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The DHC1b (DHC2) isoform of cytoplasmic dynein is required for flagellar assembly

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The DHC1b (DHC2) Isoform of Cytoplasmic Dynein Is Required for Flagellar Assembly

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Abstract. Dyneins are microtubule-based molecular motors involved in many different types of cell movement. Most dynein heavy chains (DHCs) clearly group into cytoplasmic or axonemal isoforms. However, DHC1b has been enigmatic. To learn more about this isoform, we isolated *Chlamydomonas* cDNA clones encoding a portion of DHC1b, and used these clones to identify a *Chlamydomonas* cell line with a deletion mutation in DHC1b. The mutant grows normally and appears to have a normal Golgi apparatus, but has very short flagella. The deletion also results in a massive redistribution of raft subunits from a peri-basal body pool (Cole, D.G., D.R. Diener, A.L. Himelblau, P.L. Beech, J.C. Fuster, and J.L. Rosenbaum. 1998. *J. Cell Biol.* 141:993–1008) to the flagella. Rafts are particles that normally move up and down the flagella in a process known as intraflagellar transport (IFT) (Kozminski, K.G., K.A. Johnson, P. Forscher, and J.L. Rosenbaum. 1993. *Proc. Natl. Acad. Sci. USA.* 90:5519–5523), which is essential for assembly and maintenance of flagella. The redistribution of raft subunits apparently occurs due to a defect in the retrograde component of IFT, suggesting that DHC1b is the motor for retrograde IFT. Consistent with this, Western blots indicate that DHC1b is present in the flagellum, predominantly in the detergent- and ATP-soluble fractions. These results indicate that DHC1b is a cytoplasmic dynein essential for flagellar assembly, probably because it is the motor for retrograde IFT.

Key words: cytoplasmic dynein • intraflagellar transport • flagella • cilia • *Chlamydomonas*

Dyneins use the hydrolysis of ATP to produce force within cells to carry out a wide variety of diverse activities, including ciliary and flagellar beating, axonal transport, vesicle and organelle movement, and alignment and maintenance of the mitotic spindle. Ciliated organisms from algae to mammals have ~15 different dynein heavy chain (DHC)[1] isoforms (Gibbons et al., 1994; Tanaka et al., 1995; Porter et al., 1996). Most of these isoforms can be grouped into three distinct classes: cytoplasmic dynein, axonemal outer arm dynein, and axonemal inner arm dynein. However, one isoform, DHC1b (also known as DHC2 in mammals), has been enigmatic. In phylogenetic analyses of DHC sequences, this isoform diverges from the trunk at a position nearly equidistant between the cytoplasmic and axonemal forms (Gibbons, 1995; Gibbons et al., 1994; Tanaka et al., 1995; Vaisberg et al., 1996). Expression of DHC1b is upregulated during cilia regeneration in sea urchin embryos (Gibbons, 1995; Gibbons et al., 1994). This pattern is similar to that of the axonemal dyneins, but differs from that of the conventional cytoplasmic DHC isoform (termed DHC1a), which is not upregulated during ciliary regeneration. These results raised the question of whether DHC1b is a specialized cytoplasmic dynein that plays a role in ciliary assembly, or a true axonemal dynein whose sequence simply resembles that of cytoplasmic dynein.

More recently, it was reported that DHC1b was expressed in all rat tissues examined, including tissues that produce no motile cilia or flagella (Criswell et al., 1996). Using immunofluorescence microscopy and an isotype-specific antibody, DHC1b was found to be concentrated in the apical regions of the cytoplasm of ciliated rat tracheal epithelial (RTE) cells, but was not detected in cilia. It was concluded that DHC1b is a cytoplasmic dynein that participates in intracellular trafficking in polarized cells. DHC1b also is expressed in HeLa cells and COS cells, which do not form motile cilia (Vaisberg et al., 1996). In the latter study, an antibody to DHC1b localized predominantly to the Golgi apparatus, and microinjection of the antibody caused dispersion of the Golgi complex. It was concluded

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1. *Abbreviations used in this paper:* DHC, dynein heavy chain; IFT, intraflagellar transport; RTE, rat tracheal epithelial.

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that DHC1b played a role in the organization and/or function of the Golgi apparatus.

The green alga *Chlamydomonas reinhardtii* is an excellent model system to study the function of dynein isoforms. We recently identified a *Chlamydomonas* mutant with a defect in the 8-kD dynein light chain (LC8) (Pazour et al., 1998) that is a component of cytoplasmic dynein, outer arm dynein, and the inner arm dynein I1 (King and Patel-King, 1995; King et al., 1996; Harrison et al., 1998). This mutant has short flagella that lack the retrograde component of intraflagellar transport (IFT). IFT is the movement of particles, rafts, from the base to the tip of the flagellum, and back, just beneath the flagellar membrane (Kozminski et al., 1993, 1995). Movement of rafts in the anterograde direction, i.e., from the base to the tip of the flagellum, toward the plus ends of the axonemal microtubules, is powered by the heterotrimeric kinesin FLA10 kinesin-II (Walther et al., 1994; Kozminski et al., 1995; Cole et al., 1998). However, the retrograde motor for IFT is not known. Because the LC8 mutant is defective in retrograde IFT and LC8 is a subunit of cytoplasmic dynein, we hypothesized that the retrograde IFT motor was cytoplasmic dynein, specifically the DHC1b isoform (Pazour et al., 1998). This hypothesis was supported by studies in *Caenorhabditis elegans* showing that defects in DHC1b specifically affect those sensory neurons that are modified cilia (Collet et al., 1998).

To determine directly if DHC1b has a role in retrograde IFT and flagellar assembly, we have isolated and sequenced partial cDNA clones encoding this isoform from *Chlamydomonas*, and identified a mutant with a deletion allele of the *DHC*1b gene. These mutant cells grow normally but have very short flagella that are filled with IFT rafts. An antibody to an IFT raft subunit indicates that the raft proteins are redistributed from the peri-basal body region, where they are predominantly located in wild-type cells (Cole et al., 1998), to the flagella. Apparently the rafts are moved into the forming flagella by FLA10 kinesin-II, but are not returned to the peri-basal body pool due to the defect in DHC1b. We also raised an antibody against DHC1b and found that this protein is present in the wild-type flagella, primarily in the membrane + matrix and ATP-extractable fractions. Thus, DHC1b is in the proper location to be involved in IFT. The results indicate that DHC1b is essential for flagellar assembly, probably because it is the motor for retrograde IFT.

Materials and Methods

Strains

Chlamydomonas reinhardtii strains used in the work include: g1 (*nit*1, *NIT*2, *agg*1, *mt*+) (Pazour et al., 1995), CC124 (*nit*1, *nit*2, *agg*1, *mt*−) (*Chlamydomonas* Genetics Center), V92.2 (*dhc1b*-1::*NIT*1, *nit*1, *mt*+) (obtained by transforming g1 with cloned *NIT*1 DNA), and 3088.4 (*dhc1b*-1::*NIT*1, *mt*+) (offspring of cross between V92.2 and CC124).

Transformation and Mutagenesis

Transformation was carried out by the method of Kindle (1990) as described in Pazour et al. (1995). Insertional mutants were isolated as described in Pazour et al. (1998). DNA was purified from 303 independently isolated mutant lines and examined by Southern blotting with the *DHC*1b cDNA clone as a probe. One line (V92.2) was found in which this gene was deleted.

RNA and DNA Isolation and Analysis

DNA was isolated from *Chlamydomonas* by digesting the cells with proteinase K as described in Pazour et al. (1998). For determination of the patterns of expression of genes, mRNA was obtained from wild-type (g1) cells before deflagellation and 30 min after deflagellation as described in Koutoulis et al. (1997). Gel electrophoresis, Southern blotting, and Northern blotting were performed using standard procedures (Sambrook et al., 1987).

Genetic Analysis

V92.2 and CC124 cells were induced to become gametes by nitrogen starvation. The gametes were treated with 15 mM dibutyryl-cAMP, and 0.15 mM papaverine (Pasquale and Goodenough, 1987; Wilson et al., 1997) for 1 h to induce formation of the mating structures. Afterwards the two cell types were centrifuged together into a pellet to facilitate fusion. After two additional hours, cells were plated on solid medium and zygotes allowed to mature for 6 d. Tetrads were dissected and analyzed by standard procedures (Levine and Ebersold, 1960; Harris, 1989) as described in Pazour et al. (1998).

Cloning Chlamydomonas Dynein Genes and Phylogenetic Analysis

The *Chlamydomonas DHC*1b cDNA was cloned by PCR using primers based on conserved regions of DHC1b from other species. Peptide sequences of the dynein isoforms in GenBank were aligned by CLUSTAL W (Thompson et al., 1994) and examined to identify regions that are highly conserved in the DHC1b isoform, but not conserved in other isoforms. The peptide, NPAGKGYG, which is highly specific for the DHC1b isoforms, was used to design a gene-specific antisense primer using *Chlamydomonas* codon bias. Similarly, three dynein-specific sense primers (based on CYLTLTQ, FNCDEG, and WGCFDEFNR peptides) were designed. Three sets of PCR reactions using Elongase (GIBCO BRL) were carried out on a cDNA library enriched for DHC sequences (Wilkerson et al., 1994). All three produced bands of the predicted size and all three PCR products were found by sequencing to be highly homologous to *DHC*1b from other species. One of these products, encoding amino acids Phe-776 to Gly-862 in Fig. 1 a, was used to screen the same cDNA library by hybridization. Eight positive phage clones were identified and the ends were sequenced. The sequences were compared to the *C. elegans DHC*1b gene to determine the relationship between the individual clones. The two overlapping clones (pGP638 and pGP639) that extended out the farthest were sequenced on both strands by the Iowa State University Sequencing Facility.

The *Chlamydomonas* DHC clone pcr4 (Wilkerson et al., 1994) also was used to screen the library enriched for DHC cDNAs. Two positive clones (pGP628 and pGP629) were identified and sequenced.

The predicted amino acid sequences of the P-loop regions were aligned by CLUSTAL W with a subset of the dyneins in GenBank and shaded with Boxshade (http://www.isrec.isb-sib.ch/software/BOX_form.html). A phylogenetic tree was drawn by PHYLIP (Felsenstein, 1989) using the UPGMA method.

Electron and Immunofluorescence Microscopy

Cells were fixed in glutaraldehyde for EM (Hoops and Witman, 1983) and processed as described in Wilkerson et al. (1995). Cells were fixed and stained for immunofluorescence microscopy by the alternate protocol of Cole et al. (1998). Images were acquired using Photometrix cameras on Zeiss Axioskop and Axioplan microscopes. Antibodies to p139, p172, and FLA10 were gifts of D. Cole (University of Idaho, Moscow, Idaho) and J. Rosenbaum (Yale University, New Haven, CT).

Antibody Production, Protein Isolation, and Western Blotting

A 1-kb Pst1 fragment of the *Chlamydomonas DHC*1b cDNA was inserted in the Pst1 site of pMAL-cR1 (New England Biolabs, Inc.). Expression of this construct in *Escherichia coli* produced a protein in which 315 amino acids (starting at the sequence QQFDAH and ending at NKLSFL) of *DHC*1b were fused to the maltose-binding protein. The fusion protein was purified by amylose affinity chromatography and antibodies were produced in rabbits (Research Genetics Inc.). Anti-DHC1b antibodies

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were purified by adsorption to and release from another DHC1b fusion protein made from the pThioHisB vector (Invitrogen Corp.) and bound to polyvinylidene difluoride membrane (Immobilon-P; Millipore Corp., Waters Chromatography) (Olmsted, 1981; King et al., 1996).

For Western blots, flagella were isolated and fractionated as described in Pazour et al. (1998), except that the demembranated axonemes were washed three times with buffer containing 10 mM $MgATP^{2-}$, and extracted with 0.6 M KCl to solubilize dynein arms. Whole cell extracts were made by resuspending log-phase cells in SDS-sample buffer, heating at 50°C for 10 min, and repeatedly drawing the sample through a 26-gauge needle to shear the DNA. Proteins were separated by SDS-PAGE (Pfister et al., 1982) and blotted onto polyvinylidene difluoride membrane by the two-step procedure of Otter et al. (1987). Western blotting was as described in Pazour et al. (1998).

Results

Cloning the Chlamydomonas DHC1b Gene

As an initial step in investigating the role of DHC1b in flagellar assembly, we set out to isolate cDNA clones encoding *Chlamydomonas* DHC1b. To do this, we used PCR with degenerate primers to amplify a portion of the *DHC*1b coding region (see Materials and Methods). The PCR product was used as a probe to isolate clones from a cDNA library greatly enriched for DHC sequences (Wilkerson et al., 1994). Two overlapping cDNA clones covering a total of 3.6 kb were obtained and sequenced. When the peptide encoded by this sequence (Fig. 1 a) was compared to other sequences in GenBank using BLAST, the strongest matches were the DHC1b isoforms of *Tetrahymena thermophila* (58% identical) and *Caenorhabditis elegans* (43% identical). The new sequence was aligned to the entire set of DHC sequences in GenBank. It was more similar to proteins in the DHC1b subfamily than to those in the DHC1a or axonemal DHC subfamilies, suggesting that the new *Chlamydomonas* sequence represents a DHC1b isoform. An alignment of a representative subset of these sequences is shown in Fig. 1 b. The assignment to the DHC1b subfamily was confirmed by phylogenetic analysis of the DHC family (Fig. 1 c).

To determine if the *DHC*1b gene is induced by deflagellation, RNA was isolated from control cells and cells 30 min after being deflagellated. Deflagellation induces transcription of genes that encode abundant flagellar proteins, e.g., the gene *ODA*3 that encodes a protein of the outer dynein arm docking complex (Koutoulis et al., 1997; Fig. 1 d, ODA3). The amount of *DHC*1b transcript was much higher in the deflagellated than in the control cells (Fig. 1 d, *DHC*1b), consistent with DHC1b having a role in flagellar assembly.

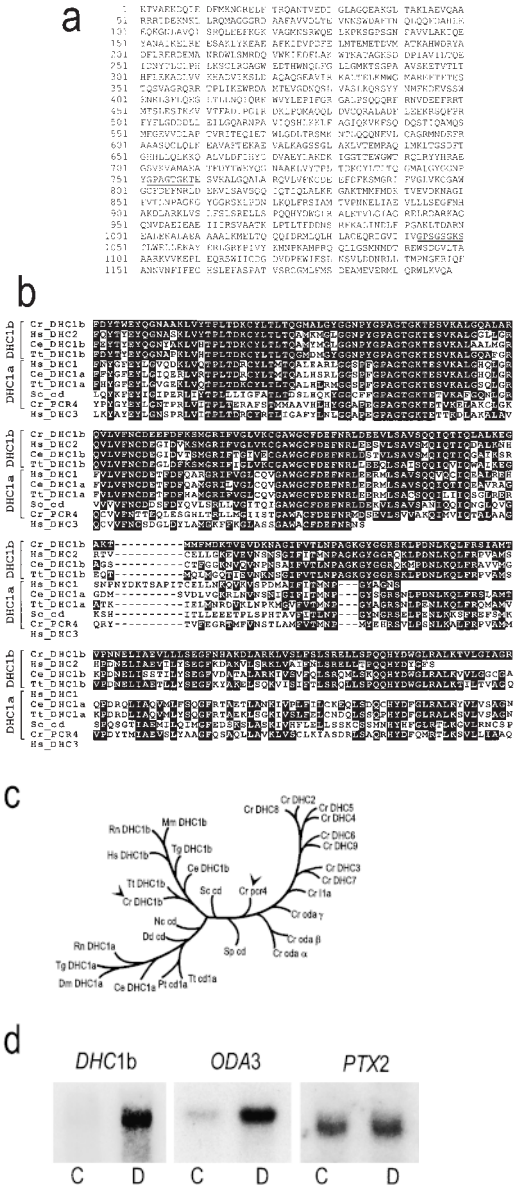

Figure 1. The *Chlamydomonas reinhardtii DHC*1b gene groups with *DHC*1b sequences from other organisms and is induced by deflagellation. (a) Partial sequence of the *C. reinhardtii* gene encoding DHC1b. P-loops 1 and 2 are underlined. Sequence data are available from GenBank/EMBL/DDBJ under accession number AF096277. (b) Alignment of the *Chlamydomonas* DHC1b and pcr4 peptides with other cytoplasmic dynein heavy chains. A representative selection of DHC sequences from GenBank were aligned with CLUSTAL W and the residues identical to *Chlamydomonas* DHC1b were shaded in black. Species names are abbreviated as described in c. A longer sequence for pcr4 is available under accession number AF106079. (c) Phylogenetic tree showing the relationship of the *Chlamydomonas* DHC1b and pcr4 sequences (arrowheads) to other DHC sequences. The predicted peptide sequences of the *Chlamydomonas DHC*1b cDNA (starting just upstream of P-loop 1 at the sequence CYLTLT and ending with the sequence FVTLNP) and pcr4 cDNA were aligned with a subset of DHC sequences in GenBank using CLUSTAL W, and a phylogenetic tree drawn with PHYLIP using the UPGMA method. Ce, *C. elegans*; Cr, *C. reinhardtii*; Dd, *Dictyostelium discoideum*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Nc, *Neurospora crassa*; Pt, *Paramecium tetraurelia*; Rn, *Rattus novegicus*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; Tg, *Tripneustes gratilla*; Tt, *Tetrahymena thermophila.* (d) The *Chlamydomonas DHC*1b gene is induced by deflagellation. mRNA was isolated from control, nondeflagellated cells (C), and from cells 30 min after deflagellation (D). The mRNA was analyzed by Northern blot using probes for the *DHC*1b, *ODA*3, and *PTX*2 genes. The latter two genes served as standards; transcription of *ODA*3 is induced by deflagellation (Koutoulis et al., 1997), whereas that of *PTX*2, a gene involved in phototaxis, is not (Pazour, G., and G. Witman, unpublished observations).

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This laboratory previously identified a DHC cDNA sequence termed pcr4 (Wilkerson et al., 1994) that we thought also potentially could encode a cytoplasmic dynein. To learn more about this isoform, we used pcr4 as a probe to isolate two partial cDNA clones covering a total of 2.0 kb, and sequenced these clones. A portion of the sequence is shown in Fig. 1 b. In the phylogenetic analysis, this dynein lies between the yeast cytoplasmic DHCs and the axonemal outer arm DHCs (Fig. 1 c), indicating that it diverged very early in the evolution of the DHC gene family. It is expressed at very low levels and does not appear to be induced by deflagellation (results not shown), so it is not likely to be involved in flagellar assembly. It probably is the *Chlamydomonas* version of the conventional cytoplasmic DHC isoform (DHC1a). However, we cannot rule out the possibility that *Chlamydomonas* contains another, still unidentified, DHC isoform that is more closely related to the conventional cytoplasmic DHC isoforms.

Identification of a Strain with a Deletion Mutation in DHC1b

In an effort to identify a *Chlamydomonas* strain with a defect in the *DHC*1b gene, a portion of one of the *DHC*1b cDNAs was used to screen a collection of insertional mutants that had been generated by transforming cells with DNA containing a selectable marker. Transformation of *Chlamydomonas* occurs by the nonhomologous integration of DNA into the genome (Kindle, 1990; Tam and Lefebvre, 1993), resulting in the disruption or deletion of genes at the site of insertion. Such mutations can be detected as restriction fragment length polymorphisms in Southern blots probed with DNAs encoding the affected genes, so that cell lines in which a cloned gene has been disrupted can be readily identified (Wilkerson et al., 1995; Koutoulis et al., 1997; Pazour et al., 1998). From a screen of >300 insertional mutants with a wide range of phenotypes (Pazour et al., 1995; Koutoulis et al., 1997), one strain (V92.2) was found with a disrupted *DHC*1b gene (Fig. 2 a). Further Southern blot analysis of this strain using the two longer *DHC*1b cDNAs as probes indicated that at least 3.6 kb of *DHC*1b coding region was deleted. Because the deleted region includes P-loops 1 and 2, this deletion allele, termed *dhc*1b-1, is likely to be a loss-of-function allele.

To determine if the mutation affected cell growth rate, growth curves were determined for the parent strain (g1), the original mutant isolate (V92.2), and a mutant offspring (3088.4) of a cross between V92.2 and wild type. All doubled at the same rate (Fig. 2 b), indicating that this mutation does not affect cell growth.

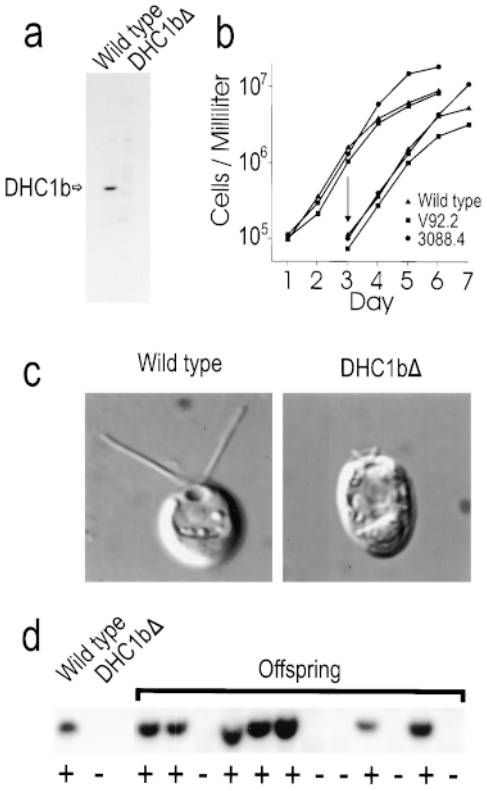

Figure 2. Phenotype of the *dhc*1b deletion mutant. (a) Identification of the *dhc*1b deletion mutant. DNA was isolated from >300 *Chlamydomonas* insertional mutants (Pazour et al., 1995; Koutoulis et al., 1997), cleaved with PstI, and analyzed by Southern blotting using a 0.3-kbp fragment of a *DHC*1b cDNA as probe. The DNA hybridized to a single band in wild-type (Wild type) and all strains except V92.2 (DHC1bΔ), which had no hybridizing band. (b) Deletion of the *DHC*1b gene does not affect growth rate. Growth of wild-type or mutant cells (V92.2 and 3088.4) in liquid medium was monitored as described previously (Pazour et al., 1998). On day 3, a second set of cultures was inoculated by diluting cells from the first series to 10^5 cells/ml (arrow). (c) Flagella are much shorter in the *dhc*1b deletion mutant (DHC1bΔ) than in wild-type cells. Cells were recorded by differential interference-contrast microscopy as described in Pazour et al. (1998). (d) The *dhc*1b deletion segregates with the flagellar defect. Strains V92.2 (DHC1bΔ) and CC124 (Wild type) were mated, tetrads were dissected, and the offspring were scored for motility by light microscopy. DNA was isolated from a single product of 49 independent tetrads and analyzed by Southern blotting as in panel a. The results for the parents and 12 progeny are shown. In all cases, progeny with the motility defect (−) lacked the *DHC*1b gene, whereas those with normal motility (+) had the *DHC*1b gene.

dhc1b Mutants Have Severe Flagellar Defects

Deletion of the *DHC*1b gene causes the cells to have very short, nonmotile flagella (Fig. 2 c). The flagella barely extend beyond the cell wall and often have a bulbous appearance. To be sure that this phenotype is the result of the *dhc*1b deletion and not of a second mutation at another site, the mutant cell line was crossed to a wild-type strain of the opposite mating type, and the resulting tetrads were dissected. One product from each of 49 tetrads was scored for the stumpy flagella phenotype by light microscopy and for the *dhc*1b deletion by Southern blotting (Fig. 2 d). In all cases the cells with short flagella had the *dhc*1b deletion, while those with normal flagella and motility had a wild-type *DHC*1b gene. These results indicate that the flagellar defect is tightly linked to the *DHC*1b deletion, and almost certainly is the result of it.

Electron microscopic analysis showed that the *dhc*1b flagella have a very aberrant structure (Fig. 3). The flagella barely extend beyond the flagellar collars (Fig. 3, d

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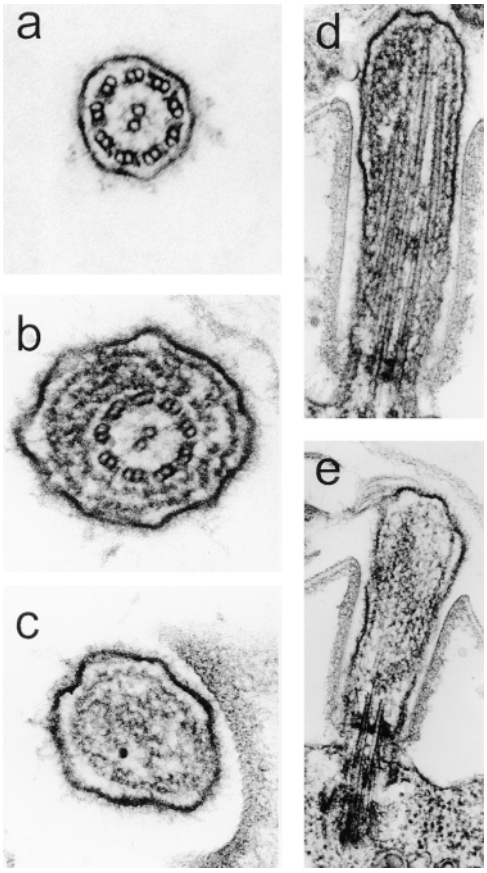

Figure 3. Ultrastructure of *dhc*1b deletion mutant flagella. In wild-type cells (a) the space between the flagellar membrane and doublet microtubules is usually devoid of material. In contrast, some *dhc*1b mutant cells (b, d, and e) have an apparently normal axoneme but the space between the doublet microtubules and the flagellar membrane is filled with electron-dense material identical in appearance to the rafts of IFT (Kozminski et al., 1993, 1995). The flagella of other *dhc*1b mutant cells (c) lack some or all of the axonemal microtubules and are completely filled with rafts. Cells were fixed as described previously (Pazour et al., 1998).

and e), and their microtubules are often disorganized. In some cross-sections, the axoneme has a normal looking 9 + 2 arrangement of microtubules (Fig. 3 b), whereas in others no microtubules are seen (Fig. 3 c). At least some inner and outer arms are present on those *dhc*1b axonemes (Fig. 3 b) that extend beyond the point where arms first appear on the doublet microtubules of wild-type axonemes (Hoops and Witman, 1983). The most distinctive feature of the *dhc*1b flagellar cross-sections is that they all contain an unusual abundance of electron-dense material that is identical in appearance to the IFT raft particles (Kozminski et al., 1993, 1995; Pazour et al., 1998). In cross-sections of wild-type flagella, rafts are occasionally seen between the doublet microtubules and the flagellar membrane (Kozminski et al., 1993, 1995), but in the *dhc*1b mutant this space is completely filled with rafts. For example, in the flagellum shown in Fig. 3 b, the axoneme is surrounded by two or three concentric rings of tightly packed rafts. In the flagellum shown in Fig. 3 c, the axoneme is missing and the entire space within the flagellar membrane is filled with rafts. In longitudinal sections (Fig. 3, d and e), the rafts have the typical appearance of a linear array of subunits (Kozminski et al., 1993).

dhc1b Cells Exhibit a Redistribution of Raft Proteins from the Peri-Basal Body Region to the Flagella

To determine if the distribution of IFT proteins in *dhc*1b cells differed in any other way from that in wild-type cells, we examined both cell types by immunofluorescence microscopy. Antibodies to raft subunits and to the FLA10 subunit of the anterograde IFT motor stain wild-type cells with very similar patterns. The antibodies localize primarily to the peri-basal body region within the cell body, and to punctate spots along the flagella (Cole et al., 1998; Fig. 4, a and c). In contrast, *dhc*1b mutant cells, stained with an antibody specific for the p172 raft subunit, lacked the peri-basal body staining but showed dramatically stronger staining of the flagella (Fig. 4 b). The latter result confirms that the electron-dense particles accumulated in the *dhc*1b flagella are IFT rafts. Antibodies to the FLA10 subunit of the anterograde motor also stain *dhc*1b flagella more strongly than they stain wild-type flagella (Fig. 4 d). However, in contrast to the raft subunit, FLA10 is retained in the peri-basal body region of *dhc*1b cells. There is little staining of the general cytoplasm or of other cell structures by these antibodies in either wild-type or mutant cells.

The Golgi Apparatus Appears Normal in Chlamydomonas DHC1b Mutants

Previous work (Vaisberg et al., 1996) showed that the mammalian DHC1b isoform was localized to the Golgi apparatus and that microinjection of DHC1b antibodies caused the Golgi complex to disperse. To determine if deletion of the *DHC*1b gene affected Golgi apparatus structure or positioning in *Chlamydomonas*, we compared wild-type and mutant cell bodies by electron microscopy. The organization of the mutant cell body was very similar to that of the wild-type parent. In both cell types, the Golgi stacks were located on the opposite side of the nucleus from the flagella, and were composed of four to eight cisternae (Fig. 5). We also did not detect defects in cell structures that presumably are dependent upon the Golgi apparatus for their formation. For example, Golgi complexes in green algae function in the formation of cell wall precursors (for review see Domozych, 1991). Cell walls in the mutant cell appear morphologically normal by electron microscopy. In addition, the cells do not lyse in the presence of 0.5% Triton X-100, indicating that the cell walls are structurally intact (data not shown).

Cytoplasmic Dynein DHC1b Is Present in the Flagella

If DHC1b powers retrograde IFT, then this isoform of cytoplasmic dynein should be present in wild-type flagella. To test this, we generated an antibody against a bacterially expressed fragment of *Chlamydomonas* DHC1b. The peptide used for antibody production constitutes ~50 kD near the NH_2-terminal end of the region that we have cloned; this region is not highly conserved among DHCs (Wilkerson et al., 1994; Gibbons, 1995; Pazour, G.J., and G.B. Wit-

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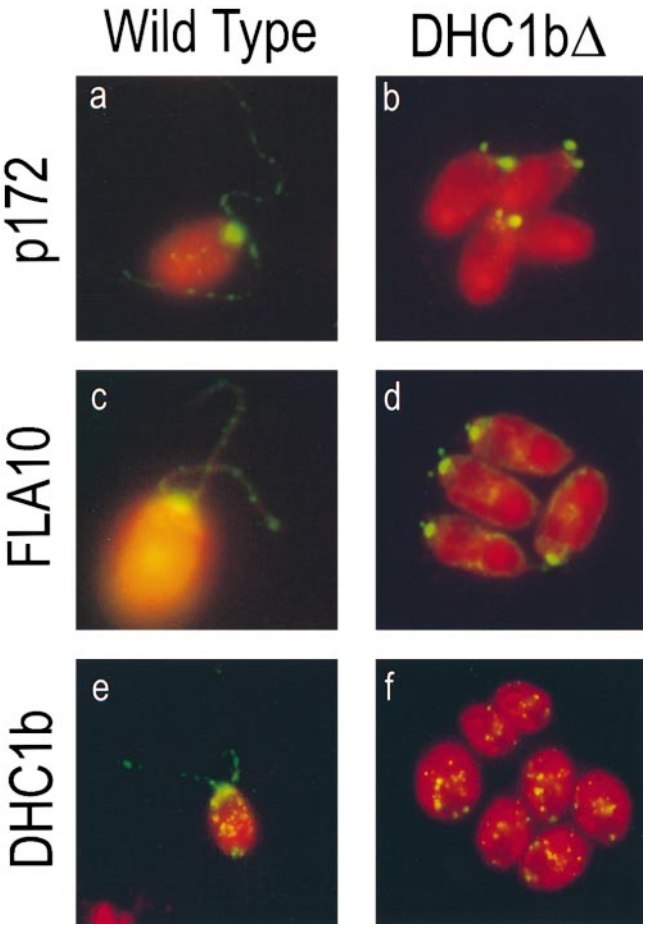

Figure 4. Localization of an IFT raft protein, FLA10, and DHC1b in wild-type and *dhc*1b mutant cells by indirect immunofluorescence. The antigens of interest are shown in green whereas autofluorescence of the cell body is shown in red. Antibody specific for the p172 subunit of the IFT rafts (Cole et al., 1998) shows that in wild-type cells, the raft proteins are located primarily in the cell body at the base of the flagella, with some punctate staining along the length of the flagella (a). In contrast, in the *dhc*1b deletion mutant (DHC1bΔ), almost no staining is seen in the peri-basal body region, but there is a very intense staining of the flagellar stubs (b). FLA10, a subunit of the anterograde IFT motor, is localized primarily in the peri-basal body region of wild-type cells with some punctate staining along the flagella (c). In the *dhc*1b deletion mutant (DHC1bΔ), FLA10 staining similarly is observed in the cell body at the base of the flagella; it is also present in the flagellar stubs (d). In wild-type cells, DHC1b is localized in the peri-basal body region with some punctate staining along the flagella (e). The *dhc*1b deletion mutant (DHC1bΔ), which lacks this antigen, shows only a small amount of nonspecific punctate staining in the cell body (f). No flagellar staining is detected.

man, unpublished results), so an antibody to it is likely to be isoform specific. To check this, we examined the ability of the antibody to react with DHCs in Western blots of extracts of wild-type vs. *dhc*1b whole cells. The antibody detected a single high molecular weight band in wild-type cells, but did not recognize any band in the cells lacking DHC1b (Fig. 6 a, DHC1b). Antibody to the outer dynein arm γ DHC detected bands in both cell lines (Fig. 6 a,

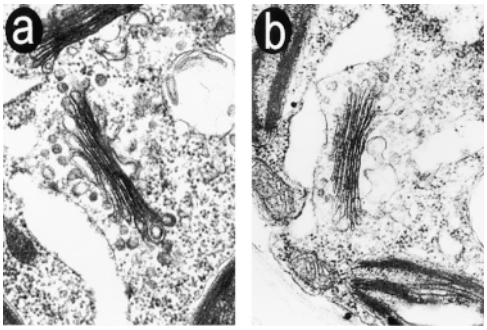

Figure 5. The location and morphology of the Golgi complex is the same in wild-type (a) and *dhc*1b (b) cells.

DHCγ), confirming that other DHCs are present in the *dhc*1b cells. Therefore, the antibody is isoform specific.

The antibody was used to determine if DHC1b is present in wild-type flagella, and if so, in which fraction. Flagella were isolated from wild-type cells and separated into the following fractions: (a) membrane + matrix, consisting of the detergent-soluble membrane proteins and any soluble proteins of the flagellar cytoplasm; (b) ATP-rinse 1, ATP-rinse 2, and ATP-rinse 3, consisting of proteins not extracted by detergent but released from the axonemes by a first, second, or third rinse with 10 mM $MgATP^{2-}$; (c) salt-extract, consisting of proteins released from the ATP-rinsed axonemes by 0.6 M KCl; and (d) axonemes, consisting of the proteins not extracted by detergent, ATP, and KCl. As shown in Fig. 6 b, the anti-DHC1b

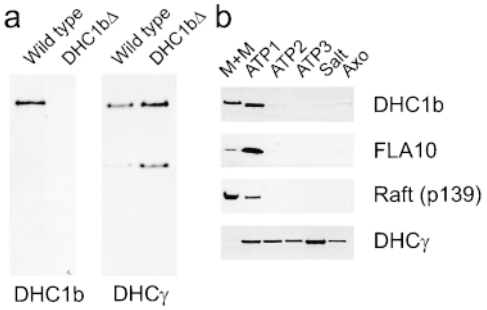

Figure 6. DHC1b is found in the flagella. (a) Whole cell extracts were made from wild-type and *dhc*1b (DHC1bΔ) cells and probed with affinity-purified DHC1b antibodies or the 12γC mAb to the γ DHC of the outer arm dynein. The DHC1b antibodies detect a single high molecular weight band in wild-type cells but not in the mutant, whereas the γ DHC antibody detects a similarly sized band in both samples. 12γC also detects a smaller unidentified band in both cell types. (b) Flagella were isolated from wild-type cells and separated into the following fractions: detergent-soluble membrane proteins and soluble proteins of the flagellar matrix (M + M); proteins released from the axoneme by a first, second, or third rinse with ATP (ATP1, ATP2, and ATP3, respectively); proteins released from the ATP-rinsed axoneme by 0.6 M KCl (Salt); and the axonemal proteins remaining after treatment with detergent, ATP and KCl (Axo). Gels were loaded with extracts from equivalent numbers of flagella and analyzed by Western blotting with antibodies to DHC1b, FLA10 (FLA10N; Cole et al., 1998), p139 [Raft (p139); Cole et al., 1998], and DHCγ (12γC; King et al., 1985).

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antibody reacts strongly with a single protein in the flagellar fractions. Nearly all of the DHC1b is in the membrane + matrix or ATP-rinse 1 fractions, with very little in the salt extract or axoneme fractions (Fig. 6, DHC1b). The distributions of the FLA10 subunit of the anterograde IFT motor and the p139 raft subunit are very similar [Fig. 6, FLA10 and Raft (p139)]. In contrast, the γ DHC of outer arm dynein is most abundant in the salt extract, with a lesser amount extracted by ATP and very little in the membrane + matrix (Fig. 6 b, DHCγ). This distribution is to be expected for an axonemal DHC. For unknown reasons, a small amount of axonemal DHCs is released from the axoneme by ATP (Goodenough and Heuser, 1984). In any case, the distribution clearly differs from that of DHC1b. These results indicate that DHC1b is present in the wild-type flagellum, and is in the fraction expected for an IFT motor.

The cellular distribution of DHC1b was further examined by immunofluorescence microscopy using the same DHC1b antibody as used in the Western blot experiments. In wild-type cells (Fig. 4 e), the antibody stained primarily the peri-basal body region and punctate spots along the flagella. Some punctate staining of the cell body was present, but this was also observed in *dhc*1b cells (Fig. 4 f), indicating that it occurred because of nonspecific binding of the antibody. Thus, the immunofluorescence localization of DHC1b in wild-type cells is virtually identical to that observed for the other IFT components, p172 and FLA10. No obvious staining of cytoplasmic microtubules or the Golgi apparatus was detected, although the presence of nonspecific punctate staining in the cell body could have obscured a specific localization to a small cell body structure.

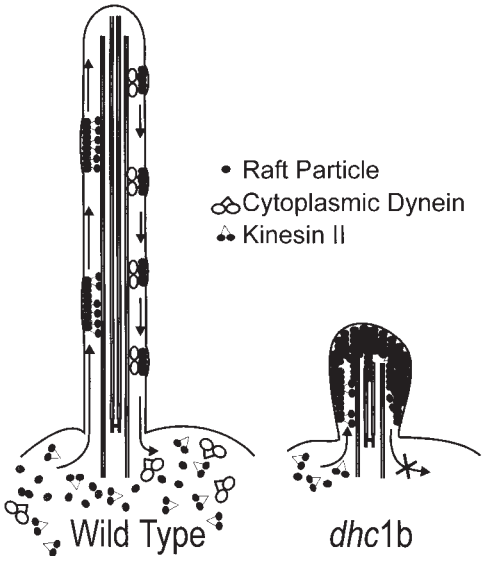

Figure 7. In wild-type cells, IFT components are continuously removed from the peri-basal body pool for IFT, and returned to the pool (modified from Fig. 13 of Cole et al., 1998). In *dhc*1b cells, IFT components are likewise transported from the peri-basal body pool into the flagella, but they then accumulate there, presumably due to a defect in retrograde IFT. As a result, the peri-basal body pool is depleted.

Discussion

Function of DHC1b

The findings presented here show that the DHC1b present in the flagella of wild-type *Chlamydomonas* is located predominantly in the detergent- and ATP-soluble fractions, and is essential for flagellar assembly. A mutant lacking the *DHC*1b gene has defective flagella, but its growth rate is normal, implying that no vital functions are compromised. The ultrastructure of the mutant is consistent with a specific defect in the retrograde component of IFT (see below). Taken together, these results indicate that DHC1b is a cytoplasmic dynein specialized for function in flagellar assembly, probably as the motor for retrograde IFT.

We reported previously that an insertional mutant of *Chlamydomonas* lacking the dynein light chain LC8 was defective in retrograde IFT (Pazour et al., 1998). This mutant accumulated large numbers of rafts at the tips of short flagella, apparently because the rafts were transported into the flagella but could not readily return to the base due to the defect in retrograde IFT. The *dhc*1b mutant has even shorter flagella filled with even more rafts. In this mutant the raft proteins are completely redistributed from the peri-basal body pool, where they are predominantly located in wild-type and the LC8 mutant (results not shown), into the flagella. Although the flagella of *dhc*1b cells are too short for IFT to be observed directly in them, the similar accumulation of rafts in the flagella of the LC8 and *dhc*1b mutants suggests that the latter also is defective in retrograde IFT. It seems likely that in wild-type cells, rafts continuously move from the peri-basal body pool up the flagella and then back down, returning to the pool (Fig. 7). In the *dhc*1b mutant, rafts presumably move into the flagella as in wild-type cells, but then accumulate there because there is no retrograde IFT to return them to the peri-basal body pool. As a result, the peri-basal body pool becomes depleted. The fact that the phenotype is stronger in the *dhc*1b mutant than in the LC8 mutant suggests that some retrograde transport of rafts remains in the absence of the light chain. However, it is possible that loss of DHC1b and LC8 affect flagellar assembly and IFT through different mechanisms. Currently, we are carrying out studies to determine if LC8 is a component of DHC1b dynein, which should clarify this matter.

Our results strongly suggest that DHC1b dynein is the motor for retrograde IFT and that loss of DHC1b impairs flagellar assembly directly via its effect on IFT. An alternative possibility is that loss of DHC1b affects IFT and flagellar assembly indirectly. IFT components, including FLA10 and the raft proteins, are concentrated in the peri-basal body pool (Cole et al., 1998; see Fig. 4). Moreover, mRNAs encoding flagellar proteins become concentrated in the peri-basal body region during flagellar assembly (Han et al., 1997; Marshall, W., and J. Rosenbaum, personal communication). Microtubules radiate from the basal body region into the cytoplasm (Ringo, 1967) and these microtubules are oriented with their minus ends toward the basal bodies. Therefore, a minus end–directed motor-like dynein could be responsible for the concentration of the IFT proteins and mRNAs encoding flagellar

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proteins in the peri-basal body region. If these components were not concentrated in this region due to a defect in DHC1b, flagellar assembly might be impaired. However, if this were the case, then one would expect the raft proteins to be distributed throughout the cytoplasm of the *dhc*1b cells. In fact, they are not distributed throughout the cytoplasm, but are packed into the flagellar stubs. Moreover, FLA10 kinesin-II is concentrated in the peri-basal body pool in the *dhc*1b mutant (Fig. 4 d), indicating that the machinery for its transport to that region is intact. Thus, it seems unlikely that the flagellar assembly defect is the result of a failure to transport components to the peri-basal body region.

Although DHC1b has been localized to the Golgi apparatus in mammalian cells, and injection of an antibody against DHC1b caused disruption of the Golgi complex (Vaisberg et al., 1996), we did not observe any effect of DHC1b loss on Golgi apparatus position, structure, or function in *Chlamydomonas*, suggesting that it may not have a role in the Golgi complex in this organism. However, our findings explain many of the other previous observations concerning DHC1b. Our data that DHC1b is necessary for flagellar assembly explain why its expression is induced during ciliogenesis (Gibbons et al., 1994; Gibbons, 1995; Criswell et al., 1996; see Fig. 1 d). Furthermore, DHC1b is likely to be necessary for the assembly and maintenance of both motile and nonmotile cilia and flagella, including the primary cilium. Because a primary cilium is found in most cell types (Wheatley, 1982), this could explain why DHC1b is expressed in cells and tissues that do not produce motile cilia (Criswell et al., 1996; Vaisberg et al., 1996). The data showing that IFT components, including DHC1b, are concentrated in the peri-basal body pool (Cole et al., 1998) explain why DHC1b was localized to the apical regions of ciliated RTE cells (Criswell et al., 1996). The fact that the peri-basal body pool stains much more brightly than the flagella with antibodies to IFT components could explain why DHC1b was not detected in the cilia of the RTE cells (Criswell et al., 1996).

The Fractionation Pattern of DHC1b and FLA10 May Reflect Their Activity States

In our Western blot analysis of flagellar fractions, DHC1b was found in nearly equal amounts in the membrane + matrix fraction and the first ATP-rinse fraction. FLA10 was predominantly in the first ATP-rinse fraction, although a considerable amount also was present in the membrane + matrix fraction. Motor molecules in the ATP-rinse fraction presumably are those that were bound to the doublet microtubules by rigor bonds. However, inasmuch as intraflagellar ATP should have been completely depleted shortly after deflagellation, it is surprising that not all of the motor molecules were attached by rigor bonds. This raises the possibility that the distribution of these motors reflects their activity states in the flagellum. Thus, dynein and kinesin in the membrane + matrix fraction may have been in an inactive state, corresponding to those motors that were being passively transported to the tip or base of the flagella, respectively. Conversely, dynein and kinesin in the ATP-rinse fraction may have been in an active state, corresponding to those motors that were actively transporting rafts to the base or tip of the flagellum, respectively. If so, the motors from these fractions may be useful for determining how dynein and kinesin activities are regulated in the flagellum.

The DHC1b Isoform Is Likely Needed for Flagellar Assembly in all Ciliated Species

The DHC1b isoform has been found in a wide range of ciliated organisms including: mammals, sea urchins, nematodes, ciliates, and algae. However, it is not present in nonflagellated yeast such as *Saccharomyces cerevisiae.* If DHC1b became specialized for flagellar assembly early in evolution, it would explain why *S. cerevisiae*, believed to have lost its flagella secondarily (Wainwright et al., 1993), has discarded the *DHC*1b gene along with all axonemal dynein genes. The nematode *C. elegans* does not have any motile cilia, but makes extensive use of sensory cilia to monitor its environment (White et al., 1976; Perkins et al., 1986). Many mutations that affect these sensory cilia have been identified. Interestingly, several of these genes encode proteins homologous to *Chlamydomonas* IFT raft subunits or motors. For example, *C. elegans OSM-1* and *OSM-6* encode proteins that are homologous to the p172 and p52 IFT raft subunits, *OSM-3* encodes a kinesin similar to the anterograde IFT motor (Cole et al., 1998), and *CHE-3* encodes the DHC1b isoform of cytoplasmic dynein (Grant, W., personal communication). The *C. elegans che*-3 phenotype (Lewis and Hodgkin, 1977; Collet et al., 1998) is very similar to the *Chlamydomonas dhc*1b phenotype. Both mutations cause the cilia to be shorter than normal and swollen with electron-dense material. As is the case in *Chlamydomonas*, the electron-dense material in *C. elegans* appears to be raft subunits, because the OSM-6 protein is highly concentrated at the ends of *che*-3 cilia (Collet et al., 1998).

In echinoderms, kinesin-II antibodies stain the midpiece of the sperm near the basal body and also stain the sperm flagella in a discontinuous punctate pattern (Henson et al., 1997) similar to what is seen in *Chlamydomonas* flagella. Furthermore, microinjection of kinesin-II antibodies into fertilized sea urchin eggs prevented the formation of cilia on the developing embryo, suggesting that a process similar to IFT occurs in echinoderms (Morris and Scholey, 1997).

Vertebrates have motile cilia (sperm tails, ciliated epithelium of the respiratory tract, etc.), sensory cilia (rods and cones in the eye, olfactory cilia) as well as primary cilia, and kinocilia that may have a role in development. DHC1b and IFT are very likely to be vitally important for the assembly of all of these types of cilia. In the mouse, knockout of Kif3A (Marszalek et al. 1998. *Mol. Biol. Cell.* 9:131a), the vertebrate homologue of FLA10, or Kif3B (Nonaka et al., 1998), the other motor subunit of vertebrate kinesin-II, results in loss of the nodal cilia. Moreover, Kif3A has been localized to the connecting cilium of the vertebrate photoreceptor (Beech et al., 1996), suggesting that kinesin-II is responsible for the anterograde transport of proteins into the rod outer segment. Very recently, photoreceptor-specific deletion of Kif3A in mice was found to cause structural abnormalities in the rod outer segment (Marszalek et al. 1998. *Mol. Biol. Cell.* 9:131a).

Therefore, defects in IFT components may be one cause of human diseases such as retinitis pigmentosa that involve the degeneration of cells bearing modified cilia.

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