Microtubule-associated Proteins (MAPs) and the Organization of Actin Filaments in Vitro

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ABSTRACT When purified muscle actin was mixed with microtubule-associated proteins (MAPs) prepared from brain microtubules assembled in vitro, actin filaments were organized into discrete bundles, 26 nm in diameter. MAP-2 was the principal protein necessary for the formation of the bundles. Analysis of MAP-actin bundle formation by sedimentation and electrophoresis revealed the bundles to be composed of ~20% MAP-2 and 80% actin by weight. Transverse striations were observed to occur at 28-nm intervals along negatively stained MAP-actin bundles, and short projections, ~12 nm long and spaced at 28-nm intervals, were resolved by high-resolution metal shadowing. The formation of MAP-actin bundles was inhibited by millimolar concentrations of ATP, AMP-PCP (β,γ-methylene-adenosine triphosphate), and pyrophosphate but not by AMP, ADP, or GTP. The addition of ATP to a solution containing MAP-actin bundles resulted in the dissociation of the bundles into individual actin filaments; discrete particles, presumably MAP-2, were periodically attached along the splayed filaments. These results demonstrate that MAPs can bind to actin filaments and can induce the reversible formation of actin filament bundles in vitro.

During the past decade, increasing evidence has indicated that there is a cytoskeleton within the cytoplasm of most eucaryotic cells and that the movements of cells and of organelles within cells occur in association with the cytoskeleton. Although the protein composition of the cytoskeleton is not well understood, the best evidence suggests that the major cytoskeletal structures include actin filaments, microtubules, and "intermediate filaments" (22, 27). If cytoplasmic movements occur in association with the cytoskeletal structures, then it is likely that the structures interact with one another to mediate the movements. For example, the apparent presence of actin, microtubules, and myosin in the mitotic apparatus (6, 12, 13, 28, 33) suggests that these structures may interact with one another to move the chromosomes. Axoplasmic transport may also require associations between microtubules, actin, intermediate filaments, or membrane vesicles (1, 3, 20, 23, 35).

Although it is tempting to speculate that associations between cytoskeletal structures are responsible for cell movements, there is little direct biochemical evidence to illustrate how these interactions may occur. Griffith and Pollard (15) recently presented viscometric and ultrastructural evidence for the interaction of actin filaments with microtubules, in addition to viscometric evidence of the interaction of actin with microtubule-associated proteins (MAPs). Moreover, the associations were reversible upon the addition of ATP, ITP, pyrophosphate, and, to a lesser extent, other nucleotides.

In this report, we present evidence that MAPs, in particular MAP-2, can reversibly induce the assembly of actin into discrete bundles of filaments comprised of ~20% MAP-2 and 80% actin. Moreover, the MAP-2 molecules are shown to be periodically attached to individual actin filaments in the filament bundles. A preliminary report of these data has been presented (29).

MATERIALS AND METHODS

Materials

Reagent-grade chemicals were purchased from the following sources: piperazine-*N*,*N*′-bis-2-ethane sulfonic acid (PIPES), GTP, AMP, ADP, ATP, ethyleneglycol-bis-(β-aminoethyl ether)*N*,*N*′-tetraacetic acid (EGTA), dithiothreitol (DTT), 2-mercaptoethanol, poly-L-lysine, and bovine serum albumin from Sigma Chemical Co., St. Louis, Mo.; β,γ-methylene-adenosine triphosphate (AMP-PCP) from Miles Laboratories, Inc., Ames Div., Elkhart, Ind.; ethylenedinitrilo-tetraacetic acid (EDTA) from Mallinkrodt Chemical Works, Inc., St. Louis, Mo.; Phosphocellulose (P-11) from Whatman Inc., Clifton, N. J.; Sephadex G-25 from Pharmacia Fine Chemicals, Piscataway, N. J.; BB411-1500 microcentrifuge tubes from Bolab Inc., Derry, N. H. Glutaraldehyde was purchased from Electron Microscopy Sciences, Fort Washington, Pa.

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Methods

ISOLATION OF MICROTUBULE PROTEINS: Bovine brain microtubule protein was purified by three cycles of temperature-dependent assembly (2) in 0.1 M PIPES, 2 mM EGTA, 1 mM $MgSO_4$, and 1 mM DTT, pH 6.4. The first polymerization step was carried out in the presence of 4 M glycerol, and subsequent cycles of assembly were performed without glycerol. Protein fractions enriched for MAP-1 and MAP-2 were prepared using phosphocellulose chromatography (16) and were dialyzed against PEMD buffer (50 mM PIPES, 1 mM EGTA, 0.5 mM $MgCl_2$, 2 mM DTT, pH 6.5) before use. Preparations enriched for MAP-2 were made by heat treatment of thrice-cycled microtubule protein (16). The heat-stable MAPs were collected by ammonium sulfate precipitation and were desalted by passage through Sephadex G-25 equilibrated with PEMD. MAPs were frozen in liquid nitrogen and stored at −70°C in 100-μl aliquots that contained 1.5–2.0 mg protein/ml.

Rabbit psoas muscle actin was purified according to the method of Spudich and Watt (36), with the addition of a second cycle of polymerization and sedimentation. Twice-cycled F-actin was then resuspended and dialyzed for 36 h against Tris-ATP buffer (2 mM Tris-Cl., pH 8.0, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM DTT), clarified by centrifugation at 100,000 *g* for 1 h, and dialyzed for an additional 15 h against Tris buffer without ATP. After a final clarification at 100,000 *g* for 1 h the actin was diluted to 3–5 mg/ml with Tris buffer and stored in 100-μl aliquots at −70°C.

MAP-ACTIN MIXING PROCEDURES: A constant amount of actin (140 μg) was mixed with various amounts of MAPs at 4°C, in a total volume of 200 μl in PEMD buffer. The mixture was then warmed to 37°C for 25 min to assemble the bundles of actin filaments. For sedimentation studies, samples were centrifuged at 28°C for 15 to 20 min at 20,000 *g*. Alternatively, polymerized mixtures were layered on a 250-μl cushion of 10% sucrose in PEMD and centrifuged in a JS-13 rotor (Beckman Instruments, Inc., St. Louis, Mo.) for 40 min at 13,000 *g*. Pellets were resuspended in distilled water or 0.1 M PIPES, pH 6.5, and their protein content was determined by the method of Schacterle and Pollack (31).

The effect of nucleotides on bundle formation was examined by incubating the MAP-actin mixtures described above in the presence of 1 mM AMP, ADP, ATP, GTP, AMP-PCP, or pyrophosphate. For electron microscopy, 100-μl samples were fixed in suspension for 1 h at room temperature after the slow injection of 10 μl of prewarmed 10% glutaraldehyde in a Hamilton syringe. The pH of the buffered mixture did not change appreciably after the addition of fixative. For shadowing experiments, aliquots of fixed material were placed on carbon- and Formvar-coated copper grids, washed with several drops of distilled water, and air-dried. A 0.3- to 0.6-nm layer of platinum/palladium (Ted Pella, Inc., Tustin, Ca.) was deposited at a 40° angle with a Denton DV 502 vacuum evaporator (Denton Vacuum, Inc., Cherry Hill, N. J.). Aliquots of MAP-actin mixtures were also placed on grids and stained for 5 s in 2% aqueous uranyl acetate. For thin-section analysis, MAP-actin mixtures were pelleted at 20,000 *g* for 15 min at 28°C and were fixed for 1 h at room temperature in PEMD buffer, pH 6.5, containing 1% glutaraldehyde and 0.2% tannic acid. Samples were stored overnight at 4°C and were then rinsed twice with 50 mM PIPES, pH 6.5, and treated with 4 mM OsO_4 in the same buffer for 25 min at room temperature. After staining with 2% uranyl acetate for 1 h, samples were dehydrated with ethyl alcohol and embedded in Spurr's resin (37). Thin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and examined and photographed in a Philips 300 electron microscope.

POLYACRYLAMIDE GEL ELECTROPHORESIS: Polyacrylamide gel electrophoresis was performed at room temperature by the method of Laemmli (19) with a 7.5% separating and 3% stacking gel poured into 0.5 × 9.5-cm tubes (in a Canalco Apparatus, Ames Co., Elkhart, Ind). Gels were stained for protein with Coomassie Blue following the procedure of Fairbanks et al. (10). For quantitative analysis, electrophoresed proteins were fixed and stained with fast green (14) and were scanned at 650 nm with a Gilford model 240 spectrophotometer equipped with linear transport (Gilford Instrument Laboratories Inc., Oberlin, Ohio).

RESULTS

When purified actin (Fig. 1*A*) was mixed with microtubule-associated proteins (MAPs) isolated from microtubule protein either by phosphocellulose chromatography (16) (Fig. 1 *C* and *D*) or by heat treatment and subsequent chromatography (16) (Fig. 1 *E* and *F*), the actin polymerized into discrete bundles of filaments, ~26 nm in diameter (Fig. 2). Because the heat-stable MAP fraction was composed principally of MAP-2, by comparison with the phosphocellulose-purified MAP, which generally contained a greater number of protein bands, and because both MAP fractions induced the formation of MAP-actin bundles (Fig. 2 *D* and *E*), the heat-stable fraction was used in all of the experiments described below.

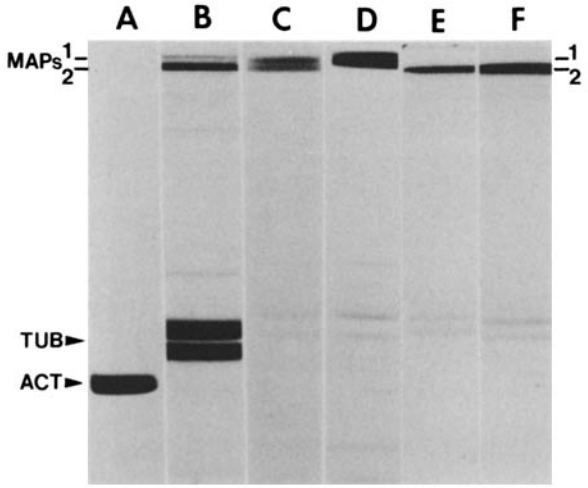

FIGURE 1 Electrophoretic analysis of purified actin (*A*), microtubule protein (*B*), MAP fraction isolated by phosphocellulose chromatography (*C* and *D*), and the heat-stable MAP fraction (*E* and *F*). The heat-stable MAP fraction contained primarily MAP-2, whereas the MAP fractions purified by phosphocellulose chromatography of microtubule protein contained both MAPs 1 and 2 as well as other proteins of lower molecular weight.

Stoichiometry of MAP-Actin Interactions

Increasing amounts of MAPs were added to a fixed amount of actin in a total volume of 200 μl. The mixtures were incubated for 25 min at 37°C, and were then centrifuged for 15 min at 20,000 *g*. As is shown in Fig. 3, relatively little of the actin alone sedimented under these conditions, and none of the MAPs alone (data not shown) sedimented. As increasing amounts of MAPs were added to the actin, a proportional increase in the amount of sedimentable protein was observed. When >120 μg of MAPs were added to 140 μg of actin, however, there was no further increase in the amount of protein sedimented. Electrophoretic analysis of the pelleted protein revealed that both actin and MAP-2 were present. Quantitative analysis of the sedimented protein (Table I) revealed that MAP-saturated actin contained ~20% MAPs and 80% actin. This corresponded to a ratio of ~1 mol of MAP-2 to 28 mol of actin. These results indicated that the interaction of MAPS with actin occurred in a defined stoichiometric ratio and suggested that the interactions between these proteins resulted in the formation of specific structures.

Because it was possible that a portion of the MAPs were nonspecifically trapped by the sedimenting actin filaments, actin (145 μg) was polymerized with 90 μg of MAP-2 and sedimented through a 10% sucrose-PEMD cushion (see Materials and Methods). As shown in Fig. 3 (gel *G*), the MAPs sedimented with the actin filament bundles in nearly the same stoichiometric ratio as when the sucrose cushion was omitted. Electron microscopy revealed that the structure of MAP-actin bundles sedimented through sucrose was identical to that shown in other experiments.

Morphology of MAP-Actin Bundles

Aliquots of mixtures of MAPs and actin were fixed with glutaraldehyde, negatively stained or shadowed with heavy

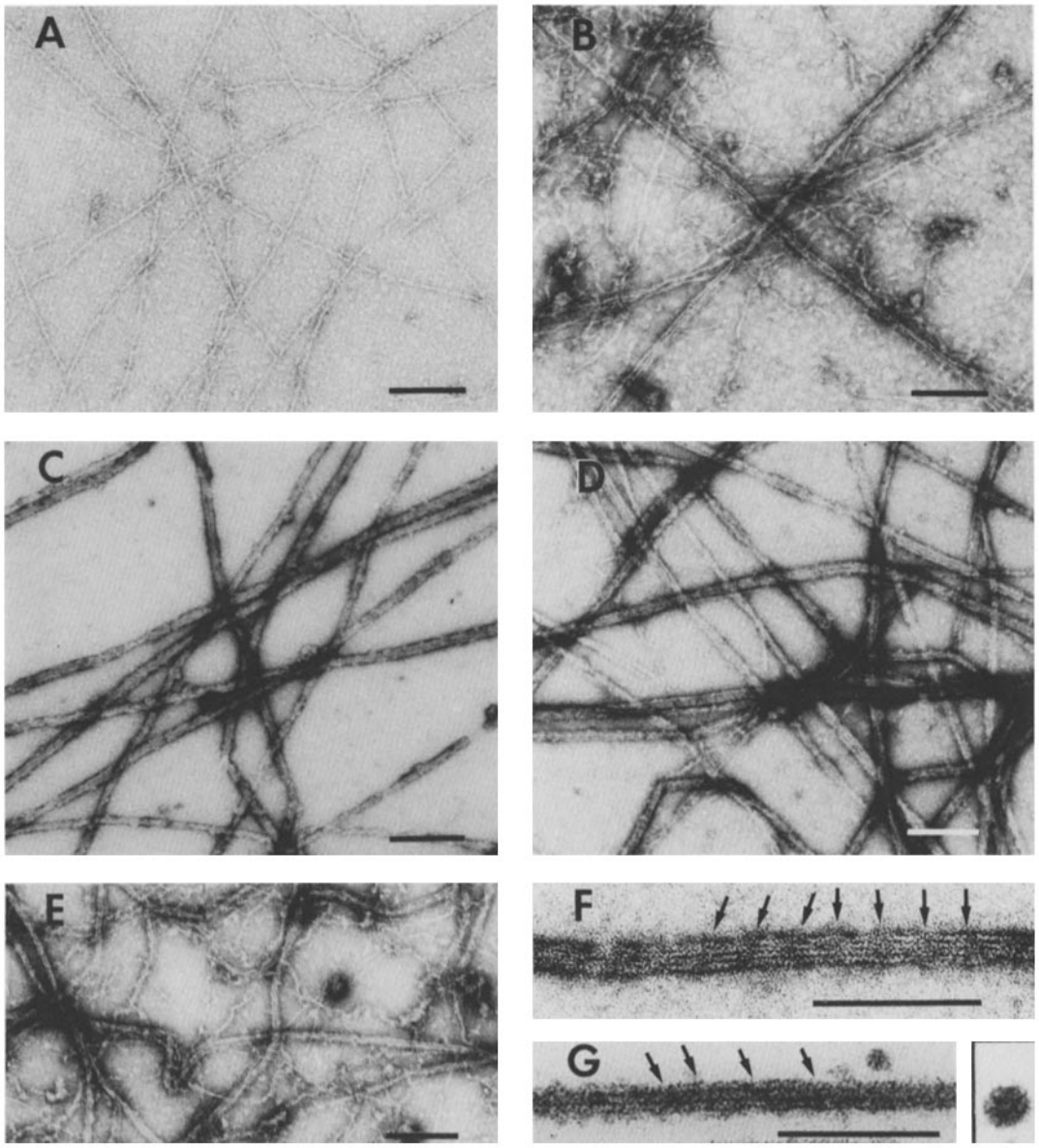

FIGURE 2 The formation of MAP-actin bundles by increasing amounts of MAP-2. Actin (140 μg) was polymerized in 200 μl of PEMD (*A*) or with the addition of 15 (*B*), 30 (*C*), or 60 μg (*D*) of the heat-stable MAP fraction, or 100 μg of phosphocellulose-purified MAPs (*E*). MAP-actin bundles were rapidly formed and contained periodic transverse striations (*F*, arrows) that could also be resolved in thin-sectioned bundles (*G*, arrows). A bundle in cross section (*inset*) reveals a hexagonal packing of actin filaments. Bars, 0.1 μm.

metal, and examined by electron microscopy. As is shown in Fig. 2 *A–D*, bundles of actin filaments were induced to form by the addition of heat-stable MAPs. As the concentration of the MAPs was increased from 75 μg/ml (*B*) to 300 μg/ml (*D*), an increasing number of 26-nm-diameter MAP-actin bundles was observed. When the MAP concentration was increased beyond 300 μg/ml, there was no evidence of an increase in either the number or the diameter of the MAP-actin bundles, although there was an increase in the granularity of the background on the Formvar film that was probably due to free MAPs (not shown). Unfractioned MAPs isolated by phosphocellulose chromatography (Fig. 2 *E*) also formed bundles; however, such preparations contained loose aggregates of actin and single filaments in addition to the bundles.

The MAP-actin bundles shown in Fig. 2 *D* were obtained from preparations that, when centrifuged, contained 83% actin and 17% MAPs (by weight) (Fig. 3 *D* and Table I). The diameter of the MAP-actin bundles was quite uniform and measured 25–30 nm in both negatively stained (*F*) and thin-sectioned bundles (*G*). Individual actin filaments could be resolved in longitudinal views of both negatively stained and thin-sectioned MAP-actin bundles. The number of filaments in a single bundle, which would be most accurately estimated from multiple cross sections, could not be determined. The *inset* of Fig. 2 *G*, however, shows an example of a cross section that suggests a hexagonal packing of seven filaments.

Periodic striations, which occurred at ~28-nm intervals, were observed along negatively stained MAP-actin bundles (Figs. 2 *F*, 4 *A–C*); similar striations were occasionally seen in thin-sectioned bundles (Fig. 2 *G*). The striations were more easily seen after translational reinforcement in which the image of the MAP-actin bundle was shifted in increments equivalent to

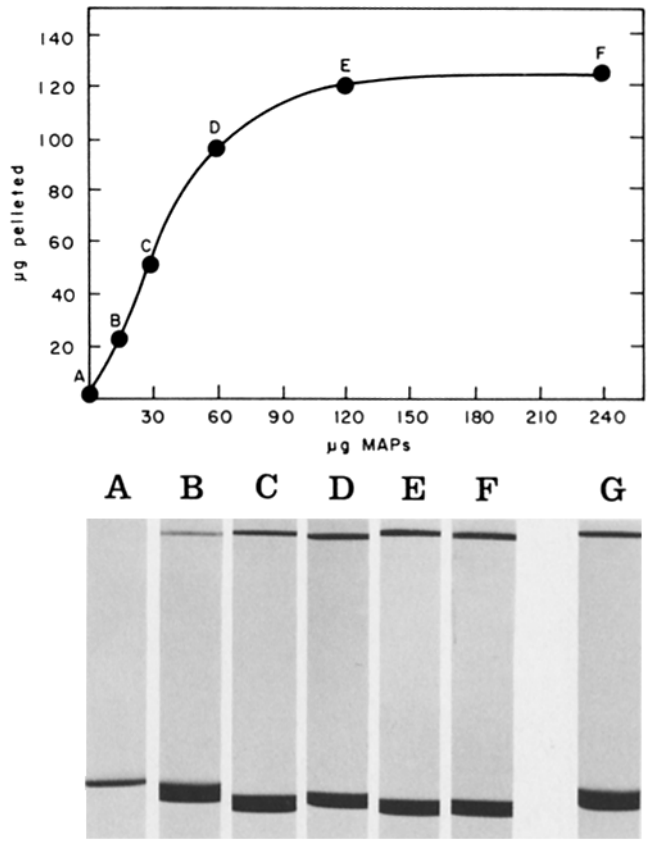

FIGURE 3 Sedimentation analysis of MAP-actin mixtures. Actin (140 µg) was polymerized in the presence of 0–240 µg of the heat-stable MAP fraction in a final volume of 200 µl of PEMD. The mixtures were then centrifuged for 15 min at 20,000 *g* and were assayed for the amount of sedimented protein. The pelleted protein was also analyzed by electrophoresis. Gels *B–F* contain aliquots of the pelleted MAP-actin mixtures shown in the graph. Gel *A* contains pure actin. Actin (145 µg) was also polymerized in the presence of 50 µg of heat-stable MAP, sedimented through a cushion of 10% sucrose in PEMD, and the pelleted protein was analyzed by electrophoresis (gel *G*).

TABLE I

Quantitation of MAP-Actin Bundle Proteins

µg MAPs	µg Pelleted	Actin	MAPs
		%	%
0	<2	100	0
15	23	96	3
30	53	92	8
60	96	83	17
120	117	81	18
240	123	80	19

Sedimented mixtures of actin and MAPs were electrophoresed on 7.5% acrylamide gels, fixed and stained with fast green, and scanned at 650 nm to determine the relative percent of actin and MAPs.

28 nm (Fig. 4*A–C*). When the MAP-actin bundles were shadowed with heavy metal (Fig. 4*D*), 12-nm projections from the surface of the bundles were resolved. These projections also were spaced at ~28-nm intervals along the bundles (Fig. 4*D–F*). Because both the striations and the projections were periodically spaced at 28-nm intervals along the bundles and because they only appeared along the surface of the MAP-actin bundles and not along bundles of actin filaments formed by other proteins,[1] we propose that they represent the MAP-2 molecules that organized the actin filaments into bundles.

Effects of Nucleotides on the Formation of MAP-Actin Bundles

The formation of MAP-actin bundles by either heat-stable or phosphocellulose-purified MAPs was inhibited by 1 mM ATP, AMP-PCP, or pyrophosphate, even though the assembly of individual actin filaments was not effected by the nucleotides. The formation of MAP-actin bundles proceeded normally in the presence of 1–3 mM AMP, GTP, or ADP, although a greater number of single actin filaments and somewhat fewer bundles were observed with ADP in comparison with the preparations that contained GTP or AMP. These observations could not be quantified by sedimentation analysis because of the entrapment of single actin filaments by intact and partially splayed bundles.

Intact MAP-actin bundles were dissociated by the addition of 1–2 mM ATP or AMP-PCP. When viewed in negatively stained preparations (Fig. 5), the addition of ATP to the MAP-actin bundles resulted in both complete and partial dissociation of the bundles into individual actin filaments, each of which was coated with small particles attached to the filaments at intervals of 25–34 nm. Because the periodicity of the particles is similar to that of the filamentous projections attached to the intact bundles (Fig. 4), it is likely that these particles represent individual MAP molecules attached to the actin filaments.

The inhibition of bundle formation by ATP, AMP-PCP, and pyrophosphate and the dissociation of intact bundles by ATP and AMP-PCP indicated that ATP hydrolysis was not necessary for the dissociation of MAPs from actin but left open the possibility that dissociation due to the chelation of magnesium ions by the nucleotides may have occurred. The addition of up to 2 mM EDTA did not, however, effect the formation of MAP-actin bundles in the absence of nucleotides. These results suggest that the inhibition of the formation of MAP-actin bundles is not due to the removal of magnesium ions but, rather, that the nucleotides have a more direct role in the inhibition, possibly by binding to sites on the MAPs or actin molecules and preventing their association with one another. In this regard, it should be recalled that hydrolysis is not required for the nucleotide-induced dissociation of myosin from actin (43, 44).

DISCUSSION

Actin has been found in virtually all eucaryotic cells (27) and is frequently organized into bundles of filaments. Discrete bundles of actin filaments have been found in the intestinal brush border microvilli (4, 25), the stereocilia of the inner ear (11, 40), acrosomal processes of invertebrate sperm (39), filopodia of sea urchin coelomocytes (9), and in cytoplasmic processes of cultured mammalian cells (21). Actin filament bundles in intestinal microvilli and in coelomocytes form in conjunction with specific actin-binding proteins (4, 5, 24, 26).

Actin filaments may also be associated with microtubules or microtubule proteins. Morphological evidence for the association of actin filaments with microtubules has been presented (15), and models have been proposed in which this kind of interaction would result in the intracellular movements of

[1] Sattilaro, R. F., and W. L. Dentler. Manuscript in preparation.

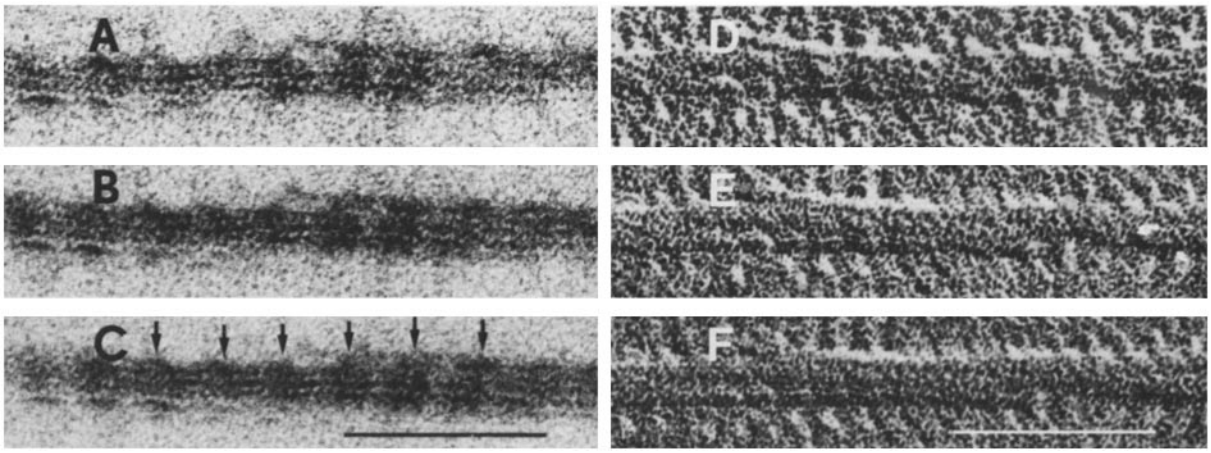

FIGURE 4 Linear translational reinforcement of MAP-actin bundles. The bundles were negatively stained (*A*–*C*) or were shadowed with platinum/palladium (*D*–*F*). Micrographs were printed with zero (*A* and *D*), one (*B* and *E*), or two (*C* and *F*) horizontal shifts that were equivalent to 28 nm. Striations (arrows, *A*–*C*) and projections (*D*–*F*) are believed to be MAP-2. Bar, 0.1 μm.

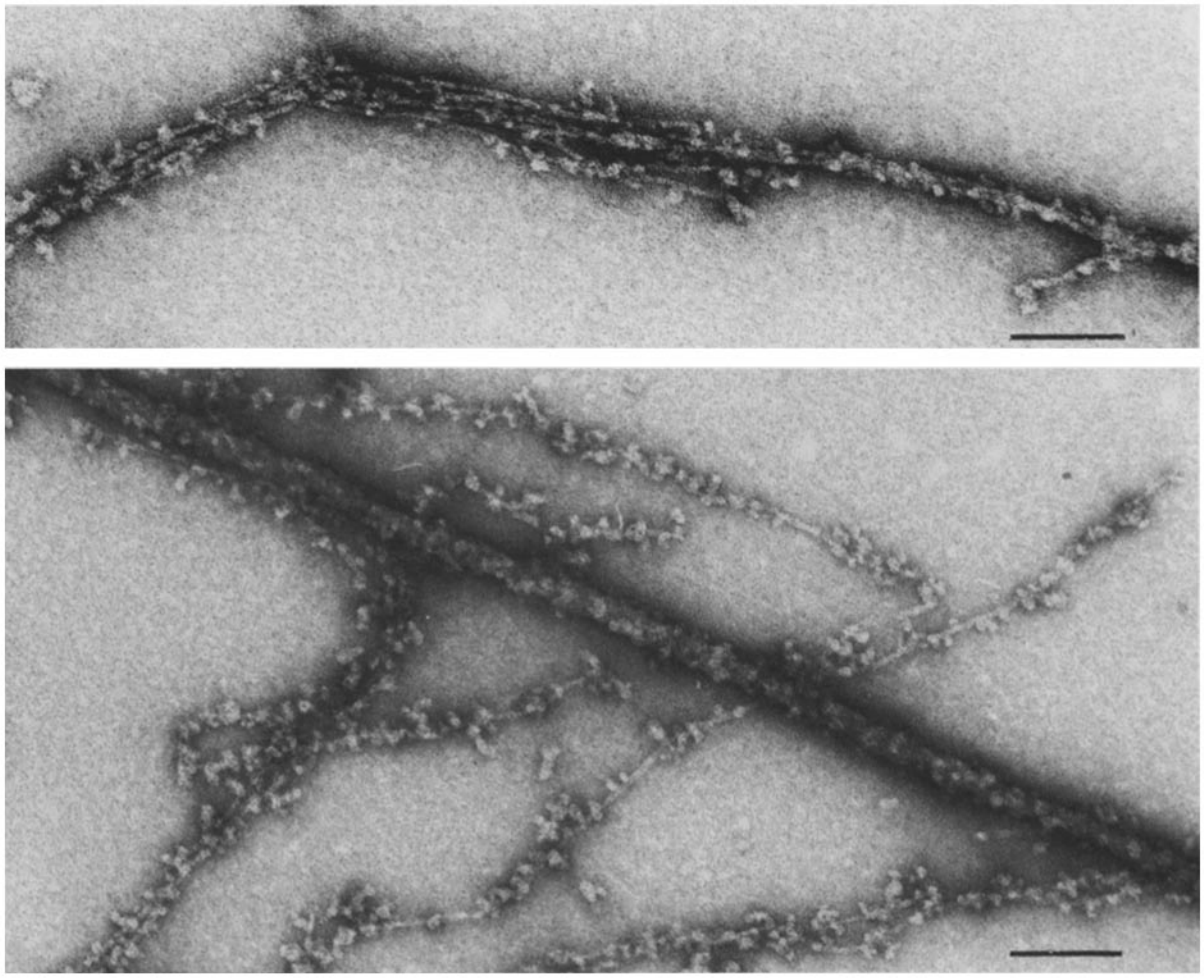

FIGURE 5 The effect of ATP on MAP-actin bundles. Bundles of actin filaments dissociate into individual filaments that are coated with globular particles presumed to be MAP-2. Bars, 0.1 μm.

organelles (5, 12). Griffith and Pollard (15) recently presented viscometric evidence that indicates that both microtubules and MAPs can interact with actin in vitro; when MAPs or microtubule protein were mixed with actin, the viscosity of the mixture was found to be greater than the sum of the viscosities of the individual components.

The results presented here show that MAPs, and particularly MAP-2, can not only bind to actin but also can induce the formation of discrete arrays of actin filaments that we have called "MAP-actin bundles." Periodic striations, visible in both thin-sectioned and negatively stained bundles, were spaced at 28-nm intervals along the long axis of the bundles. Heavy-

metal shadowing of the bundles revealed the presence of 12-nm projections from the bundle surface that were also periodically spaced at 28-nm intervals. Because both the striations and projections were seen only in the MAP-induced actin bundles and not in other aggregates of actin filaments (for example, those formed by polylysine [29]), it is likely that they represent the site of MAP-2 attachment to the F-actin.

Do the MAPs simply wrap around groups of actin filaments or do they bind to individual actin filaments? Based on the quantitative analysis of the proteins present in the sedimented MAP-actin bundles (Table I), there is ~1 MAP-2 molecule for every 28 actin molecules. This would be consistent with having the MAP-2 molecule attached at each crossover point along the actin helix. Although the binding of individual MAP molecules to individual filaments cannot be resolved in the tightly packed MAP-actin bundles, periodically spaced particles were observed to be attached to individual filaments when the bundles were partially dissociated by ATP (Fig. 5). It is likely, therefore, that the MAPs are bound to specific sites along the actin filament and that the bundling of the filaments by MAPs occurs due to interactions either between the MAPs themselves or between the MAPs and adjacent actin filaments. If the latter model is correct, then the MAPs might be expected to contain at least two different actin-binding sites.

We presume that one of the actin-binding sites is near an end of the MAP-2 molecule and that the second site is some distance from that end. The minimum separation for these sites is probably that distance required to span at least two adjacent actin filaments. The second actin-binding site may not be at the opposite end of the MAP-2 molecule, however, because filamentous projections, which presumably are the free ends of the MAPs, occur along the surface of the bundles. Preliminary evidence has indicated that coated vesicles isolated from mammalian brain can attach to the filamentous projections from the MAP-actin bundles,[1] and that purified clathrin, the major coated vesicle protein, binds to MAP-2 in vitro (30). MAP-2, therefore, may have at least three distinct sites to which actin and other proteins can attach.

Although these results suggest the possibility of distinct binding domains on MAP-2, they reveal little about the nature of the sites or of the specificity of their attachment to proteins. Furthermore, they do not reveal mechanisms by which the actin filaments can associate with MAPs that are attached to microtubules. Preliminary results (unpublished data) do indicate that one of the actin-binding sites on MAP-2 may also serve as the binding site for tubulin, because the addition of purified tubulin to a solution containing MAP-actin bundles resulted in the dissociation of the bundles. The nature of the tubulin-induced dissociation of MAP-actin bundles is currently under investigation.

Future studies of the role of MAPs and microtubules will undoubtedly be focused upon the MAP-mediated associations of microtubules with membranes, membrane vesicles, and other cytoskeletal structures. Some recent studies have shown that MAPs are necessary for the associations of secretory granule membranes and microtubules in vitro (32, 38) and it also has been shown that a high molecular weight protein is associated with the bridge observed to link ciliary outer-doublet microtubules to the ciliary membrane in vivo (8). Other studies have shown that only a small portion of the MAP-2 molecule is necessary for the observed stimulation of microtubule assembly in vitro by MAPs (18, 34, 41). The major portion of the MAP-2 molecule, therefore, projects outward from the microtubule (7, 17, 42) and presumably is available for the attachment of membranes or other cytoskeletal structures to the microtubule. The determination of the sites to which these structures bind will require new techniques with which to examine the MAP-2 molecule. It is expected that the results presented here, showing that the MAPs can induce the formation of actin bundles, may provide a new experimental system in which purified actin and MAP molecules can be studied to determine what specific sites on the MAP molecules are involved in the association of MAPs or microtubules with other cytoskeletal components.

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