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AUTOPHOSPHORYLATION OF BRAIN MICROTUBULE PROTEIN: EVIDENCE FOR ENDOGENOUS PROTEIN KINASE/PHOSPHOPROTEIN PHOSPHATASE CYCLING AND MULTIPLE PHOSPHORYLATION OF A MICROTUBULE ASSOCIATED PROTEIN*

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Summary: Brain microtubule protein, prepared by two types of recycling methods, undergoes "flash" phosphorylation in the presence of [γ-^{32}P]ATP through sequential action of protein kinase and phosphoprotein phosphatase present in microtubule protein. SDS electrophoretic analysis indicates that MAP_1, tau protein, and tubulin are poorly phosphorylated, and MAP_2 is the major site of phosphorylation. To improve [^{32}P]phosphoprotein stability in the presence of the kinase/phosphatase cycle, 3',5'-cyclicAMP, orthophosphate, or fluoride ion may be added. After separation from tubulin by phosphocellulose chromatography, the MAP fraction exhibits autophosphorylation. Finally, the maximal extent of autophosphorylation is observed with an ATP regenerating system using ADP, [^{32}P]-acetyl-P, and bacterial acetate kinase; this results in the incorporation of 3-4 phosphoryl groups per MAP_2 subunit.

Microtubules play important roles in cellular morphology and motility, and few sources are more abundant in microtubule protein than brain. For this reason, there has been considerable interest in microtubule protein phosphorylation as a mechanism for regulating microtubule assembly, disassembly, or interactions with neural cell components (1-3). Nonetheless, full characterization of such control processes is greatly impeded by the number of proteins and enzymes associated with recycled microtubule protein (4-6). Tubulin (the principal subunit of microtubules), two high molecular weight proteins, and a heat-stable protein, called tau, are the most prominent fractions of microtubules. Yet, the following enzyme activities have also been observed: nucleoside diphosphate kinase, ATPase, protein kinase, GDPase, phosphatase, 3',5'-cyclicAMP phosphodiesterase, glutamate dehydrogenase, and a cytochrome reductase (5-7). The relationship of these microtubule associated proteins to physiologic roles of microtubules remains unclear, but it is obviously desirable to examine the molecular interactions of these components to begin to unravel their regulatory potential. We have been interested in defining the dynamics of microtubule protein phosphorylation and identifying the sites of phosphorylation. In this report, we focus on the autophosphorylation reaction to

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Abbreviations used: MAP_1, MAP_2, electrophoretic separation of microtubule protein reveals two high molecular weight microtubule associated proteins, MAP_1 (310,000 M_r) and MAP_2 (280,000 M_r). See Karr, et al. (6). TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate; MTP, microtubule protein.

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understand the factors leading to retention of the phosphoryl groups by the microtubule proteins. In particular, we have found that microtubule protein obtained by a sucrose extraction technique (6) undergoes a rapid autophosphorylation which is stimulated by 3',5'-cyclicAMP and markedly increased by use of an ATP-regenerating enzyme system.

Experimental Procedures

We purified microtubule protein from bovine brain using both a modification of the Shelanski procedure (8) as described by Karr, et al. (6), and the sucrose extraction method of Karr, et al. (6). In both procedures, the protein was taken through two assembly/disassembly cycles and stored at -80°C until use.

Protein concentration was determined according to the Lowry method (9), and [^{32}P]acetyl-phosphate was prepared by the method of Stadtman (10).

Protein kinase assay reaction mixtures typically were made up to a final volume of 100µl per assay containing 50 mM 2[N-morpholino]ethane sulfonic acid (*Sigma*) pH 6.2, 10 mM $MgCl_2$, 20 µM podophyllotoxin (*Aldrich*), 10 µM 3',5'-cyclicAMP (*Sigma*), 4 mM theophylline (*Sigma*), and 4×10^5 cpm [γ^{32}P] ATP (*Amersham*) per assay. Zero and 100 µM 3'.5'-cyclicAMP were also used in the assay of cyclic nucleotide dependence. ATP concentrations were 5 µM or 100 µM as noted. When the acetate kinase ATP-regenerating system was used, 5 µM ADP (*Sigma*), 2 units acetate kinase (*Sigma*), and 1.5 mM [^{32}P]acetyl-phosphate were present. Microtubule protein (1 mg/ml) or phosphocellulose-purified microtubule-associated proteins (.25 mg/ml) was added to start the reaction and incubation carried out at 30°C. At the various time points, 100µl of reaction mix was removed and spotted onto 10% trichloroacetic acid (TCA)-soaked Whatman No. 1 paper discs (11). The zero time point was spotted before addition of protein. The discs were washed sequentially three times in cold 10% TCA, three times in cold 5% TCA, once in ethanol, and once in ethyl ether, then dried. The radioactivity was measured by Cerenkov counting in 12ml water. The samples used in SDS gel electrophoresis were removed from the assay mix in 100µl aliquots and added to 1ml cold 10% TCA. A 25µl aliquot of bovine serum albumin (100 mg/ml) was added to coprecipitate the protein. The sample was left on ice 5 minutes and the precipitate pelleted by centrifugation. The pellet was resuspended in 100µl 1 N NaOH and 1 ml cold 10% TCA added again. This precipitate was pelleted, the pellet brought to pH 7 with 1 N NaOH, and then treated as the sample for electrophoresis.

SDS gel electrophoresis was performed as described by Karr, et al. (6). After destaining, the gels containing [^{32}P]-phosphoprotein were frozen on dry ice and cut into bands corresponding to the indicated fractions. These gel segments were placed in tubes containing 0.5ml hydrogen peroxide and dissolved by oxidation at 80°C. The radioactivity of each was then measured by Cerenkov counting in 10ml water.

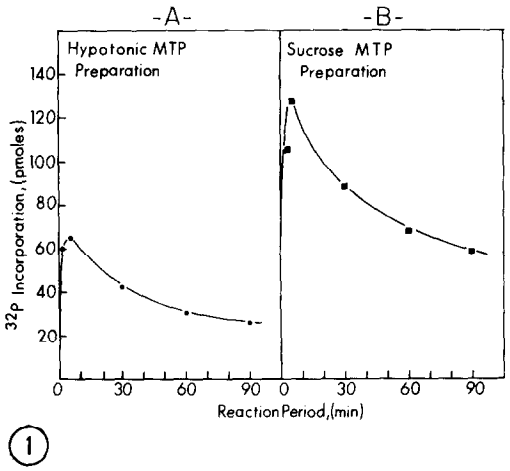
Results

During the course of experiments on the microtubule-associated ATPase, we explored the possibility that the ATP hydrolysis represented the summation of opposing reactions catalyzed by protein kinase and phosphoprotein phosphatase. We found that the ATPase (K_m=0.5-1 mM) was unrelated to such a futile cycle on the basis of lack of cyclic AMP stimulation, insensivity to heating or inhibitor protein which were found to block the kinase reaction, and failure of casein or histones to increase the ATPase activity by increasing the concentration of cosubstrate for such opposing reactions (12). Nonetheless, the

action of the endogenous protein kinase drew our attention to phosphorylation at low concentrations of ATP (5-10 μM), and we found that the microtubule protein underwent rapid autophosphorylation. Upon following the kinetics of phosphoryl group incorporation for longer periods, we observed that the actual process was one of "flash" phosphorylation. Although there is a rapid rise in the phosphorylation, the process is always followed by dephosphorylation. (This is shown in Fig. 1A-B for two types of microtubule protein preparations.) The specific activity of the phosphorylation was as high 1 nmol phosphoryl groups/mg microtubule protein/minute, exceeding the high K_m ATPase by ten to twenty-fold. Interestingly, the sucrose purification method (6) yielded the highest autophosphorylation, and this has been verified in a number of experiments. This purification procedure minimizes mitochondrial and synaptosomal contamination resulting from the osmotic shock encountered in earlier hypotonic microtubule preparation procedures. The sucrose procedure also employs 1 mM ATP in the initial extraction buffer, and this may preserve the otherwise labile protein kinase.

To learn about the proteins undergoing phosphorylation, reaction samples were quenched at peak phosphorylation by the addition of 10% TCA, and subjected to SDS gel electrophoretic separation. The ^{32}P radioactivity of protein fractions corresponding principally to MAP_1, MAP_2, T (indicated as interzone band), and tubulin was measured after peroxidation of the gel slices. As shown in Fig. 2A, ^{32}P incorporation is greatest in the MAP_2 fraction, and there is some labeling of tubulin subunits or a protein of similar molecular weight. It is also clear that the sucrose extraction method yields the highest incorporation. The extent of autophosphorylation is somewhat dependent upon added cyclic AMP (see Fig. 2B), and addition of theophylline improves the radiolabel incorporation at lower cyclic AMP levels. We have also found that 40 mM orthophosphate and/or 20 mM fluoride retard the dephosphorylation phase, but nothing has been found to completely stop this hydrolysis phase. It is also interesting to note that a microtubule-associated protein fraction isolated from tubulin by phosphocellulose chromatography undergoes phosphorylation in agreement with earlier reports (3, 13). This suggests that tubulin itself is not responsible for the kinase activity.

The time course of the phosphorylation/dephosphorylation process suggested that the extent of phosphorylation might be limited by loss of ATP and/or accumulation of ADP. To improve the extent of phosphorylation, we employed an acetate kinase regenerating system with [^{32}P]acetyl-phosphate as the radiophosphoryl donor. The value of this approach is confirmed in Fig. 3 with phosphorylations at 5 and 100 μM ATP carried out for comparison. While the slower rate of radiolabel incorporation with the acetate kinase probably reflects the well



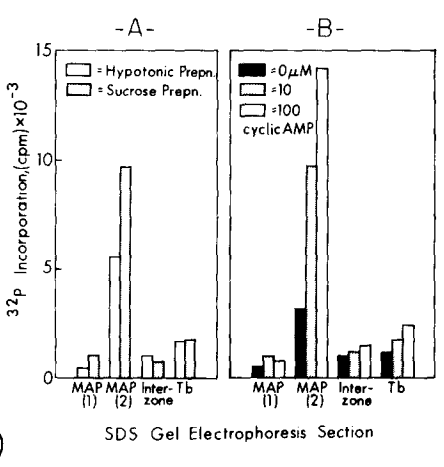
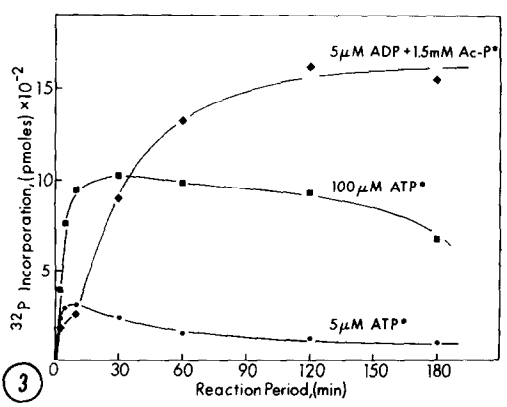


Fig. 1. Comparison of the time course of microtubule protein phosphorylation obtained by hypotonic (A) and sucrose (B) extraction methods. Microtubule protein (1 mg/ml) was incubated at 30° with 10 μM 3',5'-cyclicAMP and 5 μM [γ-^{32}P]ATP. For other details, see "Experimental Procedures."

Fig. 2. SDS gel electrophoretic analysis of autophosphorylated microtubule protein. A, comparison of hypotonic and sucrose preparations at peak phosphorylation (10 min reaction time). See Fig. 1 for experimental conditions. B, incorporation of ^{32}P into sucrose-prepared microtubule protein at several levels of 3',5'-cyclicAMP. See Fig. 1 for experimental conditions.



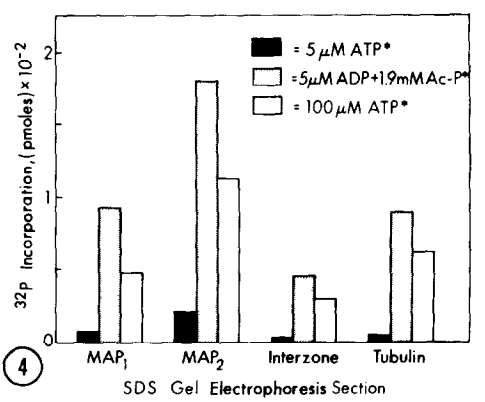


Fig. 3. Time course of microtubule protein phosphorylation in the presence of 5 μM ATP (●), 100 μM ATP (■), and 5 μM ADP with 1.5 mM [^{32}P]acetyl-P and 2 units desalted bacterial acetate kinase (◆). Microtubule protein (sucrose method) at 1 mg/ml was incubated in the presence of 10 μM 3',5'-cyclicAMP.

Fig. 4. Analysis of SDS gel sections for autophosphorylated microtubule protein. Conditions were identical to those in Fig. 3. Samples were taken for electrophoresis at peak phosphorylation time for the specific condition used: 5 μM ATP, 10 minutes; 100 μM ATP, 30 minutes; acetate kinase ATP-regenerating system, 120 minutes. For details, see "Experimental Procedures."

known hysteretic properties of this bacterial enzyme, the increased extent of phosphorylation is the important feature. We found that the autophosphorylation in the presence of 100 μM ATP fails to exceed the levels of incorporation achieved with the regenerating system using 5 μM adenine nucleotide. This suggests that the ATP/ADP ratio rather than the amount of added adenine nucleotide may be the important factor controlling phosphorylation. Assuming complete incorporation into MAP_2, the [^{32}P]-phosphoryl group incorporation would correspond to formation of 10-20 pmol of phosphoryl groups per pmol MAP_2. Nonetheless, the true phosphorylation is only revealed upon SDS electrophoresis, and the observed ^{32}P levels presented in Fig. 4 correspond to phosphorylation of three to four residues per molecule of the 300,000 M_r MAP_2 protein. This stoichiometry is estimated on the basis of densitometry tracings of the Coomassie blue dye-stained protein fractions (6). Such multiple phosphorylation of MAP_2 has been carried out on many occasions, yet its significance is not very clear.

Discussion

The results presented in this report indicate that MAP_2 phosphorylation is the principal action of the endogenous protein kinase. The acetate kinase system provides an excellent method for regenerating ATP and maintaining a high [phosphoMAP_2]/[dephosphoMAP_2] ratio. Interestingly, we have found that GTP is not an inhibitor nor an alternative substrate with the protein kinase; so use of acetate kinase to regenerate GTP during microtubule assembly (14) should be without effect on MAP_2 phosphorylation. In any case, the multiple phosphorylation of MAP_2 is indicated by the incorporation of 3-4 phosphoryl groups per 300,000 M_r polypeptide chain. The topology of phosphorylation of this large protein remains unclear.

Equally intriguing is the role of MAP_2 and its enzymatic interconversion by the kinase and phosphatase. Amos (15) and Kim, *et al.* (16) have both observed that this large polypeptide forms regular projections from the outer surface of brain microtubules. The periodicity of one MAP_2 projection per 32 nanometers suggests a stoichiometry of one MAP_2 subunit to nine tubulin dimers at saturating MAP_2 levels. Phosphorylation might be a part of a mechanism for regulating assembly, disassembly, or even the formation of these projections. Nonetheless, the literature is full of conflicting reports about phosphorylation effects on microtubules. For example, Garland (17) reported that 3',5'-cyclicAMP (with fluoride ion present) inhibits microtubule assembly from 100,000×g supernatants prepared from porcine brain homogenates. She found that the cyclic nucleotide decreased the rate and extent of both ATP- and GTP-supported assembly, but this compound had no effect on

the depolymerization rate. On the other hand, Margolis and Wilson (18) claim that ATP greatly alters the rates of microtubule turnover at steady state assembly. They conclude that disassembly is increased by as much as 20-fold, which they attribute to MAP_2 phosphorylation. These conflicting findings suggest that the conditions for phosphorylation may greatly alter the observed behavior of microtubules. We therefore believe that the identification of favorable conditions for rapid and maintained MAP_2 phosphorylation represents a first step in understanding the regulatory effects.

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