Regulation of flagellar dynein by an axonemal type-1 phosphatase in *Chlamydomonas*

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**SUMMARY**

Physiological studies have demonstrated that flagellar radial spokes regulate inner arm dynein activity in *Chlamydomonas* and that an axonemal cAMP-dependent kinase inhibits dynein activity in radial spoke defective axonemes. These studies also suggested that an axonemal protein phosphatase is required for activation of flagellar dynein. We tested whether inhibitors of protein phosphatases would prevent activation of dynein by the kinase inhibitor PKI in *Chlamydomonas* axonemes lacking radial spokes. As predicted, preincubation of spoke defective axonemes (pf/14 and pf/17) with ATPgS maintained the slow dynein-driven microtubule sliding characteristic of paralyzed axonemes lacking spokes, and blocked activation of dynein-driven microtubule sliding by subsequent addition of PKI. Preincubation of spoke defective axonemes with the phosphatase inhibitors okadaic acid, microcystin-LR or inhibitor-2 also potently blocked PKI-induced activation of microtubule sliding velocity: the non-inhibitory okadaic acid analog, 1-norokadaene, did not. ATPgS or the phosphatase inhibitors blocked activation of dynein in a double mutant lacking the radial spokes and the outer dynein arms (pf/14pf/28). We concluded that the axoneme contains a type-1 phosphatase required for activation of inner arm dynein.

We postulated that the radial spokes regulate dynein through the activity of the type-1 protein phosphatase. To test this, we performed in vitro reconstitution experiments using inner arm dynein from the double mutant pf/14pf/28 and dynein-depleted axonemes containing wild-type radial spokes (pf/28). As described previously, microtubule sliding velocity was increased from ~2 μm/second to ~7 μm/second when inner arm dynein from pf/14pf/28 axonemes was reconstituted with axonemes containing wild-type spokes. In contrast, pretreatment of inner arm dynein from pf/14pf/28 axonemes with ATPgS, or reconstitution in the presence of microcystin-LR, blocked increased velocity following reconstitution, despite the presence of wild-type radial spokes. We conclude that the radial spokes, through the activity of an axonemal type-1 phosphatase, activate inner arm dynein by dephosphorylation of a critical dynein component. Wild-type radial spokes also operate to inhibit the axonemal cAMP-dependent kinase, which would otherwise inhibit axonemal dynein and motility.

Key words: cAMP-dependent kinase, Molecular motor, ATPase, Microtubule, Cilium, Flagellar radial spoke, Dynein regulatory complex

**INTRODUCTION**

Studies of *Chlamydomonas* flagella have revealed that the flagellar radial spokes and central pair apparatus regulate dynein’s microtubule sliding activity (Smith and Sale, 1994; Porter, 1996). Mutations that disrupt the radial spokes (e.g. pf/14 or pf/17) or central apparatus (e.g. pf/16 or pf/18) result in flagellar paralysis (Huang, 1986; Curry and Rosenbaum, 1993; Dutcher, 1995). These paralyzed axonemes retain the ability to undergo dynein-driven microtubule sliding, indicating that paralysis is not a defect in the dynein-based force generating mechanism (Witman et al., 1978). However, paralysis may result from a defect in the control of dynein activity. Consistent with this interpretation, dynein-driven microtubule sliding is greatly diminished in axonemes from mutant cells lacking spokes or central apparatus (Smith and Sale, 1992b, 1994; Howard et al., 1994). Moreover, extragenic suppressor mutations have been identified in *Chlamydomonas*, which restore motility to paralyzed radial spoke or central apparatus mutants without repair of the original spoke or central apparatus defect (Huang et al., 1982). These experiments demonstrated the existence of an axonemal control system which, in the absence of radial spokes or the central apparatus, inhibits dynein throughout the axoneme. This control system includes a network of proteins referred to as the ‘dynein regulatory complex’ or DRC (Huang et al., 1982; Piperno et al., 1992), located in close association with the base of the radial spokes and the inner row of dynein arms (Mastronarde et al., 1992; Piperno et al., 1992, 1994; Gardner et al., 1994). Thus, one of the functions of the radial spokes and central apparatus is to arrest this control system (which otherwise would inhibit dynein) and activate dynein throughout the axoneme, providing wild-type flagellar motility.

The inner row of dynein arms can be selectively extracted in high salt buffers and subsequently reconstituted with dynein-depleted axonemes, resulting in precise restoration of each
inner arm dynein component to its original position, and concomitantly restoring dynein’s microtubule sliding function (Smith and Sale, 1992a; Piperno et al., 1994). Reconstitution of inner arm dynein, from axonemes lacking spokes, with axonemes containing wild-type spokes, restored wild-type dynein-driven microtubule sliding velocity (Smith and Sale, 1992b). Furthermore, these in vitro studies indicated that this change in dynein’s microtubule sliding activity was mediated by posttranslational modification of a salt extractable axoneme component that is likely to include an inner arm dynein subunit (Smith and Sale, 1992b).

Based on the observation that inhibitors of the cAMP-dependent protein kinase increase flagellar activity in reactivated Chlamydomonas axonemes (Hasegawa et al., 1987), we tested the hypothesis that dynein’s microtubule sliding activity is diminished in spoke defective mutant axonemes through phosphorylation. Protein kinase inhibitors, including PKI, a peptide inhibitor of cAMP-dependent protein kinase, restored wild-type dynein-driven sliding in axonemes from mutant cells with either defective spokes or central apparatus (Howard et al., 1994). Exogenously added type II regulatory subunit (RII) of cAMP-dependent protein kinase also restored wild-type dynein activity to spoke defective axonemes, and simultaneously restored cAMP sensitivity. We concluded that an axonemal cAMP-dependent kinase inhibits dynein’s microtubule sliding activity in spoke defective axonemes and that the axoneme must also contain a protein phosphatase required for activation of dynein. The simplest model is that the axonemal kinase is active, reducing dynein activity (illustrated in Fig. 1, and see Habermacher and Sale, 1995).

The focus of the work described here was to identify and characterize the axonemal phosphatase required for activation of dynein’s microtubule sliding activity. We predicted that inhibitors of this phosphatase would prevent activation of dynein by subsequent addition of the kinase inhibitor PKI (Fig. 1). As anticipated, okadaic acid, microcystin-LR, and inhibitor-2 blocked activation of dynein’s microtubule sliding activity by subsequent addition of PKI. Furthermore, pretreatment of spoke defective axonemes with ATPγS also blocked PKI-induced activation of dynein-driven microtubule sliding. These results are the most direct indication that phosphatase activity is required for activation of dynein. Based on specificity of inhibitors, the axonemal phosphatase required for activation of dynein by the radial spokes is a type-1 protein phosphatase. Presumably the axonemal type-1 phosphatase is located in close association with the axonemal cAMP-dependent kinase, and we predict that the target protein is an inner dynein arm component. Consistent with this proposal, preliminary studies indicate that the target protein is an intermediate chain phosphoprotein subunit of inner arm I, which becomes dephosphorylated in the presence of wild-type radial spokes (G. Habermacher and W. S. Sale, unpublished). Our results also suggest that the cAMP-dependent kinase is inactive in the presence of radial spokes, providing the active state of flagellar dynein required for wild-type motility.

**MATERIALS AND METHODS**

**Cell strains and growth conditions**

Chlamydomonas reinhardtii strains studied include 137c (wild type), pf17 (radial spoke head deficient, paralyzed), pf14 (radial spoke deficient, paralyzed), pf28 (outer arm deficient, motile), and double mutant pf14pf28 (lacking radial spokes and outer dynein arms, paralyzed). The phenotype of each cell type was verified by light and electron microscopy. With the exception of pf14pf28 cells, all strains were grown in liquid modified Medium I of Sager and Granick (1953), with aeration and a 14 hours/10 hours light/dark cycle (Witman, 1986). Due to an inability of the pf14pf28 to grow flagella in liquid culture, these cells were grown on agar plates, made in modified...
Medium I with Bacto-Agar (Difco Laboratories, Detroit, Michigan), at 22°C and over a 14 hours/10 hours light/dark cycle for 5-7 days. On the morning of an experiment, p14p/28 cells were gently scraped and resuspended into 10 mM Hepes, pH 7.4 (10 ml per plate), and the cell suspension was maintained in light for 1 hour. This procedure induced p14p/28 cells to grow half-length flagella (Kamiya et al., 1991).

Reagents, phosphatase inhibitors and PKI[6-22] amide
A 100 mM stock solution of ATPS₆ (adenosine-5′-O-(3-thiotriphospho- rate), tetralithium salt, Calbiochem, La Jolla, CA) was made in water and samples stored at −20°C. Microystin-LR (Calbiochem, La Jolla, CA) was stored as a 500 μM stock in 10% methanol at −20°C. Okadaic Acid (catalog no. 495604, Calbiochem, La Jolla, CA) was stored as a 1 mM stock in 95% ethanol at −20°C. 1-norokadaone (Calbiochem, La Jolla, CA) was stored as a 200 μM stock in 95% ethanol at 4°C. Purified recombinant rabbit inhibitor-2 (2 mg/ml stock, and stored at −20°C) was the generous gift of E. C. Lee (University of Miami School of Medicine). PKI[6-22] amide (referred to here as PKI) is a peptide with the sequence Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Asn-Ala-Ile-NH₂, which corresponds to residues 6 to 22 of the αβ isoform of the heat-stable inhibitor protein of cAPK (Glass et al., 1986). PKI was synthesized as a C-terminal amide at the Emory University Microchemical Facility, purified by reverse phase HPLC, and characterized by analytical HPLC, UV spectroscopy and amino acid analysis. PKI (500 mM stock in water) was stored at −70°C. Except as noted, all other chemicals were from Sigma Chemical Co. (St Louis, MO), and deionized water was used throughout.

Isolation of axonemes and treatment with phosphatase inhibitors and PKI
Flagella were isolated as described previously (Witman, 1986; Smith and Sale, 1992a) and resuspended in Buffer A (10 mM Hepes, 5 mM MgSO₄, 1 mM DTT, 0.5 mM EDTA, 30 mM NaCl, 0.1 mM PMSF and 0.6 TIU Aprotinin, pH 7.4). Protein concentration of the flagellar suspension was measured with the Bio-Rad Bradford reagent (Bio-Rad, Richmond, CA) (Bradford, 1976). For demembranation and axoneme isolation, flagella were adjusted to 0.85 mg/ml, with a final detergent concentration of 0.5% Nonidet P-40 (Calbiochem, La Jolla, CA). Axonemes were then pelleted at 37,000 g (18,000 rpm, Sorvall SS-34 rotor) for 20 minutes. The pelleted axonemes were resuspended to their previous volume in Buffer B (10 mM Hepes, 5 mM MgSO₄, 1 mM DTT, 1 mM EGTA, 50 mM potassium acetate, 0.1 mM PMSF, 0.6 TIU Aprotinin and 0.5% polyethylene glycol). Axonemes (~0.7 mg/ml) were then divided equally into the desired number of 1.5 ml Eppendorf tubes. The rationale for the experiments is illustrated in Fig. 1. Phosphatase inhibitors, 1-norokadaone or solvent were added to isolated axonemes, followed by a 15 minute incubation on ice. As appropriate, PKI (100 μM) or solvent was added to the axonemes and incubated for 15 minutes on ice. Then 1 mM ATP (Boehringer Mannheim, Indianapolis, IN) was added, followed by a 10 minute incubation at room temperature. For control experiments the sequence of inhibitor addition was reversed or modified as appropriate (see Results). The axonemes were then assayed for microtubule sliding.

Microtubule sliding
Measurement of the velocity of sliding between doublet microtubules was based on the methods of Okagaki and Kamiya (1986) and Smith and Sale (1992a,b) with the following modifications. Axonemes (~0.7 mg/ml) were then divided equally into the desired number of 1.5 ml Eppendorf tubes. The rationale for the experiments is illustrated in Fig. 1. Phosphatase inhibitors, 1-norokadaone or solvent were added to isolated axonemes, followed by a 15 minute incubation (Smith and Sale, 1992a), and nonadherent axonemes were washed away with at least 5 volumes (~75 μl) of Buffer B containing 1 mM ATP but minus protease inhibitors (Buffer B–PI). To initiate microtubule sliding, ‘motility buffer’ (Buffer B–PI containing 1 mM ATP and 2 mg/ml Nagarse (Type XXVII Protease; Sigma Chemical Co., St Louis, MO)) was added by perfusion. Except where noted, protein kinase inhibitors or their controls were maintained in both the wash and motility buffers. Microtubule sliding was observed by darkfield microscopy and recorded by a silicon intensified target camera (66 SIT or VE-1000 SIT, Dage-MTI, Inc., Michigan City, IN), through a time/date generator, onto videotape by a videocassette recorder equipped with a jog/shuttle device for field by field analysis (AG-1960 or AG-1970, Panasonic, Secaucus, NJ).

Microtubule sliding velocity was measured manually from calibrated video screens at a final magnification of ×3,200, using the jog/shuttle to measure displacement versus time, as described before (Howard et al., 1994). To ensure objective evaluation, the following procedures were adopted. First, only sliding events that were unequivocally occurring between a single pair of microtubule doublets were measured (Okagaki and Kamiya, 1986). Second, every measurable sliding event within a field was measured. Third, because the composition of the wash and motility buffers has pronounced effects on the velocity of microtubule sliding (Kurimoto and Kamiya, 1991; Smith and Sale, 1992b), all of the experiments reported here were performed using Buffer B containing potassium acetate at low calcium (namely <10⁻⁹ M Ca²⁺). Motility buffers were warmed to 22°C prior to use in the sliding disintegration assay. All data are presented as means ± standard error of the mean (s.e.m.). The Student’s t-test was used to determine the significance of differences between means.

Extraction and recombination of inner arm dyneins
Extraction and in vitro reconstitution of inner dynein arms and axonemes generally followed the procedure of Smith and Sale (1992b); also see Smith, 1995). Flagella were isolated from mutant cells lacking outer dynein arms (either p28 or p14p/28) as described above. Axonemes were pelleted (15,000 rpm, 20 minutes, SS-34 rotor) and resuspended in Buffer A to ~1 mg/ml. Two types of reconstitution protocols were used. Either ATPS₆ (1 mM) was added to axonemes (1 mg/ml) 20 minutes prior to extraction with high salt, or microcystin-LR (2 μM) was added to dynein-depleted axonemes prior to reconstitution with dynein-containing extract. In the first type of experiment, axonemes were divided into equal fractions and made 1 mM in either ATPS₆ or ATP, and incubated on ice for 15 minutes. Axonemes were then pelleted (18,000 rpm, 20 minutes, SS-34 rotor), supernatants removed and axonemes extracted in high salt buffer (0.6 M NaCl, 10 mM Hepes, 5 mM MgSO₄, 1 mM DTT, 0.5 mM EDTA, 0.1 mM PMSF and 0.6 TIU Aprotinin) at a protein concentration of 8 mg/ml, for 20 minutes on ice. After pelleting the extracted axonemes, the dynein-containing supernatants were collected and dialyzed (molecular mass cutoff 12-14 kDa) twice in 500 ml of Buffer A for 30 minutes at 4°C. The extracted axonemal pellets were resuspended in Buffer A to 2 mg/ml and mixed with the desired dynein extract at a 1:1 (v/v) ratio. This ratio results in a fourfold excess of dynein (Smith and Sale, 1992b). After incubating for 25 minutes on ice, the velocity of microtubule sliding was measured for each combination of isolated dynein and dynein-depleted axonemes.

RESULTS
ATPS₆ blocks PKI-induced increase in dynein activity in radial spoke defective axonemes
As discussed above, treatment of isolated, spoke defective axonemes (p14, missing spokes; p17, missing spoke heads) with the kinase inhibitor PKI resulted in an increase in microtubule sliding velocity from ~10 μm/second to ~18 μm/second (Howard et al., 1994). The results also clearly indicated that increased microtubule sliding velocity requires the activity of an axonemal phosphatase (see Fig. 1). We postulated that thio-phosphorylation of radial spoke defective axonemes with
inhibitors would also block PKI-induced activation of dynein's microtubule sliding activity in spoke-defective axonemes. Axonemes were isolated from pf17 cells, treated first with phosphatase inhibitor, then with PKI (100 nM) or buffer alone, and dynein activity assessed using the microtubule sliding assay. The prediction was that phosphatase inhibitors would block PKI-induced activation of dynein. 1 μM microcystin-LR, a cyclic heptapeptide that potently inhibits type 1 and type 2A serine/threonine phosphatases (MacKintosh et al., 1990), completely blocked the PKI-induced increase in dynein-driven microtubule sliding in pf17 axonemes (Fig. 3A). In addition, microcystin-LR pre-treatment of pf17 axonemes blocked the PKI-induced increase in dynein's microtubule sliding activity in a dose-dependent manner with a half-maximal effect at ~250 nM (Fig. 3B). As a control, the sequence of microcystin-LR and PKI addition was reversed and microtubule sliding velocity assessed. Microcystin-LR does not reverse PKI-stimulation of dynein’s microtubule sliding activity when added 5 minutes subsequent to PKI (Fig. 3C). Axonemes from both pf14 and pf17 respond identically to PKI, ATPγS and microcystin-LR (Fig. 2B and data not shown).

As a further control, we compared the effects of okadaic acid, a potent inhibitor of type-1 and type-2A serine/threonine phosphatases (Cohen et al., 1989), and 1-nor-okadaone, a non-inhibitory okadaic acid analog (Nishiwaki et al., 1990). Okadaic acid (2 μM) blocked the PKI-induced increase in dynein’s microtubule sliding activity in pf17 axonemes (Fig. 4), while 2 μM 1-nor-okadaone had no effect (Fig. 4). To further define the axonemal protein phosphatase, recombinant inhibitor-2, a potent and specific subunit of type-1 protein phosphatase, was used (Zhang et al., 1994). Inhibitor-2 blocked PKI-stimulation of dynein-driven microtubule sliding, with a half-maximal effect at ~100 nM (Fig. 5). We concluded that the axoneme contains a type-1 protein phosphatase required for the PKI-induced increase of dynein-driven microtubule sliding in spoke-defective axonemes.

### Inhibitors of type-1 phosphatase block PKI-induced increase in dynein activity

A series of phosphatase inhibitors was used to test the hypothesis that an axonemal phosphatase is required for PKI-induced increase in dynein’s microtubule sliding activity in spoke-defective axonemes. Axonemes were isolated from pf17 cells, treated first with phosphatase inhibitor, then with PKI (100 nM) or buffer alone, and dynein activity assessed using the microtubule sliding assay. The prediction was that phosphatase inhibitors would block PKI-induced activation of dynein. 1 μM microcystin-LR, a cyclic heptapeptide that potently inhibits type 1 and type 2A serine/threonine phosphatases (MacKintosh et al., 1990), completely blocked the PKI-induced increase in dynein-driven microtubule sliding in pf17 axonemes (Fig. 3A). In addition, microcystin-LR pre-treatment of pf17 axonemes blocked the PKI-induced increase in dynein’s microtubule sliding activity in a dose-dependent manner with a half-maximal effect at ~250 nM (Fig. 3B). As a control, the sequence of microcystin-LR and PKI addition was reversed and microtubule sliding velocity assessed. Microcystin-LR does not reverse PKI-stimulation of dynein’s microtubule sliding activity when added 5 minutes subsequent to PKI (Fig. 3C). Axonemes from both pf14 and pf17 respond identically to PKI, ATPγS and microcystin-LR (Fig. 2B and data not shown).

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### Inner arm dynein’s microtubule sliding activity is regulated by the axonemal type-1 phosphatase

As discussed above, we discovered, through the use of double mutants missing the outer arms, that inner arm dynein’s microtubule sliding activity is regulated by the radial spokes (Smith and Sale, 1992b) and that an axonemal cAMP-dependent kinase selectively inhibits inner arm dynein activity (Howard et al., 1994). Therefore, we postulated that the type-1 phosphatase would also specifically regulate inner arm dynein activity. We predicted that ATPγS or phosphatase inhibitors would block the PKI-induced increase of inner arm dynein’s microtubule sliding activity in the double mutant pf14/pf28, which lacks both radial spokes and the outer dynein arms. ATPγS and the phosphatase inhibitors prevented the PKI-induced increase in dynein-driven microtubule sliding (Fig. 6). Therefore, the axonemal type-1 phosphatase regulates inner arm dynein activity, independent of the outer dynein arms.
Radial spoke regulation of inner arm dynein activity is dependent on the axonemal type-1 phosphatase

The radial spokes increase inner dynein arm activity by a mechanism thought to involve posttranslational modification of a dynein subunit (Smith and Sale, 1992b; Howard et al., 1994). One hypothesis is that the radial spokes increase dynein activity through type-1 phosphatase-mediated dephosphorylation of a dynein component. Our earlier study demonstrated that reconstitution of inner arm dynein with axonemes containing wild-type spokes increased inner arm dynein activity to wild-type velocity (Smith and Sale, 1992b). Therefore, we predicted that ATPγS (added to pf14pf28 axonemes prior to high salt extraction) or phosphatase inhibitors (present during recombination) would block the radial spoke-induced increase in dynein’s microtubule sliding velocity. Inner dynein arms were extracted from pf14pf28 axonemes, reconstituted, and measured using the microtubule sliding assay. ATPγS blocked the radial spoke-induced increase in inner dynein arm activity (Fig. 7). This result indicated that the radial spoke-induced increase in inner arm dynein activity involves dephosphorylation of a salt extractable axoneme component, which rebinds to deyndepilled pf28 axonemes. Microcystin-LR also blocked the radial spoke-induced activation of reconstituted inner dynein.

Fig. 3. The protein phosphatase inhibitor microcystin-LR has no effect on the slow microtubule sliding of axonemes lacking spokes and blocks PKI-induced increase in microtubule sliding velocity in a dose-dependent manner. (A) Isolated pf17 axonemes were treated with buffer alone (control, pf17), 100 nM PKI (+PKI), 1 μM microcystin-LR (+Micro) or 1 μM microcystin-LR then 100 nM PKI (+Micro/+PKI). (B) pf17 axonemes were treated with various concentrations of microcystin-LR then 100 nM PKI and assayed using the microtubule sliding assay. (C) The order of addition of phosphatase inhibitor and kinase inhibitor affects the results. Pf17 axonemes were treated with buffer alone (control, pf17), 100 nM PKI (+PKI), 1 μM microcystin-LR then 100 nM PKI (+Micro/+PKI), or 1 μM microcystin-LR (PKI/+Micro). Each bar represents ≥25 measurements and the data represented in A and C were derived from separate experiments. Differences in the absolute value of velocity occasionally varied slightly between experiments. However, relative differences compared to controls were consistent for each experiment and were statistically significant (P>0.05).

Fig. 4. Okadaic acid (2 μM) blocks the PKI-induced increase in microtubule sliding velocity (P>0.05), but 2 μM 1-nor-okadaone does not. Isolated pf17 axonemes were treated with either buffer alone (control, pf17), 100 nM PKI (+PKI), 2 μM okadaic acid (+OA), 2 μM okadaic acid then 100 nM PKI (+OA/+PKI), 2 μM 1-nor-okadaone (+NO), or 2 μM 1-norokadaone then 100 nM PKI (NO+/PKI). Each bar represents ≥25 measurements.
The results from the reconstitution experiments indicate that radial spokes regulate dynein through the activity of the axonemal type-1 phosphatase. We also predicted the radial spokes operate to inhibit the axonemal cAMP-dependent kinase. To test this, we anticipated that phosphatase inhibitors, ATP_{γ}S and cAMP, would not alter dynein-driven microtubule sliding in wild-type axonemes. Both wild-type (137c) and outer arm deficient (pf28) axonemes were incubated with various combinations of 1 mM ATP_{γ}S, 2 μM microcystin-LR and 100 μM 8-Br-cAMP and microtubule sliding velocity was assessed. As predicted, no combination of these reagents affected microtubule sliding velocity in either wild-type or pf28 axonemes (data not shown). These results are consistent with the hypothesis that the axonemal cAMP-dependent protein kinase is inactive in the presence of wild-type radial spokes.

To further test the hypothesis that the cAMP-dependent protein kinase is inactive in the presence of radial spokes, we used an in vitro reconstitution approach. Axonemes from pf28 were treated with 1 mM ATP_{γ}S prior to salt extraction of the inner dynein arms. The ATP_{γ}S treated inner dynein arm fraction was then reconstituted with untreated, dynein-depleted pf28 axonemes and microtubule sliding velocity was assessed. Based on the hypothesis that the kinase is inactive in the presence of wild-type radial spokes, we predicted that ATP_{γ}S treatment would have no impact on inner dynein arm activity when rebound to dynein-depleted pf28 axonemes and microtubule sliding velocity was assessed. As predicted, ATP_{γ}S treatment of pf28 inner dynein arms does not reduce microtubule sliding velocity in comparison to controls (Fig. 7, compare 28/28 with 28gS/28). This result suggests that phosphorylation of axonemal substrate by the axonemal cAMP-dependent protein kinase is prevented by the presence of wild-type radial spokes.

DISCUSSION

We report three important results concerning regulation of flagellar dynein and the mechanism by which flagellar radial spokes regulate dynein’s microtubule sliding activity. First, dynein-driven microtubule sliding at wild-type velocity is
regulated by an axonemal type-1 protein phosphatase: specific inhibitors of type-1 protein phosphatase block the PKI-induced increase in microtubule sliding. Second, a target regulatory phosphoprotein is associated with the inner row of dynein arms: analysis of double mutant axonemes lacking the radial spokes or central pair shows that the radial spokes and the outer dynein arms are thought to be responsible for generation and changes in waveform (Brokaw et al., 1982) and the inner dynein arms are thought to be responsible for regulation of flagellar waveform by local regulation of subsets of inner dynein arms, located either in unique positions along each microtubule (Piperno et al., 1990; Piperno and Ramanis, 1991; Mastronarde et al., 1992) or on distinct microtubules (King et al., 1994). This model is appealing because the spokes are thought to play a role in regulation of waveform (Warner and Satir, 1974; Brokaw et al., 1982) and the inner dynein arms are thought to be responsible for generation and changes in waveform (Brokaw and Kamiya, 1987; Brokaw, 1994; Piperno, 1995).

Our results further establish that phosphorylation of an axonemal protein inhibits microtubule sliding in paralyzed *Chlamydomonas* axonemes lacking the radial spokes or central pair apparatus. ATPγS blocks the PKI-induced increase in microtubule sliding in both spoke defective axonemes and double mutants missing both the radial spokes and outer dynein arms. Also, the axoneme contains a type-1 phosphatase required for increase in microtubule sliding velocity. (Inhibitor-2 potently blocks the PKI-induced increase in dynein’s microtubule sliding activity (Fig. 5B), indicating that an axonemal type 2A phosphatase is not involved.) The simplest model is that the axonemal cAMP-dependent protein kinase inhibits microtubule sliding and, in contrast, the axonemal type-1 phosphatase is required for dephosphorylation of the critical target protein and wild-type dynein-driven motility (Fig. 1). However, we cannot yet rule out more complex models in which the kinase and the phosphatase participate in a cascade operating indirectly to affect the phosphorylation of the target regulatory protein (Tash, 1989; Dey and Brokaw, 1991; Chaudhry et al., 1995). Although we can rescue wild-type microtubule sliding in spoke or central pair defective mutants, we have been unable to rescue wild-type flagellar beating using kinase inhibitors. One possibility is that other regulatory mechanisms are yet to be identified.

Most of the data described in this paper make use of the microtubule sliding assay, developed by Okagaki and Kamiya (1986), which we presume measures dynein activity uniformly through the axoneme. However, this interpretation depends on the assumptions that microtubule sliding is unloaded and that...
all doublet microtubules and portions of doublet microtubules are equally capable of sliding under each experimental condition. We cannot yet exclude the possibility that change in sliding velocity results from changes in load. We have also assumed that changes in sliding velocity represent uniform changes in dynein activity throughout the axoneme. However, we do not know which doublets slide in the assay, nor do we know if different subsets of doublets slide in different conditions. It is possible that velocity differences are a consequence of alternations or switches in the patterns of microtubules which slide, since in our assay sliding is generally restricted to only a few sets of doublets (for further discussion of this point, see Kinoshita et al., 1995). Finally, we have assumed that changes in sliding velocity are a consequence of equivalent changes for each different inner dynein arm. However, it is equally possible that change in sliding motility is a consequence of change in activity of only one or a select subset of inner arm dyneins. Thus, immediate goals are the identification of the target regulatory phosphoproteins and the location of the axonemal kinase and phosphatase.

The simplest model is that one or more of the inner dynein arms contains the crucial phosphoprotein. The challenge is to determine where the critical protein resides among the numerous inner dynein components (for a description of the organization of the inner dynein arms, see Piperno, 1995; Porter, 1996). One approach is to use double mutants missing both the radial spokes and selected subsets of inner arm dynein components, which may contain the protein required for increased microtubule sliding velocity (Habermacher and Sale, 1995). Predictably, the axoneme missing both the radial spokes as well as the inner dynein arm containing the protein will be unresponsive to PKI. This strategy has the potential to reveal both the identity of the target phosphoprotein and the location of the kinase/phosphatase complex since, as discussed, the complex is likely to reside in close proximity to the substrate.

Our preliminary results have demonstrated that inner arm dynein II is required for PKI-induced increase in microtubule sliding velocity (Habermacher and Sale, 1995). Furthermore, we have determined that the 138 kDa intermediate chain of inner arm II is the sole phosphoprotein subunit of II (labeled in vitro with $^{32}$P[ATP], and that it becomes dephosphorylated when rebound to axonemes containing wild-type radial spokes (Habermacher and Sale, 1995; G. Habermacher, unpublished data). Furthermore, as addressed below in more detail, a mutation in inner arm II has been shown to be a suppressor of paralysis of a central pair mutant (Porter et al., 1992). One attractive model is that change in sliding velocity is a consequence of changes in phosphorylation of the 138 kDa subunit of inner arm II, either directly or indirectly affecting the activity of the other inner arm ATPases, and resulting in modulation in flagellar waveform. It is important to note that these new results in the study of inner arm dynein phosphorylation do not address or eliminate the possibility that outer arm dynein is also similarly regulated by the kinase/phosphatase complex, possibly also affecting waveform (for further discussion of this point, see King and Witman, 1995).

Functional interaction of the radial spoke/central pair complex and the dynein arms was first revealed by genetic analysis of Chlamydomonas flagellar mutants (reviewed by Huang, 1986; Smith and Sale, 1994). Huang et al. (1982) recovered extragenic suppressor mutations that rescue motility (i.e. suppress paralysis) in radial spoke and central pair mutants. One of the conclusions from this work was that the axoneme contains a control system which inhibits dynein throughout the axoneme and results in paralyzed flagella. The radial spoke/central pair complex operates to inhibit the ‘control system’. Some of the suppressors contained mutations in either the outer or inner dynein arms, which evidently compensate for defective spokes or central pair components and by-pass the ‘control system’ that would otherwise inhibit motility (Huang et al., 1982; Porter et al., 1992, 1994). Analysis also revealed that the control system, known as the dynein regulatory complex or DRC, is a network of at least six proteins (Huang et al., 1982; Piperno et al., 1992; 1994). Part of the DRC has been localized to the A-microtubule, repeating in a 96 nm interval near the base of the inner arms and radial spokes, presumably in a position to direct regulatory signals from the spokes to the dynein arms (Mastronarde et al., 1992; Gardner et al., 1994). Based on the properties of the DRC and the physiological studies we have presented, we predict the axonemal cAMP-dependent kinase is one of the components of the DRC. Furthermore, we propose that the axonemal type-1 phosphatase is also part of the network of DRC proteins. Based on our preliminary identification of the target phosphoprotein in inner arm II, we would further predict that the kinase/phosphatase complex is located near the base of inner arm II and the radial spokes. Detailed biochemical and structural analyses of existing or new suppressor mutants will provide a test of this model.

We gratefully acknowledge Dr Earnest Lee for the gift of recombinant inhibitor 2. We thank Drs H. Criss Hartzell, Lynne Quarmby and Elizabeth Smith for helpful discussion and critical comments on the manuscript. The work was supported by a grant from the NIH (GM 51173).

REFERENCES

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(Received 6 February 1996 - Accepted 28 March 1996)