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Article

Dissecting the Molecular Mechanisms of Intraflagellar Transport in *Chlamydomonas*

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Summary

Background: The assembly and maintenance of eukaryotic cilia and flagella are mediated by intraflagellar transport (IFT), a bidirectional microtubule (MT)-based transport system. The IFT system consists of anterograde (kinesin-2) and retrograde (cDynein1b) motor complexes and IFT particles comprising two complexes, A and B. In the current model for IFT, kinesin-2 carries cDynein1b, IFT particles, and axonemal precursors from the flagellar base to the tip, and cDynein1b transports kinesin-2, IFT particles, and axonemal turnover products from the tip back to the base. Most of the components of the IFT system have been identified and characterized, but the mechanisms by which these different components are coordinated and regulated at the flagellar base and tip are unclear.
Results: Using a variety of *Chlamydomonas* mutants, we confirm that cDynein1b requires kinesin-2 for transport toward the tip and show that during retrograde IFT, kinesin-2 can exit the flagella independent of the cDynein1b light intermediate chain (LIC) and IFT particles. Furthermore, using biochemical approaches, we find that IFT complex B can associate with cDynein1b independent of complex A and cDynein1b LIC. Finally, using electron microscopy, we show that the IFT tip turnaround point most likely is localized distal to the plus end of the outer-doublet B MTs.
Conclusion: Our results support a model for IFT in which tip turnaround involves (1) dissociation of IFT complexes A and B and release of inactive cDynein1b from complex B, (2) binding of complex A to active cDynein1b, and (3) reassociation of complex B with A prior to retrograde IFT.

Introduction

Cilia and flagella are dynamic microtubule (MT)-based organelles that assemble from and continuously turn over at the flagellar tip [1, 2]. These organelles emanate from the surface of many eukaryotic cells and have diverse roles in motility and sensory perception. Absent or defective cilia/flagella have been associated with severe human diseases such as respiratory disease, polycystic kidney disease, retinal degenerative disease, infertility, defective left-right-axis determination, and Bardet-Biedl syndrome [3, 4]. Proper assembly and maintenance of cilia and flagella are therefore critical to human health.

The assembly and maintenance of cilia/flagella depend on a process called intraflagellar transport (IFT; [5–7]). IFT is a bidirectional MT-based motility system that transports flagellar precursors from the cell body to the flagellar tip for assembly and returns flagellar turnover products to the cell body [8]. The IFT system consists of anterograde (base to tip) and retrograde (tip to base) motor complexes associated with groups of large protein complexes called IFT particles. Genetic and biochemical analyses in model organisms such as *Chlamydomonas* and *Caenorhabditis elegans* have led to identification of most of the IFT motor subunits, as well as most of the IFT-particle-polypeptide genes [7, 9].

In *Chlamydomonas*, anterograde IFT appears to be driven by a single motor complex, heterotrimeric kinesin-2, which consists of two motor subunits, FLA10 and FLA8, as well as a nonmotor subunit called Kinesin-Associated Protein (KAP) [10–13]. A null mutant in *FLA10* fails to assemble flagella, indicating that heterotrimeric kinesin-2 is essential for flagellar assembly in *Chlamydomonas* [14]. In *C. elegans* sensory cilia, however, two different kinesin-2 motors appear to work cooperatively during anterograde IFT: a heterotrimeric kinesin-2 and a homodimeric kinesin-2, OSM-3 [15]. Heterotrimeric kinesin-2 and OSM-3 function redundantly to build the proximal and middle segments—which contain doublet MTs—of the *C. elegans* ciliary axoneme, whereas transport of IFT particles along the distal segment, composed of singlet MTs, is carried out by OSM-3 [15]. *Chlamydomonas* flagella do not appear to contain an OSM-3 ortholog [16], and therefore anterograde IFT in *Chlamydomonas* is probably less complex than in *C. elegans*.

The retrograde IFT motor complex, cytoplasmic dynein 1 b (cDynein1b), contains heavy chain (HC) and light intermediate chain (LIC) subunits [17–22] and probably additional as-yet-unidentified subunits like intermediate and light chains [23]. Inactivation of the genes for the HC or LIC subunit in *Chlamydomonas* leads to stumpy flagella containing large accumulations of IFT particles [17, 18, 22], consistent with cDynein1b functioning in retrograde IFT. Inactivation of the dynein light chain 8 (LC8) subunit leads to a similar phenotype [24], but LC8 does not appear to be an integral part of the cDynein1b complex [21].

IFT particles can be isolated biochemically from *Chlamydomonas* flagella and separated into two complexes, A and B, which collectively contain at least 17 different polypeptides [25, 26]. Both IFT complexes A and B can

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be immunoprecipitated (IPed) and immunodepleted from flagellar extract by using a polyclonal antibody to the complex B polypeptide IFT72/74 [8], suggesting that the majority of IFT complexes A and B are bound to each other during anterograde and retrograde IFT in the flagellum. Most of the IFT polypeptide genes have been cloned and characterized and were found to encode proteins with sequence motifs/repeats associated with transient protein-protein interactions [9]. Similar to mutations in *FLA10* [11, 12, 14, 27, 28], mutations in genes encoding IFT particle proteins have indicated a role for IFT in flagellar assembly and maintenance [9, 29–32].

Although significant progress has been made in identifying and characterizing individual components of the IFT system, important questions remain. For example, the mechanisms involved in IFT tip turnaround are unknown. IFT tip turnaround involves unloading of axonemal precursors for assembly, inactivation/downregulation of kinesin-2, loading of axonemal turnover products, and activation/upregulation of cDynein1b. These events occur very rapidly, because there is no build-up of IFT particles in the tips of wild-type flagella.

We have used genetic and biochemical approaches to begin to dissect the molecular mechanisms of IFT tip turnaround in *Chlamydomonas.* Recently, we showed that *FLA11* encodes IFT particle polypeptide IFT172, a component of IFT complex B [32]. Interestingly, a temperature-sensitive (ts) mutant, *fla11*, with a single amino acid substitution in IFT172 is defective in IFT turnaround at the flagellar tip [27, 28, 32, 33], suggesting a role for IFT172 in tip turnaround. In support of this idea, IFT172 interacts with EB1 [32], which localizes to the flagellar tip [34].

By using additional *Chlamydomonas* IFT mutants, we show here that both cDynein1b HC and LIC depend on heterotrimeric kinesin-2 for transport into flagella, whereas removal of kinesin-2 from flagella can occur independent of cDynein1b LIC and IFT complexes A and B. Furthermore, we show that IFT complex B can associate, at least indirectly, with the cDynein1b HC independent of complex A and cDynein1b LIC. Finally, by electron microscopy (EM) analysis we show that the tip turnaround point for IFT particles most likely is localized distal to the plus end of the outer-doublet B MTs. On the basis of these results, as well as previously published data, we propose a model for IFT in which tip turnaround consists of three distinct steps: (1) dissociation of IFT complexes A and B and release of inactive cDynein1b from complex B into the flagellar tip compartment, (2) binding of complex A to active cDynein1b, and (3) reassociation of complex B with A prior to retrograde IFT.

Results

Kinesin-2 Is Required for Transport of cDynein1b into the Flagellum

The *fla10-1* strain harbors a ts point mutation in the region of *FLA10* corresponding to the motor domain of the FLA10 kinesin-2 subunit [11, 12]. Previous work demonstrated that FLA10 is essential for anterograde IFT of complexes A and B [26] and for cDynein1b HC [33]. Immunoblot analysis of flagella isolated from *fla10-1* cells incubated for 2 hr at the permissive (22ºC) or restrictive (32ºC) temperature confirmed these results and also showed that cDynein1b LIC, like the HC, requires FLA10 for transport into the flagella, because the flagellar level of cDynein1b LIC is reduced in *fla10-1* cells incubated at 32ºC (Figure 1, lane 6; Figure S1 in the Supplemental Data available online). Note that although there is still some kinesin-2 present in *fla10-1* flagella at 32ºC, this kinesin-2 is inactive as a result of the mutation in *FLA10*.

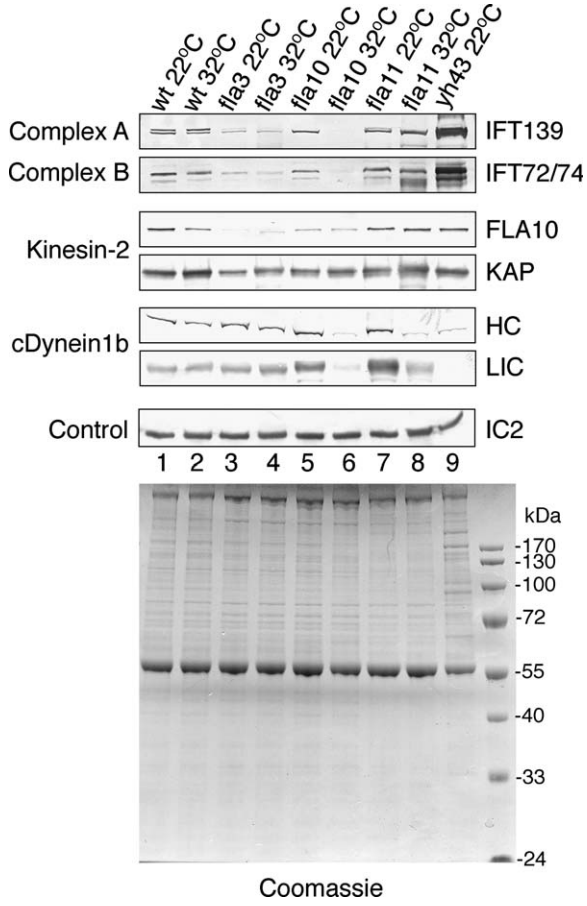

Figure 1. Levels of IFT Particle Polypeptides and Motor Subunits in Various IFT Mutant Flagella

Wild-type (wt), *fla3-1/kap*, *fla10-1*, *fla11/ift172*, and *yh43/d1blic* mutant cells grown in M1 medium were incubated for 2 hr at 22º or 32ºC prior to deflagellation. Purified flagella were analyzed by SDS-PAGE and immunoblotting with antibodies as indicated to the right. IC2 is the intermediate chain 2 of the axonemal outer-arm dynein complex and serves as a loading control. The bottom panel shows a Coomassie-blue-stained gel run in parallel. A quantitative analysis of the data is shown in Figure S1. As a result of the use of M1 medium instead of Tris-acetate-phosphate (TAP) medium for *Chlamydomonas* culture, the changes in the average flagellar levels of IFT complexes A and B observed in *fla11/ift172* mutant flagella (lanes 7 and 8) were not as prominent as those previously reported by us [32, 34], but comparable to those reported by others using M1 medium for *Chlamydomonas* culture [33].

The *fla3-1/kap* strain contains a point mutation in the region of *KAP* corresponding to the C-terminal predicted cargo binding site of the kinesin-2 KAP subunit [10]. Previous work indicated that this mutation impairs the targeting or retention of the kinesin-2 complex at the site of flagellar assembly and results in frequent and

prolonged pauses of IFT particles traveling in the anterograde direction within flagella [10]. To assess the potential effect of the *fla3-1/kap* mutation on the flagellar localization of the cDynein1b complex, we isolated flagella from *fla3-1/kap* cells incubated at 22ºC or 32ºC for 2 hr and analyzed these by immunoblotting with antibodies specific for cDynein1b HC and LIC. The results showed that the *fla3-1/kap* mutation does not significantly alter the flagellar level of cDynein1b subunits (Figure 1, compare lanes 1 through 4; Figure S1), whereas the flagellar level of the FLA10 kinesin-2 subunit is significantly reduced at both 22ºC and 32ºC in *fla3-1/kap* cells, consistent with a previous report [10]. Taken together, our data indicate that heterotrimeric kinesin-2 is required for transport of cDynein1b into the flagella, and that the small amount of (active) kinesin-2/FLA10 present in *fla3-1/kap* flagella is sufficient for this transport to occur.

Kinesin-2 Does Not Require the cDynein1b LIC Subunit to Exit the Flagellum

Previous work identified strain *yh43* as a *Chlamydomonas cDynein1b LIC* insertional mutant, and it was shown that IFT complex B polypeptides accumulate in flagella of this strain. Furthermore, it was shown that cDynein1b HC does not accumulate in *yh43/d1blic* mutant flagella, implying that the cDynein1b HC does not require the LIC to travel out of the flagella [22]. We found by immunoblot analysis that IFT complex A, similar to IFT complex B, also accumulates in *yh43/d1blic* flagella (Figure 1, compare lanes 1 and 9; Figure S1). This is consistent with previous results from *C. elegans* [20] and indicates that the LIC subunit of the cDynein1b complex is required for the transport of both IFT complexes A and B out of the flagellum.

The fate of anterograde IFT motor subunits in *yh43/d1blic* flagella has not previously been studied. We found by immunoblot analysis that the FLA10 and KAP kinesin-2 subunits accumulate only slightly in *yh43/d1blic* flagella compared to IFT particle polypeptides, which accumulate approximately 3–5-fold in these flagella compared to wild-type flagella (Figure 1, compare lanes 1 and 9; Figure S1). Because there is accumulation of both IFT complexes A and B in the flagella, kinesin-2 must be able to undergo anterograde IFT in *yh43/d1blic* cells. Therefore, the lack of kinesin-2 accumulation in *yh43/d1blic* flagella suggests that kinesin-2 can detach from the IFT particles and can be removed from flagella independent of cDynein1b LIC.

Fate of Kinesin-2 and cDynein1b in *fla11* Mutant Flagella

The *fla11* strain harbors a single ts point mutation in the gene encoding the IFT complex B protein IFT172 [32]. Previous work showed that at the permissive temperature (22ºC), the rate of IFT particles leaving the *fla11/ift172* flagellar tips is reduced [33], and IFT complex B polypeptides, but not complex A, accumulate near the flagellar tip [32–34]. At the restrictive temperature (32ºC), however, IFT complex A polypeptides, in addition to IFT complex B, accumulate near the flagellar tip of this strain [32, 34]. The effect of the *fla11/ift172* mutation on kinesin-2 and cDynein1b subunits has not previously been studied. Therefore, to study the fate of these IFT motor subunits in *fla11/ift172* flagella, we first isolated flagella from cells of this strain incubated at 22ºC or 32ºC for 2 hr and did immunoblot analysis with antibodies against KAP and FLA10 (kinesin-2 subunits) and cDynein1b HC and LIC. The results showed that kinesin-2 does not accumulate in *fla11/ift172* flagella at either 22ºC or 32ºC (Figure 1, lanes 7 and 8; Figure S1), suggesting that kinesin-2 can detach from IFT particles and travel out of the flagella of this strain whereas IFT complexes A and B are retained near the tip. This interpretation is consistent with the results of the *yh43/d1blic* mutant analysis described above in that complexes A and B are retained in *yh43/d1blic* flagella whereas kinesin-2 is not. In contrast, at the permissive temperature (22ºC), both cDynein1b HC and LIC accumulate in *fla11/ift172* flagella (Figure 1, lane 7; Figure S1) compared to at the restrictive temperature (32ºC), where the level of both the cDynein1b HC and LIC is reduced in *fla11/ift172* flagella (Figure 1, lane 8; Figure S1). Immunofluorescence microscopy (IFM) confirmed that cDynein1b accumulates in *fla11/ift172* flagella at 22ºC, but not at 32ºC, and also showed that the accumulated cDynein1b colocalizes with aggregated IFT complex B protein (IFT172) near the tip of *fla11/ift172* flagella at 22ºC (Figure 2). These observations suggest that at 22ºC, some (inactive) cDynein1b is retained near the tip of *fla11/ift172* flagella, and upon shifting of the temperature to 32ºC for 2 hr, this "retained" cDynein1b is released/activated and travels back to the cell body while a significant amount of IFT complexes A and B is retained near the flagellar tip. In combination with our immunoblot analysis of *yh43/d1blic* flagella, in which there is little or no accumulation of kinesin-2 and cDynein1b HC, but a large accumulation of IFT complexes A and B (Figure 1, lane 9; figure S1), the results indicate that kinesin-2 and cDynein1b can exit the flagella when no or little of IFT complexes A and B protein is associated with them.

Accumulation of IFT Complex A in *fla11/ift172* Flagella at 32ºC Precedes the Depletion of the cDynein1b Complex

As shown in Figure 1 and Figure S1, IFT complex A accumulates in *fla11/ift172* flagella after ~2 hr incubation at the restrictive temperature (32ºC) whereas cDynein1b levels decrease. To understand the correlation between these two events, we performed a time-course experiment. Flagella were isolated from *fla11/ift172* cells incubated at 32ºC for 0, 30, 60, and 120 min and were analyzed by immunoblotting with antibodies against IFT complex A (IFT139), complex B (IFT72/74), and cDynein1b HC and LIC. As a control, flagella were isolated from wild-type cells incubated at 32ºC for 0 min or 120 min and were analyzed in parallel. This analysis showed that IFT complex A begins to accumulate in *fla11/ift172* flagella after ~60 min incubation at 32ºC, prior to the depletion of cDynein1b subunits at ~120 min (Figure 3 and data not shown). The level of IFT complex B polypeptides in *fla11/ift172* flagella remained elevated over wild-type levels throughout the time course (Figure 3), consistent with previous reports [32, 34]. These results indicate that the accumulation of IFT complex A in *fla11/ift172* flagella at 32ºC is unlikely to result from depletion of the cDynein1b complex but most likely is due to a conformational change that occurs in the mutant

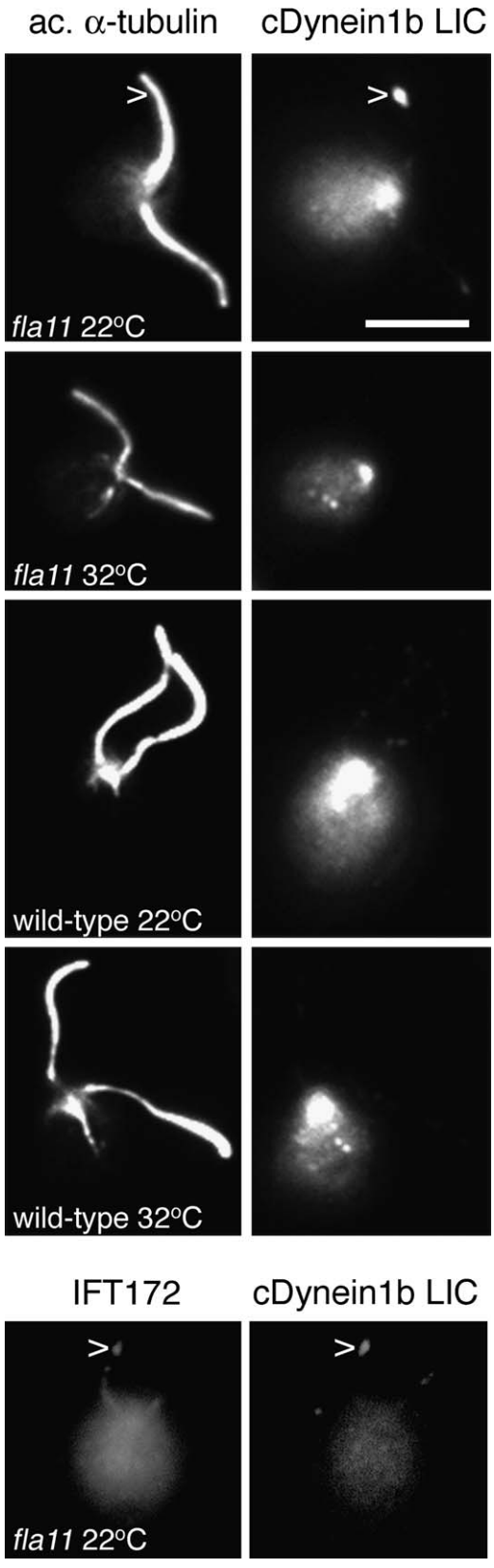

Figure 2. cDynein1b LIC Accumulates near the Tip of *fla11/ift172* Flagella at the Permissive Temperature

Cells grown in M1 medium were incubated at 22° or 32°C for 2 hr, fixed with methanol, and subjected to IFM with a polyclonal antibody against cDynein1b LIC and monoclonal anti-acetylated α-tubulin or IFT172 (complex B) antibody. Carets indicate accumulation of cDynein1b LIC near the flagellar tip of *fla11/ift172* cells at 22°C at sites of IFT complex B (IFT172) accumulation. The scale bar represents 2.5 μm.

IFT172 protein and affects the interaction between IFT complexes A and B in *fla11/ift172* flagella (see Discussion for details).

The cDynein1b HC Can Associate with IFT Complex B Independent of IFT Complex A and cDynein1b LIC

In *C. elegans*, transport of cDynein1b LIC into sensory cilia is not impaired in IFT complex A mutants [20] even though active heterotrimeric kinesin-2 appears to associate with IFT particles via complex A [35]. A likely explanation that reconciles these observations is that during anterograde IFT, the inactive cDynein1b complex is associated with IFT complex B, which in turn is associated with OSM-3 kinesin-2 [35], leading to OSM-3-mediated transport of both IFT complex B and (inactive) cDynein1b into the sensory cilia of IFT complex A mutant worms. To test this idea and whether it applies to *Chlamydomonas*, we first asked whether IFT complex B can associate with cDynein1b independent of IFT complex A. To this end, IP of wild-type *Chlamydomonas* flagellar extract with a monoclonal antibody, MAb 172.1 [26], specific for the complex B polypeptide IFT172 was performed. This antibody primarily co-IPs IFT complex B polypeptides, but not IFT complex A [32]. As shown in Figure 4A, cDynein1b HC co-IPed with MAb IFT172.1, whereas IFT complex A and the FLA10 kinesin-2 subunit did not. These results demonstrate that cDynein1b HC can associate, at least indirectly, with IFT complex B independent of IFT complex A. Because we did not observe complex-A-independent association between complex B and the FLA10 kinesin-2 subunit, the results are also consistent with *C. elegans* data indicating that heterotrimeric kinesin-2 is linked to complex A, not complex B, during anterograde IFT [35].

Next, we sought to determine whether the observed complex-A-independent association between complex B and cDynein1b HC (Figure 4A) occurs during anterograde or retrograde IFT. Retrograde transport of complex B, and hence its association with active cDynein1b, in *Chlamydomonas* requires cDynein1b LIC [22]. Therefore, if the complex-A-independent association between complex B and cDynein1b HC occurs during retrograde IFT in *Chlamydomonas*, this association should depend on cDynein1b LIC. We therefore tested whether complex-A-independent association between complex B and cDynein1b HC can occur in the absence of cDynein1b LIC. To this end, IP of *yh43/d1blic* flagella lacking cDynein1b LIC was performed with MAb 172.1. As shown in Figure 4B, immunoblot analysis of the IP samples showed that cDynein1b HC from *yh43/d1blic* mutant flagellar extract co-IPed with MAb 172.1 antibody, whereas complex A proteins did not co-IP. Therefore we conclude that the complex-A-independent association of cDynein1b HC with IFT complex B (Figure 4A) occurs independent of cDynein1b LIC (Figure 4B) and hence is unlikely to occur during retrograde IFT.

If we assume that the complex-A- and cDynein1b-LIC-independent association between cDynein1b and complex B (Figure 4) occurs during anterograde IFT,

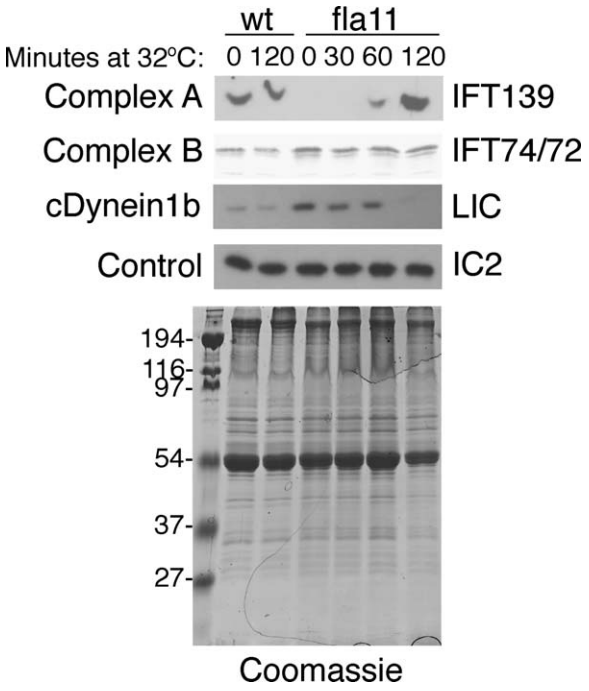

Figure 3. Time Course of Complex A Accumulation and cDynein1b Depletion in *fla11/ift172* Flagella

Wild-type (wt) and *fla11/ift172* cells grown in M1 medium were incubated at 32°C for 0, 30, 60, or 120 min prior to deflagellation. Isolated flagella were analyzed by SDS-PAGE and immunoblotting with antibodies directed against IFT139 (complex A), IFT72/74 (complex B), cDynein1b LIC, or IC2 of the axonemal outer-arm dynein complex (loading control). The bottom panel shows a Coomassie-blue-stained gel run in parallel, demonstrating equal protein loads. Note that IFT139 begins to accumulate in *fla11/ift172* flagella after 60 min, whereas cDynein1b LIC is depleted from flagella at 120 min. Complex B (IFT72/74) flagellar levels are elevated, relative to wild-type flagellar levels, in *fla11/ift172* cells throughout the time course.

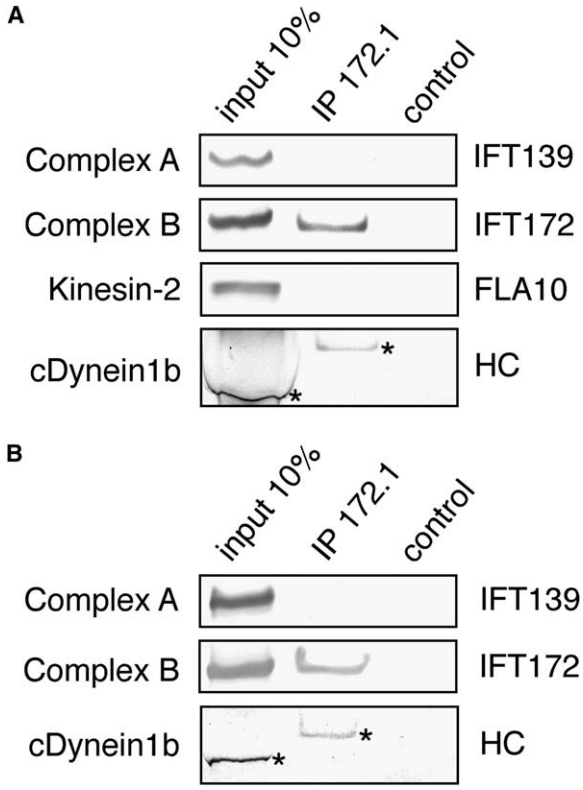

Figure 4. Association of cDynein1b HC with IFT Complex B

(A) IP of wild-type flagellar extract with a monoclonal antibody, MAb 172.1 [26], specific for the complex B polypeptide IFT172. Flagellar extract ("input 10%") and IP pellets were analyzed by SDS-PAGE and immunoblotting with antibodies as indicated. MAb 172.1 co-IPs complex B polypeptides (IFT172) and cDynein1b HC, whereas complex A (IFT139) and kinesin-2 (FLA10) do not co-IP (middle lanes). The control lane contains pellet samples from a parallel experiment in which the MAb 172.1 was omitted. Note that the cDynein1b HC that co-IPs with MAb 172.1 migrates slower than cDynein1b in the crude flagellar extract/input lane (the HC bands are marked by asterisks).

(B) IP of flagellar extract lacking the cDynein1b LIC subunit. The experiment was done as in (A) except that flagella from a *cDynein1b LIC* insertional mutant (*yh43/d1blic*) were used. Note that cDynein1b HC still co-IPs with MAb 172.1 in the absence of cDynein1b LIC.

and that during anterograde IFT, complex B in turn is associated with IFT complex A linked to (active) heterotrimeric kinesin-2 (see above), anterograde IFT of cDynein1b HC should be independent of cDynein1b LIC. This does indeed seem to be the case because the cDynein1b HC localizes normally in *yh43/d1blic* null-mutant flagella [22], indicating that its transport into the flagella is not impaired. Therefore, the simplest interpretation of our data is that the complex-A- and cDynein1b-LIC-independent association of complex B and cDynein1b HC occurs during anterograde IFT.

Interestingly, in our IP experiments (Figure 4), we observed that cDynein1b HC that co-IPed with MAb172.1 migrated much more slowly during SDS-PAGE than cDynein1b HC from crude flagellar extract ("input 10%" in Figure 4), suggesting that binding (direct or indirect) of IFT172/complex B to cDynein1b HC leads to modification and/or altered conformation of cDynein1b HC. However, the significance and nature of this modification/altered conformation of cDynein1b HC remains to be determined.

EM Analysis of IFT Tip Turnaround

In *Chlamydomonas*, the outer-doublet B MTs, along which IFT particles are known to travel [5], terminate before the end of the A subfibers, which continue on by themselves for about another 0.2 μm ([36]; Figures 5A–5D). Furthermore, the distal (plus) end of each of the A subfibers contains a plug attached to a filament that links the A MT subfiber plus end to the flagellar membrane [37]. It is presently unclear what happens to IFT particles once they reach the end of the B subfiber. To address this issue, we prepared longitudinal thin sections of wild-type flagella from flat-embedded cells (see Supplemental Experimental Procedures) and analyzed these by transmission electron microscopy (EM). Interestingly, this analysis showed that although groups of IFT particles can be observed along the length of the flagellum (Figures 5E–5H), consistent with previous observations [5], some groups of particles were also detected distal to the B MT plus end, in the vicinity of the distal filaments that connect the plus ends of the A subfiber to the flagellar membrane (Figures 5A–5D). These groups of IFT particles were of variable size, ranging from ~80 nm (Figure 5A) to ~150 nm (Figure 5B) in length. Because groups of anterograde IFT particles

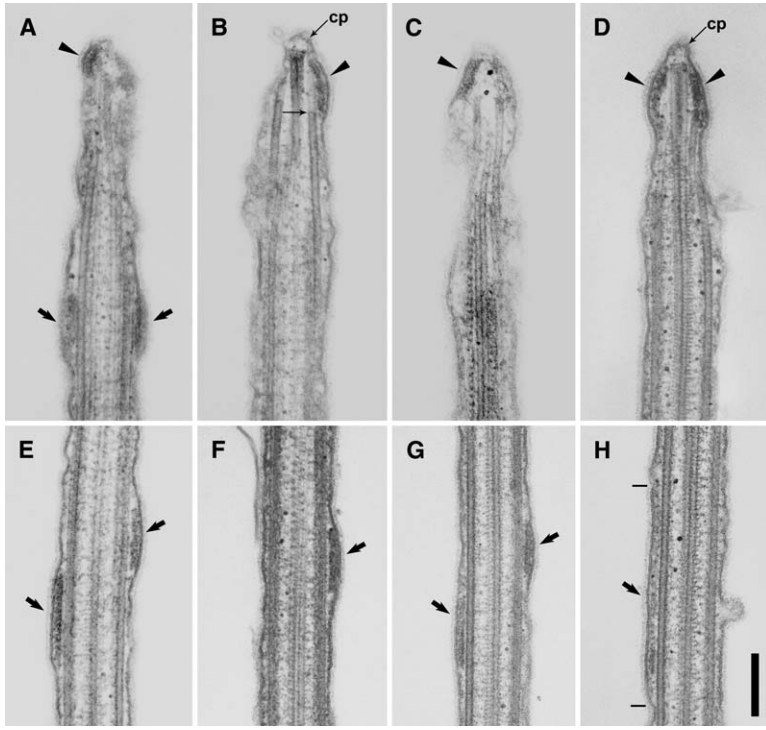

Figure 5. EM Analysis of Longitudinal Ultrathin Sections of Flagella

(A–C) Serial sections (section numbers 1, 3, and 4) of a flagellar tip. Three individual arrays of IFT particles located in the most distal compartment of the flagellum are visible (arrowheads). In (A), two groups of IFT particles in transit from/to the flagellar tip are seen (arrows). The central plug (cp) is a structure attached to the distal end of the central pair MTs [37]. In (B), the end of the B subfiber is marked by an arrow.

(D) A flagellar tip with two arrays of IFT particles located in the most distal compartment of the flagellum. The central plug (cp) is also visible.

(E–H) Arrays of IFT particles of different length and density in individual flagella.

In (H), a very long array of IFT particles is shown; the ends of the array of IFT particles are marked by short lines. The scale bar represents 200 nm.

appear about twice as large as groups of retrograde IFT particles [38], it is possible that the tip of the flagellum shown in Figure 5A contains a group of IFT particles headed in the retrograde direction, whereas that of Figure 5B shows a group of anterograde IFT particles arriving at the flagellar tip. However, more experiments are required to confirm this idea.

Immunogold EM Localization of IFT46 along the Length of the Flagellum

Although several lines of evidence indicate that the groups of particles located between the flagellar membrane and the outer-doublet B MT subfibers ([5]; Figure 5) correspond to the groups of IFT particles that are seen to move by DIC microscopy [5, 12, 33], and that can be isolated biochemically [25, 26], there is no direct evidence to support this idea. To ensure that these groups of particles, shown in Figure 5, contain IFT particle proteins, we analyzed longitudinal thin sections of flagella from flat-embedded wild-type cells by immunogold EM with a polyclonal antibody against IFT46, a complex B polypeptide [39]. As shown in Figure 6 and Figure S2, this analysis demonstrated that the groups of particles located between the flagellar membrane and the outer-doublet B MT subfibers indeed contain IFT46. Because IFT46 is a component of the core of IFT complex B, which sediments at ~16 S in a sucrose density gradient [39], we conclude that the groups of particles observed by transmission EM between the membrane and outer-doublet B MT subfibers ([5]; Figure 5) are groups of IFT particles.

Discussion

Our analysis of IFT system components of isolated wild-type and mutant *Chlamydomonas* flagella (Figure 1 and Figure S1) demonstrates that kinesin-2 is essential for anterograde IFT not only of IFT complexes A and B but also of the cDynein1b complex. During anterograde IFT, inactive cDynein1b appears to be associated with IFT complex B (this study) whereas active heterotrimeric kinesin-2 is attached to complex A [35]. On the basis of data showing that IFT complexes A and B can be co-IPed and depleted from flagellar extract by using a polyclonal antibody to the complex B polypeptide, IFT72/74 [8], we presume that the majority of IFT complexes A and B are bound to each other during anterograde (and retrograde) IFT. This would result in kinesin-2-mediated transport of both IFT complexes A and B as well as cDynein1b and other IFT cargo proteins/axonemal precursors.

During retrograde IFT, both the cDynein1b HC and LIC are clearly required for transport of IFT complexes A and B out of the flagellum ([18, 22], Figure 1 and Figure S1), but the role of the cDynein1b complex in removal of kinesin-2 from the flagellum is less obvious. Studies using a GFP-tagged version of KAP indicated that kinesin-2 does get transported in the retrograde direction following arrival at the flagellar tip [10]. Intriguingly, however, analyses of cDynein1b HC and LIC insertional mutants showed that, in contrast to IFT particles, there is minimal accumulation of kinesin-2 subunits in flagellar stumps of these strains ([18]; Figure 1 and Figure S1). Because cDynein1b is the only retrograde IFT motor complex identified to date, we presume that it mediates the observed transport of kinesin-2 out of the flagellum [10], but the exact relationship between cDynein1b and kinesin-2 appears to be complex and requires further investigation.

In our EM analysis, we observed groups of IFT particles that were localized distal to the B MT plus end (Figures 5A–5D), suggesting that the tip turnaround point for IFT particles is localized distal to the B MT plus end.

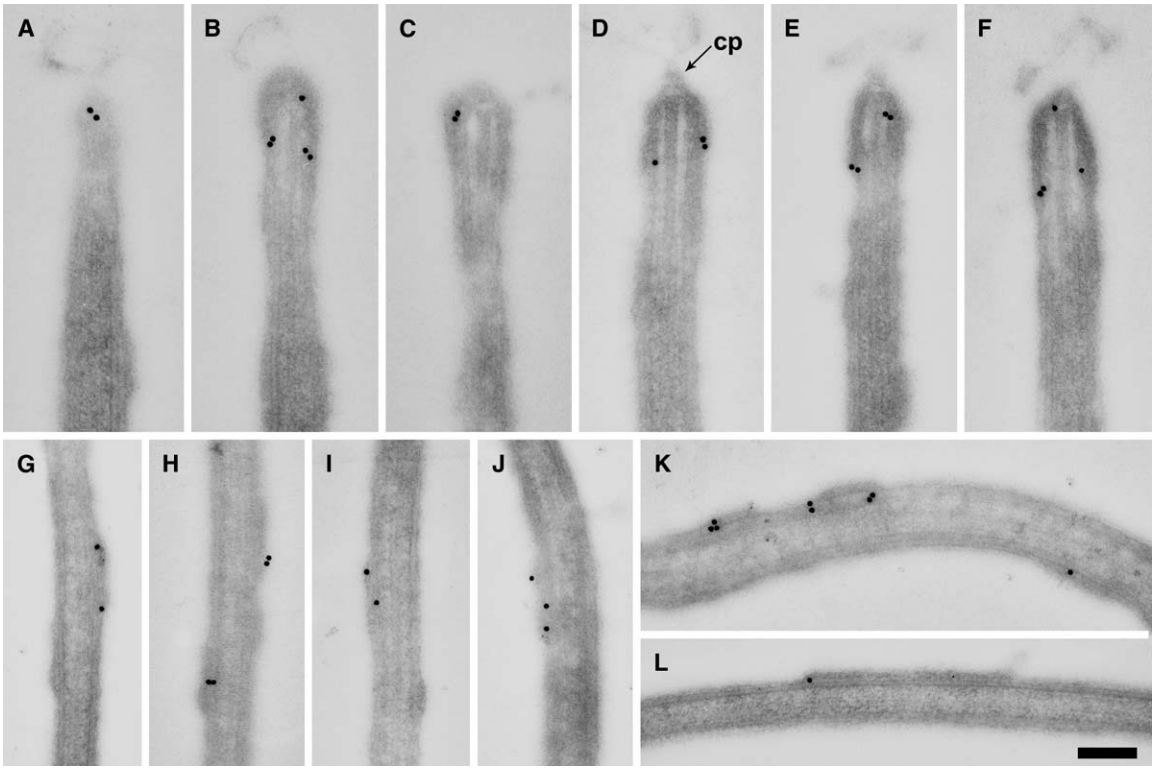

Figure 6. Postembedding Immunogold EM of Longitudinal Sections of Flagella with a Polyclonal Antibody Against IFT46, a Complex B Polypeptide

(A–C) Serial sections of a flagellar tip. The gold particles visible in all three sections likely represent the labeling of different arrays of IFT particles present in the most distal compartment of the flagella.

(D–F) Individual flagellar tips with groups of IFT particles labeled with gold particles. In (D), the central plug (cp) associated with the distal end of the central pair MTs is visible.

(G–L) Arrays of IFT particles in the flagella labeled with 15 nm gold particles. In (L), a very long array of IFT particles (compare with Figure 5H) labeled with 15 nm gold is shown. The scale bar represents 200 nm. Additional IEM micrographs are shown in Figure S2.

Because groups of anterograde IFT particles appear about twice as large as groups of retrograde IFT particles [38], significant remodeling of the IFT particle groups must occur once they reach the flagellar tip. On the basis of a number of observations, we believe that part of this "tip remodeling" involves dissociation of IFT complexes A and B. For example, in a number of ts mutants that are defective specifically in IFT tip turnaround (*fla11/ift172*, *fla15*, *fla16*, *fla17*, and *fla24*; [33]), the ratio between IFT complexes A and B in the flagella is decreased substantially at the permissive temperature, and IFT complex B polypeptides (but not complex A) accumulate near the flagellar tip [21, 32–34]. These observations indicate that complexes A and B can "uncouple" or dissociate from each other at the tip, at least under certain conditions. Furthermore, the IFT complex B polypeptide IFT172 can associate, at least indirectly, with the flagellar tip protein CrEB1 only when complex B is not bound to complex A [32]. One can therefore speculate that flagellar-tip proteins like CrEB1 may be involved in regulating the dissociation/association of IFT complexes A and B at the flagellar tip.

Model for IFT in *Chlamydomonas*

On the basis of the data presented and discussed above, we propose the following model, illustrated in Figure 7A, for IFT in *Chlamydomonas*. For simplicity, this model does not include IFT cargo proteins such as axonemal precursors and turnover products, which are known to be transported by IFT from the cell body to the flagellar tip and from the tip back to the cell body, respectively [8].

Step 1

IFT complexes A and B and the kinesin-2 and cDynein1b motor complexes gather near the basal bodies [6, 26, 30]. The mechanisms by which IFT particles and motors are transported from their site of synthesis to the basal body region is unknown. However, the KAP subunit is necessary for localization of kinesin-2 to this site [10]. The cDynein1b HC is also necessary for basal-body localization of the LIC, but the LIC is not required for basal-body localization of the HC [21, 22].

Step 2

Next, large assemblages containing IFT complexes A and B, inactive cDynein1b, and axonemal precursors are transported by kinesin-2 from the base of the outer-doublet B MT subfiber to its distal end. Active heterotrimeric kinesin-2 is attached to complex A [35], which in turn is associated with complex B that binds the inactive cDynein1b complex. The binding of complex B to inactive cDynein1b occurs independent of cDynein1b LIC and may lead to modification and/or altered conformation of cDynein1b HC.

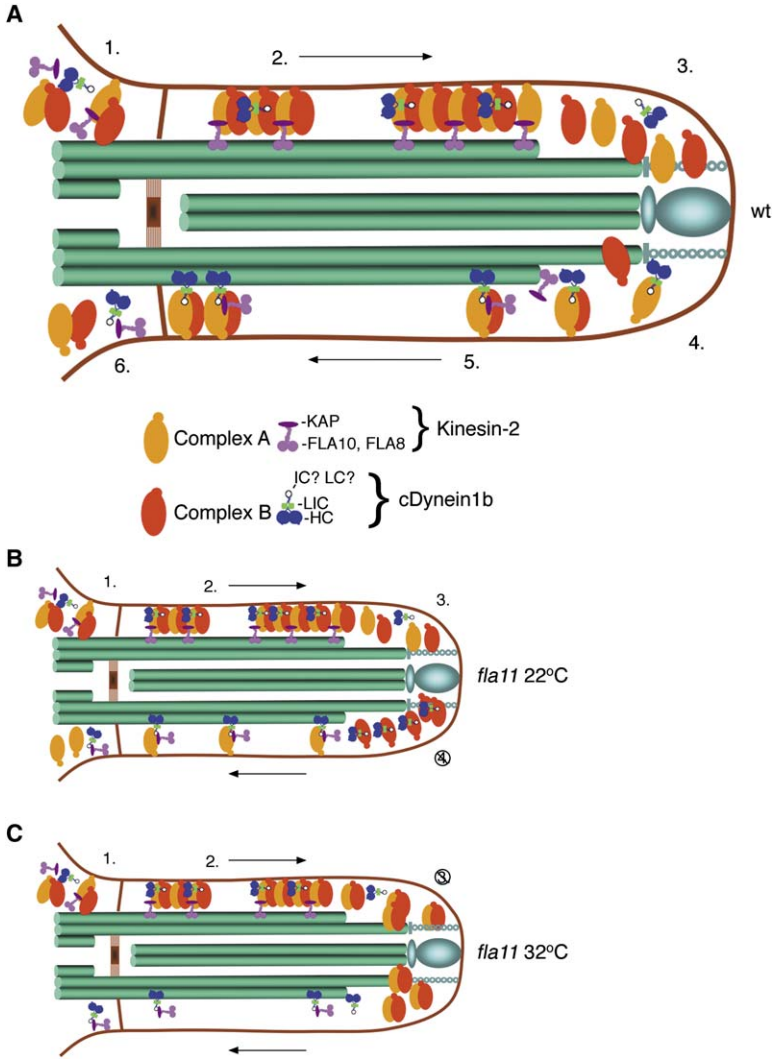

Figure 7. Model for IFT in *Chlamydomonas*

(A) Model for IFT in wild-type cells. Step 1: gathering of IFT particles and motors in the peribasal body region. Step 2: kinesin-2-mediated anterograde transport of IFT complexes A and B and inactive cDynein1b, which is attached to complex B. Active kinesin-2 is associated with complex A. Step 3: release of complexes A and B and inactive cDynein1b into the flagellar tip compartment, followed by dissociation of complexes A and B and release of cDynein1b from complex B. Step 4: complex A binds to active cDynein1b via the LIC subunit, complex B then reassociates with complex A, and kinesin-2 binds to active cDynein1b independent of complexes A and B and cDynein1b LIC. The point at which cDynein1b gets activated is not clear, but it is hypothesized that complex B maintains cDynein1b in an inactive conformation. Step 5: active cDynein1b transports everything back to the cell body. Note that, although shown here, the role of cDynein1b in removal of kinesin-2 from the flagellum is uncertain. Step 6: IFT components are recycled to the cell body.

(B) At 22ºC, *fla11/ift172* cells are proposed to be defective in step 4 of the IFT cycle (association of complex B with A and active cDynein1b), resulting in accumulation of complex B, which then sequesters some of the "free" cDynein1b released from complex B in step 3. It is hypothesized that complex B maintains cDynein1b in an inactive conformation, leading to accumulation of both complex B and cDynein1b. Retrograde transport of kinesin-2 and complex A, which are bound to some of the remaining "free" cDynein1b released from complex B in step 3, is not impaired.

(C) At 32ºC, *fla11/ift172* cells are proposed to be defective in step 3 of the IFT cycle (dissociation of complexes A and B), resulting in failure of dissociation of complexes A and B at the flagellar tip. cDynein1b can still dissociate from complex B in step 3, and when cDynein1b is not bound to B, it can be activated. Consequently, both IFT complexes A and B accumulate, whereas kinesin-2 and cDynein1b do not.

Step 3

Once kinesin-2 reaches the plus end of the B subfiber, we propose that IFT particles are released from the B tubule into the flagellar-tip compartment. Whether cDynein1b and kinesin-2 are also released into this compartment is unclear, but we tentatively propose that cDynein1b is released into the tip compartment, whereas kinesin-2 remains associated with the B subfiber, as in *C. elegans* sensory cilia, where heterotrimeric kinesin-2 only travels along doublet, not singlet MTs [15]. On the basis of analyses of various "tip" mutants (e.g., *fla11/ift172*, *fla15*, *fla16*, *fla17*, and *fla24*; [21, 32–34]), we propose that complexes A and B dissociate from each other once they are released into the tip compartment and that this dissociation is essential for the subsequent reorganization of the IFT particle and association of complex A with active cDynein1b prior to retrograde IFT. Concomitantly with the dissociation of IFT complexes A and B, inactive cDynein1b is released from complex B. The exact sequence of these events is unclear, but our time-course experiment with *fla11/ift172* flagella (Figure 3) suggests that the release of cDynein1b from IFT complex B can occur when complex A accumulates, and presumably is bound to IFT complex B, near the flagellar tip (see also below).

Step 4

After dissociation of IFT complexes A and B at the tip and release of inactive cDynein1b from IFT complex B, we propose that complex A binds (directly or indirectly) to active cDynein1b via LIC and that complex B then binds to complex A. This proposal is based on the findings that complex B cannot undergo retrograde IFT in the absence of complex A [40] or LIC [22]. How and when cDynein1b gets activated is unclear. The activation of cDynein1b does not appear to depend on its association with IFT complex A, because in *C. elegans* complex A mutant cilia, cDynein1b LIC does not accumulate (i.e., it can exit the cilia) [20]. Kinesin-2 appears

capable of exiting the flagella independent of IFT complexes A and B and cDynein1b LIC, and we therefore tentatively propose that kinesin-2 can associate with active cDynein1b independent of IFT particles and cDynein1b LIC. However, elucidating the precise link between kinesin-2 and the cDynein1b complex during retrograde IFT requires further investigation.

Step 5

Following binding (direct or indirect) of complex A to cDynein1b, association of complex B with A, and attachment (direct or indirect) of kinesin-2 to the cDynein1b complex independent of IFT complexes A and B, active cDynein1b transports everything back from the tip to the cell body.

Step 6

The IFT cycle is completed when IFT components are returned to the cytoplasm for recycling.

The Proposed IFT Model Can Account for the *fla11/ift172* Phenotype

The *fla11/ift172* mutant strain contains a single ts point mutation in the gene encoding IFT172; this point mutation leads to replacement of a conserved leucine with proline at residue 1615 [32]. As with most ts mutations, we expect that IFT172 function is somewhat compromised at 22ºC and more severely compromised at 32ºC. IFT172 appears to play a role in maintaining the integrity of IFT complex B [39] and, hence, presumably is also important for association between complexes A and B.

Given the phenotype of the *fla11/ift172* ts mutant [32–34], we propose that at the permissive temperature of 22ºC, *fla11/ift172* cells are competent to undergo steps 1–3 of the IFT cycle, but are defective in step 4 (Figure 7B). In other words, anterograde IFT is normal, complexes A and B are able to dissociate at the flagellar tip, and cDynein1b does get released from complex B. Complex A is also capable of associating with active cDynein1b under these conditions, because complex A does not accumulate in *fla11/ift172* flagella at 22ºC (i.e., A gets transported out of flagella by cDynein1b). However, because complex B and cDynein1b accumulate in *fla11/ift172* flagella at 22ºC, we propose that the reassociation of complex B with complex A at the flagellar tip is inhibited (step 4 in Figure 7A). This would lead to accumulation of complex B, which may then sequester "free" cDynein1b (released from complex B in step 3) and prevent its activation. This would result in the observed accumulation of complex B and cDynein1b subunits, whereas neither complex A nor kinesin-2 would accumulate. If the accumulation of complex B over complex A causes retention of inactive cDynein1b by "excess" complex B at the *fla11/ift172* flagellar tips at 22ºC, one would predict that any mutant in which there is an excess of complex B over complex A at the flagellar tip would similarly lead to retention of cDynein1b. This does indeed seem to be the case, because in *fla15*, *fla16*, and *fla17* ts mutant cells incubated at the permissive temperature, IFT complex B polypeptides and cDynein1b subunits accumulate in blebs at or near the flagellar tip, whereas complex A does not accumulate [21].

Upon shifting the temperature of *fla11/ift172* cells to 32ºC, we propose that IFT172 function becomes more severely compromised such that complex B can no longer dissociate from complex A upon arrival at the flagellar tip, i.e., IFT is defective in step 3 of Figure 7A, but inactive cDynein1b can still be released from complex B. If we assume that dissociation of IFT complexes A and B is necessary for subsequent binding of complex A to active cDynein1b, this would result in accumulation of complexes A and B, whereas cDynein1b and kinesin-2 would not accumulate (Figure 7C). Thus the model illustrated in Figure 7A can account for the *fla11/ift172* phenotype.

Conclusions

Our results indicate the following: (1) heterotrimeric kinesin-2 is essential for anterograde IFT not only of complexes A and B, but also of cDynein1b; (2) retrograde IFT of complexes A and B depends on both cDynein1b HC and LIC; (3) removal of kinesin-2 from the flagella can occur in the absence of cDynein1b LIC and independent of IFT complexes A and B; (4) cDynein1b HC can associate, at least indirectly, with IFT complex B independent of complex A and cDynein1b LIC; and (5) the tip turnaround point for IFT particles most likely is localized distal to the plus end of the outer-doublet B MTs. A model for IFT is proposed in which tip turnaround involves (1) dissociation of IFT complexes A and B and release of inactive cDynein1b from complex B, (2) binding of complex A to (active) cDynein1b via LIC, and (3) reassociation of complex B with A prior to retrograde IFT. Outstanding questions include the following: Which part of the cDynein1b motor is involved in its IFT complex A-independent association with complex B? How and when is cDynein1b activated at the flagellar tip? What is the relationship between kinesin-2 and cDynein1b during retrograde IFT? The model proposed here provides an important framework for resolving these issues.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and two figures and are available with this article online at: http://www.current-biology.com/cgi/content/full/16/5/450/DC1/.

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