

A Novel Microtubule-binding Motif Identified in a High Molecular Weight Microtubule-associated Protein from *Trypanosoma brucei*

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Abstract. The major component of the cytoskeleton of the parasitic hemoflagellate *Trypanosoma brucei* is a membrane skeleton which consists of a single layer of tightly spaced microtubules. This array encloses the entire cell body, and it is apposed to, and connected with, the overlying cell membrane. The microtubules of this array contain numerous microtubule-associated proteins. Prominent among those is a family of high molecular weight, repetitive proteins which consist to a large extent of tandemly arranged 38-amino acid repeat units. The binding of one of these proteins, MARP-1, to microtubules has now been characterized in vitro and in vivo. MARP-1 binds to microtubules via tubulin domains other than the COOH-termini used by microtubule-associated proteins from mammalian

brain, e.g., MAP2 or Tau. In vitro binding assays using recombinant protein, as well as transfection of mammalian cell lines, have established that the repetitive 38-amino acid repeat units represent a novel microtubule-binding motif. This motif is very similar in length to those of the mammalian microtubule-associated proteins Tau, MAP2, and MAP-U, but both its sequence and charge are different. The observation that the microtubule-binding motifs both of the neural and the trypanosomal proteins are of similar length may reflect the fact that both mediate binding to the same repetitive surface, the microtubule, while their sequence and charge differences are in agreement with the observation that they interact with different domains of the tubulins.

MICROTUBULES are intrinsically dynamic polymers that are involved in many cellular processes such as intracellular transport, cell motility, and chromosome segregation. All microtubule-based functions, as well as the stability of the microtubule polymers themselves, are mediated and regulated by a host of microtubule-associated proteins (MAPs)¹ (Olmsted, 1986). Two major groups of MAPs have been identified so far. One comprises energy-transducing MAPs, characterized by microtubule-activated ATPase activity, such as kinesin, dynein, and other mechanochemical enzymes involved in cell motility and intracellular transport phenomena (Vallee and Shpetner, 1990). A second major group of MAPs, typified by the brain MAPs Tau and MAP-2, appears to play predominantly structural roles (Wiche et al., 1991).

The microtubule-binding domains of MAP-2 and Tau were recently shown to be highly similar in that they share an 18-amino acid repeat motif which is responsible for the interaction with microtubules (Lewis et al., 1988; Himmler et al., 1989; Lewis et al., 1989). A similar sequence motif was also found to mediate binding to microtubules of a 190-kD bovine adrenal MAP (MAP-U, Aizawa et al., 1989). While all

these proteins apparently share a common microtubule-binding motif, entirely different microtubule-binding domains have been identified in other MAPs, such as a highly repetitive domain in MAPIB (Noble et al., 1989) or other, unrelated domains in the 205K MAP (Irminger-Finger et al., 1990) or kinesin (Yang et al., 1989) in *Drosophila*.

Microtubule-based structures are evolutionarily well-conserved constituents of the cellular architecture of eukaryotes. In *Trypanosoma brucei*, a parasitic protozoon (Vickerman, 1985) which is an evolutionarily old representative of the eukaryotic kingdom (Sogin et al., 1989), microtubules constitute the only cytoskeletal structure of the cell body known to date. Shape and mechanical properties of the cell body are mainly defined by a membrane skeleton which consists of a highly ordered array of microtubules (Hemphill et al., 1991a,b; Seebeck et al., 1990; Sherwin and Gull, 1989a). These microtubules exhibit properties which are very different from those of their mammalian counterparts. First, they are resistant to cold and their stability is not markedly affected by buffer composition during the preparation of cytoskeletons (Schneider et al., 1987; Sherwin and Gull, 1989a,b). Secondly, the microtubules of the membrane skeleton are unaffected by several drugs which cause rapid depolymerization of microtubules in higher eukaryotes (e.g., colchicine). In contrast, several drugs which have low

1. *Abbreviations used in this paper:* MAP, microtubule-associated protein; MARP, microtubule-associated repetitive protein.

activity against mammalian microtubules are potent disruptors of the trypanosomal membrane skeleton (Seebeck and Gehr, 1983). Thirdly, the membrane skeleton is unusual in that it remains intact throughout the cell cycle (Sherwin and Gull, 1989a). In the membrane skeleton, new microtubules do not originate from microtubule-organizing centers as in higher eukaryotes, but they are inserted into the existing array, where their polymerization is most likely nucleated by lateral interactions with MAPs of neighboring microtubules (Sherwin and Gull, 1989b).

EM studies of detergent-extracted trypanosomal cytoskeletons have revealed that microtubules are extensively cross-linked by MAPs (Hemphill et al., 1991a; Souto-Padron et al., 1984). Several recent reports have biochemically characterized MAPs from trypanosomatids (Binson et al., 1991; Schneider et al., 1988a,b). A family of high molecular weight (>300 kD) trypanosomal MAPs has been recently characterized in more detail. Its members (MARPs, for microtubule-associated repetitive proteins) essentially consist of large numbers of tandemly repeated, highly conserved 38-amino acid repeat units, and two family members, MARP-1 and MARP-2 have been identified so far (Affolter et al., in press; Schneider et al., 1988b). Quick freeze-deep etch immuno-gold EM has been used to demonstrate that the MARPs are located specifically on the microtubules of the membrane skeleton and, within this array, exclusively at the membrane-oriented face of the individual microtubules (Hemphill et al., 1991b).

The present study investigates the interaction of purified MARP-1 with microtubules *in vitro* and establishes that it binds to other domains of tubulin than do the brain MAPs Tau or MAP2. Expression of fragments of trypanosomal MARP-1 in mammalian cells demonstrates that its acidic 38-amino acid repeat motif (consensus sequence: EEVATD-MRHVDESHFLTTTTHEAYKPIDPSEYRQKRTVG) (Affolter et al., in press; Schneider et al., 1988b) represents a novel type of microtubule-binding domain.

Materials and Methods

Cell Culture

Procyclic *Trypanosoma brucei* (stock 427) were grown in SDM-79 medium (Brun and Schoenenberger, 1979) at 26°C.

CV1-fibroblasts were grown in Eagles medium supplemented with 5% FCS (Amimed Inc., Muttentz, Switzerland) at 37°C under 5% CO₂.

Purification of MARPs

One liter of trypanosomes, grown to 5×10^6 to 1×10^7 cells/ml in 500-ml batches in smoothly rocking two-liter Erlenmeyer flasks were centrifuged 10 min at 1,000 g at 4°C. The pelleted cells were gently resuspended in 30 ml of MARP buffer (100 mM morpholino propane sulfonic acid, pH 6.9, 50 mM NaCl, 5 mM MgCl₂, 1 mM EGTA) containing the following protease inhibitors: leupeptin, chymostatin, and pepstatin at 5 µg/ml each, and PMSF at 0.2 mM. The suspension was incubated on ice for 10 min, before Triton X-100 was added to a final concentration of 0.5%. After vortexing and a further incubation on ice for 5 min, the suspension was centrifuged at 6,000 g for 5 min in a precooled rotor (model SS34; Sorvall Instruments, Newton, CT) at 4°C. The pellet was resuspended in 30 ml MARP buffer plus protease inhibitors containing 0.1% Triton X-100, and centrifuged again (in the SS34 rotor at 4°C, 6,000 g for 5 min). To the pellet, which consists of cytoskeletons, 12 ml of PBS (137 mM NaCl, 2.6 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.2) containing 1 M NaCl and protease inhibitors were added. After a brief sonication (4 × 5 s in an ice/water bath), the suspension was kept on ice for ~30 min with occasional vortexing, and was then centrifuged at 10,000 g for 20 min at 4°C. The supernatant

contained the solubilized membrane skeletons, while the flagellar skeleton (axoneme and paraflagellar rod) remained insoluble. The supernatant was transferred to a fresh tube and was boiled for 5 min, quenched on ice for 10 min, and after the addition of 0.1 mM PMSF, was centrifuged at 100,000 g for 30 min at 4°C to remove thermoprecipitated proteins. The supernatant, containing the heat-resistant proteins, was then fractionated by slowly adding 1/10 vol of cold, saturated ammonium sulfate. These conditions were found to be optimal to selectively precipitate the MARPs, while most lower molecular weight proteins remained soluble. The precipitate was then sedimented at 10,000 g for 10 min at 4°C in a swing-out rotor (model HB4; Sorvall Instruments). Precipitated proteins were solubilized in 500 µl MARP-buffer containing 6 M urea and 0.1 mM PMSF, and was passed over an FPLC gel filtration column (Superose-12; Pharmacia Fine Chemicals, Piscataway, NJ) in the same buffer. Fractions containing MARPs were pooled, and urea was removed by stepwise dialyzing the protein against 4, 2, 1, 0.5, 0.25 M urea in 100 mM MOPS, 2 mM MgCl₂, 1 mM EGTA, pH 6.9, (MME) for 1 h each, before a final dialysis (three changes, 90 min each) against MME containing 0.1 mM PMSF. All dialysis steps were performed at 4°C. Protein concentrations were measured according to Bradford (1976), and purified MARP was frozen in liquid nitrogen and stored at -70°C.

Preparation of Mammalian Microtubules and *In Vitro* Binding Assays

Pig brain microtubule protein was purified by two cycles of assembly and disassembly, and tubulin was separated from MAPs by phosphocellulose chromatography according to the method of Weingarten et al. (1975). Purified tubulin and MAPs were dialyzed against MME buffer plus 0.1 mM PMSF and stored at -70°C. Heat-stable MAPs were prepared according to the method of Fellous et al. (1977).

Tubulin lacking the COOH terminus (S-tubulin) was prepared by limited proteolysis of purified pig brain tubulin with subtilisin Carlsberg (Sigma Chemical Co., St. Louis, MO) enzyme/substrate ratio 1:50 (wt/wt), for 30 min at 30°C according to the procedure described by Kanazawa and Timasheff (1989).

MAP-saturated microtubules were prepared by incubating two times-cycled microtubule protein (4.1 mg/ml) at 37°C for 20 min in the presence of 10 µM taxol and 1 mM GTP. To assure that all binding sites for pig brain MAPs were occupied under these conditions, 20-µl aliquots of the incubation mixture were supplemented with increasing amounts of MAPs (0, 200, 300, 400, and 500 µg/ml) in 50 µl MME plus 10 µM taxol, 1 mM GTP, and 0.1 mM PMSF, and were then incubated for another 30 min at 25°C. Samples were then centrifuged at 25°C through a sucrose cushion comprised of 10% sucrose in MME, 10 µM taxol, 1 mM GTP, and 0.1 mM PMSF at 30,000 g for 30 min in a TFT 80.4 rotor (Kontron Instruments, Milan, Italy). Pellets were solubilized in 6 M guanidinium chloride, protein concentrations were determined (Bradford, 1976), and each pellet was analyzed by SDS-PAGE.

Cosedimentation of MARPs with microtubules from pig brain: 100 µl of phosphocellulose-purified pig brain tubulin (1 mg/ml) was incubated at 37°C for 25 min in the presence of 10 µM taxol and 1 mM GTP. After sedimentation (see above), the pelleted microtubules were carefully resuspended in 50 µl MME containing taxol, GTP, PMSF, and purified MARPs at a concentration of 20 µg/ml. To eliminate aggregates which might have formed during storage, MARP preparations were always centrifuged at 30,000 g for 30 min before use. The MARPs microtubule-binding assay mixture was incubated at 25°C for 20 min. As a control for the solubility of the purified protein, an aliquot of MARPs was also incubated in the buffer alone, without microtubules. After sedimentation (see above), supernatants and pellets were collected, extracted with methanol and chloroform (Wessel and Flugge, 1984), and processed for SDS-PAGE.

Cosedimentation assays with MAP-saturated microtubules were performed by incubating two times-cycled microtubule protein at 37°C in the presence of 10 µM taxol, 1 mM GTP for 20 min. The resulting polymers were sedimented as above and resuspended in an equal amount of MME plus taxol, GTP, and PMSF. 20-µl aliquots were added to 50 µl MME buffer containing MARPs at 20 µg/ml, incubated at 25°C for 20 min, and were then centrifuged as described above. Supernatants and pellets were processed for SDS-PAGE.

Cosedimentation assays with polymers obtained from S-tubulin were performed by incubating S-tubulin (1 mg/ml) at 37°C in the presence of GTP and taxol and subsequently centrifuging the formed polymers as described above. The pellets were then resuspended in 50 µl of MME containing either purified MARPs or heat-stable brain MAPs at similar concentrations (~20 µg/ml). After incubating at 25°C for 20 min and subsequent centrifugation, the supernatant and pellet fractions were processed for SDS-PAGE.

Plasmid Constructs and Transfection of CV1 Fibroblasts

Recombinant DNA methods such as restriction enzyme digestions, plasmid amplification, and plasmid extractions, as well as cloning and subcloning were done according to Maniatis et al. (1983).

Expression of MARP-1 repeats in *E. coli*: A 0.8-kb EcoRI fragment of the plasmid pBS-MARP1 carrying a stretch of seven tandemly repeated 114-nucleotide units of the MARP-1 gene (Schneider et al., 1988b) was expressed under the control of a T7 polymerase promoter (Rosenberg et al., 1987) and a heat-inducible T7 RNA polymerase (Tabor and Richardson, 1985).

Transfection of MARP-1 repeats into CV1 fibroblasts: The 0.8-kb EcoRI fragment from pBS-MARP-1 was inserted into the BamHI and PvuII sites of the PSCT-GAL-X-556 eukaryotic expression vector (Rusconi et al., 1990). In the resulting construct PSCT-5/4, initiation of translation occurs at the first internal methionine codon of the MARP-1 repeat unit, while termination is achieved through the vector-coded termination signal immediately downstream from the EcoRV/PvuII fusion site. Thus, the resulting protein is coded for entirely by the MARP-1 sequence, and only the last five amino acids at the COOH terminus are derived from the vector. Plasmids to be used for transfection experiments were always purified on CsCl/ethidium bromide density gradients.

CV1 fibroblasts growing on glass coverslips were transfected (Chen and Okayama, 1987) and processed for immunofluorescence 24–72 h after transformation. Control transfection experiments were performed using the plasmid without the insert. Where indicated, transfected cells were treated with taxol (10 µg/ml) or nocodazole (200 ng/ml) in growth medium overnight before processing for immunofluorescence.

Immunofluorescence

Polyclonal antibody against MARP-1 was raised in rats using gel-purified β-galactosidase/MARP-1 fusion protein as the antigen. Antibody to be used for blotting experiments and immunofluorescence was affinity purified on nitrocellulose-immobilized β-galactosidase/MARP-1 fusion protein (Schneider et al., 1988b).

Cells were processed for immunofluorescence in two ways. Either the coverslips were briefly rinsed in PBS and fixed in methanol and acetone at –20°C for 4 min in each fixative, or cells were extracted in 0.5% Triton X-100 in a microtubule stabilizing buffer before fixation (Rickard and Kreis, 1990).

The specimens were rehydrated in PBS and incubated in PBS plus 3% BSA for 30–40 min to block unspecific binding sites. The following antibodies were applied sequentially for 30–40 min each, in PBS plus 3% BSA: Polyclonal rat anti-MARP-1, goat anti-rat-FITC (Becton Dickinson Immunocytometry Systems, Mountain View, CA), polyclonal rabbit anti-tubulin (a kind gift from Thomas Kreis from the EMBL) and goat anti-rabbit Texas red (Becton Dickinson Immunocytometry Systems). Between each incubation, coverslips were rinsed six times in PBS. At the end of the entire labeling procedure, coverslips were washed in PBS six times for 5 min before embedding in a mixture of gelvatol/glycerol (Lawson, 1983) containing 1.4-Diazobicyclo (2.2.2)octan (Merck, S. p. A., Milan, Italy) to prevent fading of FITC.

Results

Purification of MARP from *Trypanosoma brucei*

Earlier work from this laboratory has shown that the membrane skeleton of *T. brucei* contains at least two closely related, high molecular weight, repetitive proteins which are associated, in situ, with the microtubules of the membrane skeleton (MARP-1 and MARP-2) (Schneider et al., 1988b; Affolter et al., in press; Hemphill et al., 1991b). To investigate the interaction of MARPs with microtubules in more detail, these proteins were now purified by taking advantage of the observations that they remain tightly attached to the cytoskeleton during extraction with the nonionic detergent Triton X-100, and that they are heat stable, a property which they share with the neural microtubule-associated proteins MAP 2 and Tau (Vallee, 1985).

Different stages of purification of MARPs are shown in Fig. 1, A and B. Upon extraction of whole trypanosomes (lane 1) in MARP-buffer containing 0.5% Triton X-100, a large number of proteins are solubilized (lane 2) while the MARPs remain entirely in the Triton-insoluble cytoskeleton fraction (lane 3).

Cytoskeletons were resuspended in PBS containing 1 M NaCl, incubated on ice for 30 min, and vortexed vigorously. This treatment resulted in a complete disintegration of the microtubular membrane skeleton (lane 4), while the flagellar skeleton, mainly consisting of the microtubular axoneme and the paraflagellar rod, remained insoluble and could be removed by centrifugation (lane 5).

The solubilized membrane skeleton was then boiled for 5 min and centrifuged to remove precipitated protein. Under these conditions, MARPs remained soluble (lane 6). They were selectively precipitated from this heat-stable fraction by adding ammonium sulfate to 10% saturation. As a final step, MARPs that had recovered from the ammonium sulfate precipitation were subjected to gel filtration on a Superose 12 column. Lane 7 shows 3 µg of the purified preparation of MARPs which was used for in vitro binding studies (see below). Because of the similarity between MARP-1 and MARP-2 (Affolter et al., in press), the two proteins could not be separated and thus all assays with biochemically purified MARPs presented below have been performed with a mixture of both proteins. However, only MARP-1 was visualized by an appropriate antibody in all experiments described.

MARP-1 Binds to Heterologous (Pig Brain) Microtubules In Vitro

Earlier immunocytochemical studies have established that MARP-1 is localized in situ along the microtubules of the membrane skeleton (Hemphill et al., 1991b; Schneider et al., 1988b). To establish if this microtubular localization is due to a direct interaction of MARP-1 with the tubulin subunits of microtubules, its microtubule-binding properties were studied in vitro (Fig. 2). MARP-1 binds to, and cosediments with, pig brain microtubules (lanes 5 and 6). This binding is inhibited by 350 mM NaCl (lanes 7 and 8), conditions which are also known to prevent the binding of homologous MAPs to pig brain microtubules (Vallee, 1982). The presence of 0.1% Triton X-100 in the incubation buffer had no effect on the binding of MARP-1 to microtubules (lanes 9 and 10). This is in agreement with the finding that MARPs remain attached to the microtubules of the membrane skeleton during extraction of trypanosomes with Triton X-100. These observations indicate that MARP-1 can bind in vitro to heterologous (mammalian) tubulins in a very similar mode as they do in vivo to the trypanosomal tubulins.

MARP-1 Binds to Pig Brain Microtubules which Are Saturated with Endogenous MAPs

Several mammalian MAPs were shown earlier to bind to microtubules through interaction with the COOH terminus of tubulin (Paschal et al., 1989; Maccioni et al., 1988), and MAP2 and Tau were shown to compete with each other for binding to this region (Hirokawa et al., 1988). Similar approaches were now used to explore if the trypanosomal MARP-1 also competes for binding to the COOH terminus of tubulin.

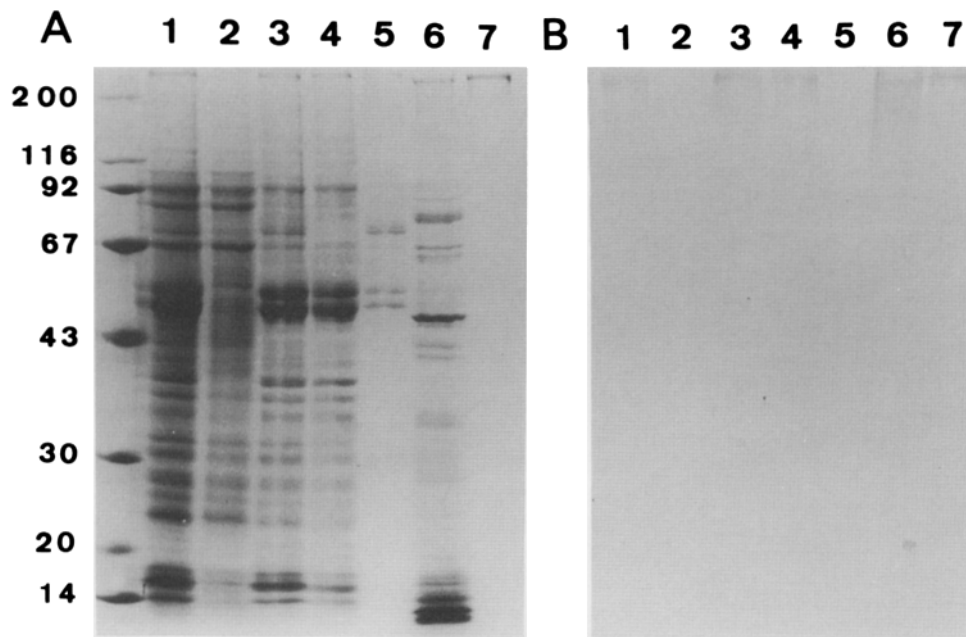


Figure 1. Different stages of MARP purification. *A*, Coomassie-stained SDS gel; *B*) Corresponding immunoblot with anti-MARP-1 antibody. Lanes 1, whole cell lysate; lanes 2, Triton-soluble fraction; lanes 3, Triton-insoluble fraction (= cytoskeletons); lanes 4, cytoskeleton components solubilized by 1 M NaCl; lanes 5, cytoskeleton components insoluble in 1 M NaCl; lanes 6, heat-stable proteins from the salt-soluble fraction; lanes 7, 3 μ g of purified MARPs after ammonium sulfate precipitation and gel filtration.

When two-times cycled pig brain microtubular protein was polymerized in the presence of taxol, the microtubules formed were not able to incorporate additional, exogenously added, brain MAPs. The addition of increasing concentrations of homologous MAPs to such microtubules did not increase the total amount of tubulin polymerized, nor did it in-

crease the relative amount of MAPs recovered in the microtubule pellets when analyzed by SDS-PAGE (data not shown). These observations indicated that in these microtubules all available binding sites for MAP2 or Tau are occupied. Such microtubules were operationally considered "MAP-saturated microtubules".

Purified trypanosomal MARP-1 can readily bind to such MAP-saturated microtubules, demonstrating that the binding site(s) for MARP-1 is/are not affected or obstructed by the bound porcine brain MAPs. Fig. 3 presents the results of such a binding experiment and demonstrates that MARP-1 quantitatively binds to, and cosediments with, MAP-saturated porcine microtubules. The interaction between MARP-1 and MAP-saturated microtubules is comparable to that shown in Fig. 2 between MARP-1 and phosphocellulose-purified, i.e., MAP-free, porcine microtubules. A more quantitative study

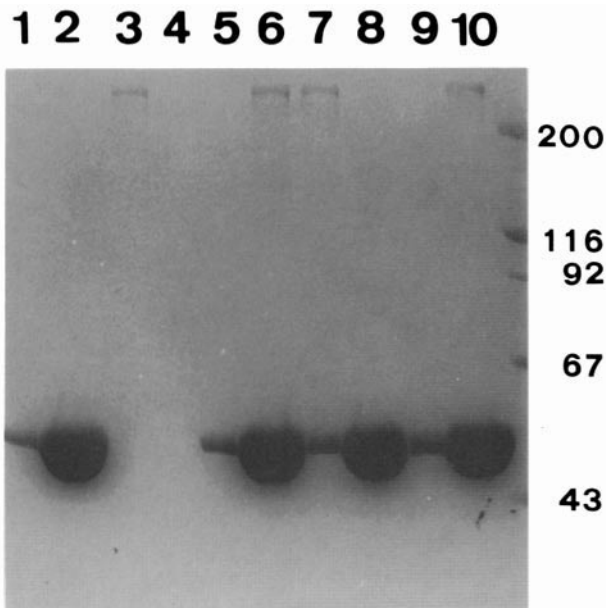


Figure 2. MARP-1 cosediments with microtubules. Coomassie-stained SDS gel. Lanes 2, 4, 6, 8, and 10, pellets; lanes 1, 3, 5, 7, and 9, supernatants. Lanes 1 and 2, pig brain microtubules incubated in buffer alone; lanes 3 and 4, MARPs incubated in buffer alone; lanes 5 and 6, MARPs incubated with pig brain microtubules; lanes 7 and 8, as in 5 and 6, but incubated in buffer containing 350 mM NaCl; lanes 9 and 10, as in 5 and 6, but incubated in buffer containing 0.1% Triton X-100.

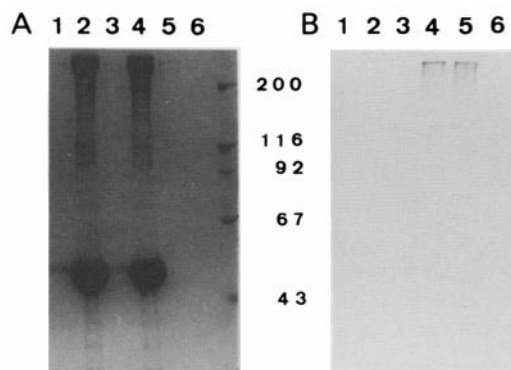


Figure 3. MARP-1 cosediments with MAP-saturated microtubules. *(A)* Coomassie-stained SDS-gel. *(B)* Corresponding immunoblot with anti-MARP-1 antibody. Lanes 2, 4, and 6, pellets; lanes 1, 4, and 5, supernatants. Lanes 1 and 2, MAP-saturated microtubules incubated in buffer alone; lanes 3 and 4, MARPs incubated with MAP-saturated microtubules; lanes 5 and 6, MARPs incubated in buffer alone.

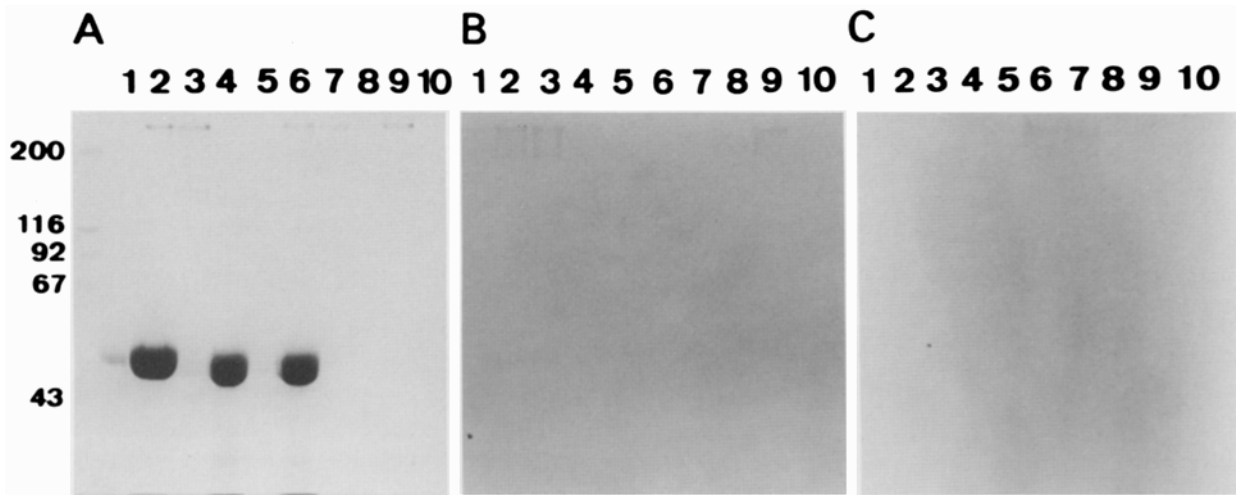


Figure 4. MARP-1 binds to S-tubulin polymers while brain MAPs do not. (A) Coomassie-stained SDS gel. (B) Corresponding immunoblot with antibody against brain MAP2. (C) Corresponding immunoblot with anti-MARP-1 antibody. Lanes 2, 4, 6, 8, and 10, pellets; lanes 1, 3, 5, 7, and 9, supernatants. Lanes 1 and 2, microtubules incubated with pig brain MAPs; lanes 3 and 4, S-tubulin polymers incubated with pig brain MAPs; lanes 5 and 6, S-tubulin polymers incubated with purified MARPs; lanes 7 and 8, purified MARPs incubated in buffer alone; lanes 9 and 10, pig brain MAPs incubated in buffer alone.

of this interaction was not possible because of the tendency of purified MARPs to aggregate at concentrations $> \sim 20 \mu\text{g/ml}$. Nevertheless, these observations suggest that MARP-1 may interact with microtubules at tubulin domains other than those used by the major brain MAPs, i.e., it may bind to the tubulins at sites different from the COOH terminus.

MARP-1 Does Not Bind to Microtubules Via the COOH Terminus of Tubulin

The results presented in Fig. 3 indicated that MARP-1 may not interact with microtubules via the COOH-terminus of tubulin. This hypothesis was verified experimentally by studying the interaction of MARP-1 with microtubules polymerized from porcine tubulin whose COOH-terminus had been removed enzymatically. Initial experiments following the protocol of Rodionov et al. (1990) were unsuccessful because the extreme sensitivity of the MARPs to proteolysis. Despite all attempts to completely inactivate the subtilisin used to cleave the COOH terminus of tubulin, MARPs added during the polymerization reaction were always degraded beyond detectability. As an alternative procedure, the method of Kanazawa and Timasheff (1989) was then used to produce S-tubulin. The purification of S-tubulin via an ion-exchange column, and the continuous presence of PMSF in all buffers, resulted in S-tubulin without residual protease activity. When polymerized S-tubulin was inspected by EM, irregularly shaped microtubules and protofilament sheets were observed (data not shown), in agreement with the polymer structures described for S-tubulin earlier (White et al., 1987). Fig. 4 presents the results of a copolymerization of S-tubulin with porcine brain MAP2 and with trypanosomal MARP-1. While MAP2 readily cosediments with microtubules formed from intact tubulin (lanes 1 and 2), it does not bind to the polymers formed by S-tubulin, and it is all recovered in the supernatant fraction (lanes 3 and 4). In clear contrast, the trypanosomal MARP-1 quantitatively cosediments with the polymers formed from S-tubulin (lanes 5 and 6). These results confirm that brain MAPs bind to different do-

main of tubulin than does the trypanosomal MARP-1 and establish that the latter does not interact with microtubules via the COOH terminus of tubulin.

The Repeat Motif of MARP-1 Represents a Microtubule-binding Domain

A previous study has shown that a major part of the MARP molecules consists of tandemly arranged, highly conserved repeat units of 38-amino acids length (Schneider et al., 1988b), a motif which is conserved between MARP-1 and MARP-2 (Affolter et al., in press). In an attempt to verify if this highly repeated motif might represent a microtubule-binding domain, a fragment of the MARP-1 gene containing seven consecutive 38-amino acid repeat units was expressed in *E. coli*. Bacterial lysates were fractionated by SDS-PAGE, blotted onto nitrocellulose, and the microtubule-binding properties were assayed by a blot-overlay procedure (Rozdzial et al., 1990). These results demonstrated that the recombinant repeat motif of MARP-1 does bind to microtubules in this assay (data not shown). In all experiments, very similar results were obtained with either taxol-stabilized microtubules or soluble-tubulin dimers in the overlay.

To corroborate these findings from in vitro experimentation, in vivo binding of the MARP-1 repeat to microtubules was analyzed by transfection.

The DNA-fragment coding for seven consecutive MARP-1 repeat units which are used for expressing MARP-1 polypeptides in *E. coli* (see above) was also cloned into the PSCT-GAL X556 eukaryotic expression vector, resulting in the MARP-1 expression plasmid PSCT-5/4 (see Materials and Methods).

In transfected CV-1 cells, the MARP-1 repeat is expressed and is present as a stable protein (Fig. 5). Immunoblotting of lysates from CV-1 cells transfected with PSCT-5/4 revealed a distinct band of an apparent molecular weight of $\sim 40,000$, with no apparent signs of degradation. The MARP-1 band detected by immunostaining does not correspond to a band detectable by silver stain, suggesting that

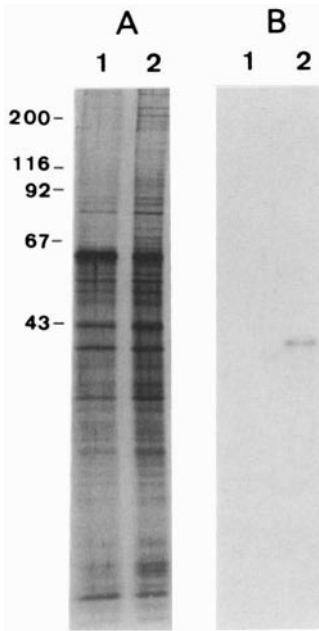


Figure 5. The MARP-1 fragment is expressed as a stable polypeptide in CV-1 cells. (A) Silver-stained SDS gel. (B) corresponding immunoblot with anti-MARP-1 antibody. Lane 1, lysate of CV-1 cells transfected with control vector PSCT-GAL-X556; lane 2, lysate of CV-1 cells transfected with PSCT-5/4.

the MARP-1 repeat is present as a low-abundance protein. No immunoreactive signal was detected in similar lysates from cells that had been infected with a control vector.

The intracellular distribution of the MARP-1 repeat polypeptides in CV1 cells was then analyzed by indirect double immunofluorescence. As a control for the specificity of MARP-1 binding, a fragment from an unrelated trypanosomal protein which is also highly repetitive, but not microtubule associated (GM6; Muller et al., 1992) was cloned into the same vector and transfected into CV-1 cells. This repetitive GM6 fragment resulted in bright and diffuse staining of the entire cytoplasm, and this staining was abolished upon extraction of the cells with Triton X-100 (data not shown). In contrast to this control, MARP-1 specific staining clearly colocalized with tubulin staining, indicating that the MARP-1 repeats are associated with the microtubules. A diffuse background staining which was consistently observed in intact cells most likely results from free, unbound MARP-1 repeats.

To reduce this cytoplasmic background, and in an effort to study whether the association of these repeats with microtubules is detergent resistant as it is in the trypanosomal cytoskeleton (Fig. 1), transfected CV-1 fibroblasts were extracted with Triton X-100 before fixation. Fig. 6 *a* shows such Triton-extracted cells labeled with anti-tubulin antibody, while Fig. 6 *b* shows the corresponding staining with anti-MARP-1 antibody. Clearly, the cytoplasmic background of MARP-1 staining is eliminated by the detergent extraction, while microtubules are still heavily stained. This clearly indicates a detergent-resistant association of MARP-1 to the CV-1 microtubules.

If the MARP-1 repeat behaves as a bona fide microtubule-associated protein in CV-1 cells, its intracellular localization should be affected by drugs that change the polymerization state of the microtubules themselves. This was explored by incubating transfected CV-1 fibroblasts with Taxol (10 μ g/ml) overnight. Taxol causes a reorganization of the microtubular

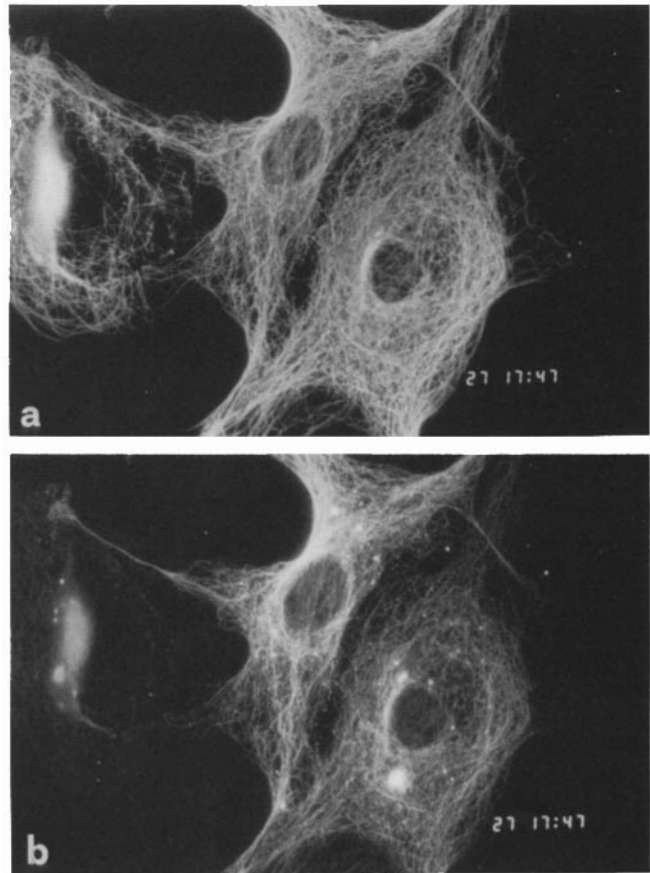


Figure 6. Distribution of MARP-1 and tubulin in transfected CV-1 cells after extraction with Triton X-100. (a) Staining with anti-tubulin antibody. (b) Staining with anti-MARP-1 antibody.

system and induces the bundling of microtubules (DeBlander et al., 1981). Such taxol-induced reorganization of the microtubule network and microtubule bundling is clearly visualized in transfected CV-1 cells by tubulin-specific immunostaining (Fig. 7 *a*). MARP-1 specific staining of the same cells demonstrates that MARP-1 repeats colocalize precisely with the reorganized microtubules (Fig. 7 *a'*).

A close codistribution of tubulin and MARP-1 specific staining was also observed when transfected cells were incubated with nocodazole (200 ng/ml) overnight to induce the complete depolymerization of the microtubular network. Labeling with anti-tubulin antibody resulted in a typical, diffuse staining of the cytoplasm indicative of the complete depolymerization of microtubules (Fig. 7 *b*). MARP-1 specific staining of the same cells revealed an essentially identical staining pattern (Fig. 7 *b'*). Very similar results have also been obtained using (murine) 3T6 or (human) HeLa cells. Thus, the trypanosomal MARP-1 repeat motive is likely to represent a novel microtubule-binding motive that interacts with domains of tubulin that are conserved between species from trypanosomes to man.

Discussion

This study reports on the microtubule-binding characteristics of MARP-1, a member of a family of high molecular

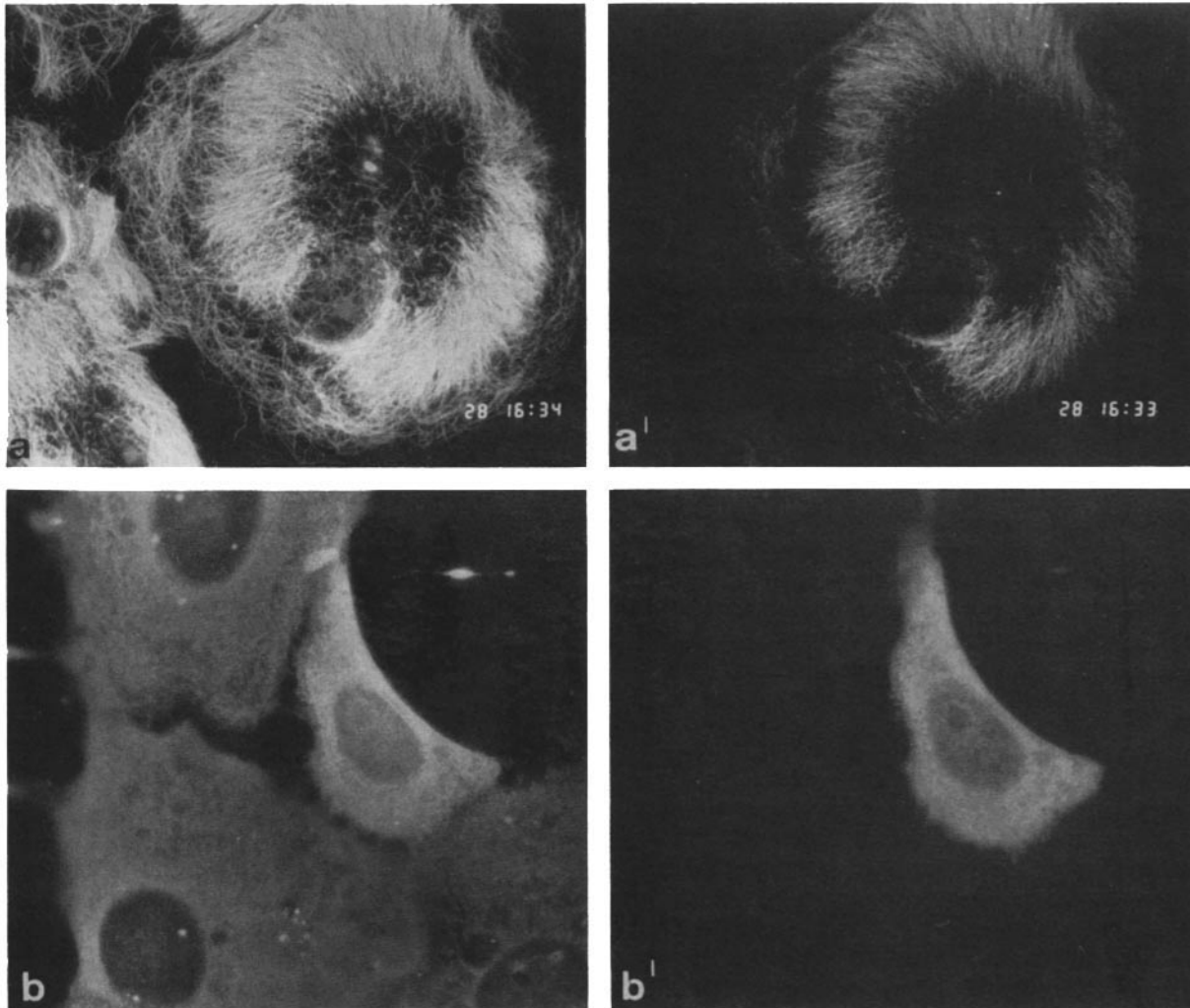


Figure 7. MARP-1 colocalizes with tubulin after drug-induced reorganization of the microtubular network. (a and a') 10 µg/ml taxol overnight. (b and b') 200 ng/ml nocodazole overnight. (a and b) Staining with anti-tubulin antibody; (a' and b') Staining with anti-MARP-1 antibody of the same cells.

weight, repetitive, microtubule-associated proteins from *T. brucei* (Affolter et al., in press; Schneider et al., 1988b). Purification of MARPs is facilitated by their heat stability, a property shared with the neural MAPs Tau and MAP2, as well as with several MAPs of non-neural origin (Albertini et al., 1990; Olmsted, 1986). On the other hand, their marked protease sensitivity not only complicates protein isolation, but it also causes experimental problems; e.g., for binding assays using crude or semipurified components that invariably contain sufficient proteolytic activity to degrade MARPs to completion.

Copolymerization and cosedimentation assays of MARPs with microtubules polymerized from purified porcine brain have demonstrated that the trypanosomal MARP-1 can interact with heterologous microtubules, and that this interaction is similarly resistant to salt and detergent as are those between MARPs and microtubules in the trypanosomal cytoskeleton. Binding of MARP-1 to porcine microtubules is unaffected by a previous saturation of these microtubules with porcine brain MAPs, suggesting that the trypanosomal MARP-1 does not bind to the same binding sites. This was

directly confirmed by determining its binding to polymers formed from tubulin from which the COOH terminus had been removed by subtilisin. In agreement with previous work (Rodionov et al., 1990; Paschal et al., 1989), the major brain MAPs, exemplified by MAP2, did not bind to these polymers, whereas trypanosomal MARP-1 did bind quantitatively. These experiments directly demonstrate that MARP-1 binds to a domain of tubulin other than the COOH terminus. Considering the observation that MARP-1 exhibits specific binding to microtubules from different species (in vitro binding: pig; in vivo binding: monkey, man, and mouse), it most likely interacts with a domain of tubulin which is well conserved between species from trypanosomes to man.

Similar in vitro binding experiments, as well as overlay assays using a recombinant fragment of MARP-1, have demonstrated that the repetitive 38-amino acid motif of MARP-1 represents a microtubule-binding domain. This conclusion is confirmed by transfection experiments where a recombinant fragment of MARP-1 comprising seven contiguous 38-amino acid repeat units was transfected into CV-1 monkey cells. The MARP-1 repeat is expressed and exists in these cells as a sta-

ble protein having molecular weight of $\sim 40,000$. Immunofluorescence demonstrated that this recombinant protein fragment colocalizes with microtubules. This interaction is stable in that the labeling pattern is not affected by detergent extraction of the cells. Essentially the same results were also obtained in transfected HeLa (human) and 3T6 (murine) cells.

Taxol treatment of mammalian cells causes a marked reorganization of the microtubular network (DeBrabander et al., 1981). When transfected CV-1 cells are treated with taxol, MARP-1 fluorescence codistributes precisely with the reorganized microtubules. In addition, when Nocodazole is used to induce depolymerization of microtubules, MARP-1 fluorescence again codistributes with tubulin and forms a diffuse staining throughout the cytoplasm. Taken together, these observations strongly indicate that MARP-1 does interact specifically and stably with microtubules of CV-1 cells, as well as with those of other mammalian cell lines, and that it behaves, during drug-induced reorganization of the microtubule network, as a bona fide MAP.

The experiments presented in this study demonstrated that the 38-amino acid repeat motif of MARP-1 represents a novel microtubule-binding domain. The amino acid sequence of this repeat unit is not related in any simple way to those of the microtubule-binding motifs of MAP2, Tau, or the 190-kD bovine adrenal MAP-U. However, it is interesting to note that the conserved 18-amino acid repeat motifs of these three latter proteins are also arranged within 36–40-amino acid repeat units, of which they form the COOH-terminal moieties. While these COOH-terminal 18 amino acids of each repeat are well conserved between individual repeats as well as between the different proteins and different species (Aizawa et al., 1989; Himmler et al., 1989; Lewis et al., 1988), the remainder of the 36–40-amino acid repeat units into which they are embedded, are not. The sequence of the conserved 18-amino acid domain may serve to specifically target the MAPs to the COOH terminus of tubulin, while the overall repeat length of 36–40 amino acids may be of importance for the spacing of these specific contacts along the repetitive surface of microtubules. A peptide of 36–40 amino acids can readily cover a distance of about 8-nm length, which corresponds to the length of a tubulin dimer along the axis of the microtubule (Amos, 1979). Thus, a protein with a repetitive sequence motif of 35–40 amino acids length may extend along the axis of microtubules and make equivalent contacts with successive tubulin dimers along the axis of the microtubule through each repeat unit. These functional constraints may account for the similar unit lengths of the microtubule binding domains of mammalian MAPs and trypanosomal MARPs. The absence of direct sequence similarity, as well as the observation that the repeat motif of trypanosomes is acidic while those of the mammalian MAPs are basic, may reflect the binding of the respective proteins to different domains of tubulin. While the (basic) repeats of MAP2, Tau, and MAP-U were shown to bind to the (acidic) COOH terminus of tubulin, no binding site for the (acidic) trypanosomal repeat has yet been identified.

Considering the potentially large number of contacts (>50 ; Schneider et al., 1988b) which an individual trypanosomal MARP molecule is able to make, these proteins can conceivably exert a tremendous stabilizing effect on the microtubules of the trypanosomal membrane skeleton, for

which stability is quintessential. The highly repetitive sequence organization of the MARPs may thus reflect a functional adaptation of these proteins to their roles as stabilizers of microtubules, and they may be regarded as the trypanosome's answer to dynamic instability.

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