Proteomic analysis of a eukaryotic cilium

Gregory J. Pazour,1 Nathan Agrin,2 John Leszyk,3 and George B. Witman2

1Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA 01605
2Department of Cell Biology, University of Massachusetts Medical School, Worcester, MA 01655
3Proteomic Mass Spectrometry Laboratory, University of Massachusetts Medical School, Shrewsbury, MA 01545

Cilia and flagella are widespread cell organelles that have been highly conserved throughout evolution and play important roles in motility, sensory perception, and the life cycles of eukaryotes ranging from protists to humans. Despite the ubiquity and importance of these organelles, their composition is not well known. Here we use mass spectrometry to identify proteins in purified flagella from the green alga Chlamydomonas reinhardtii. 360 proteins were identified with high confidence, and 292 more with moderate confidence. 97 out of 101 previously known flagellar proteins were found, indicating that this is a very complete dataset. The flagellar proteome is rich in motor and signal transduction components, and contains numerous proteins with homologues associated with diseases such as cystic kidney disease, male sterility, and hydrocephalus in humans and model vertebrates. The flagellum also contains many proteins that are conserved in humans but have not been previously characterized in any organism. The results indicate that flagella are far more complex than previously estimated.

Introduction

Defects in cilia in humans have long been known to cause the severe inherited disorder primary ciliary dyskinesia; more recent evidence has implicated cilia in polycystic kidney disease (PKD), retinal degeneration, obesity syndromes, and developmental disorders (Pazour and Rosenbaum, 2002; Ong and Wheatley, 2003; Scholey, 2003; Snell et al., 2004; Pazour, 2004). Cilia and flagella, which are essentially identical organelles and hereafter referred to interchangeably, are essential to the life cycles of many human and animal parasites, including those that cause malaria, African sleeping sickness, Chagas’ disease, and giardiosis. Some cilia, such as airway cilia and sperm tails, move; others are nonmotile and serve as sensory antennae (Pazour and Witman, 2003). Structurally, the cilium (Fig. 1) consists of a microtubular axoneme ensheathed by a ciliary membrane that is continuous with the plasma membrane of the cell. The axoneme of motile cilia includes substructures such as the dynein arms and radial spokes that generate and control axonemal bending. The cilium also contains “matrix” proteins that are not tightly associated with either the membrane or the axoneme, including proteins involved in intraflagellar transport (IFT), a process required for assembly and maintenance of the organelle (Rosenbaum and Witman, 2002).

An understanding of how cilia carry out their functions and of their role in disease will require increased knowledge of their protein composition. Previous two-dimensional gel electrophoretic analyses of isolated flagella from the green alga Chlamydomonas reinhardtii, a genetically tractable organism that has many advantages for biochemical and molecular genetic studies of the flagellum, revealed that flagella contain more than 250 proteins (Luck and Piperno, 1989; Dutcher, 1995), of which only ~100 have been characterized at the molecular level. Recently, the availability of complete genome sequences has made possible global approaches to the identification of genes encoding ciliary and flagellar proteins. For example, by comparing the genomes of ciliated versus non-ciliated organisms, Avidor-Reiss et al. (2004) and Li et al. (2004) identified 187 and 688 genes, respectively, that are present in the former but not the latter; these are candidates for encoding flagellar and basal body components. In a different approach, Stolc et al. (2005) used a gene chip to examine the level of induction of all C. reinhardtii genes after deflagellation; they identified 220 genes that were induced at least 100% and therefore likely to be involved in the assembly or function of ciliary or flagellar proteins.

Although the above approaches can provide very valuable information on proteins associated with cilia and basal bodies, they are a complement to and not a substitute for direct

G.J. Pazour and N. Agrin contributed equally to this work.
Correspondence to G.B. Witman: george.witman@umassmed.edu
Abbreviations used: FAP, flagellar associated protein; IFT, intraflagellar transport; InsP3, inositol 1,4,5-trisphosphate; MS, mass spectrometry; PKD, polycystic kidney disease; PMCA, plasma membrane Ca2⁺ ATPase.

The online version of this article contains supplemental material.
Concerns that these might prevent identification of peptides derived from the outer dynein arm were very abundant, and fraction isolated from wild-type flagella. However, peptides from less abundant proteins prompted us to use flagellar fractions isolated from an outer dynein arm mutant (oda1-1) for the remaining work. The proteins in each of the four fractions were separated by one-dimensional SDS-PAGE, each gel lane cut into 33 to 45 slices (Fig. S1), the proteins in each slice digested with trypsin, and the resulting peptides eluted, separated by HPLC, and analyzed by MS–MS using electrospray ionization and an LCQ ion trap mass spectrometer. The search engine Mascot was used to find the best matches to the MS–MS spectra in the translated C. reinhardtii genome, and the peptides also were searched against a database containing all predicted C. reinhardtii proteins to identify the origins of those that

**Results**

**Identification of flagellar proteins**

To identify the proteins that compose C. reinhardtii flagella, these organelles were released from vegetative cells by dibucaine treatment, isolated from the cell bodies by low speed centrifugation and sucrose step gradient fractionation, and harvested by high-speed centrifugation. The membranes of flagella isolated in this way generally remain intact and the matrix remains in situ (Fig. 2 A). The purified flagella were then fractionated into a Tergitol-insoluble fraction containing membrane and axonemes (Fig. 2 B), or into a Nonidet-soluble fraction containing membrane + matrix proteins, a fraction containing proteins released from the Nonidet-demembranated axonemes by KCl extraction, and a fraction containing the axonemal proteins remaining after KCl extraction (Fig. 2 D).

Electron microscopy of isolated flagella and axonemal fractions indicates that they are highly pure (Fig. 2).

Initial analysis was performed on the Tergitol-insoluble fraction isolated from wild-type flagella. However, peptides derived from the outer dynein arm were very abundant, and concerns that these might prevent identification of peptides from less abundant proteins prompted us to use flagellar fractions isolated from an outer dynein arm mutant (oda1-1) for the remaining work. The proteins in each of the four fractions were separated by one-dimensional SDS-PAGE, each gel lane cut into 33 to 45 slices (Fig. S1), the proteins in each slice digested with trypsin, and the resulting peptides eluted, separated by HPLC, and analyzed by MS–MS using electrospray ionization and an LCQ ion trap mass spectrometer. The search engine Mascot was used to find the best matches to the MS–MS spectra in the translated C. reinhardtii genome, and the peptides also were searched against a database containing all predicted C. reinhardtii proteins to identify the origins of those that
spanned exon–exon junctions. From these four fractions, 8,345 unique peptides were identified and grouped by the proteins from which they were derived. 360 proteins were identified by five or more unique peptides, 292 by two to four unique peptides (Table S1) and another 482 by a single peptide (Table S2). All protein and peptide sequences, as well as the Mascot scores for the peptides, are available at http://labs.umassmed.edu/chlamyf/index.php.

The list of flagellar proteins identified by two or more peptides is rich in motor proteins, signal transduction proteins, proteins with predicted coiled-coil domains, and predicted membrane proteins (Table I), and contains a number of proteins whose homologues are associated with disease in humans and model vertebrates. Nearly 90 proteins are highly conserved in humans (BLAST E score ≤1e-10) but have not been previously characterized in any organism. Individual proteins of interest are described in the Discussion.

The dataset contains nearly all known flagellar proteins

A measure of the completeness of the dataset can be obtained by determining what percentage of known proteins is present. At this time, 101 C. reinhardtii flagellar proteins have been identified by biochemical, genetic, and bioinformatic methods (Table S3). 97 of these are present in our dataset. This includes all known outer dynein arm subunits and associated proteins except one, all known and predicted inner dynein arm subunits except one, all known IFT components, and all known radial spoke proteins (Table S3). The gene (ODA5) encoding the outer dynein arm-associated protein that is missing is not present in the genome assembly and so could not have been found by our analysis. The gene (DHC3) encoding the putative inner dynein arm subunit that is missing was identified by PCR analysis of genomic DNA but the protein has not been shown biochemically to be in flagella. This gene was much less strongly expressed than other dynein heavy chain genes as measured by Northern blot or reverse-transcriptase PCR analysis (Porter et al., 1996). The gene encoding LF4 is mostly missing from the genome assembly. The only other missing protein is p80 katanin, which was shown genetically to be involved in central pair assembly, but the abundance of the native protein is unknown (Dymek et al., 2004). Although fractions from flagella lacking outer dynein arms were used for all but the initial analysis, all known outer arm subunits were detected in that initial sample, so any previously unidentified outer arm subunits of similar abundance also are likely to have been detected. Thus, this dataset is likely to contain nearly all flagellar proteins.

Assessment of exclusiveness of the dataset

Even though electron microscopy indicated that the flagellar fractions were highly pure, at some level the dataset is likely to contain proteins that are contaminants. The number of peptides found by electrospray MS of complex mixtures roughly correlates with the abundance and size of the proteins from which they are derived (Washburn et al., 2001). Most known flagellar proteins were identified by more than five peptides; notable exceptions include several outer dynein arm subunits, which would not have been present in flagella of the outer dynein arm-less mutant used for all but the Tergitol-insoluble membrane + axonemes fraction; Tctex1 (one peptide), which is very small; and EB1 (two peptides), which is located only at the tip of the flagellum and therefore is of relatively low abundance. Obvious contaminants in the dataset (tRNA synthetases, ribosomal proteins, and histones) were usually identified by one or two peptides, even though these proteins are highly abundant in the cell body. Thus, the 360 proteins identified by five or more peptides are highly likely to be true flagellar proteins, with possible exceptions including methionine synthase, elongation factors, arginyl-tRNA synthetase, carbonic anhydrase, and two cell wall proteins. However, even some of the latter proteins may function in the flagellum: methionine synthase gene expression is induced by flagellar adhesion (Kurvari et al., 1995); elongation factor 1a binds calmodulin and localizes to Tetrahymena cilia (Ueno et al., 2003); and arginyl-tRNA synthetase is reported to modify protein substrates to allow degradation via the ubiquitin pathway (Ferber and Ciechanover, 1987), several other components of which were found in the flagellum. Several glycolytic enzymes also are present in this data subset and are likely to function in the flagellum (see Discussion).

The 292 proteins identified by two to four peptides should be considered candidate flagellar proteins as this group contains both known flagellar proteins and likely contaminants. Within this group, the genes of 19 out of 51 (37%) randomly chosen proteins that were not previously reported to be in the flagellum were found to be induced at least 1× by deflagellation (i.e., their transcript levels doubled; see Induction of genes by deflagellation section). Flagellar proteins would include these plus known flagellar proteins plus some whose genes are not induced by deflagellation, so that the percent of true flagellar proteins in this subset is likely to be substantially higher than 37%. The group of proteins identified by single peptides was not analyzed in detail but likely contains low abundance and small flagellar proteins along with contaminants.

Distribution of proteins in flagellar fractions

In the subset of proteins identified by five or more peptides, the peptides of ~3/4 of the proteins were present predominantly in the KCl extract and extracted axoneme fractions and thus

Table I. Summary of results

| Proteins identified by five or more peptides | 360 |
| Proteins identified by two to four peptides | 292 |
| Proteins identified by one peptide | 482 |
| Major groups<sup>a</sup> | |
| Signal transduction proteins | 93 |
| Motor proteins | 41 |
| Nucleotide metabolism | 9 |
| Glycolytic enzymes + malate dehydrogenase | 8 |
| Conserved uncharacterized proteins | 87 |
| Predicted membrane proteins | 39 |

<sup>a</sup>Only proteins identified by two or more peptides are included here. Proteins may be included in more than one category.
probably represent axonemal components (Fig. 3 A). As expected, this includes the previously identified inner dynein arm proteins, central pair proteins, and radial spoke proteins. The peptides of ~1/4 of the proteins were abundant predominantly in the membrane + matrix fraction; this included all proteins known to be subunits of IFT motors or particles. Within the subset of proteins identified by two to four peptides, the proteins were approximately equally distributed between the membrane + matrix and axonemal fractions (Fig. 3 B). Therefore, the majority of proteins found in the flagellar proteome are in the axonemal fractions. The difference in the distribution of proteins in the two data subsets suggests that the membrane + matrix fraction contains a larger proportion of low abundance proteins than the axonemal fractions. Fig. 3 C shows a hierarchical cluster diagram based on the number and distribution of peptides in all four biochemical fractions. Each horizontal row represents one protein. The number of peptides in the particular fraction is represented by a color ranging from yellow (~27 peptides) to blue (0 peptides). In addition, the major known flagellar proteins are color coded under “substructure” with central pair (CP) proteins being marked in blue gray, intraflagellar transport (IFT) proteins in black, inner dynein arm (IDA) proteins in yellow, outer dynein arm (ODA) proteins in green and radial spoke proteins (RSP) in red. Note, for example, the IFT protein cluster (*) where nearly all peptides were found in the Tergitol-insoluble fraction, the inner dynein arm cluster (**) where most peptides were most abundant in the KCl extract, and the outer dynein arm cluster (***) where nearly all peptides were found in the Tergitol-insoluble fraction. Only proteins represented by 15 or more peptides were included in the cluster analysis. A detailed version of this figure including gene IDs is available at http://labs.umassmed.edu/chlamyfp/index.php.

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of proteins in the two data subsets suggests that the membrane + matrix fraction contains a larger proportion of low abundance proteins than the axonemal fractions. Fig. 3 C shows a hierarchical cluster diagram based on the number and distribution of peptides in the different fractions for proteins identified by 15 or more peptides. Proteins known to be associated with IFT, the outer dynein arm, or the inner dynein arm grouped closely together, indicating that the analysis is likely to have predictive value. Thus, novel proteins that group with these proteins may be new constituents of these structures. Proteins of previously unidentified structures or processes may similarly group with each other.

Induction of genes by deflagellation

Genes encoding proteins that are used exclusively in the flagellum are strongly induced by deflagellation in C. reinhardtii, whereas genes encoding nonflagellar proteins are not induced (Lefebvre and Rosenbaum, 1986). Proteins such as actin and calmodulin that function in both the flagellum and the cell body usually fall into the latter category or are only weakly induced. Therefore, to obtain a rough estimate of the percent of
had no close homologues in either humans or C. reinhardtii identified by two or more peptide hits. 292 C. reinhardtii flagellar proteins had no close homologues in either humans or A. thaliana.

The conserved, uncharacterized proteins that function specifically in the flagellum, as well as to investigate if other proteins of interest are likely to be specific to the flagellum, we used real-time PCR to measure changes in transcript level during flagellar regeneration for 176 proteins of the flagellar proteome, including 69 of the conserved, uncharacterized proteins (Fig. 4 and Table S1). Values were normalized relative to those for the Gβ subunit. The fold induction for known proteins believed to function specifically in the flagellum ranged from 1.8× for the mastigotene protein to 11× for the inner dynein arm subunit Tctex2b. 60 out of 69 (87%) of the conserved uncharacterized proteins tested were induced at least 1×. These results indicate that a large majority of the conserved, previously uncharacterized proteins identified in the flagellar proteome function specifically in the flagellum. In addition, large inductions were observed for genes encoding several proteins of particular interest, including the hydin homologue (7×), the inositol 1,4,5-trisphosphate (InsP3) receptor (6×), the NEK-like NIMA kinase (13×), the Pacrg protein homologue (9×), and the plasma membrane Ca2+ ATPase (PMCA) C_370080 (5×), indicating that they also are likely to function specifically in the flagellum. Transcripts encoding enzymes of the glycolytic pathway showed little change or decreased slightly following deflagellation, as expected for proteins that function in both the flagellum and cytoplasm.

**Conservation of flagellar proteins in humans and Arabidopsis**

As noted above, comparisons of genomes of organisms such as C. reinhardtii and humans that have cilia with those of organisms such as Arabidopsis thaliana that do not have cilia have resulted in the identification of new genes necessary for cilia and basal body development and function (Avidor-Reiss et al., 2004; Li et al., 2004). The C. reinhardtii proteins identified in this work were analyzed by BLAST (using a BLAST E score of ≤1e-10 as the cutoff) to identify similar proteins encoded by the human and A. thaliana genomes (Fig. 5). 336 (52%) of the proteins identified by two or more unique peptides match proteins in humans, indicating that the majority of ciliary proteins have been conserved between C. reinhardtii and humans; of these, 209 identify the same C. reinhardtii protein in a reciprocal BLAST search, providing additional support that the two proteins are orthologous. 132 proteins (20%) are conserved in humans but not in A. thaliana; these would have been identified by comparative genomics approaches. However, 204 (31%) of the proteins are conserved in both humans and A. thaliana. These proteins include the kinesins, most of the signal transduction proteins, the enzymes involved in nucleotide metabolism, and other proteins that also function in the cytoplasm or have close family members that function in the cytoplasm. Importantly, this large class would not have been readily detected by a comparative genomics approach. 24 (<4%) of the proteins are conserved in A. thaliana but not humans. Some of these are likely contaminants in the flagellar preparation (e.g., Rubisco and chlorophyll-a/b binding protein, identified by two peptides each), but two out of six tested were induced more than 1× by deflagellation (Fig. 4), indicating that some are true flagellar proteins. These may be plant-specific proteins that have been adapted by Chlamydomonas to serve flagellar functions.

**Discussion**

In this work, we used MS to characterize the C. reinhardtii flagellar proteome. We identified 360 proteins by five or more peptides. Because the preparation was highly pure as assessed by electron microscopy and this group of proteins contains very few obvious contaminants, most of these are likely to be true flagellar proteins. We also identified 292 proteins by two to four peptides; we consider these to be candidate flagellar proteins that need to be confirmed by further analysis. The best previous estimate of the number of proteins in the C. reinhardtii flagellum was ~250 based on two-dimensional gels (Luck and Piperno, 1989; Dutcher, 1995). Our data indicate that the number is substantially higher and that the complexity of the flagellum is greater than previously anticipated.

**Motor and signal transduction proteins**

In addition to the very large number of known dynein subunits and three previously known kinesin subunits, a previously uncharacterized kinesin heavy chain (C_70192) was identified. Phylogenetic analysis did not support definitive assignment of this protein to a specific kinesin family. Peptides from this protein were found primarily in the axonemal fractions and cluster analysis (Fig. 3 C) grouped it with radial spoke and central pair components. This may be the unidentified flagellar kinesin detected by immunological methods (Fox et al., 1994). No myosin heavy chains were found.

The flagellar proteome contains over 90 putative signal transduction proteins. Based on Interpro domain analyses and homology searches, the proteins identified by two or more peptides include 21 protein kinases and 11 protein phosphatases, the vast majority of which have not previously been reported to be in the flagellum. These findings are consistent with the fact that a large number of flagellar proteins are phosphorylated (Piperno et al., 1981), and with the roles of protein phosphory-
lation and dephosphorylation in the control of flagellar motility (Porter and Sale, 2000), signaling (Pan and Snell, 2000), and probably assembly (Rosenbaum and Witman, 2002). We also found 27 EF hand-containing proteins that are potential Ca\textsuperscript{2+}-binding proteins, consistent with the well-established role of Ca\textsuperscript{2+} in the control of flagellar behavior (Witman, 1993) and signaling (Bloodgood, 1992; Praetorius and Spring, 2001).

10 proteins are predicted to have IQ (calmodulin-binding) domains. An InsP\textsubscript{3} receptor was identified by 33 unique peptides in the membrane + matrix fraction, suggesting that it is a very abundant flagellar component. 10 small GTPases were identified, including five RAB subfamily members, four ARF subfamily members, and RAN. A RAB GDP-dissociation inhibitor that controls the GDP-GTP exchange reaction also is present. Two predicted 14-3-3 proteins, which mediate signal transduction by binding to phosphoserine-containing proteins, were identified. Six ion pumps or channels, including a homologue of human polycystin 2 (see Homologues of vertebrate disease proteins) and three predicted PMCAs (C_370080, C_3300001), were present. The proteome also contained a group of four closely related proteins (all encoded by scaffold 152) that are composed of 8 to 12 transmembrane helices and a PAS domain; PAS domains are thought to be sensory motifs that are involved in detecting diverse stimuli ranging from light or oxygen to redox state and small ligands (Taylor and Zhulin, 1999). We also confirmed the presence of a second type of PAS domain-containing protein, the blue-light receptor phototropin (Huang et al., 2004), in the axonemal fraction. The identification of so many signal transduction proteins underscores the importance of signaling in the function of motile as well as nonmotile cilia, and should greatly facilitate studies to understand signaling mechanisms in these organelles (Pazour and Witman, 2003).

**Nucleotide production and metabolism**

Flagellar motility, assembly and signal transduction require large amounts of ATP and GTP, and the flagellum contains numerous proteins that are likely to be involved in maintaining adequate levels of these nucleotides. Two adenylyl kinases and a nucleoside diphosphate kinase have been characterized in the past year (Patel-King et al., 2004; Wirschell et al., 2004; Zhang and Mitchell, 2004). We found two additional nucleoside diphosphate kinases (C_1090038, C_1230002), two additional adenylyl kinases (C_750038, C_10320001), and two guanylyl kinases (C_160167, C_3300001), four of which are associated with the axoneme.

Interestingly, the flagellar proteome also contains all enzymes of the late glycolytic pathway required for the conversion of fructose 1,6-bisphosphate to pyruvate, which could generate ATP for use in the flagellum (Fig. 6). Consistent with glycolysis occurring in the flagellum, we also found two isoforms of malate dehydrogenase, which could be involved in a malate–oxaloacetate shuttle to regenerate the NAD consumed by the glycolytic pathway. It might be argued that, because the early steps of the glycolytic pathway are reported to occur primarily in the chloroplast and the later steps in the cytoplasm in *C. reinhardtii* (Klein, 1986), the cytoplasmatic enzymes might simply have diffused into the flagella but are not flagellar proteins per se. Our data argue against this, because aldolase, also reported to be located primarily in the chloroplast (Klein, 1986), was identified by numerous peptides (Fig. 6). We also did not find phosphoenolpyruvate carboxylase, a cytoplasmic marker (Klein, 1986), or any enzymes of pyrimidine biosynthesis, which should be abundant in the cytoplasm. Moreover, peptides derived from glyceraldehyde-3-phosphate dehydrogenase were found almost exclusively in the axonemal fractions, indicating that this enzyme is anchored to the axoneme, and peptides derived from enolase were found in both membrane + matrix and axonemal fractions, indicating that some of the flagellar enolase is similarly assembled into the axoneme. These results suggest that there is a mechanism to exclude soluble cytoplasmic proteins from the flagellum, and that the glycolytic enzymes are true flagellar components. The localization of glycolytic enzymes to cilia may be a way to maintain a constant ATP/ADP ratio along the length of the cilium. Dynein activity is sensitive to ATP/ADP ratios (Yagi, 2000; unpublished data). If ATP were available to the cilium only via diffusion from the cell body, the ATP/ADP ratios would vary along the length of the organelle, and dynein function would be compromised. The presence of glycolytic enzymes in cilia is likely to be widespread; glyceraldehyde-3-phosphate dehydrogenase is a component of the mammalian sperm flagellum and is essential for...
sperm motility and fertilization (Miki et al., 2004), and photoreceptor rod outer segments, which are developmentally derived from cilia, are reported to contain the entire glycolytic pathway as a way of maintaining the high levels of ATP needed for the formation of cGMP in this compartment (Hsu and Molday, 1994).

Homologues of vertebrate disease proteins

Cilia have been implicated in several human disorders, including PKD (Pazour, 2004). Interestingly, the C. reinhardtii flagellar proteome contains a homologue (C_590099) of polycystin 2, a Ca^{2+} channel defective in some cases of human autosomal PKD, and a homologue (C_420012) of fibrocystin, a protein of unknown function that is defective in nearly all cases of human autosomal recessive PKD; both of these proteins are associated with cilia in mammals. We also found homologues (C_1780022 and C_10017, respectively) of scorpion and qilin, defects in which cause cystic kidney disease in zebrafish (Sun et al., 2004). Scorpion is a small GTPase in the ARF subfamily and is necessary for ciliary assembly, whereas qilin is a coiled-coil protein, but nothing more is known about these proteins. Defects in the NIMA kinases NEK1 and NEK8 result in cystic kidney disease in the mouse (Upadhya et al., 2000; Liu et al., 2002); C. reinhardtii expresses seven NIMA kinases (Bradley et al., 2004), of which three, Fa2p (C_100034), CNK2 (C_1220044), and CNK6 (C_160119) are found in the proteome. Thus, C. reinhardtii should be a useful model for learning more about the functions of these important proteins and their roles in disease.

The flagellar proteome also contains homologues (C_410060 and C_610051) of hydin and napa, which are respectively mutated in the hy3 and hyh mouse models for hydrocephalus (Davy and Robinson, 2003; Chae et al., 2004; Hong et al., 2004); the hydin gene also is a strong candidate for causing hydrocephalus in humans (Davy and Robinson, 2003). Hydrocephalus is the abnormal accumulation of cerebral spinal fluid in the ventricles of the brain. The fact that hydin and napa homologues are both present in the C. reinhardtii flagellar proteome indicates that cilia probably are involved in the development of hydrocephalus caused by defects in these proteins.

Homologues of a C. reinhardtii inner dynein arm heavy chain and a central pair protein previously were shown to cause male sterility when defective in the mouse (Neesen et al., 2001; Sapiro et al., 2002). The C. reinhardtii flagellar proteome contains homologues of additional proteins associated with male sterility in the mouse. These include C_20334, a homologue of the Pacrg (Parkin-coregulated gene) protein (Lorenzetti et al., 2004), and C_2260011, a homologue of Pp1cc encoding a protein phosphatase 1 catalytic subunit γ (Varmuza et al., 1999). In addition, C_1620015 and C_10830001 are homologues of an Arf-like protein encoded by Arl4, which results in reduced sperm count when defective in the mouse (Schurmann et al., 2002), and C_60170 is a close homologue of a protein kinase encoded by Mak, which causes reductions in litter size and sperm motility when missing (Shinkai et al., 2002). The fact that homologues of these proteins are in the C. reinhardtii flagellum indicates that the proteins are likely to exert their effects as components of the mammalian sperm flagellum.

All three of the PMCAs in the C. reinhardtii flagellar proteome are closely related to the product of the mouse wriggle/deafwaddler gene, which causes deafness and balance problems when defective. This raises the intriguing possibility that the mammalian wriggle/deafwaddler gene product may function in the kinocilium of the inner ear.

Ciliary targeting sequences

Myristoylation of NH_{2}-terminal glycine residues plays a role in directing proteins without membrane spanning domains to the ciliary membrane in Leishmania and Trypanosoma (Godsel and

| Table II. Predicted myristoylated proteins in the ciliary proteome |
|--------------------------|----------|------------------------------|----------|--------------------------------------|
| Cr Model                | M + M   | Axo  | KCl  | Terg | CrFP description |
| C_10830001              | 4       | 0    | 1    | 0    | ARFA1a, ADP-ribosylation factor 1 |
| C_1620015               | 2       | 0    | 0    | 0    | ARFA1b, ADP-ribosylation factor 1 |
| C_1120033               | 2       | 0    | 0    | 0    | Similar to protein tyrosine phosphatase |
| C_1390004               | 14      | 0    | 0    | 0    | PP2C, similar to protein phosphatase 2C |
| C_520010                | 2       | 0    | 0    | 0    | Similar to protein phosphatase 2C |
| C_60149                 | 16      | 11   | 23   | 3    | Cyclic nucleotide-dependent protein kinase |
| C_50062                 | 9       | 0    | 0    | 0    | PKG, similar to cGMP-dependent protein kinase |
| C_740056                | 3       | 0    | 0    | 0    | Similar to cAMP-dependent protein kinase |
| C_1390012               | 15      | 14   | 19   | 20   | ODAS-associated adenylate kinase |
| C_1230005               | 4       | 0    | 0    | 0    | Similar to calcium-binding protein |
| C_1150014               | 3       | 0    | 1    | 4    | Similar to carbonic anhydrase |
| C_700061                | 0       | 0    | 0    | 2    | Similar to calcium-transporting ATPases |
| C_310066                | 3       | 0    | 0    | 0    | Weak similarity to elongation factor 1 γ |
| C_780027                | 2       | 0    | 0    | 0    | Sugar phosphatase domain protein |
| C_850051                | 10      | 0    | 0    | 0    | FAP138, flagellar-associated coiled-coil protein |
| C_80226                 | 16      | 4    | 11   | 18   | FAP12, uncharacterized protein with lipase domain |
| C_90072                 | 2       | 0    | 0    | 0    | Hypothetical protein |
| C_1080051               | 2       | 0    | 0    | 1    | Hypothetical protein |
are connected to the doublet microtubules and remain connected (projecting from the extracellular surface of the flagellar membrane) to the underlying doublet microtubules. In the alga *Ochromonas*, the mastigonemes (thin, hair-like structures projecting from the extracellular surface of the flagellar membrane) are connected to the doublet microtubules and remain connected even after removal of the flagellar membrane by detergent extraction (Markey and Bouck, 1977). Finally, in *Chlamydomonas moewusii*, there is compelling evidence that a surface-exposed flagellar glycoprotein is tightly associated with the axoneme and stays attached to the axoneme after removal of the membrane by detergents (Reinhart and Bloodgood, 1988). Linkage to the axoneme may ensure that these membrane proteins remain correctly distributed along the flagellum.

Comparison with other broad studies of *C. reinhardtii* gene expression

Two recently published studies also examined expression levels of large numbers of *C. reinhardtii* genes in response to deflagellation. Li et al. (2004) used real time PCR to examine 103 genes that had been chosen based on the observation that they were conserved in ciliated species but not in nonciliated species. Of these also were examined by us and the results for these are very similar in the two datasets. Stolc et al. (2005) used a gene chip to examine genome-wide expression patterns including all of the genes we identified except FAP41. The Stolc et al. dataset has a high rate of false negatives such that only 54% of genes previously shown to be induced by deflagellation were found to be induced 100% (equivalent to 1× induction in our study) or greater. Thus, the lack of induction as measured by the gene chip cannot be interpreted as evidence for nonciliary roles. However, the Stolc et al. (2005) dataset has a very low false positive rate, as indicated by the fact that, within a subset of 45 potential flagellar genes that we examined by real-time PCR analysis and that Stolc et al. reported to be induced at least 100%, 40 were found to be induced at least 1× in our analysis. The exceptions were C_1140006, C_200160, and the genes encoding Hsp70, calmodulin, and Ran. Of 220 genes found to be induced at least 100% by Stolc et al. (2005), 155 encode proteins identified in the flagellar proteome by two or more peptides and an additional 18 encode proteins identified by one peptide. The remaining 47 genes may encode flagellar proteins not detected in our analysis, encode proteins that are involved in flagellar regeneration but not incorporated into the flagellum, encode transcripts that are not translated, or be false positives.

Conserved uncharacterized proteins

Remarkably, the flagellar proteome contains 87 proteins that have conserved homologues in humans (BLAST Expect ≤ 1e-10), but whose homologues have not yet been characterized in any organism. 60 out of 69 (87%) of these genes examined were induced by deflagellation, suggesting that they function primarily in cilia. Only eight of these were previously identified by Ostrowski et al. (2002). This confirms that the protein composition of cilia has not been well characterized, and highlights the need for further research on these proteins.

Conclusions

Our analysis of the *C. reinhardtii* flagellar proteome has identified many proteins of known function not previously ascribed to cilia and flagella, as well as many other proteins whose functions and cellular locations were completely unknown before now. The results indicate that cilia and flagella are far more complex than previously believed. The findings provide a starting point for future investigations.
studies to elucidate the roles of these proteins in the assembly and function of these ubiquitous cell organelles, to understand why defects in some of these proteins lead to human diseases, and to clarify the origin of cilia during the evolution of the eukaryotic cell.

Materials and methods

Cell culture and flagellar fractions

_C. reinhardtii_ wildtype strain 137c and mutant strain oda1-1 were grown in liquid culture as previously described (Witman, 1986). In the initial analysis (Fig. 2), flagella were isolated from cells of the dibucaine method (Witman, 1986), which yields highly intact flagella, and analyzed (Fig. 2), flagella were isolated from wild-type cells by the _C. reinhardtii_ studies to elucidate the roles of these proteins in the assembly and detection of peptide sequences of the gene models were compared to the NCBI protein database with BLAST to identify the most closely related proteins in all species and to the predicted proteomes of humans (NCBI build 33) and _Arabidopsis_ (Ahr1 pep2000304172) to determine whether the peptides were conserved in these two species. The chromosomal map position of the best human match was extracted from the NCBI Gene database. The protein was also put through Interpro domain analysis (Mulder et al., 2005), the multicoll coiled-coil predictor (Wolf et al., 1997), the MAHMH membrane-spanning helix predictor (Krogh et al., 2001), and the MYR myristoylation predictor (Maurer-Stroh et al., 2004). Cluster analysis was performed with Expander (Sharaf et al., 2003). The results of these analyses are available at our web site (http://labs.umassmed.edu/chlamyfp/index.php).

Real time PCR analysis of gene induction

To measure induction of gene expression by deflagellation, _C. reinhardtii_ 137c cells were deflagellated by pH shock, after which the cells synchronously regenerate their flagella, and RNA was isolated 30 min later from the deflagellated cells and from untreated control cells. The RNA was reverse transcribed using Clontech PowerScript Reverse Transcriptase (BD Biosciences) and the relative amount of specific messages in the two samples was determined by real-time PCR using pairs of primers that spanned introns and SYBR green to monitor amplification. The relative amount of the noninduced G protein β subunit (Schloss, 1990) was measured in each trial and used to correct for slight differences in amount of mRNA in each sample. In our current analysis, “2× induction” and “2× induction” indicate no change, a doubling, or a tripling of transcript levels, respectively. Three independent sets of RNA were isolated and analyzed. Primer pairs that spanned introns were picked using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3 www.zig).

On-line supplemental material

Table S1 shows flagellar proteins identified by two or more peptides. Table S2 shows flagellar proteins identified by a single peptide. Table S3 lists known _C. reinhardtii_ flagellar proteins. Figure S1 depicts SDS-polyacrylamide gels used for proteomic analysis. Online supplemental materials are available at http://www.jcb.org/cgi/content/full/jcb.200504008/DC1.

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