An Axonemal Dynein Particularly Important for Flagellar Movement at High Viscosity

IMPLICATIONS FROM A NEW CHLAMYDOMONAS MUTANT DEFICIENT IN THE DYNEIN HEAVY CHAIN GENE DHC9

Received for publication, August 17, 2005, and in revised form, October 12, 2005. Published, JBC Papers in Press, October 18, 2005, DOI 10.1074/jbc.M509072200

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Ciliary and flagellar axonemes contain multiple inner arm dyneins of which the functional difference is largely unknown. In this study, a *Chlamydomonas* mutant, *ida9*, lacking inner arm dynein c was isolated and shown to carry a mutation in the *DHC9* dynein heavy chain gene. The cDNA sequence of *DHC9* was determined, and its information was used to show that >80% of it is lost in the mutant. Electron microscopy and image analysis showed that the *ida9* axoneme lacked electron density near the base of the S2 radial spoke, indicating that dynein c localizes to this site. The mutant *ida9* swam only slightly slower than the wild type in normal media. However, swimming velocity was greatly reduced when medium viscosity was modestly increased. Thus, dynein c in wild type axonemes must produce a significant force when flagella are beating in viscous media. Because motility analyses in vitro have shown that dynein c is the fastest among all the inner arm dyneins, we can regard this dynein as a fast yet powerful motor.

Cilia and flagella are cell organelles for motility and are conserved in a variety of eukaryotic organisms. In mammalian cells, ciliary and flagellar movements are particularly important in respiratory and reproductive processes and in the mechanism that determines left-right asymmetry in embryos (1, 2). The beating of cilia and flagella is driven by inner arm and outer arm dyneins, the molecular motors that produce sliding forces between outer doublet microtubules. Elucidation of the functional properties of dyneins should be the key to understanding the mechanism that produces axonemal beating.

Structure and function of axonemal dyneins have been extensively studied with the green alga *Chlamydomonas*. Previous studies have shown that the inner arm and outer arm dyneins strikingly differ with regard to molecular composition and arrangement on the outer doublet microtubules (3–6). The outer arm dynein comprises a single kind of assembly composed of three dynein heavy chains (DHCs) (7, 8) (M>500,000) and several smaller proteins. On the other hand, there are seven structurally different forms (subspecies) of inner arm dynein assemblies (7). These are designated a–g. Subspecies a–e, and g each contain a single unique DHC and a few smaller proteins. In contrast, subspecies f, which is also known as I1, contains two distinct DHCs and several smaller proteins (for review see Refs. 8–12). Thus, biochemical analyses of axonemes have identified eight DHCs altogether. However, analyses of the *Chlamydomonas* genome have identified 11 DHC genes, *DHC11–DHC111*, that can be assigned to inner arm dyneins (24, 48). Thus, more DHCs may be present in the axoneme as the heavy chains of yet unidentified inner arm dynein subspecies. In accordance with multiple subspecies, the inner arms are arranged in a complex manner within a repeating unit of 96 nm along the outer doublet microtubules (see Fig. 5i). This arrangement radically differs from the outer arms, which are linearly arranged at a regular interval of 24 nm (4, 6, 13, 14).

Different axonemal dyneins appear to differ in function also (12, 15–17). For example, waveform analyses on dynein-deficient mutants suggest that the inner arm is important for producing a proper waveform, whereas the outer arm is important for beating at high frequency (18). Also, in vitro motility assays show that different dyneins translocate microtubules at significantly different velocities (7, 19–21). The presence of functionally different dyneins must be important for axonemal beating because mutant axonemes lacking certain combinations of dyneins, such as those simultaneously lacking outer arm dynein and some inner arm dyneins, completely lack motility. The question then arises as to how each kind of dynein specifically functions within the mechanism of axonemal beating.

To achieve a better understanding of the function of each dynein species, we have been seeking to obtain *Chlamydomonas* mutants lacking specific dyneins. Various mutants deficient in outer arm dynein or inner arm dynein have already been isolated (TABLE ONE), and the findings with these mutants helped us to infer the specific function of particular dynein subspecies. However, except for mutants lacking subspecies e or f, mutants that lack a single species of inner arm dynein have yet to be isolated. We isolated a mutant (*ida9*) that lacks only subspecies c, a dynein that has been extensively used in recent biophysical studies (22, 23). In this study, we found that *ida9* carries a mutation in the *DHC9* gene and determined the cDNA sequence of *DHC9* and the localization of dynein c within the axoneme. This dynein appears to be particularly important for flagella to work against a viscous load.

**EXPERIMENTAL PROCEDURES**

**Strains**—To produce mutants by insertional mutagenesis, the *Chlamydomonas reinhardtii* Nit1− strain A54-e18 was used. This strain...
Mutants deficient in inner arm dynein

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<th>Strain</th>
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<td>f</td>
<td>1α (DHC1)</td>
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<tr>
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<td>pf3</td>
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was provided by Dr. Pete Lefebvre (University of Minnesota). ida9 was originally isolated as a mutant that swims slightly more slowly than the original A54-e18, as described below. We found that A54-e18 displayed somewhat poor motility compared with the standard 137c strain under the same cultural conditions. This made it difficult to identify the mutant phenotype of ida9 in progeny between ida9 and another strain derived from 137c. We therefore extensively removed A54-e18 background from ida9 by crossing it with the 137c strain five times. The resultant strain was used as ida9. Mating of this strain to 137c always produced a clear 1:1 segregation. Double mutants of ida9 with ida1 (25), ida1, and ida4 (5) were produced using standard procedures (26). Other strains used for genetic analyses were obtained from Dr. E. Harris of the Chlamydomonas Genetics Center (Department of Botany, Duke University, Durham, NC).

**Mutant Production**—A Nit− strain, A54-e18, was mutagenized by insertional mutagenesis according to the method of Tam and Lefebvre (27). A linearized plasmid DNA, pMN56, composed of the pUC119 vector sequence and the NIT1 gene, was used for transformation (28). Each clone of Nit− transformant was cultured in 96-well plates and observed with an inverted microscope. Colonies that displayed poor motility were saved. The mutant ida9 was selected from those colonies.

**Genetic Analysis**—The genetic locus of ida9 was determined by tetrad analyses using standard procedures (26).

**Southern Blot Analysis**—DNA was isolated from wild type and ida9 using the method of Weeks et al. (29). The DNA was then digested with restriction enzymes, loaded on a 0.8% agarose gel, and transferred to a BIOGEL using the method of Weeks et al. (29). Southern blot analyses were performed using the Clustal W program (www.ddbj.nig.ac.jp/search/clustalw-e.html). The amino acid sequences used as probes were degenerate oligos corresponding to the deduced amino acid sequence of DHC9, as described below. Probes were selected from those regions of the amino acid sequence of DHC9 (see supplemental Fig. S2) that were conserved among all known DHCs.

**Isolation of Axonemes and Fractionation of Dynein**—The culture of Chlamydomonas used for isolation of poly(A)− RNA was prepared by acid guanidinium thiocyanate-phenol-chloroform extraction (31). To determine the sequence downstream of the AAA4 domain, we chose a strain that lacked inner arm dynein and was provided by Dr. Pete Lefebvre (University of Minnesota). ida9 was originally isolated as a mutant that swims slightly more slowly than the original A54-e18, as described below. We found that A54-e18 displayed somewhat poor motility compared with the standard 137c strain under the same cultural conditions. This made it difficult to identify the mutant phenotype of ida9 in progeny between ida9 and another strain derived from 137c. We therefore extensively removed A54-e18 background from ida9 by crossing it with the 137c strain five times. The resultant strain was used as ida9. Mating of this strain to 137c always produced a clear 1:1 segregation. Double mutants of ida9 with ida1 (25), ida1, and ida4 (5) were produced using standard procedures (26). Other strains used for genetic analyses were obtained from Dr. E. Harris of the Chlamydomonas Genetics Center (Department of Botany, Duke University, Durham, NC).

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Mutant Lacking Inner Arm Dynein c

from an intron of the DHC9 gene (probe A; see "Results") was contained in scaffold 1357 of the genome data base version 1. This scaffold contained a partial sequence of DHC9 up to the fourth AAA motif (AA4; supplemental Fig. S2). Four cDNA fragments (see Fig. 2a, fragments E–H) were amplified by reverse transcription-PCR using primers designed from the predicted exon sequences in this scaffold. Sense and antisense primers for each fragment are as follows: fragment E, 5′-CGGAGGTCTGGACAAATTTCGGC-3′ and 5′-TGAATGGACGCC-AGGTGTCCT-3′; fragment F, 5′-GAGACCTCGCCGCTCATCA-3′ and 5′-GCAGAATGGCCGCAACCTTTT-3′; fragment G, 5′-GAGC-ACCTCGCCGCTCATCA-3′ and 5′-GGTTAGAGGTTCTCACC-3′; and fragment H, 5′-GGTACGTCATCTGGACAA-3′ and 5′-GCC-GAAGACGACAGGTAGTTCA-3′. A fragment containing the 5′-terminal sequence (see Fig. 2a, fragment D) was amplified by 5′ rapid amplification of cDNA ends (30). The 5′ rapid amplification of cDNA ends antisense primer was 5′-GAAGAGGCTATGGTTGACGAC-3′. The cDNA used had been synthesized using Superscript III (Stratagene) from poly(A)− RNA trapped on the poly T-anchored magnet beads (DYNABEADS oligo(T)20, DYNAL Biotech.). Total RNA of wild type used for isolation of poly(A)− RNA was prepared by acid guanidinium thiocyanate-phenol-chloroform extraction (31). To determine the sequence downstream of the AAA4 domain, we took advantage of a consensus sequence for inner arm dynein genes (DHC2–8 and DHC11) present in the AAA6 domain (see supplemental Fig. S2). Reverse transcription-PCR performed using a DHC9-specific forward primer 5′-CAGAGGGTAGTCGCCAGTCTC-3′ and the consensus reverse primer 5′-GCCGATGGCGCCGCTCAG-3′ amplified a fragment (sequence), part of which was contained in scaffold 919 of the genome data base version 2 (see Fig. 2a). Finally, we sequenced the 3′-terminal part of DHC9 cDNA was determined using the information of scaffold 919 (see Fig. 2a, fragments J and K). Fragment J was obtained by reverse transcription-PCR with forward reverse primers of 5′-AACATCCGAGGAGAGAGGAGGAGG-3′ and 5′-TACCCCGCCAAACCTGCGACCA-3′ and fragment K with 5′-GGGAGGGTAGATGGAC- GC-3′ and 5′-TACCCCGCCAAACCTGAAGCGA-3′. The presence and absence of different parts of the DHC9 sequence in the ida9 genome was examined by Southern blot using blots of fragments A, B, C, F, and K as probes (indicated in Fig. 2a).

**Analysis of Deduced Amino Acid Sequence of DHC9**—Multiple alignment analysis was performed using the Clustal W program (www.ddbj.nig.ac.jp/search/clustalw-e.html). The amino acid sequences used as probes were degenerate oligos corresponding to the deduced amino acid sequence of DHC9, as described below. Probes were selected from those regions of the amino acid sequence of DHC9 (see supplemental Fig. S2) that were conserved among all known DHCs.

**Amino acid sequence comparison between DHC9 and other DHCs** were performed using the DOTMATCHER program (bioweb.pasteur.fr/seqanal/interfaces/dotmatcher.html) with a window size set at 50 and a threshold set at 18. Prediction of coiled-coil motifs in the deduced amino acid sequence was performed using the COILS program (www.ch.embnet.org/software/COILS_form.html).

**Isolation of Axonemes and Fractionation of Dynein**—The culture of Chlamydomonas used for isolation of poly(A)− RNA was prepared by acid guanidinium thiocyanate-phenol-chloroform extraction (31). The presence and absence of different parts of the DHC9 sequence in the ida9 genome was examined by Southern blot using blots of fragments A, B, C, F, and K as probes (indicated in Fig. 2a).

**Electrophoresis**—The composition of dynein heavy chains was analyzed by SDS-PAGE with a 3–5% polyacrylamide gradient and a 3–8 M urea gradient (7). For analysis of the intermediate chains and low molecu...
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FIGURE 1. Analyses of dynein composition in wild type and ida9 axonemes. a, elution patterns of extracts from wild type and mutant axonemes. Axonemes were extracted with 0.6 M KCl and the extracts were fractionated on a Mono Q column after desalination. Extracts from wild type (wt), ida9, oda1, idoda1, Peaks a–g are fractions of inner arm subspecies, and peaks η and γ are outer arm subparticles (7). Note that peak c (dashed line) is very small in ida9 and idoda1 extracts. (The separation of peaks f and g in the oda1 and idoda1 patterns is not so clear as in the wt and ida9 patterns; this is due to a slight intrinsic variability of elution pattern and is not significant.) b and c, SDS-PAGE patterns of the peak fractions from axonemal extracts. b, DHC bands separated on a 25–50% gel. The filled arrowheads indicate that a DHC band missing in the fraction c from the ida9 extract; densitometry showed that its intensity in ida9 was less than 1/10 of that in the wild type (data not shown). The small open circles in the upper panel indicate the DHC band(s) of the major dynein species contained in each peak fraction. Some DHC bands appear in multiple fractions because of incomplete separation between peaks. c, bands separated on a 5–20% gel. The open arrowheads show actin and a 28-kDa subunit (p28). The intensities of actin and p28 bands in fraction c of ida9 were estimated to be 25 and 15% of the corresponding bands of wild type, respectively. These proteins are greatly reduced in the fraction c of ida9; small amounts are present most likely because of cross-contamination of other dyneins in this fraction.

For image analysis, the negatives were first digitized with a LeafScan 2.2 scanner at a resolution of 5 μm (corresponding to 0.2 nm). The images were selected according to the clarity of the outer arms, central pairs, and radial spokes present in pairs per every repeat length. Appropriate axonemal images were separated into overlapping segments containing six outer arm images on one side, i.e. segments about 144 nm in length corresponding to 1.5 times the 96-nm repeat length of the axoneme. The segments were classified into two mirror image-related groups based on the shape of the outer arms. Next, to objectively select representative images, pair-wise correlations were made between all of the segments in each group; the images were classified into two or three groups by clustering analysis using the inverse of the cross-correlation coefficient as the “distance” between the two images (33). Average images were produced from images that form the largest cluster in one group, together with the reversed images of those forming the largest cluster in the mirror image related group. In this way, the initial average images were obtained from 145 wild type images (selected from a total of 174 images), 98 ida9 images (selected from 134 images), and 58 ida4 images (selected from 64 images). For refinement, correlation was calculated between each of the original images and the averaged images. The images that showed high correlation coefficients (with >0.65 cross-correlation coefficients with the average) were selected and used to produce the final averaged image. After this refinement procedure was repeated three times, the final averaged images for wild type, ida9, and ida4 axonemes were obtained from 90, 75, and 40 images, respectively. The statistical significance between these averaged images was assessed by t test. All of the calculations were carried out using Eos software (34).

Motility Assessment—A dark field microscope at a total magnification of ×100, a video recording system, and a personal computer were used to measure the swimming velocities of the mutant and wild type cells. A red filter with a cut-off wavelength of 630 nm was placed under the condenser lens to facilitate measurement by suppressing cellular response to light. Fifty samples, in fresh TAP medium, were measured to obtain the average velocity of a given sample. For the measurement of propulsive force at different viscosities, the medium viscosity was varied by dissolving 0–16% (w/v) Ficoll (Type 400, Sigma; M = 400,000) (35). Final viscosity was measured with an Ostwald viscometer. Propulsive force was estimated using Stokes’ formula \( F = 6\pi \eta a v \), where \( \eta \) is the medium viscosity, \( a \) is the cell body radius as approximated by a sphere, and \( v \) is the swimming velocity of the cell. Flagellar beat frequencies in swimming cells were measured as described (36). In brief, microscope images of swimming cells were projected onto a graded filter, and the intensity fluctuation of the light transmitted through the filter was analyzed with a photo detector and a fast Fourier transform analyzer. This method yields an approximate average of the beat frequencies in a large population of cells.

RESULTS

Isolation and Genetic Analyses of ida9—From about 7500 clones of Nit7 transformants, we isolated 53 clones of cells that swam more...
slowly than the parent strain (A54-e18). Axonemes were prepared from all clones and analyzed by SDS-PAGE. About half of the axonemes showed defects in either outer arm or inner arm DHC bands. The mutant ida9 axoneme showed a difference in the pattern of inner arm DHC bands (data not shown). Genetic crosses with its parent strain resulted in a 1:1 segregation of the slowly swimming phenotypes in 31 dissected tetrads. All slowly swimming daughter cells were found to be Nit−, i.e. able to grow on medium containing NO3 as the sole nitrogen source. These observations were indicative of a mutation produced by insertion of the plasmid containing the NIT1 gene. Southern blot analysis indicated that this mutant carried a single insertion of the plasmid, which was slightly shortened from the original length after incorporation (data not shown). Our attempts to recover the plasmid sequence from the ida9 genome were unsuccessful. Through genetic crosses with various mutants, the ida9 mutation was mapped to linkage group XVI/XVII, 2.2 centimorgan to the left of the centromere (parental ditype: nonparental ditype+tester type = 43:0:2 for ida9 × y1a and 48:0:9 for ida9 × ac46).

Composition of Inner Arm DHC—To examine the dynein composition, we analyzed a high salt extract of the ida9 axoneme using ion exchange chromatography with a Mono Q column, an efficient procedure for separating different species of dynein (7, 37). As shown in Fig. 1a, the elution pattern from the ida9 extract showed a significant reduction in peak c. A similar reduction was observed in the axonemal extract of double mutant ida9oda1 when compared with the extract from the oda1 axoneme. The reduction in peak c was confirmed using two different samples each of ida9 and ida9oda1 axonemes. Previous biochemical analyses indicated that dynein subspecies c is composed of a single heavy chain associated with actin and a 28-kDa protein (p28) (7, 38, 39) that may function in targeting of dynein to specific sites on the outer doublet microtubules (39). SDS-PAGE of the separated fractions showed that fraction c had only faint DHC bands with mobilities identical to those of the DHCs present in adjacent fractions (Fig. 1b). In two samples of ida9 axonemes, the intensity of the DHC band corresponding to subspecies c was always less than 10% of the band intensity in wild type, as estimated by densitometry (data not shown). It is likely that the faint DHC band appearing in fraction c was derived from the DHC of subspecies b predominantly contained in the adjacent peak. In accordance with the absence of an inner arm dynein subspecies, actin and p28 were also greatly reduced in this fraction (Fig. 1c); these faint actin and p28 bands were probably also derived from those contained in the adjacent peaks. From these observations, we suspected that the ida9 axoneme lacks inner arm dynein subspecies c. The small peak in the fraction c appearing in the elution patterns of the ida9 and ida9oda1 extracts was probably due to an unknown, non-dynein component. SDS-PAGE patterns indicated that other inner arm dynein subspecies were present in apparently normal amounts (Fig. 1, b and c).

ida9 Has a Mutation in the DHC9 Gene—A dynein heavy chain gene, DHC9, has been mapped to linkage group XVI/XVII (24), the linkage group to which DHC9 was mapped. Therefore, we speculated that ida9 might carry a mutation in this gene. To test this possibility by Southern blotting, we first isolated a genomic fragment to be used as a probe for the DHC9 gene. PCR using genomic DNA and primers designed after the registered sequence of DHC9 cDNA (24) (see “Experimental Procedures”) resulted in amplification of two products of different sizes. Sequence analysis of the products indicated that the smaller one (~300 bp) was derived from DHC2 genomic sequence, and the larger one (~600 bp) contained a registered DHC9 cDNA sequence and an inserted sequence (219 bp) other than the DHC9 sequence. Recently released data from the Chlamydomonas genome project (genome.igi-psf.org/chlre2/chlr2.home.html) indicated that the inserted sequence was an intron in the DHC9 gene. We used this intron sequence as a specific probe for DHC9 (Fig. 2a, probe A). Southern blot with the specific probe clearly showed hybridization with bands in wild type DNA, whereas the same band was missing in ida9 DNA (Fig. 2b, probe A).

The state of the DHC9 gene in ida9 was further examined using probes for different parts of this gene. For this purpose, the entire cDNA sequence of DHC9 was determined using the information from Chlamydomonas genome project (“Experimental Procedures”). Fig. 2a shows the relationship between the determined cDNA and the genomic sequence (contained in two scaffolds). Southern blot with probes for the C-terminal 80% of the molecule (fragments A, B, C, and K) showed hybridizing bands in wild type DNA but not in ida9 DNA (Fig. 2b). In contrast, Southern blot with a probe for the N-terminal sequence (fragment F) detected bands in the ida9 genome with mobilities different from those in wild type (Fig. 2b). Because fragment B, a genomic sequence containing a 3’ portion of fragment F, did not detect hybridizing bands in ida9 DNA, it is most likely that ida9 has a mutation within or near the fragment F region and lacks all of the sequence downstream of fragment B, accounting for more than 80% of the DHC9 gene.

Amino Acid Sequence of DHC9—The amino acid sequence of DHC9 protein deduced from the cDNA sequence is shown in supplemental Fig. S1. It is a 4149-residue protein with a molecular mass of 464,680 Da. Like all other DHCs reported previously, its C-terminal 2/3 region contains six consensus AAA motifs (marked in supplemental Fig. S2), of
which the first four (AAA1–4) have P-loop sequences. A BLAST search indicated that the deduced amino acid sequence of DHC9 has a high similarity (61%) to that of the human axonemal dynein heavy chain 7 (DNAH7) (40, 58). Fig. 3 shows pairwise sequence comparison between DHC9 and DNAH7, as well as between DHC9 and the two DHCs of Chlamydomonas inner arm dynein f (DHC1 and DHC10). DHC9 is similar to DNAH7 for the entire length, with especially high similarity in the C-terminal region. In contrast, DHC9 is similar to the two dynein f DHCs only in the C-terminal 3/4 region. The DHC9 amino acid sequence is shorter than the other registered sequences of Chlamydomonas genes (see “Experimental Procedures”). P3 sequence for DHC7 is not available. The accession numbers for the nucleotide sequences corresponding to DNAH7, DHC1, DHC9, and DHC10 are AF332742, AJ243806, AB232152, and AJ242523-AJ242525, respectively.

As in all other DHC sequences thus far reported, DHC9 has two ~120-amino acid coiled-coil motifs between AAA4 and AAA5 (supplemental Fig. S2). These coiled-coil sequences and the nonhelical segment between them are likely to form a microtubule-binding stalk, as suggested in other DHCs (41–43). A marked characteristic of DHC9 is that its coiled-coil forming probability in the first stalk segment is unusually low, as is evident when compared with other DHCs, such as DNAH7 (Fig. S2).

Axonemal Structure—Using thin section electron microscopy and image analyses, Mastronarde et al. (6) showed that the axoneme of the mutant ida4 lacks three discrete electron densities within the 96-nm repeat unit of the outer doublet (for an updated figure see Ref. 10). The three densities are arranged at approximately the same intervals along the length of the outer doublet, two being located near the base of the two radial spokes (S1 and S2). Because ida4 lacks subspecies a, c, and d, we expected that the ida9 axoneme, which lacks only subspecies c, might lack one of the three previously identified densities. We thus took a number of electron micrographs of longitudinal sections of wild type (Fig. 5a) and mutant axonemes. Images of 144-nm-long axonemal segments were collected from each sample and analyzed as described under “Experimental Procedures.” Averaged images of wild type, ida9, and ida4 axonemes are shown in Fig. 5 (b–d). Comparison of the averaged images revealed areas of decreased electron density in mutant axonemes (Fig. 5, e–g). The white contoured line indicates areas of significant difference at 98% confidence. In agreement with Mastronarde et al. (6), the ida4 axoneme lacked electron density at three positions within the...
repeating unit (Fig. 5e). As expected, *ida9* lacked electron density at one of the three positions: the base of the S2 radial spoke (Fig. 5f). Finally, subtraction of the *ida9* and *ida4* images confirmed that the low density area in *ida9* corresponded exactly to one of the three areas of low density areas in *ida4* (Fig. 5g). All of these results clearly indicate that dynein subspecies c in the wild type axoneme is localized at the base of the S2 radial spoke (Fig. 5, h and i).

**Motility**—The mutant *ida9* swam more slowly than wild type. However, the difference was only slight irrespective of the culture conditions or whether the cells were vegetative or gametic. TABLE TWO compares the swimming velocities, the flagellar beat frequencies, the ratio of the beat frequency to the swimming velocity (*V/F* ratio: the distance a cell moves per single flagellar beat cycle, a parameter of flagellar waveform), and the flagellar lengths in wild type and various mutants lacking different species of dynein. As reported previously, mutants *ida1* (lacking subspecies f), *ida4* (lacking a, c, and d), and *ida5* (lacking a, c, d, and e) equally swim more slowly than wild type, although their flagellar beat frequencies are not significantly reduced (5, 18, 44). The mutant *ida9* displayed normal flagellar length and beat frequency but swam at about 80% of the wild type velocity (TABLE TWO). Its slightly lower *V/F* ratio suggests that the reduced swimming velocity results from a slight change in flagellar waveform; its flagellar bend angle may be decreased but not to the extent seen in other inner arm mutants. To confirm this prediction, we observed its waveform by using a high speed video system. Measurements of the shear angles (45) in six samples yielded average standard deviations of 2.64 ± 0.44 rad (wild type) and 2.39 ± 0.26 rad (*ida9*). The wild type value was compatible with the one (2.69 ± 0.27 rad) reported by Brokaw and Luck (46). The shear amplitude in the *ida9*
flagella did not show a drastic reduction from the wild type value, compared with that of another inner arm dynein mutant ida1, 1.91 ± 0.12 rad (18). Although a more detailed analysis needs to be carried out for quantitative comparison, we can at least say that the ida9 mutation causes a less significant abnormality in the flagellar waveform than previously studied inner arm mutations.

A feature common to all the mutants lacking inner arm dyneins thus far isolated is that they become nonmotile when combined with an oda mutation that causes the loss of outer arm dynein. Unexpectedly, oda9oda1 double mutants were found to display slow swimming or, occasionally, a circling movement with only one flagellum beating effectively. The swimming velocity and beat frequency in swimming cells at increased viscosity (Fig. 6). The results showed that force generation in the flagellar beat itself. The greatly reduced flagellar beat frequency, whereas only marked reduction of propulsive force in the addition to dynein c and f, displayed slow swimming rather than total paralysis. In this regard, ida9 differs from ida4 that produces a paralyzed phenotype when combined with the oda or ida1-ida3 mutations (5).

**Motility in Viscous Media**—To evaluate the contribution of dynein c to the generation of propulsive force by cells swimming against a viscous load, we carried out velocity measurements on ida9 cells swimming in media of various viscosities (Fig. 6a). The results showed that force production in this mutant was extremely sensitive to the medium viscosity (Fig. 6b). More strikingly, although ida9 swam at fairly high speed in normal medium, it progressively slowed its speed with a modest increase of viscosity to 2–3 cP and completely stopped swimming at 8 cP. To decipher whether this decrease in propulsive force was due to a change in flagellar waveform, we measured the flagellar beat frequency (Fig. 6c) and took the V/F ratio as a parameter of flagellar waveform (Fig. 6d). With increasing viscosity in a 1–2 cP range, both ida9 and wild type displayed a similar slight decrease in beat frequency, whereas only ida9 displayed a marked decrease in the V/F ratio. At higher viscosities (3–4 cP), ida9 displayed much lower beat frequencies than wild type, whereas the V/F ratio was almost identical. Thus, the marked reduction of propulsive force in ida9 cells at increased viscosity must be mainly due to a decrease in the flagellar beat frequency.

**DISCUSSION**

**Identification of DHC9 as the Heavy Chain of Dynein c**—The ida9 mutant was found to carry a mutation in the DHC9 gene. Porter and co-workers (24, 47, 48) identified 11 genes (DHC1-DHC11) that apparently code for inner arm DHCs, as well as two genes for the DHC of cytoplasmic dynein, and showed that DHC1 and DHC10 code for the two DHCs of inner arm subspecies f (11). The DHC1 gene corresponds to the IDA1/PF9 locus (47), whereas the DHC10 gene corresponds to the IDA2 locus (48). Thus, ida9 is the third mutant shown to carry a mutation in a particular inner arm DHC gene. DHC9 is the first heavy chain of single-headed inner arm dynein whose deduced sequence has been determined. Correspondence between the rest of the DHC genes and inner arm dynein species other than c and f, including those that have been unidentified in previous biochemical analyses of axonemes, remains to be studied.

**The Primary Structure of DHC9**—The deduced sequence of DHC9 has an N-terminal 1/4 region that is diverged from the corresponding regions in DHC1 and DHC10 and a C-terminal 3/4 region containing six conservative AA domains. This general design of a variable N-terminal and a conserved C-terminal sequences is common to all other DHCs including those of cytoplasmic dyneins (for review see Ref. 49). The N-terminal 1/4 region has been thought to function in the anchoring of dynein to the outer doublet A tubule or to various cargoes. It is also the site where various light and intermediate chains bind. Because all axonemal dyneins are targeted to distinct loci within the 96-nm repeat in the axonemal structure, it is likely that the N-terminal sequence differs from one DHC to another.

The P2 loop in DHC9 has a diverged sequence with two characteristic substitutions not seen in most other DHCs. Because nucleotide binding to P2 has been suggested to regulate the motor activity of dynein (50), we speculate that the diverged P2 sequence in DHC9 may be related to the characteristic motor activity of this dynein, namely the exceptionally high gliding velocity (7), processivity (22), and high power production against viscous load (see below).

**Implications for the Function of Dynein c**—ida9 swims slightly slower than wild type and faster than ida4, which lacks subspecies a, c, and d. The slight decrease in swimming velocity of ida9 is apparently due to a slight change in waveform because its flagellar beat frequency is identical with that of wild type. Both ida9oda1 lacking the entire outer arm in addition to dynein c and ida9ida1 lacking the inner arm subspecies c and d displayed slow swimming rather than total paralysis. In this regard, ida9 differs from ida4 that produces a paralyzed phenotype when combined with the oda or ida1-ida3 mutations. The fact that the absence of subspecies c from wild type, oda1, and ida1-ida3 axonemes results in slow flagellar beating indicates that subspecies c functions to increase the fundamental flagellar activity but may not be important for generating the flagellar beating itself. The greatly reduced flagellar beat frequencies observed in ida9ida1 and ida9oda1 suggests that the function

<table>
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<th>Table Two: Motility and flagellar length in dynein mutants</th>
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<td><strong>Swimming velocity</strong></td>
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* Averages and standard deviations were calculated from the data of more than 50 cells. Temperature was 20 °C.
* Median frequency and standard deviation were estimated from the power spectrum from a population of cells (see “Experimental Procedures”). Temperature was 20 °C.
* These values are the average swimming velocity divided by average beat frequency.
* These values were from Ref. 53.

Swimming velocity and beat frequency in cells swimming forward. The cells displaying circling movements with only one flagellum beating were not used for measurements.
Mutant Lacking Inner Arm Dynein c

FIGURE 6. Effects of viscosity on the motility of wild-type cells and ida9 cells. a, swimming velocities and their standard deviation of wild-type (wt) cells and ida9 cells. About 50 cells were used for each data point. b, propulsive force and its standard deviation produced by wild type and ida9 cells. Propulsive force (F) was estimated by the Stokes’ formula, \( F = 6 \pi d \eta v \), where \( d \) is the medium viscosity, \( a \) is the radius of a cell, and \( v \) is the swimming velocity. The estimated values of propulsive force contain large errors at higher viscosities, because the errors in swimming velocity data are multiplied by large viscosity values. c, flagellar beat frequency and standard deviation measured by a fast Fourier transform method (see “Experimental Procedures”). The standard deviation was obtained from the shape of the peak, fitted with a Gaussian curve.

FIGURE 7. Viscosity and propulsive force generated by different dynein mutants. Solid line, data from the present study. Broken line, data from Ref. 35. Data for wild type, ida9, ida1, ida4, and ada1 are shown. Note that the force generation in ida9 and ida1 is extremely sensitive to viscosity.

of multiple dyneins is cooperative and that a certain combination of inner arm dyneins is important for flagella to beat at high frequency. The previously proposed idea that outer arm dynein is important for beating at high frequency and inner arm dynein is important for beating with a proper waveform (18) is apparently an oversimplification.

We found that the swimming velocity of ida9 reaches as high as 80% of the velocity of wild type under normal conditions but greatly lowers in media of modestly increased viscosity (Fig. 6). Although the lower swimming velocity appears to be due to some change in flagellar waveform, the great deceleration with viscosity appears to be caused by a great decrease in beat frequency (Fig. 6c). Thus, subspecies c is responsible for the susceptibility of the beat frequency to increased viscous loads, as well as for the slight change in the flagellar waveform in normal media. This observation suggests that, in wild type, inner arm dynein subspecies c produces a significant force when flagella are beating slowly under a viscous load. Fig. 7 shows data from our previous and present studies to compare the performance of different dynein mutants. The propulsive force in wild type cells increases with a slight viscosity increase to 2 cP but lowers with further increase. This increased force generation at slightly increased viscosity is possibly due to a cellular adaptive response (35). In contrast, the ada1 mutant without the outer arm tends to produce fairly constant force at viscosities up to 10 cP, although the magnitude of force at low viscosity is only about 1/3 of that in wild type. The mutant ida1, lacking inner arm subspecies f, shows a modest force increase at slightly increased viscosity and a decrease at higher viscosities. Strikingly, the mutant ida9, lacking subspecies a, c, and d, is extremely sensitive to viscosity, and its force production completely stops at 6 cP. These findings lead us to speculate that subspecies a, c, and d are particularly important for force production against a viscous load (35). The cessation of swimming in ida9 at ~8 cP confirms the importance of subspecies c. To determine whether this property is shared by subspecies a and d, mutants that specifically lack these dyneins must be isolated and characterized. Chlamydomonas has been found in lakes, swamps, and soils (26). The presence of dyneins such as subspecies c may be important for the cell to cope with greatly variable mechanical microenvironments.

Motility assays in vitro with isolated dyneins have shown that inner arm dynein subspecies c has an activity to translocate microtubules at high speeds (7). For example, at 0.1 mM ATP, the speed is as high as 6.8 \( \mu \text{m/s} \), the highest speed among all the Chlamydomonas dynein inner arm subspecies examined in vitro. Based on the above results, we previously speculated that subspecies c might be responsible for the fast movement of axonemes under low load conditions but not so important for force production at high viscosities. The present results indicate that this was an inaccurate prediction. Sakakibara et al. (22) carried out the first single-molecule analysis on axonemal dynein using optical tweezers and showed that subspecies c is a processive motor, i.e. a motor that undergoes multiple cycles of mechanochemical ATP hydrolyzing steps without dissociating from the microtubule. This is interesting because the force generation step in this dynein has been shown to occur within a small time fraction of the ATP hydrolysis cycle; in other words, subspecies c has a low duty ratio. For a monomeric motor protein to have such properties, it may be equipped with an intramolecular domain that constantly holds microtubules, in addition to the site responsible for mechanochemical force production (22, 51, 52). Presence of such a second microtubule-binding domain should make the motor resistant...
to external force promoting its dissociation from microtubules. In other words, such a processive motor can function under a larger load than nonprocessive motors. The present finding that subspecies c is important for axonemal motility under high viscosity supports this idea.

Dynein subspecies c has been used in a biophysical study that examined the force generation by single molecules (22) and in a detailed structural study that demonstrated a conformational change corresponding to the power stroke of the molecule (23). Hence, dynein c is one of the few dyneins in which the structure and functional properties have been well explored. Identification of DHC9 as the structural gene of the dynein c heavy chain, determination of its amino acid sequence, and the availability of a mutant lacking it should be important for further study of the structure-function relationship in dynein.

Acknowledgment—We thank Dr. Pete Lefebvre (University of Minnesota) for providing the mit− strain A54-e18 and for valuable advice on insertional mutagenesis.

REFERENCES
An Axonemal Dynein Particularly Important for Flagellar Movement at High Viscosity: IMPLICATIONS FROM A NEW CHLAMYDOMONAS MUTANT DEFICIENT IN THE DYNEIN HEAVY CHAIN GENE DHC9

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doi: 10.1074/jbc.M509072200 originally published online October 18, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M509072200

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