Casein Kinase I Is Anchored on Axonemal Doublet Microtubules and Regulates Flagellar Dynein Phosphorylation and Activity*‡

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Flagellar dynein activity is regulated by phosphorylation. One critical phosphoprotein substrate in *Chlamydomonas* is the 138-kDa intermediate chain (IC138) of the inner arm dyneins (136, 167–176). In this study, several approaches were used to determine that casein kinase I (CKI) is physically anchored in the flagellar axoneme and regulates IC138 phosphorylation and dynein activity. First, using a videomicroscopic motility assay, selective CKI inhibitors rescued dynein-driven microtubule sliding in axonemes isolated from paralyzed flagellar mutants lacking radial spokes. Rescue of dynein activity failed in axonemes isolated from these mutant cells lacking IC138. Second, CKI was unequivocally identified in salt extracts from isolated axonemes, whereas casein kinase II was excluded from the flagellar compartment. Third, Western blots indicate that within flagella, CKI is anchored exclusively to the axoneme. Analysis of multiple *Chlamydomonas* motility mutants suggests that the axonemal CKI is located on the outer doublet microtubules. Finally, CKI inhibitors that rescued dynein activity blocked phosphorylation of IC138. We propose that CKI is anchored on the outer doublet microtubules in position to regulate flagellar dynein.

The dynein ATPase is a family of molecular motors responsible for diverse cellular functions including retrograde microtubule-based transport of organelles, assembly and function of the Golgi and mitotic apparatus, and movement of cilia and flagella (1, 2). One of the primary questions is how dynein activity is regulated. The focus of this report is based on a series of studies revealing that the flagellar central pair apparatus and radial spokes play a primary role in regulation of flagellar dynein activity (3, 4). The regulatory mechanism involves a structural network of axonemal kinases and phosphatases that control phosphorylation of key subunits within the inner arm dyneins (5–8). In particular, phosphorylation of a dynein intermediate chain subunit, IC138,1 correlates with inhibition of flagellar dynein activity (7). This network of enzymes along with the central pair apparatus, radial spokes, and the inner arm dyneins operates to control flagellar waveform (9–17).

These conclusions are based on genetic analysis and in vitro functional studies of flagella from *Chlamydomonas reinhardtii*. For example, mutations that disrupt either the central pair or the radial spokes result in flagellar paralysis (2, 3, 18). These paralyzed axonemes undergo dynein-driven microtubule sliding *in vitro* (19) but at greatly reduced rates of sliding when compared with wild-type axonemes (20). Reconstitution and functional assays demonstrate that the radial spokes are required for wild-type dynein activity, and the velocity of dynein-driven microtubule sliding is mediated by posttranslational modification of the inner dynein arms (20). These results are consistent with other evidence indicating that the central pair/radial spoke apparatus along with the dynein regulatory complex controls dynein activity (14, 17, 21–26). The challenge has been to define the biochemical signaling pathway and to determine how this biochemistry can be altered by mechanical interaction between the central pair structures, radial spoke structures, and outer doublet microtubule components to control dynein activity.

Diverse experimental systems have revealed that ciliary and flagellar motility is controlled by phosphorylation and that the protein kinases and phosphatases responsible for regulation are anchored in the axoneme (27–33). Thus, we postulate that phosphorylation regulates dynein activity, and key kinases and phosphatases are anchored in the axoneme near the dynein arms or in the central pair/radial spoke structures. Consistent with this hypothesis, exogenously added protein kinase inhibitors including PKI and the type II regulatory subunit of PKA rescue wild-type microtubule sliding in isolated axonemes lacking radial spokes (5). Moreover, *in vitro* reconstitution experiments using double mutants lacking the radial spokes and selected subsets of dynein arm structures reveal that one of the dyneins, inner arm dynein I1, is required for rescue of dynein activity (7). The simplest model is that PKA is anchored in the axoneme and controls dynein activity and that the control mechanism requires changes in phosphorylation of inner arm dynein I1. I1 is important for control of flagellar waveform (12, 15), is functionally linked to the central pair apparatus/radial spoke system (14, 26), and contains the Tctex light chain postulated to play a role in meiotic drive in t-haplotype mice (34). We predict I1 contains the critical phosphoprotein subunit required for control of dynein activity, and the 138-kDa intermediate chain (IC138) of I1 is the only phosphoprotein in this complex (7). Importantly, phosphorylation of IC138 correlates with inhibition of dynein, and dephosphorylation of IC138 correlates with rescue of dynein activity.

It is now apparent that control of dynein is more complex than we initially thought. PKA inhibitors such as PKI fail to
block phosphorylation of IC138 at concentrations that rescue activity. Therefore, we conclude that additional axonal kinases are involved in the phosphorylation of IC138. Based on other studies (33, 35–37) and preliminary pharmacological analysis, we postulate one of the casein kinases (38) is anchored on doublet microtubules and directly phosphorylates IC138. In this study, we show that CKI is located in the axoneme, and inhibitors of CKI block phosphorylation of IC138 and rescue dynein activity in paralyzed axonemes. We postulate that axonal CKI operates in concert with the axonemal PKA in a network to control flagellar waveform through regulation of inner arm dynein phosphorylation. The results provide an additional connection in the physical circuitry of axonemal enzymes that control motility and offer a new opportunity to define the mechanism for anchoring CKI to the cytoskeleton (39).

**EXPERIMENTAL PROCEDURES**

Cell Strains and Growth Conditions—The double mutants pf28pf30 and pf14pf30 were described previously (7, 40, 41). Dr. E. H. Harris (Duke University and Chlamydomonas Genetics Center) provided wild-type Chlamydomonas flagellar cell strains. With the exception of pf14pf30, all cells were grown in liquid-modified medium 1 (42) with aeration over a 14:10 light/dark cycle. pf14pf30 cells were grown on agar plates for 4 days prior to harvesting and suspension in liquid medium (7).

Reagents and Kinase Inhibitors—PKI, a peptide based on residues 6–22 of the α isoform of the heat-stable inhibitor of PKA, was synthesized and stored as described previously (5), and was added to a reaction mixture to a final concentration of 40 μM (5000 cpm/pmol). The reaction was terminated at 2 min by adding electrophoresis sample buffer. For sucrose gradient fractionation of phosphorylated axonemal proteins, the reaction was scaled up to 20 μl of the DEAIE CKI-containing fraction that was diluted with an equal volume of 2X CK reaction buffer (50 mM Tris, pH 8, 0.1 mM EDTA, 0.2% β-mercaptoethanol, 7 mM magnesium acetate, 0.02% Brij 35, 20 mM NaCl, and 100 μM sodium orthovanadate). The protein samples were fixed in 5% formaldehyde overnight, and after washing with CKI buffer, the proteins sedimenting with the agarose beads were fixed by suspension in 30 μl of 1X SDS-PAGE sample buffer.

In Vitro Phosphorylation of Axonemal Proteins—For phosphorylation, purified axonemes at 5 mg/ml were suspended in CK reaction buffer in either the presence or absence of 50 μM CKI-7 or 200 μM PKI. 20-μl samples were preincubated for 20–30 min at 30 °C, and [γ-32P]ATP was added to a final concentration of 40 μM (5000 cpm/pmol). The reaction was terminated at 2 min by adding electrophoresis sample buffer. For sucrose gradient fractionation of phosphorylated axonemal proteins, the reaction was scaled up to 500 μl of 10 mg/ml axoneme. Following phosphorylation, axonemes were sedimented at 14,000 × g in a microcentrifuge and resuspended in Buffer A containing 0.57 mM NaCl and 10 μM microcystin-LR to block phosphatase activity. Salt-extracted axonemes were separated from the extract by sedimentation, and the extract was dialedyzed in Buffer A and subjected to velocity sedimentation on sucrose gradients.

For analysis of CKI autophosphorylation, each reaction contained 1X CK reaction buffer, 14 μl of the DEAIE fraction, and 100 μM [γ-32P]ATP (2000 cpm/pmol) in a total final volume of 20 μl. Before addition of ATP, 2-μl samples from gradient fractions or chromatography fractions were added to a reaction mixture to a final volume of 20 μl containing CK reaction buffer, 0.5 mg/ml CKI or CKII specific substrate, and 40 μM [γ-32P]ATP (25 mM phosphate buffer, pH 6.8, 10 mM β-mercaptoethanol, 2 mM EGTA). The reaction was incubated for 5 min at 30 °C, and the reaction was terminated by adding 1 μl of 10 mg/ml dephosphorylated casein (Sigma) in 1X SDSPAGE sample buffer. The kinase reaction was scaled up to 200 μl in a microcentrifuge and resuspended in Buffer A containing 0.57 mM NaCl and 10 μM microcystin-LR to block phosphatase activity. Salt-extracted axonemes were separated from the extract by sedimentation, and the extract was dialedyzed in Buffer A and subjected to velocity sedimentation on sucrose gradients.

Affinity purification of CKI was performed using α-casein conjugated to an agarose matrix (Sigma). α-Casein-conjugated agarose beads (40 μl) were added to 200 μl of the DEAIE CKI-containing fraction that was diluted with an equal volume of 2X CK reaction buffer (50 mM Tris, pH 8, 0.1 mM EDTA, 0.2% β-mercaptoethanol, 7 mM magnesium acetate, 0.02% Brij 35, 20 mM NaCl, and 100 μM sodium orthovanadate). The kinase reaction was scaled up to 200 μl in a microcentrifuge and resuspended in Buffer A containing 0.57 mM NaCl and 10 μM microcystin-LR to block phosphatase activity. Salt-extracted axonemes were separated from the extract by sedimentation, and the extract was dialedyzed in Buffer A and subjected to velocity sedimentation on sucrose gradients.

**In-gel Kinase Assay and Western Blot Analysis**—In-gel kinase assays were carried out as described previously (48) with modification. A stock solution of 10 mg/ml dephosphorylated casein (Sigma) in 1X resolv buffer was added to a 9% acrylamide gel mixture to a final concentration of 1 mg/ml casein. Following electrophoresis, the gel was washed twice with ~250 ml of 20% isopropyl alcohol in Buffer B (50 mM HEPES, pH 7.4, 5 mM β-mercaptoethanol) for 30 min each, and then washed with Buffer B alone for 30 min. For renaturation of proteins, the gel was immersed in ~250 ml of 0.04% Tween 20 in Buffer B at 4 °C overnight. The buffer was changed at least six times during the overnight incubation period. The gel was then incubated in 10 ml of Buffer C (50 mM Hepes, pH 7.4, 100 μM sodium orthovanadate, 10 mM MnCl2, 5 mM β-mercaptoethanol) at 30 °C for 30 min. Phosphorylation was carried out by incubating the gel in Buffer C with 7 μl of [γ-32P]ATP at 30 °C for described (47) with modification. The 0.3 mM NaCl extract was dialedyzed into DEAE buffer (20 mM Tris, pH 8, 2 mM EGTA, 2 mM EDTA, 5 mM dithiothreitol, 10% glycerol), loaded onto a DEAE column (3–5 ml) (Sigma), and washed with 30 ml of DEAE buffer, and 0.5-ml fractions were collected at a flow rate of 0.5–0.3 ml/min from 15 ml of a linear 0.0–0.7 mM NaCl gradient in DEAE buffer (50 mM Tris, pH 8, 2 mM EGTA, 2 mM EDTA, 5 mM dithiothreitol, 10% glycerol).

In some experiments, the peak CKI fractions from sucrose gradients (made in Buffer A containing 30 mM NaCl) were fractionated by P-11 phosphocellulose chromatography as described previously (47). Briefly, cellulose phosphate (Whatman, Kent, United Kingdom) was precycled following the manufacturer’s instruction and equilibrated in P-11 buffer (50 mM HEPES, pH 6.8, 10 mM β-mercaptoethanol, 1 mM EDTA, 2 mM EGTA) containing 25 mM NaCl. The peak kinase activity fractions from sucrose gradient sedimentation were pooled, diazylized in 25 ml NaCl in P-11 buffer, and loaded onto a 5–20 ml P-11 column. The column was washed in the same buffer, and fractions were eluted with 15 ml linear 0.025–1.25 mM NaCl gradient in P-11 buffer. The gradient was applied at 0.5 ml/min, and about 0.8-ml fractions were collected.
3 h. To control for ATP binding, \( \alpha^{32} \text{P} \text{ATP} \) was used, and in some cases myelin basic protein was used in place of casein. The reaction was terminated, and free nucleotides were rinsed away by gentle shaking of the gel in \( \sim 250 \) ml of fixative solution (10 mM sodium pyrophosphate and 5% trichloroacetic acid) for 20 min with 5–7 solution changes. The gel was dried for phosphor imaging following Coomassie Blue staining.

For Western blots, samples separated with SDS-PAGE were transferred to nitrocellulose membrane (Bio-Rad) and incubated with the first antibody (affinity-purified anti-CKI-(183–249) at a 1:200 dilution, anti-PP1 at a 1:5000 dilution, or anti-IC140 at a 1:5000 dilution). This was followed by addition of horseradish peroxidase-conjugated secondary antibody and ECL detection (8, 43).

Identification of a 39-kDa CKI from Purified Axonemes—Axonemes were isolated from wild-type cells and extracted in a 0.3 mM NaCl-containing buffer. The dialyzed extract was then fractionated by one of three methods, and fractions were analyzed for protein composition with an in-gel kinase assay (Fig. 2). Based on substrate specificity, CKI activity (Fig. 2, top panels, open squares) was most prominent in axonemal extracts. CKI was the first kinase to elute from a DEAE ion exchange column (Fig. 2A, top panel, fractions 9–11), and \( \alpha \)-casein was an equally effective substrate for this kinase (data not shown). PKA and CKII activities were also detectable but at much lower levels and in later fractions. Using an in-gel kinase assay with casein embedded in the gel, a prominent 39-kDa axonemal kinase was detected with peak activity in fractions 9–11 (Fig. 2A, lower panel). This same kinase activity was greatly reduced when myelin basic protein was used as a substrate. The 39-kDa axonemal kinase co-fractionated with CKI activity as an 8 S peak in sucrose gradients (Fig. 2B), and P-11 phosphocellulose chromatography of the pooled sucrose gradient fractions 15–19 also resulted in a peak of CKI activity that precisely co-fractionated with the 39-kDa axonemal kinase (Fig. 2C). In every case, either DRB or CKI-7 inhibited the phosphorylation of CKI-peptide substrate or \( \alpha \)-casein (see below and Refs. 49 and 50). DRB and CKI-7 had no effect on the phosphorylation of PKA peptide substrate; when compared with CKI, CKII activity was significantly lower with experimental variation. As described previously (20), dynein activity is greatly reduced in axonemes lacking radial spokes (pf14). Incubation of axonemes with either 100 \( \mu \)M DRB (A) or 50 \( \mu \)M CKI-7 (B) increased the velocity of dynein-driven microtubule sliding, restoring wild-type activity (\( p < 0.0005 \)). In contrast, CKI-7 (or DRB) failed to rescue dynein activity (\( p > 0.15 \)) in a double mutant, pf14pf30, lacking both the radial spokes and inner arm dynein 11 (C). Bars indicate mean \( \pm \) S.D.

**RESULTS**

Casein Kinase Inhibitors Rescue Dynein Activity in Paralyzed Axonemes Lacking the Radial Spokes—Radial spokes operate in part to control axonemal kinases that otherwise would inhibit dynein activity (5–8). We postulated that an axonemal casein kinase inhibits dynein-driven microtubule sliding in paralyzed axonemes lacking the radial spokes. The paralyzed, radial spoke mutant pf14 was selected for our experiments because dynein activity is greatly reduced in pf14 axonemes, and dynein activity can be rescued in pf14 with kinase inhibitors (5). The prediction was that using a videomicroscopic motility assay, inhibitors of casein kinases would rescue wild-type dynein-driven microtubule sliding in pf14 axonemes. We have shown that the key phosphatases required to rescue dynein activity are also built into the structure (5–8). As described previously, the velocity of microtubule sliding (\( \sim 7 \) \( \mu \)m/s) is greatly reduced in axonemes from pf14 mutants compared with microtubule sliding (\( \sim 14–16 \) \( \mu \)m/s) in axonemes from wild-type cells (Fig. 1). Treatment of isolated pf14 axonemes with the casein kinase inhibitors, DRB (100 \( \mu \)M) or CKI-7 (50 \( \mu \)M) increased the velocity of microtubule sliding to \( \sim 13 \) \( \mu \)m/s (Fig. 1, A and B), rescuing dynein-driven microtubule sliding. The effects of both DRB and CKI-7 were dose-dependent, with half-maximal activity achieved with \( \sim 50 \) \( \mu \)M DRB and \( \sim 25 \) \( \mu \)M CKI-7. Supplementation of CKI-7 with PKI had no additional effect on microtubule sliding compared with addition of CKI-7 alone. CKI-7 failed to rescue wild-type sliding in axonemes from the double mutant pf14pf30 (Fig. 1C), which lacks both the radial spokes and inner arm dynein 11 (when compared with Ref. 7). These results strongly suggest that the isolated axoneme contains a casein kinase that inhibits dynein activity in paralyzed mutants lacking radial spokes and that the inner arm dynein 11 is required for rescue. Based on the selectivity and \( K_i \) of CKI-7 for CKI, when compared with CKII, PKA, or \( \text{Ca}^{2+} / \text{calmodulin kinase} \) (49, 50), the results indicate that CKI is responsible for inhibition of dynein. We tested this hypothesis with direct biochemical approaches.

Identification of a 39-kDa CKI from Purified Axonemes—Axonemes were isolated from wild-type cells and extracted in a 0.3 mM NaCl-containing buffer. The dialyzed extract was then fractionated by one of three methods, and fractions were analyzed for CKI, CKII, and PKA activity using specific peptide substrates and an in-gel kinase assay (Fig. 2). Based on substrate specificity, CKI activity (Fig. 2, top panels, open squares) was most prominent in axonemal extracts. CKI was the first kinase to elute from a DEAE ion exchange column (Fig. 2A, top panel, fractions 9–11), and \( \alpha \)-casein was an equally effective substrate for this kinase (data not shown). PKA and CKII activities were also detectable but at much lower levels and in later fractions. Using an in-gel kinase assay with casein embedded in the gel, a prominent 39-kDa axonemal kinase was detected with peak activity in fractions 9–11 (Fig. 2A, lower panel). This same kinase activity was greatly reduced when myelin basic protein was used as a substrate. The 39-kDa axonemal kinase co-fractionated with CKI activity as an 8 S peak in sucrose gradients (Fig. 2B), and P-11 phosphocellulose chromatography of the pooled sucrose gradient fractions 15–19 also resulted in a peak of CKI activity that precisely co-fractionated with the 39-kDa axonemal kinase (Fig. 2C). In every case, either DRB or CKI-7 inhibited the phosphorylation of CKI-peptide substrate or \( \alpha \)-casein (see below and Refs. 49 and 50). DRB and CKI-7 had no effect on the phosphorylation of PKA peptide substrate; when compared with CKI, CKII activity was significantly lower with experimental variation. As discussed below, the variation in CKII activity was caused by contamination from Chlamydomonas cell bodies.

To further test whether the 39-kDa axonemal kinase is CKI, DEAE fractions were analyzed for protein composition with Western blots and \( \alpha \)-casein-agarose affinity precipitation. Sil-
ver-stained SDS-PAGE gels revealed a prominent 39-kDa protein (Fig. 3A, left arrowhead) that coincided with the CKI activity peak (Fig. 3A, fraction 15) and with the 39-kDa kinase determined by the in-gel kinase assay (Fig. 3A, bottom). The intensity of the protein stain indicates that the 39-kDa protein is a prominent axonemal component. The component was specifically enriched by affinity precipitation of α-casein-agarose (Fig. 3A, right, arrowhead). Moreover, an affinity-purified anti-CKI antibody specifically recognized the 39-kDa protein in Western blots, and immunoreactivity was also enriched by α-casein-agarose affinity precipitation (Fig. 3B). In contrast, Western blots failed to detect the catalytic or regulatory subunit of CKII in flagella or axonemes (see below).

Fraction 13 from the DEAE ion exchange chromatography (Fig. 3A) was used in in vitro phosphorylation experiments to further test for CKI activity (Fig. 3C). Addition of 100 μM \([\gamma^{-32}P]ATP\) to fraction 13 resulted in the apparent autophosphorylation of the 39-kDa protein (Fig. 3C, lane 1, arrowhead), consistent with previous studies that reveal autophosphorylation of CKI (51). The α-casein that was added to the fraction was heavily phosphorylated (Fig. 3C, lane 2), and DRB (25 μM) blocked this phosphorylation (Fig. 3C, lane 3). Addition of cAMP had no further effect on phosphorylation. We conclude that the 39-kDa axonemal kinase is CKI. The conclusion is based on the following: (a) the elution position of the 39-kDa kinase with DEAE chromatography compared with CKI studied in other systems (47), (b) substrate specificity and potency, selective inhibition by DRB and CKI-7, (c) in-gel kinase assay, (d) Western blot using a CKI-specific antibody, and (e) α-casein-agarose affinity precipitation.

**Flagellar CKI Is Exclusively Anchored on the Axoneme—** Because CKI inhibitors rescue dynein activity in isolated axonemes and the 39-kDa CKI is abundant in extracts from purified axonemes, we predict that most or all of the flagellar CKI is anchored on the axonemal microtubules. To test this hypothesis, Western blots were performed on flagella, axonemes, and the Nonidet P-40 soluble membrane/matrix fractions. Each sample was prepared from equal amounts of flagella. Strikingly, nearly all of the flagella CKI co-purified with axonemes, whereas little if any flagella CKI was found in the Nonidet P-40 membrane/matrix fraction (Fig. 4A, top panel). The same distribution was detected using the in-gel kinase assay. For comparison, as much as one-third of the flagellar type 1 protein phosphatase (PP1) was present in the detergent fraction (Fig. 4A, lower panel) (8).

We examined CKII distribution in *Chlamydomonas* using a CKII polyclonal antibody recognizing both catalytic and regulatory subunits of CKII. Although CKII was abundant in the cytoplasmic extract from the cell body, it was excluded from the flagellar fraction (Fig. 4B, right). In contrast, CKI, although enriched in flagella, was not detected in the cell body extract (Fig. 4B, left). Thus, based on subcellular localization, CKII did not directly phosphorylate flagellar dynein nor did it directly contribute to the regulation of flagellar motility.

These results demonstrate that the flagellar CKI is a stable component of the 9 + 2 axonemal structure. To determine the location of axonemal CKI, we used Western blots and the in-gel kinase assay to screen flagella and axonemes isolated from mutant cells that fail to assemble specific structures. This strategy has helped to define the composition of structures such as the dynein arms, radial spokes, and central pair and to determine the location of axonemal phosphatases (8). We postulate that axonemal CKI is anchored to one or more distinct structures. Predictably, failure to assemble the putative anchor structure should result in failure of CKI to co-purify with axonemes. However, using Western blot analysis and in-gel kinase assay, we did not find a mutant strain that lacked CKI (Fig. 4C). In each tested mutant, CKI co-purified with the axoneme. For example, in Fig. 4C an in-gel kinase assay revealed that equal activity of the 39-kDa CKI in axonemes from
An Axonemal CKI Regulates Flagellar Dynein

The 39-kDa axonemal kinase is CKI. A salt extract from isolated axonemes (p128/30) was fractionated by DEAE chromatography, and fractions were prepared for SDS-PAGE and silver staining (A, top). In-gel kinase assay (A, bottom). Western blots (B), α-casein affinity precipitation (A, right and B, far right lane), and in vitro kinase activity (C). The peak CKI activity, fraction 15 (A, arrow), corresponded to a prominent 39-kDa protein revealed by silver staining (A, top, left arrowhead) and the 39-kDa kinase revealed by in-gel kinase activity (A, bottom). The 39-kDa axonemal protein was enriched by α-casein affinity (A, right arrowhead). An anti-CKI antibody specifically bound to the 39-kDa protein, and the 39-kDa immunoreactive protein was enriched by α-casein affinity (B). C, addition of fraction 13 with [γ-32P]ATP followed by SDS-PAGE and PhosphorImager analysis revealed autophosphorylation of the 39-kDa protein (C, left lane, arrowhead). α-Ca-

wild-type or mutant cells failed to assemble the dynein regu-

latory complex (pβ), outer and inner arm dyneins (p128/30),

radial spokes (p14), and central pair apparatus (p18). The

same results were obtained using the anti-CKI antibody in

Western blots comparing proteins from both flagella and axo-

nemes (data not shown). Both assays, although not strictly quan-

titative, were performed within a linear range of detection

using identical protein loads for each sample. Thus, we con-

clude that CKI is not exclusively located in the central pair

apparatus, radial spoke, or dynein regulatory complex. The

simplest interpretation is that CKI is anchored in the outer

doublet microtubules in one or more positions to control
dynein activity. The simplest prediction is that CKI is anchored

in position to directly control dynein phosphorylation. To test this,

we determined whether CKI-7, at the same concentration used
to rescue dynein activity, will also block phosphorylation of
dynein subunits.

CKI-7 Blocks Phosphorylation of IC138—The foundation of

this project was to identify the axonemal kinase that directly

targets phosphorylation of IC138 and inhibits dynein activity.

As described in the Introduction, preliminary studies with in-
hibitors of PKA failed to block phosphorylation of IC138. Based

on the functional and biochemical analysis described above, we

tested whether the axonemal CKI is involved in phosphoryla-
tion of IC138. Axonemes were isolated from wild-type and p14

cells and treated with either PKI or CKI-7 prior to addition of

40 μM [γ-32P]ATP. The ATP concentration selected was based

on kinetic studies of CKI and the evidence that the $K_m$ for ATP

was 5–25 μM (52). However, we found no effect by the concen-

tration of ATP over a range of 0.1–150 μM. A number of axon-

eval proteins became labeled upon exposure to [γ-32P]ATP

(Fig. 5, left lane). Particularly prominent is a phosphoprotein

with a mass of about 138 kDa (Fig. 5, arrowhead). The same

138-kDa phosphoprotein was missing in axonemes from

p128/30, a mutant cell lacking inner arm dynein I1, suggesting

the 138-kDa protein is IC138 of I1 (data not shown) (see below,

Fig. 6). Addition of PKI fails to block phosphorylation of

proteins from either wild-type or p14 axonemes (Fig. 5, lanes 1

and 2). Similarly, selective inhibitors of CKII, such as hyperi-
cin, had no effect on phosphorylation. In contrast, 15–50 μM
mediating and light chains of outer dynein arms, and tubulin did not affect (Fig. 6B, arrows). Phosphorylation of a subset of other axonemal phosphoproteins including a 138-kDa protein, a 138-kDa phosphoprotein in fractions containing the I1 inner arm (Fig. 7A, left panel). IC138 is missing in a mutant (pf28pf30) that fails to assemble inner arm dynein I1 (A, right panel, arrowhead). As described before (7), IC138 is the only phosphoprotein among I1 subunits (B, left arrowhead). CKI-7 blocked phosphorylation of IC138 (B, right arrowhead) under conditions that failed to block phosphorylation of several other axonemal phosphoproteins (B, arrows). As expected, the 138-kDa phosphoprotein is missing in a mutant lacking I1 (B, right panel, arrowhead).

These results are consistent with the hypothesis that CKI plays a central role in phosphorylation of several axonemal proteins including a prominent phosphoprotein of ~138 kDa. IC138 correlates with inhibition of flagellar dynein activity (7). Thus, we postulated that the 138-kDa phosphoprotein is IC138. To test this directly, axonemes from pf14 cells were exposed to 40 μM [γ-32P]ATP in either the presence or absence of 50 μM CKI-7. The dynein complexes were extracted, dialyzed, and fractionated by velocity sedimentation on sucrose gradients. Proteins separated by 10% SDS-PAGE were revealed by Coomassie Blue staining (Fig. 6A), and phosphorylation was evaluated by phosphorimaging (Fig. 6B). As previously described (7, 15), a 138-kDa phosphoprotein in fractions containing the I1 inner arm dynein complex was strongly labeled (Fig. 6, A and B, arrowhead, fraction 3 peak). In contrast, other subunits of I1, intermediate and light chains of outer dynein arms, and tubulin did not become phosphorylated. Addition of 50 μM CKI-7 blocked phosphorylation of several proteins including IC138 (Fig. 6B). Phosphorylation of a subset of other axonemal phosphoproteins was not affected (Fig. 6B, arrows). In mutants lacking I1, IC138 was absent (Fig. 6A, right panel, arrowhead), and no phosphoprotein was present in the 20 S fraction from extracts derived from the I1 mutants (Fig. 6B, right, arrowhead).

To further confirm that the 138-kDa phosphoprotein was IC138, gradient fractions were separated on 5% gels and analyzed by Western blot analysis using an antibody against IC140 (43). As previously described, IC140 and IC138 were resolved and detected as distinctive proteins (Fig. 7A, left panel). IC140 was confirmed by Western blot analysis (Fig. 7A, far right lane), and IC138 migrated slightly faster and coincident with the 32P-labeled band (Fig. 7A, arrowhead). CKI-7 inhibited the phosphorylation of IC138 (Fig. 7B). Based on scintillation counting of the excised IC138 band, as well as quantitation by phosphor imaging, CKI-7 treatment resulted in 80% inhibition of 32P incorporation into IC138.
An Axonemal CKI Regulates Flagellar Dynein

DISCUSSION

In this study, we have addressed the hypothesis that the key kinases responsible for regulation of flagellar dyneins are anchored in the axonemal framework. We have identified an axonemal CKI and demonstrated that CKI controls phosphorylation of IC138 and dynein activity. Specifically we have shown the following. (a) Selective CKI inhibitors restore dynein activity in isolated axonemes lacking radial spokes, (b) inner arm dynein I1 is required for rescue of dynein activity, (c) the 39-kDa axonemal kinase is CKI, (d) CKI is a relatively abundant axonemal protein likely located on outer doublet microtubules, and (e) CKI inhibitors that were shown to rescue dynein activity block phosphorylation of IC138.

The simplest interpretation is that CKI is anchored on outer doublet microtubules near the base of radial spoke 1 and near the base of I1, in position to control phosphorylation of IC138. Genetic and in vitro functional assays have demonstrated that I1, through a network of structures including the radial spokes, operates in part to control flagellar waveform (Refs. 10–15 and 26). Based on results from the current study, we postulate CKI is a key enzyme in this network that is designed to control flagellar waveform.

The 39-kDa Axonemal Kinase Is CKI—The identification of CKI in the axoneme is based on a number of results. Using isolated and purified axonemes as a starting point, in-gel kinase assays revealed a 39-kDa band. In salt extracts from the axonemes, the 39-kDa band exclusively co-purified with CKI activity. This conclusion is based upon fractionation of the extracts by a number of approaches: kinase activity using selective substrates, inhibition of activity with DRB and CKI-7 (49, 50), molecular mass of the catalytic protein, Western blot analysis, and α-casein affinity precipitation (Figs. 2 and 3). Thus, the evidence is compelling that the 39-kDa axonemal kinase is CKI (38, 39). Using the Advanced Blast search algorithm of a Chlamydomonas expressed sequence tag data base, we identified the two Chlamydomonas CKI sequences that are the most homologous to the N terminus and C terminus of mammalian CKI δ (39, 53). The mammalian CKI δ is predominantly expressed in rat testis (53), suggesting that this homologue may be targeted to flagella. Moreover, the putative Chlamydomonas sequence predicts a 38.5-kDa protein, a size that matches the axonemal CKI. The sequence predicts a very basic protein (pI of 9.3), a feature that may play a role in CKI binding to the axoneme (see below) and may explain why axonemal CKI is extractable at a relatively low salt concentration. Peptide sequencing of the axonemal kinase and cloning will further test these ideas.

CKII activity is also present in some axonemal extracts; however, it is variable between axonemal preparations, and the activity is very low in the extracts (Fig. 2). More likely, the CKII activity found in the axonemal extracts results from contamination by cell bodies. Consistent with this conclusion, Western blots revealed that CKII, although present in Chlamydomonas cells, is not found in isolated flagella or axonemes (Fig. 4B). Thus, in Chlamydomonas CKII appears to be excluded from flagella. This is an important observation, indicating that CKII does not directly control flagellar motility in Chlamydomonas, and the cell has a mechanism to control protein entry into the flagellum.

CKI Is Targeted and Anchored to Axonemal Microtubules—Nearly all of the flagellar CKI co-purifies with isolated axonemes and is excluded from the detergent-soluble, membrane/matrix fraction (Fig. 4A). The simplest conclusion is that CKI is targeted and anchored to the axonemal microtubules. Alternatively, CKI may coincidentally co-purify with axonemes. However, this seems most unlikely because we have found no condition that leads to precipitation of CKI, and CKI is active both on the axoneme and in axonemal extracts. Thus, CKI appears to be specifically and exclusively attached to the axoneme. This observation is consistent with models indicating that CKI isoforms are physically positioned in cells to selectively interact with and phosphorylate precisely defined substrates (39). For example, CKI is targeted and anchored in the mitotic spindle, nuclear structures, and membrane domains (54–57). One of the challenges is to define the CKI anchor mechanisms. The Chlamydomonas axoneme offers a new opportunity to define the interacting proteins that anchor and regulate CKI in cells.

Within the axoneme, we postulate CKI is anchored to the outer doublet microtubules, possibly in a position to affect inner arm I1 phosphorylation. This model is founded on the analysis of mutants that indicate that CKI is not associated with the central pair or radial spoke structures. The only structure remaining is the outer doublet microtubules. Use of Chlamydomonas mutants has resulted in successful localization of the dyneins (2, 18), central pair components (3, 58), radial spokes (59), and the phosphatases PP1 and PP2A in the axoneme (8). A prediction of the model is that CKI is a relatively abundant axonemal component. Although we have not yet been able to precisely define the stoichiometry of CKI in the axoneme, based on Coomassie Blue staining of DEAE fractions, the 39-kDa CKI is comparable in amount to axonemal proteins of similar size found in individual inner arm dyneins (data not shown). Thus, the axoneme may contain sufficient CKI so that it is present on each doublet microtubule and anchored in each 96-nm repeat. This is precisely the position required for CKI to directly interact with and phosphorylate IC138.

Phosphorylation of IC138 and Control of Flagellar Motility—The most unique and important contribution of this study is the discovery that CKI controls flagellar dynein activity and that the mechanism for control involves phosphorylation of IC138 of the inner arm dynein I1. These conclusions are founded first on definitive pharmacological analysis of dynein function using direct, videomicroscopic motility assays. These assays have previously revealed that flagellar dynein is controlled by phosphorylation involving axonemal kinases and phosphatases and is controlled by alteration in phosphorylation of IC138 (5–8, 15). As illustrated in Fig. 1, CKI inhibitors potently restore dynein activity, and rescue of dynein activity requires IC138. The simplest model is that CKI operates directly to phosphorylate IC138. Moreover, we postulate that an axonemal PP2A operates to dephosphorylate IC138 (8). Consistent with this model is the localization of both CKI and PP2A on the outer doublet microtubules and the relatively selective inhibition of phosphorylation of IC138 by CKI inhibitors (Figs. 6 and 7). Alternatively, CKI may operate indirectly or synergistically with the axonemal PKA. For example, glyco gen synthase becomes a substrate for CKI following phosphorylation of key residues by PKA (60–62). We have discovered that radial spoke protein 3, located at the base of the spoke, is an A kinase anchor protein, AKAP (63). Thus, PKA may also be anchored in position to directly affect phosphorylation of IC138. Although PKI fails to block phosphorylation of IC138, our methods may not be sensitive enough to detect phosphorylation by PKA. More definitive tests of these models will require an understanding of IC138 structure and interaction among CKI, PKA, and IC138.

Phosphorylation of I1 correlates with inhibition of dynein activity; however, this observation alone does not explain the role of I1 and CKI in flagellar motility. Based on phenotypic analysis of motility in mutants lacking I1, it appears that I1 and phosphorylation of IC138 play a role in control of flagellar waveform (12, 15). Moreover, genetic analysis has revealed a
functional linkage between the central pair/radial spoke structures and control of waveform (14, 21–26). Although we do not yet know how alteration in dynein activity can result in changes in waveform, part of the answer is that the central pair/radial spoke system alters dynein activity via changes in phosphorylation. The network of kinases and/or phosphatases must be regulated by a mechanical interaction between the central pair structure, radial spokes, and outer double microtubules. The challenge is to develop approaches to test the idea that the activity of axonemal enzymes such as CKI or PKA can be controlled by physical strain between axonemal structures.

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