1-1-2006

The Flagellar Motility of *Chlamydomonas pf25* Mutant Lacking an AKAP-binding Protein Is Overtly Sensitive to Medium Conditions

Chun Yang  
*Marquette University*

Pinfen Yang  
*Marquette University, pinfen.yang@marquette.edu*

The Flagellar Motility of Chlamydomonas pf25 Mutant Lacking an AKAP-binding Protein Is Overtly Sensitive to Medium Conditions

Chun Yang and Pinfen Yang

Department of Biological Sciences, Marquette University, Milwaukee WI 53233

Submitted July 14, 2005; Revised October 17, 2005; Accepted October 25, 2005
Monitoring Editor: Yixian Zheng

Radial spokes are a conserved axonemal structural complex postulated to regulate the motility of 9 + 2 cilia and flagella via a network of phosphoenzymes and regulatory proteins. Consistently, a Chlamydomonas radial spoke protein, RSP3, has been identified by RII overlays as an A-kinase anchoring protein (AKAP) that localizes the cAMP-dependent protein kinase (PKA) holoenzyme by binding to the RIIa domain of PKA RII subunit. However, the highly conserved docking domain of PKA is also found in the N termini of several AKAP-binding proteins unrelated to PKA as well as a 24-kDa novel spoke protein, RSP11. Here, we report that RSP11 binds to RSP3 directly in vitro and colocalizes with RSP3 toward the spoke base near outer doublets and dynein motors in axonemes. Importantly, RSP11 mutant pf25 displays a spectrum of motility, from paralysis with flaccid or twitching flagella as other spoke mutants to wild-typelike swimming. The wide range of motility changes reversibly depending on the condition of liquid media without replacing defective proteins. We postulate that radial spokes use the RIIa/AKAP module to regulate ciliary and flagellar beating; absence of the spoke RIIa protein exposes a medium-sensitive regulatory mechanism that is not obvious in wild-type Chlamydomonas.

INTRODUCTION

The oscillatory beating of 9 + 2 cilia and flagella is tightly regulated (reviewed by Smith and Yang, 2004). However, the molecular basis of the regulation remains to be elucidated. Chlamydomonas mutants have been invaluable to address this challenge with unique approaches. Mutations in genes encoding dynein motors and the adjacent dynein regulatory complex suppress the paralysis of mutants defective in radial spokes or central pair, leading to the hypothesis that central pair and radial spokes constitute a system that controls the dynein-driven motility (Huang et al., 1982). The control system may determine the preferential sliding among the nine outer doublets (Mitchell, 2003; Wargo and Smith, 2003) and is involved in motility changes mediated by second messengers and phosphoenzymes (reviewed by Porter and Sale, 2000). In particular, cAMP-dependent protein kinase (PKA) and calcium/calmodulin-dependent protein kinases are anchored to axonemes, and the heightened kinase activities in the mutant axonemes are correlated with paralyzed flagella and inhibited motor activities (Howard et al., 1994; Habermacher and Sale, 1997; King and Dutcher, 1997; Smith, 2002; Hendrickson et al., 2004).

The coupling of second messengers, phosphoenzymes, and the control system is further illuminated by the identification of two A-kinase anchoring proteins (AKAPs), one each at radial spokes and central pair apparatus, by a comprehensive RII overlay (Gaillard et al., 2001). This well-established method using RII, the regulatory subunit of PKA as a probe, is credited for identifying numerous AKAPs (Bregman et al., 1989; reviewed by Pawson and Scott, 2005) that dock PKA as well as other signaling molecules along the molecular scaffolds, possibly for specific and integrated regulation (Dell’Acqua et al., 2002; Malbon et al., 2004; reviewed by Wong and Scott, 2004). Anchoring of PKA, the central tenant of AKAPs, is achieved by the hydrophobic association of an amphipathic region in AKAPs with RIIa domain at the N terminus of RII. The spoke AKAP that the RII probe binds to is radial spoke protein (RSP) 3, ideally located at the base of radial spoke for anchoring the structural complex to outer doublets (Dieren et al., 1993) and near dynein motors and presumably for targeting PKA regulatory pathways as well. The simplest interpretation is that RSP3 directs the cAMP-sensitive phosphoenzymes near dynein motors to locally change ciliary and flagellar motility (Gaillard et al., 2001).

However, in addition to PKA RII, certain AKAPs may interact with non-RII proteins that share the RIIa domain known for dimerization and docking to AKAPs via hydrophobic interaction (Newlon et al., 2001). A domain similar to RIIa is present in RI, an isoform of RII and binds the amphipathic helix of AKAPs as well, albeit at a lower affinity (Banky et al., 2000). Phenotypes of RI and RII knockout mice reveal distinct functions of RI and RII (reviewed by Amieux and McKnight, 2002). Most intriguingly, inhibitors of PKA enzymatic activity and the peptidase that perturb the anchorage of the regulatory subunit affect sperm flagellar motility differently (Vijayaraghavan et al., 1997). These findings suggest that the functions of some AKAPs, at least in mammalian sperm, are independent to RII or PKA.

This prediction is substantiated by the finding of non-PKA proteins that contain an RIiA domain at their N termini. ASP, ropporin, CABYR, and SP17 are abundant in testis, sperm, and the flagellar compartment and bind sperm AKAPs in...
vitro (Carr et al., 2001; Naaby-Hansen et al., 2002; Lea et al.,
2004; reviewed by Eddy et al., 2003). Intriguingly, they do not
contain the signature cAMP-binding domains that mediate
cAMP-dependent allosteric regulation of the holoenzyme.
Rather, yeast two-hybrid system shows that the C terminus of ropporin associates with rhophilin (Fujita et al.,
2000), a Rho-binding protein postulated to be a cytoskeletal
target protein for the small GTPase (Nakamura et al., 1999),
whereas CABYR and SPI17 contain calcium-signaling related
modules (Richardson, 1994; Naaby-Hansen et al., 2002).
The physiological partners and functions of these non-PKA RNA proteins remains to be established.
Given the presence of multiple RNA proteins, particularly in flagella, it is central to determine experimentally the physiologi-
ical binding partner of the spoke AKAP to elucidate the functional mechanism of radial spokes and the spoke AKAP.
Recently, a systematic characterization of new spoke proteins revealed that the 24-kDa RSP11 is a non-PKA RNA protein (submission). The RSP11 mutant pF25 has been identi-
fied previously by dikaryon rescue that cytoplasmic compo-
nents from wild-type cells complement the mutant gene product in mating heterozygotes (Huang et al., 1981). P25 is unique compared with the other spoke mutants that the assembly of the macromolecular complex is affected leading to deficiency of multiple spoke proteins, gross morphologi-
ical defect and paralyzed flagella. In contrast, pF25 lacks the 24-kDa RSP11 and has reduced amount of the 40-kDa RPS8, whereas the ultrastructure and protein composition are largely unaffected. Importantly, pF25 described as swimming actively but in an abnormal manner provides a rare oppor-
tunity to study the functional mechanism of the non-PKA RNA proteins and radial spokes. Here, we describe the cloning of RSP11 gene and present both in vitro and in vivo evidence suggesting that radial spokes use non-PKA RNA/ AKAP module for the control of ciliary and flagellar beating.

MATERIALS AND METHODS

Strains and Culture Conditions

Chlamydomonas reinhardtii strains used in this report include wild-type strains (cc124(--), cc125(+), cc620(-), and cc621(--)); the defined radial spoke mu-
tants pF14, pF17, pF24 and the available pF25 alleles pF25(+), pF25(-) pA25 and pF25D (Huang et al., 1981), and the central pair mutants pF6 and pF9. These strains are acquired from the Chlamydomonas Genetics Center (Duke Universi-
ity, Durham, NC). The pF29/p30 strain lacks both the 20S outer arm dynein and inner arm dynein I as described previously (Pierno et al., 1990) and was used for purification of 20S wild-type radial spokes. All cells were grown in liquid modified medium I under aerated phototrophic growth condi-
tion and a 14/10 light/dark cycle (Witman, 1986).

Biochemistry

Axonemal Fractionation. Preparation of axonemes and K1 axonemal extract; velocity sedimentation of K1 extract on sucrose gradients and two-dimen-
sional (2-D) electrophoresis of nonequilibrium isoelectric focusing followed by SDS-PAGE (nonequilibrium pH gel electrophoresis) were carried out as described previously (Yang et al., 2001). Blue native-PAGE was performed as described previously (Yang et al., 2005) except that the zygotic mixture was plated on 2.5% agar plate and 419–432) except that the zygotic mixture was plated into pBlueScript II KS (Stratagene). The subclone containing RSP11 gene was confirmed by restric-
tion digest and PCR. For transformation, pF25 cells were treated with autolysi-
s 105 cells/ml for 1–2 h until ~50% cell lyses by 0.5% NP-40 treatment. Cells were gently spun down and resuspended in TAP medium at 1 × 109 cell/ml (Harris, 1988). One microgram of NotI subclone plasmid (or ~5 μg of BAC DNA), 0.5 μg of pF103 (Chlamydomonas Genetics Center), 300 μg of glass beads, and 100 μl of 20% PEG 8000 were added into 0.3 ml of cells, and the mixture was vortexed at speed 8 (Mini-Vortexer; VWR, West Chester, PA) for 45 s. The cell supernatant was removed after addition of 10 ml of TAP medium. Cell pellets were resuspended in 5 ml of TAP medium, shaken gently under bright light for 24 h, and then plated on paromomycin (10 μg/ml)/TAP plates. Colonies forming in 4–5 d were streaked on TAP plates for motility evaluation under light microscope.

Backcross. Mating and tetrad analysis of pF25(–) cells with wild type strain cc124(–). S. platensis was cultured as described previously (Harris, 2000; 171–173 and 419–432) except that the zygotic mixture was plated on 2.5% agar plate (A-7921; Sigma-Aldrich) for maturation and tetrad dissection.

Motility Assessment

The percentage of swimming cells was determined by observing cell culture placed in slide chambers with three layers of Scotch tapes between slide and cover slide at 400× magnification with the Olympus compound microscope BH-2. Focal plane was centered between glass surfaces. Swimmers and cells with twitching flagella in random chosen fields were counted as separate categories. The cells that stuck to glass surface were not counted. Each of the data was derived from at least 500 cells from more than five randomly selected fields.

For imaging, cultured cells were observed at 400× magnification using Nikon Eclipse E600W compound microscope. The bright-field images were digitally captured using CoolSNAP-ES digital monochrome CCD camera (Photometrics, Tucson, AZ) and the stream mode of MetaMorph imaging system, version 6.1r5 (Molecular Devices, Sunnyvale, CA). For measurement of velocity, time-lapse images were taken at a rate of 10 frames/s for 20 s. Individual cell was tracked by MetaMorph software, and the mean velocity was derived from 20 to 30 individual swimming cells tracked from 30 to 40 sequential images at least. Statistical software program SPSS 10.0 for Win-
dows (SPSS, Chicago, IL) was used to compare the velocities.

Antibodies

Anti-RSP11 and anti-RSP8 rabbit polyclonal antibodies were raised against Ni-NTA-purified recombinant RSP11–6 His and a synthetic cysteine tagged-
RSP8 C-terminal peptide (C-DYRVHVDLPKTFIQAK). The rabbit antibodies against recombinant RSP16 and purified RSP3 from axoneme were described previously (Williams et al., 1989; Yang et al., 2005).
RESULTS

Identification of RSP11 Gene

To study RSP11 and the intriguing phenotype of RSP11 mutant pf25, we first cloned the corresponding cDNA. The cloning strategy is illustrated in Figure 1A. RSP11 was spot purified from the 2-D gel of isolated radial spokes (Yang et al., 2001) and subjected to tandem mass spectrometry. The resulting two peptide sequences were used to identify expressed sequence tag (EST) clones available in the National Center of Biotechnology Information database and the gene in *Chlamydomonas* genome v.2, C_830019. Comparison of the overlapping EST clones and genomic sequence showed that the gene including the flanking untranslated region consists of five exons and four introns. The theoretical pI/molecular weight of the predicted protein is 4.5/21.5, consistent to RSP11 spot in 2-D gel (Piperno et al., 1981; Yang et al., 2001). PCR mapping using 3′ untranslated region (UTR) sequence indicated that RSP11 gene is located distal to molecular markers GP52 and CNA26 on linkage group X, as anticipated for PF25 (http://www.chlamy.org/BAC/LG10.htm; Kathir et al., 2003).

To confirm that PF25 encodes RSP11, transformation rescue of pf25 was carried out. First, BlastN search of BAC end sequences using RSP11 gene (C_830019) flanking sequences in scaffold _83 of *Chlamydomonas* genome v.2 (http://genome.jgi-psf.org/chlre2/chlre2.home.html) identified BAC clone #1L24 containing the entire RSP11 gene. Second, the purified BAC DNA was cotransformed into pf25A along with pSi103 that confers paromomycin resistance (Sizova et al., 2001). Among 74 antibiotic-resistant transformants, four different structure prediction programs revealed a 3-strand A beta-turn-helix in the N terminus of RSP11 (bold e and h, Figure 1B).

To independently test that the new protein is a radial spoke protein, recombinant protein was synthesized for raising antibodies. The coding sequence was amplified by reverse transcription (RT)-PCR and inserted in frame into the pET28(a) vector for the expression of a recombinant protein with a C-terminal 6-His tag. The construct was confirmed by restriction digestion and sequencing. The expression and purification were evaluated by Coomassie protein gel (Figure 1C, left). An abundant ∼25-kDa His-tagged protein (arrowhead) was soluble in the IPTG-induced bacterial extract (pre) and was removed from the extract by Ni-affinity chromatography (post) and occurred in the imidazole elution buffer (Elute). The polyclonal antibody raised against the recombinant protein recognized a 24-kDa band that was present in wild-type axonemes but absent in pf25 (Figure 1C, right; compare protein stain and Western of the same blot).

Sequence Analysis of RSP11 and RIIa Proteins

Motif search using Web-based SMART program (Letunic et al., 2004) revealed a RIa domain at the N-terminal 11–53 aa of predicted RSP11 (Figure 2). Blastp homology search with default parameters revealed this region is highly homologous to the N terminus of the RI subunit of PKA as well as −20-kDa proteins from diverse organisms such as the protozoan parasite *Giardia* (GL), *Caenorhabditis elegans* (CE), zebra fish (DR), and mammals. The proteins from the last category include AKAP-binding sperm protein (ASP), ropporin (Fujita et al., 2000; Carr et al., 2001) as well as SP17 (Richardson et al., 1994). Homology of the predicted RSP11 with RI was not detected by Blastp. To illustrate the homology, the N-terminal sequences from representative proteins were aligned by Multiple Sequence Alignment and ClustalW (Figure 2A). The hallmark two helices in the RIa domain (dashed underline) as well as the preceding β-strand (double underline) are based on NMR structural study of RI (Newlon et al., 2001). Consistently, PHD and other secondary structure prediction ensembles revealed a β-sandwich followed by helix-turn-helix in the N terminus of RSP11 (bold e and h, Figure 2B).

Among the RIa proteins, the extreme N terminus of RSP11 was particularly homologous to ASP and the unknown fish and *Giardia* protein (arrow and overline, Figure 2A). Six of the nine residues were identical or similar, suggesting closer homology among the N termini of these four proteins. Sequence alignment of these proteins and ropporin showed that the proteins from vertebrates are homologous to each other largely through out the entire length of the molecules. However, the C termini of *Giardia* protein and RSP11 diverged significantly (Figure 2C).

Colocalization of RIa Protein and AKAP in Radial Spokes

To test the association of RSP11 and RSP3, the RI-binding AKAP, we analyzed the axonemes of spoke mutants by Western blots. The spoke mutants pf14, pf24, and pf17 have different levels of structural and biochemical defects (Figure 3A), allowing localization of spoke proteins (Piperno et al., 1981; Yang et al., 2005; reviewed by Curry and Rosenbaum, 1993). Control antibodies for RSP3 and RSP16 (gray asterisks) confirmed the mutant strains. As anticipated, all three proteins were present in wild-type and central pair mutants pf6 and pf19 but absent in pf14. RSP11 was also absent in pf25, whereas RSP3 and RSP16 were normal (Figure 3B).

The detailed location of RSP11 is revealed by comparing RSP11 with RSP3 and RSP16 in pf24 (Figure 3, A and B). pf24 has a polymorphic defect encompassing spokehead and the adjacent stalk area, but the basal end of the spokesstalk was normal (Yang et al., 2005; Figure 3A) as reflected by normal RSP3 and diminished RSP16 (Figure 3B, arrowhead). As for spoke AKAP but unlike RSP16, RSP11 was present at wild-type level in pf24. To further test the colocalization, pf24 spikes were extracted by Ki and fractionated by sucrose gradient velocity sedimentation. The peak spoke fraction was separated by native gel electrophoresis followed by 2-D SDS-PAGE and detected by Westerns. Compared with single intact spoke from dynein mutant (pf28sp30) and spokesstalk from pf17, the polymorphic pf24 spoke particles revealed by RSP3 immunoblot were fractionated into three smaller particles (Yang et al., 2005). Importantly, RSP11 always colocalized with RSP3, including in the smallest par-
The original first methionine is underlined. The frame for a distinct polypeptide of 72-amino acid residues (bold).

Ni-NTA affinity chromatography (post). The bound protein is eluted from the affinity matrix by imidazole buffer (Elute). Western analysis shows that the antibody raised against the recombinant RSP11–6His and GST-RSP3 were cotransformed into bacteria. Bacterial supernatant was subjected to Ni-NTA affinity purification (Figure 5B, left). The control group was transformed with the GST-RSP3 construct only (Figure 5B, right). The recombinant GST-RSP3 and RSP11–6His, revealed by Coomassie protein gel, were soluble in the extract, effectively depleted in the flow through of the Ni-NTA matrix, and copurified in the matrix fraction (Figure 5B, left, compare Pre, Post, and Ni-NTA). In contrast, there was no obvious binding of GST-RSP3 to Ni-NTA in the control lacking RSP11–6His (Figure 4B, right). GST alone also did not copurify with RSP11–6His (our unpublished data). Chemical cross-linking that demonstrated direct interaction of several axonemal proteins (Yang et al., 2001, 2005) cannot definitively reveal the interaction of RSP11 and RSP3, possibly because of the hydrophobic interaction between RIIa domain and the amphipathic helix.

Unusual Motility Phenotype of RSP11 Mutant pf25

To elucidate the function of RSP11, we investigated the motility phenotype of pf25 that was described as “swim...
actively, but in an abnormal manner" (Huang et al., 1981). Surprisingly, we initially could not distinguish wild-type and \( pf_25 \) cells taken from 8-l culture meant for axonemal preparation (Figure 3B) under light microscopy. However, careful observation revealed that a few cells have paralyzed or twitching flagella. The numbers of paralyzed cells varied among different preparations. To determine whether the mixed motility is limited to the particular allele or caused by spontaneous mutation, we examined the three available alleles, \( pf_25A, D, \) and \( F \), that were also generated by chemical mutagens and that had been backcrossed to wild type previously (Huang et al., 1981). Consistently, all of the \( pf_25 \) alleles lacked RSP11 (Figure 6A) and displayed mixed motility. Furthermore, we backcrossed \( pf_25(-) \) again with wild-type strain cc125 (+). Among the 11 sets of tetrads, as illustrated in the representative Westerns, the ratio of \( pf_25 \) and wild-type motility phenotype was 1:1, and the former always cosegregated with RSP11 deficiency, whereas RSP16 was normal (Figure 6B). Thus, the analyses of four independent alleles and backcrossed progeny strongly indicate the mixed flagellar motility of \( pf_25 \) is because of defective RSP11 gene.

To determine when the mixed motility develops, \( pf_25 \) cells were observed several times daily after the initial inoculation. \( pf_25 \) and wild-type cells generated full-length flagella within 2 h once cells maintained on agar plates were resuspended in liquid media. Interestingly, the flagella of freshly resuspended \( pf_25 \) cells predominantly were paralyzed or twitching like other radial spoke mutants. Occasionally, one or two cells were found tumbling or actively swimming. The second day, most cells had twitching flagella. Some even could circle or rotate along the longer axis of the cell body. More cells were able to swim in the signature helical pattern as wild type until the fourth day with cell density of \( 1 \times 10^6 \) cells/ml. The variation of flagellar motility is illustrated by the three panels of images taken within 1.1 s (Figure 7, live image is shown by videomicroscopy as Supplemental Material). The twitching flagella and the swimming cells are highlighted with black and white arrowheads, respectively. The trend of improvement is illustrated by increased percentage of swimmers and reduced percentage of cells with twitching flagella over the 4-d period (Figure 8A). Similar results were obtained from 16 independent cultures derived from single colonies or by using the better buffered TAP media (Tris-acetate-phosphate buffer listed in Harris, 1988) (our unpublished data). The changes were not as obvious in the same day as they were overnight.
To test whether the acquired motility was sustained, two 4-d-old cultures were continued. Surprisingly, percentage of swimmers was reduced dramatically as the culture reached stationary phase the next day, even though the flagellar length was similar (Figure 8B, white bar). On the contrary, the control p25 cells, transferred into equal amount of fresh media daily, retained the motility better (black bar). Importantly, wild-type (WT) cells cultured side by side under the same condition swim constantly (our unpublished data).

To test that medium condition determined the overall motility, a 4-d-old 300-ml culture was divided into three equal aliquots. One pellet was resuspended back into the medium from which the cells were harvested, whereas the other two pellets were transferred to fresh medium or spent medium in which paralyzed cells from the prolonged culture had been removed. Again, 1 d later, a higher percentage of cells in the fresh medium were swimming, whereas most cells in the exhausted medium were paralyzed (Figure 8C).

To investigate another allele independently and to test whether p25 cells swim slower than wild type, the swimming velocity of p25A was measured. Like p25/ and p25, none of p25A cells in the first day are swimming in the random sampling, but the number of swimmers gradually increased. For those swimming with a helical path, the averaged velocity improved over the next 3 d but slowed down dramatically on the fifth day (Figure 9, hatched bar), consistent to the changes in percentages (Figure 8). In contrast, wild-type cells grown in same condition and similar density always swam, and the velocity did not fluctuate so dramatically (Figure 9, stippled bar). Notably, the velocity of p25 was equivalent to wild-type on the fourth day (Figure 9, compare stippled and solid bars), suggesting that the deficiency in p25 does not significantly compromise mechanical property of p25 axonemes. Similarly, swimmers in day 2 culture can be stopped by 5% viscous ProtoSlow, whereas 10% ProtoSlow is required to stop p25 swimmers on day 3 and slow down p25 swimmers and wild type equally on day 4 (our unpublished data). Together, these results reveal that the motility of p25 in terms of percentages of swimmers and
swimming velocity is overtly sensitive to media conditions, whereas the output potential remains intact.

In light of the well recognized effect of medium on gametic differentiation (Martin and Goodenough, 1975), we tested the hypothesis that the shift of \( pf_{25} \) motility is related to the gametic differentiation induced by nitrogen depletion. The \( pf_{25}(-) \) cells cultured on TAP plate for a week successfully differentiated into gametes that mate with wild-type \( cc_{620}(-) \) once resuspended in nitrogen-depleted medium (Saito et al., 1993). However, no obvious difference in motility was observed between the gametic cells and vegetative control cells that were resuspended in nitrogen-containing medium in a similar manner and could not mate (our unpublished data). Most cells in both groups had flaccid or twitching flagella, whereas a small number of cells could swim actively. This result indicates that “the active swimming in an abnormal manner” of \( pf_{25} \) cells that are prepared as gametic cells freshly resuspended in nitrogen-free medium (Huang et al., 1981) resembles the motility of vegetative cells in nitrogen-containing medium in the first day or two, as shown in this study. Together, the negative results indicate that the medium-dependent shift of motility is not simply because of gametic differentiation.

**Figure 5.** RSP11 associates with RSP3, the spoke AKAP. (A) An overlay assay using RSP11-6 His as a probe shows that RSP11 binds an 83-kDa protein in axonemes prepared from various strains except the RSP3 mutant, \( pf_{14} \) (left). The probe is revealed by anti-His and anti-mouse antibodies. The 83-kDa protein comigrates with RSP3 is revealed by Western blot of axoneme samples (right). (B) Pull-down assay. GST-RSP3 (arrowheads) and RSP11–6 His (double arrowheads) are present in the supernatant (pre) of bacteria transformed with both constructs (left). Both proteins are effectively depleted in the flow through (post) after Ni-NTA chromatography. Pull down of the His-tagged protein results in the copurification of GST-RSP3. In contrast, no specific binding to Ni-NTA is detectable in the control group that expresses GST-RSP3 only (right).

**Figure 6.** \( Pf_{25} \) motility defect is tightly linked to the absence of RSP11. (A) Western analysis of axonemes reveals that RSP11 is absent in all of the \( pf_{25} \) alleles but normal in wild type and dynein mutant \( pf_{28}p30 \). (B) Western blot and motility study of tetrad meiotic progeny show a 1:1 ratio of motility phenotypes. The progeny displaying \( pf_{25} \) motility phenotypes always lack RSP11. RSP16 Western is a control. Two representatives of the 11 complete tetrad groups are shown.

**Figure 7.** \( Pf_{25} \) cells in the same culture demonstrate different motility level. Three panels of bright-field microscopy taken at the time indicated in seconds at bottom right corner show that one cell is swimming (clear arrowhead), whereas the other has twitching flagella (black arrowheads). The flagella of the other two cells stuck to glass are out of focus. Motion picture that includes the three panels is shown in the video in the Supplemental Material.
To test whether the altered motility is because of the restored proteins or phosphorylation, axonemes were prepared from paralyzed or twitching cells freshly resuspended from agar plates or from a 4-d-old liquid culture containing \( /\text{H}11022\) 70% swimmers. Compared with wild-type axonemes, RSP11 is absent and RSP8 is reduced in \( pf_{25}\) as stated by Huang et al. (1981), whereas the amount of RSP16 is normal (Figure 10A, compare first two lanes). However, there was no significant difference between the two groups of \( pf_{25}\) cells (Figure 10A, compare the second and third lanes). Phosphorylation cannot account for the \( pf_{25}\) motility phenotype either (Figure 10B; Huang et al., 1981). Two major phosphorylated proteins, RSP2 and 3, were predominantly phosphorylated in wild type, paralyzed \( pf_{25}\), and swimming \( pf_{25}\). In contrast, more dephosphorylated RSP3 (arrowhead) was

To test whether the altered motility is because of the restored proteins or phosphorylation state of radial spokes, axonemes prepared from control strains and freshly resuspended \( pf_{25}\) cells with twitching flagella (tw) and \( pf_{25}\) liquid culture with \( >70\%\) swimmers (sw) are evaluated by Western blots. (A) Neither RSP11 nor RSP8 is restored in the swimming \( pf_{25}\) cells. Compared with wild type, RSP8 and RSP11 in the axonemes of \( pf_{25A}\) are reduced and undetectable, respectively. However, there is no significant difference between twitching and swimming \( pf_{25}\) cells. RSP16 western is a control. (B) Two major phosphorylated spoke proteins, RSP2 and 3 in the two \( pf_{25}\) samples are not significantly different from those in wild type. In contrast, RSP3 is more dephosphorylated (arrowhead), and both RSP2 and RSP3 are reduced in \( pf_{27}\) as shown previously (Huang et al., 1981). Western blot of dynein IC140 shows the protein loading level.
present in p25 that has paralyzed flagella and defective phosphorylation and assembly of radial spokes (Huang et al., 1981).

DISCUSSION

This study demonstrates that RSP11 gene encodes a new RIIα protein that associates with RSP3 at the basal end of spokes. These findings strongly support previous identification of RSP3 as an AKAP (Gaillard et al., 2001) albeit the RIIα protein is not RII of PKA. Intriguingly, RSP11 mutant displays dramatic switches of motility phenotypes as a function of medium conditions, indicating that the spoke RIIα protein is essential for normal flagellar beating and possibly for regulation. These findings shed new light on the complexity of the regulatory mechanisms mediated by radial spokes.

RSP11, RSP3, and the Regulatory Mechanism of Radial Spokes

RSP11 is unequivocally an RIIα protein and p25 is an RSP11 mutant based on the following evidence. 1) The sequence founded on mass spectrometry polypeptides from spot-purified RSP11 of isolated radial spokes predicts an RIIα protein consistent to the size and pI of RSP11. 2) Mapping with 3′ UTR sequence indicates that RSP11 gene is located distal to molecular markers GP52 and CNA26 in the linkage group X, consistent with the predicted location of pF25. 3) RSP11 gene rescues p25. 4) Antibodies raised against the recombinant RIIα protein recognize a 24-kDa protein that is present in all of the strains tested except pF14 and p25. 5) Sequencing of genomic DNA reveals a point mutation at 5′ UTR of p25A. 6) Dikaryon rescue of p25 shows that p25 cells synthesize all of the spoke proteins except RSP11 (Huang et al., 1981). As predicted, RSP11 binds to spoke AKAP. Mutant analyses and biochemical extraction further indicates that both proteins colocalize toward the base of spokes toward in axonemes (Figures 3 and 5).

The finding of non-PKA RIIα protein in radial spokes is unexpected. Diverse evidence suggests that radial spokes control dynein activity through phosphoryzymes, including PKA (reviewed by Porter and Sale, 2000), and RSP3 binds PKA RII in vitro (Gaillard et al., 2001). Rather, this study demonstrated that RSP3 binds RSP11, a non-PKA RIIα protein and that phosphorylation seems unrelated to p25 abnormally regulated motility, suggesting that RSP3/RSP11 mediate a non-PKA regulatory mechanism. One interpretation is that RSP3 does not bind PKA or mediate cAMP-dependent regulation and that PKA pathway is mediated by other mechanisms.

However, equally possible is that in addition to RSP11, RSP3 may bind RII and/or other RIIα proteins. Dual-specific AKAPs interact with multiple RIIα proteins. Emerging evidence indicates that the association could be reversible and modulated. For example, fibrous sheath AKAP3 binds RII, RPI, CAYYR, SP17, ASP, and ropporin (Carr et al., 2001; Naaby-Hansen et al., 2002; Lea et al., 2004; reviewed by Eddy et al., 2003), and phosphorylation increases its binding to RIIα and PKA recruitment (Luconi et al., 2004). In addition, the RIIα domains from various proteins are not equivalent in AKAP binding. The regions N terminal to RIIα domains in RII and RIIII assume distinct secondary structures (Banky et al., 2000) that may account for the different affinity and binding sites for RII and RII on dual-specific AKAPs (Huang et al., 1997; Burns-Hamuro et al., 2004; Salvador et al., 2004). The RIIα domains of RSP11, ASP, and Giardia protein but not RII share stronger similarity (Figure 2), suggesting that RSP11

may not share the mapped binding sites for RII (Gaillard et al., 2001). It will be interesting to test whether RSP3 is a dual-specific AKAP as well. Both hypotheses support the central concept that RSP3, as a spoke AKAP, anchors regulatory pathways to the spoke complex and near dynein motors (Gaillard et al., 2001).

The Molecular Interaction of RSP11

P25 is the only known spoke mutant that does not have morphological defect and is capable of swimming (Huang et al., 1981). The limited defect in RSP11 and RSP8 indicates that these two constitutive components are not essential for the assembly and the core structure of the T-shaped complex that consists of ~23 proteins (Yang et al., 2001). Rather, they may be peripheral functional components. The indirect overlay and effective pull-down of RSP3 by RSP11 affinity (Figure 5) suggest that the affinity is likely equivalent to RII/AKAP affinity that suffices RII overlays and the purification of an oligomeric complex (Lohmann et al., 1984; Bregman et al., 1989). However, similar to the challenge of studying fibrous sheath AKAPs, the extreme stability of cytoskeletal structure and the central role of RSP3 in the assembly of radial spokes prevent further purification of RSP11/RSP3 complex. Nonetheless, the colocalization of both proteins in the basal part of the spokestalk from p25 mutants (Figure 3) is equivalent to or exceeds the current resolution of immunoprecipitation or immunolocalization of RIIα proteins and AKAPs in flagellar compartments (Carr et al., 2001; Lea et al., 2004; Rawe et al., 2004).

Despite the high affinity, RSP3 may not be the sole protein that harnesses RSP11 to the structural complex. Instead, the reduction of RSP8 in p25 (Figure 10; Huang et al., 1981) suggests that RSP8 interacts with RSP11, most likely its C terminus, as well. The defective interactions among the three proteins likely account for the abnormally regulated motility of p25.

To Beat or Not to Beat of P25 Flagella

The p25 phenotype reveals the functional significance of RSP11 and possibly a novel regulatory mechanism that is not obvious in wild-type Chlamydomonas. The motility of p25 is unique in two aspects. 1) p25 cells display a spectrum of motility in the same culture. The paralysis state resembles the other spoke mutants that have flaccid or twitching flagella correlated with gross structural defects. In contrast, p25 cells originated from the same colony in a few days can swim like wild-type cells and then become paralyzed again. 2) The dramatic swing in overall motility level occurs during regular culture period, depending on how fresh (or how old) the medium is.

To our knowledge, until this study, the flagellar motility of wild-type and mutant strains during the culture period had not changed significantly enough to inspire investigation. Wild-type Chlamydomonas cells seem to swim constantly in a smooth helical path (Harris, 1988) without obvious changes in motility (Figure 9) unless physical stimuli transiently alter flagellar beating (see below). Most of the motility mutants are generated by mutagens, X-rays, or insertional mutagenesis and have stable single phenotypes as anticipated. Mutations in motors or other axonemal complexes are manifested as reduced swimming velocity, different beating patterns, or swimming path or paralysis (Huang et al., 1981; Brokaw and Kamiya, 1987; Perrone et al., 2000). Mutants screened for defective signaling pathways (Pazour et al., 1995) fail to respond to stimuli without affect normal flagellar beating. Regardless of the severities of motility

Vol. 17, January 2006 235

Regulatory Role of Axonemal RIIα/AKAP
defects or nature of mutation, no reports have indicated shift of mixed motility as \( p/25 \).

It is not clear what causes the reversible switches. The switches seem different from the changes induced by second messengers. Light elicits phototaxis or phototroph mediated by transient fluctuation of intraflagellar \( \text{Ca}^{2+} \), maybe partly through radial spokes (Schmidt and Eckert, 1976; Bessen et al., 1980; Brokaw et al., 1982; Kamiya and Witman, 1984). Reagents that increase AMP concentration or cAMP analogues inhibit *Chlamydomonas* flagellar motility, although the physiological relevance is not clear (Rubin and Filner, 1973; Hasegawa et al., 1987). Notably, these reactions occur within milliseconds to seconds, whereas effects of medium on \( p/25 \) cells are more noticeable overnight and are observed in portions of population instead of every cell. Furthermore, switching flagella or tumbling cells seem to be a transitional stage between paralysis and wild-type swimming. Therefore, the regulatory mechanism that becomes obvious in the absence of RSP11 differs from the on-off responses induced by cAMP and calcium. Rather, reactions within cell body may be involved. Consistent to this interpretation, attempts to rescue the paralyzed \( p/25 \) cells by changing concentration of the second messengers and broad-spectrum inhibitors to kinase activated by second messengers have not succeeded. Neither can adjusting pH nor using the stronger buffered TAP medium change \( p/25 \) motility phenotypes (our unpublished data).

Additional mutation or selection of subpopulation of cells is ruled out because of the reversibility, cultures from single colonies and tight correlation of genetic and motility phenotypes among backcrossed progeny (Figures 6–9). Normal ultrastructure and composition, stability of spoke complex (Huang et al., 1981; Figure 4 and 5), and the wild-type-like velocity and smooth helical path strongly suggest that the mechanism of \( p/25 \) radial spokes and axonemes is largely intact. The simplest explanation is that the \( p/25 \) is as capable as wild type in flagellar beating, but an abnormal regulatory mechanism shuts down the beating in the unfavorable conditions. Paradoxically, the gain of sensitivity is because of loss of spoke RIIa protein. Conceivably, the heightened sensitivity could be because of a misplaced regulatory protein that normally associates with RSP11 or RSP8. Or, the regulator normally suppressed by RSP11 becomes active in \( p/25 \) flagella. Alternatively, other proteins that normally do not associate with RSP3 become accessible to RSP11 binding site when RSP11 is absent, albeit with lower affinity. Regardless the detail mechanism, the reversible nature of \( p/25 \) phenotype supports the postulated chemical signal transduction mediated by radial spokes.

**Non-PIA RIIa Proteins**

Previous studies have shown that non-PIA RIIa proteins are abundant in sperm and hence suggest that they are unique to male germ cells (Carr et al., 2001). RSP11/RSP3 complex in the typical feature of 9 + 2 cilia and flagella argues otherwise. Although RSP11 orthologues cannot be identified definitively, EST sequences homologous to RSP3 are present in different tissues in mammals and other organisms, including vertebrates, insects, *Ciona* (Gaillard et al., 2001; Koukoulas et al., 2004), and *Giardia*. The extensive homology suggests that RSP3 is conserved to target the radial spoke (Huang et al., 1981; Diener et al., 1993) as well as RIIa proteins. Thus, RIIa/RSP3 is likely a common feature of 9 + 2 cilia and flagella. In line with this prediction is the detection of one RIIa protein, SP17, in various tissues, notably in cilia as well (Frayne and Hall, 2002; Grizzi et al., 2004).

Despite the divergence, the C termini of RIIa proteins (Figure 2C) may confer special regulatory mechanism tailored for individual cell types. For example, CABYR (fibrousheathin II; AAC35373) may mediate calcium-dependent regulatory pathways via their EF-hand for calcium binding (Naaby-Hansen et al., 2002), whereas SP17 (Q15506) uses its calcium/calmodulin-binding IQ motifs (Lea et al., 2004). The C-terminal peptide of roppornin lacks recognizable modules but still associates with rhophilin, a Rho-binding protein (Nakamura et al., 1999; Fujita et al., 2000). Further study on the C terminus of RSP11 likely will reveal the new regulatory mechanism of radial spokes and nonconventional AKAPs.

The suppressor mutants inspired the hypothesis that the radial spoke is part of a system that controls flagellar motility (Huang et al., 1982). This study provides the in vivo evidence supporting the hypothesis and reveals a spoke non-PIA RIIa protein possibly mediating a new regulatory mechanism in diverse cilia and flagella. The evidence in green algae and the broad presence of non-PIA RIIa proteins sharing the targeting domain of PIA in eukaryotes indicate that they are not evolutionary mishaps or specially evolved for male germ cells. Rather, they should be considered as part of the growing repertoire of the diverse regulatory pathways that AKAPs may integrate.

**ACKNOWLEDGMENTS**

We are grateful to Dr. U. W. Goodenough (Washington University, St. Louis, MO) for advice on gametic differentiation; Nancy Haas and Dr. C. D. Stiffow (University of Minnesota, St. Paul, MN) for mapping RSP1 gene, and Dr. L. W. Tam for advice on transformation and tetrad dissection (University of Minnesota). We also thank Dr. Dennis Diener and J. L. Rosenbaum (Yale University, New Haven, CT) for anti-RSP3 and the expression construct of GST-RSP3. This study is supported by National Institutes of Health Grant GM-068101.

**REFERENCES**


C. Yang and P. Yang


