Volume 234, number 1, 189–194 FEB 06060 July 1988

Phosphorylation of the glycogen-binding subunit of protein phosphatase-1_G in response to adrenalin

Carol MacKintosh, David G. Campbell, Akira Hiraga and Philip Cohen

Department of Biochemistry, University of Dundee, Dundee, Scotland

Received 10 May 1988

The glycogen-binding (G) subunit of protein phosphatase-1 is phosphorylated in vivo. In rabbits injected with propranolol the serine residue termed site-1 was phosphorylated in 56% of the molecules isolated, and phosphorylation increased to 82% after administration of adrenalin. It is concluded that the G-subunit is a physiological substrate for cyclic AMP-dependent protein kinase. The G-subunit remained largely bound to glycogen even after injection of adrenalin, whereas half of the protein phosphatase-1 activity associated with glycogen was released into the cytosol. The results indicate that adrenalin induces dissociation of the catalytic subunit from the G-subunit in vivo.

Protein phosphatase; Glycogen; Adrenalin; Phosphopeptide; Cellular regulation

1. INTRODUCTION

Three forms of protein phosphatase-1 (PP-1) have been identified in rabbit skeletal muscle, an inactive cytosolic form (PP-1_I) [1,2] and two active forms that are bound to glycogen (PP-1_G) [3,4] and myosin (PP-1_M) [5,6]. Each form contains a very similar, if not identical, catalytic subunit (PP-1_C) complexed to an inhibitory subunit, inhibitor-2 (PP-1_I), a glycogen-binding subunit (PP-1_G) or a myosin-binding subunit (PP-1_M).

The undegraded glycogen-binding (G) subunit of PP-1_G has an apparent molecular mass of 161 kDa (Hubbard, M. and Cohen, P., in preparation) and appears to have a regulatory as well as a targetting function. It is phosphorylated very rapidly by cyclic AMP-dependent protein kinase (A-kinase) in vitro [3] and the primary structure surrounding the major phosphoserine residue (termed site-1) reveals the characteristic Arg-Arg-X-Ser- sequence [7] commonly found at the phosphorylation sites of physiological substrates for A-kinase (reviewed in [8]). Phosphorylation of the G-subunit in vitro does not seem to alter the activity of PP-1_G directly, but increases the rate at which it can be inactivated by inhibitor-1 (I-1) [3] and decreases the proportion of PP-1_G that can be sedimented with glycogen [9]. Consistent with the latter finding, injection of adrenalin in vivo decreases the amount of PP-1 activity in skeletal muscle extracts that sediments with glycogen-protein particles from ≈70% to ≈35%, and increases activity in the cytosol by a similar amount [9]. These observations suggest that translocation of PP-1 from glycogen to cytosol occurs in response to adrenalin, and may provide a mechanism for preventing dephosphorylation of the glycogen metabolising enzymes (glycogen phosphorylase and glycogen synthase) which remain tightly bound to glycogen even after administration of adrenalin [9]. In this paper we demonstrate that the G-subunit is phosphorylated at site-1 in vivo and that phosphorylation of this serine residue increases in response to adrenalin.

Correspondence address: C. MacKintosh, Department of Biochemistry, Medical Sciences Institute, The University, Dundee DD1 4HN, Scotland

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

2. MATERIALS AND METHODS

2.1. *Isolation of protein phosphatase-1_G after injection of propranolol or adrenalin*

Rabbits were injected with propranolol (2 mg/kg) 10 min prior to a lethal dose of sodium pentobarbital, or with adrenalin (0.25 mg in 10 mM ascorbic acid) injected together with pentobarbital and skeletal muscle (2000 g) from the hind limbs and back of three animals was homogenised under conditions that preserve the phosphorylation states of the glycogen metabolising enzymes. The entire procedure was carried out as described for glycogen synthase up to and including isolation of the glycogen-protein particles [10], except that a second protein phosphatase inhibitor (1 mM $Na_2P_2O_7$) was added to all solutions in addition to 50 mM NaF, and the muscle extracts were adjusted to pH 5.7, rather than 5.9, to precipitate the glycogen-protein particles. PP-1_G was purified from glycogen-protein particles as in [4] except that NaCl concentrations were reduced by 50 mM to accommodate the NaF. The enzyme was located by assaying for phosphorylase phosphatase activity [11], but with inclusion of 1 mM $MnCl_2$ to reactivate the NaF and $Na_2P_2O_7$-inactivated phosphatase [9], while the G-subunit was located by immunoblotting using the antibody described below. The G-subunit copurified with phosphatase activity at every stage and stepwise elution from each anion-exchange column (except the final chromatography on Mono Q) was employed to avoid selective purification of either the phosphorylated or dephosphorylated protein. For this reason, the final preparations were only 50–70% pure, but this did not interfere with subsequent isolation of the tryptic phosphopeptide.

2.2. *Antibody to the G-subunit*

The 103 kDa G′-fragment of PP-1_G (1.5 mg) [4] was excised from an SDS-polyacrylamide gel, lyophilised and powdered. It was emulsified with Freund's incomplete adjuvant and 0.5 mg injected subcutaneously into a sheep at multiple sites. This procedure was repeated twice more over nine weeks before bleeding. The γ-globulin fraction was isolated by fractionation with ammonium sulphate, dissolved in 50 mM Tris-HCl, pH 7.5 (0°C), 0.15 M NaCl, dialysed against this buffer and stored at −20°C.

3. RESULTS

As reported in [9], the proportion of PP-1 activity in muscle extracts that could be sedimented with glycogen-protein particles was found to decrease from ≈65% to ≈35% following intravenous injection of adrenalin, with a corresponding increase in the cytosolic content of the enzyme (table 1). However, Western blotting experiments using a polyclonal antibody directed against the G-subunit, revealed that this protein was mainly located in the glycogen-protein particles, even after injection of adrenalin, and the cytosolic content was not increase by this hormone (fig.1). This implies that PP-1_C is dissociated from the G-subunit in response to adrenalin, a finding that is considered further in section 4.

Protein phosphatase 1_G (2–4 mg, purity 50–70%), isolated from propranolol or adrenalin-treated rabbits in the presence of protein phosphatase inhibitors (see section 2), was mixed with 0.02 mg of ^{32}P-labelled PP-1_G (50–100000 cpm) that had been phosphorylated in vitro by A-kinase and freed from [γ-^{32}P]ATP as in [7]. This trace of marker protein enabled the

Table 1

Phosphate content of the site-1 tryptic peptide after injection of propranolol or adrenalin

Preparation	Injection	Phosphate content (mol/mol)		Phosphate activity (%)	
		Analysis	Area	pH 5.7 ppt	pH 5.7 supt
1	propranolol	0.48	0.53	64	36
2	propranolol	0.57	0.56	62	38
3	propranolol	0.64	0.62	67	33
Av (± SE)	propranolol	0.56 ± 0.08	0.57 ± 0.05	64 ± 3	36 ± 3
4	adrenalin	0.76	0.75	27	73
5	adrenalin	0.85	0.87	37	63
6	adrenalin	ND	0.87	22	78
Av (± SE)	adrenalin	0.81 ± 0.04	0.83 ± 0.08	29 ± 8	71 ± 8

Phosphorylation stoichiometries were calculated by quantitative amino acid analysis (analysis) of the phosphopeptide and dephosphopeptide (fig.2) and by integration of the areas under each peak (area). ND, not determined. Phosphorylase phosphatase activities were measured in the pH 5.7 precipitate and pH 5.7 supernatant in the presence of 1 mM $MnCl_2$ as in [9]

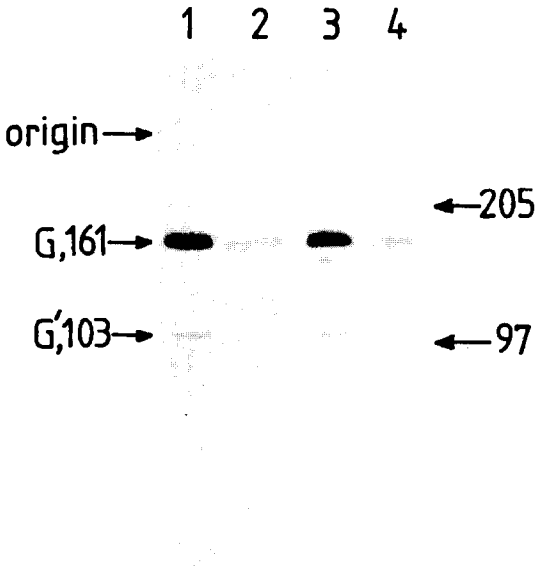

Fig.1. Localisation of the glycogen-binding subunit by Western blotting. Rabbit skeletal muscle extracts (50 ml) were adjusted to pH 5.7, and after centrifugation the supernatants were decanted, and the pH 5.7 precipitates containing the glycogen-protein particles were redissolved in 50 ml of 50 mM Tris-HCl, pH 7.5 (0°C), 1 mM EDTA, 10% (v/v) glycerol, 0.1% (v/v) 2-mercaptoethanol, 1 mM benzamidine, 0.1 mM phenylmethylsulphonyl fluoride, 50 mM sodium fluoride, 1 mM sodium pyrophosphate. The pH 5.7 supernatant and resuspended pH 5.7 precipitate (10 μl) were each denatured in 2 μl of 10% SDS at 100°C and subjected to polyacrylamide gel electrophoresis [17]. The gel was transferred to nitrocellulose and the G-subunit located by Western blotting with the polyclonal antibody as in [18]. No bands were observed if the antibody was replaced by preimmune immunoglobulin (not shown). The arrows denote the positions of the undegraded G-subunit (161 kDa) and the proteolytic G'-subfragment (103 kDa) and the marker proteins myosin (205 kDa) and phosphorylase (97 kDa). Lanes 1 and 3 show the pH 5.7 precipitates from adrenalin and propranolol-treated animals, containing 12 and 25 μU of phosphorylase phosphatase activity, respectively, and lanes 2 and 4 the pH 5.7 supernatants from adrenalin and propranolol-treated animals, containing 21 and 13 μU of phosphorylase phosphatase activity, respectively.

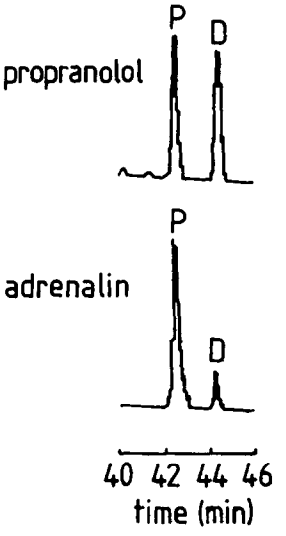

Fig.2. Reverse-phase chromatography at pH 6.5 of the site-1 tryptic peptide. The tryptic peptide obtained by reverse-phase chromatography on the Vydac C_{18} column equilibrated in 0.1% TFA (see text and [7]) was pooled, dried, redissolved in 10 mM ammonium acetate, pH 6.5, and rechromatographed on the Vydac column equilibrated in the same solvent. The column was developed with a linear acetonitrile gradient, with an increase in acetonitrile concentration of 0.36% per min. The flow rate was 1 ml/min and fractions of 0.5 ml were collected. The peptide was resolved into the phospho-(P) and dephospho-(D) forms, which were eluted at 11.5 and 12% acetonitrile, respectively. The yield of peptide (P + D) was 80%.

purification of the peptide to be followed and recoveries to be calculated. The native protein was cleaved with trypsin to release the site-1 phosphopeptide, which was purified by gel filtration on Sephadex G50 and reverse-phase chromatography on a Vydac C_{18} column equilibrated in 0.1% (v/v) trifluoroacetic acid (TFA) as in [7]. The overall recovery was 50–60%. The ^{32}P-labelled peptide was eluted as a single major peak from both columns, and coincided on the Vydac column with a major peak of 206 nm absorbance. The elution position of this peak (18% acetonitrile) and its amino acid composition were the same as the 19 residue site-1 tryptic peptide isolated previously [7].

Phosphopeptides are usually eluted at acetonitrile concentrations 0.5–1% lower than their dephosphorylated counterparts from C_{18} columns run in 0.1% TFA [12,13], but amino acid analysis of fractions eluting after the ^{32}P-radioactivity failed to detect any further material that might have corresponded to the dephosphopeptide. The peptide that coincided with the ^{32}P-radioactivity was therefore pooled, dried and resubjected to reverse-phase chromatography on the Vydac column at pH 6.5, as described in the legend to fig.2. Two major peaks of 214 nm absorbance were eluted from the column, and termed P and D. Peak P was eluted at 11.5% acetonitrile and coincided with the ^{32}P-

marker, while peak D eluted ≈1.5 min later at 12% acetonitrile. The amino acid compositions of peaks P and D were identical, except for the presence of an additional arginine residue in peak P (table 2). Fast atom bombardment mass spectrometry of the two peaks using a VG 250S machine established that peak P (MH^+ = 2075) was a monophosphorylated derivative of the site-1 tryptic peptide isolated previously [7], while peak D (MH^+ = 1839) was the dephosphopeptide lacking one of the two arginines present in peak P.

In order to locate the position of the phosphorylated residue(s), peaks P and D were incubated with NaOH and ethanethiol to convert phosphoserine residues to *S*-ethylcysteine [12,14] and analysed on a gas-phase sequencer. These experiments revealed that the N-terminal sequence of peak D was Gly-Ser-Glu-Ser-Ser-Glu-, while that of peak P was Arg-Gly-*S*-ethylCys-Glu-Ser-Ser-Glu- (fig.3). Identical results were obtained with peptides isolated from propranolol or adrenalin-treated animals. This established that serine-3 was the site of phosphorylation in peak P, and that peak D lacked the N-terminal arginine residue of the tryptic phosphopeptide. Thus phosphorylation of serine-3 prevented tryptic cleavage of the Arg-Gly bond between positions 1 and 2, as observed for other phosphorylation sites of A-kinase [15,16]. The phospho- and dephosphopeptides

Table 2

Amino acid compositions of peaks P and D from fig.2

Amino acid	Peak P	Peak D
Asp		
Glu	2.72 (3)	2.59 (3)
Ser	5.23 (5)	5.06 (5)
Gly	3.26 (3)	3.36 (3)
His	0.83 (1)	0.76 (1)
Arg	**2.15 (2)**	**1.05 (1)**
Thr	1.26 (1)	1.10 (1)
Ala	1.00 (1)	1.00 (1)
Pro		
Tyr	0.74 (1)	0.74 (1)
Val	1.58 (2)	1.59 (2)
Met		
Ile		
Leu		
Phe		
Lys		
Total	19	18

Peptides were hydrolysed for 16 h at 110°C and analysed using a Waters PICOTAG system as in [19]. Values for serine and threonine were corrected for 10% and 5% destruction, respectively, and impurities below 0.2 residues are omitted. Numbers in parentheses show values expected from the sequence analysis given in [7]

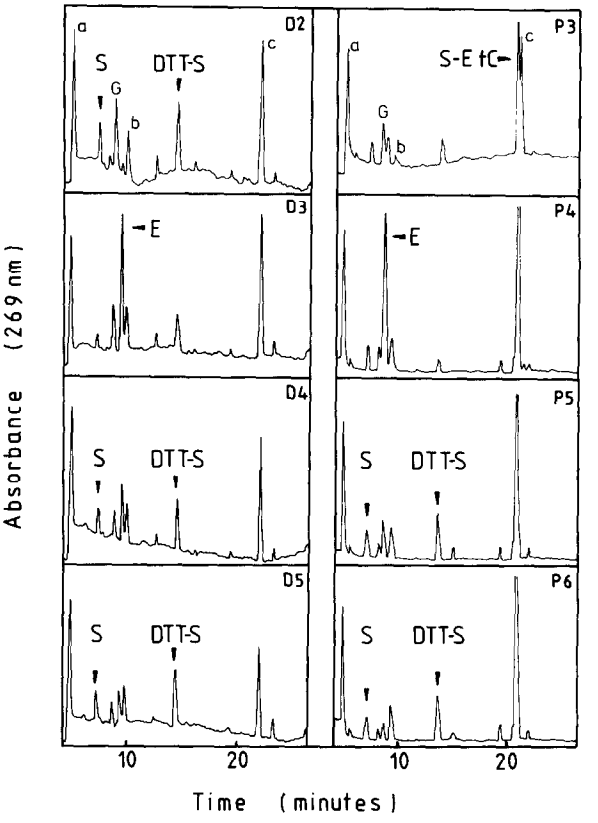

Fig.3. Sequence analysis of site-1 tryptic peptides after incubation with NaOH and ethanethiol. Following incubation with NaOH and ethanethiol [12], the reactions were terminated by addition of acetic acid and the solution dried. The residues were washed twice with water, redissolved in 0.1% TFA and passed through a Hamilton 'miniclean' C-18 cartridge. After elution of non-peptide material, peptides were eluted with 50% acetonitrile in 0.1% TFA, dried and analysed on an Applied Biosystems 470 gas-phase sequencer equipped with an on-line model 120 PTH-analyser. The figure shows reverse-phase chromatograms of PTH-derivatives corresponding to residues 2–5 of the dephosphopeptide (D2–D5) and 3–6 of the phosphopeptide (P3–P6). The amounts applied to the sequencer were approx. 350 pmol. The ultraviolet absorbance scale is 0–0.014 (D) and 0–0.025 (P). Serine was detected by the appearance of two peaks, namely PTH-Ser itself (S) and its dithiothreitol adduct (DTT-S). *Abbreviations:* S, serine; G, glycine; E, glutamic acid; *S*-EtC, *S*-ethylcysteine. a,b and c represent dithiothreitol, dimethylphenylthiourea and diphenylthiourea, respectively. The glycine present in D2 and P3 is carried over from the previous cycle.

presumably coelute during reverse-phase chromatography in TFA because the effect of the phosphoryl group to increase effective hydrophilicity and decrease retention time is counterbalanced by the presence of an additional arginine residue which increases effective hydrophobicity. At pH 6.5, the extra negative charge on phosphoserine ensures its elution at an acetonitrile concentration lower than the dephosphopeptide, despite the presence of one more arginine.

The relative amounts of the phospho- and dephosphopeptides were determined by quantitative amino acid analysis as well as by integration of the areas under the 214 nm absorbance peaks (fig.2). The results showed that the molar proportion of phosphopeptide:dephosphopeptide was about 1:1 in animals injected with propranolol and increased to nearly 5:1 after injection of adrenalin (table 1, fig.2).

The activity ratio ∓ glucose-6P of glycogen synthase was measured in glycogen-protein particles of the same preparations to check the efficacy of the hormone treatments. The activity ratios in animals injected with propranolol and adrenalin were 0.19 ± 0.01 and 0.04 ± 0.01, respectively (both ± SE for 3 preparations). These values are almost identical to those reported previously using the same assay system [10].

4. DISCUSSION

In this paper we have established that the G-subunit of PP-1_G is phosphorylated at site-1 in vivo and that its degree of phosphorylation increases in response to adrenalin, indicating that the G-subunit is a physiological substrate for A-kinase. Assuming equal recovery of the phosphorylated and dephosphorylated species throughout the protein and peptide purifications, the phosphorylation stoichiometries were 0.56 mol/mol (propranolol) and 0.82 mol/mol (adrenalin) (table 1).

The effect of adrenalin to release PP-1_C from the glycogen-protein particles is only observed if skeletal muscle is homogenised in the presence of protein phosphatase inhibitors [9], indicating that one or more phosphorylation events are required to promote dissociation from the G-subunit. However, it is not yet certain whether phosphorylation of site-1 alone is responsible for dissociation. If this was the case, the phosphorylated G-subunit associated with glycogen-protein particles should not be complexed to PP-1_C. Alternatively, the G-subunit, like glycogen synthase [20], is phosphorylated at multiple sites by several kinases, increased phosphorylation at several positions being required to promote dissociation. Another possibility is that phosphorylation of site-1 (and/or other sites) weakens the interaction between PP-1_C and the G-subunit sufficiently to allow a cytosolic protein to extract PP-1_C from the G-subunit. In this connection, it should be noted that the apparent molecular mass of PP-1 in the cytosol is >100 kDa both before and after injection of adrenalin (Hiraga, A., unpublished), indicating that PP-1_C binds to another protein upon its release from the glycogen-protein particles. The identity of this protein is currently under investigation.

It is also surprising that site-1 is phosphorylated to at least 0.5 mol/mol in propranolol-treated animals. The residue on muscle glycogen synthase phosphorylated most rapidly by A-kinase in vitro, which also has the sequence Arg-Arg-X-Ser, is not phosphorylated at all under these conditions [20]. This suggests that the G-subunit may be dephosphorylated extremely slowly in resting muscle, so that phosphorylation is still substantial even when A-kinase activity is very low. Alternatively, site-1 may be phosphorylated in vivo by an additional enzyme distinct from A-kinase.

Acknowledgements: This work was supported by a Programme Grant and Group Support from the Medical Research Council, London and by the Wellcome Trust and Royal Society. We thank Mr Barry Caudwell for performing the mass spectrometric analyses.

REFERENCES

[1] Ballou, L.M., Brautigan, D.L. and Fischer, E.H. (1983) Biochemistry 22, 3393–3399.
[2] Tung, H.Y.L. and Cohen, P. (1984) Eur. J. Biochem. 145, 57–64.
[3] Strålfors, P., Hiraga, A. and Cohen, P. (1985) Eur. J. Biochem. 149, 295–303.
[4] Hiraga, A., Kemp, B.E. and Cohen, P. (1987) Eur. J. Biochem. 163, 253–258.
[5] Chisholm, A.A.K. and Cohen, P. (1988) Biochim. Biophys. Acta 968, 392–400.
[6] Chisholm, A.A.K. and Cohen, P. (1988) Biochim. Biophys. Acta, in press.

[7] Caudwell, F.B., Hiraga, A. and Cohen, P. (1986) FEBS Lett. 194, 85–90.
[8] Cohen, P. (1988) Proc. R. Soc. (Ser. B) 234, 115–144.
[9] Hiraga, A. and Cohen, P. (1986) Eur. J. Biochem. 161, 763–769.
[10] Parker, P.J., Embi, N., Caudwell, F.B. and Cohen, P. (1982) Eur. J. Biochem. 124, 47–55.
[11] Cohen, P., Alemany, S., Hemmings, B.A., Resink, T., Stewart, A.A., Strålfors, P. and Tung, H.Y.L. (1988) Methods Enzymol. 159, in press.
[12] Holmes, C.F.B. (1987) FEBS Lett. 215, 21–24.
[13] Holmes, C.F.B., Tonks, N.K., Major, H. and Cohen, P. (1987) Biochim. Biophys. Acta 929, 208–219.
[14] Meyer, H.E., Hoffman-Posorske, E., Korte, H. and Heilmeyer, L.M.G. (1986) FEBS Lett. 204, 61–66.
[15] Yeaman, S.J. and Cohen, P. (1975) Eur. J. Biochem. 51, 93–104.
[16] Proud, C.G., Rylatt, D.B., Yeaman, S.J. and Cohen, P. (1977) FEBS Lett. 80, 435–442.
[17] Laemmli, U.K. (1970) Nature 227, 680–685.
[18] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[19] Holmes, C.F.B., Campbell, D.G., Caudwell, F.B., Aitken, A. and Cohen, P. (1986) Eur. J. Biochem. 155, 173–182.
[21] Poulter, L., Ang, S.G., Gibson, B.W., Williams, D.H., Holmes, C.F.B., Caudwell, F.B., Pitcher, J. and Cohen, P. (1988) Eur. J. Biochem, in press.