Calcium Regulates ATP-sensitive Microtubule Binding by Chlamydomonas Outer Arm Dynein*

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The Chlamydomonas outer dynein arm contains three distinct heavy chains (α, β, and γ) that exhibit different motor properties. The LC4 protein, which binds 1–2 Ca\textsuperscript{2+} with \(K_{Ca} = 3 \times 10^{-5}\) M, is associated with the γ heavy chain and has been proposed to act as a sensor to regulate dynein motor function in response to alterations in intraflagellar Ca\textsuperscript{2+} levels. Here we genetically dissect the outer arm to yield subparticles containing different motor unit combinations and assess the microtubule-binding properties of these complexes both prior to and following preincubation with tubulin and ATP, which was used to inhibit ATP-insensitive (structural) microtubule binding. We observed that the α heavy chain exhibits a dominant Ca\textsuperscript{2+}-independent ATP-sensitive MT binding activity in vitro that is inhibited by attachment of tubulin to the structural microtubule-binding domain. Furthermore, we show that ATP-sensitive microtubule binding by a dynein subparticle containing only the β and γ heavy chains does not occur at Ca\textsuperscript{2+} concentrations below pCa 6 but is maximally activated above pCa 5. This activity was not observed in mutant dyneins containing small deletions in the microtubule-binding region of the β heavy chain or in dyneins that lack both the α heavy chain and the motor domain of the β heavy chain. These findings strongly suggest that Ca\textsuperscript{2+} binding directly to a component of the dynein complex regulates ATP-sensitive interactions between the β heavy chain and microtubules and lead to a model for how individual motor units are controlled within the outer dynein arm.

Dyneins are microtubule-based motors required for many fundamental cellular processes including vesicular trafficking, mitosis, ciliary/flagellar motility, and embryonic development. In order to generate useful work, dynein motor function must be precisely controlled both in the cytoplasm and the cilium/flagellum. Signaling pathways involving cAMP and/or Ca\textsuperscript{2+} have been shown to modulate the activity of ciliary and flagellar dyneins. Indeed, Ca\textsuperscript{2+} signaling is crucial for waveform regulation of cilia and flagella in many organisms including Paramecium (1), and sea urchin (2) and mammalian (3) sperm. In Chlamydomonas, two behavioral activities (phototaxis and the photophobic or avoidance response) that require alterations in flagellar waveform are mediated by changes in intraflagellar Ca\textsuperscript{2+} (4). Ultimately, both of these responses must be controlled through modulation of inner and/or outer arm dynein function.

The photophobic response occurs when Chlamydomonas are exposed to intense light and comprises two distinct events signaled by a rise in intraflagellar Ca\textsuperscript{2+} levels. During normal forward swimming, the two Chlamydomonas flagella beat with an asymmetric waveform. However, as intraflagellar Ca\textsuperscript{2+} increases from below pCa 6 to pCa 5, \(^1\) cells stop swimming and hold the quiescent flagella in a V-shaped configuration. Upon subsequent increase in Ca\textsuperscript{2+} to pCa 4, the flagella beat with a synchronized symmetric waveform that drives backward swimming (5, 6). Thus, the Chlamydomonas flagellum must contain at least two Ca\textsuperscript{2+} sensors that respond to different metal concentrations and control flagellar waveform conversion through alterations in dynein motor function.

Several Ca\textsuperscript{2+}-binding proteins have been identified within the Chlamydomonas flagellar axoneme. Centrin, which has two high affinity Ca\textsuperscript{2+}-binding sites (\(K_{Ca} = 1.2 \times 10^{-4}\) M) and two sites of lower affinity (\(K_{Ca} = 1.6 \times 10^{-3}\) M), is a component of the inner dynein arms (7), and calmodulin has been located within the radial spoke stalk, where it may act as a signal transducer between the central pair MTs and the inner dynein arm system (8). However, reactivated axonemes lacking the radial spokes still display waveform conversion in response to changes in Ca\textsuperscript{2+} (9). In contrast, Chlamydomonas mutants lacking outer dynein arms exhibit an aberrant or missing photophobic response (10–12). Furthermore, reactivated cell models and axonemes from these mutants show little or no movement at pCa 4. These observations suggest that the outer dynein arm contains a Ca\textsuperscript{2+} sensor that is at least partly responsible for flagellar waveform conversion during the photophobic response.

The current model for arrangement of the various components within the Chlamydomonas outer dynein arm is illustrated in Fig. 1A. This dynein has a mass of ~2 MDa and contains at least 13 different protein components (Fig. 1B). The three heavy chains (\(\alpha, \beta, \) and \(\gamma\) HCs) have different ATPase and motor properties in vitro (13–15). Furthermore, mutants that assembly outer dynein arms lacking either the entire HC (oda11) or the \(\beta\) HC motor domain (oda4-s7) have been identified (16, 17). These strains exhibit different reductions in swimming velocity and flagellar beat frequency when compared with wild type (oda11, 119 \(\mu\) m s\(^{-1}\), 52 Hz; oda4-s7, 65 \(\mu\) m s\(^{-1}\), 30 Hz; wild type, 194 \(\mu\) m s\(^{-1}\), 62 Hz), further indicating that individual outer arm HCs perform distinct functions in vivo.

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\(^{1}\) pCa refers to the negative log of the Ca\textsuperscript{2+} concentration (i.e. pCa 6 = \(10^{-6}\) M Ca\textsuperscript{2+}).

The abbreviations used are: HC, heavy chain; DC, docking complex; IC, intermediate chain; LC, light chain; MT, microtubule; Pipes, 1,4-piperazinediethanesulfonic acid.
Cargo attachment by dynein requires a subcomplex of intermediate and light chain proteins and, in the case of flagellar outer arm dynein, is mediated in part through ATP-insensitive MT binding domains on the IC1 polypeptide (18) and the γ HC (13). The docking complex, consisting of proteins DC1–3, is an additional adaptor required for assembly and positioning of the outer dynein arm within the flagellar axoneme (19). Although interactions between the various components affect the enzymatic properties of the purified dynein particle (15, 20), little is known about the mechanisms of intradynein regulation. Within the outer dynein arm motor unit, each HC is associated with one or more LCs that apparently plays a regulatory role (reviewed in Ref. 21). The LC4 component is an EF-hand protein closely related to calmodulin. In vitro, LC4 binds 1–2 Ca\(^{2+}\) with a \(K_d\) of \(3 \times 10^{-5}\) M (22) and is the only Ca\(^{2+}\)-binding protein directly associated with an outer arm motor unit. LC4 interacts with the N-terminal stem domain of the γ HC possibly through one of the two IQ motifs\(^3\) that are specifically present in this HC.\(^4\) The docking complex protein DC3 also is predicted to have two Ca\(^{2+}\)-binding domains (23). However, DC3 appears to bind both Ca\(^{2+}\) and Mg\(^{2+}\), and a DC3-null strain rescued with a mutant form of the protein incapable of Ca\(^{2+}\) binding showed normal swimming behavior.\(^5\) Thus, LC4 is a strong candidate for an outer arm Ca\(^{2+}\) sensor.

Here we characterize the Ca\(^{2+}\)-dependent MT binding properties of outer arm dynein subparticles containing different HC combinations. We demonstrate that the \(-\beta\gamma\) HC subparticle exhibits a Ca\(^{2+}\)-regulated ATP-sensitive interaction with MTs. Furthermore, we observed that the α HC displays a dominant Ca\(^{2+}\)-independent ATP-sensitive MT binding activity. Together, our data support a model where, during the photophobic response, Ca\(^{2+}\) regulates ATP-sensitive interactions between the outer dynein arm and flagellar doublet MTs via the LC4 protein.

**EXPERIMENTAL PROCEDURES**

**Strains**—Intact outer arm dynein was obtained from *Chlamydomonas reinhardtii* mutant *ida1*, which lacks inner arm I1 (this dynein partly comigrates with the outer arm in sucrose density gradients). Outer arm dyneins containing only α-γ, -βγ, and -γ HC combinations were derived from mutant strains *oda11 ida1*, *oda4-s7*, and *oda4-s7 oda11*, respectively. We also examined dyneins from *sup1-1, sup1-2, oda11 sup1-1*, and *oda11 sup1-2* strains. The *sup1* mutations result in small deletions within the MT-binding stalk of the β HC (sup1-1 and *sup1-2* lack residues 3190–3196 and 3158–3167, respectively) and restore motility to paralyzed strains lacking the radial spokes or central pair MT complex (24). The β HC from *sup1* strains is \(~1\) kDa smaller than the wild type protein. This difference is difficult to detect by gel electrophoresis, so we ensured that the *oda11 sup1* strains were indeed correct by employing the polymerase chain reaction to amplify small regions around the deletion from genomic DNA using *Pfu* DNA polymerase (Stratagene, La Jolla, CA). The forward and reverse primers used to test for the *sup1-1* and *sup1-2* deletions corresponded to nucleotides 14339–14355/14353–14351 and 14002–14018/14122–14138 of the β HC.

\(^3\)The IQ motif has the consensus [(I/V/L)QXX(R/K)XXX(R/K)] and binds calmodulin-like proteins in a Ca\(^{2+}\)-independent manner (41).

\(^4\)M. Sakato and S. M. King, unpublished observations.

\(^5\)D. M. Casey and G. B. Witman, personal communication.
gene (U02963) (25) and generated wild type/mutant proteins of 212/191 and 136/106 kDa, respectively.

**Dynein Purification**—Flagellar axonemes were prepared by standard methods (26). Outer arm dynein was purified as described previously (20) with the following minor modifications. Phenylmethylsulfonyl fluoride (1 mM) was added to all solutions. NaCl (0.6 M) was used in place of KCl to extract dynein from the axoneme, so that the subsequent dialysis step could be eliminated. Sucrose density gradient centrifugation was performed at lower hydrodynamic pressure using an SW55 (Beckman Instruments) rotor for 10 h at 30,000 rpm. Peak sucrose gradient fractions containing outer arm dynein were concentrated in a Centricon 30 ultrafiltration unit (Amicon, Danvers, MA) that had previously been treated with 5% Tween 20 to prevent nonspecific protein binding.

**Microtubules**—Bovine brain tubulin (0.65 mg/ml; Cytokeleton, Inc., Denver, CO) was polymerized in 100 mM Pipes, 2 mM EGTA, 1 mM MgSO4, 1.7 mM dithiothreitol, 1.3 mM GTP, pH 6.9, at 37 °C for 30 min and stabilized by the addition of 10 μM taxol. To further elongate the MTs, they were mixed with additional tubulin to a final concentration of 1 mg/ml, incubated at 37 °C for 45 min, and then kept at room temperature until use. These taxol-stabilized MTs were stable under all Ca2+ regimes used.

**Tubulin Pretreatment of Dynein**—Dynein (0.1 mg/ml) and bovine brain tubulin (0.1 mg/ml) were mixed in HMED (50 mM Pipes, 5 mM MgSO4, 1 mM EGTA, 1 mM dithiothreitol, pH 7.4) (13) containing 1 mM ATP and incubated on ice for 1 h. The sample was concentrated by ultrafiltration using a Microsep 300 (Pall Gelman Laboratory, Ann Arbor, MI), washed three times with HMED containing 1 mM ATP, and finally washed two times in HMED without ATP. All incubation and wash buffers contained 1 mM phenylmethylsulfonyl fluoride. The final retentate was used as tubulin-pretreated dynein.

**Dynein-Microtubule Binding Assay**—The MT-binding assay was performed essentially as described by Moss et al. (27). Prior to use, MTs were washed and any aggregates removed from dynein samples by centrifugation. Protein concentrations were determined using the bicinchoninic acid assay system (Pierce). Dynein (0.05 mg/ml) and MTs (1 mg/ml) were mixed in HMED buffer containing 50 μM taxol (100-μl final volume) and incubated at 20 °C for 30 min. Samples were then centrifuged in an Airfuge at room temperature for 6 min. The supernatant (S1) was carefully removed, and the pellet was resuspended in 100 μl of HMED containing 50 μM taxol and an ATP regeneration system (10 μM creatine phosphate and 0.2 mg/ml creatine phosphokinase). Following the addition of 1 mM ATP, the resuspended pellet was incubated at 20 °C for 5 min and centrifuged a second time in the Airfuge. The second supernatant (S2) was retained, and the pellet (P2) was resuspended in 100 μl of HMED containing 50 μM taxol. To examine the effect of different cations, stock solutions of CaCl2, MgSO4, NiSO4, and LiCl were added to the desired final concentration. Free metal cation concentrations were calculated using an in-house modification of a protein (16) and I1 inner arm), and α-dynein I1 subparticles were pretreated with tubulin and ATP to block ATP-insensitive MT binding sites prior to the assay (Fig. 4A). To achieve this, dynein was incubated with bovine brain tubulin (Fig. 4A, lane T) and ATP. Subsequently, unbound tubulin was separated from dynein-tubulin complexes by ultrafiltration, resulting in a filtrate (F1) containing excess α-γ, -βγ, and --γ HC subparticles were prepared from the mutants oda4-d7 (lacking the entire α HC, the associated LC5 protein (16) and I1 inner arm), and oda4-d7 oda11 (lacking functional α and β HCs and α HC-associated LC5), respectively. Neither oda4-d7 oda11 nor oda4-d7 oda11 ida1 straws were flagellated, so the α-γ and --γ HC samples potentially contained small amounts of inner arm I1. However, analysis of the -βγ HC subparticle from both oda11 and the oda11 ida1 double mutant yielded essentially identical results (data not shown), suggesting that the level of I1 dynein present was very small. Unfortunately, no mutants are currently available that assemble an outer arm lacking only the γ HC. Axonemal dyneins contain both ATP-sensitive (motor) and ATP-insensitive (cargo) MT-binding domains. The MT sedimentation assay utilized here has the advantage of enabling us to test simultaneously for both types of MT binding (the assay scheme is shown in Fig. 2A). Dynein was first mixed with MTs in the absence of ATP and MT-bound dynein recovered following sedimentation (P1); unbound dynein remained in the first supernatant (S1). The P1 MT pellet was subsequently resuspended in the presence of an ATP regeneration system, and the MTs resedimented. This yielded a second supernatant (S2) containing dynein that had originally been bound to MTs through an ATP-sensitive (motor) interaction and consequently was released by treatment with ATP. The second pellet (P2) contained dynein that had bound to MTs through the ATP-insensitive or cargo attachment site. Electrophoretic analysis of these fractions allowed us to determine the percentage of MT-bound dynein and assess whether or not bound enzyme was associated through the motor unit in an ATP-sensitive manner. In the absence of MTs, dynein did not sediment and remained in the S1 supernatant (Fig. 2A).

Under very low Ca2+ conditions, 95% or more of the intact outer arm and α-γ HC subparticles bound MTs; ~70% was released by the addition of 1 mM ATP (Fig. 2B). In contrast, considerable amounts (40–60%) of the -βγ and --γ HC subparticles failed to bind MTs under these conditions and remained in the S1 fraction. Approximately 30–40% of all dynein subtypes exhibited ATP-insensitive MT binding, which reflects attachment via the structural MT binding domain rather than the ATP-sensitive motor site. This indicates that structural MT binding is essentially unaffected by the lack of the α HC or the β HC motor domain. Results similar to those observed following the addition of 1 mM ATP (Fig. 2B) were obtained upon the addition of either 10 or 100 μM ATP (not shown). Overall, these observations suggest that at low Ca2+, the α HC exhibits a dominant ATP-sensitive MT binding activity, whereas the β and γ HCs are mostly inactive or incapable of forming a rigid bond.

Next we tested the effects of increased Ca2+ on the MT binding properties of intact outer arm dynein (Fig. 3). The addition of Ca2+ in the range pCa 2–3 had no obvious effect on either ATP-sensitive or ATP-insensitive MT binding of intact outer arm dynein, with ~70 and ~30% of the protein being found in the S2 and P2 fractions, respectively. Previously, it has been demonstrated that ATP-insensitive MT binding by Chlamydomonas dynein could be blocked by preincubation with tubulin (32). Therefore, to more accurately assess the effect of Ca2+ on ATP-sensitive MT binding, intact dynein or the -βγ and --γ HC subparticles were pretreated with tubulin and ATP to block ATP-insensitive MT binding sites prior to the assay (Fig. 4A). To achieve this, dynein was incubated with bovine brain tubulin (Fig. 4A, lane T) and ATP. Subsequently, unbound tubulin was separated from dynein-tubulin complexes by ultrafiltration, resulting in a filtrate (F1) containing excess
tubulin (Fig. 4A, lane 1) and a retenate (R1) with the dynein-tubulin complex. The dynein-tubulin complex in the first retenate was then washed three times with ATP-containing buffer to further remove any free tubulin (electrophoretic analysis of the filtrates is shown in Fig. 4A, lanes 2–4). Subsequently, ATP was removed from the dynein-tubulin complex by washing with buffer lacking ATP. This yielded P2 filtrates (Fig. 4A, lanes 5–7) and a second retenate (R2) containing the dynein-tubulin complex in an ATP-free buffer. This procedure resulted in the attachment of mammalian brain tubulin to Chlamydomonas dynein (Fig. 4A, lane D-T). The Chlamydomonas flagellar tubulin present in the original sucrose gradient-purified dynein sample (Fig. 4A, lane D) was not dynein-associated, since it sedimented with the same S value even when the dynein particle was partially dissociated and migrated more slowly in the sucrose gradient (not shown).

The MT-binding properties of the dynein-tubulin complexes described above were then assessed (Fig. 4, B–D) using the scheme shown in Fig. 2A. As observed previously by Haimo and Fenton (32), tubulin pretreatment selectively blocked ATP-insensitive MT binding such that almost no dynein (either intact or the βγ or αγ HC subparticles) was found in the P2 fraction. Unexpectedly, the dominant ATP-sensitive interactions mediated by the α HC were also inhibited, and ~90% of intact outer arm dynein did not bind MTs under any Ca^2+ regime (Fig. 4B). This observation confirms that ATP-sensitive MT binding mediated by the α HC is not Ca^2+-sensitive. In contrast, although the pretreated βγ HC subparticle did not bind MTs at low Ca^2+ and remained in the S1 supernatant, increasing Ca^2+ above pCa 6 resulted in >60% of the enzyme attaching to MTs (Fig. 4C). Much of this dynein was released

![Figure 2](image1.png)  
**Figure 2.** Microtubule-binding properties of outer arm dynein. A, scheme for the MT binding assay is illustrated in the left panel. In this assay, dynein and MTs are mixed in the absence of ATP, and the MTs subsequently sedimented in an Airfuge. This resulted in a supernatant (S1) containing unbound dynein and a pellet (P1) containing MT-bound dynein. Subsequently, the MTs in P1 were resuspended in the presence of an ATP regenerating system and then resedimented, yielding a second supernatant (S2) containing ATP-released dynein and a MT pellet (P2) containing dynein bound in an ATP-insensitive manner. To assess the amount of dynein in S1, S2, and P2, samples were electrophoresed in an 8% polyacrylamide gel and stained with Coomassie Blue. The right panel shows a representative gel indicating the location of the HCs, tubulin and creatine kinase (from the regenerating system). In the absence of MTs, all dynein remained in the S1 supernatant (panel at lower right labeled HCs without MTs). B, the MT binding assay was performed at pCa 9 with the outer dynein arm (containing α, β, and γ HCs) and the α-γ, β-γ, and γ-γ HC subparticles derived from mutants odz4-s7, oda11 ida1, and odz4-s7 oda11, respectively. Each panel shows the upper portion of three gel lanes loaded with S1, S2, and P2 samples. Essentially all intact dynein and the α-γ HC subparticle bound MTs mainly in an ATP-sensitive manner. However, in the absence of the γ HC, a considerable amount of dynein failed to bind MTs and remained in the S1 supernatant.
from MTs in an ATP-sensitive manner, indicating that it had bound via the motor domain. Thus, the \( \beta\gamma \) HC subparticle contains ATP-sensitive MT-binding sites that are inactive at low Ca\(^{2+}\) but become activated above pCa 6.

We also observed an increase in ATP-insensitive MT binding by the \( \beta\gamma \) HC subparticle under high Ca\(^{2+}\) conditions (Fig. 4C). This increase was not found with intact dynein (Fig. 4B) and thus does not reflect Ca\(^{2+}\)-dependent ATP-insensitive MT binding. The enhanced amount of the \( \beta\gamma \) HC subparticle present in the P2 fraction above pCa 6 most likely results from dynein originally bound via an ATP-sensitive site that subsequently becomes associated through a structural binding domain (a possible mechanism is detailed under “Discussion”).

To test whether the activation of ATP-sensitive MT binding by the \( \beta\gamma \) HC subparticle was Ca\(^{2+}\)-specific, we examined the effects of adding other divalent and monovalent cations on the MT binding properties of both this subparticle and intact dynein (Fig. 5). For intact outer arm dynein, the addition of Ca\(^{2+}\) or Mg\(^{2+}\) did not affect the amount of protein (\( \leq 65\% \)) that bound MTs in an ATP-sensitive manner or the amount of unbound protein remaining in the S1 supernatant (2–4\%). Approximately 25\% of the \( \beta\gamma \) HC subparticle failed to bind MTs when 1 mM Ca\(^{2+}\) was added. Furthermore, the amount of MT-bound \( \beta\gamma \) HC subparticle released upon the

**Fig. 4. Effect of Ca\(^{2+}\) on microtubule binding by dynein pretreated with tubulin.**

A, diagram illustrating the tubulin pretreatment procedure used to block structural MT binding by dynein (left panel). Dynein was incubated with tubulin in the presence of ATP, and the sample was subjected to ultrafiltration, yielding a filtrate (F1) containing free tubulin and a retentate (R1) containing dynein plus associated tubulin. The R1 retentate was then washed three times with ATP-containing buffer. Subsequently, ATP was removed from the dynein-tubulin complex by washing three times with buffer alone. This yielded a second series of filtrates (F2) and an ATP-free dynein-tubulin complex in the second retentate (R2). The dynein-tubulin complex from the R2 retentate was then used to further assess MT-binding properties. Electrophoretic analysis of the seven filtrates from consecutive (F1 and F2) washes (lanes 1–7), the final retentate (R2) containing the dynein-tubulin complex (D-T), untreated dynein (D), and brain tubulin (T) is shown in the right panel. Note that endogenous Chlamydomonas tubulin in the untreated dynein sample (D) was exchanged for brain tubulin and that free tubulin was not detected in filtrates 3–7. B, C, and D, intact outer arm dynein (B) and the \( \beta\gamma \) (C) and \( \gamma \) (D) HC subparticles were pretreated with tubulin as described for A. The MT-binding properties of tubulin-pretreated dyneins were then assessed in the presence of Ca\(^{2+}\) (from pCa 9 to pCa 2) using the scheme illustrated in Fig. 2A. The percentage of total dynein present in the S1 (filled circles), S2 (filled squares), and P2 (open triangles) samples is shown for each Ca\(^{2+}\) concentration.
addition of ATP increased concomitantly (from \(-30\) to \(-50\%\)) in the presence of Ca\(^{2+}\). Thus, we conclude that MT binding by the \(-\beta\gamma\) HC subparticle is activated by Ca\(^{2+}\) but not by Mg\(^{2+}\). This result is consistent with our previous observation that Ca\(^{2+}\) binding by LC4 is unaffected by millimolar levels of Mg\(^{2+}\) (22).

Ni\(^{2+}\) causes the arrest of ciliary and flagellar motility (3, 33), and the addition of this cation resulted in 100\% of both intact dynein and the \(-\beta\gamma\) HC subparticle becoming bound to MTs in an ATP-insensitive manner (Fig. 5). Li\(^{+}\) is thought to interrupt the Ca\(^{2+}\)-regulatory machinery of sea urchin sperm flagella (34). However, the addition of Li\(^{+}\) did not induce any significant changes in ATP-sensitive MT binding, although it did reduce the amount of protein that bound in an ATP-insensitive manner. These observations suggest that the activation of ATP-sensitive MT binding observed for the \(-\beta\gamma\) HC subparticle is indeed Ca\(^{2+}\)-specific.

Since LC4 copurifies with the \(\gamma\) HC upon subfractionation of the outer arm (14), we also examined whether Ca\(^{2+}\)-dependent MT interactions were mediated directly through the \(\gamma\) HC by analyzing the MT-binding properties of tubulin-pretreated \(--\gamma\) HC subparticles derived from the \(oda4-s7\) \(oda11\) mutant. Because the \(--\gamma\) HC subparticle sample potentially could contain monomeric inner arm species, we used immunoblotting with the \(\gamma\) HC-specific monoclonal antibody 12\(y\)B (30) to test for the presence of this HC in the various fractions (Fig. 4D). Although this complex contains LC4, it did not exhibit Ca\(^{2+}\) dependence, with MT binding being unaffected over the range pCa 9–2. This result suggested that Ca\(^{2+}\)-dependent alteration in MT binding interactions of the \(-\beta\gamma\) HC subparticle might be mediated through the \(\beta\) HC. To further examine this possibility, we employed strains (\(sup1-1\) and \(sup1-2\)) that express a \(\beta\) HC containing small deletions of 7 and 10 residues, respectively, within the stalk emanating from the motor unit that is involved in MT binding (24). Both mutations result in suppression of paralysis caused by radial spoke or central pair defects, and it is presumed that they disrupt some \(\beta\) HC-mediated regulatory activity. Electrophoretic analysis of dyneins from these strains and the \(oda11\) \(sup1\) double mutants is shown in Fig. 6A (upper panels). As the mutant \(\beta\) HC differs from wild type by only \(<1\) kDa, we also used the PCR to amplify the genomic region containing the deletion to ensure that our stains were indeed correct (Fig. 6A, lower panels). In the presence of the \(\alpha\) HC, the \(sup1\) mutant dyneins behaved in the same manner as wild type except at extremely high nonphysiological concentrations of Ca\(^{2+}\) (Fig. 6, B and C). Dyneins from the \(oda11\) \(sup1\) double mutant strains also showed essentially no response to Ca\(^{2+}\) (Fig. 6, D and E). Similar results were obtained when these mutant dyneins were pretreated with tubulin (not shown). This suggests that the specific disruptions of the \(\beta\) HC MT-binding stalk due to the \(sup1\) suppressor mutations reduce or eliminate the Ca\(^{2+}\) response seen in the presence of intact \(\beta\) and \(\gamma\) HCs.

**DISCUSSION**

The activity of *Chlamydomonas* flagella during both phototaxis and the photophobic response is controlled by alterations in intraflagellar Ca\(^{2+}\) levels (5, 6). Ultimately, to affect swimming behavior, these signals must be propagated at the level of the dynein arms to alter beat frequency and, in the case of the photophobic response, result in waveform conversion. Previously, we identified the LC4 protein as the only Ca\(^{2+}\)-binding component that is directly associated with an outer arm dynein HC. Since the outer arm is required for waveform conversion from an asymmetric to a symmetric beat during the photophobic response, we postulated that LC4 acts as a sensor to mediate Ca\(^{2+}\)-dependent regulation of outer arm dynein motor function (22). Here we have investigated the role of Ca\(^{2+}\) in regulating ATP-sensitive interactions between outer arm dynein and MTs. The results presented support a role for outer arm dynein in Ca\(^{2+}\)-mediated control of flagellar motility and suggest a model for how individual motor units within the outer dynein arm are regulated.

Previous studies have suggested that the ATPase activity of individual HCs within the outer arm is regulated through intradynein interactions (15, 20, 35). For example, reassociation of the isolated \(\alpha\) and \(\beta\) HC subparticles resulted in a down-regulation of ATPase activity (15). We observed that *in vitro*, the \(\alpha\) HC exerted a dominant ATP-sensitive MT binding activity that was unaffected by alterations in Ca\(^{2+}\) concentration. However, when the \(\alpha\) HC was removed (using the \(oda11\) mutation), considerable amounts of the \(-\beta\gamma\) HC subparticle failed to bind MTs unless activated by increasing Ca\(^{2+}\) levels. Within the flagellum, the dominant MT-binding properties of the \(\alpha\) HC must be tightly controlled in order to allow for the generation and propagation of a flagellar wave. Indeed, we observed that incubation of intact dynein with tubulin and ATP
FIG. 6. Microtubule binding properties of sup1-1 and sup1-2 dynein. A, outer arm dynein was purified from strains sup1-1 and sup1-2, which contain small deletions within the MT-binding stalk region of the β HC and from double mutants with oda11. In the diagrams at the top, β" and β** indicate the mutant β HCs containing the sup1-1 and sup1-2 deletions, respectively. The upper panels show silver-stained acrylamide gels of the dynein samples. Since the sup1 forms of the β HC migrate very similarly to wild type, the identity of the oda11 sup1 double mutants was confirmed by PCR using primers to amplify small regions around the sections deleted in the sup1 strains. These products were separated in 2.5 and 3.0% agarose gels, respectively, and stained with ethidium bromide (left and right lower panels). B–E, Ca²⁺-dependent MT-binding properties of dyneins containing the sup1-1 (B and D) and sup1-2 (C and E) mutant forms of the β HC were assessed in the presence (B and C) and absence (D and E) of the α HC using the standard MT sedimentation assay. The amount of each dynein (expressed as a percentage of the total added) in S1 (filled circles), S2 (filled squares), and P2 (open triangles) fractions is shown.
to block the structural or ATP-insensitive MT binding activity also inhibited the dominant Ca\(^{2+}\)-independent ATP-sensitive MT binding exhibited by the α HC. In contrast, when the βγ HC subparticle was treated similarly, ATP-sensitive MT binding was found at Ca\(^{2+}\) levels above pCa 6. These results indicate that the βγ HC subparticle contains an ATP-sensitive MT binding activity that is Ca\(^{2+}\)-dependent; the Ca\(^{2+}\) concentration at which activation of MT binding occurs is consistent with the Ca\(^{2+}\)-binding properties of LC4 (22). Furthermore, these data suggest that the outer arm contains a regulatory system mediated through the α HC that impinges on the Ca\(^{2+}\)-dependent control of the β and/or γ HCs.

Based on the MT binding activities of dyneins containing different HC combinations and on the effects of tubulin/ATP pretreatment, we propose a model (depicted in Fig. 7) for how individual motor units within the outer dynein arm are regulated through intradynein interactions. In the flagellum, a negative signal is suggested to inhibit the α HC and be transmitted through that protein to impinge also on the β and γ HCs (Fig. 7A). Outer arm motor function would then be activated by a second signal that antagonizes this negative control (e.g. the β and/or γ HCs would acquire the capacity to act as motors in response to a Ca\(^{2+}\) signal). This regulatory system is disrupted when the outer arm is removed from the flagellar axoneme during purification, since under these conditions, the α HC exhibits ATP-sensitive MT-binding activity (Fig. 7B). Importantly, this control system is apparently re instituted when the structural MT binding site on the outer dynein arm is occupied by tubulin, since we observed that intact dynein pretreated with tubulin and ATP did not exhibit MT binding activity (Fig. 7C). Although the manner in which the α HC is controlled by this flagellar regulatory system remains unclear at present, it is interesting to note that this HC is the only Chlamydomonas outer arm polypeptide that is phosphorylated in vivo (37, 38). Furthermore, modification of the α HC occurs at multiple sites and is rapidly turned over both in vivo and in isolated axonemes (37).

The results presented here and elsewhere (35) together support the hypothesis that distinct regulatory inputs impinge on individual HCs within the Chlamydomonas outer dynein arm. Consequently, we propose that different motor outputs required for various flagellar beat activities are modulated by the differential activation of individual HC motors through distinct signaling inputs such as Ca\(^{2+}\), phosphorylation (37), redox poise (35), and the γ HC motor domain-associated LC1 that interacts with axonemal p45 (39).

Above pCa 6, there was a significant increase in the amount of tubulin-pretreated βγ HC subparticle that bound MTs in both an ATP-sensitive and an ATP-insensitive manner. We did not observe this increase in ATP-insensitive MT binding with wild type dynein, suggesting that it does not result from a Ca\(^{2+}\)-dependent ATP-insensitive association. This increase in ATP-insensitive MT binding probably derived from dynein that originally bound via the ATP-sensitive motor domain. Following sedimentation, the ATP-insensitive site containing prebound tubulin would experience a very high local MT concentration (possibly in excess of millimolar levels) within the MT pellet. Consequently competition for binding at this site would lead to release of the prebound tubulin and allow for MT

**Fig. 7. Model for motor unit regulation within the outer dynein arm.** In this model, the α, β, and γ HC motor units are shown in yellow, cyan, and pink, respectively. Inactive motor units (i.e. HCs that cannot act as a motor under a particular set of conditions due to an inhibitory signal) are indicated by a red cross; motor units capable of undergoing ATP-sensitive MT binding are shown by a blue tick, and those whose status is uncertain are shown by a question mark. A, intradynein regulation within the axoneme. In order to generate and propagate a flagellar bend, the motor activity of individual dyneins must be transiently activated. Furthermore, in order to obtain alterations in flagellar waveform, it is likely that the various HCs within an individual outer arm (which are known to exhibit distinct motor properties) also are differentially activated. Thus, the default situation in the axoneme must be for these motors to be inactive until modified by a specific signal. Based on analysis of the MT-binding properties of dyneins lacking various HC combinations and the effects of tubulin attachment to the structural (ATP-insensitive) MT binding site, we propose that this inhibition of motor activity is mediated through the α HC and subsequently affects the β and γ HCs. Activation of motor function would require a positive signal acting on the α HC to relieve this inhibition and lead to the dominant ATP-sensitive MT interactions mediated by this HC. This activating signal is also proposed to alter the β and γ HCs such that their motor activity may now be activated in response to another appropriate signal (such as an increase in Ca\(^{2+}\)). B, motor activity of purified dynein. Following removal of the outer dynein arm from the flagellar axoneme, the proposed negative control on MT-HC interactions is missing, and thus the α HC is activated and exhibits dominant ATP-sensitive MT binding activity. Because this MT binding activity of the α HC is Ca\(^{2+}\)-independent, the assay cannot determine whether the motor activity of the β and/or γ HCs in intact dynein is enhanced in the presence of high Ca\(^{2+}\). C, motor activity of tubulin-pretreated dynein. ATP-sensitive MT binding by intact dynein was inhibited by attachment of tubulin (green circles) to the structural MT binding domain; i.e. this treatment apparently reimposed the inhibitory signal mediated through the α HC that we propose occurs within the axoneme. In the βγ HC subparticle, however, the α HC is missing, and therefore so is the inhibitory signal mediated through this protein. Consequently, MT binding activity by at least one of the HCs within this particle could be activated following the addition of Ca\(^{2+}\).
binding such that the dynein particle became associated with two different MTs. The addition of ATP would then release the motor unit from one MT but would not affect association with a second MT via the structural MT binding domain. A similar MT bundling activity was reported previously for the α HC of sea urchin sperm flagella dynein (36) (this HC is the apparent ortholog of the Chlamydomonas γ HC).

Although LC4 copurifies with the γ HC (14), we did not observe Ca\(^{2+}\) control of MT binding by the βγ HC subparticle, suggesting that the ligand-induced interaction of the βγ HC subparticle might be mediated through the β HC. To further test this hypothesis, we isolated outer arm dyneins lacking the α HC from the \textit{oda11 sup1-1} and \textit{oda11 sup1-2} mutants. Both \textit{sup1} mutants express outer arm β HC that contain small deletions within the MT-binding stalk domain and were originally isolated because these defects suppress paralysis of radial spoke/central pair-deficient mutants (24, 40). Unlike the wild type βγ HC subparticle, both mutant dyneins failed to exhibit Ca\(^{2+}\)-sensitive interactions with MTs even following pretreatment with tubulin and ATP. This suggests that the interaction observed for the wild type βγ HC subparticle is mediated by the β HC and raises the question of how Ca\(^{2+}\) binding to the γ HC-associated LC4 protein might affect MT binding by the β HC. Since the various components of the 2-MDa outer arm dynein complex must be tightly juxtaposed so that the native complex can fit within the confines of the flagellar axoneme, one possibility is that in situ this LC actually interacts with both HCs. Indeed, we recently found that the LC3 protein, which was previously thought to be exclusively associated with the β HC (15), also interacts with the γ HC (35).

Using chromatography and electron microscopy, Haimo and Fenton (32) showed that Chlamydomonas axonemal dyneins bind to brain tubulin through both ATP-sensitive and ATP-insensitive sites and that tubulin bound to the ATP-insensitive site of dynein prevented that domain from binding to MTs. In the absence of Ca\(^{2+}\), this tubulin-treated dynein could still bind MTs in an ATP-sensitive manner, which is clearly distinct from the results reported here. The differences in MT-binding properties of outer arm dynein found in this study and those reported by Haimo and Fenton probably reflect the distinct methods used to prepare the enzyme. The dynein used here consisted of sucrose gradient-purified intact outer arm dynein with the associated docking complex. In contrast, the axonemal salt extracts utilized in the earlier work probably contained both outer and inner arm dyneins. Furthermore, these samples were desalted in a low Mg\(^{2+}\) buffer. The absence of Mg\(^{2+}\) and high hydrostatic pressure (and possibly other treatments) is now known to cause the outer arm to dissociate into three subparticles (the αβ HC dimer, the γ HC subunit, and the trimeric docking complex) (19, 20) and, consequently, could disrupt intradynem regulatory pathways.

Here we have shown that Ca\(^{2+}\) directly regulates ATP-sensitive MT binding by the βγ HC subparticle from the Chlamydomonas outer dynein arm. Furthermore, the Ca\(^{2+}\) concentration at which switching occurred corresponds to the levels needed for waveform conversion in reactivated cell models (5, 6) and to the Ca\(^{2+}\)-binding affinity of the γ HC-associated LC4 protein (22). These observations suggest that the β and/or γ HCs play a pivotal role in Ca\(^{2+}\) regulation of flagellar motility.

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**REFERENCES**

1. Naitoh, K., and Kaneko, H. (1973) Science 176, 523–524
2. Brokaw, C. J., Jesslin, R., and Bobrow, L. (1974) Biochim. Biophys. Res. Commun. 58, 795–800
3. Lindemann, C. B., and Goltz, J. S. (1988) Cell Motil. Cytoskeleton 10, 420–431
4. Witman, G. B. (1992) Trends Cell Biol. 3, 403–408
5. Bessen, M., Fay, R. B., and Witman, G. B. (1986) J. Cell Biol. 98, 446–455
6. Kamiya, R., and Witman, G. B. (1984) J. Cell Biol. 98, 97–107
7. Pipperno, G., Mead, K., and Shestak, W. (1992) J. Cell Biol. 118, 1455–1463
8. Yang, P., Diener, D. R., Rosenbaum, J. L., and Sale, W. S. (2001) J. Cell Biol. 153, 1315–1326
9. Wakabayashi, K., Yagi, T., and Kamiya, R. (1997) Cell Motil. Cytoskeleton 38, 22–28
10. Kamiya, R., and Okamoto, M. (1985) J. Cell Sci. 74, 181–191
11. Mitchell, D. R., and Rosenbaum, J. L. (1985) J. Cell Biol. 100, 1228–1234
12. Moss, A. G., and Morgan, D. B. (1999) Microsc. Anal. 34, 7–9
13. Sakakihara, H., and Nakayama, H. (1998) J. Cell Sci. 111, 1155–1164
14. Pfister, K. K., Fay, R. B., and Witman, G. B. (1982) Cell Motil. 2, 525–547
15. Pfister, K. K., and Witman, G. B. (1984) J. Biol. Chem. 259, 12072–12080
16. Sakakihara, H., Mitchell, D. R., and Kamiya, R. (1991) J. Cell Biol. 113, 615–622
17. Sakakihara, H., Takada, S., King, S. M., Witman, G. B., and Kamiya, R. (1993) J. Cell Biol. 122, 653–661
18. King, S. M., Wilkerson, C. G., and Witman, G. B. (1991) J. Biol. Chem. 266, 8401–8407
19. Takada, S., and Kamiya, R. (1994) J. Cell Biol. 126, 737–745
20. Nakamura, K., Wilkerson, C. G., and Witman, G. B. (1997) Cell Motil. Cytoskeleton 37, 338–345
21. DiBella, L. M., and King, S. M. (2001) Int. Rev. Cytol. 210, 227–268
22. King, S. M., and Patel-King, R. S. (1995) J. Cell Sci. 108, 3757–3764
23. Casey, D., Inaba, K., Pazour, G., Takada, S., Wakabayashi, K., Wilkerson, C., Kamiya, R., and Witman, G. (2003) Mol. Biol. Cell 14, 3650–3663
24. Porter, M. E., Knott, J. A., Gardner, L. C., Mitchell, D. R., and Dutcher, S. K. (1994) J. Cell Biol. 126, 1495–1507
25. Mitchell, D. R., and Brown, K. S. (1994) J. Cell Sci. 107, 635–644
26. Witman, G. B. (1986) Methods Enzymol. 134, 280–290
27. Moss, A. G., Gatti, J. L., and Witman, G. B. (1992) J. Cell Biol. 118, 1177–1188
28. Goldstein, D. A. (1979) Biophys. J. 26, 205–242
29. Sillen, I.-G., Martell, A. E., and Bjerrum, J. (1971) Stability Constants of Metal-Ion Complexes, The Chemical Society, London
30. King, S. M., Otter, T., and Witman, G. B. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 4717–4721
31. Harrison, A., Olds-Clarke, P., and King, S. M. (1998) J. Cell Biol. 140, 1137–1147
32. Haimo, L. T., and Fenton, R. D. (1988) Cell Motil. Cytoskeleton 9, 129–139
33. Naitoh, Y., and Kaneko, H. (1973) J. Exp. Biol. 58, 657–676
34. Gibbons, B. H., and Gibbons, I. R. (1984) J. Nature 309, 560–562
35. Harrison, A., Sakato, M., Tedford, H. W., Benashski, S. E., Patel-King, R. S., and King, S. M. (2002) Cell Motil. Cytoskeleton 52, 151–143
36. Moss, A. G., Sale, W. S., Fox, L. A., and Witman, G. B. (1992) J. Cell Biol. 118, 1189–1200
37. King, S. M., and Witman, G. B. (1994) J. Biol. Chem. 269, 5452–5457
38. Pipperno, G., and Luck, D. J. (1981) Cell 27 331–340
39. Benashski, S. E., Patel-King, R. S., and King, S. M. (1999) Biochemistry 38, 7253–7264
40. Huang, B., Ramanis, Z., and Luck, D. J. (1982) Cell 28, 115–124
41. Rhoads, A., and Friedberg, F. (1997) PASEJ 11, 331–340
42. Wu, H., Maciejewski, M. W., Marintchev, A., Benashski, S. E., Mullen, G. P., and King, S. M. (2000) Nature Struct. Biol. 7, 575–579