

Turnover of Tubulin in Ciliary Outer Doublet Microtubules

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ABSTRACT. Previous pulse-chase labeling studies have shown that structural proteins incorporate into fully assembled sea urchin embryonic cilia at rates approaching those of full regeneration. When all background ciliogenesis was suppressed by taxol, the turnover of most proteins, including tubulin, continued (23). The present study utilized chemical dissection to explore the route of tubulin incorporation in the presence of taxol and also in steady-state cilia from prism stage embryos. Surprisingly, in cilia from untreated embryos, the most heavily labeled tubulin was found in the most stable portion of the doublet microtubules, the junctional protofilaments. With taxol, this preferential incorporation was suppressed, although control-level turnover still took place in the remainder of the doublet. This paradoxical result was confirmed by pulse-chase labeling and immediately isolating steady-state cilia, then isolating two additional crops of cilia regenerated, respectively, from pools of high and then decreased label. In each case, the level of label occurring in the tubulin from the junctional protofilaments, compared with that from the remainder of the doublet, correlated with the level of pool label from which it must exchange or assemble. These data indicate that ciliary outer doublet microtubules are dynamic structures and that the junctional region is not inert. Plausible mechanisms of incorporation and turnover of tubulin in fully-assembled, fully-motile cilia can now be assessed with regard to recent discoveries, particularly intraflagellar transport, distal tip incorporation, and treadmilling.

Key words: tubulin/cilia/protein exchange/kinesin/dynein/chaperones

Introduction

While studying the coordinate synthesis of ciliary proteins, both at ciliogenesis and in response to experimental deciliation, I observed the incorporation of pulse-chase labeled 9+2 proteins into fully-assembled cilia of hatched blastula and gastrula stage sea urchin embryos (18, 19, 20). Interestingly, the most heavily labeled protein was tektin-A, an integral structural component of the junctional protofilaments that join the B-subfiber to the A-tubule of outer doublet microtubules. The overall level of labeling of most proteins was at least half that seen in full regeneration and could not be attributed to background ciliogenesis, apical tuft elongation, or mechanical loss and subsequent regeneration (21). Rigorous proof of true protein turnover or exchange in these fully assembled, fully functional cilia was provided by the fact that architectural protein synthesis and turnover continued unabated in colchicine

at sufficient concentrations to prevent both tubulin assembly and tubulin synthesis (23). Furthermore, when taxol was used to block tubulin assembly but not synthesis, axonemal tubulin was also labeled to nearly the same extent as in untreated embryos.

This present study carries these observations one step further by examining the labeling of tubulin in the membrane-matrix, doublet, and junctional protofilament fractions of steady-state cilia on sea urchin embryos, both in the presence of taxol and at a late stage when background ciliogenesis is insignificant. Incorporation of the most recently synthesized tubulin occurred most prominently in the stable junctional protofilaments of the outer doublets. This phenomenon, though not overall incorporation of tubulin into the doublet walls, was suppressed by taxol. Based on recent discoveries in other systems, possible mechanisms for tubulin transport, incorporation, and removal can be postulated. These include intraflagellar transport by kinesin-like proteins, distal assembly via the tip complex, basal removal by a katanin-like protein, and dimer insertion/removal via propagated surface lattice defects. Furthermore, the wide-spread presence of axoneme-bound

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chaperones suggests that these may be mediators of dynamic protein exchange.

Materials and Methods

Embryo culture and labeling

Embryos of the sea urchin *Strongylocentrotus droebachiensis* were cultured to the late gastrula stage in magnetically-stirred tissue culture flasks as described previously (20). Embryos were labeled with ^3H -leucine (New England Nuclear #460, 147 Ci/mmol) at a level of 2.5 $\mu\text{Ci/ml}$, washed with sea water, and "chased" with 0.25% unlabeled leucine for the times noted in the experiments described below.

Cilia isolation and fractionation

Embryos were harvested by manual centrifugation and deciliated with >10 volumes of double-tonicity sea water. When required, the deciliated embryos were recovered by manual centrifugation, resuspended in normal-tonicity seawater, and returned to the culture flasks for regeneration. The cilia were recovered from the high salt medium by differential centrifugation and separated into detergent-soluble membrane-matrix and insoluble axoneme fractions by extraction with 0.25% NP-40 or Triton X-100 (20). The axonemes were further fractionated into soluble axonemal tubulin and insoluble nine-fold ciliary remnants by heating at 45°C for 16 min in 1 mM EDTA, 10 mM Tris-HCl (pH 8), followed by high speed centrifugation (25).

Analysis

Proteins were resolved by SDS-PAGE on 8%T/2.5%C acrylamide (8) and equilibrium-stained with Coomassie Blue R-250 (3). Dried gels were subjected to fluorography using EN 3 HANCE or INTENSIFY fluors (New England Nuclear) and pre-flashed X-Omat AR film (Kodak) exposed at -80°C . Quantifi-

cation was by video densitometry (23). Alternatively, radioactivity was determined by scintillation counting after protein bands from dried gels were excised and solubilized with H_2O_2 (20). In all analyses described below, α -tubulin, rather than β -tubulin, was quantified since a previous study using two-dimensional gel analysis showed that no other labeled protein co-migrated with the α -subunit under the one-dimensional conditions used here (19).

Results

Tubulin turnover in cilia of taxol-treated embryos

To investigate further the incorporation of tubulin into taxol-treated steady-state cilia, a single culture of *Strongylocentrotus droebachiensis* gastrula-stage embryos was split in two. One was treated with 10 μM taxol while the other was treated with the DMSO carrier alone. Both were labeled for 4 hrs with ^3H -leucine, followed by a 4 hr chase with unlabeled leucine (8 hrs represents full regeneration at 7.5°C). Cilia were isolated, fractionated, resolved by SDS-PAGE, and the specific activity of α -tubulin was determined. The basic fractionation scheme is given in Figure 1. The results are shown in Figure 2 and Table I.

With taxol present, the amount of tubulin found in the membrane-matrix fraction was reduced by nearly a factor of two, yet its specific activity was 72% of the control, the difference probably representing background ciliogenesis in the untreated culture (23). In both cases, the specific activity of the membrane-matrix tubulin exceeded that of the axoneme by a factor of ~ 3 , as is characteristic of this presumptive precursor during steady-state turnover (20). After thermal fractionation, the specific activity of the solubilized tubulin (central pair plus all of the B-tubule and much of the A-tubule) was nearly identical in the two cases. However, the tubulin remaining with the A-B junctional

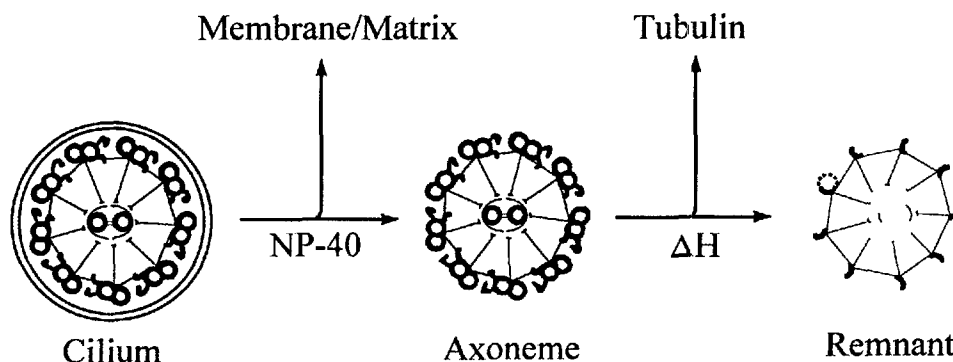


Fig. 1. Schematic diagram of detergent and thermal fractionation of sea urchin embryonic cilia into NP-40-solubilized membrane-matrix, heat-solubilized tubulin, and insoluble remnant fractions (based on 25). The "dotted" tubule on the remnant indicates the former position of an A-tubule.

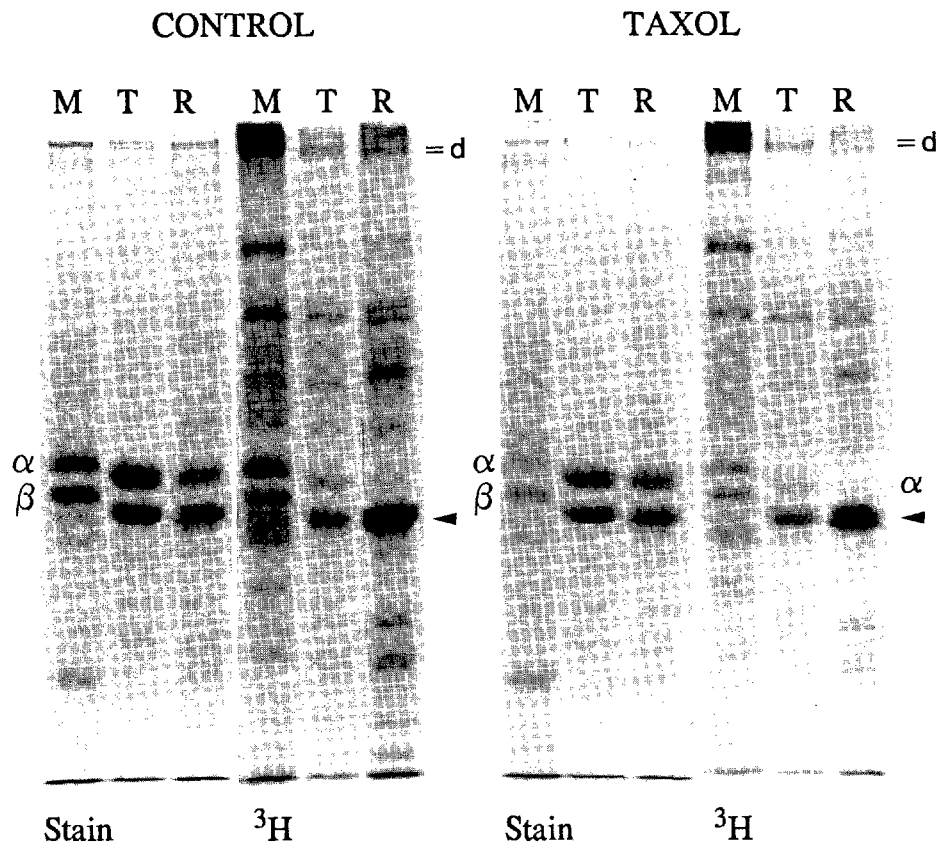


Fig. 2. SDS-PAGE (stain) and fluorographic (^3H) analysis of detergent-solubilized membrane-matrix (M), heat-solubilized tubulin (T), and insoluble 9-fold ciliary remnants (R) from cilia of control and taxol-treated late gastrula stage sea urchin embryos. Legend: d=dynein heavy chains; α and β designate the tubulin subunits; arrowhead=tektin A. Tubulin chains in the membrane-matrix lanes migrate anomalously due to NP-40 in the sample. When calibrated with internal standards, densitometry of α -tubulin protein and radioactivity signals yielded the data in Table I.

protofilaments was 1.8-fold *higher* in control cilia but 1.5-fold *lower* in the taxol treated case, even though more tubulin remained with the remnant fraction as a consequence of taxol stabilization. In spite of these differences in junctional protofilament tubulin specific activity, the label in tektin-A was essentially the same. These labeling patterns suggest preferential but taxol-suppressible incorporation of tubulin in the presumably stable junctional region.

A preferential incorporation pathway in steady-state and regenerating cilia?

To investigate this incorporation pathway paradox, the degree of tubulin labeling in steady-state and subsequently regenerating cilia was studied in untreated embryos undergoing minimal background ciliogenesis. Prism stage embryos were pulse-labeled for 6 hrs (one half the normal regeneration time at 4°C) and then deciliated. Cilia were allowed to regenerate for 12 hrs in the presence of unlabeled leucine and removed. A sec-

Table I. INCORPORATION OF ^3H -LEUCINE-LABELED α -TUBULIN INTO CILIARY FRACTIONS FROM UNTREATED CONTROL AND TAXOL-TREATED GASTRULA-STAGE SEA URCHIN EMBRYOS

	M/A	Memb-Matrix cpm/mg	Total Axoneme cpm/mg	ΔH Soluble cpm/mg	9-Fold Remnant cpm/mg	R/S
Control	2.88	307,100	106,560	80,845	149,480	1.85
Taxol	3.23	220,150	68,080	78,995	54,020	0.68

Average of two determinations. M/A is the specific activity ratio of detergent-solubilized membrane-matrix to total axonemal α -tubulin; R/S is the specific activity ratio of insoluble remnant to heat-solubilized α -tubulin.

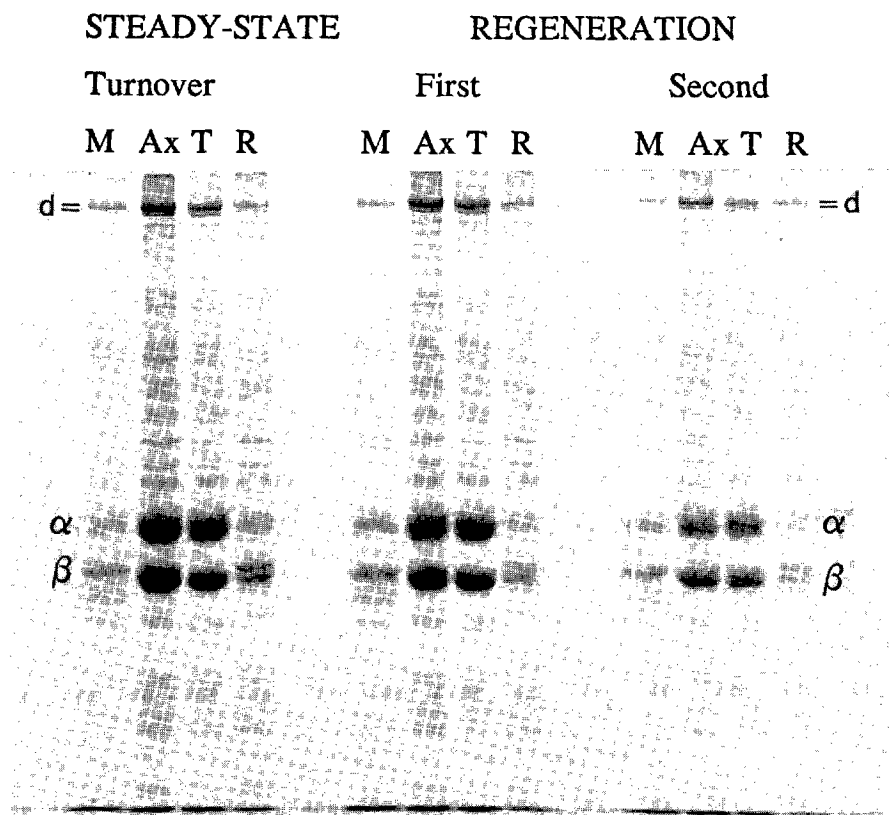


Fig. 3. SDS-PAGE analysis of detergent-solubilized membrane-matrix (M), axoneme (Ax), heat-solubilized tubulin (T), and insoluble 9-fold ciliary remnants (R) from steady-state and two regenerations of cilia from prism stage sea urchin embryos. Legend: d=dynein heavy chains; α and β designate the tubulin subunits. All samples contained NP-40, resulting in parallel migration of tubulin subunits. Steady-state samples include fully-grown somatic and apical tuft cilia and hence contain more protein; successively regenerated cilia are shorter and hence contain less protein. Densitometry of the α -tubulin protein signal, followed by scintillation counting of excised α -tubulin bands yielded the data in Table II.

and crop of regenerated cilia was allowed to form and it, too, was isolated. The consequence of this protocol was the production of 1) pre-existing, fully-assembled cilia labeled during steady-state turnover, 2) cilia regenerated from a heavily labeled pool, and 3) cilia regenerated from a label-depleted pool. As above, these sets of cilia were fractionated into detergent-solubilized membrane-matrix, heat-solubilized axonemal tubulin, and

insoluble A-B junctional remnants and resolved by SDS-PAGE. These fractions are illustrated in Figure 3 while the specific activity of α -tubulin is given in Table II. (Direct quantification was used here due to the limited dynamic range of fluorography.)

In steady-state cilia, the membrane-matrix tubulin, deriving label from the precursor pool, had much higher specific activity than the already-assembled axo-

Table II. INCORPORATION OF ^3H -LEUCINE-LABELED α -TUBULIN INTO VARIOUS 9+2 FRACTIONS FROM STEADY-STATE AND REGENERATING CILIA OF PRISM STAGE SEA URCHIN EMBRYOS

	M/A	Memb-Matrix cpm/mg	Total Axoneme cpm/mg	ΔH Soluble cpm/mg	9-Fold Remnant cpm/mg	R/S
Steady-state	3.98	124,630	31,280	21,000	56,100	2.67
1st Regeneration	0.84	245,520	292,270	270,120	380,180	1.41
2nd Regeneration	0.90	141,180	156,650	161,410	145,240	0.90

Average of two determinations. M/A is the specific activity ratio of detergent-solubilized membrane-matrix to total axonemal α -tubulin; R/S is the specific activity ratio of insoluble remnant to heat-solubilized α -tubulin.

neme, in this case by a factor of about 4 (since no chase was used during the turnover period). When the axoneme was fractionated, the solubilized tubulin had substantially lower specific activity than the axoneme as a whole while the tubulin associated with the remnant, representing only about 1/5 of the total, was labeled to nearly half the extent of the membrane-matrix.

In the first regeneration cilia, the membrane-matrix tubulin was less heavily labeled than the total axonemal tubulin but both fractions were more highly labeled than in the steady-state case. This increase in label seen in incorporated tubulin is due to the immediate upregulation of tubulin synthesis, a consequence of deciliation, while the lesser label in the membrane-matrix tubulin is due to the gradual replacement with protein synthesized primarily from unlabeled leucine during the chase (cf. 20). Upon fractionation, the remnant again contained the more heavily labeled tubulin, with its label exceeding even that of the membrane-matrix.

In the second regeneration cilia, now utilizing a pool containing a substantial fraction of proteins synthesized from unlabeled leucine, the membrane-matrix tubulin represents the lowest specific activity, closely followed by the tubulin from the remnant fraction. Here, the solubilized axonemal tubulin had the highest specific activity, again suggesting the most recently synthesized tubulin (now of the lowest specific activity) assembled preferentially at the junctional region. This is supported in all three cases by the ratio of label in the remnant to that in the soluble fraction, given as R/S in Table II. Note that the specific activity of total axonemal α -tubulin $\approx 4/5 \times$ (specific activity of ΔH soluble α -tubulin) $+ 1/5 \times$ (specific activity of remnant α -tubulin), paralleling the 4:1 tubulin distribution.

Discussion

Using this "follow the label" approach, the precursor-product relationship between tubulin in the membrane-matrix and tubulin in the axoneme was described in some detail previously and led to the initial proposal that the axonemes of fully-functional cilia may be treadmilling (20). The results presented here, in addition to confirming these basic observations with independent examples, add new insight into the incorporation process. Perhaps most surprisingly, tubulin appears to enter the outer doublet via the junctional region. This result seems less surprising, however, when one considers that the integral junctional protofilament component, tektin-A, is the most heavily labeled protein in steady-state cilia (18, 19), and it must somehow intercalate into the A-B junction. Taxol suppression of incorporation at the junction could be explained by assuming that the drug over-stabilizes this region in comparison with the remaining circumfer-

ential protofilaments of the doublets. Nevertheless, tektin-A still turns over at nearly the same rate in the presence of taxol, suggesting either independence of the two processes or some fundamental difference in the insertion of tubulin at the growing tips of the doublets.

There is considerable supporting evidence for ciliary protein turnover, most likely for maintenance purposes. For example, the levels of tektin-A mRNA remaining in sea urchin blastomeres after ciliogenesis, regeneration, animalization, or zinc-induced hyperciliation correlate with the length of cilia being maintained (14), suggesting that the cell continually provides new ciliary components. Using the same labeling approach as used with sea urchin embryos, turnover has been confirmed in molluscan gill cilia (22), *Chlamydomonas* flagella (17), and mammalian tracheal cilia (24). In the last case, the terminally differentiated ciliated epithelial cells synthesized mainly ciliary proteins.

There is no longer any question that the 9+2 structure grows by the distal addition of architectural components and tubulin itself (6). In fact, evidence for tip-to-base treadmilling has been obtained from *Chlamydomonas* flagella incorporating epitope-tagged tubulin (12). The obvious question now is how all of this action can take place in an organelle that is topologically isolated, by the basal plate and ciliary necklace, from a cell that is producing its replacement parts. It has been suggested that precursor transport takes place in association with the membrane via groups of particles observed to shuttle within the organelle (intraflagellar transport), with tipward motion utilizing a kinesin-like protein (7) and baseward transport utilizing a dynein-based motor (15).

Distal assembly must occur via a membrane-associated tip complex that exists throughout growth and remains a prominent feature of mature cilia and flagella. This structure consists of a central microtubule "cap" and carrot-like "plugs" that insert into the lumen of each A-tubule, in both cases blocking the plus end of these microtubules (5, 16). It may be that this structure directs tubulin preferentially to the junctional protofilaments since the tip complex appears to remain associated with the protofilaments after microtubule depolymerization (25).

More difficult is the question of basal disassembly. Here we are left wondering how integral structural components can be removed while maintaining the integrity of the structure. However, the protein katanin appears able to remove tubulin from microtubule walls (13) and such a protein has been shown at the base of *Chlamydomonas* flagella (11), so at least a viable candidate is available.

A novel directional incorporation mechanism for tubulin might involve insertion of dimers into discon-

tinuities at the A-B junction. While potentially also spreading radially, the surface lattice defects resulting from distal insertions should propagate primarily toward the base where, upon reaching the presumably intractable basal plate, dimers would be forced sterically to exit, annealing the defect. Since junctional protofilaments may function to accommodate different surface lattices between the two tubule subtypes (10) and since individual microtubules can have regions containing different protofilament numbers, accommodated by surface lattice defects (2), such a model is certainly plausible.

Another possibility is offered by the presence of axoneme-bound chaperones, particularly HSP70 (1, 23, 24). Not only are chaperones involved in protein folding and complex formation, they have been implicated in cytoskeletal protein exchange as well (4, 9). In cilia, these may serve as mediators of tubulin insertion or removal. However, at this stage, the only certainty is that cilia and flagella are far more dynamic than anyone had imagined.

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