

# Regulation of *Chlamydomonas* Flagellar Dynein by an Axonemal Protein Kinase

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**Abstract.** Genetic, biochemical, and structural data support a model in which axonemal radial spokes regulate dynein-driven microtubule sliding in *Chlamydomonas* flagella. However, the molecular mechanism by which dynein activity is regulated is unknown. We describe results from three different in vitro approaches to test the hypothesis that an axonemal protein kinase inhibits dynein in spoke-deficient axonemes from *Chlamydomonas* flagella. First, the velocity of dynein-driven microtubule sliding in spoke-deficient mutants (*pf14*, *pf17*) was increased to wild-type level after treatment with the kinase inhibitors HA-1004 or H-7 or by the specific peptide inhibitors of cAMP-dependent protein kinase (cAPK) PKI(6-22)amide or *N*<sup>α</sup>-acetyl-PKI(6-22)amide. In particular, the peptide inhibitors of cAPK were very potent, stimulating half-maximal velocity at 12–15 nM. In contrast, kinase inhibitors did not affect microtubule sliding in axonemes from wild-type cells. PKI treatment of axonemes from a double mutant missing both the radial spokes and the outer row of dynein arms (*pf14pf28*) also increased microtubule sliding to control (*pf28*) velocity. Second, addition of the type-II regulatory subunit of cAPK (RII) to spoke-deficient

axonemes increased microtubule sliding to wild-type velocity. Addition of 10 μM cAMP to spokeless axonemes, reconstituted with RII, reversed the effect of RII. Third, our previous studies revealed that inner dynein arms from the *Chlamydomonas* mutants *pf28* or *pf14pf28* could be extracted in high salt buffer and subsequently reconstituted onto extracted axonemes restoring original microtubule sliding activity. Inner arm dyneins isolated from PKI-treated axonemes (mutant strain *pf14pf28*) generated fast microtubule sliding velocities when reconstituted onto both PKI-treated or control axonemes. In contrast, dynein from control axonemes generated slow microtubule sliding velocities on either PKI-treated or control axonemes. Together, the data indicate that an endogenous axonemal cAPK-type protein kinase inhibits dynein-driven microtubule sliding in spoke-deficient axonemes. The kinase is likely to reside in close association with its substrate(s), and the substrate targets are not exclusively localized to the central pair, radial spokes, dynein regulatory complex, or outer dynein arms. The results are consistent with a model in which the radial spokes regulate dynein activity through suppression of a cAMP-mediated mechanism.

**D**YNEINS are a family of microtubule-based mechanoenzymes responsible for eukaryotic ciliary and flagellar motility, as well as for certain forms of cytoplasmic transport (Gibbons, 1989). Although much is known about the composition and structural organization of dyneins in flagellar axonemes (Witman, 1992), little is known about the coordinated regulation of dynein-driven microtubule sliding that produces regular bending of flagella (see Brokaw, 1994).

Genetic and biochemical approaches using *Chlamydomonas* have revealed a functional interaction between the radial spokes/central pair apparatus and the dynein arms that is likely part of a mechanism that controls microtubule sliding. For example, mutations that result in disrupted assembly of the radial spokes or the central pair apparatus also result in flagellar paralysis (reviewed in Huang, 1986; Curry and Rosenbaum, 1993). However, extragenic suppressor mutations that rescue oscillatory beating without restoration of the radial spokes or the central pair have been recovered (Huang et al., 1982; Brokaw et al., 1982; Piperno et al., 1992, 1994; Porter et al., 1992, 1994). These results revealed that the radial spokes and central pair have regulatory functions in flagellar beating that can be bypassed by other components in the axoneme. The molecular mechanism(s) by which the radial spokes and central pair regulate dynein

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is unknown. However, in each case studied so far, mutations that suppress paralysis, because of disrupted central pair or spoke components, have defects in either the outer or inner dynein arms (Huang et al., 1982; Brokaw and Kamiya, 1987; Luck and Piperno, 1989; Porter et al., 1992, 1994) or in a complex of proteins referred to as the dynein regulatory complex (drc)<sup>1</sup> (Huang et al., 1982; Piperno et al., 1992, 1994), thought to be located at the junction between the inner row of dynein arms and the radial spokes (Mastronarde et al., 1992; Piperno et al., 1992, 1994). Thus, one hypothesis is that the radial spokes, in conjunction with the central apparatus and drc, serve to regulate flagellar function by local activation of dynein-driven microtubule sliding. This hypothesis was tested by the analysis of microtubule sliding velocity in isolated axonemes from mutant strains of *Chlamydomonas* missing radial spoke components (Smith and Sale, 1992b). It was discovered that in spoke-deficient mutants the velocity of dynein-driven microtubule sliding was significantly reduced compared to wild-type controls. Furthermore, using a precise reconstitution assay and focusing on just the inner row of dynein arms, it was found that dynein extracted from spokeless axonemes gained the ability to translocate microtubules at wild-type velocities upon reconstitution with axonemes containing intact spokes. Moreover, when dynein derived from axonemes possessing wild-type spokes was reconstituted with axonemes missing spokes, the dynein retained the ability to generate rapid microtubule sliding velocity in the absence of spokes (Smith and Sale, 1992b). These results indicate that the radial spokes activate dynein-driven microtubule sliding. Furthermore, the results suggest that dynein is activated by a posttranslational modification, possibly phosphorylation or dephosphorylation. The hypothesis that phosphorylation regulates dynein activity in *Chlamydomonas* flagella is consistent with the results of Hasegawa et al. (1987), who reported that inhibition of cAMP-dependent protein kinase (cAPK) increased the percentage of wild-type axonemes that reactivate in vitro. The mechanism through which cAPK affects reactivation is unknown.

To test the hypothesis that phosphorylation regulates microtubule sliding, we used protein kinase inhibitors and a reconstitution approach combined with in vitro assay of dynein-driven microtubule sliding in spoke-deficient axonemes. We found that inhibitors of cAPK significantly increased the velocity of microtubule sliding in spoke-deficient axonemes, mimicking the effect of radial spokes in previous experiments (Smith and Sale, 1992b). Similar treatments had no effect on microtubule sliding in axonemes containing wild-type spokes. Likewise, reconstitution of spoke-deficient axonemes with the regulatory subunit of type II cAPK (RII) increased microtubule sliding to wild-type control levels. Finally, when inner arm dyneins were extracted from spoke-deficient axonemes, which were first treated with a specific cAPK inhibitor and then reconstituted onto either control or inhibitor-treated axonemes depleted of dynein, the dynein restored wild-type microtubule sliding velocity to spokeless

1. *Abbreviations used in this paper:* cAPK, cAMP-dependent protein kinase; drc, dynein regulatory complex; HA-1004, *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride; NAcPKI, *N*<sup>ε</sup>-acetyl-PKI(6-22)-amide; PKI, amino acid residues 6-22 of the  $\alpha$  isoform of the heat-stable inhibitor protein of cAPK [PKI(6-22)amide]; RII, regulatory subunit of type II cAPK; TriAlaPKI, *N*<sup>ε</sup>-acetyl-(Ala<sup>15,18,19</sup>)PKI(6-22)amide.

axonemes. We conclude that in *Chlamydomonas*, the radial spokes activate dynein-driven microtubule sliding by inhibiting or overriding a cAPK, and that this axonemal protein kinase is not exclusively located in the central pair apparatus, radial spokes, drc, or outer dynein arms.

## Materials and Methods

### Cell Strains and Growth Conditions

*Chlamydomonas reinhardtii* strains used were *pfl4* (radial spoke deficient; Piperno et al., 1977; Witman et al., 1978), *pfl7* (radial spoke head deficient; Huang et al., 1981), *pfl4pfl28* (radial spokeless/outer dynein armless; Piperno et al., 1990), and *137c* (wild type). The phenotype of each cell type was verified by electron microscopy. With the exception of *pfl4pfl28*, all cells were grown in liquid modified Medium I of Sager and Granick (1953) with aeration over a 14 h/10 h light/dark cycle (Witman, 1986). Because of an inability of the *pfl4pfl28* cells to grow flagella in liquid culture, these cells were grown on modified Medium I agar plates at 22°C during a 14 h/10 h light/dark cycle for 5-7 d. On the morning of an experiment, *pfl4pfl28* cells were gently scraped and resuspended into 10 mM sodium Hepes, pH 7.4 (10 ml/plate), and the cell suspension was stirred in the light for 1 h. This treatment induced *pfl4pfl28* cells to grow half-length flagella (5-7  $\mu$ m) (Kamiya et al., 1991).

### Reagents and Kinase Inhibitors

*N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride (HA-1004) (Research Biomedicals International, Natick, MA) was made as a 10 mM stock solution in water and stored at -20°C. The regulatory subunit (RII) of bovine cardiac type II cAPK (Promega, Madison, WI) was stored as a 1.2 mg/ml solution in Tris-buffered saline at -70°C. PKI(6-22)amide and *N*<sup>ε</sup>-acetyl-PKI(6-22)amide (referred to in this paper as PKI and NAcPKI, respectively) are peptides with the sequence Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-NH<sub>2</sub> that correspond to residues 6-22 of the  $\alpha$  isoform of the heat-stable inhibitor protein of cAPK (Glass et al., 1989; Walsh and Glass, 1991). Both of these peptides inhibit cAPK with  $K_i$  = 1.6-2.5 nM (Glass et al., 1989) and a high degree of specificity (Glass et al., 1986). PKI (200  $\mu$ M stock) and NAcPKI (100  $\mu$ M stock) were stored at -70°C as trifluoroacetic acid salts in water, and 1 mM trifluoroacetic acid alone served as a control stock solution. As a control for PKI and NAcPKI, the analogue peptide *N*<sup>ε</sup>-acetyl-(Ala<sup>15,18,19</sup>)PKI(6-22)amide (TriAlaPKI), in which alanines are substituted for the three required arginines in the PKI sequence, was used. TriAlaPKI is virtually inactive as an inhibitor of cAPK when used at submicromolar concentrations, with a  $K_i$  >125  $\mu$ M (Glass, D., unpublished results). TriAlaPKI (100  $\mu$ M stock) was stored in a solution of 4 mM NH<sub>4</sub>HCO<sub>3</sub> at 4°C. PKI, NAcPKI, and TriAlaPKI were synthesized as COOH-terminal amides at the Emory University Microchemical Facility, purified by semipreparative reverse-phase HPLC, and characterized by analytical HPLC, UV spectroscopy, and amino analysis by methods previously described (Glass et al., 1989; Katz et al., 1989). The addition of 4 mM NH<sub>4</sub>HCO<sub>3</sub> to stock solutions of NAcPKI did not alter the observed effects of NAcPKI. 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 Kinase Inhibitors

Flagella were isolated as described previously (Witman, 1986; Smith and Sale, 1992a) and resuspended in buffer A (10 mM Hepes, 5 mM MgSO<sub>4</sub>, 1 mM DTT, 0.5 mM EDTA, 30 mM NaCl, 0.1 mM PMSF, and 0.6 TIU Aprotinin, pH 7.4). The protein concentration of the flagellar suspension was measured with the Bio Rad Laboratories (Richmond, CA) Bradford assay (Bradford, 1976) and adjusted to 0.85 mg/ml. Flagella were demembrated with 0.5% Nonidet P-40 (Calbiochem-Novabiochem Corp., La Jolla, CA), and the axonemes were pelleted at 37,000 g (18,000 rpm) (SS-34 rotor; Sorvall, Wilmington, DE) for 20 min. For all experiments except extraction and reconstitution experiments (see below), the pelleted axonemes were resuspended to their previous volume in buffer B (10 mM Hepes, 5 mM MgSO<sub>4</sub>, 1 mM DTT, 1 mM EGTA, 50 mM K-acetate, 0.1 mM PMSF, 0.6 TIU Aprotinin, and 0.5% polyethylene glycol). In most experiments, protein kinase inhibitors or their respective carrier, control solutions were

added and incubated for 15 min on ice, and 1 mM ATP (Boehringer Mannheim Corp., Indianapolis, IN) was added to both inhibitor-treated and control axonemes and incubated for 10 min at room temperature. The axonemes were then used in microtubule-sliding assays.

### Assay of Microtubule Sliding Velocity

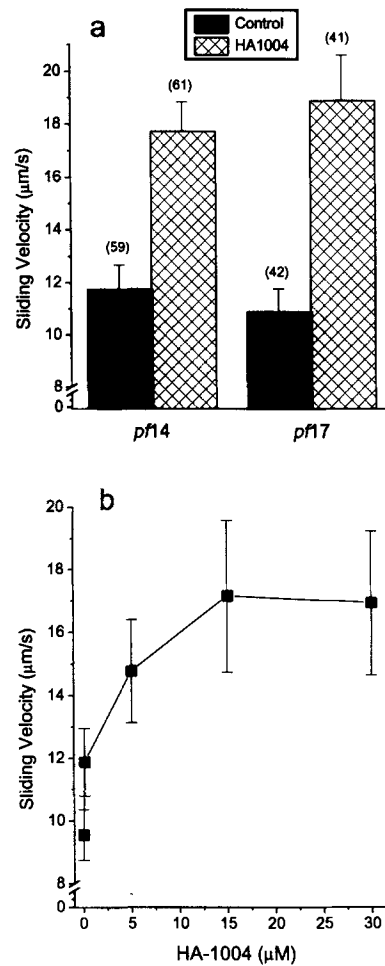
The measurement of the velocity of sliding between doublet microtubules was based on the methods of Okagaki and Kamiya (1986) and Smith and Sale (1992a,b) with the following modifications. Neither flagella nor axonemes were sonicated before sliding assays. Axonemes were applied to 10–15- $\mu$ l perfusion chambers (see Smith and Sale, 1992a), and nonadherent axonemes were washed away with  $\geq 5$  vol ( $\sim 75$   $\mu$ l) of buffer B minus protease inhibitors (buffer B-PI) with 1 mM ATP. To initiate microtubule sliding, buffer B-PI with 1 mM ATP and 2  $\mu$ g/ml Nagarse (type XXVII protease; Sigma Chemical Co.) (motility buffer) 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 dark-field microscopy and recorded by a silicon-intensified target camera (66 SIT or VE-1000 SIT; Dage-MTI, Inc., Michigan City, IN) 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).

Analysis of the velocity of microtubule sliding was from videotapes and measurements were made manually from calibrated videoscreens at a final magnification of 3,200 using the jog/shuttle to measure displacement versus time. To ensure reproducibility, 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). Sliding events that occur simultaneously between two or more pairs of microtubule doublets produce apparent velocities that are faster than individual events but are not a simple sum of the multiple events. Second, to eliminate bias, every measurable sliding event within a field was measured. To further reduce possible bias, measurements were repeated by a second observer and experiments were coded for blind measurement. Third, as demonstrated previously (Kurimoto and Kamiya, 1991; Smith and Sale, 1992b), the composition of the wash and motility buffers have pronounced effects on the velocity of microtubule sliding. All of the experiments reported here were performed using buffer B containing no added calcium (nominally  $<10^{-9}$  M  $\text{Ca}^{++}$ ). In the presence of kinase inhibitors, increased  $\text{Ca}^{++}$  had a significant effect on microtubule sliding velocity (data not shown). All data are presented as means  $\pm$  standard error of the mean. The Student's *t* test (0.05 level) was used to determine significant differences between means.

Using these procedures, the mean sliding velocities (see Fig. 2 b) are faster than those we reported previously (cf. Smith and Sale, 1992b), but they show the same relative difference between spoke-containing and spoke-deficient axonemes. The mean microtubule sliding velocity of wild-type axonemes (137c) found with this assay (21  $\mu\text{m/s}$ ) correlates well with that calculated from live beating flagella by Brokaw and Luck (1985).

### Extraction and Recombination of Inner Arm Dyneins

Flagella were isolated from *pf14*/*pf28* cells as described above. The flagellar protein concentration was adjusted to 0.35 mg/ml, and 0.5% (wt/vol) NP-40 was added to remove the flagellar membrane. Axonemes were pelleted at 11,000 rpm for 12 min (SS-34 rotor; Sorvall Instruments, Wilmington, DE) and resuspended in buffer A to half their original volume. This axonemal suspension was divided into two equal aliquots, and 100 nM PKI was added to one aliquot while the other aliquot served as the buffer control. Axonemes were then incubated for 15 min on ice. ATP (1 mM) was added to both samples, and axonemes were incubated for 10 min at 22°C. Axonemes were concentrated by centrifugation as above and resuspension to 1/10 the original volume ( $\sim 3$  mg/ml) in buffer A with 1 mM ATP  $\pm$  100 nM PKI accordingly. An equal volume of both treated and untreated axonemes were removed and used in the microtubule-sliding assay to measure the velocity of "unextracted axonemes." Dyneins were then extracted from both remaining axonemal aliquots by elevating the NaCl concentration to 0.6 M via the addition of 4 M NaCl in buffer A and incubating 15 min on ice. After pelleting the axonemes as described, the dynein-containing supernatants were collected and dialyzed (molecular mass cutoff = 12,000–14,000 D) twice against 500 ml of buffer A for 30 min at 4°C. The extracted axonemal pellets were resuspended in buffer A. The two types of inner arm dynein extracts (PKI-treated and control) were recombined 3:1 (vol/vol) with each of the



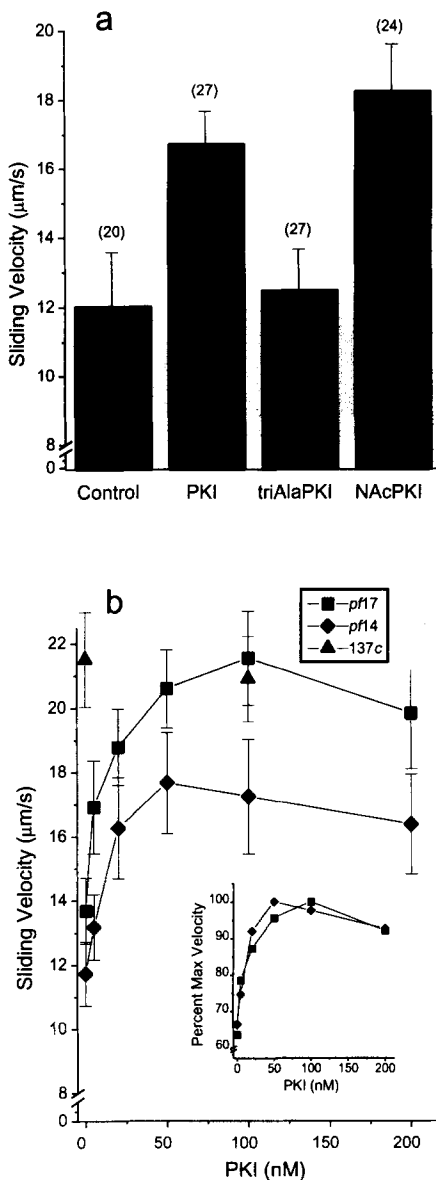
**Figure 1.** Effects of HA-1004 on the velocity of microtubule sliding in isolated axonemes. (a) Isolated axonemes from *pf14* (missing radial spokes) or *pf17* (missing radial spoke heads) were treated with 15  $\mu\text{M}$  HA-1004 for 15 min, followed by 1 mM ATP for 10 min. The sliding velocities in both *pf14* and *pf17* were significantly increased by HA-1004 ( $P < 0.001$ , Student's *t* test). The data from three (*pf14*) or two (*pf17*) independent experiments were pooled and are presented as means  $\pm$  standard errors of the mean. The total number of axonemes measured for each mean is shown in parentheses. (b) Isolated *pf17* axonemes were treated with varying concentrations of HA-1004 as described in Materials and Methods. Each point represents  $\geq 20$  measurements.

two types of extracted axonemes (PKI-treated and control) resulting in all combinations of dynein and axonemes (see Smith and Sale, 1992b). After incubating for 15 min at room temperature, the velocity of microtubule sliding was measured for each combination and for PKI-treated and control extracted axonemes without added inner arms.

## Results

### Protein Kinase Inhibitors Increase the Velocity of Microtubule Sliding in Spoke-defective Axonemes

To test the model that the radial spokes regulate dynein through phosphorylation and dephosphorylation, we began testing the effects of specific and potent inhibitors of protein kinases on in vitro microtubule sliding. As described previously (Smith and Sale, 1992b), dynein in axonemes deficient for either the entire radial spoke (*pf14*) or the spoke head



**Figure 2.** Effects of PKI peptides on microtubule sliding velocity. (a) Isolated *pf17* axonemes were treated with 100 nM PKI(6-22)-amide (PKI), *N*<sup>α</sup>-acetyl-(Ala<sup>15,18,19</sup>)PKI(6-22)amide (*triAlaPKI*), *N*<sup>α</sup>-acetyl-PKI(6-22)amide (*NAcPKI*), or buffer only (*control*). PKI and *NAcPKI* significantly increased sliding velocity compared to both the control or *triAlaPKI* ( $P < 0.01$ ). Treatment with the control peptide *triAlaPKI* did not affect microtubule sliding relative to the untreated control ( $P > 0.8$ ). The number of axonemes measured for each mean is in parentheses above its respective error bar. (b) Isolated *pf14* (diamonds), *pf17* (squares), or *137c* (triangles) axonemes were treated with 0–200 nM PKI and the microtubule sliding velocities were measured. Each point represents the mean of  $\geq 20$  individual measurements. The concentrations required for half maximal activation of sliding velocity are 12 nM for *pf14* and 15 nM for *pf17*. The inset shows the data from *pf14* and *pf17* replotted after normalization to the maximum mean sliding velocity for each experiment. The difference between the absolute velocities of *pf14* and *pf17* axonemes is caused by variation in the baseline values of these two experiments. When directly compared over a series of experiments, there is little difference in sliding velocity between axonemes from *pf14* and *pf17* (see Fig. 1).

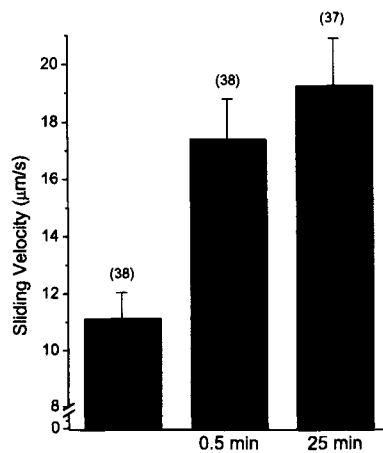
(*pf17*) translocated microtubules at 50–60% of the rate produced by dynein in wild-type axonemes (Fig. 1 a, control, vs. Fig. 2 b, *137c*). Treatment of isolated axonemes with the kinase inhibitor HA-1004 (15 µM), as described in Materials and Methods, significantly increased the velocity of microtubule sliding in spoke-deficient mutants (Fig. 1 a). In contrast, similar treatment of axonemes from wild-type cells had no effect on microtubule sliding velocity (not shown). The finding that microtubule sliding velocity in both *pf14* and *pf17* axonemes is stimulated by HA-1004 indicates that this effect is not specific to a particular mutation. Also, the ability of kinase inhibitors to increase microtubule sliding when applied to isolated axonemes indicates that their target(s) is tightly associated with axonemes.

The effect of HA-1004 on sliding velocity in spoke-deficient axonemes is dose dependent with half maximal activation of microtubule sliding velocity occurring at 1–5 µM (Fig. 1 b). The kinase inhibitor H-7 also increases sliding velocity in spoke-deficient but not spoke-containing axonemes (data not shown). Together these results suggest that an endogenous cyclic nucleotide-dependent protein kinase inhibits dynein-induced microtubule sliding in spoke-deficient axonemes. To further test this model, we examined the effect of specific and potent peptide inhibitors of protein kinases.

#### *An Endogenous cAMP-dependent Protein Kinase Inhibits Dynein Activity in Spoke-defective Axonemes*

Some of the most potent and specific protein kinase inhibitors are PKI peptides derived from the heat-stable inhibitor protein of cAPK. Unlike HA-1004 and H-7, which interact with the ATP-binding sites of protein kinases (Hidaka et al., 1984; Hagiwara, et al., 1987), PKI potently inhibits cAPK by acting as a pseudosubstrate and binding to the active site (Walsh and Glass, 1991). However, like the H-compounds, 100 nM PKI or 100 nM *NAcPKI* significantly increased the microtubule sliding velocity of *pf17* axonemes (Fig. 2 a). In contrast, the control peptide *triAlaPKI* did not affect microtubule sliding in spoke-deficient axonemes (Fig. 2 a). There was no detectable difference between the efficacies of PKI and *NAcPKI*, and similar results were found for axonemes from *pf14* (not shown) and the double mutant *pf14pf28* (see below).

PKI and *NAcPKI* both stimulated microtubule sliding velocity in a dose-dependent manner. The data from one experiment with *pf14* and one with *pf17* is shown in Fig. 2 b. The effects of PKI peptides on *pf14* and *pf17* were very similar, as can be seen when the data in Fig. 2 b are normalized to the maximal sliding velocity (Fig. 2 b, inset). Half-maximal activation of microtubule sliding velocity was achieved with 12 nM and 15 nM PKI for *pf14* and *pf17*, respectively, for these standardized conditions. These concentrations are consistent with a specific inhibition of cAPK and 3,000-fold lower than the  $K_i$  for the closely related cGMP-dependent protein kinase (Glass et al., 1986, 1992). In contrast, throughout the same concentration range, PKI did not affect the already rapid microtubule sliding velocity in wild-type *137c* axonemes (Fig. 2 b). Furthermore, the effect of the peptide inhibitor appeared to be rapid in that very short incubation with 100 nM PKI (~0.5 min, in motility buffer only) resulted in activation of microtubule sliding velocity similar to that found with 25-min preincubations

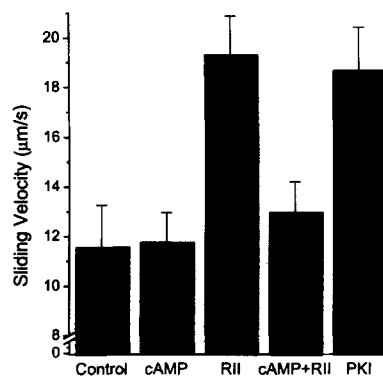


**Figure 3.** PKI rapidly affects microtubule sliding velocity in spoke-deficient axonemes. Isolated *pf14* axonemes were treated with buffer (-); 100 nM PKI only in the motility buffer used to induce microtubule sliding, which resulted in ~0.5 min treatment before the occurrence of sliding (0.5 min), or 100 nM PKI for  $\geq 25$  min. All axonemes were preincubated with 1 mM ATP for  $\geq 10$  min before induction of microtubule sliding. Both PKI treatments significantly increase the sliding velocity from the control level ( $P < 0.001$ ), and there was no difference between short and long treatments with PKI ( $P > 0.35$ ). The means represent pooled results from two separate experiments. The total number of axonemes measured for each mean are shown in the parentheses.

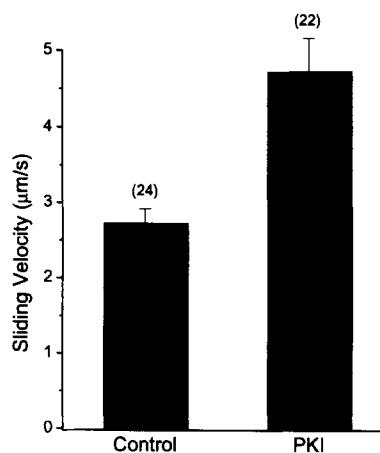
(Fig. 3). The potent effect of the two inhibitory PKI peptides and the inactivity of the control peptide strongly suggest that PKI elevates microtubule sliding velocity by specifically inhibiting a cAPK bound to *Chlamydomonas* axonemes.

#### Regulatory Subunit of cAPK Increases Microtubule Sliding Velocity in Spoke-deficient Axonemes

The finding that cAPK inhibitors increased the velocity of dynein-driven microtubule sliding in isolated axonemes sug-



**Figure 4.** Effect of RII and cAMP on the velocity of microtubule sliding in spoke-deficient axonemes. Isolated *pf14* axonemes were treated with 10  $\mu$ M cAMP (*cAMP*), 200 nM RII (*RII*), 10  $\mu$ M cAMP and 200 nM RII (*cAMP + RII*), 100 nM PKI (*PKI*), or buffer alone (*control*). The microtubule sliding velocity of axonemes treated with either RII or PKI was significantly greater than the other three treatments ( $P < 0.01$ ). Cotreatment with RII and cAMP was not different from the control ( $P > 0.5$ ). Each bar represents the mean from 20 measurements.



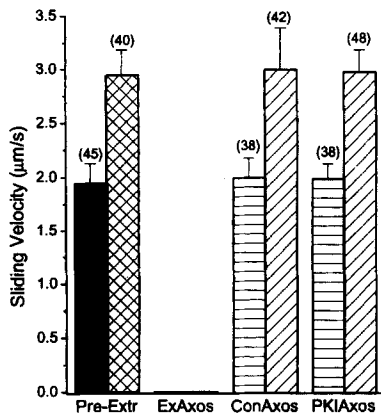
**Figure 5.** Effect of PKI on inner arm dyneins. Isolated axonemes from *pf14pf28* (missing radial spokes and outer arm dyneins) were treated with 100 nM PKI. PKI significantly increased the velocity of microtubule sliding in *pf14pf28* axonemes ( $P < 0.001$ ).

gested that a form of cAPK is active and structurally bound to isolated, spoke-deficient axonemes. In all reported cases in higher eukaryotes, cAPK exists as a heteromer of catalytic and regulatory polypeptides (Taylor et al., 1990). Cyclic AMP stimulates kinase activity by binding to the regulatory subunit and promoting subunit dissociation, thus releasing inhibition of the catalytic subunit. We found that cAMP had no effect on microtubule sliding in isolated axonemes, which would be expected if dissociated, active catalytic subunits were present (Fig. 4). Therefore, our data suggested that the putative axonemal regulatory subunit was lost during fractionation and purification of axonemes. To further examine the model that cAPK regulates dynein-driven microtubule sliding, we tested whether the addition of exogenous regulatory subunit would inhibit any free catalytic subunit present and increase the velocity of microtubule sliding in spoke-deficient axonemes.

As predicted, the addition of 200 nM bovine cardiac regulatory subunit type II (RII) significantly increased the velocity of microtubule sliding in *pf14* axonemes; the effect of RII was similar to that of PKI (Fig. 4). Furthermore, as expected for a cAPK-mediated mechanism, 10  $\mu$ M cAMP reversed the effect of RII addition (Fig. 4). As stated, cAMP alone did not affect microtubule sliding (Fig. 4). These results further support the hypothesis that cAPK inhibits microtubule sliding in spoke-deficient axonemes and suggest that a substantial fraction of the regulatory subunit of cAPK is solubilized during the isolation of axonemes. The results also corroborate the conclusion that the catalytic subunit of the kinase is not soluble, but is built into the axoneme.

#### Inner Arm Dyneins May Be the Targets of cAPK Regulation

One hypothesis to explain the results described above is that cAPK regulates microtubule sliding velocity by modulating the phosphorylation of a dynein component(s). Alternatively, cAPK may modify some other, nondynein component that affects microtubule sliding velocity. To distinguish between these possibilities and to begin to identify the sub-



**Figure 6.** The velocity of microtubule sliding in control and PKI-treated axonemes reconstituted with control and PKI-treated inner arm dyneins. Isolated *pf14pf28* axonemes were treated with buffer (solid bar) or 100 nM PKI (cross-hatched bar), and the microtubule sliding velocity was measured for an aliquot of each of these (*Pre-Extr*). The inner arm dyneins were then extracted and isolated from the remainder of the axonemes. After dialysis into low salt buffer, control dynein extracts (horizontally lined bars) were reconstituted with extracted control (*ConAxos*) or extracted PKI-treated (*PKIAxos*) axonemes. PKI-treated dynein extracts (diagonally lined bar) were reconstituted with extracted control (*ConAxos*) or extracted PKI-treated (*PKIAxos*) axonemes. The velocities of microtubule sliding in these reconstituted axonemes were measured. Extracted axonemes which did not receive dynein extract did not exhibit microtubule sliding (*ExAxos*). In all circumstances, PKI-treated dynein generated significantly faster sliding velocities than control dynein ( $P < 0.05$ ). The data from two separate experiments were pooled.

strates involved in the regulation of microtubule sliding, we used a reconstitution assay similar to that described by Smith and Sale (1992b). In this previous study, we found that wild-type spokes induced a change in an inner dynein arm component necessary for increased microtubule sliding velocity (see Introduction). One possibility is that wild-type spokes and inhibitors of cAPK effect the same changes. For reconstitution experiments, we focused on only the inner row of dynein arms for reasons previously described (Smith and Sale, 1992a,b). Therefore, we used the double mutant *pf14pf28*, which lacks both the radial spokes and the outer arms, to test the ability of the inner arms alone to respond to PKI. Treatment of *pf14pf28* axonemes with PKI in conditions identical to those used in Fig. 2 (i.e., buffer B throughout) significantly increased their microtubule sliding velocity (Fig. 5). Therefore, the inner row of dynein arms is sufficient for regulation by both cAPK and the radial spokes.

To further characterize the involvement of inner arm dyneins in regulation by cAPK, we performed dynein extraction and reconstitution experiments (Fig. 6). Axonemes were isolated from *pf14pf28* cells, and half were treated with PKI, while half were left as the control. An aliquot was taken from each of these just before salt extraction of the dynein and used in the sliding assay (Fig. 6, *Pre-Extr*). As expected, the sliding velocity of PKI-treated axonemes (Fig. 6, cross-hatched bar) was significantly greater than the velocity of control axonemes (Fig. 6, solid bar). As described in Mate-

rials and Methods, inner arm dynein was then extracted from both PKI-treated and control axonemes. The PKI-treated dynein (Fig. 6, diagonally lined bars) was reconstituted onto both extracted PKI-treated (Fig. 6, *PKIAxos*) and extracted control (Fig. 6, *ConAxos*) axonemes. The control dynein (Fig. 6, horizontally lined bars) was also reconstituted onto both extracted PKI-treated (Fig. 6, *PKIAxos*) and extracted control (Fig. 6, *ConAxos*) axonemes. The PKI-treated dynein (diagonally lined bars) generated fast microtubule sliding velocities on both control (*ConAxos*) and PKI-treated (*PKIAxos*) axonemes. In the reciprocal experiments, control dynein (horizontally lined bars) generated only slow microtubule sliding on both control (*ConAxos*) and PKI-treated axonemes (*PKIAxos*). Extracted axonemes without readded dynein (*ExAxos*) did not exhibit microtubule sliding, indicating that the dynein was functionally extracted (see also, Smith and Sale, 1992a). These results demonstrated that, as the radial spokes, PKI regulates microtubule sliding by causing a stable change in a component that is extracted from axonemes under conditions typically used to isolate dynein. Furthermore, these results suggest that cAPK inhibits microtubule sliding by phosphorylation of a salt-extractable component(s), which may include a subunit(s) of inner arm dyneins.

## Discussion

### *Chlamydomonas* Axonemes Contain a cAMP-dependent Protein Kinase That Regulates Microtubule Sliding

Diverse evidence has indicated a role for cAPK in regulation of ciliary and flagellar motility (Stephens and Stommel, 1989; Tash, 1989). For example, initial motility of many sperm flagella requires cAMP (Brokaw, 1987; Lindemann and Kanous, 1989; Morisawa and Okuno, 1982). The targets and mechanisms of cAMP and cAPK action are largely unknown. However, one hypothesis is that regulation of dynein activity is the ultimate target (Walczak and Nelson, 1994; Barkalow et al., 1994). The results of this paper indicate that *Chlamydomonas* flagellar axonemes contain a cAPK that regulates dynein-driven microtubule sliding. In *Chlamydomonas*, it appears that the protein kinase operates to reduce dynein activity and, as discussed below, may mediate radial spoke regulation of axonemal dynein activity. Furthermore, the results implicate components of the inner row of dynein arms as regulatory substrates for the axonemal kinase. Several lines of evidence with spoke-deficient axonemes support these conclusions. First, inhibitors of protein kinases increased the velocity of microtubule sliding with half-maximal activation of sliding occurring at 1–5  $\mu\text{M}$  HA-1004. This concentration is similar to the reported  $K_i$  (1–3  $\mu\text{M}$ ) for HA-1004 inhibition of cyclic nucleotide-dependent kinases and lower than the reported  $K_i$ 's for protein kinase C (40  $\mu\text{M}$ ) and myosin light chain kinase (150  $\mu\text{M}$ ) (Hidaka et al., 1984). Second, peptides that are highly specific inhibitors of cAPK increase microtubule sliding velocities to wild-type levels in a dose-dependent manner with the half-maximal effect occurring at 12–15 nM. The same results were attained using peptides synthesized in two separate batches (not shown). A related control peptide in which the essential arginine residues were replaced with alanines, thereby rendering it inactive as a cAPK inhibitor, did not

affect microtubule sliding velocity. Third, the velocity of microtubule sliding was also increased to wild-type levels by the addition of exogenous regulatory subunit of cAPK. Upon addition of exogenous RII, the velocity of microtubule sliding became cAMP dependent. Finally, inner arm dynein extracted from PKI-treated axonemes and reconstituted onto control axonemes retained the ability to translocate microtubules with accelerated velocities.

The results with HA-1004, PKI, and RII strongly suggest that the protein regulating microtubule sliding in spoke-deficient axonemes is a form of cAPK. The mechanism by which HA-1004 inhibits cAPK (Hidaka et al., 1984; Hagiwara, et al., 1987) differs from that used by PKI and RII (reviewed in Taylor et al., 1993). In addition, the sequence in PKI which dictates its high specificity of cAPK binding differs from the sequence in RII that confers specific binding, and these two sequences bind to different domains in cAPK (Taylor et al., 1993). Because of these differences in mechanisms of binding and action, it is unlikely that all three of these reagents could affect microtubule sliding through a coincidental interaction with any protein other than cAPK. In addition, the finding that cAPK inhibits the velocity of microtubule sliding in *Chlamydomonas* axonemes is consistent with previous results which indicated that cAMP (Rubin and Filner, 1973; Hartfiel and Amrhein, 1976) or cAPK (Hasegawa et al., 1987) inhibit motility in *Chlamydomonas* flagella. Our results suggest that cAPK may inhibit motility by inhibiting dynein-driven microtubule sliding.

Because all treatments were performed on isolated axonemes, the kinase is likely to be a structural component of *Chlamydomonas* axonemes. An alternative explanation is that since the action of cAPK is catalytic, small amounts of contaminating soluble kinase could influence microtubule sliding in vitro. To test this possibility, we compared the microtubule sliding velocities of *pfl7* axonemes prepared by the procedure described in Materials and Methods with axonemes washed three additional times by pelleting and resuspending in fresh buffer. No difference between the two preparations of axonemes was seen in either their baseline or PKI-treated velocities (not shown). This experiment demonstrates that the kinase was not removed by extensive washing, and it suggests that the effects we report were not caused by contaminating soluble enzyme. Similar conclusions were also reached in a recent study of the phosphorylation of the  $\alpha$ -heavy chain in the outer dynein arms of *Chlamydomonas* flagella (King and Witman, 1994). In addition, the lack of effect of cAMP alone and the ability of exogenous RII to affect microtubule sliding suggest that the catalytic subunit is bound to an axonemal component other than RII. If the catalytic subunit is a structural component of *Chlamydomonas* axonemes, physical constraints will likely require the kinase to be located within close proximity of its substrates. Since inner arm dyneins are potential targets of phosphorylation, the kinase may be located within or adjacent to the row of inner arms (see below).

Several previous studies have provided evidence that cAPK is associated with the detergent-resistant structures from rat sperm (Horowitz et al., 1984), ram sperm (San Agustin and Witman, 1994), and *Paramecium* (Bonini and Nelson, 1988; Hamasaki et al., 1989, 1991) and *Tetrahymena* (Chilcote and Johnson, 1990) cilia. In rat sperm, the catalytic subunit is probably tethered to the sperm tail via

its interaction with the regulatory subunit since cAMP releases most of the catalytic activity from demembrated sperm (Horowitz et al., 1984). In contrast, catalytic subunit in ram sperm may be bound via interactions with additional proteins. As much as 50% of PKI-sensitive kinase activity remains bound to demembrated ram sperm after treatment with cAMP (San Agustin and Witman, 1994). Furthermore, a fraction of catalytic subunit in ram sperm is apparently localized to the axoneme (Pariset et al., 1989), and therefore, the association may be analogous to that in *Chlamydomonas*.

### **Radial Spokes May Affect Microtubule Sliding through the Axonemal cAPK**

Our original goal was to elucidate the mechanism by which the radial spokes regulate dynein activity. In spoke-deficient axonemes, protein kinase inhibitors mimic the radial spokes in their ability to increase the velocity of dynein-driven microtubule sliding. In contrast, kinase inhibitors do not affect microtubule sliding in wild-type axonemes that have fast microtubule sliding velocities, even without the addition of kinase inhibitors. These findings support models in which the spokes regulate dynein activity by inactivating cAPK, activating a phosphoprotein phosphatase, or in which the spokes otherwise bypass the inhibitory effects of phosphorylation. In any case, the spokes would function to prevent the kinase from inhibiting motility and maintain permissive conditions for maximal dynein-driven microtubule sliding. At this point, we cannot rule out the possibility that the effects of kinase inhibitors and radial spokes may be coincidental. However, because the velocity of microtubule sliding in axonemes with wild-type radial spokes is insensitive to kinase inhibitors, the spokes at least are capable of bypassing the effects of the protein kinase on microtubule sliding. Measuring parameters of flagellar motility other than microtubule sliding velocity may reveal more information about the role of cAPK in wild-type axonemes.

As outlined in the Introduction, the radial spokes are thought to regulate flagellar beating through a mechanism that involves the central pair apparatus and the drc (Porter et al., 1992; Piperno et al., 1992, 1994). Mutations in the spokes and central pair lead to flagellar paralysis (Huang, 1986), and secondary mutations in the dynein arms or drc suppress this paralysis (Piperno et al., 1992, 1994; Porter et al., 1992, 1994). If cAPK activity is involved in this pathway, axonemes that are paralyzed because of mutations in the central pair might be expected to respond to protein kinase inhibitors in the way that spoke-mutant axonemes do. Our recent experiments with the paralyzed, central pairless mutant *pfl8* support the hypothesis that inhibition of dynein-driven microtubule sliding by cAPK may be fundamental to paralysis. The velocity of microtubule sliding in *pfl8* ( $13.1 \pm 1.10 \mu\text{m/s}$ ,  $n = 25$ ) is similar to that in *pfl4* and *pfl7* (e.g., Fig. 2) and significantly increased to wild-type levels by 100 nM PKI ( $21.7 \pm 1.20 \mu\text{m/s}$ ,  $n = 25$ ; Habermacher, G., unpublished results). The finding that kinase inhibitors activate microtubule sliding in both radial spoke and central pair mutants raises the possibility that certain suppressor mutations may restore beating to paralyzed flagella by inactivating the kinase or altering its substrate. However, in preliminary experiments, we were unable to reactivate in vitro

beating of *pfl7* axonemes with PKI. Although a negative result in this experiment provides little information, it may suggest that genetic restoration of beating by the suppressor mutations involves additional changes in the flagellar machinery.

To study the regulation of dynein-driven microtubule sliding, we have chosen to assay the sliding velocity of microtubule doublets in protease-treated axonemes (Okagaki and Kamiya, 1986; Smith and Sale, 1992a). This method has several advantages. First, the translocation of microtubules by dynein is uncoupled from bend generation; therefore, sliding velocity should reflect the motor activity of dynein in the axoneme. Second, dynein activity can be assessed in otherwise paralyzed axonemes (e.g., spoke-defective axonemes). Most importantly, since dynein activity is assayed *in situ* (i.e., in the axoneme), interactions between dyneins and other axonemal components are maintained and can be studied independent of membrane and cytoplasmic matrix fractions. These advantages were crucial in allowing us to identify the involvement of cAPK and the radial spokes in regulating dynein-driven microtubule sliding. However, the microtubule sliding assay has certain limitations. The extent of proteolysis and the identity of the polypeptides affected are not fully understood. Be that as it may, the sliding assay reproducibly detects changes in the velocity of microtubule sliding that are induced by both the radial spokes and protein kinase inhibitors. Therefore, the sliding assay provides information on at least these aspects of the regulation of flagellar motility. Another disadvantage of the sliding assay is that we do not know which specific microtubule doublets are undergoing sliding at any instant. In many cases, we can measure the sliding velocity of several individual doublets from one axoneme, and different doublets within one axoneme slide at similar velocities. This finding suggests that most or all doublets are active and under similar regulatory controls. However, we cannot eliminate the possibility that select subsets of microtubule doublets are sliding in the conditions used.

#### ***Inner Arm Dyneins May contain a Substrate for cAPK***

A variety of evidence has indicated that dynein from several axonemal sources may contain cAPK substrates involved in regulation of motility. For example, in *Paramecium*, increased cAMP results in faster swimming speed and increased ciliary beat frequency (Bonini and Nelson, 1988; Hamasaki et al., 1989). Experiments that measured the translocation of microtubules by purified dynein adsorbed to glass support the idea that cAMP-dependent phosphorylation of a 29-kD polypeptide in *Paramecium* outer arm dynein is involved in the regulation of beat frequency (Hamasaki et al., 1991; Barkalow et al., 1994). In mussel gill cilia, cAMP-dependent phosphorylation of presumptive 21- and 27-kD outer arm dynein light chains (Stephens and Prior, 1992) correlates with cAPK activation of motility *in vitro* (Stephens and Stommel, 1989). *Tetrahymena* outer arm dynein is phosphorylated on 78-, 37-, 34-, and 30-kD polypeptides by an axonemal cAPK, but this is not correlated with a change in motility (Chilcote and Johnson, 1990). In addition, *Chlamydomonas* outer and inner arm dyneins contain substrates for an axonemal kinase *in vivo* and *in vitro* (Piperno and Luck, 1981; Piperno et al., 1981; King and Witman, 1994).

Our inner arm dynein extraction and reconstitution experiments implicate a component of *Chlamydomonas* inner arm dyneins as one of the substrates for cAPK. Inner arm dynein extracted from PKI-treated axonemes supported fast sliding velocities on both PKI-treated and control-treated extracted axonemes. In other words, the velocity of microtubule sliding was determined by the source of the dynein extract, and the source of the axonemes was irrelevant. A simple explanation for this result is that PKI changes the phosphorylation level of at least one of the polypeptides that are extracted by high salt, and this altered polypeptide(s) dictates accelerated sliding velocities even on control axonemes. An alternative explanation is that PKI contaminates the dynein extracts. However, this latter explanation is unlikely because PKI is able to freely diffuse through the dialysis membrane used to desalt extracts. Therefore, the concentration of PKI in the dialyzed extracts should be less than that required to increase microtubule sliding.

Because inner arm dyneins actually perform the work of microtubule translocation in *pfl4pfl28*, inner arm dynein subunits are attractive candidates as regulatory substrates. However, numerous salt-extractable, axonemal polypeptides are phosphorylated *in vivo* (Piperno and Luck, 1981; King and Witman, 1994) and *in vitro* (King and Witman, 1994; Howard, D., unpublished observations) and are candidate substrates. Our results suggest two restrictions on candidate substrates involved in the regulation of motility. First, because PKI-treated axonemes reconstituted with control dynein extracts had slow microtubule sliding velocities that were indistinguishable from control axonemes with control dynein, the target of kinase regulation must be extracted from axonemes. This restriction makes the *drc*, which is resistant to salt extraction (Piperno et al., 1994), a poor candidate substrate. Second, because the PKI-treated dynein extract conferred fast sliding velocities to control axonemes, the target of the kinase must retain its function and regulated state when reassociated with axonemes. Inner arm dyneins are extracted from outer armless mutants to >90% (Smith and Sale, 1992a; Howard, D., unpublished observations). Furthermore, *Chlamydomonas* inner arm dyneins rebind to their original positions *in vitro* and restore function (Smith and Sale, 1992a,b), and as mentioned, inner arm dyneins contain phosphorylation substrates. Therefore, inner arm dyneins meet the criteria for a regulatory kinase substrate, but it is impossible to rule out other proteins in the extract as substrates involved in the regulation of microtubule sliding. In addition, the experiments reported here do not eliminate the possibility that the outer arms are also targets of cAMP-dependent regulation. Based on a comparison of the PKI-induced changes in microtubule sliding velocity in *pfl4* or *pfl7* axonemes with *pfl4pfl28* axonemes, it is clear that the outer arms contribute to the magnitude of the PKI-induced increase. However, it is not known whether outer arm activity is affected directly by phosphorylation (e.g., Hamasaki et al., 1991; King and Witman, 1994) or indirectly through interaction with other axonemal components such as the inner arms. In either case, the experiments do indicate that outer arms are not uniquely responsible for regulation of sliding velocity. Thus, one priority is to use *Chlamydomonas* flagellar mutants to determine the substrates of the axonemal cAPK. The use of PKI and RII to regulate microtubule slid-



ing in combination with in vitro reconstitution approaches provides new opportunities to identify regulatory substrates that control dynein-based motility.

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