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CYCLIC NUCLEOTIDE- AND Ca^{2+}-INDEPENDENT PHOSPHORYLATION OF TUBULIN AND MICROTUBULE-ASSOCIATED PROTEIN-2 BY GLYCOGEN SYNTHASE (CASEIN) KINASE-1

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SUMMARY: MAP-2 and tubulin are both shown to be substrates for glycogen synthase (casein) kinase-1 (CK-1). Greater than 40 mol ^{32}P is incorporated into MAP-2 by CK-1 compared to only 14 mol ^{32}P observed when cyclic AMP-dependent protein kinase (A-kinase) is the catalyst. Peptide mapping shows that CK-1 and A-kinase recognize a few common sites; the majority of the sites phosphorylated on MAP-2 by CK-1 are quite distinct. Up to 4 mol ^{32}P can be incorporated into the tubulin dimer by CK-1 compared to only 0.9 mol ^{32}P by A-kinase. The preferred substrate for both kinases is β-tubulin.

The observation that purified microtubule proteins can be phosphorylated by endogenous kinases (1, 2) has generated considerable interest in phosphorylation as a possible mechanism for the control of microtubule assembly and function. Microtubule-associated protein-2 (MAP-2) has been shown to be a major substrate for the endogenous kinases (2, 3, 4), although tubulin (2, 3) and the tau proteins (5, 6) are also phosphorylated to lesser extents.

With one exception (7-9) the nature of the kinases phosphorylating the microtubule proteins remains obscure. Cyclic AMP-dependent protein kinase (A-kinase) has been purified from microtubule preparations and shown to be bound to MAP-2, its major substrate (7, 8). In a recent study (9) the phosphorylation state of MAP-2 both before and after phosphorylation by A-kinase was evaluated. It was concluded that besides A kinase other unidentified cyclic nucleotide-independent kinases may also regulate MAP-2 phosphorylation. Tubulin has been shown to be a substrate for both A-kinase (3, 10, 11) and an unidentified cyclic nucleotide-independent protamine kinase (12). Recently two Ca^{2+}- and calmodulin-dependent kinases were isolated from

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brain and shown to phosphorylate both MAP-2 and tubulin (13, 14); in addition, one of these kinases also phosphorylated the tau proteins (15). The latter proteins can also be phosphorylated by cAMP-dependent and -independent kinases present in microtubule preparations (6).

We have recently demonstrated that a Ca^{2+} and cyclic nucleotide-independent kinase initially isolated in our laboratory as a glycogen synthase kinase (16) has a very broad substrate specificity (17). Our ongoing interest to understand the function of this enzyme, termed glycogen synthase (casein) kinase-1 (CK-1), and its mode of regulation has led us to examine the microtubule proteins as possible substrates. In this report we show that both MAP-2 and tubulin are highly phosphorylated by CK-1.

MATERIALS AND METHODS: Microtubule protein from calf brain was prepared by two cycles of temperature-dependent assembly and disassembly by a modification (18) of the method of Asnes and Wilson (19). MAP-2 (18) and tubulin (20) were purified as previously outlined. CK-1 was highly purified from rabbit skeletal muscle as described (16). The catalytic subunit of A-kinase, Staphylococcus auereus V8 protease, and [γ-^{32}P]ATP were purchased from Sigma, Miles Laboratories, and New England Nuclear, respectively. MAP-2 or tubulin was phosphorylated in a reaction mixture (0.05 ml) containing MES buffer (pH 6.8), 25 mM; dithiothreitol, 1 mM; EGTA, 0.5 mM; KF, 5 mM; magnesium acetate, 10 mM; [γ-^{32}P]ATP, 0.5 mM; protein substrates (MAP-2 or tubulin), 0.20 mg/ml; and 200 milliunits of either CK-1 or the catalytic subunit of A-kinase. The reactions were initiated at 37° by the addition of [γ-^{32}P]ATP. Aliquots of the reaction mixtures were withdrawn at timed intervals for the determination of ^{32}P-incorporation into protein (21). Other samples were quenched in SDS-buffer and analyzed by electrophoresis on slab polyacrylamide gels in the presence of 0.1% SDS by the method of Laemmli (22). After staining and destaining, gels were exposed to Kodak XAR-5 film for autoradiography. One unit of CK-1 or the catalytic subunit of A-kinase is defined as the amount of enzyme catalyzing the incorporation of 1 nmol phosphate from [γ-^{32}P]ATP into 4 mg/ml of casein per min at 37°. Protein concentrations were determined by the method of Bradford (23). For peptide mapping, MAP-2 was separately phosphorylated by CK-1 and the catalytic subunit of A-kinase for 60 min. After precipitation with 10% trichloroacetic acid (24), the protein pellets were extracted with ether and dissolved in buffer for digestion with S. aureus V8 protease as described (25). The digestion products were resolved in a 7.5% to 20% gradient polyacrylamide gel in the presence of 0.1% SDS.

RESULTS AND DISCUSSION

We have examined microtubule proteins as potential substrates for CK-1 (Fig. 1). In the absence of CK-1, MAP-2 is the major substrate for the endogenous kinase(s) with tubulin phosphorylated to a smaller extent (lane 3). When CK-1 is added to the microtubule preparation the phosphorylation of MAP-2 is stimulated 2-fold, whereas the phosphorylation of tubulin is increased 6-fold (lane 4). Besides MAP-2 and tubulin no other major protein

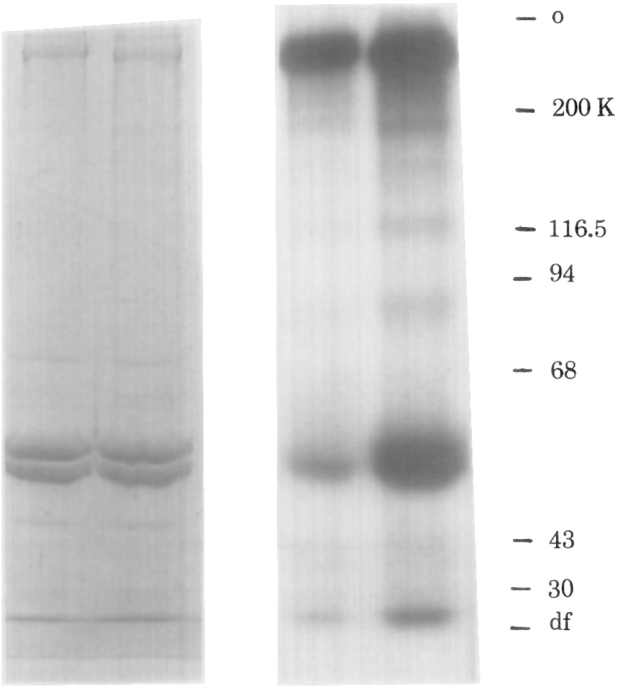

Fig. 1. Polyacrylamide slab gel electrophoresis of microtubule protein phosphorylated in the absence and presence of CK-1. Microtubule protein (1.0 mg/ml) prepared by two cycles of temperature-dependent assembly was phosphorylated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 200 mU CK-1 for 40 min. The phosphorylated proteins (10 µg) were separated in a 7.5% polyacrylamide gel in the presence of 0.1% SDS. Lanes 1 and 2, Coomassie blue stained gel; lanes 3, 4, autoradiograph. Molecular weight markers are shown on right. K, x1000; O, origin; df, dye front.

is phosphorylated by CK-1 (lane 4). To further analyze the phosphorylation of MAP-2 and tubulin we have purified these proteins from calf brain and used them as substrates for CK-1. For comparison, the phosphorylation of both proteins by the catalytic subunit of A-kinase was also examined.

The phosphorylation of MAP-2 by CK-1 and the catalytic subunit of A-kinase is shown in Fig. 2. MAP-2 is relatively free of proteolytic degradation and is not contaminated by tau proteins (Fig. 2A) which, like MAP-2, are heat-stable and shown to be substrates for different kinases (5, 6, 15). In fact we have determined that tau proteins are not substrates for CK-1 (data not shown). It can be seen that the catalytic subunit rapidly phosphorylates MAP-2, the reaction being essentially complete after 20 min. After this time 12 mol phosphate is incorporated into MAP-2, with only an

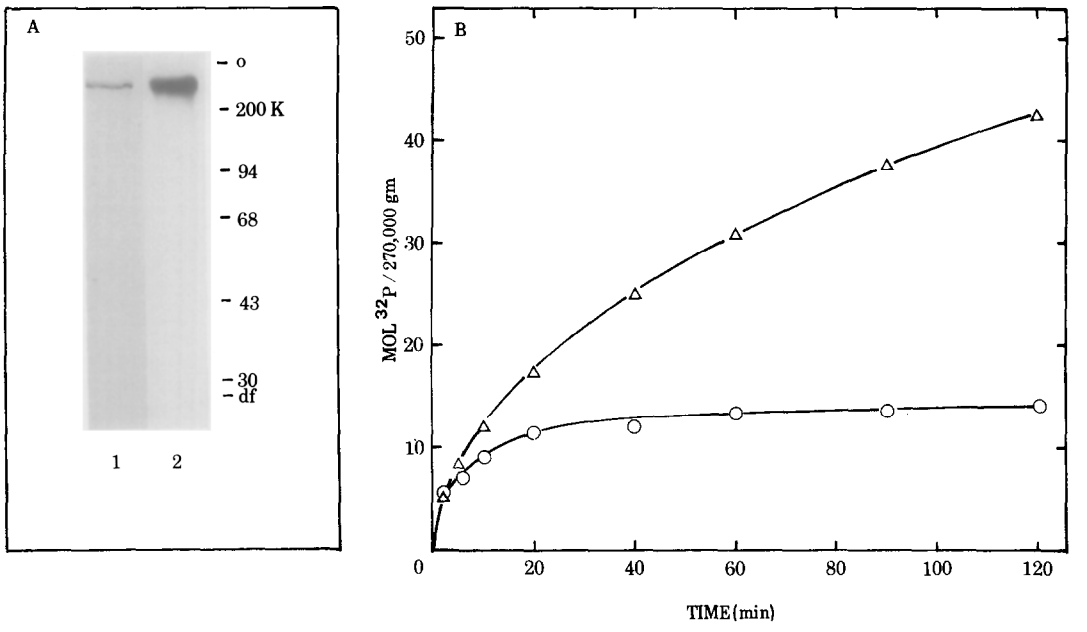

Fig. 2. Phosphorylation of MAP-2 by CK-1 and the catalytic subunit of A-kinase. MAP-2 was phosphorylated by CK-1 and the catalytic subunit of A-kinase under standard conditions. A, MAP-2 (10 µg) phosphorylated by CK-1 for 60 min is analyzed on a 7.5% SDS-polyacrylamide slab gel. Lane 1, Coomassie blue stained gel; lane 2, autoradiogram. A similar pattern is also obtained for MAP-2 phosphorylated by the catalytic subunit of A-kinase (not shown). B, Time course of the phosphorylation of MAP-2 by CK-1 (Δ) and the catalytic subunit of A-kinase (O).

additional 2 mol incorporated in the next 100 min (Fig. 2B). This level of phosphorylation of MAP-2 by the catalytic subunit of A-kinase agrees with previous findings (9, 18). By contrast, CK-1 catalyzes the incorporation of 12 mol phosphate in 10 min with an additional 30 mol being incorporated in the next 110 min, the reaction being incomplete after this time. We have repeated this experiment at least seven times with similar results. It is therefore clear that MAP-2 has many potential phosphorylation sites and that CK-1 recognizes greater than three times the number of sites phosphorylated by the catalytic subunit of A-kinase. To clarify whether CK-1 phosphorylates some of the same sites as the catalytic subunit of A-kinase we digested phosphorylated MAP-2 by Staphylococcus aureus V8 protease and analyzed the peptides on polyacrylamide gel (Fig. 3). It can be seen that the V8 protease effectively digests the 270 kilodalton MAP-2 polypeptide (lane 1) to peptides with M_r smaller than 68 kilodaltons (lanes 2 and 3). Also a greater number of ^{32}P-

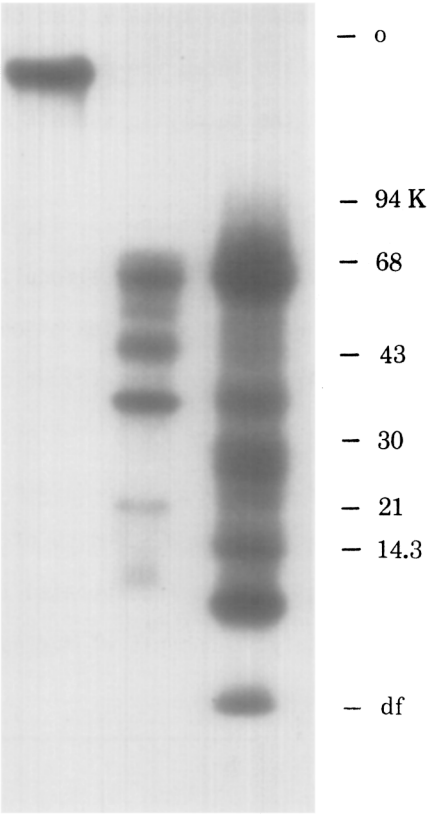

Fig. 3. Peptide mapping of the sites on MAP-2 phosphorylated by CK-1 and the catalytic subunit of A-kinase. MAP-2 phosphorylated by CK-1 (29 mol ^{32}P) or the catalytic subunit of A-kinase (12 mol ^{32}P) was digested for 20 min at 37° with S. aureus V8 protease. The ratio of MAP-2 to protease was 20:1. The reaction products were analyzed in a 7.5% to 20% gradient polyacrylamide gel containing 0.1% SDS. An autoradiograph of the gel is shown. Undigested MAP-2 phosphorylated by CK-1 (lane 1) or MAP-2 treated with protease but initially phosphorylated either by the catalytic subunit of A-kinase (lane 2) or by CK-1 (lane 3).

labeled peptides are generated from MAP-2 phosphorylated by CK-1 (lane 3) compared to MAP-2 phosphorylated by the catalytic subunit of A-kinase (lane 2). Hence, in addition to the greater incorporation of phosphate catalyzed by CK-1 (Fig. 2B) this latter result further supports the idea that CK-1 phosphorylates many more sites than does the catalytic subunit of A-kinase. A comparison of the peptides phosphorylated by the two enzymes indicates that only two of the peptides (M_r ~ 60K and ~ 35K) are overlapping (lanes 2 and 3) while others, especially those phosphorylated by CK-1, are quite distinct. It seems that CK-1 and the catalytic subunit of A-kinase may recognize some

common sites. Phosphoamino acid analysis reveals that CK-1 and the catalytic subunit phosphorylate both serine and threonine on MAP-2. The ser-P/thr-P ratio is 1.9 for CK-1 and 7.3 for the catalytic subunit of A-kinase (data not shown).

The phosphorylation of tubulin by A-kinase (3, 10, 11), an unidentified protamine kinase (12), as well as two Ca^{2+}- and calmodulin-dependent kinases (13, 14) has been demonstrated. With the exception of one of the Ca^{2+}- and calmodulin -dependent kinases (14) the stoichiometry of phosphate incorporation into tubulin was either very low or was not studied. The phosphorylation of tubulin by CK-1 and the catalytic subunit of A-kinase is shown in Fig. 4. These enzymes phosphorylate both the α and β subunits of tubulin (Fig. 4A). After 60 min 0.86 mol and 2.84 mol ^{32}P are incorporated into the tubulin dimer in reactions catalyzed by the catalytic subunit of A-kinase and CK-1,

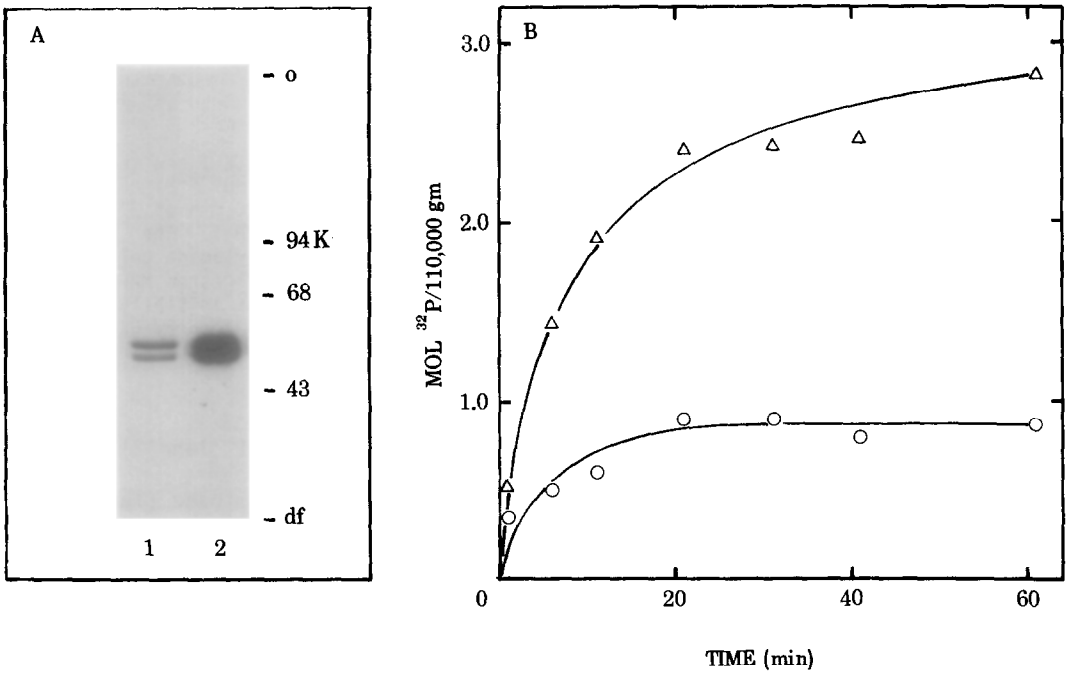

Fig. 4. Phosphorylation of tubulin by CK-1 and the catalytic subunit of A-kinase. Tubulin was phosphorylated under standard conditions by CK-1 and the catalytic subunit of A-kinase. A, Tubulin (10 µg) was phosphorylated by CK-1 for 10 min and analyzed on a 7.5% SDS-polyacrylamide slab gel. Lane 1, Coomassie blue stained gel; lane 2, autoradiogram. A similar pattern is also obtained when tubulin is phosphorylated by the catalytic subunit of A-kinase. B, Time course of the phosphorylation of tubulin by CK-1 (Δ) and the catalytic subunit of A-kinase (O).

respectively (Fig. 4B). Up to 4 mol ^{32}P were incorporated by the CK-1-catalyzed reaction after longer incubation times (2 hrs); no further phosphorylation beyond 0.90 mol was observed for the reaction catalyzed by the catalytic subunit of A-kinase. Analysis of the phosphorylation of the tubulin subunits indicated that β tubulin is phosphorylated at a faster initial rate than α tubulin by both CK-1 and the catalytic subunit (data not shown). This observation agrees with previous findings in which β-tubulin was the preferred substrate for both a protamine kinase (12) and a Ca^{2+}- and calmodulin-dependent protein kinase (10).

The results presented here further document that CK-1 has a very broad substrate specificity and may be important in controlling different regulatory processes as proposed (17). CK-1 has been purified from skeletal muscle (16) and liver (26) but not from brain. However, an enzyme that phosphorylates neurofilaments and has properties similar to CK-1 has been isolated from rat cerebellum (27). Although Ca^{2+}- and cyclic nucleotide-independent phosphorylations have been demonstrated for MAP-2 (9) and tubulin (11), the identity of the responsible kinases remains unknown. CK-1 may be one of these kinases and may therefore be important in regulating microtubule assembly and disassembly via a phosphorylation mechanism. Such CK-1-catalyzed phosphorylations may occur under conditions where neither cyclic AMP nor Ca^{2+} is elevated. Since both MAP-2 and tubulin are phosphorylated at multiple sites by multiple kinases, it is also possible that an initial phosphorylation of these proteins by CK-1 may serve to regulate their subsequent phosphorylations by other kinases. Relevant to this argument is the observation that phosphorylation of skeletal muscle glycogen synthase by kinase F_a is enhanced by a prior phosphorylation of the synthase with casein kinase-2 (28). Similarly, ATP-citrate lyase becomes a substrate for lyase kinase only after it has previously been phosphorylated by A-kinase (29).

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