Ciliary Protein Conservation during Development in the Ciliated Protozoan, *Oxytricha*

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**Abstract.** The ciliated protozoan *Oxytricha fallax* possesses multiple highly localized clusters of basal bodies and cilia, all of which are broken down and rebuilt during prefission morphogenesis—with one major exception. The adoral zone of membranelles (AZM) of the ciliate oral apparatus contains ∼1,500–2,000 basal bodies and cilia, and it is the only compound ciliary structure that is passed morphologically intact to one daughter cell at each cell division. By labeling all proteins in cells, and then picking the one daughter cell possessing the original labeled AZM, we could then evaluate whether or not the ciliary proteins of the AZM were diluted (i.e., either by degradation to constituent amino acids or by subunit exchange) during cell division.

Autoradiographic analysis demonstrated that the label was highly conserved in the AZM (i.e., we saw no evidence of turnover), and electrophoretic data illustrate that at least one of the proteins of the AZM is tubulin. We, therefore, conclude that for at least some of the ciliary and basal body proteins of *Oxytricha fallax*, AZM morphological conservation is essentially equivalent to molecular conservation.

**Materials and Methods**

**Culturing and Labeling**

*Oxytricha fallax* was cultured as previously described by Grimes (1972). To obtain labeled cells, cells were grown for several generations in the standard culture medium containing 50 μCi/ml tritiated leucine (Schwarz-Mann, Boston, MA; sp act, 13.5 Ci/mM). Prodividers were then selected, washed four times in cold nutrient medium, and opisthe (posterior fission products) from that first division product (guaranteed to have had their membranellar proteins synthesized in the presence of radioactive leucine and already formed their new adoral zone of membranelles [AZM]) were used as the starting point for study of subsequent cell divisions. At each subsequent cell division, each cell was carefully and individually observed, and only those cells known with certainty to be anterior fission products (proters) were reisolated for further cell generations. (See general protocol illustrated by Fig. 2.) This general experiment has been performed many times, and under different conditions. Variables include the amino acid used for labeling (including a tritiated yeast hydrolysate amino acid mixture), the species of the ciliate (a total of five different species have now been studied), and the number of fissions the pedigree was carried varied between 3 and 10. In all cases, data from these experiments were completely consistent with the results presented in Fig. 3, and we therefore present only the data from that one autoradiographic experiment.

**Autoradiography**

All cells represented in Fig. 3 were from the same experiment. They were individually dried down on the same albuminized slide before fixation in Carnoy's fixative for 15 min. Subsequently, they were emulsified with Ilford L-4 emulsion (Basildon, Essex, Great Britain) incubated 14 h at 4°C, and developed in Microdol-X for 4 min, washed, fixed, and air dried before observation. Thus, any variation due to emulsion thickness, incubation time, etc., cannot exist because all cells were dried and incubated on a single slide.
Figure 1. Protargol-stained preparations of *Oxytricha fallax* illustrating the fundamental aspects of structure and predivision morphogenesis essential for understanding the experimental design used. (a) A morphostatic cell illustrating the overall cortical morphology of a morphostatic cell. Note especially the prominent comma-shaped adoral zone of membranelles (AZM). (b) A cell in mid-predivision morphogenesis. Note the developing new AZM (NAZM, double arrow) whereas no change is visible in the original AZM (OAZM, single arrow). (c) A cell immediately before division. The original AZM (OAZM) is passed onto the anterior fission product (PROTER) intact whereas the posterior fission product (OPISTHE) has a completely new oral apparatus (NAZM). Bar, 20 µm.

Ciliary Axoneme Isolation

*Tetrahymena thermophila* cilia were isolated as described by Gibbons (1965). An added step in the procedure was filtration of the isolated ciliary suspension through an 8.0-µm polycarbonate filter. Demembranation of isolated cilia was achieved by resuspending a pellet of cilia in a solution containing 1% SDS and 0.1 M Tris at pH 7.8, and subjecting it to a vortex for several minutes. The suspension was kept on ice for 30 min, after which time axonemes were collected by centrifugation. The axonemes were subsequently dialyzed against 0.1 M Tris, at pH 7.3, for 12 h at 4°C. Axonemes prepared by this procedure contain α and β tubulin (Gavin, 1980).

Electrophoresis

Tritium-labeled *Oxytricha* (11 pedigreed proters and their corresponding 11 opistes) were mixed separately with 20 µg of ciliary axoneme protein in a solution containing 1.0% SDS, 2.0% mercaptoethanol, 0.1 M Tris at pH 7.3, and heated 90°C for 3 min. The mixture was then loaded onto an SDS-polyacrylamide gel. A gel containing only 20 µg of axonemal protein was used as a control for background radioactivity.

SDS-PAGE was performed in 5 × 100-mm glass tubes using the Tris-Glycine system described by Laemmli (1970). Each tube contained a 7.5-cm separation gel (10% acrylamide) and a 2.0-cm stacking gel (4.75% acrylamide).

After electrophoresis, gels were removed from the tubes and fixed overnight in 10% TCA at 4°C. The fixed gels were scanned at 280 nm in a spectrophotometer equipped with a linear gel transport and a recorder (Gilford Instrument Laboratories, Inc., Oberlin, OH). The gels were then cut into 1-mm slices. Approximately 10 consecutive slices were placed into one scintillation vial containing 10 ml of Bray's (1960) solution. Radioactivity in each vial was determined by scintillation spectrometry in an Intertechnique Scintillation Counter (Plaisir, France). A total of 4 K counts were recorded for each vial to determine cpm.

Results

The Organism

*Oxytricha fallax* was chosen exclusively because of the detailed analysis of the morphogenetic processes associated with cell division (Grimes, 1972). *Oxytricha* possesses an AZM which is composed of a complex array of cilia (~2,000/cell) and functions in locomotion and in "sweeping food" into the gullet for feeding purposes. (See Fig. 1 a and Grimes, 1972 for detailed description.)

During the typical growth and division cycle, the cells undergo an extremely complex sequence of morphogenetic events which yields two daughter cells of identical phenotype. All cortical ciliary organelles except the AZM are resorbed and new ones subsequently made to replace them. A new AZM is formed for the posterior fission product (opisthe) whereas the existing AZM is retained morphologically intact and passed on in toto to the anterior fission product (proter) (see Fig. 1, b and c). Thus, at each cell division, all ciliature is broken down and replaced except for the AZM. The question then, as raised in the introduction is, are the molecular components of the AZM conserved as is its morphology?

Experimental Design and Resultant Data

If a given cell initiated a cell cycle with X concentration of a given substance, then at the subsequent cell division, the concentration of that substance would be maximally one-half of its initial concentration, assuming that the substance were distributed equally to the two daughter cells. Thus, in two cell divisions, the substance would be only at one-fourth the initial concentration, etc. If, on the other hand, the initial substance were not distributed equally (remember that in *Oxytricha*, the AZM is passed on intact to the proter), at each division, then one daughter cell would preferentially retain a higher proportion of the substance.

With this general notion, we then could ask if this substance (protein) is preferentially retained in the proter as the
molecular components of the cilia. To answer this question, we first labeled cells in \(^{3}H\)-amino acids for several cell generations, and to be certain that the proteins of the AZM were synthesized in the presence of \(^{3}H\) amino acids, we hand-picked opisthes, washed them four times with cold nutrient medium, and allowed them to undergo multiple cell divisions in cold nutrient medium. At each cell division, each cell was carefully watched and the proter from that division isolated individually for growth to the next cell division. Opisthes from the divisions were either discarded or dried down on the slide for subsequent autoradiography (Fig. 2). Because each divider had to be observed at the time of the completion of fission, many cells were discarded because the proter could not be distinguished from the opisthe. Hence, the larger number of fissions the cells were carried, the fewer proters could not be distinguished from the opisthe. Therefore, the proteins of the AZM are highly conserved, corresponding to the morphological conservation of the AZM observed during division.

Identification of the Conserved Components of the AZM

Because of the severe limitation of the number of labeled cells obtainable (i.e., cells with highly selectively labeled AZMs), we could not use standard procedures to identify the protein(s) which were conserved by the AZM in these experiments. Therefore, we took a rather nonconventional approach, first making the assumption that one of the major proteinaceous components must be tubulin. With that thought in mind, we first prepared a tubulin fraction from the ciliary axonemes of *Tetrahymena thermophila*. This, we determined, would provide a marker for electrophoresis that could be detected easily by scanning the gel at 280 nm. If there were sufficient radioactivity associated with the AZMs that could be counted, and the detectable radioactivity comigrated with tubulin (as detected by the 280-nm scan), then we could conclude that at least one major (perhaps the major) protein conserved during cell division was indeed tubulin.

To perform this experiment, 11 pedigreed proters and 11 opisthes (the sister cells of the proters) were solubilized in 1% SDS separately at the end of seven cell generations. These solutes were then mixed with the purified *Tetrahymena* axonemal tubulin and subjected to electrophoresis. A control gel containing only isolated *Tetrahymena* tubulin was also run to determine background radioactivity. Fig. 4, *a* and *b*, shows the radioactive counts for the 11 seventh-division proters and opisthes (grown in cold medium after initial labeling) contained in the gel slices made subsequent to electrophoresis. Significant radioactivity was found in the preparation of proters, whereas no significant radioactivity above background was detectable in the preparations containing opisthes. When these numbers were plotted against the gel scan optical density at 280 nm (Fig. 4, *a* and *b*), the maximal radioactivity contained in the gel slices made subsequent to electrophoresis corresponded to the position of the tubulin isolated from *Tetrahymena* ciliary axonemes.

Discussion

The data presented herein are straightforward and present an essentially unequivocal conclusion: the tubulin molecules within the AZM are extremely stable—stable to the extent that either degradation to constituent amino acids or subunit exchange was not detected in these experiments. (Similar, but far less complete analysis and conclusions were obtained by Ruffolo, 1970.) This conclusion is a reflection of the morphological stability of the AZM and differs drastically from studies on the cytoplasmic labile (heat- and chemical-sensitive) microtubules. Thus, any discussion regarding equilibrium constants, treadmilling, etc., relative to these data is apparently unwarranted. The ciliary tubules are morphologically and molecularly conserved.

Previous evidence has been presented for turnover of ciliary tubulins in *Tetrahymena* (Williams, 1975). However, we believe this apparent turnover might only be a reflection of morphogenetic processes unique to *Tetrahymena*—features which do not occur in *Oxytricha*. In *Tetrahymena*, during any type of cortical morphogenesis, the cilia of the oral apparatus shorten (this includes prefission as well as oral replacement morphogenesis), and regrow to full length near the end of morphogenesis. Thus, the apparent turnover of the tubulin only represents turnover of tubulins at the distal ends of cilia during successive shortening and lengthening of cilia. One would then predict that the turnover would be only...
Figure 3. Autoradiographic data. Compare especially the eighth-division proter with the eighth-division opisthe. A. A., amino acids.
partial, and a point would be reached where the ciliary tubulins would not turnover any further (that point being a reflection of the degree of shortening of the cilia). This interpretation regarding the observed turnover in Tetrahymena ciliary tubulin (Williams, 1975) is completely consistent with our data.

From these data, we can only be confident about the stability of tubulins (and proteins that comigrate with tubulins). Because the cilia are so highly stable, presumably other molecular components of the cilia are equally as stable, and similar approaches can be used to test for the stability and role of other ciliary components. Obviously, we will need to develop a system with much higher yields from much less laborious experiments. We are now in the process of testing several approaches to achieve this goal.

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