RSP interactions we detected must be specific to the spokehead proteins.

Chemical crosslinking of His-tagged RSP10 yielded a product with nearly twice the molecular weight of the 6×His-RSP10 doublet bands (Supporting Information Fig. S3), suggesting that recombinant RSP10 forms dimers in solution. In support of the formation of

dimers, the bands appeared even when the sample concentration was reduced 10 fold (data not shown). Spokehead RSPs other than RSP10 did not show such higher molecular bands when treated with the same concentrations of crosslinkers (data not shown).

Protein interactions in an RSP mixture

The multiple recombinant RSPs we used in the above experiments might well interact with each other in vitro, and produce a multi-component structure. As a first step toward the in vitro reconstruction of the spokehead, we mixed the five His-tagged recombinant spokehead subunits, RSP1, RSP4, RSP6, RSP9, and RSP10, and the recombinant RSP5, which has been proposed to interact with the spokehead [Piperno, et al., 1981]. The mixture was incubated for 6 h on ice and then assayed for association products by centrifugation on a 5-20% sucrose density gradient. Individually, each spokehead protein and RSP5 was found to sediment at 3-6 S. In the mixture of all proteins, four subunits, RSP1, RSP4, RSP6, and RSP9, co-sedimented as an assembly of 7-10 S, while the sedimentation velocities of RSP5 and RSP10 were similar to, or only slightly higher than, those observed when sedimented singly (Fig. 4). These results suggest that RSP1, RSP4, RSP6, and RSP9 associate in vitro to form a partial structure of the spokehead.

To compare these results with the sedimentation pattern of the native spokehead, we examined the spokehead released from the wild type axonemes under low ionic conditions [Piperno et al., 1981]. The released spokehead proteins were found to co-sediment in two fractions: a major fraction sedimented at 7-10 S and a minor fraction sedimented at ~23 S. In this case, all of the five spokehead proteins, including RSP10, were present in both peaks. It is interesting that both the mixture of the recombinant RSPs and the extracted spokehead sample yielded similar 7-10 S products, although there are some minor differences in compositions.

Discussion

In this study we carried out the first biochemical analysis of protein-protein interactions between the five spokehead subunits, RSP1, RSP4, RSP6, RSP9 and RSP10, which were identified as the spokehead components nearly three decades ago [Piperno et al., 1981]. For these experiments, we produced recombinant proteins of all subunits and a few stalk proteins, and raised specific antibodies for some of the subunits. Protein electroporation assured that at least three of the spokehead proteins retained physiological function.

GST-pulldown assays and chemical crosslinking experiments detected discrete interactions between several proteins. The results are summarized in Fig. 5. Pulldown assays indicated that both RSP4 and RSP6 interact with RSP9 and RSP10, while no direct interactions were detected between RSP4 and RSP6 or between RSP9 and RSP10. In addition, RSP4, but not RSP6, appeared to interact with RSP1.

The RSP4-RSP10 and RSP6-RSP10 interactions were also suggested by chemical crosslinking of axonemes. Although chemical crosslinking did not detect interactions in other spokehead subunits, we surmise that this failure may be due to the absence of reactive residues in those protein pairs, or because of the inaccessibility of chemical crosslinkers to the binding site in intact spokeheads.

Spokehead-stalk interactions

GST-pulldown assays using three spoke proteins, RSP2, RSP5, and RSP23, in addition to the spokehead subunits, detected interaction only between RSP2 and RSP23. RSP2 has a GAF domain consisting of a cyclic GMP binding domain, an adenylyl cyclase domain, and an FhlA domain [Yang et al., 2004], while RSP23 has a nucleotide diphosphate kinase

(NDK) domain [Patel-King et al., 2004]. These domain structures led Patel-King et al. [2004] to suggest that RSP2 functionally interacts with RSP23. It is therefore interesting that our experiments detected a physical interaction between these proteins.

Chemical crosslinking experiments detected possible interactions between spokehead proteins and these stalk proteins: between RSP23 and RSP1, between RSP2 and RSP10, and between RSP2 and RSP4 (Fig. 3, Supporting Information Fig. S2). Because these interactions have not been detected in pulldown assays, we surmised that the interactions may be weak when the respective proteins are present by themselves; in other words, the close association detected by chemical crosslinking is realized only when these proteins are incorporated in higher-order structures. Nevertheless, the results of our chemical crosslinking experiments are consistent with the finding that the mutant *pf24*, a mutant deficient in RSP2, has greatly reduced amounts of spokehead subunits and RSP23 [Huang et al., 1981; Patel-King et al., 2004]. Although RSP5 is extracted from the axoneme under low-ionic strength conditions together with spokehead proteins [Piperno et al., 1981], our current studies using GST pulldown, chemical crosslink, or sedimentation (see below) did not detect interactions of RSP5 with any spokehead protein, RSP2 or RSP23. RSP5 may be a protein that is only weakly associated with the spokehead or the stalk, not necessarily localizing to the spokehead-stalk boundary.

Finally, chemical crosslinking of recombinant RSP10 suggested that it can form homodimers (Supplemental information Fig. S3). This is interesting in view of the two-fold symmetrical structure proposed for the RS stalk [Yang et al., 2006], which has been supported by the dimerization of RSP16 [Yang et al., 2005], RSP22 [Benashski et al., 1997], and RSP3 [Wirschell et al., 2008]. If the spoke stalk has a two-fold structure, it would be natural to assume that two spokehead complexes are attached to a single, dimeric spoke stalk. In fact, the overall structure of the RS, including the spokehead, appears to be a symmetrical T-shaped structure [e.g., Nicastro et al., 2006]. The dimeric nature of the spoke is also suggested by the asymmetric morphology of a “7-shaped” RS precursor, which contains all of the spoke head proteins and a subset of the stalk proteins; these precursors may dimerize to form the symmetric spoke (Diener et al., 2011). Our observation raises the possibility that RSP10 in the spokehead may also participate in stabilizing the dimeric spoke structure. However, it is also possible that the five-subunit assembly, as illustrated in Fig. 5, assumes a nearly symmetrical structure that can bind with the end of a dimeric stalk. Whether the spokehead has a true two-fold symmetry, stabilized by the dimerization of RSP10, should be an important issue for future studies.

Recombinant spokehead subunits assemble into a partial spokehead complex

In a mixture of recombinant spokehead proteins and RSP5, four proteins (RSP1, RSP4, RSP6, and RSP9) appeared to form a 7S-10S complex in vitro (Fig. 4A). This protein complex might correspond to a partial assembly of the spokehead; the sedimentation constant of this complex is similar to that of the lighter peak in dissociated native spokeheads (Fig. 4B). RSP5 and RSP10 are not associated with this complex. Since, in GST-pulldown assays, recombinant RSP10 displayed strong interaction with RSP6 and also with RSP4 to a lesser extent (Fig. 2), the exclusion of RSP10 from the complex is not understood. One possibility is that the assembly of the complete spokehead structure requires sequential addition of RSPs. For example, we may need to first mix RSP10, RSP4 and RSP6, and then add RSP1 and RSP9. Another possibility is that the incorporation of RSP10 into the spokehead requires proteins other than the spokehead RSPs such as a molecular chaperone, or spoke stalk proteins. Still another possibility is that the RSP10 recombinant we used was not fully functional. This is possible since the recombinant RSP10 displayed two separate bands in SDS-PAGE unlike native RSP10. Validation of the physiological activity of the recombinant RSP10 will be necessary to evaluate the result of

the sedimentation experiment. After verification of the activities of recombinant RSPs, more systematic experiments will be necessary to complete in vitro reconstruction of the spokehead. Once we succeed in reconstruction of the spokehead from recombinant proteins, we will be able to determine its structure by crystallography and obtain clues to its interaction with the central pair.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Recombinant spokehead proteins used in this study. SDS-PAGE stained with Coomassie Brilliant Blue. Recombinant proteins tagged with 6xHis or GST (arrowheads) were expressed in bacteria or in insect culture cells (RSP6) and partially purified with Ni-NTA beads or glutathione beads.

Fig. 2.

Subunit interactions detected by GST-pulldown assays. Each GST-tagged RSP indicated on the upper side and GST alone (control) were incubated with glutathione beads at 4°C for 30 min. After being washed with a buffer, the glutathione beads/GST-tagged proteins were mixed with each His-tagged RSP indicated on the left side and incubated at 4°C for 2 h. The beads were washed and eluted, and the binding was detected using anti-His tag antibody, as described in Materials and Methods. Bands that were judged positive are marked with circles.

Fig. 3.

Possible subunit interactions as detected by chemical crosslinking. Wild-type axonemes were treated with chemical crosslinkers indicated and run on SDS-PAGE. The proteins were then transferred to PVDF membranes and analyzed on immunoblots using various anti-RSP antibodies. Asterisks denote the bands of the monomeric proteins recognized by the probing antibody and arrows point to the bands of crosslinked products. Crosslinking produced several bands appearing at lower positions than the original RSP bands. Most of these bands probably represent RSPs that are internally crosslinked and therefore show higher mobilities. These include the band below the RSP6 band in the anti RSP4/6 BMH lanes, the band below the RSP1 band in the anti RSP1 BMH, and the band below the RSP23 band in the anti RSP23 BMH lanes. Unidentified bands appearing at higher positions than the uncrosslinked RSP bands possibly represent crosslinked products of the protein detected by the anti-RSP antibody and another protein that was undetectable with our battery of antibodies. Bands appearing at ~130 k and 250 k in some lanes detected with anti-RSP4/6 antibody might reflect homomeric or heteromeric interactions between RSP4 and RSP6; however, the identity of those extra bands remains to be determined.

Fig. 4.

Sedimentation analysis of the recombinant RSPs.

A. Sedimentation patterns of each recombinant RSP sample alone (upper panel) and in a mixture of RSP1, RSP4, RSP5, RSP6, RSP9 and RSP10 (lower panel). Samples were centrifuged on a 5-20% sucrose density gradient. RSP1, RSP4, RSP6 and a fraction of RSP9 (arrowheads) sedimented at 7-10 S in the mixture (asterisk), while each RSP sedimented slower when individually centrifuged. RSP5 and RSP10 apparently did not co-sediment with other RSPs, although the apparent sedimentation coefficient of RSP5 was slightly increased in the mixture. This may be due to some non-specific interactions with other proteins. B. The sedimentation pattern of the spokehead fraction released from the axoneme under low ionic-strength conditions. Sucrose density gradient was made in 50 mM Tris-HCl, 5 mM

MgCl₂, 100 mM NaCl, 10% glycerol, 1 mM DTT, pH 7.5 in all experiments. All fractions were analyzed by SDS-PAGE (10% acrylamide) and stained with silver.

Fig. 5.

Summary of the interactions detected in the present study.

Arrows indicate a pair of recombinant RSPs that co-sedimented when glutathione beads were added in the mixture. RSP(A) → RSP(B) indicates that the His-tagged RSP(A) showed interaction with the GST-tagged RSP(B). Solid lines indicate pairs of RSPs that apparently became crosslinked when treated with one or more crosslinker. A single radial spoke may be constructed by the dimerization of two sets of assemblies (see text).