

Flagellar Radial Spoke Protein 3 Is an A-Kinase Anchoring Protein (AKAP)

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Abstract. Previous physiological and pharmacological experiments have demonstrated that the *Chlamydomonas* flagellar axoneme contains a cAMP-dependent protein kinase (PKA) that regulates axonemal motility and dynein activity. However, the mechanism for anchoring PKA in the axoneme is unknown. Here we test the hypothesis that the axoneme contains an A-kinase anchoring protein (AKAP). By performing RII blot overlays on motility mutants defective for specific axonemal structures, two axonemal AKAPs have been identified: a 240-kD AKAP associated with the central pair apparatus, and a 97-kD AKAP located in the radial spoke stalk. Based on a detailed analysis, we have shown that AKAP97 is radial spoke protein 3 (RSP3). By express-

ing truncated forms of RSP3, we have localized the RII-binding domain to a region between amino acids 144–180. Amino acids 161–180 are homologous with the RII-binding domains of other AKAPs and are predicted to form an amphipathic helix. Amino acid substitution of the central residues of this region (L to P or VL to AA) results in the complete loss of RII binding. RSP3 is located near the inner arm dyneins, where an anchored PKA would be in direct position to modify dynein activity and regulate flagellar motility.

Key words: kinases • cell motility • flagella • dynein • AKAP

Introduction

The goal of this study is to determine the mechanism for localization of the cAMP-dependent protein kinase (PKA)¹ in ciliary and flagellar axonemes. In several experimental systems, cAMP or specific inhibitors of PKA alter motility of ATP-induced reactivated movement of ciliary and flagellar axonemes. For example, cAMP is required for activation of sperm tail axonemes from many species (for reviews see Brokaw, 1987; Tash, 1989; San Agustin and Witman, 1994). Addition of cAMP increases beat frequency of reactivated ciliary axonemes from *Paramecium* (for example, Hamasaki et al., 1989), and inhibits reactivated motility of flagellar axonemes from *Chlamydomonas* (Hasagawa et al., 1987). Biochemical analysis in diverse cellular systems has demonstrated that several axonemal proteins, including dynein subunits, are phosphorylated in vitro in a cAMP-dependent manner (for example, Hamasaki et al., 1991; Inaba et al., 1999; Nomura et al., 2000).

Moreover, selective inhibitors of PKA regulate dynein-driven microtubule sliding in axonemes isolated from *Chlamydomonas* flagella (Howard et al., 1994). Together the data demonstrate that PKA is a structural component of the 9 + 2 axoneme. However, the mechanism for localization and anchoring of PKA is not known.

We proposed that PKA is localized to the axoneme through association with A-kinase anchoring proteins (AKAPs). AKAPs are a family of proteins that target PKA to specific intracellular sites through interaction with type I (RI) or type II (RII) PKA regulatory subunits (for a review see Edwards and Scott, 2000; and others). In most cases, the regulatory subunits bind to AKAPs through interaction with an amphipathic helix contained within the AKAP. AKAPs can often be identified by RII blot overlays in which radiolabeled RII is incubated with blots of protein (Westphal et al., 2000). By this approach, several AKAPs have been identified in flagella of sperm (Johnson et al., 1997; Moss et al., 1999; Vijayaraghavan et al., 1999; Reinton et al., 2000). However, to date none of the AKAPs have been localized to the axoneme.

To test the hypothesis that axonemal PKA is anchored by AKAPs, we used isolated axonemes from *Chlamydomonas*. *Chlamydomonas* offers several experimental advantages

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¹Abbreviations used in this paper: AKAP, A-kinase anchoring protein; GST, glutathione-S-transferase; PKA, cAMP-dependent protein kinase; RSP, radial spoke protein.

including the ease of axoneme isolation and the availability of mutants that are immotile and defective in specific axonemal structures. Based on pharmacological analysis, *Chlamydomonas* axonemal PKA regulates reactivated motility as well as dynein-driven microtubule sliding activity (Hasagawa et al., 1987; Howard et al., 1994). We performed RII overlays in the presence or absence of specific AKAP–RII binding inhibitors to identify two AKAPs in the *Chlamydomonas* axoneme. Through detailed analysis we have determined that one of the AKAPs is radial spoke protein 3 (RSP3). By testing truncated and point mutant forms of RSP3, we have mapped the RII-binding domain of RSP3. RSP3 is the first AKAP to be identified in axonemes and is the first AKAP to be identified from a unicellular organism. This finding provides a foundation for understanding the mechanism of kinase anchoring in the axoneme, and demonstrates that AKAPs may be evolutionarily conserved components of signal transduction in the cell.

Materials and Methods

Chlamydomonas Strains and Growth Conditions

Chlamydomonas reinhardtii strains used include 137c and cc124 (wild-type), *pf14* (lacks radial spoke, paralyzed flagella), *pf17* (lacks radial spoke head, paralyzed flagella), *pf27* (radial spoke phosphorylation defect, paralyzed flagella), *pf15* (lacks central pair apparatus, paralyzed flagella), *pf16* (lacks C1 microtubule of central pair apparatus, paralyzed flagella), *pf18* (lacks central pair apparatus, paralyzed flagella), *pf19* (lacks central pair apparatus, paralyzed flagella), *pf20* (unstable central pair apparatus, paralyzed flagella), *pf6* (lacks projection on C1 microtubule of central pair apparatus, paralyzed flagella), *pf28pf30* (lacks outer dynein arms and I1 inner dynein arms, paralyzed flagella), and *pf2* (defective in the dynein regulatory complex, paralyzed flagella). All strains were obtained from the *Chlamydomonas* Genetics Center (Duke University, Durham, NC) with the exception of *pf28pf30* which was generated by crossing *pf28* with *pf30* (strains obtained from the *Chlamydomonas* Genetics Center). The double mutant was then isolated from a nonparental ditype tetrad. Cells were grown in liquid modified medium I, with aeration and a 14-h/10-h light/dark cycle (Witman, 1986).

Isolation of Axonemes

Axonemes were isolated as described previously (Howard et al., 1994). Where indicated, axonemes (at 5 $\mu\text{g}/\mu\text{l}$) were treated with 0.6 M NaCl (20 min on ice) in a buffer containing 10 mM Hepes, 5 mM MgSO_4 , 1 mM DTT, 0.5 mM EDTA, 0.1 M PMSF, and 0.6 TIU aprotinin, pH 7.4. Before fixation for SDS-PAGE, axonemes treated with 0.6 M NaCl were dialyzed to 30 mM NaCl in the same buffer. Axonemal protein samples were fixed for SDS-PAGE at a concentration of 5 $\mu\text{g}/\mu\text{l}$ (50 μg total for each lane). Protein concentration was determined using the Bradford assay. Unless otherwise stated, all reagents were obtained from Sigma-Aldrich, and deionized H_2O was used throughout.

RII Overlays

RII overlays were performed according to the method of Hausken et al. (1998) with some modifications as described below. In brief, proteins were separated by SDS-PAGE and transferred to nitrocellulose. The nitrocellulose membrane was then incubated in TBS containing 5% nonfat milk (Carnation) and 0.1% BSA. Casein kinase II (New England Biolabs, Inc.) and [γ - ^{32}P]ATP (BLU502A; NEN Life Science Products) were used to radiolabel either biochemically purified RII (Promega) or recombinant RII α (plasmid provided by Dr. J. Scott, Vollum Institute, Oregon Health Sciences University, Portland, OR). Radiolabeled RII was overlaid on the nitrocellulose blots. Blots were exposed on a phosphorimager screen, and data was recorded and analyzed using the Phosphorimager SI (Molecular Dynamics) and ImageQuant software (Molecular Dynamics). Ht31 (DLIEEAASRIV-DAVIEQVKAAGAY) and Ht31-P (DLIEEAASRPVDAVIEQVKAAGAY) peptides were synthesized by the Emory University Microchemical Facility (peptide sequences from Vijayaraghavan et al., 1997).

Western Blot Analysis

Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were treated with 3% nonfat milk in TBS to prevent nonspecific protein binding. Blots were incubated with primary antibodies: anti-RSP3 at 1:200 (Williams et al., 1989), and anti-glutathione-S-transferase (GST) at 1:500 (Amersham Pharmacia Biotech), for 1 h at 25°C. After washing in TBS, blots were incubated with HRP-conjugated secondary antibodies at 1:20,000 (goat anti-rabbit; Bio-Rad Laboratories; and rabbit anti-goat; ICN Biomedicals, respectively) for 1 h at 25°C. After a final wash, blots were developed using enhanced chemiluminescence (Amersham Pharmacia Biotech).

Identification of the RII-binding Site

RSP3 fragments were generated by PCR using the RSP3 cDNA (Diener et al., 1993) and primers that each contained one of three 5' restriction sites: BamHI, EcoRI, or SphI. The PCR products were band purified, digested with the corresponding restriction endonucleases, and ligated into the pGEX-2T expression vector (Amersham Pharmacia Biotech) that had been digested with the same restriction endonucleases. In one instance (1–220), the RSP3 fragment was generated according to the method of Diener et al. (1993). After confirming the fidelity of the constructs by DNA sequence analysis, the constructs were transformed into either *Escherichia coli* DH5 α (GIBCO BRL) or *E. coli* BL21(DE3) Gold cells (Stratagene) and expressed as GST fusion proteins. Expression was induced at mid-log phase by the addition of 0.1 mM IPTG for 1–2 h. After expression, the bacterial cells were pelleted, resuspended in SDS-PAGE sample buffer, and boiled in preparation for SDS-PAGE. Expression was monitored by Western blot analysis with an antibody to GST, and RII binding was tested by RII blot overlays.

Mutagenesis

Mutagenesis was performed using the RSP3 construct containing amino acids 104–180, generated as described above. Amino acid substitutions were made according to the protocol of the GeneEditor™ in vitro Site-Directed Mutagenesis System (Promega) with the modification of using 50 μl of GeneEditor™ antibiotic selection reagent. After mutagenesis, constructs were expressed and tested for RII binding as described above.

Results and Discussion

The Axoneme Contains Two AKAPs

To test the hypothesis that the *Chlamydomonas* axoneme contains an AKAP, we performed RII blot overlays. As illustrated in Fig. 1, the overlay resulted in the identification of several axonemal proteins that bind to RII (purified RII and recombinant RII α yielded identical results). To determine which of these proteins bind to RII in a manner specific to AKAPs, the RII overlay was performed in the presence of the inhibitor peptide Ht31 or the control peptide Ht31-P. Ht31 is a synthetic peptide derived from the RII-binding domain of a known AKAP and contains an amphipathic helix (Carr et al., 1992). The peptide acts as a competitive inhibitor of AKAP–RII binding; thus, proteins that bind to RII in an AKAP-specific manner do not bind to RII in the presence of Ht31. Ht31-P, a control peptide, contains an isoleucine to proline amino acid substitution that disrupts the amphipathic helix and renders the peptide unable to bind to RII in an AKAP-specific manner (Scott and Faux, 1998). Here we define AKAPs as proteins that bind to RII in the presence or absence of Ht31-P, but do not bind to RII in the presence of Ht31.

Ht31 inhibited RII binding to two particularly prominent axonemal proteins with masses of 240 kD (AKAP240) and 97 kD (AKAP97) (Fig. 1, compare panels). AKAP240 is partially extractable with 0.6 M NaCl, whereas AKAP97 is not extractable with 0.6 M NaCl, indi-

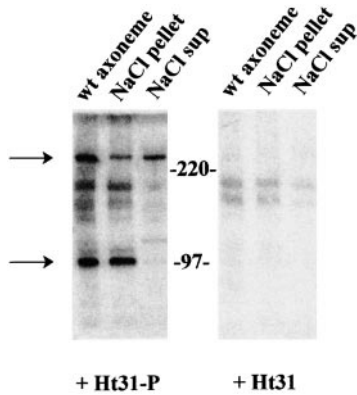


Figure 1. Identification of AKAPs in the *Chlamydomonas* axoneme. Axonemal RII-binding proteins were revealed by an RII overlay assay. RII overlays were performed in either the presence of Ht31 (right) or Ht31-P (left) to determine the specificity of RII binding. Axonemes were also treated with 0.6 M NaCl, and both the insoluble (pellet) and soluble (sup) fractions were tested to determine AKAP solubility. Two prominent axonemal AKAPs were identified of ~240 and 97 kD, designated as AKAP240 and AKAP97, respectively. wt, wild-type.

indicating that AKAP97 is very tightly associated with the axoneme (Fig. 1). In addition to AKAP240 and AKAP97, other proteins were identified in the RII overlay (Fig. 1). However, these proteins were either not consistently detected, or RII binding to the proteins was not inhibited by the Ht31 peptide. Although it is possible that these other RII-binding proteins may include additional AKAPs, they were not considered further in this study.

To localize AKAP240 and AKAP97 within the axoneme, we performed RII overlays on axonemal protein from four different classes of *Chlamydomonas* motility mutants that are defective for specific axonemal structures (Fig. 2 A). By doing so, we localized the AKAPs within the axoneme based on whether or not the AKAPs are present in the structural mutants. The analysis revealed that AKAP240 is absent in axonemes from a mutant defective for the central pair apparatus (*pf18*), and that AKAP97 is absent in axonemes from a mutant lacking the radial spokes (*pf14*) (Fig. 2 B). Both AKAPs are present in axonemes from a mutant lacking the outer dynein arms and the I1 inner dynein arm (*pf28pf30*) as well as in axonemes from a mutant defective for the dynein regulatory complex (*pf2*) (Fig. 2 B). Coomassie blue staining of a corresponding SDS-PAGE gel was performed to verify equivalent protein load. RII binding to the AKAPs was inhibited by addition of the Ht31 peptide (data not shown). The results of the mutant analysis suggest a model in which AKAP240 is associated with the central pair apparatus and AKAP97 is a component of the radial spoke.

AKAP240 Is Localized to a Substructure of the Central Pair Apparatus

To further define the location of AKAP240, RII overlays were performed on axonemes from mutants defective in various components of the central pair apparatus (for a review see Smith and Lefebvre, 1997a). RII overlays revealed that, in addition to *pf18*, AKAP240 is also absent in axonemes from other mutants that are defective for the

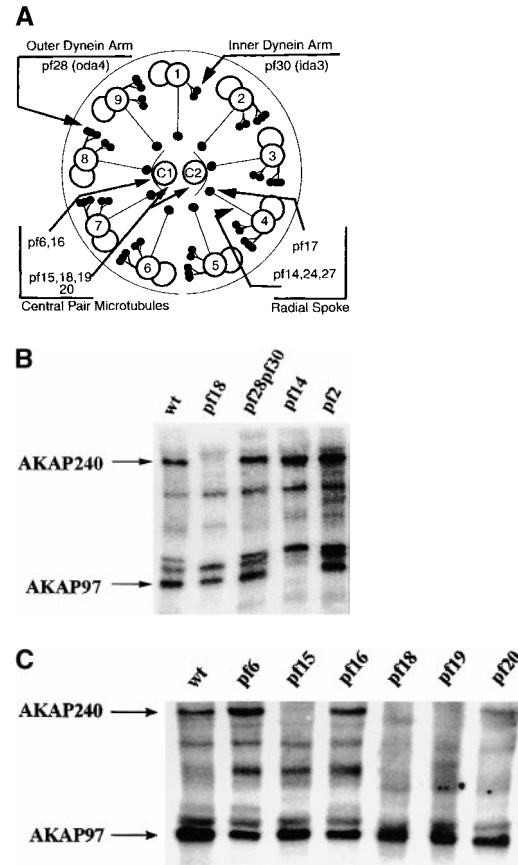


Figure 2. Deficiency of AKAPs in selected motility mutants. (A) Diagram of axoneme cross section indicating the major structural components and the corresponding mutants (adapted from Porter and Sale, 2000). (B) RII overlay of various motility mutants. wt, wild-type. (C) RII overlay of mutants defective in various structures of the central pair apparatus.

entire central pair apparatus, including *pf15* and *pf19* (Fig. 2 C). AKAP240 is reduced in axonemes from the mutant *pf20*, in which both microtubules of the central pair are unstable (Smith and Lefebvre, 1997b). However, AKAP240 is present in axonemes from a mutant defective in only the C1 microtubule (*pf16*), as well as in axonemes from a mutant that is lacking a projection of the C1 microtubule (*pf6*; Dutcher et al., 1984). Based on this mutant analysis, we propose a model in which AKAP240 is associated with the C2 microtubule of the central pair apparatus. The localization of PKA on only the C2 microtubule of the central pair apparatus is consistent with the hypothesis that regulatory components are asymmetrically localized on the central pair apparatus, a feature which may be significant in the control of flagellar waveform (Smith and Lefebvre, 1997a; Porter and Sale, 2000).

AKAP97 Is RSP3

To further localize AKAP97 within the radial spoke, we analyzed axonemes from several different radial spoke mutants by performing RII overlays. The results indicated that although AKAP97 is absent in *pf14* (lacking the entire radial spoke), AKAP97 is present in *pf17*, which is defective for the radial spoke head only (Fig. 3, A and B). This suggests that AKAP97 is associated with the radial

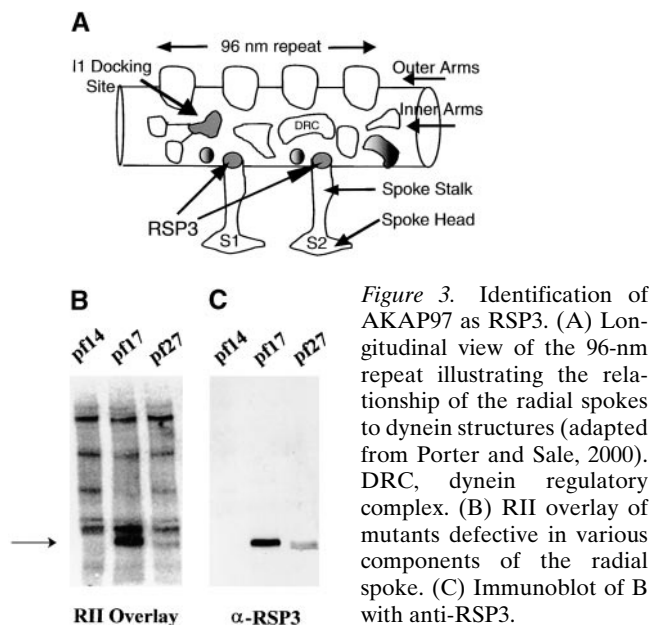


Figure 3. Identification of AKAP97 as RSP3. (A) Longitudinal view of the 96-nm repeat illustrating the relationship of the radial spokes to dynein structures (adapted from Porter and Sale, 2000). DRC, dynein regulatory complex. (B) RII overlay of mutants defective in various components of the radial spoke. (C) Immunoblot of B with anti-RSP3.

spoke stalk. We also examined *pf27*, a radial spoke mutant in which five radial spoke components that are normally phosphorylated are underphosphorylated in the mutant (Piperno et al., 1981). Three of the five proteins, RSPs 2, 3, and 13, are present in greatly reduced amounts (Huang et al., 1981). The overlay revealed that AKAP97 is greatly reduced in *pf27*, and also appears to be shifted slightly more positive on an SDS-PAGE gel, consistent with an underphosphorylated state.

Because RSP3 has been previously characterized to migrate at ~97 kD on an SDS-PAGE gel, we hypothesized that AKAP97 may be identical to RSP3. To test this hypothesis, we performed an RII overlay of radial spoke mu-

tant axonemes, and then probed the same blot with an antibody to RSP3. An identical staining pattern of AKAP97 was observed for both the RII overlay and analysis with anti-RSP3 (Fig. 3 C), indicating that AKAP97 corresponds to RSP3. Interestingly, although the predicted size of RSP3 is only 57 kD, RSP3 migrates on an SDS-PAGE gel to a position nearly two times its predicted molecular weight. This anomalous migration is a common feature among members of the AKAP family (Lester et al., 1996).

RSP3 Contains a Single RII-binding Site

To confirm that AKAP97 is RSP3, we expressed recombinant RSP3 as a GST fusion protein in *E. coli* and tested for RII binding using an RII blot overlay. The overlay demonstrated that recombinant RSP3 binds to RII (Fig. 4 A). RII binding was specifically inhibited by addition of the Ht31 peptide (data not shown). These results provided verification that RSP3 is an AKAP.

To map the location of the RII-binding domain within the amino acid sequence of RSP3, we expressed truncated forms of RSP3 as GST fusion proteins in *E. coli*. Expression of the protein fragments was monitored by Coomassie staining and Western blot analysis with an antibody to GST. Using RII blot overlays, we then tested the various RSP3 truncations for RII binding (results shown in Fig. 4 A). A COOH-terminal deletion of amino acids 221–516 had no effect on RII binding. However, an NH₂-terminal deletion of amino acids 1–193 completely abolished RII binding, indicating that the RII-binding site is contained in the NH₂-terminal portion of RSP3. By testing smaller protein fragments, we determined that RSP3 contains a single RII-binding domain located between amino acids 144–180. Curiously, RII binding to the 144–180 fragment is weaker than to larger fragments. One possible explanation for this observation is that the close proximity of

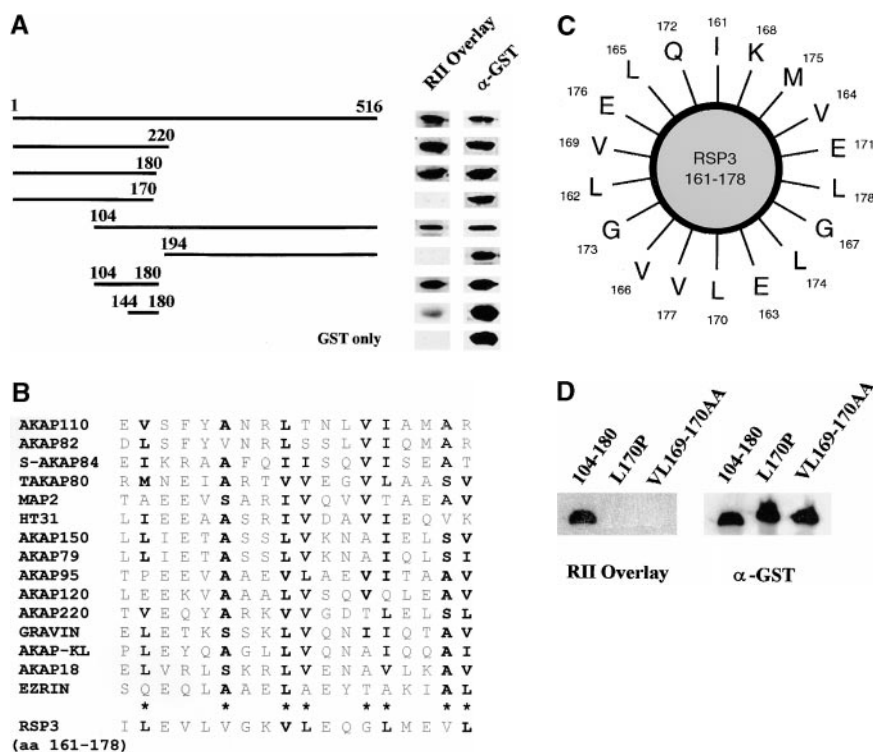


Figure 4. Mapping of the RII-binding domain in RSP3. (A) Full-length (1–516) and various truncations of RSP3 were expressed as GST fusion proteins in bacteria and tested for RII binding by RII overlays. Overlays were followed by probing with anti-GST to verify protein expression. GST only was expressed as a negative control. (B) Alignment of RSP3 residues 161–178 with the RII-binding domains of other AKAPs. Bold type indicates identical or conservatively substituted residues. Asterisks identify proposed conserved positions of the RII-binding motif (adapted from Vijayaraghavan et al., 1999). aa, amino acid. (C) Helical wheel projection of RSP3 161–178 demonstrating an amphipathic helix. (D) Site-directed mutagenesis of leucine 170 to proline and of valine 169 and leucine 170 to alanines was performed as indicated. RSP3 mutants were expressed as GST fusion proteins and tested for RII binding by RII overlays. Overlays were followed by probing with anti-GST to verify protein expression.

DROSOPHILA	I	I	D	V	L	V	D	A	C	I	E	Q	S	M	L	E	V	A
MOUSE	M	L	E	V	L	V	G	K	T	I	E	Q	S	L	L	E	V	M
HUMAN	V	L	E	V	L	V	G	K	T	I	E	Q	S	L	L	E	V	M
		*		*		*	*	*	*	*	*	*	*	*	*	*	*	*
RSP3	I	L	E	V	L	V	G	K	V	L	E	Q	G	L	M	E	V	L
(aa 161-178)																		

Figure 5. Conservation of the RSP3 RII-binding domain in orthologs of RSP3. Bold type indicates identical or conservatively substituted residues. Asterisks indicate proposed conserved positions of the RII-binding motif. aa, amino acid.

the GST protein to the RII-binding site in the 144–180 fragment results in steric hindrance by GST. However, it cannot be ruled out that residues NH₂-terminal of amino acid 144 enhance the binding of RII to RSP3.

RSP3 Contains an RII-binding Motif Similar to Other AKAPs

Recently the AKAP domain for RII binding has been refined, and conserved residues within the amphipathic helix have been proposed (Vijayaraghavan et al., 1999). Most of the conserved residues are hydrophobic, and in many cases only conservative substitutions appear to be allowed. We analyzed RSP3 amino acids 144–180 to determine whether they contain this proposed RII-binding motif. Comparative alignment of these amino acids with the RII-binding domains of other AKAPs revealed that amino acids 161–178 have sequence homology with other RII-binding domains. (Fig. 4 B). RSP3 161–178 contains 7 of 8 conserved amino acids when allowing for moderately conservative substitutions at amino acids 166 and 177 (valine for alanine/serine). Moreover, like the RII-binding domains of other AKAPs, amino acids 161–178 are predicted to form an amphipathic helix (Fig. 4 C).

To provide definitive evidence that amino acids 161–178 comprise the RII-binding domain of RSP3, we performed a point mutation at amino acid 170. Using the construct containing amino acids 104–180, the central, conserved residue, leucine 170, was replaced by a proline residue. We predicted that this substitution would disrupt the amphipathic helix and abolish RII binding. We expressed the construct as a GST fusion protein in bacteria, and tested its ability to bind to RII using an RII blot overlay. The analysis revealed that the leucine to proline substitution completely abolished RII binding to RSP3 (Fig. 4 D). To ensure that the loss of RII binding in the leucine to proline substitution did not occur as a result of nonspecific secondary structural alterations, we performed a more structurally conservative amino acid substitution in which valine 169 and leucine 170 were replaced by alanines. The substitutions again resulted in the complete loss of RII binding, indicating that these residues are essential for RII to bind to RSP3 (Fig. 4 D). Furthermore, the substitutions correspond to mutations in other AKAPs that disrupt RII binding (Glantz et al., 1993; Westphal et al., 2000), providing strong evidence that RSP3 is an AKAP with an RII-binding domain localized to amino acids 161–178.

The RII-binding Site in RSP3 Is Well Conserved in RSP3 Orthologs

Orthologs to RSP3 have been identified in human, mouse, and *Drosophila* databases. Alignment of the RII-binding

site of RSP3 with the corresponding residues in these orthologs reveals that the RII-binding site is well conserved (Fig. 5). The locations of the orthologs in their respective organisms is not known, but since the axoneme is a highly conserved structure, they most likely will be found in the radial spokes of flagellar and perhaps ciliary axonemes. It is important to test whether any of the orthologs is also an AKAP. If so, it suggests that the AKAP is an important component of the axoneme, and therefore may also be important in the regulation of motility. There is some evidence that AKAPs indeed play an important role in the control of flagellar motility. For example, cell-permeable analogues of Ht31 have been shown to inhibit sperm motility (Vijayaraghavan et al., 1997).

The Role of RSP3 in *Chlamydomonas* Flagellar Axonemes

Genetic and biochemical experiments in *Chlamydomonas* have demonstrated that the radial spokes, and in particular RSP3, are important in the regulation of flagellar motility (for reviews see Brokaw et al., 1982; Curry and Rosenbaum, 1993). A mutant defective in the gene encoding RSP3, *pfl4*, lacks radial spokes and has paralyzed flagella. Based on our data, we hypothesize that in addition to being required for assembly of the radial spokes (Witman et al., 1978; Huang et al., 1981; Diener et al., 1993), RSP3 has an additional function of localizing PKA in a position to control dynein-driven flagellar motility. Consistent with this hypothesis, several experiments have shown that the radial spokes control dynein activity by regulating axonemal PKA (Smith and Sale, 1992; Howard et al., 1994; Habermacher and Sale, 1997). In addition, genetic studies of suppressor mutations have identified a dynein control system, which, in the absence of the radial spokes, inhibits dynein activity (Huang et al., 1982).

Genetic and molecular analyses have demonstrated that RSP3 is located at the base of the radial spoke stalk, adjacent to the outer doublet microtubules and near the inner dynein arms (Fig. 3 A). A basic region of RSP3, consisting of amino acids 42–80, is necessary and sufficient for RSP3 to be targeted to the outer doublet microtubules (Diener et al., 1993). This domain is analogous to the AKAP targeting domain. In addition to the RII-binding domain, each AKAP contains a unique targeting domain that localizes the AKAP and associated PKA to a specific intracellular site (Edwards and Scott, 2000). Thus, amino acids 42–80, formerly designated as the axoneme-binding domain, can now also be considered the AKAP targeting domain. However, the axonemal protein(s) that binds to the targeting domain and facilitates the localization of RSP3 to the outer doublet microtubules remains unknown.

One disadvantage for our study of RSP3 is that, along with other proteins of the radial spoke, RSP3 is not easily solubilized and forms a strong association with the axoneme (Piperno et al., 1981). Previously thought to be insoluble, a novel extraction method has recently shown that RSPs can be solubilized by 0.5 M KI (Yang, P., D.R. Diener, J.L. Rosenbaum, and W.S. Sale, submitted for publication). The relative insolubility of RSP3 has precluded our efforts to show an association with either the endogenous regulatory or catalytic PKA subunits through traditional approaches such as cAMP-affinity purification or

immunoprecipitation. The chaotropic conditions required for the solubilization of RSP3 likely also cause a dissociation of any associated enzymes. Equally as challenging, little information is known about *Chlamydomonas* PKA, and no identifying reagents are currently available. Further studies of the role of PKA and AKAPs in the axoneme will require a dedicated effort in the development of these reagents.

In summary, we have identified an AKAP that is associated with the central pair apparatus, and an AKAP that is a component of the radial spoke. Recent studies have shown that in addition to an AKAP, the radial spoke also contains a kinase, as well as calmodulin (Yang, P., D.R. Diener, J.L. Rosenbaum, and W.S. Sale, submitted for publication). We propose that RSP3 localizes PKA to a position that, in conjunction with other components of the radial spoke, regulates dynein activity and controls flagellar motility. Future experiments will test this prediction and examine the physiological role of the AKAP in the flagellar axoneme.

The authors would like to thank John Scott (Vollum Institute, Oregon Health Sciences University) for helpful discussion, experimental advice, and the generous gift of the RII α plasmid and Ht31 peptide. We would like to gratefully acknowledge Katrina Waymire and Pinfen Yang for experimental advice, and Lynne Quarmby, Grant MacGregor, and Pinfen Yang for thoughtful discussion of the manuscript.

Submitted: 30 January 2001

Revised: 27 February 2001

Accepted: 12 March 2001

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