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Inhibition of Microtubule Assembly by Phosphorylation of Microtubule-Associated Proteins†

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ABSTRACT: ^{32}P labeling of microtubular protein by endogenous protein kinase activity is shown to result from a net increase in protein-bound phosphate and is not the result of a phosphate exchange reaction between ATP and phosphoprotein. Protein phosphorylation is maximal in the presence of 0.5 mM Mg^{2+} and 0.25 mM ATP, resulting in approximately 2.8 nmol of phosphate/mg of protein. However, phosphorylation can be increased two-to threefold by cAMP. The protein substrates for phosphorylation in either the absence or presence of cAMP are the microtubule-associated proteins which copurify with tubulin and promote microtubule assembly. Phosphorylation of microtubule-associated proteins inhibits both the rate and extent of microtubule assembly when the protein is exposed to conditions which result in dissociation of rings. These results are taken to indicate that phosphorylation modifies MAPs so that they have a reduced ability to form an assembly-competent complex with tubulin.

Microtubules are involved in a number of dynamic cellular processes which require rapid modulation of microtubule assembly and function. The observation that purified microtubular protein is phosphorylated in a reaction which is stimulated by cAMP (Goodman et al., 1970; Sloboda et al., 1975; Rappaport et al., 1976; Sandoval & Cuatrecasus, 1976a; Sheterline, 1977) has generated considerable interest in phosphorylation as a possible mechanism for the regulation of microtubule-mediated functions. Microtubular proteins are also phosphorylated in vivo (Eipper, 1974; Sloboda et al., 1975), and many studies have shown that cAMP, a well-known modulator of protein phosphorylation, affects the number (Porter et al., 1974), organization (Kram & Tomkins, 1973; Willingham & Pastan, 1975; Nath et al., 1978), and function (Williams & Wolff, 1970; Hsie & Puck, 1971; Prasad & Hsie, 1971; Kirkland & Burton, 1972; Puck, 1977) of microtubules in intact cells.

Protein kinase activity consistently copurifies with microtubular protein prepared from a variety of sources including brain (Goodman et al., 1970; Eipper, 1974; Sloboda et al., 1975), chick embryonic muscle (Piras & Piras, 1974), and platelets (Ikeda & Steiner, 1979). While the protein kinase activity is clearly distinct from tubulin (Eipper, 1974; Rappaport et al., 1976; Sheterline, 1977), the major component of microtubules, a specific association between protein kinase and tubulin has been suggested based on the observation that protein kinase activity elutes with tubulin purified by colchicine–agarose affinity chromatography (Sandoval & Cuatrecasus, 1976a).

In microtubule preparations purified by cycles of assembly and disassembly, the major substrate for the cAMP-stimulated phosphorylation reaction is a group of high molecular weight microtubule-associated proteins (MAPs)[1] (Sloboda et al., 1975; Rappaport et al., 1976; Sheterline, 1977). These proteins stimulate microtubule assembly under polymerizing conditions and promote the formation of rings under depolymerizing conditions (Murphy & Borisy, 1975; Sloboda et al., 1976; Murphy et al., 1977). Nevertheless, the state of phosphorylation of microtubular proteins has yet to be correlated with any change in microtubule assembly, disassembly, or function in vitro.

In this paper, in vitro ^{32}P labeling of microtubule-associated proteins is shown to result from the addition of new phosphate moieties and does not result from a phosphate exchange re-

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[1] Abbreviations used: Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; RB, reassembly buffer (0.1 M Mes, 0.5 mM $MgCl_2$, and 1.0 mM EGTA, pH 6.8); MAPs, microtubule-associated proteins; PEI, polyethylenimine.

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action. Phosphorylation is stimulated by cAMP and is shown to inhibit microtubule assembly when the protein is exposed to conditions which result in dissociation of rings.

Experimental Procedures

Methods

Preparation of Microtubular Protein. Microtubular protein was prepared by a modification of the Shelanski assembly–disassembly procedure (Shelanski et al., 1973). Fresh pig brains were homogenized with an equal weight of ice-cold reassembly buffer (0.1 M 2-(*N*-morpholino)ethanesulfonic acid buffer, 0.5 mM $MgCl_2$, and 1.0 mM EGTA, pH 6.8) containing 0.1 mM ATP in a Waring Blendor for 30 s. The supernatant obtained from centrifugation (30 000 rpm, 75 min, 4 °C) in a Beckman 30 rotor was diluted with an equal volume of glycerol–reassembly buffer (60.2 mL of glycerol:24 mL of reassembly buffer) containing 1 mM ATP and polymerized at 37 °C for 30 min. The assembled microtubules were then sedimented (30 000 rpm, 75 min, 30 °C), and the pellet was stored at −40 °C under glycerol–reassembly buffer.

Immediately prior to an experiment, the protein was further purified by another cycle of polymerization to assure use of active protein. The pellets were resuspended in reassembly buffer (0.1 volume of the crude supernatant) by means of a Dounce homogenizer and depolymerized at 0 °C for 30 min. After centrifugation (35 000 rpm, 20 min, 0 °C) in a Beckman 40 rotor, the supernatant was polymerized as before, and the microtubules were sedimented (35 000, 75 min, 30 °C). The pellets were resuspended in the buffer (0.025 volume of the crude supernatant) to be used for a given experiment, depolymerized (0 °C, 30 min), and centrifuged (35 000 rpm, 20 min, 0 °C) to remove aggregates.

Gel filtration was used to remove free nucleotides and glycerol from the depolymerized protein in the supernatant from the final centrifugation step. Protein (~10 mg/mL) was applied to a Sephadex G-25 column (1 mL of supernatant/15 mL bed volume) equilibrated at 4 °C with the buffer used in the final depolymerization. The protein concentration in the eluant was calculated from the absorbance at 278 nm using an extinction coefficient of 1.2 *A* unit/(mg·mL) (Jacobs et al., 1974). The ratio of absorbance at 278 nm to that at 260 nm was consistently 1.42.

The protein preparation described here is identical with that obtained when GTP is used throughout the purification by all criteria studied to date; these include microtubule yield in GTP-induced assembly; rate of microtubule assembly; kinetics for cold-induced disassembly; kinetics for dilution-induced disassembly; and average microtubule length following GTP-induced assembly, as measured by electron microscopy (B. Zeeberg and M. Caplow, unpublished experiments).

Column Chromatography. Depolymerized microtubular protein was separated into 36S rings and 6S tubulin dimers by gel filtration on Sepharose 6B. Protein (4 mg in 0.5 mL of RB) was applied to a 1 × 12 cm column equilibrated with RB at 4 °C.

Microtubule-associated proteins were separated from 6S tubulin dimers by phosphocellulose (Whatman P11) chromatography in the cold. Phosphocellulose was washed as described previously (Sloboda et al., 1976) and extensively equilibrated with RB/4 (25 mM Mes, 0.5 mM $MgCl_2$, and 0.25 mM EGTA, pH 6.8). Microtubular protein (3 mg/mL bed volume) was applied to phosphocellulose, and the unbound tubulin dimer was collected in the eluant. The fractions containing high concentrations of protein were then pooled, and the protein was immediately restored to full strength buffer by passage through a Sephadex G-25 column equilibrated with RB. After the phosphocellulose column was washed with 5 column volumes of RB/4, the adsorbed material was eluted with 0.8 M NaCl in RB/4. The pooled fractions containing protein were then desalted and restored to full strength RB by passage through Sephadex G-25 equilibrated with RB.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed according to the Laemmli method (1970). Protein samples (25–50 μg) were applied to 7.5% gels and run at 2 mA/gel. After staining with Fast Green, the gels were destained and scanned at 580 nm using an ISCO Model UA-5 absorbance monitor. Phosphorylated proteins were detected by determining the ^{32}P radioactivity in gels sliced into 2-mm sections.

Assay of Protein Phosphorylation. Protein phosphorylation was measured under conditions that were found to be optimal (see Results). Microtubular protein was incubated at 37 °C with 250 μM [γ-^{32}P]ATP (200–200 cpm/pmol) and 25 μM cAMP (when added). Reactions were initiated by the addition of [γ-^{32}P]ATP and stopped at the desired times by adding 5 μL of the reaction mixture to 50 μL of bovine serum albumin (5 mg/mL) and 500 μL of ice-cold 5% (w/v) perchloric acid. The precipitated protein was centrifuged at 2000*g* for 2 min, and the pellet was solubilized in 50 μL of 1 N NaOH. Samples were then reprecipitated with 500 μL of 5% perchloric acid and centrifuged as before. Following one further cycle of solubilization–precipitation, the final pellet was dissolved in 100 μL of 1 N NaOH, neutralized with 1 N HCl, and counted in 10 mL of Aquasol II (New England Nuclear). This procedure effectively removes added [γ-^{32}P]ATP and [γ-^{32}P]GTP formed by transphosphorylation of tubulin-bound GDP. Background radioactivity was measured by the addition of [γ-^{32}P]ATP to heat-denatured microtubular protein and was consistently less than 2% of the radioactivity incorporated into fully phosphorylated protein.

Measurement of ATPase Activity. ATPase activity was determined from hydrolysis of [γ-^{32}P]ATP. Reaction mixtures containing 1.5 mL of microtubular protein (6 mg/mL) and 87 μL of 5 mM [γ-^{32}P]ATP (4400 cpm/nmol) were incubated at 37 °C with 17 μL of RB or 2.5 mM cAMP. Aliquots (100 μL) were quenched in a boiling water bath for 2 min, and an equal volume of [^3H]ATP (2250 cpm/μL) was added to determine the recovery from subsequent steps. The solution was immediately adjusted to 0.3 M perchloric acid, and the protein was removed by centrifugation. Nucleotides in the supernatant were isolated and separated by thin-layer chromatography on PEI-cellulose as described previously (Zeeberg et al., 1977). ATP hydrolysis was determined by the decrease in [^{32}P]ATP relative to recovered [^3H]ATP.

Turbidity Measurements. Microtubule assembly was followed by the change in turbidity at 350 nm (Gaskin et al., 1974). Turbidity measurements were recorded from a thermostated (37 °C) Gilford 240 spectrophotometer at 1-min intervals or with a chart recorder, as required. Polymerization reactions (200–300 μL) were started by transferring cold protein (0 °C) to thermostated cuvettes (37 °C) containing nucleotide additions.

Electron Microscopy. Aliquots (5 μL) of protein were allowed to settle for 15 s on carbon-coated Formvar grids (provided by Dr. E. D. Salmon). The grids were washed successively with 3 drops of water and 3 drops of 1% uranyl acetate, and the excess uranyl acetate was drawn off with filter paper. Grids were examined after they had been air-dried. The magnification of the microscope was calibrated by using a standard line grating with 11 340 lines/cm. Microtubule

lengths were measured from about 50 microtubules, and, in the case where the length extended beyond the edge of the micrograph, the observed length was doubled, based upon the assumption that the edge of the micrograph is randomly located along the microtubule length (Johnson & Borisy, 1977).

Materials

[^{3}H]ATP, [^{3}H]cAMP, and $^{32}P_i$ were obtained from New England Nuclear. All nonradioactive nucleotides were purchased from Sigma. [γ-^{32}P]ATP was synthesized by a modification of the Glynn & Chappell method (1964) in which activated charcoal was used to isolate the radioactive nucleotide (Zeeberg et al., 1977). [γ-^{32}P]ATP synthesized by this method was analyzed by thin-layer chromatography and found to be greater than 98% pure.

Results

Optimal Conditions for Protein Phosphorylation. (*a*) *ATP Concentration Dependence for Protein Phosphorylation.* Protein phosphorylation was maximal using 250 μM ATP. At lower concentrations of ATP (20–200 μM), phosphorylation did not reach completion, while at higher ATP concentrations (300–600 μM), maximal phosphorylation was attained, but the rate was reduced by approximately 30%. ATP is a more effective phosphoryl donor than GTP. Using 250 μM [γ-^{32}P]ATP, the maximal extent of protein phosphorylation was 2.8 nmol of phosphate/mg of protein, as compared to 1.2 nmol of phosphate/mg of protein with 250 μM [γ-^{32}P]GTP.

(*b*) *Effect of cAMP.* Both the rate and final extent of protein phosphorylation were stimulated by cAMP; the apparent K_m for cAMP enhancement of the rate was 10 μM. In the presence of 25 μM cAMP, the maximal extent of protein phosphorylation was increased two- to threefold (6.0–10.0 nmol of phosphate/mg of protein) relative to reactions carried out in the absence of cAMP (2.5–3.0 nmol of phosphate/mg of protein).

The decreased level of protein phosphorylation in the absence of cAMP could not be accounted for by ATP hydrolysis prior to complete phosphorylation, since approximately 50% of the initial ATP remains unhydrolyzed in either the absence or presence of cAMP after phosphorylation is complete (20 min). Furthermore, addition of 0.5–2.5 mM [γ-^{32}P]ATP following complete phosphorylation in the absence of cAMP did not bring about any increase in phosphorylation; however, addition of cAMP to this protein resulted in an immediate twofold increase in the level of phosphorylation. This suggests that cAMP stimulates phosphorylation of sites in microtubular protein which are not phosphorylated in the absence of cAMP.

(*c*) *Effect of Divalent Cations.* Protein kinase activity is stimulated by Mg^{2+} but inhibited by Ca^{2+}. Maximal phosphorylation was attained with 0.5 mM Mg^{2+}, with inhibition occurring at concentrations greater than 20 mM Mg^{2+}. Using a Ca^{2+}–EGTA buffer, it was found that calcium effectively inhibits protein kinase activity at concentrations above 0.5 mM; maximal inhibition was obtained by using 8 mM Ca^{2+}. Under these conditions, phosphorylation did not exceed 0.4 nmol of phosphate/mg of protein on prolonged incubation using the optimal concentrations of ATP and Mg^{2+}.

Characterization of the Phosphorylation Reaction. (*a*) *Reaction Mechanism for ^{32}P Labeling of Microtubular Protein.* Earlier reports (Rappaport et al., 1976) that cAMP-stimulated phosphorylation did not affect microtubule assembly led us to consider the possibility that microtubular protein had already been phosphorylated either in vivo or during purification and that the observed ^{32}P labeling was the result of an exchange of labeled phosphate in ATP for unlabeled phosphate in protein, without a net change in the actual number of protein-bound phosphate groups. Thus, incorporation of ^{32}P into microtubular protein could be accounted for by either of the mechanisms shown:

$$\text{protein} + \text{AT*P} \rightleftharpoons \text{protein–*PO}_4 + \text{ADP} \quad (1)$$

$$\text{protein–PO}_4 + \text{AT*P} \rightleftharpoons \text{protein–*PO}_4 + \text{ATP} \quad (2)$$

According to the mechanism shown in eq 1, transfer of labeled phosphate from [γ-^{32}P]ATP to protein results in phosphorylation of a previously unphosphorylated site. Alternatively, as shown in eq 2, ^{32}P labeling could arise from a phosphate exchange reaction between ATP and phosphoprotein. In this case, a trace amount of ADP, formed by either ATP hydrolysis or transphosphorylation with GDP, can catalyze the exchange of labeled phosphate into a previously phosphorylated site. According to either mechanism, addition of [γ-^{32}P]ATP results in ^{32}P-labeled protein.

These mechanisms can be distinguished by determining the rate of ^{32}P loss from previously labeled protein following addition of unlabeled ATP. Under these conditions, protein which has been ^{32}P labeled by mechanism 1 would retain its label, while protein which has been labeled by mechanism 2 should lose ^{32}P at a rate comparable to that at which ^{32}P was incorporated using [γ-^{32}P]ATP. Microtubular protein was labeled by using [γ-^{32}P]ATP at 37 °C in the presence of 100 μM colchicine (Figure 1A). After complete labeling, [γ-^{32}P]ATP was removed by gel filtration on Sephadex G-25 (first arrow), and the protein was further incubated with either unlabeled ATP or without additional nucleotide (second arrow). The rate of loss of ^{32}P following addition of unlabeled ATP was very slow (0.01 nmol of phosphate per mg of protein per 10 min) relative to the rate of ^{32}P incorporation (2.0 nmol of phosphate per mg of protein per 10 min) into protein which had been treated identically but without exposure to ATP during the previous incubation (dotted line, Figure 1A). Furthermore, the rate of loss of ^{32}P following addition of unlabeled ATP was indistinguishable from that in which no ATP was added following gel filtration. Similar results were obtained when these reactions were carried out in the presence of cAMP. These results clearly indicate that ^{32}P incorporation is not due to a phosphate exchange reaction (mechanism 2) and that ^{32}P labeling reflects a net increase in the number of phosphate groups in microtubular protein.

(*b*) *Phosphorylation during Protein Purification.* Since both ATP and GTP serve as phosphate donors for phosphorylation of microtubular protein, it was interesting to find that purified protein could still be phosphorylated after it had been exposed to millimolar concentrations of nucleoside triphosphates throughout the purification procedure. This problem was analyzed by using [γ-^{32}P]ATP in place of ATP in each purification step (Methods) and determining the amount of ^{32}P incorporation after each procedure. Very little phosphorylation was observed following polymerization of the crude brain supernatant (0.3 nmol of phosphate/mg of protein). The same stoichiometry was observed when this step was carried out with 0.1 or 0.2 mM [γ-^{32}P]ATP. This indicates that the low stoichiometry for phosphate incorporation was not due to a decrease in the specific activity of added [γ-^{32}P]ATP by contaminating unlabeled ATP from the crude brain supernatant. Although a significant amount of phosphorylation took place during the second cycle of polymerization (1.8 nmol of phosphate/mg of protein), greater than half of this phosphate was lost during the remainder of the purification procedure. The amount of ^{32}P-labeled protein discarded during purification was negligible, indicating that the observed decrease

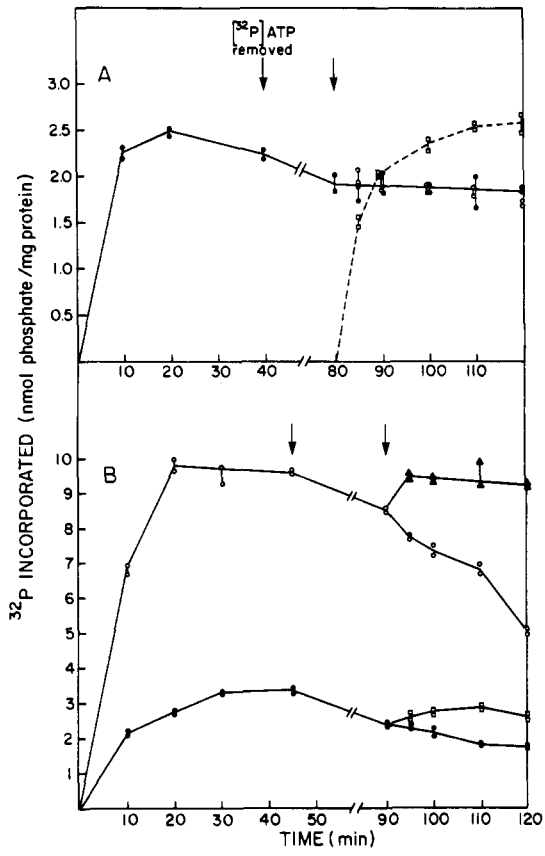

FIGURE 1: Incorporation of ^{32}P into purified microtubular protein. (A) Lack of phosphate exchange between ^{32}P-labeled microtubular protein and unlabeled ATP. Microtubular protein (4.2 mg/mL) was incubated at 37 °C with either 250 μM [γ-^{32}P]ATP (●) or without ATP. After 40 min (first arrow), each reaction mixture (1 mL) was applied to a Sephadex G-25 column (1 × 18 cm) which had been equilibrated with RB. The protein from the reaction mixture incubated without ATP eluted at 2.8 mg/mL and was labeled (second arrow) by using 250 μM [γ-^{32}P]ATP (□, dotted line). The protein from the reaction mixture previously labeled by [γ-^{32}P]ATP eluted from the column free of ATP. This protein was adjusted to 2.8 mg/mL and incubated at 37 °C (second arrow) with either 250 μM unlabeled ATP (○) or without further additions (●). (B) Protein phosphatase activity in purified microtubular protein. Microtubular protein (6.4 mg/mL) was incubated at 37 °C with 250 μM ATP in either the absence (●) or presence (○) of 25 μM cAMP. After 45 min (first arrow), the protein in each reaction mixture (1 mL) was depolymerized at 0 °C and [γ-^{32}P]ATP removed by passage through a Sephadex G-25 column (1 × 18 cm) which had been equilibrated with RB or RB containing 25 μM cAMP for the reaction carried out in the presence of cAMP. The protein obtained from each column was adjusted to 2.8 mg/mL and incubated at 37 °C (second arrow) without further additions [(●) without cAMP; (○) with cAMP] or with 250 μM [γ-^{32}P]ATP [(□) without cAMP; (▲) with cAMP].

in phosphorylation of purified protein was not due to selective cycling out of highly phosphorylated proteins. These results suggest that the ability to phosphorylate protein which has been purified in the presence of ATP is due to the presence of phosphatase activity (Sandoval & Cuatrecasus, 1976b; Sheterline, 1977).

(*c*) *Protein Phosphatase Activity.* Protein phosphatase activity is, in fact, present in our preparations of microtubular protein. As shown in Figure 1B, when [γ-^{32}P]ATP is removed by gel filtration following phosphorylation, protein is dephosphorylated in either the absence or presence of cAMP. However, when [γ-^{32}P]ATP is added back to the protein following gel filtration (second arrow, Figure 1B), phosphorylation is maintained at the maximal level.

Addition of cAMP to purified microtubular protein results in a two- to threefold increase in phosphorylation relative to

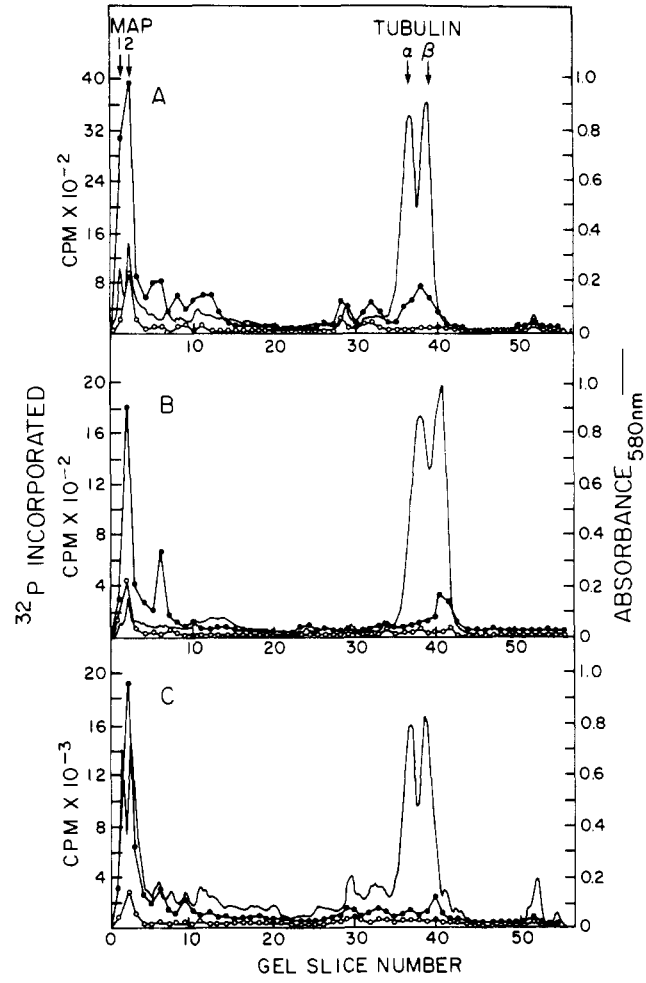

FIGURE 2: Electrophoresis of phosphorylated microtubular proteins. Microtubular protein (6.5 mg/mL) was incubated at 37 °C for 30 min with 250 μM [γ-^{32}P]ATP in either the absence (○) or presence (●) of 25 μM cAMP. ^{32}P-Labeled protein was subjected to electrophoresis on 7.5% polyacrylamide–sodium dodecyl sulfate gels as described under Methods. The gels were stained with Fast Green, scanned at 580 nm (solid line), sliced into 2 mm wide sections, and counted to determine ^{32}P-labeled proteins. (A) Unfractionated microtubular protein (25 μg); (B) unbound protein from phosphocellulose (25 μg); (C) protein eluted from phosphocellulose with 0.8 M NaCl (35 μg).

reactions carried out without cAMP (Figure 1B). The possibility that cAMP increased phosphorylation by inhibiting a protein phosphatase was excluded by showing that protein, which had been phosphorylated in the presence of cAMP and passed through Sephadex G-25 to remove [γ-^{32}P]ATP and cAMP,[2] had the same rate of dephosphorylation during a subsequent incubation at 37 °C (0.69 nmol of phosphate per mg of protein per 10 min) as protein that was passed through a column equilibrated with cAMP such that only [γ-^{32}P]ATP was removed (0.71 nmol of phosphate per mg of protein per 10 min).

(*d*) *Proteins Phosphorylated by the Microtubular Protein Kinases.* Depolymerized microtubular protein consists of 36S rings and 6S tubulin dimers (Borisy & Olmsted, 1972). When phosphorylated microtubular protein was separated into rings

[2] This procedure assumes that removal of free cAMP is sufficient to bring the cAMP concentration below that at which cAMP enhances phosphorylation. Control experiments using [^3H]cAMP indicated that less than 0.1% of the added cAMP was retained following column passage. No stimulation of phosphorylation is observed with this concentration of cAMP.

and tubulin dimers by Sepharose 6B gel filtration, approximately 95% of the phosphorylated protein was in the faster eluting ring fraction. Since 36S rings consist of a number of microtubule-associated proteins in addition to tubulin (Erickson, 1974; Dentler et al., 1975), identification of the phosphorylated species required further characterization. Protein was phosphorylated in either the absence or presence of cAMP, and the phosphorylated proteins were analyzed by polyacrylamide gel electrophoresis (Figure 2A). The major phosphorylated bands consisted of two high molecular weight peptides (MAPs 1 and 2). Very little radioactivity was incorporated into either tubulin subunit. There were no major peptides phosphorylated in the presence of cAMP that were not also phosphorylated without cAMP. Further fractionation of the protein by phosphocellulose chromatograpy separated the protein into 6S tubulin dimer (97% pure, Figure 2B) and the microtubule-associated proteins (Figure 2C), having a composition comparable to that found previously (Sloboda & Rosenbaum, 1979; Runge et al., 1979). This additional fractionation also revealed no major differences in the specificity for phosphorylation in the presence of cAMP.

Effect of Phosphorylation on Microtubule Assembly. (*a*) *Effect of Phosphorylation on GTP-Induced Microtubule Assembly.* Microtubular protein solutions (2.5 mg/mL) containing either 250 μM ATP, 25 μM cAMP, 250 μM ATP and 25 μM cAMP, or no nucleotide additions were incubated with 1 mM GTP at 37 °C. The effect of phosphorylation on microtubule assembly was analyzed by measuring the change in absorbance at 350 nm during the incubation. No significant differences in either the rate or the extent of microtubule assembly were found for reactions polymerized in the presence of ATP, cAMP, or ATP and cAMP compared to the control polymerized with GTP alone (data not shown).

Since microtubule assembly is very rapid relative to the rate of phosphorylation, it was possible that phosphorylation was occurring too late to significantly affect the assembly process. However, when the protein was polymerized after it had been maximally phosphorylated during a preceding cycle of assembly–disassembly (30 min of incubation at 37 °C with 250 μM ATP and 25 μM cAMP followed by 10 min at 0 °C), the rate and extent of assembly were unchanged.

(*b*) *Inhibition of Microtubule Assembly following Phosphorylation under Conditions Which Dissociate Rings.* The rate and extent of microtubule assembly are decreased at low concentrations as compared to higher concentrations of sulfonate buffers (Olmsted & Borisy, 1975; Himes et al., 1979). In addition, rings can be dissociated by reducing the concentration of Mes buffer. When microtubular protein is depolymerized in buffer containing 10 mM Mes, 0.1 mM EGTA, and 0.5 mM $MgCl_2$, the amount of protein in the form of rings is reduced to approximately 15%,[3] as compared to 55% when the protein is in reassembly buffer. However, protein phosphorylation is not changed under these conditions when the Mg^{2+} concentration is maintained at 0.5 mM. Therefore, complete phosphorylation can be carried out under conditions in which rings are dissociated, and polymerization does not occur. When ionic strength is increased, microtubule assembly takes place, and the effect of phosphorylation on this process can be analyzed.

Microtubular protein was incubated in low ionic strength buffer at 37 °C with either cAMP, ATP and cAMP, or without nucleotide additions. After 30 min, phosphorylation was found to be complete and turbidity measurements indicated the absence of microtubule assembly in any of the reactions. Microtubule assembly was then induced by addition of a concentrated buffer solution containing the appropriate nucleotides such that the final nucleotide compositions of the reaction mixtures were identical (except for one of the controls (reaction 1), which contained only GTP). The rate and final extent of microtubule assembly were unaffected by a 30-min preincubation with cAMP or by adding ATP and cAMP at the start of the GTP-induced assembly (Figure 3). However, when the protein was preincubated with ATP and cAMP for 30 min to allow phosphorylation to occur, the rate of subsequent microtubule assembly was markedly decreased, as compared to control reactions (Figure 3). Also, the final extent of microtubule assembly (not shown) after phosphorylation was less than 70% of that in the control reactions. In separate experiments it was found that phosphorylation with ATP alone inhibited both the rate and extent of subsequent microtubule assembly, although less so than when the phosphorylation was done in the presence of cAMP. Electron microscopy revealed characteristic microtubules when a protein solution at reduced ionic strength was induced to polymerize by raising the ionic strength (Figure 4). The lengths of microtubules that had been assembled after phosphorylation with ATP and cAMP were greater than in control reactions (Table I). Similar electron microscopy results were obtained in two experiments.

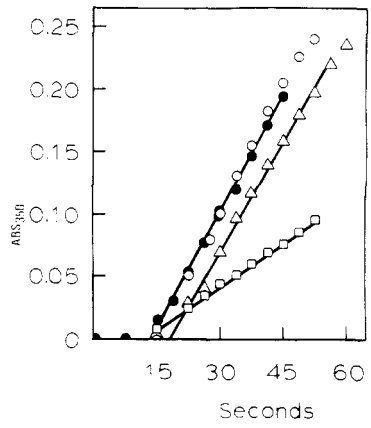

FIGURE 3: Effect of phosphorylation at low ionic strength on subsequent microtubule assembly. Microtubular protein (0.8 mg in 220 μL) in low ionic strength buffer (10 mM Mes, 0.5 mM $MgCl_2$, and 0.1 mM EGTA, pH 6.8) was preincubated at 37 °C with ATP and cAMP, cAMP, or without nucleotide additions. After 30 min, polymerization was induced by the addition of a concentrated buffer (1.6 M Mes, 8 mM $MgCl_2$, and 16 mM EGTA, pH 6.8) with a nucleotide composition as described. Reaction 1 (●): preincubated without nucleotide addition and polymerized by the addition of 15 μL of concentrated buffer containing 15.7 mM GTP. Reaction 2 (○): preincubated without nucleotide additions and polymerized by addition of 15 μL of concentrated buffers containing 15.7 mM GTP, 3.92 mM ATP, and 293 μM cAMP. Reaction 3 (△): preincubated with 25 μM cAMP and polymerized by the addition of 15 μL of concentrated buffer containing 15.7 mM GTP and 3.92 mM ATP. The time curve has been offset by 5 s for greater visibility. Reaction 4 (□): preincubated with 250 μM ATP and 25 μM cAMP and polymerized by the addition of concentrated buffer containing 15.7 mM GTP. Microtubule assembly was measured from the change in absorbance at 350 nm.

(*c*) *Inhibition of MAP-Stimulated Microtubule Assembly by Phosphorylation.* Microtubular protein was incubated at 37 °C with ATP, ATP and cAMP, or without nucleotide additions. After time for complete phosphorylation (30 min), the protein from each reaction mixture was applied to identical phosphocellulose columns to convert rings to 6S tubulin and to allow isolation of the MAPs. The unbound 6S tubulin from

[3] Electron microscopy reveals that the protein eluted in the void volume of Sepharose 6B consists exclusively of rings. Reducing the concentration of Mes buffer from 100 to 10 mM reduces the amount of protein eluted in the ring peak by greater than 75%.

Table I: Effect of Phosphorylation on Microtubule Length

control reaction				phosphorylated protein			
range (μ)	%	range (μ)	%	range (μ)	%	range (μ)	%
0.4–0.97	0	5.82–6.77	0	0.4–2.6	20	15.51–18.0	5.5
0.98–1.92	12.5	6.78–7.74	4.2	2.61–5.2	23.6	18.01–20.6	0
1.93–2.50	25	7.75–8.71	8.3	5.21–7.7	20	20.61–23.2	3.6
2.91–3.87	14.6	8.72–9.68	0	7.71–10.3	16.4	23.21–25.8	0
3.88–4.84	14.6	9.69–10.64	4.2	10.31–12.9	7.3	25.81–28.4	0
4.84–5.81	12.5	10.65–11.61	4.2	12.91–15.5	1.8	28.41–31	1.8

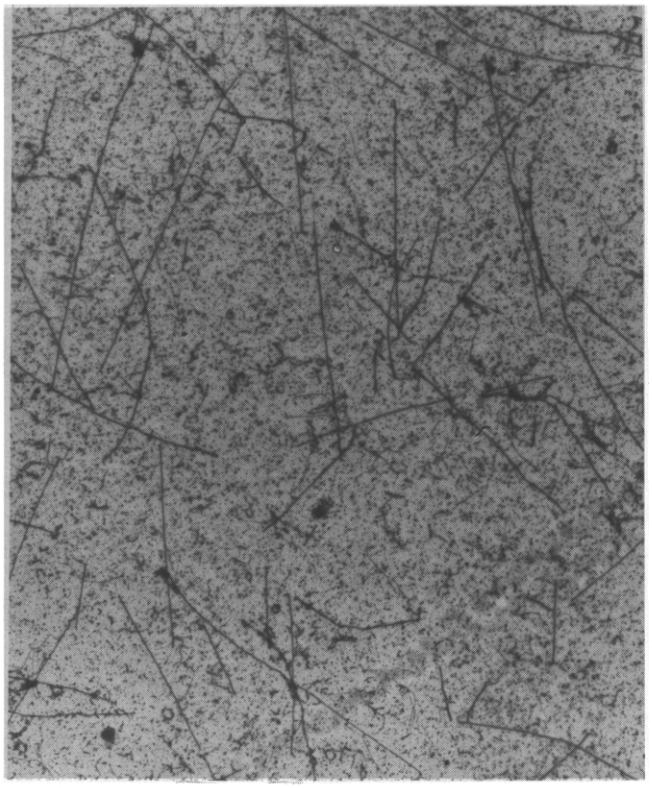

FIGURE 4: Electron micrograph of microtubules formed in reaction 4 of Figure 3.

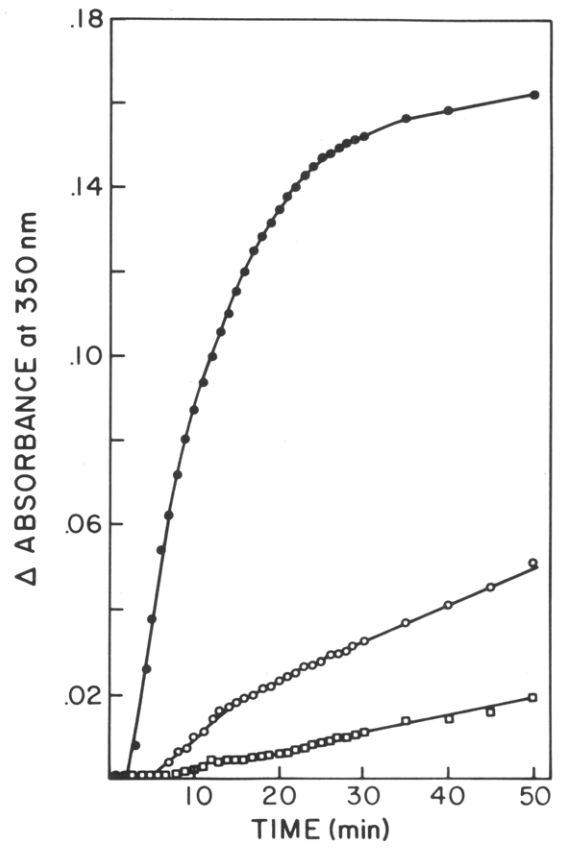

FIGURE 5: Inhibition of MAP-stimulated microtubule assembly by phosphorylation. Microtubular protein (7 mg/mL) in RB/4 (25 mM Mes, 0.5 mM $MgCl_2$, and 0.25 mM EGTA, pH 6.8) was incubated at 37 °C for 30 min with either 250 μM [γ-^{32}P]ATP (○), 250 μM [γ-^{32}P]ATP and 2 μM cAMP (□), or without nucleotide additions (●). The protein in each reaction was then fractionated on identical phosphocellulose columns as described under Methods. The unbound 6S tubulin from the three columns was pooled and immediately applied to a Sephadex G-25 column equilibrated with RB to restore the protein to full strength buffer. The microtubule-associated proteins (MAPs) eluted from each phosphocellulose column with RB/4 containing 0.8 M NaCl were desalted on Sephadex G-25 columns equilibrated with RB. Protein concentrations were determined by the Bradford procedure (Bradford, 1976). All polymerization reactions contained tubulin (1.9 mg/mL), MAPs (0.4 mg/mL), and 1 mM GTP. Microtubule assembly was measured by the change in absorbance at 350 nm. There was no polymerization in controls containing 1 mM GTP with either tubulin or MAPs alone.

the three reaction mixtures was pooled, and the MAPs were eluted with 0.8 M NaCl. Polyacrylamide gel electrophoresis profiles of the proteins eluted from the different phosphocellulose columns with 0.8 M NaCl were indistinguishable. However, more phosphate was incorporated into the protein phosphorylated with cAMP than without cAMP (Figure 2C).

The MAP proteins from each phosphocellulose column were passed through Sephadex G-25 equilibrated with RB to remove excess NaCl and added nucleotides. Microtubules were then assembled using GTP by reconstituting the MAP proteins incubated under different conditions with the 6S tubulin pooled from all three columns. As shown in Figure 5, both the rate and extent of microtubule assembly were reduced for the proteins that had been phosphorylated in either the absence or presence of cAMP prior to reconstitution. The proteins that had been phosphorylated in the presence of cAMP promoted less polymerization than those which had been phosphorylated to a lesser extent in the absence of cAMP. Since added ATP and cAMP are removed prior to reconstitution, inhibition cannot be attributed to the presence of these nucleotides during microtubule assembly.

Discussion

Phosphorylation of microtubular protein has been proposed as a possible mechanism for the regulation of microtubule assembly or interactions with other cellular components. However, the endogenous protein kinase activity in microtubular protein preparations has only been partially characterized, and phosphorylation has no known effects on the properties of microtubular protein. We have further characterized the protein kinase activity in microtubular protein and investigated the effects of phosphorylation on microtubule assembly.

The protein kinase activity isolated with microtubular protein varies with respect to such properties as cAMP stimulation and protein substrate, depending upon the source and

method of isolation. The properties of the protein kinase studied in this report are similar to those described previously (Sloboda et al., 1975; Rappaport et al., 1976; Sheterline, 1977) for microtubular protein isolated from brain by the method of assembly–disassembly of Shelanski et al. (1973). We observe that cAMP increases the maximal extent of protein phosphorylation two- to threefold (Figure 1B). Furthermore, we find that the MAPs are the major phosphorylated components of microtubular protein, with very little ^{32}P incorporated into tubulin (Figure 2).

The mechanism for ^{32}P incorporation into microtubular protein is of interest in that a phosphate exchange reaction between ^{32}P-labeled ATP and phosphoprotein could result in ^{32}P labeling of protein without a net change in the number of protein-bound phosphate groups. Reversibility of the protein kinase reaction has been demonstrated with phosphorylated casein (Shizuta et al., 1975), and a similar process may occur with the protein kinase reacting with microtubular protein. A hypothetical exchange reaction resulting from significant reversibility of the protein kinase could account for the observations that: (1) ^{32}P incorporation has no known effect on the in vitro properties of microtubular protein and (2) microtubular protein that has been purified in the presence of ATP is readily ^{32}P labeled.

A phosphate exchange mechanism was, however, excluded by our demonstration that the rate of ^{32}P loss from labeled protein was not increased by the addition of unlabeled ATP (Figure 1A). Therefore, incorporation of ^{32}P into purified protein represents a net increase in the number of protein-bound phosphate groups. The fact that protein purified in the presence of ATP can be further phosphorylated after purification was shown to be the result of protein phosphatase activity both during and after purification (Figure 1B).

It has been reported previously that phosphorylation of microtubular protein under conditions developed to maximize tubulin assembly has no effect on tubulin polymerization (Rappaport et al., 1976). We have explored the possibility that phosphorylation would affect microtubule assembly after the protein was exposed to conditions which result in dissociation of rings. This was investigated in two ways:

(1) At low ionic strength, rings are converted to MAPs and tubulin dimer. Under these conditions, phosphorylation by ATP proceeds normally, but polymerization does not occur. When polymerization was induced by increasing the ionic strength, both the rate and extent of microtubule assembly were inhibited, and the microtubules formed were longer in reactions previously incubated with ATP and cAMP relative to those incubated with cAMP alone or without nucleotide additions (Figure 3 and Table I). Since the nucleotide composition of the reaction mixtures during microtubule assembly was identical, the inhibition observed after preincubation with ATP and cAMP can be attributed to a reaction which takes place during this time. The greater length of the microtubules formed and the slower assembly rate with protein which had been phosphorylated suggest that phosphorylation reduces the number of nucleation centers for microtubule assembly. It is postulated that these centers are dissociated at low ionic strength, and their re-formation is inhibited with phosphorylated protein. It has previously been found in studies of microtubule assembly with purified MAPs and tubulin dimer that with low concentrations of MAPs, the rate of microtubule formation is decreased, and the average length of the microtubules formed is increased (Sloboda et al., 1976).

(2) The effect of phosphorylation on the formation of microtubules was analyzed following fractionation of microtubular protein into MAPs and tubulin dimers by phosphocellulose chromatography. MAP-stimulated assembly of tubulin was significantly inhibited when the MAPs had been phosphorylated with ATP (Figure 5). Since the nucleotides used for phosphorylation are removed by gel filtration prior to reconstitution and since the tubulin dimer in each reaction mixture was identical, the reduced microtubule assembly observed in the phosphorylated reaction can be attributed to the MAPs fraction.

These studies indicate that phosphorylation of MAPs inhibits both the rate and extent of microtubule assembly. Inhibition of microtubule assembly occurs after the protein is exposed to conditions which result in ring dissociation. The results are taken to indicate that phosphorylation modifies MAPs so that they have a decreased ability to form an assembly-competent complex with tubulin.

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Properties of a β-*N*-Acetylgalactosaminyl Transferase and Its Mobilization from an Endogenous Pool to the Cell Surface of Embryonic Chick Neural Retina Cells†

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ABSTRACT: We have analyzed the incorporation of [^{14}C]-GalNAc from UDP-[^{14}C]GalNAc among whole cells and homogenates of embryonic chick neural retinas. Single cells prepared by trypsinization incorporate [^{14}C]GalNAc from UDP-[^{14}C]GalNAc into a product released from the intact cells into the reaction medium. Little or no incorporation into cell-associated products is observed. The possibility of misleading results due to sugar–nucleotide breakdown or cell lysis has been excluded. The reaction is dependent on Mn^{2+} and is stimulated by Ca^{2+}. Colchicine added at zero time completely blocks incorporation. Cells which have been "repaired" in culture in the presence of cycloheximide also incorporate [^{14}C]GalNAc from UDP-[^{14}C]GalNAc into a cell-free product, but the reaction is neither stimulated by Ca^{2+} nor blocked by colchicine. Incorporation of [^{14}C]GalNAc from UDP-[^{14}C]GalNAc in whole tissue homogenates prepared in 1% Triton X-100 is linear for at least 1 h and is unaffected by cycloheximide, Ca^{2+}, or colchicine. Homogenates of trypsin-dispersed cells show a 30% reduction in activity when compared to homogenates of whole tissues. Transferase activity is further reduced to ~10% when single cells are allowed to repair in cycloheximide and then are retrypsinized and assayed as homogenates. The product of the reaction in both homogenates and intact cells is a high molecular weight glycoprotein containing terminal GalNAc residues. The results suggest that a β-*N*-acetylgalactosaminyl transferase is present in both a cell surface associated and an endogenous form. Following tissue trypsinization, the surface-associated activity is destroyed. The endogenous complement of the enzyme acceptor is then mobilized to the cell surface by a colchicine-sensitive process where transfer of [^{14}C]GalNAc occurs followed by release of the glycosylated acceptor into the reaction medium.

The presence of glycosyl transferases at the cell surface has been indicated in a variety of different systems [see Shur & Roth (1975) for a review]; however, it is still a matter of controversy whether these enzymes play a specific role at the surface or represent the passive result of fusion of Golgi vesicles with the plasma membrane [see Cook (1977)]. Cell surface glycosyl transferases have been implicated in such physiological roles as homeostasis (Jamieson, 1978; Bosman, 1972), biosynthesis of complex polysaccharides (Pat & Grimes, 1975), and cell–cell adhesion [see Shur & Roth (1975) for a review].

Previous work in this laboratory (McDonough & Lilien, 1978) has provided evidence that a β-*N*-acetylgalactosaminyl transferase is involved in the turnover of a tissue-specific ligand from the surface of embryonic chick neural retina cells. Release of the ligand into the extracellular fluid is stimulated by Mn^{2+} and inhibited by EDTA,[1] UDP (but not other nucleotides), hydroxyurea, and cytosine arabinoside. Soluble ligand preparations treated with β-*N*-acetylhexosaminidase, which liberates GalNAc and inactivates ligand (McDonough & Lilien, 1975, 1978), are reactivated upon incubation with intact cells. Like release, reactivation is Mn^{2+} dependent and is inhibited by EDTA and UDP. It was suggested that ligand exists as an endogenous pool; treatment of the cell surface with trypsin triggers the mobilization of this pool to the cell surface, where release of the ligand molecule into the medium is accomplished via a glycosylation reaction.

In this paper we demonstrate biochemically the existence of a β-*N*-acetylgalactosaminyl transferase active at the surface of embryonic chick neural retina cells and identify the products

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[1] Abbreviations used: GalNAc, *N*-acetyl-D-galactosamine; EDTA, ethylenediaminetetraacetic acid (tetrasodium salt); GalNAc-P, *N*-acetyl-D-galactosamine 1-phosphate; LDH, lactic dehydrogenase; PMSF, phenylmethanesulfonyl fluoride; Gal, galactose; $GalNH_2$, galactosamine; GluNAc, *N*-acetylglucosamine; PTA, phosphotungstic acid; Cl_3AcOH, trichloroacetic acid; BSA, bovine serum albumin; DNase, deoxyribonuclease.

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