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.

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) 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). 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 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), but this is most probably because of its incomplete separation from dynein d (Fig. 1B). Because dynein d DHC is clearly the product of DHC2, the dynein e DHC gene should be DHC8.

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

After identifying the genes corresponding to eight known inner-arm DHCs, three unassigned DHC genes remained: DHC3, DHC4 and DHC11. 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 (fzDHC of dynein f11; 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 spectroscopy of these bands showed that the upper band was encoded by DHC11 and the lower band by DHC4 (supplementary material Table S1).

Compared with other inner-arm DHCs, DHC4 and DHC11 are of low abundance (Fig. 2A). Assessment of band intensities using a gel stained with Sypro Ruby, a fluorescent dye that has a wide range of staining linearity, indicated that the axonemal content of each of these DHCs was only about 10% of that for DHC1 (fzDHC of dynein f11; Fig. 2B). We also found that DHC4 and DHC11, unlike other major DHCs, tended to remain in the axoneme after high-salt extraction (data not shown). These features probably account for the previous difficulty in detecting these DHCs.

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 photo-cleavage 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 before and after UV irradiation, 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 (fox DHC of *Chlamydomonas*; Fig. 3B), 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 nucleo-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...
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/11) 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 axoneme 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 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.

Fig. 6. Distribution of DHC along the length of flagella. (A) (Upper panels) Distribution of DHC11, DHC9 and DHC5 along the length of flagella. 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. (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.
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 odalidae axonemes, which lack p28 (Fig. 3B). DHC3 and DHC4 are present in reduced amounts in odalidae 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 μm 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.

Fig. 7. Phylogenetic tree of Chlamydomonas dynein heavy chains. A phylogenetic tree was constructed for cytoplasmic and flagellar DHCs in Chlamydomonas using full-length sequences. For dyneins with cDNA sequences not yet determined, the predicted sequences from the Chlamydomonas genome database were used (see Materials and Methods). The single-headed DHC type can be classified into three subgroups, IAD3, IAD4, and IAD5 types, as reported previously (Morris et al., 2006; Wickstead and Gull, 2007). Minor DHCs identified in this study are indicated by arrowheads. The protein ID 206178, which was putatively assigned to conventional cytoplasmic dynein (Porter et al., 1999), might belong to the axonemal type (Wickstead and Gull, 2007).

Table 1. Chlamydomonas inner-arm dyneins

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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 distributed distally and more proximally, respectively. One possibility is that 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, AGG2, 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

Isolation of dynein and fractionation of dye

Preparation of flagellar axonemes and high-salt extract of axonemes, and purification of dye were carried out according to the method of Kamiya and Kamiya (Kamiya and Kamiya, 1992). Briefly, flagella were detached from the cell body by treatment with dibucaine-HCl, purified through a differential centrifugation series, and 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 −20°C acetone. The fixed samples were stained with 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, Tokyo) or Rhodamine-labeled anti-mouse IgG (Sigma) was used as the secondary antibody. 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.html) and 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 (AJ132478), DHC4 (L52049), DHC6 (U02963), DHC9 (AJ243806), DHC9 (AB232152), and DHC10 (AJ24253-AJ242525). For dynems with DHC sequences not yet determined, the predicted sequences registered in the Chlamydomonas genomes (version 1), http://genome.jgi-psf.org/chlre2/chlre2.home.html; version 2, http://genome.jgi-psf.org/chlre3/chlre3.home.html; and version 3: http://genome.jgi-psf.org/chlre3/chlre3.home.html) were used.

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References

Fliegel, M., Olbrich, H., Horvath, J., Wildhaber, J. H., Zhang, A., Kennedy, M., Knowles, M. R. and Ono, H. (2005). Mislocalization of DNAH5 and DNAH9 were amplified by RT-PCR as described previously (Yagi et al., 2005). PCR primers were constructed using the determined or predicted cDNA sequences registered in the Chlamydomonas genome database version 2.0 (http://genome.jgi-psf.org/chlre2/chlre2.home.html). The forward and reverse primers for DHC5 were ATCGACGGCAGTGCTC and TAGCGAGCTGAAAGGCCTC, respectively (designated on the basis of the C270078 gene model); for DHC4, AAGGCAGCA- GCCAGCAGCCTG (C20225); for DHC5, TCAGGAT- GTGTCAGATCTTC and GCCGTACCTAATGCTACCTG (C20308); and for DHC9, CTGTCAGCCCAAAGGCCTAGG and CAGCGTCACGTCACGCGC. The DHC11 gene has been registered in the database as two separate genes (C950023 and C950024). Here, we used GCAGCGGATGGGTCATG (C950023) as the forward primer and CATGATGATGGGCACGTACTC (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 C20308, 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 antisera 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 −20°C acetone. The fixed samples were stained with 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, Tokyo) or Rhodamine-labeled anti-mouse IgG (Sigma) was used as the secondary antibody. 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.html) and 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 (AJ132478), DHC4 (L52049), DHC6 (U02963), DHC9 (AJ243806), DHC9 (AB232152), and DHC10 (AJ24253-AJ242525). For dynems with DHC sequences not yet determined, the predicted sequences registered in the Chlamydomonas genomes (version 1, http://genome.jgi-psf.org/chlre2/chlre2.home.html; version 2, http://genome.jgi-psf.org/chlre3/chlre3.home.html; and version 3: http://genome.jgi-psf.org/chlre3/chlre3.home.html) were used.

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<table>
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<tr>
<th>DHC band</th>
<th>Peptides Match/Total</th>
<th>Score</th>
<th>Sequence coverage</th>
<th>JGI name</th>
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¹ Protein IDs and gene model names from the JGI database ver. 2 are shown, along with protein IDs from the database ver. 3 (in parenthesis).

² DHC7 is listed in the database (JGI, ver. 2) as two separate gene models corresponding to its N-terminal half and C-terminal half.

³ The two minor bands shown in Fig. 2.