An axonemal PP2A B-subunit is required for PP2A localization and flagellar motility

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Abstract

Analysis of *Chlamydomonas* axonemes revealed that the protein phosphatase, PP2A, is localized to the outer doublet microtubules and is implicated in regulation of dynein-driven motility. We tested the hypothesis that PP2A is localized to the axoneme by a specialized, highly conserved 55-kDa B-type subunit identified in the *Chlamydomonas* flagellar proteome. The B-subunit gene is defective in the motility mutant *pf4*. Consistent with our hypothesis, both the B- and C-subunits of PP2A fail to assemble in *pf4* axonemes, while the dyneins and other axonemal structures are fully assembled in *pf4* axonemes. Two *pf4* intragenic revertants were recovered that restore PP2A to the axonemes and re-establish nearly wild-type motility. The revertants confirmed that the slow-swimming Pf4 phenotype is a result of the defective PP2A B-subunit. These results demonstrate that the axonemal B-subunit is, in part, an anchor protein required for PP2A localization and that PP2A is required for normal ciliary motility.

Keywords

Cilia; flagella; dynein; axonemes; protein phosphatases; microtubules

Introduction

Cilia, also called flagella, are responsible for a surprisingly wide range of essential motile and sensory / signaling functions (Berbari et al. 2009; Gerdes et al. 2009; Goetz and Anderson 2010; Satir and Christensen 2007; Smith and Rohatgi 2011). Motile cilia play a central role in the embryonic node where they contribute to normal pattern formation and in the adult where they are required for normal mucociliary transport in the airway, movement of cerebral spinal fluid in the brain ventricles, fluid movement in the oviducts and sperm motility (Brokaw 2009; Lindemann and Lesich 2010; Satir and Christensen 2008; Wallingford 2010). Defects in motile cilia can result in the ciliopathy called primary cilia dyskinesia (PCD), randomization of pattern formation, chronic respiratory infection and infertility (Basu and Brueckner 2008; Hashimoto and Hamada 2010; Hirokawa et al. 2006;

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Leigh et al. 2009). However, we are only beginning to understand the mechanisms that regulate ciliary motility.

Diverse studies in a number of experimental systems have revealed that ciliary / flagellar motility is regulated by phosphorylation (Elam et al. 2009; Salathe 2007). One of the best studied examples revealed that the inner dynein arm, I1 (or dynein f), is required for control of flagellar waveform (Bayly et al. 2010; Brokaw and Kamiya 1987) and phototaxis (King and Dutcher 1997; Okita et al. 2005). Analysis of isolated *Chlamydomonas* axonemes demonstrated that dynein-driven microtubule sliding is regulated by phosphorylation of the I1 intermediate chain, IC138 (Porter and Sale, 2000; Wirschell et al. 2007). One of the surprises was that protein kinases and phosphatases are localized to the axoneme and include conserved, ubiquitous kinases (PKA, CK1) and phosphatases (PP1, PP2A) (Gokhale et al. 2009; Habermacher and Sale 1995; Habermacher and Sale 1996; Howard et al. 1994; Yang et al. 2000; Yang and Sale 2000). However, with the exception of the A-kinase anchoring proteins, AKAPs (Gaillard et al. 2001; Gaillard et al. 2006), it is not known how CK1, PP1 or PP2A are anchored in the axoneme.

Biochemical analysis of *Chlamydomonas* axonemes revealed that PP2A is localized to the outer doublet microtubules (Yang et al. 2000). PP2A is a versatile and ubiquitous serine / threonine phosphatase that plays a role in many vital cellular processes including cell growth, cell cycle, motility and apoptosis (Basu 2010; Eichhorn et al. 2009; Mumby 2007; Shi 2009b; Sontag and Sontag 2006; Virshup and Shenolikar 2009). The holoenzyme is composed of three subunits; the A- scaffolding or structural, C-catalytic and the B-regulatory subunits (Basu 2010). The A- and C-subunits form an obligate heterodimer, which then interacts with a B-type subunit (Janssens et al. 2008; Shi 2009a; Virshup and Shenolikar 2009). In mammals, four different families of B-subunits have been defined (PR55/B, PR61/B', PR72/B", and PR93/PR110/B'''). Furthermore, in each family, at least three different genes encode several different B-type subunit isoforms (Eichhorn et al. 2009). The common feature of each family member is the ability to bind to the PP2A A- and C- dimer (Xu et al. 2008): otherwise, there is very little sequence or structural homology among the four families (Eichhorn et al. 2009). However, within each family, the sequences and structures are very similar. One prevalent idea is that B-subunits function to provide specificity by targeting and anchoring PP2A to precise positions, organelles and substrates in the cells (Eichhorn et al. 2009; Lechward et al. 2001). We previously identified A- and C- subunits in *Chlamydomonas* axonemes (Yang et al. 2000). Thus, we postulated the axoneme contains a specialized B-type subunit for localization of PP2A.

Taking advantage of the *Chlamydomonas* flagellar proteome (Pazour et al. 2005), we identified a highly conserved axonemal PP2A B-subunit in the B/PR55, WD-repeat family (Supplemental Fig. 1) (Eichhorn et al. 2009). Using an antibody to a polypeptide in the N-terminus, we confirmed the B-subunit is localized to the axoneme. The B-subunit gene maps to the *PF4* locus on chromosome I (McVittie 1972) and sequencing confirmed the *pf4-1* strain has a mutation in the B-subunit gene. Immunoblots revealed the axonemal B-subunit is missing from *pf4* axonemes. Consistent with our hypothesis, the axonemal PP2A C-subunit also fails to assemble in *pf4* axonemes. The *pf4* cells swim slowly and fail to phototax, a phenotype similar to mutants that lack the inner dynein arm I1 (Brokaw and Kamiya 1987; King and Dutcher 1997; Okita et al. 2005). However, *pf4* axonemes show no defect in assembly of the dynein arms or other axonemal structures. Through genetic analysis of *pf4*, two intragenic revertants were recovered that restore assembly of PP2A in the axoneme, rescue phototaxis and near wild-type swimming, thus confirming the *pf4* mutant phenotype is a consequence of the B-subunit mutation. This data demonstrates that the B-subunit is necessary for localization of PP2A in the axoneme and that PP2A is required for normal flagellar motility.

Results and Discussion

A PP2A B-subunit is localized to the axoneme

To identify a potential B-type subunit in *Chlamydomonas* flagella, we analyzed the flagellar proteome database and identified a conserved member of the B/PR55 family of B-subunits (Pazour et al. 2005). The proteome data indicates the B-subunit is an axonemal protein that is solubilized with high salt. The B-subunit gene is 3792 base pairs with nine exons and eight introns (Fig. 1A) and encodes a highly conserved 52.6-kDa protein with seven WD-repeats that form a seven bladed propeller-like structure (Fig. 1B; Sup. Fig. 1, see Xu et al. 2008). While there are other B-type subunits found in the *Chlamydomonas* genome (Table I), only this B-subunit isoform was identified in the flagellar proteome. Predictably, since PP2A is a ubiquitous and functionally versatile phosphatase (Shi 2009b), the other, non-flagellar B-type subunit proteins are responsible for targeting PP2A to other cellular locations in *Chlamydomonas*.

To confirm that the identified B-subunit localizes to the flagellar axoneme, we generated a B-subunit specific antibody that recognizes a ~50-kDa band on immunoblots of isolated flagella and axonemes, consistent with the predicted size of the B-subunit protein (Fig. 2A; Supplemental Fig. 2). The B-subunit is partially solubilized with detergent (M-M fraction), while the fraction of the B-subunit in the axoneme is solubilized in high salt buffers, confirming the fractionation profile reported in the flagellar proteome and consistent with the solubility of the axonemal A- and C-subunits (Pazour et al. 2005; Yang et al. 2000).

The flagellar mutant, *pf4*, is defective in the B-subunit gene

The B-subunit gene was mapped to Chromosome 1 using BAC clone, 14G12, identified in the *Chlamydomonas* genome database (http://genome.jgi-psf.org/Chlre4/Chlre4.home.html). The gene maps near the *pf4* mutant (Levine and Goodenough 1970; McVittie 1972), a slow-swimming mutant that otherwise had not been characterized. Sequence analysis revealed that *pf4* contains a base pair substitution/deletion at positions 2239 and 2240 in exon seven of the B-subunit gene (Fig. 2B; Supplemental fig. 2A, B). This mutation predicts a F306A change in the fifth WD repeat and a frame shift resulting in the coding of a premature stop codon; the mutation predicts a 37-kDa truncated protein product.

To verify the phenotype associated with defects in the B-subunit gene, we backcrossed *pf4* to wild-type cells three times and selected one progeny, *pf4 9-1*, as the representative *pf4* mutant strain for all experiments. The *pf4 9-1* mutant displayed full-length flagella and reduced swimming speed (see below). From here on, we will refer to *pf4 9-1* as *pf4*.

The B-subunit is required for targeting of PP2A to the axoneme

We predicted that the B-subunit would be missing in *pf4* cells and, as a consequence, the entire PP2A complex would fail to assemble in the *pf4* axoneme. Immunoblots failed to detect the B-subunit in *pf4* axonemes (Fig. 2C) or flagella (Supplemental Fig. 2D). Analysis of additional cell and flagellar fractions from the *pf4* mutant demonstrate that *pf4* does not express any full length or truncated B-subunit protein. Thus, the *pf4* mutant appears to be a null allele for the B-subunit. Notably, the C-subunit also fails to assemble in *pf4* axonemes (Fig. 2C) and flagella (Supplemental Fig. 2D). Given that the A- and C-subunits are an obligate heterodimer and consistent with our model, these data indicate that assembly of the B-subunit is necessary for localization of the PP2A C-, and presumably A-, subunits to the outer doublet microtubules. Since in *pf4* the PP2A C-subunit fails to assemble in flagella and axonemes, the axonemal B-subunit may be the predominant or sole PP2A B-type subunit in the flagellar compartment.

The *pf4* mutant has defective motility and fails to phototax

The *pf4* swimming phenotype is similar to the phenotype of mutant cells lacking I1 dynein (Fig. 3A); *pf4* swimming velocities are reduced to velocities exhibited by *ida3* mutants (shown here) and other I1-dynein mutants including *ida7* (Perrone et al. 1998). Similar to inner dynein arm mutants (Kamiya et al., 1991), flagellar beat frequency is only slightly reduced in *pf4* compared to wild-type cells. Thus, like inner dynein arm mutants (Brokaw and Kamiya, 1987; Bayly et al., 2010), reduced swimming speed in *pf4* is primarily a consequence of altered flagellar waveform. We performed immunoblot analysis and thin section electron microscopy to determine whether the dynein arms are assembled in *pf4* axonemes. Immunoblots determined the dynein arms and radial spokes are fully assembled in *pf4* axonemes (Fig. 3B). Transmission electron microscopy also confirmed that there are no obvious structural defects in the dynein arms, radial spokes or central pair apparatus (Fig. 3C). Thus, the motility phenotype in *pf4* appears to be a consequence of a failure in PP2A assembly that results in a failure in regulation of dynein motor function.

Diverse genetic and pharmacological evidence has revealed an axonemal phospho-regulatory mechanism that controls dynein activity (Porter and Sale 2000; Wirschell et al. 2007). As indicated above, like the *pf4* mutant, mutations that lead to a failure in assembly of I1 dynein results in a slow-swimming phenotype (Fig. 3A; Bower et al. 2009; Hendrickson et al. 2004; Myster et al. 1997; Myster et al. 1999; Perrone et al. 2000; Perrone et al. 1998; Porter et al. 1992; Toba et al. 2011) and defective phototaxis (Fig. 4A; King and Dutcher 1997; Okita et al. 2005). To test whether PP2A may be part of dynein regulatory pathway, we examined phototaxis in *pf4* cells. A photo-accumulation assay revealed that *pf4* cells do not respond to light; failing to accumulate on one side of a dish, demonstrating that *pf4* mutant cells are defective in phototaxis (Fig. 4A). Thus, assembly of PP2A is required for normal motility and phototaxis. One model is that PP2A is part of the phospho-regulatory mechanism that regulates I1 dynein by control of the regulatory phosphoprotein IC138 (discussed below and see Bower et al. 2009; Habermacher and Sale 1997; Hendrickson et al. 2004; King and Dutcher 1997).

Intragenic *pf4* revertants restore PP2A assembly and rescue phototaxis and near wild-motility

To verify that the defect in the B-subunit gene is responsible for the Pf4 mutant phenotype and to potentially define an axonemal regulatory pathway that includes PP2A, we performed a suppressor screen and recovered two independent intragenic *pf4* revertant strains, *pf4rV5* and *pf4rV11*, that harbor an identical second site mutation in the *PF4* gene (Fig. 4B, Supplemental Fig. 2A). Mapping analysis revealed that *pf4* and the revertants are intragenic based on no recovery of the Pf4 phenotype in 350 tetrads. Sequence analysis revealed a 7-bp insertion (AGAAGAA) upstream of the original *pf4* mutation which restores the reading frame. The new sequence in the *pf4rV5* and *pf4rV11* revertants predicts changes in amino acids 300-308 with the insertion of a valine and a proline, and the alteration of an alanine to a glycine; these amino acids may introduce kinks in the B-sheet structure (Fig. 4B; Supplemental Fig. 2). Thus, the predicted structure of the revertant B-subunit protein should differ from the wild-type PP2A B-subunit.

To determine whether *pf4* revertants axonemes assemble PP2A, isolated axonemes were analyzed by immunoblots using the PP2A B- and C-subunit antibodies. The revertant PP2A B-subunit and C-subunit were fully assembled at wild-type levels in *pf4* revertant axonemes (Fig. 4C), thus providing additional evidence that the B-subunit is required for localization of the PP2A holoenzyme to the axonemal structure. The revertant B-subunit migrates slightly slower on SDS-PAGE gels compared to the wild-type B-subunit (Fig. 4C). This observation is consistent with the prediction that the revertant protein differs slightly from

the wild-type protein, however retains the ability to localize the PP2A holoenzyme in the axoneme.

Since the revertant axonemes assemble PP2A, we examined phototaxis and motility in the *pf4* revertant cells. The revertant cells have restored phototaxis (Fig. 4A) and increased swimming velocities relative to the *pf4* mutation alone (Fig. 4D). However, the swimming velocities were not fully restored to wild-type levels (Fig. 4D); which indicates that the modified B-subunit expressed in *pf4rV5* and *pf4rV11* is partially defective. The revertant B-subunit contains a VP insertion, an A302G substitution and several additional amino acid substitutions in the fifth WD repeat which may disrupt the β-sheet structure (Fig. 4B). Perhaps PP2A is accessible only to a subset of its axonemal substrates or the function of PP2A is partially compromised in the revertants. Consistent with this idea, and as an example, a B-type subunit has been shown to interact with Tau to contribute to interaction with the catalytic subunit (Xu et al. 2008). Thus, the inability to fully restore wild-type swimming is most likely a direct result of the modified B-subunit protein. These data also suggest that the phototaxis pathway may be separate from the pathway controlling swimming velocity. Consistent with this interpretation, recent analysis of *Chlamydomonas* null mutations in the IC138 gene (*bop5*) revealed that IC138 is required from normal flagellar bending, but not required for phototaxis (See Discussion below and our unpublished data).

The *pf4* double mutants display more severe phenotypes

To better understand the role of PP2A in regulation of motility, we generated double mutants between *pf4* and dynein arm mutations. As discussed, diverse evidence suggest PP2A may function in a conserved signaling pathway that involves the central pair, radial spokes and at least one ciliary dynein motor, the inner arm I1 dynein (Porter and Sale 2000; Smith and Yang 2004; Wirschell et al. 2007). The Pf4 motility phenotype is consistent with defects in regulation of the inner dynein arms; *pf4* and inner dynein arm mutations exhibit slow-smooth swimming phenotypes. If the motility defect in *pf4* is due to a mis-regulation of I1 dynein, then we predict the motility phenotype of a *pf4*, I1 double mutant will be similar to or no worse than the parental motility phenotypes. Furthermore, we predict that double mutants between *pf4* and other dynein motors, which are unaffected by PP2A, will display a more severe phenotype.

Table II lists the *pf4* double mutants generated in this study and their motility phenotypes. When the *pf4* defect is combined with defects in inner arm dyneins a, c, and d (*ida4*), inner arm dynein e (*ida6*), the outer dynein arm (*oda6*) or with defects in regulation of I1 dynein (*mia1*, *mia2*), the resulting motility phenotypes were significantly worse than the parental phenotypes. These results indicate that PP2A does not play a unique role in regulation of the single headed inner dynein arms or the outer arms and that axonemal PP2A does not function in regulatory mechanisms unique to the pathways defined by the *mia1* and *mia2* mutants. In spite of several attempts, we could not isolate double mutants between *pf4* and any I1-dynein mutant (*ida1*, *ida7*, *bop5*). The significance of this result is unclear, but is possibly technical or that these combinations are lethal for unknown reasons. It is possible we may be revealing additional, non-ciliary functions for the PP2A B-subunit and I1 dynein.

Based on pharmacological evidence for a role for PP2A in regulation of I1 dynein and our current results, PP2A may operate, at least in part, in a pathway that includes I1 dynein and possibly IC138 (Bower et al. 2009; Wirschell et al. 2007). Consistent with this hypothesis, we determined that IC138 is highly phosphorylated in *pf4* axonemes compared to wild-type axonemes; a phenotype similar to that observed for mutants that are defective in the phospho-regulatory pathway that controls I1 dynein (Fig. 4E). IC138 also appears to be highly phosphorylated in *pf4* revertant axonemes (Fig. 4E). As discussed above, this

observation may also indicate that the revertant PP2A B-subunit differs in structure from wild-type, and as a consequence, IC138 is abnormally phosphorylated in the revertants resulting in only partial restoration of swimming speed. To date, the key phospho-residues in I1 dynein are not known and, thus, sites of phosphorylation in IC138 may differ in *pf4* axonemes compared to the *pf4* revertants. Further tests of this model will require identification of specific IC138 phospho-residues and detailed analysis of point mutations in these residues. Nevertheless, these results are consistent with a role for PP2A in regulation of I1 dynein.

What is the role of PP2A in signaling and regulation of motility?

Targeting of PP2A to specific sub-cellular domains is an important model for control of substrate specificity. A general role for the B-subunits may be for targeting PP2A to specific sites within the cell for interaction with specific substrates also localized in the same position (Matre et al. 2009; Price et al. 1999; Schmidt et al. 2002; Strack et al. 1998). Based on our current results, we demonstrate that a B/PR55 type B-subunit functions to target PP2A to the flagellar compartment and axoneme where it plays a role in regulating motility. Members of the B/PR55 family are enriched in the ciliated tissues such as the brain and testis (Hatano et al. 1993; Mayer et al. 1991) and play a role in targeting PP2A to microtubules (Nunbhakdi-Craig et al. 2007; Sontag et al. 1999; Tar et al. 2004). An anchoring factor binding PP2A to the microtubules has also been reported (Price et al. 1999). It will now be important to localize PP2A on the outer doublet microtubules and define the axonemal B-subunit interacting proteins that localize PP2A in the axoneme.

Because pharmacological evidence implicates PP2A in control of dynein-driven microtubule sliding (Wirschell et al. 2007) and *pf4* mutants strongly phenocopy I1-dynein mutants, our results are consistent with a role of PP2A in regulation of I1 dynein and control of flagellar bending. However, we cannot exclude the possibility that PP2A also regulates additional dyneins. Important questions include identifying specific PP2A substrates in the axoneme and determining if PP2A is directly regulating I1 dynein and IC138.

Materials and methods

Strains, genetic analyses and culture conditions

Chlamydomonas reinhardtii strains include CC124 and CC125 (wild-type), *pf4* mt− (CC-880), *pf4* mt+ (CC-1027) from the *Chlamydomonas* Genetics Center (Univ. of MN, St. Paul, MN), *pf4 9-1* (recovered from a parental tetrad between wild type and *pf4* crosses), *pf4rV5* and *pf4rV11* (recovered from non-parental tetrad, see below). All experiments utilized the backcrossed progeny *pf4 9-1* and referred to as pf4 in this manuscript.

Backcrosses and tetrad dissection were performed as described (Dutcher 1995). Cells harboring the *pf4* mutation are palmelloid, which is a phenotype often displayed in mutants with short flagella or a slow-swimming phenotype. Suppressors of *pf4* were generated by treating *pf4* cells with a UV light dose of 700 μJ/cm^2 and screening for individual colonies that hatched. An identical second site mutation in the B-subunit gene was identified in two clones, *pf4V5* and *pf4V11*. In addition to the two intragenic revertants described here, several extragenic suppressor mutations were isolated and will be characterized elsewhere.

Hatching of the palmelloid cells is promoted by resuspending *pf4* cells in water or gametic autolysin. Cells were grown in L-medium with aeration on a 14:10 hr light: dark cycle.

Molecular Biology

A restriction fragment containing the first 134 amino acids of the B-subunit was cloned into the pET28A vector (EMD) using *Bam*HI and *Xho*I to create pCEBsub134 and protein expression induced with 1mM IPTG. The expressed fusion protein was purified using a metal affinity resin (Clontech, Mountain View, CA) and used as an antigen for production of a B-subunit antibody (Spring Valley Labs, Inc, Woodline, MD). B-subunit antibodies were blot purified using the fusion protein. Specific antibodies were eluted using 100 mM citric acid pH 3.0 followed by neutralization with 100 mM Tris pH 9.0.

The *pf4* and *pf4* revertant mutations (*pf4v5* and *pf4v11*) were identified by sequencing the entire *PF4* gene. All primers were designed using Primer3 program (http://frodo.wi.mit.edu/primer3/) and sequencing was performed by Iowa State DNA sequencing facility (http://www.dna.iastate.edu/).

Flagella Isolation

Flagella were isolated from three liters of cells as described (Witman 1986) and resuspended in Na-low buffer (30mM HEPES, pH 7.4; 5mM MgSO4·7H$_2$0; 1mM DTT; 0.5mM EDTA; 30Mm NaCl; 1mM PMSF; 0.6 trypsin inhibitory unit [TIU] Aprotinin). NP-40 was added to a final concentration of 1% and axonemes and detergent soluble membrane-matrix fractions separated by centrifugation. To extract the PP2A B-subunit, proteins were extracted from axonemes with 0.6 M NaCl for 30 min and the salt-soluble protein and extracted axonemes were separated by centrifugation. Samples from each step were saved for SDS-PAGE analysis.

SDS-PAGE and immunoblot analysis

Flagellar samples were run on 5%, 7.5% or 10% SDS-PAGE gels and transferred to a nitrocellulose membrane (BioRad, Hercules, CA) for analysis. Equivalent sample loads of each flagellar fraction were loaded. Immunoblots were probed with the following antibodies: affinity purified B-subunit (1:50 – this study), IC140 (1:10,000 – Yang and Sale, 1997), IC138 (1:10,000 – Hendrickson et al., 2004), IC97 (1:10,000 – Wirschell et al., 2009), FAP120 (1:1000 – Ikeda et al., 2009), RSP3 (1:1000 – Williams et al., 1989), LC8 (1:50 – King et al., 1996), IC1 (1:1000 – King et al.,1986), actin (1:100 – Sugase et al., 1996) and p28 (1:250 – LeDizet and Piperno, 1995). For analysis of IC138 phosphorylation, axonemes were treated with calf intestinal phosphatase (CIP) as described (Hendrickson et al. 2004) or buffer alone and resolved on 5% gels and analyzed by immunoblots using the IC138 antibody.

Motility analyses

Swimming velocity measurements were determined as described (Elam et al. 2009). Briefly, cells were placed on a microscope perfusion chamber and visualized using a 10X Plan-Apochromat lens (Carl Zeiss, Inc.) and a darkfield condenser using an Axiovert 35 inverted light microscope (Carl Zeiss, Inc.). Cells were recorded using a silicon intensified camera (VE-1000; Dage-MTI) and converted to a digital format using Labview 7.1 software (National Instruments). Swimming velocity was calculated using manual tracings of twenty cells for each strain from three independent experiments.

Photo-accumulation assays were performed as follows: cells (2.5×10^6 cells/ml in 35 mm petri dishes) were dark adapted for 10 min, then exposed to either ambient or fluorescent light located 30 cm away ($2 W/m^2$) with the same photo-accumulation results. Dishes were photographed after 5 min to determine relative photo-accumulation ability.

Electron Microscopy

Electron microscopy samples were prepared as described (Mitchell and Sale 1999). Embedded axonemes were thin sectioned, stained with lead citrate and uranyl acetate and viewed using a Hitachi H7500 transmission electron microscope. Images were acquired usiing a Gatan 792 BioScan CCD camera with Gatan Digital Micrograph software (Gatan, Pleasanton, CA) and adjustments were made using Adobe Photoshop (Adobe Systems, Mountain View, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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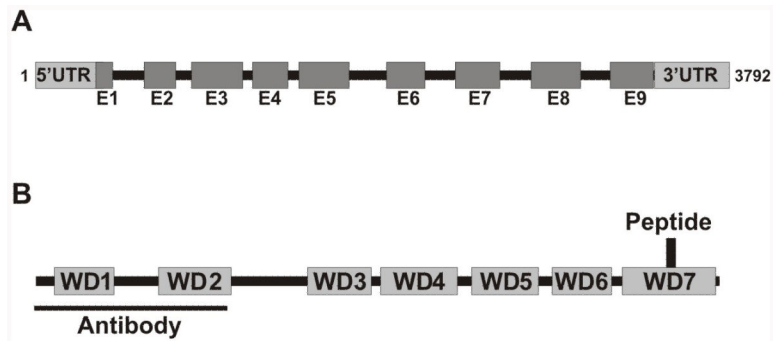
Figure 1. The*Chlamydomonas* **PP2A B-subunit**
(A) The B-subunit gene is 3792 base pairs and contains nine exons – E1-E9 - and eight introns. **(B)** The predicted *Chlamydomonas* B-subunit protein is 479 amino acids with a mass of 52.6 kDa. The protein contains seven WD-repeats throughout its protein sequence, forming a seven bladed propeller-like structure. Amino acids 1-134 were used to design an antibody to the B-subunit protein. The peptide (amino acids 439-452) identified in the *Chlamydomonas* Flagellar Proteome resides in the seventh WD repeat domain located in the C-terminus.
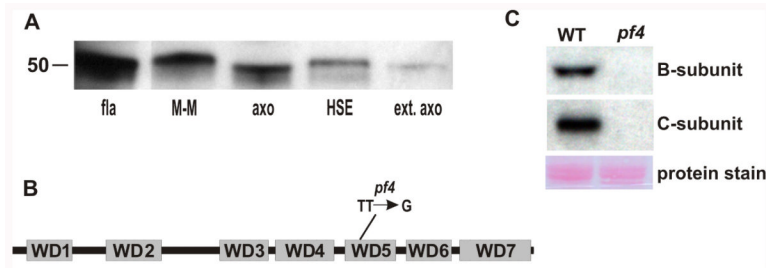
Figure 2. The B-subunit gene is mutated in *pf4* cells and the PP2A holoenzyme fails to target to the axoneme

(A) Immunoblots performed with the B-subunit antibody detect a band in isolated wild-type flagella (fla) with a relative mobility of ~50 kDa, consistent with the predicted size of the B-subunit protein. The B-subunit is partially soluble in detergent (membrane-matrix fraction; M-M); while a fraction of the B-subunit remains associated with the axoneme (axo). The axonemal fraction of the B-subunit is solubilized with high salt buffers (HSE), leaving very little left on the extracted axonemes (ext. axo). Variations in B-subunit migration is a consequence of protein abundance, detergent or salt concentrations in the samples. **(B)** Schematic of the B-subunit protein shows the location of the *pf4* mutation (wild-type sequence is TT; *pf4* sequence is G) in the fifth WD repeat. **(C)** Immunoblots of wild-type and *pf4* axonemes demonstrate that the PP2A B-subunit is not present *pf4* axonemes. Furthermore, the C-subunit also fails to target to the axoneme in the absence of the B-subunit. A protein stain showing tubulin from these samples is shown below.
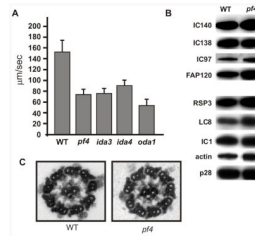
Figure 3. The *pf4* axonemes are structurally similar to wild-type
(A) Swimming velocities of wild-type and mutant cells were analyzed. The *pf4* mutant has a reduced swimming velocity that is comparable to mutants lacking I1 dynein (*ida3*). Also analyzed are *ida4*, a mutant lacking inner arms a, c, and d, and *oda1* lacking the outer dynein arm. **(B)** Immnoblots of wild-type and *pf4* axonemes demonstrate that all of the axonemal dynein motors are assembled in *pf4* including the I1 dynein (IC140, IC138, IC97, FAP120), dyneins a, b, c, d, e, and g (actin and p28) and the outer dynein arm (IC1). In addition, there are no defects in assembly of the radial spokes (RSP3). LC8 levels are also unaltered (LC8 is a component of I1, the outer arm and the radial spoke). **(C)** Transmission electron microscopy of wild-type and *pf4* axonemes demonstrates that *pf4* assembles all the major axonemal structures including the central pair, the radial spokes, the dynein arms and the outer doublet microtubules.
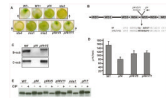
Figure 4. The *pf4rV5* and *pf4rV11* revertants assemble the PP2A B- and C-subunits in the axoneme and partially rescue the Pf4 phenotype

(A) Photo-accumulation assays reveal that *pf4* is defective in phototaxis. Wild-type cells swim towards (WT+; *AGG1*) or away (WT−, *agg1*) from a light source (black arrows) and accumulate on one side of a culture dish, whereas *pf4* cells do not. This defect is also present in *ida3, mia1* and *mia2* mutants—defective in I1 dynein assembly or regulation, but is not a consequence of slow swimming, as another inner arm mutant and an outer arm mutant (*ida4* and *oda1* respectively) photo-accumulate with wild-type kinetics. These assays revealed that phototaxis is rescued in the *pf4* revertants; the revertant cells swim away from the light source and accumulate on one side of the culture dish, similar to wild-type cells **(B)** Schematic of the B-subunit protein shows the location of the original *pf4* mutation and the upstream *pf4* revertant mutation—a 7-bp insertion (AGAAGAA) at positions 1802-1808 in exon 6 in the fifth WD repeat domain. **(C)** Immunoblots of isolated axonemes probed with the B- and C-subunit antibodies demonstrate that PP2A assembly is restored in the revertants (*pf4rV5*) as compared to wild-type and the *pf4* mutant. **(D)** Analysis of swimming speed demonstrates that the *pf4rV5* and p*f4rV11* revertant cells swim with a velocity intermediate between wild-type and *pf4* cells. The velocity of swimming in the revertants *pf4V5* and *pf4V11* was significantly faster than the swimming velocity of *pf4* (P < 0.001 by t-test). **(E)** Immunoblots of axonemes treated with buffer alone or CIP were probed with the IC138 antibody as described (Hendrickson et al. 2004; Wirschell et al. 2009). Wild-type axonemes display relatively little IC138 phosphorylation (compare axonemes treated with CIP to untreated axonemes) while axonemes from mutants defective in regulation of I1 dynein (*mia1, pf17*) show excessive IC138 phosphorylation due to a global inactivation of I1-dynein activity. Axonemes from *pf4* and the *pf4* revertants also show increased IC138 phosphorylation, consistent with a role for PP2A in regulation of I1 dynein.

Table I
Potential PP2A subunits in the *Chlamydomonas* Genome Database

(JGI version 4) reveals several PP2A subunits including 2 A-subunits, 4 C-subunits and 5 B-type subunits. Both A-subunits and one of the C-subunits were found in the *Chlamydomonas* flagellar proteome (Pazour et al., 2005). Of the five B-type subunits in the genome, only one, PP2A1, was identified in the flagellar proteome. This gene is defective in the *pf4* mutant.

Type	Name[1]	Protein ID[2]	Domain/Motif/Family[3]	Flagellar Proteome[4]
A (Scaffold)	PP2A-2r	131550	HEAT	YES
	FAP14	194683	HEAT	YES
B, B', B", B'" (Regulatory)	PP2A-1r (B")	112343	---	NO
	PP2A2 (B')	127638	B56	NO
	TAP42-like protein	148948	TAP42	NO
	PP2A1/*PF4*(B)	185509	WD repeats	YES
	Not annotated (B")	405966	---	NO
C (Catalytic)	PP2A3	807	Metallophosphatase/PP2Ac	NO
	PP2A-c4	132486	Metallophosphatase/PP2Ac	YES
	PPA1	144867	Metallophosphatase/PP2Ac	NO
	PP2A-1c	193562	Metallophosphatase/PP2Ac	NO

[1] Name and/or description in JGI *Chlamydomonas reinhardtii* v4.

[2] Protein ID in JGI *Chlamydomonas reinhardtii* v4.

[3] Domain, motif, and family found in SMART and/or pfam analyses.

[4] Presence or absence in flagellar proteome (Pazour *et al*., 2005).

Table II

Phenotype of *pf4* double mutants

The *pf4* mutant phenocopies I1-dynein mutants that are defective in assembly or regulation of I1 dynein. Double mutants generated between *pf4* and other dynein deficiencies show more severe phenotypes consistent with the observation that PP2A is involved in a phospho-regulatory pathway that controls I1 dynein.

Mutant	Defect	Phenotype
pf4	PP2A	Slow swimming
pf4 x oda6	PP2A, Outer arm dyneins	Very slow swimming or paralyzed
pf4 x ida4	PP2A, Dynein a, c, and d	Very slow swimming
pf4 x ida6	PP2A, Dynein e, DRC	Sporadic twitching, nearly paralyzed, short flagella
pf4 x ida9	PP2A, Dynein c	Very slow swimming
pf4 x mia1	PP2A, Mia1p	Very slow swimming
pf4 x mia2	PP2A, Mia2p	Sporadic twitching, nearly paralyzed