Original

Identification of an XMAP215-related 60 kDa polypeptide as a major microtubule-associated protein in *Tetrahymena pyriformis*

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SUMMARY

We have investigated the microtubule-associated proteins (MAPs) of *Tetrahymena pyriformis*. Three polypeptides, with apparent molecular masses of 90 kDa, 60 kDa, and 37 kDa, co-sedimented with Taxol-stabilized mammalian microtubules. Partial amino acid sequencing of the 60kDa MAP revealed that it was a newly identified *Tetrahymena* protein. The sequence was homologous to *Xenopus* XMAP215, suggesting that the 60kDa MAP was a member of the XMAP215 family. The purified 60kDa MAP promoted the polymerization of mammalian tubulin in vitro. The 37kDa MAP was identical to *Tetrahymena* glyceraldehyde-3-phosphate dehydrogenase. The 90 kDa polypeptide was not characterized further, because of its low abundance.

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Received: 29 Oct 2003; Accepted: 5 Dec 2003.

INTRODUCTION

The ciliated protozoan *Tetrahymena* has elaborate microtubular structures. During amitosis, microtubules assemble inside the macronucleus, and between the macronucleus and the cortex (Fujiu and Numata, 2000), while various intracytoplasmic microtubular structures are observed during conjugation. Microtubules also associate with the contractile vacuole pore and the oral apparatus (for review, see Frankel, 2000). *Tetrahymena* is one of the best experimental systems for studying the functions of cellular microtubules.

Microtubule dynamics are regulated by various microtubule-associated proteins (MAPs), and this is probably also true for *Tetrahymena*. In many eukaryotic cells, XMAP215, originally identified in amphibian cells, and its related proteins play a key role in regulating microtubule dynamics (for review, see Ohkura et al., 2001). In *Tetrahymena*, microtubule-dependent motor molecules and molecular chaperones reportedly bind to cortical microtubules (Frankel, 2000), and one of them was shown to mediate the oral apparatus formation (Frankel et al., 2001), possibly by interacting with

cortical microtubular structures. Yet, most of the functions of *Tetrahymena* cellular microtubules have remained unclear, in part because the molecules that modulate microtubule dynamics have not been characterized.

We aimed to clarify the cellular microtubule functions by investigating how MAPs influence microtubule dynamics in *Tetrahymena*. For this purpose, *Tetrahymena* MAPs were biochemically characterized in this study. We successfully isolated a novel *Tetrahymena* MAP with potent microtubule assembly activity. The relationship between this newly identified MAP and XMAP215 is discussed.

MATERIALS AND METHODS

Materials

Taxol was generously supplied by Dr. N. Lomax, of the Natural Products Branch, National Cancer Institute, Bethesda, MD. Anti-MAP1, anti-MAP2, anti-MAP4, and anti-tau antibodies were prepared against mammalian MAP1, MAP2, MAP4, and tau as described previously (Kotani et al., 1986). Anti-(AP sequence) antibody was prepared against a bacterially expressed MAP4 fragment as described (Tokuraku et al., 2003). Anti-(chick α-tubulin) monoclonal antibody (clone no. DM1A) was purchased from Sigma-Aldrich Japan K.K. Anti-(*Tetrahymena* GAPDH) antiserum was generously supplied by Dr. A. Soukri (the University of Hassan II, Morocco). All other materials were of reagent grade.

Protein preparations

Twice-cycled microtubule protein and tubulin were purified from bovine or porcine brain as described previously (Kotani et al., 1986). The *Tetrahymena pyriformis* strain W was cultured according to Watanabe et al. (Watanabe et al., 1994). To prepare the cell extract, the cells were harvested, washed twice with reassembly buffer (RB) (100 mM 2-(N-morpholino)ethanesulfonic acid (MES) (pH 6.8), 0.1 mM EGTA, and 0.5 mM $MgCl_2$), suspended in RB supplemented with 0.5 mM GTP and protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 0.2 mM 1-chloro-3-tosylamido-7-amino-L-2-heptanone, 0.1 mg/ml leupeptin, and 0.02 mg/ml pepstatin A), homogenized, and centrifuged at 100,000 × g for 60 min. For the isolation of MAPs, Taxol-stabilized mammalian microtubules were added to the cell extract, and the mixture was centrifuged at 100,000 x g for 60 min at 2°C through a cushion of 10% sucrose in RB. The subsequent procedures for the Taxol-dependent preparation of MAPs, and the heat-treatment of the MAP fraction, were as described (Kotani et al., 1986).

The 60kDa MAP was purified as follows. The *Tetrahymena* extract was heat-treated, applied to a DEAE-cellulose column equilibrated with a buffer (20 mM MES (pH6.8), 0.1 mM EGTA, and 0.5 mM $MgCl_2$) containing 0.5 mM PMSF, and eluted with a linear gradient of NaCl (from 0 to 0.5 M). The 60kDa MAP-rich fractions were collected, and subjected to the Taxol-dependent MAP preparation procedure (Kotani et al., 1986). The residual tubulin in the MAP fraction was removed by heat-treatment.

The 37kDa MAP (*Tetrahymena* glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) was prepared from the *Tetrahymena* extract by the method of Hafid et al. (Hafid et al., 1998), except that we used a single phosphocellulose column chromatography step, instead of the series of Blue Sepharose CL-6B and Polybuffer Exchanger PBE-94 column chromatographies.

Assay and other procedures

Microtubule assembly was monitored by either the change in absorbance at 350 nm or the amount of polymerized tubulin, as described (Tokuraku et al., 2003). Samples for electron microscopy were negatively stained and observed as

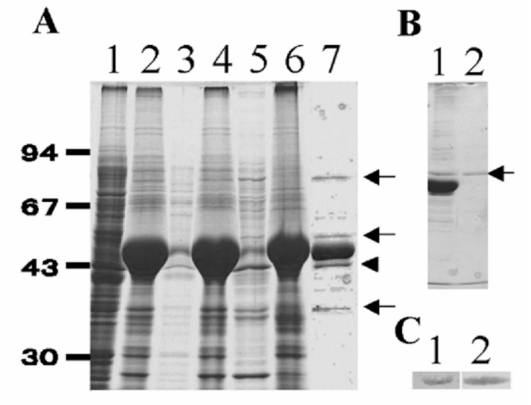

Fig.1. Isolation and characterization of *Tetrahymena* MAPs. A: Taxol-dependent MAPs preparation. *Tetrahymena* soluble extract (lane 1) was mixed with taxol-stabilized mammalian microtubules and centrifuged, and the microtubule pellet (lane 2) was obtained. The pellet was washed with RB to remove the non-specifically bound proteins, and was separated into the supernatant (lane 3) and the pellet (lane 4). The second microtubule pellet (lane 4) was washed with RB containing 0.35 M NaCl to release the MAPs, and was separated into the MAP-containing supernatant (crude MAPs) (lane 5), and the remaining microtubule pellet (lane 6). The Taxol-stabilized microtubules were mixed again with the dialyzed crude MAPs fraction (lane 5), collected by centrifugation, and washed with the high salt buffer to release the final MAP fraction (lane 7). Samples were electrophoresed and stained with Coomassie Brilliant Blue (CBB). The molecular masses (kDa) are indicated on the left side of the figure. Arrows indicate the three MAPs that appeared reproducibly in the final MAP fraction. The arrowhead indicates degraded tubulin (see text). B: Heat stability of *Tetrahymena* MAPs. The crude MAPs fraction (prepared similarly to lane 5 of A) (lane 1) was heat-treated, and the heat-stable fraction was electrophoresed (lane 2). The arrow indicates the 60kDa MAP. C: Immunoreactivity of the 37kDa MAP with anti-(*Tetrahymena* GAPDH) antiserum. The purified 37kDa MAP was electrophoresed, transferred onto a polyvinylidene fluoride membrane, and stained with CBB (lane 1), or immunodetected with anti-GAPDH antiserum (lane 2).

described (Tokuraku et al., 2003). Preparation of the rabbit anti-(60kDa MAP) antiserum and immunoblotting were according to Katsuki et al. (2000). The protein concentration was determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was according to Laemmli (1970). In-gel digestion of proteins was done by the method of Cleveland et al. (1977). The amino acid sequences were determined by automated Edman degradation with a PROCISE^{TM} protein sequencer (Applied Biosystems, USA).

RESULTS

Isolation of *Tetrahymena* MAPs

Although the concentration of the intrinsic *Tetrahymena* tubulin was too low for the Taxol-dependent MAPs preparation procedure (data not shown), we successfully identified the proteins that coprecipitated with Taxol-stabilized mammalian microtubules (Fig. 1A, lane 2). Since the crude MAPs fraction (Fig. 1A, lane 5) still contained many protein bands, some of which were not reproducible, the fraction was subjected to an additional coprecipitation-desorption cycle. The resultant MAP fraction (Fig. 1A, lane 7) reproducibly contained three major polypeptides, with apparent molecular masses of 90 kDa, 60 kDa and 37 kDa (Fig. 1A, arrows). The prominent 50 kDa band and the 45 kDa band (arrowhead) were tubulin and its degradation product, since they reacted with the anti-(chick α-tubulin) monoclonal antibody (data not shown). None of the 90 kDa, 60 kDa, and 37 kDa polypeptides were reactive to anti-MAP1, anti-MAP2, anti-MAP4, anti-tau, or anti-(AP sequence) antibodies (data not shown). Among the detectable bands in the crude MAP fraction (Fig.

Fig. 2. Partial amino acid sequences of the 60kDa and 37kDa MAPs. The two proteins were proteolyzed, and the N-terminal portions of the two 60kDa MAP-derived peptides and the single 37kDa MAP-derived peptide were sequenced. Homologous regions of XMAP215 (EMBL accession number AJ251130) and *Tetrahymena* GAPDH (Zhao et al., 1997) are aligned. Residue numbers are indicated in parentheses. Asterisks indicate identical residues.

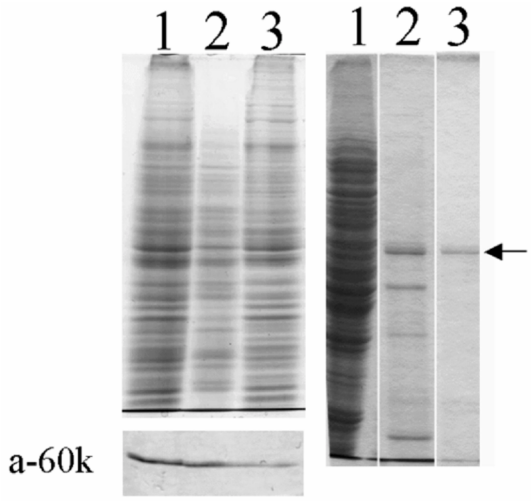

Fig.3. Fractionations of the 60kDa MAP. A: Subcellular localization of the 60kDa MAP. *Tetrahymena* cell homogenate (lane 1) was centrifuged, and separated into soluble (lane 2) and insoluble (lane 3) fractions. Samples were electrophoresed and either stained with CBB (upper panel) or detected with anti-(60kDa MAP) antiserum (lower panel). B: Electrophoretic patterns of fractions obtained during the purification of the 60kDa MAP. Lane 1, *Tetrahymena* crude extract; lane 2, fraction after DEAE-cellulose chromatography; lane 3, the purified 60kDa MAP. The 60kDa MAP is indicated by an arrow.

1A, lane 5), only the 60kDa MAP was heat-stable (Fig. 1B).

Amino acid sequence analysis

The N-terminal amino acid sequences of the intact 60kDa and 37kDa MAPs could not be analyzed, so their N-termini may have chemical modifications. Consequently, the two MAPs were subjected to in-gel digestions, and the internal sequences were determined (Fig. 2). As shown in Fig. 2, two sequences of the 60kDa MAP were highly homologous to *Xenopus* XMAP215 (EMBL accession number AJ251130), while the sequence of the 37kDa MAP was identical to *Tetrahymena* GAPDH (Hafid et al., 1998), one of the glycolytic enzymes. *Tetrahymena* GAPDH shows an apparent molecular mass of 36 kDa (Hafid et al., 1998), and the anti-(*Tetrahymena* GAPDH) antiserum (Hafid et al., 1998) reacted with the 37kDa MAP (Fig. 1C). In addition, mammalian GAPDH reportedly binds to microtubules (for review, see Sirover, 1999). Consequently, the 37kDa MAP was considered to be identical to *Tetrahymena* GAPDH. *Tetrahymena* proteins homologous to the 60kDa MAP have not been reported so far. The amino acid sequence of the 90kDa MAP was not determined, because the content of this protein was too low for a sequence analysis.

Characterizations of the 60kDa MAP and the 37kDa MAP (GAPDH)

The 60kDa MAP existed abundantly in the soluble fraction, rather than the membranous one (Fig. 3A). From the soluble fraction, it was purified to homogeneity (Fig. 3B).

The 60kDa MAP enhanced both the initial rate and the plateau level of bovine tubulin polymerization, in a concentration-dependent manner (Fig. 4A). Electron microscopic observations showed that the assembled structures were normal microtubules (Fig. 4B). Sedimentation assays (Tokuraku et al., 2003) also revealed that the

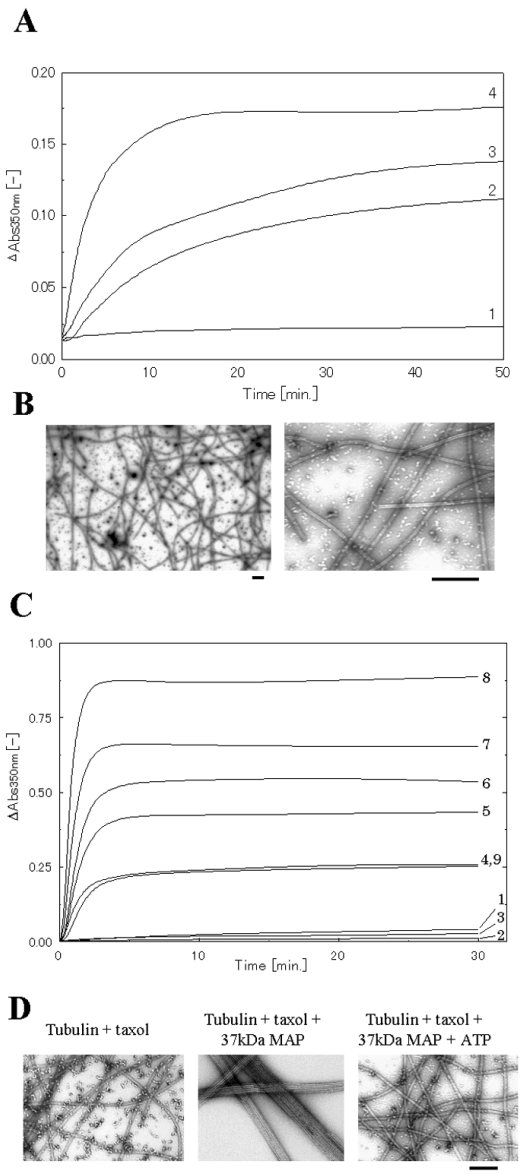

Fig.4. Effects of the 60kDa, and the 37kDa MAPs on microtubules. A: Microtubule assembly induced by the 60kDa MAP. Bovine brain tubulin (1.50 mg/ml) was mixed with 0.00 mg/ml (curve 1), 0.05 mg/ml (curve 2), 0.10 mg/ml (curve 3), and 0.20 mg/ml (curve 4) of the 60kDa MAP. B: Electron micrographs of microtubules reconstituted from 1.50 mg/ml of tubulin and 0.20 mg/ml of the 60kDa MAP. Bars indicate 200 nm. C: Microtubule bundling induced by the 37kDa MAP. The sample represented in curve 1 contained the 37kDa MAP alone (0.80 mg/ml). Bovine brain tubulin (1.50 mg/ml), in the absence (curves 2 and 3) or presence (curves 4-9) of 30 μM Taxol, was mixed with 0.00 mg/ml (curves 2 and 4), 0.10 mg/ml (curve 5), 0.20 mg/ml (curve 6), 0.40 mg/ml (curve 7), and 0.80 mg/ml (curves 3, 8, and 9) of the 37kDa MAP. In curve 9, 1 mM ATP was also present. D: Electron micrographs of microtubules and the 37kDa MAP-induced bundles. The conditions for the left, center, and right panels were the same as those of curve 4, curve 8, and curve 9 of C, respectively. Bar indicates 200 nm.

amount of polymerized tubulin and the microtubule-bound 60kDa MAP increased with higher concentrations of the 60kDa MAP (data not shown).

Mammalian GAPDH bundles microtubules, rather than assembling them (Sirover, 1999). Since the homology between mammalian and *Tetrahymena* GAPDH is low (61%) (Zhao et al., 1997), and the effects of *Tetrahymena* GAPDH on microtubules were not tested, we purified the 37kDa MAP and analyzed its activity on microtubule assembly (Fig. 4C, D). The 37kDa MAP did not promote bovine microtubule assembly in the absence of Taxol (Fig. 4C, curves 1-3). When the assembly was initiated by Taxol, the turbidity of the microtubules steadily increased, depending

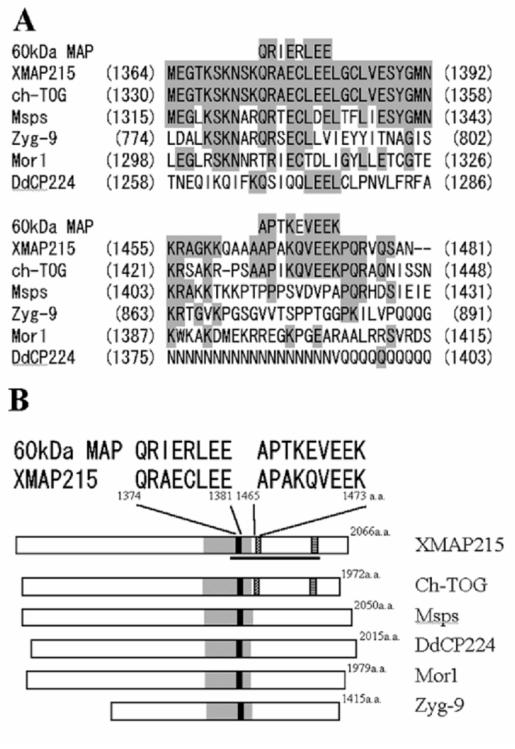

Fig.5. Sequence comparison of the 60kDa MAP and XMAP215 family members. A: Partial amino acid sequence alignments of the 60kDa MAP, *Xenopus laevis* XMAP215 (EMBL accession number AJ251130), *Homo sapiens* ch-TOG (EMBL accession number X92474), *Drosophila melanogaster* Msps (EMBL accession number AJ249115), *Caenorhabditis elegans* Zyg-9 (EMBL accession number AF035197), *Arabidopsis thaliana* Mor1 (EMBL accession number AF367246), and *Dictyostelium discoideum* DdCP224 (EMBL accession number AJ012088). Residues identical to XMAP215 are shadowed. Residue numbers are indicated in parentheses. B: Schematic diagrams. In each of the molecules, the black box and the hatched line box designate the regions homologous to QRIERLEE and APTKEVEEK of the 60kDa MAP, respectively, while the horizontal-striped box indicates the microtubule-target motif (Popov et al., 2001), and the gray box shows a region highly conserved among family members (Popov et al., 2001). The region of XMAP215 that putatively corresponds to the 60kDa MAP is underlined.

on the 37kDa MAP concentration (Fig. 4C, curves 4-8). The microtubule-bound 37kDa MAP also increased in a concentration-dependent manner (data not shown). Many microtubule bundles were observed in the presence of the 37kDa MAP (Fig. 4D). The turbidity increase was inhibited by adding ATP (Fig. 4C, curve 9), which was a characteristic similar to the mammalian GAPDH-induced microtubule bundling (Sirover, 1999).

DISCUSSION

In this study, we identified three MAPs (90 kDa, 60 kDa and 37 kDa) in a *Tetrahymena* extract. Two of them, the 60kDa and 37kDa MAPs, were further characterized. The 60kDa MAP was a newly identified *Tetrahymena* MAP that facilitated tubulin polymerization. The 37kDa MAP was *Tetrahymena* GAPDH. Its activities on microtubules were similar to those of mammalian GAPDH (Sirover, 1999).

The molecular mass and the heat-stability of the 60kDa MAP were similar to those of the major heat-stable mammalian MAP, tau, but no immunocrossreactivity between them was detected (data not shown). In addition, the partial amino acid sequences of the 60kDa MAP were not homologous to those of tau; instead, they were homologous to those of XMAP215. XMAP215 belongs to a well-known, evolutionarily conserved MAP family, whose members are regulators of microtubule dynamics (Ohkura et al., 2001). As shown in Fig. 5A, the sequence homologous to QRIERLEE is conserved among the family members, while APTKEVEEK is conserved only in XMAP215 and ch-TOG; the two proteins are homologous along the entire length (Popov et al., 2001). Judging from the molecular masses of the sequenced peptides, the 60kDa MAP approximately corresponds

to the C-terminal third of XMAP215 (Fig. 5B, underline). The marked difference in the molecular size is a unique feature of the 60kDa MAP, as a member of the XMAP215 family (Fig. 5B). Since the N-terminus of the 60kDa MAP is chemically modified (see Results), it is not a degradation product of a larger molecule. Popov et al. (2001) reported that XMAP215 contains a microtubule-target motif, on its C-terminal region. The putative 60kDa MAP homologous region on XMAP215 includes this motif (Fig. 5B, horizontal-striped box).

XMAP215 family members are usually associated with the elongation of centrosome-nucleated microtubules and the formation of the mitotic apparatus (Ohkura et al., 2001). Although *Tetrahymena* does not contain typical mitotic apparatus observed in vertebrate cells, functionally similar structures were reported, such as the perimicronucleus microtubules of *Tetrahymena thermophila* (Gaertig and Fleury, 1992), and intramacronucleus microtubules which appear during cell division (Fujiu and Numata, 2000). It is probable that the 60kDa MAP regulates the formation of these microtubular structures during nuclear division. In addition, *Tetrahymena* contains elaborate cytoplasmic microtubule networks (see introduction). Since the 60kDa MAP was mostly recovered from the cytoplasmic protein fraction (Fig. 3A), and it promoted microtubule assembly under centrosome-free conditions (Fig. 4A), the 60kDa MAP may also regulate the dynamics of cytoplasmic microtubules.

Full length sequencing of the 60kDa MAP is now in progress, in order to reveal further similarities and differences between the 60kDa MAP and XMAP215.

ACKNOWLEDGEMENTS

We wish to thank Dr. O. Numata (University of Tsukuba, Japan) for providing the *Tetrahymena*, and Dr. A. Soukri (University of Hassan II, Morocco) for the anti-(*Tetrahymena* GAPDH) antiserum. Thanks are also due to Dr. T. Takeda (Columbia University, USA) for valuable advice, and to Dr. B. Guthrie (SKYBAY Scientific Editing) for editing the manuscript. This work was supported in part by the Sasakawa Scientific Research Grant from The Japan Science Society.

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