IFT20 Links Kinesin II with a Mammalian Intraflagellar Transport Complex That Is Conserved in Motile Flagella and Sensory Cilia*

Sheila A. Baker‡, Katie Freeman‡, Katherine Luby-Phelps‡, Gregory J. Pazour§, and Joseph C. Besharse¶

‡From the Department of Cell Biology, Neurobiology, and Anatomy, Medical College of Wisconsin, Milwaukee, Wisconsin 53226 and the §Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, Massachusetts 01605

Intraflagellar transport (IFT) is an evolutionarily conserved mechanism thought to be required for the assembly and maintenance of all eukaryotic cilia and flagella. Although IFT proteins are present in cells with sensory cilia, the organization of IFT protein complexes in those cells has not been analyzed. To determine whether the IFT complex is conserved in the sensory cilia of photoreceptors, we investigated protein interactions among four mammalian IFT proteins: IFT88/Polaris, IFT57/Hippi, IFT52/NGD5, and IFT20. We demonstrate that IFT proteins extracted from bovine photoreceptor outer segments, a modified sensory cilium, co-fractionate at ~17 S, similar to IFT proteins extracted from mouse testis. Using antibodies to IFT88 and IFT57, we demonstrate that all four IFT proteins co-immunoprecipitate from lysates of mouse testis, kidney, and retina. We also extended our analysis to interactions outside of the IFT complex and demonstrate an ATP-regulated co-immunoprecipitation of heterotrimeric kinesin II with the IFT complex. The internal architecture of the IFT complex was investigated using the yeast two-hybrid system. IFT20 exhibited a strong interaction with IFT57/Hippi and the kinesin II subunit, KIF3B. Our data indicate that all four mammalian IFT proteins are part of a highly conserved complex in multiple ciliated cell types. Furthermore, IFT20 appears to bridge kinesin II with the IFT complex.

Intraflagellar transport (IFT) involves bidirectional motility of a large protein complex along axonemal microtubules of cilia and flagella. As originally described in the green alga, Chlamydomonas, the 17 S IFT complex is composed of at least 15 different polypeptides and is thought to assemble near the basal body (1, 2). The IFT complex is then carried in an anterograde direction along the axoneme by kinesin-II (3) and in a retrograde direction by a cytoplasmic dynein containing the dhc2/1b heavy chain (4). Cycling of the IFT complex between axoneme and cell body is thought to be associated with transport of essential “cargo” proteins (5), a concept supported by the finding that FLA10, a motor subunit of Chlamydomonas kinesin-II, is required for both IFT and transport of inner dynein arms (6). IFT has been directly observed in the motile flagella of Chlamydomonas reinhardtii (5) as well as the sensory cilia of Caenorhabditis elegans (7–10). Furthermore, mutations in IFT particle proteins in Chlamydomonas, C. elegans, and mice prevent ciliary assembly (3, 4, 9, 11–18), suggesting that this mechanism is important for the maintenance of all axonemal structures.

Most mammalian cell types develop either a cilium or flagellum (19), and several lines of evidence suggest a role for IFT in ciliated mammalian cells (reviewed in Ref. 20). Mammalian homologues to four of the Chlamydomonas IFT proteins (IFT88, -57, -52, and -20) have been localized in the basal body and cilia in multiple ciliated cells (16, 21), and mutations in the IFT88 gene lead to defects in at least three ciliary structures. A hypomorphic mutation in IFT88 (Tg737/orpk) causes both polycystic kidney disease accompanied by shortened primary cilia and retinal degeneration due to abnormalities of the photoreceptor outer segments (12, 16, 22), while replacement of the IFT88 gene by β-galactosidase results in mouse embryos that lack the motile cilium on the ventral nodal cells and fail to develop past mid-gestation (23).

In addition, we have previously reported that IFT88, -57, -52, and -20 from mouse testis co-fractionate at ~17 S (12), formally extending the concept of an IFT complex from Chlamydomonas to mammalian motile flagella. However, the characterization of IFT particles as ~17 S protein complexes is based exclusively on studies of the motile flagella of Chlamydomonas and mouse testis, while the IFT complexes of sensory and primary cilium have not yet been characterized. An IFT particle utilized in the sensory outer segment of photoreceptors could be grossly different from the IFT particle in motile cilia in its size or architecture as a reflection of a very different cargo pool. For example, components of the phototransduction cascade, such as rhodopsin, transducin, and arrestin (24, 25), are potential cargo candidates for IFT, but they are unique to photoreceptor cells. An IFT particle in such cells may require additional adaptor proteins that could increase the sedimentation value of the complex. Conversely, the IFT particle in sensory cilium could be smaller and more simplified as a reflection of the simplified organization of an axoneme lacking large protein complexes involved in regulated motility such as radial spokes and dynein arms.

The principal goal of this study was to determine whether IFT88, -57, -52, and -20 are conserved in the IFT complex in...
tissues with multiple types of cilia. Using a combination of velocity sedimentation and co-immunoprecipitation studies, we report a large, ~17 S complex containing these four proteins is present in tissues containing sensory cilia, motile flagella, and primary cilia. Additionally, we report that the anterograde motor, kinesin II, is co-immunoprecipitated with the IFT complex in multiple tissues in an ATP-regulated manner. IFT20 appears to function in bridging these two complexes by directly interacting with both IFT57 in the IFT complex and KIF3B, a minus-end motor of kinesin II. Our analysis indicates that an IFT complex is present in multiple ciliary types and provides insight into the interaction between the complex and the anterograde motor, kinesin II

EXPERIMENTAL PROCEDURES

Antibodies and Western Blotting—Rabbit polyclonal antibodies directed against IFT88, -57, -52, and -20 were generated as previously described (12). Additional peptide antibodies were generated at Bethyl Laboratories (Montgomery, TX) by immunizing goats with synthetic peptides corresponding to a region near the N terminus of IFT57 (EE-LLQLYNPRLPSGK (BFPSSR)), to a region near the N terminus of IFT88 (ED-DLGYSGFDYNPANAY), to a region near the C terminus of IFT58 (DDFADEELGDDLIKE) and affinity purified. KIF3A was detected with the mouse monoclonal antibody, R2.4 (Covance, Berkeley, CA). KIF3B and KAP3 were detected with mouse monoclonal antibodies from BD Biosciences (Palo Alto, CA). Conventional kinesin heavy chain was detected with a mouse monoclonal antibody from Chemicon (Temecula, CA). Antibodies against cytoplasmic dynein heavy chain 2 and light intermediate chain 3 were a generous gift from Richard Vale (Columbia University, New York) (26). Western blotting was performed according to standard procedures with horseradish peroxidase-conjugated secondary antibodies and detected with either ECL Western blotting detection reagents (Amersham Biosciences) or the SuperSignal West Femto chemiluminescent system (Pierce).

Preparation of ROS Extract and Velocity Sedimentation—Bovine eyes were obtained fresh from Eumpak Foods (Milwaukee, WI). Retinas were either dark-adapted for one hour or immediately harvested and stored at −80 °C. Minimums of four retinas were thawed in 50% sucrose in HMEK buffer (10 mM HEPES, pH 7.2, 5 mM MgSO4, 0.5 mM EDTA, 25 mM KCl) supplemented with a protease inhibitor mixture (1 µg/ml pepstatin A, 1 µg/ml leupeptin, 4 µg/ml aprotinin, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride). The thawed retinas were vortexed at medium speed for 1 min to break off the rod outer segments (ROS) and then filtered through cheesecloth. After centrifugation at 15,000 × g for one hour at 4 °C, the crude ROS were collected and extracted using an equal volume of 2% Nonidet P-40 extraction buffer (0.5% Surfact-Amp Nonidet P-40 (Pierce) in HMEK buffer), with and without 300 mM NaCl. After incubation on ice for at least 10 min, the ROS extract was clarified by a 10-min spin at 20 psi in a Beckman tabletop centrifuge. The clarified extract was then diluted 2:1 with HMEK buffer to reduce the sucrose concentration, and 1 ml was loaded on a 10.6-ml 5–20% sucrose gradient prepared in 10 mM HEPES, pH 7.2. In preparations treated with salt, 300 mM NaCl was added to the sucrose gradients. Centrifugation was carried out in an SW41Ti rotor at 35,000 RPM for 14 h at 4 °C (Beckman Optima LE 80 K ultracentrifuge). Following centrifugation, 0.5-ml fractions were collected and analyzed by Western blotting. Sedimentation standards were run in parallel gradients and included thyroglobulin (19 S), catalase (11.5 S), and bovine serum albumin (4.2 S). Western blotting for cytoplasmic dynein intermediate chain, which sediments at ~20 S (27), was often used as an internal size standard. Six frozen mouse testes were homogenized in 1× Nonidet P-40 extraction buffer, with or without 300 mM NaCl, clarified, and analyzed on sucrose gradients as above.

Immunoprecipitation—Tissues were harvested fresh from C57Bl6 adult mice and homogenized, followed by sonication in IP lysis buffer (0.5% Triton X-100, 5 mM EDTA in phosphate-buffered saline) supplemented with protease inhibitor mixture (see above) or mammalian protease inhibitor mixture (Sigma). Lysates were then clarified by centrifugation at 20,000 × g for 20 min at 4 °C and incubated with an antibody against the IFT57-N peptide, the IFT58-N peptide, or pre-immune serum and incubated on a rotator at 4 °C for a minimum of 2 h. Sixty microliters of protein G-Sepharose 4 Fast Flow (Amersham Biosciences) was added to each lystate/antibody mixture, and the incubation was continued for a minimum of 2 h. The beads were then washed twice in phosphate-buffered saline, followed by four washes in IP lysis buffer. Proteins were eluted from the beads by boiling in 2× Laemmli buffer and analyzed by Western blotting. In salt experiments, 5 mM NaCl was added to the tissue lysate prior to incubation with the antibody to the desired final concentration; all washes were performed with IP lysis buffer at the same NaCl concentration as the lysate. To test the effect of nucleotides, ATP, AMP-PNP, and ATPγS (Sigma) were incubated with the tissue lysates for one hour at 4 °C prior to proceeding with the IP protocol outlined above.

Yeast Two-hybrid Analysis—Vectors, yeast, and major reagents were supplied as part of the Matchmaker Gal4 Two-Hybrid System 3 (Clontech, Palo Alto, CA). The entire open reading frame or deletion constructs of IFT88, -57, -52, and -20 were cloned into both the pGADT7 and pGBK7T vectors using standard cloning procedures (28). Constructs encoding the C terminus of KIF3A (amino acids 338–701) and KIF3B (amino acids 470–747) were obtained by RT-PCR from C57Bl mouse testis RNA and subcloned into pGADT7 and pGBK7T. KAP3 constructs were a generous gift from Tetsu Akiyama (University of Tokyo, Tokyo, Japan) (29). Plasmids were transformed into yeast strain AH109 using the LiAc-mediated yeast transformation as described in the Yeast Protocols Handbook (PT3024–1, Clontech). Transformed yeast cells were isolated by growth on S.D.-leucine-trypotphan plates at 30 °C for 3 days. For high stringency selection, cells were then transferred to S.D.-adine-histidine-leucine-trypotphan plates, supplemented with 20 µg/ml X-a-Gal (5-bromo-4-chloro-3-indolyl-a-D-galactopyranoside), and allowed to grow at 30 °C for 3–5 days.

α-Galactosidase Quantitative Assay—S-D-leucine-trypotphan cultures were inoculated with a single, fresh yeast colony and incubated overnight at 30 °C. The A590 was measured, and the supernatant was
rabbit antibodies to the respective proteins. The upper band either IFT57, IFT88, or pre-immune sera (IgG heavy chain. precipitation of IFT52 with anti-57 shows cross-reactivity with the goat

Harvested by centrifugation at 14,000 × g for 2 min. Sixteen microliters of the supernatant was combined with 48 μl of fresh assay buffer (2:1 ratio of 0.5 M sodium acetate, pH 4.5, to 100 mM p-nitrophenyl α-D-galactopyranoside (Sigma) and incubated at 30 °C for 1 h. The reaction was stopped with the addition of 136 μl of 1 M Na2CO3. Optical density was measured at 405 nm in a micro titer plate, and absorbance was calculated from the following formula: (milliunits/ml × cell) = (Afinal assay volume/1000)/(10.5 ml/umol/60 min)/16 μl of culture volume/Ainitial). Each yeast colony was assayed in triplicate.

RESULTS

An IFT Particle in the Sensory Cilia of Photoreceptors—We have extended the velocity sedimentation analysis of IFT complexes to photoreceptors in order to determine whether an ~17 S particle is present in cells with sensory cilia. The abundance of IFT proteins is lower in the retina compared with the testis, which previously made it difficult to demonstrate a complex associated with photoreceptors (12). To counter this problem, we prepared crude bovine ROS by mechanical disruption and broken outer segments as well as the basal body, the axoneme, and variable amounts of the inner segment but lacks other retinal neurons and glia. The crude ROS were then extracted in 0.25% Nonidet P-40, generating a supernatant fraction. After centrifugation on a 5–20% sucrose gradient, Western blots revealed that IFT57 and -88 co-fractionate at ~17 S (Fig. 1A). Comparison of the fractionation pattern of extracts from bovine photoreceptor to mouse testis (Fig. 1, A and C) demonstrated that the hydrodynamic properties of IFT proteins from both tissues were similar. The principal difference between bovine photoreceptors and testis was that IFT proteins were much more abundant in testis extracts, which permitted analysis of IFT52 and -20 (Fig. 1C); these two components are difficult to detect reliably in the gradients derived from photoreceptors. In testis both IFT20 and -52 co-fractionate with IFT57 and -88 at ~17 S. However, we also observed that only a portion of IFT20 co-fractionates with the remaining three IFT proteins; a substantial pool of unassembled IFT20 remained near the top of the gradient. In Chlamydomonas the homologues of all four mammalian IFT proteins are part of complex B, which dissociates from complex A at moderate ionic strength. We found that when both bovine ROS (Fig. 1B) and testis extracts (Fig. 1D) were prepared in moderate ionic strength buffer (300 mM NaCl), the IFT particle was partially dissociated, causing a similar shift in the peak of IFT protein from ~17 S to ~12 S. In addition, the extraction of total IFT protein was enhanced in the presence of 300 mM NaCl. This was most clearly seen in the photoreceptor samples (compare Fig. 1, A and B) but was also routinely observed in the testis samples. Similar to the low salt condition (Fig. 1C), only a portion of IFT20 co-fractionated with the remaining IFT components (Fig. 1D), while the remainder sedimented near the top of the gradient. However, a larger proportion of IFT20 was found co-fractionating with IFT88, -57, and -52 in the presence of 300 mM NaCl (compare Fig. 1, C and D). This suggests that moderate ionic strength favors association of IFT20 with a smaller, partially dissociated IFT complex. The continued co-fractionation of these mammalian homologues at increasing ionic strength suggests that an arrangement of subcomplexes similar to that proposed for Chlamydomonas is conserved in mammalian ciliated tissues.

Immunoprecipitation of IFT Components from Multiple Ciliated Cell Types—Co-immunoprecipitation assays demonstrated that the four IFT proteins that co-fractionate on velocity sedimentation gradients are components of the same complex in both photoreceptors and testis. We found that affinity-purified goat antibodies against two different IFT proteins, IFT88 and -57, precipitated all four IFT components in extracts of mouse retina, kidney, and testis (Fig. 2). The kidney lysates were included in these experiments to demonstrate that the IFT complex is also present in a tissue containing primary

Fig. 2. Co-immunoprecipitation of IFT components from multiple tissues. Lysates prepared from mouse testis (A), retina (B), or kidney (C) were incubated with goat antibodies to the N terminus of either IFT57, IFT88, or pre-immune sera (PI) or left untreated and then incubated with protein G beads. The specific precipitation of IFT57, -20, -88, and -52 (lower band) was demonstrated by Western blotting using rabbit antibodies to the respective proteins. The upper band in the precipitation of IFT52 with anti-57 shows cross-reactivity with the goat IgG heavy chain.
cilia, an organelle structurally and functionally different from photoreceptor cilia and motile flagella. Although IFT52 and -20 were difficult to detect in the velocity sedimentation analysis of photoreceptors (Fig. 1A), enrichment of IFT proteins by immunoprecipitation confirmed their presence in the photoreceptor IFT complex. Note that the upper band in the precipitation of IFT52 with anti-IFT57 shows cross-reactivity with the goat IgG heavy chain.

The immunoprecipitation of IFT proteins in different tissues is qualitatively similar, but the lower abundance of IFT proteins in the retina compared with the testis (Fig. 2, A and B) is apparent, particularly when IFT52 and -20 are analyzed. In addition, the anti-IFT57 immunoprecipitates pull down more IFT57 than is pulled down with anti-IFT88. Similarly, the anti-IFT88 immunoprecipitates pull down more IFT88 than is pulled down with anti-IFT57. This difference suggests that unassembled pools of both proteins are present. In summary, the immunoprecipitation data support the velocity sedimentation results of demonstrating that the IFT proteins are part of a complex and that this complex is conserved in multiple types of ciliated cells.

IFT57 Interacts Directly with IFT20—We utilized the yeast two-hybrid system to look for direct interactions among the four mammalian IFT proteins. IFT88, -57, -52, and -20 were expressed in yeast as fusion proteins with either the GAL4 activation or DNA-binding domain. Each pair was tested for interaction by growth on high stringency (S.D.-ade) medium (data not shown) and in quantitative #galactosidase assays. The intraflagellar transport model predicts that interactions between each pair were tested by #galactosidase quantitative assays as described in Fig. 3. C, the IFT20 deletion constructs and full-length IFT57 tested for interactions by #galactosidase quantitative assays.

![Diagram](image)

**Fig. 4.** Coiled-coil domains mediate the interaction between IFT57 and IFT20. A, deletion constructs used to map the IFT57-IFT20 interaction domains. Numbers indicate amino acids encoded by each clone; full-length IFT57 is 429 amino acids, and full-length IFT20 is 132 amino acids. Gray boxes indicate the position of predicted coiled-coil domains. B, the IFT57 deletion constructs and full-length IFT20 were expressed in AH109 yeast cells as fusion proteins with either the GAL4 activation or DNA-binding domain. Interactions between each pair were tested by #galactosidase quantitative assays as described in Fig. 3. C, the IFT20 deletion constructs and full-length IFT57 tested for interactions by #galactosidase quantitative assays.

coiled-coil domains near the C terminus (31), previously identified as a myosin-like domain and a pseudo-death effector domain (32). The strong interaction with IFT20 was abolished by a truncation of IFT57 lacking the C-terminal coiled-coil domains. Truncations encoding either of the two coiled-coil domains were sufficient to maintain a weaker but significant interaction with IFT20 (Fig. 4B). We conclude that the entire extended coiled-coil region, including the pseudo-death effector domain, in IFT57 is required for optimal interaction with IFT20.

Secondary structure analysis of the IFT20 sequence also predicted a coiled-coil domain near the C terminus of the protein as the only candidate protein-protein interaction motif (31). Deletion of this region caused a greatly reduced interaction but did not abolish it completely. In contrast, the coiled-coil domain by itself was sufficient for a reduced, but significant, interaction with IFT57 (Fig. 4C). Thus, the primary interaction domain between IFT57 and IFT20 are the coiled-coil domains in each protein, although secondary contacts outside of these domains may further stabilize the interaction.

**Regulated Interaction Between Heterotrimeric Kinesin II and the IFT Complex**—The salient feature of the IFT complex is its association with motility within cilia and flagella. Genetic studies in *Chlamydomonas* have demonstrated that the motility of IFT particles or individual IFT components requires the activity of kinesin II for anterograde movement and cytoplasmic dynein 1b for retrograde movement (2–4, 13, 17). It has also been reported that kinesin II co-immunoprecipitates with an IFT complex in *Chlamydomonas* (2). To determine whether either of the motors was associated with the mammalian IFT complex in our preparations, we probed IFT immunoprecipitates with antibodies against the subunits of kinesin II or cytoplasmic dynein. Neither cytoplasmic dynein heavy chain 2 nor light intermediate chain 3 was detected (data not shown). We did observe that all three subunits of kinesin II, KIF3A, KIF3B, and KAP3, co-immunoprecipitated with the IFT components from testis and retina (Fig. 5, A and B). Similar results were obtained from kidney (data not shown).

The intraflagellar transport model predicts that interactions between the IFT complex and motors would be transient. To determine whether the interaction between the IFT complex
and kinesin II was affected by ionic strength, testis lysate was supplemented with increasing amounts of NaCl prior to immunoprecipitation. The co-immunoprecipitation of IFT proteins remained stable at the highest salt concentration tested (Fig. 5C). However, raising the salt concentration above physiological conditions disrupted the interaction between kinesin II and the stable, IFT subcomplex containing IFT88, -57, -52, and -20.

We also tested the effect of nucleotides on the association between IFT components and kinesin II. The addition of 1 mM ATP to the testis lysate prevented the co-immunoprecipitation of kinesin II with the IFT complex, whereas the non-hydrolyzable ATP analog, AMP-PNP, had no effect (Fig. 6). However, ATPγS, a stable ATP analog that can be used to generate thiophosphorylated proteins, mimicked the action of ATP, releasing kinesin II from the IFT complex. None of these treatments disrupted interactions among the four IFT proteins. Also note that we did not detect conventional kinesin heavy chain (KHC) or tubulin in these immunoprecipitation experiments (Fig. 6). In conclusion, we have demonstrated a physical interaction between the mammalian IFT complex and heterotrimeric kinesin II that is regulated by ATP hydrolysis.

The Kinesin II Subunit, KIF3B, Interacts Directly with IFT20—We used yeast two-hybrid assays to determine whether kinesin II interacted with the IFT complex directly via one of the four known mammalian IFT subunits. Constructs encoding the C terminus of KIF3A, KIF3B, or the full open reading frame of KAP3 were expressed in yeast as fusion proteins with either the Gal4 activation domain or DNA-binding domain (Fig. 7B). These fusion proteins were tested pair-wise against each other and the four IFT proteins. Each pair was tested for interaction by growth on high stringency (S.D.-ade-nine-histidine-leucine-tryptophan + X-a-gal) medium (data not shown) and in quantitative α-galactosidase activity assays. KIF3A and KIF3B interacted with each other as expected (Fig. 7A). However, interaction between KAP3 and either of the heavy chains was not detected in this assay, possibly because both heavy chains are required to stabilize the KAP3 interaction. Of the four IFT proteins, only IFT20 showed an interaction with a kinesin II subunit. IFT20 binds to KIF3B in an interaction that was quantitatively weaker than IFT20 paired against IFT57 (Fig. 7A) but similar to the positive control. The kinesin II motor chains are similar in sequence and domain structure, being composed of an N-terminal motor domain, central helical rod, and C-terminal globular tail domain. However, IFT20 interacted specifically with KIF3B, not KIF3A. A deletion construct of KIF3B encoding just the globular tail domain, being composed of an N-terminal motor domain, central helical rod, and C-terminal globular tail domain. However, IFT20 interacted specifically with KIF3B, not KIF3A. A deletion construct of KIF3B encoding just the globular tail domain did not maintain the interaction with IFT20, suggesting that IFT20 overlaps with the KIF3A binding regions in the helical rod (Fig. 7C). The deletion constructs of IFT20 used to map the interaction domain with IFT20 were also tested against KIF3B. Unlike the case with IFT57 (compare with Fig. 4C), any disruption of IFT20 prevented association with KIF3B, suggesting that KIF3B makes multiple contacts with IFT20.

DISCUSSION

The 17 S IFT Complex Is Conserved in Mammalian Sensory Cilia and Motile Flagella—Intraflagellar transport is a highly conserved mechanism for the assembly and maintenance of cilia and flagella. The IFT particle described in Chlamydomo-
is a large complex composed of at least 15 polypeptides (1, 2). It has previously been shown that mammalian homologues of the IFT proteins, IFT88/Polaris, IFT57/Hippi, IFT52/NGD5, and IFT20 also co-sediment at an ~17-S complex in extracts of mouse testis (12). Because IFT has been implicated in the maintenance of diverse classes of cilia, including primary cilia and the modified sensory cilium of photoreceptors, we examined interactions among these four proteins to determine whether the architecture of the IFT particle was conserved or modified in cells with specialized cilia.

Velocity sedimentation analysis demonstrated that IFT proteins from bovine photoreceptors co-fractionate as an ~17 S complex similar to the motile flagella of *Chlamydomonas* and mouse testis. We observed that antibodies against two different IFT proteins immunoprecipitate the other three components from tissues containing motile flagella (testis), primary cilia (kidney), and sensory cilia (retina), verifying that these four IFT subunits are part of the same complex.

**Organization of the IFT Complex**—We demonstrate that the IFT proteins from both the sensory cilium of photoreceptors and the motile flagella of testis continue to co-fractionate in velocity sedimentation gradients at an increased salt concentration even though the IFT complex is partially dissociated. Furthermore, high salt concentrations do not disrupt the co-immunoprecipitation of the four known IFT subunits, verifying that IFT88, -57, -52, and -20 are part of a stable subcomplex. This is consistent with the analysis of IFT particles in *Chlamydomonas* that revealed that the IFT particle was composed of two complexes, A and B, that could be dissociated by an increase in ionic strength (2). However, complex A and B each sedimented at ~16 S, whereas the salt dissociated particle we observed was at ~12 S. Although all four of the IFT proteins examined in our analysis are part of complex B in *Chlamydomonas* and the 12 S particle in mouse testis, the difference in hydrodynamic properties suggests that the 12 S complex is not identical to complex B in *Chlamydomonas*. Further studies that include mammalian homologues of *Chlamydomonas* complex A proteins will be necessary to elucidate the overall properties of mammalian IFT complexes.

Interestingly, our velocity sedimentation analysis of mouse testis demonstrates that IFT20 behaves differently from the other three IFT subunits. We always observe a peak of IFT20 co-fractionating with IFT88, -57, and -52, but even in low salt conditions where the IFT complex is large, a second peak of IFT20 fractionates near the top of the gradient. We conclude that only a fraction of IFT20 is part of the IFT complex.

Our yeast two-hybrid analysis indicates that the IFT20 that is a part of the IFT complex is bound via a direct interaction with IFT57. Interestingly, a point mutation in CHE-13, the *C. elegans* homologue of IFT57, generates a truncated protein lacking the coiled-coil domains (the IFT20 interaction domain) (33). This mutation prevents the assembly of sensory cilia, strongly indicating that the link between IFT57 and IFT20 is required for functional IFT.

Because we have shown that the amount of IFT88 protein pulled down by IFT57 is not representative of the entire IFT88 population, and *vice versa*, we suggest that there are additional pools of both proteins that are not fully assembled into the IFT...
complex. However, this pool of free IFT88 and IFT57 must be very small because it is not readily detected in the gradients.

This is consistent with a model in which IFT proteins accumulate near the basal body before assembling into a complex that is transported along the flagellum, originally suggested to account for the immunocytochemical localization of IFT proteins in Chlamydomonas and mammals (2, 12, 21, 34, 35).

Regulation of the Interaction between Kinesin II and the IFT Complex by ATP—It is well established that one of the functions of heterotrimeric kinesin II is intraflagellar transport, based largely on genetic experiments in multiple systems reviewed in Refs. 20, 36, and 37). Kinesin II is composed of two heavy chains, KIF3A and KIF3B, and an accessory subunit, KAP3. Deletion of either Kif3a or Kif3b in mice leads to the absence of primary cilia on the ventral node and embryonic lethality (38, 39). Furthermore, photoreceptor-specific deletion of Kif3a causes disruption of the outer segment structure and cell death (24); similarly, kidney-specific deletion of this gene prevents assembly of the primary cilium (41). This clearly establishes that one of the roles of kinesin II is the assembly and maintenance of cilia. However, the direct link between kinesin II and the IFT complex is not understood. We provide co-immunoprecipitation and yeast two-hybrid data that demonstrate a physical interaction between these two complexes.

The interaction between kinesin II and IFT occurs at physiological salt conditions, but kinesin II is readily dissociated at high salt even though the IFT subcomplex containing IFT88, -57, -52, and -20 is maintained. Additionally, we observe that the interaction at physiological salt is regulated by ATP hydrolysis. Kinesin II does not co-immunoprecipitate with the IFT complex in the presence of ATP or ATPγS; it does co-immunoprecipitate in the presence of the non-hydrolyzable ATP analog, AMP-PNP. The motor domains of kinesin motors hydrolyze ATP to produce mechanical force, and ATP can be used to release kinesin from microtubules. In our experiments conventional kinesin heavy chain, a component of kinesin I, was not detected in the co-immunoprecipitates, demonstrating that the ATP-regulated interaction with IFT is specific to kinesin II. Furthermore, the ATP-mediated release of kinesin II from the IFT complex must be distinct from kinesin-microtubule interactions because tubulin is not co-immunoprecipitated in our preparations. The effect of ATP hydrolysis that we observe may be because of the activation of a kinase that directly phosphorylates either IFT20 or KIF3B. Alternatively, the ATPase may be a chaperone such as Hsc70, which has been shown to release kinesin I from vesicles (42).

Further studies are underway to elucidate this mechanism.

IFT20 Functions as an Adaptor between the IFT Complex and Heterotrimeric Kinesin II—IFT is thought to assemble and maintain the length of cilia and flagella by facilitating the transport of cargo proteins to the site of incorporation in the distal axoneme (20). However, the functions of the individual IFT subunits have not been identified. In general, an IFT subunit may play one or more of three different roles in this process. An IFT subunit may function in a structural role such that it is required for the assembly or stabilization of the entire complex. Alternatively, an IFT subunit may function as an adaptor between the complex and the microtubule motors or as an adaptor between the complex and the cargo that is being transported.

Our yeast two-hybrid analysis showing an interaction between the KIF3B subunit of kinesin II and IFT20 leads us to suggest that the specific function of IFT20 is to link the kinesin II motor to the IFT complex. We propose that IFT57 recruits IFT20 to the IFT complex, and then IFT20 recruits the heterotrimeric kinesin II complex by binding to KIF3B. According to this hypothesis, IFT20 would be essential for anterograde transport in cilia and flagella (Fig. 8).

The direct interaction of IFT20 with KIF3B is unique because all of the previously described binding partners bind to the motor through an interaction with the accessory subunit, KAP3 (29, 43–46). In this respect IFT20 resembles some of the binding partners that have been described for other kinesin family members, such as mLin10 binding directly to KIF17 (47) or RanBP2 binding directly to the stalk region of KIF5B and KIF5C (40).

The deletion constructs of IFT57, IFT20, and KIF3B allow us to describe the interactions among these proteins in greater detail. The coiled-coil domains in IFT57 and IFT20 mediate their interaction. KIF3B also interacts with the coiled-coil domain of IFT20, but unlike IFT57 our deletion analysis indicated that additional contacts in the N terminus of IFT20 might be required for the interaction with the motor. The KIF3B construct used in these studies lacks the motor domain but contains the C-terminal portion of the coiled-coil domain required for dimerization with KIF3A and the globular tail domain. The deletion construct encoding only the globular tail domain of KIF3B could not maintain the interaction with either KIF3A or IFT20, indicating that these two proteins share an overlapping interaction domain with KIF3B in the coiled-coil domain.

In conclusion, we have demonstrated that the features of a large IFT particle are conserved from green algae to the retina, kidney, and testis of mammals, representing the three major classes of ciliary organelles. We also provide biochemical and yeast two-hybrid data that physically link the IFT complex to the anterograde motor, kinesin II, an interaction that is regulated by ATP hydrolysis. Furthermore, we propose that IFT20 specifically functions as an adaptor between the IFT complex and kinesin II by directly binding to both IFT57 and KIF3B.

Acknowledgments—We thank George Witman, Win Sale, Virgil Muresan, and Douglas Cole for helpful discussions.

Note Added in Proof—Additional recent studies on Chlamydomonas reporting co-immunoprecipitation of kinesin II with the IFT complex (Qin, H., Diener, D. R., Geimer, S., Cole, D., and Rosenbaum, J. (2002) Mol. Biol. Cell 13, 473 (abstract)) and defining additional protein interactions within the complex (Lucker, B. F., Blackman, P., Qin, H., Rosenbaum, J. L., and Cole, D. (2002) Mol. Biol. Cell 13, 190 (abstract); Cole, D. (2003) Traffic 4, 436–442) further emphasize the highly conserved nature of IFT among eukaryotes.

REFERENCES
1. Pipergo, G., and Moad, K. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 4457–4462
2. Cole, D. G., Diener, D. R., Himelblau, A. L., Beech, P. L., Fuster, J. C., and Rosenbaum, J. L. (1998) J. Cell Biol. 141, 985–1008
3. Kozminski, K. G., Beech, P. L., and Rosenbaum, J. L. (1995) J. Cell Biol. 131, 1517–1527
4. Pazour, G. J., Dickert, B. L., and Witman, G. B. (1999) J. Cell Biol. 144,
