Flagellar Protein Dynamics in *Chlamydomonas*

Lin Song and William L. Dentler‡

From the Department of Molecular Biosciences, University of Kansas, Lawrence, Kansas 66045

Cilia and flagella appear to be stable, terminal, microtubule-containing organelles, but they also elongate and shorten in response to a variety of signals. To understand mechanisms that regulate flagellar dynamics, *Chlamydomonas* cells with non-growing flagella were labeled with $^{35}$S, and flagella and basal body components were examined for labeled polypeptides. Maximal incorporation of label into the flagella occurred within 3 h. Twenty percent of the flagellar polypeptides were exchanged. These included tubulins, dyneins, and 80 other axonemal and membrane plus matrix polypeptides. The most stable flagellar structure is the PF-ribbon, which comprises part of the wall of each doublet microtubule and is composed of tubulin and three other polypeptides. Most $^{35}$S was incorporated into the high molecular weight ribbon polypeptide, rib240, and little, if any, $^{35}$S is incorporated into PF-ribbon-associated tubulin. Both wild-type (9 + 2) and 9 + 0 flagella, which lack central microtubules, exhibited nearly identical exchange patterns, so labeling is not due to turnover of relatively labile central microtubules. To determine if flagellar length is balanced by protein exchange, $^{35}$S incorporation into disassembling flagella was examined, as was exchange in flagella in which microtubule assembly was blocked by colchicine. Incorporation of $^{35}$S-labeled polypeptides was found to occur into flagellar axonemes during wavelength-dependent shortening in *pf18* and in *fla10* cells induced to shorten flagella by incubation at 33 °C. Colchicine blocked tubulin addition but did not affect the exchange of the other exchangeable polypeptides; nor did it induce any change in flagellar length. Basal bodies also incorporated newly synthesized proteins. These data reveal that *Chlamydomonas* flagella are dynamic structures that incorporate new protein both during steady state and as flagella shorten and that protein exchange does not, alone, explain length regulation.

Cilia and eukaryotic flagella are ubiquitous microtubule-containing organelles that mediate a variety of functions and are prominently displayed on cells in the heart, kidney, ovary, uterus, liver, central nervous system, spleen, inner ear, and olfactory epithelium and in adrenal, thyroid, pancreatic, thymus, and pituitary glands. Cilia propel vertebrate sperm, display visual pigments in vertebrate photoreceptors and sensory molecules on olfactory and chemosensory neurons. During development, they are prominent on embryonic surfaces.

Pathological conditions associated with defects in ciliogenesis include liver disorders, drug-induced cirrhosis, gastritis, hyperparathyroidism, retinal dysplasia, Mulvihill-Smith progeria-like syndrome, Oak Ridge polykystic kidney disease (1), Usher’s syndrome and retinitis pigmentosa. Patients with primary ciliary dyskinesia have abnormally aligned cilia or cilia with motility defects, which leads to infertility and decreased resistance to lower respiratory tract infections, chronic rhinitis, sinusitis, and otitis media. Ciliary movement is essential for the development of asymmetry in developing mammals and is particularly important for development and positioning of the heart (2, 3). There are likely to be many unknown links between cilia and embryonic development. Cilia line embryonic surfaces of developing neural tubes and ventral and notochordal plate cells in developing mouse embryos (4, 5), and they appear during neural tube closure (6). Cell- or tissue-specific defects in cilia formation may cause currently unidentified defects during early embryonic development.

The growth, maintenance, and disassembly of cilia and flagella is a highly orchestrated process in which the length of each of the nine doublets and the two central microtubules is precisely regulated as is the addition of more than 250 different polypeptides that attach to the microtubules or are associated with the membrane and soluble matrix. Isolated cilia and flagella are extremely stable microtubular structures that can only be dissociated by ionic detergents, heat, or chaotropic agents (7). Despite their stability in *vitro*, fully grown flagella in many cells can be completely disassembled (8). In sea urchin embryos, cilia can be stimulated to grow by the application of several different agents, including concanavalin A (9) and theophylline (10). Some of the most dramatic examples of flagellar dynamics occur in *Chlamydomonas*, in which flagella normally disassemble prior to cell division and new flagella grow after division is completed. If one flagellum is amputated, the remaining flagellum shortens and then elongates once a new flagellum has started to grow on the “amputated” basal body (11). *Chlamydomonas* flagellar growth can be stimulated with lithium (12, 13), induced to disassemble by IBMX and other agents known to be involved with signal transduction pathways (13), and destabilized by cytochalasin D, which induces periods of rapid shortening and elongation (14). A temperature-sensitive mutant, *fla10*, shortens its flagella at 33 °C but not at 22 °C (15, 16). *Chlamydomonas pf18* mutants shorten flagella under reduced red light (13), and long flagellar (lf) mutants produce up to 3 times longer than normal flagella (17). When the cytoplasm of cells with abnormally long flagella is mixed with that of cells with normal length flagella, the long flagella rapidly shorten (17), indicating that signaling mechanisms that regulate flagellar length are dominant in cells with normal length flagella. Although isolated flagellar microtubules are the most stable microtubules known, these microtubules clearly can be dynamic in *vitro*. Little is understood about the mechanisms that regulate flagellar dynamics, although recent
Flagellar assembly, or elongation, requires the transport of subunits from the cytoplasm, where they are synthesized, to the tips of the flagellar microtubules, where they are added to the microtubule ends (19). Some of the transport to the distal tips requires the heterotrimeric kinesin, kinesin-II, which is associated with intraflagellar transport (IFT) (20). Early studies by Morris and Scholey (21) revealed that sea urchin embryonic cilia could grow nearly half-length if IFT is blocked by kinesin-II antibodies but that further growth could not occur. Subsequent studies have confirmed that kinesin-II is required for flagellar assembly in Chlamydomonas (16, 22), C. elegans (23–26), Tetrahymena (27), tracheal epithelia (28), and mouse embryonic nodal cilia (29, 30). Kinesin-II appears to deliver flagellar protein to the distal tips of the flagellum, the plus-ends of the microtubules, where they are added to the microtubules.

The components transported by kinesin-II require recycling to flagellar bases for flagellar growth or maintenance to occur, and this movement requires cytoplasmic dyneins (31–33). Direct movement of cytoplasmic dynein has been observed in sensory ciliated neurons in C. elegans (26). Additionally, cytoplasmic dynein is required for the delivery of rhodopsin to vertebrate retinal cilia, and the failure to do so may lead to retinitis pigmentosa (34, 35).

The dependence of flagellar length on kinesin-II and cytoplasmic dynein indicates that flagellar proteins and/or signal transduction pathway components are shuttled back and forth along the flagellum and that some flagellar components are likely to be exchanged in the fully assembled flagellum. One indication of the importance of this shuttling is provided by examination of Chlamydomonas cytoplasmic dynein mutants. These cells can assemble short flagella, but shortly after they are formed, the flagella shorten (31–33) and, in many cases, the microtubules start to fray into disorganized filamentous structures (31). Marshall and Rosenbaum (36) showed that epitope-tagged tubulin could be added to steady state flagella and revealed the presence of retrograde tubulin flux. Moreover, the retrograde flux was balanced by IFT (37). Taken together, these studies clearly indicate that the balance of anterograde and retrograde IFT and, possibly, some flagellar protein turnover is essential for the maintenance of steady state length. Imbalances in anterograde transport (as in kinesin-II mutants) or in retrograde transport (as in cytoplasmic dynein mutants) may lead to flagellar disassembly or, possibly, elongation.

Several early studies of pulse-labeled flagella indicated that a low amount of protein exchange, or turnover, occurred in steady state flagella. Low levels of protein turnover were detected in whole flagella and in the membrane plus matrix and axonemal fractions (11, 38–41), and some tubulin turnover was detected in Chlamydomonas flagella (40) and Tetrahymena cilia (42). The only prominent axonemal protein detected to turnover in Chlamydomonas flagella was a nontubulin 55-kDa “protein X” (43). However, none of these studies rigorously ruled out the possibility that the incorporation of label was not due to protein exchange but, rather, was due to the assembly of new flagella due to flagellar amputation, which up-regulates flagellar protein synthesis. In contrast to protozoans, embryonic cilia on marine organisms are considerably more dynamic. Steady state cilia on sea urchin blastulae exhibit exchange of numerous membrane plus matrix and axonemal polypeptides, including tubulin and tektin A (44, 45).

The recent studies of sea urchin ciliary turnover and the importance of IFT motors in maintaining flagellar length prompted us to critically reexamine flagellar protein turnover in steady state Chlamydomonas flagella. Moreover, with the availability of mutants that shorten flagella due to light or temperature shifts and long flagellar mutants, we could compare the dynamics of protein turnover in shortening or abnormally long flagella. The results reported here show that steady state flagella exhibit a considerable amount of protein exchange, with a minimum 20% of flagellar proteins being exchanged within a 6-h period. Although tubulin turnover occurs, tubulin turnover could be blocked without affecting the turnover of nontubulin components. Turnover of most flagellar proteins continues to occur as flagella shorten, thus demonstrating that flagellar length is not strictly coupled to the turnover of major flagellar proteins. The single peptide exhibiting the greatest exchange is rib240, a component of the axonemal PF-ribbons. Unlike the ribbon fractions in sea urchin cilia, neither significant tubulin nor tektin-like proteins are exchanged in the PF-ribbons, but a new high molecular weight component, rib240, is exchanged. Since the ribbons have been suggested to be important stabilizers or length determinants in flagella, the role of rib240 in flagellar assembly deserves serious consideration.

**Experimental Procedures**

**Cell Culture and 35S Labeling.—**For all experiments, Chlamydomonas reinhardtii (wild-type CC-2929, pYB (CC-1090), fla10 (CC-1919), and fla4 (P. Lefebvre, University of Minnesota) were grown in minimal medium (M medium) bubbled with air and were synchronized by growing cells on a 12-h light/12-h dark cycle. For each experiment, t = 0 was the start of the light period.

Vegetative cells were grown to a density of 5 × 10⁶ cells/ml, harvested in sterile bottles, and incubated in low sulfur M medium (MgSO₄, 10 mM, and MgCl₂, added to 1.18 mM for 24 h. For each labeling experiment, 4 μCi/ml ³⁵S (as sulfuric acid: PerkinElmer Life Sciences) was added to starved cells from a 1 mM/ml stock solution in sterile distilled water. For colchicine experiments, freshly prepared colchicine was added to cells at a final concentration of 2 mg/ml. Each batch of colchicine was tested to ensure that it inhibited flagellar growth after deflagellation of cells by pH shock.

For chase experiments, 1 liter of starved cells were labeled with 4 μCi/ml ³⁵S for up to 6 h and then pelleted at 1100 × g for 5 min at 20 °C in a JA-10 (Beckman) rotor and washed twice with 400 ml of low sulfur M medium, and cells were gently resuspended in 1 liter of low sulfur M medium supplemented with 30 ml of 10% MgSO₄. The final cell suspension was examined by phase microscopy to ensure that all cells were uniformly flagellated. After the chase period, cells were pelleted, and flagella were isolated and fractionated as described below.

To induce shortening in light-synchronized pf18 cells, cells were harvested and incubated under low red lights with gentle shaking at 1000 rpm, described by Tuxhorn et al. (13).

To measure flagellar length, cells were fixed in an equal volume of 2% glutaraldehyde for 20 min. Flagellar length was determined by phase-contrast microscopy using an ocular micrometer. For each sample, flagellar lengths were averaged from 50 biflagellated cells.

Flagellar Isolation and Fractionation.—Flagella were amputated using dibucaine and were isolated following procedures described by King (46). Cells were collected by centrifugation (1100 × g, 5 min) and were suspended in 50 ml of ice-cold HDMDS (10 mM HEPES, 5 mM MgSO₄, 1 mM dithiothreitol, 4% sucrose, pH 7.5). Dibucaine was added to a final concentration of 1 mM, and cells were swirled for 1–2 min and examined by phase-contrast microscopy to ensure that all cells were deflagellated. All subsequent steps were carried out at 4 °C. Cell bodies were pelleted (1100 × g, 5 min), and flagella were recovered from the supernatant at centrifugation at 7500 × g for 10 min. Flagellar pellets were suspended and centrifuged over HMD (10 mM HEPES, 5 mM MgSO₄, 1 mM dithiothreitol) with 25% sucrose at 1100 × g for 20 min. Purified flagella were harvested at the interface. The membrane plus matrix fractions were isolated by incubating flagella with 0.1% Nonidet P-40 (Sigma) for 20 min on ice and then centrifuging the suspension at 12,000 × g for 10 min. The pelleted axonemes were then twice suspended in HDMDS plus 0.6 M KCl, incubated for 20 min on ice, and centrifuged at 12,000 × g for 10 min.

PF-Ribbons were purified as described by Norrlander et al. (47). Iso-

---

The abbreviations used are: M medium, minimal medium; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.
labeled axonemes were incubated in 0.7% Sarkosyl in TED (10 mM Tris, pH 8, 0.1 mM EDTA, 1 mM dithiothreitol) overnight with gentle shaking. Ribbons were pelleted 100,000 × g for 1 h, washed with 1 ml of TED, and pelleted a final time.

Basal bodies were isolated using the method of Snell (48) with the following modifications. Sulfur-starved cells were incubated with H235SO4 for 2 or 6 h as described above. Cells were collected by centrifugation (11000 × g, 5 min, 20 °C), suspended in 100 ml of TE (10 mM Tris, 1 mM EDTA, pH 7.5), and deflagellated with 1 mM dibucaine (Sigma). After centrifugation (15000 × g, 3 min, 20 °C), the supernatant was collected to purify flagella, and the pellet (cell bodies) was suspended in autolysin (49) for 40 min to remove any cytoplasm and cell debris. Cell lysates were layered over 20 ml of 25% sucrose-TE in 50 ml of conical polycarbonate tubes and centrifuged in a swinging bucket rotor (15000 × g, 10 min, 4 °C) to sediment cells. The supernatant was then layered over 25% sucrose-TE and recentrifuged. This supernatant was layered over a 40–50% sucrose-TE gradient and centrifuged in a swinging bucket rotor (14000 × g, 60 min, 4 °C). Basal bodies were collected from the 40–50% interface, diluted with equal volume of TE, and pelleted by centrifugation (35000 × g, 30 min, 4 °C). The basal bodies were suspended in 3 ml of 0.5% Nonidet P-40 in TE, homogenized in a 5 ml glass-Teflon homogenizer, layered over a 50–55% sucrose-TE gradient, and centrifuged in a swinging bucket rotor (14000 × g, 45 min, 4 °C). Basal bodies from the 45–60% interface and treated with 0.6 M NaCl on ice for 20 min. After centrifugation (35000 × g, 30 min, 4 °C), basal bodies were collected from the pellet.

Flagellar Regeneration—Synchronized wild-type cells were starved in low sulfur M medium for 24 h and deflagellated by pH shock. Cells were concentrated in 200 ml of low sulfur M medium, and 0.5% acetic acid was added dropwise until pH 4.5 was reached. After 30 s, the pH was raised to 7.0 by adding 0.5 N KOH. Cells were then pelleted and resuspended in low sulfur M medium. 35S-Labeled sulfuric acid was added to dissolve the gel slices. After incubation at room temperature overnight, samples were counted as described above.
cells were fixed, and flagellar lengths were measured to ensure that there was no change in flagellar length during the labeling period (Fig. 2A).

More than 80 $^{35}$S-labeled polypeptides were resolved by SDS-PAGE (Fig. 2B). Prominent polypeptides in the membrane plus matrix fractions included a 350-kDa membrane protein, 230-kDa mastigonemes, a major anomalously migrating membrane protein (~150 kDa), several peptides comigrating with IFT proteins (S3, 57/55, 46, and 20 kDa), and a 90-kDa polypeptide that comigrated with FLA10 kinesin. Prominently labeled polypeptides released from axonemes with 0.6M KCl included high molecular weight polypeptides that comigrated with flagellar dynein heavy chains. The axonemes contained numerous labeled polypeptides, including tubulins and radial spoke polypeptides (Fig. 2B).

To ensure that flagellar $^{35}$S incorporation was due to protein exchange and not to flagellar regeneration by cells deflagellated during handling, $^{35}$S was added to cells incubated in colchicine, under conditions that prevent flagellar regeneration after deflagellation. Fully grown flagella did not shorten during colchicine treatment (Fig. 2A). Flagella isolated from $^{35}$S-labeled and colchicine-treated cells contained the same labeled polypeptides that were found in untreated cells (Fig. 2B), with the exception that no labeled tubulin was incorporated into flagella on colchicine-treated cells. These results confirm that newly synthesized protein is incorporated without flagellar growth and demonstrate that tubulin exchange is not necessary for the exchange of other flagellar polypeptides.

After a 3-h incubation with $^{35}$S, flagella were isolated and fractionated, and the amount of radioactivity in each fraction was measured as a percentage of the total labeled flagella. The membrane plus matrix fraction contained 45 ± 17% (196 ± 48 cpm/µg; four experiments) of the total labeled protein (478 ± 190 cpm/µg), while 55 ± 17% (281 ± 186 cpm/µg) of the labeled protein was in the axoneme. Of the axonomal proteins, 60% (147 ± 26 cpm/µg) were solubilized by KCl, and 40% (134 ± 41 cpm/µg) were in the KCl-insoluble, axonomal fraction.

**Flagellar Protein Exchange in a High Molecular Weight Component of PF-ribbons**—In sea urchin embryonic cilia, an integral outer doublet microtubule component, tektin-A, was shown to undergo rapid turnover in steady state cilia (44, 45, 52). To examine the possibility that stable axonomal structures in Chlamydomonas also may exhibit high levels of turnover, we isolated PF-ribbons (47) from cells that were labeled for 6 h or without colchicine, and flagella were isolated from equal numbers of cells at 1- or 3-h intervals. In both cases, $^{35}$S incorporation into flagella reached a maximum by 3 h (Fig. 3A). Incorporation of proteins into the membrane plus matrix fraction reached a maximum within 3 h, while labeling in the polypeptides during the labeling period. Most of the label (42%) was incorporated into a single 240-kDa polypeptide, rib240 (Fig. 3A). To confirm that the labeled protein was an axonomal component, a polyclonal antibody was generated against rib240 (asterisk), and tubulins (<) in detergent- and KCl-extracted axonemes (A). Tubulins were not incorporated in flagella isolated from colchicine-treated cells. C, cell bodies; M, membrane plus matrix; K, KCl-soluble protein; A, KCl-insoluble axonomes.

**FIG. 3.** Incorporation of labeled polypeptides into PF-ribbons. Cells were incubated with $^{35}$S for 6 h, and PF-ribbons were isolated from the outer doublets. A, SDS-PAGE of PF-ribbons, stained with Coomasie Blue (S) and $^{35}$S-labeled ribbon polypeptides (P) analyzed with a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). B and C, negatively stained fractions of PF-ribbons in low (B) and high (C) magnification. Bar, 100 nm.
Isolated from 1.25
29758
filled circle
open circles (,, immunofluorescence staining of isolated axonemes using anti- postimmune serum containing a polyclonal antibody against rib240 (c).

Axonemes isolated from 1.25
29758
filled circle
open circles (,, immunofluorescence staining of isolated axonemes using anti- postimmune serum containing a polyclonal antibody against rib240 (c).

B, immunofluorescence staining of isolated axonemes using anti-β-tubulin antibody (a), buffer alone (b), and a polyclonal antibody against rib240 (c).

The exchange rate of individual polypeptides did not vary during the time of labeling. Synchronized cells with fully grown flagella were labeled for 3, 6, and 9 h, and flagella were isolated at 3-h intervals during a 12-h incubation and fractionated into membrane plus matrix, KCl-soluble fraction, and the KCl-insoluble axoneme fraction. The incorporation of 35S into 18 individual polypeptides also varied significantly (Fig. 7 and Table II). Some polypeptides, including the membrane polypeptides M1 and M4, were completely exchanged with newly synthesized proteins, while another polypeptide (M2) exhibited little significant exchange. In the axonemes, some polypeptides, such as A2, A3, A4, and A5, exchanged completely, while others, including A1 and A8, showed no significant exchange. Polypeptides A6 and A7 are likely α- and β-tubulins, because the incorporation of these labeled polypeptides is abolished when cells are incubated with colchicine. Thus, exchange of axonemal polypeptides is not uniform.

One of the major polypeptides that was exchanged in non-growing flagella is rib240 (R2 in Table II), a component of the PF-ribbon complex, the most insoluble component of microtubule walls. During a 6-h labeling period, 3% of the 35S incorporated into axonemes was found in the PF-ribbons, which comprise less than 2% of the total axonemal protein. Most 35S in PF-ribbons was found in a single polypeptide, rib240 (Fig. 3). rib240 composed 0.1% of total flagellar protein and 9% of the PF-ribbon complex, but this minor flagellar protein also incorporated 20% of the label incorporated into regenerating flagella over a 6-h period (during which time the maximum amount of exchange occurred) with the amount of 35S incorporated into flagella after cells were deflagellated and incubated in 35S for 2 h, as flagella grew to the length of steady state labeled flagella. Labeling of regenerating cells was carried out for 2 h to minimize any incorporation due to protein turnover once the flagella reached full length.

Axonemes isolated from fully grown flagella contained 19% of the 35S incorporated in axonemes in regenerating flagella (Table I). The membrane plus matrix in fully grown flagella contained 20% of the label incorporated into regenerating flagella. Thus, during a 6-h labeling period, ~40% of the flagellar polypeptides exchanged with newly assembled polypeptides.

The incorporation of 35S into 18 individual polypeptides also was examined. The exchange rate of individual polypeptides varied significantly (Fig. 7 and Table II). Some polypeptides, including the membrane polypeptides M1 and M4, were completely exchanged with newly synthesized proteins, while another polypeptide (M2) exhibited little significant exchange. In the axonemes, some polypeptides, such as A2, A3, A4, and A5, exchanged completely, while others, including A1 and A8, showed no significant exchange. Polypeptides A6 and A7 are likely α- and β-tubulins, because the incorporation of these labeled polypeptides is abolished when cells are incubated with colchicine. Thus, exchange of axonemal polypeptides is not uniform.

One of the major polypeptides that was exchanged in non-growing flagella is rib240 (R2 in Table II), a component of the PF-ribbon complex, the most insoluble component of microtubule walls. During a 6-h labeling period, 3% of the 35S incorporated into axonemes was found in the PF-ribbons, which comprise less than 2% of the total axonemal protein. Most 35S in PF-ribbons was found in a single polypeptide, rib240 (Fig. 3). rib240 composed 0.1% of total flagellar protein and 9% of the PF-ribbon complex, but this minor flagellar protein also incorporated 20% of the 35S incorporated into PF-ribbons and 1% of the total flagellar 35S. Incorporation of 35S into rib240 is easily visualized in fluorograms of Coomassie Blue-stained gels in which rib240 cannot be detected (Fig. 2, asterisk).

Flagellar Proteins Exchange as Flagella Disassemble—Is the exchange we observe solely located in the relatively labile central microtubules (54)? To examine this, pf18, a Chlamydomonas “9 + 0” mutant that lacks central microtubules, was lab-
FIG. 6. Chase of incorporated polypeptides in wild-type cells. A, cells were labeled for 3 h and chased for another 3 h in unlabeled sulfur medium. Flagella were isolated and precipitated by perchloric acid. Radioactivity at each time point was calculated as the percentage of radioactivity after a 3-h incubation with $^{35}$S. The arrow indicates the time point when label was replaced by cold sulfate. B, cells were transferred to regular medium after a 6-h incubation with $^{35}$S (6). Flagella were isolated and fractionated after 1 (1) or 6 h (6).

Fig. 7. Coomassie Blue-stained SDS-PAGE showing selected polypeptides that were sliced from the gel for radioactivity analysis, shown in Table II. Membrane plus matrix (M), axonemes (A), and PF-ribbons (R) are shown. A6 and A7 comigrated with tubulins. R2, rib240.

beled with $^{35}$S and compared with $^{35}$S incorporation into $9 + 2$ wild type flagella. As shown in Fig. 8B, the same major labeled polypeptides were found in the axonemes and in the detergent- and salt-soluble fractions of pf18 flagella and in wild-type flagella. Thus, turnover does not occur in the central microtubules alone but, rather, in all of the axonemal microtubules and associated proteins. More important, these data revealed that subunit exchange occurred as flagella shortened. During the labeling period, pf18 cells were incubated under low red light, which induces flagellar shortening (13) (Fig. 8A). Despite shortening by $\sim 14\%$, labeled polypeptides were found in each of the flagellar compartments.

Since flagellar subunit exchange occurred in shortening pf18 flagella, we examined exchange in more rapidly shortening flagella, using the temperature-sensitive flagellar assembly mutant fla10. Synchronously cultured fla10 cells were labeled for 2 h at 20 °C, at which flagellar length was constant, or for 2 h at 33 °C, at which flagella shortened to half-length during the labeling period (Fig. 9A). Flagella isolated from 20 °C cultures contained the same labeled polypeptides found in wild type cells (Fig. 9B). As flagella shortened, new polypeptides continued to be added to the flagellar membrane plus matrix, salt-soluble components, and the salt-insoluble axonemal microtubules. The rate of incorporation of newly synthesized proteins into shortening flagella was reduced to $\sim 25\%$ that of control cells at 20 °C (Table III). However, incorporation of $^{35}$S into total cell body protein also decreased to $\sim 24\%$ of the control level (605 cpm/μg versus 2529 cpm/μg). Since the reduction of newly incorporated flagellar protein matched the decrease in $^{35}$S incorporation into total cell body proteins, it appears that flagellar polypeptide turnover continues to occur as flagella disassemble at nearly the same rate as it occurs in nonshortening flagella.

Flagellar disassembly in fla10 is due to a temperature-sensitive mutation in a kinesin-II microtubule motor required for IFT (15, 22). Since IFT continues for up to 90 min after the temperature shift (22), the labeling study described above was carried out while IFT continued. To determine if protein turnover continues after IFT cessation, fla10 cells were incubated at 33 °C for 90 min, $^{35}$S was added, and cells were incubated for an additional 2 h. During this time, flagella shortened from 12 μm to 4.6 μm. When the short flagella were isolated, no significant labeling was detected (data not shown). However, incubation at 33 °C also reduced $^{35}$S incorporation into cell body protein by 90% (from 2529 to 251 cpm/μg). Whether incubation at 33 °C

### Table I

<table>
<thead>
<tr>
<th>Proteins labeled in steady state flagella for 6 h</th>
<th>Proteins labeled in regenerating flagella</th>
<th>Percentage of labeled proteins of regenerating flagella</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpm/μg</td>
<td>cpm/μg</td>
<td>%</td>
</tr>
<tr>
<td>Flagella</td>
<td>2793</td>
<td>12,152</td>
</tr>
<tr>
<td>Membrane + matrix</td>
<td>658</td>
<td>3505</td>
</tr>
<tr>
<td>Axonemes</td>
<td>1129</td>
<td>5862</td>
</tr>
<tr>
<td>PF-ribbons</td>
<td>978</td>
<td>2786</td>
</tr>
</tbody>
</table>

### Table II

<table>
<thead>
<tr>
<th>Steady state, 6 h</th>
<th>Regeneration</th>
<th>Label incorporated during steady state</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpm/μg</td>
<td>cpm/μg</td>
<td>%</td>
</tr>
<tr>
<td>Membrane + matrix</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>578</td>
<td>3162</td>
</tr>
<tr>
<td>M1</td>
<td>66</td>
<td>33</td>
</tr>
<tr>
<td>M2</td>
<td>16</td>
<td>311</td>
</tr>
<tr>
<td>M3</td>
<td>64</td>
<td>110</td>
</tr>
<tr>
<td>M4</td>
<td>32</td>
<td>24</td>
</tr>
<tr>
<td>M5</td>
<td>17</td>
<td>43</td>
</tr>
<tr>
<td>Axonemes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>750</td>
<td>3891</td>
</tr>
<tr>
<td>A1</td>
<td>7</td>
<td>27</td>
</tr>
<tr>
<td>A2</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>A3</td>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td>A4</td>
<td>46</td>
<td>20</td>
</tr>
<tr>
<td>A5</td>
<td>164</td>
<td>14</td>
</tr>
<tr>
<td>A6</td>
<td>10</td>
<td>59</td>
</tr>
<tr>
<td>A7</td>
<td>34</td>
<td>99</td>
</tr>
<tr>
<td>A8</td>
<td>0</td>
<td>38</td>
</tr>
<tr>
<td>PF-ribbons</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>760</td>
<td>2081</td>
</tr>
<tr>
<td>R1</td>
<td>5</td>
<td>18</td>
</tr>
<tr>
<td>R2</td>
<td>15</td>
<td>29</td>
</tr>
<tr>
<td>R3</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>R4</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>R5</td>
<td>0</td>
<td>22</td>
</tr>
</tbody>
</table>
resulted in an inhibition of $^{35}$S uptake into cytosine or inhibition of protein synthesis was not determined. The lack of incorporation of $^{35}$S into protein made it impossible to determine if turnover occurred after IFT had completely stopped.

**Does Exchange Occur in Long Flagella?**—Flagellar outer doublet microtubules are continuous with basal bodies, so it was of interest to determine if basal bodies underwent protein turnover during the light period, when no new basal body assembly occurs (55). Cells were labeled with $^{35}$S for 2 and 6 h, and basal bodies were isolated and fractionated by SDS-PAGE. Numerous polypeptides were labeled, some of which comigrated with flagellar polypeptides and may be components of the axoneme (Fig. 11A, dots). Some polypeptides were not labeled during a 2-h period but were labeled after a 6-h labeling period (Fig. 11A, asterisks). Labeled polypeptides were also found in basal bodies if cells were labeled for 1–3 h and then chased with cold sulfate for 3–4 h (data not shown). Thus, the basal bodies, like flagella, exchange subunits with newly synthesized polypeptides in the absence of new basal body assembly. Whether these polypeptides exchange directly from a soluble cytoplasmic pool or enter from the flagellar compartment remains to be discovered.

**DISCUSSION**

Previous studies of sea urchin embryos provided important insight into the dynamics of protein turnover in cilia (44, 45). Early studies of *Chlamydomonas* flagella (11, 38–41) and *Tetrahymena* (42) cilia indicated the possibility of protein turnover. However, the reported turnover was low, and it was possible that labeled polypeptides found in isolated flagella and cilia could have been due to new protein synthesis induced during flagellar assembly in a small proportion of the cells and not protein turnover in steady state flagella. Remillard and Witman (43) reported the labeling of a nontubulin 55-kDa flagellar polypeptide in pulse-labeled flagella, and Marshall

---

**TABLE III**

Radioactivity of labeled proteins in each flagellar compartment after fla10 cells were incubated with $^{35}$S for 2 h

<table>
<thead>
<tr>
<th>Equal numbers of cells were used at each temperature.</th>
<th>22°C</th>
<th>33°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flagella</td>
<td>83,457</td>
<td>19,539</td>
</tr>
<tr>
<td>Membrane + matrix</td>
<td>45,717</td>
<td>12,272</td>
</tr>
<tr>
<td>Axonemes</td>
<td>49,776</td>
<td>6874</td>
</tr>
<tr>
<td>KCl-soluble proteins</td>
<td>26,400</td>
<td>4592</td>
</tr>
<tr>
<td>KCl-insoluble proteins</td>
<td>13,724</td>
<td>3055</td>
</tr>
</tbody>
</table>

**TABLE IV**

Comparison of labeled proteins in *lfa* and wild type cells after a 4-h incubation with $^{35}$S

<table>
<thead>
<tr>
<th>cpm/µg ± range</th>
<th>/f4 (n = 2)</th>
<th>Wild type (n = 2)</th>
<th>Ratio (lfa/wild type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axonemes</td>
<td>2149 ± 154</td>
<td>707 ± 474</td>
<td>3.0:1</td>
</tr>
<tr>
<td>KCl-soluble</td>
<td>1496 ± 265</td>
<td>594 ± 461</td>
<td>5:0:1</td>
</tr>
<tr>
<td>KCl-insoluble</td>
<td>654 ± 131</td>
<td>113 ± 16</td>
<td>5:8:1</td>
</tr>
<tr>
<td>Membrane + matrix</td>
<td>1243 ± 259</td>
<td>494 ± 53</td>
<td>2.5:1</td>
</tr>
<tr>
<td>Cell bodies</td>
<td>5895 ± 1179</td>
<td>1698 ± 879</td>
<td>5:4:1</td>
</tr>
</tbody>
</table>
and Rosenbaum (36) reported the incorporation of epitope-tagged tubulin in nongrowing flagella, but the exchange of other flagellar components was not examined. Recent discoveries of the importance of the IFT motors kinesin-II and cytoplasmic dynein in flagellar assembly and maintenance in a variety of organisms (16, 21–30) combined with earlier studies showing that flagellar growth and/or shortening can be induced by light (13), various drugs and ions (12–14), and temperature (15, 24), prompted a careful examination of protein turnover in steady state *Chlamydomonas* flagella.

Our experiments confirm that more than 80 different polypeptides are exchanged with a cytoplasmic protein pool in steady state flagella. However, many polypeptides are not exchanged in these flagella, and the turnover of some components is not necessarily linked to turnover of other components. For example, tubulin incorporation was blocked by colchicine without affecting the exchange of most of the other axonemal and membrane plus matrix polypeptides. It is possible that the lack of labeled tubulin incorporation in the presence of colchicine was due to the lack of new tubulin synthesis, due to tubulin autoregulation (56). However, we also tested our colchicine to ensure that the batch and concentration of colchicine used in each experiment completely blocked flagellar regrowth after deflagellation. Even if colchicine blocked new tubulin synthesis, there would be a sufficient pool of tubulin to assemble half-length flagella (11), so we are confident that the turnover observed in the presence of colchicine occurred in the absence of microtubule assembly.

Polypeptides associated with the most insoluble component of the axoneme, the PF ribbons (44, 47, 52, 53), were labeled, but rib43a and a 70-kDa ribbon polypeptide were lightly labeled. Tubulins were not labeled in the ribbon fractions, in contrast to the significant labeling of tubulin found in sea urchin embryonic ciliary ribbons (44, 52). The lack of labeling of individual polypeptides might reflect low numbers of cysteine or methionine residues, but tubulin is prominently labeled in the axonemes and is rich in each of these amino acids. Rib43a lacks cysteines but contains 10 methionine residues (47), so the lack of rib43a labeling in our experiments is unlikely to be due to a lack of $^{35}$S incorporation due to its amino acid composition. Rib240 exhibits a high level of exchange in *Chlamydomonas* and, after a 6-h labeling period, it contained 1% of the total flagellar $^{35}$S while comprising less than 0.1% of the axonemal protein. It will be interesting to further characterize this protein and to determine why it exchanges so rapidly and how it moves in and out of the insoluble and, presumably, stable PF-ribbons. The lack of tubulin exchange in PF-ribbons in *Chlamydomonas* but the presence of high levels of tubulin exchange in sea urchin junctional protofilalements may reveal differences in length regulation or in the positions of these insoluble flagellar components in the doublet microtubules.

Based on $^{35}$S incorporation, 20% of the exchangeable flagellar protein is replaced in steady state flagella within a 6-h period. On synchronized cells, flagella remain at constant length for greater than 12 h, so nearly 50% of the flagellar proteins would be expected to undergo replacement during the normal 12-h light period for *Chlamydomonas*. This is a low estimate of the turnover that occurs in vivo, because flagellar protein synthesis is relatively low in fully flagellated cells (57), and significant turnover would be expected to occur with unlabeled flagellar polypeptides synthesized before the addition of $^{35}$S in our experiments. New synthesis is not required to maintain flagella, because cells can be maintained in the presence cycloheximide or LiCl, both of which block protein synthesis required for flagellar regeneration, without any detectable changes in flagellar length or motility (13). Protein exchange also exceeds the rate at which flagella shorten, since fla10, at restrictive temperatures, and *pf18*, with reduced red light, incorporate newly synthesized flagellar proteins, including tubulin, at essentially the same rates as control cells even as the flagella shorten at rates up to 3.5 μm/h.

We expected that flagellar turnover would occur more slowly or not at all in mutants with exceptionally long flagella, but we discovered that proteins turn over at the same rate or, in some experiments, at a slightly faster rate than wild-type flagella. Flagellar protein exchange might occur throughout the flagella, in which case flagellar length would not be relevant to the rate of exchange. Alternatively, signals that regulate flagellar growth or maintenance might be more active in long flagellar mutants, and these might increase the rate at which new proteins are transported to the flagellar tips. The latter explanation is consistent with the rapid shortening of long flagella in dikaryons formed during mating of *If* mutants with cells with normal length flagella (17), which suggests that one or more of the mutations responsible for long flagella may be in a signaling system.

Signals that regulate the addition or removal of tubulin and, therefore, flagellar microtubule length are probably independent of those that regulate flagellar protein turnover. Although colchicine blocked flagellar growth and did not stimulate shortening, it did not affect the exchange of other axonemal or membrane plus matrix components. Colchicine induces flagellar shortening in *pf18* cells treated with low levels of red light and in *pf18* and wild-type cells treated with cytochalasin D (14), but these appear to reflect unique properties of *pf18* (13, 14) and a yet unexplained effect of cytochalasin D on flagellar length. Axonemal protein exchange may make the axonemes more responsive to signals that regulate length, but turnover alone does not appear to drive flagellar length changes.

Where does flagellar protein exchange occur? It is well es-
established that flagellar growth occurs by the addition of tubulin at the distal tips of flagella (11, 19), in association with the microtubule-capping structures that link the microtubules to the membrane (58–61). In fully grown flagella, new tubulin also is added to the distal tips of each microtubule (36). Radial spokes are added to the distal tips, and the addition of spokes to the axonemes proceeds proximally (19). Each of these results supports the existence of tubulin treadmilling (62) in the axoneme, and Marshall and Rosenbaum (37) have presented evidence that retrograde flux of epitope-tagged tubulin is balanced by IFT.

Our data are consistent with a treadmilling model with several important exceptions. First, tubulin exchange or treadmilling in the flagellum must occur independently of the turnover of other axonemal components, because most exchangeable axonemal components continue to turnover when tubulin addition is blocked by colchicine under conditions in which colchicine blocks flagellar regeneration after amputation. Moreover, if tubulin exchange occurs at the distal microtubule tips, one would expect that colchicine would lead to flagellar shortening; tubulin would be removed from one end of a microtubule, but no new tubulin could be added at the distal tip. We reported that colchicine increases flagellar shortening in a Chlamydomonas mutant, pft18, incubated under reduced red light (13, 14) and that colchicine induces flagellar shortening in cytochalasin D-treated pft18 and wild type Chlamydomonas cells (14). In each of these experiments, colchicine had no effect on wild type Chlamydomonas cells incubated under normal light. Based on our cytochalasin D results, we first proposed that flagellar microtubules are dynamic and undergo substantial tubulin exchange at the tips (14). However, since colchicine does not lead to significant flagellar disassembly, except in an unusual example (13, 14), it is unlikely that the maintenance of flagellar length is solely due to tubulin exchange being balanced by IFT.

IFT (and the kinesin-II and cytoplasmic dynein motors that drive it) is essential for the growth and maintenance of flagellar length. Disruption or inhibition of kinesin II blocks ciliary assembly in sea urchin blastulae (21), Tetrahymena (27), Caenorhabditis elegans (23–26), and mammalian embryos (29, 30). In Chlamydomonas, mutations of kinesin-II or of cytoplasmic dynein and associated proteins prevent flagellar assembly or make it impossible to maintain flagella (16, 31–33). The role of IFT in flagellar protein turnover is unclear, since data reported here revealed that Chlamydomonas cells that contain normal level of kinesin-II still resorb their flagella at restrictive temperature (15). Data reported by Piperno et al. (63) and our data clearly show that flagellar protein turnover continues to occur in fla10 kinesin-II mutants at restrictive temperatures.

In our experiments, 35S incorporation into flagellar proteins continued to occur as fla10 cells were maintained at restrictive temperature. Flagella shortened during this period, although IFT continues for 60–90 min after shifting cells to restrictive temperature (22). We attempted to label cells by adding 35S to cells after 60 or 90 min at restrictive temperature, but these cells failed to incorporate 35S into protein. These experiments do, however, clearly show that IFT alone is not responsible for flagellar protein exchange. This increases the importance of identifying the specific components carried by IFT, because IFT is essential for flagellar length control, and IFT probably carries signals that regulate length, not simply mediate polypeptide exchange and providing valuable suggestions about the manuscript as well as possible future directions for examining the dynamics of steady state flagella. We also thank Dr. Richard Lincke for providing rib43 antibodies and Dr. Richard Himes for tubulin antibodies.

**Acknowledgments**—We thank Dr. Ray Stephens for critically reviewing and providing valuable suggestions about the manuscript as well as pointing out the intellectual values for examining the dynamics of steady state flagella. We also thank Dr. Richard Lincke for providing rib43 antibodies and Dr. Richard Himes for tubulin antibodies.

**REFERENCES**


**Figures and Table**

Figure 1. Light micrographs of Chlamydomonas flagella.

Table 1. Properties of Chlamydomonas flagella.

**Tables**

Table 1. Properties of Chlamydomonas flagella.

**Figures**

Figure 1. Light micrographs of Chlamydomonas flagella.
Flagellar Protein Dynamics in *Chlamydomonas*
Lin Song and William L. Dentler

doi: 10.1074/jbc.M103184200 originally published online May 30, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M103184200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 63 references, 28 of which can be accessed free at
http://www.jbc.org/content/276/32/29754.full.html#ref-list-1