Amino acid sequence of a region on the glycogen-binding subunit of protein phosphatase-1 phosphorylated by cyclic AMP-dependent protein kinase

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The amino acid sequence of a region on the glycogen-binding (G)-subunit of protein phosphatase-1_G that is phosphorylated by cyclic AMP-dependent protein kinase has been determined. The sequence is:

P
|
K P G F S P Q P S R R G S E S S E E V Y V H T A S S G G R

This finding will facilitate studies of the effects of hormones on the phosphorylation state of the G-subunit in vivo.

Glycogen metabolism *cyclic AMP* *Protein phosphorylation* *Protein phosphatase*

1. INTRODUCTION

Protein phosphatases in the cytoplasm of mammalian cells that are capable of dephosphorylating seryl and threonyl residues have been classified into two types. Type-1 protein phosphatases dephosphorylate the β-subunit of phosphorylase kinase preferentially and are inhibited by the thermostable proteins inhibitor-1 and inhibitor-2, while type-2 protein phosphatases dephosphorylate the α-subunit of phosphorylase kinase preferentially and are insensitive to inhibitors-1 and -2 [1,2].

Two type-1 protein phosphatases have been purified to homogeneity from rabbit skeletal muscle, termed protein phosphatase-1_I and protein phosphatase-1_G [3]. Protein phosphatase-1_I, also termed the Mg-ATP-dependent protein phosphatase [4], is an inactive species that consists of a 37 kDa catalytic (C)-subunit and inhibitor-2 in a 1:1 molar ratio [5–8]. Activation of protein phosphatase-1_I is triggered by the phosphorylation of inhibitor-2 on a threonyl residue [5,8], catalysed by a protein kinase termed glycogen synthase kinase-3 [9,10] or Factor F_a [4].

A major proportion of the protein phosphatase-1_G in rabbit skeletal muscle is bound to glycogen. This species is composed of a 37 kDa catalytic (C)-subunit and a 103 kDa glycogen-binding (G)-subunit in a 1:1 molar ratio [3]. The C-subunits of protein phosphatase-1_I and protein phosphatase-1_G appear to be identical, as judged by peptide mapping [3,7,11].

Recently, we reported that protein phosphatase-1_G was an excellent substrate for cyclic AMP-dependent protein kinase in vitro. Its initial rate of phosphorylation was similar to that of glycogen synthase, a well established physiological substrate of this protein kinase, and near stoichiometric

This paper is dedicated to Prakash Datta on the occasion of his retirement as Managing Editor of FEBS Letters

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phosphorylation of the G-subunit could be achieved [3]. The covalently bound phosphate was attached to a seryl residue(s) [3].

Here, the amino acid sequence surrounding the phosphorylation site on the G-subunit of protein phosphatase-1_G has been determined. This information will facilitate analysis of the phosphorylation state of this protein in vivo, and further analysis of its physiological role(s).

2. MATERIALS AND METHODS

2.1. *Protein preparations*

Protein phosphatase-1_G [3] and the catalytic subunit of cyclic AMP-dependent protein kinase [12] were purified from rabbit skeletal muscle. Trypsin (treated with tosylphenyl chloromethyl ketone) and chymotrypsin (3 times crystallised) were products of Worthington, purchased from Cambrian Chemicals (Croydon, England).

2.2. *Phosphorylation of protein phosphatase-1_G*

Protein phosphatase-1_G (5 mg) was incubated for 5–10 min at 30°C with cyclic AMP-dependent protein kinase (20 U; see [13] for definition of units) in 25 mM sodium glycerol-1-phosphate, 4 mM magnesium acetate and 0.1 mM [γ-^{32}P]ATP (6×10^7 cpm/μmol). The total volume was 20 ml. The solution was made 5 mM in EDTA, 10 mM in sodium pyrophosphate and 80 mM in sodium fluoride to terminate the phosphorylation reaction and prevent dephosphorylation. The solution was concentrated to 1–2 ml by vacuum dialysis at 4°C and dialysed for a further 20 h at 4°C against 25 mM sodium glycerol-1-phosphate, 1.0 mM EDTA, 10 mM sodium pyrophosphate, 80 mM sodium fluoride, 0.1% 2-mercaptoethanol, pH 7.0. After dialysis, residual [γ-^{32}P]ATP comprised 25–30% of the ^{32}P radioactivity, the remainder being protein-bound. This solution was digested with either trypsin or chymotrypsin as described in section 3.

3. RESULTS

3.1. *Isolation and structure of the tryptic phosphopeptide*

^{32}P-labelled protein phosphatase-1_G (1.1 ml, 4 mg/ml) containing 0.9 mol phosphate/mol G-subunit in 25 mM sodium glycerol-1-phosphate, 1.0 mM EDTA, 10 mM sodium pyrophosphate, 80 mM NaF, 0.1% (v/v) 2-mercaptoethanol, pH 7.0, was incubated with trypsin (0.11 ml, 1.0 mg/ml) for 10 min at 30°C. The digestion was terminated by addition of 0.07 ml of 100% (w/v) trichloroacetic acid, and after standing in ice for 5 min, the suspension was centrifuged for 2 min at $13\,000\times g$. The supernatant was collected, and the pellet re-extracted with 0.3 ml of 5% (w/v) trichloroacetic acid, and recentrifuged. The combined trichloroacetic acid supernatants, containing >90% of the ^{32}P radioactivity, were extracted three times with ether to remove trichloroacetic acid, made 0.2 M in NH_4HCO_3 and redigested for 16 h at 37°C with 25 μg trypsin. The solution was then subjected to gel filtration on Sephadex G-50 superfine (Pharmacia) as described in the legend to

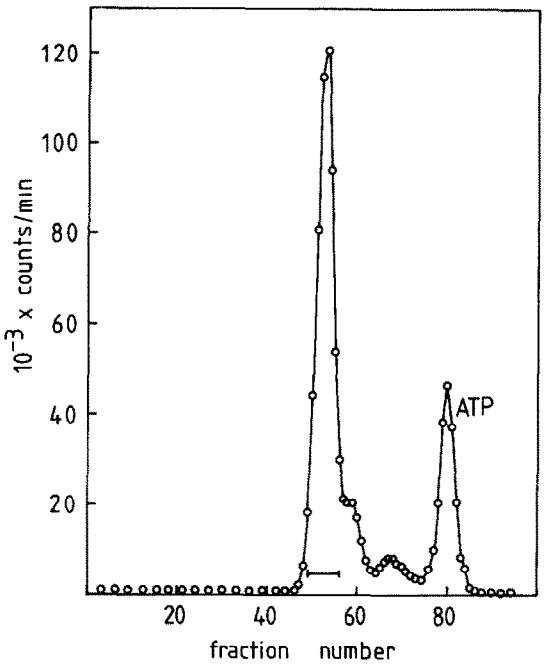

Fig.1. Gel-filtration on Sephadex G-50 (superfine) of trichloroacetic acid-soluble ^{32}P radioactivity released by brief tryptic digestion of native protein phosphatase-1_G. ^{32}P-labelled enzyme was digested with trypsin and prepared for gel filtration as described in the text. The sample (1.5 ml) was applied to the column (120×1.3 cm) equilibrated at 20°C in 0.1 M NH_4HCO_3. The flow rate was 10 ml/h and fractions of 1.15 ml were collected. The horizontal bar indicates the fractions pooled after elution. The minor peak corresponds to [γ-^{32}P]ATP. The void volume was at fraction 1.

fig.1. ^{32}P radioactivity was recovered from the column as a major peak (V_e/V_0 = 2.3) and a minor peak (V_e/V_0 = 2.85), the latter corresponding to [γ-^{32}P]ATP that had been incompletely removed by dialysis (section 2.2).

The major radioactive peak from Sephadex G-50 was dried in a vacuum concentrator (Savant Instruments), dissolved in 0.1% (v/v) trifluoroacetic acid (TFA) and subjected to reverse-phase HPLC using a Vydac C_{18} column (Separations Group, CA) as described in the legend to fig.2. ^{32}P radioactivity was separated by HPLC into two peaks that were just resolved from one another (fig.2). The major component T1 (85%) eluted just after the minor component T2 (15%), and both radioactive species coincided with peaks of 206 nm absorbance (fig.2).

The composition of peptide T1 is shown in table 1. A sample of T1 (4 nmol) was analysed on a Beckman sequencer as described in the legend to fig.3, and its complete primary structure was established by this technique. The analysis was repeated using a second aliquot of peptide T1 with identical results. The sequence of the peptide was found to be:

R-G-S-E-S-S-E-E-V-Y-V-H-T-A-S-S-G-G-R.

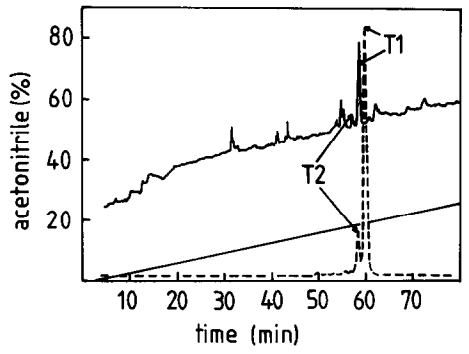

Fig.2. Fractionation of the major tryptic phosphopeptide from Sephadex G-50 (fig.1) on a Vydac C_{18} column. Reverse-phase chromatography was carried out using a Gilson HPLC system equipped with an ultraviolet detector (Gilson) and a radioactive on-line monitor (Reeve Analytical). The delay time between the detectors was 1 min. The full line shows absorbance at 206 nm; and the broken line ^{32}P radioactivity in arbitrary units. The column was developed with a water/acetonitrile gradient (0–40%) containing 0.1% (v/v) TFA, with an increase in acetonitrile concentration of 0.3% per min (——). No radioactivity was eluted after 80 min.

Table 1

Amino acid compositions of peptides T1, C1 and C2

Amino acid	Amount in		
	T1	C1	C2
Asx	0.25(0)	0.64(0)	0.46(0)
Glx	3.28(3)	4.01(4)	4.33(4)
Ser	4.71(5)	4.60(5)	5.13(5)
Gly	3.24(3)	2.09(2)	2.11(2)
His	0.55(1)		
Arg	2.00(2)	1.80(2)	1.19(2)
Thr	0.79(1)	0.34(0)	
Ala	1.05(1)	0.37(0)	
Pro		2.91(3)	3.00(3)
Tyr	0.80(1)	0.62(1)	0.70(1)
Val	1.64(2)	0.62(1)	0.79(1)
Met			
Ile			
Leu		0.32(0)	
Phe		0.86(1)	0.85(1)
Lys		1.10(1)	1.15(1)
Total	19	20	20

Peptides were hydrolysed in pyrex tubes for 18 h at 110°C in the presence of 6 M HCl and 2 mM phenol. The hydrolysates were dried, converted to phenylthiocarbamyl derivatives and analysed on a Waters PICOTAG amino acid analysis system. Serine and threonine were corrected for 10 and 5% destruction, respectively and impurities below 0.2 mol are omitted. Numbers in parentheses indicate residues determined by sequencer analysis

Tryptic peptides *commencing* with arginine have been isolated from many proteins phosphorylated by cyclic AMP-dependent protein kinase, because the sequence Arg-X-Ser(P) is resistant to tryptic attack (e.g. [14,15]). This suggested that Ser-3 was the site of phosphorylation, an idea confirmed by the 'burst' of ^{32}P radioactivity observed after the third cycle of Edman degradation (fig.3). The thiazolinone derivative of phosphoserine is unstable and breaks down rapidly to yield inorganic phosphate, which is poorly extracted from the sequencer cup by the organic solvents employed. Hence the burst of radioactivity is not quantitative and a 'trail' of radioactivity occurs at subsequent cycles due to continued extraction of traces of inorganic phosphate [16].

Although the quantity of peptide T2 was too low

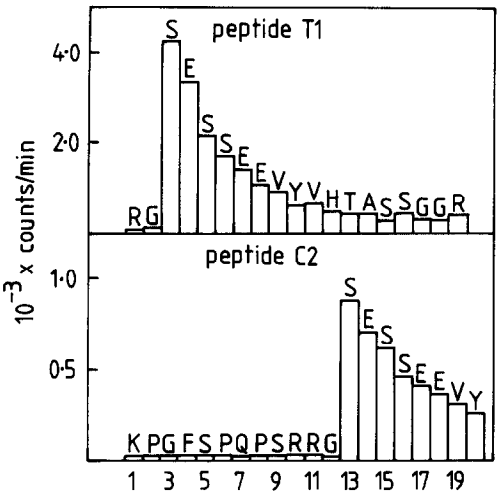

Fig.3. Sequencer analysis of peptide T1 and peptide C2. Tryptic peptide T1 from fig.2 (4 nmol, 95 000 cpm) and chymotryptic peptide C2 from fig.4 (4 nmol, 60 000 cpm) were analysed on a Beckman 890D sequencer using a 0.1 M quadrol programme, with a single coupling followed by a double cleavage. Polybrene (3 mg) was added in aqueous solution to the spinning cup and dried down as a film. Conversion to phenylthiohydantoins was by incubation with 20% (w/v) TFA, 0.02% (w/v) dithioerythrytol for 8 min at 80°C. The figure shows ^{32}P radioactivity extracted from the sequencer cup at each cycle of Edman degradation measured by Cerenkov counting and residues detected by HPLC analysis of the phenylthiohydantoin derivatives [18]. The average repetitive yield per cycle was 94% for both peptides.

for an accurate amino acid analysis, its composition was very similar to that of T2 (not shown). Furthermore, when T2 (0.5 nmol) was subjected to sequencer analysis, its first 14 residues could be assigned unambiguously and were identical to those of peptide T1. The relationship between T1 and T2 is considered further in section 4. The overall recovery of peptides T1 and T2 from the starting material was 45%.

3.2. *Isolation and structure of a chymotryptic phosphopeptide*

The finding that tryptic peptide T1 commenced with the sequence Arg-Gly-Ser(P) indicated the presence of two adjacent basic residues immediately N-terminal to the phosphoserine, a characteristic of many of the best substrates for cyclic AMP-dependent protein kinase (reviewed [17]). In order to extend the sequence of peptide T1 in the N-terminal direction, a second preparation of ^{32}P-labelled protein phosphatase-1_G was digested with chymotrypsin. The initial steps of purification, up to and including gel-filtration on Sephadex G-50 were identical to those described in section 3.1, except that chymotrypsin (1.0 mg/ml) replaced trypsin (1.0 mg/ml). As observed with trypsin, brief chymotryptic attack of the native enzyme released >95% of the protein-bound radioactivity as trichloroacetic acid-soluble peptides within 10 min. Three ^{32}P-labelled peptides were eluted from Sephadex G-50, a major component at V_e/V_0 of 2.25 and two minor components at V_e/V_0 of 2.4 and 2.65. A fourth peak at V_e/V_0 = 2.9 corresponded to [γ-^{32}P]ATP. The major peak (V_e/V_0 = 2.25) was dried, redissolved in 0.1% (v/v) TFA and subjected to reverse-phase HPLC (fig.4). This fraction was separated by HPLC into two ^{32}P-labelled peaks, C1 and C2, that were just resolved from one another. The two radioactive species were present in similar amounts and coincided with peaks of ultraviolet absorbance.

The amino acid compositions (table 1) and primary structures of peptides C1 and C2 were identical and their possible relationship is considered further in section 2. Both peptides were se-

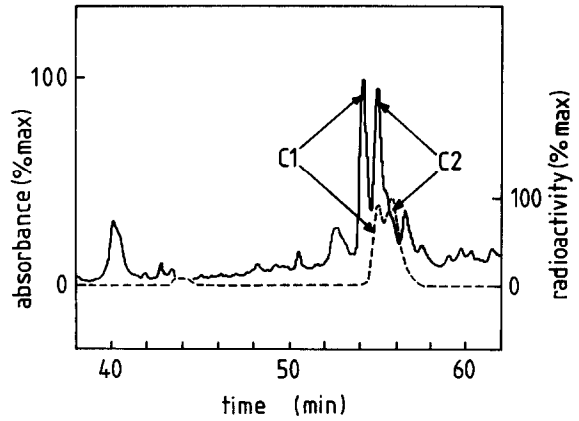

Fig.4. Fractionation of the major chymotryptic peptide from Sephadex G-50 on a Vydac C_{18} column. HPLC was carried out as described for the tryptic peptides (fig.2). The full line shows absorbance at 214 nm and the broken line ^{32}P radioactivity in arbitrary units. Note the 1 min delay between the ultraviolet and radioactive detectors (see fig.2). No radioactivity was eluted before 40 min or after 60 min.

quenced to their C-termini, and their primary structures were found to be:

K-P-G-F-S-P-Q-P-S-R-R-G-S-E-S-S-E-E-V-Y

The last 10 residues were identical to the first 10 residues of peptides T1 and T2, and a burst of ^{32}P radioactivity was observed at the 13th cycle, as expected (fig.3). This established the presence of two adjacent arginyl residues N-terminal to the phosphorylation site, and demonstrated that one of the sites of chymotryptic attack was the Tyr–Val bond between residues 10 and 11 of peptides T1 and T2.

4. DISCUSSION

The results of tryptic digestion demonstrate that the G-subunit is phosphorylated by cyclic AMP-dependent protein kinase at one major site. However, following either tryptic or chymotryptic digestion two phosphopeptides were separated by reverse-phase HPLC (figs 2 and 4) that had identical compositions, specific radioactivities (cpm/nmol) and primary structures. Why these peptides were resolved into two forms is unclear. One possibility is that a second seryl residue in the peptide was already partially phosphorylated even before incubation with cyclic AMP-dependent protein kinase and Mg-ATP. We have observed that diphosphorylated forms of peptides are eluted from reverse-phase HPLC columns earlier than monophosphorylated peptides, and that monophosphorylated species frequently elute just before the dephosphorylated forms (unpublished). If this suggestion is correct, it would imply that the G-subunit is a target for at least two protein kinases.

The sequence Arg-Arg-X-Ser is common to many of the best substrates for cyclic AMP-dependent protein kinase, and it is well established that the adjacent arginyl residues play a key role in substrate recognition (reviewed in [17]). This explains why protein phosphatase-1_G is phosphorylated at a similar rate to glycogen synthase [3]. However, the presence of several acidic residues immediately C-terminal to the phosphoserine is unusual [17]. We have previously suggested that acidic residues C-terminal to phosphorylation sites may act as negative specificity determinants for protein phosphatases [1]. Thus the three glutamyl residues may play an important role in decreasing the rate at which the G-subunit is dephosphorylated by the C-subunit, thereby prolonging the biological effects of phosphorylation.

The G-subunit is extraordinarily susceptable to proteolysis. Despite the inclusion of several proteinase inhibitors and a rapid purification procedure [3], the G-subunit is undetectable by SDS-polyacrylamide gel electrophoresis in a number of preparations, even though the C-subunit appears completely undegraded [3]. However, such preparations are phosphorylated by cyclic AMP-dependent protein kinase in an identical manner to undegraded preparations of protein phosphatase-1_G. Following phosphorylation, SDS-polyacrylamide gel electrophoresis of degraded preparations reveals a multiplicity of ^{32}P-labelled bands (sometimes more than 25) ranging in apparent molecular mass from 100 kDa to <20 kDa. These bands are derived from the G-subunit, since they are immunoprecipitated by antibodies raised against the undegraded G-subunit (purified by SDS-polyacrylamide gel electrophoresis), but not by preimmune IgG. Similarly, tryptic digestion of degraded preparations generates a single 19-residue phosphopeptide, i.e. the tryptic phosphopeptide described in this paper (not shown). Such extensive fragmentation of the G-component presumably explains the failure to detect this subunit, until recently. Nevertheless, despite the cleavage of many peptide bonds, the fragments of the G-subunit must interact with one another and with the C-subunit, since the gel-filtration behaviour of degraded and undegraded preparations is very similar (unpublished).

Two roles for the G-subunit in the regulation of protein phosphatase-1 have so far been identified. Firstly, the G-subunit is the component responsible for targetting the C-subunit to the glycogen-protein particles [3]. Secondly, phosphorylation of the G-subunit by cyclic AMP-dependent protein kinase increases the rate at which the G-subunit can be inactivated by inhibitor-1. Since inhibitor-1 is only inhibitory if phosphorylated by cyclic AMP-dependent protein kinase (reviewed in [2]), phosphorylation of the G-subunit may amplify the effects of cyclic AMP. Investigation of the in vivo phosphorylation state of the G-subunit will be necessary to evaluate the physiological significance of this control mechanism. The results presented here will facilitate this analysis.

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REFERENCES

[1] Ingebritsen, T.S., Foulkes, J.G. and Cohen, P. (1983) Eur. J. Biochem. 132, 255–261.
[2] Ingebritsen, T.S. and Cohen, P. (1983) Science 221, 331–338.
[3] Stralfors, P., Hiraga, A. and Cohen, P. (1985) Eur. J. Biochem. 149, 295–303.
[4] Goris, J., Defreyn, G. and Merlevede, W. (1979) FEBS Lett. 99. 279–282.
[5] Hemmings, B.A., Resink, T.J. and Cohen, P. (1982) FEBS Lett. 150, 319–324.
[6] Resink, T.J., Hemmings, B.A., Tung, H.Y.L. and Cohen, P. (1983) Eur. J. Biochem. 133, 455–461.
[7] Tung, H.Y.L. and Cohen, P. (1984) Eur. J. Biochem. 145, 57–64.
[8] Ballou, L., Brautigan, D.L. and Fischer, E.H. (1983) Biochemistry 22, 3393–3399.
[9] Hemmings, B.A., Yellowlees, D., Kernohan, J.C. and Cohen, P. (1981) Eur. J. Biochem. 119, 443–451.
[10] Woodgett, J.R. and Cohen, P. (1984) Biochim. Biophys. Acta 788, 339–347.
[11] Tung, H.Y.L., Resink, T.J., Hemmings, B.A., Shenolikar, S. and Cohen, P. (1984) Eur. J. Biochem. 138, 635–641.
[12] Beavo, J.A., Bechtel, P.J. and Cohen, P. (1974) Methods Enzymol. 39, 299–308.
[13] Cohen, P., Nimmo, G.A. and Antoniw, J.F. (1977) Biochem. J. 162, 435–444.
[14] Cohen, P., Watson, D.C. and Dixon, G.H. (1975) Eur. J. Biochem. 51, 79–92.
[15] Proud, C.G., Nimmo, H.G., Yeaman, S.J. and Cohen, P. (1977) FEBS Lett. 80, 435–442.
[16] Embi, N., Parker, P.J. and Cohen, P. (1981) Eur. J. Biochem. 115, 405–413.
[17] Cohen, P. (1985) Bioessays 1, 63–68.
[18] Aitken, A., Bilham, T., Cohen, P., Aswad, D. and Greengard, P (1981) J. Biol. Chem. 256, 3501–3506.