Interaction of Microtubule-Associated Protein 2 with Actin Filaments†

Richard F. Sattilaro
Center for Biomedical Research, The University of Kansas, Lawrence, Kansas 66046
Received September 3, 1985; Revised Manuscript Received December 6, 1985

ABSTRACT: The interaction of unphosphorylated and phosphorylated microtubule-associated protein 2 (MAP-2) with actin filaments was examined by electron microscopic, electrophoretic, and dark-field light microscopic techniques. Unphosphorylated MAP-2 was observed to cross-link and bundle individual actin filaments. Chymotryptic fragments of MAP-2 protein were produced which bound to, but could not cross-link, actin polymer; these fragments encompassed the tubulin binding domain of MAP-2. The phosphorylation of intact MAP-2, by means of endogenous protein kinases, inhibited the ability of this molecule to cross-link and bundle actin filaments. Phosphorylation did not, however, inhibit the binding of MAP-2 to F-actin. The chymotryptic fragments of phosphorylated MAP-2 that retained their ability to bind to actin and promote microtubule assembly also encompassed the tubulin binding domain of this molecule. An analysis of MAP-2 fragments by nonequilibrium pH gradient electrophoresis indicated that most of the polypeptide backbone is relatively acidic with the exception of the tubulin binding domain. This region was determined to be the most basic (positively charged) region of the MAP-2 molecule. Biochemical and morphological evidence is presented to demonstrate that both unphosphorylated MAP-2 and phosphorylated MAP-2 have the capacity to use actin, in addition to microtubules, as a separate anchoring substrate. The presence of tubulin, however, strongly inhibits the interaction of MAP-2 with actin filaments.

Microtubule-associated proteins (MAPs)[1] are non-tubulin components that copurify with microtubules through repetitive cycles of polymerization in vitro. One of the principal MAPs from brain tissue is MAP-2, a filamentous phosphoprotein (Vallee, 1980; Nishida et al., 1981; Burns & Islam, 1982; Selden & Pollard, 1983) of approximately 280000 daltons that projects from the surface of purified microtubules (Dentler et al., 1975; Kim et al., 1979). The biological function of this molecule is unknown, but it does have the capacity to promote the polymerization of microtubules from tubulin in vitro (Dentler et al., 1975; Keates & Hall, 1975; Murphy & Borisy, 1975; Weingarten et al., 1975).

MAP-2 has been shown to be preferentially associated with dentritic microtubules (Matus et al., 1981; Caceres et al., 1983) and appears to be concentrated in the distal regions of developing dendrites, often preceding the appearance of microtubules (Bernhardt & Matus, 1982). Therefore, this protein may be involved in the coordination of several cellular functions. There is evidence, for example, that MAP-2 and other high molecular weight MAPs may be involved in the association of microtubules with other cytoplasmic organelles, including various membrane-bound vesicles (Sherline et al., 1977; Sattilaro & Dentler, 1982; Suprenant & Dentler, 1982), neurofilaments (Leterrier et al., 1982; Heimann et al., 1985), intermediate filaments (Pytela & Wiche, 1980; Bloom & Vallee, 1983), and F-actin (Griffith & Pollard, 1978; Sattilaro et al., 1981; Griffith & Pollard, 1982a; Sattilaro & Dentler, 1982). The observation that portions of the molecule can be phosphorylated (Sloboda et al., 1975; Vallee, 1980; Burns & Islam, 1982) even suggests a basic mechanism by which these associations may be regulated.

On the molecular level, it is essential to carefully examine the structural/functional aspect of this molecule. Specifically, which peptide domains have the capacity to interact with each cytoskeletal element? Are these interactions found in vivo? To this end, purified MAP-2 has been enzymatically cleaved into a number of fragments, one class of which binds to and induces the assembly of microtubules (Vallee, 1980). The non-tubulin binding fragments of MAP-2 are presumed to represent that portion of the molecule extending from the surface of microtubules (the projection domain). The present report describes both biochemical and morphological studies concerned with determining the identity and nature of the actin and tubulin binding sites on both unphosphorylated and phosphorylated MAP-2.

Materials and Methods

Materials

Piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), GTP, ATP, cAMP, phenylmethanesulfonyl fluoride (PMSF), pepstatin A, and leupeptin (each dissolved in Me_2SO_4) and ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), dithiothreitol (DTT), 2-mercaptoethanol, α-chymotrypsin (type I-S), and acrylamide were purchased from Sigma Chemical Co., St. Louis, MO; phosphocellulose (P-11) was from Whatman Inc., Clifton, NJ; Serval ampholytes AG 2-11 were from Polysciences, Inc., Warrington, PA; Bio-Gel A-15m agarose was from Bio-Rad, Richmond, CA; [γ-^{32}P]-ATP (25 Ci/mmol) was from ICN Radiochemicals, Irvine, CA; sodium dodecyl sulfate (SDS) was purified from MCB Inc., Cincinnati, OH.

Methods

Isolation of Proteins. Bovine brain microtubule protein was purified by three cycles of temperature-dependent assembly in the presence of 2 M glycerol and 0.2 mM PMSF as described elsewhere (Borisy et al., 1974; Sattilaro et al., 1981).

† This work was supported by NIRA Grant NS 20560 and National Institutes of Health Grant AM 21672/GM 32556.

[1] Abbreviations: MAP-2, microtubule-associated protein 2; NEPHGE, nonequilibrium pH gradient electrophoresis; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PMSF, phenylmethanesulfonyl fluoride; Me_2SO_4, dimethyl sulfoxide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate.

0006-2960/86/0425-2003$01.50/0

Phosphocellulose-purified tubulin (PC-tubulin) was prepared from thrice-cycled microtubule protein (Weingarten et al., 1975). A crude heat-stable MAP fraction was prepared by exposure of microtubule protein to 100 °C for 5 min (Herzog & Weber, 1978). MAP-2 was purified by gel filtration chromatography in the presence of 0.2 mM PMSF, 2 μg/mL pepstatin A, and 2 μg/mL leupeptin using Bio-Gel A-15m resin (Vallee, 1980; Vallee et al., 1981). Rabbit psoas muscle actin was purified according to the method of Spudich and Watt (1971) as described by Sattilaro et al. (1981). Actin was stored at −80 °C in 100-μL aliquots at a concentration of 2–4 mg/mL.

Protein Phosphorylation. To produce phosphorylated MAP-2, slight modifications of the procedure of Nishida et al. (1981) were employed. Microtubule protein was incubated for 30 min at 37 °C in the presence of 20 mM PIPES (pH 6.8), 5 mM $MgCl_2$, 90 mM KCl, 0.1 mM GTP, 1.1 mM $CaCl_2$, 0.5 mM ATP, 10 μM cAMP, 0.2 mM PMSF, 2 μg/mL pepstatin A, and 2 μg/mL leupeptin. For some experiments, [γ-^{32}P]ATP at 25 Ci/mmol was included at 50 μCi/mL. After the incubation period, the crude MAP solution was brought to 0.8 M NaCl and 80 mM 2-mercaptoethanol, heated in a 100 °C water bath for 5 min, and cooled on ice. Fresh PMSF was then added to 0.2 mM, and the precipitated proteins were removed by centrifugation at 20000*g* for 25 min at 4 °C. The supernatants were collected and concentrated to approximately 1.5 mL with type 50 Amicon conical filters and fractionated on a column (90 × 1.6 cm) of Bio-Gel A-15m as previously described (Vallee, 1980; Vallee et al., 1981). Fractions enriched for MAP-2 were pooled, concentrated as above, dialyzed against 50 mM PIPES (pH 6.8) containing 1 mM EGTA, 0.5 mM $MgCl_2$, and 2 mM DTT (PEMD), and frozen as droplets in liquid N_2 and stored at −80 °C. The total inorganic phosphate content of protein preparations was estimated by using modifications (Selden & Pollard, 1983) of the methods of Stull and Buss (1977).

Digestions of MAP-2. Purified MAP-2, at a concentration of 0.6 mg/mL, was digested with 0.8 or 1.6 μg/mL α-chymotrypsin at 37 °C for predetermined intervals. The reaction was terminated by the addition of PMSF to 2 mM, and the MAP-2 fragments were incubated with actin and tubulin in the presence of 2 mM PMSF as described below.

MAP-2 Binding Assay. PC-tubulin (0.8 mg/mL) was polymerized in the presence of intact or enzymatically cleaved MAP-2 (0.2 mg/mL) by the addition of 0.5 mM GTP and incubation at 35 °C for 30 min. Actin was polymerized at a concentration of 0.8 mg/mL in PEMD buffer containing 0.2 mg/mL MAP-2 for 20 min at 30 °C. Microtubules and actin bundles were then sedimented in microcentrifuge tubes at 18000*g* for 30 min at 30 °C. Solutions of F-actin were sedimented at 18000*g* for 1 h at 30 °C.

Dark-Field Light Microscopy. MAP–actin bundle formation was observed in solution by means of a Zeiss microscope equipped with a Zeiss 63 × N.A. 1.4 objective lens and a Zeiss oil-immersion ultracondensor (N.A. 1.2/1.4). Illumination was provided by a 75-W xenon arc lamp in a Zeiss lamp housing with a Zeiss 75-W power supply (Zeiss Opticals, Scarsdale, NY). Photographs were made with 15-s exposures on Kodak Tri-X film and were developed in Kodak HC-110.

Electrophoresis. One-dimensional and two-dimensional polyacrylamide gels were prepared as described (Laemmli, 1970; O'Farrell, 1975; O'Farrell et al., 1977). Proteins were separated in the presence of wide-range carrier ampholytes, pH 2–11, in cylindrical (9.0 × 0.5 cm) gels. Protein samples were made 0.5% in SDS, 9.5 M in urea, 5% in 2-mercaptoethanol, and 0.2% in ampholine and processed exactly as described (O'Farrell et al., 1977) on 3% gels. All samples were loaded at the anodic end (top) of the gel and were overlayed with 40 μL of 5% NP-40, 8 M urea, and 1% ampholytes (pH 2–11). Nonequilibrium pH gradient electrophoresis (NEPHGE) was carried out at 150 V for 6 h on 3% gels containing 0.01% bis(acrylamide). All second-dimension SDS–polyacrylamide slab gels were stained either with Coomassie Blue by the procedure of Fairbanks et al. (1971) or with silver (Merrill et al., 1980). To determine the relative amount of ^{32}P incorporated into MAP-2 fragments, undigested MAP-2 or fragment bands were excised from stained and dried gels. Their ^{32}P activity was then determined by liquid scintillation counting in Aquasol (New England Nuclear).

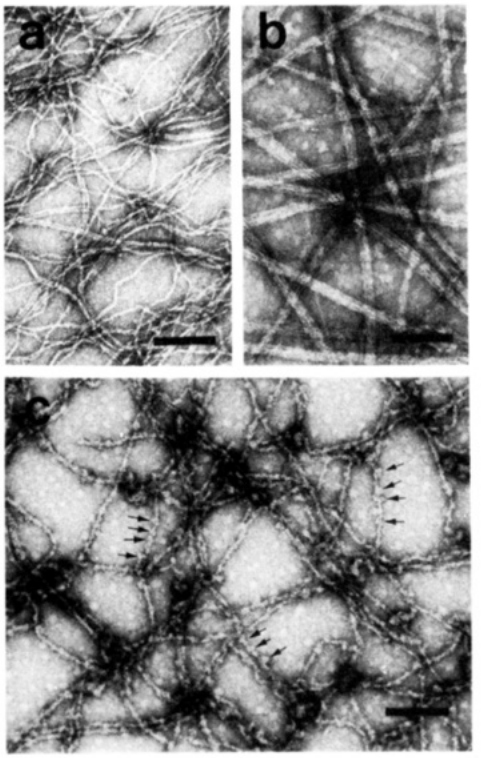

FIGURE 1: Electron micrographs of negatively stained specimens of actin filaments alone (a), actin filaments polymerized in the presence of intact MAP-2 (b), and actin filaments polymerized in the presence of enzymatically cleaved MAP-2 (c). Arrows indicate MAP-2 fragments. Intact MAP-2 at 0.6 mg/mL was incubated with 1.6 μg/mL chymotrypsin at 37 °C for 3 min. Magnification 75000×. Bars, 1.0 μm.

Electron Microscopy. For electron microscopy, 100-μL samples were fixed in suspension for 30 min at room temperature after the slow injection of 10 μL of prewarmed 5% glutaraldehyde with a Hamilton syringe. Aliquots of fixed material were placed on carbon- and Formvar-coated copper grids and stained for 5 s in 2% aqueous uranyl acetate. All samples were examined and micrographs obtained with a Philips 300 electron microscope.

RESULTS

The inherent ability of MAP-2 to organize F-actin into discrete bundles measuring 20–26 nm in diameter is depicted in Figure 1b. This kind of morphological evidence indicated that the molecule could bind to and cross-link individual actin filaments. Consequently, it was reasoned that appropriate enzymatic cleavage of MAP-2 into smaller peptides would inhibit such bundle formation and allow for the direct observation of the fragments that bind to F-actin. Indeed, when MAP-2 solutions were digested briefly in the presence of

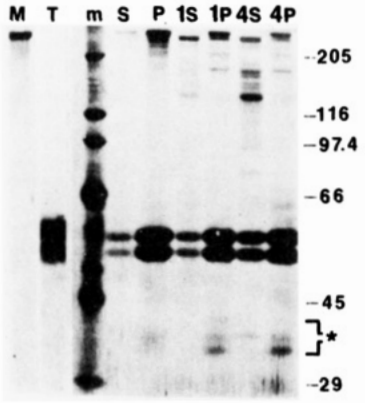

FIGURE 2: Electrophoretic analysis of tubulin binding and projection (nonbinding) fragments of MAP-2. Purified MAP-2 (M), at 0.6 mg/mL, was exposed to 0.8 mg/mL chymotrypsin at 37 °C for 1 or 4 min. Microtubules were assembled from tubulin (T) in the presence of intact or digested MAP-2 and sedimented in order to separate tubulin binding (1P and 4P pellets) and nonbinding MAP-2 fragments (1S and 4S supernatants). Lanes S and P are the respective supernatant and pellet fractions of microtubules assembled from tubulin and undigested MAP-2. Lane m is a set of standards whose molecular masses are indicated in daltons × 10^{-3}. The asterisk indicates the location of the 32–39-kilodalton tubulin binding domain of MAP-2. The 8% acrylamide separating gel was silver stained.

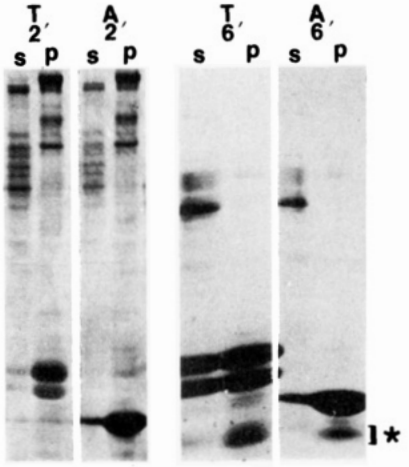

FIGURE 3: Electrophoretic comparison of actin and tubulin binding fragments of MAP-2. Purified MAP-2 at 0.6 mg/mL was exposed to 1.6 μg/mL chymotrypsin at 37 °C for 2 or 6 min. Tubulin (T) or actin (A) was polymerized in the presence of early (2 min) or late (6 min) chymotryptic digests of MAP-2 and sedimented in order to separate polymer binding (p) and nonbinding (s) fragments. The asterisk indicates the location of the 32–39-kilodalton tubulin binding domain of 6-min MAP-2 digests. Both sets of 7% separating gels were silver stained.

α-chymotrypsin and incubated with actin, the cleaved molecule lost its ability to organize F-actin into bundles (Figure 1c). More importantly, an examination of negatively stained preparations indicated that individual actin filaments were now decorated with small globular particles (Figure 1c, arrows) spaced at approximately 14-nm intervals. Electrophoretic analysis of sedimented mixtures of F-actin and MAP-2 digests (not shown) suggested that these globules were composed of MAP-2 fragments. Similar results are presented in Figure 3.

Interaction of MAP-2 Fragments with Microtubules and Actin. Since α-chymotrypsin can cleave MAP-2 into distinct tubulin binding and projection (nonbinding) domains (Vallee, 1980), the above morphological result (Figure 1c) suggested that it should be possible to identify actin binding fragments with respect to their approximate location on the intact MAP-2 molecule. Figure 2 shows the chymotryptic generation of tubulin binding (lanes 1P and 4P) and nonbinding (lanes 1S and 4S) fragments of MAP-2 as analyzed by one-dimensional gel electrophoresis. The distribution of each class of fragments was found to be in good agreement with the results presented by Vallee (1980); in particular, a class of chymotryptic fragments of M_r 32K–39K (bracket) has been described as encompassing the microtubule assembly promoting portion of MAP-2. Larger tubulin binding fragments in lanes 1P and 4P arise from peptides that overlap the tubulin binding domain of MAP-2. The peptides in the supernatants (lanes 1S and 4S) arise from the portion of MAP-2 which projects from the microtubule surface (Vallee, 1980).

The above sedimentation technique was therefore used to compare the binding of MAP-2 fragments to actin and microtubules. After polymerization of actin and tubulin in the presence of digested MAP-2, the preparations were sedimented, and the respective supernatants and pellets were examined by gel electrophoresis (Figure 3). It was observed that for both early (2 min) and late (6 min) digests, the fragments which sedimented with microtubules (lanes T2′p and T6′p) appeared to be quite similar to those which sedimented with F-actin (lanes A2′p and A6′p). None of the fragments pelleted in the absence of tubulin or actin (not shown). These results indicated that the particles attached to F-actin in Figure 1c were composed of MAP-2 fragments. In addition, all of the fragments of MAP-2 which failed to bind to microtubules (lanes T2′s and T6′s) also failed to bind to actin (lanes A2′s and A6′s). Of particular interest was the observation that the 32–39-kilodalton fragments (brackets), which arise from the tubulin binding domain of MAP-2, also sedimented quite readily with actin (lanes T6′p and A6′p).

Competition of Tubulin for Actin-Associated MAP-2. Since the MAP-2 molecule is generally considered to be a microtubule binding protein, it was important to determine how actin and microtubules might then compete for intact MAP-2. To this end, purified tubulin was added to preformed MAP–actin bundles in buffer conditions which favored microtubule polymerization but did not affect actin bundle integrity. When tubulin was present below its critical concentration, samples from such preparations showed diminished numbers of bundles (compare panels b and c of Figure 4), in addition to partially dissociated bundles. When the tubulin concentration was raised to 0.5 mg/mL, the bundles completely dissociated within 20 min and microtubules polymerized (Figure 4d). The addition of excess actin to 2 mg/mL did not reverse the competition of tubulin for MAP-2 (not shown). Since both actin and tubulin bind the same major chymotryptic fragments of MAP-2 (Figure 3), this result clearly suggested that the primary actin binding site on MAP-2 may be identical with or very close to the tubulin binding domain of the molecule and that tubulin has a greater affinity for MAP-2 than does actin.

Effect of Phosphorylation on MAP–Actin Interactions. Since it has been established that the phosphorylation of MAPs or MAP-2 (Nishida et al., 1981; Selden & Pollard, 1983) in the presence of ATP and cyclic AMP inhibits their viscometric interaction with F-actin, the effect of phosphorylation on the actin binding and bundling capacity of MAP-2 was examined. The total inorganic phosphate content of unphosphorylated MAP-2 preparations was determined to be 4.2 ± 0.7 mol mol^{-1} (300 000 g)$^{-1}$. Upon phosphorylation, the net amount was increased to 9.5 ± 1.7 mol mol^{-1} (300 000 g)$^{-1}$. As shown in Figure 5, the complex of unphosphorylated MAP-2 (–) with F-actin gave rise to the appearance of long wispy fibers when viewed by dark-field microscopy (middle row). Extensive areas

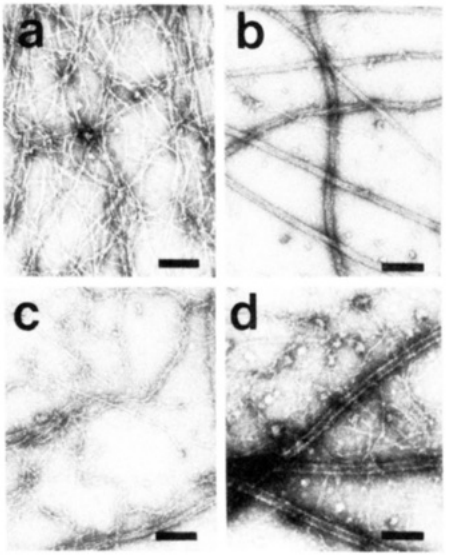

FIGURE 4: Electron micrographs (negative stain) depicting the competition of tubulin for MAP-2. Actin (0.8 mg/mL) was polymerized in the absence (a) or presence (b) of 0.2 mg/mL purified MAP-2 in PEMD buffer. Tubulin was then added, at a final concentration of 0.1 mg/mL (c) or 0.5 mg/mL (d), to preformed MAP–actin bundles and incubated for 20 min at 37 °C in the presence of 0.2 mM GTP. Magnification 32250×. Bars, 0.1 μm.

of densely packed refractile fibers were quite common in all samples examined. Samples from the same preparations were also negatively stained and examined by electron microscopy. Discrete bundles of F-actin were observed, and the surface of such bundles was studded with globular particles as previously reported (Sattilaro et al., 1981; Sattilaro & Dentler, 1982; Griffith & Pollard, 1982a). The particles were presumed to arise from the attached MAP-2 protein that sedimented with the bundles (accompanying gel). Solutions of actin polymerized in the presence of unphosphorylated MAP-2 were of sufficient viscosity to trap numerous bubbles formed during gelation (Figure 5, curvette, middle row). By comparison, individual actin filaments could not be seen by dark-field microscopy, nor were bubbles trapped during the polymerization of purified actin (Figure 5, top row). The use of these complementary assays provided several ways in which to examine the interaction of MAP-2 with F-actin in a given experiment.

When phosphorylated MAP-2 (+) was incubated with actin, only numerous refractile particles were observed in dark field (Figure 5, bottom row) along with a number of barely visible linear structures. When these preparations were negatively stained and examined by electron microscopy, numerous 7–8-nm globular particles were observed to be attached at 12–14-mm intervals to individual actin filaments. Occasionally, intact bundles could be found (not shown). When the F-actin in these solutions was sedimented and analyzed by SDS-polyacrylamide gel electrophoresis, it was evident that phosphorylated MAP-2 was associated with this polymer (accompanying gel). In striking contrast to unphosphorylated MAP-2, mixtures of actin and phosphorylated MAP-2 were not of sufficient viscosity to trap bubbles during incubation (cuvette, bottom row).

From these data, it appeared that the phosphorylation of MAP-2 inhibited its ability to cross-link and bundle F-actin. Phosphorylation did not, however, prevent MAP-2 from binding to individual actin filaments (Figure 5, bottom row). This suggested that one or more actin binding sites on MAP-2 were suppressed and that at least one actin binding site was not, by the addition of phosphate.

Effect of Phosphorylation on the Sedimentation of MAP-2 Fragments with Actin and Microtubules. Before investigating how phosphorylation affected the interaction of MAP-2 domains with actin, the chymotryptic fragments of unphosphorylated (−) and phosphorylated (+) protein were first compared by two-dimensional electrophoretic techniques.

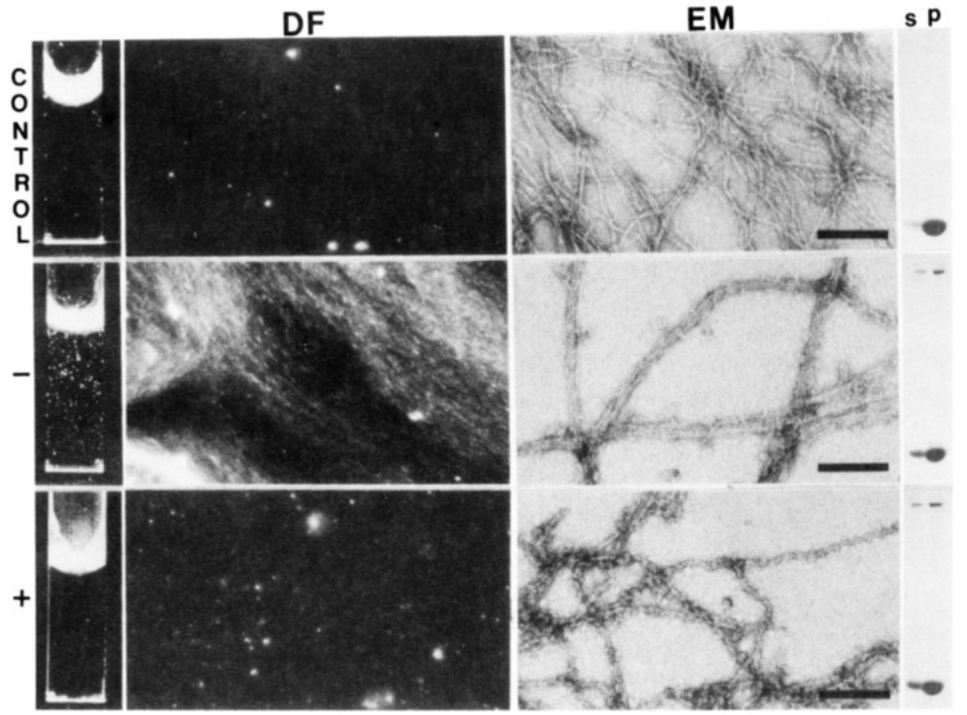

FIGURE 5: Interaction of unphosphorylated (−) and phosphorylated (+) MAP-2 with actin filaments. Actin was polymerized in PEMD alone (top row) or in the presence of unphosphorylated (middle row) or phosphorylated (bottom row) MAP-2 in glass microcuvettes. Fixed aliquots of each reaction were also examined by dark-field (DF) and negative-stain electron microscopy (EM). Unfixed mixtures were separated into supernatant (s) and pellet (p) fractions by centrifugation and examined by polyacrylamide gel electrophoresis (Coomassie Blue stain). Dark-field magnification 1200×. Negative-stain magnification 63750×. Bars, 0.1 μm.

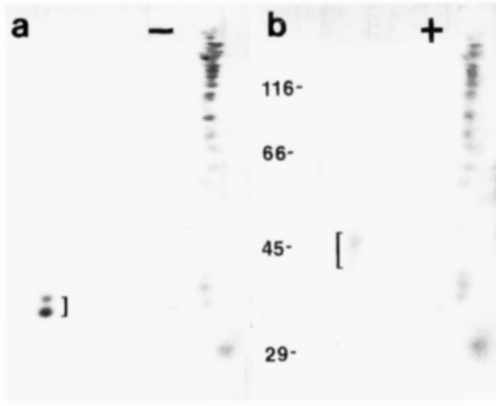

FIGURE 6: Two-dimensional electrophoretic analysis of MAP-2 fragments. Unphosphorylated (–) MAP-2 and phosphorylated (+) MAP-2 at 1.4 mg/mL were digested with 1.6 μg/mL α-chymotrypsin at 37 °C for 4 min and resolved in the first dimension for 6 h at 150 V in the presence of pH 2–11 ampholytes. MAP-2 peptides were further separated in the second dimension (a and b) on 8% acrylamide–SDS gels. The right side of the gel patterns represents the top (acidic end) of the first-dimension gels. The relatively basic peptides arising from the tubulin binding domain of MAP-2 are indicated by brackets. The standard scale represents molecular masses indicated in daltons × 10^{-3}. Gels were silver stained.

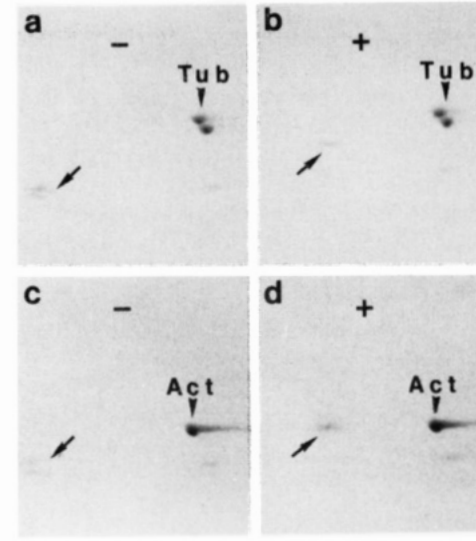

FIGURE 7: Two-dimensional electrophoretic analysis of the binding of MAP-2 fragments by microtubules and F-actin. Both unphosphorylated (–) MAP-2 and phosphorylated (+) MAP-2 were digested as in Figure 6. Microtubules and actin were polymerized in the presence of these chymotryptic fragments, sedimented, and analyzed to two-dimensional gel electrophoresis. Arrows point to the tubulin and actin binding fragments of MAP-2 (Coomassie Blue stain).

Digested fragments were subject to NEPHGE in the first dimension, followed by SDS–polyacrylamide gel electrophoresis in the second dimension. Most of the 32–39-kilodalton tubulin binding domain in unphosphorylated MAP-2 migrated as a relatively basic set of peptides (Figure 6a, brackets). The remainder of the larger fragments, which included the projection domain of MAP-2, migrated as relatively acidic components. The basic 32–39-kilodalton domain observed in Figure 6a was absent in chymotryptic digests of phosphorylated MAP-2 (Figure 6b). Instead, a more acidic and diffuse spot with an average molecular weight of 45K was observed (bracket). This apparent charge and size modification was presumed to be a consequence of chymotryptic inhibition by multiple organophosphate residues on the tubulin binding domain of MAP-2. Indeed, when the diffuse 45-kilodalton cluster was excised from duplicate gels containing 50 μg of protein, it was found to contain nearly 30% (1200 cpm) of the ^{32}P activity present in an equivalent amount of undigested MAP-2 (4100 cpm).

To preclude the possibility that any of the stained patterns of phosphorylated fragments were concealed in one-dimensional gels by the presence of actin, mixtures of actin (or microtubules) polymerized in the presence of unphosphorylated (–) and phosphorylated (+) MAP-2 fragments were pelleted and analyzed by two-dimensional NEPHGE (Figure 7). An examination of stained gel patterns (Figure 7) indicated that both microtubules and actin filaments were able to bind the same 32–39-kilodalton site on the MAP-2 molecule (compare panels a and c of Figure 7, arrows). In addition, it was observed that phosphorylation did not affect the binding of this domain to either microtubules or F-actin (Figure 7b,d, arrows).

Discussion

There are a number of macromolecules which appear to bind to actin filaments in vitro and form a gel, a few of which have been noted elsewhere (Griffith & Pollard, 1982b). For the most part, these macromolecules have a characteristic basic isoelectric point in contrast to the acidic isoelectric point of actin. Therefore, they may not have any relative in vivo significance with regard to actin interactions. It has also been pointed out, however, that such molecules may mimic the biochemistry of true actin regulatory proteins isolated from cells (Craig & Pollard, 1982).

With regard to MAP-2, cytological evidence reported over the last 15 years has constantly suggested the possibility of in vivo interactions between microtubules and actin filaments. This present report does not confirm the potential for actin–microtubule interactions but rather indicates that MAP-2 can bind to actin filaments by means of the same domain that binds to microtubules. This conclusion depends on the assumption that MAP-2, isolated by a procedure which involves heat treatment at 100 °C for 5 min, is not altered from its "natural" state. Findings presented elsewhere (Voter & Erickson, 1982) suggest that it is not.

An examination of MAP-2 and actin interactions by means of morphological and biochemical techniques demonstrates that at least one F-actin binding site is contained within the region of the MAP-2 molecule that binds to and promotes the polymerization of tubulin. This region of MAP-2 will be referred to as the microtubule/actin binding domain (M/A domain). In addition, it was determined that phosphorylation inhibits the cross-linking but not the binding of actin by MAP-2.

The 32–39-kilodalton M/A domain was determined to be the most positively charged chymotryptic fragment of MAP-2, on the basis of its relative migration in nonequilibrium pH gradient electrophoresis. In experiments where the basic end of the pH gradient displayed minimal collapse after 5000 V-h, the isoelectric point of the M/A domain appeared to be on the order of 8.5 (unpublished results). Such a relatively strong positive charge on one end of the MAP-2 molecule undoubtedly assures its ionic interaction with an acidic site on the tubulin molecule. In this regard, it has been observed that approximately 14 of the last 36 amino acids of the carboxyl terminal of both α- and β-tubulin are glutamic acid and that the C-terminal fragments can bind to MAP-2 (Serrano et al., 1984).

It must be emphasized that the results of the present study do not exclude the possibility that other actin binding sites exist on MAP-2 which may be inactivated when the protein is cleaved by chymotrypsin. This qualification is essential since MAP-2 has the capacity to cross-link and organize individual

actin filaments into discrete bundles (Sattilaro et al., 1981; Griffith & Pollard, 1982a; Sattilaro & Dentler, 1982). In addition, it has been reported that mixtures of actin, tubulin, and microtubule-associated protein can form gelled networks (Griffith & Pollard, 1978; Nishida et al., 1981; Selden & Pollard, 1983). Such observations suggest that there are at least two actin binding sites on MAP-2 and that they are separated (minimally) by the width of a single actin filament. Furthermore, the diameter of MAP-2-saturated actin bundles does not vary by more than a few nanometers nor do individual filaments become cross-linked into continuous sheets. Instead, MAP-2 organizes F-actin into discrite packets of four to seven filaments (Sattilaro et al., 1981; Griffith & Pollard, 1982a; Sattilaro & Dentler, 1982). An alternate explanation for actin bundling would be possible if MAP-2, in addition to having only one actin binding site, could interact with itself. However, there is no evidence that MAP-2 can undergo dimerization.

It is evident from the MAP-2 competition data presented in this report that the M/A domain has a greater affinity for tubulin than for actin. The presence of 100 μg/mL tubulin was sufficient to disrupt preformed MAP–actin bundles in vitro. Therefore, if MAP-2 interacts with actin in vivo, it may be able to do so only in the absence of tubulin or MAP-free microtubules. These results, which demonstrate how dramatically tubulin can disrupt preformed MAP–actin bundles, appear to contradict the observations by others (Griffith & Pollard, 1978, 1982a; Selden & Pollard, 1983) that mixtures of tubulin, actin, and MAPs or MAP-2 form a highly viscous complex. It may not be appropriate, however, to compare two distinctly different experimental procedures in light of the unique conditions of each assay. Indeed, the difference may be related to the sequence of polymer formation. In the present study, tubulin was incubated in the presence of preformed MAP–actin bundles. It is possible that actin became bridged to the microtubules once tubulin polymerized; however, this would not be detected by the negative staining techniques employed. Nevertheless, distinct associations between actin and microtubules were not apparent when examined by thin-section electron microscopy even when gentle osmium tetraoxide treatment (Sattilaro et al., 1981) was employed.

Several studies have demonstrated the immunochemical localization of actin (Matus et al., 1982; Caceres et al., 1983) and high molecular weight MAPs (Matus et al., 1981; Bernhardt & Matus, 1982) or MAP-2 (De Camilli et al., 1984) in dendritic processes. In one of these studies (Bernhardt & Matus, 1982), high molecular weight MAPs were found to be present in developing dendrites before the appearance of tubulin. Consequently, it is possible that in the absence of tubulin, MAP-2 may regulate the gel–sol transformation of the actin present in these structures. One way in which the interaction of MAP-2 with actin might be regulated is by phosphorylation.

The MAP-2 component of purified microtubules was first shown to be capable of being phosphorylated by means of an intrinsic protein kinase that appeared to copurify with microtubule protein through several cycles of assembly–disassembly (Sloboda et al., 1975). In addition, a cAMP-dependent protein kinase, similar to type II kinase from bovine heart, has been demonstrated to be associated with purified microtubules (Vallee, 1980; Vallee et al., 1981; Theurkauf & Vallee, 1982). In fact, the phosphorylation of MAP-2 has been previously determined to interfere with its viscometric interaction with actin (Nishida et al., 1981; Selden & Pollard, 1983). Several lines of evidence from this present study indicate that phosphorylation inhibits the ability of MAP-2 to cross-link and bundle F-actin yet does not interfere with the binding of MAP-2 to individual actin filaments. Specifically, actin bundle formation rarely occurred in mixtures of phosphorylated MAP-2 and actin. Instead, the persistent binding of phosphorylated MAP-2 to individual actin fibers was observed by electron microscopy. Furthermore, the MAP-2 content of sedimented mixtures of actin and phosphorylated MAP-2 was essentially similar to that found in bundles cross-linked by unphosphorylated MAP-2 (Figure 5). Finally, the enzymatically cleaved tubulin binding domains of both phosphorylated MAP-2 and unphosphorylated MAP-2 were observed to be capable of binding to and sedimenting with F-actin (Figures 6 and 7).

Since MAP-2 isolated directly from thrice-cycled microtubule protein contained 4.2 ± 0.7 mol of phosphate per mole of MAP-2, it is clear that partial phosphorylation of unknown sites had already occurred prior to experimental in vitro phosphorylation. Such "untreated" MAP-2 could, nevertheless, bind to and cross-link F-actin. Following acid or alkaline phosphatase treatment, untreated MAP-2 has been found to be approximately 40% more active in forming a viscous complex with F-actin (Selden & Pollard, 1983). This suggests that there may exist other actin binding sites on MAP-2 that have not been addressed by this present study or that phosphorylation modifies the relative binding affinity of only one actin binding domain on MAP-2.

In essence, the results presented in this paper suggest that MAP-2 has the unique potential to use microtubules or actin filaments as a substrate for some as yet undefined cellular function(s). In particular, it would appear that MAP-2 can bind to either cytoplasmic polymer by means of its positively charged M/A domain. In addition, there is also evidence that MAP-2 can bind to intermediate filaments and neurofilaments in vivo (Bloom & Vallee, 1983; Papasozomenos et al., 1985) and neurofilaments in vitro (Leterrier et al., 1982; Heimann et al., 1985). This suggests that MAP-2 may participate in the organization of as many as three different cytoskeletal lattice systems.

The present study also indicates that the distribution of charge along the primary structure of this molecule is highly asymmetric. It is likely that the positioning of such a charge is related to the conformation and function of MAP-2. It is also possible that the phosphorylation of one site may alter the conformation of MAP-2 in such a way so as to modify its interaction with other cellular structures. If and how MAP-2 or perhaps other MAPs use these binding characteristics in the cell are not at all clear. Even less clear is the way in which these cytoskeletal associations might be related to the functioning of the neuronal cytoskeletal system.

ACKNOWLEDGMENTS

I thank Dr. W. L. Dentler for his encouragement and interest in this research and Drs. P. R. Burton, R. Himes, and W. L. Dentler for their valuable comments on the manuscript. The technical assistance of E. L. Le Cluyse is sincerely acknowledged.

REFERENCES

Bernhardt, R., & Matus, A. (1982) *J. Cell Biol. 92*, 589–593.
Bloom, G. S., & Valle, R. B. (1983) *J. Cell Biol. 96*, 1523–1531.
Borisy, G. G., Olmstead, J. B., Marcum, M. M., & Allen, C. (1974) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33*, 167–174.
Burns, R. G, & Islam, K. (1982) *Eur. J. Biochem. 122*, 25–29.
Caceres, A., Payne, M. R., Binder, L. I., & Steward, O. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1738–1742.

Craig, S. W., & Pollard, T. D. (1982) *Trends Biochem. Sci. (Pers. Ed.) 7*, 88–92.
De Camilli, P., Miller, P., Navone, F., Theurkauf, W. E., & Vallee, R. B. (1984) *Neuroscience (Oxford) 11*, 819–846.
Dentler, W. L., Granett, S., & Rosenbaum, J. L. (1975) *J. Cell. Biol. 65*, 237–241.
Fairbanks, G. T., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2617.
Griffith, L. M., & Pollard, T. D. (1978) *J. Cell Biol. 78*, 958–965.
Griffith, L. M., & Pollard, T. D. (1982a) *J. Biol. Chem. 257*, 9143–9151.
Griffith, L. M., & Pollard, T. D. (1982b) *J. Biol. Chem. 257*, 9135–9142.
Heimann, R., Shelanski, M. L., & Liem, K. H. (1985) *J. Biol. Chem. 260*, 12160–12166.
Herzog, W., & Weber, K. (1978) *Eur. J. Biochem. 92*, 1–8.
Keates, R. A. B., & Hall, R. H. (1975) *Nature (London) 257*, 418–421.
Kim, H., Binder, L. I., & Rosenbaum, J. L. (1979) *J. Cell Biol. 80*, 266–276.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Leterrier, J. F., Liem, R. K. H., & Shelanski, M. C. (1982) *J. Cell Biol. 95*, 982–986.
Matus, A., Bernhardt, R., & Hugh-Jones, T. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3010–3014.
Matus, A., Ackerman, M., Pehling, G., Byers, H. R., & Fujiwara, K. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7590–7594.
Merril, C. R., Goldman, D., Sedman, S. A., & Ebert, M. H. (1980) *Science (Washington, D.C.) 211*, 1437–1438.
Murphy, D. B., & Borisy, G. G. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2696–2700.
Nishida, E., Kuwaki, T., & Sakai, H. (1981) *J. Biochem. (Tokyo) 90*, 575–578.
O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007–4021.
O'Farrell, P. Z., Goodman, H. M., & O'Farrell, P. H. (1977) *Cell (Cambridge, Mass.) 12*, 1133–1142.
Papasozomenos, S. Ch., Binder, L. I., Bender, P. K., & Payne, M. R. (1985) *J. Cell Biol. 100*, 74–85.
Pytela, R., & Wiche, G. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4808–4812.
Sattilaro, R. F., & Dentler, W. L. (1982) in *Biological Functions of Microtubules and Related Structures* (Sakai, H., Mohri, H., & Borisy, G. G., Eds.) pp 297–309, Academic Press, New York and Tokyo.
Sattilaro, R. F., Dentler, W. L., & Le Cluyse, E. L. (1981) *J. Cell Biol. 90*, 467–473.
Selden, S. C., & Pollard, T. D. (1983) *J. Biol. Chem. 258*, 6064–6071.
Serrano, L. Avila, J., & Maccioni, R. B. (1984) *Biochemistry 23*, 4675–4681.
Sherline, P., Lee, Y. C., & Jacobs, L. S. (1977) *J. Cell Biol. 72*, 380–389.
Sloboda, R. D., Rudolph, S. A., Rosenbaum, J. L., & Greengard, P. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 177–181.
Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866–4871.
Stull, J. T., & Buss, J. E. (1977) *J. Biol. Chem. 252*, 851–857.
Suprenant, K. A., & Dentler, W. L. (1982) *J. Cell Biol. 93*, 164–174.
Theurkauf, W. E., & Vallee, R. B. (1982) *J. Biol. Chem. 258*, 7883–7886.
Vallee, R. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3206–3210.
Vallee, R. B., Di Bartolomeis, M. J., & Theurkauf, W. E. (1981) *J. Cell Biol. 90*, 568–576.
Voter, W. A., & Erickson, H. P. (1982) *J. Ultrastruct. Res. 80*, 374–382.
Weingarten, M. D., Lockwood, A. H., Hwo, S., & Kirschner, M. W. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1858–1862.

Reduction of the Buried Intrachain Disulfide Bond of the Constant Fragment of the Immunoglobulin Light Chain: Global Unfolding under Physiological Conditions

Hirohiko Kikuchi, Yuji Goto, and Kozo Hamaguchi*
Department of Biology, Faculty of Science, Osaka University, Toyonaka, Osaka 560, Japan
Received August 5, 1985

ABSTRACT: The constant (C_L) fragment of the immunoglobulin light chain contains only one intrachain disulfide bond buried in the interior of the molecule. The kinetics of reduction with dithiothreitol of the disulfide bond were studied at various concentrations of guanidine hydrochloride at pH 8.0 and 25 °C. It was found that the disulfide bond is reduced even in the absence of guanidine hydrochloride. The results of the reduction kinetics were compared with those of the unfolding and refolding kinetics of the C_L fragment previously reported [Goto, Y., & Hamaguchi, K. (1982) *J. Mol. Biol. 156*, 891–910]. It was shown that the reduction of the disulfide bond proceeds through a species with a conformation very similar to that of the fully unfolded one and that the C_L fragment undergoes global unfolding transition even in water.

Fluctuations of the protein molecule are believed to play an important role in biological functions and have been studied extensively by various methods such as X-ray crystallography, nuclear magnetic resonance, fluorescence quenching, fluorescence anisotropy, and hydrogen isotope exchange (Gurd & Rothgeb, 1979; Karplus & McCammon, 1981; Ringe & Petsko, 1985). The immunoglobulin molecule consists of two regions, Fab and Fc, which are flexibly linked through a hinge

0006-2960/86/0425-2009$01.50/0