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Acetylated α-Tubulin in the Connecting Cilium of Developing Rat Photoreceptors

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Purpose. To examine the distribution of acetylated α-tubulin in the connecting cilium of rat rod photoreceptors during different stages of photoreceptor development.

Methods. An antibody found to be specific for the acetylated form of α-tubulin was used in immunoelectron microscopy of retinas from animals of different ages.

Results. All the microtubules of the connecting cilium, including those of the basal bodies, were found to contain acetylated α-tubulin at the earliest stage of outer segment development, before the cilium has begun to grow out from the cell, and at all subsequent stages.

Conclusions. These results indicate that the α-tubulin of the connecting cilium is acetylated either before, or at least very soon after, its assembly into microtubules. Given that acetylation of α-tubulin is correlated with stable microtubules, the results suggest that stable microtubules might be important in creating the foundation for the formation of the outer segment, as well as in helping maintain the polarity of the mature photoreceptor. Invest Ophthalmol Vis Sci. 1993;34:2145–2149.

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The outer and inner segments of a vertebrate photoreceptor are connected by a cilium that contains nine outer microtubule doublets and is comparable to the transitional zone of conventional cilia.[1] In a mature photoreceptor, the connecting cilium is important for maintaining the separation of the outer and inner segment compartments.[2] It is also the site of the morphogenesis of new membrane disks, which are added to replace those shed from the distal end of the outer segment.[3] During development of a photoreceptor cell, the connecting cilium is involved in the generation of the restricted outer segment domain.[4]

To understand the function of the microtubules of the photoreceptor connecting cilium, it is important to define their molecular composition. Most evidence suggests that the sequence differences among the tubulin isotypes do not specify function.[5,6] In determining function, post-translational modifications appear to be more important. These modifications, of which detyrosinylation and acetylation are two of the most significant, affect the location of microtubules, thereby creating potentially important functional subsets of cytoplasmic microtubules. Acetylation is ac-

complished by tubulin acetyltransferase, which catalyzes the transfer of an acetyl group from acetylCoA to the lysine 40 of α-tubulin.[7–9] In many types of cells, the acetylation of α-tubulin is correlated with microtubule stability.[10]

Immunofluorescence microscopic studies have shown that the connecting cilium of adult Xenopus rod photoreceptors contains acetylated α-tubulin.[11] The microtubules of this cilium were found to be more stable than the nonacetylated microtubules present in the rod inner segment.[11] In the current study, we have carried out immunoelectron microscopy and focused on the distribution of acetylated α-tubulin at different stages during development of the connecting cilium and outer segment of rat rod photoreceptors.

MATERIALS AND METHODS. *Animals and Tissue.* Immunocytochemical localization of acetylated α-tubulin was performed on retinal sections of Long Evans rats of the following ages: 36 hours, 5 days, 10 days, 14 days, and 21 days. All animals were kept under a 12-hour light/12-hour dark cycle. They were decapitated in room light during the light period and eyes were surgically removed. Procedures conform to the ARVO resolution on the use of animals. The posterior segments of each eye were fixed by immersion in 0.5% glutaraldehyde and 2% paraformaldehyde plus 1% tannic acid in 0.1 M sodium phosphate buffer, pH 7.4, for an hour at 24°C. The eyecups were then cut into small pieces, dehydrated by a graded methanol series, and infiltrated and embedded in L. R. White resin. Ultrathin sections were cut with a diamond knife and collected on nickel grids.

For Western blot analysis, isolated rat rod outer segments were denatured by the addition of Laemmli sample buffer, containing 2 mM EGTA and 5% 2-mercaptoethanol. Proteins were separated in a 10% SDS-polyacrylamide slab gel and electrophoretically transferred to Immobilon (Millipore, Marlborough, MA) in buffer containing 25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3. Rod outer segments were isolated using Percoll gradients.[12]

Immunolabeling. We used a monoclonal antibody (1–6.1) that was raised against sea urchin tubulin and has been shown to recognize only acetylated α-tubulin.[10] Ascites serum was passed through a mono Q FPLC column, and the IgG fraction was used at 1 μg/ml on sections and 0.1 μg/ml on immunoblots. The immunolabeling procedure followed that of Arikawa and Williams.[13] Briefly, the primary antibody was detected on the L. R. White sections and on the immunoblots by goat-anti-mouse IgG conjugated to colloidal gold (5 nm) or to alkaline phosphatase, respectively. Each time sections or blots were labeled, negative controls were also prepared. They were processed by omitting incubation with the primary antibody, or by replacing the primary antibody with 10 μg/ml normal mouse IgG. Sections were not examined unless the negative controls were found to contain no label.

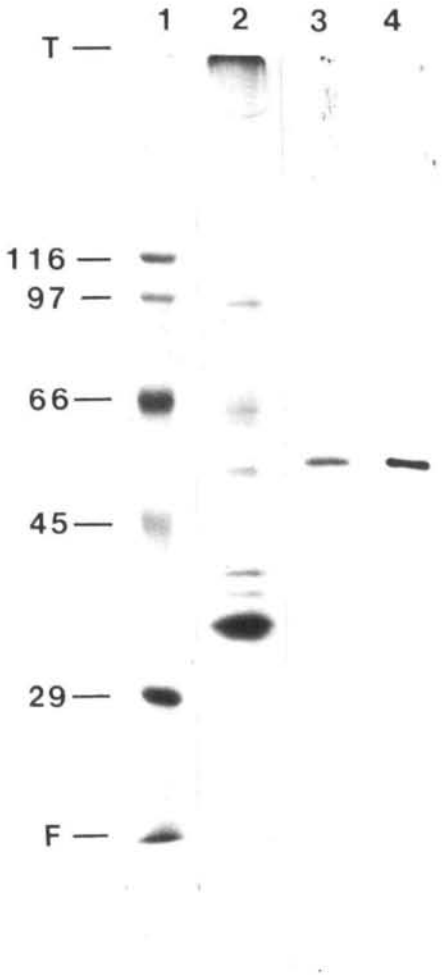

FIGURE 1. Western blot of rat retina and purified rat rod outer segments. Electrophoresis was done under reducing conditions in an SDS polyacrylamide 10% gel (T, top; F, front). Lanes 1–2: Coomassie blue stain of molecular mass standards (lane 1) and purified rat rod outer segments (lane 2). Lanes 3–4: Immobilon, containing purified rat rod outer segment proteins (lane 3) and whole rat retinal proteins (lane 4) transblotted and incubated with antiacetylated α-tubulin (1–6.1), followed by goat antimouse IgG conjugated to alkaline phosphatase.

RESULTS. *Characterization of Antibody.* The monoclonal antibody, 1–6.1, has been found to be specific for acetylated α-tubulin, recognizing only the acetylated domain of α-tubulin[10] (also H. Keating and D. Asai, personal communication). On Western blots of rat retina and purified rat rod outer segments, the

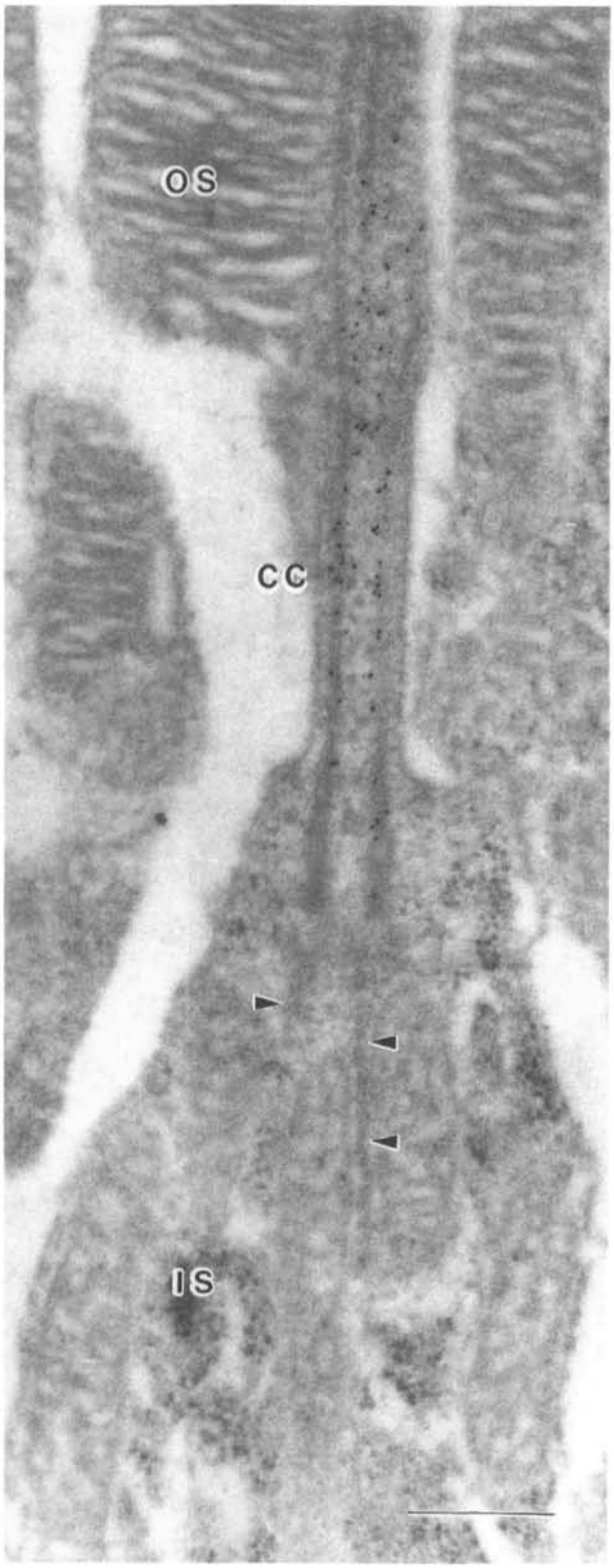

FIGURE 2. Indirect immunogold labeling of a 21-day postnatal rat retina with antiacetylated α-tubulin. The section shows the connecting cilium (CC) between the outer segment (OS) and the distal part of the inner segment (IS) of a rod photoreceptor. The structures indicated by arrowheads in the inner segment are most likely microtubules, which are not labeled. Scale = 0.5 μm.

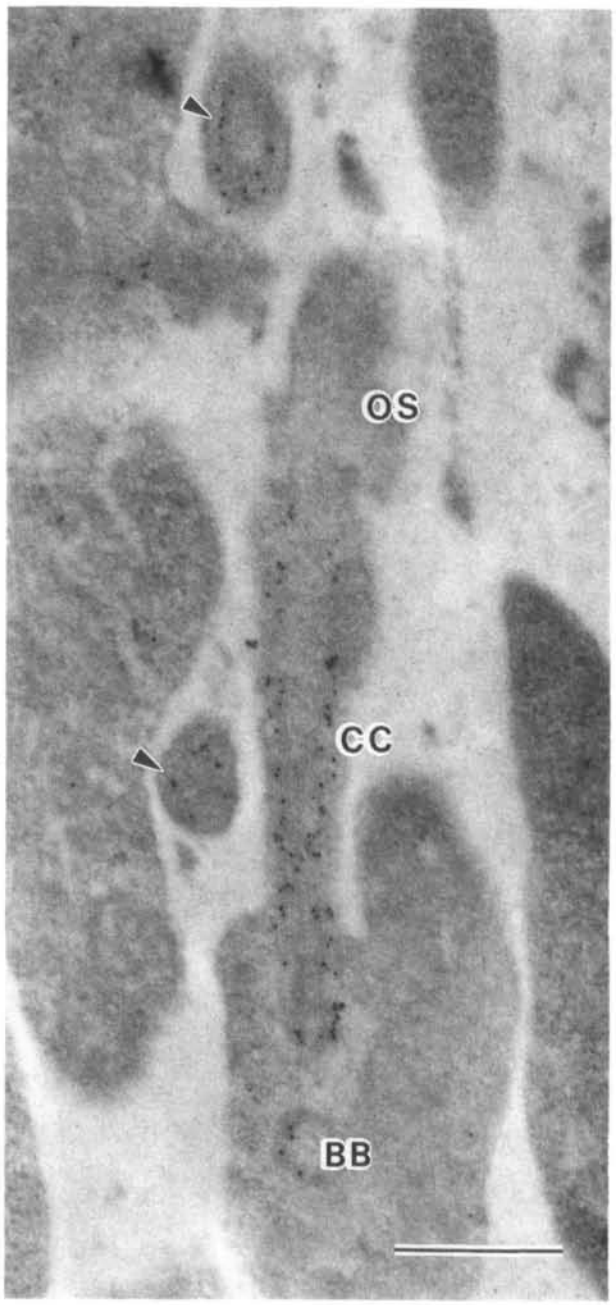

FIGURE 3. Indirect immunogold labeling of a 5-day postnatal rat retina with antiacetylated α-tubulin. A connecting cilium (CC) extends from an inner segment. The outer segment (OS) has just begun to form. Arrowheads indicate oblique sections of the connecting cilia of adjacent photoreceptors. BB, basal bodies. Scale = 0.5 μm.

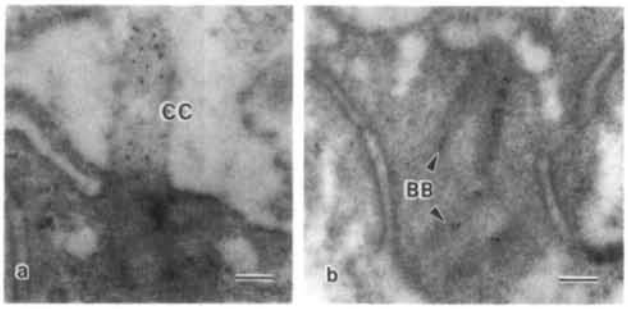

FIGURE 4. Indirect immunogold labeling of a 36-hour postnatal rat retina with antiacetylated α-tubulin. (a) A developing connecting cilium (CC); (b) basal bodies (BB) in a photoreceptor precursor cell, at an even earlier stage than that in a. Scales = 0.1 μm.

antibody reacted with a single band of M_r 50 kD (Fig. 1), the known M_r of tubulins, indicating that it does not recognize any other polypeptide in these tissues.

Mature Photoreceptors. In 21-day old rat retinas, all the microtubules of the photoreceptor connecting cilia, including those in the basal bodies, were labeled by antiacetylated α-tubulin (Fig. 2). Label was absent from the inner segment microtubules (arrowheads in Fig. 2), which were not acetylated,[11] indicating that the antibody did not detect any tubulin that was not acetylated. Although the outer segments are shorter in 10- and 14-day old rats, antiacetylated α-tubulin was also found to label their connecting cilia (data not shown).

Immature Photoreceptors. Photoreceptors from rat retinas fixed on the fifth postnatal day possessed a developing outer segment that is evident at the tip of the connecting cilium. All the microtubules in the connecting cilia of these immature photoreceptors were labeled by antiacetylated α-tubulin (Fig. 3).

About 36 hours after the birth, rat photoreceptors begin to differentiate. Photoreceptor precursor cells are located beneath the retinal pigment epitheretina, cells at slightly different stages of development could be observed. The microtubules of developing connecting cilia were found to be labeled by antiacetylated α-tubulin at this early stage (Fig. 4a). In cells whose cilium was not evident, the basal bodies were still labeled (Fig. 4b).

DISCUSSION. Previous work showed by immunofluorescence microscopy that the connecting cilium of lium, with their cilia extending toward it. In any one adult frog rod photoreceptors contains acetylated tubulin.[11] In the current study, we examined the connecting cilium of rat photoreceptors, in both mature and developing stages, by immunoelectron microscopy. We found that during all stages of connecting cilium and outer segment development, all the axonemal microtubules, including those of the basal bodies, contained acetylated α-tubulin. Even before the outgrowth of the connecting cilium, the microtubules of the basal bodies were labeled with the antibody against acetylated α-tubulin.

These results indicate that the α-tubulin of the connecting cilium must be acetylated either before, or at least very soon after, assembly into microtubules. The timing of α-tubulin acetylation in the connecting cilium thus appears similar to that in the flagellar axonemes of Chlamydomonas, but contrasts with that in axonal microtubules and microtubules in vitro. In differentiating axons, polymerization of tubulin precedes its acetylation by several days,[14] and in vitro studies show that polymerized tubulin is acetylated more readily than the dimer.[8] These observations indicate that the acetyltransferase prefers assembled microtubules as its substrate. However, during regeneration of Chlamydomonas flagella, acetylated α-tubulin can be detected biochemically in the axoneme as soon as regeneration had begins.[7] This finding, as well as the present one, suggests that the acetyltransferase might also act on tubulin that is not yet polymerized.

Taking microtubule acetylation as a general marker for microtubule stability,[10] our results indicate that the microtubules along the length of the connecting cilium, including the basal bodies, are stable even before the development of the outer segment, as well as after. It follows that it might be important to have stable microtubules during the formation of the cilium and outer segment, as well as for maintaining the polarity of the mature photoreceptor.

Key Words

photoreceptor, connecting cilium, microtubule, acetylated α-tubulin, immunocytochemistry

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