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Multisite phosphorylation of the glycogen-binding subunit of protein phosphatase-1_G by cyclic AMP-dependent protein kinase and glycogen synthase kinase-3

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The glycogen-binding (G) subunit of protein phosphatase-1_G is phosphorylated stoichiometrically by glycogen synthase kinase-3 (GSK3), and with a greater catalytic efficiency than glycogen synthase, but only after prior phosphorylation by cyclic AMP-dependent protein kinase (A-kinase) at site 1. The residues phosphorylated are the first two serines in the sequence AIFKPGFSPQPSRRGS-, while the C-terminal serine (site 1) is one of the two residues phosphorylated by A-kinase. These findings demonstrate that (i) the G subunit undergoes multisite phosphorylation in vitro; (ii) phosphorylation by GSK3 requires the presence of a C-terminal phosphoserine residue; (iii) GSK3 can synergise with protein kinases other than casein kinase-2.

Protein kinase; Protein phosphatase; cyclic AMP; Glycogen metabolism; Glycogen synthase

1. INTRODUCTION

Insulin stimulates glycogen synthesis within minutes in mammalian skeletal muscle by promoting the dephosphorylation and activation of glycogen synthase, the rate limiting enzyme in this process. Phosphate is released relatively specifically from a region containing several serine residues (the 'sites 3 region') which are phosphorylated by glycogen synthase kinase-3 (GSK3) [1]. Interaction of insulin with its receptor should therefore lead to inhibition of GSK3 and/or to activation of the phosphatase(s) acting on the sites 3 region.

Protein phosphatase-1 (PP-1) and protein phosphatase-2A (PP-2A) account for all the phosphatase activity in mammalian skeletal muscle extracts towards the sites 3 region of glycogen synthase [2,3]. Two lines of evidence suggest that PP-1 is the enzyme that dephosphorylates these serines in vivo. Firstly, it accounts for 70% of the glycogen synthase phosphatase activity when muscle extracts are assayed under standard conditions [3]. Secondly, like glycogen synthase, PP-1 is specifically associated with glycogen-protein particles, whereas PP-2A is not [3,4].

The glycogen-associated form of PP-1 (PP-1_G) is a heterodimer composed of the phosphatase catalytic (C) subunit and a glycogen-binding (G) subunit [5]. The G subunit is phosphorylated in vitro by cyclic AMP-dependent protein kinase (A-kinase) at two serines (site 1 and site 2) ([6], fig.1), which are 19 residues apart in the primary structure [7]. Site 1 is phosphorylated in vivo and phosphorylation increases from 0.57 to 0.83 mol/mol in response to adrenalin [8]. The in vivo phosphorylation state of site 2 is unknown.

Phosphorylation of the G subunit by A-kinase in vitro [7], or injection of adrenalin in vivo [8,9], promotes dissociation of the C subunit and its translocation from the glycogen-protein particles to the cytosol. Released C subunit is much less effective than glycogen-bound PP-1_G in dephosphorylating glycogen synthase at physiological ionic strength [7]. Furthermore, released C subunit

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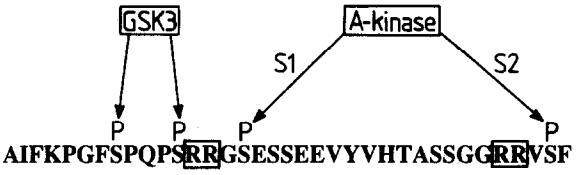

Fig.1. Phosphorylation sites on the G subunit of PP-1_G. Tryptic peptide T1 comprises residues 1–12 and chymotryptic peptide C1/C2 residues 4–23. The sequences are taken from the present study and [7,16]. The figure indicates the residues phosphorylated by GSK3, the serines, site 1 (S1) and site 2 (S2), phosphorylated by A-kinase, and the pairs of adjacent arginine residues that determine the substrate specificity of A-kinase.

is likely to be inactivated by inhibitor-1, a cytosolic protein which after phosphorylation by A-kinase becomes a potent inhibitor of PP-1 (review [4]). Inactivation of PP-1 by these two mechanisms may account for the increased phosphorylation of the sites 3 region and decreased activity of glycogen synthase that occurs in response to adrenalin [10].

Phosphorylation of the sites 3 region of glycogen synthase by GSK3 and its dephosphorylation by PP-1_G, raised the question of whether PP-1_G might itself be phosphorylated by GSK3. In this paper, we report that the G subunit of PP-1_G is an even more effective substrate for GSK3 than glycogen synthase, but only if the G subunit is first phosphorylated by A-kinase.

2. MATERIALS AND METHODS

2.1. *Protein preparations*

A-kinase purified from bovine cardiac muscle [11], and GSK3 [12] and the glycogen synthase-glycogenin complex [13,14] from rabbit skeletal muscle were provided by Dr Carl Smythe and Miss Sara Nakielny in this laboratory. PP-1_G, comprising the C subunit of PP1 complexed to the 103 kDa G′ fragment of the G subunit, was purified from rabbit skeletal muscle as in [6] with some minor modifications [7]. A peptide comprising residues 5–22 of the specific protein inhibitor of A-kinase (PKI) [15] was a gift from Dr Bruce Kemp, St Vincent's Institute of Medical Research, Melbourne, Australia. Trypsin (treated with tosylphenylchloromethyl ketone) and chymotrypsin (3 times crystallised) were Worthington products, purchased from Lorne Laboratories (Reading, England).

2.2. *Phosphorylation of PP-1_G and glycogen synthase*

Incubations were carried out at 30°C, and unless indicated otherwise contained 50 mM Tris-HCl (pH 7.0), 0.1 mM EGTA, 0.1% (by vol.) 2-mercaptoethanol, 25 mM NaF, PP-1_G (0.18 mg/ml, 1.3 μM) or glycogen synthase (0.36 mg/ml, 2.9 μM), A-kinase (10 mU/ml) and/or GSK3 (0.75 mU/ml), 2 mM magnesium acetate and 0.1 mM [γ-^{32}P]ATP (10^6 cpm/nmol). Reactions were initiated with MgATP and stopped by addition of either trichloroacetic acid (TCA) to 5%, or (for phosphopeptide isolation) by addition of 0.1 vol. of a solution containing 100 mM EDTA, 500 mM NaF and 100 mM sodium pyrophosphate, pH 7.0. One unit of GSK3 or A-kinase is that amount which catalyses the phosphorylation of 1 μmol of glycogen synthase per min in the standard asssay.

2.3. *Generation of phosphopeptides*

Native ^{32}P-labelled PP-1_G (2.0 ml, 2.6 nmol) was freed from [γ-^{32}P]ATP by centrifugal ultrafiltration (Centricon 30 from Amicon) [7] and incubated for 20 min (trypsin) or 10 min (chymotrypsin) under conditions that release site 1 peptides as TCA-soluble species [16]. Phosphopeptides were further purified and analysed as described in section 3.

2.4. *Other analytical procedures*

Protein concentrations were determined by colorimetric assay [17] using bovine serum albumin as standard. In calculating phosphorylation stoichiometries, the molecular mass of PP-1_G was taken as 140 kDa [5] and that of the glycogen synthase-glycogenin complex as 124 kDa [14]. Reverse phase chromatography of peptides was carried out using a Vydac 218TP54 C_{18} column (Phase Separations Group, Hesperia, CA, USA) and amino acid sequencing on an Applied Biosystems 470A gas-phase sequencer equipped with an on-line reverse-phase chromatography system for identification of phenylthiohydantoin amino acids.

3. RESULTS

3.1. *Phosphorylation of PP-1_G by A-kinase and GSK3*

Purified preparations of PP-1_G were phosphorylated rapidly to a level approaching 2 mol/mol by A-kinase, but not by GSK3 (fig.2), as reported previously [5,6]. However, following phosphorylation by A-kinase, PP-1_G became an excellent substrate for GSK3, phosphorylation reaching a plateau within 5 min, and at a stoichiometry exceeding 1.0 mol/mol PP-1_G (fig.2). SDS/polyacrylamide gel electrophoresis and autoradiography showed that all the ^{32}P-label was incorporated into the G subunit (not shown).

3.2. *Identification of the serine residues phosphorylated by GSK3*

PP-1_G was phosphorylated by A-kinase using unlabelled ATP, and then with GSK3 using [γ-^{32}P]ATP. Following digestion of native ^{32}P-labelled PP-1_G with chymotrypsin, 90% of the ^{32}P-radioactivity was released as TCA-soluble peptides within 10 min. When this material was subjected to gel filtration on Sephadex G50 Superfine (120 ×

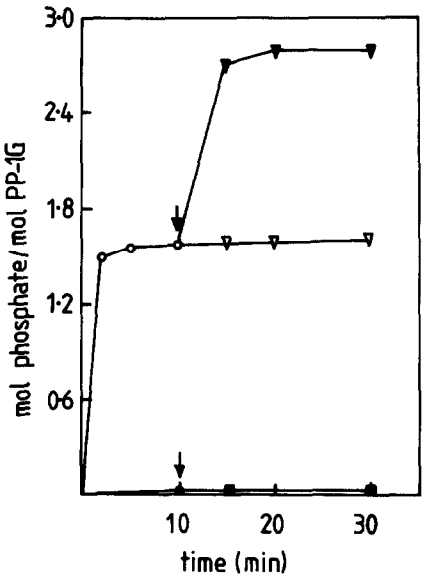

Fig.2. Phosphorylation of PP-1_G by A-kinase and GSK3. PP-1_G (1.3 μM) was phosphorylated for 10 min under the standard conditions in a 0.1 ml incubation containing either 10 mU/ml A-kinase (○—○) or 0.75 mU/ml GSK3 (●—●). At the point denoted by arrows, 2.5 μl of PKI (0.1 mM) was added to both incubations to inhibit the A-kinase. The incubation containing A-kinase was divided in two, and one half supplemented with 2.5 μl of 15 mU/ml GSK3 (▼—▼), while the other received the same volume of buffer (▽—▽). Conversely, the original incubation which contained GSK3 was supplemented after 10 min with 5 μl of 200 mU/ml A-kinase (■—■). Aliquots were removed at the times indicated and analysed for incorporation of ^{32}P-radioactivity into protein [27].

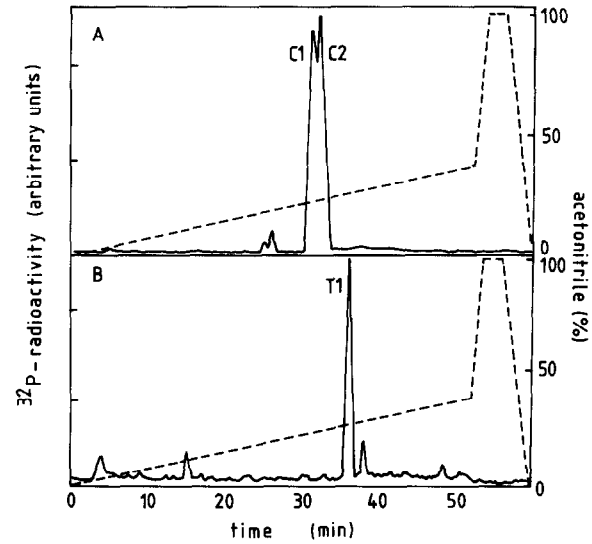

Fig.3. Purification of chymotryptic (A) and tryptic (B) phosphopeptides containing the residues phosphorylated by GSK3. Reverse phase chromatography was carried out on a Vydac C_{18} column using a Gilson HPLC system equipped with an on-line radioactivity monitor. The column was equilibrated in 0.1% TFA and developed at 1.0 ml/min with an acetonitrile gradient (0–40%) increasing at 0.6% per min. Fractions of 0.5 ml were collected. ^{32}P-radioactivity is shown by the solid line and the acetonitrile gradient by the broken line. The major chymotryptic (C1 and C2) and tryptic (T1) phosphopeptides are marked.

1.2 cm) equilibrated in 0.1 M NH_4HCO_3, 82% of the applied radioactivity was recovered in a single peak that eluted at the same position (V_e/V_0 = 1.8) as the chymotryptic phosphopeptide containing site 1 [16]. This material was dried, dissolved in 0.5% (by vol.) trifluoroacetic acid (TFA), and chromatographed on a C_{18} reverse phase column equilibrated in 0.1% TFA and developed with an acetonitrile gradient. Of the applied ^{32}P-radioactivity, 72% was recovered in two peaks, C1 and C2, that were incompletely resolved (fig.3A) and which eluted at the same position as the chymotryptic peptide containing site 1 [16]. The amino acid compositions of C1 and C2 (table 1) were very similar to that of the site 1 chymotryptic peptide. Both had the N-terminal sequence KPGFSPQPSRR-, confirming their identity as forms of the site 1 chymotryptic peptide (see fig.1).

In peptide C2, ^{32}P-radioactivity was stoichiometric with amino acids present as single residues, while ^{32}P-radioactivity in peptide C1 was present at a stoichiometry near 2.0 (table 1). This indicated that C1 and C2 were the same peptide phosphorylated by GSK3 to 2.0 and 1.0 mol/mol, respectively. In this case, C1 should be a triphosphorylated and C2 a diphosphorylated derivative, since site 1 had already been phosphorylated by A-kinase using unlabelled ATP. Consistent with this view, peptide C1 migrated more rapidly to the anode than C2 in isoelectric focussing experiments (fig.4), while C2 migrated more rapidly than the ^{32}P-labelled monophosphorylated chymotryptic peptide phosphorylated by A-kinase alone (termed peptide C3 in fig.4).

The C1 phosphopeptide contains four serines in addition to site 1. In order to identify which two serines were phosphorylated by GSK3, PP-1_G phosphorylated by A-kinase using unlabelled ATP and then GSK3 using [γ-^{32}P]ATP, was subjected

Table 1

Amino acid compositions of peptides C1, C2 and T1

Amino acid	C1 (mol/mol)	C2 (mol/mol)	T1 (mol/mol)
Asx	0.7	1.0	–
Glx	4.8 (4)	4.6 (4)	1.0 (1)
Ser	5.0 (5)	4.1 (5)	2.0 (2)
Gly	2.6 (2)	2.4 (2)	1.1 (1)
Arg	2.0 (2)	2.0 (2)	1.1 (1)
Thr	0.6	0.7	–
Ala	0.7	1.1	1.0 (1)
Pro	3.5 (3)	2.6 (3)	3.4 (3)
Tyr	1.4 (1)	1.0 (1)	–
Val	1.1 (1)	0.8 (1)	–
Ile	–	–	0.8 (1)
Leu	0.6	0.6	–
Phe	0.9 (1)	0.6 (1)	1.9 (2)
Lys	1.3 (1)	1.4 (1)	1.0 (1)
^{32}P phosphate	1.82 (2)	0.96 (1)	2.06 (2)

Peptide C1 was the 30.5 min fraction and peptide C2 the 31.5 min fraction from fig.3. Samples were hydrolysed for 1 h at 150°C in 6 N HCl containing 2 mM phenol, dried, converted to phenylthiocarbamyl derivatives and analysed on a Waters PICOTAG system. Values for serine, threonine and tyrosine were corrected for 17%, 13% and 16% destruction, respectively, and values below 0.4 mol are omitted. Numbers in parentheses indicate residues determined by sequence analysis [16]. Peptide C1/C2 comprises residues 4–23 of the sequence shown in fig.1

to brief tryptic digestion (see section 2). 90% of the ^{32}P-radioactivity was released as TCA-soluble peptides and subsequent reverse phase HPLC revealed a major ^{32}P-peptide, T1, which eluted at 25% acetonitrile (fig.3B). T1 was further purified by rechromatography on the C_{18} column equilibrated in 10 mM ammonium acetate (pH 6.5), and its composition is shown in table 1. Sequence analysis showed the following primary structure: AIFKPGFSPQPS(R). This corresponds to the first 10 residues of peptide C1/C2 preceded by three additional residues at the N-terminus. ^{32}P-radioactivity was present at a stoichiometry of 2.1, indicating that T1 was a diphosphorylated derivative. Since peptide T1 only contains two of the five serines present in peptide C1/C2, this establishes that the first two serines in C1/C2 are the residues phosphorylated by GSK3. The absence of significant amounts of a monophosphorylated tryptic peptide is explained by more efficient phosphorylation in this particular preparation.

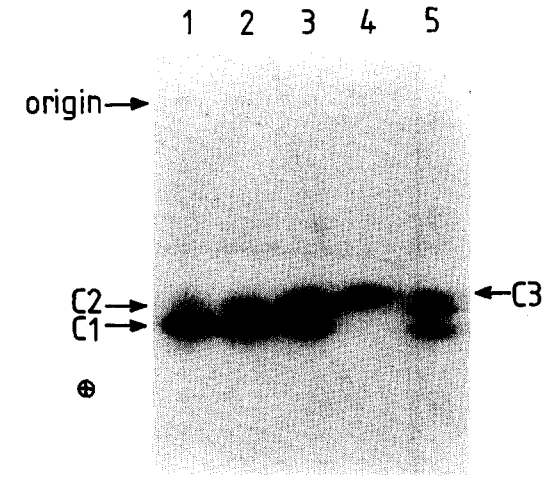

Fig.4. Isoelectric focussing of chymotryptic phosphopeptides. Isoelectric focussing [28] of the ^{32}P-labelled peptides shown in fig.3 was carried out using a Bio-Rad Model 111 Mini IEF Cell followed by autoradiography. Lane 1, 30.5 min fraction from fig.3; lane 2, 31 min fraction; lane 3, 31.5 min fraction; lane 4, monophosphorylated site 1 chymotryptic peptide C3 obtained by ^{32}P-labelling with A-kinase alone [16]; lane 5, mixture of peptides C1 + C2 + C3.

The complete amino acid sequence in this region of the G subunit is shown in fig.1.

3.3. *Further analysis of the phosphorylation of PP-1$_G$ by GSK3*

Kinetic analyses showed that after phosphorylation by A-kinase, PP-1$_G$ and glycogen synthase were phosphorylated by GSK3 with apparent K_m values of 0.3–0.5 μM and 2–3 μM, respectively, which are similar to the intracellular concentrations of each enzyme [4,5,18]. The V_{max} values for both substrates were similar.

Further experiments were carried out to determine whether the phosphorylation of site 1, site 2, or both sites, by A-kinase was required to promote phosphorylation of PP-1$_G$ by GSK3. In these experiments PP-1$_G$ was maximally phosphorylated at site 1 and site 2, and site 2 was then dephosphorylated selectively by using protein phosphatase-2A as in [7]. Over 98% of the ^{32}P-radioactivity was released from site 2 at a time when only 50% of the ^{32}P-radioactivity had been released from site 1. Subsequent incubation with GSK3 yielded phosphorylation to 50% of the control level. These experiments indicate that the extent of phosphorylation by GSK3 is determined by the amount of phosphate in site 1, and not site 2.

4. DISCUSSION

Glycogen synthase is phosphorylated on nine serine residues in vivo [10], five of which are clustered within 17 residues near the C-terminus of the molecule. The primary structure of this region is:

1 2 3 4 5
SVPPSPSLSRHSSPHQSEDEEE [19]

The C-terminal serine is phosphorylated by casein kinase-2 (CK2) without decreasing activity, while phosphorylation of serines 1–4 by GSK3 is accompanied by inactivation (review [18]). It is well established that phosphorylation of glycogen synthase by CK2 is a prerequisite for phosphorylation by GSK3 [20,21], and the recent studies of Fiol et al. [22] using synthetic peptides corresponding to this region have elegantly demonstrated that phosphorylation occurs sequentially in an N-terminal direction, the phosphorylation of serine-5 being followed by phosphorylation of serine-4 and so on. This suggested that, in this instance, GSK3 recognises the sequence Ser-X-X-X-Ser(P).

Although we were unable to detect phosphorylation of PP-1_G by GSK3 initially [5], our interest in this possibility was rekindled by the presence of the motif Ser-X-X-X-Ser-X-X-X-Ser immediately N-terminal to one of the serines (site 1) phosphorylated by A-kinase (fig.1). The results presented in this paper establish that the first two serines in this sequence are indeed phosphorylated by GSK3, provided that the C-terminal serine (site 1) is phosphorylated by A-kinase. Nevertheless, phosphorylation of these two serines by GSK3 may not occur sequentially in an N-terminal direction. If this were the case, then peptide C2 (which is phosphorylated at only one serine by GSK3) should be phosphorylated exclusively at the second serine from the N-terminus (fig.1). However, when C2 was subjected to gas phase sequencer analysis using the procedure of Wang et al. [23], over half of the ^{32}P-radioactivity was associated with the N-terminal serine (unpublished).

In terms of k_{cat}/K_m the G subunit of PP-1_G is approximately 5-fold more effective as a substrate for GSK3 than glycogen synthase, previously the best known substrate for this protein kinase [19]. Since the serines on glycogen synthase phosphorylated by GSK3 are phosphorylated in vivo [10], the G subunit is also likely to be a physiological substrate for this protein kinase, and the information presented here will facilitate analysis of the in vivo phosphorylation states of these serine residues. The high level of phosphorylation of site 1 in vivo, even in the absence of adrenalin (see section 1), suggests that a protein kinase distinct from A-kinase may phosphorylate this serine, allowing GSK3 to phosphorylate PP-1_G even under basal conditions.

Besides glycogen synthase, two other proteins have been identified where phosphorylation by CK2 is required to form the recognition site for GSK3, namely the regulatory (R_{II}) subunit of A-kinase [24] and protein phosphatase inhibitor-2 [25]. The present work is therefore important in demonstrating that protein kinases other than CK2 may be able to synergise with GSK3. This, in turn, suggests that more substrates for GSK3 may exist than have hitherto been identified.

While this manuscript was in preparation Fiol et al. [26] reported that a synthetic peptide corresponding to residues 4 to 24 of the G subunit sequence (fig.1) is phosphorylated by GSK3 after prior phosphorylation by A-kinase.

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