Phosphorylation of Microtubule-associated Proteins Regulates Their Interaction with Actin Filaments*

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We have determined the absolute phosphate content of microtubule-associated proteins (MAPs) and established that phosphorylation inhibits the actin filament cross-linking activity of MAPs and both of the major MAP components, MAP-2 and r. Similar results were obtained with actin from rabbit muscle, hog brain, and Acanthamoeba castellanii. We used the endogenous phosphatases and kinases in hog brain microtubule protein to modulate MAP phosphate level before isolating heat-stable MAPs. MAPs isolated directly from twice-cycled microtubule protein contain 7.1 ± 0.1 (S.E.) mol of phosphate/300,000 g protein. After incubating microtubule protein without ATP, MAPs had 4.9 ± 0.6 phosphates. After incubating microtubule protein with 1 mM ATP and 5 mM cAMP in 2 mM EGTA, MAPs had 8.6 ± 0.5 phosphates but there was also exchange of three more [32P]phosphates from γ-labeled ATP for pre-existing MAP phosphate. Incubation of microtubule protein with ATP and cAMP in 5 mM CaCl2 resulted in exchange but no net addition of phosphate to MAPs. We fractionated the MAP preparations by gel filtration and obtained MAP-2 with 4.3 to 7.5 and r with 1.5 to 2.2 mol of phosphate/mol of protein depending on how we treated the microtubule protein prior to MAP isolation. The actin filament cross-linking activity of whole MAPs, MAP-2, and r depended on the MAP-phosphate content. In all cases, phosphorylation of MAPs inhibited actin filament cross-linking activity. The concentration of high phosphate MAPs required to form a high viscosity solution with actin filaments was 2 to 4 times more than that of low phosphate MAPs. During incubation of microtubule protein with [γ-32P]ATP, only MAP peptides are labeled. Treatment of these MAPs with either acid or alkaline phosphatase removes phosphate mainly from MAP-2, with an increase in actin filament cross-linking activity. Thus, both MAP phosphorylation and the effect of phosphorylation on actin cross-linking activity of MAPs are reversible.

We report here experiments demonstrating that the extent of phosphorylation of MAPs1 is one factor that can regulate the interaction of microtubules with actin filaments. This finding suggests a physiological function for MAP phosphorylation. Further, the existence of such a regulatory mechanism provides indirect support for the idea that actin filament-microtubule interactions observed in vitro with isolated proteins may also occur in the cell.

There have been many suggestions that actin filament-microtubule interactions are important for cellular structure, or some types of microtubule-dependent movement, or both (1–4). Griffith and Pollard (5) provided the first biochemical evidence for such interactions. They found that mixtures of purified actin filaments and microtubules have a higher viscosity (especially at low shear rates) than expected from the viscosity of the individual components. Since pure tubulin polymers failed to form a high viscosity complex with actin filaments and MAPs alone caused actin filaments to gel, they concluded that MAPs are required to cross-link microtubules and actin filaments (5, 6). Both of the major MAPs, MAP-2 (6, 7) and r protein (6), can interact with actin filaments.

It was known that various nucleoside triphosphates inhibit the viscosity of mixtures of MAPs with actin filaments (5), but it was Nishida et al. (8) who first suggested that this might be mediated by reversible phosphorylation of MAPs. Most microtubule protein preparations are contaminated with kinases which can phosphorylate MAPs (9–12). Under conditions where 32P from [γ-32P]ATP is incorporated into MAPs, the MAPs have reduced ability to stimulate microtubule polymerization (13, 14) and to cross-link actin filaments (8). These two effects of incubating microtubule protein with ATP can be partially separated by carrying out the reaction under polymerizing conditions (8). Together with Valle’s (15) evidence for multiple phosphorylation sites on MAP-2, these experiments suggest that tubule polymerization and actin interaction could be regulated by separate sites, at least on MAP-2.

In all of the experiments where “phosphorylation” was judged by the extent of the incorporation of 32P from ATP into MAPs, it was assumed that there was a net increase in the phosphate content of the MAPs, but this was never shown. Furthermore, the starting level of phosphorylation was not known. Although improbable, it is even possible due to the antagonistic action of phosphatases that the level of MAP phosphorylation would actually decrease during incubation with [γ-32P]ATP even though some 32P was transferred to the protein.

We show here by direct measurement of protein-bound phosphate that heat-stable MAPs isolated from pig brain microtubule protein have about 7 mol of phosphate/300,000 g of protein. Incubation of the microtubule protein with ATP and cAMP in EGTA results in a net increase in phosphate content up to 12 mol/300,000 g of protein with an average of 8.6. During incubation without the nucleotides, the phosphate content declines to 5. The incorporated phosphate is distrib-

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1 The abbreviation used are: MAPs, microtubule-associated proteins (MAP-1, \( M_t = 350,000 \)); MAP-2, \( M_t = 300,000 \); MAP components; CGC, critical gelation concentration; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N’N’-tetraacetic acid; PIPES, pipera
dine-N,N’-bis(2-ethanesulfonic acid); MES, 4-morpholinolinesulfonic acid; HEPES, 4-(2-hydroxyethyl) -1-piperazinethanesulfonic acid; MeSO, dimethyl sulfoxide.
utted among all of the MAPs. There is an inverse, nonlinear relationship between the extent of MAP phosphorylation and the actin filament cross-linking activity of the MAPs. This effect of phosphorylation on MAPs-actin filament interaction is entirely reversible. Preliminary accounts were presented at the 1981 Oji Conference on Microtubules in Tokyo (16) and at the 1982 Annual Meeting of the American Society for Cell Biology (17).

MATERIALS AND METHODS

Reagent grade chemicals were obtained from the following sources: Tris, MES, PIPES, HEPES, diithiothreitol, 2-mercaptoethanol, GTP (type II S), ATP (grade I), cAMP, paranitrophenyl phosphate, phenylmethylsulfonyl fluoride, diisopropyl fluorophosphate, bovine pancreatic trypsin inhibitor (type IP), pepstatin A (dissolved in Me$_2$SO), leupeptin (synthetic, hemisulfate, dissolved in Me$_2$SO), ME$_2$SO, EGTA, orthophosphoric-monooesther phosphorohydrolase (alkaline optimum; EC 3.1.3.2) (phosphatase type III from potato, orthophosphoric-monooester phosphorohydrolase (alkaline optimum; EC 3.1.3.1) (alkaline phosphatase) type VII-S from bovine intestinal mucosa, Sephadex G-150, and Coomassie brilliant blue R obtained from Sigma, St. Louis, MO. Lactate, glycerol, trichloroacetic acid, malachite green hydrochloride, ammonium molybdate 4H$_2$O, and other salts were from J. T. Baker Chemical Co., Phillipsburg, NJ; Folin-Ciocalteau phenol reagent was from Fisher Scientific Co., Fair Lawn, NJ; adenosine 5'-triphosphate, tetra(triethylammonium) salt, 1,23-P$_2$ATP, 60 Ci/mmol was from New England Nuclear, Boston, MA. All other chemicals, activity, 4,000 CPM per increment of 1.6% sodium dodecyl sulfate, Triton X-100, were obtained from Easkey Quality Meat Co., Baltimore, MD. Ashing tubes were Pyrex No. 9860, 14 x 100 mm, from Corning Glass Works (Corning, NY).

Isolation of Proteins—Conventional actin for viscometric assays was isolated from rabbit skeletal muscle by a modification of the method of Spudich and Watt (18) as described by Griffith and Pollard (5). This was stored at 4°C in 0.2 mM ATP, 0.2 mM CaCl$_2$, 0.5 mM dithiothreitol, 3 mM Na azide, 2 mM Tris-Cl, pH 8 (actin buffer). Hog brain actin was prepared as described by Weir and Fredericksen (19). 4.5 mg of whole heat-stable MAPs from MTP incubated in EGTA either with or without adenine nucleotides as described above. Then the MAPs were precipitated by addition of dry (NH$_4$)$_2$SO$_4$ to 60% saturation, dialyzed in 2 ml of 100 mM KCl, 2 mM HEPES, pH 7.5, and then resuspended by gel filtration on a column (45 x 1.6 cm) of Bio-Gel A-15m equilibrated with MAPs buffer.

Phosphoprotein Phosphatase Experiments—Heat-stable MAPs (1.3 to 2 mg/ml) in 100 mM KCl, 25 mM HEPES at pH 7.5, plus 0.36 mg/ml diisopropyl fluorophosphate, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 2 mM EGTA, 2 µg/ml pepstatin, and 2 µg/ml leupeptin, were treated with phosphatases for 1 h at 37°C as follows: (a) with 10 µg/ml acid phosphatase (the specific activity in the same buffer was 17 µmol of paranitrophenyl phosphate mg$^{-1}$ min$^{-1}$) plus enough 0.5 M HCl to give pH 6.0; or (b) 10 µg/ml alkaline phosphatase (specific activity, 37 µmol paranitrophenyl phosphate mg$^{-1}$ min$^{-1}$). A control was prepared the same way, but with an amount of 3.2 M (NH$_4$)$_2$SO$_4$ equivalent to the volume of phophatase suspension added to the test tubes. The reaction was terminated by heating at 100°C for 5 min followed by centrifugation and dialysis as described above for MAPs.

Phosphate Assays—Protein-bound phosphate was measured by a modification of the method of Stull and Buss (22). Protein samples containing 0.5 to 4 nmol of phosphate were placed in ashing tubes. Protein was precipitated with an equal volume of cold 10% trichloroacetic acid and pelleted by centrifugation. The supernatant was used in all experiments except when 0.5 µl of cold 29% trichloroacetic acid. The subsequent pellet was resuspended and washed twice with 1 ml of cold 5% trichloroacetic acid and then resuspended in 16% polyethylene glycol. After heating for 20 min at 90°C, the sample was cooled on ice and the precipitate was pelleted, dissolved in 0.3 µl of 0.2 M NaOH, mixed, and immediately reprecipitated with 0.6 ml of cold 29% trichloroacetic acid. The ash was dissolved in 0.3 ml of 1.2 M HCl, and then 0.3 µl phosphate reagent (23) was added. After 5 min, the KH$_2$PO$_4$ standards (0.2 to 4.5 nmol) were dried and ashed also. All glassware was soaked in 6 M HCl for several hours before use.

Protein Quantitation—Protein concentrations were determined by the method of Hartree (24) with bovine serum albumin as a standard. Viscometry—Low shear miniature falling ball viscometry was performed as described by MacLean-Fletcher and Pollard (25) in a 22°C water bath. Samples were mixed as follows: 150 µl of an appropriate dilution of MAPs in MAP buffer, 10 µl of a 20x salt solution composed of 452 mM KCl, 50 mM EGTA, 20 mM MgCl$_2$, 12 mM MES, 1 mM Na azide, 0.4 mM ATP, pH 6.15, and 0.1 M of 20x salt solution in actin buffer. The final buffer composition, then was 100 mM KCl, 2.5 mM EGTA, 2 mM HEPES, 1 mM MgCl$_2$, 0.6 mM MES, 0.4 mM Tris-Cl, 0.1 mM dithiothreitol, 0.06 mM ATP, 0.04 mM CaCl$_2$, pH 6.35. The sample preparation tube was shaken immediately, briefly, by hand. A 100-µl micropipette was loaded and one end was plugged and then placed in the water bath for 10 min before measuring the rate at which a stainless steel ball falls through the sample. After polymerization of the actin, these samples had an apparent viscosity between 30 and 60 centipoise in the absence of MAPs.

Polyacrylamide Gel Electrophoresis—Slab gel electrophoresis was performed in 10% polyacrylamide gels containing 1% sodium dodecyl sulfate (26). Samples were first dialyzed for 4 h against distilled water, lyophilized, dissolved in the appropriate volume of preheated sample buffer containing 1.6% sodium dodecyl sulfate and 8% 2-mercaptoethanol, and then heated in boiling water for 2 min. Gels were stained as described by Fairbanks et al. (27) and destained in 10% acetic acid in the presence of a polyfoam sponge. Autoradiography was performed using X-Omat AR film (Eastman Kodak) and a Cronex Lighting Plus AA intensifying screen (DuPont).

Exposure times ranged from 5 to 21 days. A Joyce double beam multichannel spectrophotometer (Joyce-Loebl and Co., Ltd., Gateshead, England) was used to quantitate band densities on autoradiograms and photographically positive films of Coomassie blue-stained gels. Some stained gels were scanned directly at 510 or 560 nm in a Gilford spectrophotometer (Gilford Instruments Div., Corning Glass Co., Oberlin, OH). Peak area integration was quantitated by cutting and weighing or by peak area integration with a Hewlett-Packard digitizer (Hewlett-Packard, Palo Alto, CA).
RESULTS

Polypeptide Composition of Microtubule Protein and Heat-stable MAPs—The polypeptide compositions of the twice-cycled hog brain microtubule protein (Fig. 1, lanes 1 and 2) and the heat-stable microtubule-associated proteins (lane 3) are similar to those reported previously (28, 29). MAP-1 and a minor polypeptide of about 70,000 Da (lanes 1 and 2) are not present in supernatants after boiling microtubule protein (lanes 3–7). MAP-2, about 300,000 Da, is partially recovered in heat-stable supernatants. The usual four bands of τ are more apparent after boiling. Similarly, most of the peptides between 200,000 and 280,000 Da and the bands at about 125,000 Da present in whole microtubule protein are also present, but in a higher ratio to MAP-2, after boiling. Densitometry of lane 3 shows heat-stable MAPs to be 61% MAP-2 and 15% τ.

Effect of Incubation Conditions on the Phosphate Content of Microtubule-associated Proteins—Heat-stable MAPs isolated directly from microtubule protein (C₈) have a remarkably high protein-bound phosphate content of 7.1 mol/300,000 g of protein (Table I). If the microtubule protein is incubated at 37°C in EGTA without ATP for 30 min before isolating the heat-stable MAPs, the protein phosphate content declines to 4.9 (Table I). This establishes the existence of protein phosphatase activity in the microtubule protein preparation. After incubation in 5 mM CaCl₂, the phosphate content is not significantly different from the starting material. When microtubule protein is incubated with ATP and cAMP in EGTA, there is a net increase in the protein-bound phosphate of the heat-stable MAP fraction to 8.6 mol/300,000 g and the incorporation from [γ-³²P]ATP of 4.1 mol of ³²P/300,000 g (Table I). Thus, in addition to the net increase of about 1.5 mol of phosphate/300,000 g, there is exchange of about 2.6 mol of phosphate. When the same experiment is carried out in CaCl₂, there is incorporation of 2.9 mol of ³²P from [γ-³²P]ATP, but no change in the protein phosphate content (Table I). All of the phosphates must add in exchange reactions.

During the incubation of whole microtubule protein with [γ-³²P]ATP, all the MAP bands, except one of 70,000 Da but including MAP-1, are labeled with ³²P (Fig. 1, lane 2). Tubulin is not labeled. Before the microtubule protein is boiled, MAP-2 contains 62% of the radioactivity as measured by densitometry. This result agrees with Nishida et al. (8). After boiling, the radioactivity of heat-stable MAPs remains distributed among all bands roughly in proportion to the Coomassie blue staining, including new bands that appear only after boiling. This suggests that the bands with molecular masses between 200,000 and 280,000 Da are fragments produced by or subsequent to heating the microtubule protein.

With one interesting exception, the electrophoretic mobility of the MAP polypeptides is not altered by incubation with or without nucleotides. The exception is a pair of bands of about 125,000 Da which migrate slower and more diffusely after the incubation of whole microtubule protein with [γ-³²P]ATP, all the MAP bands, except one of 70,000 Da but including MAP-1, are labeled with ³²P (Fig. 1, lane 2). Tubulin is not labeled. Before the microtubule protein is boiled, MAP-2 contains 62% of the radioactivity as measured by densitometry. This result agrees with Nishida et al. (8). After boiling, the radioactivity of heat-stable MAPs remains distributed among all bands roughly in proportion to the Coomassie blue staining, including new bands that appear only after boiling. This suggests that the bands with molecular masses between 200,000 and 280,000 Da are fragments produced by or subsequent to heating the microtubule protein.

Table I

<table>
<thead>
<tr>
<th>Incubation condition</th>
<th>Phosphate/protein (± S.E.)</th>
<th>³²P/protein (± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not incubated</td>
<td>7.1 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>2 mM EGTA</td>
<td>4.9 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>2 mM EGTA, 1 mM [γ-³²P]ATP</td>
<td>8.6 ± 0.5</td>
<td>4.1 ± 0.2</td>
</tr>
<tr>
<td>5 μM cAMP</td>
<td>6.2 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>5 mM CaCl₂</td>
<td>7.2 ± 0.5</td>
<td>2.9 ± 0.5</td>
</tr>
</tbody>
</table>

* Mean of three experiments.
* Mean of four experiments.

**TABLE 1** Effects of microtubule protein incubation conditions on MAP phosphate content

Conditions are means of eight experiments unless otherwise noted. Experimental details are given under "Materials and Methods."
The phosphate content of the MAPs was varied by incubating phosphorylation and the critical gelation concentration is nonlinear. Low phosphate MAPs have the highest cross-linking activity. The viscometer amplifies differences in the apparent viscosity of non-Newtonian samples. Both phosphatases remove $^{32}$P from all bands on autoradiographs (Fig. 1). With figures corrected for changes in Coomassie blue staining, acid phosphatase removes 46% of the label from MAP-2, 14% from the bands at 125,000 Da, and 21% from the $\gamma$ bands. Alkaline phosphatase removes 60% from MAP-2, 45% from bands at 125,000 Da, and 21% from the $\gamma$ bands.

There is partial proteolysis of the MAPs during the incubation with the phosphatases. Acid phosphatase reduces the amount of MAP-2 (300,000 Da) by 28% (from 61 to 44% of total MAP). Bands of molecular masses greater than 200,000 Da increase enough to account for the loss. Alkaline phosphatase reduced the amount of MAP-2 by 57% (from 61 to 26% of total MAP); bands of approximately 200,000, 230,000, and 270,000 Da do not increase enough to account for the loss at 300,000. Two bands at about 80,000 Da also increase, from less than 1% of MAP proteins before incubation with alkaline phosphatase to more than 4% after. The proteolysis is likely due to a non-serine protease in the commercial phosphatase preparation because a variety of serine protease inhibitors do not prevent this degradation. In data not shown, (a) diisopropyl fluorophosphate is required to prevent an extensive proteolysis and loss of actin gelation activity during incubation with acid phosphatase, and (b) the initial level of MAP phosphorylation does not affect the amount of MAP-2 proteolysis.

**Effect of MAP Phosphorylation on MAP Cross-linking of Actin.** We measured actin filament cross-linking activity of MAPs by a simple low shear viscosity assay with a falling-ball viscometer. This semiquantitative assay gives an apparent viscosity for non-Newtonian solutions like the actin filament samples. For all actin filament cross-linkers tested, including MAPs (5), the dependence of the apparent viscosity on cross-linker concentration is highly nonlinear, rising exponentially between 50 and 10,000 centipoise (Fig. 2). The nonlinearity is due to a combination of two factors: first, the viscometer amplifies differences in the apparent viscosity of non-Newtonian samples (25, 31), and second, the absolute viscosity of polymer solutions is a nonlinear function of cross-linker concentration (32). The cross-linker concentration at the viscosity transition is called the critical gelation concentration and can be used as a quantitative measure of cross-linker specific activity (31). We use 1000 centipoise as the measure of the CGC because this apparent viscosity is in the early part of the viscosity transition and in the midrange of the viscometer.

The relationship between the extent of MAP phosphorylation and the critical gelation concentration is nonlinear (Fig. 3). Note that the CGC is inversely proportional to the activity of the MAPs, so that the low phosphate MAPs have the highest cross-linking activity. The phosphate content of the MAPs was varied by incubating microtubule protein in four different ways as described above. Curve A in Fig. 3 is from eight experiments with MAPs from 2×-cycled microtubule protein (C$_2$s). MAPs from microtubule protein incubated without ATP in either 5 mM Ca$^{2+}$ or 2 mM EGTA have low phosphate contents. There is no statistical difference in their actin filament cross-linking activities. Microtubule protein incubated with 1 mM ATP, 5 mM cAMP, and Ca$^{2+}$ gives MAPs with less activity (higher CGC; p < 0.05). Although this material has only slightly more phosphate compared with incubation with Ca$^{2+}$ alone (p < 0.18), 3 phosphates are exchanged, as shown above. The largest effect on activity is seen in MAPs from microtubule protein incubated in 2 mM EGTA plus the adenine nucleotides. Both phosphate content and gelation activity are significantly different (p < 0.05) from that of material incubated without adenine nucleotides. The data in Fig. 3 indicate that, in the range of 6 to 9 phosphates/300,000 g of protein, two phosphate moieties can alter the CGC substantially. Curve B in Fig. 3 is the averaged data from two experiments with MAPs isolated after incubating 3×-cycled microtubule protein (C$_3$s) as with

<table>
<thead>
<tr>
<th>First incubation condition</th>
<th>Second incubation condition</th>
<th>Phosphate/protein</th>
<th>$^{32}$P/protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mM EGTA</td>
<td>No second incubation</td>
<td>6.2 m mole/10$^6$ g</td>
<td>12.1 dpm/300 $\mu$g</td>
</tr>
<tr>
<td></td>
<td>No enzyme</td>
<td>6.1</td>
<td>10.3</td>
</tr>
<tr>
<td></td>
<td>Acid phosphatase</td>
<td>3.6</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td>Alkaline phosphatase</td>
<td>3.5</td>
<td>7.4</td>
</tr>
<tr>
<td>2 mM EGTA, 0.5 mM [γ-$^{32}$P]ATP, 5 $\mu$M cAMP</td>
<td>No second incubation</td>
<td>12.1</td>
<td>25,900</td>
</tr>
<tr>
<td></td>
<td>No enzyme</td>
<td>10.3</td>
<td>12,800</td>
</tr>
<tr>
<td></td>
<td>Acid phosphatase</td>
<td>5.4</td>
<td>14,500</td>
</tr>
<tr>
<td></td>
<td>Alkaline phosphatase</td>
<td>7.4</td>
<td>14,500</td>
</tr>
</tbody>
</table>

**TABLE II**

Effect of phosphatases on MAP phosphate content

All values are means of two separate experiments. Experimental details are given under "Materials and Methods."
Moles phosphate per 3×10⁵ g protein

Fig. 3. The critical gelation concentration of MAPs mixed with actin filaments depends on the phosphate content of the MAPs. The points on curve A are the mean values from eight separate experiments per data point (+S.E.) with heat-stable MAPs from twice-cycled microtubule protein. Curve B represents two experiments with heat-stable MAPs from three times-cycled microtubule protein. For both curves, the MAPs were isolated from microtubule protein that was first incubated with buffered EGTA (○), buffered 5 mM CaCl₂ (●), EGTA plus 1 mM ATP and 5 μM cAMP (■), or CaCl₂ plus the adenine nucleotides (●) as described under "Materials and Methods." The critical gelation concentrations were determined as described in Fig. 2; the phosphate quantities were determined by direct chemical analysis as described in the text.

Fig. 4. Reversal of the effect of MAP phosphorylation on the critical gelation concentration. Heat-stable MAPs from twice-cycled microtubule protein that was first incubated in buffered EGTA (○) or EGTA plus 0.5 mM ATP and 5 μM cAMP (■) was then dephosphorylated by incubation with acid or alkaline phosphatase (filled symbols). Again, the critical actin gelation concentration of the MAPs is higher when they are more heavily phosphorylated, with a sharp transition in CGC in the range of 6 to 9 mol of phosphate/300,000 g of protein.

Reversibility of Phosphorylation and Reactivation of MAP–Actin Cross-linking—Acid and alkaline phosphatases remove phosphate from MAPs and reverse the effect of phosphorylation on the critical actin gelation concentration of MAPs. In these experiments, 3 to 5 phosphates are added per 300,000 g of MAP protein during the incubation of microtubule protein in EGTA plus ATP and cAMP. These MAPs have a high critical gelation concentration (e.g. are relatively poor actin cross-linkers (Fig. 4, top right)). During incubation with the phosphatases, 3 to 5 mol of phosphate are removed per 300,000 g of protein. Concomitantly, viscometry shows that these MAPs are reactivated; the critical gelation concentration is reduced (Fig. 4, closed squares, center of graph). Similarly,
MAPs isolated from microtubule protein incubated without ATP or cAMP are even more active after phosphatase treatment: phosphate is removed, with a concomitant decrease in cross-linking activity so that the critical gelation concentration is doubled. However, the phosphorylation of MAP-2 with no ATP or cAMP produces a lower concentration of MAPs than 1% MAP-2; other peptide bands individually account for 0.3–0.7 mol. Data are from three separate experiments, expressed as means ± S.E.; multiple fractions of MAP-2 analyzed give n ≥ 3.

### Table III

<table>
<thead>
<tr>
<th>MAP fraction</th>
<th>Conditions of microtubule protein incubation</th>
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<tr>
<td></td>
<td>Buffered EGTA only</td>
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<tr>
<td></td>
<td>Phosphate*</td>
</tr>
<tr>
<td>MAP-2</td>
<td>4.3 ± 0.7 (n = 7)</td>
</tr>
<tr>
<td>r</td>
<td>1.5 ± 0.4 (n = 3)</td>
</tr>
</tbody>
</table>

*Phosphate in units of moles/mol of protein.

### Table IV

MAP interaction with actin from three different species

Hog brain and Acanthamoeba actins were isolated using gel filtration as a final preparative step. Rabbit muscle actin was not gel-filtered. The microtubule protein was incubated 1 h as described in more detail under "Materials and Methods" before heating to isolate MAPs. Experiments with each actin preparation were performed on different days with fresh MAPs prepared from the same isolate of microtubule protein.

<table>
<thead>
<tr>
<th>Actin</th>
<th>Source</th>
<th>Concentration</th>
<th>Apparent viscosity</th>
<th>Microtubule protein incubation condition</th>
<th>Phosphate/protein</th>
<th>CGC</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Rabbit muscle</td>
<td>552</td>
<td>17 ± 4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hog brain</td>
<td>302</td>
<td>33 ± 9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acanthamoeba</td>
<td>290</td>
<td>39 ± 2</td>
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</table>

### Discussion

**MAP Phosphorylation**—Our observations confirm the existence of multiple phosphorylation sites on MAP-2 (35) and add the finding that r is also multiply phosphorylated. There are at least 10 mol of phosphorylation sites/300,000 g of whole MAP protein, with at least 7.5/300,000-Da MAP-2 molecule and 2.2/60,000–68,000-Da r molecule. Freshly isolated MAPs are partially phosphorylated at these multiple sites. The antagonistic action of three endogenous kinases (9–11), one of which co-purifies with MAP-2 (12), and two phosphoprotein phosphatases (10,37–39), can result in either simple exchange of phosphates or net addition of phosphates depending on whether the microtubule protein is incubated with ATP. The partial phosphorylation of MAPs when isolated from brain and the presence of phosphatase activity in the preparation necessitated the chemical measurement of protein-bound phosphate in our work. Previous work where MAP "phosphorylation" was measured by incorporation of 32P from ATP (9, 13–15, 38, 40–42) will have to be re-evaluated, because, due to exchange reactions, there is no way to relate the resulting 32P content of the MAPs to the extent of their phosphorylation. The exchange of phosphate on MAPs was relatively rapid under our conditions, but may be slow under other conditions. For example, Jameson et al. (13) found that microtubule protein prelabeled with 32P slowly lost the 32P when incubated with excess cold ATP.
Regulation of MAP-Actin Interaction by Phosphorylation—
Our work also establishes that reversible phosphorylation of heat-stable microtubule-associated proteins can regulate the ability of these proteins to cross-link actin filaments. There is a complex, nonlinear relationship between the actin filament cross-linking activity and the extent of phosphorylation, with highly phosphorylated MAPs having the lowest activity. The most active MAPs are obtained by removing phosphates with phosphoprotein phosphatases. Fractionation experiments show that phosphorylation inhibits the cross-linking activity of both of the major MAP components, MAP-2 and r. The mechanism by which phosphorylation regulates actin cross-linking by MAPs is still under investigation, but we can rule out an effect of phosphorylation on the self-association of MAPs: the level of phosphorylation does not affect the elution position of any of the MAP components on gel filtration. Alternatively, phosphorylation may inhibit cross-linking by reducing the affinity of MAP-2 and r for binding to actin filaments, however, neither high nor low phosphate MAP-2 or r pelletted with actin filaments in a centrifugation assay.²

The nonlinear relationship between extent of phosphorylation of the MAPs and their actin cross-linking activity suggests that the mechanism by which phosphorylation regulates activity may be complex. The curves in Figs. 3 and 4 show that each additional MAP-phosphate causes increasingly more inhibition of cross-linking, at least by the criteria of the critical gelation concentration. We offer two interpretations. Either the MAP-phosphates act synergistically with each other or the first phosphates were added to MAPs at functionally less important sites, while the last phosphates are added to sites where they inhibit actin cross-linking. This latter interpretation is consistent with reports of other systems where phosphorylation of certain amino acids has no effect on protein function (43). The results of the experiments where MAPs were phosphorylated with [γ-32P]ATP and then dephosphorylated with exogenous phosphatases are consistent with the idea of synergism, but are also difficult to interpret mechanistically. In these experiments, only about half of the newly added [32P]phosphate is removed by the phosphatases, yet cross-linking activity is restored. The problem is that exchange of [32P]- for [33P]phosphate occurs during the labeling-phosphorylation step, as mentioned above. Then when the phosphatases are used, they may remove some of the exchanged [32P] as well as some newly added [33P] and some of the [33P] initially present. Therefore, while the simplest mechanism may be a total phosphate number-dependent inhibition of cross-linking activity with synergistic interaction of the phosphates, multiple alternative interpretations exist. This interesting question will only be resolved with further characterization of MAP phosphorylation sites.

This work provides an explanation for an earlier observation on actin filament-microtubule interactions. It now appears highly likely that the inhibitory effect of ATP on actin filament cross-linking with microtubules and by MAPs observed by Griffith and Pollard (5) was due to phosphorylation of the MAPs as suggested by Nishida et al. (8).

We presume, but need to prove in future experiments, that the inhibition of actin filament cross-linking by phosphorylation of MAPs reflects a similar effect of phosphorylation on the interaction of actin filaments with microtubules where the MAPs are bound tightly to the tubule. We used the system of MAPs and actin for the present work to simplify the experimental design. It is unproven, but free MAPs may also function as actin filament cross-linkers under some circumstances in vivo and phosphorylation may regulate, at least in part, this second possible function of MAPs. Other covalent modifications of MAPs, such as methylation, sulfation, and hydroxylation, may also occur in cells. Sulfation and phosphorylation would make cationic MAPs more anionic, and may reduce electrostatic interactions of MAPs with actin and, probably, tubulin as well. Such modifications might be expected to function in time frames of seconds to minutes, times within which changes of cell shape, cell motility, and chromosome movements occur. In this way, MAP phosphorylation could be one way that cells control both tubulin polymerization (13, 14) and actin-microtubule interactions and, thus, structure of the cytoplasmic matrix during cell motility.

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REFERENCES

Phosphorylation of microtubule-associated proteins regulates their interaction with actin filaments.
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