Identification of microtubule-associated proteins (MAPs) in *Xenopus* oocyte

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Received 13 September 1985

Microtubules were isolated from prophase-blocked oocytes of *Xenopus laevis* with the use of the anti-tumor drug taxol. In addition to tubulin, 5 microtubule-associated proteins (MAPs) were characterized. Among them, 2 high molecular mass proteins (200–300 kDa) are phosphorylated in ovo. The oocyte MAP extract promotes the assembly of rat brain 6 S purified tubulin.

(Xenopus *oocyte*) *Microtubule* *Taxol* *Microtubule-associated protein*

1. INTRODUCTION

Microtubules have been isolated from a variety of cells and found to be composed of tubulin subunits as well as of a number of associated proteins. These microtubule-associated proteins (MAPs) were first identified on the basis of their affinity for microtubules in vitro. However, although MAPs have been immunocytochemically localized with microtubules in cultured cells, their physiological significance and function remain speculative [1]. MAPs from mammalian brain tissue have been the most extensively studied; they appear on electrophoretic gels as 2 groups: a group of high molecular mass (HMM) proteins (250–300 kDa) known as MAP1, MAP2, MAP3 and MAP4, and the group of tau proteins (60–67 kDa) [2–5]. Recent investigations have shown that HMM proteins, essentially MAP1, copurify with tubulin in numerous cultured cells [6].

The *Xenopus* oocyte provides an attractive experimental system for the elucidation of the roles of MAPs during cell division; first because the subcellular arrangement of microtubules changes during meiotic maturation [7–11] and after fertilization [12], second because it was recently shown that the microinjection of fluorescent MAPs isolated from rat brain into the oocyte interfered with the organization of oocyte microtubules [13]. The identification of oocyte MAPs is therefore essential to better understanding of their functions in early development.

Vallee and Bloom [14] report the isolation of a variety of MAPs from unfertilized eggs of sea urchin with the use of the anti-tumor drug taxol. Immunochemical analysis furthermore suggests that these proteins may be either structural or functional components of the spindle. Here we have utilized a similar isolation procedure which takes advantage of the microtubule assembly promoting activity of taxol, to characterize biochemically MAPs from the *Xenopus* oocyte.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Collagenase type I, trypsin, trypsin inhibitor from soybean, and dispase II were from Boehringer. [^{32}P]Orthophosphate (carrier-free, 10 mCi/ml, 150 Ci/mg) and L-[^{35}S]methionine (16 mCi/ml, 6.8 Ci/mg) were obtained from the Radiochemical Center (Amersham). Taxol was a gift from Drs A. Fellous (CNRS, Bicetre, France) and E. Karsenti (CNRS, Ivry, France).

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2.2. Animals

X. laevis adult females (de Rover, The Netherlands) were bred and maintained under laboratory conditions. Rats were male albino Sprague-Dawley of 250 g body wt (Charles River).

2.3. *Oocyte preparation*

Animals were anesthetized with MS 222 (Sandoz) at 1 g/l. Ovaries were removed and transferred to medium A composed of 88 mM NaCl, 0.33 mM $Ca(NO_3)_2$, 1 mM KCl, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2 mM Tris, pH 7.4, penicillin (50000 units/l) and streptomycin (1 mg/l).

After dispase digestion (0.4 mg/l) for 4 h at laboratory temperature and collagenase digestion (0.8 mg/l) for 1 h at 25°C with continuous stirring, stage VI oocytes, 1.3 mm in diameter [15], were collected.

2.4. *[^{32}P]Phosphoprotein and ^{35}S-protein labeling procedures*

Oocytes were preincubated for 16 h at laboratory temperature with gentle continuous shaking in medium A containing [^{32}P]orthophosphate (500 μCi/ml, 400 oocytes(8 ml) or [^{35}S]methionine (500 μCi/ml, 300 oocytes/8 ml) and unlabeled methionine (10^{-3} M). Then the oocytes were washed 4 times in medium A at room temperature.

2.5. *Preparation of rat brain 6 S purified tubulin*

Rats were killed by decapitation. The brains were removed, immediately chilled in the assembly buffer of Weisenberg [16] containing 0.1 M Mes, 1 mM EGTA, 0.1 mM EDTA, 1 mM GTP, 0.5 mM $MgCl_2$, 0.2 mM phenylmethylsulfonyl fluoride, 2 mg/l leupeptin (pH 6.5 at 4°C), and first cycle tubulin was prepared using the procedure of Shelanski et al. [17]. Pure tubulin was prepared by the method of Weingarten et al. [3].

2.6. *Preparation of* Xenopus *oocyte microtubules*

Microtubules were prepared in the presence of taxol by using a modification of the procedure described by Vallee and Bloom [14]. 1000 stage VI oocytes were homogenized in 3 ml lysis buffer (0.1 M Pipes, 5 mM EGTA, 1 mM $MgSO_4$, 0.9 M glycerol, 2 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 100 μg soybean trypsin inhibitor/ml, 2 mg leupeptin/l, pH 6.6 at 4°C) and centrifuged at 30000 × *g* for 30 min at 2°C. The supernatant was recovered and centrifuged at 135000 × *g* for 90 min at 2°C. Taxol was added to the supernatant (total proteins: 20 mg) to 20 μM and GTP was added to 1 mM. The extract was incubated at 37°C for 10 min to assemble microtubules and centrifuged at 22500 × *g* for 45 min at 2°C through a 1 ml cushion of 10% sucrose in lysis buffer containing 20 μM taxol and 1 mM GTP. The first microtubule pellet (total proteins: 500 μg) was resuspended (total volume, 1/4 the volume of the preceding extract) and washed in lysis buffer containing 20 μM taxol and 1 mM GTP. The pellet was resuspended and washed in lysis buffer containing 20 μM taxol, 1 mM GTP and 0.35 M NaCl. The final pellet was resuspended in lysis buffer containing 20 μM taxol and 1 mM GTP, and MAPs were dissociated from the microtubules by addition of 1 M NaCl (total proteins: 100 μg). In the case of radioactive oocyte MAPs, this MAP extract was dialyzed against the assembly buffer of Weisenberg [16] and added (0.2 mg/ml) to first cycle rat brain tubulin (1 mg/ml). This solution was warmed for 30 min at 30°C to assemble microtubules and centrifuged at 150000 × *g* for 90 min at 25°C through a cushion of 6 M glycerol in the assembly buffer of Weisenberg [16]. The radioactive oocyte MAPs present in the microtubule pellet were then analyzed by electrophoresis and autoradiography.

2.7. *Turbidimetry measurements*

Turbidimetry measurements during the tubulin polymerization at 37°C were performed at 345 nm using a Beckman DU-7 spectrophotometer equipped with an automatic thermostatted 6 sample changer. Rat brain 6 S purified tubulin (1 mg/ml) was incubated in the presence of *Xenopus* oocyte MAP extracts in the assembly buffer of Weisenberg [16]. The oocyte MAP extracts were previously dialyzed against this medium.

2.8. *Gel electrophoresis and autoradiography*

SDS-solubilized fractions (20–50 μl) were submitted to electrophoresis, according to Laemmli [18] adapted for 7 × 8.5 cm slab gels, 1 mm thick, 0.1% SDS/5–20% polyacrylamide; slab gels were run for 3 h at room temperature at a constant voltage of 160 V. Gels were stained with

Coomassie blue. For the detection of [^{35}S]methionine-labeled proteins, gels were incubated in Amplify (Amersham) at room temperature with agitation for 15–30 min. Gels were dried and exposed in contact with Kodak X-Omat AR film in a Cromex cassette Du Pont, with intensifying screen, at −70°C.

2.9. *Electron microscopy*

One drop of the oocyte microtubule first pellet was applied for 30 s to a glow-discharged, 400 mesh grid coated with carbon and formvar. The grid was then washed, placed on top of a drop of 1% uranyl acetate for 30 s, and then dried with filter paper. Specimens were examined in an electron microscope.

3. RESULTS

3.1. *Isolation of* Xenopus *oocyte microtubules*

Soluble tubulin in a high-speed supernatant of *Xenopus* oocyte homogenate was polymerized into microtubules with taxol and GTP (fig.1). Polyacrylamide gel electrophoresis of the microtubule pellet revealed 2 prominent bands which comigrated with α- and β-tubulins from rat brain. In addition to tubulin, numerous minor bands were stained with Coomassie blue. To characterize more accurately these minor MAPs, microtubules were isolated from a limited number of [^{35}S]methionine-labeled oocytes (generally 300 completed to 1000 with unlabeled oocytes). The first microtubule pellet was highly enriched in radioactive tubulin (fig.2, lane 2); 5 prominent radioactive proteins (1–5) of higher molecular mass copurified with tubulin (80, 94, 100 kDa; and 2 between 200 and 300 kDa). These 5 MAPs do not appear to represent cytosolic contaminants because they were not released when the pellet was washed with the lysis buffer containing taxol and GTP, and/or treated with 0.35 M NaCl. In contrast, the radioactive MAPs of oocyte can be dissociated from the microtubules by addition of 1 M NaCl.

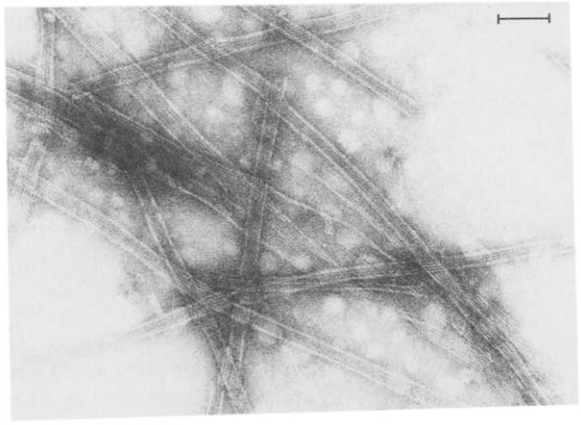

Fig.1. Negatively stained taxol polymerized microtubules. The first microtubule pellet is negatively stained with uranyl acetate. Microtubules have a subunit structure consisting of protofilaments. Bar = 52 nm.

The radioactive proteins present in the 1 M NaCl extract copolymerized with first cycle tubulin from rat brain (fig.2, lane 3). Proteins 1–4 were incorporated into brain microtubules. To determine if these MAPs are thermostable, the first [^{35}S]methionine radioactive pellet was treated at 100°C for 5 min, and then analyzed by autoradiography. Fig.2, lane 4, shows that pro-

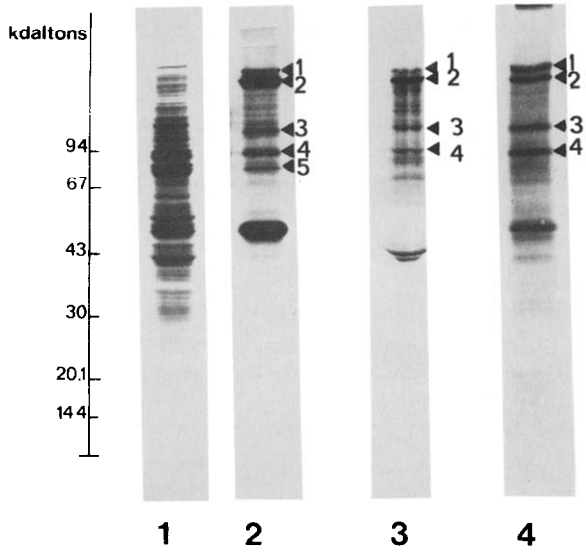

Fig.2. Autoradiographs of MAPs from *Xenopus* oocytes. Oocytes were preincubated for 16 h in [^{35}S]methionine and taxol-stabilized microtubules were prepared as described in section 2. Lanes: 1, microtubule-depleted supernatant (corresponding to 1 oocyte); 2, first microtubule pellet; 3, copolymerization of 1 M NaCl oocyte MAP extract with non-radioactive first cycle rat brain tubulin; 4, thermostable proteins isolated from the heat-treated first microtubule pellet. Proteins corresponding to the same amount of oocytes (100) were loaded in channels 2–4.

teins corresponding to proteins 1–4 of lanes 2 and 3 appear to be resistant to heat treatment.

3.2. *In vivo phosphorylation of MAPs*

It is well established that mammalian brain MAPs, essentially MAP2, tau and a 94 kDa protein, are phosphorylated in vitro [18]. It was therefore of interest to know if the oocyte MAPs could be phosphorylated in ovo. In the next experiment, oocytes were preincubated in the presence of [^{32}P]orthophosphate for 16 h at room temperature. They were then washed out. The tubulin pellets were prepared by the taxol procedure, and the [^{32}P]phosphoproteins present in the microtubule pellet were separated by gel electrophoresis and detected by autoradiography. In our experimental conditions, tubulin dimer was not phosphorylated (fig.3, lane 1). Interestingly, rat brain tubulin and *Xenopus* brain tubulin, prepared with the taxol procedure in the presence of [γ-^{32}P]ATP, are phosphorylated in vitro, whereas the *Xenopus* oocyte tubulin was phosphorylated neither in ovo nor in vitro (not shown). In contrast, numerous non-tubulin bands were found to be phosphorylated (fig.3, lane 1); among them, the 2 HMM proteins may correspond to the 2 HMM ^{35}S-labeled MAPs shown in fig.2, lanes 2–4. After heat treatment of the 1 M NaCl extract of ^{32}P-labeled microtubules, the thermostable oocyte proteins again copolymerized with first cycle rat brain tubulin (fig.3, lane 2). The preparation appears to be enriched in 2 main HMM phosphoproteins. Two low molecular mass phosphoproteins were also present, since they were not observed after staining with Coomassie blue nor the ^{35}S autoradiographs (fig.2, lanes 2–4); they probably correspond to non-specific contaminants.

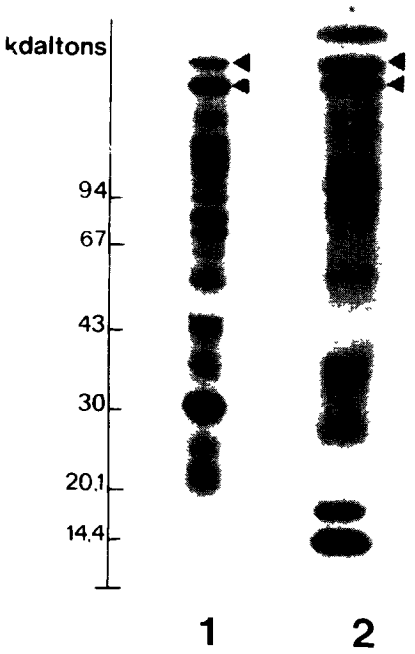

Fig.3. Autoradiographs of in ovo microtubule-associated phosphorylated proteins from *Xenopus* oocytes. Oocytes were preincubated for 16 h in [^{32}P]orthophosphate and taxol-stabilized microtubules were prepared as described in section 2. Lanes: 1, first microtubule pellet; 2, NaCl MAP extract, heat treated (100°C, 5 min) and then copolymerized with non-radioactive first cycle rat brain tubulin. Proteins corresponding to the same amount of oocytes (100) were loaded in each channel.

3.3. Xenopus *oocyte MAPs induce in vitro polymerization of rat brain 6 S tubulin*

The ability of oocyte MAPs to promote the assembly of the purified 6 S rat brain tubulin was

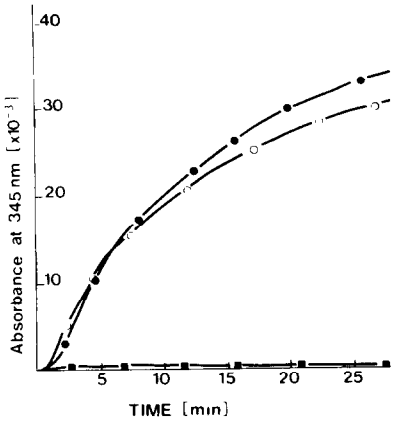

Fig.4. Turbidimetry time course of assembly of 6 S purified brain tubulin in the presence of oocyte MAP extract. The first microtubule pellet was obtained by the taxol procedure as described in section 2. Oocyte MAPs were dissociated from the microtubules by addition of 1 M NaCl. Rat brain 6 S purified tubulin (1 mg/ml) was incubated in the presence of MAP extract (0.2 mg/ml, ●—●), trypsin-treated MAP extract (0.2 mg/ml, ■—■) and heat-treated MAP extract (0.2 mg/ml, 100°C, 5 min, ○—○). For trypsin treatment, the MAP extract was incubated in the presence of trypsin (1 mg/ml) at 37°C during 45 min and then with soybean trypsin inhibitor (3 mg/ml).

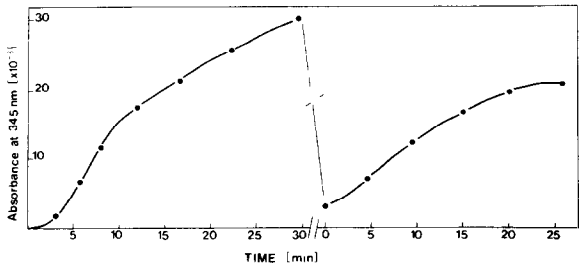

Fig.5. Turbidimetry time course of 2 cycles of 6 S purified brain tubulin assembly and disassembly in the presence of oocyte MAPs. Rat brain 6 S purified tubulin assembly (1 mg/ml) was monitored at 37°C in the presence of the MAP extract (0.2 mg/ml). After 30 min, the solution was chilled at 4°C for 45 min until the absorbance return at his initial value. Then the solution was warmed again at 37°C.

tested. Oocyte MAPs from first taxol microtubule pellet were dissociated by exposure to 1 M NaCl and tested after extensive dialysis. As shown in fig.4, the oocyte MAPs induce tubulin polymerization. After heat treatment (100°C, 5 min) the thermostable MAPs were again able to promote tubulin assembly (fig.4). Since traces of taxol may remain present in this fraction, it was important to show that the tubulin assembly was due to proteins and not to remaining taxol. This possibility was excluded following treatment of the MAP extract with trypsin (fig.4). Furthermore, the microtubules assembled in vitro by the oocyte MAP extract could be disassembled by cold, and reassembled (fig.5). This again indicates that microtubule assembly was not induced by taxol, since taxol microtubules are cold stable [20].

4. DISCUSSION

Microtubules were isolated from [^{35}S]methionine-labeled prophase blocked *Xenopus* oocytes with the use of taxol. In addition to tubulin, these microtubules contain 4 other protein components which can be dissociated from microtubules by 1 M NaCl treatment and appear to be thermostable; among them are 2 HMM proteins (200–300 kDa) which are phosphorylated in ovo. Preliminary experiments performed with monospecific antibodies raised against rat brain MAPs (gift of Dr A. Fellous, Bicetre, France) strongly suggest that the oocyte salt extract contains proteins sharing antigenic determinants with rat brain HMM proteins (not shown). When the salt extract was further added to purified (6 S) rat brain tubulin, the 4 thermostable proteins promoted tubulin assembly and cosedimented with brain microtubules. It seems therefore reasonable to assume that the protein components isolated from oocyte microtubules correspond to MAPs.

It is interesting that the MAPs we have identified were originally isolated from prophase blocked oocytes (stage VI [15]). When taxol or centrosomes were microinjected into these oocytes, no cytoplasmic microtubules were induced; in contrast, when these components are microinjected into eggs (mature metaphase II oocytes), they induced the formation of numerous asters [7,8,11]. Our results show that although tubulin and MAPs are present in prophase blocked oocytes, they are incapable of assembling in ovo into microtubules. This observation shows that the critical concentration for microtubule assembly is high enough to prevent microtubule nucleation and elongation in the prophase blocked oocytes. As soon as the nuclear envelope breaks down, new microtubules appear at the basal part of the nucleus [9,10]. Are oocyte MAPs implicated during the formation of these new microtubules and during the organization of the meiotic spindles? To answer this question partially, we are now preparing antibodies against oocyte MAPs with the aim to identify and localize them by immunological methods. In recent experiments using monoclonal antibodies (gift of Dr M. De Brabander, Belgium), we observed that the *Xenopus* oocyte contains immunologically related proteins to rat brain MAP1, whose cellular distribution changes after the breakdown of the nuclear membrane (in preparation).

ACKNOWLEDGEMENTS

We acknowledge Dr E. Karsenti for help with the electron microscopy. This work was supported by grants from the Ministère de la Recherche et de l'Industrie, Centre National de la Recherche Scientifique and Institut National de la Santé et de la Recherche Médicale.

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