A Proteomic Analysis of Human Cilia

IDENTIFICATION OF NOVEL COMPONENTS*§

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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 ≥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.

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),† in which genetic defects cause dysfunctional cilia and result in chronic otitis media, sinusitus, 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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* 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 axonal 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 axoneme 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, 5–10 × 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 × g, the ciliary axonemes were collected by centrifugation at 16,000 × g and frozen at −80 °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 Heps (pH 7.3), 1 mM EGTA, 0.1 mM EDTA, 25 mM NaCl, 5 mM 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 Immobiline DryStrips (pH 3–10; Amersham Biosciences) on an IPGphor (Amersham Biosciences) according to the manufacturer's instructions. Axonemal pellets (40–70 μg) 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 μg of axonemes were loaded, and the gel was stained with Pro-Blue colloidal Coomassie stain (Owl Separation Systems, Portsmouth, 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 μg/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-μg 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/MS/MS, a 30-μg aliquot was initially solubilized using 5 μl of hexafluoroacetone and enough 6 M urea to solubilize the pellet completely. The sample was diluted to 2 μl 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–μm inner diameter × 5-mm strong cation exchange column (LC Packings, San Francisco, CA) packed with polyacrylamide material (PolyLC, Columbia, MD) onto a reversed-phase trapping cartridge using a 5 mM KH_2PO_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/MS/MS/MS datafiles from each salt step were processed initially using the Proteinlynx module of Masslynx 3.4 (Micromass) to generate Mascot-searchable *.psl_ 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-
formed as described previously (10) with the following modifications. Mouse monoclonal anti-annexin 1 (Biogenesis, Brentwood, NH) was used at 0.05 μg/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 μg/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′-TGAAGGCCGCTTATTG-GCTCT-3′ (forward) and 5′-AAGGGGTAGTCAGTTCCCTCG-3′ (reverse). Cycling conditions for amplification included an initial denaturation at 95 °C for 105 s, 4 cycles of 95 °C for 15 s; 60 °C for 30 s, 31 cycles of 95 °C for 15 s; 58 °C for 30 s, followed by a 1-min extension at 72 °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.

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 sequenced 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...
| Spot | Description | Accession No. | No. of peptides |
|------|-------------|---------------|----------------|
| 1    | cDNA FLJ23305 FIS | Q9H5M3 | 8 |
| 2    | hb08b03 × 1 cDNA Clone | AW473770 | 1 |
| 3    | P97 ATPase-related sequence | CAB41525 | 12 |
| 4    | KIAA0356 Protein | Q9Y4G2 | 1 |
| 5    | TER ATPase | P46462 | 1 |
| 6    | Heat shock protein | HS70 | 3 |
| 7    | Heat shock cognate 70-kDa protein | 3HSC | 2 |
| 8    | 602350570F1 cDNA Clone | BG122387 | 1 |
| 9    | Stratagene fetal retinal | AA176890 | 1 |
| 10   | Heat shock protein 70 | P06107 | 4 |
| 11   | HS7C Mouse heat shock cognate 71-kDa | P06109 | 4 |
| 12   | TER human endoplasmic reticulum ATPase | P55072 | 1 |
| 13   | Testicular microtubules-related protein Tektin 3 | AAK15340 | 5 |
| 14   | Unnamed protein product | BAA92077 | 5 |
| 15   | α-Tubulin | Q06455 | 6 |
| 16   | β-Tubulin | Q06455 | 6 |
| 17   | NEB protein | AF022650 | 1 |
| 18   | β-Tubulin | A085030 | 1 |
| 19   | Canine Tektin | Q0TTW5 | 8 |
| 20   | Unnamed protein product | BABB71402 | 3 |
| 21   | β-Tubulin | Q06455 | 5 |
| 22   | Reticulocyte-binding protein | Q00798 | 1 |
| 23   | KIAA0356 Protein | Q9Y4G2 | 1 |
| 24   | SPAM | Q54064 | 1 |
| 25   | α-Tubulin | Q9DT4 | 1 |
| 26   | Similar to Tektin-3 | AAH21716 | 1 |
| 27   | Unnamed protein product | BABB71402 | 1 |
| 28   | α-Tubulin | Q06455 | 5 |
| 29   | Similar to Tektin-3 | AAH21716 | 2 |
| 30   | Similar to Tektin-3 | AAH21716 | 2 |
| 31   | β-Tubulin | Q06455 | 5 |
| 32   | H-Tektin-T | Q06455 | 5 |
| 33   | H-Tektin-T | Q06455 | 5 |
| 34   | Similar to Tektin-3 | AAH21716 | 1 |
| 35   | H-Tektin-T | Q06455 | 5 |
| 36   | CGI-53 Protein | AF151811 | 3 |
| 37   | β-Actin | Q06455 | 5 |
| 38   | β-Actin | Q06455 | 5 |
| 39   | CGI-53 protein | AF151811 | 3 |
| 40   | β-Tubulin | Q06455 | 5 |
| 41   | H-Tektin-T | Q06455 | 5 |
| 42   | Similar to Tektin-3 | AAH21716 | 1 |
| 43   | β-Tubulin | Q06455 | 5 |
| 44   | β-Tubulin | Q06455 | 5 |
| 45   | Similar to Tektin-3 | AAH21716 | 1 |
| 46   | α-Tubulin | Q06455 | 5 |
| 47   | β-Tubulin | Q06455 | 5 |
| 48   | α-Tubulin | Q06455 | 5 |
| 49   | Novel protein isoform 1 | Q9H1X0 | 2 |
| 50   | Similar to Tektin-3 | AAH21716 | 1 |
| 51   | Heat shock protein J2 | Q5190 | 7 |
| 52   | β-Tubulin | Q06455 | 5 |
| 53   | AKAP-associated sperm protein | AGS59587 | 1 |
| 54   | HU EST cDNA | BG323215 | 1 |
| 55   | HU EST cDNA | BF65422 | 2 |
| 56   | H-Tektin-T | Q06455 | 5 |
| 57   | Human nucleoside diphosphate kinase homolog | P56597 | 5 |
| 58   | EIF-3-β | Q13347 | 1 |
| 59   | Similar to myeloid leukemia factor | AAH03975 | 2 |
| 60   | Heat shock protein J2 | AAH02446 | 5 |
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 α- and β-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 determine 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 (≥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 I-continued

| Spot | Description | Accession No. | No. of peptides |
|------|-------------|---------------|----------------|
| 61   | D8 β-Tubulin | P09206        | 3              |
| 62   | D9 β-Tubulin | P09206        | 4              |
| 63   | D10 β-Tubulin | P09206 | 4              |
| 64   | D10 14-3-3 Protein ε | 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 | Q9VYNY1 | 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 β-Tubulin | Q8JJY6 | 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 | AAQ95897 | 2              |
| 82   | F12 α-Tubulin | Q06331 | 2              |
| 83   | F12 Phenylalanyl-tRNA synthetase β-chain | Q9ZDB4 | 1              |
| Description | Accession No. | 2-D gel | 1-D gel | Direct LC/MS/MS | Multi-dimensional LC/MS/MS |
|-------------|--------------|---------|---------|----------------|---------------------------|
| α-Tubulin   | Multiple     | X       | X       | X              | X                         |
| β-Tubulin   | Multiple     | X       | X       | X              | X                         |
| 70-kDa Heat shock protein(s) | Multiple | X | X | X |
| Heat shock protein(s) J2 | Multiple | X | X | X |
| Actin       | Multiple     | X       | X       | X              | X                         |
| 14–3-3 Proteins | Multiple | X | X | X |
| Stratagene fetal retinal | AA176890 | 1 |
| Fusion gene | AAA99997    | 3       | 2       | 1              |                           |
| AKAP-associated sperm protein | AAG59587 | 2 |
| Putative GTP-binding protein | AAH00566 | 3 |
| Similar to myeloid leukemia factor | AAH03975 | 2 |
| Unknown protein | AAH04522 | 4 |
| Testicular microtubules-related protein Tektin 3 | AAK15340 | 5 |
| Kinesin-like protein 38B (NEB protein) | AF022650 | 1 |
| CGI-53 Protein | AF151811 | 5 |
| Mus Musculus male testis cDNA | AK005609 | 1 |
| Mus Musculus male testis cDNA | AK005793 | 3 |
| Mus Musculus male testis cDNA | AK015697 | 1 |
| hb08b031 ×1 cDNA clone | AW473770 | 1 |
| Unnamed protein product | BAB71402 | 4 |
| Similar to Riken cDNA 4933428D01 | BC007374 | 2 |
| P97 ATPase-related sequence | CAB41525 | 12 |
| H-Tektin-T | Multiple     | X       | X       |                |                           |
| KIAA0477 Protein | O75065 | 1 |
| LEPS BACSU signal peptidase | P28628 | 1 |
| Tera human endoplasmic reticulum ATPase | P55072 | 1 |
| Human nucleoside diphosphate kinase homolog (testis-specific) | P56597 | 5 |
| Reticulocyte-binding protein | Q00798 | 1 |
| EIF-3-β | Q13347 | 1 |
| SPAM | Q54084 | 1 |
| c-Myc-binding protein | Q99417 | 4 |
| Novel protein isoform 1 | Q9H1X0 | 2 |
| Unnamed protein product | BAA92077 | 5 |
| KIAA0356 Protein | Q9VYN1 | 1 |
| Phenylalanyl-tRNA synthetase β-chain | Q9ZDB4 | 1 |
| cDNA FLJ23305 FIS | Q9H5M3 | 8 |
| Predicted protein | XP_088490 | 1 |
| Annexin 1 | Multiple     | X       | X       | X              | X                         |
| 1CMI_A Protein inhibitor of neuronal nitric oxide PDB | 1CMIA | 2 |
| Galectin-7 | 3GALA | 1 |
| CLP-like protein fragment | AAF64302 | 1 |
| HU ciliary dynein heavy chain 9 | AA69004 | 32 |
| AAF97774 J4R | AA97774 | 1 |
| ICl Homolog | AAG28497 | 5 |
| Putative glutathione S-transferase OSGSTU4 | AAG32471 | 1 |
| HU DNA sequence clone RP1-261G23 | AL136131 | 6 |
| cDNA DKFZp434N2435 | AL136858 | 2 |
| cDNA FLJ20753 FIS | BAA91365 | 1 |
| cDNA FLJ10466 FIS | BAA91628 | 12 |
| KIAA1374 Protein (fragment) | BAA92612 | 2 |
| KIAA1430 Protein (fragment) | BAA92668 | 1 |
| LUNX protein | BAA93633 | 1 |
| Unnamed protein product | BAB01602 | 9 |
| Genomic DNA chromosome 5, TAC clone:K3K7 | BAB08743 | 1 |
| Putative cysteine proteinase | BAB17096 | 1 |
| Similar to part of several eg. ACDS MEGEL Q06319 | CAB09628 | 1 |

Acyl-CoA dehydrogenase
|    | 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 | CAB92087DJ34K7.2 (novel protein)                                            | CAB92087      | 3       | 1       |                |                           |
| 61 | DJ4121.1 (similar to radial spokehead protein) (fragment)                  | CAC00771      | 4       |         |                |                           |
| 62 | Hypothetical 37.1-kDa protein                                                | CAC09526      | 12      |         |                |                           |
| 63 | Intermediate dynein chain                                                   | CAC17464      | 1       |         |                |                           |
| 64 | Canis β-galactosides-binding lectin                                         | L23429        | 2       |         |                |                           |
| 65 | HU chloride intracellular channel protein 1                                 | O00299        | 2       |         |                |                           |
| 66 | Probable dolichyl-phosphate-mannose–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 DJ206015.3                                                           | O60780        | 1       | 1       |                |                           |
| 74 | Hypothetical 20.4-kDa protein                                                | O60897        | 3       |         |                |                           |
| 75 | Hypothetical 50.3-kDa protein                                                | O69823        | 1       |         |                |                           |
| 76 | hsp89-α-Δ-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_BPH2 DNA terminal protein                                               | P03681        | 1       |         |                |                           |
| 83 | HU heat shock 27-kDa protein                                                 | P04792        | 3       |         |                |                           |
| 84 | Human annexin II (lipocortin II) (calpain I heavy chain) (chromobin 8)      | P07384        | 1       |         |                |                           |
| 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, α-catalytic subunit                          | P27791        | 4       |         |                |                           |
| 92 | Mouse muringoglobin 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 | P30153        | 2       |         |                |                           |
| 95 | SORC Human sorcin                                                           | P30626        | 2       |         |                |                           |
| 96 | Human transformation-sensitive protein IEF SSP 3521                         | P31948        | 1       |         |                |                           |
| 97 | Guanine nucleotide-binding protein G(1), α-2 subunit (adenylate cyclase-inhibiting G α-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, α-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 α-subunit                            | Q13765        | 1       |         |                |                           |
| 105| CAYP Human calphosphine                                                     | Q13938        | 5       |         |                |                           |
| 106| Human Neddy 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       |         |                |                           |
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 protein 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 β-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 Q9UGG0 | 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 Q9Y413 | 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 Homo sapiens laminin-binding protein X61156 | 2 | | | | |
| 148 Nasopharyngeal epithelium-specific protein 1 Q9UL16 | 2 | 2 | 4 | | |
| 149 hsP162/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 β-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 protein Q15701 | 1 | | | | |
| 158 Dynein γ-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 GLT5 AAH05152 | 1 | | | | |
| 163 DPY-30-like protein AAK00840 | 1 | | | | |
| 164 MHS42 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 | | | | |
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

| 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 ARCUF 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 | P16515 | | | 1 | |
| 185 Stomatgin | P27105 | | | 2 | |
| 186 PP11_DROME Serine/Threonine protein phosphatase | P48461 | | | 1 | |
| 187 FLGK_BUCAI flagellar hook-associated protein | P57428 | | | 1 | |
| 188 Phenylalanine-IRNA synthetase α-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 | Q9HIV9 | | | 1 | |
| 197 Novel protein | Q9H1X1 | | | 5 | |
| 198 FLJ22843 FIS | Q9H5X5 | | | 2 | |
| 199 FLJ22801 FIS | Q9H653 | | | 1 | |
| 200 cDNA FLJ14117 | Q9H7X7 | | | 1 | |
| 201 cDNA FLJ13946 | Q9H849 | | | 1 | |
| 202 KIAA1640 protein | Q9HCD2 | | | 2 | |
| 203 KIAA1603 protein | Q9HC9G | | | 2 | |
| 204 Similar to radial spokehead protein | Q9NQ10 | | | 2 | |
| 205 C11ORF16 protein | Q9NQ32 | | | 2 | |
| 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 | Q9NYY9 | | | 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 |
### 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 α-Tubulin  | Multiple      | X       | X       | X              | X                         |
| 2 β-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 | | | |
| 13 Human nucleoside diphosphate kinase homolog | P56597 | X | X | X | X |
| 14 c-Myc-binding protein | Q99417 | X | | | |
| 15 Annexin 1 | Multiple | X | | | X |
| 16 1CMI_A protein inhibitor of neuronal nitric oxide PDB | 1CMI | X | | | |
| 17 HU ciliary dynein heavy chain 9 | AAFA69004 | X | | | X |
| 18 IC1 Homolog | AAG28497 | X | | | X |
| 19 CAB71195 DJI11C20.1 (similar to chlamydomonas radial spoke protein) | X | | | | |
| 20 DJ41217.1 (similar to radial spokehead protein) (fragment) | CAC00771 | X | | | |
| 21 Intermediate dynein chain | CAC17464 | X | | | |
| 22 Axonemal dynein light chain | Q14645 | X | | | X |
| 23 Probable dynein protein (fragment) | Q43352 | X | X | X | |
| 24 Hsp89-α-Δ-N | Q75322 | X | | | |
| 25 Type II peroxiredoxin 1 | Q83376 | X | | | |
| 26 HU heat shock 27-kDa protein | P04792 | X | | | |
| 27 cAMP-dependent protein kinase, α-catalytic subunit | P27791 | X | | | |
| 28 Human Serine/Threonine protein phosphatase 2A, 65-kDa regulatory subunit A, α-isofrom | P30153 | X | | | X |
| 29 Human transformation-sensitive protein IEF SSP 3521 | P31948 | X | | | |
| 30 Guanine nucleotide-binding protein G(1), α-2 subunit (adenylate cyclase-inhibiting G α-protein) | P38400 | X | | | |
| 31 Kinesin-like protein FLA10 (KHAP1 protein) | P46869 | X | | | |
| 32 Casein kinase I, α-isofrom | 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_1CAEL probable dynein light chain 1 cytoplasmic | Q22799 | X | | | |
| 37 Axonemal dynein light chain p33 | Q26630 | X | | | |
| 38 Dynein heavy chain isoype 3A | Q27803 | X | | | X |
| 39 Dynein heavy chain isoype 7A | Q27811 | X | | | |
| 40 Cytoplasmic dynein 3 heavy chain (fragment) | Q92816 | X | | | |
| 41 β-Dynein heavy chain | Q9MBF8 | X | | | |
| 42 Dynein intermediate chain DNA1 | Q9UJ46 | 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 β-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 γ-chain, flagellar outer arm | Q39575 | X | | | |
| 53 Dynein intermediate chain DNA1 | Q9UJ45 | X | | | |
| 54 Dynein-associated protein | AAK18711 | X | | | |
| 55 Radial spoke protein 3 | AAK26432 | 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 nanoelectrospray 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 α- and β-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...
flagella contain the same highly conserved 9 + 2 axonal 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 a ~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), or Sp17 may be bound tightly to other axonal components. Finally, differentiated cultures of HBE cells were immunostained with the affinity-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 right panels).
spotted with DAPI. shows no staining. Texas Red-labeled secondary antibody, nuclei section) and center were stained with affinity-purified anti-Sp17 antisera (left panel). The pre-absorbed control antisera 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.

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 analyzes of the same complex protein sample by LC/MS/MS resulted in different peptides being sequenced in each analyses (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 β- and δ-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 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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REFERENCES

1. Wanner, A., Salathe, M., and O’Riordan, T. G. (1996) Mucociliary clearance in the airways. Am. J. Respir. Crit. Care Med. 154, 1868–1902
2. Meeks, M., and Bush, A. (2000) Primary ciliary dyskinesia (PCD). Pediatr. Pulmonol. 29, 307–316
3. Wagner, M. K., and Yost, H. J. (2000) Left-right development: the roles of nodal cilia. Curr. Biol. 10, R149–R151
4. Praetorius, H. A., and Spring, K. R. (2001) Bending the MDCK cell primary cilium increases intracellular calcium. J. Membr. Biol. 184, 71–79
5. Piperno, G., Huang, B., and Luck, D. J. (1977) Two-dimensional analysis of flagellar proteins from wild-type and paralyzed mutants of Chlamydomonas reinhardtii. Proc. Natl. Acad. Sci. U. S. A. 74, 1600–1604
6. Luck, D. J. (1984) Genetic and biochemical dissection of the eucaryotic flagellum. J. Cell Biol. 98, 789–794
7. Gray, T. E., Guzman, K., Davis, C. W., Abdullah, L. H., and Nettesheim, P. (1996) Mucociliary differentiation of serially passaged normal human tracheobronchial epithelial cells. Am. J. Respir. Cell Mol. Biol. 14, 104–112
8. Bernacki, S. H., Nelson, A. L., Abdullah, L., Sheehan, J. K., Harris, A., Davis, C., and Randell, S. H. (1999) Mucin gene expression during differentiation of human airway epithelia in vitro. Muc4 and muc5b are strongly induced. Am. J. Respir. Cell Mol. Biol. 20, 595–604
9. Link, A. J., Eng, J., Schietz, D. M., Carmack, E., Mize, G. J., Morris, D. R., Garvik, B. M., and Yates, J. R. I. (1999) Direct analysis of protein complexes using mass spectrometry. Nat. Biotechnol. 17, 676–682
10. Zhang, Y. J., Ong, W. K., Randell, S. H., Blackburn, K., Moyer, M. B., Berson, E. L., and Dryja, T. P. (1999) Mutations in a gene encoding a novel retina-specific gene cause autosomal dominant retinitis pigmentosa. Nat. Genet. 22, 248–254
11. Reed, W., Carson, J. L., Moats-Staats, B. M., Moir, T., Hu, P., White, L., Gamblin, T. M., Huang, C. H., Leigh, M. W., and Collier, A. M. (2000) Characterization of an axonemal dynein heavy chain expressed early in airway epithelial ciliogenesis. J. Cell Sci. 113, 734–741
12. Hastie, A. T., Dicker, D. T., Francis, S. K., Kueppers, F., Higgins, M. L., and Weinbaum, G. (1986) Isolation of cilia from porcine tracheal epithelium and extraction of dynein arms. Cell Motil. Cytoskeleton 6, 25–34
13. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chem. 68, 850–858
14. Burkhardt, W. (1993) in Techniques in Protein Chemistry IV (Angelettii, R., ed) pp. 399–406, Academic Press, New York
15. Koc, E. C., Burkhardt, W., Blackburn, K., Moyer, M. B., Schlatter, D. M., Moseley, A., and Spremulli, L. L. (2001) The large subunit of the mammalian mitochondrial ribosome. Analysis of the complement of ribosomal proteins present. J. Biol. Chem. 276, 43958–43969
16. Perkins, D. N., Pappin, D. J., Creasy, D. M., and Cottrell, J. S. (1999) Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 20, 3551–3567
17. Chen, R., Perrone, C. A., Amos, L. A., and Linck, R. W. (1993) Tektin B1 from ciliary microtubules: primary structure as deduced from the cDNA sequence and comparison with tektin A1. J. Cell Sci. 106, 909–918
18. Williams, N. E., and Nelsen, E. M. (1997) HSP70 and HSP90 homologs are associated with tubulin in hetero-oligomeric complexes, cilia and the cortex of Tetrahymena. J. Cell Sci. 110, 1665–1672
19. Stephens, R. E., and Lemieux, N. A. (1999) Molecular chaperones in cilia and flagella: implications for protein turnover. Cell Motil. Cytoskeleton 44, 274–283
20. Yangasawa, H. A., and Kamiya, R. (2001) Association between actin and light chains in Chlamydomonas flagellar inner-arm dynein. Biochem. Biophys. Res. Commun. 288, 443–447
21. Pazour, G. J., Dickert, B. L., Vucica, Y., Seeley, E. S., Rosenbaum, J. L., Witman, G. B., and Cole, D. G. (2000) Chlamydomonas IFT88 and its mouse homologue, polycystic kidney disease gene 7q37/37, are required for assembly of cilia and flagella. J. Cell Biol. 151, 709–718
22. Haycraft, C. J., Swoboda, P., Taulman, P. D., Thomas, J. H., and Yoder, B. K. (2001) The C elegans homolog of the murine cystic kidney disease gene 7q37 functions in a ciliogenic pathway and is disrupted in otsm-5 mutant worms. Development 128, 1493–1505
23. Mayran, N., Traverso, V., Maroux, S., and Massey-Harroche, D. (1996) Cellular and subcellular localization of annexins I, IV, and VI in lung epithelia. Am. J. Physiol. 270, L863–L871
24. Pierce, E. A., Quinn, T., Meehan, T., McCue, T. L., Berson, E. L., and Dryja, T. P. (1999) Mutations in a gene encoding a new oxygen-regulated photoreceptor protein cause dominant retinitis pigmentosa. Nat. Genet. 22, 248–254
25. Sullivan, L. S., Heckenlively, J. R., Bowne, S. J., Zuo, J., Hide, W. A., Gal, A., Denton, M., Ingles, C., Blanton, S. H., and Daiger, S. P. (1999) Mutations in a novel retina-specific gene cause autosomal dominant retinitis pigmentosa. Nat. Genet. 22, 255–259
26. Bonneau, D., Raymond, F., Kremer, C., Klosek, J. M., Kaplan, J., and Patte, F. (1993) Usher syndrome type I associated with bronchiectasis and immotile nasal cilia in two brothers. J. Med. Genet. 30, 253–254
27. Andrews, K. L., Nettesheim, P., Asai, D. J., and Ostrowski, L. E. (1996) Identification of seven rat axonemal dynein heavy chain genes: expression during ciliated cell differentiation. Mol. Biol. Cell 7, 71–79
28. Watson, Y., Richardson, R. T., Widgren, E. E., and O’Rand, M. G. (2001) Characterization of Sp17: a ubiquitous three domain protein that binds heparin. Biochem. J. 367, 25–31
29. Lea, I. A., Richardson, R. T., Widgren, E. E., and O’Rand, M. G. (1996) Cloning and sequencing of cDNAs encoding the human sperm protein, Sp17. Biochim. Biophys. Acta 1307, 263–266
30. Carr, D. W., Fujita, A., Stenz, C. L., Liberty, G. A., Olson, G. E., and Narumiya, S. (2001) Identification of sperm-specific proteins that interact with A-kinase anchoring proteins in a manner similar to the type II regulatory subunit of PKA. J. Biol. Chem. 276, 17332–17338
31. Neilsen, L., I., Schneider, P. A., Van Deerinck, P. G., Kirisakido, M., Driscoll, D. A., Pellegrini, M. C., Millinder, S., Yamamoto, K. C., French, C. K., and Strauss, J. F., III (1999) cDNA cloning and characterization of a human sperm antigen (SPAG6) with homology to the product of the Chlamydomonas PF16 locus. Genomics 60, 272–280

Molecular & Cellular Proteomics 1.6 465