Mammalian Cells Express Three Distinct Dynein Heavy Chains That Are Localized to Different Cytoplasmic Organelles

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Abstract. We describe two dynein heavy chain (DHC)–like polypeptides (DHCs 2 and 3) that are distinct from the heavy chain of conventional cytoplasmic dynein (DHC1) but are expressed in a variety of mammalian cells that lack axonemes. DHC2 is a distant member of the "cytoplasmic" branch of the dynein phylogenetic tree, while DHC3 shares more sequence similarity with dynein-like polypeptides that have been thought to be axonemal. Each cytoplasmic dynein is associated with distinct cellular organelles. DHC2 is localized predomi- nantly to the Golgi apparatus. Moreover, the Golgi disperses upon microinjection of antibodies to DHC2, suggesting that this motor is involved in establishing proper Golgi organization. DHC3 is associated with as yet unidentified structures that may represent transport intermediates between two or more cytoplasmic com- partments. Apparently, specific cytoplasmic dyneins, like individual members of the kinesin superfamily, play unique roles in the traffic of cytomembranes.

MICROTUBULE-dependent motor enzymes play crit- ical roles in many types of intracellular trans- port. There are two principal classes of microtu- bule motors: kinesins and dyneins. The kinesins comprise a superfamily of proteins, defined by sequence identity in a "motor domain." They are associated with a surprising diversity of cytoplasmic movements: spindle pole separa- tion, chromosome movement, axonal transport, Golgi ves- icle traffic, migrations in and out of axonemes, and proba- bly a wealth of additional movements that are still to be characterized (Goldstein, 1993; Skoufias and Scholey, 1993). The family of dyneins is less diverse, but even so, it includes more than a dozen heavy chain polypeptides that contribute to making an axoneme (Gibbons et al., 1994; Rasmusson et al., 1994) and a cytoplasmic isoform that has been suggested to play roles in vesicle transport, mem- brane dynamics, chromosome movement, and the posi- tioning of some organelles (for review see Holzbaur and Vallee, 1994).

The importance of dynein function in axoneme motility is unequivocal, but our understanding of dynein's role in intracellular motility is less complete. Several lines of evi- dence implicate this enzyme in retrograde axonal trans- port (Schroer, 1992). Localization studies have placed both the heavy chain and a 74-kD intermediate chain of cytoplasmic dynein at kinetochores, where a minus-end– directed motor enzyme could contribute to anaphase A (Steuer et al., 1990; Pfarr et al., 1990), but there is as yet no evidence that dynein plays such a role. Microinjection of function-blocking antibodies raised against bacterially ex- pressed fragments of cytoplasmic dynein heavy chains from two species blocked the separation of mitotic centrosomes, but they had no effect on chromosome attachment to the spindle (Vaisberg et al., 1993). Genetic analysis of cyto- plasmic dynein function in fungi has implicated this en- zyme in both the positioning and the elongation of mitotic nuclei in *Saccharomyces cerevisiae* (Li et al., 1993) and in postmitotic nuclear movements in other fungi (Xiang et al., 1994; Plamann et al., 1994). Cytoplasmic dynein has also been implicated in the motion of vesicles derived from the Golgi apparatus to the centrosomal region of lysed cell models (Corthesy-Theulaz et al., 1992), and in the trans- port of endocytic vesicles (Bomsel et al., 1990; Fath et al., 1994; Lafont et al., 1994; Oda et al., 1995), but the relation- ship between these in vitro systems and membrane move- ments in vivo is still undefined.

The diversity of functions attributed to cytoplasmic dy- nein, together with the ineffectiveness of our motility- blocking dynein antibodies in disrupting both chromo- some movement and Golgi dynamics in vivo (Vaisberg, E.A., and J.R. McIntosh, unpublished results), led us to suspect that cytoplasmic dynein might be more complex than was previously thought. We hypothesized that there were novel isoforms of cytoplasmic dynein involved in sev- eral aspects of cell motility, so experimental approaches focused on the one known isozyme were bound to fail. We have therefore undertaken a search for novel dynein heavy chains that are expressed in cells making neither cilia nor flagella. Here we report the identification of two such iso- forms, cytoplasmic dynein heavy chains (DHCs)[1] 2 and 3

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1. *Abbreviations used in this paper.* BFA, brefeldin A; DHC, dynein heavy chain; RT, reverse transcription.

(DHC2 and 3), whose intracellular localizations differ markedly from that of "conventional" cytoplasmic dynein heavy chain (DHC1) and from each other. DHC2 is associated predominately with the Golgi apparatus and is likely to be involved in the organization of this organelle. DHC3 localizes to cytoplasmic structures that do not correspond well to any of the well-defined membranous compartments of cytoplasm.

Materials and Methods

Reagents and Antibodies

Unless otherwise specified, biochemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Enzymes for molecular biology were purchased from Promega Corp. (Madison, WI) or Boehringer Mannheim Biochemicals (Indianopolis, IN). The mAb p58-9 (Bloom and Brashear, 1989) was a gift from Dr. G. Bloom (University of Texas Southwestern Medical Center, Dallas, TX); the mAb to mannose-6-phosphate receptor (Lombardi et al., 1993; Dintzis et al., 1994) was a gift from Dr. S. Pfeffer (Stanford University, Stanford, CA); the mAb to α-mannosidase II (Burke et al., 1982) was purchased from BAbCO (Richmond, CA). Texas red–conjugated human transferrin vital fixable markers for lysosomes (LysoTracker Red DHD-99) and mitochondria (MitoTracker Green FM) were purchased from Molecular Probes, Inc. (Eugene, OR).

Reverse Transcription, PCR, and Analysis of Clones

Purification of mRNA, cDNA synthesis, and PCR with degenerate primers were performed as described previously (Vaisberg et al., 1993) with two modifications: PCR was performed with an annealing temperature of 37°C, and the pDK101 vector (Kovalic et al., 1991) (No. 77406; American Type Culture Collection, Rockville, MD) was used to clone the resulting reaction products.

RNA Walk, Library Screening, Molecular Biological Techniques, and Sequence Analysis

A random primed cDNA library from a human adenocarcinoma cell line (Stratagene, La Jolla, CA) was screened with the DHC1-specific probe Hp22 (Vaisberg et al., 1993) using the Genius 1 labeling system (Boehringer Mannheim Biochemicals) according to the manufacturer's protocol. cDNA for a 5′ "walk" was primed with a degenerate oligonucleotide that encodes the MNPGYAG sequence (primer "b" in Vaisberg et al., 1993). Subsequent tailing with dCTP and amplification by two rounds of PCR with sequence-specific primers (1.1 and 1.2 for DHC1 and 2.1 and 2.2 for DHC2; see Fig. 1 B) and an anchor dG oligonucleotide were performed as described in (Lee et al., 1993), except dITP residues were incorporated into the adapter (Schuster et al., 1992). The resulting products were cloned into the pDK101 vector and sequenced using the ΔTaq Cycle Sequencing kit (United States Biochemical Corp., Cleveland, OH).

To account for possible PCR errors during reverse transcription (RT)–PCR and RACE, sequences encoding DHC2 were verified by sequencing three independently isolated clones. The sequence of the 3′ 2 kb of the library clone for DHC1 was additionally verified by sequencing the corresponding RACE clone. Sequence alignment and analysis was performed using GCG Wisconsin Package Version 8.1-UNIX (Program Manual for the Wisconsin Package, Version 8, September 1994; Genetics Computer Group, Madison, WI). For Southern blot analysis, genomic DNA was isolated from HeLa cells as described in Sambrook et al. (1989). 20 μg of human genomic DNA was digested with restriction endonucleases as indicated, blotted, and hybridized with DHC probes at high stringency as described in Vaisberg et al. (1993). For Northern blotting, total RNA was isolated using RNA Isolator (Genosys Biotechnologies Inc., The Woodlands, TX). 10 μg of RNA was separated on a 0.8% formaldehyde-denaturing agarose gel, transferred to a nylon membrane, and probed as described in Vaisberg et al. (1993).

Preparation of Immunogens, Purification of Antibodies, and Immunoblotting

cDNA clones used for the expression of the antigens were obtained by direct PCR from HeLa RNA using isoform-specific primers, thus minimizing the possibility of accumulating PCR errors in cycles of amplification.

The primer pairs used were: 1.6–1.3 to produce DHC1/1; 2.6–2.3 to produce DHC2/1; 3.4–3.3 to produce both DHC3/1 and DHC3/2; and 1.5–1.4 and 2.5–2.4 to produce DHC1/2 and DHC2/2, respectively (Fig. 1). The identity of the clones was verified by end sequencing. Gene fragments encoding the antigens were cloned into the pET5b expression vector (Studier et al., 1990) (DHC1/1, DHC1/2, DHC2/2, and DHC3/2) or pRSET vector (Invitrogen, San Diego, CA) (DHC2/1 and DHC3/1) and expressed in *Escherichia coli* strain BL21(DE3). Inclusion bodies containing the expressed protein were purified (Lin and Cheng, 1991) and fractionated by gel electrophoresis. Bands of expressed proteins were excised and electroeluted using an Elutrap device (Schleicher & Schuell, Inc., Keene, NH).

Immunization, preparation of columns for affinity depletion and purification, and chromatography were performed as previously described (Vaisberg et al., 1993). Serum from rabbits immunized with each of the smaller (see Fig. 4) DHC fragments was depleted for cross-reactivity with the two other isoforms by repeated passages through the columns with coupled larger fragments until no cross-reactivity was detected by immunoblotting. This preparation was affinity purified on a column with the larger antigen representing the same isoform used for immunization. For example, anti-DHC2 antibody was prepared by immunization of rabbits with DHC2/2 fragment, sequential immunodepletion of the resulting serum on columns with DHC1/1 and DHC3/1 polypeptides, and then affinity purification on the column with DHC2/1 polypeptide. Immunoblotting was performed with 4% dry milk as a blocking reagent and developed using an ECL development reagent (Amersham Corp., Arlington Heights, IL) according to manufacturer's protocol. Antibodies to DHC1, 2, and 3 were used at concentrations of 0.2–0.5 μg/ml.

Preparation of Cell Extracts and Fractionation on Sucrose Gradients

HeLa and NRK cells were collected by scraping into ice-cold PBS; washed two times with PBS and once with PME buffer (100 mM Pipes, 5 mM $MgSO_4$, 1 mM EGTA, 1 mM DTT; pH 6.9); resuspended in PME, supplemented with protease inhibitors (10 μg/ml of leupeptin, pepstatin A, and aprotinin; 1 mM PMSF) to the final density 10^8 cells per ml; lysed by gentle sonication or by passing 10–15 times through a 26-gauge needle; and clarified by centrifugation in an SS34 rotor (Sorvall Instruments Div., Newton, CT) at 17,000 g_{av} for 15 min. 250 μl of the resulting extract was loaded on the top of 5–20% gradient of sucrose in PME and centrifuged in an SW60Ti rotor (Beckman Instruments, Inc., Fullerton, CA) at 360,000 g_{av} for 5.2 h. Thyroglobulin (19S), catalase (11S), and cytochrome C (2.2S) were used as standards. Fractions from the gradients were analyzed by immunoblotting as described above.

Cell Culture, Immunofluorescence, and Microinjection

NRK, COS-7, and HeLa cells were grown in DME (Sigma Chemical Co.), supplemented with 10% FCS (GIBCO BRL, Gaithersburg, MD), 50 U/ml penicillin G, and 50 U/ml streptomycin sulfate. A431 cells were grown in DME, supplemented with 10% calf serum (GIBCO BRL), and CFPAC-1 cells were cultured in IMEM (Sigma Chemical Co.), supplemented with 10% FCS. For immunofluorescence, cells were plated on glass coverslips, and allowed to grow for 24–36 h, fixed with 4% paraformaldehyde in PBS for 15 min, permeabilized with 0.1% Triton X-100 in PBS for 5 min, and processed for immunofluorescence as previously described (Nislow et al., 1990). All anti-DHC antibodies were used at a concentration of 5–10 μg/ml.

During the course of this study we experimented with several fixation protocols for immunofluorescence. Our experience with the microtubule cytoskeleton had led us to favor fixation in cold (−20°C) methanol, usually followed by cold acetone. Upon application of this protocol to cells destined for staining by DHC3 antibodies, we found label specifically associated with mitochondria. Aldehyde fixation, followed by detergent permeabilization, on the other hand, showed little or no such association. By comparing these localizations with various marker antibodies, we discovered that cold methanol fixation makes staining with several markers, such as βCOP, congruent with mitochondrial markers, a result that is almost certain to be erroneous. We therefore fixed specimens by ultrarapid freezing and freeze substitution. Cultured cells were plated upon formvarcoated, carbon-stabilized gold grids, as used for EM. Individual grids were plunge frozen in liquid ethane, freeze substituted in acetone at −90°C, and fixed in paraformaldehyde and glutaraldehyde as the specimen was warmed to 0°C; then the cells were rinsed in pure acetone, rehydrated in PBS, and processed for immunofluorescence. These specimens showed the expected distributions for βCOP, mitochondrial, and Golgi markers, and DHC staining was far more similar to that described here with alde-

hyde fixation. We therefore settled on this as the more reliable way of preserving cytomembranes for immunofluorescent localization.

Microinjection of antibodies to DHC2 and preimmune immunoglobulins (both at 4.7 mg/ml) was performed as previously described (Vaisberg et al., 1993). After injection, NRK cells were incubated at 37°C for 3 h, and then fixed and processed for immunostaining with antibody to α-mannosidase II (FITC) and to the injected antibody (Texas red). The dispersion of the Golgi apparatus in injected and control cells was assessed by eye.
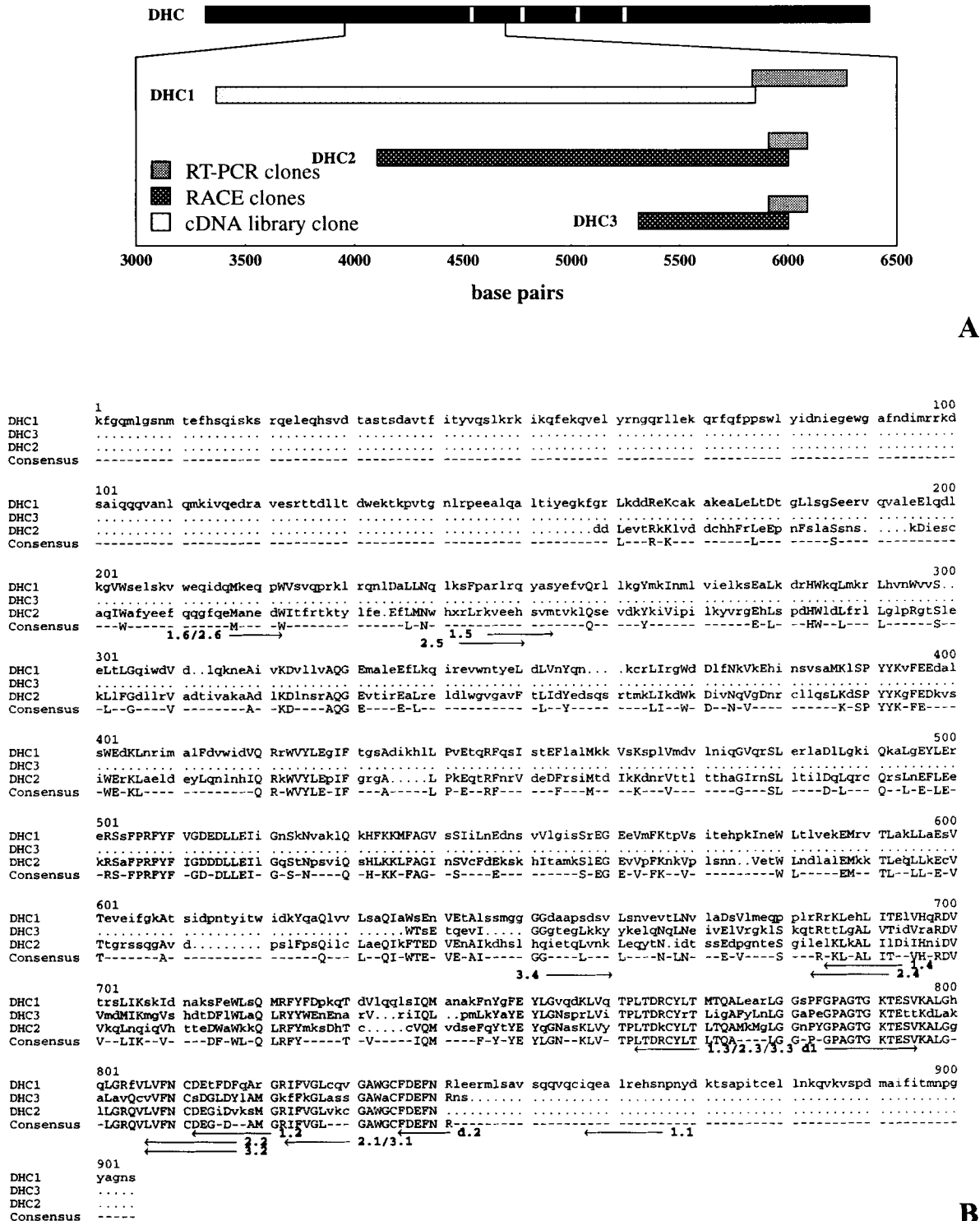
DHC

DHC1

RT-PCR clones
RACE clones
cDNA library clone

DHC2

DHC3

3000   3500   4000   4500   5000   5500   6000   6500

base pairs

A

```
          1                                                                                      100
DHC1      kfgqmlgsnm tefhsqisks rqeleqhsvd tastsdavtf ityvqslkrk ikqfekqvel yrnggqrllek qrfqfppswl yidniegewg afndimrrkd
DHC3      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
DHC2      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
Consensus ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

          101                                                                                    200
DHC1      saiqqqvanl qmkivqedra vesrttdllt dwektkpvtg nlrpeealqa ltiyegkfgr LkddReKcak akeaLeLtDt gLlsgSeerv qvaleElqdl
DHC3      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
DHC2      .......... .......... .......... .......... .......... ........dd LevtRkKlvd dchhFrLeEp nFslaSsns. ....kDiesc
Consensus ---------- ---------- ---------- ---------- ---------- ---------- L---R-K--- ------L---- -----S---- ----------

          201                                                                                    300
DHC1      kgVWselskv weqidqMkeq pWVsvqprkl rqnlDaLLNq lksFparlrq yasyefvQrl lkgYmkInml vielksEaLk drHWkqLmkr LhvnWvvS..
DHC3      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
DHC2      aqIWafyeef qqgfqeMane dWItfrtkty lfe.EfLMNw hxrLrkveeh svmtvklQse vdkYkiVipi lkyvrgEhLs pdHWldLfrl LglpRgtSle
Consensus ---W----- -----M--- -W-------- ------L-N- ---------- -------Q-- ---Y------ ------E-L- --HW--L--- L------S--
                         1.6/2.6 ------->            2.5   1.5 ------->

          301                                                                                    400
DHC1      eLtLGqiwdV d..lqkneAi vKDvllvAQG EmaleEfLkq irevwntyeL dLVnYqn... .kcrLIrgWd DlfNkVkEhi nsvsaMKlSP YYKvFEEdal
DHC3      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
DHC2      kLlFGdllrV adtivakaAd lKDlnsrAQG EvtirEaLre ldlwgvgavF tLIdYedsqs rtmkLIkdWk DivNqVgDnr cllqsLKdSP YYKgFEDkvs
Consensus -L--G----V --------A- -KD----AQG E----E-L-- ---------- -L--Y----- ----LI--W- D--N-V---- ------K-SP YYK-FE----

          401                                                                                    500
DHC1      sWEdKLnrim alFdvwidVQ RrWVYLEgIF tgsAdikhlL PvEtqRFqsI stEFlalMkk VsKsplVmdv lniqGVqrSL erlaDlLgki QkaLgEYLEr
DHC3      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
DHC2      iWErKLaeld eyLqnlnhIQ RkWVYLEpIF grgA.....L PkEqtRFnrV deDFrsiMtd IkKdnrVttl tthaGIrnSL ltilDqLqrc QrsLnEFLEe
Consensus -WE-KL---- ---------Q R-WVYLE-IF ---A-----L P-E--RF--- ---F---M-- --K---V--- ----G---SL ----D-L--- Q--L-E-LE-

          501                                                                                    600
DHC1      eRSsFPRFYF VGDEDLLEIi GnSkNvaklQ kHFKKMFAGV sSIiLnEdns vVlgisSrEG EeVmFKtpVs itehpkIneW LtlvekEMrv TLakLLaEsV
DHC3      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
DHC2      kRSaFPRFYF IGDDDLLEIl GqStNpsviQ sHLKKLPAGI nSVcFdEksk hItamkSlEG BvVpFKnkVp lsnn..VetW LndlalEMkk TLeQLLkEcV
Consensus -RS-FPRFYF -GD-DLLEI- G-S-N----Q -H-KK-FAG- -S----E--- ------S-EG E-V-FK--V- ---------W L-----EM-- TL--LL-E-V

          601                                                                                    700
DHC1      TeveifgkAt sidpntyitw idkYqaQlvv LsaQIaWsEn VEtAlssmgg GGdaapsdsv LsnvevtLNv laDsVlmeqp plrRrKLehL ITElVHqRDV
DHC3      .......... .......... .......... ......WTsE tqevI..... GGgtegLkky ykelqNqLNe ivElVrgklS kqtRttLgAL VTidVraRDV
DHC2      Ttgrssqg Av d........ pslFpsQilc LaeQIkFTED VEnAIkdhsl hqietqLvnk LeqytN.idt ssEdpgnteS gilelKLkAL IlDiIHniDV
Consensus T-------A- ---------- ------Q--- L--QI-WTE- VE-AI----- GG----L--- L----N-LN- --E-V----S ---R-KL-AL IT--VH-RDV
                                                                                  3.4 ------->            <------ 2.4

          701                                                                                    800
DHC1      trsLIKskId naksFeWLsQ MRFYFDpkqT dVlqqlsIQM anakFnYgFE YLGvqdKLVq TPLTDRCYLT MTQALearLG GsPFGPAGTG KTESVKALGh
DHC3      VmdMIKmgVs hdtDFlWLaQ LRYYWEnEna rV...riIQL ..pmLkYaYE YLGNsprLVi TPLTDRCYrT LigAFyLnLG GaPeGPAGTG KTEttKdLak
DHC2      VkqLnqiqVh tteDWaWkkQ LRFYmksDhT c.....cVQM vdseFqYtYE YgGNasKLVy TPLTDkCYLT LTQAMkMgLG GnPYGPAGTG KTESVKALGg
Consensus V--LIK--V- ---DF-WL-Q LRFY-----T -V-----IQM ----F-Y-YE YLGN--KLV- TPLTDRCYLT LTQA----LG G-P-GPAGTG KTESVKALG-
                                                                                             <------ 1.3/2.3/3.3 d1 ------>

          801                                                                                    900
DHC1      qLGRfVLVFN CDEtPDFqAr GRIFVGLcqv GAWGCFDEFN Rleermlsav sqqvqciqea lrehsnpnyd ktsapitcel lnkqvkvspd maifitmnpg
DHC3      aLavQcvVFN CsDGLDYlAM GkfPkGLass GAWaCFDEFN Rns....... .......... .......... .......... .......... ..........
DHC2      lLGRQVLVFN CDEGiDvksM GRIFVGLvkc GAWGCFDEFN .......... .......... .......... .......... .......... ..........
Consensus -LGRQVLVFN CDEG-D--AM GRIFVGL--- GAWGCFDEFN R--------- ---------- ---------- ---------- ---------- ----------
                        <----- 1.2         <------ 2.1/3.1   d.2 ------>            <------ 1.1

          901
DHC1      yagns
DHC3      .....
DHC2      .....
Consensus -----
```

B

Figure 1. (*A*) Schematic representation of clones described in the paper. Black bar at top depicts a whole dynein heavy chain with four P-loops from the consensus sequence for ATP-binding marked with white. Lighter cross-hatched bars show the position and sizes of the initial PCR clones; darker spotted bars depict RACE clones; light gray bar indicates a clone obtained by cDNA library screening. (*B*) Deduced amino acid sequences for parts of DHC1 and DHC2. Shown below the sequences are the positions of oligonucleotide primers used for initial RT-PCR amplification (degenerate primers *d.1* and *d.2*), subsequent 5' RACE (primers *1.1, 1.2, 2.1, 2.2*), and amplification of cDNA for the expression of immunogens (primers *1.3–6* and *2.3–6*).

Based on the morphology of the Golgi, cells were assigned to one of three classes: not dispersed, dispersed, and unclear. The latter category comprised a small minority of cells (10–15%) and was excluded from further analysis. Confidence intervals for the percentage of cells with the dispersed Golgi were calculated assuming a binominal distribution.

Results

Three Isoforms of Dynein Heavy Chain Are Expressed in Cells That Do Not Make Cilia or Flagella

We have performed a rigorous screen for cytoplasmic DHCs by reverse transcription of mRNA from HeLa cells, followed by the PCR (RT-PCR). HeLa cells do not form cilia or flagella, so it is likely that any dynein expressed in these cells performs a "cytoplasmic" function.

The degenerate oligonucleotide primers used for RT-PCR were designed to amplify the most conserved region surrounding the P-loop of the presumed ATP-binding site of the DHC polypeptide (Fig. 1). The majority of ~250 independently cloned and sequenced RT-PCR products represented a fragment of a conventional cytoplasmic dynein heavy chain (DHC1), identical to the previously described hp22 fragment of human cytoplasmic DHC (Vaisberg et al., 1993). Four additional clones, however, encoded dynein-related polypeptides, clearly different from DHC1. Three of those identified DHC2 and one identified DHC3.

While the PCR clones of DHCs 1, 2, and 3 were sufficiently distinct to show that they represented different human genes, they were obtained from the most highly conserved portions of the dynein heavy chain molecule, suggesting that we would need additional sequence information to construct isotype-specific reagents for protein biochemical work. Comparisons of existing dynein heavy chain sequences revealed that the regions lying 5' from the sequences already in hand were the least conserved parts of the polypeptides, so we employed a combination of cDNA library screening and 5' RACE walking on cDNA from HeLa cells to obtain additional sequence (Fig. 1).

The resulting DHC nucleotide sequences were used to make probes with which to study DHC genes and their expression. Southern blot analysis was consistent with the notion that DHCs 1, 2, and 3 are the products of different unique genes (Fig. 2 A), although an unambiguous interpretation of Southern blot data is complicated by the presence of several introns of unknown sequence in the relevant region of genomic DNA (Vaisberg, E.A., and P.M. Grissom, unpublished observations). Northern blots with RNA isolated from several cell lines and human tissues, including those that make neither cilia nor flagella, revealed bands hybridizing with each of the three dynein genes; all showed molecular sizes of ~15 kb (Fig. 2 B). In some cell lines the DHC1 probe recognized a doublet of mRNA bands, suggesting alternative splicings of this message. The levels of expression of the three DHCs varied significantly between different cells and tissues. While DHC1 mRNA was prevalent in the majority of cells and tissues, DHC2 predominated in COS cells, and DHC3 in both foreskin and liver. These results confirm that all three dynein heavy chains are expressed in cells that do not make cilia or flagella.

DHC2 Is a Distant Member of a Family of Cytoplasmic Dyneins, while DHC3 Is More Similar to Axonemal Dyneins Than to Any of the Previously Identified Cytoplasmic Polypeptides

The amino acid sequence predicted from the cloned region of human DHC1 is almost identical to its rat counter-
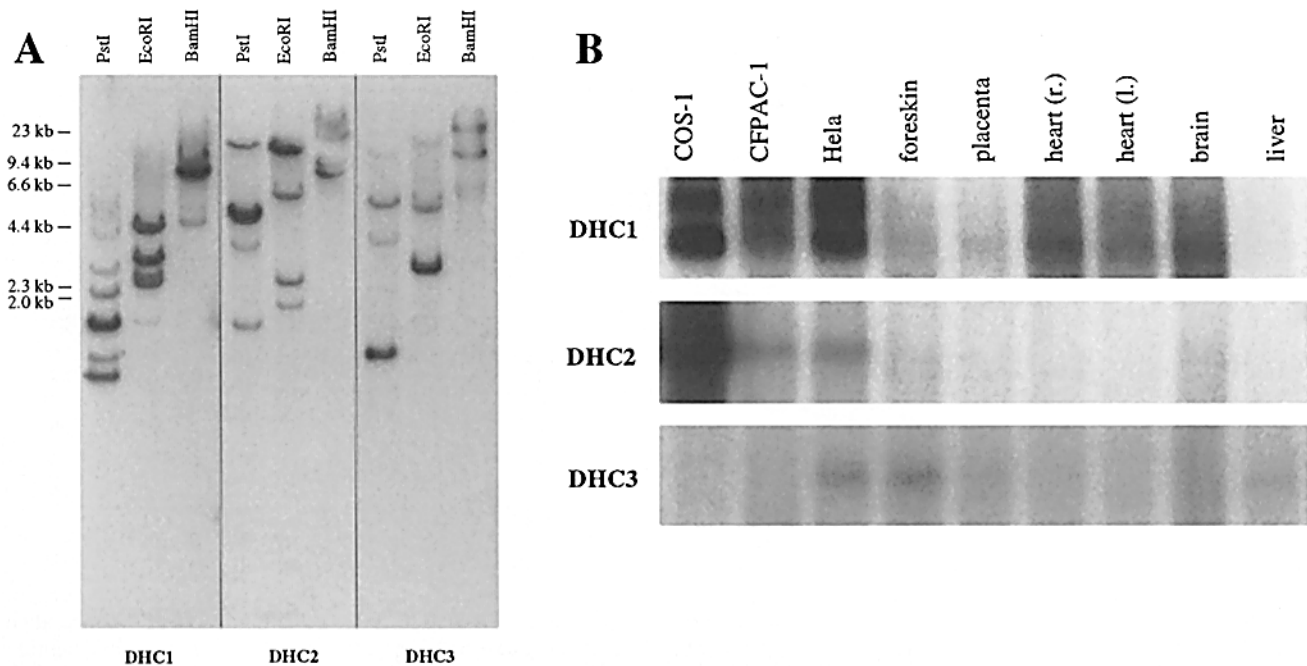
Figure 2. (A) Southern blot analysis of human genomic DNA probed with sequences from DHCs 1, 2, and 3. Enzymes used for digestion are shown on top of the lanes; positions of molecular size markers are indicated at left. (B) Northern blots of RNA from monkey and human cell lines and human tissues probed with DHCls 2 and 3. Only the ~15-kb region of the gel is shown. No other regions bound the probe.

part (Mikami et al., 1993; Zhang et al., 1993) (98.5% identity). Levels of pairwise similarity between the human DHCs in the regions from which sequence is available are presented in Table I. The similarity of DHCs 2 and 3 to DHC1 and to the DHCs from other species drops significantly towards the NH_2 terminus of the predicted protein, as was observed from comparisons of cytoplasmic and axonemal dyneins (Koonce et al., 1992; Mikami et al., 1993; Zhang et al., 1993; Eshel et al., 1993; Li et al., 1993; Xiang et al., 1994; Plamann et al., 1994; Mitchell and Brown, 1994; Lye et al., 1995; Ogawa, 1991; Gibbons et al., 1991; Kandl et al., 1995). Nonetheless, amino acid residues conserved among recently identified members of a DHC family in sea urchin (Gibbons et al., 1994), *Drosophila*, and rat brain (Tanaka et al., 1995) are also conserved in DHCs 2 and 3. In the region where DHC2 clones overlap with the short PCR fragments obtained from sea urchin embryos and rat brain, the sequence is most similar to that of DLP4 from rat brain (Tanaka et al., 1995) (100% identical) and DHC1B (Gibbons et al., 1994) from sea urchin (91.7% identical). Thus, DHC2 probably is a human homologue of these DHC-like polypeptides. In agreement with the analysis by Tanaka et al. (1995) and Gibbons et al. (1994), based on shorter sequence fragments, DHC2 is a highly di-

Table I. Sequence Comparisons of Human Cytoplasmic Dynein Isoforms

	DHC1	DHC2	DHC3	βDHC
	%	%	%	%
DHC1		42.7/60.2	36.9/54.2	39.0/58.6
DHC2	34.1/55.9		38.9/61.1	44.7/60.0
DHC3	NA	NA		50.5/67.0
βDHC	31.1/55.5	31.8/52.1	NA	

Sea urchin axonemal β-dynein heavy chain (βDHC) is included for comparison. The first value in each box represents percent identity, and the second represents percent similarity between corresponding polypeptides. Comparisons in the upper-right part of the table are based on the region of DHC from amino acids 636–839 on Fig. 1 *B*. Comparisons in the lower left part of the table are based on a larger region from amino acids 1–839 on Fig. 1 *B*, for which DHC3 sequence information is not available.

verged member of the cytoplasmic DHC family (Fig. 3 and Table I).

DHC3 sequence is more similar to known axonemal dyneins than to any of the cytoplasmic DHCs (Fig. 3 and Table I). It is most similar to dyneinlike protein 12 (DLP12) from rat brain (Tanaka et al., 1995) (100% identity in a 50–amino acid overlap) and to sea urchin DHC isotype 7C (DHC7C) (Gibbons et al., 1994) (93% identity over a 97–amino acid overlap). Based on sequence comparisons with known dynein heavy chains and, in the case of DHC7C, on the upregulation of mRNA upon sea urchin embryo deciliation, these dynein isotypes previously have been considered to be axonemal (Gibbons et al., 1994).

DHC2 and DHC3 Polypeptides Are Expressed in a Variety of Mammalian Cell Lines

To investigate the expression and function of these human cytoplasmic DHC isoforms, we have generated antibodies that would discriminate among them. The lower level of similarity among the sequences in the segments upstream from the ATP-binding domain suggested that we could use this region to generate isoform-specific antibodies. Nonetheless, we anticipated that the polypeptides expressed from these gene fragments might contain some common epitopes, so our strategy has been designed to allow both affinity purification and affinity depletion of those anti-
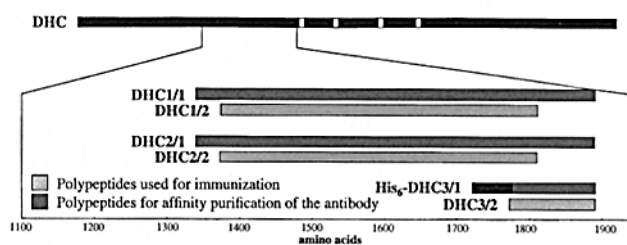

Figure 3. A tree of sequence similarities showing human DHC isoforms with cytoplasmic dynein heavy chains from various organisms (Koonce et al., 1992; Mikami et al., 1993; Zhang et al., 1993; Eshel et al., 1993; Li et al., 1993; Lye et al., 1995; Xiang et al., 1994; Plamann et al., 1994; Kandl et al., 1995) and axonemal dynein heavy chains from sea urchin (Ogawa, 1991; Gibbons et al., 1991) (*S.u. βDHC*), *Paramecium* (Kandl et al., 1995) (*P. βDHC*), and *Chlamydomonas reinhardtii* (Mitchell and Brown, 1994) (*Ch. αDHC*). The tree was generated using the Pileup program (using an UPGMA algorithm) from GCG with the following parameters: gap creation penalty = 3.0; gap extension penalty = 0.1. A region of DHC corresponding to amino acids 636–839 on Fig. 1 *B* was used for the comparison.

Figure 4. Diagram of the antigens used to produce and to purify antibodies. Black bar at top depicts the whole dynein heavy chain. Smaller polypeptides denoted DHC1/2 (52 kD), DHC2/2 (51 kD), and DHC3/2 (15 kD) (*light gray bars*) represent analogous regions of DHC1, 2, and 3, as inferred by sequence alignment using several different alignment algorithms. Polypeptides used for the affinity purification and depletion of the cross-reactive antibodies (DHC1/1 [67 kD], DHC2/1 [65.5 kD], and DHC3/1 [17.3 kD; *darker bars*]) completely overlap with the region that encodes each immunogen; for DHC1 and 2, they also contain extra sequences flanking the immunogen.

bodies that interact with epitopes shared by two or three of the isoforms. Fig. 4 depicts the clones that were expressed in bacteria to generate the immunogens (*light gray bars*), as well as those that were used for subsequent affinity purification (*darker bars*). For DHCs1 and 2, the latter polypeptides added sequences flanking the immunogen at both ends. By using smaller antigens for immunization and larger ones for immunodepletion, we ensured that antibodies to the common epitopes could be removed and that we could control for cross-reactivity of the resulting antibodies by immunoblotting.

The immunoblots presented in Fig. 5 *A* demonstrate the lack of cross-reactivity among the resulting antibodies. Each of the antibodies recognized only its corresponding

polypeptide (and the products of its degradation), but even on overexposed blots, they did not show reactivity with polypeptides from the other dynein isoforms.

Immunoblotting analysis with proteins from cultured mammalian cells showed that all three DHC polypeptides are expressed in HeLa, NRK, PtK₁, and COS-7 cells. In cell lysates these antibodies specifically recognized polypeptides with molecular masses characteristic of DHCs (Fig. 5 *B*). In some cell lines the antibodies detected two bands of similar molecular weight (i.e., DHC1 in COS-7 cells, DHC2 in PtK₁ cells, or DHC3 in NRK cells). This complexity may result from alternative splicing, posttranslational modification of the DHCs, and/or the presence of additional cytoplasmic DHCs whose genes are not yet in
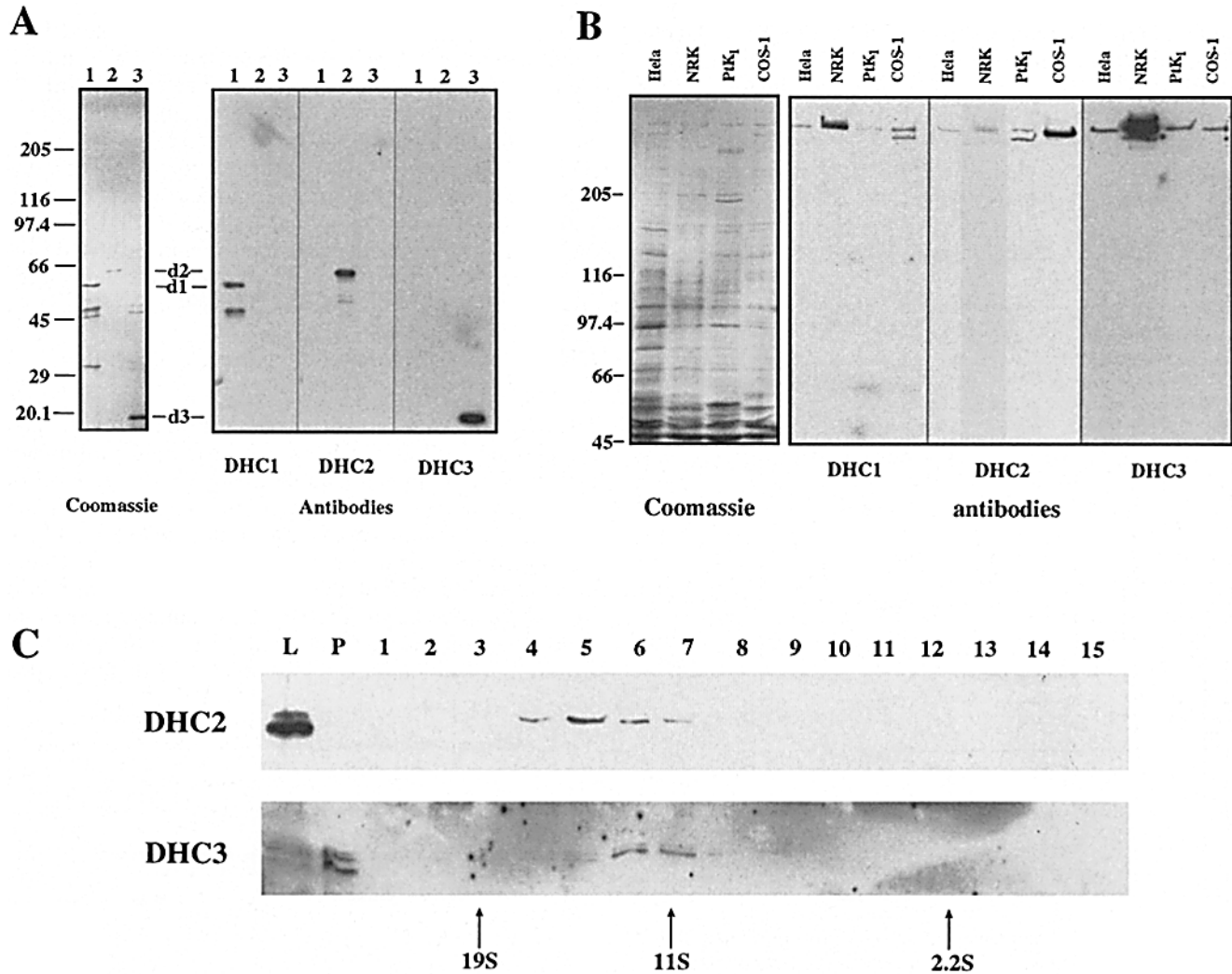
Figure 5. Antibodies raised against bacterially expressed fragments of human DHCs are isoform specific and recognize all three DHCs in lysates of several mammalian cell lines. (*A*) Equal amounts of inclusion body protein isolated from bacteria-expressing fragments DHC1/1, DHC2/1, and DHC3/1 (lanes *1, 2,* and *3,* respectively) were separated by SDS-PAGE (*left-most panel*), transferred to nitrocellulose, and probed with affinity-purified anti-DHC1 (*left panel*), DHC2 (*middle panel*), or anti-DHC3 (*right panel*) antibodies. The positions of the bands corresponding to each of the dynein antigens are marked between the panels. Positions of molecular weight markers are shown at right. (*B*) All three isoforms are expressed in different mammalian cells but at different levels. 20 μg of proteins from whole cell lysates was resolved by SDS-PAGE, transferred to nitrocellulose, and probed with one of the antibodies as indicated. Each of the antibodies reacts specifically with a band, or a doublet of bands, with high apparent molecular mass characteristic of a DHC. (*C*) Fractionation of cell extracts on a 5–20% sucrose gradient. HeLa and NRK cell extracts were prepared and fractionated on sucrose gradients as described in Materials and Methods. Extracts (*L*), individual fractions of the gradients (numbered beginning at the bottom of the gradient), and pellets from the bottom of the gradients (*P*). Arrows below the panels indicate the positions of standards.

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hand. The amount of each DHC polypeptide varied from cell type to cell type, just like the levels of the corresponding mRNAs. Both DHC1 and 3 are expressed at highest levels in NRK cells, while DHC2 is expressed at a high level in COS fibroblasts. Since the immunoblots were probed with different antibodies and developed with different exposures, we are unable to draw quantitative conclusions about the absolute amounts of different DHC isoforms expressed in different cells, but the relative level of each protein in different cell types is consistent with the results of Northern blotting. For example, both mRNA and protein of DHC1 are expressed at higher levels in HeLa cells than in COS cells, while both mRNA and protein of DHC2 are more abundant in COS fibroblasts than in HeLa cells.

Molecules of all characterized species of the conventional cytoplasmic dynein (DHC1) are comprised of two heavy chain polypeptides and a number of intermediate and light chains; they sediment at $\sim$20S (Holzbaur and Vallee, 1994). To get an initial insight into the structure of the molecules that contain DHC2 and DHC3, we examined the sedimentation of the soluble fraction of these polypeptides on a 5–20% sucrose gradient (Fig. 5 C). Both DHC2 ($\sim$15S) and DHC3 ($\sim$13S) sedimented significantly slower than conventional cytoplasmic dyneins, implying different sizes and/or structures of the molecules. The low sedimentation coefficient of DHC3 suggests that this holoenzyme may contain only one heavy chain, unlike conventional cytoplasmic dynein but similar to some species of axonemal dyneins. Under the conditions used for this experiment (relatively high ionic strength PME buffer; see Materials and Methods), almost all DHC2 is soluble. The amount of DHC2 associated with membranes in cell lysates depended strongly on the ionic conditions and the divalent cation composition of the buffers used for homogenization (not shown). Much of the DHC3 remained associated with rapidly sedimenting particles, even at relatively high ionic strength (lane P on Fig. 5 C). Detailed studies will be required to address the important problem of the mechanism by which different dynein isoforms associate with different cellular membranes.
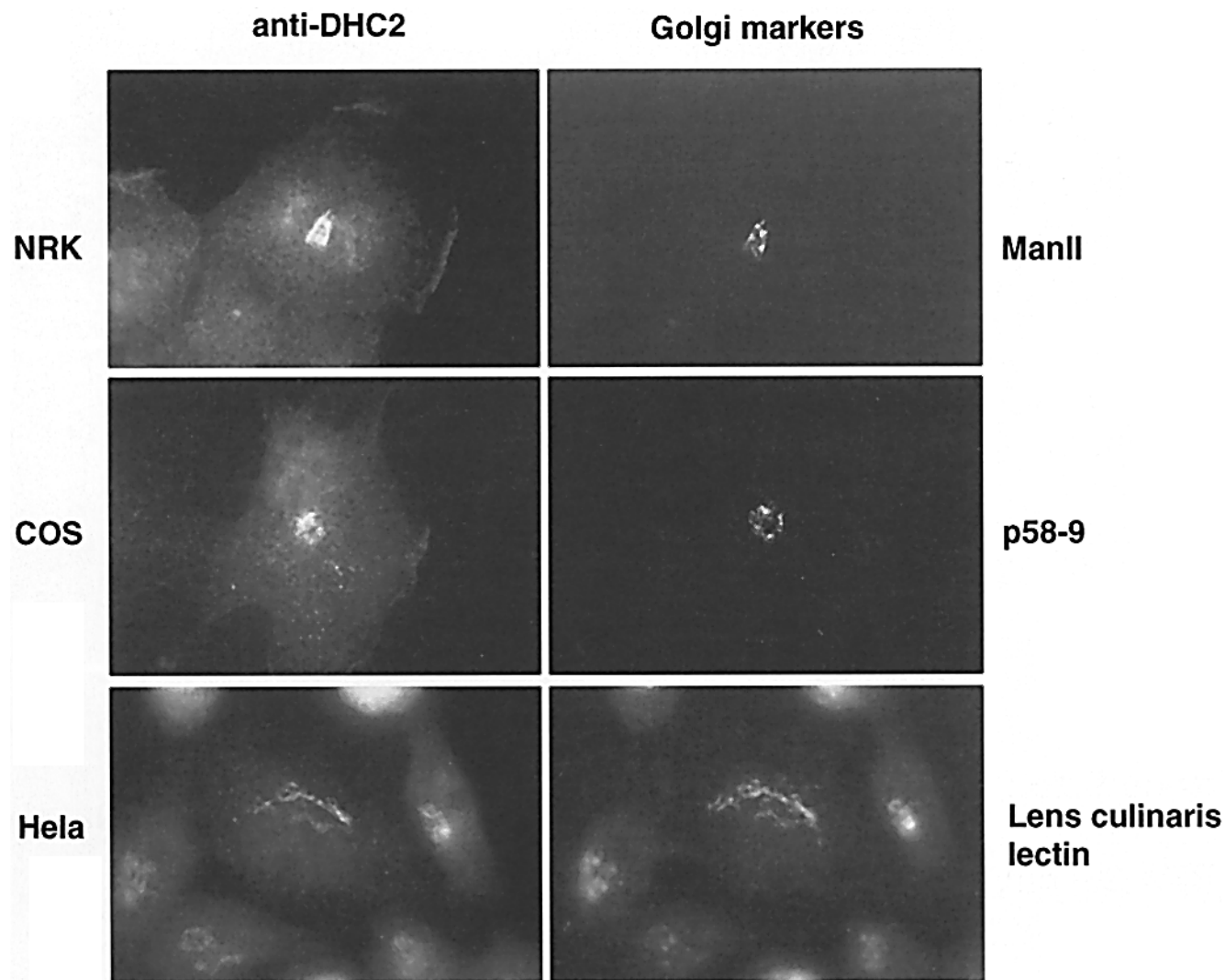
Figure 6. Immunolocalization of DHC2 in several cell lines. DHC2 is predominantly associated with the Golgi apparatus. NRK, COS7, and HeLa cells were costained with DHC2 antibody (left panels) and one of the following Golgi markers as indicated: anti-α-mannosidase II, mAb p58-9, or FITC-conjugated Lens culinaris lectin.

DHC2 Is Associated with the Golgi Apparatus and Participates in Proper Golgi Organization

We used our isotype-specific antibodies to examine the intracellular distribution of each DHC in several mammalian cell lines and found that all three isoforms displayed distinct cytoplasmic localizations. DHC1 localizations are similar to those previously reported for cytoplasmic dynein in cultured cells (Steuer et al., 1990; Pfarr et al., 1990). Interphase cells show a punctate staining of the cytoplasm, which is generally brighter in the perinuclear area and dimmer near the cell periphery. As previously described
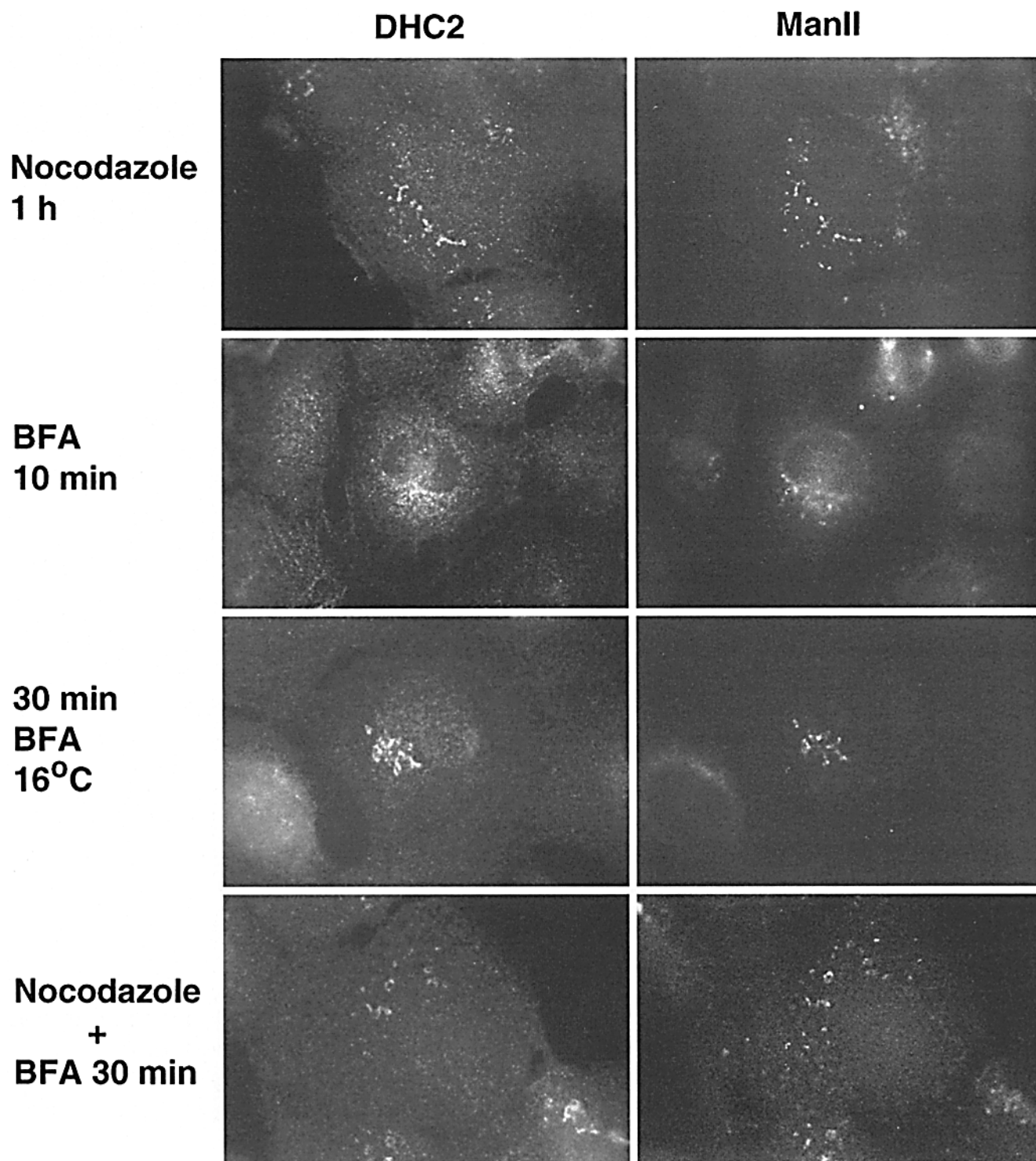
Figure 7. Effects of nocodazole and brefeldin A treatments on DHC2 localization in NRK cells. After the treatment indicated at left, NRK cells were fixed and processed for immunofluorescence with DHC2 antibody (*left panels*) or anti-mannosidase II.

(Steuer et al., 1990), DHC1 redistributes during mitosis to the kinetochores and mitotic spindle (not shown).

The intracellular localization of DHC2 differs from that of DHC1. In all cell types examined (NRK, COS-1, HeLa, 3T3, and BHK), it colocalizes mainly with markers for the Golgi apparatus, although some cytoplasmic vesicles are also stained (Fig. 6). In fine details, however, Golgi localization of DHC2 is different from that of such markers as α-mannosidase II, p58, or *Lens culinaris* lectin, suggesting that DHC2 is associated only with a subset of the Golgi compartments.

Additional evidence about the relationship between DHC2 and the Golgi apparatus has been obtained by a pharmacological investigation of DHC2's colocalization with several Golgi markers during and after treatment of the cells with drugs. When cytoplasmic microtubules are depolymerized by Nocodazole, the Golgi apparatus loses its association with the centrosomal region and appears as small fragments scattered throughout the cytoplasm (for reviews see Thyberg and Moskalewski, 1985; Kreis, 1990). DHC2 remains associated with these scattered fragments (Fig. 7, *top panels*).

The fungal metabolite brefeldin A (BFA) inhibits membrane traffic between the Golgi and the ER. It causes fast (<15 s) dissociation of coatomer proteins from Golgi membranes (Donaldson et al., 1990), followed by an escape of Golgi membranes and proteins into the ER (for review see

Klausner et al., 1992). In cells treated with BFA, DHC2 disappears from the Golgi apparatus; it reappears at the Golgi upon recovery from BFA with approximately the same kinetics as the *cis*-medial Golgi enzyme α-mannosidase II (Fig. 7). The distribution of DHC2 in BFA-treated cells resembles that described for escaped Golgi proteins (e.g., Lippincott-Schwartz et al., 1990), suggesting that during BFA treatment, this motor enzyme becomes at least partially associated with the ER. Loss of DHC2 localization from the Golgi depends upon active transport from Golgi to ER, because a block of the escape of Golgi material in BFA-treated cells by either low temperature (16°C) or microtubule depolymerization with nocodazole causes DHC2 to remain associated with the Golgi or its fragments (Fig. 7).

From these results we infer that the proximity of DHC2 and Golgi staining in normal cells is not simply the result of the enzyme moving along microtubules to the centrosomal region. The persistence of the association through drug treatment and recovery suggests that DHC2 participates in ER to Golgi transport and/or in establishing the Golgi's organization in cells.

To further test this hypothesis, we injected anti-DHC2 antibody into NRK cells. In $43 \pm 12.4\%$ of cells ($n = 62$; $P = 0.05$), this injection led to the dispersal of the Golgi (Fig. 8). The fraction of cells with dispersed Golgi upon injection with preimmune immunoglobulins at the same concentration was similar to the fraction with dispersed Golgi
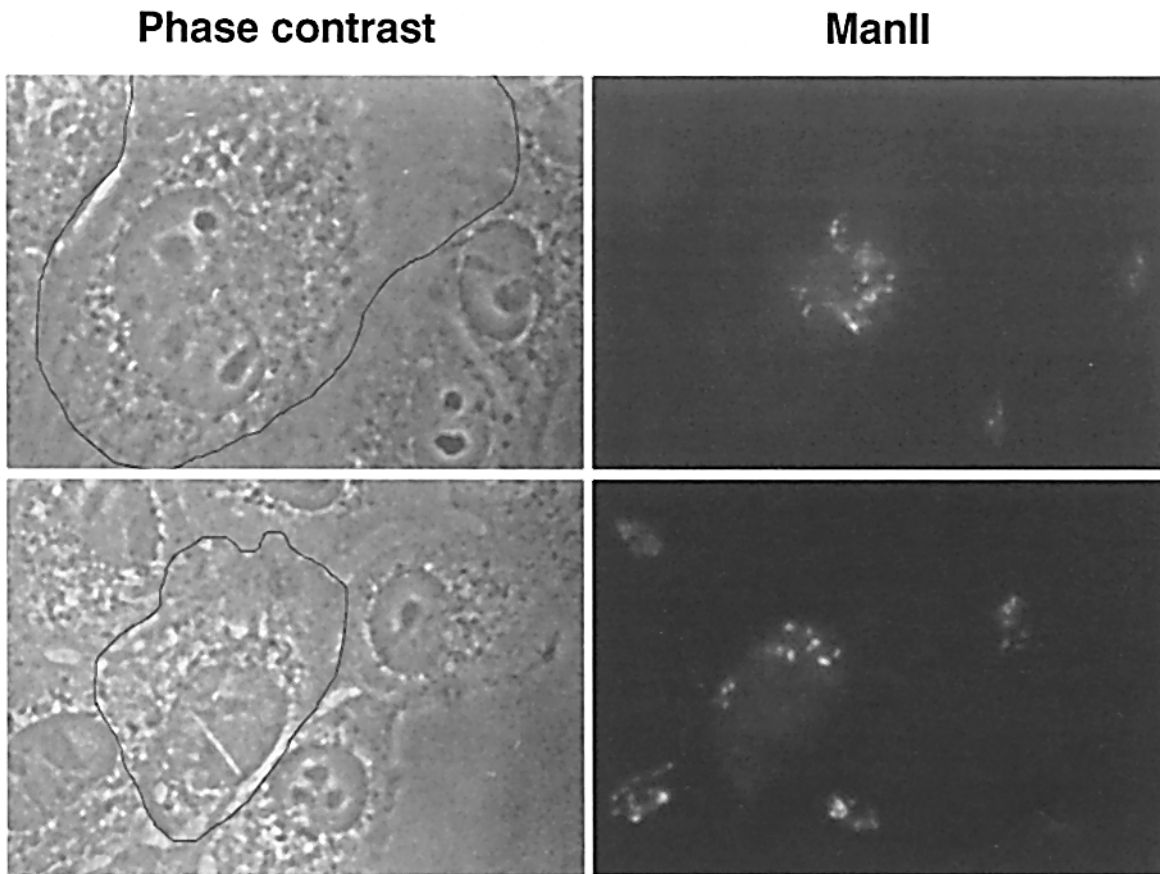
Phase contrast

ManII

Figure 8. Microinjection of anti-DHC2 causes fragmentation of the Golgi apparatus. NRK cells were injected with DHC2 antibody (at 4.7 mg/ml), incubated at 37°C for 3 h, and processed for immunofluorescence with antibody to α-mannosidase II (*right panels*). Injected cells are outlined with a black line on left panels.

in an uninjected population of cells (17.1 ± 4.4%; $n = 287$; $P = 0.05$ vs 15.4 ± 6.4%; $n = 124$; $P = 0.05$). Thus, antibody injection induced a 2.5–2.8-fold increase in the fraction of cells with a fragmented Golgi apparatus. Apparently the presence of active DHC2 is necessary for cells to maintain the normal arrangement of their Golgi membranes.

DHC3

The intracellular localization of DHC3 is different from that of both DHCs 1 and 2. In all the cell lines examined (COS-7, HeLa, PtK$_1$, A431, and 3T3), antibodies to DHC3 stained both small vesicles almost uniformly distributed in the cytoplasm and an accumulation of small vesicles or tubules that was usually located near the microtubule-organizing center (Fig. 9). Unlike DHC1, "background" cytoplasmic staining with anti-DHC3 was very low, even with fixation procedures that immobilize a significant fraction of the soluble cytoplasmic proteins, suggesting that the level of soluble DHC3 in the cytoplasm is relatively low. This observation is consistent with the sedimentation behavior of DHC3, which showed that a significant fraction

of DHC3 is associated with the insoluble fraction of the extract (see Fig. 5 C).

We have performed either double labeling or simultaneous staining, using antibodies to DHC3 and a variety of markers for different parts of the cytoplasmic membrane system. From this work it is evident that the organelles that bind DHC3 are not congruent with any of the major cytoplasmic compartments: mitochondria, ER, the Golgi apparatus, early or late endosomes, or lysosomes (Fig. 9 and not shown). Such a localization is consistent with the hypothesis that DHC3 contributes to the transport system that moves intermediate structures between these major cellular compartments.

Discussion

There Are Multiple Cytoplasmic Dynein Heavy Chains

Our RT-PCR screen has identified three dynein heavy chain–like proteins that are expressed in a wide variety of somatic mammalian cells. Both Northern and Western blotting confirm that all of these dynein isoforms are expressed in cells that do not appear to make either cilia or
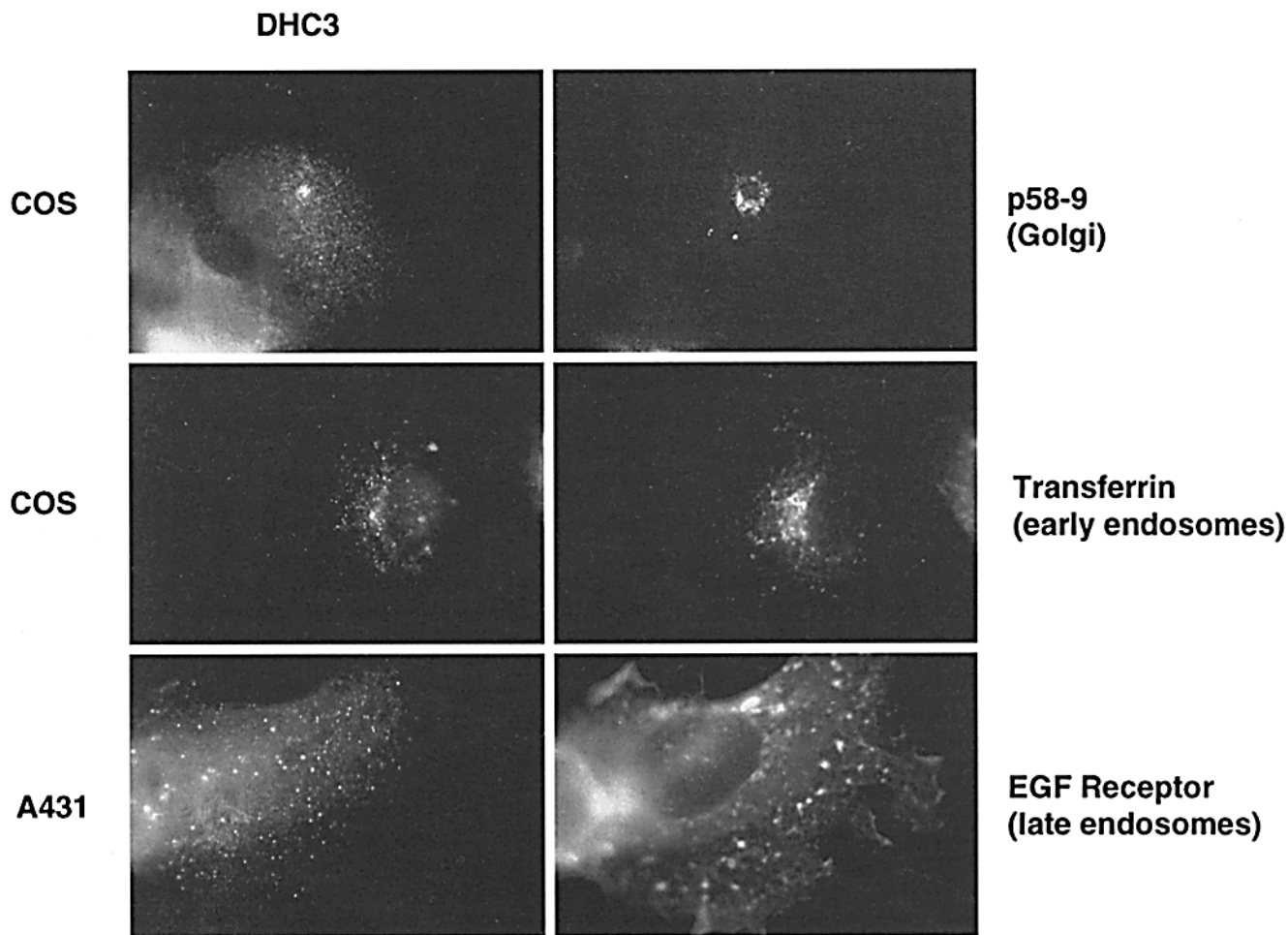
DHC3

Figure 9. Intracellular localization of DHC3 in COS and A431 cells. (*Left panels*) Staining with anti-DHC3. To identify early endosomes, cells were incubated for 15 min in DME supplemented with 0.2% BSA and 5 µg/ml Texas red–conjugated transferrin, and then fixed for immunolocalization. Late endosomes were localized with an antibody to human EGF receptor after incubating A431 cells in DME with 0.1% calf serum for 12 h, DME with 0.2% BSA for 1 h, and treatment with EGF (at 100 ng/ml) for 30 min.

flagella. One of these genes (DHC1) encodes the cytoplasmic dynein that has already been studied extensively, the second (DHC2) contains sequence elements that have led to the suggestion that its homologues might be cytoplasmic isoforms (Gibbons et al., 1994; Tanaka et al., 1995), while the third (DHC3) is among a group of sequences that previously have been interpreted as components of the axoneme. Moreover, the low sedimentation velocity of DHC3 is more similar to the sedimentation velocity of single-headed axonemal dyneins (Witman, 1989, 1992) than to double-headed cytoplasmic dynein. In its native form this polypeptide may be part of a bigger protein complex, not yet identified, that includes additional cytoplasmic dynein heavy chains. The more complete studies of multiple dynein isoforms recently published for sea urchins and rodent brain identified several DHC sequences that are closely related to our DHC3, e.g., DHC7A and B in sea urchin, DHC62B in *Drosophila*, and DLPs 3 and 7 in rat. We infer that there may be additional DHCs that, similar to DHC3 (and its homologues in other species), are components of dyneins that play roles in cytoplasmic motility (see e.g., Fig. 5 in Tanaka et al., 1995).

An axonemal assignment for 11 of the 13 dynein isoforms identified in sea urchins was made on the basis of an increase in their expression when blastomeres were deciliated (Gibbons at al., 1994). Homologues of the human DHC3 isoform fulfilled this definition of an axonemal dynein and may contribute to axonemal function, as well as playing some role in intracellular transport. Alternatively, their increase in expression upon deciliation may result from the cell's need to increase its cytoplasmic transport capabilities at a time when axonemal precursors are transferred to the cell surface and assembled to form cilia. Further work will be required to distinguish between these possibilities.

The Functions of Different Cytoplasmic Dyneins

DHC1 corresponds both in protein sequence and in localization to the cytoplasmic dynein previously studied by us (Pfarr et al., 1990; Lye et al., 1987; Koonce et al., 1992; Vaisberg et al., 1993) and others (Steuer et al., 1990; Hays et al., 1994; Mikami et al., 1993; Zhang et al., 1993; Eshel et al., 1993; Li et al., 1993; Xiang et al., 1994; Plamann et al., 1994; Lye et al., 1995). Since this dynein isoform is comparatively well characterized, we have not pursued its function or localization in the current study.

DHC 2 is likely to play a role in the organization and/or the function of the Golgi apparatus. A role for dynein in Golgi function was previously suggested by experiments using lysed cells and Golgi membrane fractions (Corthesy-Theulaz et al., 1992). However, antibodies raised in our lab that inhibited the motility of DHC1 both in vitro and in vivo (Vaisberg et al., 1993) failed to influence Golgi organization, either in interphase, in cells recovering from Nocodazole treatment, or in postmitotic cells (Vaisberg, E.A., unpublished results). This result is compatible with the finding presented here that the isoform of cytoplasmic dynein involved in Golgi activity is the novel DHC2 and not the conventional cytoplasmic dynein isoform.

This interpretation is supported both by the localization of DHC2 in normal and drug-treated cells and by the re-

sponse of several cultured cell lines to injection of DHC2-specific antibodies. The fraction of injected cells that showed Golgi dispersal was not large (~50%), but on the other hand, these antibodies were affinity purified for isotype specificity, rather than a function-perturbing effect. In sum, these data provide strong support for the hypothesis that DHC2 plays a role in Golgi organization.

The same dynein isoform may also contribute to traffic to or from the Golgi. The colocalization of DHC2 with α-mannosidase II during both prolonged treatments with brefeldin A and during reversal of this treatment suggest that this motor enzyme can disperse with other Golgi membrane components to the ER. The immunofluorescence image of fixed cells may represent a steady-state concentration distribution, in which the majority of dynein is Golgi resident, while some functions to bring membranes from some pre-Golgi compartments to the Golgi itself. Further studies of fractionated cells may help to elucidate the role that this motor enzyme plays in intracellular membrane traffic.

The immunolocalization of DHC3 leaves the function of this motor enzyme an enigma. It may well act to bring vesicles from one endomembrane compartment to another, but in the absence of a specific compartment assignment, it is difficult to design simple cell biological experiments, such as antibody injection, that can elucidate the role this enzyme plays in membrane traffic. Future work will focus on the association of DHC3 with various endocytic compartments in pulse-chase experiments that could reveal some aspect of the enzyme's motor role.

In conclusion, our data unambiguously identify three cytoplasmic dynein heavy chains, each with a unique localization in several different cultured cell types. The phylogenetic placement of DHC3 rather strongly suggests that there may be more cytoplasmic dyneins involved in other aspects of intracellular motility. It is now clear that the problem of isoform diversity must be confronted for the family of cytoplasmic dyneins, as well as for the kinesin superfamily. When one adds to this the diversity recently established for the myosin superfamily, it becomes evident that cells have at their disposal an almost bewildering variety of motor enzymes for moving their components. It remains a fascinating challenge to characterize this complexity and to categorize the different motions that are associated with each of these elements of the cytoplasmic machinery.

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