Journal of Cell Science 109, 1891-1898 (1996)
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JCS8002

A novel cytoplasmic dynein heavy chain: expression of DHC1b in mammalian ciliated epithelial cells

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SUMMARY

Organisms that have cilia or flagella express over a dozen dynein heavy chain genes. Of these heavy chain genes, most appear to encode axonemal dyneins, one encodes conventional cytoplasmic dynein (MAP1C or DHC1a), and one, here referred to as DHC1b, encodes an unclassified heavy chain. Previous analysis of sea urchin DHC1b (Gibbons et al. (1994) *Mol. Biol. Cell* 5, 57-70) indicated that this isoform is either an axonemal dynein with an unusual protein sequence or a cytoplasmic dynein whose expression increases during ciliogenesis. In the present study, we examined the expression of DHC1b in rat tissues. The DHC1b gene is expressed in all tissues examined, including unciliated liver and heart cells. In contrast, rat axonemal dyneins are only expressed in tissues that produce cilia or flagella. In cultured rat tracheal epithelial (RTE) cells, DHC1b is expressed in undifferentiated cells and increases in expression during ciliogenesis. In contrast, the expression of conventional cytoplasmic dynein, DHC1a, does not change during RTE differentiation and axonemal dynein is not expressed until after differentiation commences. In order to examine the expression of DHC1b protein, we produced an isoform-specific antibody to a synthetic peptide derived from the rat DHC1b sequence. The antibody demonstrated that DHC1b is a relatively minor component of partially purified cytoplasmic dynein. Indirect immunofluorescence microscopy revealed that DHC1b is not detected in cilia and remains in the cytoplasm of ciliated RTE cells, often accumulating at the apical ends of the cells. These results suggest that DHC1b is a cytoplasmic dynein that may participate in intracellular trafficking in polarized cells.

Key words: Cytoplasmic dynein, Dynein isoform, Ciliogenesis

INTRODUCTION

Dyneins are high molecular mass motor proteins that produce directed movement along microtubules towards their proximal or (–) ends. Dynein transduces the free energy derived from ATP hydrolysis into mechanical work to carry a molecular cargo along the microtubule. There are two functional classes of dynein: axonemal dynein produces the active sliding of outer doublet microtubules that underlies ciliary bending (Satir, 1965; Summers and Gibbons, 1971); non-axonemal or cytoplasmic dynein is thought to participate in the intracellular trafficking of a variety of organelles (reviewed by Holzbaur and Vallee, 1994).

In cells that have cilia or flagella, there are multiple isoforms of dynein heavy chains encoded by separate genes. Each of the many (probably >10) axonemal isoforms is structurally customized to contribute in a precise way to the initiation and propagation of ciliary bending (reviewed by Asai and Brokaw, 1993; Brokaw, 1994; Asai, 1995). In contrast to the wide diversity of axonemal dyneins, cytoplasmic dynein is much simpler. In organisms that do not have cilia or flagella, including yeast, filamentous fungi, slime mold, and nematode, only a single dynein heavy chain has been identified (Eshel et al., 1993; Li et al., 1993; Xiang et al., 1994; Plamann et al., 1994; Koonce et al., 1992; Lye et al., 1995). In organisms that have cilia, the major cytoplasmic dynein, called MAP1C (Paschal et al., 1987), is the homologue of the dynein found in the unciliated organisms; examples of cytoplasmic dynein sequences from ciliated organisms include those from rat (Mikami et al., 1993; Zhang et al., 1993), *Drosophila* (Li et al., 1994), sea urchin (Gibbons et al., 1992), and *Paramecium* (Kandl et al., 1995).

The two classes of heavy chains exhibit distinct mechanochemical properties. Cytoplasmic dynein, but not axonemal dynein, has a high CTPase activity (Pallini et al., 1983; Shpetner et al., 1988; Collins and Vallee, 1989) although only ATP supports movement. Further, the duty cycles of the two classes of dynein appear to be different (Leibler and Huse, 1993). The evidence from in vitro motility experiments suggests that axonemal dynein is in a weakly bound state for significant periods of time whereas cytoplasmic dynein remains tightly associated with the microtubule over most of its duty cycle (Vale et al., 1992).

Because of the observed differences in enzymological prop-

erties, one place to look for sequences that contribute to the specialization of axonemal and cytoplasmic dyneins is the centrally located catalytic domain of the heavy chain. Two sequence motifs located immediately adjacent to the catalytic P-loop, termed motifs A and B, accurately distinguish between axonemal and cytoplasmic isoforms among all dynein sequences whose functional classification is known (Asai and Brokaw, 1993; Asai et al., 1994). For example, the axonemal dyneins of sea urchin and rat tracheal epithelial cells contain the sequence motif A (Gibbons et al., 1994; Andrews et al., 1996), whereas the cytoplasmic dyneins (MAP1C and its homologues) from the organisms listed earlier contain the sequence motif B.

A possible exception to the classification by motifs A and B was discovered in the study of sea urchin dyneins (Gibbons et al., 1994). A novel heavy chain gene, called DHC1b, has the 'cytoplasmic' sequence motif B but its mRNA increases in concentration during embryo reciliation. Dynein 1b is also found in other organisms that have cilia. As described in this report, DHC1b mRNA is expressed in rat pheochromocytoma (PC12) cells and in rat brain. The same rat gene was previously described by Tanaka et al. (1995) who called it DLP4. In addition, dynein 1b has been identified in other organisms, including human, where it has been called hcDHC2 (Vaisberg et al., 1994; also B. H. Gibbons and I. R. Gibbons, unpublished, accession no. U20552), and *Tetrahymena* (D. J. Asai, J. Chen, and J. D. Forney, unpublished). An extensive bootstrapping analysis (Gibbons, 1995) of many of the available dynein sequences identified three groups: (i) one large group consists of the known and putative axonemal dyneins and is subdivided into an outer arm cluster and an inner arm cluster; (ii) the second group is composed of all the known cytoplasmic dyneins; and (iii) the third group is DHC1b, which falls approximately midway between the axonemal and cytoplasmic groups. The function of dynein 1b is not known, nor can it be accurately inferred from its sequence or the pattern of mRNA accumulation in deciliated embryos. Dynein 1b may be an axonemal dynein with a sequence unlike all of the other identified axonemal heavy chains. Or dynein 1b may be a cytoplasmic dynein whose expression is induced during reciliation. This report summarizes our efforts to understand the expression and function of DHC1b during mammalian ciliogenesis.

MATERIALS AND METHODS

Cloning and sequencing of fragments of dynein heavy chain genes

RNA-directed polymerase chain reaction (RT-PCR) utilizing degenerate oligonucleotide primers, as previously described (Asai and Criswell, 1995), was used to amplify 300 bp fragments of rat dynein DHC1a and DHC1b genes. Approximately 1.5 μg of poly(A)$^+$ RNA from adult rat brain and cultured pheochromocytoma cells (PC12; Greene and Tischler, 1976) were used in separate RT-PCR reactions. The amplified products were ligated into pUC118, transformed into JM101, cloned, and sequenced using single-stranded templates (Sequenase 2.0, United States Biochemicals, Cleveland, OH).

Southern and northern blotting

Genomic DNA was isolated from powdered rat brain by standard methods (Graham, 1978). The DNA, ~20 μg per digestion, was digested with restriction endonucleases (Promega, Madison, WI), electrophoresed on 1% agarose gels, and transferred to Genescreen Plus membrane (New England Nuclear, Boston, MA). Total cellular RNA was obtained using guanidinium thiocyanate (Chomczynski and Sacchi, 1987), and poly(A)$^+$ RNA was isolated using an oligo-dT kit (Pharmacia LKB Biotechnology, Piscataway, NJ). Approximately 20 μg of poly(A)$^+$ RNA was electrophoresed in a 1% agarose/formaldehyde gel (Sambrook et al., 1989) at 2 V/cm for 8 hours and transferred to Genescreen Plus membrane. Hybridization was performed at 65°C in 100 mM sodium phosphate, 1% bovine serum albumin (BSA), 5% sodium dodecyl sulfate (SDS) and 1 mM EDTA (Church and Gilbert, 1984). The DHC1a and 1b probes were the 300 bp cloned inserts, excised from the plasmids, gel-purified, and labeled with [α-^{32}P]dCTP (Amersham, Arlington Hts, IL) using the Random Priming kit (Gibco BRL, Gaithersburg, MD). Specific activities of the two probes were 3.9×10^8 cpm/ng (DHC1a) and 5.7×10^8 cpm/ng (DHC1b). The northern blot shown in Fig. 5 was performed as described by Andrews et al. (1996).

RNA-directed PCR analyses

RNA-directed PCR (RT-PCR) analysis of DHC1b was carried out essentially as described by Andrews et al. (1996). For the analysis of expression in different rat tissues, samples of total RNA were reverse transcribed into cDNA using random hexamers and an RNA PCR kit (Perkin-Elmer, Branchburg, NJ). The cDNA samples were divided into two portions and, following addition of axonemal or DHC1b primers, PCR amplification was performed as follows: 1 minute at 95°C; 35 cycles of 95°C (15 seconds), 52°C (30 seconds), 72°C (30 seconds); and 7 minutes at 72°C. The same conditions were used for the analysis of dynein expression during ciliogenesis in RTE cells except that the amplification of axonemal dyneins was for 36 cycles and that of DHC1b was for 28 cycles. Primers for DHC1b were 5′-CAGTAAAGGCTCTAGGTGGAC-3′ (the sense strand primer encoding VKALGG) and 5′-GATAAAAATTCCAGAATTGGC-3′ (the antisense primer corresponding to ANSGIFI). Axonemal primers were as described previously (Andrews et al., 1996). Negative controls included reactions performed in the absence of reverse transcriptase and reactions performed with no added template.

RTE cultures

Rat tracheal epithelial cells were isolated from adult male Fisher 344 rats and cultured as described elsewhere (Andrews et al., 1996; Ostrowski et al., 1995; Clark et al., 1995). Briefly, isolated tracheas were filled with a 1% solution of protease (Sigma) and incubated at 4°C overnight. The isolated epithelial cells were seeded onto Transwell-col culture inserts (Costar, Cambridge, MA) which had been coated with Type I collagen. The cells were kept submerged in supplemented DMEM/F12 medium until day 7-8, at which time an air-liquid interface was created in some cultures to induce ciliated cell differentiation. Parallel cultures were maintained in the submerged state or grown in the absence of growth factors, conditions which have been shown to inhibit or promote, respectively, ciliogenesis (Ostrowski and Nettesheim, 1995; Clark et al., 1995).

Site-directed antibodies

Two peptides were synthesized: peptide 1a, CEHSNPNYDKTSAPITCELLNKQVKVSPDM; peptide 1b, CNHRSVCELLGKEVEINANS (see Fig. 1). Each peptide was coupled through the N-terminal cysteine to porcine thyroglobulin using sulfo-SMCC (Pierce, Rockford, IL). The peptide-thyroglobulin conjugates were utilized as antigens in rabbits. The first injection was delivered in Freund's complete adjuvant, and all subsequent injections were delivered in Freund's incomplete adjuvant. The rabbits were injected with approximately 50 μg per injection on the following schedules: anti-1a, days 1, 22, 29, 37, 44, and 65; anti-1b, days 1, 22, 29, 44, 50, and 58. The antisera to peptides 1a and 1b used in this study were obtained on days 65 and 64, respectively. Sera were stored at –20°C

in 0.1% sodium azide. Antibodies were purified from the antisera by affinity chromatography in which the synthetic peptide was coupled to cyanogen bromide-activated agarose (Sigma).

Solid-phase binding assays

Antisera were assayed for peptide specificity using an alkaline phosphatase-based solid phase binding assay (Asai and Wilder, 1993) in which 50 ng of synthetic peptide were coated to the bottom of each microtiter well. Antibody binding was measured at 405 nm with a vmax microplate reader (Molecular Devices, Menlo Park, CA). Competitive binding assays were performed in the same way as the standard binding assay except that the diluted antiserum was mixed with different quantities of competing peptide immediately prior to its application to the wells.

Cytoplasmic dynein isolation and western blotting

Rat brain cytoplasmic dynein was partially purified by a modification of published methods (Amos, 1989). Taxol-induced microtubules were obtained from the brains of adult male Sprague-Dawley rats. The microtubules were pelleted through 40% sucrose, washed twice, and extracted with 0.5 M NaCl and 10 mM $MgATP^{2-}$. The ATP extract contained cytoplasmic dynein.

The ATP extract from rat brain microtubules was electrophoresed by SDS-PAGE (Dreyfuss et al., 1984), transferred to nitrocellulose, and the blots blocked overnight at room temperature with 1% gelatin dissolved in TBST (10 mM Tris, 0.15 M NaCl, 2.5% Tween-20, 0.05% sodium azide). The blots were incubated with antisera diluted in TBST, then washed in TBST without azide, and incubated at room temperature with donkey anti-rabbit IgG conjugated to horseradish peroxidase, diluted 1:10,000 (Amersham, Arlington Hts, IL). The blots were washed in TBST and then TBS (TBST without the Tween-20). The antibody binding was visualized using the LumiGLO kit (Kirkegaard and Perry, Gaithersburg, MD) and exposed to X-ray film.

Indirect immunofluorescence microscopy

Rat tracheal epithelial cells, obtained by pronase digestion as described above, were washed, resuspended in PBS, and fixed by adding an equal volume of 4% formaldehyde solution. The cells were pelleted, resuspended in PBS, and stored at 4°C until use. The cells were attached to poly-L-lysine coated glass coverslips, then sequentially treated as follows: 0.5% Triton X-100 in microtubule stabilizing buffer (MTSB; Thompson et al., 1984) for 5 minutes at 37°C, 3.7% formaldehyde in MTSB for 10 minutes at 37°C, and methanol for 10 minutes at −20°C. The cells were rehydrated in PBS and then incubated with affinity-purified antibodies, washed in PBS, and stained with rhodamine-conjugated goat anti-rabbit antibodies (Kirkegaard and Perry). Cells were examined by epiillumination fluorescence microscopy and photographed on Kodak TMAX film. The anti-axonemal dynein antiserum (see Fig. 8e and f) was raised against bacterial fusion protein representing a portion of sea urchin β heavy chain including the catalytic P-loop. On western blots, this antiserum reacted well with axonemal dyneins from a variety of organisms (Tjandra et al., 1995).

RESULTS

Characterization of the rat DHC1b gene

Fragments of the rat DHC1a and DHC1b genes were obtained by RNA-directed polymerase chain reaction (RT-PCR) using poly$(A)^+$ RNA from rat pheochromocytoma (PC12) cells and, in separate experiments, from rat brain. The procedure amplified products of ~300 bp which were then cloned and sequenced. The deduced amino acid sequences divided into two species: one sequence was that of MAP1C (Mikami et al., 1993; Zhang et al., 1993) and here is referred to as DHC1a; the second sequence was identical to DLP4, previously cloned from rat brain (Tanaka et al., 1995), and here is referred to as DHC1b (Fig. 1). Rat DHC1b is homologous to sea urchin DHC1b (Gibbons et al., 1994). In the portions of the proteins shown in Fig. 1, rat 1b is 83% identical and 89% similar to sea urchin 1b, but shares only 66% identity with sea urchin 1a. Altogether, 22 clones of DHC1a and 16 clones of DHC1b were found. The DHC1b from PC12 cells had the same sequence as brain DHC1b except for the following substitutions: R in the PC12 sequence for an A in the rat brain sequence at residue 12, I for a V at residue 23.

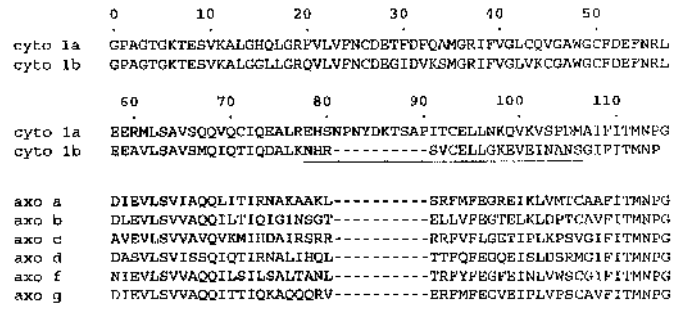

Fig. 1. Partial sequences of DHC1a and DHC1b. The deduced amino acid sequences of fragments of rat brain DHC1a and DHC1b are aligned. The peptides to which antibodies were raised in this study are indicated with an underline. The partial rat 1a sequence shown here represents residues 1,904-2,019 of MAP1C (Mikami et al., 1993). Below: the corresponding regions of six rat axonemal dynein heavy chains (Andrews et al., 1996).

The 300 bp cloned fragments of rat DHC1a and DHC1b were used to probe Southern and northern blots of rat DNA and RNA. Genomic DNA was digested with different restriction enzymes, electrophoresed, and blotted onto nitrocellulose. The resulting Southern blots, shown in Fig. 2a, revealed that each probe generally hybridized with a single restriction fragment and that the patterns produced with the two probes were different. The simplest interpretation of these data is that DHC1a and DHC1b are separate, single-copy genes. Northern blots of poly$(A)^+$ RNA isolated from rat brain revealed that each probe hybridized with a very high molecular mass RNA, ~15 kb (Fig. 2b). Densitometry of the exposed films indicated that the DHC1a signal was greater than 20-fold stronger than the DHC1b signal (note: in Fig. 2b, the two X-ray films were exposed for different lengths of time). This suggests that the DHC1b transcript is far less abundant than DHC1a in rat brain; this is the same conclusion made by Tanaka et al. (1995).

Patterns of DHC1b expression

Utilizing gene-specific oligonucleotide primers, RT-PCR was performed using RNAs obtained from various rat tissues. The RT-PCR provided a qualitative analysis of DHC1b expression. The DHC1b specific primers amplified a product from all tissues examined, including the heart and liver (Fig. 3). In contrast, axonemal dynein-specific primers amplified a product from only those tissues in which axonemes are found: trachea, testis, and brain. These results demonstrate that the DHC1b gene is expressed in ciliated and unciliated rat tissues in a pattern distinct from that of axonemal dynein.

A cell culture system, in which primary rat trachea epithelia (RTE) cells differentiate into a mucociliary epi-

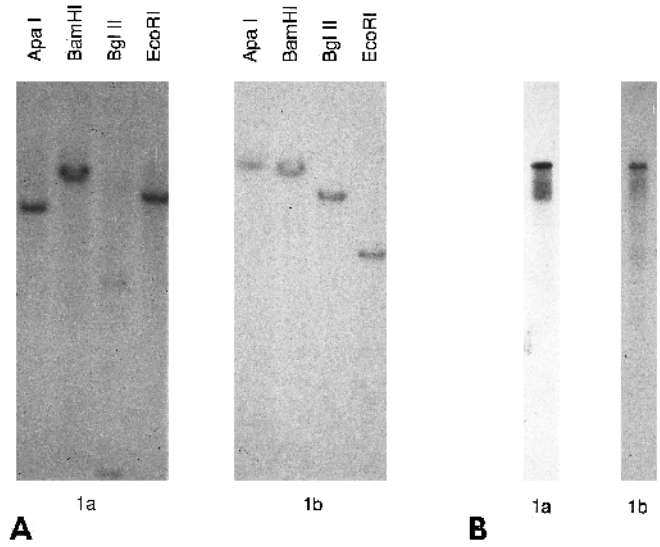

Fig. 2. Southern and northern blots of DHC1a and DHC1b. (A) Two identical Southern blots of rat genomic DNA digested with four different enzymes were probed with the DHC1a and DHC1b fragments. (B) Two identical northern blots of rat brain poly(A)$^+$ RNA were probed with the DHC1a and DHC1b fragments. In both cases, the major RNA species hybridizing with the probe was ~15 kb. In B, the 1a blot was exposed to the X-ray film for 1 day; the 1b blot was exposed for 10 days.

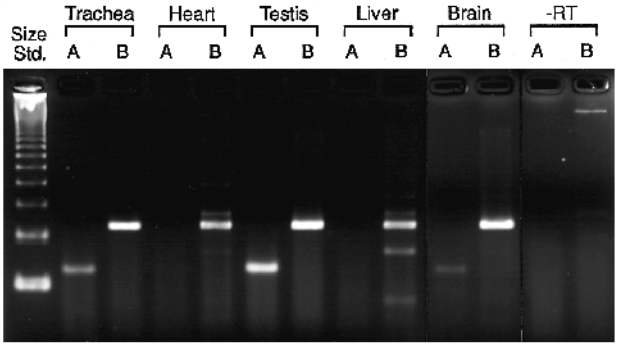

Fig. 3. RT-PCR analysis of the expression of DHC1b in rat tissues. RNA-directed PCR, using axonemal dynein-specific primers (lane A in each tissue set) and DHC1b-specific primers (lane B), was performed as described in Materials and Methods, and the amplified products analyzed on an agarose gel stained with EtBr. Axonemal dynein products (~150 bp) were found only in tissues known to produce cilia or flagella: trachea, testis, and brain. The DHC1b product (~250 bp) was found in all tissues examined. Control amplifications of tracheal RNA with no reverse transcriptase (–RT) did not yield any products.

thelium (Kaartinen et al., 1993; Clark et al., 1995), was utilized to examine the expression of DHC1b during mammalian ciliogenesis. This model system is particularly powerful because the extent of ciliated cell differentiation can be regulated positively or negatively by altering the culture conditions (reviewed by Ostrowski et al., 1995). After reaching confluence, the RTE cells were induced to begin differentiation by the creation of an air-liquid interface in the culture. Using gene-specific primers, RT-PCR revealed that

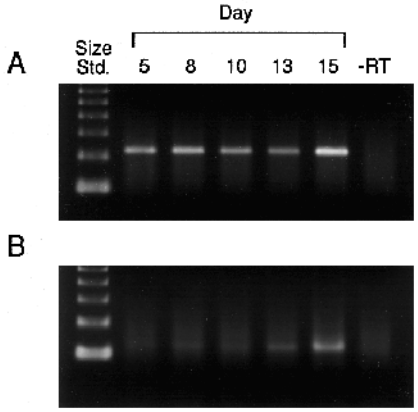

Fig. 4. RT-PCR analysis of the expression of DHC1b during ciliogenesis of cultured RTE cells. RNA was isolated at different timepoints from cultured RTE cells. The air-liquid interface was introduced on day 7 in this experiment. (A) Expression of DHC1b was apparent prior to as well as during ciliogenesis. (B) Expression of axonemal dynein was not seen until after differentiation was induced and appeared to increase during ciliogenesis. Control amplifications of day 15 RNA with no reverse transcriptase (–RT) did not yield any products.

DHC1b mRNA was present in the cultures prior to differentiation as well as through ciliogenesis (Fig. 4a). In contrast, axonemal dynein mRNA was not detected prior to differentiation and only appeared after the introduction of the air-liquid interface. The very sensitive RT-PCR was performed under conditions that provided a qualitative, but only semi-quantitative, view of dynein expression. A more exact measure of dynein expression was obtained in northern blotting experiments, an example of which is shown in Fig. 5. The DHC1b mRNA was detected at a constant low level in undifferentiating (submerged) cells. The expression of DHC1b mRNA increased during differentiation (control); this increase in DHC1b mRNA was substantially more rapid and greater in cultures from which epidermal growth factor (EGF) and cholera toxin (CT) had been removed, a treatment that stimulates ciliogenesis. In parallel experiments, axonemal dynein expression increased during differentiation whereas DHC1a expression was high and remained unchanged throughout the experiment (see Fig. 5 in Andrews et al., 1996). Taken together, these data demonstrate: (1) that DHC1b mRNA is present in undifferentiated RTE cells, unlike axonemal dynein which is detected only after differentiation has commenced; (2) that DHC1b is expressed in a different pattern from DHC1a; and (3) that rat DHC1b mRNA, like sea urchin 1b, increases in its apparent steady state concentration during ciliogenesis.

Isoform-specific antibodies

In order to understand the cellular function of DHC1b, it is necessary to examine the 1b protein. Two peptide antisera were produced against the 1a and 1b sequences indicated in Fig. 1. The rabbit antisera were first characterized in solid-phase binding experiments. Anti-1a reacted with 1a peptide and, more weakly (i.e. at a higher antibody concentration), with 1b peptide (Fig. 6a). In contrast, anti-1b reacted only with 1b peptide and did not cross-react with the inappropriate peptide (Fig. 6b). The specificity of the antisera was also examined in competitive solid-phase binding assays in which the antisera were pre-incubated with increasing quantities of soluble peptide during the binding assay. In the competition experiments, anti-1a binding to 1a was inhibited with 1a peptide but

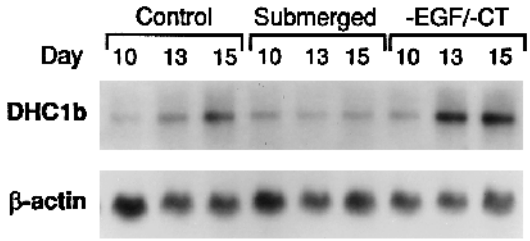

Fig. 5. Northern blot analysis of DHC1b expression in cultured RTE cells. RNA was isolated at different timepoints from cultured RTE cells and probed with DHC1b and, as a control, actin. The air-liquid interface was introduced in the control culture on day 7 in this experiment. The steady-state concentration of DHC1b mRNA clearly increased during ciliogenesis in the control culture; this accumulation was more rapid in the culture from which growth factors had been withdrawn (–EGF/–CT), a treatment which stimulates ciliogenesis. In contrast, DHC1b mRNA was present in submerged (not ciliating) cultures, but its concentration did not change over time in the submerged culture.

not 1b peptide (Fig. 6c), and the anti-1b antiserum was competed with 1b peptide but not with 1a peptide (Fig. 6d). The anti-1a antiserum reacted at different titers with both peptides (Fig. 6a), but its binding to 1a peptide was completely competed with 1a and not at all with 1b (Fig. 6c). This suggests that the anti-1a antiserum contains two separate populations of antibodies; one population binds an epitope only on 1a and the other population binds an epitope common to 1a and 1b. In contrast, the anti-1b antiserum recognized only 1b epitope(s). These results demonstrate that the anti-1b antiserum is specific for the 1b peptide whereas the anti-1a antiserum, although preferentially binding the 1a peptide, is unable to discriminate completely between 1a and 1b.

These antisera were then tested on western blots of the ATP extract from rat brain microtubules (Fig. 7). The binding of anti-1a to the dynein heavy chain region of the blot was mostly, but not completely, eliminated by preabsorbing the antiserum with excess 1a peptide (Fig. 7, lane 2). This was expected because the anti-1a antiserum contained a low titer of reactivity against 1b (Fig. 6a). The western blots of anti-1b demonstrated that this antiserum reacted with cytoplasmic dynein heavy chain; this binding was completely abolished by preabsorbing the antiserum with 1b peptide (Fig. 7, lane 6). Because the signal obtained with anti-1b antiserum was much weaker than that obtained with anti-1a, and because the binding of the 1a-absorbed anti-1a antiserum (presumably detecting 1b) was also very weak, it appears that DHC1b is a relatively minor component of rat brain cytoplasmic dynein.

Immunolocalization of DHC1b in ciliated RTE cells

Freshly isolated RTE cells were fixed in formaldehyde and stained by indirect immunofluorescence. The affinity-purified anti-1b antibody stained the cytoplasm of ciliated cells, but only very weakly stained unciliated cells (Fig. 8a-d). Often, the anti-1b staining was concentrated at the apical ends of the cells, sometimes forming a bright crescent near the base of the cilia. In contrast, an antiserum reactive with axonemal dyneins (Tjandra et al., 1995) brightly stained the cilia and the apical ends of the RTE cells (Fig. 8e, f). Anti-1a antiserum weakly stained the cytoplasm of all cells, ciliated and unciliated; this

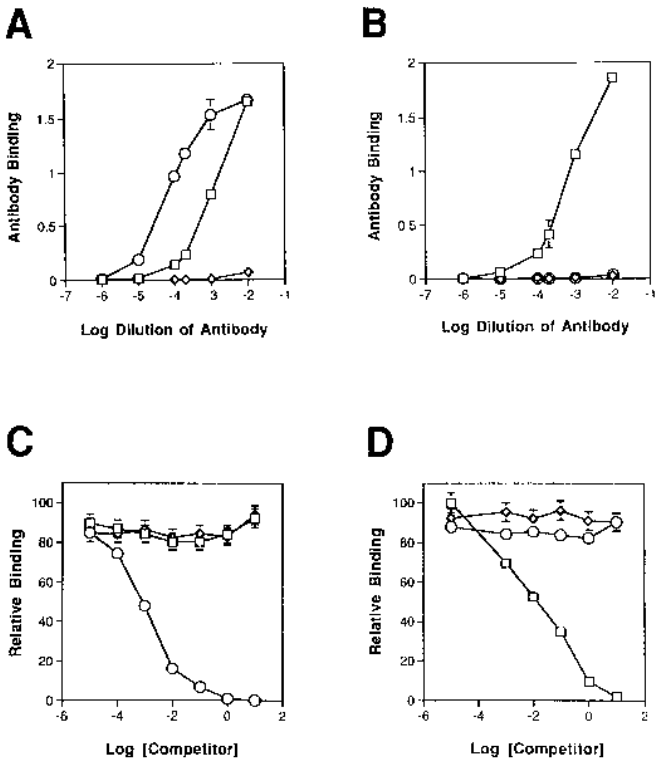

Fig. 6. Solid-phase binding assays with peptide antisera. (A,B) Binding of anti-1a antiserum (A) and anti-1b antiserum (B) to 50 ng peptide 1a (circles) and 50 ng peptide 1b (squares) immobilized on the plates. Antibody binding is reported in units of absorbance at 405 nm after the alkaline phosphatase reaction. In both panels, preimmune serum binding to the peptides (diamonds) was negligible. Under these conditions, anti-1a antiserum reacted with both 1a and 1b peptides, exhibiting a higher titer to peptide 1a; in contrast, anti-1b antiserum reacted only with 1b peptide. (C,D) Competitive binding experiments. (C) Anti-1a binding to immobilized peptide 1a was competed only with peptide 1a (circles) and not affected by peptide 1b (squares) and bovine serum albumin (BSA, diamonds). (D) Anti-1b binding to immobilized peptide 1b was competed only with peptide 1b (squares) and not affected by peptide 1a (circles) and BSA (diamonds). The stock concentration of each competitor was 1 mg/ml. In all panels, the averages of three separate measurements are plotted.

staining was usually diffuse throughout the cytoplasm (data not shown).

DISCUSSION

We have cloned and sequenced fragments of DHC1a and DHC1b from rat tissues; DHC1a is the same as the previously sequenced conventional cytoplasmic dynein, MAP1C (Mikami et al., 1993; Zhang et al., 1993), and DHC1b is the same as DLP4 which was previously identified in rat brain (Tanaka et al., 1995). Both dynein heavy chain sequences contain the B motif adjacent to the catalytic ATP-binding site, a feature that is found in all confirmed cytoplasmic dyneins (Asai and Brokaw, 1993). Southern blotting indicated that DHC1a and DHC1b are separate, single copy genes. Northern blotting showed that DHC1b mRNA is far less abundant in rat brain

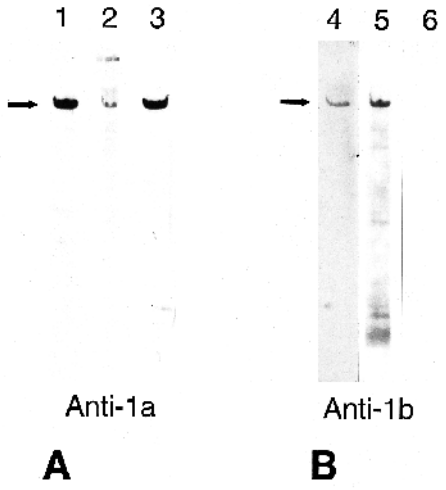

Fig. 7. Western blots of rat brain cytoplasmic dynein. Partially purified rat brain cytoplasmic dynein was electrophoresed, transferred to nitrocellulose, and probed with anti-1a (A) or anti-1b (B). Binding to dynein heavy chain (arrow) was examined. Lanes 1 and 4, strips probed with unabsorbed antisera. Lanes 2 and 5, strips probed with antisera preabsorbed with 1 mg peptide 1a; this treatment only partially eliminated anti-1a binding to dynein (lane 2) and did not inhibit anti-1b binding (lane 5). Lanes 3 and 6, strips probed with antisera preabsorbed with 1 mg peptide 1b; this treatment completely eliminated anti-1b binding (lane 6) and did not affect anti-1a binding (lane 3).

than is DHC1a. Qualitative RT-PCR revealed that DHC1b is expressed in cilia containing tissues (trachea, testis, brain) as well as in the non-ciliated tissues heart and liver. The RT-PCR analysis also showed that DHC1b is present in undifferentiated RTE cells. This was in marked contrast to the expression of axonemal dynein, which only was detected after differentiation had commenced. Northern blotting showed that the steady state concentration of rat DHC1b, like sea urchin 1b, increased during ciliogenesis. In contrast, in the same experiments, rat DHC1a did not change (see Andrews et al., 1996).

In order to examine the cellular expression of the two dynein isoforms, we produced antisera to synthetic peptides whose sequences were derived from DHC1a and DHC1b. Western blotting indicated that DHC1b is expressed as a protein, but is only a relatively minor component of rat brain cytoplasmic dynein. Indirect immunofluorescence microscopy of RTE cells revealed that DHC1b is present in the cytoplasm of ciliated cells and often is concentrated at the apical ends of the cell. Importantly, DHC1b does not appear to be a component of RTE cilia.

There are several lines of evidence that indicate that DHC1b is a cytoplasmic dynein. One, DHC1b RNA is expressed in tissues that contain no cilia, including heart and liver. Two, in cultured rat tracheal epithelial cells, DHC1b RNA is expressed prior to the onset of ciliogenesis. Three, the DHC1b-specific antibody stained the cytoplasm but not the cilia of ciliated tracheal cells. Four, using the procedure of Neely et al. (1990), we have found that DHC1b from rat testis resides in the soluble fraction after tissue homogenization (P. S. Criswell and D. J. Asai, unpublished). We conclude that DHC1b is a second isoform of cytoplasmic dynein.

To date, DHC 1b has been identified only in organisms that produce cilia. In unciliated organisms, only the DHC1a isoform has been found. However, DHC1b is not restricted in its expression to ciliated cells. For example, rat DHC1b is expressed in testis, trachea, and brain (which contains ciliated ependymal cells) and is also expressed in unciliated cell types, including those from the heart and liver and PC12 cells. One of the functions of DHC1b in unciliated cells is revealed in a recent study of human fibroblast cells. In these cells, DHC1b appears to mediate the organization and trafficking of secretory organelles (Vaisberg et al., 1996).

What is the function of DHC1b in ciliated cells? Two lines of evidence indicate that DHC1b plays a special role during ciliogenesis. The first indication is the increase of DHC1b mRNA expression during ciliogenesis, which was first

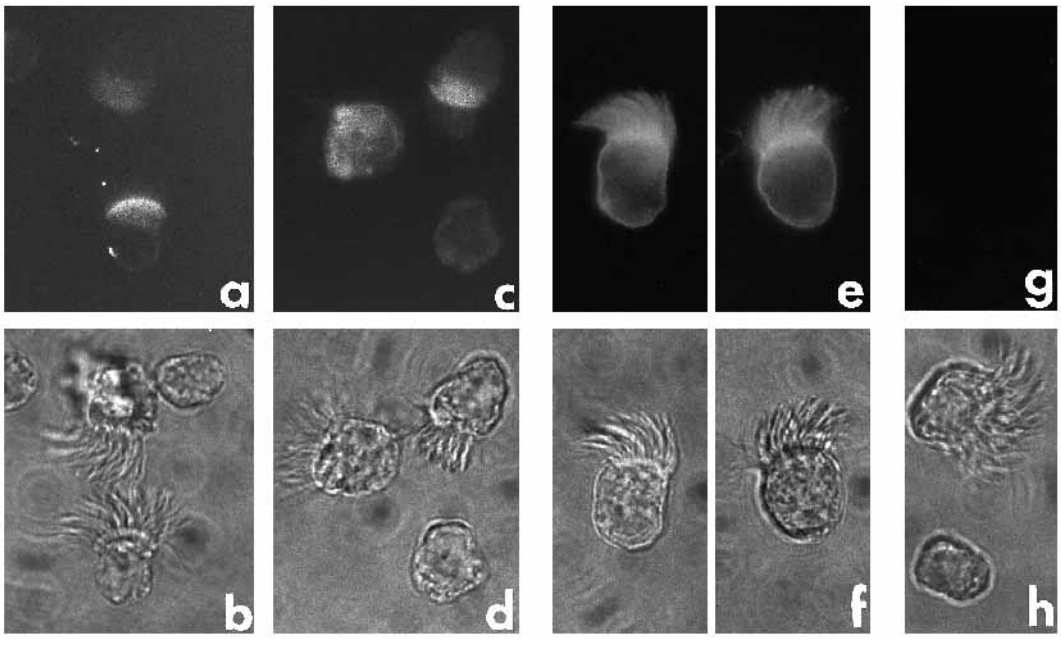

Fig. 8. Indirect immunofluorescence microscopy of isolated RTE cells. (a-d) Cells stained with affinity-purified anti-1b antibody (a and c) and the corresponding brightfield images (b and d). In each photograph, there is an unciliated cell that did not stain brightly with the antibody. (e and f) Cells stained with anti-axonemal dynein antibody (Tjandra et al., 1995). (g and h) Cells stained with preimmune serum from the rabbit that produced anti-1b antibody.

observed in sea urchin embryos (Gibbons et al., 1994). In the RTE cells studied in the present report, DHC1b expression parallelled cilia formation: when ciliogenesis was accelerated, DHC1b was more rapidly expressed; and when ciliogenesis was suppressed, DHC1b expression was detected but did not change (see Fig. 5). The second line of evidence is the immunolocalization of the DHC1b isoform in RTE cells. In ciliated cells, the DHC1b antigen often is concentrated at the apical end, which may reflect the accumulation of DHC1b at the proximal ends of the cytoplasmic microtubules. Perhaps the DHC1b isoform participates in the transport of ciliary precursors from the interior to the apical end of the polarized epithelial cells (LaFont et al., 1994; Fath et al., 1994).

Finally, it is intriguing to speculate about the functional specialization of dyneins 1a and 1b. Does the axonemal dyneins paradigm, in which each dynein isoform is highly specialized in its contribution to the overall movement, apply to cytoplasmic dynein isoforms (see Asai, 1996)? Specialization of cytoplasmic dyneins might be manifested in the tethering of a particular isoform to a specific cargo, or, as in the case of axonemal dyneins, each cytoplasmic isoform may produce a different force, perhaps to move cargoes at different rates. It will be interesting to apply the methods of in vitro motility assays and molecular genetics to define the properties of each of the cytoplasmic dyneins.

We would like to especially acknowledge Ian Gibbons, whose laboratory initially identified DHC1b and who has provided us with many helpful comments throughout this study. We also thank Kaya Andrews, Jim Forney, Paul Nettesheim, Hendri Tjandra, and John Wilder for reagents and good advice. The Purdue University Cancer Center (supported by a research core grant from the NCI) partially subsidized the production of oligonucleotides, peptides, and antibodies. This study was supported by grants from the NIH and American Cancer Society (D.J.A.) and the Division of Intramural Research of NIEHS (L.E.O.).

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(Received 7 February 1996 - Accepted 9 April 1996)