Distinct roles of 1α and 1β heavy chains of the inner arm dynein I1 of Chlamydomonas flagella

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ABSTRACT The Chlamydomonas I1 dynein is a two-headed inner dynein arm important for the regulation of flagellar bending. Here we took advantage of mutant strains lacking either the 1α or 1β motor domain to distinguish the functional role of each motor domain. Single-particle electron microscopic analysis confirmed that both the I1α and I1β complexes are single headed with similar ringlike, motor domain structures. Despite similarity in structure, however, the I1β complex has severalfold higher ATPase activity and microtubule gliding motility compared to the I1α complex. Moreover, in vivo measurement of microtubule sliding in axonemes revealed that the loss of the 1β motor results in a more severe impairment in motility and failure in regulation of microtubule sliding by the I1 dynein phosphoregulatory mechanism. The data indicate that each I1 motor domain is distinct in function: The I1β motor domain is an effective motor required for wild-type microtubule sliding, whereas the I1α motor domain may be responsible for local restraint of microtubule sliding.

INTRODUCTION Eukaryotic cilia and flagella are conserved organelles required for motile and sensory functions vital for development and the function of most organs (Satir and Christensen, 2007). Failure in assembly or regulation of cilia results in a wide range of human diseases called “ciliopathies” (Badano et al., 2007; Fliegauf et al., 2007; Marshall, 2008; Pazour and Witman, 2008; Gersdes et al., 2009; Leigh et al., 2009; Nigg and Raff, 2009), yet our understanding of the assembly and mechanism of cilia is incomplete. Here we focus on the motile ciliary/flagellar axoneme and the mechanism and functional interactions of the dynein motors that power motility (Kamiya, 2002; Oiwa and Sakakibara, 2005; King and Kamiya, 2008).

Motile cilia and flagella share a common “9 + 2” structure, in which nine peripheral doublet microtubules surround two central singlet microtubules. Outer and inner dynein arms projecting from each peripheral doublet microtubule are capable of extending to the neighboring doublet microtubule and, coupled with ATP hydrolysis, induce microtubule sliding (Oiwa and Sakakibara, 2005; King and Kamiya, 2008). Based on analysis of mutant phenotypes, the outer dynein arms regulate beat frequency and power motility, whereas the inner dynein arms regulate the size and shape of the bend (Brokaw and Kamiya, 1987; King and Kamiya, 2008). This view of independent functions for different axonemal dyneins may be an oversimplification, however. We are just beginning to understand the functional interactions among the different dyneins (Kamiya, 2002; Brokaw, 2008; Kikushima, 2009) and between each dynein heavy chain (HC) motor (e.g., Furuta et al., 2009).

The present study focuses on the inner arm dynein I1, also called dynein-f (Goodenough et al., 1987; Piperno et al., 1990; Kamiya et al., 1991; Kagami and Kamiya, 1992; Porter et al., 1992). I1 dynein is an exceptionally interesting dynein: It is required for normal regulation of axonemal bending, and, unlike the other inner dynein arms, is composed of two distinct motor domains (Porter and Sale, 2000; Wirschell et al., 2007). Studies of flagellar...
mutants from *Chlamydomonas* have demonstrated that cells either lacking I1 dynein or exhibiting altered I1 dynein intermediate chain (IC) phosphorylation have defects in flagellar waveform (Brokaw and Kamiya, 1987) and phototaxis (King and Dutcher, 1997; Okita et al., 2005). Thus I1 dynein plays important roles in the regulation of motility. The isolated I1 complex does not efficiently translocate microtubules in vitro motility assays (Smith and Sale, 1991; Kagami and Kamiya, 1992; Smith and Sale, 1992b; Kotani et al., 2007). Moreover, in vitro evidence indicates that I1 dynein can function to inhibit microtubule translocation, possibly indicating a novel role for I1 dynein in the local control of microtubule sliding and regulation of axonemal bending (Kotani et al., 2007). Additional tests of this idea, however, require a detailed understanding of the molecular structure and functional capability of each motor domain.

I1 dynein is located near the base of the S1 radial spoke, at the proximal end of the axonemal 96-nm repeat (Goodenough and Heuser, 1985; Piperno et al., 1990; Mastronarde et al., 1992; Porter et al., 1992; Smith and Sale, 1992a; Nicastro et al., 2006; Bui et al., 2008, 2009; Heuser et al., 2009), and is composed of two HCs (1α-HC and 1β-HC), three ICs (IC140, IC138, and IC97), FAP120, and several light chains (LCs) (Figure 1 and reviewed in Wirschell et al., 2007; King and Kamiya, 2008). Relative to HCs of other dyneins, the sequences of the 1α- and 1β-HCs of I1 dynein are highly conserved (Morris et al., 2006; Wilkes et al., 2008; Yagi, 2009). In *Chlamydomonas*, four independent loci, when defective, result in a failure to assemble the I1 dynein complex in the axoneme (reviewed in Myster et al., 1997; Perrone et al., 1998; Perrone et al., 2000; Wirschell et al., 2007; King and Kamiya, 2008). Two of these loci encode the I1 dynein HC subunits; I1DA1(PF9) encodes the 1α-HC, and I1DA2 encodes the 1β-HC (Table 1; Myster et al., 1997; Perrone et al., 2000). Importantly, mutant strains containing genes that express truncated 1α-HC or 1β-HC HCs lacking the motor domains but retaining the tail domains still assemble the remaining I1 dynein subunits (Figure 1 and Myster et al., 1999; Perrone et al., 2000). Thus these I1 dynein motor domain mutants offer an opportunity to examine the role of each motor domain in the regulation of axonemal bending.

Here we take advantage of these *Chlamydomonas* mutant strains that assemble I1 dynein lacking either one or the other HC motor domain (see Table 1 and Figure 1). We refer to each mutant strain based on the full-length dynein HC remaining in the I1 complex (i.e., the protein complex and the mutant strain that contains the intact HC, the truncated HC, or both). The mutants are illustrated in Figure 1. As described earlier in text, we refer to each mutant strain based on the intact HC remaining in the I1 complex (i.e., the protein complex and the mutant strain that contains the intact 1α-HC or the intact 1β-HC). In assessing the functional capability of each motor domain, it was important to determine whether the motor domain mutations were also accompanied by defects in the assembly of other subunits in I1 dynein. Assembly of the I1 dynein and truncated HCs in the axoneme was confirmed as shown previously for I1α (Myster et al., 1999) and I1β (Perrone et al., 2000). Additionally, Western blot analyses confirmed that the
**Table 1:** Strains used in this study.

| Strain name/genotype | Molecular phenotype | Motility | References |
|----------------------|---------------------|----------|------------|
| WT (137c)            | –                   | WT       | Harris, 1989 |
| oda2-6 pf28 (CC-1877)| Lacks outer dynein arm | Slow, jerky | Kamiya, 1988; Mitchell and Rosenbaum, 1985 |
| pf9-2 (CC-3899)      | Lacks I1 dynein, mutation in 1α–HC gene (Dhc1) | Slow, smooth | Porter et al., 1992 |
| pf17 (CC-1035)       | Lacks radial spoke heads | Paralyzed | Lewin, 1954 |
| ida2–6 (27B3; CC-3922)| Lacks I1 dynein, mutation in 1β–HC gene (Dhc10) | Slow, smooth | Perrone et al., 2000 |
| ida2–7 (J6H9; CC-3923)| Lacks I1 dynein, mutation in 1β–HC gene (Dhc10) | Slow, smooth | Perrone et al., 2000 |
| ida4 (CC-2670)       | Lacks inner arm dyneins a, c, d—mutation in the inner arm p28 LC | Medium, smooth swimming | Kamiya et al., 1991; LeDizet and Piperno, 1995 |
| ida2–6::IDA2ΔN       | Truncated I1β–HC; restores I1α–HC | Faster than ida2–6 | Perrone et al., 2000 |
| ida2–7::IDA2ΔN       | Truncated I1β–HC, restores I1α–HC, lacks the outer dynein arm | Slow, jerky | Perrone et al., 2000 |
| ida7–1 (5b10; CC-3921)| Lacks I1 dynein, mutation in the IC140 gene | Slow, smooth | Perrone et al., 1998 |
| bop5–1 (CC-4080)     | Truncated IC138 | Medium, smooth | Hendrickson et al., 2004; Dutcher et al., 1988 |

I1 dynein ICs and LCs are fully assembled in axonemes from I1α, I1β, and the double mutants I1α x oda and I1β x oda (Figure 2A). Thus the only known difference between WT and the I1α and I1β mutants is the absence of either the 1β- or 1α- HC motor domain. These results indicate that ICs and LCs in I1 dynein are not directly associated with the motor domains (see also Myster et al., 1999; Perrone et al., 2000). This organization is in contrast to the LC1 subunit of the Chlamydomonas outer dynein arm that interacts with the γ HC motor domain (Patel-King and King, 2009).

I1 dynein and the truncated motor complexes were isolated from oda mutant strains (lacking the outer dynein arms) using the ion exchange procedure described previously (Kotani et al., 2007). The I1 dynein complex (dynein-f) eluted at approximately 325 mM KCl, as described before (Sakakibara et al., 1999; Kotani et al., 2007), and contains the two distinct 1α- and 1β-HCs (Figure 2B, top panel, “purified I1”). The truncated HC complexes also eluted at approximately 325 mM KCl (the dynein-f peak). SDS–PAGE confirmed that the 1α complex contains the full-length 1α-HC (Figure 2B, top panel, “purified I1α”) and that 1β contains the full-length 1β-HC (Figure 2B, top panel, “purified I1β”). The N-terminal fragment of the truncated 1α-HC was also observed in the I1β fraction (Figure 2B, arrowheads) and the N-terminal fragment of the 1β-HC was also observed in the I1α fraction (Figure 2B, bottom panel, arrowhead). The I1, I1α, and I1β dynein complexes each contain IC140, IC138, and IC97 (Figure 2B, bottom panel). Dynein-c and dynein-g fractions are included as controls (Figure 2B), and, judging from these observations, we conclude that there was no significant contamination of the I1 dyneins with these other dynein subspecies.

**Structural analysis of the I1α and I1β head domains**

Negative stain electron microscopy was used to assess the structure of the isolated I1 dynein and truncated motor domain complexes. As described previously, electron microscopic analysis revealed that I1 dynein is a two-headed structure with a prominent tail domain (Figure 3A, left panels, and Goodenough et al., 1987; Smith and Sale, 1991; Kotani et al., 2007). Electron microscopy of the purified mutant I1 complexes revealed that they are single-headed dynein structures with a tail domain that is morphologically similar to intact I1 dynein (Figure 3B, middle and right panels). These observations are consistent with the structures predicted from HC sequence analysis and seen previously by transmission electron microscopy and image analysis of I1 mutant axonemes (Figure 1) (Myster et al., 1999; Perrone et al., 2000). The results are also consistent with the model...
in which the N-terminal domains of both HCs are necessary and sufficient for assembly and docking of 11 dynein in the axoneme. To examine the conformations of the two heads, single-particle image analysis was performed using electron micrographs of negatively stained 11α and 11β head domains (Figure 3B). Class averages of the “right-view” images were used for comparison of the two heads because the right-view images of other dynein head domains are well characterized and reveal conserved, structural landmarks (Burgess et al., 2003, 2004; Roberts et al., 2009). Figure 3B shows class averages of the right view of the 11α head domains (Figure 3B, b–e) and 11β head domains (Figure 3B, g–j) and shows global averages of the right view of the 11α head domain (Figure 3B, a) and 11β head domain (Figure 3B, f). Alignment of the head domains of negatively stained molecules clearly shows that they display an asymmetric ringlike morphology similar to that of Chlamydomonas flagellar inner arm subspecies dynein c, cytoplasmic dyneins, and the head domains of intact 11 dynein (Figure 3B) (Burgess et al., 2003; Kotani et al., 2007; Roberts et al., 2009). The diameter of the globular heads was approximately 15 nm, and the deposit of stain in the center of the head domain is clearly observed as reported in dynein-c (compare Figure 3B, right panel, and Burgess et al., 2003). The groove on the left side, observed on cytoplasmic dynein (Roberts et al., 2009), is also observed in 11α and 11β head domains (Figure 3B, red bars). Similar to cytoplasmic dynein and dynein-c, the three globular domains are observed on the right side (Figure 3B, yellow bars; compare to Burgess et al., 2003, 2004; Roberts et al., 2009). Thus the purified, truncated 11 dyneins have retained their molecular configuration, and the globular motor domains of each HC are similar to each other and to other dyneins (see Discussion and Burgess et al., 2003, 2004; Mizuno et al., 2004; Samso and Koonce, 2004; Roberts et al., 2009). Although the right view of the 11β head has features similar to other dyneins, in several of particle class images examined (Figure 3B, f, h, i, and j), the pattern of stain density surrounding the dynein head is different from that of other dyneins: Stain density at the bottom of the 11β head is heavier than that at the top. This observation may reveal subtle differences in the structure of the 11β motor-head domain compared to the motor domain in other dyneins.

**Isolated 11 dynein can induce the formation of microtubule bundles**

A microtubule bundling assay was used to assess the ability of the 11 dynein, 11α, and 11β complexes to interact with microtubules in an ATP-sensitive manner (Haimo et al., 1979; Smith and Sale, 1991; Moss et al., 1992b; Sakakibara and Nakayama, 1998; Toba and Toyoshima, 2004). As described previously, dark field light microscopy can be used to resolve individual microtubules in the absence of added dyneins (Figure 4, top left panel). Addition of the purified, two-headed WT 11 dynein resulted in the rapid cross-linking of microtubules into large bundles, but these bundles were dispersed into single microtubules following the addition of ATP (Figure 4, I1 dynein). These observations are consistent with previous analysis of I1 dynein-microtubule bundling by electron microscopy (Smith and Sale, 1991) and indicated that the two-headed dynein cross-links microtubules through the ATP-sensitive microtubule binding site in each HC. When microtubules were mixed with the single-headed...
I1 dynein complexes, however, microtubule bundles were never formed, irrespective of the presence or absence of ATP.

The simplest interpretation of these data is that the two-headed I1 dynein is capable of cross-bridging microtubules through the microtubule-binding domains present in both the I1α and I1β dynein HC s. Presumably, the I1α and I1β single-headed dyneins fail to cross-link microtubules because each single-headed complex has only one microtubule-binding site. These observations also suggest that the ATP-insensitive microtubule-binding site observed in the axoneme requires a docking protein or complex specialized for binding the base of the I1 dynein. This docking complex is apparently not present in microtubules assembled from purified tubulin (Smith and Sale, 1991).

**Distinct MgATPase activities of the I1α and I1β complexes**

The basal ATPase activities of the isolated I1 dynein, I1α, and I1β complexes were measured at various ATP concentrations in the absence of microtubules (Figure 5A). The steady-state ATPase rates were fitted with Michaelis–Menten-type kinetics. I1β has a higher maximal velocity (k_{cat}) compared to the two-headed, WT I1 dynein, whereas the maximal velocity of I1α is approximately one eighth the maximal velocity of I1β. The I1α complex also has a higher K_m, value than either the I1 or I1β dynein complex, indicating a lower affinity for ATP. Thus I1β has a much higher basal ATPase activity than I1α. Importantly, the ATPase activity of the two-headed I1 dynein is lower than the combined value of its I1α and I1β motor domains. These observations suggest that the I1α motor domain may exert an inhibitory effect on the ATPase activity of the I1β complex motor domain in the intact I1 dynein complex.

The ATPase activities of the two-headed I1 dynein and the single-headed I1β dynein were activated by microtubules (Figure 5B). Again, the k_{cat} of I1β is higher than that of the intact I1 dynein. This difference is due primarily to its higher basal ATPase activity, as in both cases the addition of microtubules stimulated ATPase activity only 1.5-fold (in I1β) to 1.6-fold (in I1 dynein). K_m, MT is the microtubule concentration at the half saturation of microtubule-activated ATPase activity. I1β has a lower K_m, MT value than I1α, indicating that I1β alone has a higher affinity for microtubules than does the two-headed I1 dynein. These results suggest that the I1α motor domain modulates the ATPase and microtubule affinity of the I1β motor domain in the WT I1 dynein complex. Surprisingly, I1α did not show any activation of ATPase activity by the addition of microtubules, even though I1α can support the translocation of microtubules (see next section). The failure to stimulate I1α activity further reveals novel properties of this dynein HC motor domain. Thus, although the structure of I1α-HC is strikingly similar to that of other dyneins, the structural analysis alone is not sufficient for assessing functional capability.

**Microtubule gliding produced by purified I1α or I1β complexes**

To characterize the mechanical properties of the I1 dynein complexes, we used conventional in vitro motility assays in which microtubules glide over glass surfaces coated with dyneins. The velocity of I1 dynein (1.8 ± 0.6 µm/s) is similar to the value reported previously (Kotani et al., 2007). The I1α dynein translocates microtubules at a considerably reduced speed (0.7 ± 0.2 µm/s), whereas the microtubule gliding velocity of I1β (3.3 ± 0.7 µm/s) is nearly two times higher than that of the intact I1 dynein. The velocity of two-headed I1 dynein is close to the average of the individual heads. These observations suggest that the I1α motor domain may suppress the activity of I1β in the intact I1 dynein.

Curiously, I1α did not show any microtubule activation on its ATPase activity. We then tested the possibility that the microtubule gliding activity observed with the I1α was due to contamination with other dynein isoforms. On the basis of silver-stained SDS-PAGE gels, we estimate that contamination of the I1 fraction with other dynein isoforms is <1% of I1α. We then performed the microtubule gliding assay at a low protein concentration using the I1, I1β, and dynein-g diluted to ~1% of the concentration of I1α. None of the diluted dyneins (I1, I1β, and dynein-g) supported robust gliding activity.
The 1β-HC motor domain is required for regulation of microtubule sliding by the axonemal radial spoke–phosphorylation pathway

Several lines of evidence indicate that the assembly of I1 dynein, in particular its regulatory ICs (IC138 and IC97), is required for regulation of microtubule sliding by a signaling pathway that involves the central pair apparatus, radial spokes, and axonemal kinases and phosphatases (Bower et al., 2009; Gokhale et al., 2009; Wirschell et al., 2009). The regulatory pathway was revealed by functional and pharmacological analysis of microtubule sliding in paralyzed axonemes from central pair or radial spoke mutants (reviewed in Porter and Sale, 2000; Smith and Yang, 2004; Wirschell et al., 2007).

Dynein-driven microtubule sliding is globally inhibited in isolated, paralyzed axonemes from radial spoke mutants such as p14 or p17, and normal microtubule sliding velocity can be rescued by pretreating these axonemes with kinase inhibitors such as PKA inhibitor (PKI), casein kinase 1–7 (CK1–7), or 5,6-dichloro-β-D-ribofuranosylbenzimidazole (DRB) (Smith and Sale, 1992a; Howard et al., 1994; Yang and Sale, 2000; Gokhale et al., 2009) (Figure 6B, p17 + PKI and p17 + DRB). Rescue of microtubule sliding requires assembly of I1 dynein, indicating that I1 dynein plays an essential role in this pathway (Habermacher and Sale, 1997; Yang and Sale, 2000; Bower et al., 2009). The mechanism of inhibition and the rescue of microtubule sliding correlate with phosphorylation and dephosphorylation of IC138 (Smith and Sale, 1992a; Howard et al., 1994; Habermacher and Sale, 1996, 1997; Yang and Sale, 2000; Hendrickson et al., 2004; Bower et al., 2009; Wirschell et al., 2009). To test whether either of the I1 motor domains is required for regulation of microtubule sliding by the central pair–radial spoke phosphorylation pathway, we crossed the I1α and I1β strains to the paralyzed, radial spoke mutant, p17, to recover triple mutants containing the original HC mutant allele and expressing the truncated motor domain mutant constructs in a radial spoke–defective background (I1α × p17, containing the ida2–6 mutant allele, the Dhc10 transgene lacking the 1β-HC motor domain and the radial spoke heads, and I1β × p17, containing the p9–2 mutant allele, the Dhc1 transgene lacking the 1α-HC motor domain and the radial spoke heads). Molecular and biochemical analyses were performed to confirm the genotypes and phenotypes of the triple mutant strains (Supplemental Figure S1). We then measured microtubule sliding velocities in axonemes in the absence or presence of the kinase inhibitors PKI or DRB or the phosphatase inhibitor microcystin-LR (MC).

Microtubule sliding is greatly reduced in p17 axonemes, and the addition of PKI or DRB restores microtubule sliding to WT levels (Figure 6B), whereas MC blocks rescue by PKI (PKI/MC, Figure 6B). As expected, microtubule sliding velocity is also greatly reduced in axonemes from the mutants I1α × p17 and I1β × p17 (Figure 6B). PKI or DRB treatment, however, only increases microtubule sliding velocity in axonemes from I1β × p17 and fails to rescue sliding velocity in axonemes from I1α × p17 (Figure 6B). These results demonstrate that the 1β-HC motor domain is required for regulation of I1 dynein–mediated microtubule sliding by the central pair–radial spoke phosphorylation pathway.

The 1β-HC motor domain is required for normal microtubule sliding in situ

We previously showed that each I1 dynein motor domain contributes to forward swimming speed in Chlamydomonas (Myster et al., 1999; Perrone et al., 2000). In particular, the deletion of the 1β-HC motor domain reduces forward swimming velocity more significantly than does the deletion of the 1α-HC motor (see Table 2). To further assess the relative contributions of each I1 motor domain, we measured microtubule sliding in isolated axonemes, where dynein activity is uncoupled from the production of flagellar bending. In 1 mM MgATP, microtubules in WT axonemes slide at ∼18 µm/s (Figure 6A; Table 2). Similarly, microtubules slide rapidly in axonemes from the inner arm dynein mutant ida4 (Figure 6A; Table 2). As previously described (Okagaki and Kamiya, 1986; Smith and Sale, 1992a), microtubule sliding velocity is greatly reduced in axonemes lacking the outer dynein arms (p28; Table 2; Figure 6A) or defective in radial spoke assembly (p17; Table 2; Figure 6B). Similarly, microtubule sliding is reduced in mutants lacking I1 dynein (ida2–6, ida2–7, ida7, and p9–2; Table 2; Figure 6A, and see Discussion), in I1 dynein mutants that lack the 1β-HC motor domain (I1α; Table 2; Figure 6A), or in double mutants that lack the outer dynein arms as well as either the 1α or 1β-HC motor domain (Table 2; Figure 6A). Microtubule sliding velocity is nearly WT, however, in axonemes from the 1β strain that lacks only the 1α motor domain (Table 2; Figure 6A), suggesting that the 1α motor domain does not contribute significantly to microtubule sliding velocities. Moreover, despite the assembly of outer dynein arms in the 1α strain, its microtubule sliding velocity is slow, equivalent to complete loss of I1 dynein (compare I1α and I1β in Figure 6A). These observations suggest that the 1α-HC motor domain does not contribute significantly to microtubule sliding in the absence of the 1β-HC motor domain.

FIGURE 4: Dark-field microscopy of I1 dynein induced microtubule bundles: Single-headed complexes I1α and I1β do not induce microtubule bundling. Microtubules polymerized in vitro (top left; microtubules only). The addition of intact I1 dynein formed microtubule bundles in the absence of ATP (top panel). Microtubule bundles formed by I1 dynein dissociated into single microtubules upon addition of 2 mM ATP (bottom panel; I1 dynein). In contrast, microtubules are not bundled in the presence of the single-headed I1α and I1β irrespective of ATP addition (right panels; I1α and I1β). Bar = 10 µm.
The results indicate that each motor domain in I1 dynein is distinct in motor and regulatory activity. As described before (Kotani et al., 2007), I1 dynein may regulate the pattern and speed of microtubule sliding in the axoneme by locally constraining sliding driven by other axonemal dyneins. One hypothesis is that the α-motor domain may resist the faster microtubule sliding driven by the β-motor domain, an activity similar to that proposed for the α HC motor in the outer dynein arm from sea urchin sperm tail axonemes (Sale and Fox, 1988; Moss et al., 1992a, 1992b). This model must be tested directly, but may be consistent with observations of swimming phenotype in the I1 dynein mutants: Cells that lack the β-HC motor domain swim slower (107.9 ± 15.3 µm/s; Table 1, ida2–6::D11), (Perrone et al., 2000) than cells that lack the α-HC motor domain.

FIGURE 5: ATPase activity of I1 mutants. (A) ATPase activity of I1, I1α, and I1β dyneins at various ATP concentrations in the absence of microtubules. The Mg-ATPase activities of I1, I1α, and I1β were fitted by the Michaelis–Menten equation:

\[
k = (k_{cat} \cdot [ATP]) / (K_m + [ATP])
\]

I1: \(k_{cat} = 3.36 \pm 0.17 \text{ s}^{-1} \text{molecule}^{-1}, K_m = 3.22 \mu M\), I1α-HC: \(k_{cat} = 0.62 \pm 0.06 \text{ s}^{-1} \text{molecule}^{-1}, K_m = 8.96 \mu M\), I1β-HC: \(k_{cat} = 4.73 \pm 0.12 \text{ s}^{-1} \text{molecule}^{-1}, K_m = 3.77 \mu M\). The intact I1 dynein shows a characteristic increase in ATPase activity with increasing concentrations of ATP (I1 dynein, left panel). I1β shows higher ATPase activity in the absence of the I1α-HC (I1β, right panel). In contrast, I1α shows low ATPase activity (I1α, middle panel). Error bars indicate standard deviations. The inset is an expanded graph of the activity of I1α. (B) Microtubule activation of ATPase activity. The ATPase assay was performed exactly as for basal ATPase activity, and the activities were measured at various microtubule concentrations. The microtubule-activated Mg-ATPase activities of I1 and I1β were fitted by the modified Michaelis–Menten equation:

\[
k = (k_{cat} - k_{base}) \cdot ([\text{MT}] / (K_m, \text{MT} + [\text{MT}]) + k_{base})
\]

I1: \(k_{cat} = 5.41 \pm 0.31 \text{ s}^{-1} \text{molecule}^{-1}, k_{base} = 2.23 \pm 0.43 \text{ s}^{-1} \text{molecule}^{-1}, K_m, \text{MT} = 0.42 \mu M\), I1β: \(k_{cat} = 7.32 \pm 0.41 \text{ s}^{-1} \text{molecule}^{-1}, k_{base} = 2.31 \pm 0.62 \text{ s}^{-1} \text{molecule}^{-1}, K_m, \text{MT} = 0.16 \mu M\). The intact I1 dynein and I1β both show microtubule-stimulated ATPase activity, whereas I1α does not. The average value of ATPase activity over the whole range of microtubule concentration is 0.52 ± 0.22 s⁻¹·molecule⁻¹. kbase is the basal ATPase activity. Error bars indicate standard deviations.

DISCUSSION
Here we took advantage of mutant cells that lack one or the other I1 dynein motor domain to address whether the α- and β-HC motor domains play distinct roles in control of flagellar movement. We determined that although the α- and β-HC motor domains are similar in structure, they display different activities. In vitro analysis revealed that the I1β complex is similar to other dynein motors with significant ATPase and microtubule translocation capability, thus possibly contributing to net microtubule sliding in the axoneme. Additionally, analysis of microtubule sliding in isolated axonemes revealed that assembly of the β-HC motor domain is required for regulation of microtubule sliding by the central pair–radial spoke I1 dynein phosphoregulatory pathway (reviewed in Wirschell et al., 2007). In contrast, the I1α complex displays unusually low ATPase and microtubule translocation, and assembly of the α motor domain is not required for regulation of microtubule sliding by phosphorylation.

The results indicate that each motor domain in I1 dynein is distinct in motor and regulatory activity. As described before (Kotani et al., 2007), I1 dynein may regulate the pattern and speed of microtubule sliding in the axoneme by locally constraining sliding driven by other axonemal dyneins. One hypothesis is that the α motor domain may resist the faster microtubule sliding driven by the β motor domain, an activity similar to that proposed for the α HC motor in the outer dynein arm from sea urchin sperm tail axonemes (Sale and Fox, 1988; Moss et al., 1992a, 1992b). This model must be tested directly, but may be consistent with observations of swimming phenotype in the I1 dynein mutants: Cells that lack the β-HC motor domain swim slower (107.9 ± 15.3 µm/s; Table 1, ida2–6::D11), (Perrone et al., 2000) than cells that lack the α-HC motor domain.
The 1β-HC motor domain is required for regulated microtubule sliding in the axoneme. (A) Microtubule sliding measurements reveal that assembly of the 1β-HC motor domain is required for WT microtubule sliding velocity. In particular, microtubule sliding velocity is greatly reduced in axonemes from the I1α mutant (lacking the 1β-HC motor domain; I1α or I1α oda). In contrast, microtubule sliding velocity in the 1ββ axonemes (lacking the 1α-HC motor domain) is nearly WT. Note that, based on these assays, there is no significant difference in the slow sliding in axonemes from oda2 and the double mutants I1α x oda and I1β x oda. (B) The 1β-HC motor domain is required for rescue of microtubule sliding by kinase inhibitors. Compared to WT axonemes, microtubule sliding velocity in pf17 axonemes (defective in the radial spokes) or in the triple mutants (I1α x pf17 and I1β x pf17) is reduced by ~50%. As previously shown, the kinase inhibitors PKI and DRB rescue sliding in pf17 axonemes, and the phosphatase inhibitor MC blocks rescue (Howard et al., 1994; Yang et al., 2000; Gokhale et al., 2009). In the I1β x pf17 mutant, kinase inhibitors also rescue sliding. In the I1α x pf17 mutant (lacking the 1β-HC motor domain), however, kinase inhibitors fail to rescue microtubule sliding. The average microtubule sliding velocity for each sample was calculated from three independent experiments with a total sample size of at least 80 axonemes and plotted as a percentage of the sliding velocity relative to WT axonemes. Values shown are means and standard deviations.

Functional domains and the role of I1 dynein in axonemal motility

Functional assays reveal large differences in activity between I1α and I1β. In particular, the I1α dynein exhibits some unusual properties. In contrast to I1β, I1α can translocate microtubules in vitro, but its ATPase activity is not activated by microtubules. Additionally, although the 1α and 1β motors have distinct properties, they do not appear to work independently in the intact I1 complex. One model is that the 1α motor domain regulates the activities of the 1β motor domain. In vitro experiments, all the properties measured for I1β are higher than those of intact I1: i) basal ATPase activities, I1α < I1β; ii) microtubule-activated ATPase activities, 0 = I1α < I1β; and iii) in vitro motility assay, I1α < I1β. Thus the activity of the 1β-HC is modulated by the presence of 1α-HC. Given the reduced ATPase...
activity of the 1α-HC, the primary function of the 1α-HC may be to modulate 11β activity. Refined understanding of I1 dynein structure in the axoneme and understanding of interactions between the I1 dynein motor domains and their respective stem domains is required to further test these ideas.

Diverse observations also indicate that assembly of I1 dynein and, in particular, assembly of the I1β motor domain and the IC138 regulatory complex (Bower et al., 2009) are required for full outer dynein arm activity. Thus, in addition to other unexpected functional features, I1 dynein may operate to regulate the outer dynein arms or possibly other inner dynein arms. For example, in the absence of outer dynein arms, microtubules slide slowly (compare WT and ptf28, Figure 6A). This observation is consistent with other studies of microtubule sliding in axonemes indicating that the outer dynein arms are required for rapid microtubule sliding (reviewed in Kamiya, 2002; King and Kamiya, 2008). Microtubule sliding, however, is also reduced to approximately half of WT sliding velocity when I1 dynein or the I1β motor domain fails to assemble (Figure 6A). This reduced sliding velocity occurs despite full assembly of the outer dynein arms in the I1 dynein mutants (see Figure 2A, IC78). Moreover, microtubule sliding velocity is also greatly reduced in other I1 dynein mutants that fail to assemble the IC138 regulatory complex (see Figure 6A in Bower et al., 2009). In contrast, microtubule sliding velocity is nearly WT in axonemes from I1β, indicating that the outer dynein arms are fully active in I1β axonemes (Figure 6A). The mechanism for how I1 dynein could contribute to regulation of the other dynein arms is not understood. Recent reports by cryoEM tomography, however, reveal a structural linkage between the tails of the outer dynein arm and I1 dynein and also between I1 dynein and other components in the inner arm region (Nicastro et al., 2005; Bui et al., 2008; Heuser et al., 2009). These linkages through the base of the I1 dynein may be designed to relay regulatory information between dynein isoforms.

As introduced earlier in this article, assembly of I1 dynein and the IC138 complex is critical for regulation of dynein-driven microtubule sliding by the central pair–radial spoke phosphoregulatory pathway (Wirschell et al., 2007; Bower et al., 2009). Our new data also indicate that assembly of the I1β motor domain is required for regulation of the phosphoregulatory pathway, suggesting a functional interaction between the IC138 complex and the I1β-HC motor domain (Figure 6B). One model is that change in IC138 phosphorylation alters I1 dynein motor activity; however, in vitro analysis of I1 dynein motor activity, using purified I1 dyneins either in the presence or absence of phosphorylated IC138, does not support this model (unpublished data, H. Sakakibara). Thus I1 dynein phosphorylation and the I1β-HC motor domain may also regulate dynein-driven microtubule sliding through regulation of other dynein arms, including the outer dynein arm.

### MATERIALS AND METHODS

**Chlamydomonas strains**

Chlamydomonas strains used for this study are listed in Table 1. Intact I1 dynein was purified from flagella of an outer-armless mutant, oda1 (Kamiya and Okamoto, 1985; Kamiya, 1988). I1 dynein with a truncated I1β-HC was purified from strain 11α x oda (9A, CC-4079) (Perrone et al., 2000), and I1 dynein with a truncated 1α-HC was purified from I1β x oda (4G, CC-3917) (Myster et al., 1999). The I1 dynein HC mutants (11α and I1β) were crossed to ptf17, and “triple” mutants (containing the original HC mutation, the truncated HC transgene mutation, and the radial spoke defect) were recovered from nonparental tetrads. These triple mutants were verified and characterized by either Western blotting or PCR as described in Supplemental Figure S1. The inner dynein arm mutants ida4 (Kamiya et al., 1991; LeDizet and Piperno, 1995), bop5–1 (Dutcher et al., 1988; Hendrickson et al., 2004), and ida7–1 (Perrone et al., 1998) were used in control experiments.

**Preparation of proteins**

Chlamydomonas I1 dynein was purified as described previously (Kotani et al., 2007) by using two cycles of anion exchange column chromatography in HMED buffer (30 mM HEPES–KOH, 5 mM MgSO4, 1 mM ethylene glycol tetraacetic acid [EGTA], 1 mM dithiothreitol [DTT], pH 7.4). The I1 dynein and truncated HC complexes eluted from the column at approximately 325 mM KCl. For the

### TABLE 2: Swimming and microtubule sliding velocity.

| Strain name | Microtubule sliding velocity (µm/s) ±SD | Swimming velocity (µm/s) ±SD |
|-------------|---------------------------------------|-----------------------------|
| WT          | 17.3 ± 2.5                            | 144.2 ± 17.1 x               |
| oda2 (pt28) | 7.5 ± 0.4 x                           | 51.5 ± 6.9 x                |
| ptf17       | 8.2 ± 0.8                             | N/A x                       |
| ida4        | 18.5 ± 2.3                            | 102 ± 11.0 x                |
| ida7–1      | 9.6 ± 1.7                             | 81.5 ± 14.0 x               |
| bop5–1      | 12.3 ± 1.2                            | 92 x                        |
| ida2–6      | 9.8 ± 0.6                             | 77.6 ± 15.4 x               |
| ida2–7      | 11.4 ± 1.9                            | 53.7 ± 10.7 x               |
| l1α         | 10.5 ± 2.0                            | 107.9 ± 15.3 x              |
| l1α x oda   | 9.8 ± 3.0 x                           | ND x                        |
| ptf9–2      | 8.5 ± 0.1                             | 73.4 ± 12.4 x               |
| 11β         | 16.0 ± 2.5                            | 136.9 ± 16.5 x              |
| 11β x oda   | 9.3 ± 2.0 x                           | 41.2 ± 5.6 x                |

a Velocity determined in Perrone et al., 1998. b N/A = not applicable; ND = not determined. c Swimming velocity determined in Kamiya et al., 1991. d Velocity determined in Hendrickson et al., 2004. e Velocity determined in Perrone et al., 2000. f Velocity measured in Myster et al., 1999. *Based on the sliding assay, there is no significant difference the slow sliding in axonemes from oda2 and the double mutants 11α x oda and 11β x oda.
measurement of ATPase activity, the purified dynein fractions were pooled and assayed immediately. For other in vitro assays, 20% sucrose was added to the fractions, which were frozen in liquid nitrogen and stored at −80°C. Porcine brain microtubules were prepared by cycles of assembly and disassembly (Weingarten et al., 1975). Tubulin was separated from the microtubule-associated proteins (MAPs) by chromatography through phosphocellulose (P-11; Whatman, Maidstone, UK) chromatography (Sloboda and Rosenbaum, 1982). MAP-depleted tubulin (4–5 mg/ml) was assembled at 30°C for 30 min and stabilized by 20 µM Taxol (Sigma, St. Louis, MO). Protein concentrations were determined by the TONEIN-TP kit (Otsuka Pharmaceutical Co., Tokyo, Japan) based on the method of Bradford (Bradford, 1976). Protein samples were analyzed by SDS–PAGE (Laemmli, 1970).

Electron microscopy and single-particle analysis in electron micrographs
To observe the molecular configuration of 11 dynein, purified dyneins were analyzed by negative staining and electron microscopy (Burgess et al., 2003). One drop of a freshly prepared specimen containing intact or motor head-truncated dynein at ~20 µg/ml was applied to a carbon film and stained with 1% uranyl acetate. Samples were observed with a JEM 2000EX electron microscope (JEOL, Tokyo, Japan) with a magnification of 50,000× operating at 80 kV. Electron micrographs were digitized on an EPSON GTX-700 scanner (Seiko Epson, Nagano, Japan) at 1000 dpi, corresponding to a pixel size of 0.54 nm on the grid. For investigation of precise configurations of the 1α- and 1β-HC head domains, the digitized images of those heads were further analyzed by using the single-particle image-processing technique (Frank, 2006). Well-isolated head images were extracted and subjected to single-particle image processing using the SPIDER software programs (Frank, 2006). The number of particles analyzed is as follows: 6877 (1α head); 7316 (1β head). Images were aligned by reference-free algorithms and classified into homogeneous groups as described (Burgess et al., 2004). A total of 1805 particles for 1α and 1040 particles for 1β were classified as the right view of the head domain based on positions of the tail and stalk protrusions. These images of the right view were aligned again and further classified. The averaged images in each particle class were used for comparison of configurations.

Microtubule-bundling assays
MAP-depleted and Taxol-stabilized microtubules were mixed with dynein to a final concentration of 4 µg/µl and 30 µg/ml, respectively, in HMED buffer containing 20 µM Taxol and 50 mM KCl. Microtubule bundling by dynein in the absence ATP was induced by incubating the mixture for 5 min at room temperature and then observed by dark-field microscopy. To observe the dissociation of microtubules, 2 mM ATP was added to the mixtures, and they were observed after 5 min.

Mg-ATPase activity
Mg-ATPase activities of dynein fractions were measured using the EnzChek phosphate assay kit (E-6646; Molecular Probes, Eugene, OR) in a temperature-controlled cell at 25°C. Released inorganic phosphate was measured by continuously monitoring the absorbance at 360 nm for 20 min. To measure microtubule-activated ATPase activity, we used GTP-free microtubules made by sedimenting stabilized microtubules through a 25% sucrose cushion containing HMED buffer and 10 µM Taxol. Control assays using GTP-free microtubules alone indicated that phosphate release from microtubules did not significantly contribute to the total ATPase activity of the dyneins.

In vitro microtubule gliding assays
In vitro microtubule gliding assays were performed as previously described (Kotani et al., 2007). We used untreated glass slides #1 glass slide, 26 mm × 76 mm, S-1126; Matsunami, Osaka, Japan). 5 µl of thawed dynein was mixed with an equal volume of bovine serum albumin (BSA) at 0.5 mg/ml, then applied to a flow chamber and absorbed onto the glass for 5 min. The following were then added in sequence: 1) two volumes of BSA at 0.5 mg/ml to remove unabsorbed dynein and to block the surface of the glass; 2) one volume of Taxol-stabilized microtubules (10–20 µg/ml) in HMED buffer with 1 mM ATP, 20 µM Taxol, and 0.1% methylcellulose. The gliding of the microtubules was observed by dark-field illumination with a 40x objective, captured with a digital CCD camera (excel-V, Dage-MTI, Michigan City, IN), and recorded on a personal computer. The recorded movies were analyzed by custom software (Furuta et al., 2008). All microtubules in the visual field were identified, and the displacement of microtubules was measured. The velocities of individual microtubules the length of which was > 10 µm and that traveled at least 10 µm were measured. Approximately 40 microtubules were used to measure the average and standard deviation for each dynein.

In situ axonemal microtubule sliding assay
Microtubule sliding velocities were measured using the method of Okagaki and Kamiya (Okagaki and Kamiya, 1986) and as previously described (Howard et al., 1994; Habermann and Sale, 1996, 1997; Hendrickson et al., 2004; Bower et al., 2009; Gokhale et al., 2009; Wirschell et al., 2009). Briefly, isolated flagella were resuspended in buffer without protease inhibitors and demembranated with buffer containing 0.5% Nonidet P-40 in 10 mM HEPES, pH 7.4; 5 mM MgSO4; 1 mM DTT; 0.5 mM EDTA; 1% polyethylene glycol (20,000 MW); and 25 mM potassium acetate. The axonemes were added to a perfusion chamber, and microtubule sliding was initiated by the addition of buffer containing 1 mM ATP and subtilisin A Type VIII protease at 3 µg/ml (Sigma Aldrich, St. Louis, MO). Sliding was recorded using a Zeiss Axiovert 35 microscope equipped with dark-field optics, a 40x Plan-Apo lens (Zeiss, Thornwood, NY) and a silicon intensified camera (VE-1000; Dage-MTI). The video images were converted to a digital format using Labview 7.1 software (National Instruments, Austin, TX), and sliding velocity was determined manually by measuring microtubule displacement on tracings calibrated with a micrometer.

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