

γ -Tubulin in *Leishmania*: cell cycle-dependent changes in subcellular localization and heterogeneity of its isoforms

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Received 24 September 2003, revised version received 15 December 2003

Abstract

A panel of six anti-peptide antibodies recognizing epitopes in different regions of the γ -tubulin molecule was used for the characterization and localization of γ -tubulin during cell cycle in *Leishmania* promastigotes. Immunofluorescence microscopy revealed the presence of γ -tubulin in the basal bodies, posterior pole of the cell, and in the flagellum. Furthermore, the antibodies showed punctuate staining in the subpellicular microtubule. This complex localization pattern was observed in both interphase and dividing cells, where staining of posterior poles and the subpellicular corset was more prominent. In posterior poles, γ -tubulin co-distributed with the 210-kDa microtubule-interacting protein and the 57-kDa protein immunodetected with anti-vimentin antibody. Immunogold electron microscopy on thin sections of isolated flagella showed that γ -tubulin was associated with the paraflagellar rod (PFR) that runs adjacent to the axonemal microtubules. Under different extraction conditions, γ -tubulin in *Leishmania* was found only in insoluble cytoskeletal fractions, in contrast to tubulin dimers that were both in soluble and cytoskeletal pool. Two-dimensional electrophoresis revealed multiple charge variants of γ -tubulin. Posttranslational modifications of *Leishmania* γ -tubulin might therefore have an important role in the regulation of microtubule nucleation and interaction with other proteins. The complex pattern of γ -tubulin localization and its properties indicate that γ -tubulin in *Leishmania* might have other function(s) besides microtubule nucleation.

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Keywords: Antibodies; Cell cycle; Gamma-tubulin; *Leishmania*

Introduction

Leishmania are protozoan parasites from the family Trypanosomatidae. Members of this family (*Leishmania* spp., *Trypanosoma brucei*, *Trypanosoma cruzi*) are deadly human pathogens that affect millions of people in countries worldwide. Leishmaniasis comprises a group of diseases with a wide spectrum of clinical manifestations ranging from self-healing cutaneous ulcers to severe visceral disease and even death. *Leishmania* are digenetic organisms shuttling between a slender flagellated promastigote in the gut of the dipteran sandfly and an intracellular round-shaped immobile amastigote in the mammalian host. *Leishmania*

tropica belongs to the species of the Old World and causes mainly cutaneous leishmaniasis.

Flagellated promastigote of *Leishmania* comprises four major highly organized microtubule arrays: the subpellicular cortical microtubules, the flagellar axoneme, the basal body, and the intranuclear mitotic spindle. Tubulin is also the most abundant protein in *Leishmania* [1]. *Leishmania* therefore represents a particularly interesting model when studying proteins that participate in microtubule nucleation and organization. The microtubular corset of the cell is formed by parallel arrays of microtubules closely apposed to the inner surface of plasma membrane [2]. Promastigotes carry a single flagellum that protrudes from the cell through a flagellar pocket. The flagellum consists of a canonical 9 + 2 microtubule array and a unique cytoskeletal structure called the paraflagellar rod (PFR, also known as paraxonemal rod). The PFR is a complex lattice of filaments that run parallel to the axoneme once the flagellum emerges from the flagellar

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pocket. Two major protein components of the PFR (called PFR 1 and PFR 2 in *Leishmania*) are essential for proper motility and attachment to insect vector [3,4]. Apart from the well-characterized major components of PFR, other constituents have also been described, but their role in the PFR structure is unknown [5,6]. The basal body of the flagellum not only controls the assembly of flagellar axoneme but is also involved in mitochondrial DNA (called kinetoplast) segregation. During division, the basal body of the flagellum divides first, and a new flagellum is generated from the daughter basal body. Next, the kinetoplast and subsequently the nucleus divide. The basal body as a microtubule-organizing center (MTOC) plays a prominent part in the orchestration of microtubule arrays and regulation of microtubule assembly. Despite its important role, only few proteins were so far located to the region of basal body of trypanosomatids. A homolog of human centrin 2 was localized to the basal body of *Leishmania donovani* [7]. The 210-kDa microtubule-interacting protein and the 57-kDa protein co-distribute in the region of basal body and on posterior poles of *L. tropica* cells [8]. In *T. brucei*, the γ -tubulin, the 170-kDa *T. brucei* basal body component protein (TBBC), and the 43-kDa protein BBA4 closely associate with the basal body complex [9,10].

γ -Tubulin, a highly conserved member of the tubulin superfamily, is widely accepted as a marker of MTOC in diverse eukaryotes [11]. In mammalian cells, the majority of γ -tubulin is associated with other proteins in soluble cytoplasmic complexes. Large γ -tubulin-ring complexes (γ -TuRCs) were identified in various species [12,13]. Smaller complexes (γ -tubulin small complex; γ TuSC) were also found in different cells [14]. The γ -TuRCs are formed by small complexes and by other proteins. In addition to the nucleation from MTOC, γ -TuRCs are also involved in the regulation of the dynamics of microtubule (–) ends [15].

γ -Tubulin itself has been recognized as a microtubule (–)-end binding molecule in non-anchored microtubules [16], and the binding sites for α - and β -tubulin subunits on γ -tubulin were identified using synthetic peptides [17]. Membrane-bound forms of γ -tubulin with nucleation activity were also described [18,19]. Phosphorylation of the γ -tubulin on tyrosine has been reported [20] and multiple isoelectric variants of γ -tubulin were defined in different species [21–23].

In *T. brucei*, γ -tubulin appears to be present as a single copy gene per haploid genome. Immunostaining revealed its localization in the basal body, and also in nucleus, on the anterior tip of the cell body, along four stable microtubules that underline the flagellum attachment zone and low level punctuate staining over the microtubular corset [9]. RNAi experiments implicated γ -tubulin in nucleation of the central pair microtubules during flagellum morphogenesis in *T. brucei* [24]. In comparison with *T. brucei*, *Leishmania* has different morphology and different position and attachment of the flagellum. We wanted to find out whether γ -tubulin, a key component for microtubule nucleation, is in *Leishmania*

also in multiple nucleation sites and whether its distribution changes during cell cycle. Because multiple γ -tubulin isoforms were identified in different species, we searched for such variants in *Leishmania*.

Using a panel of antibodies against different γ -tubulin antigenic determinants, we collected data on complex, cell cycle-dependent distribution patterns of γ -tubulin in *L. tropica*.

Materials and methods

Cell cultures

L. tropica, strain Z-K (MHOM/JO/99/Z-K), an original isolate from human cutaneous leishmaniasis was provided by Dr. E. Nohýnková (Charles University, Prague, Czech Republic). The species identification was done by Dr. C. L. Eisenberger using a permissively primed intergenic PCR [25]. Promastigotes were grown at 28°C in Schneider's *Drosophila* Medium (Sigma-Aldrich, Prague, Czech Republic) supplemented with 10% (v/v) fetal calf serum, penicillin (500 units/ml), and amikacin (200 μ g/ml). Cells from the logarithmic phase of growth were used for immunofluorescence and preparation of cell lysates.

Antibodies

The following anti-peptide antibodies against γ -tubulin were used: monoclonal antibodies TU-30 (IgG2b), TU-31 (IgG2b), and TU-32 (IgG1) were prepared against the EYHAATRPDYISWGTQ peptide corresponding to the human γ -tubulin conservative sequence 434–449 [26]; monoclonal antibody GTU-88 (IgG1) prepared against the EEFATEGTDRKDVFFY peptide corresponding to human γ -tubulin sequence 38–53, and affinity-purified rabbit antibody γ -TUB raised against the same peptide. The last two antibodies were bought from Sigma-Aldrich. Rabbit antibody No. 9 was raised against the EEFATEGGDRKDV peptide corresponding to *Aspergillus nidulans* γ -tubulin sequence 38–50 [27]. The antibody was affinity purified on recombinant 6 \times His-tagged γ -tubulin immobilized on a Ni-NTA agarose column (Qiagen, Hilden, Germany) [28]. The amino acid γ -tubulin sequences in regions where the peptides used for immunizations are located are compared in Fig. 1.

The antibody TU-01 (IgG1) recognizes the epitope that is in phylogenetically highly conserved region (amino acids 65–97) in the N-terminal structural domain of α -tubulin [29]. In double-label immunofluorescence, the microtubule structures were visualized by a rabbit affinity-purified TUB antibody against $\alpha\beta$ -tubulin dimer [30]. The MA-01 antibody (IgG1) was raised against porcine brain microtubule-associated proteins MAP2ab and recognizes the 210-kDa protein in different nonneural cells of various species including *L. tropica* [8,31]. The mouse



Fig. 1. Comparison of γ -tubulin sequences in regions where the peptides used for immunizations are located. H.s., *Homo sapiens*; L.m., *Leishmania major*; A.n., *Aspergillus nidulans*. Identical amino acid positions are highlighted. Full line: human peptide used for preparation of monoclonal antibody GTU-88 and polyclonal antibody γ -TUB; dashed line: *A. nidulans* peptide used for preparation of polyclonal antibody No. 9; dotted line: human peptide used for preparation of monoclonal antibodies TU-30, TU-31, and TU-32.

monoclonal VI-01 antibody (IgM) is directed against the antigenic determinant on vimentin and smooth muscle desmin [32]. In *L. tropica*, it recognizes the 57-kDa protein [8]. The rabbit affinity-purified antibody M8 was raised against pericentrin [33]. As negative controls served the rabbit affinity-purified anti-actin antibody (Sigma-Aldrich; catalogue number A2066), mouse monoclonal TU-20 antibody (IgG1) against neuron-specific class III β -tubulin [34] and mouse monoclonal MT-02 antibody (IgM) against microtubule-associated proteins MAP2ab [35]. Secondary anti-mouse and anti-rabbit antibodies conjugated with horseradish peroxidase were purchased from Promega Biotec (Madison, WI, USA). Indocarbocyanin 3 (Cy3)-conjugated anti-mouse and anti-rabbit Ig antibodies, fluorescein isothiocyanate (FITC)-conjugated anti-mouse and anti-rabbit Ig antibodies, and immunoglobulin class-specific antibodies conjugated with Cy3 or FITC were from Jackson Immunoresearch Laboratories (West Grove, PA, USA). The 10 nm gold-conjugated anti-mouse Ig antibody was from British BioCell (Cardiff, UK).

Subcellular fractionation and preparation of cell extracts

Isolation of flagella was performed according to Bastin et al. [36]. Briefly, cells were washed twice in phosphate-buffered saline (PBS) and extracted with 1% NP-40 (v/v) in PEME buffer (0.1 M Pipes, pH 6.9, 2 mM EGTA, 1 mM MgSO₄, and 0.1 mM EDTA) on ice for 5 min. The insoluble material was pelleted, resuspended in 1% NP-40 (v/v) supplemented with 1 M NaCl in PEME buffer, and incubated on ice for 20 min. The pelleted material contained a highly enriched flagellar fraction as confirmed by phase contrast microscopy.

To prepare total extracts, cells or flagella were washed twice in PBS and solubilized in 6 M guanidine thiocyanate in H₂O at room temperature for 10 min. After centrifugation at 20,000 \times g for 15 min at room temperature, the collected supernatant was dialyzed against H₂O. After dialysis the

suspension was mixed with twice concentrated SDS-sample buffer and boiled for 5 min.

For the preparation of soluble and insoluble fractions, washed cells were extracted with cold 1% Nonidet P-40 (v/v) in microtubule-stabilizing buffer (MSB; 20 mM MES, pH 6.9, 2 mM EGTA, 2 mM MgCl₂) supplemented with protease inhibitor cocktail Complete (Roche Diagnostic, Mannheim, Germany) and phosphatase inhibitors (1 mM NaF and 1 mM Na₃VO₄). After 10 min incubation at 4°C under mild shaking, the samples were centrifuged at 24,000 \times g for 15 min at 4°C. One-fourth volume of four times concentrated SDS-sample buffer was added to the supernatant. The pelleted material was washed in cold MSB, resuspended in 1% Nonidet P-40 (v/v) in MSB to the original volume, and one-fourth volume of four times concentrated SDS-sample buffer was added. Samples were boiled for 5 min. Protein quantification in SDS-sample buffer was performed using the colloidal silver method [37].

Gel electrophoresis and immunoblotting

Details of the protein separation on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and the immunostaining procedure are described elsewhere [38]. Primary monoclonal antibodies, in the form of supernatants, were used undiluted. Antibody GTU-88 was diluted 1:5000. Rabbit antibodies against γ -tubulin (γ -TUB) and pericentrin (M8) were diluted 1:2000 and 1:500, respectively. After incubation of the immunoblots with secondary antibodies conjugated with horseradish peroxidase (diluted 1:10,000), bound antibodies were detected by a chemiluminescent reaction (SuperSignal WestPico Chemiluminescent reagents; Pierce, Rockford, IL, USA) according to the manufacturer's recommendation. Autoradiography films X-Omat K were purchased from Eastman Kodak (Rochester, NY, USA).

Two-dimensional PAGE was performed essentially as described previously [39]. The pelleted flagellar fraction was solubilized in sample buffer containing 7 M urea, 2 M thiourea, 20 mM Tris, 4% CHAPS (w/v), 1% Triton X-100 (w/v), and 1% dithiothreitol (w/v) for 30 min at room temperature. Suspension was centrifuged at 24,000 \times g for 20 min at 24°C and the supernatant was diluted 1:5 in solubilization buffer supplemented with 2% IPG (v/v), pH 4–7 (Amersham Biosciences, Uppsala, Sweden). Immobilized DryStrip gels with linear pH 4–7 gradient, 7 cm long (Amersham Biosciences), were rehydrated using 170 μ l of prepared sample containing 30–50 μ g of proteins. Each strip was overlaid with mineral oil and left overnight at room temperature. Strips were focused for the total of 22 kV h on Multiphor II apparatus (LKB, Bromma, Sweden). The second dimension was performed using 7.5% SDS-PAGE, and separated proteins were transferred onto nitrocellulose by electroblotting. Markers for 2D electrophoresis were from BioRad Laboratories (München, Germany).

Immunofluorescence microscopy

Cells were harvested by centrifugation, washed three times in PBS, resuspended in PBS, and settled on poly-L-lysine-coated coverslips. Attached cells were treated with 2% Triton X-100 (v/v) in MSB supplemented with 4% PEG 6000 (w/v) for 5 min, washed in MSB, fixed with 3% formaldehyde (w/v) for 30 min, and treated with 0.3% SDS (w/v) in MSB for 10 min. In further text, this extraction–fixation method is called the Triton X-100 extraction and formaldehyde fixation. Alternatively, cells were extracted with 1% Nonidet P-40 (v/v) and fixed in cold methanol. In some experiments, cells extracted with Triton X-100 were incubated for 10 min with MSB containing 150 mM, 250 mM, 500 mM, 750 mM or 1 M NaCl. After washing in MSB, the cells were fixed by formaldehyde. Immunofluorescence staining was then performed as described [40]. In double-label immunofluorescence stainings, the fixed cells were first incubated with the corresponding anti- γ -tubulin antibody for 45 min, washed, and then incubated with the other primary antibody. The anti- γ -tubulin antibodies TU-30, TU-31, and TU-32 were used as undiluted supernatants, GTU-88 was diluted 1:400, polyclonal antibody γ -TUB 1:300 and polyclonal antibody No.9 1:500. The monoclonal antibodies TU-01, MA-01, and VI-01, and negative control antibodies TU-20 and MT-02 were all used as undiluted supernatants. Rabbit antibodies TUB against $\alpha\beta$ -tubulin dimer and against pericentrin were diluted 1:5 and 1:500, respectively. The slides were washed three times in PBS and incubated with a mixture of secondary antibodies. The secondary antibodies conjugated with Cy3 were diluted 1:1000 and the secondary antibodies conjugated with FITC

were diluted 1:200. Coverslips were mounted in Mowiol 4.88 (Calbiochem AG, Lucerne, Switzerland) containing 2 μ g/ml 4,6-diamidino-2-phenylindole (DAPI) and 6.25% propyl gallate (w/v) (Fluka AG, Buchs, Switzerland). The preparations were examined with an AX-70 PROVIS (Olympus) fluorescence microscope equipped with 100/1.35 fluorescence oil-immersion objective. Images were recorded using a Life Science Resources KAF 1400 cooled CCD camera.

In some immunofluorescence experiments, the antibodies TU-32 and GTU-88 were preabsorbed with the peptides used for immunization; that is, the 16 amino acid peptide corresponding to human γ -tubulin sequence 434–449 (TU-30) or 38–53 (GTU-88). Two molar antibody to peptide ratios were used, 1:10 and 1:100. Mixtures of antibodies and peptides were incubated for 30 min at room temperature.

Immunogold electron microscopy

Isolated flagella were washed in Sørensen buffer (SB, 0.1 M Na/K phosphate buffer, pH 7.3), pelleted, fixed for 30 min at room temperature in 3% paraformaldehyde (w/v) with 0.05% glutaraldehyde (w/v) in SB, and washed twice in SB. After centrifugation into 1% agarose (w/v), blocks were incubated for 20 min in 0.02 M glycine in SB, washed in SB, and dehydrated in ethanol. Ethanol was replaced in two steps by LR White acrylic resin (Sigma-Aldrich) and blocks were polymerized by UV light (20 h, 4°C). Eighty nanometer sections were prepared on Reichert UltraCuts Microtome (Leica, Germany) and nonspecific labelling was blocked for 30 min by 10% normal goat serum (v/v) in PBTB (PBS supplemented with 0.1% Tween

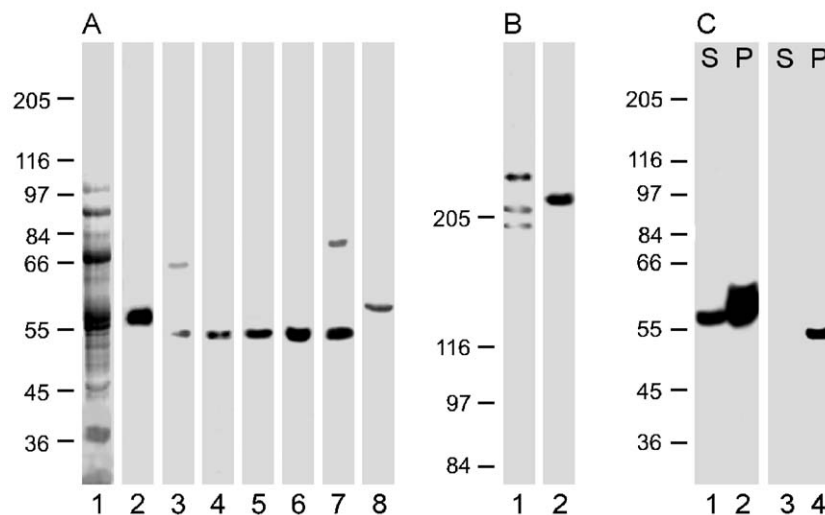


Fig. 2. Immunoblot analysis of *L. tropica* cell extracts with monoclonal antibodies against cytoskeletal proteins. (A and B) Whole cell lysates, (C) insoluble and soluble fractions after 1% NP-40 extraction. (A) Lane 1: Coomassie Blue staining; lanes 2–8: immunostaining with antibodies TU-01, TU-30, TU-31, TU-32, GTU-88, γ -TUB, and VI-01. 7.5% SDS-PAGE. (B) Lanes 1–2: immunostaining with anti-pericentrin antibody and MA-01 antibody. 5% SDS-PAGE. (C) To compare the relative distribution of immunoblotted proteins, the pelleted material was resuspended in a volume equal to that of the corresponding supernatant. Lanes 1–2: antibody TU-01, lanes 3–4: antibody GTU-88. S and P denote supernatant and pellet. 7.5% SDS-PAGE. The same amount of protein (10 μ g) was loaded into each lane. Scale bars on the left margins indicate positions of molecular-mass markers in kDa.

20 (w/v) and 1% BSA (w/v)). Sections were incubated 45 min with primary antibodies diluted twice in PBTB, washed three times with PBT (PBS supplemented with 0.005% Tween 20), and incubated for 30 min with 10 nm gold-conjugated secondary antibody diluted 1:30 in PBT. After washing in PBT and water, sections were contrasted 5 min with a saturated solution of uranyl acetate in water and observed in Philips Morgagni electron microscope equipped with Megaview II CCD camera. Control samples were incubated as above except that the primary antibody was omitted.

Results

Antibody characterization

Antibodies were characterized by immunoblotting on whole cell lysates of *L. tropica*. All antibodies directed against the C-terminal (TU-30, TU-31, and TU-32) and N-terminal (GTU-88, γ -TUB) regions of γ -tubulin reacted dominantly with proteins of the same electrophoretic mobility corresponding to the molecular weight around 53 kDa (Fig. 2A, lanes 3–7). Faint cross-reactivity was observed in

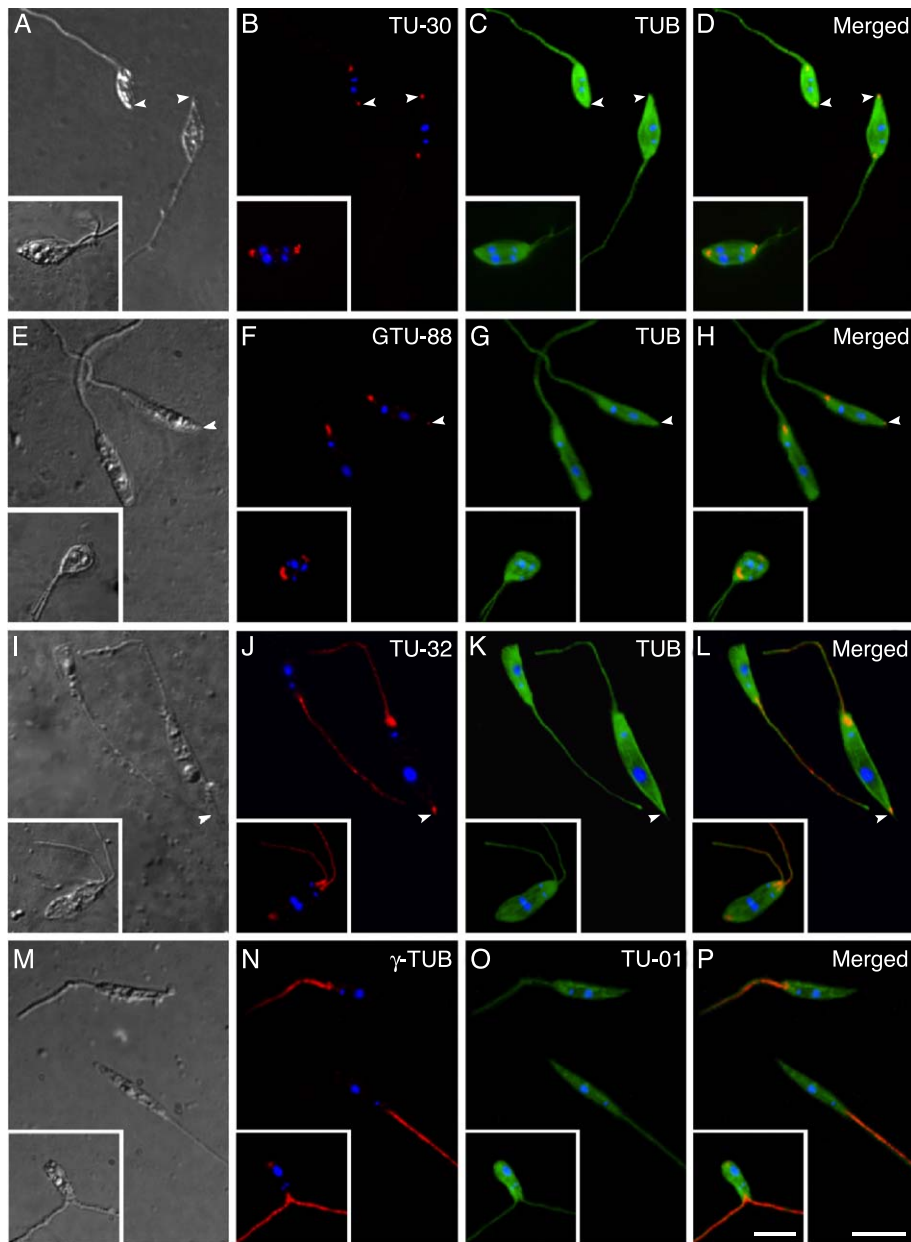


Fig. 3. Distribution of γ -tubulin in interphase and mitotic (inserts) cells of *L. tropica*. Cells were stained with anti- γ -tubulin antibodies (red) TU-30 (B), GTU-88 (F), TU-32 (J), or γ -TUB (N); antibodies against tubulin dimer (green) TUB (C, G, K) or TU-01 (O) and DNA-binding dye (blue). Pictures (D, H, L, P) show superpositions of stainings in each row. Phase-contrast images (A, E, I, M) correspond to each row of fluorescent images. Arrowheads denote staining of posterior poles in cells. Cells were extracted with Triton X-100 and fixed by formaldehyde. Scale bar = 5 μ m.

the case of antibodies TU-30 (around 66 kDa) and γ -TUB (around 80 kDa). No reactivity was found in position of tubulin dimers, as demonstrated by staining with anti- α -tubulin antibody TU-01 (Fig. 2A, lane 2). The TU-01 antibody recognized a protein with a molecular weight of around 55 kDa and the anti-vimentin antibody VI-01 stained a 57-kDa protein (Fig. 2A, lane 8). The anti-pericentrin antibody reacted mainly with a protein of electrophoretic mobility around 220 kDa and also gave a weaker staining with proteins of lower molecular weight, representing either proteolytic fragments of pericentrin-related protein or cross-reacting protein(s) (Fig. 2B, lane 1). The MA-01 antibody recognized the 210-kDa protein (Fig. 2B, lane 2). No reactivity with blotted proteins was detected when negative control mouse monoclonal antibodies of IgG and IgM class, or negative control rabbit antibody, were applied. To compare the relative distribution of γ -tubulin and tubulin dimers in soluble and insoluble (cytoskeletal) fractions, cells were extracted with 1% Nonidet P-40 and the pelleted material was resuspended in a volume equal to that of the corresponding supernatant. Immunoblotting analysis showed that α -tubulin was present both in soluble and insoluble (cytoskeletal) fractions (Fig. 2C, lanes 1 and 2), whereas γ -tubulin under the same conditions was found only in the insoluble fraction (Fig. 2C, lanes 3 and 4).

Localization of γ -tubulin in interphase and mitotic cells

A panel of anti- γ -tubulin antibodies was used to assign the subcellular localization of γ -tubulin in *L. tropica*. For a better orientation in cell structures, DNA-binding dye was used to visualize the nucleus and kinetoplast. Flagella and subpellicular microtubular arrays were decorated with polyclonal antibody against tubulin dimers (Figs. 3C, G, and K) or with monoclonal antibody TU-01 against α -tubulin (Fig. 3O). Immunofluorescence staining with antibodies TU-30 (C-terminal region of γ -tubulin) and GTU-88 (N-terminal region of γ -tubulin) showed labelling of two major sites: basal body region and posterior pole of the cell opposite the flagellar pocket (Figs. 3B and F). The intensity of labelling at the posterior poles varied in the cell population; some interphase cells were not decorated in this position. However, all mitotic cells were stained at the posterior poles, and the stained area was generally larger than in interphase cells (inserts in Figs. 3B and F). Overexposition of TU-30 labelling revealed a faint decoration of flagella, while no flagellar staining was observed with overexposition of GTU-88 labelling. Staining of the basal body region and posterior pole of the cell was also observed with antibodies TU-32 (C-terminal region of γ -tubulin) and γ -TUB (N-terminal region of γ -tubulin). Again, staining on the posterior pole was cell-cycle dependent with the strongest reaction in mitotic cells (inserts in Figs. 3J and N). In addition, these antibodies gave a strong discontinuous staining along the flagellum in both mitotic and interphase cells (Figs. 3J and N). Similar results were also obtained

with antibodies No. 9 (N-terminal region of γ -tubulin) and TU-31 (C-terminal region of γ -tubulin) (not shown). The latter only gave a weak staining of all mentioned structures. Superposition of stainings with anti- γ -tubulin antibodies and antibodies against tubulin dimers revealed that γ -tubulin was not present at the tip of the flagella and did not colocalize with tubulin in this structure (Figs. 3L and P). The described staining patterns with the mentioned anti- γ -tubulin antibodies were similar, no matter which fixation procedure was applied. This was true not only for immunofluorescence data shown in Fig. 3 but also for subsequent immunofluorescence experiments performed on fixed cells. A speckled distribution of γ -tubulin along the whole length of the flagellum, visualized by TU-32 antibody, is shown at higher magnification in Fig. 4A. All anti- γ -tubulin antibodies also gave a weaker punctuate staining over the cell body, as demonstrated at higher magnification in Fig. 4C.

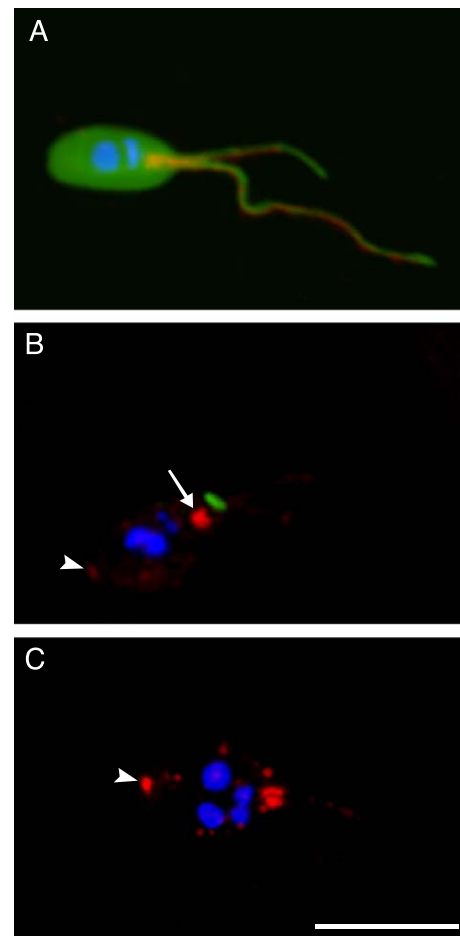


Fig. 4. Details of γ -tubulin localization in *L. tropica* cells. (A) γ -Tubulin staining with TU-32 antibody (red) in flagellum does not match the staining of tubulin dimers with TUB antibody (green). (B) Immunostaining with anti-pericentrin antibody (green) and anti- γ -tubulin antibody TU-30 (red) in the anterior pole of the cell. (C) Dot-like staining of microtubular corset with anti- γ -tubulin antibody TU-30 (red). All cells were stained with DNA-binding dye (blue). Arrowheads denote staining of posterior poles in cells. Arrow shows staining of the basal body region. Cells were extracted with Triton X-100 and fixed by formaldehyde. Scale bar = 5 μ m.

Neither the negative control mouse monoclonal antibodies of the IgG and IgM classes, nor the negative control rabbit antibody or the conjugated secondary antibodies alone gave any staining.

To corroborate the specificity of immunofluorescence staining, antibodies TU-32 and GTU-88 were pre-absorbed with the corresponding peptides which had been used for antibody preparation. No staining of *L. tropica* cells was observed with preabsorbed antibodies. On the other hand, preabsorption of the TU-32 antibody with the peptide used for GTU-88 preparation or preabsorption of GTU-88 antibody with the peptide used for TU-32 preparation caused no reduction in immunofluorescence intensity. The same results were obtained irrespective of the used molar ratio of antibody to peptide (1:10 or 1:100).

To learn more about the nature of γ -tubulin association with the flagellum and posterior pole, cells were first extracted with 2% Triton X-100 in MSB and then incubated for 10 min in MSB containing 150 mM, 250 mM, 500 mM or 1 M NaCl. γ -Tubulin staining at those particular areas

was observed after incubation of cells in buffer containing up to 500 mM NaCl. The majority of extracted cells incubated in 1 M NaCl became detached from the coverslips, but in some of the remaining cells, γ -tubulin was still present at the posterior pole and flagellum.

Comparison of γ -tubulin distribution with other cytoskeletal proteins

To compare γ -tubulin distribution with that of other cytoskeletal proteins previously localized at the posterior pole of *L. tropica* cell [8], double-label immunofluorescence was performed with antibodies VI-01 and MA-01. Merged pictures of γ -tubulin stained with TU-30 antibody that decorated only basal bodies and posterior poles of cells, and vimentin-like protein stained with VI-01 antibody, showed a striking colocalization of these two proteins both in the basal body and at the posterior pole of the cell (Fig. 5D). Prominent co-distribution of these proteins was also found in mitotic cells (insert in Fig. 5D). Colocalization of

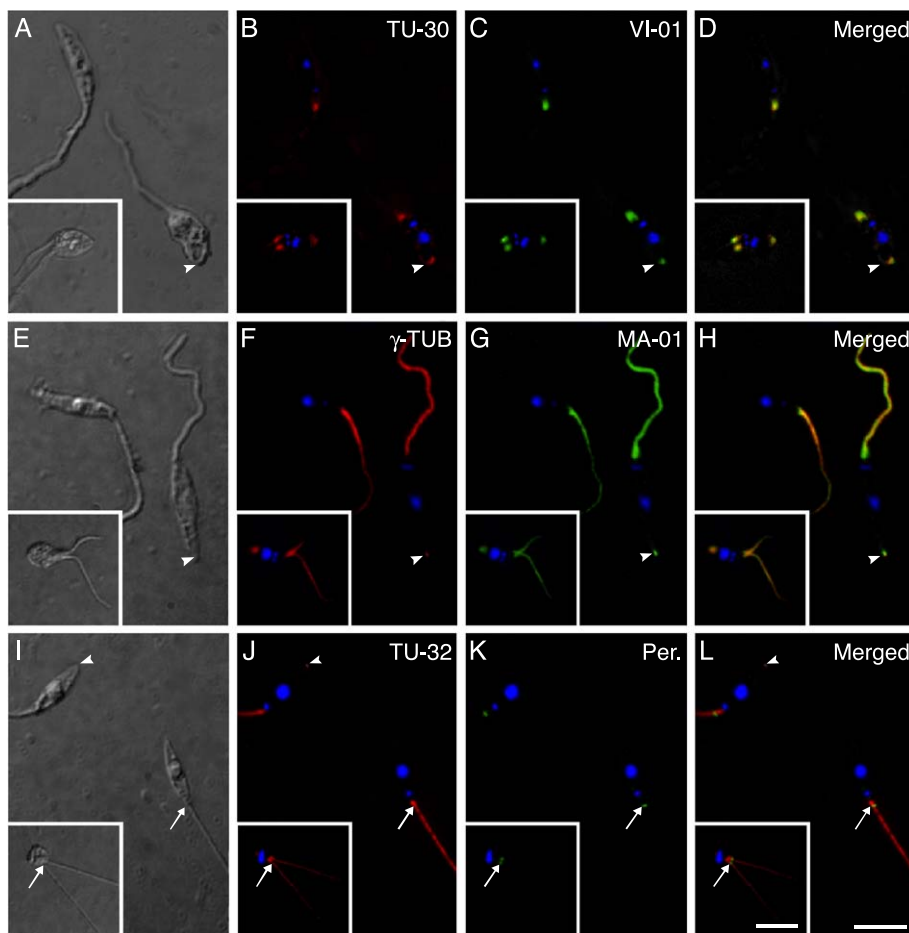


Fig. 5. Comparison of γ -tubulin distribution with other cytoskeletal proteins in interphase and mitotic (inserts) cells of *L. tropica*. Cells were stained with anti- γ -tubulin antibodies (red) TU-30 (B), γ -TUB (F), or TU-32 (J); anti-vimentin antibody VI-01 (C; green), antibody against 210-kDa microtubule-interacting protein MA-01 (G; green), anti-pericentrin antibody (K; green), and DNA-binding dye (blue). Pictures (D, H, L) show superpositions of staining in each row. Phase-contrast images (A, E, I) correspond to each row of fluorescent images. Arrowheads denote staining of posterior poles in cells. Arrows pinpoint the localization of immunostaining with anti-pericentrin antibody. Cells were extracted with Triton X-100 and fixed by formaldehyde. Scale bar = 5 μ m.

γ -tubulin, stained with polyclonal γ -TUB antibody, and the 210-kDa microtubule-interacting protein, detected by MA-01 antibody, is shown in Fig. 5H. Again, co-distribution of both proteins was found in posterior poles and basal body regions. The co-distribution was also found in mitotic cells (insert in Fig. 5H). Superposition of staining patterns with either of the two antibodies that decorated the flagella revealed colocalization of γ -tubulin and MA-01 antigen in this region. A comparison of γ -TUB staining in Fig. 3P with that in Fig. 5H indicates that both γ -tubulin and MA-01 antigen are localized outside the axonemal microtubules. Co-distribution was also observed when anti- γ -tubulin antibody TU-32 and biotin-conjugated MA-01 antibody were used for double labelling (not shown).

Pericentrin, like γ -tubulin, is an established marker of MTOCs in different model systems. We therefore performed double-labelling experiments with anti- γ -tubulin and anti-pericentrin antibodies. The anti-pericentrin antibody gave discrete staining at the anterior pole of interphase and mitotic cells (Fig. 5K), but superposition with anti- γ -tubulin staining disclosed a difference in localization (Fig. 5L). The protein immunologically related to pericentrin (pericentrin-related protein) was absent in the basal body, but was found somewhat closer to the anterior tip of the cell. Higher magnification (Fig. 4B) showed that the pericentrin-related protein (green) was located at the anterior pole more distant from the nucleus than γ -tubulin (red), probably in the area of the flagellar pocket opening. No staining at the posterior pole was detected with the used anti-pericentrin antibody.

Flagellar γ -tubulin

To characterize the flagellar γ -tubulin in more detail, we have isolated *L. tropica* flagella. Immunoblotting analysis of flagellar samples containing the same amount of protein demonstrated that it was possible to detect both α -tubulin by antibody TU-01 (Fig. 6A, lane 2) and γ -tubulin by antibody GTU-88 (Fig. 6A, lane 3) or TU-32 antibody (Fig. 6A, lane 4). The two-dimensional analysis of isolated flagella revealed that γ -tubulin existed in multiple charge variants that have their isoelectric points more basic in comparison with α -tubulin (Fig. 6B). Three isoelectric variants of γ -tubulin were identified on immunoblots with antibody GTU-88. The pI value of the most acidic variant was 6.0.

Immunofluorescence and immunogold electron microscopy confirmed the presence of γ -tubulin in isolated flagella. Flagella detected by phase contrast microscopy (Fig. 7A) were discontinuously labelled along the flagellum by anti- γ -tubulin antibody (Fig. 7B). Staining with anti- α -tubulin antibody TU-01 (Fig. 7F) was stronger and more homogeneous. Immunoelectron microscopy with anti- γ -tubulin antibody TU-32 on thin sections of isolated flagella showed that γ -tubulin was present on the paraflagellar rod but absent on microtubular axoneme. Staining of cross-sections (Fig. 7C) and longitudinal sections (Fig. 7D) showed no reactivity with nine outer doublets and central pair of

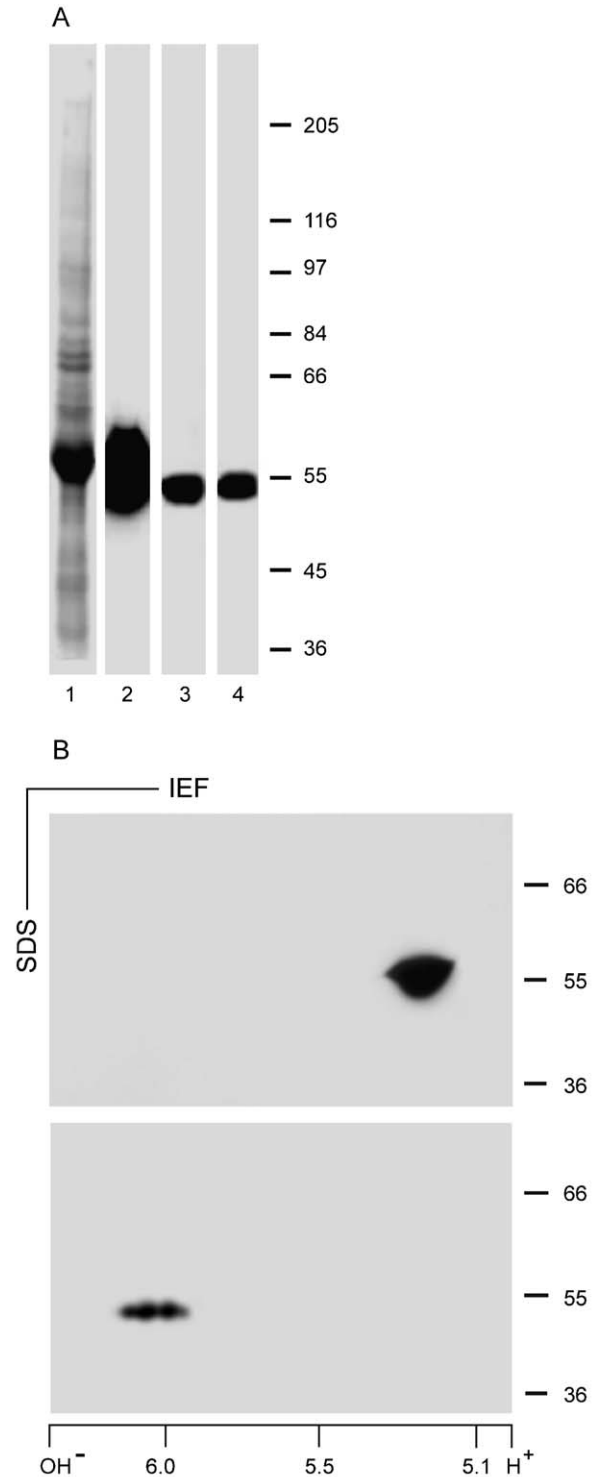


Fig. 6. Immunoblot analysis of *L. tropica*-isolated flagella. (A) Lane 1: Coomassie Blue staining; lanes 2–4: immunostaining with anti- α -tubulin antibody TU-01, anti- γ -tubulin antibody GTU-88, and anti- γ -tubulin antibody TU-32. Protein (10 μ g) was loaded into each lane. 7.5% SDS-PAGE. (B) Immunostaining of isolated flagella extract separated by two-dimensional electrophoresis with anti- α -tubulin antibody TU-01 (top) and anti- γ -tubulin antibody GTU-88 (bottom). The pI scale is shown along the bottom of the picture. Scale bars on the right margin indicate positions of molecular-mass markers in kDa.

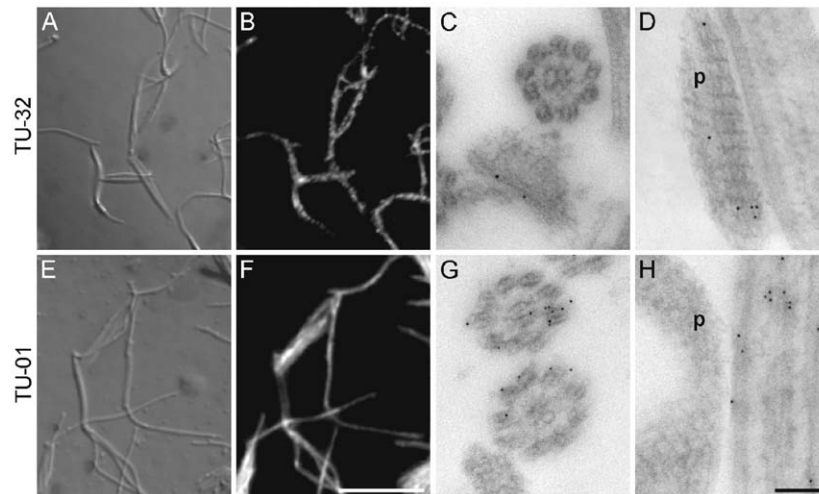


Fig. 7. Distribution of γ -tubulin in *L. tropica* flagella. Isolated flagella were stained with anti- γ -tubulin antibody TU-32 (A–D) and anti- α -tubulin antibody TU-01 (E–H). Immunofluorescence staining (B, F) and corresponding phase-contrast images (A, E). Flagella were extracted with Triton X-100 and fixed by formaldehyde. Scale bar = 5 μ m. Immunoelectron microscopy of isolated flagella on cross (C, G) and longitudinal (D, H) sections. p, paraflagellar rod. Scale bar = 100 nm.

microtubules. On the other hand, anti- α -tubulin antibody TU-01 decorated axonemal microtubules on cross-sections (Fig. 7G) and longitudinal sections (Fig. 7H). The paraflagellar rod was not stained. These data are consistent with the findings obtained with fluorescence microscopy (Fig. 4A).

Discussion

To characterize γ -tubulin and its distribution in *L. tropica*, we made use of a panel of anti-peptide antibodies recognizing epitopes in different regions of the molecule. Monoclonal antibodies TU-30, TU-31, TU-32 are directed against the C-terminal region of γ -tubulin, while the monoclonal antibody GTU-88 is directed against the N-terminal region. Both polyclonal antibodies (γ -TUB and No. 9) recognize the N-terminal region. As anti-peptide antibodies were used in the study, one can expect that they will recognize linear epitopes. These can be mapped to small linear peptide sequences of 5–20 amino acids [41]. A comparison of the γ -tubulin sequences in *H. sapiens*, *A. nidulans*, and *L. major* revealed substantial conservation in the N-terminal region of the molecule. On the other hand, there is a lower homology between *H. sapiens* and *L. major* in the C-terminal region of the molecule. It is known, however, that linear epitopes can involve closely spaced but noncontiguous amino acids [41]. Because antibodies directed against the C-terminal and the N-terminal regions of γ -tubulin reacted alike on immunoblots with the 53-kDa protein, and the staining patterns in immunofluorescence were generally very similar for both groups of antibodies, we believe that monoclonal antibodies directed against the C-terminal region of human γ -tubulin can detect γ -tubulin in *L. tropica*. γ -Tubulin was found only in insoluble cytoskeletal fraction of cells extracted in 1% NP-40 at

4°C. On the other hand, tubulin $\alpha\beta$ -dimers were found in both soluble and cytoskeletal pools. After extraction in 2% or 0.5% Triton X-100 at 28°C or 4°C, γ -tubulin was again detectable only in the insoluble fraction (Libusová, unpublished). Soluble forms of γ -tubulin are therefore either absent in *L. tropica* or their quantities are below the detection limit of the method used. This contrasts with the situation in animal cells, where up to 80% of γ -tubulin participates in large (γ TuRCs; 2 MDa) and small (γ TuSCs; 240 kDa) soluble complexes [14,42]. Soluble γ -tubulin-containing complexes were also described in plants [43,44], fungi [45], and *Dictyostelium discoideum* [46]. In animal cells, γ TuSCs comprise two molecules of γ -tubulin and one molecule each of GCP2 and GCP3 (γ -tubulin complex protein), which are homologues of *Saccharomyces cerevisiae* proteins Spc97p and Spc98p [47]. An open question still is whether insoluble γ -tubulin in *L. tropica* also exists in the form of such complexes. Up to now γ -tubulin interaction partners from the Spc family were not described in kinetoplastida.

γ -Tubulin in *L. tropica* shows a complex localization pattern as determined by immunofluorescence. In interphase cells, it was located in the basal body region and the posterior pole of the cell. A weak punctuate staining was observed over the whole cell body, and some antibodies also decorated the flagellum. In dividing cells, both old and new basal bodies were decorated, and so were the flagella. There was a very intensive staining at posterior poles. Punctuate labelling of the subpellicular corset was also more intensive. Intensive flagellar staining was seen with both the monoclonal TU-32 antibody against the C-terminal region of γ -tubulin and the polyclonal antibodies against the N-terminal region (γ -TUB, No. 9). Other antibodies directed against the C-terminal region (TU-30, TU-31) also decorated the flagellum, but the signal was far less obvious. Monoclonal

antibody GTU-88, directed against the N-terminal region of γ -tubulin, failed to stain flagellum under the used fixation conditions. Differential staining intensity of the flagellum with diverse antibodies cannot be attributed to an unspecific attachment of antibodies because the negative control monoclonal antibodies of IgG and IgM class, as well as the polyclonal antibody, did not provide any staining of these structures. The entire staining pattern was also preserved after the high salt treatment. Moreover, preabsorption of TU-32 with the peptide used for immunization abolished the staining. On the other hand, evidence exists that antibodies TU-30, TU-31, and TU-32 do not recognize identical epitopes (Dráber, unpublished). Differential staining of flagella could thus reflect the masking of corresponding epitopes by associated proteins, by potential posttranslational modification(s) of the γ -tubulin molecule, or by conformational changes occurring during fixation. The staining pattern in *L. tropica* partially resembled that seen in closely related *T. brucei*. In this case, γ -tubulin was immunodetected in the basal body, on the tip of the cell body, and along the flagellum. γ -Tubulin was also discernible in the form of discrete dots within the nucleus in regions corresponding to the poles of intranuclear spindle. A weak punctuate staining was found over the cell body [9]. It was suggested that staining of the cell body could reflect the presence of γ -tubulin on subpellicular corset microtubules, as new microtubules of the corset are intercalated into the lattice of old microtubules without any obvious MTOC [48].

The localization of γ -tubulin to the basal body is in agreement with previous reports on γ -tubulin presence in the basal body of *Tetrahymena thermophila* [49], *T. brucei* [9], *Chlamydomonas reinhardtii* [50], or *Giardia intestinalis* [51]. In the basal body region of *L. tropica*, γ -tubulin colocalized with the 210-kDa microtubule interacting protein and the 57-kDa vimentin-like protein that had previously been localized in this region [8]. On the other hand, γ -tubulin did not colocalize with the pericentrin-related protein(s) immunodetected with the anti-pericentrin antibody at the anterior pole of the cell in the flagellar pocket area. A special “desmosome-like” thickening has been described in the pocket opening, and a quartet of specialized microtubules run along the flagellar pocket [52]. Whether the pericentrin-related protein interacts with these microtubules remains questionable. In animal cells, pericentrin forms soluble complexes with γ -tubulin, and these are located on centrosomes [53]. The pericentrin-related protein was recently described in amoeba *Naegleria gruberi* during de novo formation of the basal body [54]. The role of γ -tubulin in the formation of kinetoplast basal body and flagellum axoneme was investigated by the RNAi approach. γ -Tubulin was found to be essential for the formation of the central doublet of microtubules in new flagellum axoneme. Cells with suppressed γ -tubulin expression carried an old motile flagellum and a new immobile flagellum that lacked both of the central pair of microtubules, but possessed nine outer doublet microtubules [24].

γ -Tubulin colocalized with the 210-kDa microtubule-interacting protein and the 57-kDa vimentin-like protein also at the posterior pole of the cell. The role of γ -tubulin at the posterior pole is not clear, but it could be involved in the anchoring or stabilizing of the subpellicular corset of microtubules. A higher number of microtubules are to be anchored in dividing cell and a correspondingly more concentrated γ -tubulin signal is found on the posterior pole. The tapering posterior tip of the cell is the only position of the corset where many microtubules end. The bright dot of γ -tubulin staining at this position could reflect the presence of γ -tubulin on the ends of microtubules rather than a sign of discrete MTOCs. In *T. brucei*, the (+) ends of microtubules are at the same pole of the cell where the basal body of flagellum is located [55], and discrete γ -tubulin staining was found on the opposite cell pole. It remains an open question if the same holds true for *Leishmania*, as the orientation of microtubules has not yet been determined in these protozoa.

The unexpected finding of γ -tubulin along the flagellum was verified by immunoblotting, immunofluorescence, and immunogold electron microscopy on isolated flagella. Immunoelectron microscopy revealed that γ -tubulin was not present on axonemal microtubules but on the paraflagellar rod (PFR). Staining along the whole length of the flagellum in *T. brucei* was the result of decoration of four microtubules underlining the flagellar attachment zone of PFR [9]. However, the flagellar attachment zone is absent in *L. tropica*, and four specialized microtubules are found only in the flagellar pocket region. The filamentous structure of PFR lies alongside the whole length of axoneme under the membrane and consists of three distinct regions—a proximal, intermediate, and distal zone relative to the axoneme [56]. The proximal zone is attached to axonemal microtubules. Detailed localization of γ -tubulin to the zones was not possible because the method of isolation of flagella partially impaired the structure of PFR. Two major protein components, PFR-1 (74 kDa) and PFR-2 (69 kDa), were described in PFR of *Leishmania mexicana* [57]. Secondary structural prediction suggested high alpha-helical content and the potential of these helices to form coiled-coil motifs in all members of the PFR1/PFR2 family [58]. It was reported that noncentrosomal γ -tubulin colocalized with intermediate filaments in CACO-2 epithelial cells, and cytokeratins specifically co-immunoprecipitated with γ -tubulin [59]. It is possible that γ -tubulin interacts with some proteins of the filamentous PFR structure. Alternatively, γ -tubulin could be associated with rafts of proteins that become attached to PFR building components during intraflagellar transport [60]. γ -Tubulin was found in association with membranes both in animal [18] and in plant cells [19].

Two-dimensional electrophoresis revealed the presence of at least three distinguishable isoelectric variants of γ -tubulin in flagella, with isoelectric points more basic when compared to α -tubulin. The pI of the most acidic variant was 6.0, which is close to the pI calculated for the amino acid

sequence of *L. major* γ -tubulin (6.39). Although the leishmanial genome has been almost fully sequenced, only a single γ -tubulin gene per haploid genome was found up to now in databases. This is in contrast to multiple α - and β -tubulin genes present in trypanosomatida [61]. Thus, the number of detected γ -tubulin isoforms exceeds the number of γ -tubulin genes and suggests the presence of posttranslational modification. Cell cycle-dependent phosphorylation of γ -tubulin on tyrosine was reported in yeast [20]. γ -Tubulin was found in association with serine–threonine polo-like kinase [62] and with protein tyrosine kinase p53/p56Lyn [63]. It is possible that phosphorylation or other posttranslational modification of *L. tropica* γ -tubulin could play an important role in the regulation of microtubule nucleation or its interaction with other proteins.

In conclusion, we demonstrate a unique subcellular localization of γ -tubulin in *Leishmania*. γ -Tubulin is localized to four distinct structures: basal body, PFR of the flagellum, posterior end of the cell body, and discrete dots in the subpellicular microtubular array. The cell cycle-dependent distribution of γ -tubulin in the posterior end of the cell could imply its important role in microtubule anchorage during interphase and mitosis. γ -Tubulin in *L. tropica* is posttranslationally modified, and these modifications could have a significant role in the regulation of microtubule nucleation or interaction with other proteins.

Acknowledgments

We thank Dr. E. Nohýnková (First Faculty of Medicine, Charles University, Prague, Czech Republic) for providing the cell line, Dr. T.H. MacRae (Dalhousie University, Halifax, Canada) for the generous gift of anti- γ -tubulin antibody, Dr. S.J. Doxsey (University of Massachusetts, Medical Center, Worcester, MA, USA) for the generous gift of anti-pericentrin antibody, and Dr. B.R. Oakley (The Ohio State University, Columbus, OH, USA) for a full-length human γ -tubulin cDNA used for expression of 6 \times His-tagged γ -tubulin. This work was supported in part by grants from the Ministry of Education of the Czech Republic (ME310 and LN00A026).

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