

γ -Tubulin in differentiated cell types: localization in the vicinity of basal bodies in retinal photoreceptors and ciliated epithelia

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SUMMARY

γ -Tubulin, a newly discovered member of the tubulin superfamily required for microtubule nucleation, is associated with the centrosome(s) throughout the vertebrate cell cycle. We have used a polyclonal antibody, generated against a highly conserved segment of γ -tubulin, to localize this protein in postmitotic, ciliated cells, in which the major microtubule organizing centers are the basal bodies. Single-cilium photoreceptor cells from bovine retina contained a strongly immunoreactive species, with molecular characteristics of γ -tubulin, in association with a detergent-resistant, cytoskeletal fraction devoid of cytoplasmic microtubules. γ -Tubulin was discretely localized throughout the basal body region,

extending opposite to the axonemal shaft, in mechanically detached rod outer segments and whole-mounted, connecting cilium-derived axonemes. In multiciliated epithelia from bovine trachea and oviduct, γ -tubulin immunoreactivity was detected at the base of the cilia, where basal bodies are located. These results suggest that this key centrosomal protein of mitotically active cells is also an integral component of microtubule organizing centers, required for the generation of the microtubule network in terminally differentiated cells.

Key words: basal body, cilia, γ -tubulin, microtubule organizing center, photoreceptor cell

INTRODUCTION

The microtubular framework is a part of the cytoskeleton that combines stability of the structure with an ability to reorganize rapidly in response to internal and external signals (Burnside and Dearry, 1986; Mitchison, 1988; Sandoz et al., 1988; Besharse and Horst, 1990). During mitosis, microtubules participate, as principal structural elements, in the organization of the mitotic spindle, and may function in chromosome segregation (Mitchison, 1988; Gorbsky, 1992). In interphase and postmitotic cells, microtubules have important functions (e.g., in the generation and maintenance of cell shape and polarity, morphogenesis of specific organelles, vesicle and organelle anchoring and movement, cell motility), which are carried out by populations of microtubules of different composition, stability and properties (Sale et al., 1988; Adoutte et al., 1991; Joshi and Cleveland, 1990; Pagh-Roehl et al., 1991; Avila, 1992).

With a few exceptions, the different microtubular networks are organized around one or more discrete foci (Porter, 1966), known as microtubule organizing centers (MTOCs) (Pickett-Heaps, 1969; see for reviews, Brinkley, 1985; Vorobjev and Nadezhdina, 1987), which also establish a common polarity of microtubules, with their fast-growing (plus) end away from the MTOC (Heidemann and McIntosh, 1980; Saltys and Borisy, 1985). Despite their het-

erogeneous morphology in different species and cell types, all MTOCs have the property of nucleating microtubules at a tubulin concentration below that required for spontaneous polymerization, and of stabilizing their slowly-growing (minus) end (Himes et al., 1977; Mitchison and Kirschner, 1984).

Recent work from several laboratories has shown that γ -tubulin, a newly discovered member of the tubulin superfamily (Oakley and Oakley, 1989), is associated with MTOCs (Oakley et al., 1990; Zheng et al., 1991; Stearns et al., 1991; Horio et al., 1991; Joshi et al., 1992; Baas and Joshi, 1992) and is required for microtubule nucleation throughout the cell cycle (Joshi et al., 1992). It has been suggested that γ -tubulin may be a universal component of MTOCs responsible for both microtubule nucleation and establishment of microtubule polarity (Oakley, 1992). Although present in a wide range of eukaryotic organisms (i.e. fungi, insects, mammals, plants, algae), the association of γ -tubulin with MTOCs other than centrosomes and spindle pole bodies is still not determined.

The two most frequently described MTOCs, the perinuclear centrosome and ciliary (or flagellar) basal body, are homologous, although distinct structures (Brinkley, 1985; Vorobjev and Nadezhdina, 1987). In numerous postmitotic or quiescent cells, the centrosomal centrioles move from their perinuclear location to one beneath the plasmalemma,

and become basal bodies, from which rudimentary, immotile cilia grow (Tucker et al., 1979; Wheatley, 1982). In multiciliated epithelial cells, such as those from the vertebrate respiratory and reproductive tracts, active multiplication of centrioles in a terminally differentiated cell gives rise to 200-300 basal bodies which, when fully developed, generate ciliary axonemes at the apical surface of the cell (Lemullois et al., 1988; Dirksen, 1991). In several cell types, basal bodies appear to be the only structures with MTOC activity, being responsible for the nucleation of the cytoplasmic microtubular network of the cell body as well. This is the case of the retinal photoreceptor cell, the outer segment of which is a modified, non-motile cilium, generated from a basal body during cell differentiation (reviewed by Besharse and Horst, 1990). In the mature, terminally differentiated cell, the cytoplasmic microtubules of the cell body appear to originate from the basal body, located at the distal end of the photoreceptor inner segment (Troutt et al., 1990; Pagh-Roehl et al., 1991). As a consequence, microtubules in the ellipsoid and myoid segment of the cell have their plus ends oriented toward the nucleus, and not toward the cell periphery as in most cells (Troutt and Burnside, 1988). We hypothesize that (1) these microtubules are generated from the basal body via a mechanism similar to that which occurs at centrosomes, and (2) α -tubulin is involved in the nucleation of cytoplasmic microtubules from the basal body. In this article, we demonstrate that α -tubulin is indeed associated with basal bodies in retinal photoreceptors and ciliated epithelia.

MATERIALS AND METHODS

Antibodies

A rabbit polyclonal antibody generated against a highly conserved peptide of α -tubulin was used, as a purified IgG fraction, throughout this study (Joshi et al., 1992). A similarly purified IgG fraction, obtained from the preimmune serum, served as control for nonspecific binding. α - and β -tubulin were detected with mouse monoclonal antibodies: B-5-1-2, specific for all α -tubulin isoforms, was a gift of Dr Gianni Piperno (Piperno et al., 1987); the other antibody was from Amersham Corp. (Arlington Heights, IL) and recognized all α -tubulin gene products (Joshi and Cleveland, 1989).

Primary antibodies raised in rabbit were detected with an anti-rabbit IgG (whole molecule)-alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO) (for western blotting), affinity-purified, goat anti-rabbit IgG-L-rhodamine (Boehringer Mannheim Biochemicals, Indianapolis, IN), or goat anti-rabbit IgG-fluorescein isothiocyanate (FITC) (Miles, Inc., Elkhart, IND) (for immunofluorescence). Mouse monoclonal antibodies were detected with a goat anti-mouse IgG-FITC conjugate (Zymed Laboratories, Inc., South San Francisco, CA), or anti-mouse Ig-digoxigenin (affinity-purified F(ab)₂ fragment), followed by anti-digoxigenin-rhodamine (Boehringer Mannheim Biochemicals, Indianapolis, IN). In western blots, mouse antibodies were reacted with NANOGOLD™ gold-anti-mouse IgG conjugate (Nanoprobes, Inc., Stony Brook, NY), followed by silver enhancement.

Buffers

The following buffers were used in this study: *buffer A* (10 mM Pipes, pH 7.0, 5 mM MgCl₂, 0.1 mM phenylmethylsulfonyl fluoride), for rod inner segment-outer segment (RIS-ROS) preparation; *buffer B* (10 mM Pipes, pH 7.0, 5 mM MgCl₂, 1 mM dithio-

threitol, 0.1 mM phenylmethylsulfonyl fluoride, 2% Triton X-100), for RIS-ROS extraction; *transfer buffer* (192 mM glycine, 25 mM Tris, pH 8.3, 20% methanol, 0.05% sodium dodecyl sulfate [SDS]); *blot washing buffer* (250 mM triethanolamine, pH 7.4, 0.5% Triton X-100, 0.1% SDS, 100 mM NaCl, 2 mM EDTA); *antibody incubation buffer* (4% PTX: 10 mM sodium phosphate, pH 7.5, 0.2% Triton X-100, 0.15 M NaCl, 1 mM EGTA, 4% bovine serum albumin [BSA]); *fixation/permeabilization buffer* (0.1 M sodium phosphate, pH 7.4, 1 mM MgCl₂, 4% formaldehyde, 0.1% glutaraldehyde, 0.05% Triton X-100); and *phosphate-buffered saline* (PBS: 10 mM sodium phosphate, pH 7.3, 0.14 M NaCl, 1 mM MgCl₂).

Preparation of photoreceptor axoneme fraction from frozen bovine retinas

A cytoskeletal fraction enriched in photoreceptor axonemes was obtained from dark-adapted, frozen retinas (George A. Hormel & Co., Boston, MN), by a modification (Horst et al., 1990) of the procedure of Fleischman and Denisevich (1979). Briefly, a purified RIS-ROS population was collected in sucrose-containing buffer A, and extracted with detergent for 1 h, by mixing with an equal volume of buffer B. The Triton X-100-insoluble residue (i.e. the axoneme fraction) was separated from the solubilized material by a step gradient centrifugation, at the interface of the 50% and 60% sucrose layers. Since all steps of the procedure were carried out on ice, cold-sensitive cytoplasmic microtubules (e.g. those in the ellipsoid) were not preserved in the fraction.

Electrophoretic separation and immunoblot

Samples of axoneme fraction were subjected to SDS-polyacrylamide gel electrophoresis, according to Laemmli (1970), using 5% to 15% polyacrylamide gradients and minigels. Proteins were transferred for 35 min at 23°C onto Immobilon™-P transfer membranes (Millipore Corp., Bedford, MA), using the TE70 Semi-Phor™ Semi-Dry Blotter (Hoefer Scientific Instruments, San Francisco, CA), set at 80 mA constant current. Strips cut from the membrane blot were individually immunostained at 23°C, in Mini-Incubation Trays (Bio-Rad Laboratories, Richmond, CA), as follows. Blots were blocked for 30 min in 4% PTX, then incubated for 3 h or overnight in primary antibody diluted 1:5000 (for anti- α -tubulin) or 1:2000 (for anti- β -tubulin) in 4% PTX. Membrane strips were washed three times with blot washing buffer and once with 4% PTX, before incubation in secondary antibody solution. This was anti-rabbit IgG-alkaline phosphatase, diluted 1:1250 in 4% PTX for α -tubulin, and gold-anti-mouse IgG (1:150) for β -tubulin detection. Blot strips were washed, and antibody binding was visualized either with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (Glycan Differentiation Kit, Boehringer Mannheim Biochemicals, Indianapolis, IN) (for α -tubulin), or by silver enhancement (LI SILVER enhancement kit, Nanoprobes, Inc, Stony Brook, NY) (for β -tubulin), according to manufacturer's instructions. Two strips, containing transferred axonemal proteins and molecular size standards (low range, Bio-Rad Laboratories, Richmond, CA) respectively, were stained for protein with AuroDye™ forte (Amersham Corp., Arlington Heights, IL).

Immunolabeling of rod outer segments (ROS) and intact axonemes

Whole-mount preparations

RIS-ROS or ROS photoreceptor fragments were obtained by mechanical detachment from freshly dissected bovine retinas, as a suspension in Hanks' balanced salt solution (Sigma Chemical Co., St. Louis, MO) buffered with 25 mM Hepes. After gentle homogenization by five passes through a Pasteur pipet, cell fragments in suspension were allowed to adsorb for 3 min to a

Vectabond™ (Vector Laboratories, Burlingame, CA)-treated glass slide. Adhered cell fragments were fixed for 5 min in methanol at -20°C , and washed three times with PBS, before processing for immunostaining. In preparation for immunolabeling, samples of the axoneme-enriched fraction were diluted 1:5 in buffer B, permitted to dry onto glass slides (Horst et al., 1990), and washed three times with PBS to remove dried salts and sucrose.

Immunolabeling

Whole-mounted photoreceptor fragments and axonemes were blocked for 1-3 h with 1% BSA in PBS, then incubated with anti- γ -tubulin antibody diluted 1:200 or 1:500 in the same buffer, for an additional 3 h at 23°C . After three 10 min washes with PBS, 0.1% Tween-20, and one with PBS, 1% BSA, slides were treated with fluorescently labeled secondary antibody (1:400 dilution for the rhodamine-coupled antibody and 1:100 dilution for the FITC conjugate), for 1 h at 23°C , washed again three times, and coverslipped. In controls, normal rabbit IgG or nonimmune rabbit serum was used instead of anti- γ -tubulin antibody. In some experiments, antibody dilutions and specimen washings were done in 4% PTX, to create more stringent conditions for antibody binding, but with no effect on the results.

Double fluorescence labeling

Whole mounted axonemes were successively labeled for γ -tubulin and either α - or β -tubulin. Antibody dilutions were 1:10 for anti- α - and 1:200 for anti- β - and anti- γ -tubulin. In the second labeling step, primary antibodies were detected either with an anti-mouse IgG-FITC conjugate (1:40) or with anti-mouse Ig-digoxigenin (1:10) and anti-digoxigenin-rhodamine (1:10), applied successively, each for 1 h. Specimens were examined with a Zeiss Photomicroscope III, under epifluorescence illumination, and photographed on Kodak Ektachrome 400 film.

Immunohistochemistry of frozen tissue sections

Small tissue blocks, removed from bovine trachea and oviduct, freshly collected at a local slaughterhouse, were fixed in fixation/permeabilization buffer for 90 min at 23°C . The blocks were washed in PBS, then transferred successively to buffers containing increasing sucrose concentrations, before embedding by freezing in a 2:1 (v/v) mixture of 20% sucrose-PBS and Tissue-Tek O.C.T. compound (Miles, Inc., Elkhart, IN) (Barthel and Raymond, 1990). 7 μm sections were collected on Vectabond™-treated slides, air-dried and preserved at -20°C until use. Occasionally, 1 and 3 μm sections were obtained by ultracryotomy.

In preparation for immunolabeling, sections were treated for 15 min at 23°C with 0.5 mg/ml sodium borohydride in PBS to reduce tissue fluorescence produced by glutaraldehyde, washed with PBS, and blocked with PBS, 1% BSA, 10 mM glycine, for up to 4 h. Incubations in primary antibody solutions (1:200 dilution in PBS, 1% BSA for anti- γ -tubulin, 1:50 dilution for anti- α -tubulin) were carried out, usually overnight at 4°C (or 3 h at 23°C), and were followed by three washes with PBS, 0.1% Tween-20. Secondary (and tertiary) antibodies were applied in conditions described for whole-mounted specimens. In double-labeling experiments, γ -tubulin was probed essentially as described above, after completion of α -tubulin labeling steps. Specimens were mounted in glycerol-PBS 1:1 (v/v), and examined by fluorescence microscopy, using either narrow- or broad-band excitation filters.

RESULTS

Photoreceptor cell fragments bind anti- γ -tubulin antibodies at the distal end of the inner segment

Vertebrate retinal photoreceptors are highly polarized neu-

roepithelial cells, with four morphologically and functionally distinct compartments: the outer segment, inner segment (composed of myoid plus ellipsoid), cell body and synaptic terminal (see Besharse and Horst, 1990). The outer segment, containing the phototransduction machinery, maintains a persistent but highly regulated connection with the inner segment, necessary for its proper functioning. The structure which is interposed between these two compartments, the connecting cilium, has the main features of the transition zone of motile cilia and flagella, to which it morphologically corresponds (Röhlich, 1975; Besharse and Horst, 1990). The basal body, situated at the distal end of the ellipsoid, generates a typical non-motile axoneme (missing the central pair of microtubules), which extends towards the outer segment.

Mechanical detachment of photoreceptor cells from fresh bovine retinas produced a heterogeneous population of RIS-ROS fragments. While some of the specimens were composed of the outer segment and the ellipsoid region of the inner segment (i.e. intact RIS-ROS), others contained only outer segments with no or only a portion of the ellipsoid (Fig. 1). However, even when the inner segment was completely absent, a characteristic extension, at a position corresponding to the connecting cilium of intact photoreceptors, was frequently detectable by phase microscopy (data not shown).

When probed with the anti- γ -tubulin antibody, methanol-fixed RIS-ROS or ROS fragments showed bright staining of a small region at the base of the presumptive connecting cilium (Fig. 1A-H). A mostly diffuse background fluorescence of the ellipsoid in intact RIS-ROS, due probably to autofluorescent mitochondria, occasionally obscured the specific labeling at the distal end of the inner segment (Fig. 1I). The position of the labeled area generally corresponded to that of the basal body, but the fluorescent spot often extended for a variable length and faded out towards the inner segment (Fig. 1C,E,G). ROS fragments missing the connecting cilium/basal body extension did not bind the antibody (Fig. 1K). We conclude that epitopes recognized by the anti- γ -tubulin antibody are present in the basal body region of bovine photoreceptors.

Photoreceptor axonemes contain a γ -tubulin-like molecule

Previous studies have shown that, in mammalian cells, γ -tubulin is localized exclusively to the detergent-resistant cytoskeleton (Joshi et al., 1992). Therefore, we have used a cytoskeletal preparation, obtained by Triton X-100 extraction of RIS-ROS fragments (Horst et al., 1987), to further investigate the presence of γ -tubulin-like molecules in retinal photoreceptors. This fraction was highly enriched in intact photoreceptor axonemes still attached to the basal body (Horst et al., 1990). In transblots of axonemal proteins, the rabbit polyclonal anti- γ -tubulin antibody (IgG fraction) bound to a single species, with an electrophoretic mobility slightly higher than that of γ -tubulin, and an apparent molecular mass of 50-52 kDa (Fig. 2). We conclude that the antibody recognizes γ -tubulin in axonemal preparations from bovine photoreceptors.

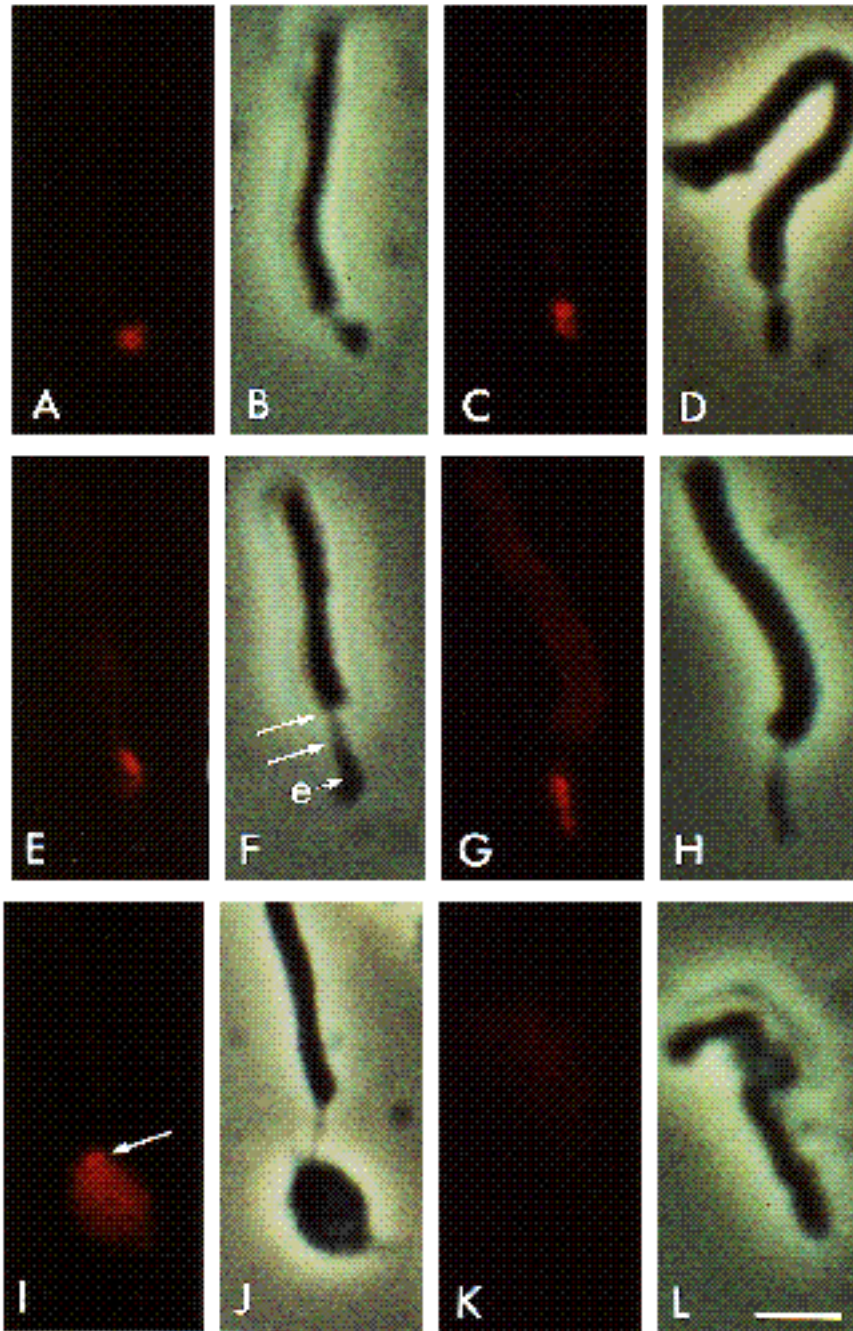


Fig. 1. Immunolocalization of γ -tubulin on isolated, methanol-fixed ROS. The gallery shows paired fluorescence (A, C, E, G, I, K) and phase contrast (B, D, F, H, J, L) images of ROS with a characteristic extension, delineated by arrows in (F), corresponding to the connecting cilium. Remnants of the ellipsoid region of the inner segment are also present (e). Note that the fluorescent labeling is localized to the proximal end of the presumptive connecting cilium, but occasionally extends, for a variable length, through the ellipsoid remnants (C, E, G). In (I), the characteristic fluorescent dot (arrow) is superposed on a diffuse fluorescent background in the distal ellipsoid. ROS with no connecting cilium are not labeled (K). Bar, 3 μ m.

Anti- γ -tubulin antibodies stain the basal body and accessory structures in photoreceptor axonemes

We have used fluorescence microscopy of whole-mounted specimens to localize γ -tubulin on the axoneme-basal body complex of photoreceptor cells. In phase contrast microscopy, the basal body appeared as a dark spot, with filamentous and amorphous extensions towards the inner segment, and a thin shaft, corresponding to the connecting cilium region of the axoneme, emanating distally (Fig. 3B). Occasionally, the companion centriole was isolated with the axoneme, and appeared as a second phase-dark spot.

γ -Tubulin, as detected by antibody binding, was always localized to the basal body region. The labeling appeared

either as a bright, mostly oval spot superposable to the basal body (Fig. 3A-F), or as an elongated projection (up to 1-2 μ m in length) emanating from the basal body and pointing towards the inner segment of the intact photoreceptor cell (Fig. 3G-P). In most cases, the filiform, fluorescent structure was also detectable by phase contrast microscopy (Fig. 3H,J,L); in others, it appeared embedded in the amorphous material, vicinal to the basal body (data not shown). Double probing of the specimens for γ -tubulin and either α - or β -tubulin indicated that the γ -tubulin-positive projection did not usually contain α - or β -tubulin accessible to the antibody (Fig. 3K,O). However, antibodies to α - or β -tubulin labeled the entire axoneme, including the basal body region

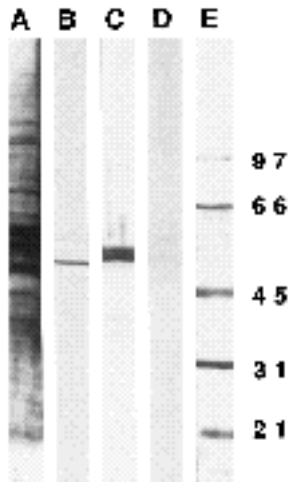


Fig. 2. Immunodetection of α - and γ -tubulin in the photoreceptor axoneme-enriched fraction. Protein samples from the axonemal fraction, separated by SDS-PAGE and transferred to ImmobilonTM-P membrane, were probed with anti- α -tubulin (lane B) or anti- γ -tubulin (lane C) antibodies. The membrane strip shown in lane D was processed similarly to that in lane B, but in the absence of primary antibody. Total protein in the axoneme fraction (lane A) and molecular size markers (in kDa) (lane E) were stained with AuroDyeTM forte.

(Fig. 3E,K,O), a result consistent with the presence of tubulin in the fibrogranular material associated with basal bodies (Dirksen, 1991). No labeling was obtained when an IgG fraction, prepared from nonimmune rabbit serum, was substituted for the γ -tubulin-specific antibody (Fig. 3Q). We conclude that, in photoreceptor axonemes, γ -tubulin is localized to an extended region in the vicinity of the basal body.

Anti- γ -tubulin antibodies label the basal body region in ciliated epithelial cells in situ

To investigate the in situ location of γ -tubulin in other ciliated cells, we have applied the anti- γ -tubulin antibody to permeabilized frozen sections from bovine trachea and oviduct. In both tissues, the mucosa is lined by an epithelial layer (pseudostratified columnar and simple columnar, respectively), in which ciliated and secretory cells predominate. The ciliated cells contain motile cilia which are formed from numerous basal bodies located underneath the apical plasmalemma. In both cell types, immunoreactivity was detected in a region at the base of the cilia, and appeared as a fluorescent line running parallel to the cell contour (exemplified for the tracheal epithelium in Fig. 4). At closer examination, this line was clearly seen to be formed of regularly spaced, fluorescent dots, which corresponded to the basal bodies (Fig. 4C). Ciliary axonemes were brightly labeled with anti- α -, but not anti- γ -tubulin antibody (Fig. 4E). Occasionally, γ -tubulin was detected in the cell matrix, localized probably to cytoplasmic microtubules, which appeared to emanate from a region just below the basal bodies (data not shown). We conclude that γ -tubulin is a general component associated with basal bodies, but not microtubules, in ciliated epithelial cells.

DISCUSSION

γ -Tubulin is localized to MTOCs in postmitotic cells

We have shown that γ -tubulin is present in terminally differentiated cells, where it is associated with MTOCs. Our study was focused on single-cilium sensory cells (for which we selected the vertebrate photoreceptor cell as prototype) but included also multiciliated cells; in all these cells, the major MTOCs are the basal bodies (Sandoz et al., 1988; Troutt et al., 1990). The study extends recently reported results (Baas and Joshi, 1992) on the presence of γ -tubulin at the centrosome of postmitotic cells (i.e. rat sympathetic neurons) in culture.

In photoreceptors, the basal body of the connecting cilium, derived from the apical centriole of the cell during ontogeny (Kunz et al., 1983), is the only MTOC of distinct morphology, functionally active under various experimental conditions (Troutt et al., 1990). The ciliated epithelial cells of the respiratory and reproductive tract are derived through differentiation of nonciliary precursor cells (Sandoz et al., 1976; Evans et al., 1986). The numerous basal bodies of the mature, nondividing cell are produced as centriolar structures in the early stages of ciliogenesis, from aggregates of fibrogranular material (associated or not with mature centrioles), and migrate to the apical surface, where they generate ciliary axonemes (Vorobjev and Nadezhdina, 1987; Lemullois et al., 1988; Dirksen, 1991).

Basal bodies and centrosomes are distinct MTOCs, which generate different cytoskeletal assemblages, and nucleate microtubules by different mechanisms (Gould and Borisy, 1977; Dentler, 1987). In spite of these differences in their function, the two MTOCs have a common major structural component, the centriole(s) (Wheatley, 1982), have similar associated appendages, and share enzymatic activities and immunologically-related epitopes (see for reviews, Brinkley, 1985; Vorobjev and Nadezhdina, 1987; Kimble and Kuriyama, 1992). In addition, centrosomal centrioles may serve as basal bodies and vice versa (Wheatley, 1982), each of them being capable of organizing microtubule arrays characteristic for the other MTOC.

Our finding that basal bodies contain γ -tubulin is consistent with the above-described relationship between basal bodies and centrosomal centrioles. The presence of γ -tubulin, both at the basal body of postmitotic cells and at the centrosome in numerous actively dividing and quiescent cells (Zheng et al., 1991; Stearns et al., 1991; Joshi et al., 1992), as well as in cultured neurons (Baas and Joshi, 1992), suggests that it may have a similar function in both MTOCs. It also indicates that γ -tubulin is still required in cells with a fairly stable, but less abundant and less complex cytoplasmic microtubular network, such as those which are terminally differentiated.

Basal body-associated material, possibly involved in nucleation of cytoplasmic microtubules, contains γ -tubulin

Our results demonstrate unambiguously that, in both retinal photoreceptors and multiciliated epithelial cells, γ -tubulin is localized to a region which corresponds to the posi-

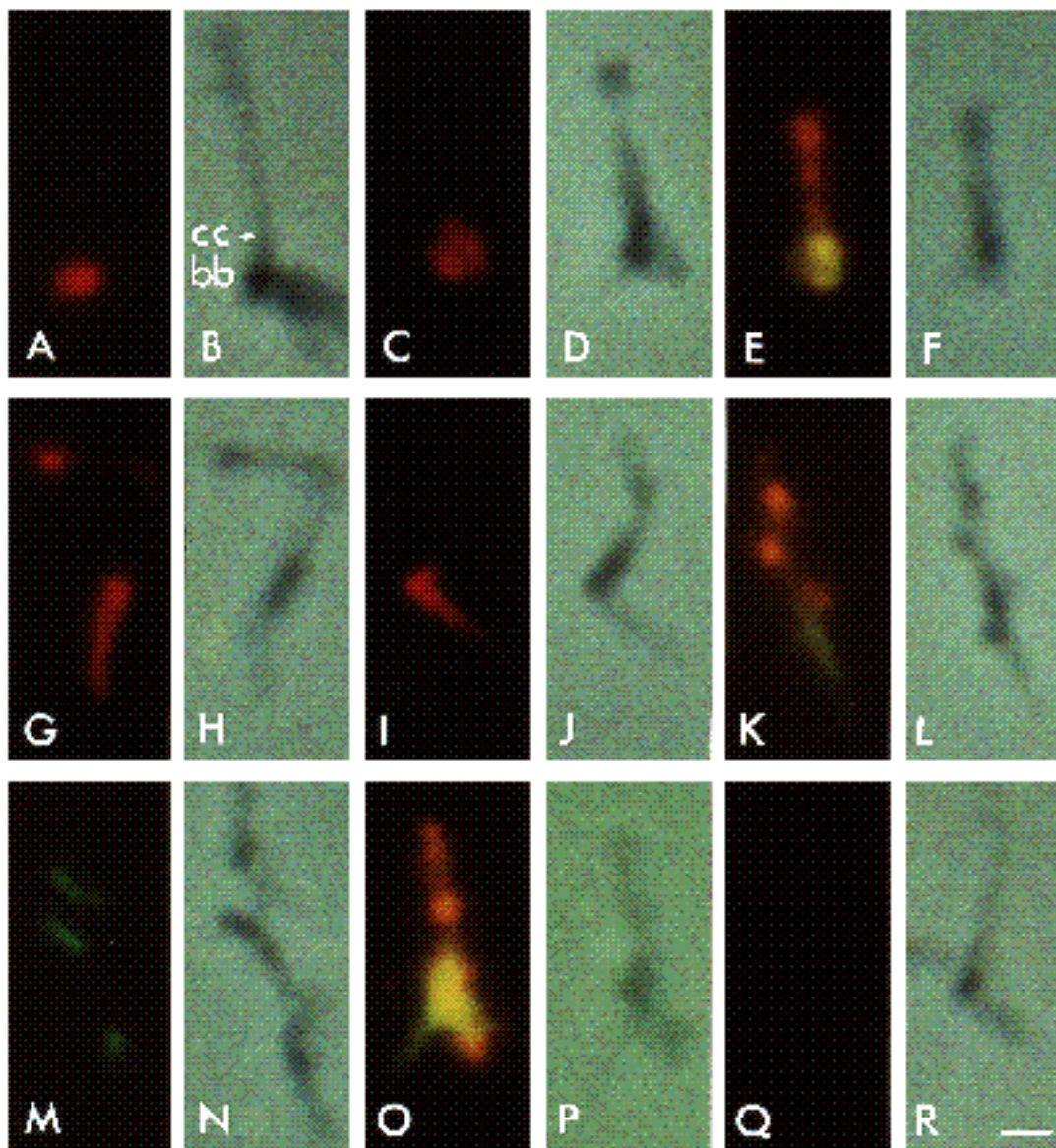


Fig. 3. Immunolocalization of α -, β - and γ -tubulin on isolated photoreceptor axonemes. Whole-mounted axonemes possess a phase-dark basal body (bb), from which filamentous and amorphous material extends toward the inner segment (B). A thin shaft, corresponding to the connecting cilium (cc) emanates in the opposite direction. The gallery contains pairs of phase (B, D, F, H, J, L, N, P, R) and fluorescence images, obtained through rhodamine (A, C, G, I, Q), FITC (M), or FITC-rhodamine (E, K, O) filter sets. Axonemes were stained with anti- α -tubulin antibody (A, C, G, I, M), rabbit IgG fraction obtained from preimmune serum (Q), or double-stained for either α - and β -tubulin (O) or α - and β -tubulin (E, K). The color code for the double-label images is: β -tubulin (green); α - or γ -tubulin (orange-red). The yellow color results from the superposition of green and red. Upper row: α -tubulin is localized at and around the basal body; middle row and (O): β -tubulin labels an elongated structure emerging from the basal body. The two axonemes in (G) and (H), and the three axonemes in (M) and (N), show different distribution patterns of β -tubulin. Bar, 1 μ m.

tion of the basal bodies. A more detailed, ultrastructural localization of this protein by electron microscopy was not attempted in this study. However, based on the results of Stearns et al. (1991) and Baas and Joshi (1992), who found a pericentriolar location for β -tubulin in centrosomes, we assumed that, in ciliated cells, β -tubulin is also localized to the cytoplasmic material associated with basal bodies. Such a location is consistent with the extended area, surrounding and including the basal body, which contains β -tubulin in photoreceptor (ROS) fragments.

The frequent localization of β -tubulin, in isolated retinal

photoreceptor axonemes, to a filiform structure emanating and fading away from the basal body region (see Results), suggests the possible association of β -tubulin with the striated rootlet, in the distal end of the ellipsoid. In mammalian photoreceptors, the striated rootlet extends from the basal body into the inner segment to the level of the outer limiting membrane (Sjöstrand, 1953), and may continue through the cell body and axon down to the synaptic terminal (Spira and Milman, 1979). The function of striated rootlets remains largely unknown, although it is likely that they may be involved in the association of the basal body

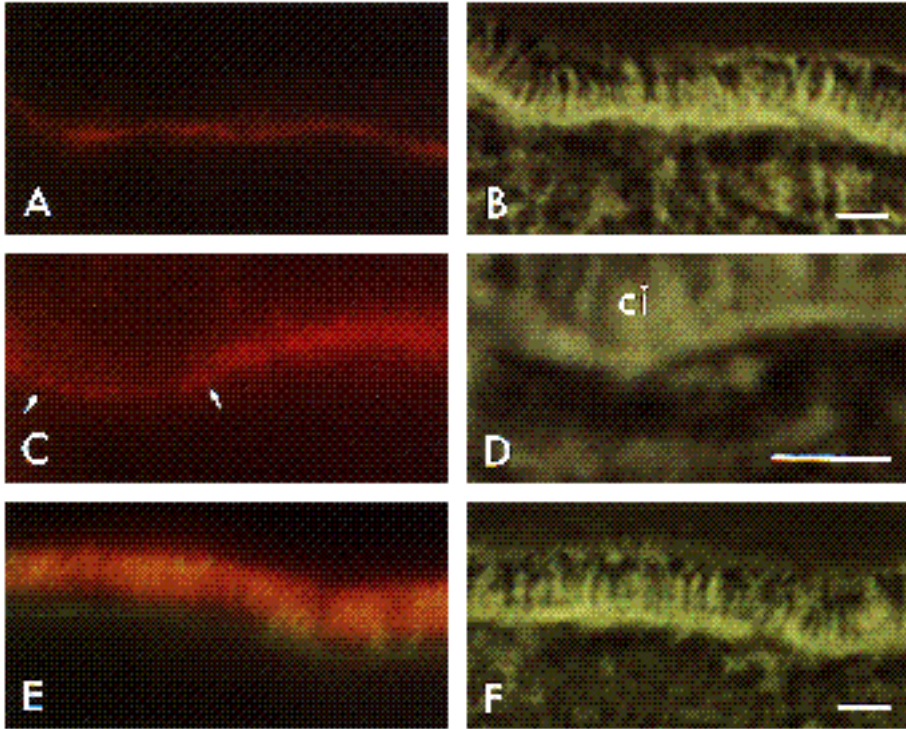


Fig. 4. Immunohistochemical localization of α - and γ -tubulin on fixed and permeabilized frozen sections of bovine trachea. Cryosections were stained with anti- α -tubulin (A, C), or double-stained for α - and γ -tubulin (E). α -Tubulin labeling (rhodamine, red in A and C, and FITC, green in E) is localized to the base of the cilia. Note the punctate appearance of the staining, at higher magnification (area between arrows in C). Anti- γ -tubulin (rhodamine, orange-red in E) binds to axonemal microtubules of the cilia. (B), (D) and (F) are corresponding phase contrast micrographs. Cilia (ci) appear out of focus at high magnification (D). Bars, 5 μ m.

with the cytoskeleton (Lemullois et al., 1991). However, in several organisms and cell types, appendages associated with the basal body (eg. basal foot, rootlets) have been described to nucleate cytoplasmic microtubules (Stearns et al., 1976; Gordon, 1982; Burton, 1985; Sandoz et al., 1988). Based additionally on the fact that striated rootlets share common epitopes with the major MTOC of eukaryotic cells, the centrosome (Salisbury et al., 1986; Baron and Salisbury, 1988), we suggest that the region of the striated rootlet proximal to the basal body, to which γ -tubulin is localized, may be involved in the nucleation of inner segment microtubules, in mammalian photoreceptors. Such a role is consistent with the proposed function of γ -tubulin at the MTOC.

The highly asymmetric distribution of γ -tubulin at the basal body, generated by its localization to basal body appendages, may have important consequences for cell morphogenesis. This may explain why cytoplasmic microtubules extend unidirectionally, towards the nucleus, in the mature photoreceptor cell. In more general terms, the pattern of distribution of γ -tubulin within the MTOC may explain how MTOCs determine a particular spatial distribution of the microtubular network in each cell type, and thereby control cell morphology.

γ -Tubulin colocalizes with the minus end of cytoplasmic and axonemal microtubules

We have shown that, in multiciliated epithelia, γ -tubulin is localized to a submembrane region, in the apical cortex of the cells. Similarly, this protein was found attached to a structure which resides in the distal ellipsoid of retinal photoreceptors, in the vicinity of the plasma membrane. This result is in contradistinction to the perinuclear location of γ -tubulin in all other eukaryotic cells investigated up to now (Oakley et al., 1990; Zheng et al., 1991; Stearns et al., 1991;

Horio et al., 1991; Joshi et al., 1992; Baas and Joshi, 1992), but is consistent with the position of MTOCs and microtubule polarity in each particular cell type.

In retinal photoreceptors, cytoplasmic microtubules of the inner segment are oriented with their plus ends towards the nucleus (Trout and Burnside, 1988; Trout et al., 1990), as opposed to microtubules in most cells, where their fast-growing ends point away from the perinuclearly located MTOC (Brinkley, 1985; Soltys and Borisy, 1985). However, γ -tubulin colocalizes, as expected, with the minus end of both cytoplasmic and axonemal microtubules. In mature ciliated epithelia, such as those from the trachea and oviduct, cytoplasmic microtubules are concentrated mainly in the apical part of the cell, where most of them appear connected to basal body appendages (Sandoz et al., 1988; Lemullois et al., 1988). However, there is still no clear demonstration of the polarity of these microtubules with regard to the basal body. According to the proposed function of γ -tubulin in microtubule nucleation, and based on our results showing γ -tubulin localization to the basal body region, it is conceivable that all apical microtubules (i.e. both those extending parallel to the cell surface, or penetrating in the supranuclear cytoplasm) of multiciliated cells are oriented with their slowly growing end towards basal bodies.

The MTOC assembled around basal bodies organizes simultaneously two microtubule networks, which are generated differently, and have different functions and properties (see Satir and Sleight, 1990). Cytoplasmic microtubules are nucleated from the matrix or structures associated with the basal body, apparently by a process similar to that occurring at the centrosome, which may require the presence of γ -tubulin. Axonemal microtubules respect the polarity rule in that that the rapidly polymerizing end is most distal to the basal body (Allen and Borisy, 1974). However,

axonemal assembly occurs at the cell surface, in close association with the cell membrane, with tubulin added directly on the basal body microtubules (Dirksen, 1982; Dentler, 1990). According to the present model for microtubule nucleation, in which γ -tubulin molecules are necessary to attach the first tubulin dimer of each protofilament to the MTOC (Oakley, 1992; Avila, 1992), there is no obvious requirement of γ -tubulin for nucleation of axonemal outer doublet microtubules (although its localization corresponds to the position of their minus ends). Little is known about the nucleation of the central pair of microtubules, originating in the transition zone of motile cilia, from a plate of granular material. It is possible that the process resembles that which occurs at the centrosome, and may involve γ -tubulin.

Summary and perspectives

We have shown that γ -tubulin is generally present in post-mitotic, terminally differentiated ciliated cells, where it associates with the major MTOCs, the basal bodies. We have reinforced the assumption that γ -tubulin may be a required component of all MTOCs, by adding basal bodies and their associated material to the family of already proven γ -tubulin-containing MTOCs. Future studies will indicate if γ -tubulin is also present at the nucleation site of microtubular arrays which do not originate at a compact, spatially restricted and well-defined MTOC, such as the centrosome, spindle pole body, or basal body.

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