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Radial spoke proteins of *Chlamydomonas* flagella

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Summary

The radial spoke is a ubiquitous component of '9+2' cilia and flagella, and plays an essential role in the control of dynein arm activity by relaying signals from the central pair of microtubules to the arms. The *Chlamydomonas reinhardtii* radial spoke contains at least 23 proteins, only 8 of which have been characterized at the molecular level. Here, we use mass spectrometry to identify 10 additional radial spoke proteins. Many of the newly identified proteins in the spoke stalk are predicted to contain domains associated with signal transduction, including Ca^{2+}-, AKAP- and nucleotide-binding domains. This suggests that the spoke stalk is both a scaffold for signaling molecules and itself a transducer of signals. Moreover, in addition to the recently described HSP40 family member, a second spoke stalk protein is predicted to be a molecular chaperone, implying that there is a sophisticated mechanism for the assembly of this large complex. Among the 18 spoke proteins identified to date, at least 12 have apparent homologs in humans, indicating that the radial spoke has been conserved throughout evolution. The human genes encoding these proteins are candidates for causing primary ciliary dyskinesia, a severe inherited disease involving missing or defective axonemal structures, including the radial spokes.

Keywords

Axoneme; Chaperones; Calcium; Primary ciliary dyskinesia

Introduction

The radial spoke is a structurally conserved macromolecular complex that is an essential and ubiquitous component of the '9+2' axoneme of motile cilia and flagella. This T-shaped complex repeats in pairs or triplets, depending on the organism, every 96 nm along each outer

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doublet microtubule. It consists of a thin 'stalk' that is anchored to the doublet microtubule adjacent to the inner dynein arms and projects towards the center of the axoneme, where it terminates in a bulbous 'head' that interacts with the projections of the central pair of microtubules (e.g. Goodenough and Heuser, 1985;Warner and Satir, 1974;Witman et al., 1978). Thus, the spoke is perfectly positioned to relay signals from the central pair of microtubules to the dynein arms.

The importance of the radial spoke in ciliary and flagellar motility is highlighted by the phenotypes of human (Sturgess et al., 1979) and *Chlamydomonas reinhardtii* (Witman et al., 1978;Huang et al., 1981) mutants lacking the entire complex or all or part of the spoke head; in these mutants, the cilia and flagella are paralyzed or display abnormal motility. Ultrastructural studies in conjunction with genetic and motility studies of *Chlamydomonas* mutants have provided evidence that the radial spoke transmits signals from the central pair of microtubules to the dynein arms through mechanical and/or mechanochemical interactions (Warner and Satir, 1974;Witman et al., 1978;Huang et al., 1981;Huang et al., 1982;Brokaw et al., 1982;Kamiya, 1982;Goodenough and Heuser, 1985;Omoto et al., 1999;Mitchell and Nakatsugawa, 2004;Smith and Yang, 2004). Measurement of inter-doublet microtubule sliding in the presence of pharmacological reagents has revealed that the control system is modulated by a network of kinases, phosphatases and potential sensors of second messengers that signal motility changes (Smith and Sale, 1992) (reviewed by Porter and Sale, 2000).

Much of our knowledge of the composition of the radial spoke has come from comparisons, using two-dimensional (2D) gels (Piperno et al., 1981), of the proteins of wild-type *C. reinhardtii* versus radial-spoke-defective axonemes; more recently, information has been gained from the analysis of isolated radial spokes (Yang et al., 2001). These studies reveal that the *C. reinhardtii* radial spoke, which sediments as a 20S particle, contains at least 23 distinct polypeptides, termed radial spoke protein (RSP)1 to RSP23 (Piperno et al., 1981;Yang et al., 2001;Patel-King et al., 2004), with a combined molecular mass of approximately 1200 kDa (Padma et al., 2003). Five of these proteins are located in the spoke head and the rest are in the spoke stalk.

Among the 23 RSPs, genes and cDNAs encoding RSPs 2, 3, 4, 6, 16 (HSP40), 20 (calmodulin), 22 [dynein light chain 8 (LC8)] and 23 [p61 nucleotide diphosphate kinase (NDK)] have been cloned (Yang et al., 2004;Williams et al., 1989;Curry et al., 1992;Yang et al., 2005;Zimmer et al., 1988;King and Patel-King, 1995;Patel-King et al., 2004). The predicted amino acid sequences have provided hints as to the possible functions of these proteins. For example, RSP3, which anchors the radial spoke to the outer doublet microtubule (Diener et al., 1993), contains an AKAP (for 'A-kinase anchoring protein') domain and binds the cyclic (c)AMP-dependent protein kinase (PKA) regulatory subunit in vitro (Gaillard et al., 2001). RSPs 2 and 23 contain calmodulin-binding domains and bind calmodulin (RSP20) in vitro (Yang et al., 2001;Yang and Sale, 2004;Patel-King et al., 2004). RSP23 also contains a Ca^{2+}-stimulated NDK activity.

A complete understanding of the architecture, assembly and function of the radial spokes will require a detailed knowledge of the entire ensemble of RSPs. Moreover, although defects in radial spokes are known to be one cause of the severe, genetically heterogeneous, human disorder termed primary ciliary dyskinesia (PCD) (Sturgess et al., 1979;Antonelli et al., 1981), the genes responsible for PCD in patients lacking the radial spokes have not been identified, and discovery of these genes will most probably require a candidate gene approach that begins with genes known to encode RSPs. The development of large databases of *C. reinhardtii* expressed sequence tags (ESTs) (Asamizu et al., 1999;Shrager et al., 2003) and the recent sequencing of the *C. reinhardtii* genome by the US Department of Energy Joint Genome Institute (JGI) (http://genome.jgi-psf.org/Chlre2/Chlre2.home.html) has now made possible

the accurate identification of RSPs from isolated radial spokes or spots on 2D gels using mass spectrometric methods. Here, we report the sequences of 10 new *C. reinhardtii* RSPs, analyze their potential structural and functional motifs, and identify their most likely human homologs.

Results

Identification of new RSPs

To identify the previously unknown RSPs, in most instances we purified spoke proteins from 2D gels of isolated axonemes or 20S radial spokes (Fig. 1) based on the well-established 2D map of RSPs (Piperno et al., 1981;Williams et al., 1986;Yang et al., 2001). The excised gel spots for each protein were subjected to tryptic digestion and the resulting peptides were analyzed by matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS). The MS and MS/MS data were used to search *C. reinhardtii* genomic and EST databases to identify nucleotide sequences encoding the peptides. RSPs 1, 5, 7, 8, 9, 10, 11, 12, 14 and 17 (labeled in red in Fig. 1) are identified here for the first time. Table 1 lists the gene models for these proteins in the JGI *C. reinhardtii* v. 2.0 genome, as well as the National Center for Biotechnology Information (NCBI) accession numbers for the previously identified RSPs. All new proteins were initially identified on the basis of two or more peptide hits (Table 1;Fig. 2, peptides are indicated by bold letters in the predicted protein sequences); thus, the chance of a coincidental match is remote. In no case did peptides from a spot or band match more than a single gene, indicating the high purity of the protein and the reliability of the search. For each of these proteins except RSP14 and 17, the complete coding sequence plus some 5′-and 3′-untranslated region sequence was determined from ESTs or by amplification of cDNAs followed by sequencing of the products (Fig. 2). The complete coding region of RSP17 obtained from the JGI v. 2.0 gene model was supported by ESTs corresponding to the 5′- and 3′-ends of the message and a total of 15 matching peptides from this study, and a proteomic analysis of the flagellum (Pazour et al., 2005). The C-terminal three-quarters of the predicted RSP14 sequence has been confirmed by RT-PCR, but the sequence may not be complete at the N-terminus.

For RSP15, MS/MS yielded a single high-scoring peptide sequence predicted by JGI v. 1.0 Genie.2667.0 and v. 2.0 Bonus_Scaffold_7104. Although the gene model is unlikely to be complete, the available sequence indicates that RSP15 is a leucine-rich repeat (LRR) protein, probably homologous to the LRR protein in *Ciona intestinalis* radial spokes (Padma et al., 2003). A single high-scoring peptide for RSP18 matched JGI v. 2.0 genomic sequence between gene models C_30269 and C_30270.

The protein previously designated RSP19 co-sediments with 20S radial spokes and 15S spoke stalks (Yang et al., 2001), migrates at M_r ~140,000 in SDS-PAGE, and has a pI similar to that of tubulin (Fig. 1). Six peptides from this spot were analyzed and all were identified as originating from β-tubulin. Western blot analysis confirmed that β-tubulin migrates as a discreet band at M_r ~140,000 in SDS gels of radial spoke and spoke stalk fractions (Fig. S1, supplementary material). Although no non-tubulin peptides were identified from this spot, it is possible that β-tubulin is covalently associated with a spoke stalk protein whose peptides were not recovered. RSPs 13 and 21 were not identified because the MS/MS spectra obtained could not be matched to sequence in the *C. reinhardtii* databases. The theoretical molecular weights and isoelectric points for the predicted RSPs are consistent with the experimental values determined by 2D gel analyses (Table 1).

Validation of newly identified RSPs

To confirm that the MS analysis identified the correct proteins, antibodies based on the predicted sequences were generated for six of the new proteins. Western blot analyses using

these antibodies confirmed that the cloned proteins identified as RSPs 7, 8, 9, 10, 11 and 12 are missing in axonemes of the well-characterized spokeless mutant *pf14* (Fig. 3A), and that those identified as RSPs 7 and 11 cosediment with solubilized 20S radial spokes and 15S spoke stalks (Fig. 3B; results for RSP7 not shown). Antibodies previously shown specifically to recognize natural RSP1 and RSP5 (Williams et al., 1986;Qin et al., 2004) recognized bacterially expressed recombinant RSP1 and RSP5, respectively (Fig. 3C), confirming that these sequences have been correctly identified; as expected, the proteins recognized by these antibodies are also missing in axonemes of *pf14* (Fig. 3C). Finally, in a separate proteomic analysis of *C. reinhardtii* axonemal fractions, multiple peptide hits were obtained to sequences identified as RSPs 1, 5, 7, 8, 9, 10, 11, 12, 14, 15 and 17 (Pazour et al., 2005), providing independent evidence that the predicted genes encode axonemal proteins.

Predicted domains

The newly identified proteins were analyzed to identify predicted domains and structural motifs; these, together with those of the previously identified RSPs, are diagrammatically illustrated in Fig. 4. RSP1 has at least six MORN (for 'membrane occupation and recognition nexus') domains in its C-terminal half. RSP5 contains an aldoketo reductase domain and a short region that is predicted to form a coiled-coil domain. RSP7 has an RIIa domain and seven EF-hand domains, five of which match the consensus sequence for Ca^{2+} binding (PROSITE accession number PS00018) (Fig. 5). RSP8 has at least two armadillo repeats. RSP9 has no obvious structural or functional domains. RSP10 has at least four MORN domains in its N-terminal half. RSP11 has an RIIa domain. RSP12 has a conserved cyclophilin/peptidyl-prolyl cis-trans isomerase (PPI) domain that includes most of the protein. RSP14 has at least three armadillo repeats. RSP15 has LRRs. RSP17 has a possible GAF domain (E-value, 5.21e-01; the GAF domain is named for its presence in cGMP-regulated phosphodiesterases, certain adenylyl cyclases and the bacterial transcription factor FhlA), as well as a predicted coiled-coil domain that is similar to those of some members of the intermediate filament superfamily. The possible significances of the predicted domains are addressed in the Discussion.

Human homologs

BLAST searches were carried out for each RSP to identify homologs in other organisms. At least 12 of the RSPs have probable homologs in diverse other organisms. For each of these RSPs, Table 1 lists the representative human homolog and its chromosomal location, as well as the length of the aligned region, the percentage identity and similarity, and the BLAST E value. *C. reinhardtii* RSP1 is most similar to *Homo sapiens* meichroacidin, which is expressed primarily in the testis (Tsuchida et al., 1998); much of this similarity is a result of MORN repeats in both proteins. *C. reinhardtii* RSP3 is closely related to human radial spoke head-like protein RSHL2. *C. reinhardtii* RSP4 and RSP6, which are homologs of each other (Curry and Rosenbaum, 1992), are equally closely related to human RSHL1 and RSHL3; RSHL1 was previously identified as a homolog of *C. reinhardtii* RSP4/RSP6 (Eriksson et al., 2001). RSP9 is similar to an uncharacterized predicted protein encoded by human chromosome 6. RSP10, like RSP1, is similar to human meichroacidin, and also to a hypothetical protein encoded at 7p22.2; the similarities are due primarily to MORN repeats in all three proteins. RSP11 is weakly similar to the human AKAP-associated sperm protein ASP; this similarity is primarily in the RIIa domain. RSP12 is 38% identical throughout its length to the hypothetical human protein peptidyl-prolyl isomerase (cyclophilin)-like 6 (PPIL6). RSP16 is 41% identical over its entire length to the similarly sized human TSARG6 (for 'testis spermatogenesis apoptosis-related protein 6'); it is only slightly less similar to a human DnaJ (Hsp40) homolog. As previously reported, RSP20, RSP22 and RSP23 are homologous to human calmodulin, LC8 and testis-specific NDK, respectively. No highly similar human proteins were identified for RSPs 2, 5, 7, 8, 14 and 17.

Discussion

Structural and functional domains

Previous comparisons of *C. reinhardtii* mutants lacking the entire radial spoke or just the spoke head have allowed the RSPs to be assigned to either the spoke head or stalk (Piperno et al., 1977;Piperno et al., 1981). Because location within these two morphologically distinct subdomains is important in considering the proteins' structural and functional roles, the proteins of the spoke head and stalk are discussed separately here. Fig. 6 illustrates the predicted locations of the spoke proteins (Fig. 6A) and their domains (Fig. 6B) relative to spoke ultrastructure and proximity to the inner dynein arms and central microtubules. Strikingly, proteins in the spoke stalk (RSPs 2, 3, 5, 7, 8, 11, 12, 14, 15, 16, 17, 20 and 23) are rich in predicted functional motifs, including many associated with signal transduction, whereas proteins in the spoke head (RSPs 1, 4, 6, 9 and 10) contain no obvious functional motifs and few predicted structural motifs. The results suggest that the spoke stalk is likely to serve as a scaffold for signaling molecules, and probably has an important role in transducing chemical and mechanical signals.

Spoke stalk proteins

Spoke stalk proteins RSP7 and RSP11 have RIIa domains, which bind with high affinity to AKAP domains and also mediate homodimerization (Colledge and Scott, 1999). RSP3, which is located at the base of the spoke stalk (Diener et al., 1993), was previously shown to contain an AKAP domain that binds the RII regulatory subunit of PKA in vitro (Gaillard et al., 2001). Thus, it was proposed that RSP3 anchors PKA adjacent to inner arm dyneins to regulate their motor activity, a role consistent with the effects of PKA inhibitors on inter-doublet sliding velocity (Howard et al., 1994). However, no RSP was identified as the RII subunit. It is possible that RII dissociated from the radial spokes during the potassium iodide (KI) extraction used to isolate the spokes. Alternatively, the AKAP domain of RSP3 might interact with the RIIa domain of RSP7 and/or RSP11. Consistent with this, these two RIIa proteins co-sediment with RSP3 in partial spoke particles from the mutant *pf24* (Yang et al., 2005), in which the spokes are unstable and dissociate into subparticles when extracted from the axoneme. The unique motility phenotype of the RSP11 mutant *pf25*, which swims actively but in an abnormal fashion (Huang et al., 1981), suggests a regulatory role for this novel RIIa protein.

At least four spoke stalk proteins are likely to be involved in Ca^{2+}-mediated signaling. RSP20 was previously identified as calmodulin (Yang et al., 2001). RSP2 binds calmodulin through 1-8-14 motifs (Yang et al., 2004), and RSP23, which contains an NDK domain, binds calmodulin through IQ motifs (Patel-King et al., 2004). The newly identified RSP7 contains five predicted EF-hands that match exactly the consensus for Ca^{2+} binding (Fig. 5). Ca^{2+} is a regulator of flagellar waveform in *Chlamydomonas* (Witman, 1993), and the radial spokes together with the central pair apparatus are predicted to be involved in the Ca^{2+}-induced waveform changes (Brokaw et al., 1982;Smith, 2002). Thus, these RSPs might have a key role in the Ca^{2+} control of flagellar motility.

RSPs 2 and 17 are predicted to contain GAF domains, which bind cyclic nucleotides or small ligands in many sensory and signaling proteins (Hurly, 2003). cAMP has been shown to inhibit the motility of demembranated, reactivated axonemes of *Chlamydomonas* (Hasegawa et al., 1987), and both cGMP and cAMP have been shown to affect ciliary motility in detergent-permeabilized, reactivated *Paramecium* (Bonini and Nelson, 1988). It has been assumed that these effects are mediated by cAMP- and cGMP-dependent protein kinases. Therefore, it will be of great interest to determine whether cyclic nucleotides bind to the predicted GAF domains of these two RSPs and, if so, if the binding is responsible for any of the observed effects of cyclic nucleotides on flagellar motility.

Two spoke stalk proteins are predicted to function as molecular chaperones. RSP16 was previously shown to have conserved DnaJ-J and DnaJ-C domains found in the HSP40 (DnaJ) family of molecular chaperones (Yang et al., 2005;Satouh et al., 2005). HSP40 family members cooperate with HSP70 to suppress protein aggregation (Fink, 1999), and HSP70 is indeed present in the *C. reinhardtii* flagellum and concentrated at the flagellar tip (Bloch and Johnson, 1995), where final assembly of the spoke occurs (Qin et al., 2004). The newly identified RSP12 is predicted to be a member of the cyclophilin family of peptidyl-prolyl isomerases, which help protein folding by catalyzing proline cis-trans isomerization (Fink, 1999). Thus, these two co-chaperones might be involved in converting radial spoke precursors into mature spokes. In addition, the peptidy-prolyl isomerase Pin1 has been shown to bind specifically to Ser-Pro or Thr-Pro motifs when the serine or threonine is phosphorylated (Yaffe et al., 1997), and then affect a conformational change that might alter the activity of the target protein (Zacchi et al., 2002;Zheng et al., 2002); other peptidy-prolyl isomerases might function similarly (Ryan and Vousden, 2002). Spoke stalk proteins 2, 3, 5 and 17 are phosphorylated (Piperno et al., 1981), and have Ser-Pro and/or Thr-Pro motifs, so the possibility that RSP12 functions as a regulatory protein should be considered.

RSP5 consists of an aldo-keto reductase domain, which is common to a diverse family of oxidoreductases (Sanli et al., 2003). Outer arm dynein contains thioredoxin homologs with perfect copies of the redox-active site (Patel-King et al., 1996), and the Ca^{2+}-binding activity of the DC3 subunit of the outer dynein arm docking complex is redox sensitive (Casey et al., 2003), suggesting that outer arm activity is regulated by the redox state of the cell. Radial spoke function might be similarly regulated through RSP5 to ensure coordination between the radial spokes and the outer arms.

RSP8 and RSP14 have armadillo repeats and RSP15 has LRRs, both of which are believed to function in protein-protein interactions. RSPs 2 and 17 are predicted to have long coiled-coil domains. Coiled coils commonly mediate homodimer and heterodimer formation (Lupas, 1996). Therefore, it is possible that these proteins form homodimers, or heterodimers with each other, through coiled coils. In either case, the coiled coils might form part of the structural framework of the spoke stalk, which is ~30 nm long and very thin (Witman et al., 1978). Assuming approximately 1.5 Å of coiled coil per amino acid residue (Fraser and MacRae, 1973), a coiled coil of 123 residues as predicted for RSP17 would be ~18 nm long and could account for over 60% of the length of the spoke stalk, whereas one of 103 residues as predicted for RSP2 would be ~15 nm long and could account for half of the length of the spoke stalk. RSP2 also contains a Dpy-30 motif, not previously reported, which might function in dimerization (http://pfam.wustl.edu/cgi-bin/getdesc?name=Dpy-30).

Spoke head proteins

Among the five spoke head proteins, RSPs 1, 9 and 10 are newly identified. The latter two appear to be the orthologs of proteins recently reported in a spoke complex from *C. intestinalis* sperm (Satouh et al., 2005). In contrast to the spoke stalk proteins, the only domains identified in spoke head proteins are multiple MORN motifs present in RSP1 and RSP10. These motifs are thought to represent a protein folding module and, in the junctophilins, to mediate binding of the protein to the plasma membrane (Takeshima et al., 2000). It will be of interest to determine whether they mediate interactions between the spoke head and the central microtubule projections in the axoneme.

There is cytochemical evidence for ATPase activity in the center of the axoneme (Gordon and Barrnett, 1967), and it has been postulated that interaction between the radial spoke and central pair might be mediated by an ATPase (Burton, 1973;Ogawa and Gibbons, 1976). Thus, it is interesting that none of the spoke head proteins resembles a known ATPase, or has motifs, such as a P-loop, associated with ATP binding and hydrolysis. If an ATPase is involved in

these interactions, it is likely to be a component of the central pair complex. One such candidate is Klp1, a kinesin-like protein associated with a projection of one of the central pair microtubules and thus in a position to contact the spoke heads (Bernstein et al., 1994;Yokoyama et al., 2004).

Relationship to PCD loci

At least 12 RSPs appear to have homologs in other organisms, including *H. sapiens*, indicating that not only the structure, but also many of the proteins of the radial spoke have been conserved throughout evolution. The identification of probable human homologs for many of the RSPs provides new candidates for causing the human disease PCD, which involves missing or defective axonemal structures (Afzelius and Mossberg, 1995), including the radial spokes (Sturgess et al., 1979). Over 16 PCD loci have been mapped (Blouin et al., 2000;Jeganathan et al., 2004), including one at chromosomal region 19q13.3 (Blouin et al., 2000;Meeks et al., 2000). Eriksson et al. (Eriksson et al., 2001) previously noted that *RSHL1*, a human homolog of the closely related *C. reinhardtii RSP4* and *RSP6* genes, maps to 19q13.3, and therefore suggested that it was a candidate gene for PCD. However, Meeks et al. (Meeks et al., 2000) reported dynein arm deficiencies in two of the families used in their mapping of a PCD locus to 19q13.3, so it seems unlikely that the defect in these families involves an RSP gene. Some families have a PCD disease gene on chromosome 17 (Blouin et al., 2000), which contains a potential homolog of RSP10 at 7p22.2; however, defects in a dynein heavy chain gene at 7p21 are likely to be the cause of PCD in these families (Bartoloni et al., 2002). The gene encoding TSARG6, which is highly similar to RSP16, maps to 11q13.3; in some families, 11q potentially harbors a gene linked to situs inversus (Blouin et al., 2000), which is frequently associated with PCD. It would be of interest to examine the cilia and flagella of patients from these families for radial spoke defects. None of the other human homologs of *Chlamydomonas* RSP genes maps to known PCD loci, and no PCD gene that encodes an RSP has been identified yet. This undoubtedly reflects both a paucity of studies of PCD families with defects in radial spokes, and a hitherto lack of candidate PCD genes encoding RSPs. The identification here of eight new human genes that potentially encode RSPs should greatly facilitate discovery of the defective genes in future studies of such patients.

Conclusion

The identification and sequence analysis of 10 *C. reinhardtii* RSPs previously known only as spots on 2D gels has yielded intriguing and important new information on predicted structural and functional domains within these proteins, consistent with the predicted role of the radial spoke as a major signal-transducing structure within the axoneme. This information provides a strong foundation for future studies on the roles of individual RSPs in radial spoke assembly and function, on protein-protein interactions within the radial spoke, and on how the radial spokes integrate both mechanical and chemical signals to ensure coordinated flagellar motility during a variety of behavior responses. Finally, the identification of homologs of *C. reinhardtii* RSPs in humans and other organisms indicates that the radial spoke is conserved at the molecular level, and provides new candidate genes for causing PCD in patients with defects in the radial spokes.

Materials and Methods

Cell strains and culture

C. reinhardtii wild-type strain CC125 and radial spoke mutant strains were obtained from the *Chlamydomonas* Genetics Center (Duke University, Durham, NC). The double mutant *pf*28*pf*30 was obtained from G. Piperno (Mount Sinai School of Medicine, New York, NY); although the flagella of double mutants of outer and inner dynein arms are usually short

(Hayashi et al., 2001), this strain of *pf28pf30* carries an uncharacterized suppressor mutation (LeDizet and Piperno, 1995) that allows assembly of full-length flagella. All cells were cultured as described (Yang et al., 2001). ESTs for RSPs 5, 7, 9 and 10 were obtained from the Kazusa DNA Research Institute, Japan.

Protein purification

Isolation of flagella and axonemes, extraction of axonemes with 0.6 M KI, purification of 20S radial spokes and 15S spoke stalks, and 2D gel electrophoresis were carried out as described previously (Williams et al., 1986;Yang et al., 2001). Briefly, 20S radial spokes were typically obtained from the double mutant *pf28pf30*, which lacks the ~20S dyneins. Proteins of the isolated radial spokes were separated by non-equilibrium pH gradient gel electrophoresis in tube gels using an ampholine range of pH 3.5–9.5. Proteins in the acidic portion (~pH 3.5–7.5) of the tube gels were further fractionated by SDS-PAGE in mini-gels followed by non-formaldehyde silver staining (BioRad Laboratories); 6, 10 or 12% polyacrylamide gels were used as necessary to obtain optimum separation of RSPs in the range of 15–200 kDa. For RSPs 9 and 10, spokes from the 20S fraction of the sucrose gradient were further purified by fast-performance liquid chromatography using a HiTrap Q (Pharmacia Biosciences) column. The eluted proteins were then separated by 1D SDS-PAGE.

Mass spectrometry

Protein bands or spots excised from 2–8 silver-stained gels were digested with trypsin. The peptide mixture was then eluted and analyzed using a Kratos Analytical Axima CFR MALDI-TOF mass spectrometer (Shimadzu Biotech) to obtain a peptide mass fingerprint; selected peptides were further analyzed by MS/MS following post-source-decay to determine the sequence of the peptide. The MS and MS/MS data were then used to search a *C. reinhardtii* EST database (http://www.chlamy.org/search.html) and the *C. reinhardtii* genome (JGI v. 1.0 or v. 2.0, http://genome.jgi-psf.org/chlre1/chlre1.home.html or http://genome.jgi-psf.org/chlre2/chlre2.home.html, respectively) using the search algorithms ProteinProspector (http://prospector.ucsf.edu) and Mascot (http://www.matrixscience.com) to identify EST or genomic sequence that encoded each peptide. Matches to genomic sequence were examined to identify predicted gene models encoding the peptides, and to confirm that the peptides were predicted by the correct reading frame.

Gene and structural predictions and identification of human homologs

Domains within proteins were predicted by the internet-available programs SMART (http://smart.embl-heidelberg.de/), SUPERFAMILY (http://supfam.mrc-lmb.cam.ac.uk/SUPERFAMILY/), Scansite (http://scansite.mit.edu/), MOTIF (http://motif.genome.jp/) and COILS (http://www.ch.embnet.org/software/COILS_form.html), using default parameters. The BLAST server at NCBI was used to search nucleotide and protein databases for the human homologs of *C. reinhardtii* RSPs.

DNA cloning and sequencing

RT-PCR was carried out to confirm coding sequences and recover cDNA clones for RSPs 1, 5, 7, 8, 9, 10, 11, 12 and 14. 5′-rapid amplification of cDNA ends was used to obtain a portion of the RSP7 sequence that was not contained in the databases. The genomic clone RSb1, encoding RSP1 (Williams et al., 1986), was also sequenced and the sequence used to isolate a partial cDNA clone. All cloning and sequencing was carried out using standard procedures.

Antibodies

Rabbit polyclonal antibodies were raised against bacterially expressed recombinant proteins (RSPs 7, 9, 10, 11 and 12) and a synthetic C-terminal peptide (RSP8). The rabbit antibody

against recombinant RSP16 was described previously (Yang et al., 2005). The rabbit anti-RSP1 and anti-RSP5 sera**,** raised against the purified axonemal proteins, were previously described (Williams et al., 1986;Qin et al., 2004).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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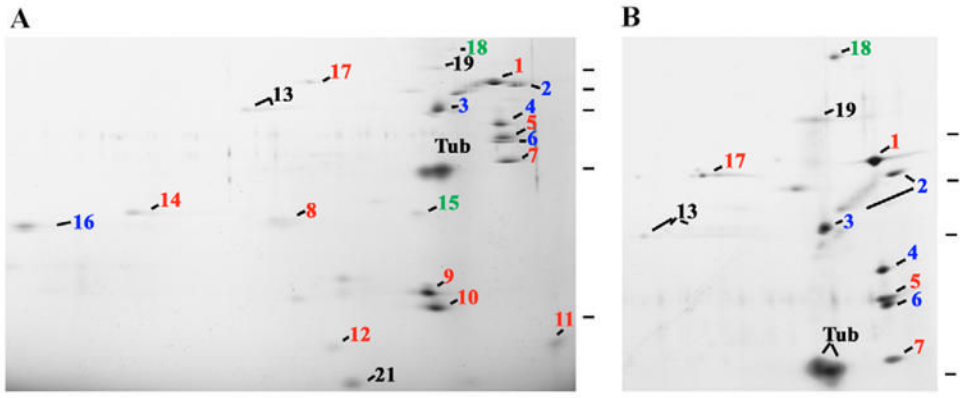

Fig 1.

2D maps of RSPs in 20S radial spoke fractions. Newly identified proteins whose sequences were completely determined are labeled in red. RSPs 15 and 18, marked in green, yielded peptides that matched regions of the genome where no gene model currently exists. Proteins identified previously are in blue. Those that remain to be identified are in black. RSPs 20, 22 and 23, which also were identified previously, are not visible because they are poorly stained by the non-formaldehyde silver stain (RSPs 20 and 23) or are too small to be retained in the gel (RSP22). The proteins were resolved in 12% (A) and 6% (B) gels following non-equilibrium isoelectric focusing. Figures only show the relevant area of pH 4.6–7.3 (A) and 5–6.5 (B). The acidic end is to the right. Horizontal bars indicate molecular markers of 130, 100, 83, 54, 40 and 24 kDa, from top to bottom.

>RSP1 (DQ298254)
MALGPFVLPFRGDQYSFGINFKSSPEEKLNFDLSCVAFDVK**GQLHDTLHA**
RKPTALDGALVKGFEKQALPEETVQVEGDDVIYMFPKKFERQVEVLLFVA
SAPSIPGKKHDLDSSSKLEFAVSYSDVGGQAFNQSFDLKPLAAQGGVSSI
IVAVMYLQAEGGWTLRSVGDCHPFDSPGLIVPELKQTILNLRDHHGVQLD
AADAIQAIDPAERVPVTRQFQDQSLDEASAGRAAEPAPVKKLRIDLSWTF
WPPPPPTEEGEEPPEEPALEYNLVMYNKDGEEVQSISTGNREATGARAGR
PEPEEDEEEEKEEEKEEPEEGEEGEEGEGGEPKEPPPPPPAPKVDPYEFK
ER**DVIYLDVPDLPAEVR**SMVLLVTNYDEENGFTRVRTVRCR**LVDVSNGEA**
PLPGSKAAVAAAAAAAAEQGLAAPPNPERVLADYGVLSKYEDDKATTQVAL
MK**LYKEYADSAFNVFRGAGVDNVAAFIGQEPDTIINQLK**AYLEATKKQK**A**
AEAAAAAAAAEESGEEITADPKPHVWRFRALGLNFGGDSLEAIEHDLKNLF
AFDGDLAPGAARDSDTSRSSFPNGDTYFGSYADDVK**HGPGLYAFATGAGY**
AGEYAGGKRHGRGVMVFPDGGTYVGEFVADKFEGQGQYRYPDGSVY**TGSW**
AAGQKHGPGVYWDTARGCLRGEWKKGLLVGKGTYEQPALRFEGEFVR**GMP**
AGTATYTLTGHRTLDMPCFAAQHIQAEEGPTLALPCAYGIPPGSGDEPQL
DEEGQPIEDTDKPPLPAHPKYEGLTFTAEQLPGAAPDTVFPPEEGKPVPI
TAVPAFSVSTGLVA

>RSP5 (DQ298247)
MSEPGEEPVAAPAGPAPDPVLNELYGSERPAVELLPGVPLSPIVNSCWLP
ADAKAMLAESWIPVPPEDAGEEAGPPPPAFEAAAPEYNELVRRLAKTAPF
R**KWNELTIQAK**QLEQEVAGLKGPDAEAKQAELENVKVQIADAEAAVAEVK
QSFSDDPLSLTGWMQALTDLADGGMTTFEVSGQGWPYCSLRQLFGEMPSA
APPAGFFDGVERVLGTFKRRYEKERGPGSVQLMLKLAPNVFSDAWSTGGA
PAAVAAVEAYVERARANVFGPDGGVTPEGVPEPLDLVQLVWWDFAAADPL
PVLKALQR**MATDQLQVDEDSGEVSVSEP**KKIRGIGLVDFPADRLKAAIQA
GVPITCVQVEHSVLVRSAQPVLDLCAKYGIKVLARGGTLGGLLSAKYLGA
PPPDPVRGDADLDSVPGCLDAVNNVGGWARLQAALAVIKGIADKHGVKPE
TVALRWQINAGCFPLVTTRWSSRVWRQFGYEGWSSFEVSGGRPGVDGPLF
QVESFLDVEDVRALAGLAAVHLGPKAG

>RSP7 (DQ298255)
MSSTYQKPITIPGDFPAILKAFTREILR**AQPSNIYEFGARYFSGLQGQAE**
PEGLGRAAPVEPASTSHAATSKATDVEETAVMFDIAALTPAELEPILMKL
FIEADADRSGFLDRHEFTAVLRNANLKLSDRQIRQILAEADENDDDVIQY
KEFLPIMVDILQSIKAKEQAKAMMHGVETMVRTEVETMLLHGLPQEELQA
LMLKVFKKADADGSGQLNRHEFKEALKAAELGLTPKDINLILSHIDVDRD
GLVSYEEFIPVCFQVLVERFKDEIVVNDILGNADELQQMLLGAFRDADPD
NTGLLSQRQVKSIFKELSYKALGLTTLQMVSLISQAPTTPDGMVQYIQFV
PQAASIIRSMYDVETMKGRMHAIKAVAEAGGIAALGALDLDQLR**GVLEQA**
FQRVDTEGAGQLTLPQVTQVLDGLNSLAPDANLALSDQHMKAMFAAIDAD
ESGTVDWTELVNFICDALEHIEREAYVANMRDGGAGGAGEAEASPGDEEA

>RSP8 (DQ298248)
MQSHSSRHVVSEHLKDLHPDLSEPFP**KHVIEDEVAEAHGRR**AIP**KLVAVL**
ALPELPDDQRAHAL**RVLNGLLSTQEHKTNAVAEGAAPPLCQLASQCRDDE**
VRRLSCSALASLGQVMAGRNGIVAAGGLPVLTEALQTTPEQAAAAL**KSFA**
ASNDGAAQLNLERAAIVPALVTLLSQPTDPAFTLTAFSNALSTLEGMTRT
DDGVLAALDGGVPACLVALARRGLEGDLKFEGRLMELLQLVATCLEQICH
HADGKAACRQAEAHKVLAELLTLQHREIIKHAAAALMGLAVEKESKVNVM
LYAGVSLVRLMRGSDAELAANA**RDTVAAAAEHLEARR**TAEMLLSMEEREL
LLW**RGPPPETPPDYRY**HVDLPKFTPQAK

>RSP9 (DQ298249)
MVQLEPNITLVLKHLASCGAVVSAEQQAALDHSIPIKRIEAGLRSLTLWG
RLTTLNGKDYLVAEGYNVASSKEGAAVYETK**YFYSQDGAR**WSDLQPVDSE
TATRCARIKGMLSGDPAKNYELEEKDPNAPEPSPEAEEEVKPLVFQIPEL
AVLRCRVDAIATATSVIPTDSTILNAASQVVPNR**LFAGAAYPEKLESYQH**
RFSLPGSGVTLSQDLRGTWAVQYDAFKGVAQVRSLLFPGYFFYYAANELT
WGSLYVGDGLRNNDLIFML

>RSP10 (DQ298250)
MADDELPPQPVWEGPLDEDGKPHGLGKMEYPPPPMGEDDEEEKPGDKFEG
TMEHGVRTGKGTYTWGVSGAVYTGDYVNGKKHGKGKMVYPDKGVYEGDWV
EDVMQGQGTYTYPNGDIYQGAFWAGKRHGK**GMYHYK**GPCCQLVGDWADGG
FTYGRW**VYADGSMFMGK**FGGAAADSKPTAGSYFYSSSSLVQEGHFAKDGS
WVGHRDPAVGKEFSVA

>RSP11 (DQ298251)
MDVEPIFCAEQIVIPHNLADILKAYTKEVIR**RQPTDLIAFSAKYFTNLAN**
VASGVSNSSAPAKEQLRQVYTRGGSGGATLTESQVTGLCQQAGIADAVVA
KVMEVGAFTPAAVDLSKFVFLCLAMSCEDFNRVCMGVFDVFSDNGSLPAQ
DLLTLIAHLGPDMDPEVTPAFLDAVAAELPAGGGAVTYMELCEAPSLKPK
LGLS

>RSP12 (DQ298252)
MDFESNDAMMEYCKSTGR**TYCAFSIQQSSK**LLGTVVLELFTDIAPATCAN
FIK**YIKDGYQGTPLHRIVPNGWVQGGDIVDGSGKGDPGFVLPDETYSVK**H
DAPGVLGMATGGQPHTANTQFYISLSPLPFLDGK**RVAFGR**VLNKQSLENL
LALQTLPTFQNERPVPDVVIASCHVIYNPNS

>RSP14 (DQ298253)
VGARDLAQHSGLDALAAALEDPSEGVRDEAYGALIEAARFDSTRRALEAC
GSGAVLPR**LMELALLEAQGGAAGRAQQGLVLLFTCTQAR**HNAGILSQLVD
VAQAIPHLAGLLKPELPMPVRHAAAELLGALATREDAKIQAVQVGAVPLL
LLAASPSVPVPFATSAVAALGAITIRREGKYAALESPGGLAGLVSVLDPC
HEQLCINAMTAVSNVAEAPEARAILVASGAGPKLQHIFETATVEVVKRAA
AQAIRQCRFKHLPYEVLPGAPPINEE

>RSP17** (DQ298256)
MPWLRYFFPKDFDLREGQPVQPYYQFFDVVQQPLQEDTSVPPPDWAPNAY
FQLSFTQVHVPKDMVLATRIPRPPPPPDGSIDVTADNSNILKPGNEKLMA
DILAFCRSVATFRARTWKDALPLVDLLRGVLAHLLNHYTWGAALVREQAK
KDAEAALAAEKKALKAWYDKQLEEAMARARQEYDEKLEARAEELRMTLAE
DLTLQLRAEREEKNQLRSQATGLESQLKK**AAEAQLTLSR**NLEHMQQQLDL
EAQRRAEALAGKDAQMTAMSEQAASLRRRLAAAQGAVVEALNAKTKEDAL
NVAVKALAGVLPAGSASYVAELRTAADVDGDDPSAALLAQAAAAPDPAAL
APALAALRGVTPGFMVSPLAALVAATHEQRAAQSQDGEVGAAAAGAAGRR
RSSATGAAANLAAVISGAALNEIKESLVFKYIKASKGHEFMIGEKLAWGS
GASWEIVERGTPALVVPDVAAQGNMWFFRHKQPPAAPGGNYMAVPILGTQ
GEVVGILAADTMGAAAAEAATAAAAAGGADTTQGTR**AAAAAAAAADTSVT**
DAAFMRGLAAAIGARARADAEAYHEAMLAAAIAGEEAPEPEPSAVDPSQF
VVGASGEAGEGGNLSGGLKLFADALSLLRELTDEELEALRNLDEPDDVTL
AVIKSILAAVGEIAGGEDWAAARQHLSRELLERLMSMDPQAAWAEWQALC
RAFRALNELSMASLDAASTAASDPSAAPSVRASGAFARWAAGVRAVSAAR
ALQQVLEVKLQKANAALMKTFSGDSCNALTELRSYRVPPPLTFGVLQCVL
MLSGNVDEEDCRNWARMRTLANYRLIKRLVTLRPAQVPRKIFRAVKRLVH
DIVEGDVKQESVATLSLYRWLADFTRLASVAK

Fig 2.

Predicted sequences of the 10 newly identified RSPs. The GenBank accession numbers for the nucleotides are listed in parentheses. Peptide sequences obtained from MS are in bold font. The underlined polypeptide sequences in RSP17 (asterisks) were described previously (Pazour et al., 2005). The N-terminal end of the RSP14 sequence may not be complete.

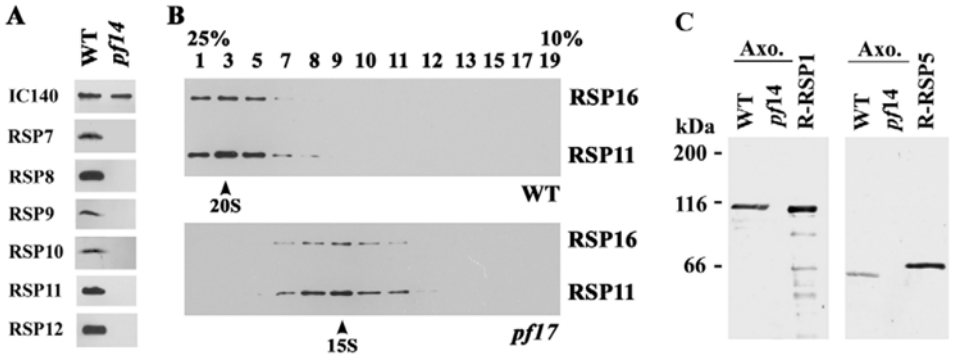

Fig 3.

Confirmation that the MS analysis identified the correct RSPs. (A) Western blot showing that antibodies based on the sequences predicted for RSPs 7, 8, 9, 10, 11 and 12 recognize proteins present in wild-type (WT) axonemes but absent in axonemes from the spokeless mutant *pf14*. (B) Western blot of sucrose density gradient fractions showing that an antibody based on the sequence predicted for RSP11 recognizes a protein that co-sediments with solubilized 20S WT radial spokes (upper panel) or 15S spoke stalks from the spoke-head-less mutant *pf17* (lower panel). The blot was also probed with an antibody to RSP16 (Yang et al., 2005) as a marker for the 20S spokes and 15S spoke stalks. Similar results were obtained for RSP7 (data not shown). (C) Antibodies raised against RSPs 1 and 5 purified from spots on 2D gels recognize the recombinant proteins (R-RSP1 and R-RSP5). In addition, the recombinant proteins, which are His tagged, migrate at the expected size relative to the native proteins in WT axonemes. The proteins are absent in *pf14* axonemes.

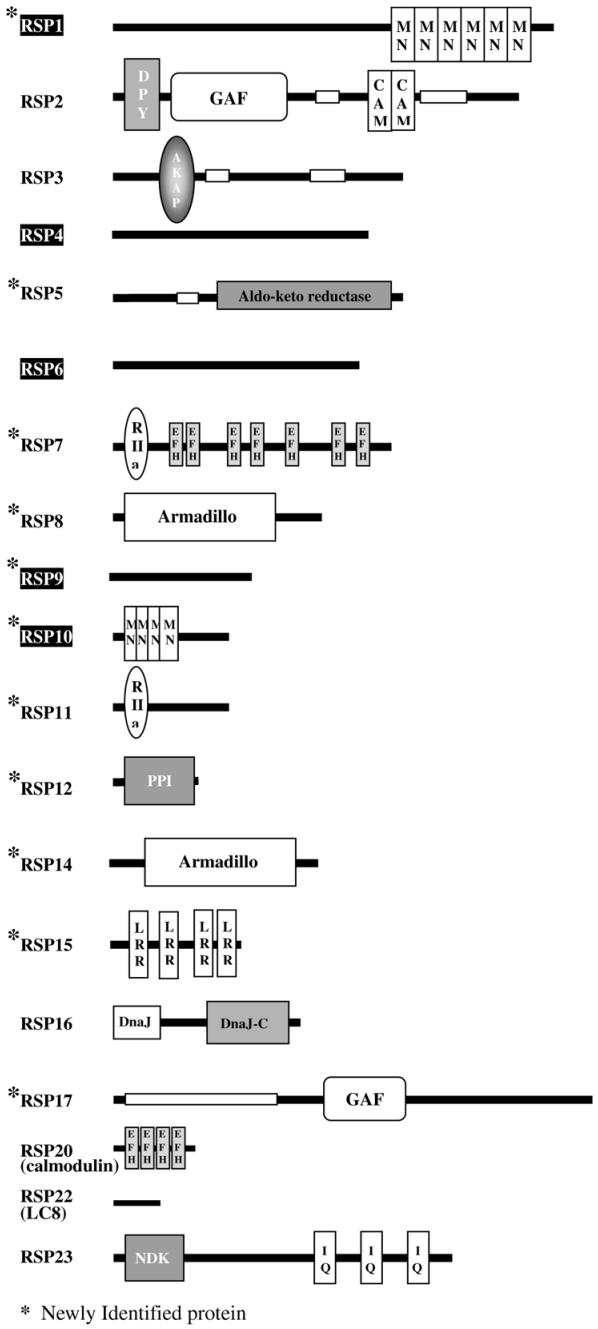

Fig 4.

Predicted domain architecture in RSPs. MN, MORN domain; DPY, Dpy-30 motif; GAF, cyclic GMP, adenylyl cyclase, FhlA domain; CAM, 1-8-14 calmodulin-binding motif; AKAP, A-kinase anchoring protein motif; RIIa, RII alpha motif; EFH, EF-hand domain; PPI, peptidyl-prolyl isomerase motif; LRR, leucine-rich repeat; DnaJ and DnaJ-C, DnaJ-J and DnaJ-C molecular chaperone homology domains; NDK, nucleotide diphosphate kinase domain; IQ, IQ calmodulin-binding motif. Coiled-coil domains are indicated by an open bar. Spoke head proteins are indicated by white characters on a black background.

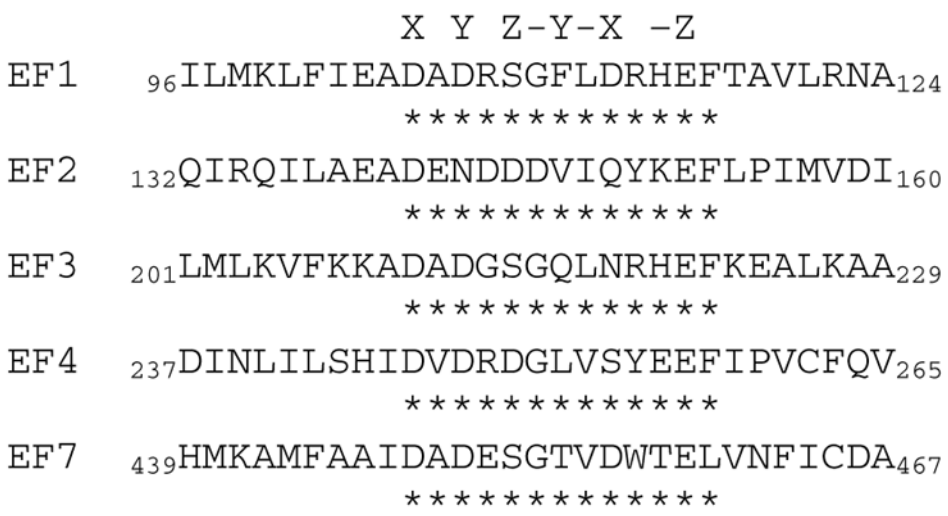

Fig 5.

The five canonical EF-hand motifs in RSP7. An asterisk indicates a precise match to the Ca^{2+}-binding loop consensus sequence D-x-[DNS]-{ILVFYW}-[DENSTG]-[DNQGHRK]-{GP}-[LIVMC]-[DENQSTAGC]-x(2)-[DE]-[LIVMFYW], where x = any amino acid, [ ] = either/or, and { } = any amino acid except those in brackets (PROSITE accession number PS00018). The Ca^{2+} ligands that contribute oxygen atoms are denoted as X, Y, Z, −Y, −X and −Z.

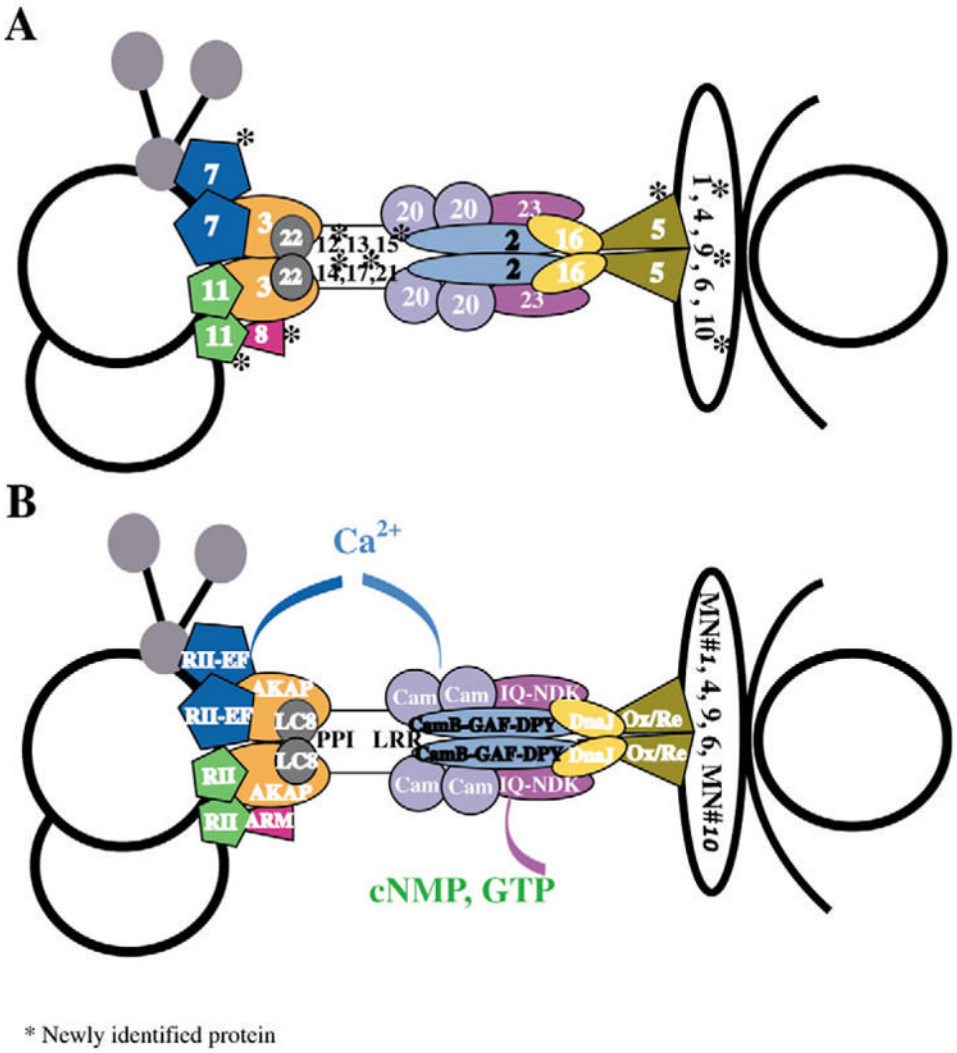

Fig 6.

Model for radial spoke structure. The diagram illustrates the probable locations of the RSPs (A) and their molecular modules (B) relative to a central pair microtubule (right) and an inner dynein arm on an outer doublet microtubule (left). LC8, dynein light chain 8; MN, MORN domain Ox/Re, aldo-keto reductase domain. The locations of the RSPs are based on studies of mutants (Huang et al., 1981;Patel-King et al., 2004;Yang et al., 2005) and chemical dissection of the spoke (Piperno et al., 1981); RSPs 18 and 19 are not included because of insufficient experimental evidence to locate them within the radial spoke. The stoichiometry of subunits is speculative but is based on the homodimerization of RSPs 3, 16 and 22 (Benashski et al., 1997;Yang et al., 2005) (W.S. and P.Y., unpublished data), the presence of homodimerization domains in RSPs 2, 7 and 11, and other data discussed in Yang et al. (Yang et al., 2004).

Table 1

Chlamydomonas RSPs and predicted human homologs

RSP*	Number of peptides or Ref.†	pI/M_r‡ (2D-NEPHGE)	pI/M_r (theoretical)	JGI v. 2.0 gene model or NCBI I.D.§	Linkage group (and mutant)	Similar to H.s. I.D.¶	H.s. Entrez Gene I.D., chromosomal location	NCBI-BlastP result			
								aligned region (a.a. no.)	% identity	% positive	E value
Newly identified RSPs (see Fig. 2 for peptide and protein sequences)											
RSP1	11	5.2/123	4.55/78.6	C_120055	III	NP_543136 (meichroacidin/ TSGA2)	89765, 21q22.3	568-708	40	50	3e-21
RSP5	2	5.1/69	4.68/55.9	C_70095	XII/XIII	NI					
RSP7	3	5.1/58	4.54/55	C_100190	VII	NI					
RSP8	7	6.5/40	5.76/40.5	C_50211	II	NI					
RSP9	3	5.7/26	5.02/29.5	C_450112	VII (*pf*17)	NP_689945 (C6orf206)	221421, 6p21.1	7-269	29	45	1e-18
RSP10	2	5.6/24	5.02/23.5	C_900027	I	NP_543136 (TSGA2)	89765, 21q22.3	12-149	40	55	5e-25
						NP_775836	222967, 7p22.2	45-211	28	47	2e-18
RSP11	2	4.8/22	4.53/21.5	C_830019	X (*pf*25)	NP_114122 (ASP)	83853, 5p15.31	4-90	36	51	9e-07
RSP12	6	6.3/20	5.46/19.7	C_20323	II	NP_775943 (PPIL6)	285755, 6q21	2-165	38	54	1e-24
RSP14	2	6.8/41	5.84/28.3	C_240045	XV	NI					
RSP17	3	6.2/124	5.56/98.5	C_420011	VII	NI					
Previously identified RSPs											
RSP2	Yang et al., 2004	5.0/118	4.48/77.4	AAQ92371	X (*pf*24)	NI					
RSP3	Williams et al., 1989	5.5/86	4.85/56.8	P12759	VI (*pf*14)	NP_114130 (RSHL2)	83861, 6q25.3	11-306	47	66	2e-54
RSP4	Curry et al., 1992	5.1/76	4.58/49.8	A44498	V (pf1)	XP_294004 (RSHL3)	345895, 6q22	6-425	32	48	4e-35
						NP_110412 (RSHL1)	81492, 19q13	6-463	32	45	2e-36
RSP6	Curry et al., 1992	5.1/67	4.53/48.8	B44498	V (*pf*26)	NP_110412 (RSHL1)	81492, 19q13	5-426	29	43	2e-45
						XP_294004 (RSHL3)	345895, 6q22	5-426	30	44	6e-45
RSP16	Yang et al., 2005	7.1/34	6.78/39.0	C_490039	XII/XIII	NP_705842 (TSARG6)	374407, 11q13.3	4-311	41	57	6e-54
						NP_006136 (DNAJB1)	3337, 19p13.2	4-306	38	54	1e-56
RSP20 (calmodulin)	Zimmer et al., 1988 Yang et al., 2001	4.3/18	4.3/18.3	AAA33083	III	Multiple	Multiple				
RSP22 (LC8)	King et al., 1995 Yang et al., 2001	6.8/8	6.89/10.3	Q39580	III	NP_542408 (Dlc2)	140735, 13q23.2	7-91	94	98	2e-39
RSP23	Patel-King et al., 2004	5.4/102	4.67/61	AY452667	XVIII	NP_003542 (NME5)	8382, 5q31	4-202	43	62	4e-34
Incompletely characterized RSPs											
RSP13		6.3/98		NI							
RSP15	1	5.7/38		Bonus_Scaffold_7104**		NI					
RSP18	1	5.4/210		C_30269 to C_30270		NI					
RSP19 (β-tubulin)	6	5.5/140	4.82/49.6	P04690		Multiple	Multiple				
RSP21		6.2/16		NI							

*Radial spoke head proteins are indicated by bold characters.

[†] The number of peptide hits (see Fig. 2) is given for each newly identified protein; the reference is given for each previously identified protein.

[‡] pI and M_r are from Pipemo et al. (1981),Yang et al. (2001), and Patel-King et al. (2004).

[§] The JGI v. 2.0 gene model is given for each newly identified protein; the NCBI identification number is given for each previously identified protein.

[¶] H.s., *Homo sapiens*; NI, none identified.

[**] A more complete sequence for RSP15 is predicted by the gene model Genie.2667.0 in JGI v. 1.0.