Identification of dyneins that localize exclusively to the proximal portion of Chlamydomonas flagella

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Summary
The movements of cilia and flagella are driven by multiple species of dynein heavy chains (DHCs), which constitute inner- and outer-dynein arms. In Chlamydomonas, 11 DHC proteins have been identified in the axoneme, but 14 genes encoding axonemal DHCs are present in the genome. Here, we assigned each previously unassigned DHC gene to a particular DHC protein and found that DHC3, DHC4 and DHC11 encode novel, relatively low abundance DHCs. Immunofluorescence microscopy revealed that DHC11 is localized exclusively to the proximal ~2 μm region of the ~12 μm long flagellum. Analyses of growing flagella suggested that DHC3 and DHC4 are also localized to the proximal region. By contrast, the DHC of a previously identified inner-arm dynein, dynein b, displayed an inverse distribution pattern. Thus, the proximal portion of the flagellar axoneme apparently differs in dynein composition from the remaining portion; this difference might be relevant to the special function performed by the flagellar base.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/122/9/1306/DC1

Key words: Cilia, Mass spectroscopy, Immunofluorescence microscopy, Phylogeny

Introduction
Cilia and flagella are cell organelles that produce fluid flow over the surface of cells or propel cells in liquid media. In mammals, ciliary and flagellar movements are particularly important in respiratory and reproductive processes, and recent studies have revealed their involvement in extracellular signal transduction related to various key steps in development. For example, cilia in the mammalian embryo convey signals that determine the left-right asymmetry of the body (Afzelius, 1985; Nonaka et al., 1998; Hirokawa et al., 2006). The movement of cilia and flagella is driven by multiple dynein molecules that constitute the inner and outer dynein arms of axonemes. Dyneins generate sliding force between adjacent outer doublets, which is converted to axonemal bending. The mechanism that converts the sliding movement into cyclical axonemal bending is not known, but most probably involves coordinated activities of dynein molecules with diverse properties.

Studies using Chlamydomonas mutants have shown that inner- and outer-dynein arms are strikingly different in molecular organization, arrangement on the doublet microtubules, and force production properties (for a review, see Kamiya, 2002). Outer-arm dynein comprises a single kind of huge complex of three dynein heavy chains (DHCs) and several intermediate and light chains (for a review, see DiBella and King, 2001; King and Kamiya, 2009). This complex is linearly aligned on the doublet microtubule at 24 nm intervals. By contrast, inner-arm dynein has been shown to comprise seven different species, designated dynein a-g (Kagami and Kamiya, 1992). Each of these dyneins contains one (all species except dynein f, which is also called I1 dynein) dynein; this dynein will be hereafter called f/I1 or two (dynein f/I1) distinct DHCs and several smaller proteins (for a review, see Witman et al., 1994; Porter, 1996; Porter and Sale, 2000; DiBella and King, 2001; Kamiya, 2002).

Inner-arm dyneins are arranged on the doublet microtubule in a complex manner with a unit repeat length of 96 nm (Mastronarde et al., 1992; Porter and Sale, 2000; Nicastro et al., 2006). The arrangement of inner-arm dyneins might be more complex, because analysis of dynein composition in short flagella has suggested that some dyneins are preferentially localized to distal or proximal axoneme regions (Piperno and Ramanis, 1991). Non-uniform distribution of outer-arm DHCs in human cilia has been observed by immunofluorescence microscopy (Fliegauf et al., 2005; Samant et al., 2005). However, no direct observation of any region-specific DHC has been made in the flagella of Chlamydomonas, a model organism that has offered the most extensive information about the structure and function of cilia and flagella.

In Chlamydomonas, 11 DHC genes encoding putative inner-arm DHCs have been identified by mRNA analysis (Porter et al., 1996; Porter et al., 1999; Perrone et al., 2000), and eight inner-arm DHC proteins have been found in the axoneme (Kagami and Kamiya, 1992). Transcription levels of these genes (with the exception of DHC11, which has not been examined) have been shown to increase after deflagellation, indicating that they encode flagellar proteins (Porter et al., 1996; Perrone et al., 2000). Therefore, there must be three more inner-arm DHCs that have not yet been detected. Two DHCs of dynein f/I1 are known to be encoded by the DHC1 and DHC10 genes (Myster et al., 1997; Perrone et al., 2000), and the DHC of dynein c is encoded by the DHC9 gene (Yagi et al., 1992). However, the DHC genes corresponding to the remaining five known inner-arm species (dynein a, b, d, e and g) have not yet been assigned. Furthermore, genes encoding the three putative inner-arm DHCs yet to be identified are also unknown.

In this study, we determined the corresponding genes encoding the DHC of the five remaining known species by mass spectroscopy. In addition, we found three, novel DHCs, encoded by DHC3, DHC4 and DHC11 genes, present in small amounts in axonemes. Thus,
each of the 11 putative inner-arm DHC genes has been assigned to a particular dynein protein species. Of special interest, we found by immunofluorescence microscopy that DHC11 preferentially localizes to the proximal ~2 μm portion of the axoneme. The other two novel DHCs are also likely to localize in the proximal region. We propose that the minor inner-arm dyneins substitute for major inner-arm dyneins in the flagellar proximal portion to perform special functions in the beating mechanism.

**Results**

**Mass-spectroscopic analysis of inner-arm DHCs**

The seven inner-arm dynein species previously identified in *Chlamydomonas* are made up of a total of eight DHCs (Kagami and Kamiya, 1992), of which only three have been correlated with particular DHC genes (see Introduction). To identify the genes of the remaining five DHCs, each DHC band was analyzed by mass spectroscopy. Inner-arm dyneins were purified from the high-salt extract of *oda1* outer-armless axonemes by ion-exchange chromatography (Fig. 1A). Each fraction was subjected to SDS-PAGE, and the DHC bands were cut out from the silver-stained gel (Fig. 1B, arrowheads). Mass-spectroscopic analyses of the DHCs in the fractions of dynein a, b, d and g identified the proteins as the gene products of *DHC6, DHC5, DHC2* and *DHC7*, respectively (supplementary material Table S1). Two heavy chain genes, *DHC2* and *DHC8*, were detected for dynein e (supplementary material Table S1). However, for the DHC4 and DHC11 genes, we surmised that they were also DHCs. Mass-spectroscopy of these bands showed that the upper band was encoded by *DHC11* and the lower band by *DHC4* (supplementary material Table S1).

**Identification of two novel DHC bands in the SDS-PAGE patterns**

Identification: After identifying the genes corresponding to eight known inner-arm DHCs, three unassigned DHC genes remained: *DHC3, DHC4* and *DHC11*. (1) To determine whether or not the products of these genes are present in the axoneme, we used two strategies: (1) a search for novel bands with apparent molecular masses of about 500 kDa in SDS-PAGE bands; and (2) immunoblot analysis using antibodies raised against the sequences encoded by these unassigned DHC genes.

When SDS-PAGE was carried out with a large amount of *oda1* axonemes, two novel, faint bands appeared above the band of DHC1 (Fig. 2A). Interestingly, the upper band was absent in samples prepared from the axonemes of double mutants *oda1ida4* and *oda1ida5*, and the lower band was reduced in intensity only in the axonemes of *oda1ida5*. Mutant *ida4* has a mutation in the gene of inner-arm dynein light chain p28, and lacks dyneins a, c and d (Kagami and Kamiya, 1992; LeDizet and Piperno, 1995). Mutant *ida5* lacks the gene encoding conventional actin, and lacks dyneins a, c, d and e (Kato et al., 1993; Kato-Minoura et al., 1997). Because the appearance of the two novel protein bands depended on the presence of p28 and conventional actin, as in other inner-arm dyneins, we surmised that they were also DHCs. Mass spectrometry of these bands showed that the upper band was encoded by *DHC11* and the lower band by *DHC4* (supplementary material Table S1).

**Immunological detection of DHC3**

We raised polyclonal antibodies against DHC3, DHC4 and DHC11 using bacterially produced polypeptides corresponding to the N-
terminal sequences characteristic of the respective DHCs. Immunoblot analyses of *oda1* axonemes using these antibodies gave rise to bands representing large molecular mass proteins in the same area as other DHCs, in addition to several non-specific bands in a lower molecular mass region (Fig. 3A). The bands in the DHC region disappeared or decreased in intensity when the sample had been irradiated with UV in the presence of ATP and vanadate; a condition that induces photobleaching of DHC (Lee-Eiford et al., 1986) (Fig. 3A). After UV irradiation, each antibody detected one (in DHC3 and DHC4) or two (in DHC11) 150-200 kDa bands not present before irradiation. These bands most probably correspond to the N-terminal DHC fragment(s) produced by the cleavage. This result clearly indicates that each novel band corresponds with a distinct DHC, and that DHC3 is also present in the axoneme. The lower-mass bands in the DHC region detected by DHC3 and DHC11 antibodies (Fig. 3A, filled circles) might represent degraded products or splice variants. In the original SDS-PAGE pattern of axonemes, a fairly strong band was present at the DHC3 band position (Fig. 3A, leftmost two lanes). No change in density of this band was observed before and after UV irradiation, but the DHC3 band detected by immunoblot analysis underwent photocleavage (Fig. 3A, arrowhead). The DHC3 band is thus most probably masked by a band of an unidentified non-dynein protein. We conclude that the amount of DHC3 must be very low, as the band intensity did not show a detectable change after the cleavage of DHC3 because of this overlapping band. An interesting feature of DHC3 is its unusually large size. Using 5% acrylamide SDS-PAGE without urea (supplementary material Fig. S1A), we estimated its apparent molecular mass to be ~100 kDa larger than that of DHC1 (fzDHC of dynein f11), which has the largest molecular mass (535 kDa) of any of the previously known *Chlamydomonas* axonemal DHCs.

The presence of the novel DHCs was confirmed by immunoblot analysis of the axonemes of mutants deficient in outer-arm dynein and p28 (*oda1ida4*) or actin (*oda1ida5*; Fig. 3B). The DHC3 antibody detected two bands in *oda1* and *oda1ida4* axonemes, and a very faint band in *oda1ida5* (Fig. 3B). Thus, DHC3 is present in all of the mutant axonemes, although its amount in *oda1ida5* must be very low. The DHC4 antibody detected a modest band from *oda1* and *oda1ida4* and a weak band from *oda1ida5*; the DHC11 antibody detected a band from *oda1*, but no bands from either *oda1ida4* or *oda1ida5*.

### Localization of DHC11 to the proximal portion of the axonemes
The high specificity of DHC11 antibody (Fig. 3A; supplementary material Fig. S2) allowed us to locate DHC11 by immunofluorescence microscopy. For comparison, we also examined the localization of two previously identified dynein species, dynein b (DHC5) and dynein c (DHC9), for which we could obtain specific antibodies (supplementary material Fig. S2).

The DHC11 antibody preferentially stained a short proximal region of axonemes in the nucleus-flagellar apparatuses, the complexes of two axonemes, two basal bodies and a nucleus (Fig. 4A). The staining was uniform in the proximal fifth of the total axoneme, but sharply decreased to <10% beyond this region (Fig. 4B). The average length from the proximal end to the point of the sharp intensity change was 2.1±0.3 μm (supplementary material Fig. S3). By contrast, the DHC9 antibody uniformly stained the entire length of the axoneme (Fig. 4A,B). Unexpectedly, DHC5, another major DHC, displayed a distribution pattern that was roughly inverse to the DHC11 pattern; in about 60% of axonemes, DHC5 staining was observed along the axoneme length, but its intensity decreased in the proximal ~2 μm portion (Fig. 4A,B). Thus, these three inner-arm DHCs display distinct localization patterns.

### Change in the relative contents of novel DHCs during flagellar elongation
To examine localization of DHC3 and DHC4, for which available antibodies lacked the necessary specificity for use in immunolocalization, we measured the relative change in the amounts of these DHCs during flagellar regeneration after amputation. We reasoned that if these minor DHCs were localized to a particular section of the axoneme, the content ratio of these and other dyneins would change according to flagellar length. For comparison, we also examined the amounts of DHC5 (dynein b), DHC9 (dynein c) and DHC11.

The relative band intensity of DHC11 was found to be higher in short flagella, isolated 30 minutes after amputation, than in normal-length flagella isolated 300 minutes after amputation (Fig. 5B,C). By contrast, the relative band intensity of DHC9 was constant throughout the time course of flagellar regeneration (Fig. 5B,C). These results are consistent with the proximal localization of DHC11 and the uniform localization of DHC9 in the axoneme as seen by immunofluorescence microscopy. Like DHC11, DHC3 and DHC4 thus most probably distinguished by the presence of a non-dynein protein, which is most likely the N-terminal DHC fragment produced by photocleavage.

![Fig. 3. Immunoblot analysis using specific DHC antibodies. (A) Analysis of *oda1* axonemes before and after photocleavage of DHCs. SDS-PAGE was carried out on 4% acrylamide and 6 M urea gel. The gel was stained with silver (far left two lanes), or analyzed by immunoblot using DHC3, DHC4, and DHC11 antibodies. UV+ and − denote axoneme samples with or without UV irradiation in the presence of ATP and vanadate, a condition that induces photocleavage of DHCs. With each antibody, a band in the DHC region (filled arrowheads) was detected in the sample without UV irradiation. After UV irradiation, the band disappeared or weakened, and a new, 150-200 kDa band (open arrowheads) was detected. The band appearing after UV irradiation is most probably the N-terminal DHC fragment produced by photocleavage. In DHC3 and DHC11, weak bands migrating below the DHC bands (filled circles) also appeared to undergo photocleavage. These weak bands might be DHC degradation products or splice variants. Note that the DHC3 band appears at a significantly higher position than DHC4 and DHC11 bands. (B) Immunoblot analyses of *oda1, oda1ida4* and *oda1ida5* axonemes using DHC3, DHC4, DHC11 and DHC5 antibodies. DHC5 antibody was used as a positive control. Only the high molecular mass region of the blot containing the area around DHC bands is shown. DHC3 and DHC4 antibodies detected their respective bands in all three axoneme samples, but the band intensities were significantly reduced in *oda1ida5* (filled and open arrowheads). By contrast, the DHC11 antibody detected a band only in *oda1* axonemes. DHC5 antibody detected a band in all three samples.](image-url)
also showed higher staining densities in shorter flagella (Fig. 5B,C). Essentially the same features were observed for the band densities of DHC4 and DHC11 in SDS-PAGE gels stained with Sypro Ruby (supplementary material Fig. S4). By contrast, the band density of DHC1 (the α-DHC of dynein f/i1) did not show any length-dependent change. These results strongly suggest that DHC3 and DHC4 localize to the proximal portion of the flagella, as does DHC11.

The band intensity of DHC5 changed with flagellar length in a different manner. An extremely weak band was detected 30 minutes after amputation (Fig. 5B,C), suggesting that a much smaller amount of DHC5 is present in the proximal portion of the flagella than in the distal portions. This observation is qualitatively consistent with the low DHC5 content in the axoneme proximal portion observed by immunofluorescence microscopy (Fig. 4A,B). However, the band density detected 30 minutes after flagellar amputation seemed to be too low to account for the intensity of the DHC5 signal in the image of long flagella (Fig. 4A,B). This suggests that DHC5 is incorporated into the axoneme more slowly than the rate of flagellar growth.

DHC5 slowly localizes to the distal region of the axoneme

The above-mentioned discrepancy in the DHC5 band intensity prompted us to compare its localization patterns in flagella of various lengths. To ensure comparison under identical staining conditions, we examined a mixture of axonemes isolated from cells that had undergone flagellar regeneration for different time periods (Fig. 6A). Fig. 6B shows examples of DHC distribution patterns along the length of short and long axonemes. DHC11 and DHC9 distribution patterns were unchanged irrespective of the length of axoneme: in both short and long flagella, DHC11 signals were fairly strong in the proximal ~2 μm region, and DHC9 signals were constant along the entire length (Fig. 6A,B). Thus, apparently, DHC11 becomes incorporated into the axoneme with flagellar growth until the flagellar length exceeds ~2 μm, and DHC9 becomes incorporated into the axoneme concomitantly with flagellar growth. In sharp contrast to DHC11 and DHC9 distribution, the DHC5 distribution pattern along the length of axonemes in the same mixed-length flagella depended on the axonemal length: the shorter axoneme displayed extremely low DHC5 staining, and the longer axoneme showed fairly strong DHC5 signals except for the proximal ~2 μm region. By contrast, DHC5 antibody also stained axonemes along their entire length; but in ~60% of axonemes, DHC5 antibody signal was weaker in a short region near the proximal end of the axoneme (white arrowheads) than in the rest.

In the remaining 40% of axonemes, the axonemes appeared to be uniformly stained along their entire length. Scale bar: 10 μm. (B) Examples of DHC distribution. (Upper and middle panels) Higher magnification immunofluorescence images with DHC (green) and α-tubulin (magenta) signal distributions along the length of the axonemes. The regions analyzed are indicated by double arrows in upper panels. Strong DHC11 signals were present in the proximal ~2 μm region. The signal in the more distal region was ~10% that of the proximal region. By contrast, DHC9 signals were uniformly intense along the entire length of the flagellum. DHC5 signals appear to be present along the entire flagellar length; however, the signal intensity was weaker in the proximal ~2 μm region of the axoneme than in the distal region. The signal intensity in the proximal region was ~50% that of the distal region. Because of experimental limitations such as non-specific binding of antibodies, we cannot conclude whether DHC11 and DHC5 are completely absent from the distal and proximal regions, respectively, of the axoneme. However, the variability of DHC5 distribution as mentioned above appears to suggest that the change in localization of these DHCs does not occur in an all-or-none manner. Scale bar: 10 μm.

Fig. 4. Immunolocalization of DHC11, DHC9 and DHC5 in the axoneme. (A) (Upper panels) DHC11 localization. Nucleoflagellar apparatuses (see Materials and Methods) were stained with (left) DHC11 antibody and (middle) an α-tubulin antibody. The far right panel shows the merged images. The DHC11 antibody preferentially stained a portion near the proximal end of each axoneme, and the α-tubulin antibody uniformly stained the entire length of the axoneme. (Middle and lower panels) DHC9 and DHC5 localization. DHC9 antibody showed staining of axonemes along their entire length. This antibody also stains the nucleus. DHC5 antibody also stained axonemes along their entire length; but in ~60% of axonemes, DHC5 antibody signal was weaker in a short region near the proximal end of the axoneme (white arrowheads) than in the rest. In the remaining 40% of axonemes, the axonemes appeared to be uniformly stained along their entire length. Scale bar: 10 μm. (B) Examples of DHC distribution. (Upper and middle panels) Higher magnification immunofluorescence images with DHC and α-tubulin antibodies, respectively. (Lower panels) DHC (green) and α-tubulin (magenta) signal distributions along the length of the axonemes. The regions analyzed are indicated by double arrows in upper panels. Strong DHC11 signals were present in the proximal ~2 μm region. The signal in the more distal region was ~10% that of the proximal region. By contrast, DHC9 signals were uniformly intense along the entire length of the flagellum. DHC5 signals appear to be present along the entire flagellar length; however, the signal intensity was weaker in the proximal ~2 μm region of the axoneme than in the distal region. The signal intensity in the proximal region was ~50% that of the distal region. Because of experimental limitations such as non-specific binding of antibodies, we cannot conclude whether DHC11 and DHC5 are completely absent from the distal and proximal regions, respectively, of the axoneme. However, the variability of DHC5 distribution as mentioned above appears to suggest that the change in localization of these DHCs does not occur in an all-or-none manner. Scale bar: 10 μm.
In this study we assigned five dynein heavy chain genes (DHCs) to particular inner-arm species (Fig. 1; supplementary material Table S1). Taken together with those DHC genes previously determined for dynein l1 (Myster et al., 1997; Perrone et al., 2000) and dynein c (Yagi et al., 2005), the present study has completed the gene assignment of all known *Chlamydomonas* inner-arm dyneins. In addition, we identified the products of the DHC3, DHC4 and DHC11 genes as novel DHCs that are present in small amounts in axonemes. These novel dyneins are most probably localized in a short, proximal segment of the flagellum. This raises an important possibility that the proximal portion of cilia and flagella might generally consist of specialized DHCs.

**Discussion**

In this study we assigned five dynein heavy chain genes (DHCs) to particular inner-arm species (Fig. 1; supplementary material Table S1). Taken together with those DHC genes previously determined for dynein l1 (Myster et al., 1997; Perrone et al., 2000) and dynein c (Yagi et al., 2005), the present study has completed the gene assignment of all known *Chlamydomonas* inner-arm dyneins. In addition, we identified the products of the DHC3, DHC4 and DHC11 genes as novel DHCs that are present in small amounts in axonemes. These novel dyneins are most probably localized in a short, proximal segment of the flagellum. This raises an important possibility that the proximal portion of cilia and flagella might generally consist of specialized DHCs.

**Relationship among DHCs in *Chlamydomonas* flagella**

The *Chlamydomonas* genome contains 16 DHC genes (Pazour et al., 2006) (Fig. 7), of which seven had been correlated to particular DHCs: three outer-arm DHCs (Mitchell and Brown, 1994; Wilkerson et al., 1994), three inner-arm DHCs (Myster et al., 1997; Perrone et al., 2000; Yagi et al., 2005), and one cytoplasmic DHC that functions in retrograde intra-flagellar transport (Pazour et al., 1999; Porter et al., 1999). In this study we identified the products of eight DHC genes, resulting in the assignment of 15 of the 16 DHCs in this organism. The identity of the remaining DHC (ID 206178 registered in *Chlamydomonas* genome database version 3) remains to be determined; it was classified as a cytoplasmic dynein (Fig. 7) (Porter et al., 1999; Pazour et al., 2006), although a recent phylogenetic study suggested that it is an axonemal dynein (Wickstead and Gull, 2007).

Assignment of all inner-arm DHCs to particular DHC genes enabled us to correlate the properties of various inner-arm dyneins with their phylogenies (Fig. 7, Table 1). As shown in recent studies (Morris et al., 2006; Wickstead and Gull, 2007; Wilkes et al., 2008), single-headed DHCs as a whole makes up a single large family which can be further classified into three groups: group IAD3 comprising DHC4, DHC5 (the DHC of dynein b), DHC6 (dynein a), DHC8 (dynein e), DHC9 (dynein c) and DHC11; group IAD4 comprising DHC2 (dynein d); and group IAD5 comprising DHC3 and DHC7 (dynein g) (Fig. 7). This phylogenetic classification of various dyneins might well be related to their structural or functional properties. For example, DHC2 is the only DHC that belongs to IAD4 and it is the only DHC that is assembled with two widely conserved subunits, p38 and p44 (Yamamoto et al., 2006; Yamamoto et al., 2008). With regard to functional properties, only dyneins belonging to IAD4 (dynein d) and IAD5 (dynein g) have been shown to produce torques that cause microtubule bending (Kikushima and Kamiya, 2008).

Thus our results have provided a basis for further studies on the structure-function relationship of various dyneins. However, the roles and distributions specific to these DHCs might not be directly correlated with those of DHC species in other eukaryotes; although the three main groups of single-headed IADs are present in all groups of eukaryotes, the diversification of multiple IADs within each group, including minor dyneins, took place late in eukaryotic evolution (Morris et al., 2006). Further functional and distribution studies will be needed in each eukaryote for a comprehensive understanding of dynein function.

**Characteristics of the novel DHCs**

The DHC phylogeny indicates that the novel ‘minor’ DHC species have similar counterparts in the ‘major’ single-headed inner-arm DHCs: DHC3 is most similar to DHC7 (dynein g), DHC4 to DHC5 (dynein b), and DHC11 to DHC9 (dynein c). If we assume that each major and minor pair shares subunit composition, then it follows that DHC3 and DHC4 contain centrin and actin, but that DHC11 contains p28 and actin as subunits. This idea is consistent with the presence of DHC3 and DHC4, and the absence of DHC11, in *odaiidae*4 axonemes, which lack p28 (Fig. 3B). DHC3 and DHC4 are present in reduced amounts in *odaiidae*5 axonemes, which lack conventional actin most probably because NAP (an unconventional actin) can partially substitute for actin (Kato et al., 1997) in these dyneins.

Despite the presence of closely similar counterparts in the major dynein group, the three novel DHCs appear to have significantly larger molecular masses than the other inner-arm DHCs (Fig. 2, Fig. 3A). DHC3 has a particularly high molecular mass (Fig. 3A). The position of this DHC band in the SDS-PAGE pattern suggests that its molecular mass is about 100 kDa larger than that of any other DHCs (supplementary material Fig. S1A). Such a large DHC, either axonemal or cytoplasmic, has not been previously reported in any organism. Its size is consistent with the previous observation that its mRNA, detected by northern blot analysis, is significantly larger than the mRNAs of other inner-arm DHCs [figure 5 in Porter et al. (Porter et al., 1996)]. After UV-photocleavage of DHC3, immunoblot analysis detected an N-terminal fragment of 200 kDa (Fig. 3A). N-terminal fragments of this size are also observed in the photocleavage products of other dyneins. Therefore, the unusually large size of DHC3 must be due to a large sized motor
domain. In accordance with this idea, several extra sequences, not seen in other DHCs, are present in the predicted amino acid sequence in the AAA2, AAA5, AAA6 and C-terminal domains of its motor domain (supplementary material Fig. S1B). The extra sequences add up to approximately 90 kDa. Determining the role of these unique sequences in the motor domain is an interesting future problem.

Localization of novel DHCs in the axoneme
Immunofluorescence microscopy revealed distinct localization of DHC11, DHC9 (DHC of dynein c) and DHC5 (DHC of dynein b; Fig. 6C). DHC11 was localized to the proximal ~2 μm region of the flagellum (Figs 4 and 6). This is the first report of any particular dynein heavy chain being localized exclusively near the proximal end of the axoneme, although some outer-arm DHCs of mammalian cilia and flagella have been shown to be present only in the axoneme proximal or distal half (Fliegauf et al., 2005; Samant et al., 2005).

We also observed that the relative amounts of DHC3, DHC4 and DHC11 in the axoneme are greater in shorter flagella during the process of regeneration after amputation. These results suggest that DHC3 and DHC4, as well as DHC11, localize to a short proximal region of the flagellum. Previous studies using various techniques of electron microscopy and image analysis have indicated that five to seven discrete inner-arm dyneins are arranged within the 96 nm repeat unit of the outer doublet (Goodenough and Heuser, 1985; Mastronarde et al., 1992; Nicastro et al., 2006). If the minor species of inner-arm dyneins in the proximal ~2 μm portion are arranged in the same pattern as the major inner-arm dyneins in the remaining part of the axoneme, the amount of a single minor DHC in the axoneme would be about 15-20% of a major inner-arm DHC that is uniformly present along the length of the 10-12 μm axoneme. This estimate is roughly consistent with our experimental finding that the amounts of DHC11 and DHC4 in the axoneme are each about 10% that of DHC1 (Fig. 2B).
These considerations led us to speculate that the minor inner-arm dyneins might replace some of the major dyneins in the proximal region. We first thought that such replacement would take place between the most phylogenetically similar pairs: DHC3 and DHC7; DHC4 and DHC5; DHC11 and DHC9. However, the observation that DHC9 is present along the entire length of the axoneme (Figs 4 and 6) does not support this idea. At the same time, the finding that DHC5 is preferentially localized distal to the proximal 2 \( \mu \text{m} \) region (Figs 4 and 6) supports the general idea that the proximal portion is composed of minor DHCs in place of some major DHCs. Determination of the exact mode of replacement between the major and minor DHCs awaits further studies.

Piperno and Ramanis (Piperno and Ramanis, 1991) previously suggested that different sets of inner-arm dynein species are present in the proximal, mid and distal portions of the flagella. In their analyses, the inner-arm dyneins are largely classified into three groups, I1, I2 (I2') and I3 (I3'), and sets of the three dynein groups are arranged every 96 nm from the proximal to distal end in the order of I1I2'I1I2I3-I1I2I3. Considering the correspondence between these dyneins and dynein a-g (Kagami and Kamiya, 1992; Piperno, 1995), the suggested pattern is translated as a proximal to distal arrangement of f/e/c/b-f/e/c/g-f/ad/g. Because later image analyses localized dyneins a, d and e in the same 96 nm repeating unit of the axoneme (Mastronarde et al., 1992; Gardner et al., 1994) and dynein b (DHC5) is localized distally in the axoneme (Figs 4 and 6), this dynein arrangement scheme is clearly incorrect. However, our present study supports the general idea that some inner-arm dyneins are specifically localized near the proximal end. At the same time, it remains possible that some dyneins, minor and/or major species, are attached only to a subset of outer-doublet microtubules, or that two species of dyneins are randomly mixed in some region of the axoneme. Determining the arrangement of inner-arm dyneins at an ultrastructural level requires further studies.

**Function of minor DHCs and mechanism of their localization**

The present study raises questions regarding the function and location of the novel dynein species. First, what is the function of these minor dyneins in the mechanism of flagellar beating? It is possible that dyneins in the proximal region might serve a specialized function such as bend initiation (Brokaw, 1994). However, it is unlikely that the minor dyneins are absolutely necessary for flagellar beating, because the mutant \textit{ida5} displays moderate flagellar beating, even though it completely lacks DHC11, has greatly reduced amounts of DHC3 and DHC4, and lacks dynein a, c, d and e (Kagami and Kamiya, 1992). Currently, direct evaluation of minor dynein functions is not possible, because no mutants lacking only minor dyneins are available. However, the low efficiency flagellar beating of mutant \textit{ida5} has led us to speculate that these dyneins might function to optimize flagellar waveform at the flagellar basal portion.

**Table 1. \textit{Chlamydomonas} inner-arm dyneins**

| Type: | Major | 1-headed |
|------|-------|---------|
| Species | f | a | b | c | d | e | g |
| Other name* | I1 | I2' | I3' | I2A | I2' | I2B | I3 |
| DHC gene | 1', 10§ | 6 | 5 | 9§ | 2 | 8 | 7 |
| Phylogenetic group** | IAD-1a-IAD-1b | IAD-3 | IAD-3 | IAD-3 | IAD-4 | IAD-3 | IAD-5 |
| Composition of IC/LC‡‡ | 140 kDa | 138 kDa | 97 kDa |
| Actin | p28 | Actin | p28 | 44 kDa | Actin | Actin | (Actin) | (Actin) | (Actin) |
| Centrin | Centrin | Centrin | (Centrin) | (Centrin) |
| MT rotation§§ | – | + | – | + | + | + | + |
| MT bending¶¶ | – | – | – | + | + | + | + |

IC/LC, intermediate chain/light chains; parentheses denote those proteins inferred from circumstantial evidence.

References: *Piperno (1995); †Myster et al. (1997); ‡Perrone et al. (2000); ¶Yagi et al. (2005); **Morris et al. (2006), Wickstead and Gull (2007), Wilkes et al. (2008); ††King and Kamiya (2009); ‡‡Kagami and Kamiya (1992); ¶¶Kikushima and Kamiya (2008).
A second question is how are minor dyneins localized exclusively to the proximal portion of the axoneme, but another dynein, DHC5, is localized exclusively to the distal portion. One possibility is that specific docking structures for these dyneins are present in particular regions in the axoneme, although this assumption raises a new question of how the docking structures switch at a particular position (e.g., 2 μm from the base) during flagellar growth. Another possibility is that certain pairs of DHCs compete for the same docking site, and that the expression of the DHC pairs switch some time after the onset of flagellar growth. For example, after flagellar amputation, the protein DHC5 might be expressed later than a minor DHC species that competes with DHC5 for the docking site. This mechanism explains the distal localization and the lag in appearance of DHC5 in growing flagella.

Recently, several proteins were reported to localize only to the proximal region of the axoneme. For example, A GG2, a membrane protein involved in phototactic signal transduction (Iomini et al., 2006), and Fa2p, an axoneme-bound NIMA-related kinase (Mahjoub et al., 2004), were localized to a short (<1 μm) proximal region of the flagellum. These data, taken together with our present findings, demonstrate that the axoneme is longitudinally differentiated. The proximal region of cilia and flagella might well perform specific functions other than propagating bending waves. Elucidation of the physiological significance of longitudinal compartmentalization, as well as the mechanism that produces it, are important points of future study.

Materials and Methods

Strains and culture

Chlamydomonas reinhardtii 137C (wild type), cw92 (a mutant lacking a cell wall), oda1 (a mutant lacking outer-arm dynein) (Kamya, 1988) and double mutants oda1ida4 and oda1ida5 were used. Mutant ida4 carries a mutation in the dynein light chain p28 gene (Kamya et al., 1991; Ledizet and Piperno, 1995) and lacks inner-arm dynein species a, c, and d. Mutant ida5 lacks the gene encoding conventional actin (Kato-Minoura et al., 1997) and inner-arm species a, c, d, and e. Cells were grown at 25°C in Tris acetate phosphate medium (Gorman and Levine, 1965) with aeration on a 12:12 hour light-dark cycle.

Isolation of axonemes and fractionation of dynein

Preparation of flagellar axonemes and high-salt extract of axonemes, and purification of dynein were carried out according to the method of Kagami and Kamya (Kagami and Kamya, 1992). Briefly, flagella were detached from the cell body by treatment with dibucaine-HCl, purified through a differential centrifugation series, and demembranated with HMDE solution [30 mM HEPES (pH 7.4), 5 mM MgSO4, 1 mM DTT, 1 mM EGTA] containing 0.5% Nonidet P40 (NP-40). For dynein extraction, axonemes were suspended in HMDE containing 0.6 M KCl, and precipitated by centrifugation. The supernatant fraction, referred to as crude dynein extract, was fractionated into dynein species by high-pressure liquid chromatography on a Mono-Q column (GE Healthcare Bioscience, Tokyo, Japan). For analysis of flagella of various lengths, flagella were isolated from oda1 cells undergoing flagellar regeneration after pH shock demembrallation (Witman et al., 1972).

SDS-PAGE

The composition of DHCs was analyzed by SDS-PAGE gel, and subjected to peptide-mass fingerprinting (PMF) analysis using MALDI-TOF mass spectrometry (Applied Biosystems, Foster, CA). Search for the most plausible candidate for the protein contained in each band was performed in the database of Chlamydomonas release 2.0 predicted proteins at the JGI website (http://genome.jgi-psf.org/chil2/chil2.download.ipfi.html) using the Mascot Search program (Matrix Science, Boston, MA).

Mass-spectroscopic analysis

The band containing the DHC polypeptide of each dynein species was cut out from the SDS-PAGE gel, and subjected to peptide-mass fingerprinting (PMF) analysis using MALDI-TOF mass spectrometry (Applied Biosystems, Foster, CA). Search for the most plausible candidate for the protein contained in each band was performed in the database of Chlamydomonas release 2.0 predicted proteins at the JGI website (http://genome.jgi-psf.org/chil2/chil2.download.ipfi.html) using the Mascot Search program (Matrix Science, Boston, MA).

Polycatonic antibodies

Polycatonic antibodies were produced against the N-terminal polypeptides of DHC3, DHC4, DHC5, DHC9 and DHC11. The DNA fragments coding for these polypeptides were amplified by RT-PCR as described previously (Yagi et al., 2005). PCR primers were chosen and constructed using the determined or predicted cDNA sequences registered in the Chlamydomonas genome database version 2.0 (http://genome.jgi-psf.org/chil2/chil2.home.html). The forward and reverse primers for DHC3 were ATCAAGCGCCTAGTGCACCTCC and TAGCAGGTCGACGCCGCT, respectively (designed on the basis of the C270078 gene model); for DHC4, AAGGGCCAGA- TCGTGATGCTGGTCAAGGCGGCT (C20225); for DHC5, GTGCTGTTG- TGGTCAGATCTTT and GCCGTACTACATGACCTGACTC (C20038); and for DHC9, CTGTCAGGCCCAAGGCGTGAAG and CAGCGTCAGTCAGCACGGCGC. The DHC11 gene has been registered in the database as two separate genes (C950023 and C950024). Here, we used GCAGGCGTGATGAGCCTATCA (C950023) as the forward primer and CATGATGATGCGACGCTACTC (C950024) as the reverse primer. These fragments correspond to amino acids 821-1020 in the C270078 gene product, amino acids 620-771 in C20225, amino acids 88-649 in C20038, amino acids 88-772 in DHC9, and amino acids 834-915 in the gene product of C950023 joined to amino acids 1-531 in the gene product of C950024. The DNA sequences were cloned into the plasmid pCold (Takara, Tokyo, Japan) for His-tagged fusion protein expression in E. coli cells. Polypeptides were affinity-purified using Ni-NTA agarose beads (Qiagen, Tokyo, Japan), and used as antigens to immunize rabbits.

Immunoblotting

Immunoblotting was carried out using the method of King et al. (King et al., 1986). Immunoreactive bands were detected using horseradish peroxidase-conjugated secondary antibody and an ECL Advance Kit (GE Healthcare Bioscience, Tokyo, Japan).

Immunofluorescence microscopy

Immunofluorescence microscopy was performed according to the method of Taillon et al. (Taillon et al., 1992). The samples of whole cells or flagella were first demembranated with 1% NP-40. Nucleoflagellar apparatuses (complexes of two axonemes, two basal bodies and a nucleus) were produced by the demembranation of cw92 cells (Wright et al., 1985). These samples were fixed with 2% paraformaldehyde for 5 minutes at room temperature, followed by treatment with 10°C acetone. The fixed samples were stained with an α-tubulin monoclonal antibody (T5168, Sigma) and with either preimmune serum or affinity-purified DHC5, DHC9 and DHC11 antibodies diluted in the blocking buffer. FITC-labeled anti-rabbit IgG (Sigma-Aldrich, Japan) or Rhodamine-labeled anti-mouse IgG (Sigma) was used after secondary antibody incubation. After blocking, the samples were washed, mounted, and observed under a fluorescence microscope. Images were acquired using an Axioscope microscope with a ×63/1.4 NA objective lens (Carl Zeiss Microimaging) and a CoolSNAP cooled-CCD camera (Roper). Images were prepared for publication using Photoshop Elements 5.0 (Adobe).

Phylogenetic analysis

Phylogenetic analyses were carried out for all DHC genes in the Chlamydomonas genome using the sequence of the entire molecule. Multiple-alignment analysis was performed using the ClustalW program (http://www.ddbj.nig.ac.jp/search/clustalw). A phylogenetic tree was constructed with the Tree View program (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). The DHC sequences (cDNA accession numbers) used were cytoplasmic DHC1b (AJ312478), DHCα (LC52049), DHCβ (U20963), DHCγ (U25303), DHC1 (AJ2123806), DHC9 (AB232152), and DHC10 (AJ242535-AJ242525). For dynes with cDNA sequences not yet determined, the predicted sequences registered in the Chlamydomonas genome databases (version 1, http://genome.jgi-psf.org/chil2/chil2.home.html; version 2, http://genome.jgi-psf.org/chil2/chil2.home.html; and version 3: http://genome.jgi-psf.org/chil2/chil2.home.html) were used.

We thank Masaumi Hirono (University of Tokyo, Tokyo, Japan) for help with molecular analysis and antibody production; Masahide Kikawa (Kyoto University, Kyoto, Japan) for encouragement and critical reading of the manuscript; and Yoshinori Fujiyoshi (Kyoto University, Kyoto, Japan) for allowing us to use facilities in his laboratory. This study was supported by a Grant-in-Aid for Scientific Research on Priority Areas to T.Y., and a Grant-in-Aid for Scientific Research (A) to R.K.

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