Characterization of an Axonemal Dynein Heavy Chain Expressed Early in Airway Epithelial Ciliogenesis

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The most conspicuous evidence of airway epithelial maturation and vitality is the presence of motile cilia. In an effort to generate genetic and antigenic markers of airway maturation, injury, and repair, we characterized airway epithelial expression of a gene identified by two human expressed sequence tags that encoded peptides with sequence similarity to an invertebrate ciliary dynein heavy chain (DHC). Molecular analyses showed that the gene has a very large RNA transcript that encodes a very high molecular weight polypeptide with biochemical properties that are characteristic of a dynein heavy chain. Expression of the gene transcript correlated with the presence of ciliated cells in tissues, and immunohistochemical localization of the gene product confirmed its presence in the cilia of mature airway epithelium. In epithelium undergoing ciliogenesis *ex vivo*, expression of the gene transcript preceded ciliation of the epithelium and the gene product was present in the cytoplasm and at the apical border of nonciliated cells. These data suggested that the gene encodes an axonemal DHC that is expressed early during ciliogenesis, before the appearance of cilia.

Approximately 80% of the epithelial cells of healthy human upper and lower airways are ciliated, whereas the loss of normal ciliated cell phenotype is characteristic of airway disease and injury. Thus, protein components of the cilium and their messenger RNAs (mRNAs) may be useful markers of airway maturation, injury, and repair. At the core of each cilium is an axoneme, a motile organelle composed of at least 250 distinct polypeptides (1). The most abundant are α and β tubulin, which assemble into the nine outer doublet microtubules and the central pair of singlet microtubules characteristic of most axonemes. Ciliary motion is powered by axonemal dyneins, which are microtubule-activated, Mg^{2+}-adenosine triphosphatases. Each axonemal dynein is an armlike structure affixed to the A subfiber of each doublet microtubule. Each A subfiber has two longitudinal arrays of dynein arms, a row of outer arms with a periodicity of 24 nm, and the more complex row of inner arms with a periodicity of 96 nm (2). In addition to axonemal dyneins, there are cytoplasmic dyneins, distinct isoforms that transport molecular cargoes along cytoplasmic microtubules and participate in aspects of cell division (3). Together, axonemal and cytoplasmic dyneins make up a family of molecular motor proteins that usually translate the cargoes to which they are anchored toward the minus end of the microtubule with which they transiently interact.

A functional dynein is composed of one to three heavy chains (DHCs) and a variable number of intermediate and light chains (4). Each heavy chain has a molecular weight of 400 to 500 kD and has four nucleotide-binding domains. The first (amino-terminal) domain mediates nucleotide-binding to the principal adenosine triphosphate (ATP) hydrolytic site. Recently, sequences of the first nucleotide-binding domain of a large number of DHC genes expressed in diverse phyla have been described (5–13). Phylogenetic analyses of these sequences confirm that DHCs can be grouped into three broad categories: cytoplasmic, outer arm axonemal, and inner arm axonemal (10, 11, 13, 14). Thus, a given DHC has greater sequence similarity to its homologs in other species than it has to the other DHCs of the same species. The phylogenetic analyses also suggest that species from diverse phyla that employ cilia and flagella express 12 to 15 DHC genes (5, 8, 10, 11, 13).

Cytoplasmic DHCs probably have a broad, if not universal, tissue distribution because they perform essential cellular functions. In contrast, the tissue distribution of axonemal DHCs is likely to be more restricted, a factor enhancing their potential as tissue-specific markers of differentiation and function. In an effort to generate genetic and antigenic markers of airway epithelial maturation, injury, and repair, we characterized the expression of the RNA transcript and protein product of a gene originally identified by two expressed sequence tags (ESTs) isolated from a human testis complementary DNA (cDNA) library. Sequence analyses of the polypeptides encoded by the ESTs suggested that the gene is homologous to the β-heavy chain of sea urchin outer arm ciliary dynein. We demonstrate here that this gene is expressed in human airway epithelium, that the molecular properties of the gene's RNA transcript and protein product are typical of an axonemal DHC gene, and that its expression precedes the appearance of cilia in epithelium undergoing ciliogenesis *ex vivo*.

Materials and Methods

Tissue Acquisition Procedures

Normal human nasal, tracheal, and bronchial epithelium and alveolar macrophages were obtained from nine healthy, nonsmoking adult male volunteers participating in inhalation toxicology studies in the E. P. A. Human Studies Facility at the University of

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Abbreviations: adenosine triphosphate, ATP; beta heavy chain, βHC; complementary DNA, cDNA; dynein heavy chain, DHC; ethylenediaminetetraacetic acid, EDTA; expressed sequence tag, EST; immunoglobulin, Ig; monoclonal antibody, mAb; relative molecular mass, M_r; phosphate-buffered saline, PBS; polymerase chain reaction, PCR; reverse transcriptase polymerase chain reaction, RT-PCR; sodium dodecyl sulfate, SDS.

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North Carolina at Chapel Hill (Chapel Hill, NC). Epithelial specimens were obtained by cytologic brushing at bronchoscopy and alveolar macrophages by bronchoalveolar lavage.

Porcine tracheas and normal human sperm were excess tissues obtained from a local abbatoir and human sperm bank, respectively.

Human Airway Epithelial Cell Culture Techniques

The human BEAS-2B bronchoepithelial cell line (15) was cultured in keratinocyte growth medium (Clonetics Corp., San Diego, CA). Normal human tracheal and bronchial epithelial cells were expanded on tissue culture–treated, plastic dishes (Costar, Cambridge, MA) in bronchial epithelial growth medium (Clonetics Corp.). These conditions promoted cell growth and the eventual loss of the ciliated phenotype. Cultures of primary human airway epithelial cells were induced to undergo ciliogenesis *ex vivo* using media and conditions described by Gray and coworkers (16). Passages 2 through 5 primary epithelial cells were plated onto Transwell-COL tissue culture supports (Costar) coated with Vitrogen 100 (Collagen Corp., Palo Alto, CA) and cultured submerged beneath the surface of the medium for 4 d. After 4 d, cultures were fed exclusively from the lower compartment while the upper compartment was left exposed to the 95% air/5% CO_2 atmosphere of the incubator.

Cloning of a Partial cDNA Encoding a Human Axonemal DHC-Like Protein

A fragment of a human axonemal DHC-related cDNA was cloned by long-range polymerase chain reaction (PCR) using a human tracheal cDNA library propagated in bacteriophage λgt11 (Stratagene, La Jolla, CA) as template and oligonucleotides corresponding to sequences flanking the cloning site in λgt11 (5′-GGTGGCGACGACTCCTGGAGCCCGTCAGTATCGGCGGAATTC-3′) and the sequence of a human testis EST (GenBank accession no. Z21269; 5′-GTGGAGTCCGAATGTCCTGAGAAAG-3′). Five microliters of the λgt11 tracheal cDNA library (titer $\sim 10^8$ plaque-forming units/ml) were subjected to long-range PCR in a total reaction volume of 25 μl using Taq polymerase (Boehringer Mannheim, Indianapolis, IN) and Vent polymerase (New England Biolabs, Beverly, MA) according to Barnes (17). One amplified product, 1.13 kb in length, was obtained, cloned into the TA cloning vector pCR2.1 (Invitrogen Corp., San Diego, CA), and sequenced using the dideoxy termination method (18) by the DNA Sequencing Facility of the Program in Molecular Biology and Biotechnology of the University of North Carolina. This sequence of this partial tracheal cDNA was submitted to GenBank (accession no. AF015265).

Northern Hybridization Analyses

A commercial human multiple tissue poly-(A)+ RNA blot (Clontech, Palo Alto, CA) was hybridized with a ^{32}P-labeled antisense riboprobe as described previously (19). The tracheal cDNA was cloned into the pCR2.1 vector and then used as the template to generate a riboprobe by *in vitro* transcription according to the vector manufacturer's instructions (Invitrogen Corp.).

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

In some cases mRNA abundance was monitored by sequential reverse transcription and DNA amplification (RT-PCR) of total RNA isolated from cultured human airway epithelial cells and freshly isolated human respiratory tract cells recovered by airway lavage and biopsy. Total RNA was isolated by solubilization of cultures, cells, and tissues in guanidine thiocyanate (Boehringer-Mannheim) and ultrasedimentation through cesium chloride. Total RNA from human testis was purchased from Clontech. First-strand cDNAs were synthesized from each specimen by reverse transcription and amplified using the PCR. For the amplification of the tracheal cDNA (DNEL1), initially, we used a pair of primers: forward 5′-AACCTTCACTCAGGACAC-3′ and reverse 5′-CAAATCATCATAGGGGAC-3′ (Figure 3). In subsequent experiments, more stringent primers were used: forward 5′-CCTAGATTTTGCAACCTCATTTGAAGAATCG-3′ and reverse 5′-GGTGGCTGTTCTCACTGTGCTCCTC-3′ (Figure 7A). Primers for DNEL2 (20) (GenBank accession no. AJ000522) were forward 5′-GGAGTGTTGAGTTTTCTAAGTCCTACGAGGAGAG-3′ and reverse 5′-CATGTCTGCCCGTGCTGTAGCG-3′. Human cytoplasmic DHC 1 cDNA (21) (GenBank accession no. L23958) was amplified using the following primer pair: forward 5′-GACAATGACACAAGCCTTGG-3′ and reverse 5′-TTCACGCAGTGCTTCCTGTA-3′.

Antibody Generation

To prepare a fusion protein for the generation of antibodies, a 375-bp fragment of the tracheal cDNA flanked by *Bam*HI and *Hin*dIII restriction sites was generated by DNA amplification using the tracheal cDNA as template and a pair of primers: forward 5′-GGATCCCCTGGAGAAGCTGGAGGAG-3′ and reverse 5′-AAGCTTTCCAGGAAGTTGTAGAGGACATTC-3′. The primers flank a region of the tracheal cDNA open reading frame that encodes a 124-amino-acid polypeptide with relatively high hydrophilicity and antigenicity indices. The amplification products were cloned into plasmid pGEMT (Promega, Madison, WI) and screened for positive clones. A suitable subclone (pS-12) was isolated and digested with *Bam*HI and *Hin*dIII, and the insert was gel-purified and ligated into the 6× His fusion vector pQE-31 (Qiagen, Chatsworth, CA) for the production of a fusion protein containing the desired 124-amino-acid antigen. The polypeptide was expressed in *Escherichia coli* strain M15(pREP4), isolated by nickel-nitrilotriacetate resin chromatography according to the vector manufacturer's instructions (Qiagen), and used as an antigen to immunize mice in the generation of a primary antiserum identified as PQD and in the preparation of a monoclonal antibody identified as PQD189 using previously described procedures (22).

Isolation of Axonemes and Extraction and V1 Photolysis of Axonemal Dyneins

All reagents were chilled on ice before use, and all operations were performed at 0 to 4°C unless indicated otherwise. Porcine tracheal axonemes were isolated by the method of Hastie and coworkers (23). Human axonemes were isolated from airway epithelial cultures induced to undergo ciliogenesis *ex vivo.* Cultures were deciliated by gently shaking for 30 s in a minimum of deciliation buffer (23) supplemented with 2.5 μg/ml each of aprotinin, leupeptin, and pepstatin (Boehringer-Mannheim), and 0.5 mM phenylmethylsulfonylfluoride (Sigma, St. Louis, MO). Axonemes were recovered by differential sedimentation (23).

A subset of axonemal dyneins from pig tracheal axonemes were isolated by incubation of the axonemes for 30 min in a high salt extraction buffer (HSEB; 30 mM *N*-2-hydroxyethylpiperazine-*N*′-ethane sulfonic acid [HEPES], pH 7.4, 5 mM $MgSO_4$, 1 mM ethyleneglycol-*bis*-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid [EGTA], 0.1 mM ethylenediaminetetraacetic acid [EDTA], 625 mM NaCl, 1 mM dithiothreitol [DTT], 70 mM β-mercaptoethanol, 0.1 mg/ml soybean trypsin inhibitor, 2.5 μg/ml each of aprotinin, leupeptin, and pepstatin, 500 μM ATP, and 100 μM $NaVO_3$). This buffer also contained the ATP and $NaVO_3$ necessary for V1 photolysis of DHC (24). Insoluble dyneins that remained attached to the axonemes were removed by sedimentation at 12,000 × *g* for 10 min and resuspended in HSEB. For V1 photolysis, a portion of the soluble dyneins and extracted axonemes were irradiated on ice with 254 nm ultraviolet light in a Stratalinker (Stratagene). Irradiated and nonirradiated soluble dyneins

and extracted axonemes were analyzed by immunoblotting as described subsequently.

Immunoblotting

Protein components of porcine tracheal axonemes, human bronchial axonemes, and human sperm were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) in 5% gels and either stained with coomassie blue or transferred to nitrocellulose using transfer buffer supplemented with 0.01% SDS to facilitate the transfer of DHCs. Blots were probed with primary antisera or ascites, stained with ^{125}I-labeled, affinity-purified goat antimouse immunoglobulins and autoradiographed.

Immunohistochemistry and Immunoelectron Microscopy

Tissues and cultures were fixed in 4% buffered paraformaldehyde for 1 h or 20 min, respectively, rinsed in phosphate-buffered saline (PBS), dehydrated and embedded in paraffin. Four micrometer sections were mounted on Superfrost plus microscope slides (Fisher Scientific, Pittsburgh, PA). Sections were probed with empirically optimized concentrations of PQD primary antisera or ascites and stained with Vectastain ABC reagents (Vector Laboratories, Burlingame, CA) and ImmunoPure metal-enhanced 3,3′-diaminobenzidine substrate (Pierce, Rockford, IL) according to the manufacturer's instructions. An anticytokeratin 19 antibody of the same isotype as PQD189 (immunoglobulin [Ig] G_{2b}) was obtained from Amersham Pharmacia Biotech (Piscataway, NJ).

Whole mounts of cultures were fixed as described previously, probed with PQD ascites and stained with a fluorescein isothiocyanate (FITC)-labeled rabbit antimouse secondary antibody (Molecular Probes, Eugene, OR). Control specimens were incubated with only the FITC-labeled secondary antibody. The specimens were examined and digital images acquired using a Leica TCS 4D (true confocal scanning) microscope (Leica, Exton, PA).

Human axonemes isolated as described previously were resuspended in 0.5 mg/ml trypsin, 0.53 mM EDTA, and digested for 30 s at room temperature. Digestion was halted by the addition of one-tenth volume of 20 mg/ml soybean trypsin inhibitor (Sigma). The digested axonemes were sedimented at 12,000 × *g* for 10 min, resuspended in 50 μl HMEEN buffer (30 mM Hepes, pH 7.4, 5 mM $MgSO_4$, 1 mM EGTA, 0.1 mM EDTA, 25 mM NaCl) supplemented with 1 mM DTT and 2.5 μg/ml each of the protease inhibitors aprotinin, leupeptin, and pepstatin, and applied in 5-μl drops to formvar-coated gold grids. The grid was drained by wicking onto filter paper, incubated for 10 to 60 s in reactivation buffer (HMEEN + DTT + PI + 5 mM ATP), drained, and fixed for 10 to 60 s by inversion on a drop of 4% paraformaldehyde/0.25% glutaraldehyde in 0.1 M phosphate buffer. The fixed axonemes were blocked for 2 h with blocking buffer (PBS + 0.05% Tween-20 + 1% bovine serum albumin [Sigma]), incubated for 2 to 12 h on a drop of undiluted PQD189 ascites, rinsed with four drops of wash buffer (PBS + 0.05% Tween-20), and stained for 1 to 2 h by incubation on a drop of goat $F(ab')_2$ antimouse IgG + IgM (H+L) adsorbed to 10 nm colloidal gold particles (Vector Laboratories) diluted 1:10 with blocking buffer. The stained grids were washed sequentially with four drops wash buffer, four drops PBS, and four drops distilled water, and negative stained with 1% uranyl acetate as described (25). The specimens were examined in a Zeiss EM-900 transmission electron microscope (Zeiss, Thornwood, NY) operating at an accelerating voltage of 80 kV.

Results

A systematic partial sequence analysis of a human adult testis cDNA library conducted by Affara and colleagues (26) identified a cDNA clone (clone 192) whose 5′ and 3′ ends produced two ESTs (GenBank acession nos. Z21174 and Z21269), both of which encoded polypeptides with significant sequence similarity to the β heavy chain (βHC) of sea urchin ciliary outer arm dynein. Because there is evidence that some DHCs are employed by both sperm flagella and cilia of the same organism (27), expression of the testis ESTs was investigated in human tracheal epithelium, a heavily ciliated tissue. DNA amplification using a human tracheal cDNA λgt11 library as template and a pair of oligonucleotide primers, one specific for the putative 5′ testis EST and the other specific for the λgt11 cloning site (*see* MATERIALS AND METHODS), yielded a 1.13-kb cDNA that begins with the 5′ EST and includes the 3′ EST and additional 3′ sequence (Figure 1). The tracheal cDNA had a single open reading frame that encoded a polypeptide which shared 81% sequence identity with the βHC of sea urchin but only 31% sequence identity with rat cytoplasmic DHC1 (Figure 1), suggesting that it is related to the axonemal subfamily of DHCs. The sequence of the tracheal cDNA was identical to the central region of a larger cDNA obtained from testis whose sequence was published subsequent to our cloning of the tracheal cDNA, which has been identified as DNEL1 (28). The polypeptide encoded by the tracheal cDNA was 78% identical to another axonemal DHC-like cDNA also cloned from testis identified as DNEL2 (20) (Figure 1).

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DNEL1        SECPEKEKLPQEWKNKT.......ALQRLCMLRAMRPDRMTYALRDPVEEK
DNEL2        --A----IF-K-------.......---K---V-CL---------IKN-----
Axonemal     ---------F--------S.......---K---M--L-A---S--V-N-I---
Cytoplasmic  -SS--.QTV-YL-TEE-PATPIGQ-IH--LLIQ-F----LLAMAHM--STN

DNEL1        LGSKYVVGRALDFATS...PEESGPATPMFFILSPGVDPLKDVESQGRKLG
DNEL2M       ---F-E--SVE-SK-...Y---S-S-SI-------~~~~~~~~~AL-K---
Axonemal     ------E--QVE--K-...Y--TD----V----------------AL-K---
Cytoplasmic  --HSPMSIMEQPLDLTHIVGT-VK-N--VLMCSV--Y-ASGH--DLAARQN

DNEL1        YTFNNQNFHNVSLGQGQEVVAEAALDLAAKKGHWVILQNIHLVAKWLSTLE
DNEL2        F-ID-GKL--------------N---V--E--------------R--G--D
Axonemal     F--D-N------------I---QCM-----E--------------------
Cytoplasmic  TQITSIAIG.SAE-FN-...-DK-INT-V-S-R--M-K-V--APG--MQ--

DNEL1        KKLEEHSENSHPEFRVFMSAEPAPSPEGHIIPQGILENSIKITNEPPTGMH
DNEL2        ----RY-TGR-EDY---IR--------T----------A-----------Y
Axonemal     ----QY-VG--DSY--Y------G---A---------S-------------F
Cytoplasmic  ---HSLQP..-AC--L-LTM-IN-R.....V-VNL-RAGRIFVF---P-VK

DNEL1        ANLHKALDNFTQDTLEMCSRETEFKSILFALCYFHAVVAERRKFGPQGWNR
DNEL2        ---Y----L---------TK-M---CM-----------------A-----
Axonemal     -------Y--N-------A--A---V------------C--Q----------
Cytoplasmic  --MLRTFS..SIPVSR--KSPN-RARLY-L-AW----IQ--LRYA-L--SK

DNEL1        SYPFNTGDLTISVNVLYNFLEANA.........KVPYDDLRYLFGEIMYGG
DNEL2        -----N------I-----Y----P..........---W--------------
Axonemal     ------------------Y----S.....------WQ--------------
Cytoplasmic  K-E-GES--RSACDTVDTW-DDT-KGRQNISPD-I-WSA-KT-MAQSI---

DNEL1        HITDDWDRRLCRTYLGEFIRPEMLEGELSLAP......GFPLPGNMDYNGY
DNEL2        ---------------A-Y--T-----DVL---.....--QI-P-L--K--
Axonemal     ---------------E-YMA----D---Y---.....---V-P-S--K--
Cytoplasmic  RVDNEF-Q--LN-F-ERLPTTRSFDS-FK--CKVDGHKDIQM-DGIRREEF

DNEL1        HQYIDAELPPESPYLYGLHPNAEIGFLTQTSEKLPRTVLELQPR
DNEL2        -E---EN--------------------V-----------M--K
Axonemal     -----EI---------------------TE-DN--KI-------
Cytoplasmic  V-WVELLPDAQT-SWL--PN---RVL--TQGVDMISKM-KM-ML
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Figure 1. The tracheal cDNA is more closely related to axonemal DHCs than to cytoplasmic DHCs. A comparison of the amino-acid sequence encoded by the sole open reading frame of the tracheal cDNA to the sequences of an axonemal DHC (GenBank accession no. [GB] CAA42170, amino-acid [aa] residues 3810–4185) and a cytoplasmic DHC (GB AAA41103, aa 3961–4348) is shown. The tracheal cDNA is identical to the midregion of DNEL1 (GB NP_004653, aa 142–517). The sequence of another closely related putative DHC from testis, DNEL2 (GB CAA04165, aa 523–898), is also shown. A dash indicates identity with the sequence of the sole open reading frame of the tracheal cDNA. The sequence of the fusion protein used as antigen in the preparation of antibodies is underlined.

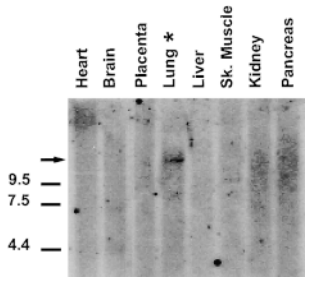

Figure 2. The tracheal cDNA hybridizes to a large mRNA transcript. A Northern blot analysis of eight human tissues using a riboprobe derived from the tracheal cDNA is shown. An *arrow* indicates the position of a transcript greater than 9.5 kb in size present only in whole lung tissue that hybridized to the riboprobe. Of the tissues screened, ciliated epithelium is present only in whole lung, which includes ciliated bronchi, and brain in which the ependyma is ciliated. Molecular size standards are indicated in kilobases on the left.

A Northern blot of poly(A)+ RNAs isolated from eight human tissues was hybridized under stringent conditions using an antisense riboprobe derived from the tracheal cDNA. A single transcript greater than 9.5 kb in size was detected in human lung (Figure 2). The large size of the transcript was appropriate for a DHC mRNA. No hybridization was detected to RNAs from any of the other tissues.

Expression of the mRNA complementary to the tracheal cDNA was investigated in greater detail using sequential reverse transcription and DNA amplification (RT-PCR) of total RNA isolated from tissues and cells derived from human airways. The mRNA was detected in total RNAs isolated from fresh biopsies of the nasal turbinate and lower trachea (Figure 3). In contrast, total RNA obtained from bronchoalveolar macrophages and nonciliated human airway epithelial cell cultures yielded no detectable products, although these cells expressed human cytoplasmic DHC mRNA at levels comparable to that of ciliated epithelium (Figure 3).

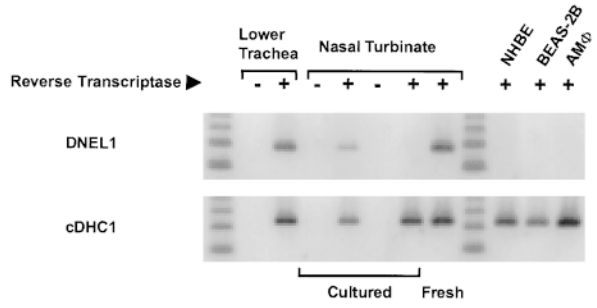

Figure 3. The mRNA corresponding to the tracheal cDNA is preferentially expressed in ciliated airway epithelium. Abundance of the tracheal cDNA (DNEL1) gene transcript and a cytoplasmic DHC transcript (cDHC1) were estimated by RT-PCR. DNA products resulting from 36 cycles of amplification, separated by electrophoresis through 2% alkaline-agarose gels, and stained with ethidium bromide are shown. Total RNA from a biopsy of lower trachea, from nasal turbinate biopsies of three different individuals, from nonciliated primary tracheobronchial epithelial cells grown out of a biopsy in 100 pM retinoic acid (NHBE), from a human bronchoepithelial cell line (BEAS-2B) grown in the absence of retinoic acid, and from freshly isolated alveolar macrophages (AMϕ) were analyzed. RNA was isolated immediately from AMϕ, lower trachea, and one of the nasal turbinate biopsies (fresh). Cells from two of the nasal turbinate biopsies were cultured for several weeks before total RNA was isolated. All the biopsies were obtained from different individuals.

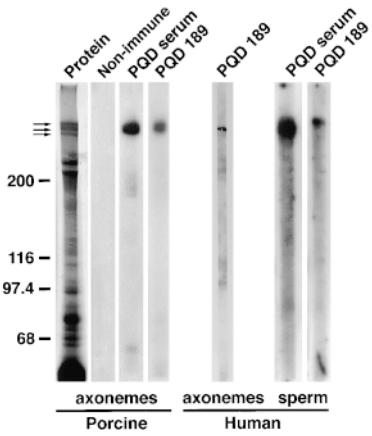

Figure 4. Antibodies to a polypeptide encoded by the tracheal cDNA recognize DHC-like components of isolated axonemes and whole sperm. Protein components of porcine tracheal cilia, human bronchial cilia, and whole human sperm, separated in 5% SDS-polyacrylamide gels and blotted to nitrocellulose, were probed with an antiserum (PQD serum) or a mAb (PQD189) to a peptide encoded by the tracheal cDNA or antisera prepared from a mouse that was not immunized (nonimmune) The M_r of molecular weight standards are indicated in kilodaltons on the left.

To characterize the protein product of the axonemal DHC-like mRNA, mouse antisera (PQD) and a monoclonal antibody (PQD189) were generated using a 6× His-tagged fusion protein containing a 124-amino-acid region of the open reading frame of the tracheal cDNA as antigen (*underline*, Figure 1). Both the antiserum and a monoclonal antibody specifically recognized an antigen of a very high relative molecular mass (M_r) on immunoblots of isolated porcine tracheal axonemes, human bronchial axonemes and whole human sperm (Figure 4). The M_r of the antigen was appropriate for a DHC.

The antigen present in porcine tracheal cilia was characterized in greater detail. The outer dynein arms of isolated pig tracheal cilia were selectively extracted from the axoneme by brief treatment with high salt (29). Sedimentation of the axonemes separated the soluble dyneins in the supernatant from the insoluble dyneins still associated with the axonemes. As previously observed (23), the two largest of the abundant heavy chains were selectively extracted from the axonemes (compare *lanes I* and *S*, Figure 5A). Immunoblotting with the PQD189 monoclonal antibody (mAb) demonstrated that the antigen is extracted along with these two heavy chains (Figure 5A). To determine whether the antibody was specific for one or both of the high M_r polypeptides, the solubilized dyneins were ultraviolet-irradiated (254 nm) in the presence of ATP and orthovanadate. This causes the scission of DHCs near the ATP-binding domain termed the V1 site (24). Depending on the molecular weight of the heavy chain, photolytic cleavage produces a smaller amino terminal fragment with an M_r of 155 to 200 kD and a larger carboxy terminal fragment with an M_r of 225 to 290 kD (Figure 5B). Heavy chains of different molecular weights from the same cilium usually yield scission fragments of different sizes that are readily resolved from each other by SDS-PAGE (30). Immunoblots of the V1 photolysis products of the soluble heavy chains demonstrated that the PQD189 mAb recognized a single species (*arrowhead*, Figure 5A), suggesting

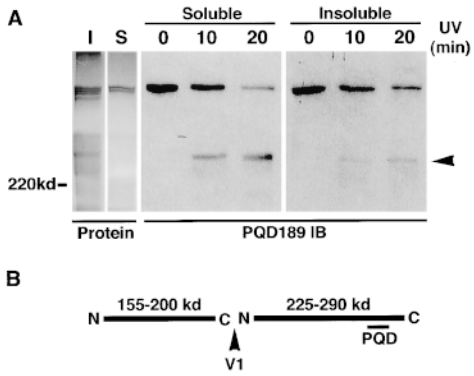

Figure 5. The PQD189 antibody is specific for one of two dynein arm heavy chains of porcine tracheal axonemes. (*A*) The outer dynein arms of porcine tracheal axonemes were selectively solublized by brief treatment with a high salt buffer. The insoluble (I) and soluble (S) fractions separated by electrophoresis through 5% reducing SDS-polyacrylamide gels and stained with coomassie blue are shown (Protein). Immunoblots using PQD189 of the soluble and insoluble fractions after ultraviolet irradiation for the indicated duration in minutes are shown (PQD189 IB). The immunoreactive V1 photolysis product is indicated (*arrowhead*). Only the high molecular weight region of the gels is shown. The M_r of a 220-kD molecular weight standard is indicated on the left. (B) A diagram of the expected products of the photolytic cleavage of DHCs is shown. The predicted location of the PQD189 epitope on the carboxy terminal fragment is indicated (PQD).

that it is specific for one of the two major heavy chains that co-elute with the outer dynein arms. When the insoluble dyneins were subjected to V1 photolysis and analyzed by immunoblotting, a single immunoreactive photolytic fragment was observed (Figure 5A). This photolytic fragment was the same size as that observed in the soluble dyneins subjected to V1 photolysis, indicating that a fraction of the PQD189 antigen remained associated with the axoneme. The absence of additional immunoreactive species suggested that the antibody does not cross-react with any of the other abundant DHCs in porcine tracheal cilia. The immunoreactive photolysis product had an $M_r > 220$ kD (Figure 5A), indicating that the carboxy terminal fragment of the heavy chain bore the PQD189 epitope. This was in agreement with the predicted location of the sequence homologous to the PQD189 epitope in sea urchin βHC (Figure 5B).

Immunohistochemical localization of the PQD189 antigen to paraffin sections of normal adult human bronchial epithelial biopsies revealed immunoreactivity concentrated in the cilia on the lumenal surface of the epithelium and weak cytoplasmic staining (Figure 6B). No staining of cilia was observed when PQD189 was omitted (not shown). An anticytokeratin 19 antibody of the same isotype as PQD189 localized to cell bodies but did not stain cilia (Figure 6C), indicating that the PQD189 localization was not due to the interaction of mouse IgG_{2b} with ciliary components.

The localization of PQD189 immunoreactivity to cilia was investigated in greater detail by electron microscopic immunohistochemistry. Axonemes isolated from ciliated human bronchial epithelial cultures were briefly treated with trypsin, adsorbed to formvar-coated grids, and reactivated with ATP. This caused the disintegration of the axonemes into individual doublet microtubules (25). PQD189 immunoreactivity was preferentially localized to one side of the doublet microtubules (Figure 6D), demonstrating that the antigen is associated with only one of the doublet subfibers. This asymmetric distribution is consistent with the distribution of axonemal dyneins that are attached to the A subfiber only.

To determine the temporal relationship between DNEL1 mRNA expression and the appearance of motile cilia, ciliogenesis was examined in human bronchial epithelial

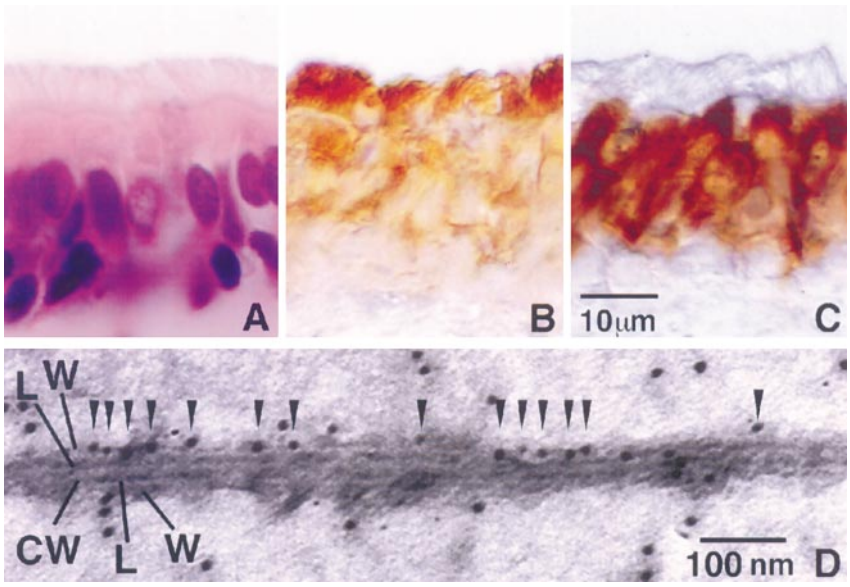

Figure 6. The PQD189 antibody localizes to one subfiber of human axonemal doublet microtubules. Immunoreactivity to the PQD189 mAb and to an isotype-matched (IgG_{2b}) anticytokeratin 19 was localized in serial sections of a paraffin-embeded adult human bronchial biopsy by indirect immunohistochemistry. Hematoxylin and eosin staining showed a pseudostratified ciliated epithelium (*A*). Strong PQD189 immunoreactivity was present in the cilia (*B*), whereas anticytokeratin 19 immunoractivity was restricted to the cell body (*C*). (*D*) Indirect immunoelectron microscopic localization of PQD189 to a whole mount of one of the nine microtubule doublets of a disintegrated human axoneme is shown. The doublet was negatively stained with uranyl acetate so that it appears as a five-layer structure composed of two electron-lucent outer walls (W) and a central wall (CW) separated by two electron-dense lumens (L). Immunoreactivity detected using a secondary antibody adsorbed to 10 nM colloidal gold particles was localized to one subfiber of the doublet microtubule (*arrowheads*).

cultures grown at an air–liquid interface in the presence of 250 nM retinol. In these cultures, ciliogenesis occurred asynchronously as it does *in vivo* (31). Motile cilia were first observed on a small subpopulation of cells and the percentage of ciliated cells increased thereafter for more than 1 wk. In the example shown in Figure 7A, DNEL1 mRNA was significantly upregulated beginning sometime between Days 4 and 8 and increased in abundance for 8 d. In contrast, expression of transcripts encoding a cytoplasmic DHC and glyceraldehyde-3-phosphate dehydrogenase was unchanged (cDHC1 and G3PDH, Figure 7A). When ciliogenesis was suppressed by maintaining cultures submerged in 250 nM retinol, DNEL1 mRNA was not upregulated (S, Figure 7A). Expression of DNEL2, the axonemal DHC-like gene cloned from testis that is closely related to DNEL1, was observed in testis but was undetected in bronchial epithelial cultures (DNEL2, Figure 7A). Daily light microscopic inspection of the live cultures revealed that motile cilia were present beginning on Day 10. Inspection of paraffin sections of a subset of the same cultures showed that ciliated cells appeared between Days 8 and 12 and were not abundant until Day 20 (Figure 7B). Thus, expression of the DNEL1 gene transcript preceded the appearance of cilia.

The DNEL1 gene product was localized in whole mounts of cultures undergoing ciliogenesis (23 d after ALI) using indirect immunofluorescent localization and laser scanning confocal microscopy. Optical sections in a plane at an acute inclination to the surface of the epithelium and taken at the level of the nucleus revealed diffuse cytoplasmic staining in many cells (*arrows* and *open arrowhead*, Figure 8A) but no nuclear staining (e.g., *solid arrowhead*, Figure 8A). Strong punctate staining was observed in optical sections passing through the cell apex reflecting the presence of cilia (e.g., *open arrowhead*, Figures 8B through 8D). In some cells, however, cytoplasmic staining was observed in the absence of ciliary staining (e.g., *arrows*, Figures 8A through 8D), suggesting that during ciliogenesis, the antigen is present in the cytoplasm before the appearance of cilia.

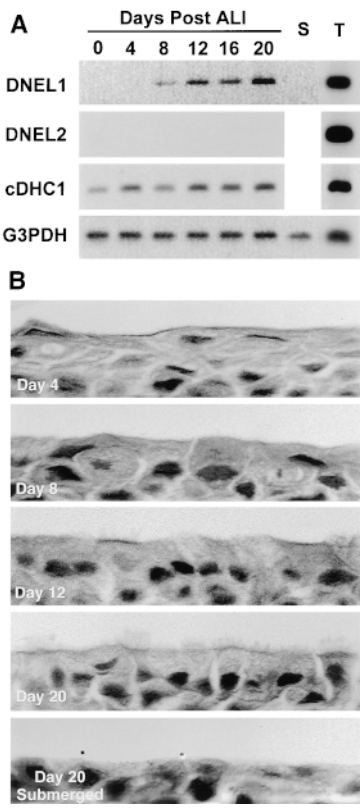

Figure 7. The tracheal mRNA is expressed before the appearance of cilia in cultures undergoing ciliogenesis *ex vivo*. (*A*) The abundance of transcripts corresponding to DNEL1, a closely related testis DHC (DNEL2), a cytoplasmic DHC (cDHC1), and glyceraldehyde-3-phosphate dehyrogenase (G3PDH) in bronchial epithelial cultures harvested at the indicated days after establishment of an air–liquid interface (ALI) in the presence of 250 nM retinol were estimated by RT-PCR. Expression of the same transcripts in total RNA from adult human testis was also assessed (T). Expression of DNEL1 and G3PDH in a bronchial epithelial culture grown in 250 nM retinol for 20 d, but maintained submerged is also shown (S). DNA products obtained after 31 (DNEL1, DNEL2, cDHC1) or 27 (G3PDH) amplification cycles, separated by electrophoresis through 2% alkaline-agarose gels, and stained with ethidium bromide are shown. (*B*) Hematoxylin and eosin–stained paraffin sections of epithelium cultured in parallel cultures with those analyzed by RT-PCR in *A* are shown.

Discussion

In this study, a fragment of a human tracheal cDNA was isolated based upon its DNA sequence identity to two human testis ESTs, and the expression of its corresponding mRNA and protein product were characterized in airway epithelium. The data strongly suggested that the tracheal cDNA is derived from an axonemal DHC gene. A 3.16-kb region of the mRNA transcript of the gene from which the testis ESTs were derived has been assembled from overlapping clones from a testis cDNA library, and the gene has been assigned to chromosome 17 (28). The DNA sequence of the entire 1.13-kb tracheal cDNA characterized here is identical to the sequence of the midregion of this testis cDNA, suggesting that both are derived from the same gene, DNEL1.

DNEL1 has a 3.2-kb mRNA in testis and was not detected in lung by Millisav and associates (28), whereas the tracheal cDNA corresponded to an mRNA > 9.5 kb and was present in lung (Figure 2). The reasons for these discrepancies are unclear. Our probe and the probe used by Millisav and coworkers (28) completely overlap. The radiolabeled probe used in this study was a single-stranded riboprobe that may be capable of detecting smaller transcript abundances than the double-stranded DNA probe used by Milisav and colleagues (28). This may account for the detectable hybridization of the RNA probe but not the DNA probe to total RNA from lung. The difference in mRNA size may be due to differential processing of the DNEL1 transcript in lung and testis.

Immunoblotting using PQD189, a monoclonal antibody to a fusion protein encoded by the tracheal cDNA, recognized antigens with similar very high molecular weights in both isolated human bronchial axonemes and whole mature human sperm (Figure 4). The protein product of DNEL1 is predicted to have a molecular weight of about 100 kD in testis (28), much smaller than the immunoreactive species we observed in sperm. The PQD189 antibody may cross-react with the protein product of DNEL2, another axonemal DHC-like gene expressed in human testis (20). DNEL2 encodes a polypeptide with significant sequence similarity to the polypeptide used as antigen in this study. However, the full-length transcript and protein product of DNEL2 have not been determined, nor have they been localized in testis or sperm. Given the available information, the identity of the high molecular weight an-

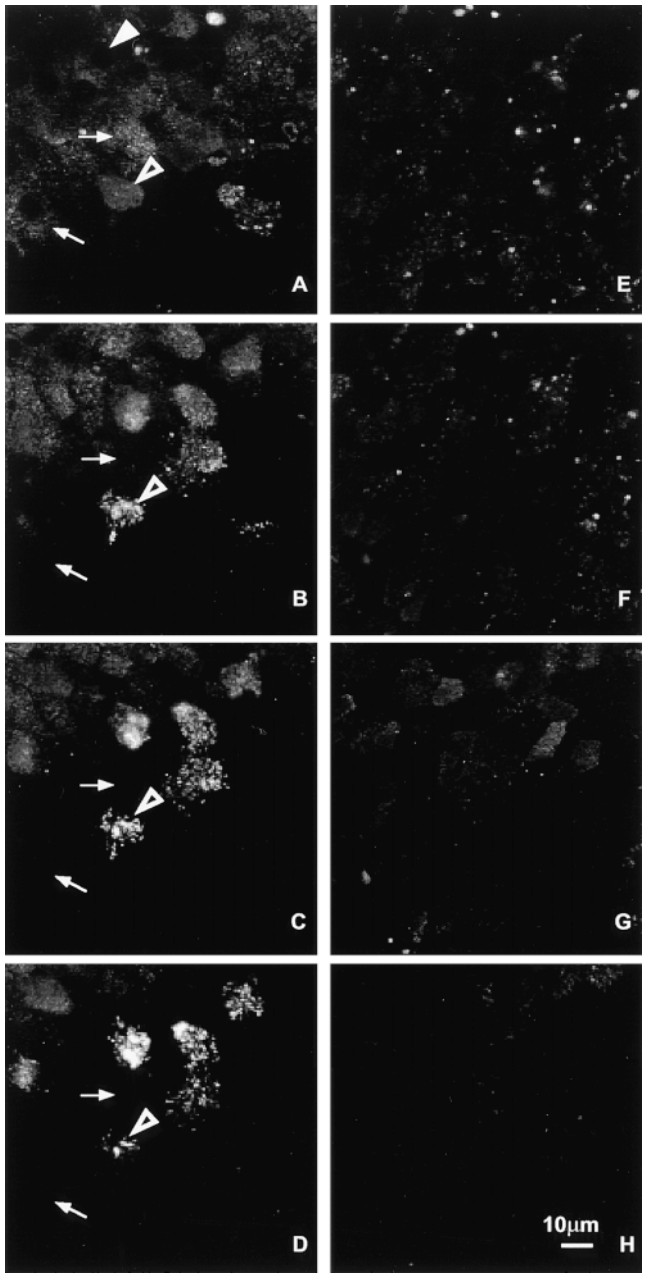

Figure 8. The PQD189 antibody localizes to the cytoplasm of a subset of nonciliated cells in epithelium undergoing ciliogenesis. PQD189 immunoreactivity in whole mounts of human bronchial epithelium undergoing ciliogenesis *ex vivo* at 23 d after establishment of the air–liquid interface was localized by indirect immunofluorescence and analyzed by laser scanning confocal microscopy. The epithelium is viewed *en face* from above in serial optical sections starting at the basal surface and progressing toward the apical surface (*A* through *D*). In some cells, diffuse cytoplasmic staining (*A*, *open arrowhead*) gave way to strong punctuated staining at the apical border, indicative of the presence of cilia (*B* through *D, open arrowheads*). Other cells exhibited cytoplasmic staining, but no staining indicative of cilia (*A* through *D, arrows*). PQD189 immunoreactivity was excluded from the nucleus (*A*, *arrowhead*). No staining was observed in serial optical sections of a companion epithelium for which the PQD189 was omitted during staining (*E* through *H*).

tigen in whole mature sperm recognized by the PQD189 antibody remains uncertain.

The transcript of DNEL2 was undetected in bronchial epithelium undergoing ciliogenesis *ex vivo* (Figure 7A), making it unlikely that the protein product of DNEL2 is a DHC of major abundance in these cultures. Thus, the PQD189 immunoreactivity on immunoblots (Figure 4) and microtubule doublet whole mounts (Figure 6D) of axonemes isolated from these cultures and in the cytoplasm and cell apex of nonciliated cells from these cultures (Figure 8) most likely corresponds to the protein product of DNEL1. In preliminary experiments, we have observed that the single PQD189 immunoreactive species in axonemes from these cultures is cleaved by V1 photolysis (W. Reed, unpublished observations). Together, these data provided the strongest evidence that the protein product of DNEL1 in airway epithelium is an axonemal DHC.

Ciliogenesis in invertebrates has been addressed at the ultrastructural and molecular levels by numerous studies (1), whereas vertebrate ciliogenesis has been studied predominantly at the ultrastructural level (31). Currently, molecular markers temporally correlated with vertebrate ciliogenesis include tubulins, especially β-tubulin IV, and hepatocyte nuclear factor-3/forkhead homolog-4, a member of the forkhead/winged helix family of transcription factors (31, 32). In this study, we sought to develop additional molecular probes that could be used to phenotype ciliated cells early in their differentiation, before the appearance of overt morphologic features indicative of cilia. We pursued axonemal DHCs because studies have documented that they are immunologically distinct from their cytoplasmic isoforms, whose expression is ubiquitous (33, 34). Moreover, we hypothesized that axonemal DHCs may undergo greater relative increases in expression levels than tubulin levels do and therefore may be more sensitive indicators of ciliogenesis. In preliminary studies using an anti-DNEL1 antibody, we have demonstrated that DNEL1 is present in the cytoplasm of a subset of cells in epithelium undergoing ciliogenesis *ex vivo* (Figure 8). DNEL1 was present in the cytoplasm of both ciliated and nonciliated cells in the same epithelium, raising the possibility that axonemal dyneins are synthesized before axonemal assembly begins. A cytoplasmic pool of DNEL1 is not unprecedented because cytoplasmic pools of assembled axonemal dyneins have been demonstrated in invetebrates (35). One interpretation of our data suggests that events subsequent to the synthesis of DNEL1 are necessary for initiation of axonemal assembly in airway epithelium. However, at this time it is not known whether the observed pool of cytoplasmic DNEL1 is a consequence of artificial culture conditions or is representative of ciliogenesis *in vivo.* In future studies, we will investigate the kinetics of DNEL1 mRNA and protein expression in airway epithelium undergoing ciliogenesis *in vivo* with the objective of confirming and extending these observations.

The probes used in this study and additional molecular probes generated using the rapidly growing database of molecular sequences of ciliary protein components have a number of potential uses in addition to the characterization of vertebrate ciliogenesis at a molecular level. They may be useful in the diagnosis and therapeutic treatment

of human disorders characterized by congenital ciliary dysfunction, such as primary ciliary dyskinesia. They also may be used to isolate ciliated cell-specific gene promoters of value in studying epithelial cell fate (phenotype) during airway maturation, injury, and repair or in achieving ciliated cell type–specific expression of transgenes.

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Note added in proof: Since this manuscript was submitted, the complete amino acid coding sequence of DNEL1 as it is expressed in airway epithelium has been determined (GenBank accession no. AF257737, gene symbol DNAH9).

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