A multifunctional calmodulin-dependent protein kinase

Similarities between skeletal muscle glycogen synthase kinase and a brain synapsin I kinase

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Calmodulin-dependent glycogen synthase kinase isolated from skeletal muscle and synapsin I kinase II isolated from brain have several properties that are very similar. These properties include: substrate and site-specificities, immunological cross-reactivity, and phosphopeptide maps following limited proteolysis. Both enzymes phosphorylate a wide variety of substrate proteins. The two kinases may represent different isozymes of a multifunctional calmodulin-dependent protein kinase that mediates many of the actions of Ca^{2+} in various tissues. Therefore, we propose the name 'calmodulin-dependent multi-protein kinase' for this broad specificity enzyme.

Ca^{2+} *Calmodulin* *Protein phosphorylation*

1. INTRODUCTION

Most, if not all, of the actions of extracellular signals that increase the intracellular concentration of cyclic AMP are mediated by cyclic AMP-dependent protein kinase [1–3]. Many of the actions of extracellular signals that elevate the cytoplasmic concentration of calcium (Ca^{2+}) ions are mediated by calmodulin or structurally related proteins [3,4]. The Ca^{2+}–calmodulin complex is capable of activating many classes of enzyme including protein kinases. In contrast to the situation with cyclic AMP, where a single protein kinase catalytic subunit phosphorylates multiple substrates, several Ca^{2+}–calmodulin-dependent protein kinases, have been found. In the case of two of these, myosin light chain kinase [5,6] and phosphorylase kinase [7], a narrow substrate specificity has been demonstrated. However, the existence of a Ca^{2+}–calmodulin-dependent protein kinase of much broader specificity remains an interesting possibility.

We have here compared a Ca^{2+}–calmodulin-dependent protein kinase isolated from skeletal muscle that phosphorylates glycogen synthase [8,9] with a Ca^{2+}–calmodulin-dependent protein kinase isolated from brain that phosphorylates synapsin I [10–12]. The two enzymes demonstrate broad and remarkably similar substrate specificities and share a number of other properties.

2. MATERIALS AND METHODS

Calmodulin-dependent glycogen synthase kinase was purified from rabbit skeletal muscle up to and including affinity chromatography on calmodulin–Sepharose [9]. Synapsin I kinase II (also referred to as '30 K region' synapsin I kinase [11] and as calmodulin-dependent protein kinase II [4]) was purified from rat brain by a modification [12,13] of the method in [11]. Calmodulin was purified from sheep brain [14] and glycogen synthase from rabbit skeletal muscle [15,16]. Synapsin I was purified from bovine brain by a modification

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of the method in [17]. Microtubule-associated protein-2 (purified through 3 cycles of assembly–disassembly) was a gift from Dr Joel Rosenbaum. The sources of all other substrate proteins and the procedures for phosphorylating substrate proteins are given in [8,9]. Similar results were obtained when protein phosphorylation was carried out as in [11]. Linear rate conditions were met in both procedures for all substrate proteins. Transfer of proteins from dodecyl sulphate polyacrylamide gels to nitrocellulose and subsequent immunoblotting were performed as in [18]. Phosphopeptide mapping following limited proteolysis was carried out as in [19] using *S. aureus* V8 protease (Miles Lab).

3. RESULTS

The substrate specificities of calmodulin-dependent glycogen synthase kinase and synapsin I kinase II are compared in table 1. The specificities of the two enzymes were virtually identical. Synapsin I proved to be an excellent substrate for calmodulin-dependent glycogen synthase kinase from skeletal muscle and, conversely, glycogen synthase proved to be a good substrate for synapsin I kinase II from brain. Furthermore, the apparent K_m for synapsin I was 0.44 μM for glycogen synthase kinase, in agreement with that reported previously (0.4 μM) for synapsin I kinase II [11]. Similarly, the apparent K_m for glycogen synthase was identical (3.0 μM) for both enzymes.

It was previously reported that calmodulin-dependent glycogen synthase kinase did not phosphorylate mixed histones (2 mg/ml) [8]. It is now known that this observation was due to inhibition of the enzyme at high histone concentrations, caused by the binding of these basic proteins to the acidic calmodulin molecule. If the histone concentration is reduced to 0.2–0.3 mg/ml, significant rates of phosphorylation are observed (table 1).

The substrates glycogen synthase and synapsin I both contain multiple phosphorylation sites [20–22]. Analysis of the sites phosphorylated by glycogen synthase kinase and by synapsin I kinase II demonstrated similar site specificities for the two enzymes. Calmodulin-dependent glycogen synthase kinase phosphorylates two serine residues on glycogen synthase, the rapid phosphorylation of site-2 being followed by a slower phosphorylation of site-1b. At moderate levels of phosphate incorporation (<0.7 mol/mol subunit), about 90% of the ^{32}P-radioactivity is incorporated into site-2 [9]. When glycogen synthase was phosphorylated to 0.5 mol/mol subunit using synapsin I kinase II, digestion with cyanogen bromide followed by dodecyl sulphate polyacrylamide gel electrophoresis [20] showed that 90% of the radioactivity was located in peptide CB-1. The only known phosphorylation site in CB-1 is site-2 [20]. To confirm that site-2 was phosphorylated by synapsin I kinase II, the tryptic peptide containing this site was isolated by high-performance liquid chromatography (HPLC) [23,24]. The results showed that the tryptic peptide containing site-2 (eluting at 47% acetonitrile) accounted for almost

Table 1

Comparison of the substrate specificities of calmodulin-dependent glycogen synthase kinase from skeletal muscle (GSK) and calmodulin-dependent synapsin I kinase II (SK) from brain

Substrate	Concentration		Relative rates of phosphorylation (%)	
	mg/ml	μM	GSK	SK
Synapsin-I	0.1	1.2	710	760
Myosin P-light chain (smooth muscle)	0.8	40	115	125
Glycogen synthase	0.4	4.6	100[a]	100
Histone H1	0.2	10	27	23
Microtubule-associated protein-2	0.8[b]		17	17
Acetyl-CoA carboxylase	0.3	1.3	8.0	7.8
ATP-citrate lyase	0.4	3.5	4.0	5.0
Myosin P-light chain (skeletal muscle)	0.2	10	1.0	1.0
Phosphorylase *b*	1.5	1.5	<0.1	<0.1
Phosvitin	2.0	50	<0.1	<0.1

[a] Some preparations of glycogen synthase gave somewhat (up to 1.9-fold) higher rates of phosphorylation than the standard preparation used in this series of experiments

[b] Concentration refers to concentration of 3-times assembly–disassembly purified microtubule protein

Phosphorylation of each protein was carried out as in [8,9]

all of the ^{32}P-radioactivity eluting from the column. When the peptide was subjected to Edman degradation on a Beckman 890C sequencer, a burst of ^{32}P-radioactivity was observed after the third cycle, as expected if the phosphate was located at site-2. These experiments demonstrate that synapsin I kinase II phosphorylates the same serine residue as calmodulin-dependent glycogen synthase kinase, rather than one of the other 6 known phosphorylation sites on glycogen synthase [20].

In the case of synapsin I, synapsin I kinase II phosphorylates one or two serine residues, depending on the species from which the substrate was purified ([11,22], unpublished). These serines are contained in an M_r 30000 peptide recovered after digestion of synapsin I with *S. aureus* V8 protease [10,22]. A distinct calmodulin-dependent synapsin I kinase (calmodulin-dependent synapsin I kinase I) from brain [10] phosphorylates a different serine residue contained in an M_r 10000 proteolytic peptide. Calmodulin-dependent glycogen synthase kinase from skeletal muscle was found to phosphorylate only the M_r 30000 peptide fragment, again indicating site specificity similar to that of synapsin I kinase II.

Calmodulin-dependent glycogen synthase kinase and synapsin I kinase II were compared with respect to several other properties. In the dodecyl sulphate polyacrylamide gel system used in our experiments, calmodulin-dependent glycogen synthase kinase exhibited a major protein-staining doublet (M_r 58000/59000) and a minor protein-staining band (M_r 54000) (not shown). Both polypeptides become phosphorylated to 5 mol phosphate/mol subunit when the enzyme is incubated with Mg^{2+}-ATP and Ca^{2+} plus calmodulin [9]. Synapsin I kinase II also exhibits a major protein-staining band (M_r 50000) and a minor protein-staining band (actually a doublet of M_r 58000/60000) on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE). These polypeptides also become phosphorylated when the enzyme is incubated with Mg^{2+}-ATP and Ca^{2+} plus calmodulin. However, the initial rate of phosphorylation per mol subunit is greater for the minor M_r 58000/60000 doublet than for the major M_r 50000 band [11,13].

The polypeptides of the two kinases were examined for possible immunological similarity. For this purpose a monoclonal antibody (C42.1), which had been prepared against synapsin I kinase II, and had previously been shown [13,18,25] to cross-react with the M_r 50000 and M_r 58000/60000 bands of synapsin I kinase II, was used. A Western immunoblot (fig.1) showed that the antibody cross-reacted with both the M_r 58000/59000 and the M_r 54000 components of glycogen synthase kinase.

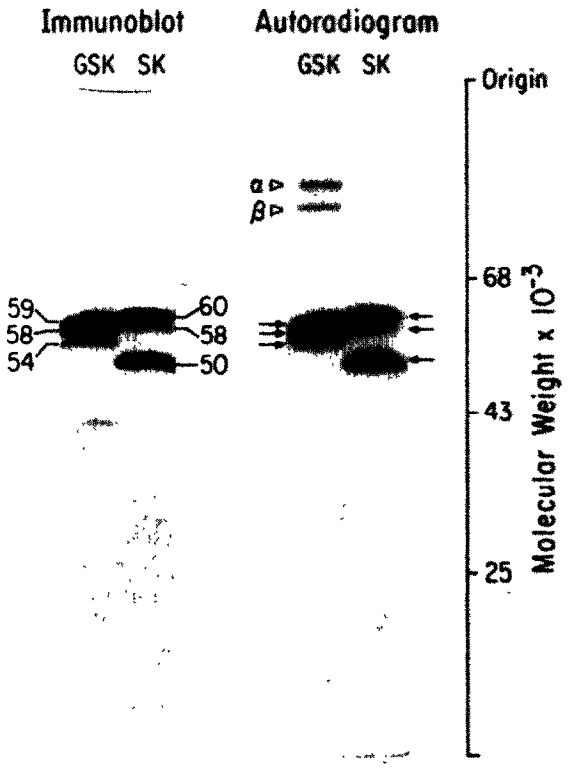

Fig.1. Immunoblot and autoradiogram of glycogen synthase kinase (GSK) and synapsin I kinase II (SK). GSK (6 μg) and SK (2.5 μg) were incubated with [γ-^{32}P]ATP (50 μM), Ca^{2+} (300 μM) and calmodulin (0.03 mg/ml) for 15 s as described in [11]. The proteins were subjected to SDS–PAGE and transferred to nitrocellulose. Immunoblotting was performed using monoclonal antibody C42.1. Arrows point to the M_r 59000, M_r 58000 and M_r 54000 bands of GSK and to the M_r 60000, M_r 58000 and M_r 50000 bands of SK; α and β refer to subunits of phosphorylase kinase, which contaminate the GSK purified through calmodulin–Sepharose [9]. Approximate M_r-values are indicated in the margin.

The possible similarity between the two protein

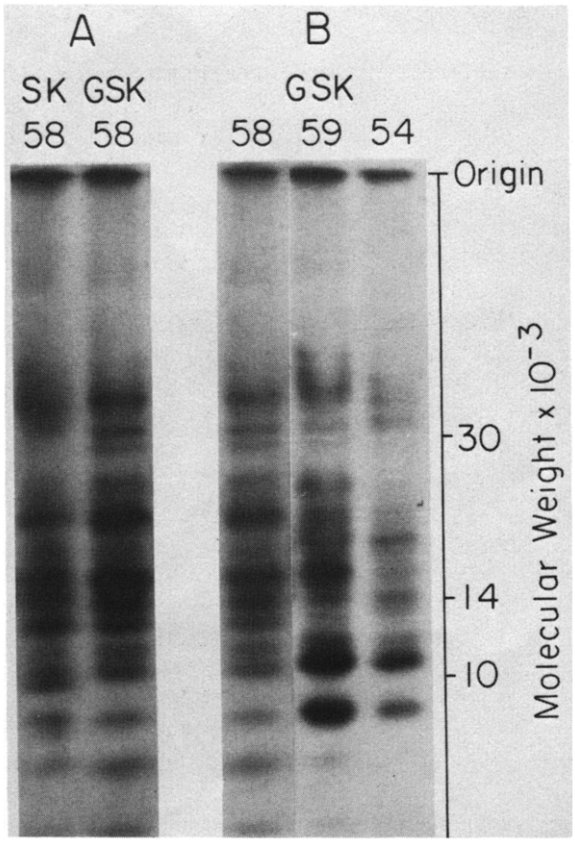

Fig.2. One-dimensional phosphopeptide maps of: (A) the M_r 58000 polypeptide from glycogen synthase kinase (GSK,58) and from synapsin I kinase II (SK,58); (B) polypeptides from GSK of M_r 58000 (58), M_r 59000 (59) and M_r 54000 (54). GSK (0.5 μg) and SK (3.8 μg) were incubated with [γ-^{32}P]ATP (2 μM), Ca^{2+} (300 μM) and calmodulin (0.03 mg/ml) for 15 min as in [11]. The phosphorylated bands were located by autoradiography, cut from the dried gel, and then subjected to limited proteolysis with *S. aureus* V8 protease (5 μg) in a second SDS polyacrylamide gel, followed by autoradiography. Approximate M_r-values are indicated in the margin.

kinases was further examined in experiments involving phosphopeptide mapping of the components of the two enzymes using *S. aureus* V8 protease. Fig.2A shows that the phosphopeptide pattern generated from the glycogen synthase kinase M_r 58000 band was almost identical to that generated from the synapsin I kinase II M_r 58000 band. In comparing the phosphopeptide maps of the two kinases, it was noted that the glycogen synthase kinase M_r 59000 and M_r 54000 polypeptides exhibited several phosphopeptides in common with the glycogen synthase kinase M_r 58000 polypeptide (fig.2B).

4. DISCUSSION

We here demonstrate that calmodulin-dependent glycogen synthase kinase from skeletal muscle and synapsin I kinase II from brain share remarkably similar substrate and site specificities. Moreover, the two enzymes exhibited immunological cross-reactivity. In addition, the M_r 58000/59000 polypeptide doublet of calmodulin-dependent glycogen synthase kinase and the M_r 58000/60000 polypeptide doublet of synapsin I kinase II were found to have similar phosphopeptide maps.

Although there are several marked similarities between the two enzymes, certain differences do exist that require further investigation. First, in the case of calmodulin-dependent glycogen synthase kinase, the major protein-staining band is the higher M_r polypeptide doublet, whereas in the case of synapsin I kinase II, the major protein-staining band is the lower M_r polypeptide. Secondly, when the enzymes are incubated with Mg^{2+}-ATP and Ca^{2+} plus calmodulin, the polypeptides of glycogen synthase kinase incorporate phosphate at equivalent rates per mol subunit, whereas the higher M_r polypeptide of synapsin I kinase II incorporates phosphate at a faster rate per mol subunit than does the lower M_r polypeptide. The significance of these differences is not clear at this time.

The relationship of the individual polypeptides present in each kinase preparation also remains to be clarified. The M_r 50000 and the M_r 58000/60000 polypeptides of synapsin I kinase II co-elute with enzyme activity during a wide variety of purification procedures and these polypeptides appear to bind calmodulin and ATP [11–13]. Phosphopeptide mapping studies raise the possibility that the M_r 58000 component may be a proteolytic degradation product of the M_r 60000 polypeptide, but have failed to provide evidence that the M_r 50000 polypeptide is derived from either of the higher M_r components [11]. It is not known whether the individual polypeptides of synapsin I kinase II are subunits of a single enzyme

or constitute different isozymes. Phosphopeptide mapping of the glycogen synthase kinase polypeptides revealed several phosphopeptides common to the M_r 58000/59000 doublet and M_r 54000 polypeptide, suggesting that these polypeptides may be related.

The results presented here, indicating similar properties and broad substrate specificities of calmodulin-dependent glycogen synthase kinase and synapsin I kinase II, suggest that the two enzymes may be isozymes of a multifunctional Ca^{2+}–calmodulin-dependent protein kinase. In support of this interpretation, several other Ca^{2+}–calmodulin-dependent protein kinases have recently been purified from various tissues that have properties in common with these two enzymes. In [26] and [27] a calmodulin-dependent protein kinase was purified from liver that phosphorylates glycogen synthase and smooth muscle myosin P-light chain. In [28] a protein kinase was purified from brain using smooth muscle myosin P-light chain as substrate. This enzyme also phosphorylates myelin basic protein, casein, arginine-rich histones, microtubule protein, and synaptosomal membrane proteins. Recently, glycogen synthase was also shown to be phosphorylated by this kinase, the major site being cyanogen bromide peptide CB-1 containing site-2 [29]. In [30] a calmodulin-dependent kinase from brain was partially purified using a microtubule preparation as substrate. This enzyme was reported to phosphorylate tubulin, microtubule-associated protein-2 and smooth muscle myosin P-light chain [30,31]. In [32] and [33] a protein kinase from brain was purified that phosphorylates smooth muscle myosin P-light chain, microtubule protein, casein, and tyrosine- and tryptophan-5-monooxygenases. It was also reported [32] that phosphorylation of tyrosine and tryptophan-5-monooxygenases allows these enzymes to be stimulated by an 'activator protein', thereby enhancing the synthesis of the neurotransmitters dopamine, noradrenaline, and serotonin. In [34] the partial purification of a protein kinase from brain that phosphorylates the spectrin-like protein termed fodrin or calspectin was reported. In [35] a protein kinase was partially purified from the electric organ of *Torpedo californica* which preferentially phosphorylated synapsin-I at the same site as did calmodulin-dependent glycogen synthase kinase–synapsin-I kinase II.

Most of these enzymes appear to exist as high-M_r complexes of 500000–800000 by gel filtration and to contain autophosphorylatable subunits of M_r 50000–60000. It will be of considerable interest to establish whether these Ca^{2+}–calmodulin-dependent protein kinases are closely related to calmodulin-dependent glycogen synthase kinase and synapsin I kinase II. The demonstration of such a relationship would strengthen the interpretation, based on our results, that many of the actions of Ca^{2+} are mediated by a widely distributed, multifunctional calmodulin-dependent protein kinase.

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