Identification of a calmodulin-dependent glycogen synthase kinase in rabbit skeletal muscle, distinct from phosphorylase kinase

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A glycogen synthase kinase that is completely dependent on Ca^{2+} and calmodulin has been identified in mammalian skeletal muscle, and purified ~3000-fold by chromatography on phosphocellulose and calmodulin–Sepharose. The presence of 50 mM NaCl in the homogenisation buffer was critical for extraction of the enzyme. The calmodulin-dependent glycogen synthase kinase (app. M_r 850 000) is distinct from myosin light-chain kinase and phosphorylase kinase, but phosphorylates the same serine residue on glycogen synthase as phosphorylase kinase. The physiological role of the enzyme is discussed.

Calmodulin *Glycogen synthase* *Protein phosphorylation* *Cyclic AMP*

1. INTRODUCTION

Several years ago we reported that highly purified preparations of rabbit skeletal muscle glycogen synthase were contaminated with protein kinase activity distinct from cyclic AMP-dependent protein kinase [1]. This activity, initially termed glycogen synthase kinase-2, was stimulated by calmodulin (2–10-fold) and phosphorylated a serine located 7 residues from the N-terminus of glycogen synthase [2,3]. Subsequently, it was concluded that glycogen synthase kinase-2 was identical to phosphorylase kinase [4], a calmodulin-stimulated enzyme [5] that also phosphorylates serine-7 of glycogen synthase [4,6]. Here, we demonstrate the existence of a calmodulin-dependent glycogen synthase kinase in skeletal muscle distinct from phosphorylase kinase, and discuss the reasons for the failure to recognise this activity previously.

2. MATERIALS AND METHODS

2.1. *Materials*

Phosphocellulose P-11 was obtained from Whatman and treated before use as in [7]. Calmodulin was purified from sheep brain and coupled to CNBr-activated Sepharose 4B (Pharmacia) as in [8]. Phosphorylase *b* [9], phosphorylase kinase [10], glycogen synthase *a* [11,12], myosin light chains [13], myosin light-chain kinase [14] and the specific protein inhibitor of cyclic AMP-dependent protein kinase, termed the *inhibitor protein* [15], were purified from rabbit skeletal muscle by standard procedures. Heparin, phosvitin, and partially dephosphorylated and hydrolysed casein were purchased from Sigma, *E. coli* β-galactosidase from Boehringer and[γ-^{32}P]ATP from Amersham International. Trifluoperazine was a gift from Smith, Kline and French.

2.2. *Purification of the calmodulin-dependent glycogen synthase kinase*

All operations were carried out at 0–4°C. Rabbits were killed with a lethal dose of sodium pentobarbitone. The muscle from the hind limbs and back (800 g) was minced, and homogenised in a Waring Blender for 25 s at low speed with 4.0 mM EDTA (pH 7.0)–0.1% (v/v) 2-mercaptoethanol–50 mM NaCl. The homogenate was centrifuged at 6000 × *g* for 45 min, and the supernatant decanted through glass wool and recentrifuged at 100 000 × *g* for 60 min. One litre of the supernatant was loaded on to a column of phosphocellulose (6 × 5 cm) equilibrated in 50 mM Tris–HCl (pH 7.5, 0°C)–0.1 mM EGTA–10% (v/v) glycerol–0.1% (v/v) 2-mercaptoethanol (solution A)

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containing 50 mM NaCl. The column was washed with solution A + 200 mM NaCl (3 litres), and the calmodulin-dependent glycogen synthase kinase eluted with solution A + 450 mM NaCl. The solution (~90 ml) was concentrated by vacuum dialysis to ~10 ml, dialysed against solution A containing 50 mM NaCl, and made 0.4 mM in $CaCl_2$ and 3.0 mM in $MgCl_2$. It was then chromatographed on calmodulin–Sepharose as in fig.1. The entire procedure could be completed within 3 days.

2.3. *Assay of the calmodulin-dependent glycogen synthase kinase*

Preparations of glycogen synthase *a* were passed through phosphocellulose to remove endogenous protein kinase activities [16], and dialysed against 50 mM sodium glycerol 1-phosphate (pH 7.0) containing 0.5 mM EGTA and 0.1% (v/v) 2-mercaptoethanol. The standard assays (0.1 ml) were carried out at 30°C (pH 7.0) and contained 0.4 mg glycogen synthase *a*/ml, 10 mM sodium glycerol 1-phosphate, 0.1 mM EGTA, 0.15 mM $CaCl_2$, 0.01 mg calmodulin/ml, calmodulin-dependent glycogen synthase kinase, 4.0 mM magnesium acetate and 0.1 mM [γ-^{32}P]ATP. The assays also contained the inhibitor protein and heparin (5 μg/ml) to inhibit any trace cyclic AMP-dependent protein kinase or glycogen synthase kinase-5 activity [17], respectively, in the glycogen synthase *a* or glycogen synthase kinase preparations. The reactions were initiated with ATP and terminated and analysed as in [18]. One unit of activity was that amount which catalysed the incorporation of 1.0 nmol phosphate into glycogen synthase/min. The phosphorylation stoichiometry was calculated as in [19].

The phosphorylation of other proteins was studied in the same assay, except that glycogen synthase was replaced by the protein being tested.

2.4. *Preparation and assay of mouse muscle extracts*

These were prepared from adult ICR/IAn mice, which lack skeletal muscle phosphorylase kinase [20] and C3H/He-mg mice that have normal activity. The muscle was homogenised as described for rabbit skeletal muscle with 2.5 vol. 4.0 mM EDTA–0.1% (v/v) 2-mercaptoethanol (pH 7.0). In some experiments 50 mM or 100 mM NaCl was added to the homogenisation buffer. The homogenates were centrifuged at 15 000 × *g* for 20 min, and the supernatant (extract) decanted.

The extracts were assayed for glycogen synthase kinase activity at pH 6.8 or 8.2, employing the standard assays for phosphorylase kinase [21,22], except that glycogen synthase (0.5 mg/ml) was substituted for phosphorylase *b* (5 mg/ml), and the [γ-^{32}P]ATP (80 000 cpm/nmol) concentration was reduced from 3.0–1.0 mM. Measurements were performed in the presence of EGTA (1.0 mM) or at saturating concentrations of Ca^{2+} (50 μM). Calcium-dependent glycogen synthase kinase was the activity in the presence of EGTA subtracted from the activity in the presence of Ca^{2+}. The assays were carried out for 2 min at 30°C in duplicate, and at a 1:30 final dilution of the extracts. Appropriate reaction blanks were included in which either extract or glycogen synthase were omitted. Reactions were initiated with ATP and terminated and analysed as in [18].

2.5. *Measurement of protein concentration*

The concentrations of glycogen synthase and phosphorylase *b* were determined by absorbance measurements at 280 nm, using values of 13.4 [11] and 13.1 [23], respectively, for 1% solutions. Protein concentrations of other solutions were carried out according to [24] using bovine serum albumin ($A^{1\%}_{280\,nm}$ = 6.5) as standard.

3. RESULTS

3.1. *Purification of the calmodulin dependent glycogen synthase kinase from rabbit skeletal muscle*

This enzyme cannot be measured in skeletal muscle extracts because of the presence of phosphorylase kinase. The latter enzyme is not retained by phosphocellulose at 200 mM NaCl and is therefore separated from the calmodulin-dependent glycogen synthase kinase, which is eluted at 450 mM NaCl. The calmodulin-dependent glycogen synthase kinase binds to calmodulin–Sepharose in the presence of Ca^{2+} and can be displaced with EGTA (fig.1). This separates it from contaminating glycogen synthase kinase-3 and glycogen synthase kinase-4 activity [17], traces of which are found in the flow through fractions (not shown). The residual phosphorylase kinase activity in the purified enzyme is very low or absent (fig.1). The specific activity of the preparation (700 U/

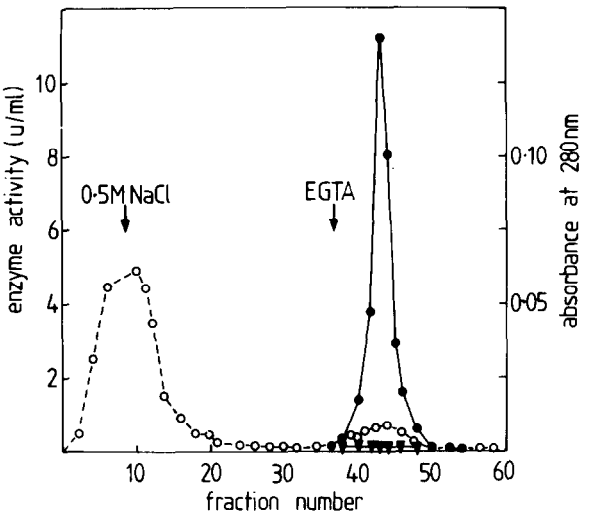

Fig.1. Affinity chromatography of the calmodulin-dependent glycogen synthase kinase on calmodulin–Sepharose. Enzyme from chromatography on phosphocellulose was loaded on to the column (6×2 cm) equilibrated in 40 mM Tris–HCl (pH 7.5, 0°C)–0.3 mM $CaCl_2$–3.0 mM $MgCl_2$–0.1 mM dithiothreitol–50 mM NaCl. At the point marked with an arrow, [NaCl] was increased to 500 mM. The enzyme was eluted by replacing 0.3 mM $CaCl_2$ with 2.0 mM EGTA. The flow rate was 75 ml/h and fractions of 3.2 ml were collected: (•—•) calmodulin-dependent glycogen synthase kinase; (▾—▾) phosphorylase kinase; (○---○) absorbance at 280 nm. Phosphorylase kinase was assayed at pH 7.0 as described for the calmodulin-dependent glycogen synthase kinase, except that phosphorylase *b* (0.5 mg/ml) replaced glycogen synthase (0.4 mg/ml).

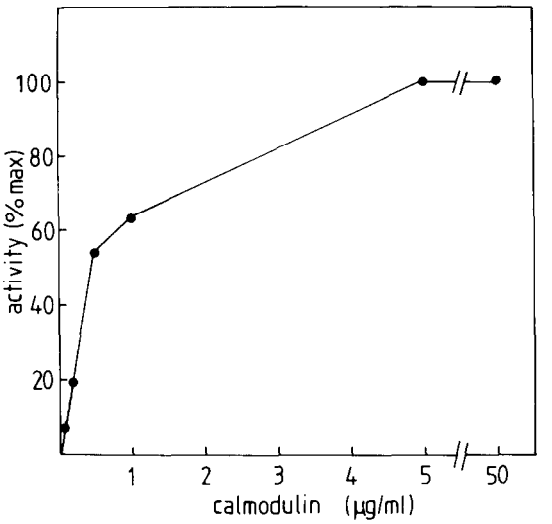

Fig.2. Regulation of the calmodulin-dependent glycogen synthase kinase by calmodulin. The assays were done as in section 2 except that the calmodulin concentration was varied.

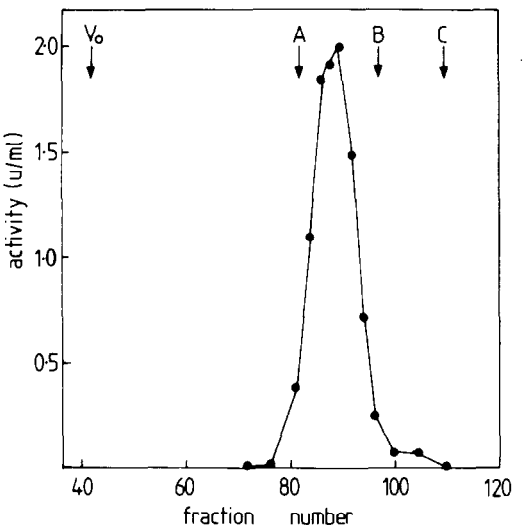

Fig.3. Gel filtration of the calmodulin-dependent glycogen synthase kinase on Sepharose 4B. The column (140 × 2.5 cm) was equilibrated in solution A + 500 mM NaCl. The sample (2.0 ml) was applied at a flow rate of 20 ml/h and fractions of 4.6 ml were collected. The arrows V_o, A, B and C denote the void volume and the positions at which the marker proteins (M_r) phosphorylase kinase (1 300 000), β-galactosidase (464 000) and phosphorylase *b* (195 000) were eluted.

mg), corresponds to a purification of 3000-fold, assuming that the specific activity in rabbit skeletal muscle extracts is comparable to that in extracts prepared from ICR/IAn mice that lack phosphorylase kinase activity (see below).

The presence of 50 mM NaCl in the homogenisation buffer was critical in order to obtain a high yield of calmodulin-dependent glycogen synthase kinase. If NaCl was omitted, the yield from phosphocellulose was 3–6-fold lower. The effect of NaCl appears to be specific for this enzyme, since the activities of phosphorylase, phosphorylase kinase and phosphorylase phosphatase were not increased by the inclusion of salt in the extraction buffer (not shown).

Table 1

Substrate specificity of the calmodulin-dependent glycogen synthase kinase (GSK) and myosin light-chain kinase (MLCK)

Substrate	[Protein]		Rel. act. (%)	
	mg/ml	μM	GSK	MLCK
Glycogen synthase	0.4	4.6	100	0.3
Casein	2.0	~100	4.5	0.4
Phosvitin	2.0	~50	<1	<0.1
Myosin P-light chain	0.1	5	1.0	100
Phosphorylase *b*	5.0	50	<0.1	<0.1
Mixed histones	2.0		<1	

3.2. *Properties of the calmodulin-dependent glycogen synthase kinase*

The purified enzyme was completely dependent on both Ca^{2+} and calmodulin for activity. Half-maximal activation was observed at 0.45 μg/ml (27 nM) calmodulin (fig.2). The activity was completely inhibited by the inclusion of trifluoperazine (50 μM) in the assays. The K_m-values for ATP and glycogen synthase were 40 μM and 3 μM (0.27 mg/ml), respectively. The K_a for Mg^{2+} was 0.8 mM, and maximal activity was observed above 2.0 mM Mg^{2+}.

The enzyme was eluted as a single symmetrical peak from Sepharose 4B and an apparent M_r of 850 000 ± 50 000 was determined from these experiments (fig.3).

The purified enzyme phosphorylated casein at a low rate, but had no activity toward the P-light chain of myosin, phosphorylase *b*, phosvitin or mixed histones (table 1).

The phosphorylation of glycogen synthase by the calmodulin-dependent glycogen synthase kinase was unaffected by the presence of a 25-fold molar excess of phosphorylase *b* (fig.4). In contrast, the phosphorylation of glycogen synthase by phosphorylase kinase was inhibited completely under these conditions (fig.4).

3.3. *Identification of serine-7 as the major residue phosphorylated by the calmodulin-dependent glycogen synthase kinase*

The phosphorylation of glycogen synthase reached different plateau values depending on the

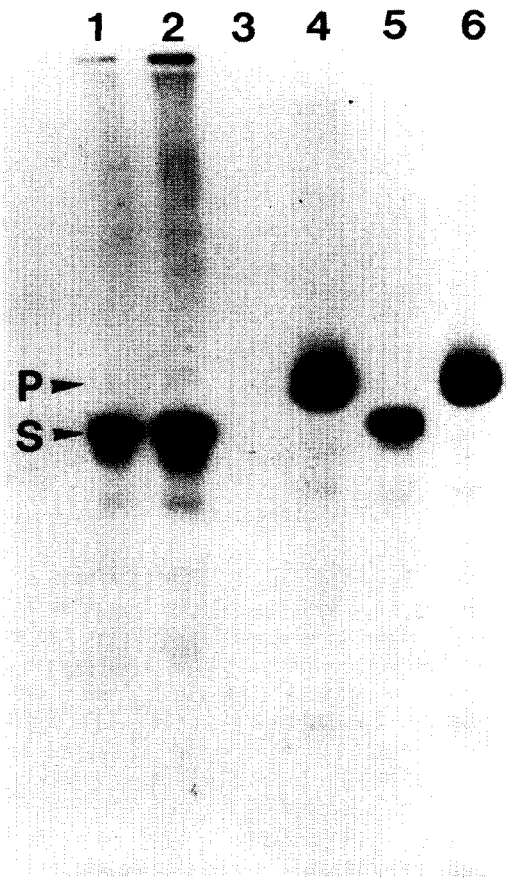

Fig.4. Phosphorylation of glycogen synthase *a* and phosphorylase *b* by the calmodulin-dependent glycogen synthase kinase and phosphorylase kinase. Phosphorylations were carried out at pH 7.0 as described for the assay of the calmodulin-dependent glycogen synthase kinase, except that the glycogen synthase *a* was 0.25 mg/ml and phosphorylase *b* 7.0 mg/ml. The concentration of the calmodulin-dependent glycogen synthase kinase was 1.0 U/ml, while that of phosphorylase kinase was 1.2 μg/ml, and phosphorylations were limited to <0.1 mol/mol subunit. The reactions were terminated by the addition of sodium dodecyl sulphate and electrophoresed on 7.5% polyacrylamide gels according to [25]. Channels 1 and 2 show phosphorylation of glycogen synthase *a* by the calmodulin-dependent glycogen synthase kinase and phosphorylase kinase, respectively, and channels 3 and 4 phosphorylation of phosphorylase *b* by these two enzymes. Channels 5 and 6 show phosphorylation of a glycogen synthase *a* + phosphorylase *b* mixture by the calmodulin-dependent glycogen synthase kinase and phosphorylase kinase, respectively. The arrows P and S denote the positions of glycogen phosphorylase (subunit M_r = 97 400) and glycogen synthase (subunit M_r = 86 000), respectively.

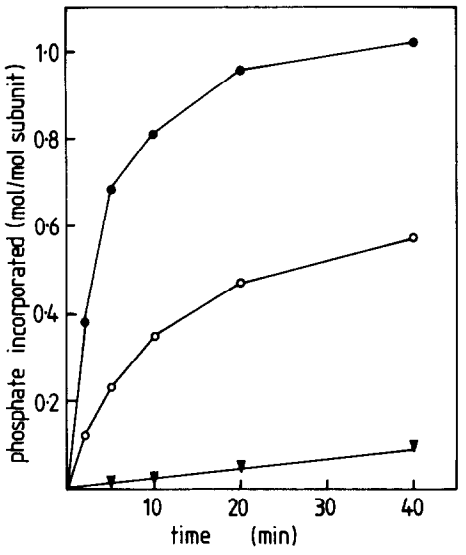

Fig.5. Phosphorylation of glycogen synthase by the calmodulin-dependent glycogen synthase kinase. Assays were carried out in the presence of Ca^{2+} and calmodulin as in section 2.3, at 9.0 U/ml (•—•), 1.8 U/ml (○—○), or in the absence (▾—▾) of added protein kinase.

amount of protein kinase added (fig.5). Phosphorylation approached 1.0 mol/mol subunit (M_r = 86 000) after incubation for 40 min with high concentrations of protein kinase (9 U/ml). Digestion with CNBr followed by SDS–polyacrylamide gel electrophoresis as in [26] showed that ~90% of the phosphate was located in peptide CB-1 (app. M_r = 9000) and the remainder in peptide CB-2 (app. M_r = 24 000) up to an incorporation of 0.7 mol/mol subunit. The distribution was ~80% in CB-1 and ~20% in CB-2 when the phosphorylation approached 1.0 mol/mol subunit (not shown).

Since the only known phosphorylation site in CB-1 is serine-7 [26] the tryptic peptide containing serine-7 was isolated by high-performance liquid chromatography as in [27]. This showed that the tryptic peptide containing serine-7 (eluted at 47% acetonitrile) was the major peptide phosphorylated by the calmodulin-dependent glycogen synthase kinase. The chymotryptic peptide containing serine-7 was then isolated as in [2]. In this peptide, serine-7 is the N-terminal residue. One cycle of the Edman degradation was therefore carried out, followed by electrophoresis on thin-layer cellulose at pH 3.5 [2]. This showed that all the ^{32}P-radioactivity had been removed from the peptide, appearing as P_i. The result established that serine-7 was the only residue in the peptide that was phosphorylated.

The effect of phosphorylation of serine-7 on the activity of glycogen synthase is discussed in [4,17].

3.4. *Assay of calcium-dependent glycogen synthase kinases in murine skeletal muscle extracts*

The calmodulin-dependent glycogen synthase kinase could be detected in muscle extracts prepared from ICR/IAn mice that lack phosphorylase kinase (< 0.2% normal activity). These studies confirmed that the specific activity of the enzyme in extracts prepared by homogenisation with 50 mM NaCl (0.24 ± 0.02 U/mg at pH 6.8) was several-fold higher than in extracts prepared by homogenisation in the absence of salt. Addition of 100 mM NaCl to the extraction buffer did not increase the specific activity further.

Calcium-dependent glycogen synthase kinase activity was measured at pH 8.2 in skeletal muscle extracts of ICR/IAn mice and a control strain (C3H/He-mg) with normal phosphorylase kinase activity. In extracts prepared by homogenisation in the absence of NaCl, the specific activity was ~4-fold lower in ICR/IAn mice (0.055 U/mg) than C3H/He-mg mice (0.23 U/mg).

The addition of exogenous calmodulin did not increase the calcium-dependent glycogen synthase kinase in skeletal muscle extracts. This is as expected, since the endogenous calmodulin in the extracts is sufficient for maximal activation [5].

4. DISCUSSION

These results demonstrate the existence in skeletal muscle of a calmodulin-dependent protein kinase, distinct from phosphorylase kinase (fig.1) and myosin light chain kinase (table 1). This enzyme preferentially phosphorylated the same residue on glycogen synthase (serine-7) as phosphorylase kinase, raising the question of the need for such an enzyme. A possible reason is suggested by the results presented in fig.4. This showed that physiological concentrations of phosphorylase *b* (7.0 mg/ml) suppressed the phosphorylation of physiological concentrations of glycogen synthase

(0.25 mg/ml) by phosphorylase kinase. In contrast, the phosphorylation of glycogen synthase by the calmodulin-dependent glycogen synthase kinase was unaffected by phosphorylase *b*. Therefore, following electrical excitation of muscle and elevation of the cytoplasmic concentration of Ca^{2+}, the calmodulin-dependent glycogen synthase kinase might initially be the most active enzyme towards serine-7, phosphorylase kinase only phosphorylating this site to an appreciable extent after the conversion of phosphorylase *b* to *a* has been accomplished. Phosphorylase *a* (7.0 mg/ml) inhibits the phosphorylation of glycogen synthase (0.25 mg/ml) by phosphorylase kinase by 60% (not shown).

We have recently separated 3 enzymes termed glycogen synthase kinase-3, glycogen synthase kinase-4 and glycogen synthase kinase-5 by chromatography on phosphocellulose, whose activities are unaffected by cyclic AMP or Ca^{2+}-calmodulin [17]. The calmodulin-dependent glycogen synthase kinase was not detected in this work for two reasons:

(i) The chromatography on phosphocellulose was preceded by a 33% ammonium sulphate precipitation which removes the enzyme (unpublished).

(ii) NaCl at 50 mM was not included in the homogenisation buffer, which decreases the activity extracted 3–6-fold.

In [4] we failed to find significant amounts of calcium-dependent glycogen synthase kinase activity in skeletal muscle extracts of ICR/IAn mice that lack phosphorylase kinase. These assays were carried out near the pH optimum for phosphorylase kinase (8.2) using extracts prepared by homogenisation in the absence of NaCl. The present work demonstrates that such activity *can* be detected under these conditions, although it is 4-fold lower than in extracts prepared from mice with normal levels of phosphorylase kinase. The calmodulin-dependent glycogen synthase kinase is, however, easily detectable in muscle extracts of ICR/IAn mice prepared by homogenisation in the presence of NaCl. It seems probable that the calmodulin-stimulated glycogen synthase kinase originally discovered in purified preparations of glycogen synthase [2,3] contained traces of the enzyme described here, in addition to phosphorylase kinase.

A calmodulin-dependent glycogen synthase kinase has been described in rabbit liver by two laboratories [28–30]. This enzyme has a similar K_m for ATP and K_a for Mg^{2+} to the skeletal muscle enzyme, and is also retained by phosphocellulose at 200 mM NaCl [30]. On the other hand, the apparent M_r of the liver enzyme determined by gel filtration is 500 000 [30] and the K_a for calmodulin is 80–100 nM [28–30]. These values differ significantly from those obtained in the present work with the skeletal muscle enzyme. The liver enzyme was reported by one laboratory to phosphorylate casein and phosvitin at a comparable rate to glycogen synthase [30], whereas another laboratory found that casein was not phosphorylated at a significant rate [28]. The latter report is consistent with the present work (table 1).

The liver enzyme was reported by one laboratory to phosphorylate CNBr peptides CB-1 and CB-2 of glycogen synthase at similar rates [30], whereas another group reported that the initial rate of phosphorylation of CB-1 was 3-fold faster [28]. This site specificity does not appear to correspond to that of the skeletal muscle enzyme, which initially phosphorylates CB-1 (containing serine-7) at least 10-fold more rapidly than CB-2. Further studies are clearly required to clarify the relationship between the skeletal muscle and liver calmodulin-dependent glycogen synthase kinases.

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REFERENCES

[1] Nimmo, H.G. and Cohen, P. (1974) FEBS Lett. 47, 162–167.

[2] Rylatt, D.B. and Cohen, P. (1979) FEBS Lett. 98, 71–75.

[3] Rylatt, D.B., Embi, N. and Cohen, P. (1979) FEBS Lett. 98, 76–80.

[4] Embi, N., Rylatt, D.B. and Cohen, P. (1979) Eur. J. Biochem. 100, 339–347.

[5] Shenolikar, S., Cohen, P.T.W., Cohen, P., Nairn, A.C. and Perry, S.V. (1979) Eur. J. Biochem. 100, 329–337.
[6] Soderling, T.R., Sheorain, V.S. and Erickson, L.H. (1979) FEBS Lett. 106, 181–184.
[7] Kish, V.M. and Kleinsmith, L.J. (1975) Methods Enzymol. 40, 198–208.
[8] Klee, C.B. and Krinks, M.H. (1978) Biochemistry 17, 120–126.
[9] Fischer, E.H. and Krebs, E.G. (1958) J. Biol. Chem. 231, 65–71.
[10] Cohen, P. (1973) Eur. J. Biochem. 34, 1–14.
[11] Nimmo, H.G., Proud, C.B. and Cohen, P. (1976) Eur. J. Biochem. 68, 21–30.
[12] Caudwell, F.B., Antoniw, J.F. and Cohen, P. (1978) Eur. J. Biochem. 86, 511–518.
[13] Blumenthal, D.K. and Stull, J.T. (1980) Biochemistry 19, 5608–5614.
[14] Ingebritsen, T.S. and Cohen, P. (1982) Eur. J. Biochem. in press.
[15] Nimmo, G.A. and Cohen, P. (1978) Eur. J. Biochem. 87, 341–351.
[16] Soderling, T.R., Srivastrava, A.K., Bass, M.A. and Khatra, B.S. (1979) Proc. Natl. Acad. Sci. (USA) 76, 2536–2540.
[17] Cohen, P., Yellowlees, D., Aitken, A., Donella-Deana, A., Hemmings, B.A. and Parker, P.J. (1982) Eur. J. Biochem. 124, 21–35.
[18] Guy, P.S., Cohen, P. and Hardie, D.G. (1981) Eur. J. Biochem. 114, 399–405.
[19] Embi, N., Rylatt, D.B. and Cohen, P. (1980) Eur. J. Biochem. 107, 519–527.
[20] Cohen, P.T.W., Burchell, A. and Cohen, P. (1976) Eur. J. Biochem. 66, 347–356.
[21] Cohen, P. (1982) Methods Enzymol. in press.
[22] Cohen, P. (1980) Eur. J. Biochem. 111, 563–574.
[23] Cohen, P., Duewer, T. and Fischer, E.H. (1971) Biochemistry 10, 2683–2694.
[24] Bradford, M. (1976) Anal. Biochem. 72, 248–254.
[25] Laemmli, U.K. (1970) Nature 227, 680–685.
[26] Picton, C., Aitken, A., Bilham, T. and Cohen, P. (1982) Eur. J. Biochem. 124, 37–45.
[27] Parker, P.J., Caudwell, F.B. and Cohen, P. (1982) Eur. J. Biochem. in press.
[28] Payne, M.E. and Soderling, T.R. (1980) J. Biol. Chem. 255, 8054–8056.
[29] Soderling, T.R. and Payne, M.E. (1981) Cold Spring Harbor Conf. Cell. Prolif. 8, 413–423.
[30] Ahmad, Z., DePaoli-Roach, A.A. and Roach, P.J. (1982) J. Biol. Chem 257, 8348–8355.