STRUCTURES LINKING THE TIPS OF CILIARY AND FLAGELLAR MICROTUBULES TO THE MEMBRANE

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SUMMARY
The distal tips of both central pair and outer doublet microtubules are linked to flagellar membranes of *Chlamydomonas* and to ciliary membranes of both *Tetrahymena* and *Aequipecten* by the central microtubule cap and by distal filaments, respectively. The distal filaments are linked to ciliary membranes at their distal ends and, at their proximal ends, form a carrot-shaped plug which inserts into the lumen of the A-tubule of each outer doublet. Both types of tip structures are present in regenerating cilia and flagella. At least two different types of distal filaments are present in *Tetrahymena*. Certain cilia in *Tetrahymena* oral apparatuses and in certain molluscan gills contain distal filaments which join with the central microtubule cap and with the ciliary membrane to form a cap-filament-membrane complex.

INTRODUCTION
Although the 9 + 2 microtubular structure of cilia and flagella is relatively well understood, the relationship between the microtubules and the surrounding membrane has received little attention. Recently it has been demonstrated that each of the 9 outer doublet microtubules is linked to ciliary and flagellar membranes by lateral bridges (Allen, 1968; Dentler, Stephens & Pratt, 1978; Ringo, 1967; Sattler & Staehelin, 1974) and that these lateral bridges may be involved in ciliary motility (Dentler et al. 1978). Moreover, the distal ends of both the central pair and each of the A-tubules of the outer doublets terminate in the central microtubule cap (Dentler & Rosenbaum, 1977; Sale & Satir, 1977) and distal filaments (Dentler & Rosenbaum, 1977), respectively. It was proposed that the central microtubule cap attaches the central microtubules to the distal tip of the flagellar membrane and serves as an initiation centre for their assembly and that the central microtubules, therefore, assemble by the addition of subunits to their proximal ends, near the basal body (Dentler & Rosenbaum, 1977). The relationship of either the central microtubule cap or the distal filaments to the surrounding membrane was not, however, clear, since the tip structures were observed only in negatively stained flagella after the flagellar membranes were dissolved by nonionic detergent. Results reported here demonstrate, using both thin-sectioned and negatively-stained material, that the central microtubule cap and the distal filaments link the distal tips of *Chlamydomonas* flagellar and *Tetrahymena* ciliary microtubules to the membrane. The distal filaments are linked to the membrane and insert into the lumen of the A-tubules of...
each outer doublet, thereby linking the outer doublet microtubules to the membrane. In some *Tetrahymena* oral apparatus cilia and in certain scallop gill cilia the distal filaments appear to link the outer doublets to the central microtubule cap which is, in turn, linked to the membrane. Finally, these results demonstrate that after amputation and prior to ciliary and flagellar regeneration, the central microtubule cap and the distal filaments are the initial structures which are formed prior to the assembly of the microtubules and that these structures link the microtubules to the membrane throughout ciliary and flagellar growth.

A preliminary report of some of these data was presented to the American Society for Cell Biology (Dentler, 1977).

**MATERIALS AND METHODS**

**Cell culture**

*Chlamydomonas reinhardtii* (strain 21gr) were grown in 125-ml flasks in Medium I (Sager & Granick, 1953) with constant aeration at room temperature on a cycle of 12 h of light and 12 h of dark. For best results the cells were grown to a low density and were used within 4 h after the lights were turned on.

*Tetrahymena pyriformis* (strain BIV) were grown and harvested as previously described (Dentler & Cunningham, 1977).

*Aequipecten irradians*, the bay scallop, were obtained from the supply room at the Marine Biological Laboratory, Woods Hole, MA, and were stored in running seawater until used.

**Electron microscopy**

*Chlamydomonas* cells were attached to grids coated with poly-L-lysine and Formvar, treated with deflagellation solution (5 mM MgSO₄, 0.35 mM DTT, 0.5 mM EDTA, 10 mM HEPES, pH 7.5) with 0.002–0.01 % Nonidet P-40 (Particle Data Laboratories, Elmhurst, IL), and negatively stained as previously described (Dentler & Rosenbaum, 1977). To preserve distal filaments and plugs, the detergent extraction was kept to less than 10 s. In order to splay open the A-tubules, extracted flagella were occasionally rinsed with several drops of distilled water prior to negative staining with freshly prepared 1 % aqueous uranyl acetate.

*Tetrahymena* cells were harvested during early log phase of growth and were fixed and embedded as described elsewhere (Dentler & Cunningham, 1977). Thin sections were cut with a diamond knife and were stained with methanolic uranyl acetate and lead citrate and were viewed and photographed using a Philips EM 300.

For negative staining, *Tetrahymena* cilia were isolated using the dibucaine procedure of Thompson, Baugh & Walker (1974). Isolated cilia were then applied to poly-L-lysine-coated Formvar films and were demembranated and negatively stained as described above for *Chlamydomonas* flagella. Since *Tetrahymena* ciliary membranes were more resistant to detergent, the deflagellation solution contained 0.1–1 % Nonidet P-40.

Gills from the bay scallop *Aequipecten* were removed by dissection and were fixed in 1 % glutaraldehyde in 0.1 M sodium phosphate, pH 7.8 for 1 h at 4 °C. Gills were rinsed in cold phosphate buffer and were then postfixed with 1 % osmium tetroxide in phosphate buffer for 1 h at 4 °C, rinsed with distilled water, stained en bloc with 1 % aqueous uranyl acetate, and were dehydrated with ethanol and propylene oxide and embedded in Epon–Araldite.

**Ciliary and flagellar amputation and regeneration**

*Chlamydomonas* cells were deflagellated using the pH shock method of Witman, Carlson, Berliner & Rosenbaum (1972). Cell bodies, after amputation of flagella, were recovered by centrifugation (5 min at 600 g) and were resuspended in fresh medium. Cells were continuously aerated in room light and samples were taken at 5-min intervals during flagellar regeneration. Regenerating cells were either fixed in 1 % glutaraldehyde in phosphate buffer for light microscopy and measurement of flagellar lengths or they were applied to electron-
microscope grids, extracted with detergent, and negatively stained as described above. Flagellar lengths were measured using an ocular micrometer in a Nikon phase-contrast microscope.

*Tetrahymena* cells were deciliated using the dibucaine procedure; cell bodies were recovered by centrifugation and were resuspended in fresh 2% proteose peptone. Ciliary regeneration, as assayed by an increase in cell motility and the observation of individual cilia by phase microscopy, was generally complete within 90 min after deciliation. As contrasted with *Chlamydomonas* cells, in which all flagella regenerated at the same rate, ciliary regeneration in *Tetrahymena* was neither synchronous in the whole population nor on any single cell. Aliquots of cells were removed at 10-min intervals during regeneration and were fixed and embedded for electron microscopy as described elsewhere (Dentler & Cunningham, 1977).

**RESULTS**

**Distal filaments**

Detergent-extraction of the distal tips of cilia and flagella revealed that the central microtubule cap was attached to the 2 central microtubules and the distal filaments were attached to the A-tubule of each outer doublet microtubule (Fig. 1). Two 4-nm diameter distal filaments are attached to the tip of each A-tubule in *Chlamydomonas* flagella (Fig. 1) and each of these filaments is, in turn, composed of several 1-2-nm filaments (Fig. 1, inset). When distal filaments were released from the A-tubules or when the A-tubules were splayed open it could be seen that the filaments were attached to a small plug, approximately 9 nm in diameter and 70 nm long, which was inserted in the lumen of each A-tubule (Figs. 1-3). Although the plugs and filaments were loosely associated with the A-tubules and popped out during brief extraction with low concentrations of detergent, a small piece of the plug was often observed to remain in the microtubule lumen, presumably attached to the tubulin protofilaments (Fig. 3).

The distal filaments and plugs, therefore, appear similar to carrots which insert into the hollow distal tips of the A-microtubules.

Previous results (Dentler & Rosenbaum, 1977) showed that the central microtubule cap and distal filaments remain associated with flagellar microtubules throughout pyrophosphate-induced resorption of most of the flagellum and during regrowth following resorption. It was possible, however, that the tip structures were added after the flagella had fully grown and that they were present in the regenerating flagella only because the flagella had been partially resorbed prior to regeneration. In order to determine if the tip structures were present throughout the assembly of new flagella, *Chlamydomonas* cells were deflagellated and analysed for the presence of tip structures during flagellar growth. The results in Fig. 4 show that prior to deflagellation 100% of the central microtubules were attached to the caps and 98% of the A-tubules contained distal filaments. After deflagellation and throughout regeneration, the central microtubule cap was found to be attached to all central microtubules. Although the percentage of outer doublets with distal filaments was as low as 50% during early stages of flagellar assembly, the percentage of doublets containing distal filaments increased up to the initial value of 98% as the rate of flagellar growth reached a plateau. Even though distal filaments were found to be more frequently associated with outer doublets in fully grown rather than in rapidly assembling flagella (Fig. 4), at all times at least 50% of the A-tubules contained distal filaments and, moreover, in rapidly growing flagella, distal filaments were generally associated with one or more of
Fig. 1. Detergent-extracted and negatively stained distal tip of a *Chlamydomonas* flagellum. The central microtubule cap (cmc) at the tip of the central microtubules and distal filaments (arrowheads) protrude from the distal tips of the A-tubules ($A$) of each outer doublet microtubule. Paired distal filaments can be seen on the third doublet from the bottom of the micrograph. $B$, B-tubule. Inset: frayed distal filaments at the tips of A-tubules. $\times 70000$. Inset, $\times 150000$. 
Fig. 2. Distal tips of detergent-extracted and negatively stained *Chlamydomonas* flagella. The distal tips of the A-microtubules are splayed open to reveal the distal filament plugs which lay inside the lumen of the A-microtubules. Although the A-microtubules are severely disrupted, the central microtubule cap remains attached to the central pair microtubules. ×80,000.

Fig. 3. Distal filaments and plugs (large arrowheads) which have popped out of the A-microtubules; occasionally, a small piece of the plug remains attached to the inner wall of the A-microtubules (small arrowheads). ×80,000.
the 9 outer doublet microtubules in any single flagellum. Since distal tips of rapidly assembling outer doublets were more frequently disrupted by the extraction procedure than were fully grown outer doublets (Dentler & Rosenbaum, 1977), it is likely that distal filaments were associated with outer doublets throughout assembly; the lower frequency with which distal filaments were observed in growing outer doublets is probably due to the relative fragility of the growing microtubule tips.

![Graph showing the fate of distal filaments and central microtubule cap](Image)

**Fig. 4.** Fate of the distal filaments (DFs) and the central microtubule cap (CMCs) during Chlamydomonas flagellar regeneration. Following amputation and throughout regeneration the CMCs were present on all of the central microtubules. Under the same extraction conditions, DFs were associated with 98% of the outer doublets prior to amputation, only 50-60% of the outer doublets during rapid flagellar growth (solid line), and 98% of the outer doublets as the rate of flagellar growth reached a plateau. An average of 50 microtubules were scored for each point on the graph.

**Relationship of the central microtubule cap and distal filaments with ciliary and flagellar membranes**

Although the cap and distal filaments were occasionally observed to be attached to a piece of membrane (Dentler & Rosenbaum, 1977), negative staining of detergent-extracted flagella was not sufficient to permit analysis of the relationship of the tip structures with the membrane in intact flagella. Since the central microtubule cap (Sale & Satir, 1977; Fig. 5) and distal filaments (Fig. 5) are attached to the tips of Tetrahymena cilia, thin-section electron microscopy was used to examine Tetrahymena cilia, since the large number of cilia per cell made thin-section analysis more feasible.

Thin sections of Tetrahymena cilia revealed that the bead portion of the central microtubule cap is linked to the ciliary membrane by small bridges (Figs. 6-9). Other results suggest that this linkage must be relatively strong, since isolated cilia retain pieces of membrane attached to the cap even after detergent extraction or extensive mechanical agitation (Dentler, 1979). Thin sections also revealed that the distal filaments attach to both the ciliary membrane and the outer doublet microtubules.
The filaments extend from the distal tips of the A-tubules and lie along the cytoplasmic face of the ciliary membrane: short bridges extend from the membrane and appear to stitch the distal filaments to the membrane (Figs. 6–8, 13, 16–18). These results demonstrate that the ciliary membrane is attached to both central and outer microtubules. 

Fig. 5. Isolated, detergent-extracted and negatively stained *Tetrahymena* cilium. The central microtubules terminate in the central microtubule cap (large arrowhead); pieces of the membrane remain attached to the cap. A distal filament (small arrowhead) is attached to an A-microtubule. × 56000.

Figs. 6–9. Thin sections of distal tips of cilia fixed *in situ* (compare with the negatively stained ciliary tip in Fig. 1). Central microtubule caps (large arrowheads) are linked to ciliary membranes by small bridges and distal filaments (small arrowheads) are linked to both the A-microtubules and to the membrane. Fig. 6, × 100000; Fig. 7, × 60000; Fig. 8, × 100000; Fig. 9, × 90000.
doublet microtubules at their distal tips by the central microtubule cap and by the distal filaments.

Although the distal filaments in the body cilia of *Tetrahymena* appear virtually identical to their counterparts in *Chlamydomonas* flagella, there are significant differences in the tip structures of *Tetrahymena* body cilia when compared with cilia in the oral apparatus of the same cell. Some of the oral apparatus cilia contain knob-shaped distal filaments which appear to attach directly to the membrane (Figs. 17, 18),

Fig. 10–16. Thin sections of regenerating *Tetrahymena* cilia after dibucaine-induced deciliation. Distal filaments (small arrows) and central microtubule caps (large arrows) are present at the distal tips of the regenerating cilia. Fig. 10, × 24,000; Fig. 11, × 33,000; Fig. 12, × 42,000; Fig. 13, × 30,000; Figs. 14–16, × 36,000.
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Fig. 17. Distal filaments in some *Tetrahymena* oral apparatus cilia are club-shaped structures which attach to the ciliary membrane by small bridges (arrows). Some of the distal filaments appear to be closely associated with the central microtubule (ct) cap and with filaments attached to adjacent outer doublets (top left). $\times 100,000$.

Fig. 18. Thin section showing the fine structure of the central microtubule cap in *Tetrahymena*. Both the 2 plates attached to the central microtubules (large arrows) and bridges linking the cap to the membrane (small arrows) are visible. $\times 100,000$.

Fig. 19. Distal filaments (arrowhead) connecting the A-microtubules to the central microtubule cap in *Aequipecten* cilia. $\times 100,000$. 
while others contain distal filaments which appear to link the outer doublets to the central microtubule cap which is, in turn, connected to the membranes (Fig. 17).

Since both the cap and the distal filaments were attached to flagellar microtubules during their assembly, it was of interest to determine if the tip structures attached ciliary microtubules to the membrane throughout their assembly and, if so, how the tip structures originated. Tetrahymena cells were deciliated using the method of Thompson et al. (1974) and cells with regenerating cilia were fixed and embedded at various times during ciliary growth. Although the tip structures were very difficult to preserve and resolve, the results (Figs. 10–16) suggested that the caps and distal filaments attach the growing microtubules to the membrane. Preliminary results suggest that the central microtubule cap may be formed in association with the basal granule (axosome).

Distal tip structures in molluscan gill cilia

Since the central microtubule caps and distal filaments were found in both Tetrahymena cilia and in Chlamydomonas flagella, it was important to examine cilia from higher organisms to determine if these structures may be common to all cilia and flagella. Thin sections of cilia from the gills of the bay scallop Aequipecten showed both distal filaments and central microtubule caps (Fig. 19). Although the tips of actively motile cilia in scallop gills appeared similar to the tips of Tetrahymena body cilia, gill cilia which are specialized to hold adjacent gill filaments together and which are, therefore, relatively non-motile, contain distal filaments which link the outer doublets to the central microtubule cap which is, in turn, linked to the ciliary membrane (Fig. 19).

DISCUSSION

Relationship of the distal tip structures with membranes

Previous reports have shown that the central microtubules of both Chlamydomonas flagella (Dentler & Rosenbaum, 1977) and Tetrahymena cilia (Sale & Satir, 1977) terminate in the central microtubule cap. Although pieces of flagellar membrane were often found attached to the cap in detergent-extracted flagella, the relationship of the cap to the membrane was not determined (Dentler & Rosenbaum, 1977). Data presented here shows that the bead portion of the cap is linked to the ciliary membrane by small bridges which appear similar to the bridges which link the long axis of outer doublets to ciliary membranes (Dentler, Pratt & Stephens, 1979).

Both the structure of the distal filaments and their relationship to the membrane is also now clarified. The distal filaments are bound together at their proximal ends by a knot-like structure to form a carrot-shaped plug which is then inserted into the lumen of the A-tubule of each outer doublet. The plug is most likely equivalent to the electron-dense dot which is found in the lumen of the most distal portions of A-tubules (Ringo, 1967; Dentler, unpublished observation). The distal filaments, which are connected to the plug, link the distal tips of the A-tubules to the cytoplasmic face of the membrane. A diagram illustrating this association is presented in Fig. 20.

Although distal filaments and central microtubules caps have been found in all cilia
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which have been examined, it is interesting that these tip structures vary somewhat from species to species and even within a single *Tetrahymena* cell. Whereas most of the *Tetrahymena* body cilia and *Chlamydomonas* flagella had distal tips consistent with the model presented in Fig. 20, certain of the *Tetrahymena* oral apparatus cilia contained knob-shaped distal filaments. Moreover, in certain of these oral cilia as well as in certain specialized cilia in scallop gills, the distal filaments appeared to link the outer doublets to the central microtubule cap which was, in turn, attached to the surrounding

Fig. 20. Diagram illustrating the associations of the distal filaments and the central microtubule cap with the ciliary membrane and the outer doublet microtubules. A portion of the wall of an A-microtubule has been omitted to illustrate the insertion of the distal filament into the lumen of the A-microtubule. The helical filament attached to the central microtubule cap is distinct from the apparent sheath which has been reported to wrap around the proximal portion of the central pair microtubules.
membrane; these cilia, however, contained outer doublet microtubules which were approximately equal in length to the central pair microtubules. In both *Chlamydomonas* flagella and in the body cilia of *Tetrahymena*, however, the central pair microtubules are usually longer than the outer doublets. Although Sattler & Staehelin (1974) reported other differences between the body and oral cilia of *Tetrahymena*, no correlation between the structures reported by them and the distal tip structures reported here was made.

**Distal tip structures and flagellar microtubule assembly**

We previously reported that the central microtubule cap remained attached to the central microtubules throughout pyrophosphate-induced flagellar disassembly and regeneration of the partially resorbed flagella after removal of pyrophosphate (Dentler & Rosenbaum, 1977). Furthermore, the caps prevented the addition of tubulin subunits to the ends of the central microtubules *in vitro*, even though uncapped microtubules in the same preparation readily nucleated microtubule assembly (Dentler & Rosenbaum, 1977). We proposed, therefore, that the central microtubule cap is a nucleation centre for the assembly of the central microtubules and that these microtubules assemble by the addition of subunits to their proximal ends. The results reported here are consistent with this proposal: in both *Chlamydomonas* and *Tetrahymena* the central microtubule cap is one of the first structures to appear during the regeneration of cilia and flagella following amputation and, moreover, the cap attaches the distal tips of the central microtubules to the membrane throughout growth. These results strongly suggest that the central microtubule cap is a nucleation centre for the central microtubules. Additional studies, using pulse-chase labelling and autoradiography, will, however, be necessary to determine clearly the *in vivo* directionality of central microtubule assembly.

The distal filaments also remain attached to the outer doublets throughout microtubule assembly. The filaments, however, appear to be less tightly attached to the outer doublets than are the caps to the central microtubules: in any single preparation the distal filaments tend to be released from the A-microtubules while the central microtubule caps remain attached to the central microtubules. Since other studies have demonstrated that the outer doublet microtubules assemble by the addition of subunits to their distal tips (Rosenbaum & Child, 1967; Rosenbaum, Moulder & Ringo, 1969; Witman, 1975) the distal filaments must not be tightly linked to the outer doublets or it would be expected that they would block the addition of subunits to the growing ends of the A-tubules. Since the distal filaments are, however, linked to the membrane as well as to the A-tubules, the plugs may simply dip into the lumen of the growing A-tubules during microtubule assembly. This would necessitate either close coordination of the assembly of the outer doublets with the assembly of the membrane or that the distal filaments be loosely anchored in the membrane bilayer and, therefore, be able to float along during ciliary and flagellar growth and resorption.

The presence of distal filaments forming a complex with the central microtubule cap in certain *Tetrahymena* oral apparatus cilia and in certain molluscan gill cilia is interesting. If the association is very strong then the rate of assembly of outer doublet
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and central microtubules must be the same and, therefore, the complexes may help to regulate the rate of assembly of the ciliary microtubules. Although the body cilia of *Tetrahymena* and *Chlamydomonas* flagella do not exhibit linkages between central microtubule cap and distal filament, both the distal filaments and the caps are attached to the surrounding membrane. It is possible that the tip structures may be linked to one another by proteins in the membranes and, therefore, that the rate of assembly of each of the ciliary and flagellar microtubules may be coordinated by these structures. Although the composition and arrangement of membrane proteins at the distal tips of cilia and flagella is unknown at present, other studies have demonstrated extensive linkage of the sides of outer doublets with protein complexes in ciliary membranes by high molecular weight dynein-like proteins (Dentler *et al.* 1979).

In addition to their possible role in the coordination of assembly of outer doublet microtubules, the distal filaments may act as siphons which could possibly funnel proteins from the membrane on to the growing tips of the outer doublet microtubules (Stephens, 1977). While this mechanism may possibly occur in cilia, whose membranes appear to contain tubulin (Dentler, 1978; Stephens, 1977), it would be less likely to occur in flagella, since neither *Chlamydomonas* (Snell, 1976; Witman *et al.* 1972), nor scallop sperm (Stephens, 1977) flagellar membranes appear to contain very much tubulin. Another possibility is that these tip structures may simply link the membrane to the axonemal microtubules, either to prevent billowing of the membrane which could occur during ciliary and flagellar movement or to protect the distal tips of the microtubules from frictional forces which must be developed between the microtubules and the membrane during ciliary and flagellar beating.

Since the outer doublet microtubules slide relative to one another during ciliary and flagellar movement, the presence of distal filaments attaching the A-tubules of the outer doublet microtubules to the central microtubule cap is somewhat puzzling. If the association is very strong then it must restrict the degree to which microtubule sliding can occur. It may be significant that these complexes occur in cilia which do not beat as actively as do *Chlamydomonas* flagella and *Tetrahymena* body cilia; i.e. they are found in *Tetrahymena* oral apparatus cilia and in regions of scallop and mussel gills in which the cilia do not beat but serve to hold adjacent gill filaments together. Since the interlocking molluscan gill cilia become more active if the gill filaments are teased apart, it will be important to examine the tips of these cilia under motile and non-motile conditions.

I would like to thank L. Hammer for technical assistance and J. Marr for the artwork. This research was supported by grants from The National Institutes of Health (AM 21672) and from the University of Kansas (3505 and RRO 7037).

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(Received 9 May 1979 – Revised 6 October 1979)