EVIDENCE FOR ACTIN FILAMENT-MICROTUBULE INTERACTION MEDIATED BY MICROTUBULE-ASSOCIATED PROTEINS

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ABSTRACT

We have used low shear viscometry and electron microscopy to study the interaction between pure actin filaments and microtubules. Mixtures of microtubules having microtubule-associated proteins (MAPs) with actin filaments have very high viscosities compared with the viscosities of the separate components. MAPs themselves also cause a large increase in the viscosity of actin filaments. In contrast, mixtures of actin filaments with tubulin polymers lacking MAPs have low viscosities, close to the sum of the viscosities of the separate components. Our interpretation of these observations is that there is an interaction between actin filaments and microtubules which requires MAPs. This interaction is inhibited by ATP and some related compounds. Electron micrographs of thin sections through mixtures of actin and microtubules show numerous close associations between the two polymers which may be responsible for their high viscosity.

KEY WORDS microtubule · actin · viscosity

Although microtubules are required for a variety of intracellular movements including axoplasmic transport, pigment granule migration, saltatory movements, and chromosome movement, the energy-transducing mechanism responsible for these movements has not been identified. Microtubule assembly and disassembly is suggested as one possibility (13). There could be a dynein-like ATPase, or, alternatively or in addition, some other force-generating system could be associated with cytoplasmic microtubules (7). The presence of actin (2, 7, 9, 16) and myosin (8) together with microtubules in the mitotic spindle suggests that actin and myosin might be this other force-generating system. A minimum requirement for actomyosin to generate microtubule-dependent movements is that the proteins interact with each other.

To approach the question of possible actin-microtubule interaction at the molecular level, we have used low shear viscometry and electron microscopy to study the properties of mixtures of purified actin filaments and microtubules. We report here evidence that these two polymers can interact and that this interaction depends upon the presence of microtubule-associated proteins (MAPs).

MATERIALS AND METHODS

Materials

Reagent grade chemicals were obtained from the following sources: 2-(N-morpholino)-ethane sulfonic acid (MES), GTP, adenosine, AMP, ADP, ATP, UTP, cyclic AMP (cAMP), and dibutyryl cAMP from Sigma Chemical Co., St. Louis, Mo.; AMP-P-C-P, AMP-P-N-P from Boehringer Mannheim Biochemicals, Indianapolis, Ind.; ITP, CTP from P-L Biochemicals, Inc., Milwaukee, Wis.; cytochalasin B from Aldrich Chemical Co., Inc., Milwaukee, Wis.; phosphocellulose (P-11) from Whatman, Inc., Clifton, N. J.; Sepharose 4B (4% agarose beads) from Pharmacia Fine Chemicals, Inc., Piscataway, N.J.

Methods

PURIFICATION OF PROTEINS: Rabbit skeletal muscle actin was purified (18) by a single cycle of

polymerization and sedimentation from 0.8 M KCl. Stock solutions of 10–12 mg/ml were dialyzed 2–4 days vs. a depolymerizing buffer (2 mM Tris-Cl, pH 8.0, 0.2 mM ATP, 0.2 mM CaCl$_2$, 0.5 mM dithiothreitol), clarified by ultracentrifugation at 100,000 g for 1 h, and used within 2–3 days.

Crude microtubule protein from calf brain grey matter was purified by one cycle of polymerization in glycerol according to a slight modification of the method of Shelanski et al. (17) and stored in liquid N$_2$ for up to 3 wk. On the day of an experiment, the crude microtubule protein was processed through a second cycle of polymerization, depolymerization, and clarification in microtubule buffer (0.1 M MES-K pH 6.4, 1 mM EGTA-K, 0.5 mM MgCl$_2$, 1 mM GTP). We will refer to this twice-polymerized and -depolymerized material consisting of tubulin and MAPs as microtubule protein (MTP). Stock solutions of 8–15 mg/ml retained their polymerizing ability for up to 12 h at 0°C. Purified tubulin and MAPs were obtained by fractionating ~120 mg of microtubule protein on a 1.5 × 17-cm column of phosphocellulose (20), equilibrated with quarter-strength microtubule buffer. Tubulin was eluted with this buffer, at a peak concentration of ~9 mg/ml. After washing the column with GTP-free buffer, MAPs were eluted at a peak concentration of 2 mg/ml with 0.8 M KCl in GTP-free column buffer. Isolated MAPs were dialyzed vs. GTP free-microtubule buffer and used within 30 h. Microtubule fragments were made from polymerized microtubule protein by three rapid passages through a 25-gauge 5/8-inch syringe needle.

PROCEDURE FOR COMBINING PROTEIN FOR VISCOMETRY AND PELLETING EXPERIMENTS: Because microtubules require exacting buffer conditions for polymerization and actin does not, experiments were done in microtubule buffer unless otherwise indicated. Samples were mixed at 0°C in the following order: (a) H$_2$O, (b) stock 8× microtubule buffer, (c) additions, if used, such as ATP or cAMP, (d) microtubule protein or MAPs and/or purified tubulin, (e) actin, and, if used, (f) dimethyl sulfoxide (DMSO). Actin was added last because it polymerizes readily under experimental buffer and salt conditions, even at 0°C. Except where stated differently, these samples were immediately drawn into capillary tubes (ID 1.3 mm, 12.6 cm long) and warmed to 37°C for exactly 20 min, when their viscosity was measured by determining the rate at which a 0.64-mm stainless steel ball falls through the sample. In this simple viscometer, the velocity of the ball is inversely proportional to the viscosity over a range of 1 to 12,000 cp.[1]

PREPARATION OF ACTIN AFFINITY RESIN: 200 mg of actin was coupled covalently to 80 ml of cyanogen-bromide-activated Sepharose 4B (3). The beads were washed successively with water, 0.1 M glycine, 0.5 M NaCl in 0.1 M NaHCO$_3$, pH 10, 0.5 M NaCl in 0.1 M sodium acetate, pH 4, water, and microtubule buffer.

[1] This viscometer will be described in detail in another publication by MacLean and Pollard.

BIOCHEMICAL METHODS: Protein concentrations were measured during experiments by UV absorption using the following extinction coefficients: actin $E_{290}^{1\%}$ = 6.5, microtubule protein $E_{290nm-1.74\times333nm}^{1\%}$ = 5.2, MAPs $E_{290}^{1\%}$ = 3.1, and purified tubulin $E_{290}^{1\%}$ = 5.3. They were later verified by protein analysis (11), using serum albumin or egg albumin as a standard.

Sodium dodecyl sulfate polyacrylamide electrophoresis gels (19) were stained with Coomassie Brilliant Blue R (5) (ICI United States, Inc., Wilmington, Del.).

ELECTRON MICROSCOPY: Samples were negatively stained with 1% uranyl acetate on hydrophilic Formvar-carbon-coated grids. Pellets of microtubules and tubulin polymers were prepared by fixation at room temperature in 2.5% glutaraldehyde (TAAB Laboratories, Reading, England) in phosphocellulose column buffer lacking GTP, rinsing twice with 5 ml of 100 mM Na phosphate, pH 7, 50 mM KCl, and 5 mM MgCl$_2$, treatment with 4 mM OsO$_4$ in rinse buffer for 20 min at 22°C, rapid dehydration with EtOH followed by two changes of propylene oxide, and embedding in Epon. High viscosity mixtures of 2 mg/ml of actin and 4 mg/ml of microtubule protein polymerized at 37°C for 30 min were fixed without stirring by layering on top warm 1% glutaraldehyde, 0.2% tannic acid in 50 mM sodium phosphate, 50 mM KCl, 5 mM MgCl$_2$, pH 7.0 (1). After 1 h at 37°C, the solidified samples were moved to 4°C overnight, then treated with 4 mM OsO$_4$ in the same buffer, pH 6.0, for 20 min at 20°C and embedded as described above.

RESULTS

Preparation of Proteins

The purity of the proteins used in our experiments is illustrated in Fig. 1. The actin is electrophoretically pure. The microtubule protein consists mainly of tubulin and high molecular weight MAPs and is free of actin. The tubulin is electrophoretically pure. The MAPs are heterogeneous, consisting of high molecular weight proteins (mol wt 270,000–300,000), tau proteins (mol wt 55,000–70,000), and at least a dozen other minor polypeptides.

Viscosity of Polymers of Actin and Microtubule Protein

Both actin and microtubule protein polymerize rapidly under the conditions of our experiments. When measured at high shear in an Ostwald capillary viscometer, an equilibrium viscosity is reached for 1 mg/ml of actin in 2 min and for 4 mg/ml of microtubule protein in 7 min. The high shear reduced viscosity of the equilibrium mixture of actin filaments is ~1 cp-ml/mg. For microtubule, the value is ~0.2 cp-ml/mg. In contrast, when the viscosity is measured at low shear in the
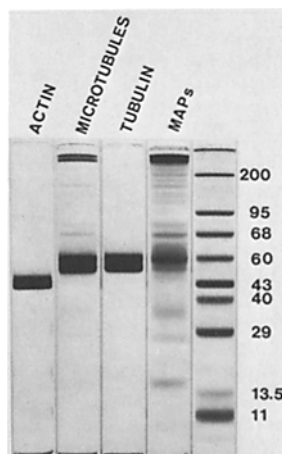
FIGURE 1 Electrophoresis on 7.5% polyacrylamide in sodium dodecyl sulfate. The gel on the right is a standard. Molecular weights are given in thousands.

falling ball viscometer, the reduced viscosities of both actin filaments and microtubules are much higher. This variation of the viscosity with shear rate demonstrates that neither polymer solution is a Newtonian fluid. Moreover, the reduced viscosities depend on protein concentration (Figs. 2 and 3 *inset*). At low concentration, the reduced viscosity of actin filaments is ~50 cp-ml/mg, whereas at 6 mg/ml it is >120 cp-ml/mg. The reduced viscosity of microtubules is ~2 cp-ml/mg at low concentrations and >50 cp-ml/mg at high concentrations (Fig. 2).

Viscosity of Actin Filament-Microtubule Mixtures

The viscosity of mixtures of actin filaments and microtubules is much higher than expected from the viscosities of the components (Figs. 2 and 3; Table I).[2] This observation, supported by various controls described below, is our main evidence that actin filaments and microtubules form a complex.

The viscosity of an actin-microtubule mixture depends on the manner in which the mixture is formed (Table I). The highest viscosities are attained when the monomers are mixed and allowed to polymerize together. When polymerized actin filaments and polymerized microtubules are mixed

[2] Throughout this paper, we use as a reference the viscosities of the components measured at approximately the same shear as the mixture when describing the viscosity of mixtures because all of these samples are non-Newtonian.

together, the viscosity is low initially but increases with time. Mixtures of actin with microtubules fragmented by shearing give the same high viscosity as mixtures of actin with intact microtubules.
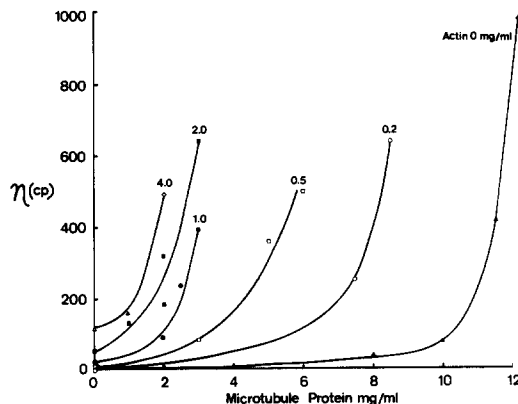

FIGURE 2 Concentration dependence of the viscosity of mixtures of actin and microtubule protein. The concentration of actin in mg/ml is given next to each curve.
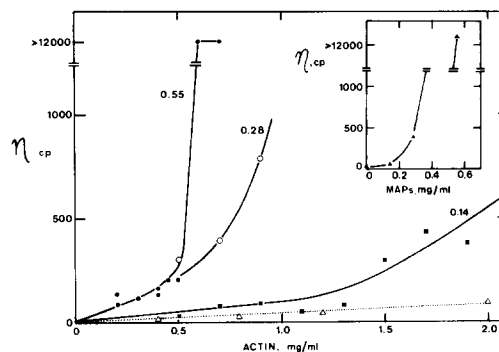

FIGURE 3 Comparison of the viscosity attained by mixtures of actin filaments with polymers of either microtubule protein or purified tubulin. The conditions were normalized by using concentrations of microtubule protein and tubulin which had viscosities of 15 cp when polymerized separately (*inset*). The concentrations were 5.8 mg/ml of microtubule protein without DMSO, 4.2 mg/ml of microtubule protein in 10% DMSO, or 5.5 mg/ml of purified tubulin in 10% DMSO. (O——O) Copolymerization of microtubule protein and actin, (□·····□) copolymerization of microtubule protein and actin in DMSO, (△·····△) copolymerization of purified tubulin and actin in DMSO, (●——●) actin filaments alone, (■·····■) actin filaments in DMSO. *Inset*: The protein concentration dependence of the viscosity of polymers of microtubule protein with or without 10% DMSO and purified tubulin with 10% DMSO. (O——O) microtubule protein polymers, (□·····□) microtubule protein polymers in DMSO, (△·····△) purified tubulin polymers in DMSO.

TABLE I

Influence of Shearing on the Viscosity of Actin Filament-Microtubule Mixtures

	Viscosity
	cp
Component	
Actin, 1 mg/ml	90
Microtubules, 2 mg/ml	4
Mixtures of actin and microtubule protein	
Polymerized together for 20 min	780
Polymerized together for 20 min, mixed by vortexing, and then measured immediately	23
Polymerized separately for 20 min, mixed together by vortexing, and measured immediately	12
Polymerized separately for 20 min, mixed together by vortexing, incubated for 20 min, and then measured	210
Polymerized separately for 20 min, microtubules fragmented by shearing, actin filaments mixed with sheared microtubules by vortexing, incubated for 20 min, and then measured	190

Because shearing decreases the viscosity of the mixture (Table I) and the falling ball shears the sample, we routinely make a single measurement on each sample.

The viscosity of actin-microtubule mixtures increases with time at 37°C. This occurs although the polymerization of the filaments, as measured by Ostwald viscometry, is complete within 7 min. The low shear viscosity of the mixtures doubles every 20 min for over an hour, after which it plateaus. In comparison, the low shear viscosity of microtubules is stable after 5 min, and the low shear viscosity of pure actin filaments increases only slowly with time. To simplify experiments, we routinely take viscosity measurements 20 min after mixing and initiating polymerization.

The viscosity of mixtures of actin filaments and microtubules depends on the shear rate during measurement. For example, a mixture of 1 mg/ml of actin and 4 mg/ml of microtubule protein polymerized together in 100 μM GTP for 30 min has a specific viscosity of 2.5 cp when measured in an Ostwald viscometer and 3,100 cp in the falling ball viscometer. In both cases, the viscosity is higher than expected from the viscosity of the components; but this difference is obviously much easier to measure at low shear.

The viscosity of actin-microtubule mixtures depends in a nonlinear fashion upon the concentra-

tion of both proteins (Figs. 2 and 3). If the mixture contains a high concentration of either or both polymers, the viscosity exceeds the 12,000-cp limit of our viscometer.

Actin-microtubule mixtures do not attain high viscosity when microtubule protein is polymerized in the presence of polymerization incompetent monomeric actin made in Ca^{++}-free buffer.

Role of MAPs in Actin-Microtubule Interaction

MAPs are required for microtubules to form a high viscosity complex with actin filaments. This is demonstrated by comparing the viscosity of mixtures of actin filaments and microtubules consisting of tubulin and MAPs with the viscosity of mixtures of actin filaments and pure tubulin polymers (Fig. 3 and additional experiments with pure tubulin nucleated with microtubule fragments). The MAPs-tubulin microtubules form a high viscosity complex with actin filaments, but neither of the pure tubulin polymers does. Pure tubulin polymers can form a high viscosity mixture with actin filaments only if MAPs are added back (Table II).

These experiments require the following special considerations. The proteins were polymerized in microtubule buffer with 10% DMSO, because this buffer supports pure tubulin polymerization (12) without perturbing actin polymerization. Other conditions which promote the polymerization of pure tubulin, such as high glycerol and/or high $MgCl_2$, alter the polymerization of actin. Pure tubulin polymerized in DMSO forms coiled sheets of protofilaments (Fig. 4 a), although when

TABLE II

Reconstitution Experiments

Protein (s)	η
	cp
Actin filaments	80
Microtubule protein microtubules	15
Actin + microtubule protein microtubules	>12,000
MAPs	<5
Tubulin polymers	10
MAPs + tubulin microtubules	10
Actin filaments + MAPs	560
Actin filaments + MAPs + tubulin microtubules	>12,000

Buffer: 0.1 M MES-K, pH 6.4, 1.0 mM EGTA, 0.5 mM $MgCl_2$, 1.0 mM GTP, 10% DMSO. Protein concentrations: 1.6 mg/ml actin, 4.6 mg/ml microtubule protein, 3.3 mg/ml purified tubulin, 1.0 mg/ml MAPs. Samples were incubated for 20 min at 37°C.

nucleated with a low concentration of microtubule fragments it will form ~75% closed tubules (Fig. 4b). Neither of these pure tubulin polymers formed a high viscosity complex with actin filaments like microtubule protein polymers consisting of tubulin and MAPs.

A further indication that MAPs participate in the interaction of microtubules with actin filaments is the ability of MAPs to increase the viscosity of actin in the absence of tubulin (Fig. 5). The viscosity of actin-MAPs mixtures depends on the concentrations of both constituents.
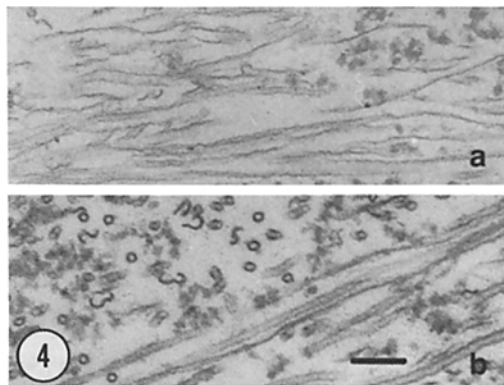
FIGURE 4   Electron micrographs of thin sections of pellets of purified tubulin polymerized in 10% DMSO (a) and in 10% DMSO with 8% of the total protein present as fragments of microtubules (b). Without nuclei, tubulin forms sheets of protofilaments. When nucleated by microtubule fragments, tubulin forms 75% closed microtubules. Bar, 0.1 μm.
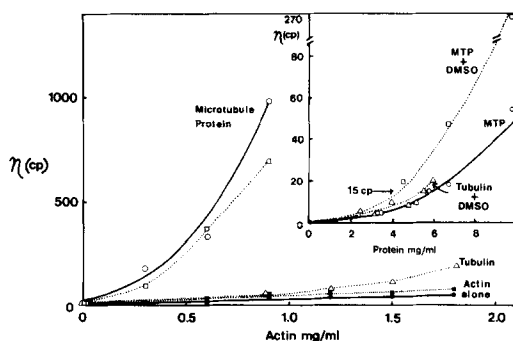
FIGURE 5   Dependence of viscosity of mixtures of actin and MAPs on the concentrations of both constituents. Actin and MAPs were combined in 0.1 M MES-K, pH 6.4, 1 mM EGTA-K, 0.5 mM MgCl₂. The concentration of MAPs in mg/ml is indicated next to each plot. (△······△) actin alone. Inset: Dependence of actin-MAPs viscosity on MAPs concentration at an actin concentration of 0.7 mg/ml.
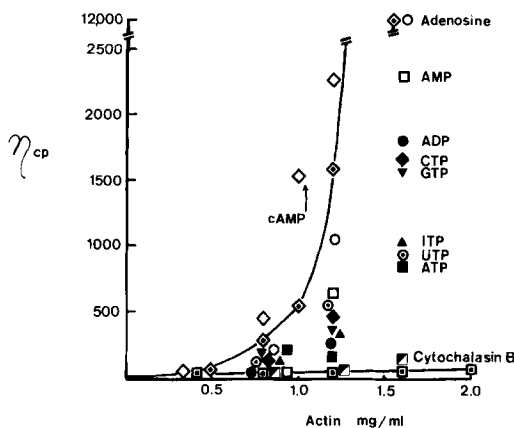
FIGURE 6   Influence of various compounds on the viscosity of mixtures of actin filaments and microtubules. Various concentrations of actin were polymerized together with 2 mg/ml of microtubule protein in microtubule buffer with 50 μM GTP and 1 mM of the added compounds (except 10 μM cytochalasin B). All of the symbols are defined on the graph except for square within diamond (no additions) and square within square (actin without microtubule protein).

Factors Influencing the Viscosity of Mixtures of Actin Filaments with Microtubules or MAPs

The viscosity of mixtures of actin filaments with microtubules (Fig. 6) or MAPs (Table III) is sensitive to the addition of nucleotides and related compounds. In both cases, ATP inhibits strongly, GTP inhibits moderately, AMP has little effect, and cAMP increases the viscosity of the mixture. Some compounds, such as the nonhydrolyzable ATP analogues, could be tested only with actin and MAPs, inasmuch as they decrease the low shear viscosity of microtubules alone. The effects of additions on actin-MAPs viscosity and actin-microtubule viscosity were in general similar, except that cytochalasin B inhibits actin-MAPs viscosity less strongly than that of actin-microtubules. These effects are not due to an increase in ionic strength because 10 mM KCl has no effect on the viscosity of actin-microtubule mixtures.

Affinity of Microtubule Proteins and MAPs for Actin

Two types of preliminary experiments indicate that the interaction between actin filaments and microtubules is one of low affinity. (a) Microtubules with MAPs bind to actin coupled covalently

TABLE III

Effects of Various Compounds on the Viscosity of MAPs-Actin Mixtures

Effect	Compound
Enhancement	cAMP
No effect	Adenosine
	AMP
Moderate inhibition	ADP
	AMP-PNP
	AMP-PCP
	GTP
	UTP
	CTP
	Cytochalasin B
Strong inhibition	ATP
	ITP
	Pyrophosphate

The influence of various compounds on the viscosity of mixtures of actin filaments and MAPs was determined at a fixed MAPs concentration of 0.8 mg/ml and various actin concentrations up to 2 mg/ml as in Fig. 5. The concentration of the added compounds was 1 mM except for cytochalasin B which was 10 μM. Enhancement: viscosity values greater than 150% those of (MAPs + action). No effect: viscosity values equal to those of (MAPs + actin) ± 20%. Moderate inhibition: viscosity values less 50% those of (MAPs + actin) but greater than 120% those of actin alone. Strong inhibition: viscosity values equal to those of actin alone ± 20%.

to agarose beads, but at saturation only 0.2 mg of 1.0 mg total microtubule protein binds to beads containing 2.5 mg of immobilized actin.[3] (*b*) Purified MAPs (0.9 mg/ml) do not pellet with actin filaments (1.5 mg/ml) in centrifugation experiments performed in 0.1 M MES-K, pH 6.4, 1 mM EGTA, 0.5 mM $MgCl_2$, with or without 1 mM GTP.

Electron Microscopy of High Viscosity Actin Filament-Microtubule Mixtures

Microtubules and actin filaments are frequently seen in close association with each other in samples fixed without stirring (Fig. 7). A few actin

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[3] J. Izant and J. R. McIntosh (14) of the University of Colorado have carried out related affinity chromatography experiments with immobilized microtubule protein and found evidence for a weak binding of actin in crude cellular extracts. This work was presented in their poster at the Annual Meeting of the American Society for Cell Biology in November 1977, but is not described in their abstract.

filaments can be seen extending radially from microtubules, and lateral associations between the polymers are quite common. >80% of microtubule profiles have at least one actin filament within 20 nm.

DISCUSSION

We interpret our viscometric and electron microscopic observations as evidence for actin-microtubule interactions mediated by MAPs. Both approaches have shortcomings which make our present conclusions only qualitative. In the following paragraphs, we first discuss these experimental difficulties and then comment briefly on the possible significance of actin-microtubule interactions.

Viscometry

Analysis of actin-microtubule interactions by low shear falling ball viscometry is certainly convenient, but the quantitative interpretation of these data is limited by two factors. (*a*) The main problem is that none of the samples (including both pure actin and microtubules) are Newtonian fluids. Consequently, it is impossible to record precise viscometric data without being able to control and vary at will the shear rate in the viscometer. The problem is amplified with samples like actin-microtubule mixtures which lose viscosity and do not recover completely when sheared, because the measurement itself irreversibly alters the viscosity. (*b*) Secondly, the theory of weakly cross-linked gels is not developed well enough to allow quantitative interpretations of the data.

In spite of these limitations, we can make some qualitative conclusions from the measurements. (*a*) The high viscosity of the actin-microtubule mixtures is probably due to cross-linking of the fibers, as in other types of protein gels (6). The high viscosity is not due to tangling of the polymers, because mixtures of actin filaments and pure tubulin polymers do not have high viscosities, because the viscosity of the mixtures is decreased by various compounds which do not alter the viscosity of either component fiber, and because short microtubules cause the same large increase in actin viscosity that long microtubules do. (*b*) The cross-linking of the two fibers appears to be mediated by MAPs. The specific microtubule-associated protein(s) responsible for this cross-linking remains to be purified, but the high molecular
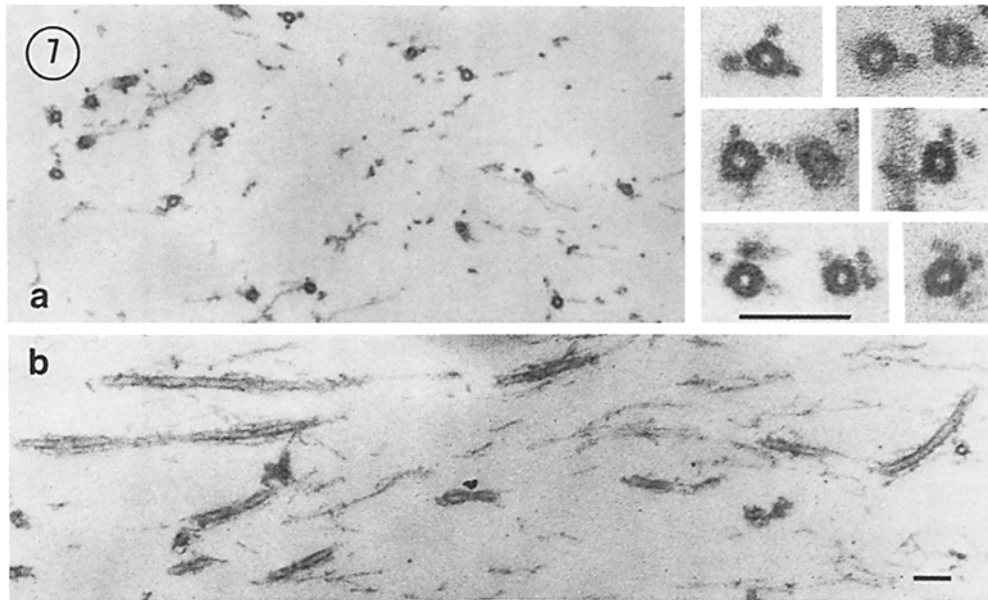
FIGURE 7 Electron micrographs of thin sections of a mixture of 2 mg/ml of actin and 4 mg/ml of microtubule protein polymerized together in microtubule buffer with 0.7 mM GTP and then fixed *in situ*. Insets show cross sections of microtubules with associated actin filaments at higher magnification. Bars, 0.1 μm.

weight proteins bound to the surface of microtubules (4, 15) seem like good candidates for binding actin. The mechanism by which isolated MAPs increase the viscosity of actin is unknown, but it is suspected that it involves the cross-linking of actin filaments by aggregated MAPs. (c) The affinity of MAPs for actin is low compared with their affinity for tubulin. On the basis of their failure to pellet with actin, the association constant of MAPs for binding to actin is probably $<10^5$. It may be questioned whether such a low affinity association is specific. One indication of specificity is the differential sensitivity of the MAPs-actin viscosity to a variety of nucleotides, but the final answer will depend on the purification and characterization of the active protein(s).

Electron Microscopy

The electron microscopy of actin filament-microtubule mixtures reveals close associations between the two fiber types, but, without the independent viscometric data, it would not be convincing evidence for interaction. For example, there is no simple way of knowing whether these links exist before fixation. On the other hand, we do not know whether some additional links between the two fibers are lost during preparation for electron microscopy.

With these reservations in mind, we cautiously interpret the close association of actin filaments with microtubules as cross-links between the fibers. As in other protein gels (6), it is likely that these actin-microtubule links bind the fibers into a continuous network, so that at high fiber concentrations a gel forms.

The micrographs suggest that a short segment of an actin filament is bound along the microtubule surface, whereas other parts of the same actin filament extend radially from the microtubule surface. If one of these extended actins connected each pair of microtubules, a gel would form (6). Similar actin-microtubule bonds have been observed in the mitotic spindle (9). The relatively constant separation of the two fibers suggests that they are attached by multiple bonds of fixed length.

Significance

These observations on purified actin filaments and microtubules provide some biochemical evidence for interaction of these important cellular fibers. The experiments reveal some new infor-

mation about this interaction, including the participation of MAPs and inhibition by nucleotides. Now that it is possible to measure these interactions, rapid progress should be made in the analysis of their molecular basis. It will be important to reach this goal, because it seems possible that actin filaments anchored to microtubules in cells may be responsible for some vital microtubule-dependent movements including mitosis.

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