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Differential Phosphorylation of MAP-2 Stimulated by Calcium-Calmodulin and Cyclic AMP

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Comparison of cyclic AMP- and calcium-dependent phosphorylation in rat brain cytosol reveals MAP-2 to be a common endogenous substrate. Examination of limited protease digestion patterns indicates that the two kinases phosphorylate MAP-2 at distinct sites and suggests that such phosphorylation may have differential effects on MAP-2 function.

Proper function of cytoplasmic microtubules requires controlled and orchestrated assembly and disassembly of their tubulin subunits (24). Efforts at understanding this regulation have focused attention on several groups of proteins termed microtubule-associated proteins, designated so by virtue of their copurification with microtubules through several cycles of assembly and disassembly (2, 7, 10, 29). In the brain, a rich source of microtubules, these proteins include MAP-1 (23, 28), a 350,000-dalton doublet; MAP-2 (23, 28), a 280,000-dalton doublet; tau factor, consisting of several major proteins ranging from 55,000 to 62,000 daltons (4); and several other as yet uncharacterized proteins. In vitro, these proteins promote nucleation, increase the assembly rate of microtubules, and increase interaction of microtubules with microfilaments (8, 12–14, 16–18, 21, 22). MAP-2 has been shown to be phosphorylated by a cyclic AMP (cAMP)-dependent protein kinase in microtubule preparations from brain (23, 27). In view of the importance of calcium to microtubule function (6), it was of interest to determine whether MAP-2 phosphorylation could be regulated by calcium.

The effect of cAMP and calcium on endogenous protein phosphorylation in brain cytosol is shown in Fig. 1. A 150,000 × *g* supernatant from a homogenate of rat brain was assayed for incorporation of [^{32}P]phosphate from [γ-^{32}P]ATP into protein and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The autoradiograph of the proteins shown on the left side of Fig. 1 reveals that stimulation of the cAMP-dependent protein kinases by the addition of cAMP leads to an enhanced incorporation of ^{32}P into a large number of substrate proteins relative to basal phosphorylation. Those proteins whose phosphorylation was consistently stimulated are indicated by an arrow and approximate molecular mass in kilodaltons. The most prominent substrates for the cAMP-dependent protein kinases are at 18,000, 47,000, 51,000, and 280,000 daltons. The 280,000-dalton substrate has been identified in chick brain and bovine brain as a microtubule-associated protein and has been designated as MAP-2 (23, 27). Addition of calcium also stimulated ^{32}P incorporation into numerous endogenous substrates. The extent of ^{32}P incorporation and the number of substrates seen upon stimulation with calcium was comparable to that seen with the better-characterized cAMP-dependent protein kinases. The most prominent substrates for the calcium-dependent kinase have molecular masses of 46,000, 48,000, a group of poorly resolved proteins ranging from 51,000 to 60,000, and 280,000 daltons. Although most endogenous substrates are not shared by the two classes of kinase, the most prominent substrate, at 280,000 daltons, appears to have similar if not identical mobility.

The potential importance of the regulation of microtubule function by calcium via protein phosphorylation led me to investigate the possibility that the 280,000-dalton protein phosphorylated by the calcium-dependent kinase was MAP-2. Cytosolic proteins were phosphorylated in the absence or presence of calcium as above. Authentic bovine microtubules containing MAP-1 and MAP-2 were phosphorylated by the addition of a calcium- and calmodulin-dependent protein kinase recently purified from rat brain cytosol (J. Cell Biol., in press). Protein staining of purified microtubules (Fig. 2A) indicated the position of MAP-1, a widely spaced protein doublet, and MAP-2, a closely spaced doublet at lower molecular weight. The electrophoretic mobility of the four major high-molecular-weight proteins present in rat brain cytosol is nearly indistinguishable. Autoradiography of the stained gel (Fig. 2B) revealed that the 280,000-dalton phosphoprotein in rat brain cytosol exactly coincided with the lower doublet of stained protein. The purified calcium-dependent kinase phosphorylated MAP-2 and not MAP-1 in the preparation of bovine microtubules. The 280,000-dalton protein from rat brain cytosol and bovine MAP-2 can also be phosphorylated by a purified cAMP-dependent protein kinase (data not shown), thus fulfilling another criteria needed for its identification. Electrophoretic blotting and immunostaining of the rat cytosol lanes for MAP-1 and MAP-2 (Fig. 2C) revealed that, indeed, the 280,000-dalton phosphoprotein corresponds to MAP-2 and the higher-molecular-weight doublet corresponds to MAP-1. Similar results were seen when bovine microtubules were immunostained.

Since, in general, the substrate specificity of the cAMP- and calcium-dependent protein kinases appeared to be distinct (Fig. 1), it was of interest to compare the sites of phosphorylation on MAP-2 phosphorylated by the two protein kinases. Cytosolic MAP-2 was phosphorylated under basal, cAMP-, and calcium-stimulated conditions, and sites of phosphorylation were compared by partial proteolysis (3). Whereas several proteases did not clearly distinguish between sites of cAMP- and calcium-dependent phosphorylation, suggesting that the phosphoproteins are similar, the use of V8 protease from *Staphylococcus aureus* revealed a number of differences. Protease digestion after cAMP-dependent phosphorylation generated large fragments as well

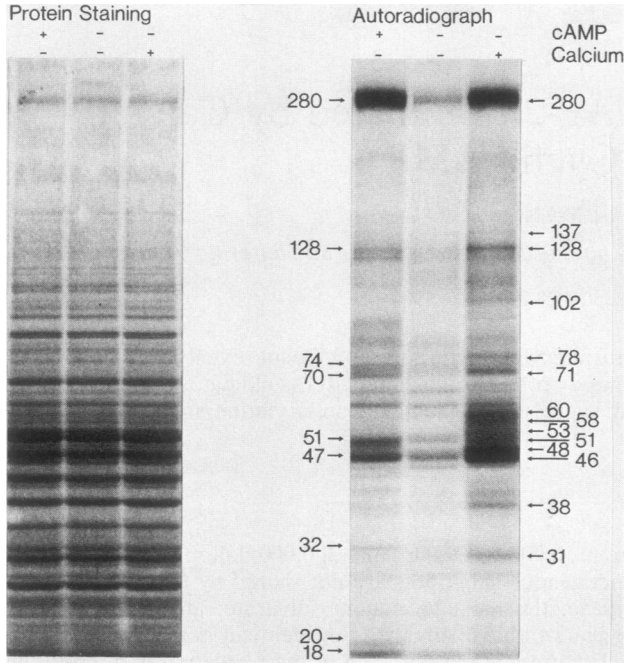

FIG. 1. Effect of calcium and cAMP on endogenous protein phosphorylation of rat brain cytosol. Male Sprague-Dawley rats (120 to 180 g) were decapitated, and the brains (less cerebellum) were used for preparation of cytosol. Brains were homogenized in 3 volumes of 25 mM PIPES [piperazine-*N*,*N*'-bis(2-ethanesulfonic acid)] buffer (pH 7.4)–5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid]–400 μM phenylmethylsulfonyl fluoride with 12 strokes in a glass-Teflon homogenizer at 12,000 rpm at 4°C. Cytosol fraction (13 mg/ml) was obtained by centrifugation at 150,000 × *g* for 60 min. Freshly prepared cytosol was used in these experiments although similar results can be obtained with cytosol kept at 4°C for up to 3 days. Calmodulin was purified to homogeneity from bovine brain (20). The standard phosphorylation mixture for assay of endogenous phosphorylation (final volume, 100 μl) contained the following: 50 mM PIPES buffer (pH 7.0), 5 mM $MgCl_2$, 0.2 mM EGTA (minus calcium and minus cAMP), 0.2 mM EGTA plus 0.5 mM $CaCl_2$ plus 0.5 μg of calmodulin (plus calcium) or 10 μM cAMP plus 1 mM 3-isobutyl-1-methylxanthine (plus cAMP), 20 μM [γ-^{32}P]ATP (1 Ci/mmol), and 65 μg of cytosol protein. After preincubation for 30 s at 30°C, the reaction was initiated by the addition of [γ-^{32}P]ATP. The initial rate of phosphate incorporation was obtained by carrying out the reaction for 30 s. The reaction was terminated by the addition of 50 μl of an SDS stop solution, the mixture was heated for 2 min at 100°C, and a 75-μl portion of the sample was analyzed by SDS-polyacrylamide gel electrophoresis, using a 6 to 12% gradient of acrylamide, and autoradiography (19, 20). Numbers are defined in the text.

as three reproducible phosphopeptides of ca. 19,000, 16,000, and 12,000 daltons (Fig. 3). Digestion after calcium-dependent phosphorylation generated four characteristic phosphopeptides; three that were similar to those above and a fourth one at 9,000 daltons that is unique. Another distinct band, migrating above the dye front, was seen upon prolonged digestion. Although the three bands were similar they had slight differences in electrophoretic mobility and appeared to be distinct based on additivity of phosphorylation and differential rates of dephosphorylation (data not shown). Nearly identical phosphopeptide patterns, with differential phosphorylation by the two protein kinases, were obtained after maximal phosphorylation of MAP-2 accomplished by longer incubation times or higher concentrations of ATP. Differential phosphorylation was apparent over a broad range of V8 protease concentrations. A preliminary report of this study has been published (H. Schulman, J. A. Kuret, and K. H. Spitzer, Fed. Proc. **42**:2250, 1983). Phosphorylation of MAP-2 by calcium-dependent phosphorylation has recently been reported (9, 11). It has been shown that the cyclic AMP-dependent protein kinase can at most account for half of the phosphate in MAP-2 (25). The findings above suggest that incorporation of some of the other phosphate may be cata-

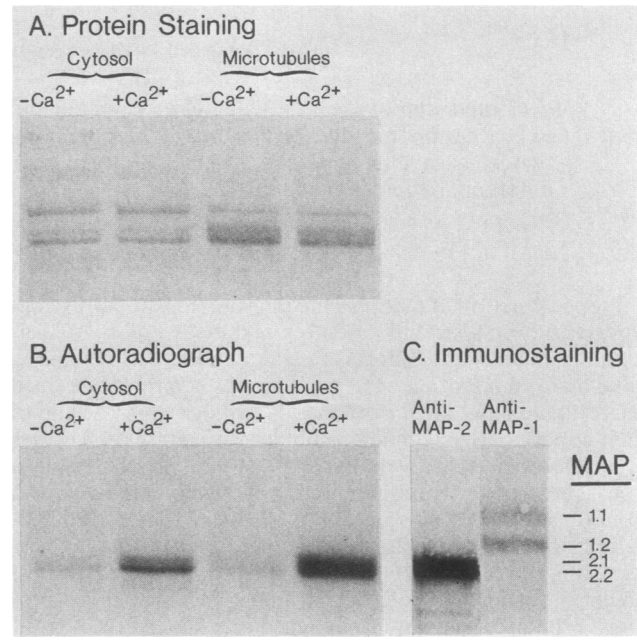

FIG. 2. Comparison of high-molecular-weight phosphoprotein with authentic microtubule-associated protein. Endogenous calcium-dependent phosphorylation was carried out with rat brain cytosol and standard phosphorylation conditions for 2 min at 30°C. Authentic bovine brain microtubules, consisting of ca. 70% tubulin and 30% microtubule-associated proteins, were isolated without glycerol by a modification of the procedure of Asnes and Wilson (1) and were generously provided by L. Wilson. Three cycles of assembly and disassembly and a final centrifugation through a 50% sucrose cushion were used to purify the microtubules. Bovine brain microtubules (3.3 μg) were phosphorylated by the calcium- and calmodulin-dependent protein kinase purified from rat brain cytosol (in press) under standard phosphorylation conditions for 2 min at 30°C in the absence or presence of calcium and calmodulin. ^{32}P incorporation into the 280,000-dalton doublet was 100% in the presence of both calcium and calmodulin and less than 5% in the absence of either calcium or calmodulin alone or in the presence of cAMP alone. (A) Coomassie blue staining pattern of phosphorylation mixture analyzed by 5% acrylamide gels. Only the upper portion of the SDS gel is shown. (B) Autoradiograph of (A). (C) Immunostaining for MAP-1 and MAP-2. A duplicate SDS gel of (A) was electrophoretically transferred to a nitrocellulose filter (26). Transfer was carried out for 800 V-h with a conductance buffer consisting of 0.15 M glycine (pH 6.2) and 0.02% SDS in a BioRad Trans-Blot Cell. Approximately 80% of MAP-2 was transferred. Immunostaining of lanes containing rat brain cytosol was performed by a biotin-avidin-antibody method (Vector Laboratories). First antibody consisted of (i) rabbit antibody against high-molecular-weight microtubule-associated proteins (3) which selectively reacts with MAP-2, kindly provided by M. W. Kirschner, and (ii) a mouse monoclonal antibody (clone 7-1.1) (W. C. Thompson, D. J. Asai, C. Dresden, D. L. Purich, and L. Wilson, J. Cell Biol., abstr. no. 765, p. 201a, 1983) against bovine MAP-1 developed by D. J. Asai, W. C. Thompson, C. F. Dresden, L. Wilson, and D. L. Purich and kindly provided by them. The positions of MAP-1.1, MAP1.2, MAP2.1, and MAP2.2 determined by protein staining are indicated.

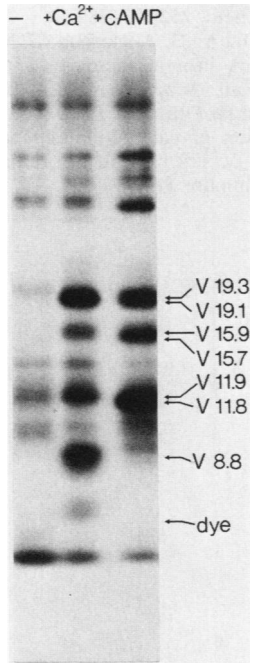

FIG. 3. Partial proteolysis of ^{32}P-labeled MAP-2. Endogenous phosphorylation with rat brain cytosol was carried out as described in the legend to Fig. 1. ^{32}P-labeled MAP-2 was located on the stained SDS gel by autoradiography. Gel pieces corresponding to the MAP-2 doublet phosphorylated in the presence of EGTA, calcium, or cAMP were excised from the gel and rehydrated before proteolytic digestion. Partial proteolysis was performed with 5 μg of *S. aureus* V8 protease per sample in 15% SDS-polyacrylamide gels (3). Approximate molecular mass of the V8 protease-generated peptides is designated in kilodaltons.

lyzed by the calcium- and calmodulin-dependent protein kinase.

Examination of calcium sensitivity of cold labile microtubules demonstrated that calmodulin concentrations severalfold in excess of that of tubulin were required for disassembly (15). I now show that a calcium- and calmodulin-dependent protein kinase with broad substrate specificity can phosphorylate MAP-2. This may provide a catalytic mechanism for affecting microtubule function. It has been suggested that cAMP- and calcium-dependent phosphorylation of MAP-2 favors disassembly or increased flux of microtubules (14, 16, 30). Phosphorylation of distinct sites on MAP-2 by these two enzyme systems offers the possibility for differential regulation of MAP-2 function by the two second messengers.

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