

# A Proteomic Analysis of Human Cilia

IDENTIFICATION OF NOVEL COMPONENTS\*<sup>†</sup>

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Cilia play an essential role in protecting the respiratory tract by providing the force necessary for mucociliary clearance. Although the major structural components of human cilia have been described, a complete understanding of cilia function and regulation will require identification and characterization of all ciliary components. Estimates from studies of *Chlamydomonas* flagella predict that an axoneme contains  $\geq 250$  proteins. To identify all the components of human cilia, we have begun a comprehensive proteomic analysis of isolated ciliary axonemes. Analysis by two-dimensional (2-D) PAGE resulted in a highly reproducible 2-D map consisting of over 240 well resolved components. Individual protein spots were digested with trypsin and sequenced using liquid chromatography/tandem mass spectrometry (LC/MS/MS). Peptide matches were obtained to 38 potential ciliary proteins by this approach. To identify ciliary components not resolved by 2-D PAGE, axonemal proteins were separated on a one-dimensional gel. The gel lane was divided into 45 individual slices, each of which was analyzed by LC/MS/MS. This experiment resulted in peptide matches to an additional 110 proteins. In a third approach, preparations of isolated axonemes were digested with Lys-C, and the resulting peptides were analyzed directly by LC/MS/MS or by multidimensional LC/MS/MS, leading to the identification of a further 66 proteins. Each of the four approaches resulted in the identification of a subset of the proteins present. In total, sequence data were obtained on over 1400 peptides, and over 200 potential axonemal proteins were identified. Peptide matches were also obtained to over 200 human expressed sequence tags. As an approach to validate the mass spectrometry results, additional studies examined the expression of several identified proteins (annexin I, sperm protein Sp17, retinitis pigmentosa protein RP1) in cilia or ciliated cells. These studies represent the first proteomic analysis of the human ciliary axoneme and have identified many potentially novel components of this complex organelle. *Molecular & Cellular Proteomics* 1:451–465, 2002.

Mucociliary clearance is an essential mechanism for defending the airways against exposure to inhaled toxins and pathological organisms (1). The continuous, coordinated beating of the cilia transports mucus and foreign material out of the airways to help maintain a sterile environment. The importance of this process is clearly illustrated by the disease primary ciliary dyskinesia (PCD),<sup>1</sup> in which genetic defects cause dysfunctional cilia and result in chronic otitis media, sinusitis, and bronchitis (2). In addition to their role in mucociliary clearance, cilia and other axonemal structures are clearly essential to many other specialized cell biological functions. Nodal cilia have been shown to be important for the determination of situs (3), and recently the primary cilium of renal epithelial cells has been demonstrated to be responsive to flow (4). Motile cilia are also present in the oviduct, the epididymis, and the ependymal cells of the brain. Sperm flagella possess a 9 + 2 axoneme, and the photoreceptor cells of the retina contain a modified cilium. Although these diverse axonemes may share many common structural features, it is clear that each is specialized for a particular function.

The structure of respiratory tract cilia has been the subject of many previous microscopic studies. These studies have demonstrated the 9 + 2 arrangement of microtubules, the inner and outer dynein arms, the radial spokes, and other features common to all motile cilia. However, much of our understanding of cilia structure and function has been derived by analogy to model systems, such as *Chlamydomonas* and *Paramecium*. Although these models have provided a wealth of information, to understand the structure and regulation of human cilia it is clear that complementary studies of human material are required.

An important step toward a complete understanding of ciliary function is the identification of all the protein components that comprise the ciliary axoneme (a ciliary proteome). Early studies of *Chlamydomonas* suggested that an axoneme may be composed of over 250 individual proteins (5, 6), and a number of these have been identified and studied. However, whereas the sequencing of the human genome has allowed the identification of homologs of these *Chlamydomonas* proteins, biochemical

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<sup>1</sup> The abbreviations used are: PCD, primary ciliary dyskinesia; HBE, human bronchial epithelial; DHC, dynein heavy chain; LC/MS/MS, liquid chromatography-tandem mass spectrometry; LC/LC/MS/MS, two-dimensional liquid chromatography-tandem mass spectrometry; EST, expressed sequence tag; 2-D, two-dimensional; 1-D, one-dimensional.

evidence is still necessary to demonstrate their axonemal nature in human cilia. Further, it is likely that human cilia will contain important novel proteins not found in simpler model systems.

Although the difficulty of obtaining human cilia has limited previous studies, recent advances in cell culture techniques allow ciliated cell differentiation of human airway epithelial cells to occur *in vitro* (7, 8), providing a reliable source of material. Coupled with the improvements in sensitivity and throughput of mass spectrometry, the identification of the ciliary proteome is now feasible. Because it is not yet clear which separation technique, if any, is optimal, we employed both one- and two-dimensional gel electrophoresis, as well as multidimensional liquid chromatography (9), in this initial characterization of the ciliary axoneme.

### EXPERIMENTAL PROCEDURES

**Cell Culture**—Human bronchial epithelial (HBE) cells were obtained from excess surgical tissue under protocols approved by the University of North Carolina Institutional Review Board. HBE cells were obtained primarily from normal subjects (transplant donors) and cystic fibrosis patients undergoing lung transplant. No differences were observed between cilia from the normal and cystic fibrosis cells. Passage 1 or passage 2 HBE cells were grown at an air/liquid interface using procedures described previously (7, 8) with only minor modifications. Briefly,  $5\text{--}10 \times 10^5$  HBE cells were plated on 30-mm-diameter Millicell-CM culture inserts (Millipore) coated previously with rat tail collagen (BD Pharmingen). After reaching confluence, medium was removed from the apical surface, and cultures were fed from only the basal surface every 2–3 days. Newly differentiated ciliated cells appeared after 10–14 days. After 4–6 weeks ~50–80% of the culture surface was ciliated.

**Isolation of Cilia**—Cilia were isolated as described previously (10–12). Briefly, heavily ciliated cultures were washed with cold phosphate-buffered saline to remove mucus and cell debris. Deciliation buffer (12) was added to the surface, and the culture was rocked gently for 1 min. The supernatant was collected, and the procedure was repeated. The two washings were pooled, and after pelleting debris at  $1000 \times g$ , the ciliary axonemes were collected by centrifugation at  $16,000 \times g$  and frozen at  $-80^\circ\text{C}$ . During the course of these studies, an additional wash step was included in some preparations to further reduce contamination of the axonemes. Ciliary pellets were resuspended gently in 30 mM Hepes (pH 7.3), 1 mM EGTA, 0.1 mM EDTA, 25 mM NaCl, 5 mM  $\text{MgSO}_4$ , 1 mM dithiothreitol, and 1% volume of protease inhibitor mixture (Sigma). Triton X-100 was added (0.5%) to the suspension, and the axonemes were incubated for 15 min on ice, followed by centrifugation. Protein concentrations were estimated using the BCA reagent (Pierce).

**2-D Gel Electrophoresis and Analysis**—First-dimension isoelectric focusing was carried out using 18-cm NL Immobilized DryStrips (pH 3–10; Amersham Biosciences) on an IPGphor (Amersham Biosciences) according to the manufacturer's instructions. Axonemal pellets (40–70  $\mu\text{g}$ ) were resuspended directly in sample buffer and focused at 20 volts for 12 h; at 100, 250, 500, 1000, and 2500 volts for 1 h each; and at 8000 volts for 32,000 V-h. Electrophoresis in the second dimension was performed on a 12% SDS-PAGE gel using a Bio-Rad Protean II apparatus. Gels were silver-stained (Amersham Biosciences), scanned, and analyzed using Imagemaster 2-D software, Version 2.0 (Amersham Biosciences). For mass spectrometry, axonemes were electrophoresed under identical conditions, except 120  $\mu\text{g}$  of axonemes were loaded, and the gel was stained with Pro-Blue colloidal Coomassie stain (Owl Separation Systems, Ports-

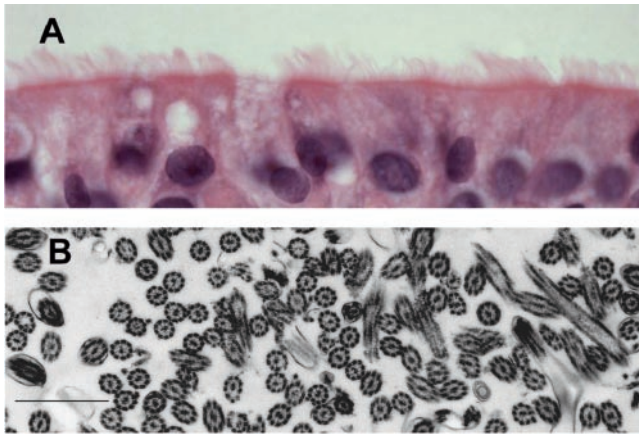
mouth, NH) with omission of the first fixation step. Individual spots were excised, digested with trypsin as described previously (13), and analyzed by LC/MS/MS (see below).

**1-D Gel and Analysis**—Purified ciliary axonemes (50  $\mu\text{g}/\text{lane}$ ) were separated on a 16-cm pre-cast 8–16% SDS-PAGE gel (Bio-Rad) and stained with Pro-Blue. The gel was sliced into 46 individual bands, including one large band containing tubulin. Bands from two lanes were pooled, and in-gel digestion was performed as above.

**Digestion of Axonemes for Direct Analysis**—For direct analysis by LC/MS/MS, a 30- $\mu\text{g}$  aliquot of purified ciliary axonemes was solubilized using 6 M guanidine HCl (pH 8.0). The solubilized axonemes were then loaded onto a reversed phase cartridge and digested *in situ* with endoprotease Lys-C (14). For direct analysis by LC/LC/MS/MS, a 30- $\mu\text{g}$  aliquot was initially solubilized using 5  $\mu\text{l}$  of hexafluoroacetone and enough 6 M urea to solubilize the pellet completely. The sample was diluted to 2 M urea with a 20 mM Tris buffer (pH 8.5) and digested in solution with endoprotease Lys-C. Lys-C was used for digestion, because it remains active in the solutions used.

**Peptide Sequencing by Mass Spectrometry**—In-gel and solution digests of ciliary axonemal proteins were analyzed by nanoscale capillary LC/MS/MS using an Ultimate™ capillary LC system, Switchos valve switching unit, and Famos autosampler (LC Packings, San Francisco, CA) coupled to a quadrupole time-of-flight mass spectrometer (Q-TOF2; Micromass, Manchester, United Kingdom) as described previously (15). In addition to the LC/MS/MS analysis, a multidimensional LC/LC/MS/MS approach similar to that described by Yates and co-workers (9) was performed using strong cation exchange in the first LC dimension and reversed-phase in the second dimension. Peptides were sequentially step-eluted from the 300- $\mu\text{m}$  inner diameter  $\times$  5-mm strong cation exchange column (LC Packings, San Francisco, CA) packed with polysulfoethyl aspartamide material (PolyLC, Columbia, MD) onto a reversed-phase trapping cartridge using a 5 mM  $\text{KH}_2\text{PO}_4$  buffer (pH 3.0) containing 0, 25, 35, 50, 60, 75, 100, or 200 mM KCl. Peptides from each salt fraction were then analyzed by sequential reversed phase LC/MS/MS analyses. Following data acquisition, LC/LC/MS/MS datafiles from each salt step were processed initially using the Proteinlynx module of Masslynx 3.4 (Micromass) to generate Mascot-searchable \*.pkl files. A peptide QA filter of 20 was used in Proteinlynx to eliminate poor quality spectra prior to database searching. This filter takes into account the presence of immonium ions, the intensity of the product ion peaks above precursor mass for multiply charged species, the base peak intensity, and amino acid mass differences between peaks. Proteinlynx-processed data (containing quality product ion spectra) from each salt step were then searched against an in-house nonredundant protein database using the Mascot program (16). Spectra with no matches from the protein database were subsequently searched against a database containing human expressed sequence tags (ESTs). Parameters used for all Mascot searches were as follows: allow up to one missed proteolytic cleavage; peptide mass tolerance, 2 Da; product ion mass tolerance, 0.3 Da. For samples analyzed from 1-D or 2-D gels, the following variable modifications were used in the searches: pyridylethyl-Cys, acrylamide-Cys, methionine oxidation. For samples analyzed from solution or *in situ* digests, only pyridylethyl-Cys was used as a variable modification. All peptide matches returned by Mascot with MOWSE scores above 12 were examined individually to assess the correctness of the match. Criteria that were used to accept matches include quality of the match (presence of an extended y- or b-ion series; sequence-specific fragmentation features such as unique immonium ions, preferential fragmentation at proline, etc.), as well as biological information about a particular protein that might point toward its presence in cilia. In many cases, a protein could be identified confidently on the basis of a single peptide match.

**Western Blotting of Annexin/Sp17**—Western blotting was per-



**FIG. 1. Isolation of cilia from cultures of human bronchial epithelial cells.** *A*, section of a well differentiated culture with an abundance of cilia (hematoxylin and eosin stain, 100 $\times$ ). *B*, electron micrograph showing a highly enriched preparation of ciliary axonemes after isolation from cultured human bronchial epithelial cells. Scale bar, 1.1  $\mu$ m.

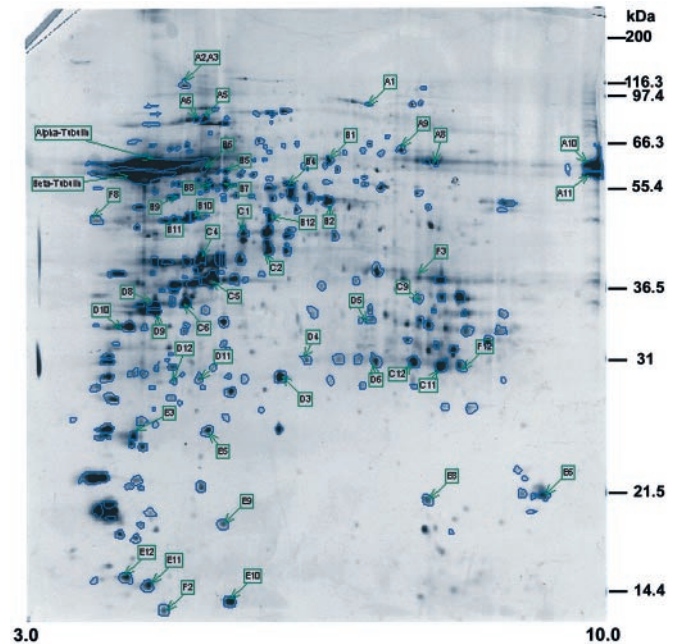
formed as described previously (10) with the following modifications. Mouse monoclonal anti-annexin 1 (Biogenesis, Brentwood, NH) was used at 0.05  $\mu$ g/ml. Rabbit anti-Sp17 was a generous gift from Dr. M. O'Rand and was used at a dilution of 1:5000. Membranes were blocked with 1% non-fat milk in Tris-buffered saline containing 0.05% Tween 20, and detection was with ECL reagents according to the manufacturer (Amersham Biosciences).

**Immunostaining of Sections and Cultures**—Paraffin sections of HBE cultures were immunostained with mouse anti-annexin 1 as described previously (10) using a concentration of 0.5  $\mu$ g/ml. For immunolocalization of Sp17, cultures of well differentiated HBE cells were fixed in 4% paraformaldehyde, washed in phosphate-buffered saline, and permeabilized in 0.2% Triton X-100 for 15 min. Cultures were then incubated with affinity-purified anti-Sp17 (1:500) or with an equal concentration of rabbit antiserum depleted of Sp17-reacting antibodies by pre-absorption. Additional cultures were incubated with an identical concentration of normal rabbit IgG. Detection was performed using Texas Red-conjugated goat anti-rabbit and a Leica scanning confocal microscope.

**RT-PCR of Retinitis Pigmentosa Protein RP1**—Total RNA was isolated from cultures of HBE cells using an RNeasy kit (Qiagen), and RT-PCR was performed using the GeneAmp RNA PCR kit (Applied Biosystems, Branchburg, NJ), both according to the manufacturer's instructions. Primers used were 5'-TGAAGGGCAGTCATTTG-GCTCT-3' (forward) and 5'-AAGGGTTGAGTCAGTTCCTCG-3' (reverse). Cycling conditions for amplification included an initial denaturation at 95  $^{\circ}$ C for 105 s, 4 cycles of 95  $^{\circ}$ C for 15 s; 60  $^{\circ}$ C for 30 s, 31 cycles of 95  $^{\circ}$ C for 15 s; 58  $^{\circ}$ C for 30 s, followed by a 1-min extension at 72  $^{\circ}$ C.

## RESULTS

HBE cells were grown on collagen-coated membranes at an air/liquid interface. Under these conditions, HBE cells differentiate to produce a heavily ciliated epithelium (Fig. 1A). Ciliary axonemes were isolated from these cultures using a non-ionic detergent in the presence of calcium. As reported previously (10, 11), these preparations are highly enriched for intact ciliary axonemes (Fig. 1B) and are well suited for biochemical analysis.



**FIG. 2. 2-D gel electrophoresis of human ciliary axonemes.** Axonemal proteins were separated by 2-D gel electrophoresis as detailed under "Experimental Procedures." The gel was silver-stained and analyzed using ImageMaster software. Protein spots outlined appeared reproducibly in 80% of the gels analyzed. Numbered spots were excised, digested with trypsin, and sequenced by LC/MS/MS. Acidic polypeptides are on the left.

As a first step toward developing a ciliary proteome, axonemes isolated from several different cultures were separated by 2-D PAGE, silver-stained, and analyzed using image analysis software. In general, the overall pattern of proteins was highly reproducible between experiments. One gel (Fig. 2) was chosen as a reference gel. By comparing four other gels to the reference gel, an average gel was created. This average gel consists of over 240 protein spots (outlined in Fig. 2) that appeared in at least 80% of the gels analyzed (four of five).

To facilitate identification of these proteins by mass spectrometry, axonemal proteins were again resolved by 2-D PAGE, but the amount of protein loaded was increased, and the gel was stained with colloidal Coomassie Blue. The pattern of proteins observed was identical to that obtained with silver staining, although the overall staining was fainter. Seventy-two individual spots of different intensities were isolated from throughout the gel, digested with trypsin, and analyzed by LC/MS/MS. Sequence information was obtained on 62 of the spots, and over 200 individual peptides were sequenced. For the studies reported here, peptide matches to entries in the databases have been organized by accession number. Duplicate hits to the same accession number have been eliminated, and where it is clear that more than one accession number exists for the identical protein, the results have been combined. A summary of all the matches identified for each spot is presented in Table I, and the corresponding spots are

TABLE I  
 Identification by LC/MS/MS of human axonemal proteins separated by 2-D PAGE

	Spot	Description	Accession No.	No. of peptides
1	A1	cDNA FLJ23305 FIS	Q9H5M3	8
2	A2	hb08b03.×1 cDNA Clone	AW473770	1
3	A2	P97 ATPase-related sequence	CAB41525	12
4	A2	KIAA0356 Protein	Q9Y4G2	1
5	A3	TER ATPase	P46462	1
6	A5	Heat shock protein	HS70	3
7	A5	Heat shock cognate 70-kDa protein	3HSC	2
8	A5	602350570F1 cDNA Clone	BG122387	1
9	A5	Stratagene fetal retinal	AA176890	1
10	A6	Heat shock protein 70	P08107	4
11	A6	HS7C Mouse heat shock cognate 71-kDa	P08109	4
12	A6	TERA human endoplasmic reticulum ATPase	P55072	1
13	A8	Testicular microtubules-related protein Tektin 3	AAK15340	5
14	A9	Unnamed protein product	BAA92077	5
15	A10	$\alpha$ -Tubulin	AAH04949	4
16	A11	$\beta$ -Tubulin	Q60455	6
17	B1	NEB protein	AF022650	1
18	B1	$\beta$ -Tubulin	AF083031	1
19	B2	Canine Tektin	Q9TTW3	8
20	B4	Unnamed protein product	BAB71402	3
21	B4	$\beta$ -Tubulin	Q60455	5
22	B4	Reticulocyte-binding protein	Q00798	1
23	B4	KIAA0356 Protein	Q9Y4G2	1
24	B4	SPAM	Q54064	1
25	B4	$\alpha$ -Tubulin	Q9DFT4	1
26	B4	Similar to Tektin-3	AAH21716	1
27	B4	Unnamed protein product	BAB71402	1
28	B5	$\alpha$ -Tubulin	Q9GLW6	2
29	B5	Similar to Tektin-3	AAH21716	2
30	B6	Similar to Tektin-3	AAH21716	2
31	B6	$\beta$ -Tubulin	Q9JJY6	2
32	B7	H-Tektin-T	Q9UIF3	5
33	B8	H-Tektin-T	O60638	6
34	B9	Similar to Tektin-3	AAH21716	1
35	B9	H-Tektin-T	Q9UIF3	1
36	B9	CGI-53 Protein	AF151811	3
37	B10	$\beta$ -Actin	Q61276	2
38	B11	$\beta$ -Actin	X03765	3
39	B12	CGI-53 protein	AF151811	3
40	B12	$\beta$ -Tubulin	P04350	1
41	B12	H-Tektin-T	Q9UIF3	1
42	B12	Similar to Tektin-3	AAH21716	1
43	C1	$\beta$ -Tubulin	Q60455	5
44	C2	$\beta$ -Tubulin	Q60455	3
45	C2	Similar to Tektin-3	AAH21716	1
46	C4	$\alpha$ -Tubulin	Q08114	1
47	C5	$\beta$ -Tubulin	Q60455	9
48	C6	$\alpha$ -Tubulin	Q43605	1
49	C6	Novel protein isoform 1	Q9H1X0	2
50	C9	Similar to Tektin-3	AAH21716	1
51	C11	Heat shock protein J2	O75190	7
52	C11	$\beta$ -Tubulin	Q9JJY6	2
53	C11	AKAP-associated sperm protein	AAG59587	1
54	C12	HU EST cDNA	BG423215	1
55	C12	HU EST cDNA	BF965422	2
56	C12	H-Tektin-T	Q9UIF3	7
57	D3	Human nucleoside diphosphate kinase homolog	P56597	5
58	D4	EIF-3- $\beta$	Q13347	1
59	D5	Similar to myeloid leukemia factor	AAH03975	2
60	D6	Heat shock protein J2	AAH02446	5

TABLE I—continued

Spot	Description	Accession No.	No. of peptides	
61	D8	$\beta$ -Tubulin	P09206	3
62	D9	$\beta$ -Tubulin	P09206	4
63	D10	$\beta$ -Tubulin	P09206	4
64	D10	14-3-3 Protein $\epsilon$	U53882	2
65	D10	KIAA0477 Protein	O75065	1
66	D11	Similar to Riken protein	BC007374	2
67	D12	Similar to Riken protein	BC007374	2
68	E3	Unknown protein	AAH04522	4
69	E5	Putative GTP-binding protein	AAH00566	3
70	E6	CG11245 Protein	Q9VYN1	1
71	E8	Mus Musculus male testis cDNA	AK005609	1
72	E9	Predicted protein	XP_088490	1
73	E10	c-Myc-binding protein	Q99417	4
74	E12	$\beta$ -Tubulin	Q9JJY6	1
75	F2	Mus Musculus male testis cDNA	AK015697	1
76	F3	Fusion gene	AAA99997	2
77	F4	Fusion gene	AAA99997	2
78	F5	Fusion gene	AAA99997	3
79	F8	Mus Musculus male testis cDNA	AK005739	3
80	F8	LEPS BACSU signal peptidase	P28628	1
81	F12	AKAP-associated sperm protein	AAG59587	2
82	F12	$\alpha$ -Tubulin	Q06331	2
83	F12	Phenylalanyl-tRNA synthetase $\beta$ -chain	Q9ZDB4	1

labeled in Fig. 2. The number of matching peptides obtained for each protein is also included in Table I. Many of the proteins were identified by only one peptide; however these may represent axonemal components and have therefore been included. Some spots yielded only keratin, and several of the spots contained more than one protein. Also, some proteins were identified in more than one location, suggesting that the protein was modified or exists in different isoforms (e.g. peptides in spots C11 and F12 both matched an AKAP-associated sperm protein).

From this analysis, 38 unique proteins were identified. Table II contains a composite list of all proteins identified by each of the separate approaches used in this study. The data from the analysis of the 2-D gel constitutes the first 38 entries in this table. Analyses of these 38 matches identified 14 entries that were considered to be “known” or “likely” components of cilia, based on the literature or the description in the database. These 14 proteins are listed in Table III, which also includes known or likely axonemal components found in each of the other approaches used in this study. In addition to the major  $\alpha$ - and  $\beta$ -tubulin spots, other known ciliary components identified by 2-D analysis included tektins (17), heat shock proteins (18, 19), and actin (20). Several proteins were considered potentially novel components of human cilia, including the AKAP-associated sperm protein, three mouse testis cDNAs, a testis-specific nucleoside diphosphate kinase, a c-Myc-binding protein found in sperm, and an EST from a human retinal library. Four of the remaining proteins are most likely contaminants, signal peptidase, endoplasmic reticulum ATPase, EIF-3B, and the phenylalanyl-tRNA synthetase. For the remaining 21 protein matches, there is insufficient information to deter-

mine whether they are associated with the ciliary axoneme or if they are cellular proteins that co-purify with the axoneme.

Although 2-D PAGE provides much important information about a protein sample, it will not resolve all of the proteins predicted to be present in an axoneme. For example, dynein heavy chains (DHCs), which are known to be a major axonemal component, are not resolved on standard 2-D systems because of their large molecular mass ( $\geq 500$  kDa). To obtain a more complete representation of the proteins comprising the human cilium, a preparation of purified axonemes was separated on a 1-D gradient gel (Fig. 3). The gel lanes were then sliced into 46 individual bands of a few mm each. Each of the bands was subjected to trypsin digestion, and the purified tryptic peptides were then sequenced by LC/MS/MS. This approach yielded sequence data on over 890 tryptic peptides that matched 195 protein entries and 148 ESTs. Combining redundant entries and removing several suspected contaminants (e.g. keratin, ribosomal proteins) resulted in a set of 123 possible axonemal proteins (Table II). Many ciliary proteins were detected that were not found in the 2-D PAGE experiment. For example, whereas no peptides were identified from DHCs in the 2-D gel, over 40 peptides were found to match DHC sequences in the 1-D experiment. Matches were found to dynein light and intermediate chains, as well. Many potentially novel ciliary components were also identified by this approach, including a brain-specific protein, a sperm surface protein, and the retinitis pigmentosa protein, RP1. Thirty-nine of the 123 matches were assigned to the known/likely axonemal group (Table III), whereas 17 were considered probable contaminants, leaving 67 proteins as potential newly identified axonemal proteins. Many of these

TABLE II  
Combined results of four different proteomic analyses of human ciliary axonemes

	Description	Accession No.	2-D gel	1-D gel	Direct LC/MS/MS	Multi-dimensional LC/MS/MS
1	$\alpha$ -Tubulin	Multiple	X	X	X	X
2	$\beta$ -Tubulin	Multiple	X	X	X	X
3	70-kDa Heat shock protein(s)	Multiple	X	X	X	X
4	Heat shock protein(s) J2	Multiple	X	X	X	X
5	Actin	Multiple	X	X	X	X
6	14-3-3 Proteins	Multiple	X	X		X
7	Stratagene fetal retinal	AA176890	1			
8	Fusion gene	AAA99997	3	2		1
9	AKAP-associated sperm protein	AAG59587	2			1
10	Putative GTP-binding protein	AAH00566	3			2
11	Similar to myeloid leukemia factor	AAH03975	2			
12	Unknown protein	AAH04522	4			
13	Testicular microtubules-related protein Tektin 3	AAK15340	5			
14	Kinesin-like protein 38B (NEB protein)	AF022650	1			
15	CGI-53 Protein	AF151811	5			1
16	Mus Musculus male testis cDNA	AK005609	1			
17	Mus Musculus male testis cDNA	AK005739	3			
18	Mus Musculus male testis cDNA	AK015697	1			
19	hb08b03. $\times$ 1 cDNA clone	AW473770	1			
20	Unnamed protein product	BAB71402	4			
21	Similar to Riken cDNA 4933428D01	BC007374	2			1
22	P97 ATPase-related sequence	CAB41525	12			
23	H-Tektin-T	Multiple	X	X		
24	KIAA0477 Protein	O75065	1			
25	LEPS BACSU signal peptidase	P28628	1	1		
26	Tera human endoplasmic reticulum ATPase	P55072	1			1
27	Human nucleoside diphosphate kinase homolog (testis-specific)	P56597	5	5	1	1
28	Reticulocyte-binding protein	Q00798	1			
29	EIF-3- $\beta$	Q13347	1			
30	SPAM	Q54064	1			
31	c-Myc-binding protein	Q99417	4	1		
32	Novel protein isoform 1	Q9H1X0	2			
33	Unnamed protein product	BAA92077	5	2		
34	CG11245 Protein	Q9VYN1	1	1		
35	KIAA0356 Protein	Q9Y4G2	1			
36	Phenylalanyl-tRNA synthetase $\beta$ -chain	Q9ZDB4	1			
37	cDNA FLJ23305 FIS	Q9H5M3	8			1
38	Predicted protein	XP_088490	1			
39	Annexin 1	Multiple		X	X	X
40	1CMI_A Protein inhibitor of neuronal nitric oxide PDB	1CMIA		2		
41	Galectin-7	3GALA		1		
42	CLP-like protein fragment	AAF64302		1		
43	HU ciliary dynein heavy chain 9	AAF69004		32		
44	AAF97774 J4R	AAF97774		1		
45	IC1 Homolog	AAG28497		5		1
46	Putative glutathione S-transferase OSGSTU4	AAG32471		1		
47	HU DNA sequence clone RP1-261G23	AL136131		6		
48	cDNA DKFZp434N2435	AL136858		2		
49	cDNA FLJ20753 FIS	BAA91365		1		
50	cDNA FLJ10466 FIS	BAA91628		12	1	
51	KIAA1374 Protein (fragment)	BAA92612		2		
52	KIAA1430 Protein (fragment)	BAA92668		1		
53	LUNX protein	BAA93633		1		
54	Unnamed protein product	BAB01602		9		
55	Genomic DNA chromosome 5, TAC clone:K3K7	BAB08743		1		
56	Putative cysteine proteinase	BAB17096		1		
57	Similar to part of several eg. ACDS_MEGEL Q06319 Acyl-CoA dehydrogenase	CAB09628		1		

TABLE II—continued

	Description	Accession No.	2-D gel	1-D gel	Direct LC/MS/MS	Multi-dimensional LC/MS/MS
58	CAB70790 Hypothetical 32.7-kDa protein	CAB70790		3		
59	CAB71195 DJ111C20.1 (similar to chlamydomonas radial spoke protein)	CAB71195		2		
60	CAB92087DJ534K7.2 (novel protein)	CAB92087		3	1	
61	DJ412I7.1 (similar to radial spokehead protein) (fragment)	CAC00771		4		
62	Hypothetical 37.1-kDa protein	CAC09526		12		
63	Intermediate dynein chain	CAC17464		1		
64	Canis $\beta$ -galactosides-binding lectin	L23429		2		
65	HU chloride intracellular channel protein 1	O00299		2		
66	Probable dolichyl-phosphate-mannose-protein mannosyltransferase	O13898		1		
67	Axonemal dynein light chain	O14645		1		1
68	Nephrocystin (fragment)	O14837		2		
69	Mouse carnitine deficiency-associated (CDV-1) protein	O35594		2		
70	Tegument protein/FGARAT	O41977		1		
71	Probable dynein protein (fragment)	O43352		7	1	2
72	Hypothetical 16.9-kDa protein	O58143		1		
73	O60780 DJ206D15.3	O60780		1		1
74	Hypothetical 20.4-kDa protein	O60897		3		
75	Hypothetical 50.3-kDa protein	O69823		1		
76	hsp89- $\alpha$ - $\Delta$ -N	O75322		1		
77	Dega-like protein	O88161		1		
78	Type II peroxiredoxin 1	O88376		1		
79	Hypothetical 363.6-kDa protein	O97278		1		
80	PHNA-like protein	O9PIU4		1		
81	Yeast threonine dehydratase precursor	P00927		1		
82	TERM_BPPH2 DNA terminal protein	P03681		1		
83	HU heat shock 27-kDa protein	P04792		3		
84	Human annexin II (lipocortin II) (calpactin I heavy chain) (chromobindin 8)	Multiple		9		
85	Human calpain 1, large (catalytic) subunit	P07384		1		
86	Annexin V	P08758		8		
87	HU glutathione S-transferase P	P09211		1		
88	HU annexin 4	P09525		5		
89	Ezrin (P81) (cytovillin)	P15311		4		2
90	Human annexin VII (synexin)	P20073		5		
91	cAMP-dependent protein kinase, $\alpha$ -catalytic subunit	P27791		4		
92	Mouse murinoglobulin 1 precursor (MUG1)	P28665		1		
93	Human squamous cell carcinoma antigen 1 (SCCA-1)	P29508		1		
94	Human Serine/Threonine protein phosphatase 2A, 65-kDa regulatory subunit A, $\alpha$ -Isoform	P30153		1		2
95	SORC Human sorcin	P30626		2		
96	Human transformation-sensitive protein IEF SSP 3521	P31948		1		
97	Guanine nucleotide-binding protein G(1), $\alpha$ -2 subunit (adenylate cyclase-inhibiting G $\alpha$ -protein)	P38400		1		
98	Human caltractin	P41208		1		1
99	MYCMY RECA protein	P42466		1		
100	Kinesin-like protein FLA10 (KHP1 protein)	P46869		1		
101	Casein kinase I, $\alpha$ -Isoform	P48729		3		
102	HU oxygen-regulated protein 1 (retinitis pigmentosa RP1 protein)	P56715		1		1
103	Glucose-regulated protein/BIP precursor	Q12752		1		
104	Nascent polypeptide-associated complex $\alpha$ -subunit	Q13765		1		
105	CAYP Human calcyphosine	Q13938		5		
106	Human Nedd5 protein homolog (KIAA0158)	Q15019		1		1
107	HU sperm surface protein Sp17	Q15506		1	1	1
108	Human 110-kDa cell membrane glycoprotein	Q16186		1		
109	ORF2 5' to PD-ECGF/TP protein	Q16193		1		
110	DY13_ANTCR dynein intermediate chain 3, ciliary	Q16960		1		

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TABLE II—continued

	Description	Accession No.	2-D gel	1-D gel	Direct LC/MS/MS	Multi-dimensional LC/MS/MS
111	C07B5.4 protein	Q17777		1		
112	DYL_1CAEEL probable dynein light chain 1 cytoplasmic	Q22799		2		
113	Axonemal dynein light chain p33	Q26630		1		
114	Dynein heavy chain isotype 3A	Q27803		2		1
115	Dynein heavy chain isotype 7A	Q27811		2		
116	Q52643 ORF2	Q52643		1		
117	Cytoplasmic dynein 3 heavy chain (fragment)	Q92816		1		
118	B7 protein	Q92977		4		
119	$\beta$ -Dynein heavy chain	Q9MBF8		1		
120	DJ622L5.6 (novel protein) (fragment)	Q9NU80		2		
121	cDNA FLJ10466 FIS	Q9NVW6		1		4
122	Related to TOM1 protein	Q9P4Z1		1		
123	F22D16.14 protein	Q9SRX9		1		
124	Hypothetical 35.9-kDa protein (fragment)	Q9UF43		1		
125	Hypothetical 27.1-kDa protein (fragment)	Q9UF45		5		
126	Hypothetical 34.8-kDa protein (fragment)	Q9UFG8		5		1
127	Hypothetical 186.7-kDa protein (fragment)	Q9UG01		2		
128	Hypothetical 36.8-kDa protein (fragment)	Q9UGA2		1		
129	Hypothetical 19.3-kDa protein	Q9UGQ0		5		
130	Rhabdoid tumor deletion region protein 1	Q9UHP6		7		
131	Dynein intermediate chain DNAI1	Q9UI46		13		
132	Novel protein similar to WORM E04F6.2 (fragment)	Q9UJF8		4		
133	CDV-1 protein	Q9UNY8		2		
134	KIAA1023 protein (fragment)	Q9UPX7		1		
135	Q9VGA0 CG10091 protein	Q9VGA0		1		
136	CG9492 protein	Q9VH97		1		
137	KIAA0944 protein (fragment)	Q9Y2F3		3	1	
138	Axonemal dynein heavy chain protein DNAH	Q9Y3I6		1		
139	Hypothetical 18.0-kDa protein	Q9Y4I3		1		
140	Hypothetical 18.4-kDa protein	Q9Y4P9		2		
141	NM23-H7	Q9Y5B8		9		
142	Brain-specific protein	Q9Y6H0		1		
143	MAP kinase phosphatase-like protein MK-STYX	Q9Y6J8		1		
144	Factor C protein precursor	Q9Z4K0		1		
145	Mus musculus AC39/physophilin mRNA	U21549		2		
146	Hypothetical 35.9-kDa protein (fragment)	X03284		1		
147	<i>Homo sapiens</i> laminin-binding protein	X61156		2		
148	Nasopharyngeal epithelium-specific protein 1	Q9UL16		2	2	4
149	hspc162/bithoraxoid	AAF29126			1	1
150	Growth hormone receptor	AAF67170			1	
151	Axonemal dynein heavy chain	CAB94756			6	
152	Y17D7C.1 protein	O62407			1	
153	WUGSC:H_RG252K19.1 protein (fragment) (dynein $\beta$ -chain, ciliary by FASTA)	O95705			1	
154	Predicted integral membrane protein	O96120			1	
155	Human calgizzarin (S100C protein)	P31949			1	
156	TG737	Q13099			1	
157	HU dynein light chain 1, cytoplasmic	Q15701			1	
158	Dynein $\gamma$ -chain, flagellar outer arm	Q39575			1	
159	Dynein intermediate chain DNAI1	Q9UI45			1	
160	Putative movement protein	Q9XG72			1	
161	Unknown protein	AAH03024				1
162	Mouse GLTS	AAH05152				1
163	DPY-30-like protein	AAK00640				1
164	MHS4R2	AAK13588				1
165	Dynein-associated protein	AAK18711				1
166	Radial spoke protein 3	AAK26432				1
167	PtnD protein	AAK02916				1
168	Predicted protein	AE006351				1



TABLE II—continued

	Description	Accession No.	2-D gel	1-D gel	Direct LC/MS/MS	Multi-dimensional LC/MS/MS
169	Mus Musculus male testis cDNA	AK017056				1
170	KIAA1770 protein	BAB21861				1
171	Dynein light chain, Hp28	CAC36087				1
172	Novel protein	CAC37303				1
173	KIAA0357 protein	O15064				1
174	Hypothetical protein	O17121				1
175	ARCFU putative translation initiation factor	O28214				1
176	Pyruvate dehydrogenase	O43106				1
177	PP32R1.GPM	O43423				1
178	T27E7.6 protein	O45859				1
179	Hypothetical 99.5-kDa protein	O67796				1
180	KIAA0643 protein	O75138				1
181	Sperm flagellar protein, SPAG6	O75602				1
182	Hypothetical protein 56.0 kDa	O84054				1
183	Growth arrest protein	O95995				1
184	Pyruvate dehydrogenase	P10515				1
185	Stomatin	P27105				2
186	PP11_DROME Serine/Threonine protein phosphatase	P48461				1
187	FLGK_BUCAI flagellar hook-associated protein	P57428				1
188	Phenylalanyl-tRNA synthetase $\alpha$ -chain	P94282				1
189	Dynein light chain	Q15763				1
190	Hypothetical protein 11.7 kDa	Q51572				1
191	Hypothetical protein 133.1 kDa	Q94185				1
192	Kinesin-like calmodulin-binding protein	Q9FQL7				1
193	Hypothetical 55.2-kDa protein	Q9H0C0				1
194	Hypothetical protein 76.7 kDa	Q9H0K5				5
195	Dynein	Q9H179				5
196	Novel protein	Q9H1V9				1
197	Novel protein	Q9H1X1				5
198	FLJ22843 FIS	Q9H5X5				2
199	FLJ22601 FIS	Q9H653				1
200	cDNA FLJ14117	Q9H7X7				1
201	cDNA FLJ13946	Q9H849				1
202	KIAA1640 protein	Q9HCD2				2
203	KIAA1603 protein	Q9HCG9				2
204	Similar to radial spokehead protein	Q9NQ10				2
205	C11ORF16 protein	Q9NQ32				1
206	Hypothetical protein 34.3 kDa	Q9NQC8				2
207	Hypothetical protein 47.7 kDa	Q9NTC0				1
208	Novel Serine/Threonine protein kinase	Q9NUH7				1
209	Dynein heavy chain	Q9NYC9				6
210	Hypothetical protein 77.2 kDa	Q9P1V8				1
211	KIAA1374 protein	Q9P2H3				1
212	CG6892 protein	Q9VC38				1
213	Hypothetical protein 66.0 kDa	Q9Y4Q1				1
214	cDNA FLJ10466	QPNVW6				1

matches were to predicted or hypothetical proteins and probably represent novel ciliary components. Many peptides that only matched ESTs were also identified (Supplemental Material contains a list of all ESTs identified in this study), and these may also represent novel axonemal components.

Although the use of 1- and 2-D gel separations coupled with LC/MS/MS yielded significant information about the proteins that make up the ciliary axoneme, both techniques have limitations. As noted above, standard 2-D gel procedures do not resolve all proteins, and both the 1-D and 2-D approach are labor-intensive. As an alternative approach to identifying

all the proteins in the ciliary axonemes, total axonemal preparations were digested, and the resulting peptides were then analyzed directly, eliminating the gel electrophoresis step. In one experiment, axonemal proteins were digested with Lys-C, and peptides were separated by reverse phase LC and sequenced by MS/MS. Although this experiment produced a limited amount of sequence data (60 peptides), the results confirmed the presence of several of the proteins found in the analysis of the 1- and 2-D gels (Table II). Twelve peptides matched entries in the database not identified in the 1- and 2-D experiments, including a single peptide that matched

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TABLE III  
Proteins identified by MS/MS that are "known" or "likely" axonemal components

Description	Accession No.	2-D gel	1-D gel	Direct LC/MS/MS	Multi-dimensional LC/MS/MS
1 $\alpha$ -Tubulin	Multiple	X	X	X	X
2 $\beta$ -Tubulin	Multiple	X	X	X	X
3 70-kDa heat shock protein(s)	Multiple	X	X	X	X
4 Heat shock protein(s) J2	Multiple	X	X	X	X
5 Actin	Multiple	X	X	X	X
6 Stratagene fetal retinal	AA176890	X			
7 AKAP-associated sperm protein	AAG59587	X			X
8 Testicular microtubules-related protein Tektin 3	AAK15340	X			
9 Mus Musculus male testis cDNA	AK005609	X			
10 Mus Musculus male testis cDNA	AK005739	X			
11 Mus Musculus male testis cDNA	AK015697	X			
12 H-Tektin-T	Multiple	X	X		
13 Human nucleoside diphosphate kinase homolog	P56597	X	X	X	X
14 c-Myc-binding protein	Q99417	X	X		
15 Annexin 1	Multiple		X	X	X
16 1CMI_A protein inhibitor of neuronal nitric oxide PDB	1CMIA		X		
17 HU ciliary dynein heavy chain 9	AAF69004		X		
18 IC1 Homolog	AAG28497		X		X
19 CAB71195 DJ111C20.1 (similar to chlamydomonas radial spoke protein)	CAB71195		X		
20 DJ412I7.1 (similar to radial spokehead protein) (fragment)	CAC00771		X		
21 Intermediate dynein chain	CAC17464		X		
22 Axonemal dynein light chain	O14645		X		X
23 Probable dynein protein (fragment)	O43352		X	X	X
24 Hsp89- $\alpha$ - $\Delta$ -N	O75322		X		
25 Type II peroxiredoxin 1	O88376		X		
26 HU heat shock 27-kDa protein	P04792		X		
27 cAMP-dependent protein kinase, $\alpha$ -catalytic subunit	P27791		X		
28 Human Serine/Threonine protein phosphatase 2A, 65-kDa regulatory subunit A, $\alpha$ -isoform	P30153		X		X
29 Human transformation-sensitive protein IEF SSP 3521	P31948		X		
30 Guanine nucleotide-binding protein G(1), $\alpha$ -2 subunit (adenylate cyclase-inhibiting G $\alpha$ -protein)	P38400		X		
31 Kinesin-like protein FLA10 (KHP1 protein)	P46869		X		
32 Casein kinase I, $\alpha$ -isoform	P48729		X		
33 HU oxygen-regulated protein 1 (retinitis pigmentosa RP1 protein)	P56715		X		X
34 HU sperm surface protein Sp17	Q15506		X	X	X
35 DY13_ANTCR dynein intermediate chain 3, ciliary	Q16960		X		
36 DYL_1CAEEL probable dynein light chain 1 cytoplasmic	Q22799		X		
37 Axonemal dynein light chain p33	Q26630		X		
38 Dynein heavy chain isotype 3A	Q27803		X		X
39 Dynein heavy chain isotype 7A	Q27811		X		
40 Cytoplasmic dynein 3 heavy chain (fragment)	Q92816		X		
41 $\beta$ -Dynein heavy chain	Q9MBF8		X		
42 Dynein intermediate chain DNAI1	Q9UI46		X		
43 KIAA0944 protein (fragment)	Q9Y2F3		X	X	
44 Axonemal dynein heavy chain protein DNAH	Q9Y3I6		X		
45 Brain specific protein	Q9Y6H0		X		
46 Hspc162/bithoraxoid	AAF29126			X	X
47 Axonemal dynein heavy chain	CAB94756			X	
48 WUGSC:H_RG252K19.1 protein (fragment) (dynein $\beta$ -chain, ciliary by FASTA)	O95705			X	
49 Human calgizzarin (S100C protein)	P31949			X	
50 TG737	Q13099			X	
51 HU dynein light chain 1, cytoplasmic	Q15701			X	
52 Dynein $\gamma$ -chain, flagellar outer arm	Q39575			X	
53 Dynein intermediate chain DNAI1	Q9UI45			X	
54 Dynein-associated protein	AAK18711				X
55 Radial spoke protein 3	AAK26432				X

TABLE III—continued

Description	Accession No.	2-D gel	1-D gel	Direct LC/MS/MS	Multi-dimensional LC/MS/MS
56 Mus Musculus male testis cDNA	AK017056				X
57 Dynein light chain Hp28	CAC36087				X
58 KIAA0357 protein	O15064				X
59 Sperm flagellar protein SPAG6	O75602				X
60 PP11_DROME Serine/Threonine protein phosphatase	P48461				X
61 FLGK_BUCAI flagellar hook-associated protein	P57428				X
62 Dynein light chain	Q15763				X
63 Dynein	Q9H179				X
64 Similar to radial spokehead protein	Q9NQ10				X
65 Dynein heavy chain	Q9NYC9				X

Tg737, a polycystic kidney disease gene known to be present in cilia (21, 22). Several ESTs were also identified by this approach (included in Supplemental Material).

To increase the amount of sequence information obtained from a single sample, axonemal proteins were again digested with Lys-C but were subjected to an additional LC separation step before analysis by MS/MS. Peptide fragments were applied to a strong cation exchange resin and sequentially eluted with a series of increasing salt concentrations. The eluted peptides were further resolved on an in-line reverse phase column and then sequenced by nano-electrospray MS/MS. This approach yielded sequence data on an additional 250 peptides. Matches were obtained to 84 separate entries in the databases, 30 of which were also found in the earlier studies, thereby confirming these results (Table II). Of the remaining 54 entries, 12 were added to the known/likely axonemal protein list (Table III), whereas six were considered likely contaminants. As in the above studies, the majority of the remaining 36 matches are to novel or hypothetical proteins, for which there is at present no evidence to determine whether they are axonemal proteins.

Combining the data from the four experiments described above results in peptide matches to over 210 proteins (Table II). As expected, matches were obtained to many known components of the axoneme, including  $\alpha$ - and  $\beta$ -tubulin, the light, intermediate, and heavy chains of axonemal dynein, and the radial spoke proteins (Table III). Others are most likely cellular contaminants that co-purify along with the axonemes, including ribosomal and nuclear proteins. Of greatest interest are the many proteins that have not been described previously as being part of the ciliary apparatus. To assess whether the above experiments had identified potentially novel components of human cilia, strategies were explored to test for the expression of selected proteins identified by MS/MS in cilia or ciliated cells.

In the analysis of ciliary proteins after 1-D gel electrophoresis, many peptides were obtained that matched the calcium-dependent phospholipid-binding proteins known as annexins. This is not surprising, because annexins can be precipitated by the concentrations of calcium used during the cilia isolation procedure (23), and this preparation of cilia was not

washed extensively to remove ciliary membranes. To test whether annexin was present in the cilia preparations used for MS analysis, isolated axonemes were separated on an SDS-PAGE gel and transferred to nitrocellulose for Western blotting. Probing the membrane with a monoclonal antibody to annexin I resulted in a strong signal of the expected size, clearly demonstrating that annexin I was present in preparations of isolated cilia (Fig. 4A). Next, cultured HBE cells were immunostained with the anti-annexin I antibody to examine the cellular location of the protein. Cilia were clearly labeled by the anti-annexin 1 antibody (Fig. 4B), whereas cultures labeled with control mouse IgG showed little background staining. Interestingly, the nuclei of ciliated cells also stained positively for annexin I. A similar localization of annexin I has been previously reported in the rabbit trachea (23).

Peptides that matched the oxygen-regulated protein, retinitis pigmentosa protein RP1 (24, 25), were also found in the proteomic screen of ciliary axonemes. Retinal photoreceptor cells contain a modified cilium, and an association between retinitis pigmentosa, an inherited disease resulting in progressive blindness, and PCD, an inherited disease of abnormal ciliary structure, has been reported (e.g. (26)). Therefore, the expression of RP1 was examined in cultures of HBE cells. No antibodies were available to RP1; therefore, RT-PCR was used to analyze the expression of the RP1 mRNA. Total RNA isolated from cultured HBE cells at different stages of differentiation was amplified using primers designed to be specific for RP1. At early times, when ciliated cells have not yet developed, a weak signal for RP1 was detectable (Fig. 5). At days 14 and beyond, when newly differentiated ciliated cells became visible in these cultures, a much stronger signal for RP1 was produced. This pattern of expression has been observed for other axonemal proteins, including DHCs (10, 11, 27). Control amplifications in the absence of reverse transcriptase produced no product, whereas amplification with control primers demonstrated the integrity of the RNA samples. These results demonstrate that RP1 is expressed by HBE cells and that an increase in RP1 expression may be correlated with ciliogenesis in airway epithelial cells.

Several of the peptides sequenced matched proteins identified previously as sperm- or testis-specific. Because sperm

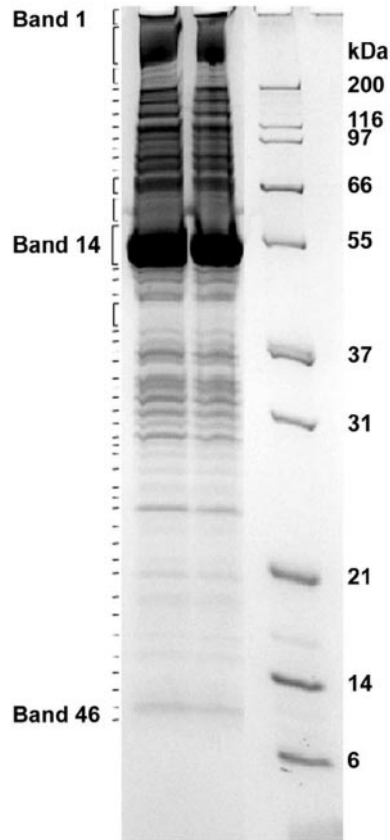


FIG. 3. **1-D gel electrophoresis of human ciliary axonemes.** Axonemal proteins were separated by SDS-PAGE, and 46 individual bands, indicated on the left, were excised, pooled, digested with trypsin, and analyzed by LC/MS/MS.

flagella contain the same highly conserved 9 + 2 axonemal structure found in cilia, it is likely that proteins identified as sperm-/testis-specific may also be components of airway cilia. Sp17 is a well characterized sperm protein that is present in the principal piece and midpiece of the sperm flagella and may also function in binding sperm to the extracellular matrix of the oocyte (28, 29). Western blotting of cell lysates from differentiated (ciliated) cultures with an affinity-purified rabbit anti-Sp17 antisera demonstrated the presence of an ~26-kDa protein that migrated with the same mobility as purified recombinant human Sp17 (Fig. 6A, lanes 2 and 4) whereas no signal in this region was detected in lysates from undifferentiated cultures (Fig. 6A, lane 1). To determine whether Sp17 was localized to the cilia of airway epithelial cells, isolated ciliary axonemes were probed by Western blotting (Fig. 6A, lane 3). A strong signal of the expected size was observed, as well as many higher molecular weight bands. These may be the result of Sp17 aggregation (recombinant human Sp17 is known to aggregate),<sup>2</sup> or Sp17 may be bound tightly to other axonemal components. Finally, differentiated cultures of HBE cells were immunostained with the affinity-

<sup>2</sup> M. O’Rand, personal communications.

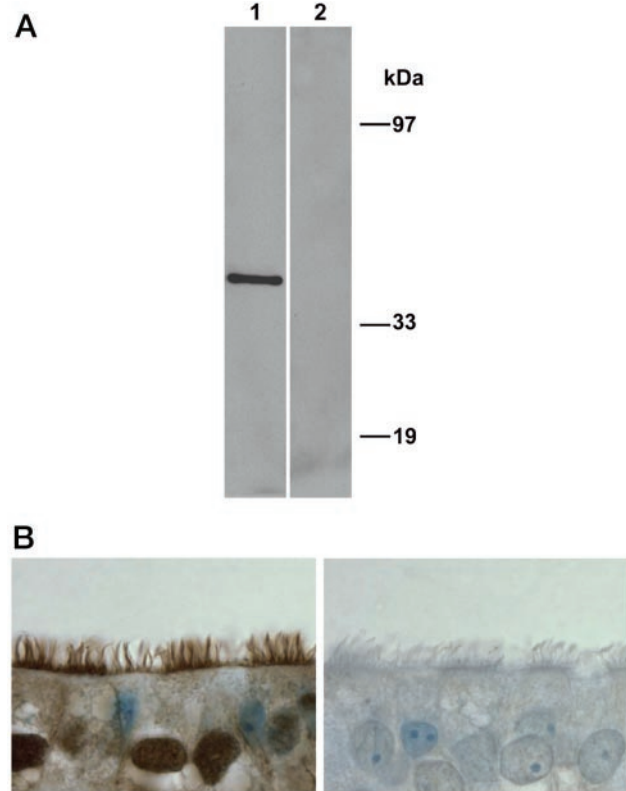


FIG. 4. **Expression of annexin I in human bronchial epithelial cells.** A, Western blot of axonemal proteins probed with a monoclonal antibody to annexin I (lane 1) or a control mouse IgG (lane 2). A single band of the expected size is present in the cilia preparation. B, cultured HBE cells were immunostained with anti-annexin I (left panel) or a control IgG (right panel). The cilia and nuclei are stained clearly with the anti-annexin I antibody.

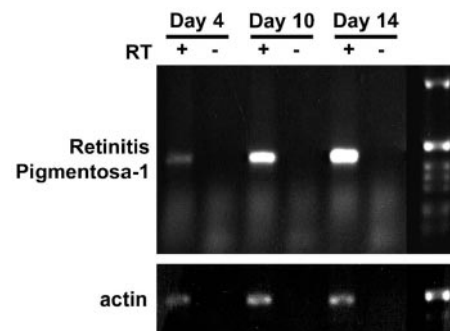
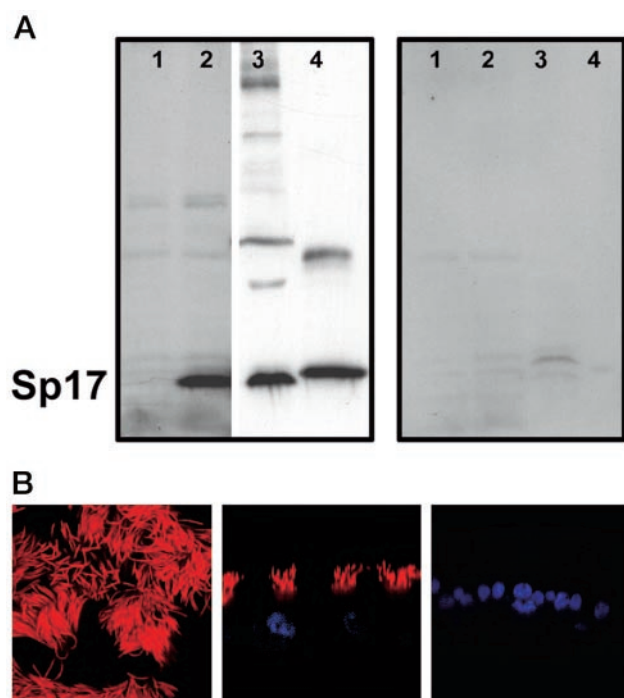


FIG. 5. **Expression of retinitis pigmentosa protein RP1 in human bronchial epithelial cells.** Total RNA was isolated from cultures of HBE cells on days 4, 10, and 14 of culture. RT-PCR was performed using primers specific for RP1 (top panel) or  $\gamma$ -actin (lower panel) with (+) or without (-) the addition of reverse transcriptase. Amplification with the RB-1-specific primers generated a clear product of the expected size, demonstrating the expression of RP1 in HBE cells. The signal appeared to increase at later time points, when more ciliated cells are present.

purified Sp17 antisera. Although the control pre-adsorbed serum showed no specific staining (Fig. 6B, right panel), the Sp17 antisera strongly labeled the cilia (Fig. 6B, left and



**FIG. 6. Expression of Sp17 in human bronchial epithelial cells.** *A*, total cell lysates from undifferentiated (*lane 1*) or differentiated (*lane 2*) cultures of HBE cells, isolated ciliary axonemes (*lane 3*), or recombinant human Sp17 (*lane 4*) were probed with affinity-purified anti-Sp17 antisera (*left panel*) or with pre-absorbed control antisera (*right panel*). A signal of the expected size (~26 kDa) was detected in the cell lysate from the differentiated (ciliated) cultures but not in the lysate from the undifferentiated cultures. A 26-kDa signal was also detected in the ciliary axonemes, along with a number of higher molecular weight bands. The pre-absorbed control antisera showed no specific staining. *B*, cultures of well ciliated HBE cells were stained with affinity-purified anti-Sp17 antisera (*left and center panels*) or the antisera depleted of Sp17-reacting antibodies (*right panel*), and immunoreactivity was visualized by confocal microscopy. The anti-Sp17 antisera reacts clearly with cilia in the *left* (xz section) and *center* (xy section) panels, whereas the control antisera shows no staining. Texas Red-labeled secondary antibody, nuclei stained with DAPI.

*center panels*). These results clearly demonstrate that Sp17 is a component of human airway cilia, validating the result obtained by mass spectrometry.

#### DISCUSSION

Although the axonemes of cilia and flagella have been the subject of many detailed studies using model animals such as *Chlamydomonas* and *Paramecium*, only recently have detailed studies of human material become feasible. In this study, we have begun to assemble a complete proteome of the ciliary axoneme from human airway cells. Identifying the entire complement of ciliary components will be required for a complete understanding of the mechanics and regulation of ciliary beat. A complete proteome would also allow for rapid identification of proteins that are modified in response to treatments that alter ciliary function or in diseases, such as

primary ciliary dyskinesia, in which defects in ciliary structure are commonly observed.

It is clear from the studies reported here and elsewhere that the technique of 2-D gel electrophoresis followed by mass spectrometry to identify individual protein spots is useful but suffers from several limitations. A clear example from this study is the inability of 2-D gels to resolve the high molecular weight dynein heavy chains that are essential components of the ciliary apparatus. Although 1-D gels avoid some of the problems associated with 2-D gels, this approach is also very labor-intensive. In addition, most gel slices contained multiple proteins, and proteins of low abundance may be difficult to identify if they migrate similarly to a more abundant protein, such as tubulin. In contrast, digesting a sample of isolated axonemes in solution eliminates many of the variables and labor involved in gel electrophoresis. Further, the use of multidimensional liquid chromatography to resolve and concentrate the resulting peptide fragments allows for the efficient identification of the proteins present in the original mixture. However, each of the four approaches utilized in the current study identified peptides not identified by any of the other techniques. Similarly, very few of the proteins were identified by more than one approach. This is not surprising, because previous studies demonstrated that repeated analyses of the same complex protein sample by LC/MS/MS resulted in different peptides being sequenced in each analysis (15). Some of the differences in the present study are also no doubt because of sample variation. However, it seems unlikely that a single approach will be able to resolve all the proteins present in a complex sample such as an axoneme. Therefore although it is useful to compare the results obtained by the different techniques, they can also be viewed as complementary approaches that can be combined to achieve better sample coverage.

The combination of these different approaches has resulted in significant progress toward obtaining a complete proteome of human ciliary axonemes. The only available estimate of the total number of proteins in an axoneme is from studies of *Chlamydomonas*. By analyzing isolated flagella using 2-D PAGE, Luck and co-workers (5, 6) estimated that an axoneme consists of  $\geq 250$  proteins. This number may represent a minimum value, because it is clear that 2-D PAGE does not resolve all proteins (e.g. DHCs), and multiple proteins may be present in a single spot. Alternatively, the total number of individual axonemal proteins may be  $< 250$ , because the same protein may exist in multiple forms, and some of the proteins are cellular constituents that co-purify with the axoneme.

As a first step toward obtaining a human axonemal proteome, we have developed a 2-D map of isolated cilia that consists currently of over 240 well resolved components. This number is similar to the estimate of 250 proteins in a *Chlamydomonas* flagellum. Although this map does not include all axonemal components (see above), this map will be useful for

the targeted identification of specific axonemal components, for example, proteins absent from the cilia of PCD patients. In-gel digestion of individual spots followed by LC/MS/MS allowed us to identify 38 proteins present throughout this map, which will provide useful landmarks for future experiments.

Second, by taking advantage of additional proteomic approaches, peptide sequences to other potential axonemal proteins were obtained. Separation of axonemal proteins on a 1-D gradient gel allowed us to identify proteins not resolved on the 2-D gels, including DHCs. By digesting axonemes directly in solution, we were able to omit the time-consuming gel electrophoresis and analysis steps. Analyzing these samples, by LC/MS/MS or LC/LC/MS/MS, generated additional data sets that contained proteins both unique and common to each of the other approaches. In total, peptide sequences were obtained to over 210 potential axonemal proteins. If an axoneme is assumed to be comprised of ~250 proteins, this data set could represent ~85% of the axonemal proteins. In addition, we obtained peptide matches to over 200 human ESTs. Although many of these may be redundant, it is likely that some of them represent additional novel axonemal proteins. Further characterization of these ESTs will therefore increase the percentage of axonemal proteins identified.

Sixty-five of the 214 matches (~30%) have been assigned as known or likely axonemal components based on literature or database annotations that are consistent with the protein having a potential axonemal location (Table III). These results confirm the presence of many proteins that are expected to be components of cilia, including  $\alpha$ - and  $\beta$ -tubulin, radial spoke proteins, and light, intermediate, and heavy chains of dynein. For example, many peptides were obtained that matched the sequence of human dynein heavy chain 9. This is not surprising, because this outer arm DHC has been completely sequenced from human. In contrast, two peptides were obtained that perfectly matched the sequence for sea urchin DHC isotype 3a, although showing significant homology to several DHCs from other species. Because there are at least 12 highly homologous axonemal DHCs, some with multiple isoforms, additional studies will be needed to identify the full-complement of human axonemal DHCs present in cilia. These data also demonstrate that when analyzing highly conserved families of proteins, which may have different splice variants, a single peptide sequence may not provide unambiguous identification.

Many proteins were identified that were not known previously to be components of airway cilia. For some of these, the available information about these proteins suggests that they are axonemal proteins. For example, several sperm- or testis-specific proteins were identified, including an AKAP-associated sperm protein (30), SPAG 6, and Sp17. SPAG6 demonstrates 85% similarity to the *Chlamydomonas* protein pf16, which localizes to the flagellar central pair microtubules (31), whereas Sp17 has been localized to both the acrosomal

region and throughout the sperm tail. Proteins from other tissues that contain axonemal structures were also identified, including the retinitis pigmentosa protein, RP1, and a brain-specific protein. These data demonstrate the remarkable conservation of axonemal structure, even between diverse cellular tissues. In the future, a proteomic comparison of different axonemal structures will be useful to not only identify conserved proteins but to identify the unique proteins that are responsible for the specialized function of each particular axoneme.

The remaining proteins, which constitute the majority of proteins identified, are proteins for which there is insufficient information to determine whether they are axonemal components or cellular contaminants. Although the isolation procedure used results in a highly enriched preparation of ciliary axonemes, it is clear that other cellular structures were pelleted along with the axonemes during centrifugation. Therefore, it is not surprising that peptides were obtained from proteins known to be components of the nucleus and ribosome. However, the number of proteins identified in these studies known to be non-axonemal is low (~10%), suggesting that the majority of the proteins identified may be axonemal components.

Finally, to test whether that these experiments had in fact resulted in the identification of novel axonemal components, we explored several methods to test whether the proteins identified in the proteomic screen are associated with cilia or ciliated cells. In the case of previously characterized proteins for which antibodies are available (e.g. annexin 1, Sp17), Western blotting of isolated axonemes was used to demonstrate the presence of these proteins in the ciliary preparation. Immunostaining was also used to demonstrate that annexin 1 and Sp17 localized to the cilia in differentiated cultures of HBE cells. For unknown proteins or proteins for which antibodies are not available, RT-PCR can be used to determine whether the mRNA for the protein is expressed in ciliated cultures and also if the expression of the mRNA correlates with ciliogenesis. For example, the expression of retinitis pigmentosa protein RP1 mRNA was demonstrated clearly in ciliated HBE cells, and the level of RP1 expression appeared to correlate with ciliated cell differentiation. Thus selected proteins, identified as being components of ciliary axonemes by proteomic techniques, were confirmed by biochemical studies. Although each of the potentially novel axonemal components identified in this study will require similar verification, these data provide approaches to validate the results of the proteomic analysis.

In summary, we have used proteomic techniques to identify many potentially novel components of human cilia. Future studies will focus on optimization of these techniques to identify additional axonemal proteins. In addition, similar strategies will be applied to analyze both the ciliary membrane and the soluble matrix components. The results of these studies will lead to a greater understanding of cilia structure and regulation and will form a basis for investigating changes in

the ciliary proteome in response to experimental variables. A complete ciliary proteome will also be invaluable for comparing cilia from normal and diseased specimens and for identifying proteins that are conserved/unique between axonemes from different tissues, e.g. airway cilia and sperm flagella.

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