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SUBSTRATE SPECIFICITY OF LIVER CALMODULIN-DEPENDENT GLYCOGEN SYNTHASE KINASE*

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A number of proteins were tested as potential substrates for purified rabbit liver calmodulin-dependent glycogen synthase kinase. It was found that liver phenylalanine hydroxylase and several brain proteins including tyrosine hydroxylase, microtubule-associated protein 2, and synapsin I were readily phosphorylated. Brain tubulin was very poorly phosphorylated. These results suggest that calmodulin-dependent glycogen synthase kinase may be a more general protein kinase involved in the regulation of several cellular Ca^{2+}-dependent functions.

In 1980 we described the existence of a liver calmodulin[1]-dependent protein kinase which phosphorylates glycogen synthase (1,2). This CaM-dependent glycogen synthase kinase, which has an apparent Mr of approximately 300,000 and subunits of 50,000 and 53,000 daltons (3,4,5), is distinct from myosin light chain kinase (6) and phosphorylase kinase (7), two well characterized CaM-dependent kinases. Liver CaM-dependent glycogen synthase kinase rapidly phosphorylates glycogen synthase (sites 2 and 1b) and also isolated smooth muscle myosin light chain. It does not phosphorylate the smooth muscle myosin light chain in intact myosin, nor does it phosphorylate isolated cardiac or skeletal muscle myosin light chains, skeletal muscle or liver phosphorylase, or liver pyruvate kinase (4). It was, therefore, our hypothesis that glycogen synthase may be the only physiological substrate for this kinase and hence the name CaM-dependent glycogen synthase kinase.

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[1]Abbreviations used: calmodulin, CaM; microtubule associated protein 2, MAP-2; SDS/PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

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Recently two groups have described the purification from brain of a CaM-dependent protein kinase of subunit Mr 50,000-55,000 (8,9). This kinase actively phosphorylates isolated smooth muscle myosin light chain as well as the neural proteins tryptophan hydroxylase, microtubule protein, and synaptosomal proteins. To test whether our liver CaM-dependent glycogen synthase kinase may be similar to the brain CaM-dependent protein kinase, we tested several of these brain proteins as substrates for the liver kinase.

MATERIALS AND METHODS

Rabbit liver CaM-dependent glycogen synthase kinase was purified as described previously (4). Rat liver phenylalanine hydroxylase was provided by Drs. Anna and Stein Døskeland (Univ. of Bergen, Norway) (10); tyrosine hydroxylase from pheochromocytoma cells was provided by Drs. Tank and Weiner (Univ. of Colorado Medical School, Denver) (11); protein I from synaptosomes (called synapsin I) was provided by Dr. Ueda (Univ. of Michigan, Ann Arbor) (12); rat brain tubulin was provided by Dr. R. Williams (Vanderbilt University) (13). Prior to phosphorylation, the partially purified tubulin preparation was incubated at 80°C for 5 min to inactivate endogenous kinase activity. Skeletal muscle glycogen synthase was purified as described previously (14) except that the glucosamine-6-P affinity column was replaced by precipitation with polyethylene glycol between 5% and 10% (15). [γ-^{32}P]ATP was made by the method of Walseth and Johnson (16).

Phosphorylation reaction mixtures (90 µl) contained 50 mM Hepes buffer (pH 7.5), 10 mM magnesium acetate, 0.5 mM [γ-^{32}P]ATP (700 cpm/pmol), 0.5 mM $CaCl_2$, 0.6 µM CaM, CaM-dependent glycogen synthase kinase, and the substrate protein at the indicated concentration. In some reactions the CaM was omitted and 100 µM trifluoperazine was added. Controls containing kinase without substrate or substrate without kinase did not incorporate significant amounts of phosphate. Reactions were terminated by pipetting 15 µl aliquots on 1 cm^2 Whatman 3M chromatography papers which were then subjected to multiple washes in trichloroacetic acid (17) prior to liquid scintillation counting. A 15 µl aliquot at 60 min was analyzed by polyacrylamide gel electrophoresis according to the method of Laemmli (18) using 10% slab gels.

RESULTS AND DISCUSSION

It is known that α-adrenergic agonists, vasopressin, and angiotensin II promote the phosphorylation of several cellular proteins in a calcium-dependent manner in rat liver (19,20,21). Included among these are phosphorylase, pyruvate kinase, glycogen synthase, and phenylalanine hydroxylase. Since we had already established that phosphorylase and pyruvate kinase were not substrates for our CaM-dependent glycogen synthase kinase (2), we were interested in testing liver phenylalanine hydroxylase. Liver CaM-dependent glycogen synthase kinase did incorporate up to 0.7 mol ^{32}P per mol phenylalanine hydroxylase over 60 minutes as well as 1.1 mol ^{32}P per mol

glycogen synthase (not shown). Both reactions were completely dependent on the presence of CaM-dependent kinase and was blocked by trifluoperazine (Figs 1A and 1B). Phenylalanine hydroxylase is also phosphorylated by cAMP-dependent protein kinase (22) resulting in activation of the enzyme. We are currently investigating the effects on phenylalanine hydroxylase activity of phosphorylation by CaM-dependent glycogen synthase kinase.

Tyrosine hydroxylase, the rate-limiting enzyme in the biosynthesis of catecholamines, is also phosphorylated and activated by cAMP-dependent protein kinase (11). Recent evidence suggests a role for calcium-dependent phosphorylation in response to acetylcholine (23). Since tyrosine hydroxylase has many similarities to phenylalanine hydroxylase, we examined it as a substrate for CaM-dependent glycogen synthase kinase. There was incorporation of about 0.5 to 0.6 mol ^{32}P per mol tyrosine hydroxylase (Fig 1C).

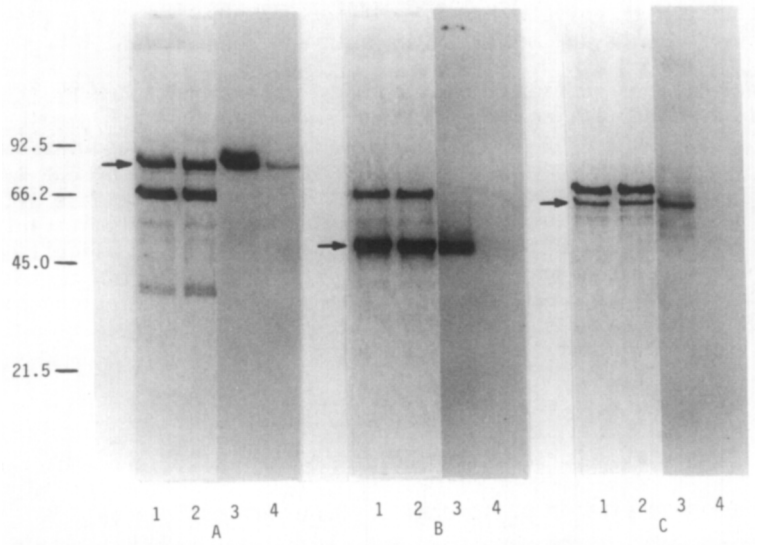

Fig 1. SDS/PAGE analysis of protein phosphorylations catalyzed by CaM-dependent glycogen synthase kinase. A 25 µl aliquot of the protein phosphorylation reactions (Methods) at 60 min was resolved by SDS/PAGE. Reactions corresponding to lanes 1 and 3 contained 0.5 mM $CaCl_2$; those represented by lanes 2 and 4 contained 0.1 mM trifluoperazine. The gels were stained with Coomassie blue (lanes 1 and 2) and subjected to radioautography (lanes 3 and 4). Proteins analyzed, their concentrations in the phosphorylation reactions, and their subunit Mr were as follows: A = glycogen synthase, 0.20 mg/ml, 90,000; B = phenylalanine hydroxylase, 0.18 mg/ml, 51,000; C = tyrosine hydroxylase, 0.04 mg/ml, 60,000. The arrow indicates the protein of interest. The protein band at 66,200 daltons present in all reactions is bovine serum albumin which was in the CaM-dependent kinase preparation to stabilize it. The scale to the extreme left shows the relative migrations of standard proteins of the indicated Mr (kilodaltons).

Next, a partially purified preparation of brain tubulin was phosphorylated by the CaM-dependent glycogen synthase kinase. The reaction mixture was examined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. It was apparent that although tubulin (Mr 55,000) comprised the majority of the protein, most of the ^{32}P was incorporated into microtubule associated protein 2 (MAP-2) of Mr 270,000 (Fig 2A). When highly purified tubulin was utilized as substrate, less than 0.1 mol ^{32}P/mol tubulin subunit was incorporated in 1 hr (Fig 2B). Because the MAP-2 was a minor component of the partially purified tubulin preparation, accurate phosphorylation stoichiometries could not be calculated. It was apparent, however, that more than 1 mol ^{32}P per mol MAP-2 was incorporated. MAP-2 can incorporate up to 20 mol ^{32}P/mol subunit catalyzed by cAMP-dependent and cAMP-independent protein kinases (24).

Synapsin I, a 80,000 to 86,000 dalton protein associated with synaptosomes, is subject to phosphorylation by cAMP-dependent (12) and CaM-dependent protein kinases (25). Liver CaM-dependent glycogen synthase kinase did phosphorylate this protein in a CaM-dependent manner (Fig 2C). An approximate stoichiometry of 5 mol ^{32}P per mol synapsin I was estimated.

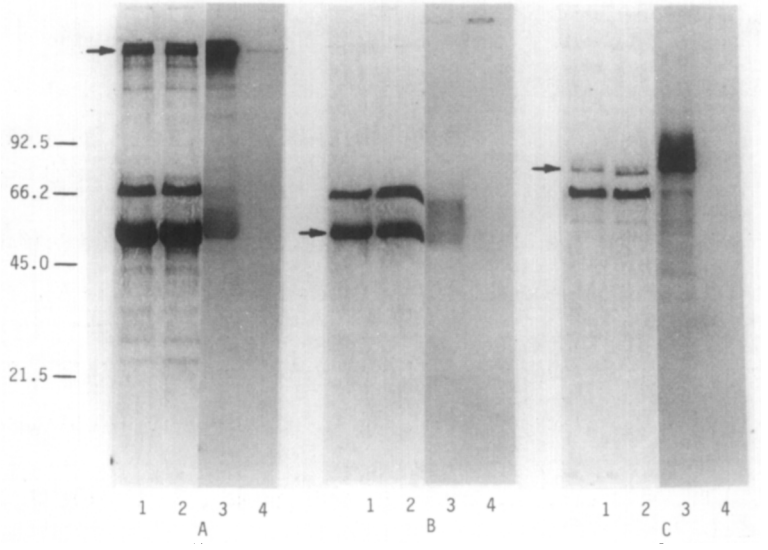

Fig 2. SDS/PAGE analysis of protein phosphorylations catalyzed by CaM-dependent glycogen synthase kinase. For details refer to the legend of Fig 1. A = tubulin preparation containing MAP-2, 1.0 mg/ml, 270,000; B = tubulin, 0.21 mg/ml; 55,000; C = synapsin I, 0.02 mg/ml, 80,000.

These studies clearly establish that liver CaM-dependent glycogen synthase kinase can catalyze phosphorylation of a number of proteins in addition to glycogen synthase. More detailed investigations of these reactions are in progress to determine stoichiometries and sites of phosphorylation, Km values for the substrates, and effects of phosphorylation on the respective protein functions. These studies should give a better understanding of the potential physiological significance of these reactions.

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