Dissecting the Axoneme Interactome
THE MAMMALIAN ORTHOLOGUE OF CHLAMYDOMONAS PF6 INTERACTS WITH SPERM-ASSOCIATED ANTIGEN 6, THE MAMMALIAN ORTHOLOGUE OF CHLAMYDOMONAS PF16*

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The axoneme central apparatus is thought to control flagellar/ciliary waveform and maintain the structural integrity of the axoneme, but proteins involved in these processes have not been fully elucidated. Moreover the network of interactions among them that allows these events to take place in a compact space has not been defined. PF6, a component of the Chlamydomonas central apparatus, is localized to the 1a projection of the C1 microtubule. Mutations in the Chlamydomonas Pf6 gene result in flagellar paralysis. We characterized human and murine orthologues of PF6. The murine Pf6 gene is expressed in a pattern consistent with a role in flagella and cilia, and the Pf6 protein is indeed localized to the central apparatus of the sperm flagellar axoneme. We discovered that a portion of PF6 associates with the mammalian orthologue of Chlamydomonas Pf16 (sperm-associated antigen 6 (SPAG6)), another central apparatus protein that is localized to the C1 microtubule in algae. A fragment of PF6 corresponding to the Pf6 domain that interacts with SPAG6 in yeast two-hybrid assays and colocalizes with SPAG6 in transfected cells was missing from epididymal sperm of SPAG6-deficient mice. SPAG6 binds to the mammalian orthologue of PF20, which in Chlamydomonas is located in bridges connecting the C2 and C1 microtubules. Thus, Pf6, SPAG6, and PF20 form a newly identified network that links together components of the axoneme central apparatus and presumably participates in its dynamic regulation of ciliary and flagellar beat. Molecular & Cellular Proteomics 4:914–923, 2005.

Cell motility and the movement of surface fluids are dependent upon flagella and cilia. The core structure of these organelles, the axoneme, is remarkably conserved across species, having in common nine doublets of microtubules with the associated force-generating dynein arms and radial spokes surrounding two central singlet microtubules (the central apparatus), which contain associated proteins (1–10). The central apparatus is thought to play a key role in translating the microtubule sliding caused by dynein into the flagellar waveform and maintaining structural integrity of the axoneme (9, 10). However, the central apparatus proteins involved in these processes have not been fully elucidated, and the network of interactions among them that allows these events to take place in a compact space has not been defined. PF6, a component of the Chlamydomonas central apparatus, is localized to the 1a projection of the C1 microtubule (11). The Chlamydomonas pf6 mutant has paralyzed flagella and lacks the 1a projection, indicating that Pf6 has a critical role in axoneme structure and function. We characterized the human and murine orthologues of PF6 and demonstrated that the murine Pf6 gene is expressed in a pattern consistent with a role in flagella and cilia and that Pf6 protein is indeed localized to the central apparatus of the sperm flagellar axoneme. We discovered that murine Pf6 directly associates with the mammalian orthologue of Chlamydomonas Pf16 (SPAG6)1 (12), a central apparatus protein that is also localized to the C1 microtubule in algae. SPAG6 also binds to the mammalian orthologue of PF20, which in Chlamydomonas is located in bridges connecting the C2 and C1 microtubules (1, 13, 14). Thus, Pf6, SPAG6, and PF20 form a network that links together components of the axoneme central apparatus and presumably participate in its dynamic regulation of ciliary and flagellar beat (15).

MATERIALS AND METHODS
Determination of the Nucleotide and Amino Acid Sequences of Mouse and Human PF6 Protein and Comparison of Amino Acid Sequences among Species—The nucleotide sequences of the human and mouse Pf6 cDNAs were extracted from public data bases searching first with the Chlamydomonas reinhardtii Pf6 protein sequence and then with the sequence of the human orthologue. PCR was used to generate human and murine cDNAs from human testis and mouse testis total RNA, respectively, and the PCR products were sequenced. The cDNA nucleotide sequences have been deposited in GenBankTM (accession numbers AY555274 (human), AY555275 (mouse 97-kDa protein), and AY792594 (mouse 250-kDa protein)). The ClustalW program (MacVector) was used to construct multiple alignments of conserved regions in Pf6 from different eukaryotes.

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* The abbreviations used are: SPAG6, sperm-associated antigen 6; CHO, Chinese hamster ovary; GFP, green fluorescent protein.
Northern Blot and RT-PCR Analyses—Human and mouse multiple tissue RNA blots were purchased from Clontech. The blots were hybridized with the PF6 probes generated by PCR based on the human and mouse PF6 cDNA sequences as described previously (13). The primer set used to generate the human 2-kb cDNA fragment was: forward, 5'-GATTGTTCACTGTTGATCG-3'; reverse, 5'-CTA-AGTGAGAAACTTTC-3'. The primer set used to generate the mouse PF6 cDNA fragment was: forward, 5'-ATGGAGAACAAGAGAGAGAGACCAAAAC-3'; reverse, 5'-CAGAGAATCATCTTCTGGTGAC-3'.

Total RNA from the indicated tissue was reverse transcribed, and PF6 and glyceraldehyde-3-phosphate dehydrogenase sequences were amplified. The PF6 primers used were: forward, 5'-CCTT-TCCGGTTCAACCTTTC-3'; reverse, 5'-CAGCCTGACAACTGTTAAATGC-3'. The PCR conditions were incubation at 95 °C for 4 min followed by 35 cycles of 95 °C for 30 s, 60 °C for 45 s, and 72 °C for 1 min ending at 72 °C for 10 min. Amplification products were resolved on agarose gels stained with ethidium bromide.

Generation of Anti-PF6 Antibodies and Analysis of PF6 Protein Expression—A cDNA encoding a C-terminal portion of mouse PF6 (amino acid residues 548–875) was amplified from a PF6 cDNA clone with the following primers: forward, 5'-CATATGGCAGGAGGATG-TGACCCCAAG-3'; and reverse, 5'-AAGCCTGCTATTGCGCTTCTGTA-3'. The cDNA was inserted into Ndel/HindIII sites of the pET28a vector (Novagen). The resulting fusion protein contained His$_6$ tags at both the N and C termini. The construct was transformed into BL-21(DE3) cells, and the fusion protein was induced and subsequently purified using a protocol previously described (13). A second recombinant protein consisting of the N terminus of PF6 (amino acid residues 1–305) was generated using a similar strategy. The N-terminal sequence cDNA was generated with the following primers: forward, 5'-CATATGGCAGGAGGATG-TGACCCCAAG-3'; and reverse, 5'-AAGCCTGCTATTGCGCTTCTGTA-3'. The purified recombinant proteins were used to generate polyclonal antisera in rabbits by a commercial organization (Rockland).

Extracts of testis, epididymal sperm, and heart were made as described previously (13). Germ cells were separated by the Staput method (13). Examination of the purity of isolated fractions under Nomarski optics revealed that the pachytenite spermatocyte, round spermatid, and condensing spermatid fractions were, respectively, 85, 90, and 45% pure. For Western blotting, the cells were dissolved in immunoprecipitation buffer, and equal amounts of protein (50 µg/lane) were heated to 95 °C for 10 min in sample buffer, loaded onto 10% sodium dodecyl sulfate-polyacrylamide gels, electrophoretically separated, and transferred to poly(vinylidene difluoride) membranes (Millipore). Membranes were blocked (Tris-buffered saline solution containing 5% nonfat dry milk and 0.05% Tween 20 (TBST)) and then incubated with the anti-rabbit PF6 antibody (1:1000 dilution) at 4 °C overnight. After washing in TBST, the blots were incubated with an anti-rabbit immunoglobulin conjugated to horseradish peroxidase (1:2000 dilution) at 4 °C overnight. After washing by centrifugation, the blots were incubated with Super Signal chemiluminescent substrate (Pierce).

Immunolocalization of PF6—Testes from adult males were fixed with 4% paraformaldehyde in 0.1 M PBS (pH 7.4), rinsed in 30% sucrose in PBS at 4 °C overnight, frozen in OCT medium with dry ice/2-methylbutane, sliced to 10-µm sections on a cryostat, and collected on gelatin chromosome-coated glass slides. Immunostaining was performed by the avidin-biotin complex technique with a PF6 polyclonal antibody. Briefly sections were treated with 0.05% Triton X-100, 1% horse serum, and 1% BSA for 45 min to block nonspecific staining and sequentially incubated with anti-PF6 antibody at a dilution of 1:400 in PBS with 0.05% Triton X-100 overnight, biotinylated goat anti-rabbit immunoglobulins, and finally avidin-biotin complex reagent (Vector Laboratories, catalog number PK-6101). The avidin activity was visualized by using the 3-amino-9-ethylcarbazole kit (Vector Laboratories, catalog number SK-4200). The reaction was terminated by rinsing the sections with PBS. The sections were counterstained with hematoxylin and then mounted with Fluoromount-G (Southern Biotechnology Associates Inc.).

Sperm were obtained from mouse cauda epididymis and centrifuged at 3,000 × g, washed twice in PBS, resuspended in PBS, and layered onto polylysine-coated slides. The preparations were fixed with 4% paraformaldehyde and permeabilized with 1% Triton X-100. Following permeabilization, slides were blocked in PBS containing 10% goat serum (1 h at 37 °C) and incubated with primary antibody (rabbit anti-PF6) overnight at 4 °C. The secondary antibody, fluorescein-conjugated goat anti-rabbit immunoglobulin G, was applied, and PF6 staining was visualized by using an Olympus IX-70 epifluorescence microscope and Meta Morph Imaging System software (Universal Imaging Corp.). Electron microscope immunocytochemistry was performed as described previously (12, 13) to determine the subcellular localization of PF6.

Co-localization and Co-immunoprecipitation Studies—A cDNA containing the full coding sequence of the mouse 97-kDa PF6 protein (875 amino acids) was generated by RT-PCR with the following primers: forward, 5'-CTCGAGACGGCCACCCACAGGAGAAA-3'; and reverse, 5'-GGATCCGGACATTGTCGAGAAGACCA-3'. The PCR product was cloned into the pCR2.1 TOPO vector. After sequencing to verify fidelity of the PCR product, the cDNA was subcloned into BamHI/XhoI sites of the pEGFP-N2 vector, creating the PF6/pEGFP-N2 plasmid. The full-length SPAG6 coding sequence was cloned into BamHI/SalI sites of pTarget vector, creating the SPAG6 mammalian expression vector. SPAG6/DSRed-N$_2$ was generated as described previously (13). CDNAs containing the N-terminal (amino acids 1–460) and C-terminal (amino acids 465–875) regions of mouse PF6 were also cloned into the same sites of pEGFP-N$_2$ vector to create the PF6N/pEGFP-N$_2$ and PF6C/pEGFP-N$_2$ plasmids. Chinese hamster ovary (CHO) cells were cultured in 2-well chamber slides with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum at 37 °C. PF6/pEGFP-N$_2$ plasmid was transfected to these cells, and 48 h after transfection, the cells were photographed with a fluorescence microscope followed by Western blotting to assess protein expression with anti-GFP and -PF6 antibodies.

Lysates from untransfected CHO cells or co-transfected cells were immunoprecipitated with anti-SPAG6 antibody or preimmune serum and subjected to Western blotting with an anti-GFP antibody.

For co-immunoprecipitation studies, 48 h after transfection the cells were harvested with IP buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1% Triton X-100, 1 mM PMSF, and proteinase inhibitor mixture), and the lysates were passed through 20-gauge needles. After centrifugation at 12,000 rpm for 5 min, the supernatants were precleared with Protein A beads at 4 °C for 30 min. After washing by centrifugation, the supernatants were incubated with polyclonal anti-SPAG6 antibody or preimmune serum (negative control) at 4 °C for 2 h, and protein A beads were then added followed by another incubation at 4 °C overnight. The beads were washed with IP buffer three or four times, and 1× protein loading buffer was added to the beads, which were then boiled at 100 °C for 10 min, and the samples were loaded for Western blotting with monoclonal anti-GFP antibody.

**Yeast Two-hybrid Experiments**—The interaction of SPAG6 and PF6 was confirmed in yeast with the Matchmaker Lex A system (Clontech). Briefly the full-length SPAG6 (forward primer, 5'-GAATTCATGACCCAGCGCGTGGTGC-3'; and reverse primer, 5'-CTCAGTACAGCTGGTGGTATAGTC-3') was cloned into EcoRI/XhoI sites of pB42AD vector. Because the 97-kDa PF6 protein is too big for interaction in the yeast, it was divided into N-terminal (amino acids 1–460; forward primer, 5'-GGATCCGATATGACCCAGCGCGTGGTGC-3'; and reverse primer, 5'-GAATTCATGACCCAGCGCGTGGTGC-3') and C-terminal (amino acids 465–875; forward primer, 5'-CTCAGTACAGCTGGTGGTATAGTC-3'; and reverse primer, 5'-GGATCCGATATGACCCAGCGCGTGGTGC-3') fragments. The fusion proteins were then co-transformed into the yeast strains containing the corresponding plasmids. The activity of SPAG6/pEGFP-N$_2$ and PF6/pEGFP-N$_2$ plasmids was assessed by Western blotting with anti-GFP and -PF6 antibodies. The SPAG6/DSRed-N$_2$ and PF6/pEGFP-N$_2$ plasmids were cotransfected into these cells, and 48 h after transfection, the cells were photographed with a fluorescence microscope followed by Western blotting to assess protein expression with anti-GFP and -PF6 antibodies.
Fig. 1. Alignment of the human, mouse, and *Chlamydomonas* PF6 proteins. The boxed amino acids are identical or similar. The underlined sequence represents the sequence encoding the 97-kDa PF6 protein.
FIG. 1—continued
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GAGAAACCTAC-3'; and reverse primer, 5'-CTCGAGTCCTCCAC-
CAAACTTGGGGATGAG-3'; and C-terminal (amino acids 456–
875; forward primer, 5'-GGATCCGTCTCATCCCGCCAAGTTTGGTG-
GAG-GAG-3'; and reverse primer, 5'-CTCGAGTCGCGATTCCTTGGCCTC-
CTGGTACT-3') parts. The two PF6 cDNAs were cloned into BamHI/
XhoI sites of pLexA vector. The SPAG6/pB42AD plasmid was
transformed with either of the two PF6/pLexA plasmids into yeast
(EGY48) containing p8op-lacZ plasmid, and the yeasts were grown on
synthetic dropout plates lacking His, Trp, and uracil with or without
galactose and raffinose as inducers. The interaction of p53 and large
T antigen was used as positive control; the SPAG6/pB42AD and
empty pLexA were also transformed as negative controls.

Studies on SPAG6-deficient Mice—Mice lacking SPAG6 were gen-
erated by homologous recombination as described previously (12).
Extracts were prepared from testes and epididymal sperm for West-
erm blot studies for the indicated proteins as described previously
(13).

RESULTS AND DISCUSSION

Identification of the Mammalian Orthologues of PF6—The
alignment of human (encoded by a gene on chromosome 1),
mouse (encoded by a gene on chromosome 3 in a syntenic
region), and Chlamydomonas PF6 is shown in Fig. 1. Human
and mouse PF6 are 67% identical and 76% similar when
conservative replacements are considered. The alignment
shown in Fig. 1 reveals that the two mammalian PF6 proteins
and Chlamydomonas PF6 have several short regions that have
been highly conserved over 1 billion years since plants and
animals diverged. Regions conserved over this time are of great
interest because they are likely to be functionally important in
PF6. To investigate further the conservation of these regions,
we looked for other PF6 orthologues in vertebrates using
BLAST. At this time, only partial sequences of PF6 orthologues
were present in the GenBank™, Ensemble, and TIGR (The
Institute for Genomic Research) data bases. BLAST searches of
these data bases retrieved rat PF6 (accession number XP_227526)
and chicken PF6 (accession number XP_416538.1) for comparison
with human, mouse, and Chlamydomonas PF6. These
comparisons identified two segments corresponding to
residues 512–547 and 2323–2380 (Fig. 2). Domain 1 is just after
a proline-rich domain at the N terminus of human and Chlamy-
domonas PF6. Domain 2 is at the C terminus.

Mouse and Human PF6 Are Expressed in a Pattern Consis-
tent with a Gene Encoding an Axonemal Protein—Human
and mouse PF6 cDNAs were hybridized to human and mouse
multiple tissue RNA blots (Fig. 3A) revealing a 7.5-kb tran-
script that is highly expressed in testis. To determine whether
PF6 message is expressed at lower levels in tissues with
ciliated cells, total RNA was isolated, and RT-PCR was carried
out (Fig. 3B). Compared with testis, PF6 appears to be ex-
pressed at more modest levels in organs that contain cilia-
bearing cells including brain, oviduct, lung, and uterus. An
examination of PF6 mRNA expression during the first wave of
spermatogenesis by Northern blotting of total testis RNA in-
dicated that PF6 mRNA accumulates at the time round sper-
matids appear on day 20 of life, a time when the sperm
flagellum is assembled (Fig. 3C). However, RT-PCR analysis
of the same RNA samples detected PF6 message on day 16
when pachytene spermatocytes are present (Fig. 3D). This
suggests that expression of the mouse PF6 gene is initiated at
about the same time that other axoneme genes including P20
and Spag6 are expressed (12, 13).

A rabbit anti-mouse PF6 polyclonal antibody was generated
against a recombinant fragment of PF6 representing amino
acid residues 548–875 adjacent to one of the conserved PF6
domains. In Western blot analyses on testis extracts two
bands were detected, a 250-kDa protein consistent with the
size of the translated protein encoded by the 7.5-kb cDNA
sequence and a more abundant 97-kDa protein consistent
with the predicted molecular mass of a second PF6 cDNA
clone generated by PCR from mouse testis RNA that may be
an alternatively spliced variant (Fig. 4A). There was no detect-
able PF6 immunoreactivity in mouse heart (Fig. 4A) or spleen
and liver (data not shown). In epididymal sperm the most
prominent immunoreactive band was at 28 kDa, suggesting
that there is proteolytic processing of the larger PF6 protein
isoforms (Fig. 4B). PF6 protein was not detected in pachyty-
tene spermatocytes (Fig. 4C). The 97-kDa protein was found in
round spermatids, and both the 250- and 97-kDa proteins
were detectable in greatest abundance in condensing sper-
matids. The conclusion that the 97-kDa PF6 protein is pro-
teolytically processed during spermatogenesis is supported
by Western blot analysis using an antibody against the PF6 N
terminus (Fig. 4B). This antibody detected the 250- and 97-
kDa PF6 proteins in testis and a 170-kDa band, whereas in
sperm it reacted only with a 72-kDa protein, which is likely to be the N terminus of PF6 protein remaining after the cleavage event that generated the 28-kDa protein detected by the anti-C terminus antibody.

The patterns of PF6 expression described above are consistent with the patterns of PF6 mRNA expression and the immunohistochemical studies on testis sections described below. Because the tails are generally lost from condensing spermatids during isolation, the immunoreactive protein in condensing spermatids probably reflects a pool of cytoplasmic protein not yet assembled into the tail. The nature of the processing and the potential interactions among the processed PF6 components in sperm remain to be elucidated. This may be a unique feature of PF6 in mammals or specific to male germ cells. A number of sperm tail proteins are known to undergo proteolytic processing as they are assembled into the flagellum (16, 17). Immunohistochemical studies on testis sections demonstrated that PF6 is present in the cytoplasm of round spermatids, and more intense staining was found in the condensing spermatids (Fig. 5A).

The antibody detected PF6 antigen along the tail of permeabilized epididymal sperm. No signal was detected with the preimmune serum or when the antibody was neutralized with the purified recombinant protein (Fig. 5B). Electron microscope immunocytochemistry revealed that PF6 was localized to the central pair of axoneme, a location that is consistent with the Chlamydomonas PF6 protein (Fig. 5C). Interestingly the asymmetric distribution of colloidal gold particles suggests that, as in Chlamydomonas, mammalian PF6 may be localized to projections of a single microtubule.

**PF6 Interacts with SPAG6**—When CHO cells were transfected with PF6/pEGFP-N2, the fusion protein was localized in the cell cytoplasm (Fig. 6A). Western blotting was performed with the cell lysates, and a 123-kDa protein, representing the 97-kDa PF6 sequence coupled to GFP, was identified using both the anti-PF6 and anti-GFP antibodies, demonstrating that the fusion protein remained intact in the transfected cells (Fig. 6B). It has been shown previously in our laboratory that SPAG6 binds with high affinity to microtubules, particularly those surrounding the nucleus, in transfected cells (Fig. 6A).
To investigate whether PF6 can interact with SPAG6, CHO cells were cotransfected with PF6/pEGFP-N2 and SPAG6/DsRed-N1. In the co-transfected cells PF6 protein decorated the microtubules and co-localized with SPAG6 (Fig. 6A).

CHO cells were transfected with SPAG6/pTarget and PF6/pEGFP-N2 plasmids, and co-immunoprecipitation studies were performed. We found that PF6 protein can be pulled down by anti-SPAG6 antibody, suggesting that PF6 and SPAG6 do indeed interact (Fig. 6D). These studies are consistent with the presence of PF6 and SPAG6 proteins on the same structures (microtubules). Because we have previously reported that SPAG6 decoration of microtubules increases their stability and renders them resistant to depolymerization by cold (incubation at 4 °C) and nocodazole, it is likely that the immunoprecipitated PF6 was bound to SPAG6 on microtubules (13, 18).

We created two different constructs, one encoding the N terminus of the 97-kDa PF6 protein and the other encoding its C terminus, and tested them for the ability to interact with SPAG6 in a yeast two-hybrid system. The PGF6 N-terminal protein did not interact with SPAG6 in this assay, whereas the C terminus did (Fig. 7). To verify that the C terminus is responsible for the binding of PF6 to SPAG6-decorated microtubules, we produced expression plasmids for GFP-tagged proteins corresponding to the two fragments and examined their localization in CHO cells co-transfected with a SPAG6-DsRed construct. The PF6 N terminus-GFP was distributed in the cytoplasm (Fig. 6B, panels a and b) and did not decorate the SPAG6-containing microtubules (Fig. 6B, panels c–f), whereas the PF6 C terminus-GFP when expressed alone was localized to perinuclear vesicles (Fig. 6C, panels a and b), but it was co-localized with SPAG6-DsRed in co-transfected cells (Fig. 6C, panels c–f).

The 28-kDa PF6 Fragment but Not the 72-kDa Fragment Is Missing from Epididymal Sperm of SPAG6-deficient Mice—Extracts of testes and epididymal sperm from SPAG6-deficient mice and wild-type littermates were prepared, and Western blotting was performed. The 97-kDa PF6 band was observed in both groups with no apparent differences in abundance (Fig. 8, left). The 28-kDa PF6 band was readily detected in wild-type sperm but was virtually absent from the
epididymal sperm of SPAG6-deficient mice (Fig. 8, right). In contrast, the 72-kDa fragment was present in both wild-type and SPAG6-deficient sperm. This finding is consistent with the notion that the C terminus of the 97-kDa PF6 protein interacts with SPAG6. Confirming our previous findings, the expression of PF20 was decreased in sperm of SPAG6-defi-
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Fig. 7. Interaction of the C terminus of 97-kDa PF6 with SPAG6. Yeast cells were transformed with the indicated plasmids and grown in the absence or presence of galactose. LacZ activity was detected reflecting specific interactions between the C terminus of PF6 and SPAG6 and the p53/large T antigen control. There was no detectable LacZ activity in the negative control (pLexA + SPAG6/pB42D) or between PF6 N terminus and SPAG6.

Fig. 8. PF6 protein expression in testis and epididymal sperm from SPAG6-deficient mice. Left, testis extracts. Right, epididymal sperm extracts. +/-, wild type; -/-, SPAG6-deficient. 50 μg of protein were loaded in each lane. Blots were probed with the indicated antibodies detecting SPAG6 PF20, the N and C termini of 97-kDa PF6, and AKAP4.

cient mice, but there was little change in the levels of AKAP4.

We have identified the mammalian orthologues of Chlamydomonas PF6, a protein that is localized to the 1a projection arising from the C1 microtubule. Consistent with an important role for PF6 in flagella and cilia, the Pf6 gene was most highly expressed in testis but its expression was detectable at lower levels in RNA extracted from organs that contain cilia-bearing cells. Western blotting and immunocytochemistry localized PF6 expression to developing male germ cells with increased abundance at the time of assembly of the sperm flagellum. The PF6 protein was found along the length of the tail of epididymal sperm and localized to the central pair of the axoneme.

Proteomic and genomic studies to identify axonemal proteins and genes have not discovered PF6 as a component of mammalian axonemes, but this may be due to the fact that the amino acid sequences were not previously known (19, 20). We obtained evidence that strongly suggests that PF6 is indeed a component of the central apparatus based on immunocytochemistry, immunoelectron microscopy, and evidence for association with SPAG6, a Chlamydomonas central apparatus protein that we have previously localized to the central pair of microtubules of the mouse sperm axoneme using immunoelectron microscopy (12). Notably PF6 was attracted from the cytoplasm to polymerized microtubules that were decorated with SPAG6, and PF6 and SPAG6 could be co-immunoprecipitated from transfected cells. Further evidence for PF6-SPAG6 interaction was derived from the study of epididymal sperm from SPAG6-deficient mice. These sperm have axonemal defects including loss of the central pair of microtubules (12). Western blotting revealed that, in the absence of SPAG6, the 28-kDa fragment of PF6 was also missing as was PF20, another central apparatus protein that we previously demonstrated binds to SPAG6 (13). However, a piece of the 97-kDa PF6 protein, the 72-kDa N terminus, was not affected in the epididymal sperm of SPAG6-deficient mice. The central apparatus is unstable in SPAG6-deficient mice and missing in many axonemes. Consequently a number of proteins that are not directly linked may be lost. Thus, the absence of the 28-kDa fragment of PF6 in SPAG6-deficient mice cannot be taken in itself as proof of a PF6-SPAG6 interaction. However, these observations do support the conclusion that the C terminus of the 97-kDa PF6 protein is responsible for the microtubule association in the presence of SPAG6 and are consistent with a PF6-SPAG6 association. In Chlamydomonas, PF20 is associated with the C2 microtubule in the bridges connecting it to the C1 microtubule (1). Because both PF6 and PF20 were detectable in testicular extracts, one interpretation of our findings is that instability of the axoneme resulting from the absence of SPAG6 leads to loss and presumably degradation of proteins to which SPAG6 normally binds, including PF6 and PF20.

Studies on Chlamydomonas mutants provided no evidence for direct interactions between PF6, the SPAG6 orthologue, and PF6 or PF20 (7). Several polypeptides are missing from the flagella of the pf16 Chlamydomonas, but these do not correspond in molecular weight to the full-length PF6 or PF20 proteins. Studies on the pf6 mutant suggested that PF6 is associated/co-sediments with a number of proteins including polypeptides of 40, 80, 135, and 180 kDa (11). This cohort of polypeptides does not appear to include PF16 (SPAG6). At this writing it is not clear whether structural differences in the algal and mammalian central apparatus account for these apparent discrepancies or whether the proteins that co-sediment with Chlamydomonas PF6 are processed or posttranslationally modified forms of PF16.

The specific domains of PF6 and SPAG6 that mediate interactions remain to be determined. However, we can predict that they are encompassed in the C terminus of the 97-kDa PF6 protein based on our yeast two-hybrid and co-localization studies. The absence of the PF6 28-kDa band, which is detected by the antibody directed against the 97-kDa PF6 protein C terminus (residues 548–875) in SPAG6-deficient epididymal sperm, in the face of retention of a PF6
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fragment reacting with an antibody against the N terminus further supports this notion. The anti-C terminus antibody was generated against a sequence immediately adjacent to a highly conserved sequence downstream from a proline-rich domain. This conserved sequence is a candidate domain for mediating protein–protein interaction.

Our studies suggest the presence of a network of interacting proteins in the mammalian central apparatus that includes PF6, SPAG6, and PF20. SPAG6 binds to both PF6 and PF20 and may therefore be a nexus between the C1 and C2 microtubules and the 1a projection off the C1 microtubule of the central apparatus essential for the dynamic changes that modulate axoneme motility and waveform. The functional significance of these interactions is inferred from the fact that deficiency in two of these proteins results in defects in spermatogenesis and sperm motility as well as ciliary dysfunction in ependymal cilia in the case of absent SPAG6 (12, 21).

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The nucleotide sequences reported in this paper have been submitted to the GenBank™ EBI Data Bank with accession numbers AY555275, AY555274, and AY792594.

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