INTRODUCTION

The major challenge in understanding ciliary and flagellar motility is to determine the mechanisms that locally control the multiple dynein motors (Satir, 1985; Brokaw, 1994; Kamiya, 1995). Structural and biochemical analysis of Chlamydomonas mutants has revealed that the central pair apparatus and radial spokes are central regulators of dynein arm activity (Smith and Lefebvre, 1997a). For example, mutations that disrupt either the central pair or the radial spokes result in paralysis by a mechanism involving the inactivation of flagellar dynein activity (Huang et al., 1982; Piperno et al., 1992; Porter et al., 1992). Mutant, paralyzed axonemes can still undergo dynein-driven microtubule sliding in vitro (Witman et al., 1978), but at greatly reduced rates of microtubule sliding compared to wild-type axonemes (Smith and Sale, 1992). In vitro reconstitution and functional assays demonstrated the radial spokes are required for wild-type dynein activity and that the velocity of dynein-driven microtubule sliding is mediated by posttranslational modification of the inner dynein arms (Smith and Sale, 1992). The results of the in vitro experiments are consistent with other evidence indicating the central pair/radial spoke complex controls dynein activity (Huang et al., 1982; Porter et al., 1992; Piperno et al., 1992, 1994; Gardner et al., 1994; Rupp et al., 1996; Yoshimura and Shingyoji, 1999).

Several studies have revealed that ciliary and flagellar activity is controlled by phosphorylation (Brokaw, 1987; Satir et al., 1993; Walzak and Nelson, 1994; Tash and Bracho, 1994). Furthermore, in vitro analysis has indicated the protein kinases and phosphatases responsible for regulation are anchored in the axoneme (Hasagawa et al., 1987; Hamasaki et al., 1989; San Agustin and Witman, 1994; Chaudhry et al., 1995). For example, based on the studies of Hasegawa et al. (1987), we predicted Chlamydomonas flagellar dyneins are controlled by kinases and phosphatases anchored in the axoneme. To test this, a pharmacological approach was taken with the prediction that inhibitors of kinases and/or phosphatases could rescue wild-type dynein activity in isolated, paralyzed axonemes (Howard et al., 1994). Using this approach, specific inhibitors of cAMP-dependent kinase (PKA) rescue wild-type dynein activity in isolated axonemes lacking the radial spokes (Howard et al., 1994). Evidently in the absence of these axonemal structures, an axonemal PKA blocks dynein-driven microtubule sliding. In the same study, in vitro reconstitution experiments also demonstrated that one of the target proteins is located in the inner arm dynein fraction. Thus, it was concluded that PKA is anchored in the axoneme, possibly in position to control inner arm dynein driven motility. Furthermore, since the kinase inhibitors resulted in rescue of wild-type dynein activity, it was postulated the axoneme must

SUMMARY

We postulated that microcystin-sensitive protein phosphatases are integral components of the Chlamydomonas flagellar axoneme, positioned to regulate inner arm dynein activity. To test this, we took a direct biochemical approach. Microcystin-Sepharose affinity purification revealed a prominent 35-kDa axonemal protein, predicted to be the catalytic subunit of type-1 protein phosphatase (PP1c). We cloned the Chlamydomonas PP1c and produced specific polyclonal peptide antibodies. Based on western blot analysis, the 35-kDa PP1c is anchored in the axoneme. Moreover, analysis of flagella and axonemes from mutant strains revealed that PP1c is primarily, but not exclusively, anchored in the central pair apparatus, associated with the C1 microtubule. Thus, PP1 is part of the central pair mechanism that controls flagellar motility. Two additional axonemal proteins of 62 and 37 kDa were also isolated using microcystin-Sepharose affinity. Based on direct peptide sequence and western blots, these proteins are the A- and C-subunits of type-2A protein phosphatase (PP2A). The axonemal PP2A is not one of the previously identified components of the central pair apparatus, outer arm dynein, inner arm dynein, dynein regulatory complex or the radial spokes. We postulate PP2A is anchored on the doublet microtubules, possibly in position to directly control inner arm dynein activity.

Key words: Dynein, Cilium, Flagellum, Phosphatase, PP1, PP2A, Chlamydomonas, Cell motility
also bear protein phosphatases anchored in position to control inner arm dynein activity.

Based on a pharmacological approach using axonemes isolated from wild-type and mutant cells, an axonemal protein phosphatase type 1 (PP1) was discovered that either directly or indirectly acts to control inner arm dynein activity (Habermacher and Sale, 1996). This conclusion was based on specific inhibitors of PP1, but the data did not exclude the presence and involvement of additional axonemal protein phosphatases in the activation of dynein activity. Moreover, genetic and biochemical analyses revealed that one target substrate is the 138-kDa intermediate chain (IC138) of inner arm dynein I1, and that an axonemal microcystin-sensitive protein phosphatase, such as PP1, is either directly or indirectly responsible for de-phosphorylation of IC138 and rescue of wild-type dynein driven motility (Habermacher and Sale, 1997). The simplest model is that a microcystin-sensitive protein phosphatase, or phosphatases, is anchored in the axoneme in position to regulate inner arm dynein activity. Alternatively, the microcystin sensitive axonemal phosphatases may act indirectly (King and Dutcher, 1997).

To test this model, a direct biochemical and molecular approach was selected to define the presence and location of microcystin-sensitive protein phosphatases in isolated axonemes from both wild-type and mutant *Chlamydomonas* flagella. As predicted from the physiological and pharmacological studies, we determined the axoneme contains two microcystin-sensitive phosphatases, protein phosphatase type 1 (PP1) and protein phosphatase type 2A (PP2A). The major fraction of axonemal PP1 is located in the central pair apparatus associated with the C1 microtubule (Dutcher et al., 1984; Smith and Lefebvre, 1996, 1997a,b; Mitchell and Sale, 1999). The axonemal PP2A is likely anchored on the outer doublet microtubules, possibly in position to directly control phosphorylation of inner arm dynein. This system of control, which involves the central pair, radial spokes, and inner arm dynein I1, is likely to regulate flagellar waveform (Brokaw et al., 1982; Brokaw and Kamiya, 1987; King and Dutcher, 1997). The discovery that PP1 and PP2A are anchored in the wild-type 9+2 axoneme, but not anchored in specific *Chlamydomonas* mutants, provides a new opportunity to define subunits that anchor phosphatases in the cell.

**MATERIALS AND METHODS**

**Cell strains**

Cell strains unless otherwise specified were provided by *Chlamydomonas* Genetics Center (E. H. Harris, Duke University). Wild-type CC-125 was used for preparation of DNA and RNA. Drs E. F. Smith and P. Lefebvre (University of Minnesota and Dartmouth College) provided pJ16 and pJ20 (Smith and Lefebvre, 1996, 1997a,b). All cells were grown in L-medium with aeration over a 14/10 light/dark cycle (Witman, 1986).

**Molecular characterization of PP1c**

For RT-PCR and northern analysis, total RNA was prepared from wild-type cells and/or at timed intervals following deflagellation as described previously (Yang and Sale, 1998). Genomic DNA was prepared as described (Wilkinson et al., 1995). For cloning PP1c, primers for RT-PCR were designed based on published peptide sequences (Song et al., 1993). These sequences included KIKYPENF and EDGYEFF. The degenerate primer pairs for RT-PCR included Ps1 and Pa1: Ps1[CGCGAATTCARATI194TCCGGAATTCY]; Pa1[CGCGCTCGAGGAAATCTRTAC]{CTCCTCCTCCTCC}.

The additional primer pair, Ps2 and Pa2, was designed based on the genomic sequence and used for RT-PCR to identify the 5’ end of the PP1c cDNA sequence: Pa2[CAAGAGGGACCACACTGACCTGC]C; Ps2[GAAGAAGTGCATCCCTTCCCAC].

*The RT-PCR reactions were carried out as described previously (Yang and Sale, 1998). The 480 bp product from primers Ps1 and Pa1, was cloned into the EcorI and XhoI site of pBlueScript II KS(−) (Strategene, La Jolla, CA) and designated pRT1. The 840-bp product from primers Ps2 and Pa2 was cloned into pGemEasy (Promega, Madison, WI) and designated pRT2. The insert from pRT1 was used as a probe for library screening and for Southern and northern blots.*

**Library screening**

cDNA and genomic libraries were screened as described previously using the insert of the pRT1, the RT-PCR clone, as a probe (Yang and Sale, 1998). A AzapII cDNA library was kindly provided by C. G. Wilkerson and G. Witman (University of Massachusetts; Wilkerson et al., 1995) and a *fixII* genomic library was kindly provided by E. F. Smith and P. Lefebvre (University of Minnesota; Smith and Lefebvre, 1997b). The cdNA clone pC1 was excised from purified plaques with helper phage (Wilkerson et al., 1995). The inserts from genomic clones were released from the phage vector by digestion with *XhoI, SacI* or *NotI* in separate reactions. The products of restriction digestion were subcloned into pBlueScript KS II(−).

**Biochemistry**

The *Chlamydomonas* and *A. simplex* axonemes from various strains were prepared as described (Smith and Sale, 1992). Briefly, cells were de-flagellated with dibucaine, and axonemes were isolated by suspension of flagella in Buffer A (30 mM NaCl, 10 mM Hepes, pH 7.4, 5 mM MgSO4, 1 mM DTT, 0.5 mM EDTA, PMSF and aprotinin) containing 0.5% Nonidet-P 40 (NP-40, Calbiochem, LaJolla, CA) for SDS-PAGE. Flagella and axonemes from duplicate aliquots were resuspended with Buffer A to a final axoneme concentration of 5 mg/ml. Protein was determined using the Coomassie binding assay (Bio-Rad, Hercules, CA) using BSA as a standard. For SDS-PAGE, samples were fixed in the appropriate volume of 5× sample buffer. For peptide sequencing, proteins were separated by SDS-PAGE, transferred to PVDF membrane and band purified as described (Yang and Sale, 1998). Dr John Leszynski (Protein Chemistry Core, University of Massachusetts) performed tryptic digestion, peptide separation by HPLC and microsequencing.

Cross-linking of axonemes with EDC (1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride, Pierce, Rockford, IL) was carried out as described (Yang and Sale, 1998). Briefly, axonemes (suspended at 2 mg/ml in buffer A without DTT) were treated for 1 hour at room temperature with EDC freshly dissolved in water. The reaction was then terminated by a 20-fold molar excess of β-mercaptoethanol. Western blot analysis was carried out as described (Yang and Sale, 1998). Unless stated otherwise, an 8% mini-gel was used for SDS-PAGE.

**Antibodies**

Polyclonal antibodies were raised in rabbits (Spring Valley laboratories, Sykesville, MD) against a synthetic peptide containing cysteine, tyrosine and the C-terminal 11 amino acids residues from the *Chlamydomonas* PP1c (CYKPAETKAKRR). For immuno-fluorescence localization, antibodies to PP1c were affinity purified to the peptide conjugated to Affi-Gel 10 following directions supplied by the manufacturer (Bio-Rad, Hercules, CA). Dr D. Pallas (Emory University) provided the monoclonal antibodies against the catalytic subunit of PP2A (1D6, 4G7; Orgis et al., 1999). Drs E. F. Smith and P. Lefebvre (Dartmouth College and the University of Minnesota) kindly provided the monoclonal antibodies against the axonemal PP1c (CYKPAETKAKRR). For immuno-fluorescence localization, antibodies to PP1c were affinity purified to the peptide conjugated to Affi-Gel 10 following directions supplied by the manufacturer (Bio-Rad, Hercules, CA). Dr D. Pallas (Emory University) provided the monoclonal antibodies against the catalytic subunit of PP2A (1D6, 4G7; Orgis et al., 1999). Drs E. F. Smith and P. Lefebvre (Dartmouth College and the University of Minnesota) kindly provided the monoclonal antibodies against the axonemal PP1c (CYKPAETKAKRR). For immuno-fluorescence localization, antibodies to PP1c were affinity purified to the peptide conjugated to Affi-Gel 10 following directions supplied by the manufacturer (Bio-Rad, Hercules, CA). Dr D. Pallas (Emory University) provided the monoclonal antibodies against the catalytic subunit of PP2A (1D6, 4G7; Orgis et al., 1999). Drs E. F. Smith and P. Lefebvre (Dartmouth College and the University of Minnesota) kindly provided the monoclonal antibodies against the axonemal PP1c (CYKPAETKAKRR). For immuno-fluorescence localization, antibodies to PP1c were affinity purified to the peptide conjugated to Affi-Gel 10 following directions supplied by the manufacturer (Bio-Rad, Hercules, CA). Dr D. Pallas (Emory University) provided the monoclonal antibodies against the catalytic subunit of PP2A (1D6, 4G7; Orgis et al., 1999). Drs E. F. Smith and P. Lefebvre (Dartmouth College and the University of Minnesota) kindly provided the monoclonal antibodies against the axonemal PP1c (CYKPAETKAKRR). For immuno-fluorescence localization, antibodies to PP1c were affinity purified to the peptide conjugated to Affi-Gel 10 following directions supplied by the manufacturer (Bio-Rad, Hercules, CA). Dr D. Pallas (Emory University) provided the monoclonal antibodies against the catalytic subunit of PP2A (1D6, 4G7; Orgis et al., 1999). Drs E. F. Smith and P. Lefebvre (Dartmouth College and the University of Minnesota) kindly provided the monoclonal antibodies against the axonemal PP1c (CYKPAETKAKRR).
provided the antibodies to the pf16 and pf20 gene products (Smith and Lefebvre, 1996, 1997b).

**Immunofluorescence microscopy**

For immuno-localization, axonemes were prepared by two different protocols with identical results. For both procedures, cells were suspended in 10 mM HEPES, were attached to the glass coverslips. In the first method, cells were then permeabilized with 0.5% Nonidet in a microtubule stabilizing buffer (MTSB; Johnson and Rosenbaum, 1992) for 10 minutes at room temperature and then fixed in 4% para-formaldehyde/MTSB for 30 minutes at room temperature. In the second method, cells were fixed in 4% para-formaldehyde/MTSB for 30 minutes at room temperature and then immersed in 0.5% Nonidet/MTSB/4% para-formaldehyde for 20 minutes at room temperature. In each case, coverslips were rinsed in MTSB, immersed in –20°C methanol and briefly air-dried. Cells were re-hydrated in 3 changes of PBS, blocked with 3% BSA in PBS, and incubated in primary antibodies in PBS/3% BSA overnight at 4°C. Following extensive rinses with PBS, cells were incubated with FITC-conjugated goat anti-rabbit antibodies (1:400 dilution in PBS/10% goat serum, ICN, Costa Mesa, CA) or TRITC-conjugated goat anti-mouse antibodies (1:100 dilution in PBS/10% goat serum, Zymed Labs, Inc., ICN, Costa Mesa, CA) for 2 hours at room temperature. After extensive washing in PBS, coverslips were mounted in Citifluor (Ted Pella, Inc., Redding CA). Data were collected using a 100× Zeiss plan-neofluar lens and air-cooled CCD camera (DAGE MTI, Michigan City, IN City) using Scion Image software (Scion Corporation, Fredrick, MD) to capture images. Digital images were analyzed and prepared for publication using Photoshop (Adobe Systems, Inc., San Jose, CA).

**Microcystin-Sepharose affinity**

Two methods were used to prepare extracts for microcystin-Sepharose affinity. First a salt extract from isolated axonemes was prepared. Isolated axonemes were suspended at 8 mg/ml in Buffer A supplemented with 0.57 M NaCl, 10 mM NaF, 100 μM ortho-vanadate, and 0.1% β-mercaptoethanol (replacing 1 mM DTT). This buffer, when containing 0.12 M NaCl, is referred to as Buffer B. The resultant salt extract was separated from the extracted axonemes by centrifugation and dialyzed in Buffer B. Second, in some cases, an extract from whole flagella was prepared by a novel procedure as follows. Isolated flagella were suspended at 10 mg/ml in Buffer B, drop-frozen in liquid nitrogen and the extract prepared for microcystin-Sepharose affinity as described above.

For microcystin affinity, 0.5 ml extract from isolated axonemes or whole flagellar extract (~2-4 mg/ml) was either pretreated with 50 μM microcystin-LR (Calbiochem, La Jolla, CA) or with buffer alone for two hours at 4°C. The samples were then incubated with ~20 μl microcystin-Sepharose (15 μM, final microcystin concentration, Upstate Biotechnology, Lake Placid, NY) overnight at 4°C. Sepharose beads were then washed extensively with buffer B and then re-suspended to 1/5 the original volume in 1× SDS-PAGE sample buffer. For microcystin-Sepharose precipitation of the EDC cross-linked proteins, the EDC treated axonemes were extracted with 0.6 M NaCl and the extract prepared for microcystin-Sepharose affinity as described above.

**RESULTS**

**Isolation of axonemal protein phosphatases by microcystin-Sepharose affinity**

Based on pharmacological analysis of dynein-driven microtubule sliding, we predicted isolated axonemes must contain microcystin-sensitive protein phosphatases (Habermacher and Sale, 1996, 1997). To directly test this hypothesis, we performed the microcystin affinity-precipitation on salt extracts from isolated axonemes. This approach has been used to identify PP1, PP2A, and associated proteins, in several cell types (Moorhead et al., 1995; Campos et al., 1996; Colbran et al., 1997; Damer et al., 1998). Three axonemal proteins, with apparent masses of 35, 37 and 62 kDa (lane 2, Fig. 1), specifically bound to the microcystin-Sepharose matrix compared to the control treated with excess, soluble microcystin-LR (lane 1, Fig. 1). The same three proteins specifically bound microcystin-Sepharose using extracts from whole flagella prepared by the freeze-thaw method described in Materials and Methods. Based on molecular size and microcystin affinity, we speculated the prominent 35-kDa protein is the catalytic subunit of PP1 (PP1c). To test this, we cloned the *Chlamydomonas* PP1c, and produced polyclonal, peptide antibodies specific to a unique sequence for localization studies.

**Molecular characterization of *Chlamydomonas* PP1**

For cloning, we took advantage of the highly conserved protein sequence of PP1c. A pair of degenerate oligonucleotide primers was designed from two regions of PP1c containing residues completely conserved in PP1c, but not found in the catalytic subunit of PP2A (Materials and Methods). RT-PCR with this primer pair (designated Ps1 and Pa1, Fig. 2) resulted in a 480-bp product designated the pRT1 clone (Fig. 2A). Sequence analysis indicated that the predicted protein sequence of pRT1 is nearly identical to that of other known PP1cs. Thus the pRT1 clone was used as a probe for screening the cDNA and genomic libraries as well as for Southern and northern blot analysis.

Three cDNA clones and six distinct genomic clones were recovered. The largest clone was subcloned and sequenced with the primers used in the PCR reaction (Fig. 2A). Sequence comparison of this cDNA clone revealed a high degree of homology with *Chlamydomonas* PP1c, and its deduced amino acid sequence was identical to that of other known PP1cs. To confirm the identity of this clone, we used degenerate oligonucleotide primers to design a second primer pair (designated Ps2 and Pa2, Fig. 2) that would only amplify the pRT1 clone (Fig. 2A). Sequence analysis of the PCR product obtained with these primers revealed the deduced amino acid sequence of the cDNA clone was identical to that of other known PP1cs. Thus, the pRT1 clone was used as a probe for screening the cDNA and genomic libraries as well as for Southern and northern blot analysis.

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**Fig. 1.** Microcystin-Sepharose affinity of salt extracted axonemal proteins. Silver stained SDS-PAGE revealed that 35, 37 and 62 kDa axonemal proteins specifically bound to microcystin-Sepharose (arrowheads, lane 2). In control experiments, in which the extract was pre-incubated with soluble microcystin, these proteins failed to bind to microcystin-Sepharose (lane 1).
Step 1. RT-PCR

Step 2. Screen a cDNA library

Step 3. Screen a genomic library

Step 4. RT-PCR

Fig. 2. Cloning of the *Chlamydomonas* PP1c gene. (A) Stepwise strategy for cloning *Chlamydomonas* PP1c gene and message. Key: arrows designate primers used for RT-PCR; solid bar designates the exons; and the open box designates the un-translated region. (B) Genomic and the predicted protein sequence for *Chlamydomonas* PP1c. Arrows designate primer positions; bold italicized amino acids designate peptides used to design the degenerate primers; bases in capitals designate the exons; bold bases designate potential polyadenylation signal; the asterisk (*) indicates the predicted stop codon; the arrowhead designates the 5' end of the pC1 cDNA clone; and the boxed letters designate the synthetic peptide used to raise anti-PP1 peptide antibody. These sequence data are available from GenBank under accession number (AF156101).
Phosphatases anchored in flagellar axonemes

The cDNA clone was 1.3 kb (pC1, Fig. 2A), which contained the C-terminal half of the coding sequence and the complete 3′ untranslated region, including a polyadenylation signal (TGTTA) located 19 bp upstream of a poly(A) tail (Fig. 2B). Mapping of six genomic clones indicated that the PP1c gene is located within two SacI subclones, a 5′, 5.5-kb fragment (designated pG1 in Fig. 2A) and a 3′, 1.9-kb fragment (designated pG2 in Fig. 2A). The pC1, pG1 and pG2 clones were sequenced. To recover the 5′ end of the cDNA, a second RT-PCR was performed using the primer pair Ps2 and Pa2. An 820-bp product, designated pRT2 (Fig. 2A), was recovered. An open reading frame of 912 bp was identified in the pRT2 and pC1 clones, and the first ATG is preceded by an in-frame stop codon-21 bp upstream. Thus, it is likely that the complete coding sequence has been recovered (Fig. 2B).

The Chlamydomonas PP1c gene consists of six exons, which, when combined, predict a 1.8 kb message. The 912 bp coding sequence predicts a 35-kDa protein with pI 4.8. Sequence analysis predicts >90% of the amino acid residues are identical to the PP1 from other species (Fig. 3). The Chlamydomonas PP1c is most similar to the PP1c from Acetabularis, and among mammalian isoforms most similar to PP1g. Greatest sequence diversity is found at the N- and C-terminus as described for PP1c from earlier studies. As described below, we took advantage of unique sequence to produce a peptide antibody that specifically reacts with Chlamydomonas PP1c. Southern blot analysis of genomic DNA, digested with either HindIII and Psrl, revealed only one band suggesting that a single gene encodes PP1c in Chlamydomonas (Fig. 4A). Based on RFLP analysis using pC1 as a probe, the gene is located on the right arm of linkage group VI (Silflow, 1998). Northern blot analysis revealed the predicted 1.8-kb message (Fig. 4B). Moreover, the transcript is more abundant following experimental deflagellation, a characteristic common to the mRNA of many axonemal proteins (Lefebvre et al., 1980).

**Chlamydomonas PP1c is anchored in the axoneme**

A rabbit polyclonal antibody was raised against a synthetic peptide containing the unique C-terminal 11 amino acids (boxed sequence in Fig. 2B), with cysteine and tyrosine added at the N terminus for conjugation and phosphorylation respectively. Western blot analysis demonstrated that the antibody bound to a 35-kDa protein in isolated flagella (arrow, Fig. 5A), and, as predicted, the major fraction of the 35-kDa protein remained bound to isolated axonemes (lane 3, Fig. 5). The 35-kDa protein is extractable with 0.6 M NaCl buffer, permitting functional analysis and purification as a soluble protein. Thus, using microcystin-Sepharose affinity, we were able to confirm that the 35-kDa immuno-reactive protein is PP1c (Fig. 5B).

Immuno-fluorescence localization using affinity purified PP1c antibodies revealed that PP1c is located along the length of both flagellar axonemes, as well as being abundant in the cell body (Fig. 5C). Pre-immune serum did not stain the axonemes. Notably, compared to many other axonemal proteins including PP2A (see below) the staining of PP1c appeared punctate irrespective of mode of fixation, permeabilization or irrespective of the source of secondary antibody. One explanation for the punctate staining of PP1c is...
that in the axoneme, PP1c is not easily accessible to the antibodies. Consistent with this interpretation, we have not produced a strong signal using the PP1c antibody and electron microscopic immuno-gold localization with either pre- or post-embedding methods. This failure with electron microscopic localization is despite successful positive controls using antibodies to other axonemal proteins. Thus, direct localization will require new reagents and/or approaches or discovery of previously characterized axonemal proteins that interact with and anchor PP1c.

**Chlamydomonas axonemal PP1 is located in the central pair apparatus**

To further determine the location of PP1c in the axoneme, western blot analysis was performed using axonemes isolated from flagellar mutants. We postulated that axonemal PP1c is anchored to a distinct structure, and that failure to assemble the anchor structure would result in failure of PP1c to co-purify with the axoneme, despite the presence of PP1c in flagella. To test this, we analyzed and compared PP1c in both flagella and axonemes from various mutants using the PP1c antibody. Among the mutants analyzed, only the axonemes from mutants that fail to assemble the central pair structures lack a significant fraction of the axonemal PP1c (Fig. 6). Axonemes lacking PP1c were derived from pf15, pf16, pf18, pf19 and pf20 (Fig. 6A). Each of these mutants fails to assemble all or part of the central pair apparatus, and, in particular, in pf16 the ‘C1’ microtubule and associated structures are unstable (Dutcher et
al., 1984; Smith and Lefebvre, 1996; Mitchell and Sale, 1999). In contrast, axonemes from mutants lacking the outer or inner dynein arms, radial spokes, dynein regulatory complex, or the ‘mbo’ gene products all contain the wild-type amount of PP1c. The isolated flagella and axonemes from ‘mia’ mutants, which are defective in phosphorylation of IC138 (King and Dutcher, 1997), also contain wild-type levels of PP1c. The simplest interpretation is that a major fraction of axonemal PP1c is anchored in the central pair apparatus, associated with the C1 microtubule. Moreover, axonemes from pf6, which lack one projection from the C1 microtubule (Smith and Lefebvre, 1996; Mitchell and Sale, 1999), retain the wild-type levels of PP1c. Thus, PP1 is not likely to be anchored to that projection.

Despite the lack of PP1c in isolated axonemes from central pair mutants, PP1c is found in the whole flagella of these mutants at similar concentrations found in wild-type flagella (top row, Fig. 6A). This indicates that none of the central pair mutants analyzed are defective in PP1c expression, and that PP1c is correctly targeted and transported to the flagellar compartment. In contrast, blots probed with antibodies to two other central pair proteins, the pf16 and pf20 gene products (Smith and Lefebvre, 1996; 1997A), revealed these proteins are absent in both flagella and axonemes of mutants that fail to assemble the central pair complex (compare axonemes and flagella for pf18 and pf19, Fig. 6A). This experiment indicated that PP1c is transported to the flagella and axoneme differently from the pf16 and pf20 proteins.

To determine whether the axonemal PP1c is solely located in the central pair apparatus, we used larger protein loads and compared the proteins from isolated flagella, Nonidet extract, and axonemes from wild-type and pf18 (Fig. 6B). Western blots revealed the axonemes from pf18, which lack the central pair structure, retain a small amount of PP1c (compare axonemes from pf18 with wild type, Fig. 6B). The simplest interpretation is that PP1c is anchored in more than one axonemal location. Of potential relevance, an outer arm dynein light chain with sequence homology to the PP1 binding protein SDS22 has been reported (Patel-King et al., 1997).

**PP1c forms a molecular complex with a ~60 kDa axonemal protein**

To further confirm that PP1 is docked in the axoneme and identify PP1 interacting proteins, western blots were used to analyze axonemes treated with the ‘zero-length’ cross-linker, EDC, which has proven useful in identification of interactions among protein subunits in dynein and the radial spokes (King et al., 1991; Diener et al., 1993; Yang and Sale, 1998). Following exposure of isolated axonemes to 1.5 mM EDC, a new band with a mass of ~97 kDa appeared in blots probed with the PP1c antibody (closed circle, Fig. 7A). As a further control, the cross-linked 97 kDa axonemal product was solubilized with 0.6 M NaCl, and following dialysis, was specifically precipitated by microcystin-Sepharose (Fig. 7B). This result confirmed the 97 kDa cross-linked product is a complex containing axonemal PP1c tightly associated with an axonemal protein of about 60 kDa. Furthermore, the distinctive cross-linked product produced, rather than multiple, spurious cross-linked products or lack of product, supports the model that PP1c is anchored in specific positions in the axoneme. The 97-kDa cross-link product did not react on western blots using antibodies to tubulin or dynein intermediate chain subunits.

However, based on the preponderance of PP1c in the central pair complex, it is likely the ~60 kDa protein is the central pair component that secures PP1c in the structure. The 97 kDa cross-linked product failed to appear when EDC cross-linking

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**Fig. 6.** The major axonemal fraction of PP1c is located in the central pair apparatus, associated with the C1 microtubule. (A) Top pair of panels: western blot analyses revealed that PP1c is dramatically reduced in the axonemes isolated from mutants that fail to assemble central pair apparatus. In contrast, PP1c is present in the flagella of all strains examined. Middle and lower pairs of panels: in control experiments, two other central pair proteins, the pf16 and pf20 gene products, were found in similar quantities in both flagella and axonemes from the various cell types illustrated. Phenotypes: pf15, pf18 and pf19 axonemes each lack the entire central pair apparatus; pf6 axonemes lack a projection of the C1 central microtubule; pf16 axonemes lack the C1 microtubule and associated projections; and pf20 axonemes predominantly lack the central pair components. (B) PP1c is not exclusively located to the central pair apparatus. PP1c distribution was compared with higher protein loads between flagella, axonemes and detergent extract (NP-40 Ext.) from wild type and mutants lacking the central pair (pf18). Notably, in the absence of the central pair, a small amount of PP1c remains in the axoneme (compare lanes 5 and 6).
was performed in the salt extract, following 0.6 M NaCl extraction. This suggested that without cross-linking, PP1c and the 60-kDa axonemal protein become dissociated following exposure to the 0.6 M NaCl extraction buffer.

**PP2A is also anchored to the axoneme**

In addition to PP1, microcystin-Sepharose affinity purification revealed two other axonemal proteins with masses of 37 and 62 kDa (Fig. 1). Based on molecular weight and microcystin affinity, we postulated the proteins are the C- and A-subunits of PP2A. To test this prediction, the microcystin binding proteins were blotted to the PVDF membrane and the 62-kDa protein was band purified for peptide sequencing. One of the peptides recovered was nearly identical to the A-subunit of PP2A (Fig. 8A), strongly suggesting the 62 kDa axonemal protein is the A-subunit. To test whether the 37 kDa protein is the catalytic subunit of PP2A, two different monoclonal antibodies to the C-subunit were used for western blot analysis (Orgis et al., 1999). In both cases, the antibodies to the C-subunit specifically bound to the 37-kDa, axonemal protein (Fig. 8B). The identical blot re-probed with anti-PP1 antibody showed that the 37-kDa protein is slightly larger than PP1 (Fig. 8B and Fig. 1). Moreover, the PP1c antibody selectively precipitated the 35 kDa PP1c: the 37 and 62-kDa microcystin binding proteins were not found in such immune precipitates (data not shown). Immuno-fluorescence microscopy using the monoclonal antibody to the C-subunit revealed PP2A is found along the length of both flagellar axonemes and bright staining of the cell body. Bar, 5 μm.

**PP2A is localized to the outer doublet microtubules**

To further determine the location of PP2A in the axoneme, western blot analysis was performed using the flagella and axonemes isolated from flagellar mutants. As described above for axonemal PP1, we postulated that axonemal PP2A is anchored to distinct structures, and that failure to assemble the anchor structure would result in failure of PP2A to co-purify with the axoneme. To test this, we analyzed PP2A from both flagella and axonemes from each mutant using the monoclonal antibody to the C-subunit of PP2A. Based on western blots, isolated flagella and axonemes from mutants lacking outer or
inner arm dyneins, radial spokes, and the dynein regulatory complex all contained wild-type levels of PP2A. In contrast to PP1, PP2A is found in both the flagella and axonemes of mutants that fail to assemble the central pair apparatus. This is best illustrated in Fig. 9A, comparing the staining of the PP1 to the staining of PP2A for flagella, Nonidet extract, and axonemes from pf19 and wild-type cells. Notably, PP2A is distributed in an equivalent manner in wild type and pf19, indicating the axonemal PP2A is not located in the central pair apparatus. The isolated flagella and axonemes from ‘mia’ mutants, which are defective in phosphorylation of IC138 (King and Dutcher, 1997), also contain wild-type levels of PP2A. Among all the mutants analyzed, only flagella and axonemes from pf15 contained reduced amount of PP2A (Fig. 9B). Although pf15 lacks the central pair, it is not likely that the axonemal PP2A is located in the central pair structure since other central pair mutants, including pf6, pf16, pf18, pf19, and pf20, all contain wild-type concentration of PP2A (Fig. 9B). Moreover, other studies have shown that lack of the central pair complex in pf15 is likely to be a secondary consequence of the defect in pf15 (Smith and Lefebvre, 1998). Since the mutants lacking dyneins, central pair, radial spoke, dynein regulatory complex all contain a similar concentration of PP2A, we conclude that PP2A is anchored to the nine outer microtubule doublets.

**DISCUSSION**

We determined that two protein phosphatases, PP1 and PP2A, are transported to the flagellar compartment and anchored in the axoneme. This conclusion is founded on microcystin-affinity of extracts from flagella and isolated axonemes. The conclusion is also based on western blot analysis of flagellar fractions including the membrane-matrix and isolated axonemes and based on light microscopic immunolocalization. Localization of the phosphatases to the axoneme is consistent with previously published pharmacological analysis revealing PP1 and possibly PP2A are anchored to the microtubules of the axoneme in positions to control the phosphorylation and activity of inner arm dynein (Habermacher and Sale, 1996, 1997). The results are also consistent with immuno-localization analysis of PP1 in cilia (Momayezi et al., 1996). Not surprisingly, additional fractions of flagellar PP1c and PP2A are found in the detergent soluble, membrane-matrix fraction where they may play additional roles.

The axonemal fraction of PP1c is predominantly localized to the central pair apparatus and is associated with the C1 microtubule. This conclusion is founded on analysis of both flagella and axonemes from mutant cells that fail to assemble specific axonemal structures (Fig. 6). In particular, localization of PP1c to the C1 microtubule of the central pair apparatus is based on analysis of axonemes from pf16, which fails to assemble the C1 central pair structure (Dutcher et al., 1984; Smith and Lefebvre, 1996, 1997b; Mitchell and Sale, 1999). Importantly, in each central pair mutant examined the isolated flagella retain the wild-type concentration of PP1c despite the lack of PP1c in the isolated axonemes. The simplest interpretation is that the majority of axonemal PP1c is anchored in the central pair apparatus, and failure to assemble the key anchor structures results in failure of PP1c to sediment with the axoneme. The significance of the central pair to axonemal function is discussed below.

The axonemal fraction of PP2A is localized to the outer doublet microtubules. This conclusion is also founded on western blot analysis of both flagella and axonemes from mutant cells that fail to assemble specific structures. Based on this analysis, PP2A is found at identical concentration in axonemes from wild-type cells and cells lacking the central pair apparatus, radial spokes, outer or inner arm dyneins, or the dynein regulatory complex. By elimination, PP2A is likely to be associated with the outer doublet microtubules. Thus, one hypothesis is that PP2A is anchored in position to directly control phosphorylation of IC138 in inner arm dynein I1 (see Habermacher and Sale, 1997). The minor fraction of axonemal
PP1c, not located in the central pair, may also be anchored in position to control dynein activity, but this may be unlikely since only a very small amount of axonemal PP1c is found outside of the central pair. To test these models, it will be necessary to develop new reagents or approaches to directly localize PP1 and PP2A in the axoneme. It will also be necessary to determine the relative stoichiometry and determine the anchor proteins for the axonal fractions of PP1 and PP2A.

Diverse evidence has revealed that regulation of dynein is imposed in part by activities of the central pair apparatus, the radial spokes and dynein regulatory complex (Huang et al., 1982; Smith and Sale, 1992; Piperno et al., 1992, 1994; Porter et al., 1992, 1994; Yoshimura and Shingyoji, 1999). The mechanism is not understood, but at least one of these interactions involves phosphorylation of inner dynein arms (Howard et al., 1994; Habermacher and Sale, 1996, 1997; King and Dutcher, 1997). The mechanism appears to involve control of flagellar waveform (Brokaw et al., 1982; Hosokawa and Miki-Noumura, 1987; Brokaw and Kamiya, 1987; King and Dutcher, 1997), however, other axonomal components are also involved in this control (Frey et al., 1997; Wakabayashi et al., 1997). In other examples, the interactions involve outer arm dynein (Huang et al., 1982; Porter et al., 1994) and control of beat frequency (Mitchell and Sale, 1999). The challenge in each case is to determine how signals in the central pair apparatus become transmitted through the radial spokes and dynein regulatory complex, to alter dynein activity.

Based on localization of PP1c in the central pair apparatus, the central pair apparatus may operate, in part, through changes in phosphorylation of central pair proteins, consequently leading to change in the structural interaction with the radial spokes. As described above, this model is consistent with genetic studies that revealed the central pair/radial spoke system controls dynein activity. The model is also consistent with structural studies showing a transient interaction between the spokes and central pair projections (Warner and Satir, 1974; Goodenough and Heuser, 1985), and with discovery that in many cases the central pair rotates relative to the radial spokes (Omoto et al., 1999). In vivo analysis has revealed that at least eight of twenty-three central pair proteins and at least five of seventeen radial spoke proteins are phosphoproteins (Piperno et al., 1981; Huang et al., 1981; Adams et al., 1981). Therefore, predictably, the central pair complex and radial spokes contain protein kinases. Mammalian sperm tail axonemes bear a unique catalytic subunit for PKA with structure that may play a role in anchoring PKA and restricting mobility (San Agustin et al., 1997). The mechanism appears to involve control of flagellar waveform (Brokaw et al., 1982; Hosokawa and Miki-Noumura, 1987; Brokaw and Kamiya, 1987; King and Dutcher, 1997), however, other axonomal components are also involved in this control (Frey et al., 1997; Wakabayashi et al., 1997). In other examples, the interactions involve outer arm dynein (Huang et al., 1982; Porter et al., 1994) and control of beat frequency (Mitchell and Sale, 1999). The challenge in each case is to determine how signals in the central pair apparatus become transmitted through the radial spokes and dynein regulatory complex, to alter dynein activity.

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The axoneme offers a new opportunity to define mechanisms that anchor protein phosphatases in the cell. Protein phosphatases 1 and 2A dephosphorylate serine and threonine residues on proteins that control a wide range of cellular functions (Cohen, 1989; Wera and Hemmings, 1995). Thus, the question is how diverse substrates, within a cell, are regulated independent of one another. In many cases substrate specificity of PP1c appears to be conferred by localization of the catalytic subunit through regulatory or targeting subunits (Hubbard and Cohen, 1993; Brautigan, 1997; Campos et al., 1996; Egloff et al., 1997; Colbran et al., 1997; Damer et al., 1998; Liao et al., 1998). However, it is likely that we are just beginning to identify such anchor mechanisms.

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