Purification and Characterization of a 190-kD Microtubule-associated Protein from Bovine Adrenal Cortex

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Abstract. A heat-stable microtubule-associated protein (MAP) with molecular weight of 190,000, termed 190-kD MAP, was purified from bovine adrenal cortex. This MAP showed the same level of ability to promote tubulin polymerization as did MAP2 and tau from mammalian brains. Relatively high amounts of 190-kD MAP could bind to microtubules reconstituted in the presence of taxol. At maximum 1 mol of 190-kD MAP could bind to 2.3 mol of tubulin. 190-kD MAP was phosphorylated by a cAMP-dependent protein kinase prepared from sea urchin spermatozoa and by protein kinase(s) present in the microtubule protein fraction prepared from mammalian brains. The maximal numbers of incorporated phosphate were ∼0.2 and ∼0.4 mol per mole of 190-kD MAP, respectively. These values were lower than that of MAP2, which could be heavily phosphorylated by the endogenous protein kinase(s) up to 5 mol per mole of MAP2 under the same assay condition. 190-kD MAP had no effects on the low-shear viscosity of actin and did not induce an increase in turbidity of the actin solution. It was also revealed that 190-kD MAP does not co-sediment with actin filaments. These data clearly show that, distinct from MAP2 and tau, this MAP does not interact with actin. Electron microscopic observation of the rotary-shadowed images of 190-kD MAP showed the molecular shape to be a long, thin, flexible rod with a contour length of ∼100 nm. Quick-freeze, deep-etch replicas of the microtubules reconstituted from 190-kD MAP and brain tubulin revealed many cross-bridges connecting microtubules with each other.

MICROTUBULES prepared by repeated cycles of temperature-dependent polymerization and depolymerization contain, in addition to tubulin, several accessory proteins termed microtubule-associated proteins (MAPs)[1]. Brain tissues are rich in tubulin and contain a larger amount of MAPs than do other tissues and organs. Therefore most of the MAPs that have been purified and characterized well are those from mammalian brains (Dustin, 1984; Vallee et al., 1984). Brain MAPs fall into three categories, namely MAP1, MAP2, and tau (Sloboda et al., 1976; Murphy et al., 1977; Cleveland et al., 1977). These three MAPs have been shown not only to decrease the critical concentration of tubulin for polymerization in vitro but also to bind to the reconstituted microtubules.

Immunohistochemical studies revealed that MAP2 and tau exist almost exclusively in the neuron, the former being located in the dendrite and cell body (Matus et al., 1981) and the latter in the axon (Binder et al., 1985). On the other hand, MAP1 or proteins that cross-react with anti–MAP1 antibodies were found to be present in a wide variety of cells and tissues (Bloom et al., 1984; Wiche et al., 1984; Sato et

al., 1985). Electron microscopic observation revealed that MAP1 and 2 are in the shape of a flexible thread (Voter and Erickson, 1982; Hirokawa et al., 1985) connecting microtubules with other cytoskeletal components or membranous organelles (Griffith and Pollard, 1978; Shelanski et al., 1981; Suprenant and Dentler, 1982; Hirokawa, 1982).

Several reports have been published on the isolation of MAPs from sources other than brain. For instance, MAPs with molecular weights of 200,000–220,000 were prepared from HeLa cells (Bulinski and Borisy, 1980a; Weatherbee et al., 1980). Conflicting results were presented on the identification of MAPs in other cell lines such as C_6 glioma cells (Nagle et al., 1977; Wiche et al., 1979), 3T3 cells (Nagle et al., 1977; Weber et al., 1977; Klein et al., 1978; Cleveland et al., 1979), neuroblastoma cells (Nagle et al., 1977; Olmsted and Lyon, 1981), and others (Solomon et al., 1979; Doenges et al., 1979), even though the same cell lines were used. MAPs from mammalian organs other than brain were detected by several authors (Berk and Hinkle, 1980, 1981; Bloom et al., 1985), but detailed analyses of those MAPs have not yet been performed.

Adrenal medulla, developmentally derived from the neural crest, has been used as a tool for neurochemical (Noda et al., 1982; Gubler et al., 1982) and neurophysiological

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1. *Abbreviations used in this paper*: MAP, microtubule-associated protein; MEM, 20 mM MES (pH 6.8)-1 mM EGTA-0.5 mM MgCl₂; PEM, 0.1 M Pipes (pH 6.8)-2 mM EGTA-1 mM MgCl₂.

studies (Livett, 1984; Livett et al., 1983). Several reports have shown that microtubules have an important role in the differentiation of chromaffin cells (Bader et al., 1981) or pheochromocytoma cells (Greene et al., 1983; Heidemann et al., 1985; Drubin et al., 1985) into a neuron-like shape mediated by treatment with nerve growth factor. It is also suggested that microtubules take part in the secretion in the adrenal gland (Magalhaes et al., 1985). To understand the roles of microtubules in these phenomena, it is important to characterize MAPs in the adrenal gland in detail. In the course of the study on MAPs in the bovine adrenal gland, we found that the medulla contained large amounts of MAP1 and small amounts of MAP2 and tau. In addition to these MAPs, adrenal medulla contained a new kind of MAP with a molecular weight of 190,000, which we termed 190-kD MAP. It was also revealed that this MAP was the major one in the adrenal cortex and that it was heat-stable like both MAP2 and tau in brain (Kotani et al., 1986). Judging from its molecular weight and heat stability, the 190-kD MAP appears to be a member of a class of widely distributed heat-stable MAPs of ~200,000 mol wt (Bulinski and Borisy, 1980a; Weatherbee et al., 1980).

In the present study, we succeeded in the purification of 190-kD MAP from bovine adrenal cortex using heat treatment, column chromatography, and co-sedimentation with brain tubulin. This is the first report on the purification of a non-neural MAP in milligram quantities. In this paper some characteristics of 190-kD MAP, including the promotion of tubulin polymerization, binding to reconstituted microtubules, phosphorylation by protein kinases, and the molecular shape observed by rotary shadowing and quick-freeze, deep-etch replica techniques, are presented.

Materials and Methods

Materials

Bovine adrenal glands and bovine and porcine brains were obtained from Teikoku Zoki Co. Ltd., Tokyo.

Taxol was a generous gift from Natural Product Branch, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health. DEAE-cellulose (DE 52) and Butyl-Toyopearl 650C were purchased from Whatman and Toyo Soda Co. Ltd. (Tokyo), respectively. Pipes, 2-(N-morpholino)ethane-sulfonic acid (MES), EGTA, phenylmethysulfonyl fluoride (PMSF), and adenosine 3':5'-cyclic monophosphate were from Sigma Chemical Co. (St. Louis, MO). Leupeptin and pepstatin were obtained from Peptide Institute Inc. (Osaka). GTP and ATP were from Yamasa Shoyu Co. (Choshi). [γ-^{32}P]ATP was purchased from ICN Radiochemicals (Div. ICN Biomedicals Inc., Irvine, CA).

Preparation of Microtubule Proteins from Mammalian Brains

Microtubule proteins were prepared from bovine or porcine brains by three cycles of temperature-dependent polymerization and depolymerization by the method of Shelanski et al. (1973). Tubulin was purified from the three-cycled microtubule protein preparation with the aid of phosphocellulose column chromatography according to the method of Weingarten et al. (1975). MAP2 and tau were prepared from the heat-supernatant fraction of the microtubule protein preparation by DEAE-cellulose column chromatography after Kotani et al. (1985).

Preparation of Actin

Rabbit skeletal muscle actin was prepared by the method of Spudich and Watt (1971) and further purified by gel filtration on a Sephadex G-100 column. The actin concentration was determined by ultraviolet light absorption measurement using $A_{290}^{1\%} = 6.5$.

Preparation of Heat-treated Extract of Bovine Adrenal Cortex

Unless otherwise indicated, all the procedures for the purification of 190-kD MAP were performed at 0–4°C. Bovine adrenal glands were removed and chilled on ice immediately after the slaughter of the animals and were brought to the laboratory within an hour. The cortex was carefully separated from the medulla with a surgical blade and was homogenized with a Waring blender in an equal volume of a buffer solution consisting of 0.1 M Pipes (pH 6.8), 2 mM EGTA, and 1 mM MgCl$_2$ (PEM) supplemented with 1 mM PMSF, 10 µg/ml leupeptin, and 10 µg/ml pepstatin. The homogenate was centrifuged at 40,000 g for 30 min, and the supernatant was centrifuged at 150,000 g for 1 h to obtain a crude extract. NaCl and 2-mercaptoethanol were added to the crude extract at final concentrations of 0.8 M and 1% (vol/vol), respectively, and the extract was heated at 100°C for 3 min. After chilling in an ice-water bath, the heat-treated extract was centrifuged at 12,000 g for 30 min to remove denatured proteins. The resultant supernatant was dialyzed overnight against 20 mM MES (pH 6.8)-1 mM EGTA-0.5 mM MgCl$_2$ (MEM) supplemented with 0.2 mM PMSF, 1 µg/ml leupeptin, and 1 µg/ml pepstatin to yield the heat-treated extract. The heat-treated extract containing ~800 mg of proteins was routinely prepared from 60 adrenal glands.

DEAE-Cellulose Column Chromatography

The heat-treated extract was applied to a DEAE-cellulose column (1.3 × 22 cm) equilibrated with MEM containing 0.2 mM PMSF, 1 µg/ml leupeptin, and 1 µg/ml pepstatin. After unadsorbed materials were washed out with the same buffer solution, a linear gradient of NaCl (from 0 to 0.5 M, total 200 ml) in MEM containing 0.2 mM PMSF, 1 µg/ml leupeptin, and 1 µg/ml pepstatin was applied collecting 5 ml of fractions. The content of 190-kD MAP in each fraction was checked by PAGE.

Hydrophobic Column Chromatography

All the procedures of hydrophobic chromatography were carried out at 4°C. DEAE-cellulose column fractions containing 190-kD MAP were combined and dialyzed against MEM containing 1 M ammonium sulfate, 0.2 mM PMSF, 1 µg/ml leupeptin, and 1 µg/ml pepstatin followed by centrifugation to remove insoluble materials. The supernatant containing ~20 mg of protein was applied to a hydrophobic column (Butyl Toyopearl 650C, 1 × 8 cm) equilibrated with the above buffer solution. After unadsorbed materials were washed out with the same buffer solution, adsorbed materials were eluted with a linear gradient of ammonium sulfate (from 1 to 0 M, total 42 ml) in MEM supplemented with 0.2 mM PMSF, 1 µg/ml leupeptin, and 1 µg/ml pepstatin. 1.5-ml fractions were collected.

Co-polymerization with Tubulin

Hydrophobic column fractions containing 190-kD MAP were combined and dialyzed against PEM containing 0.2 mM PMSF, 1 µg/ml leupeptin, and 1 µg/ml pepstatin. An equal volume of 5 mg/ml bovine brain tubulin in PEM containing 1 mM GTP was added to the dialyzed preparation (6 ml, 4 mg protein), and the mixture was incubated at 36°C for 30 min to reconstitute microtubules. The solution was layered onto a pre-warmed cushion consisting of 10% (wt/vol) sucrose, 0.5 mM GTP, 0.2 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and PEM, and the microtubles formed were collected by centrifugation at 30°C and 50,000 g for 20 min. Microtubules were disassembled by homogenization in 1 ml of PEM containing 0.5 mM GTP, 0.2 mM PMSF, 1 µg/ml leupeptin, and 1 µg/ml pepstatin at 0°C. Insoluble materials were removed by centrifugation at 100,000 g and 2°C for 30 min. The supernatant was supplemented with NaCl and 2-mercaptoethanol (final 0.8 M and 1% [vol/vol], respectively) and was heat-treated at 100°C for 3 min. After the solution was incubated at 0°C for 30 min, denatured proteins consisting mainly of tubulin were removed by centrifugation at 50,000 g for 20 min, leaving 190-kD MAP in the supernatant. The supernatant was dialyzed against PEM supplemented with 0.2 mM PMSF, 1 µg/ml leupeptin, and 1 µg/ml pepstatin to yield purified 190-kD MAP fraction (1.5 ml, 1 mg protein).

Turbidity Measurement of Microtubules

Microtubule assembly was monitored by measurement of the change in turbidity of the solution containing tubulin and MAP at 350 nm and 36°C using a Gilford 260 spectrophotometer (Gilford Instrument Laboratories, Inc.,

Oberlin, OH) as described (Kotani et al., 1984). PEM containing 0.5 mM GTP was used as a buffer solution for the microtubule assembly.

Co-Sedimentation of 190-kD MAP with Microtubules

Various concentration of 190-kD MAP was incubated with tubulin purified from bovine brain at 36°C for 20 min. The buffer solution used was the same as that of the turbidimetric assay of microtubule assembly with the exception that 20 μM taxol was supplemented. After incubation microtubules formed were precipitated by centrifugation at 35,000 g and 2°C for 20 min, followed by electrophoresis and protein determination of the supernatants and precipitates.

Low-Shear Viscometry of Actin

Low-shear viscosity of an actin solution was measured by the falling ball viscometric technique developed by MacLean-Fletcher and Pollard (1980). PEM was used for the solution of actin polymerization.

Co-Sedimentation of 190-kD MAP with Actin Filaments

0.3 mg/ml of skeletal muscle actin was incubated with 0.1 mg/ml of 190-kD MAP in the same buffer condition as in the viscosity experiment. After incubation of the solutions at 25°C for 40 min, polymerized actin was recovered by centrifugation at 120,000 g for 60 min, followed by an analysis of the proteins in the supernatants and precipitates by electrophoresis.

Electrophoresis

PAGE in the presence of SDS was carried out by the method of Laemmli (1970) using 7.5% acrylamide gels with the following molecular weight markers: rabbit skeletal muscle myosin heavy chain (200,000), rabbit skeletal muscle phosphorylase b (94,000), bovine serum albumin (BSA; 68,000), bovine brain tubulin (55,000), rabbit skeletal muscle actin (42,000), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (35,000), and bovine erythrocyte carbonic anhydrase (29,000). Gels were stained with Coomassie Brilliant Blue R-250.

Phosphorylation of 190-kD MAP

Phosphorylation of 190-kD MAP was performed using a cAMP-dependent protein kinase purified from sea urchin spermatozoa after Ishiguro et al. (1982) and protein kinases that co-purify with brain microtubule proteins (Sloboda et al., 1975; Theurkauf and Vallee, 1982; Burns et al., 1984). Adrenal 190-kD MAP (0.15 mg/ml) was incubated at 30°C for 15 min with 80 U/ml of sperm protein kinase or 0.5 mg/ml of three-cycled bovine brain microtubule proteins in a buffer solution consisting of 50 mM Pipes (pH 6.8), 1 mM EGTA, 4 mM MgCl₂, and 0.05 mM [γ-³²P]ATP (0.32

Ci/mmol). The reaction was terminated by the addition of an equal volume of 0.125 M Tris (pH 6.8)-4% (wt/vol) SDS-20% (vol/vol) glycerol-10% (vol/vol) 2-mercaptoethanol immediately followed by heating the samples at 100°C for 1 min. Phosphorylated proteins were analyzed by electrophoresis and autoradiography.

Protein Determination

The amount of proteins was determined according to the method of Lowry et al. (1951) using BSA as a standard.

Electron Microscopy

Low angle rotary shadowing of 190-kD MAP was performed according to the method of Tyler and Branton (1980). 0.1 mg/ml of 190-kD MAP was mixed with 3/7 vol of glycerol, and the mixture was sprayed onto pieces of freshly cleaved mica. The mica sheets were dried in vacuo at room temperature for 5 h. Platinum-carbon was then evaporated onto the mica by an electron beam gun at 5° followed by an overcoating of carbon.

Microtubules were assembled by incubating purified 190-kD MAP and bovine brain tubulin in PEM containing 0.5 mM GTP at 36°C for 20 min. The assembled microtubules were collected by centrifugation at 100,000 g and 30°C for 20 min. Quick-freeze, deep-etch replicas of the microtubules were prepared according to the method of Hirokawa and Heuser (1981). Electron micrographs were taken using a JEOL 1200 EX electron microscope.

Results

Purification of 190-kD MAP

It has been shown in the previous paper that the major MAP in the bovine adrenal cortex was 190-kD MAP (Kotani et al., 1986). It was also revealed that this MAP was heat-stable and that the contents of the other heat-stable MAPs, MAP2 and tau, in the cortex were lower than those in the medulla. Therefore cortices were used for the source of 190-kD MAP. As a starting material 60 adrenal glands were routinely used, from which ∼400 g of cortices were obtained. The heat stability of adrenal 190-kD MAP was used for the first step of the purification. Heat treatment could remove more than 80% of the proteins in the crude extract. Electrophoresis of the heat-treated extract showed that 190-kD MAP was a minor though distinct electrophoretic species (Fig. 3, lane 2). This band co-precipitated with bovine brain tubulin upon in-
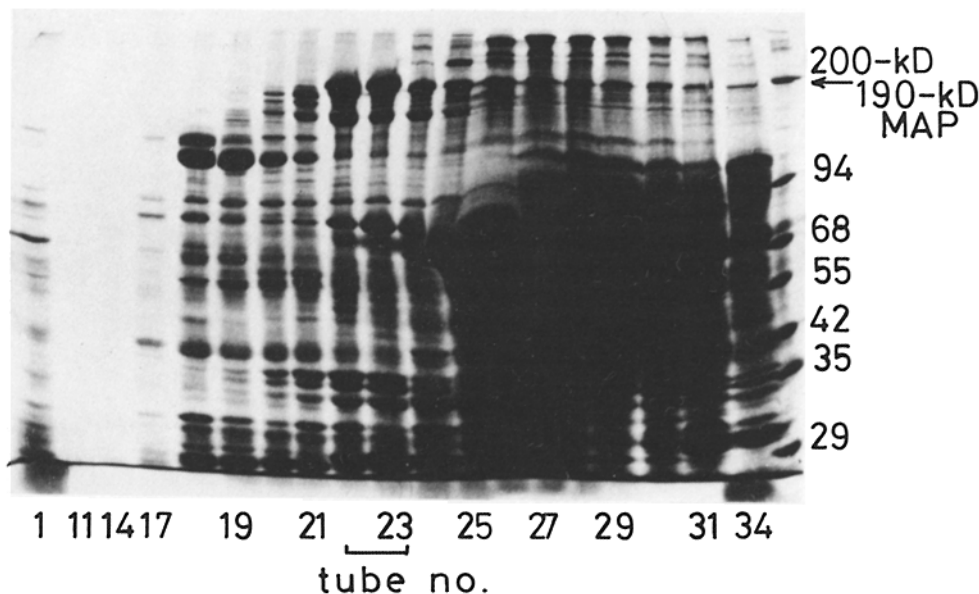

Figure 1. DEAE-cellulose column chromatography of heat-treated extract of bovine adrenal cortex. Heat-treated extract of adrenal cortex was chromatographed on a DEAE-cellulose column. 30 μl each of the fractions was subjected to electrophoresis. The bracket indicates the position of 190-kD MAP.
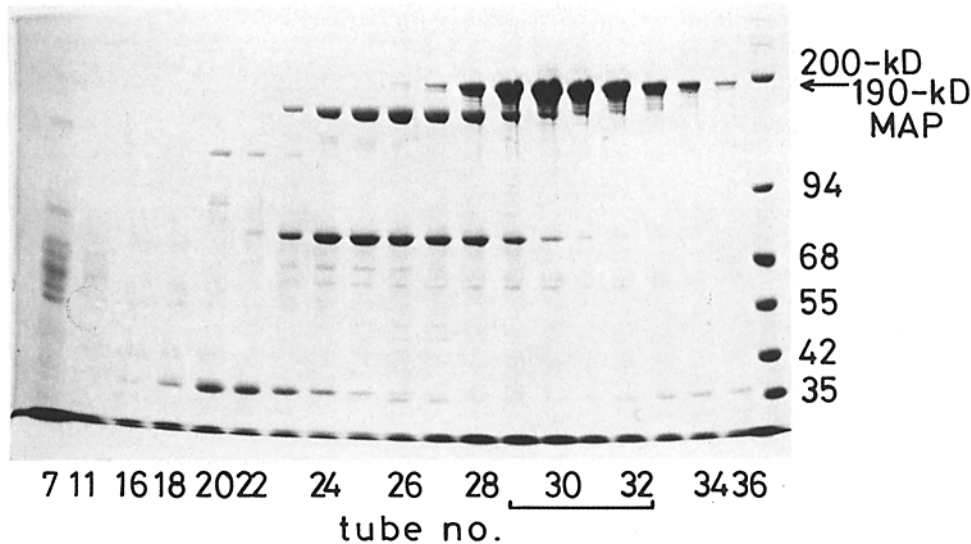
Figure 2. Butyl Toyopearl hydrophobic column chromatography of the DEAE-cellulose fraction. The DEAE-cellulose fraction was chromatographed on a Butyl Toyopearl column. Adsorbed materials were eluted with a linear gradient of ammonium sulfate. 20 μl from each tube were used for electrophoresis. The bracket indicates the position of 190-kD MAP.

cubation at 36°C (data not shown), and therefore the content of 190-kD MAP in the fractions resulting from subsequent chromatography was monitored by electrophoresis.

When heat-treated extract was chromatographed on a DEAE-cellulose column, 190-kD MAP was eluted with ∿0.15 NaCl (Fig. 1, tubes *22* and *23*). Many of the major polypeptides present in the heat-treated extract were removed in this step. Immunoblot analysis using anti–tau antiserum (Kotani et al., 1986) revealed that a small amount of tau, which could not be detected well by protein staining of the gel, was eluted from the DEAE-cellulose column along with 190-kD MAP.

The DEAE-cellulose fraction was dialyzed against 1 M ammonium sulfate for the next chromatography. 190-kD MAP did not precipitate in the presence of 1 M ammonium sulfate. When the fraction was applied to a Butyl-Toyopearl column at 4°C, most of the proteins were absorbed to the column. 190-kD MAP was eluted from the column with a linear gradient of ammonium sulfate from 1 to 0 M (Fig. 2). Tau proteins, which had contaminated the DEAE-cellulose fraction in a small amount, were removed in this step, being eluted at a higher concentration of ammonium sulfate (tube 7) than for 190-kD MAP. Tubes 29–32 were pooled and dialyzed against PEM to facilitate subsequent purification by in vitro microtubule assembly.

When the Butyl-Toyopearl fraction was mixed with bovine brain tubulin free from brain MAPs and was incubated at 36°C, an increase in turbidity of the solution was observed, showing that microtubule assembly occurred from brain tubulin and adrenal 190-kD MAP. The microtubules formed were precipitated by centrifugation through a sucrose cushion. Reconstituted microtubules were depolymerized by homogenization with ice-cold PEM containing 0.5 mM GTP. A small amount of cold-insoluble material was removed by centrifugation. This temperature-dependent polymerization and depolymerization removed all the contaminating proteins present in the Butyl-Toyopearl fraction. A large amount of tubulin present in the cold supernatant was removed by heat treatment of the fraction in the presence of 0.8 M NaCl and 1% (vol/vol) 2-mercaptoethanol and subsequent centrifugation. About 1 mg of purified 190-kD MAP was routinely obtained from 400 g of adrenal cortices.

The electrophoretic pattern of each fraction obtained in the course of the purification of 190-kD MAP from bovine adrenal cortex is shown in Fig. 3. Lane *5* shows that the purified 190-kD MAP fraction is almost homogeneous with a slight contamination of polypeptides with molecular weights <190,000. It was shown that they reacted with an antiserum raised against electrophoretically purified 190-kD MAP (Kotani et al., 1986) (data not shown). Moreover the staining intensities of the lower molecular weight bands increased with aging of the MAP preparation. These results strongly suggest that these polypeptides are proteolytic products of 190-kD MAP. Complete inhibition of the degradation of 190-kD MAP was not achieved even in the presence of three kinds of protease inhibitors, namely PMSF, leupeptin, and pepstatin. Immunoblot analysis using antisera against MAP1,
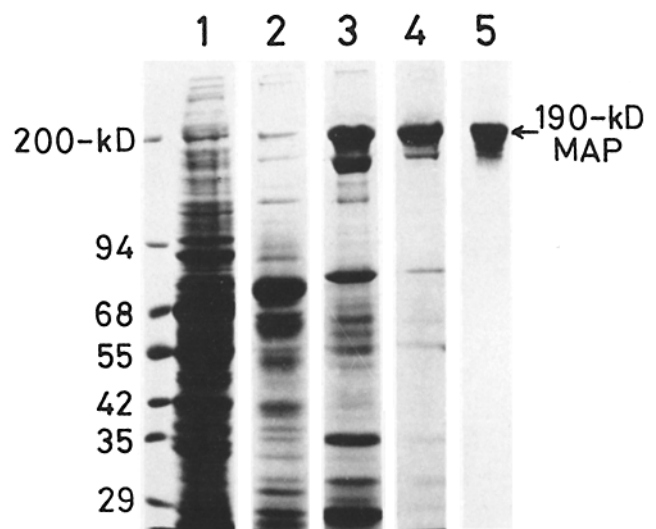

Figure 3. Electrophoresis of proteins in the fractions obtained in the course of the purification of 190-kD MAP. Lane *1*, crude extract of bovine adrenal cortex; lane *2*, heat-treated extract; lane *3*, DEAE-cellulose column chromatography fraction; lane *4*, hydrophobic column chromatography fraction; lane *5*, 190-kD MAP purified by co-polymerization with brain tubulin and subsequent heat treatment.
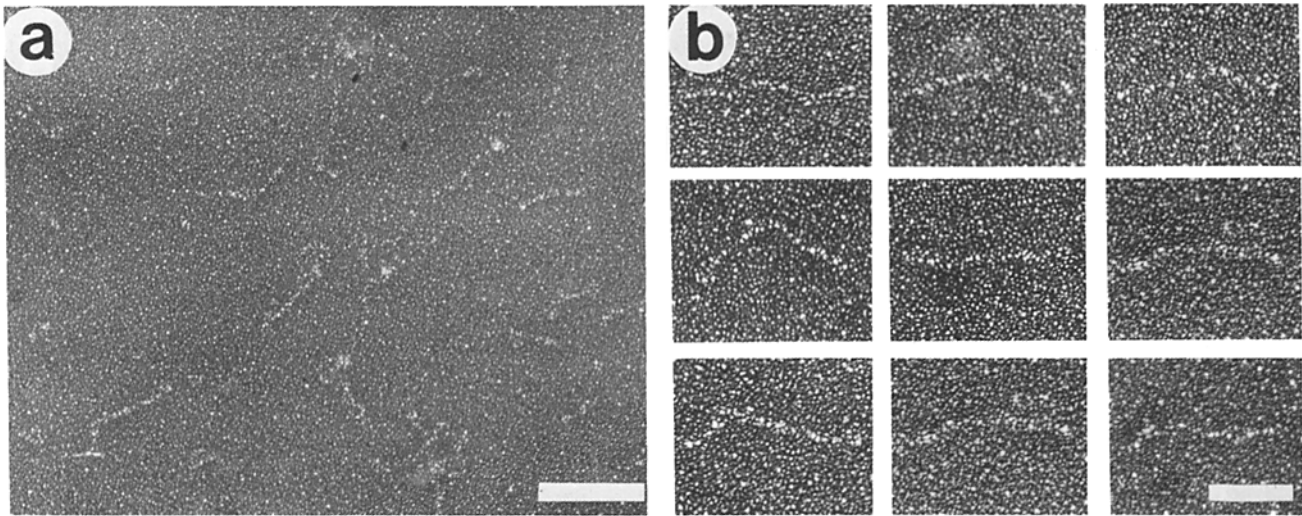
Figure 4. Rotary-shadowed images of purified 190-kD MAP. (*a*) Electron micrograph of 190-kD MAP. Bar, 100 nm. (*b*) Higher magnification images of the typical 190-kD MAP. Bar, 50 nm.

MAP2, and tau from mammalian brain (Kotani et al., 1986) revealed that the purified 190-kD MAP fraction was not contaminated by polypeptides that react with these antisera (data not shown).

Properties of 190-kD MAP

Polymerization of tubulin was promoted by 190-kD MAP with a linear relationship between the concentration of 190-kD MAP and the mass concentration of assembled microtubules measured by turbidimetry. Apparent critical concentration of 190-kD MAP for polymerization of 1.5 mg/ml tubulin was 57 μg/ml with nucleating activity close to those of MAP2 and tau.

Binding of 190-kD MAP to microtubules in the presence of taxol was demonstrated by the centrifugation assay. Electrophoretic analysis revealed that at most ∼1 mol of 190-kD MAP bound to 2.3 mol of tubulin in microtubules.

190-kD MAP was phosphorylated by cAMP-dependent protein kinase purified from sea urchin sperm in a cAMP-dependent fashion as well as by protein kinases present in the brain microtubule protein fraction (Sloboda et al., 1975; Vallee et al., 1981; Theurkauf and Vallee, 1982; Burns et al., 1984) with respective incorporation values of 0.2 and 0.4 mol at maximum. This was in contrast to a 5 mol incorporation for MAP2.

Brain MAPs interact with actin in vitro to form a gel or bundles of actin filaments (Griffith and Pollard, 1978; Kotani et al., 1985). In contrast, 190-kD MAP did not interact with actin filaments, showing no effect on the low-shear viscosity of an actin solution measured by the falling ball technique (MacLean-Fletcher and Pollard, 1980), no activity to bundle actin filaments measured by turbidity change at 320 nm (Kotani et al., 1985), and no co-sedimentation with actin filaments.

Electron Microscopic Observation of 190-kD MAP

Molecular shape of the purified adrenal 190-kD MAP was examined with the rotary shadowing technique. Fig. 4 *a* shows the electron micrograph of 190-kD MAP. Typical molecular shapes of the MAP at higher magnification are presented in *b*. From these figures it is apparent that the adrenal 190-kD MAP had a long, thin, flexible structure. The contour length of the molecule was calculated to be ∼100 nm.

Electron Microscopic Observation of Microtubules Reconstituted from 190-kD MAP and Brain Tubulin

Microtubules were reconstituted from adrenal 190-kD MAP and brain tubulin and were collected by centrifugation. Quick-freeze, deep-etch replicas of the microtubules were prepared and were visualized by electron microscopy (Fig. 5). Many projections extending outward from the microtubule wall are clearly seen. Adjacent microtubules appear to be cross-connected by these projections. It is considered that the projection is 190-kD MAP itself because no projections can be observed in the quick-freeze, deep-etch replica image of the microtubules reconstituted from tubulin without MAPs with the aid of taxol (Hirokawa et al., 1985). Distance between the neighboring projections on the same microtubule was 40–60 nm, and the average length of the projection which apparently cross-connects microtubules was ∼30 nm (Fig. 5).

Discussion

In the present study we developed the method for the purification of a non-neural MAP in milligram quantities. Our previous study revealed that the adrenal gland contains high amounts of MAP (Kotani et al., 1986). It was also shown that the major MAP in the adrenal cortex was a new kind of MAP with a molecular weight of 190,000 and that the contents of other MAPs such as MAP1, MAP2, and tau were very low, which makes the purification of 190-kD MAP easier. Another advantage of the adrenal for the purification of non-neural MAPs is that a large amount of the tissue can be obtained more easily than other sources that have been used, e.g., HeLa cells (Bulinski and Borisy, 1980; Weatherbee et al., 1980), thyroid (Berk and Hinkle, 1980, 1981), and anterior pituitary gland (Bloom et al., 1985).

It was revealed in this study that the adrenal 190-kD MAP had characteristics both similar to and distinct from brain
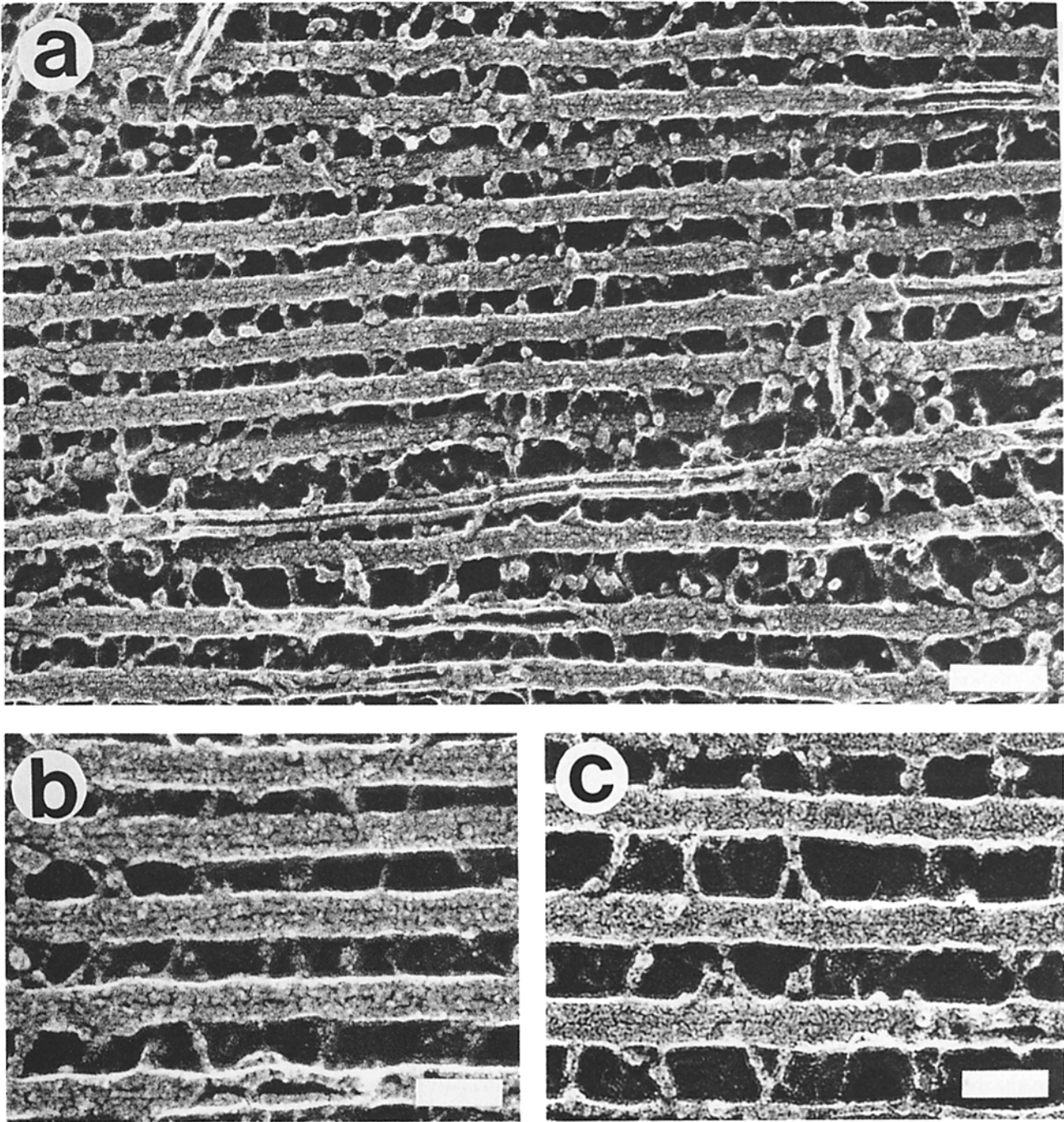
Figure 5. Electron micrographs of quick-freeze, deep-etch replicas of microtubules reconstituted from 190-kD MAP and brain tubulin. 1.5 mg/ml of brain tubulin was incubated with 0.3 mg/ml of 190-kD MAP. After collecting microtubules by ultracentrifugation, quick-freeze, deep-etch replicas of the microtubule pellet were made. (*a*) Lower magnification image. Bar, 100 nm. (*b* and *c*) Higher magnification images. Bar, 50 nm.

MAPs. It has been shown in a previous report (Kotani et al., 1986) that the 190-kD MAP is immunologically distinct from MAP1, MAP2, and tau. Like MAP2 and tau but unlike MAP1, this MAP is heat-stable, and this property was exploited in the purification. In this study the advantage of this property was taken for the purification of the MAP. 190-kD MAP was shown to have the ability to promote polymerization of brain tubulin with about the same efficiency as do MAP2 and tau. The maximal molar ratio of 190-kD MAP to tubulin in the reconstituted microtubules was calculated

to be ∼1:2.3. This value is higher than that of MAP2 (1:9 in Kim et al., 1979; 1:8 in Herzog and Weber, 1978), strongly suggesting that 190-kD MAP is multimer. It has been shown that HeLa MAP of ∼200-kD exists as a dimer in a native form (Bulinski and Borisy, 1980*a*).

It was shown that 190-kD MAP was phosphorylated by a cAMP-dependent protein kinase purified from sea urchin spermatozoa or protein kinase(s), which co-purify with brain microtubule proteins. The number of phosphorylated sites in 190-kD MAP was smaller than that of MAP2, which

could be phosphorylated up to 5 mol per mole of MAP2 by protein kinases in the microtubule protein fraction in the same assay condition. However, the possiblity still remains that most of the sites in 190-kD MAP had already been occupied by phosphate before purification.

It has been demonstrated that MAP2 and tau interact not only with microtubules but also with actin filaments (Griffith and Pollard, 1978; Kotani et al., 1985). The former greatly enhances the low-shear viscosity of actin filaments with "critical gelling concentration" of ∼0.1 mg/ml (Kotani et al., 1985). Tau, which has a gelling activity lower than MAP2, enhances the turbidity of actin solution by bundling a part of actin filaments (Kotani et al., 1985). These data have led to the hypothesis that those MAPs have a role in cross-linking microtubules with actin filaments to construct a structure of higher order in vivo. In the present study it was shown that 190-kD MAP did not cross-link actin filaments to form a gel or bundles. It was also shown that 190-kD MAP did not co-sediment with actin. These results clearly indicate that 190-kD MAP has no binding sites for actin. In this respect adrenal 190-kD MAP is distinct from MAP2 and tau.

The rotary-shadowing technique revealed that adrenal 190-kD MAP has a long, thin, flexible structure with a contour length of ∼100 nm (Fig. 4). Using the same technique Voter and Erickson (1982) reported that MAP2 prepared from porcine brains had a similar thread-like structure with a length of ∼185 nm at the maximum, which is about twice as large as that of adrenal 190-kD MAP. On the other hand Gottlieb and Murphy (1985) reported that MAP2 has a contour length of 90 ± 30 nm. Hirokawa (1986) also reported about the same length (104 ± 22 nm) on a MAP2-like 270-kD protein prepared from the crayfish peripheral nerve axon. These values are similar to that of bovine adrenal 190-kD MAP.

Electron microscopic observation of quick-freeze, deep-etch replicas of microtubules reconstituted from adrenal 190-kD MAP and brain tubulin revealed the presence of many long, thin rods, which are considered to be a part of 190-kD MAP, projecting outward from the microtubule wall. From the limited proteolytic digestion of MAP2, Vallee (1980) suggested that at least ∼⅓ of the total length of the molecule is attached to the microtubules, leaving the rest of the molecule extending from the microtubule wall. According to Voter and Erickson (1982), rotary-shadowed images of the microtubules reconstituted from tubulin and MAP2 revealed that about half of the MAP2 molecule was extended outward from the microtubule wall. These data indicate that MAP2 is composed of two domains, one that binds to microtubules and the other that extends from the microtubule wall. This seems also to hold true for 190-kD MAP. The actual length of the projecting domain of 190-kD MAP, however, cannot be calculated from our data, because our electron microscopic images show apparent cross-bridging of microtubules by 190-kD MAP. It can be said, however, that the length of the projecting domain is longer than 30 nm, which is the length of the projections between adjacent microtubules (Fig. 5).

Similar apparent cross-linking of microtubules by MAPs was observed using thin sections (Kim et al., 1979; Herzog and Weber, 1978) and quick-freeze, deep-etch replicas (Hirokawa et al., 1985; Hirokawa, 1986) of the ultracentrifugal pellet of microtubules. The average length of the cross-bridges was reported to be ∼30 nm in the microtubules reconstituted from calf brain microtubule proteins (Hirokawa et al., 1985) and those from crayfish axonal 270-kD MAP and rat brain tubulin (Hirokawa, 1986). This value is similar to the length of the cross-bridge observed in the microtubules reconstituted from bovine adrenal 190-kD MAP and brain tubulin.

We do not consider that bundling of microtubules reconstituted from tubulin and the 190-kD MAP occurs in suspension, because no bundles were detected in unfixed suspensions of reconstituted microtubules under a light microscope equipped with a dark-field condenser and a high-voltage Hg arc lamp (Murofushi et al., 1983). Also, in support of this view no abnormal turbidity of the microtubule suspension was observed during turbidimetric assay of 190-kD MAP-induced microtubule assembly. Moreover, no bundling of microtubules by 190-kD MAP was detected by electron microscopy of negatively stained microtubules after dilution and fixation with a Pipes-based reassembly buffer containing glutaraldehyde.

There are two possibilities that explain the apparent cross-bridging of microtubules by the 190-kD MAP when the quick-freeze, deep-etch replicas of the microtubule pellet were observed. One is that the projecting part of the 190-kD MAP only attaches to a neighboring microtubule when microtubules are forced together into a pellet by centrifugation. In this case there is no need to postulate the presence of two microtubule-binding sites on a single molecule of the 190-kD MAP. However, another possibility is that the 190-kD MAP possesses two binding sites for microtubules, one with higher affinity which serves to bind the MAP to the microtubule even at lower polymer concentrations in suspension, and the other with a lower affinity being detectable only at a very high concentration of microtubules like in the pellet after centrifugation. We cannot distinguish these two possibilities only from electron microscopic observations of microtubules reconstituted from tubulin and 190-kD MAP. However, the quick-freeze, deep-etch studies of the axonal cytoskeleton in vivo indicated that there are cross-bridges ∼30-nm long between microtubules and that they could be composed of MAP1 (Hirokawa et al., 1985) or 270-kD MAP (Hirokawa, 1986). These data favor the latter possibility. Detailed biochemical studies are necessary to determine the number of binding sites by which 190-kD MAP attaches to microtubules and thus resolve this question.

There are several reports on the presence of MAPs having molecular weights of ∼200,000 in several kinds of cells and tissues. It has been reported that HeLa cells contain a MAP with a molecular weight of 200,000–220,000 which are heat-stable (Weatherbee et al., 1982). MAP4 consisting of three polypeptides of 215,000–240,000 mol wt isolated from neuroblastoma is also reported to be heat-stable (Parysek et al., 1984a). A heat-stable 210,000-mol-wt MAP was found in cultured sympathetic neurons (Peng et al., 1986). Huber et al. (1985) detected a doublet of polypeptide bands with a molecular weight of ∼180,000 which reacts with a monoclonal antibody raised against brain microtubule proteins. Several bands at molecular weights of 200,000–220,000 were detected in the MAP preparation prepared from anterior pituitary gland (Bloom et al., 1985). Immunohistochemical experiments using an antibody raised against HeLa MAP revealed that polypeptides that reacted with this antibody were present in many kinds of cells and tissues (Bulinski and

Borisy, 1980b). The same results were obtained using an antibody against MAP4 (Parysek et al., 1984b). We have obtained data showing that polypeptides with molecular weights of ~190,000 which reacted with an antiserum raised against bovine adrenal 190-kD MAP were present in bovine brain, pituitary gland, liver, kidney, adrenal medulla, adrenal cortex (Kotani et al., 1986), and several lines of cultured cells of human and rodent origins (manuscript in preparation). Our data along with that of others may lead to the conclusion that those MAPs having molecular weights of ~200,000, which are presumably heat-stable, can be classified as a family of proteins that exist in a wide variety of cells and tissues.

The function of 190-kD MAP in the adrenal gland is not known at present. It is well known that both adrenal cortex and medulla are typical secreting organs. One possible role of this MAP may be related to secretion. Further studies with the aid of immunohistochemical, electron microscopic, and pharmacological techniques are necessary to have an insight into the functions of 190-kD MAP in the adrenal cells and in other cells.

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