Limited Chymotryptic Digestion of Bovine Adrenal 190,000-\textit{M}_r Microtubule-associated Protein and Preparation of a 27,000-\textit{M}_r Fragment Which Stimulates Microtubule Assembly*

(Received for publication, October 7, 1986)

Hiroyuki Aizawa, Hiromu Murofushi, Susumu Kotani, Shin-ichi Hisanaga, Nobutaka Hirokawa, and Hikoichi Sakai;

From the Department of Biophysics and Biochemistry, Faculty of Science and the Department of Anatomy, School of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan.

A heat stable microtubule-associated protein of \textit{M}, 190,000 (190-kDa MAP) has been purified from bovine adrenal cortex (Murofushi, H., Kotani, S., Aizawa, H., Hisanaga, S., Hirokawa, N., and Sakai, H. (1986) J. Cell Biol. 103, 1911-1919). Limited chymotryptic digestion of 190-kDa MAP produced a fragment of \textit{M}, 27,000 (27-kDa fragment), which bound to microtubules reconstituted in the presence of taxol. This fragment was purified with the aid of cosedimentation with microtubules. The purified 27-kDa fragment showed an ability to stimulate tubulin polymerization in the absence of taxol. Electron microscopic observation of microtubules reconstituted from purified 27-kDa fragment and tubulin revealed that the microtubules were in the form of thick bundles and that lateral projections which can be seen in microtubules reconstituted from intact 190-kDa MAP and tubulin were not observed. These results indicate that 27-kDa fragment includes or is a part of microtubule-binding domain of 190-kDa MAP and that this fragment is active in stimulating microtubule assembly. Amino acid analysis revealed that the 27-kDa fragment was rich in lysine, proline, and alanine, the sum of these three being about 45% of the total amino acids and that the contents of methionine, tyrosine, phenylalanine, and histidine were very low. These data suggest that the microtubule binding domain of the 190-kDa MAP comprises an unique structure.

Microtubules prepared by cycles of assembly and disassembly in vitro contain tubulin and a number of microtubule-associated proteins (MAPs). 1 Today three classes of MAPs, i.e. MAP1 (300 kDa), MAP2 (280 kDa), and tau (55-70 kDa) were purified from mammalian brain and characterized (1-4). Many kinds of MAPs have been identified in several culture cells and organs other than brain (5-10). Among these MAPs, MAP2 has been well studied. It was reported that MAP2 had a flexible rod-like structure under electron microscopic observation (11, 12). Protease treatment revealed that MAP2 consisted of two domains, that is, microtubule binding domain and projection domain (12, 13). In addition, it was reported that limited chymotryptic digestion of MAP2 produced a fragment of \textit{M}, 35,000 (35-kDa fragment) which had an ability to promote microtubule assembly, and it was suggested that the fragment derived from the microtubule binding domain of MAP2 (13). This suggestion was supported by the fact that the addition of the antibody against 35-kDa fragment to the mixture of tubulin and MAP2 prevented the latter from stimulating tubulin polymerization (12).

Recently, we succeeded in the purification of a MAP with \textit{M}, 190,000 (190-kDa MAP) from bovine adrenal gland (14). 190-kDa MAP differs from well-studied MAPs such as MAP1, MAP2, and tau in molecular weight and immunological cross-reactivity (15). 190-kDa MAP is heat-stable and stimulates tubulin polymerization with almost the same efficiency as MAP2 and tau. It is interesting that in contrast to MAP2 and tau, 190-kDa MAP fails to interact with actin filament (14).

Electron microscopic observation of quick-freeze deep-etch replicas of microtubules reconstituted from purified 190-kDa MAP and tubulin revealed the presence of lateral projections, which are considered to be a part of 190-kDa MAP, attached to the microtubule wall (14). Because of the similarity of the electron microscopic pictures of microtubules made from 190-kDa MAP and tubulin to those of microtubules from MAP2 and tubulin, we speculated the presence of microtubule binding domain and projection domain in a single molecule of 190-kDa MAP.

In order to study the structure of 190-kDa MAP, we applied a method of limited chymotryptic digestion of the MAP and found a fragment of \textit{M}, 27,000 which had an ability to bind to microtubules. This fragment was purified and it was revealed that it stimulated microtubule assembly. We report these results in this paper.

EXPERIMENTAL PROCEDURES

Preparation of Microtubule Proteins—Microtubule proteins were prepared from bovine brains by the method of Shelanski et al. (16). Tubulin was purified from the microtubule protein fraction by the method of Weingarten et al. (17). The 190-kDa MAP was purified by the method of Murofushi et al. (14). MAP2 was purified from bovine brains by the method as previously described (18).

Chymotryptic Digestion—Chymotrypsin (type VII) was a product of Sigma. The concentration of 190-kDa MAP was adjusted to 0.25 mg/ml in a reassembly buffer solution consisting of 0.1 M Pipes (pH 6.8), 2 mM EGTA, and 1 mM MgCl2. Chymotrypsin was added at a concentration of 2.5 \textmu g/ml and digestion was carried out at 25°C. MAP2 at a concentration of 0.4 mg/ml in the reassembly buffer was digested with chymotrypsin (4 \textmu g/ml) at 25°C. The reaction was...
stopped by the addition of 100 mM phenylmethylsulfonyl fluoride (PMSF) in dimethyl formamide to a final concentration of 2 mM.

**Purification of 27-kDa Fragment**—Digestion of 190-kDa MAP was performed for 10 min according to the above-mentioned method. 190-kDa MAP (0.75 mg in 3 ml) digested with chymotrypsin (7.5 μg) for 10 min was mixed with purified tubulin (6.6 mg in 2 ml) and adjusted to an additional 0.5 mM and 20 μM taxol (20 μM taxol, from the Natural Product Branch, Division of Cancer Institute, MD). The mixture was incubated at 37°C for 30 min and the microtubules formed were centrifuged at 30,000 × g for 20 min at 4°C through a cushion consisting of 2 M NaCl, 0.5 mM GTP, and 1 mM PMSF, and centrifuged at 30,000 × g for 20 min. The supernatant was supplemented with NaCl and 2-mercaptoethanol to a concentration of 0.8 M and 1% (v/v), respectively, heat-treated at 100°C for 2.5 min, and then chilled at 0°C for 30 min. Precipitates mostly consisting of denatured tubulin were removed by centrifugation at 30,000 × g for 30 min. The supernatant was dialyzed against the reassembly buffer containing 0.1 mM PMSF followed by centrifugation, to yield the 27-kDa fragment fraction.

**Polymerization Assay**—Various amounts of 27-kDa fragment were mixed with purified tubulin in the reassembly buffer containing 0.5 mM GTP and 0.5 mM taxol at 37°C. Microtubule formation was monitored by measurement of the change in turbidity at 350 nm using a Gilford 280 spectrophotometer as described (19). Then the mixtures were centrifuged at 40,000 × g for 30 min at 37°C, and the proteins in the supernatants and precipitates were analyzed by electrophoresis.

**Protein Determination**—Protein concentration was determined by the method of Lowry et al. (20) using bovine serum albumin as a standard.

**Electron Microscopy**—In order to observe negatively stained microtubules, the mixture of tubulin (1.5 mg/ml) and 27-kDa fragment (0.06 mg/ml) was incubated and microtubules formed were fixed with the addition of 20 volumes of 1% glutaraldehyde in the reassembly buffer. Aliquots were mounted on Formvar-coated grids and negatively stained with 1.5% uranyl acetate. Observations were performed with a Hitachi HU-11E electron microscope.

**Identification of Microtubule-binding Fragment of the 190-kDa MAP**—Limited chymotryptic digestion of purified 190-kDa MAP (Fig. 1a) for a short time produced fragments of M<sub>W</sub> 150,000–170,000, 120,000, 100,000, 70,000, 42,000, and 27,000 (Fig. 1b; Digest, lanes 0', 1', and 2'). Fragments of M<sub>W</sub> 36,000 and 32,000 appeared, and the amount of fragment of M<sub>W</sub> 27,000 increased with longer digestions (Fig. 1b; Digest, lanes 5', 10', and 20'). The 190-kDa MAP was partially digested even at time 0 (Fig. 1b; Digest, lane 0'). This was because proteolytic digestion proceeded to some extent before the complete inhibition of the enzyme by PMSF. When 1 μg of chymotrypsin which had been treated with 2 mM PMSF for 10 min at 0°C was added to 100 μg of 190-kDa MAP, no degradation of the MAP was observed (data not shown). After chymotryptic digestion was stopped, the samples were mixed with tubulin. The mixtures were incubated at 37°C in the presence of 0.5 mM GTP and 20 μM taxol, followed by centrifugation to collect microtubules. In the presence of taxol, most of the tubulin was precipitated by centrifugation along with peptide fragments which have affinity to microtubules. In the early stage of digestion (0–2 min), fragments of M<sub>W</sub> 150,000–170,000, 120,000, 100,000, 70,000, 42,000, and 27,000 cosedimented with microtubules (Fig. 1b; Pellet, lanes 0', 1', and 2'). Fragments of M<sub>W</sub> 120,000 did not cosediment with microtubules (Fig. 1b; Sup, lanes 0', 1', and 2'). In the later stage of digestion, the 42-kDa fragment fraction co-sediments with microtubules (Fig. 1b; Digest, lanes 5', 10', and 20'). They were then centrifuged at 40,000 × g for 30 min at 4°C. Supernatant fractions were removed, and the precipitates were suspended in a volume of the reassembly buffer equal to that of the supernatant. Aliquots of 30 μl from each sample were electrophoresed using 10% polyacrylamide gel (lanes labeled Digest). Tubulin (6.2 mg/ml, 18 μl) and taxol (final 20 μM) were added into each of digested MAP (82 μl) to make final concentrations of tubulin and fragments 1.1 mg/ml and 0.20 mg/ml, respectively. The mixtures were incubated at 37°C for 20 min to allow microtubules to assemble. They were then centrifuged at 40,000 × g for 30 min at 4°C. Supernatant fractions were removed, and the precipitates were suspended in a volume of the reassembly buffer equal to that of the supernatant. Aliquots of 30 μl from each sample of the supernatants (lanes labeled Sup) and precipitates (labeled Pellet) were electrophoresed. Times of chymotryptic digestion were presented on the top of the lanes. M<sub>W</sub> marker proteins. Molecular weights are in thousands.

**RESULTS**

**Electrophoresis of chymotryptic fragments of 190-kDa MAP which cosediment with microtubules.** a, electrophoretic pattern of undigested bovine adrenal 190-kDa MAP. 7.5% polyacrylamide gel was used. b, electrophoretic patterns of total chymotryptic fragments of the 190-kDa MAP and those which cosediment with microtubules in the presence of taxol. Purified 190-kDa MAP at 0.25 mg/ml was digested with chymotrypsin at 2.5 μg/ml for 0, 1, 2, 5, 10, and 20 min. For time 0 sample PMSF was added just after the addition of chymotrypsin. Aliquots of 30 μl from each sample were electrophoresed using 10% polyacrylamide gel (lanes labeled Digest). Tubulin (6.2 mg/ml, 18 μl) and taxol (final 20 μM) were added into each of digested MAP (82 μl) to make final concentrations of tubulin and fragments 1.1 mg/ml and 0.20 mg/ml, respectively. The mixtures were incubated at 37°C for 20 min to allow microtubules to assemble. They were then centrifuged at 40,000 × g for 30 min at 4°C. Supernatant fractions were removed, and the precipitates were suspended in a volume of the reassembly buffer equal to that of the supernatant. Aliquots of 30 μl from each sample of the supernatants (lanes labeled Sup) and precipitates (labeled Pellet) were electrophoresed. Times of chymotryptic digestion were presented on the top of the lanes. M<sub>W</sub> marker proteins. Molecular weights are in thousands.

**FIG. 1.** Electrophoresis of chymotryptic fragments of 190-kDa MAP which cosediment with microtubules. a, electrophoretic pattern of undigested bovine adrenal 190-kDa MAP. 7.5% polyacrylamide gel was used. b, electrophoretic patterns of total chymotryptic fragments of the 190-kDa MAP and those which cosediment with microtubules in the presence of taxol. Purified 190-kDa MAP at 0.25 mg/ml was digested with chymotrypsin at 2.5 μg/ml for 0, 1, 2, 5, 10, and 20 min. For time 0 sample PMSF was added just after the addition of chymotrypsin. Aliquots of 30 μl from each sample were electrophoresed using 10% polyacrylamide gel (lanes labeled Digest). Tubulin (6.2 mg/ml, 18 μl) and taxol (final 20 μM) were added into each of digested MAP (82 μl) to make final concentrations of tubulin and fragments 1.1 mg/ml and 0.20 mg/ml, respectively. The mixtures were incubated at 37°C for 20 min to allow microtubules to assemble. They were then centrifuged at 40,000 × g for 30 min at 4°C. Supernatant fractions were removed, and the precipitates were suspended in a volume of the reassembly buffer equal to that of the supernatant. Aliquots of 30 μl from each sample of the supernatants (lanes labeled Sup) and precipitates (labeled Pellet) were electrophoresed. Times of chymotryptic digestion were presented on the top of the lanes. M<sub>W</sub> marker proteins. Molecular weights are in thousands.
of digestion (5-20 min), only a fragment of M, 27,000 (27-
kDa fragment) cosedimented with microtubules (Fig. 1b; Pel-
let, lanes 5', 10', and 20'), and fragments of M, 36,000 and
32,000 did not (Fig. 1b; Sup, lanes 5', 10', and 20'). The 27-
kDa fragment appeared resistant against chymotryptic diges-
tion for 20 min.

In addition to the above-mentioned experiment of chymo-
trypsin treatment of free 190-kDa MAP, we carried out an-
other experiment of digesting 190-kDa MAP bound to micro-
tubules reconstituted from tubulin in the presence of taxol
(Fig. 2). Chymotrypsin-treated microtubules were collected
by centrifugation, followed by an analysis of supernatants
and precipitates by electrophoresis. The digestion pattern of
190-kDa MAP was not identical with that in Fig. 1. However,
a fragment of M, 27,000 which had an ability to bind to
microtubules was the most prominent one in this case, too
(Fig. 2; Pellet, lanes 4', 8', and 12'). This result is consistent
with previous work which reported that both chymotryptic
digestions of free MAP2 (13) and MAP2 bound to microtu-
bules (25) produced fragments of M, 27,000 which are consid-
ered to be derived from microtubule binding domain of MAP2.

To test whether the 190-kDa MAP and the 27-kDa frag-
ment occupy the same sites on the microtubule surface, the
following experiment was performed. The 190-kDa MAP
was digested by chymotrypsin for 10 min as described under
“Experimental Procedures.” Digested 190-kDa MAP was
mixed with purified tubulin and incubated in the presence of
20 μM taxol to allow microtubules to assemble. After incuba-
tion with various concentrations of intact 190-kDa MAP,
microtubules were collected by centrifugation, and the amount of
the 27-kDa fragment in the supernatants and precipitates
was determined by electrophoresis and densitometric scan-
ning (Fig. 3). In the absence of 190-kDa MAP, almost all
the 27-kDa fragment cosedimented with microtubules. As the
concentration of the 190-kDa MAP increased, the amount of
the 27-kDa fragment bound to microtubules decreased. At
that time the displaced 27-kDa fragments were recovered in
the supernatants. This result suggests that the 27-kDa frag-
ment occupies the same site(s) on the microtubule surface as
does the intact 190-kDa MAP molecule.

The 27-kDa fragment obtained from digestion of free 190-
kDa MAP can bind to microtubules and digestion of 190-kDa

![Fig. 2. Chymotryptic digestion of the 190-kDa MAP bound to microtubules reconstituted in the presence of taxol. Mixtures of 190-kDa MAP (0.25 mg/ml) and tubulin (1.0 mg/ml) were incubated at 37 °C for 20 min in the reassembly buffer containing 0.5 mM GTP and 20 μM taxol. Then the mixtures were treated with chymotrypsin (2.5 μg/ml) at 25 °C for 0, 4, 8, and 12 min. For time 0 sample PMSF was added just after the addition of chymotrypsin. They were then centrifuged at 40,000 × g for 30 min at 4 °C to collect supernatant fractions. Precipitates were suspended in equal volume of the reas-
sembly buffer. Aliquots of 20 μl from each sample of the supernatant (lanes labeled Sup) and precipitates (labeled Pellet) were electrophoresed. Times of chymotryptic digestion are presented on the top of the lanes. M, marker proteins. Molecular weights are in thousands.](image2)

![Fig. 3. Release of 27-kDa fragment from microtubules by the addition of undigested 190-kDa MAP. 190-kDa MAP (0.25 mg/ml) was digested by chymotrypsin (2.5 μg/ml) for 10 min. Digested 190-kDa MAP (50 μl) was mixed with purified tubulin (3.9 mg/ml, 9 μl) and incubated at 37 °C for 20 min in the presence of 20 μM taxol and 0.5 mM GTP to allow microtubule assembly. Various concentrations of undigested 190-kDa MAP (30 μl) were added to the microtubules and incubated at 37 °C for 20 min, followed by centrifugation at 40,000 × g for 30 min at 4 °C. The precipitates were electrophoresed, and the amount of the 27-kDa fragment bound to microtubules was measured by densitometer.](image3)
positions of bovine adrenal 190-kDa MAP and MAP2 are between microtubules (Fig. 4). Percentage of glutamine, alanine, and proline. High percentage of lysine, glutamic acid + glutamine and aspartic acid + asparagine gives a low average hydrophobicity to the 190-kDa MAP. The amino acid analysis of bovine brain MAP2 was very similar to that of porcine brain MAP2 (26). It is apparent from Table I that the amino acid composition of 190-kDa MAP resembles that of MAP2.

**Amino Acid Composition Analysis**—The amino acid compositions of bovine adrenal 190-kDa MAP and MAP2 are shown in Table I. The 190-kDa MAP is rich in glutamic acid + glutamine, alanine, and proline. High percentage of lysine, glutamic acid + glutamine and aspartic acid + asparagine gives a low average hydrophobicity to the 190-kDa MAP. The amino acid composition of bovine brain MAP2 was very similar to that of porcine brain MAP2 (26). It is apparent from Table I that the amino acid composition of 190-kDa MAP resembles that of MAP2.

On the other hand, the 27-kDa fragment has a higher percentage of lysine and arginine residues and a much lower percentage of glutamine + glutamic acid residue than the intact 190-kDa MAP (Table I). This suggests that the 27-kDa fragment derives from a very basic region of the 190-kDa MAP. It is interesting that the percentage of proline residue of both the 190-kDa MAP and the 27-kDa fragment is high. Especially in the 27-kDa fragment, the content of proline residue is as high as 14% of the total amino acid residues. The amino acid analysis of the 35-kDa fragment of MAP2 which derives from the microtubule binding domain of MAP2 was also performed to compare with the amino acid composition of the 27-kDa fragment (Table I). It is true that like the 27-kDa fragment, 35-kDa fragment is richer in arginine and poorer in glutamic acid + glutamine than intact MAP2. But the overall amino acid composition of 35-kDa fragment is not so similar to that of the 27-kDa fragment.

**DISCUSSION**

It is shown in the present paper that the 27-kDa fragment is a part of or includes microtubule binding domain of the 190-kDa MAP. Moreover, purified 27-kDa fragment is active in stimulating microtubule assembly in vitro. The microtubules reconstituted from 27-kDa fragment and tubulin were cold-sensitive like those from intact 190-kDa MAP and tubulin (data not shown). Under electron microscopic observation, quick-freeze deep-etch replica images of microtubules reconstituted from the 27-kDa fragment and tubulin revealed the absence of lateral projections which were observed in microtubules reconstituted from intact 190-kDa MAP and tubulin (Fig. 4, c, e, and d). No special structures which are considered to be the 27-kDa fragment were observed on the microtubule surface in the former case. It is considered that the 27-kDa fragment is too small to be detected by electron microscopy.

Electron microscopic observation of negatively stained samples showed that most of the microtubules assembled in the presence of the 27-kDa fragment were in the form of thick bundles (Fig. 5, a and b). Bundling of microtubules was confirmed by dark-field light microscopic observation of the unfixed samples (data not shown). On the contrary, microtubules reconstituted from intact 190-kDa MAP and tubulin did not show any bundles both under dark-field light microscopic observation and under electron microscopic observation (data not shown). When purified 27-kDa fragment (40 μg/ml: 0.15 μM) and tubulin (1.5 mg/ml) were mixed, less than half of total tubulin was polymerized (Fig. 4c, Sup, lane 6 and Pellet, lane 6) and the microtubules showed a turbidity of 1.0 at the plateau (Fig. 4b, curve 6). On the other hand, when intact 190-kDa MAP (280 μg/ml: 0.15 μM) and tubulin (1.5 mg/ml) were mixed, more than half of the total tubulin was polymerized, and the turbidity of the microtubules was 0.2. This five times higher turbidity of the former microtubules than the latter was derived from bundling of microtubules as was described in previous work (27, 28).

One possible explanation of the formation of microtubule bundles by 27-kDa fragment is as follows. In the case of microtubules consisting of intact 190-kDa MAP and tubulin, the projection domain of 190-kDa MAP inhibits tubulin-
Microtubule-binding Domain of 190-kDa MAP

FIG. 5. Electron microscopy of reconstituted microtubules. Microtubules were reconstituted from purified tubulin and purified 27-kDa fragment (a, b, and c) or tubulin and intact 190-kDa MAP (d). Samples for electron microscopy were prepared as described under “Experimental Procedures.” a and b, negatively stained samples. c and d, quick-freeze deep-etch replicas. Magnifications are × 15,000 (a), × 150,000 (b), × 134,000 (c and d).

It was revealed that amino acid composition of 190-kDa MAP is similar to that of MAP2 (Table I). It was also shown that the 27-kDa fragment has an unique composition (Table I). The 27-kDa fragment contains few aromatic amino acid residues: that might explain the resistance of the fragment against chymotryptic digestion. The 27-kDa fragment is rich in basic amino acids and poor in acidic amino acids. It was reported recently that tubulin interacts with MAPs on its carboxyl-terminal regions which were abundant in glutamic acid (29–32). Because the 27-kDa fragment is rich in lysine residue, microtubule binding domain of the 190-kDa MAP seems to interact with tubulin electrostatically. It was also suggested that tubulin polymerization and depolymerization might be modulated by the charge neutralization of tubulin carboxyl-terminal regions by polycationic microtubule binding domain of 190-kDa MAP. It is also considered that the presence of a high percentage of proline residue makes it difficult for the 27-kDa fragment to form ordered structure.

A chymotryptic fragment of MAP2 with  
\[ M, 35,000 \] (35-kDa fragment) has been identified as the microtubule binding domain of the MAP (12, 13). The molecular mass of this fragment is not so different from that of the 27-kDa fragment. In order to compare the 35-kDa fragment derived from MAP2 with the 27-kDa fragment of 190-kDa MAP, we analyzed the amino acid composition of 35-kDa fragment of MAP2. Like the 27-kDa fragment, the 35-kDa fragment has a higher percentage of arginine residue and lower percentage of glutamine residue than intact MAP2. This supports the idea that polycationic microtubule binding domain of MAPs interacts with tubulin electrostatically. But as a whole, the amino acid compositions of the 35-kDa fragment are not similar to that of the 27-kDa fragment. This may be due to the differences in the structure of microtubule binding domains of these two MAPs. It is shown that microheterogeneity of the tubulin molecule is clustered in the carboxyl-terminal region which is supposed to interact with MAPs (33). It is supposed that this heterogeneity makes one species of tubulin molecule interact with a selected type of MAP (34). It can be considered that the possible difference in the structure of microtubule binding domains of 190-kDa MAP and MAP2 makes these MAPs interact with other species of tubulin. However, we cannot rule out the possibility of the existence of partial specific structures which are common to the microtubule binding domain of the 190-kDa MAP and MAP2. To make a detailed study, determination of amino acid sequence of the fragments is needed.

### Table I

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TABLE I

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Acknowledgments—We would like to express our thanks to Sachiko Endo for electron microscopy of negatively stained microtubules and Osamu Matsuzaki for amino acid analysis. We are also indebted to Dr. Matthew Suffness, the Natural Products Division of the National M. W. of taxol. Biol. Chem. 109, 99, 1257-1264.