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Cell Cycle-Dependent Changes in Localization of a 210-kDa Microtubule-Interacting Protein in *Leishmania*

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Using the monoclonal antibody MA-01, a new 210-kDa microtubule-interacting protein was identified in *Leishmania* promastigotes by immunoblotting and by immunoprecipitation. The protein was thermostable and was located on microtubules prepared by taxol-driven polymerization *in vitro*. On fixed cells the antibody gave specific staining of flagellum, flagellar pocket, and mitotic spindle. Subpellicular microtubules were basically not decorated but posterior poles of the cells were labeled in a cell-cycle-dependent manner. In anterior and posterior poles of cells the 210-kDa protein codistributed with the 57-kDa protein, immunodetected with anti-vimentin antibody, that was located only on cell poles. Immunolocalization of the 57-kDa protein was most prominent in dividing cells. The presented data suggest that the 210-kDa protein is a newly identified microtubule-interacting protein of *Leishmania* that could be involved in anchoring the microtubules in posterior poles of these cells. The striking codistribution of the microtubule-interacting protein and the 57-kDa protein in protozoa is described for the first time.

Key Words: **antibodies; cell cycle; *Leishmania;* microtubule-interacting protein.**

INTRODUCTION

Protozoan parasites of the genus *Leishmania* cause a variety of diseases in humans. Leishmaniasis comprises a variety of syndromes from asymptomatic infections to those with a very high mortality. It may be confined to skin lesions or it could cause the severe visceral disease "kala-azar," which is very often fatal. In recent years *Leishmania*/HIV co-infection has emerged as an extremely serious new disease. The life cycle of *Leishmania* includes two distinct stages. Promastigotes are slender actively motile flagellated cells that live and multiply extracellularly in the lumen of the sand fly's gut. The second stage is round-shaped immobile intracellular amastigotes that inhabit parasitophorous vacuoles within the mammalian host's macrophages [1]. *L. tropica* belongs to *Leishmania* species of the Old World and causes mainly cutaneous leishmaniasis. The cutaneous forms are most common and represent more than 50% of all new cases of leishmaniasis with about 1.5 million new cases reported annually worldwide [2].

Because millions of people live under the risk of *Leishmania*-caused diseases, safe and effective chemotherapeutic agents are needed. Since tubulin is the most abundant protein in *Leishmania*, attention has also focused on widely used anti-microtubule drugs. However, colchicine-site agents show little or no effect on *Leishmania* tubulin [3], and vinca alkaloids (vincristine and vinblastine) have only a temporary, reversible effect on kinetoplastid protozoa [4]. Taxol seems to be more promising, but a taxol-resistant mutant has already been isolated [5]. A better understanding of microtubules of *Leishmania* and identification of new microtubule proteins could thus lead to the development of new field-applicable methods of diagnosis and more effective treatment with specific drugs.

Microtubules form various arrangements in *Leishmania*. Subpellicular parallel arrays of microtubules are present just under the plasma membrane. These microtubules are connected to each other as well as to the plasma membrane [6]. Promastigotes carry a single flagellum that protrudes from the cell through a flagellar pocket. A subpellicular microtubular coat is not present in the vicinity of the flagellar pocket. The basal body of the flagellum lies within the cytoplasm next to the kinetoplast, an electron-dense material containing all mitochondrial DNA. Near the basal body, four

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unique microtubules of the subpellicular corset originate. They remain attached to the basal body region even after high-salt treatment [7]. During division, the basal body of the flagellum divides first and a new flagellum is generated from the daughter basal body. Meanwhile the kinetoplast and subsequently the nucleus divide. The process of cytokinesis starts from the anterior (flagellar) end of the cell [1].

Although tubulin polymorphism is well documented in *Leishmania* [8, 9], our knowledge about microtubule-associated proteins (MAPs) in *Leishmania* is limited. Up to now only the 230-kDa kinesin-related antigen had been identified in *L. chagasi*, but its subcellular distribution is unknown [10]. On the other hand, in the closely related kinetoplastid parasites *Trypanosomes* a number of proteins associated with tubulin have been described. For example, a heavily phosphorylated thermolabile 210-kDa protein (WCB210) was identified in *T. brucei* [11]. The WCB210 protein is specifically associated with the subpellicular array of microtubules. This protein could be involved in the regulation of microtubular cross bridges. A family of high-molecular-weight microtubule-associated repetitive proteins (MARPs) associated with subpellicular microtubules was also described in *T. brucei* [12]. One member, MARP-1, binds to microtubules via tubulin domains other than the carboxy-termini used by MAPs from mammalian brain. With the subpellicular array is also associated the 36-kDa repetitive protein I/6 [13]. In contrast, the *T. brucei* Gb4 protein has a restricted location within the subpellicular corset at the posterior end of microtubule array [14].

The presence of an intermediate filament class of proteins or structures in kinetoplastid protozoa is poorly documented. In *L. mexicana* intermediate filament-like proteins have been detected using cross-reacting heterologous antibodies [15].

Here we describe the immunological characterization of a new microtubule-interacting protein and the 57-kDa protein in *L. tropica* and their distribution in interphase and dividing cells. We show for the first time that the 57-kDa protein is uniquely distributed in anterior and posterior cell poles and codistributes with the newly characterized *L. tropica* 210-kDa microtubule-interacting protein in cell-cycle-dependent manner.

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). Dr. C. L. Eisenberger (Hebrew University, Jerusalem, Israel) did the species identification using a permissively primed intergenic PCR [16]. 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 amykacin (200 μg/ml). For immunofluorescence and preparation of cell lysates, cells from the logarithmic phase of growth were used.

Antibodies. The following monoclonal antibodies were used: The MA-01 antibody (IgG1) was raised against porcine brain microtubule-associated protein MAP2ab, M_r ~280 kDa, and recognizes the 210-kDa protein in different nonneural cells of various species [17, 18]. The antibody MA-02 (IgG1) is directed against a different epitope of the same protein (E. Dráberová, unpublished). The antibodies TU-01 (IgG1) and TU-06 (IgM) are directed against conservative epitopes in N-terminal structural domains of α-tubulin or β-tubulin, respectively [19]. The antibody TU-16 (IgM) is directed against α-tubulin and is suitable for immunoprecipitation experiments [20]. Mouse monoclonal antibody VI-01 (IgM) is directed against the antigenic determinant on vimentin and smooth muscle desmin [21], and VI-10 (IgM) is directed against vimentin [22]. In double-label immunofluorescence tests the microtubule structures were detected by a rabbit affinity-purified polyclonal antibody against $\alpha\beta$-tubulin dimer [23]. The MT-02 (IgM) monoclonal antibody against microtubule-associated protein MAP-2ab [24] and the HTF-14 (IgG1) monoclonal antibody against human transferrin [25] were used as negative controls. Antibodies of IgG class were purified from ascitic fluid by DEAE-chromatography following ammonium sulfate precipitation. The VI-01 antibody was purified from ascitic fluid by polyethylene glycol precipitation followed by hydroxyapatite chromatography. Purified VI-01 was conjugated with biotin using sulfosuccinimidyl-6-(biotinamido)hexanoate (Pierce, Rockford, IL) according to the manufacturer's instruction. Secondary anti-mouse antibodies conjugated with alkaline phosphatase or horseradish peroxidase were purchased from Promega (Madison, WI). Indocarbocyanin 3 (Cy3)-conjugated anti-mouse Ig antibody and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit Ig were from Jackson Immunoresearch Laboratories (West Grove, PA).

Preparation of cell extracts. To prepare the whole-cell extract, cells were centrifuged, washed twice in phosphate-buffered saline (PBS), solubilized in SDS sample buffer [26], and boiled for 5 min. For preparation of soluble and insoluble fractions, washed cells were extracted with 1 volume of 0.5% Triton X-100 (v/v) in microtubule-stabilizing buffer (MSB; 20 mM Mes, pH 6.9, 2 mM EGTA, 2 mM $MgCl_2$) supplemented with protease and phosphatase inhibitors (1 μg/ml each of leupeptin, aprotinin, pepstatin, antipain, 1 mM NaF, and 1 mM Na_3VO_4). After 5 min of mild shaking at 28°C, the samples were centrifuged at 20,000*g* for 15 min at 4°C.

For preparation of high-speed supernatants, washed cells were resuspended in MSB supplemented with inhibitors. Suspended cells were disrupted by sonication at 4°C 3 $\times$ 20 s (amplitude 20) with a 500-W ultrasonic homogenizer (Cole–Parmer, Vernon Hills, IL) equipped with a microtip probe. The homogenate was centrifuged at 20,000*g* for 15 min at 4°C, the supernatant was collected and centrifuged again at 40,000*g* for 15 min at 4°C. High-speed supernatant was obtained by further centrifugation at 200,000*g* for 40 min at 4°C.

To prepare a heat-stable fraction, NaCl and 2-mercaptoethanol were added to 0.7 ml of the high-speed extract to a final concentration of 0.8 M and 1% (v/v), respectively, and the extract was boiled for 3 min. After chilling on ice, the heat-treated extract was centrifuged at 20,000*g* for 30 min at 4°C to remove denatured proteins. The supernatant was transferred to MSB and concentrated to 50 μl using a Vivaspin 500 μl concentrator (Vivascience Limited, Lincoln, UK). After concentration to 50 μl, five-times-concentrated SDS sample buffer was added and the samples were boiled. Centrifuged denatured proteins were dissolved in 50 μl of SDS sample buffer.

Preparation of microtubule proteins. The high-speed extract was polymerized in the presence of 20 μM taxol and 1 mM GTP for 15 min at 37°C and thereafter 15 min at 4°C. Control samples were treated under the same conditions in the absence of taxol and GTP. Microtubules were spun down at 30,000*g* for 30 min at 4°C through a 10% (w/v) sucrose cushion in MSB containing 20 μM taxol and 1 mM GTP [27]. Samples of supernatants were mixed with five-times-concentrated SDS sample buffer. Polymerized microtubules were gently resuspended in cold MSB with 20 μM taxol and 1 mM GTP and spun

down at 30,000*g* for 30 min at 4°C. Two-times-washed microtubules were resuspended in MSB containing 1.2 M NaCl, spun down at 30,000*g* for 20 min, and dissolved in SDS sample buffer.

Immunoprecipitation. Sheep anti-mouse antibody was covalently coupled to CNBr-activated Sepharose 4B according to the manufacturer's directions and the gel was equilibrated in TBST (10 mM Tris, pH 7.4, 150 mM NaCl, 0.05% Tween 20). The 80-μl aliquot of sedimented gel with immobilized anti-mouse antibody was incubated under rocking with 1 ml of primary antibody at a concentration of 0.5 mg/ml in TBST. The beads were pelleted by centrifugation and washed four times in cold TBST. Then the beads were further incubated under rocking for 1 h with 1 ml of high-speed extract diluted 1:1 with TBST. The pelleted beads were washed four times and thereafter boiled for 5 min in 100 μl of SDS sample buffer. The HTF-14 monoclonal antibody was used as negative control.

Gel electrophoresis and immunoblotting. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the method of Laemmli [26]. Separated proteins were transferred onto nitrocellulose filters with 0.4-μm pores (Schleicher & Schuell, Dassel, Germany) by electroblotting [28] for 80 min in the presence of 0.1% SDS in blotting buffer. Details of the immunostaining procedure are described elsewhere [29]. Primary antibodies, in the form of ascitic fluid, were diluted in the range from 1:500 to 1:5000, whereas supernatants were used undiluted; secondary anti-mouse antibody labeled with alkaline phosphatase was diluted 1:7500. Anti-mouse antibody conjugated with horseradish peroxidase was diluted 1:10,000. SuperSignal WestPico chemiluminescent reagents (Pierce) were used according to the manufacturer's recommendation to detect horseradish peroxidase. Autoradiography films X-Omat K were purchased from Eastman Kodak (Rochester, NY).

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 0.5% Triton X-100 in MSB supplemented with 4% PEG 6000 for 5 min, washed in MSB, and fixed with 3% formaldehyde for 30 min. The immunofluorescence staining was then performed as described [30]. For double-label immunofluorescence staining with polyclonal anti-tubulin and MA-01 or VI-01 antibodies, the fixed cells were first incubated with a mixture of the rabbit anti-tubulin antibody (dilution 1:5) and the corresponding mouse monoclonal antibody for 45 min. Purified MA-01 antibody was used at a concentration of 20 μg/ml or as undiluted supernatant, and VI-01 was used as undiluted supernatant. The slides were then washed three times in PBS and incubated with a mixture of secondary antibodies. The anti-mouse antibody conjugated with FITC was diluted 1:200 and the anti-rabbit antibody conjugated with Cy3 was diluted 1:1000. For double-label immunofluorescence with MA-01 and VI-01 antibody, the slides were incubated with MA-01 antibody, washed, and stained with anti-mouse antibody conjugated with FITC. The remaining binding sites of the secondary antibody were blocked with normal mouse serum, diluted 1:5, prior to incubation with biotin-conjugated VI-01 antibody (dilution 1:50). After three washes, slides were incubated with Cy3-conjugated streptavidin (dilution 1:1000) from Jackson Immunoresearch Laboratories. Coverslips were mounted in Mowiol 4.88 (Calbiochem AG, Lucerne, Switzerland) containing 2 μg/ml 4,6-diamidino-2-phenylindole and 6.25% (w/v) propyl gallate (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. Neither the control antibodies of IgG or IgM classes nor the conjugates alone gave any detectable staining.

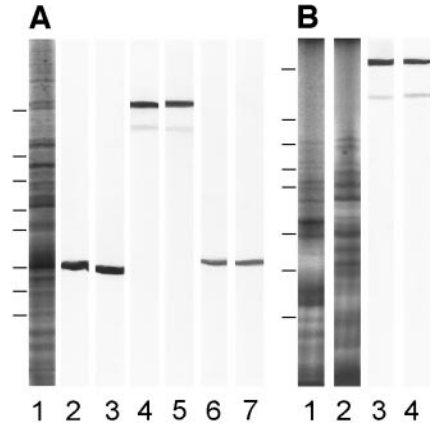

FIG. 1. Immunoblot of *L. tropica* cell extracts with monoclonal antibodies against cytoskeletal proteins. (A) Whole-cell lysate. Lane 1, Coomassie blue staining; lanes 2–7, immunostaining with antibodies TU-01, TU-06, MA-01, MA-02, VI-01, and VI-10. 5–15% SDS–PAGE. (B) Insoluble (lanes 1, 3) and soluble (lanes 2, 4) fractions of cells treated for 5 min at 28°C in 0.5% Triton X-100. Lanes 1 and 2, Coomassie blue staining; lanes 3 and 4, immunostaining with antibody MA-01. 7.5% SDS–PAGE. Bars on the left margins indicate positions, from top to bottom, of molecular mass markers in kDa (205, 116, 97.4, 84, 66, 55, 45, and 36).

RESULTS

Antibody Characterization

Monoclonal antibodies were characterized by immunoblotting on whole-cell lysates of *L. tropica*. The antibodies TU-01 and TU-06 directed, respectively, against α- and β-tubulin reacted specifically with proteins of relative electrophoretic mobilities around 55 kDa. The TU-01 antibody stained a band with slightly lower mobility than that recognized by the TU-06 antibody (Fig. 1A, lanes 2 and 3). No cross-reactivity with other proteins was observed. The antibodies MA-01 and MA-02 reacted strongly with proteins of relative electrophoretic mobility approximating 210 kDa. Both antibodies also showed weaker staining of proteins with relative molecular weight around 160 kDa, which could imply proteolytic degradation during the process of sample preparation. No reactivity was detected in positions corresponding to brain high-molecular-mass MAP1 and MAP2 proteins or tubulin (Fig. 1A, lanes 4 and 5). The anti-vimentin antibodies VI-01 and VI-10 stained only a single 57-kDa band with slightly lower mobility compared with the immunoreactive proteins recognized by anti-tubulin antibodies (Fig. 1A, lanes 6 and 7). The same results were obtained with monoclonal antibodies used in the form of diluted ascitic fluids and spent culture supernatants. No reactivities with blotted proteins were detected when negative control IgG-and IgM-class monoclonal antibodies or secondary antibodies alone were applied. Extraction of cells in 0.5% Triton X-100 in microtubule-stabilizing buffer at 28°C showed that MA-01 antigen was present in insoluble (cytoskeletal) as well as in soluble fractions (Fig. 1B).

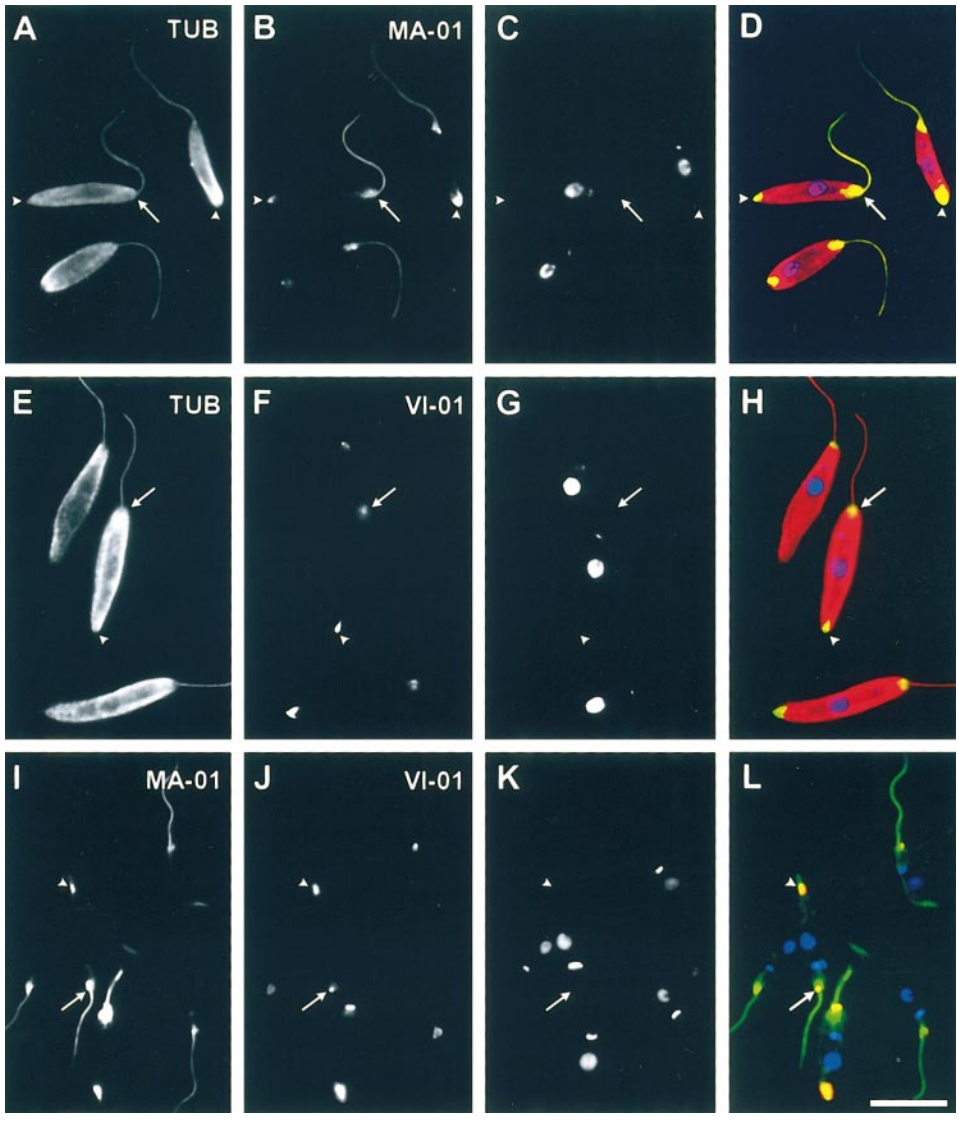

FIG. 2. Fluorescence triple-label staining of *L. tropica* interphase cells. Cells were stained with polyclonal anti-tubulin antibody TUB (A, E, red), MA-01 antibody (B, I, green), anti-vimentin antibody VI-01 (F, green; J, red), and DNA-binding dye (C, G, K, blue). Color pictures (D, H, L) show superpositions of staining in each row. Arrows denote anterior poles and arrowheads denote posterior poles in cells. Bar, 5 μm.

Localization of MA-01 Antigen and 57-kDa Protein in Interphase Cells

Triple-label fluorescence staining of fixed interphase cells of *L. tropica* was used to estimate the subcellular distribution of MA-01 antigen and the 57-kDa protein (Fig. 2). For a better localization of cell structures, DNA in the nucleus and in kinetoplast was stained by DNA-binding dye (Figs. 2C, 2G, and 2K). Polyclonal anti-tubulin antibody gave specific staining of flagellum and subpellicular array of microtubules (Figs. 2A and 2E). Similar staining was obtained with monoclonal antibodies TU-01 and TU-06 directed against α-tubulin or β-tubulin, respectively (not shown). The MA-01 antibody stained the flagellum in all cells and the anterior pole of cells in the basal body region, where strong immunofluorescence surrounded the flagellar pocket. Subpellicular microtubules were basically not decorated and only the posterior pole of the cells was labeled. The intensity of labeling, however, varied and in some cells this part of the cell body was not stained at all (Figs. 2B and 2I). The MA-02 antibody gave the same staining pattern (not shown). The anti-vimentin antibody VI-01 did not stain the flagellum and subpellicular region, but stained in all cells the anterior poles of the cells (Figs. 2F and 2J). The labeling of posterior poles varied in intensity and in some cells they were not decorated, similar to the case of MA-01 antibody staining. When the other anti-vi-

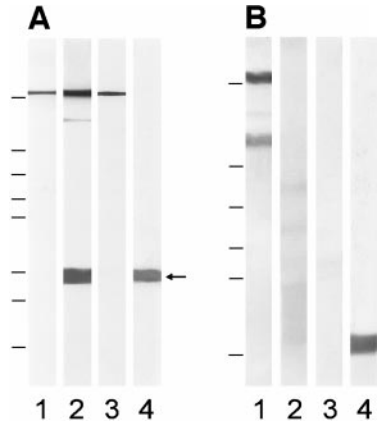

FIG. 3. Characterization of MA-01 antigen by immunoprecipitation and by immunoblotting of thermostable proteins. (A) Immunoblot of *L. tropica* cells immunoprecipitated by MA-01 antibody. Cell extracts were precipitated with MA-01 antibody (lanes 1 and 2) or with control HTF-14 antibody (lanes 3 and 4). Proteins remaining after precipitation (lanes 1 and 3) and immunoprecipitated proteins (lanes 2 and 4) were separated on 7.5% SDS–PAGE and stained with MA-01 antibody and secondary antibody labeled with alkaline phosphatase. Arrow indicates position of heavy chains of immunoglobulins stained with secondary anti-mouse antibody. Bars on the left margin indicate positions, from top to bottom, of molecular mass markers in kDa (205, 116, 97.4, 84, 66, 55, 45, and 36). (B) Immunoblot of *L. tropica* thermostable proteins. Concentrated thermostable proteins (lanes 1 and 3) and pelleted thermolabile proteins (lanes 2 and 4) were separated on 6% SDS–PAGE. Lanes 1 and 2, immunostaining with MA-01 antibody; lanes 3 and 4, immunostaining with anti-tubulin antibody. Secondary antibodies were labeled with peroxidase. Bars on the left margin indicate positions, from top to bottom, of molecular mass markers in kDa (205, 116, 97.4, 84, 66, and 55).

mentin antibody (VI-10) was used in immunofluorescence assay, no specific staining was observed at the antibody concentrations tested. Direct comparison of the distributions of the MA-01 antigen and 57-kDa protein detected by VI-01 antibody clearly demonstrated that the proteins codistributed in anterior and posterior poles of the cell body. However, MA-01 decorated a wider region of flagellar pocket in the anterior pole of the cell, while VI-01 staining was restricted to the basal body region (Figs. 2I–2L). The same staining patterns were observed when monoclonal antibodies were used in the form of spent culture supernatants.

Characterization of MA-01 Antigen

To identify the protein(s) recognized in *L. tropica* by antibody MA-01, cell extracts were prepared and used for immunoprecipitation, thermostability experiments, and *in vitro* preparation of microtubule proteins. The 210-, 160-, and sometimes 130-kDa proteins could be specifically precipitated from the high-speed fraction of sonicated cells. The amount of precipitated 210-kDa protein, however, substantially exceeded those of the 160- (Fig. 3A, lanes 1 and 2) or 130-kDa proteins (not shown). When a control antibody was used, no precipitation of MA-01 antigen was detected (Fig. 3A, lanes 3 and 4). The prominent labeling of a band around 55 kDa (arrow in Fig. 3A) is caused by heavy chains of immunoglobulins stained with the secondary anti-mouse antibody. When the precipitated material was probed with anti-vimentin antibody VI-01 or anti-tubulin antibody, no reactivity was observed. Similarly, when anti-tubulin antibody TU-16 was used for precipitation and precipitated material was probed with MA-01 antibody, no staining was observed. The TU-16 antibody, however, did precipitate the tubulin as confirmed by staining with polyclonal anti-tubulin antibody (not shown). These experiments indicated that the MA-01 antigen in prepared extracts was not in soluble complex with tubulin or 57-kDa protein.

The behavior of the MA-01 antigen at high temperature was also examined, as it is known that the corresponding protein in nonneuronal mammalian cells is thermolabile. Surprisingly, in *L. tropica* the 210-kDa protein and lower molecular weight proteins 160 and 130 kDa were detected in the concentrated heat-stable fraction and no staining was seen in the heat-labile fraction, even when a sensitive detection method was applied, using anti-mouse antibody conjugated with horseradish peroxidase and chemiluminescence development. However, as expected, control anti-tubulin antibody labeled the 55-kDa band in the thermolabile fraction and no labeling was observed in the heat-stable fraction (Fig. 3B). When the microtubule proteins were prepared by taxol-driven polymerization, the 210-kDa protein, as well as the 160- and 130-kDa proteins, was found on microtubules washed twice in isolation buffer and subsequently in buffer containing 1.2 M NaCl. The immunoreactive proteins, however, were not substantially enriched (Fig. 4, lanes 1 and 2). In samples prepared by procedures from which taxol and GTP were omitted, the immunoreactive proteins were found only in supernatants (Fig. 4, lanes 3 and 4), indicating that the observed binding was not just the result of sedimentation of clustered protein material. If the same samples were probed with anti-tubulin antibody, tubulin was found in greater quantity in isolated microtubule proteins (Fig. 4, lanes 5–8). Probing of microtubule proteins with VI-01 antibody did not reveal copolymerization of 57-kDa protein with microtubules.

Localization of MA-01 Antigen and 57-kDa Protein in Dividing Cells

Since the MA-01 antigen and 57-kDa protein codistributed in the anterior and posterior poles of cells, but the labeling intensity of the posterior poles differed among individual interphase cells, we examined in more detail their immunolocalization in dividing cells.

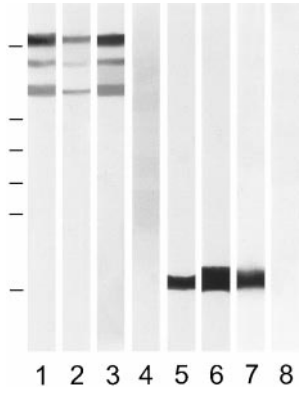

FIG. 4. Immunoblot of *L. tropica* microtubule proteins prepared by taxol-driven polymerization. Cell extracts were polymerized in the presence of 20 μM taxol and 1 mM GTP (lanes 1 and 2, 5 and 6) or in the absence of taxol and GTP (lanes 3 and 4, 7 and 8). After centrifugation through sucrose cushion, supernatants (lanes 1, 3, 5, 7) and pelleted material (lanes 2, 4, 6, 8) were separated on 6% SDS–PAGE. Lanes 1–4, immunostaining with MA-01 antibody; lanes 5–8, immunostaining with anti-tubulin antibody. Secondary antibodies were labeled with peroxidase. Bars on the left margin indicate positions, from top to bottom, of molecular mass markers in kDa (205, 116, 97.4, 84, 66, and 55).

Anti-tubulin antibody decorated not only the flagellum and subpellicular array of microtubules, but also the mitotic spindle (arrowhead in Fig. 5A). In cells the MA-01 antibody labeled strongly both poles and flagellum as well as mitotic spindle (arrowhead in Fig. 5B). In the anterior pole it decorated the whole area surrounding the flagellar pocket. The anti-vimentin antibody strongly decorated cells only at both poles (Figs. 5F and 5J). In contrast with interphase cells, both antibodies reacted with posterior poles in all dividing cells. No cells were detected showing mitotic spindle and lacking immunolabeling of posterior poles. A direct comparison of the distribution of MA-01 antigen and 57-kDa protein clearly demonstrated that the corresponding proteins codistributed in anterior and posterior poles of the cell body. However, like in the anterior pole of interphase cells, the MA-01 decorated a wider area, whereas the VI-01 staining was restricted to the basal body region (Figs. 2I–2L). The same staining patterns were observed when monoclonal antibodies were used in the form of spent culture supernatants.

DISCUSSION

Previously it has been shown that MA-01 antibody recognizes an epitope on MAP2ab proteins [17, 31]. The epitope was not detected on the other structural microtubule-associated proteins MAP2c, MAP2d, MAP4, and tau (E. Dráberová, unpublished results) that share conserved microtubule-binding repeats [32]. On immunoblots of various types of nonneuronal cells this antibody reacted with a 210-kDa protein [17, 18, 33]. A protein of the same relative mobility was detected also by immunoblotting on whole lysates of *L. tropica* and *L. major* (not shown) and by immunoprecipitation of high-speed extracts of *L. tropica*. Neither the MA-01 and MA-02 antibodies nor the other monoclonal antibodies prepared against different epitopes on porcine MAP2ab [24, 34] reacted with proteins in the 280–300 kDa region on *L. tropica* immunoblots. These data suggest that MAP2 and 210-kDa protein detectable by MA-01 and MA-02 antibodies share a common epitope. However, in contrast to the immunoreactive proteins in nonneuronal mammalian cell lines tested so far, the 210-kDa protein in *L. tropica* is thermostable. Two 320-kDa thermostable proteins, MARP-1 and MARP-2, were identified in *T. brucei*. They are characterized by highly conserved 38-amino-acid repeat units and by a distinct proteolytic pattern detected after electrophoretic separation. They were located on subpellicular arrays of microtubules but not in axoneme [12, 35]. Due to the different subcellular location and the observed relative molecular weight, we think it unlikely that the MA-01 antigen belongs to the MARP family of proteins. Moreover, we never observed on SDS–PAGE the typical proteolytic pattern when protease inhibitors proteases were not applied (not shown). The MA-01 antigen also differs from the 210-kDa protein WCB210 identified in *T. brucei*. The WCB210 is a thermolabile, heavily phosphorylated protein that is located on subpellicular microtubules and absent in flagellum [10]. To the group of high-molecular-weight proteins belongs also the 2G4 antigen (700 to 2500 kDa), which is confined to microtubules of the flagellar attachment zone in *T. cruzi* [36]. The MA-01 antigen has a different subcellular distribution and does not thus resemble the 2G4 protein(s). The MA-01 antigen is present both in cytoskeletal and in soluble form and associates with tubulin upon taxol-induced microtubule formation. However, we did not observe its accumulation in microtubule proteins prepared *in vitro*. Similarly, the MA-01 antigen was not enriched in microtubule proteins prepared from 3T3 cells [30]. Immunofluorescence labeling of interphase and dividing cells indicates that it could as well be associated with microtubules *in vivo*. It was detected in the flagella, basal body regions, and posterior poles of the cells containing subpellicular microtubules and on mitotic structures of *L. tropica* (Figs. 2 and 4) and *L. major* (not shown). Combined data indicate that the MA-01 antigen might possibly represent a new microtubule-interacting protein of *Leishmania*.

When whole-cell lysates were probed with monoclonal anti-vimentin antibodies VI-01 and VI-10, a specific staining of the 57-kDa protein was observed. But in immunofluorescence only the VI-01 antibody gave specific reaction. As the antibodies recognize different epitopes on the vimentin molecule, the reactivity of the

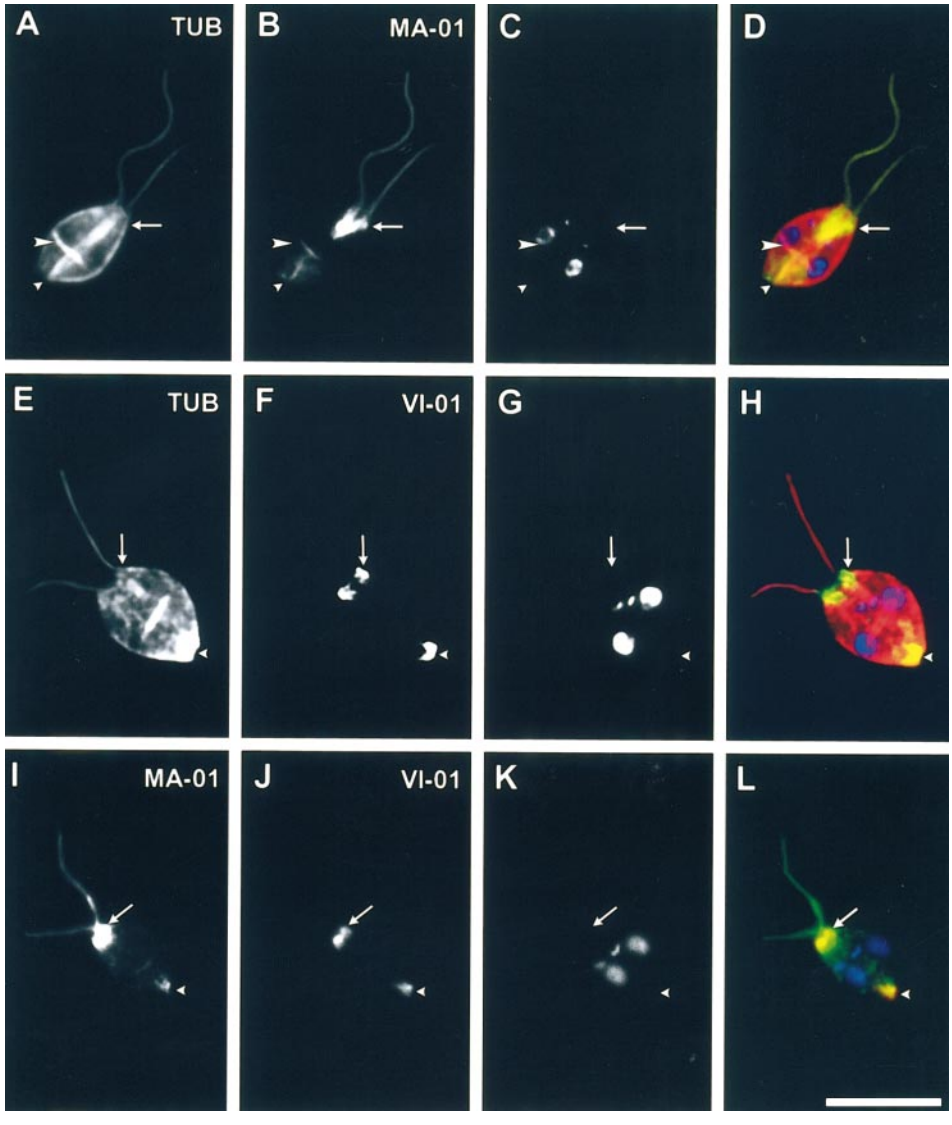

FIG. 5. Fluorescence triple-label staining of *L. tropica* mitotic cells. Cells were stained with polyclonal anti-tubulin antibody TUB (A, E, red), MA-01 antibody (B, I, green), anti-vimentin antibody VI-01 (F, green; J, red), and DNA-binding dye (C, G, K, blue). Color pictures (D, H, L) show superpositions of staining in each row. Arrows denote anterior poles and arrowheads denote posterior poles in cells. Bigger arrowheads in A–D show position of mitotic structure. Bar, 5 μm.

VI-10 antibody could be the result of specific masking of the corresponding region in fixed cells. Intermediate filament-like proteins were already identified in *L. mexicana* with polyclonal antibodies against desmin and vimentin. In immunoblots the anti-desmin antibody reacted with the 66- and 60-kDa proteins and anti-vimentin antibody with the 60-kDa protein. The anti-desmin antibody stained the basal body region, the flagella, and, diffusely, the cell body. The anti-vimentin antibody decorated the basal body region and nucleus [15]. Similarly, VI-01 antibody stained the basal body region, but no staining of the nucleus or flagella was seen. On the other hand, the antibody stained the posterior region of the cell. Although intermediate filaments were not identified in protozoa [37, 38], fibrils of approximately 11 nm, linking the kinetoplast to basal bodies, were found in *T. cruzi* [39]. It was suggested that those fibrils could be intermediate filament-related structures [15]. The common structural principle of all intermediate filament proteins is a central α-helical rod domain of coiled-coil-forming ability, which is flanked by a hypervariable head-and-tail domain. The rod is divided into subdomains which are connected by nonhelical linkers. Type III intermediate filament proteins exhibit a highly conserved amino acid motif at the C-terminal end of their central α-helical rod domain [40], as well as in the head domain [41]. However, such domain structure and consensus

motifs were not found in *Leishmania* proteins so far deposited in databases. As the epitope recognized by the VI-01 antibody is present on vimentin as well as on desmin [21], one cannot exclude that a corresponding epitope is located in the coiled-coil region of type III intermediate filament proteins. The antibody could then react in *Leishmania* with a similarly structured protein that is not homologous to type III intermediate filament proteins.

What the function of MA-01 antigen and 57-kDa protein in the posterior poles of *Leishmania* could be is unclear. Interestingly, while in the anterior pole the MA-01 antibody decorated a wider region of the flagellar pocket and the VI-01 antibody stained a smaller basal body region, both antibodies stained approximately the same area in the posterior pole of interphase as well as dividing cells (Figs. 2L and 5L). When interphase cells were examined, the posterior poles were stained by both antibodies with varying intensities and some cells were not stained at all in this region. In contrast, the posterior poles in dividing cells with duplicated kinetoplast and nuclei were decorated in all examined cells. This suggests that the staining of this part of the cell is possibly dependent on the cell cycle phase or that the corresponding epitopes are more exposed in mitotic cells. In *T. brucei*, subpellicular microtubules converge on the posterior pole of the cell body, creating a ring-shaped opening that is delimited by the plus (+) ends of microtubules [42]. A repetitive 28-kDa protein, Gb4, which caps the microtubules at the posterior end microtubules, was described in this protozoon [14]. It was proposed that the Gb4 protein could regulate microtubule elongation and/or it might be involved in mediating the contact of these microtubule ends with the cell membrane. In connection with the striking codistribution of the MA-01 antigen and the 57-kDa protein containing the vimentin epitope in the posterior poles, it is worth mentioning that the MA-01 antigen is directly or indirectly involved in the interaction of microtubules with vimentin-type intermediate filaments of 3T3 cells [30]. It is possible that some kind of similar interaction(s) could be involved in anchoring the *Leishmania* microtubules in the posterior pole of the cell.

In conclusion, the presented data indicate that in *Leishmania* the newly described 210-kDa thermostable microtubule-interacting protein codistributes with the 57-kDa protein in various stages of the cell cycle. The striking codistribution of these two kinds of cytoskeletal protein is described in protozoa for the first time.

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