Polarity of Flagellar Assembly in *Chlamydomonas*

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Abstract. During mating of the alga *Chlamydomonas*, two biflagellate cells fuse to form a single quadriflagellate cell that contains two nuclei and a common cytoplasm. We have used this cell fusion during mating to transfer unassembled flagellar components from the cytoplasm of one *Chlamydomonas* cell into that of another in order to study in vivo the polarity of flagellar assembly.

In the first series of experiments, sites of tubulin addition onto elongating flagellar axonemes were determined. Donor cells that had two full-length flagella and were expressing an epitope-tagged α-tubulin construct were mated (fused) with recipient cells that had two half-length flagella. Outgrowth of the shorter pair of flagella followed, using a common pool of precursors that now included epitope-tagged tubulin, resulting in quadriflagellates with four full-length flagella. Immunofluorescence and immunoelectron microscopy using an antiepitope antibody showed that both the outer doublet and central pair microtubules of the recipient cells' flagellar axonemes elongate solely by addition of new subunits at their distal ends.

In a separate series of experiments, the polarity of assembly of a class of axonemal microtubule-associated structures, the radial spokes, was determined. Wild-type donor cells that had two full-length, motile flagella were mated with paralyzed recipient cells that had two full-length, radial spokeless flagella. Within 90 min after cell fusion, the previously paralyzed flagella became motile. Immunofluorescence microscopy using specific antiradial spoke protein antisera showed that radial spoke proteins appeared first at the tips of spokeless axonemes and gradually assembled toward the bases. Together, these results suggest that both tubulin and radial spoke proteins are transported to the tip of the flagellum before their assembly into flagellar structure.
Wild-type cells (CC124 rat-) and the radial spokeless mutant pfl4 (CC1024 University, Durham, NC). The transformed Chlamydomonas Strains and Culture coding nitrate reductase into genomic copy of the acids, including a 9-amino acid epitope from influenza virus hemagglutinin mutant was present in unassembled form in the cytoplasm to study the polarity of assembly of one class of axonemal microtubule assembly studies, transformant cells expressing publication) were used to donate a pool of marked tubulin D. R. Diener, and J. L. Rosenbaum, 1992, submitted for publication. 

We have used this procedure to reinvestigate the polarity of assembly of the flagellar microtubules, and, in addition, to study the polarity of assembly of one class of axonemal microtubule-associated structures, the radial spokes. For microtubule assembly studies, transformant cells expressing an epitope-tagged α-tubulin construct (Kozminski, K. G., D. R. Diener, and J. L. Rosenbaum, 1992, submitted for publication) were used to donate a pool of marked tubulin subunits to recipient cells that had half-length flagella. After cell fusion, the half-length flagella grew to full length using a common pool of unassembled subunits that now included tagged α-tubulin. The epitope-tagged α-tubulin was then localized using immunofluorescent and immunogold techniques using an epitope-specific mAb (Field et al., 1988). Similarly, the assembly of radial spokes onto spokeless, but full-length, axonemes during dikaryon rescue of a spokeless mutant was followed by immunofluorescent analysis using antibodies to specific radial spoke proteins (Williams et al., 1986).

The results of these experiments show that both the flagellar outer doublet and central pair microtubules assemble solely at their distal tips. Surprisingly, radial spokes first appear at the tips of spokeless, but full-length, axonemes during dikaryon rescue, and assembly progresses toward the base of the axonemes. These results indicate that both tubulin and radial spoke proteins are transported to the tip of the flagellum before their assembly into flagellar structure.

**Materials and Methods**

**Chlamydomonas Strains and Culture**

Wild-type cells (CC124 mt-) and the radial spokeless mutant pfl4 (CC1024 mt+) were obtained from the Chlamydomonas Genetics Center (Duke University, Durham, NC). The transformed Chlamydomonas cell line 4A5, which expresses an epitope-tagged α-tubulin construct, is described elsewhere (Kozminski, K. G., D. R. Diener, and J. L. Rosenbaum, 1992, submitted for publication). Briefly, an oligonucleotide encoding 12 amino acids, including a 9-amino acid epitope from influenza virus hemagglutinin (Field et al., 1988), was inserted near the 3' end of the coding region of a genomic copy of the Chlamydomonas α-tubulin gene (Silflow et al., 1985). This construct was cotransformed with the Chlamydomonas gene encoding nitrate reductase into nit-305 mt+ cells (Diener et al., 1990); nitrate reductase-positive cells were screened for expression of epitope-tagged tubulin by Western analysis using the epitope-specific mAb 12CA5 (Field et al., 1988). Immunofluorescent analysis showed that epitope-tagged tubulin was assembled into both the cytoplasmic and axonemal microtubules of expressing cell lines (Kozminski, K. G., D. R. Diener, and J. L. Rosenbaum, 1992, submitted for publication).

Preparation of Cells with Partially Regenerated Flagella

Pool-depleted cells with partially regenerated flagella were produced by deflagellating wild-type mt- gametes by pH shock (Witman et al., 1972) and allowing them to begin flagellar regeneration. When the new flagella were approximately one-third to one-half length, further protein synthesis was inhibited by the addition of cycloheximide (10 μg/ml) to the medium and the cells were allowed to continue regeneration for 1 h. The flagella of these cells reach about one-half to two-thirds of their final length; assembly ceases as the cells deplete a preexisting cytoplasmic pool of unassembled axonemal components (Rosenbaum et al., 1969; Lefebvre et al., 1978). It has been shown that if similarly treated cells are washed out of cycloheximide, flagellar regeneration resumes as new subunits are synthesized (Lefebvre et al., 1978). Partial regeneration prior to the addition of cycloheximide was necessary as the preexisting pool of unassembled flagellar precursors in gametes is not large enough to produce flagella of sufficient length to allow for efficient mating.

**Immunofluorescence Microscopy**

Chlamydomonas cells were processed for immunofluorescence following a modification of the procedure of Holmes and Dutcher (1989). Cells were adhered to poly-l-lysine-coated multiwell slides (Carlson Scientific, Peotone, IL). Cells were washed in microtubule stabilization buffer (MTSB): 30 mM Pipes, 25 mM KCl, 5 mM MgSO4, 5 mM EGTA, and 12% hexylene glycol (Aldrich Chem. Co., Milwaukee, WI; vol/vol), pH 6.8, and then were simultaneously fixed and permeabilized in MTSB + 4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) + 0.5% NP-40 for 30 min. Unreacted aldehydes were blocked with 10 mM NaBH4, and the slides were washed with distilled water and briefly air dried. The wells were rehydrated in PBS containing 1% BSA (PBSB), and this was replaced by a dilution of primary antibody in PBSB. The anti-epitope mAb 12CA5 (Field et al., 1988) was used at a dilution of 1:10, whereas affinity-purified radial spoke protein polyclonal antisera (Williams et al., 1986) were used at dilutions of 1:100–1:500. After incubation in primary antibody, wells were washed several times with PBSB, and incubated with secondary antibody (fluorescein-conjugated goat anti-rabbit or goat anti-mouse; Zymed Labs. Inc., South San Francisco, CA) diluted 1:200–1,100 in PBSB. After washing, slides were mounted in 90% glycerol containing phenylamine diamine (Pringle et al., 1989). Cells were viewed on a Zeiss Universal microscope equipped for epifluorescence and photomicrography; micrographs were taken on TMAX 400 (Eastman Kodak Co., Rochester, NY) and developed according to the manufacturer's directions.

**Immunoelectron Microscopy**

Axonemes were obtained for immunoelectron microscopy by allowing cells to settle onto poly-l-lysine-coated formvar-covered nickel grids. Flagella were simultaneously separated from the cell bodies and demembranated by inverting the grids onto a solution of HMDEK (30 mM Hepes, 25 mM KC1, 5 mM MgSO4, 1 mM DTT, 0.5 mM EDTA, pH 7.4) + 1% NP-40 The

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1. Abbreviations used in this paper: MTSB, microtubule stabilization buffer; PBSB, PBS containing 1% BSA.
resulting axonemes were fixed in HMDEK + 1% glutaraldehyde (Electron Microscopy Sciences) and immunogold labeled (Johnson and Rosenbaum, 1990) using 10 nm gold conjugated to goat anti-mouse antibodies (Zymed Labs. Inc.). After immunogold labeling, the grids were washed briefly in distilled water, dried, and viewed in an electron microscope (model EM201; Philips Electronic Instrs. Co., Mahwah, NJ). Although negative staining was omitted to accentuate the immunogold label, the axonemes are sufficiently electron dense to be seen clearly without additional contrast. Electron micrographs were taken using Estar film 4489 (Eastman Kodak Co.) and developed according to the manufacturer's directions.

Results

Sites of Tubulin Addition in Regenerating Axonemes

Cells expressing the epitope-tagged tubulin construct and having full-length flagella were mated with pool-depleted cells that had partially regenerated flagella of one-half to two-thirds full-length in the continued presence of cycloheximide (10 μg/ml). The donor cells contained unassembled flagellar components, including epitope-tagged α-tubulin, that became available to the regenerating cells after fusion. The recipient cells’ pool of unassembled flagellar subunits had been depleted by partial regeneration in the absence of protein synthesis. This reduced the dilution of the tagged tubulin from the donor cell pool after cell fusion.

Immediately after mating, the dikaryons had one pair of full-length flagella and one pair of shorter flagella. Within 1 h after mating, regeneration of the shorter pair of flagella was completed using the pool of unassembled precursors from the donor cell, leaving the dikaryons with four flagella of equal length (Fig. 1 A). When these quadriflagellates were examined by immunofluorescence for the distribution of epitope-tagged tubulin, two of the four axonemes were labeled along their full lengths and two axonemes were labeled only along their distal portions. Before mating, the antiepitope antibody labels all microtubules of donor gametes (expressing the epitope-tagged tubulin) but does not label any structures in wild-type recipient gametes (data not shown). Bar, 10 μm.

Figure 2. Immunofluorescent localization of tubulin in quadriflagellates. (A) Using a polyclonal anti-α-tubulin antiserum, all four axonemes as well as the cytoskeletal microtubules of a dikaryon are labeled. (B-D) Using the antiepitope mAb, two of the axonemes of a quadriflagellate are labeled along their full lengths and two axonemes are labeled only along their distal portions. Before mating, the antiepitope antibody labels all microtubules of donor gametes (expressing the epitope-tagged tubulin) but does not label any structures in wild-type recipient gametes (data not shown). Bar, 10 μm.

Figure 1. (A) Introduction of epitope-tagged tubulin subunits into cells with regenerating flagella. The recipient cells were pool-depleted gametes with one-half to two-thirds full-length flagella. The donor cells were expressing epitope-tagged α-tubulin and had full-length flagella. Within 1-2 h after mating, regeneration of the shorter pair of flagella was completed using the pool of unassembled precursors from the donor cell, leaving the quadriflagellate with four equal-length flagella. These flagella were examined for the distribution of tagged tubulin. (B) Diagram of dikaryon rescue of the radial spokeless mutant pf14. Radial spokeless, paralyzed gametes were mated with wild-type gametes. Immediately after mating, the quadriflagellates have a pair of paralyzed flagella and a pair of motile ones. Within 1-2 h after mating, all four flagella of the quadriflagellates begin beating. During the time course of rescue, quadriflagellate flagella were examined for the distribution of radial spoke proteins.

subunits only became available for
incorporation into the recipient axonemes after cell fusion during mating. Note that no labeling occurs along the proximal portions of the microtubules of the partially labeled pair. When these axonemes splay apart at their distal ends, as they sometimes do during the labeling procedure, it is clear that, during the later stages of regeneration, tagged tubulin had been incorporated into the distal ends of both the outer doublet and the central pair microtubules (Fig. 4).

Pattern of Radial Spoke Addition during Dikaryon Rescue

The mutant pf14 has paralyzed, full-length flagella and cannot swim because of the complete absence of radial spokes (Witman et al., 1978; Piperno et al., 1981). In wild-type cells, radial spokes are attached in a row along the A-tubules of each of the outer doublet microtubules and extend toward

Figure 3. Immunogold localization of epitope-tagged tubulin in quadriflagellate axonemes. The four axonemes shown are from a single quadriflagellate cell. In this whole mount, the axonemal bases (where they have been detached from the cell) are oriented to the left and the axonemal tips to the right. Of the four axonemes of a quadriflagellate, two are labeled along their full lengths and two are labeled only at their distal ends (brackets). Bar, 1 μm.
the central pair microtubules (Hopkins, 1970; Warner, 1970). In the mutant pfl4, no radial spokes, nor the 17 different radial spoke proteins that comprise these structures, are present in the flagella. When paralyzed pfl4 cells are mated with wild-type cells, the resulting quadriflagellate initially has one pair of paralyzed flagella and one pair of motile ones (Fig. 1 B). However, within 90 min after mating, all four flagella of the quadriflagellate are motile (Piperno et al., 1981). The pool of unassembled axonemal components provided by the wild-type cytoplasm includes the radial spoke protein 3 (rsp3) that had been missing in the mutant. Radial spokes are assembled onto the previously spokeless axonemes, restoring motility (Piperno et al., 1981). Using antibodies against radial spoke proteins (Williams et al., 1986), we have examined cells during the process of dikaryon rescue to establish the temporal pattern of radial spoke protein appearance on the previously spokeless axonemes.

Immunofluorescent analysis, using an affinity-purified polyclonal antiserum to rsp3, of dikaryons fixed at different times after mating is shown in Fig. 5. Each dikaryon initially has a pair of flagella from the wild-type parent that contained radial spokes (appearing fluorescent along their full lengths) and a pair of flagella from the pfl4 parent that lacked radial spokes (appearing dark by immunofluorescence, data not shown). In cells fixed at ~15 min after mating, some fluorescence appeared over the tips of some of the otherwise dark, spokeless axonemes (arrows, Fig. 5); at subsequent time points, the fluorescent signal brightened and extended proximally. The border between the fluorescent and nonfluores-
Discussion

The results of experiments examining the in vivo incorporation of epitope-marked tubulin onto axonemes after cell fusion show that both the outer doublet and central pair microtubules are assembled from their distal ends. Although the distal assembly of flagellar outer doublet microtubules might have been inferred from earlier pulse labeling and autoradiographic studies in *Ochromonas* (Rosenbaum and Child, 1967) and *Chlamydomonas* (Rosenbaum et al., 1969; Witman, 1975), this is the first study to show directly that the microtubules themselves are assembled distally. These experiments also show, for the first time, that the central pair microtubules assemble distally during flagellar growth. It had been proposed that the central pair microtubules might assemble through addition to their bases (Dentler and Rosenbaum, 1977) since their proximal ends are not continuous with the basal body microtubules and do not appear to be structurally blocked. Moreover, the 35% of the label that was localized proximally in the original autoradiographic studies could have been due, in part, to proximal addition of tubulin to the central pair microtubules. In contrast to these earlier autoradiographic studies (Rosenbaum and Child, 1967; Rosenbaum et al., 1969; Witman, 1975), the patterns of both immunofluorescent and immunogold localization of epitope-tagged tubulin observed in the present study show labeling only over the distal portions of the microtubules. These results also indicate that, within the limits of sensitivity of the techniques used, there is no turnover of the flagellar outer doublet or central pair microtubules along their lengths, or some epitope-tagged tubulin would have been localized in the proximal portions of the flagella of the recipient cells (Figs. 2 and 3).

The results of assembly of radial spoke proteins onto spokeless, full-length flagella were more surprising. The flagella were not elongating and radial spoke sites presumably were available for assembly along the full lengths of the outer doublet microtubules of the axonemes. However, radial spoke proteins first appeared near the distal ends and then in a zone that extended proximally. Consistent with these data, the flagella of the paralyzed radial spokeless mutant first recover motility at the flagellar tips at \( \sim 30 \) min after fusion with a wild-type cell (K. A. Johnson, unpublished observations).

Distal assembly, not only of the outer doublet and central pair microtubules, but also of the radial spokes, raises questions concerning the targeting of proteins to the flagellar compartment and the mechanism(s) by which they are transported to the distal tips of flagella. It is possible that the assembly process has more than one component, because earlier pulse-labeling studies (Rosenbaum and Child, 1967; Rosenbaum et al., 1969; Witman, 1975) demonstrated that some newly synthesized proteins are incorporated into the basal half of the axoneme late in regeneration. The identities of these proteins remain unknown, although from the present study it is clear that they are neither tubulin nor radial spoke proteins. Because the genes for several flagellar proteins have now been cloned and sequenced (Curry et al., 1992; Mitchell and Kang, 1991; Schloss et al., 1984; Silflow et al., 1985; Williams et al., 1986, 1989), problems of targeting, transport, and assembly now can be approached directly through deletion analysis and site-directed mutation of these genes followed by their reintroduction to cells by transformation (Ang, L. H., D. R. Diener, and J. L. Rosenbaum, manuscript in preparation).

The results presented in this study suggest two alternative mechanism(s) by which flagellar precursors may move to the tip of the flagellum before their assembly. It is possible that flagellar precursors enter the flagellar compartment at its base and diffuse passively through the space between the flagellar membrane and the outer doublets of the axoneme to tip assembly sites. The observed tip-to-base pattern of radial spoke restoration onto full-length but previously spokeless axonemes might be a consequence of the ranks of dynein arms that closely interconnect adjacent outer doublets. Radial spoke proteins may have to travel the length of the axoneme, past the distal ends of the outer doublet microtubules, before gaining access to assembly sites on the inner faces of the outer doublets. An alternative hypothesis is that precursors are transported in a more active, directed process. The presence of putative carriers associated with the flagellar membrane and/or axoneme can be inferred from...
(a) descriptions of the rapid movements of latex beads up and down the external surface of the flagellar membrane (Bloodgood, 1977), (b) observations of particle movement underneath the flagellar membrane when viewed using high resolution differential interference contrast microscopy (Kozminski, K. G., K. A. Johnson, P. Forscher, and J. L. Rosenbaum, 1992, submitted for publication), and (c) reports of molecular motors, other than flagellar dynein, in the flagellum (Kozminski, K. G., K. A. Johnson, P. Forscher, and J. L. Rosenbaum, 1992, submitted for publication; Sale, W. S., L. A. Fox, K. E. Sawin, and J. Heuser, submitted for publication). Once unassembled axonemal proteins arrive at the flagellar tips, they may be targeted to capping structures that link the ends of the axonemal microtubules to the flagellar membrane (Dentler and Rosenbaum, 1977; Dentler, 1980). At this point, the precursors may be released from their carriers and become available for assembly into axonemal structure.

We would like to thank Dennis Diener and Keith Kozminski for the transformed Chlamydomonas cell line 4A5.

Work in the authors' laboratory has been supported by grants from the National Institutes of Health (GM-13758) and the American Cancer Society (ACS NP-723). K. A. Johnson has been supported by a National Institutes of Health postdoctoral fellowship (GM-13758).

Received for publication 23 June 1992 and in revised form 25 August 1992.

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