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Identification of three in vivo phosphorylation sites on the glycogen-binding subunit of protein phosphatase 1 from rabbit skeletal muscle, and their response to adrenaline

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The in vivo phosphorylation stoichiometries of 4 serines on the glycogen-binding (G)-subunit of protein phosphatase 1 (PP1) have been determined. In fed rabbits injected with propranolol stoichiometries (mol/mol) were: site 1 (0.67 ± 0.09), site 2 (0.20 ± 0.07), site 3a (0.23 ± 0.01) and site 3b (0). After injection with adrenalin they became: site 1 (0.90 ± 0.02), site 2 (0.72 ± 0.01), site 3a (0.23 ± 0.02) and site 3b (0). These results, together with other data, establish that site 2 phosphorylation by cyclic AMP-dependent protein kinase triggers dissociation of PP1 from the G-subunit in vivo. They also demonstrate that a residue phosphorylated in vitro by glycogen synthase kinase 3 (site 3a) is phosphorylated in vivo.

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

1. INTRODUCTION

The glycogen-bound form of protein phosphatase 1 ($PP1_G$) is a heterodimer comprising the 37 kDa catalytic (C) subunit and a 161 kDa glycogen-binding (G)-subunit, whose interaction is regulated by the cyclic AMP-dependent protein kinase (PKA) catalysed phosphorylation of the G-component [1-3]. Initial investigations suggested the presence of a single phosphoserine [4], now termed site 1, that was shown to be phosphorylated in vivo [5], but subsequent in vitro studies revealed 3 further phosphoserines. One, termed site 2 (19 residues C-terminal to site 1), was also phosphorylated by PKA, and missed in earlier work for reasons discussed in [3,6]. The other serines, termed sites 3a and 3b, are located 4 and 8 residues N-terminal to site 1 and phosphorylated by glycogen synthase kinase 3 (GSK3), provided that site 1 is phosphorylated by PKA [7,8]. The sequence of this 48 residue regulatory domain is: AIFKPGFSPQPSRRGSESSEEVYVHT ASSGGRRVSFADNFGFNLVSVK [3,7], where the 1st, 2nd, 3rd and 8th serines correspond to sites 3a, 3b, 1 and 2, respectively. Here, we extend analysis of the in vivo phosphorylation state of the G-subunit to encompass all 4 potential phosphorylation sites using fed animals injected with either adrenaline or the antagonist propranolol.

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2. MATERIALS AND METHODS

2.1. *Materials*

The sources of all reagents and proteins used in this study are given in [5,7].

2.2. *Generation of tryptic phosphopeptides from protein phosphatase 1_G after injection of fed rabbits with propranolol or adrenaline*

Hormone injection, followed by isolation of $PP1_G$ from skeletal muscle extracts in the presence of phosphatase inhibitors was carried out as in [5], with slight modifications to the purification procedure [2]. $PP1_G$ (2-4 mg, purity 50-70%) was mixed with 0.02 mg of ^{32}P-labelled $PP1_G$ ($\approx 300\,000$ cpm) that had been phosphorylated to ≈ 3 mol/mol in vitro by the combined action of PKA and GSK3 [8]. This trace of 'marker' protein enabled the purification and recovery of each peptide to be followed. $PP1_G$ (0.5 ml) was then incubated with trypsin (0.05 mg) for 10 min at 37°C, and digestion terminated by addition of 0.05 ml of 100% (w/v) trichloroacetic acid. After centrifugation for 2 min at $13\,000 \times g$ the supernatant, containing $\approx 70\%$ of the ^{32}P-radioactivity and the tryptic peptides corresponding to sites 1, 3a and 3b, was removed. The pellet, containing 30% of the ^{32}P-radioactivity and the trichloroacetic acid-insoluble tryptic peptide containing site 2, was resuspended in 0.1 ml of 100% (v/v) trifluoroacetic acid, placed in a sonicating water bath for 10 min and, after addition of 0.1 ml of water, neutralised with solid ammonium bicarbonate. Following centrifugation for 2 min at $13\,000 \times g$, the supernatant was removed, the pellet resuspended once more in 0.1 ml of trifluoroacetic acid, and the procedure repeated. The combined supernatants containing $\approx 70\%$ of the site 2 tryptic phosphopeptide were added to the supernatant containing the tryptic peptides corresponding to sites 1, 3a and 3b and analysed.

2.3. *Other analytical procedures*

Protein concentrations were determined by colorimetric assay [9]. Chromatography of peptides was carried out using a 250×4 mm Vydac 218TP54 reverse-phase C_{18} column (Separations Group, Hesperia, CA, USA) equilibrated in either 0.1% (v/v) trifluoroacetic acid, pH 1.9, or 10 mM ammonium acetate, pH 6.5. The chromato-

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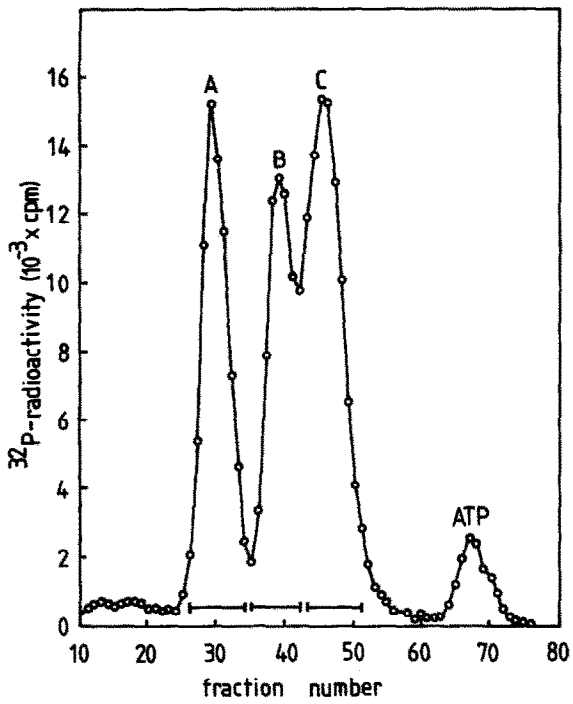

Fig.1. Gel filtration of tryptic phosphopeptides on Sephadex G50 Superfine. Phosphopeptides (1.0 ml) released by brief tryptic digestion of native $PP1_G$ (section 2.2) were applied to column (110 × 1.3 cm) equilibrated in 0.1 M NH_4HCO_3. The flow rate was 13 ml/h and fractions of 1 ml were collected. The graph shows ^{32}P-radioactivity derived from the trace of 'marker' peptide that was used to locate the positions of the tryptic peptides containing sites 1 + 3a + 3b (peak A), site 1 (peak B), site 2 and sites 3a/3b (peak C). The horizontal bars indicate the fractions pooled and the position of ^{32}P-ATP (traces of which contaminated the 'marker') is also shown.

grams were developed with linear water/acetonitrile gradients with an increase in acetonitrile concentration of 0.33% per min and analysed on-line for 214 nm absorbing material and ^{32}P-radioactivity. The flow rates were 1 ml/min and fractions of 0.5 ml were collected. Amino acid analysis was carried out with a Waters PICOTAG system [10], protein sequencing using an Applied Biosystems 470A gas phase sequencer equipped with an on-line reverse-phase chromatography system for identification of phenylthiohydantoin (Pth) amino acids, and fast atom bombardment mass spectrometry (FABMS) in the positive ion mode using a VG Analytical 70SE machine [11].

3. RESULTS

3.1. *Isolation and analysis of tryptic phosphopeptides from the G-subunit*

The mixture of tryptic peptides containing site 1, site 2, and sites (3a + 3b) were applied to Sephadex G50 (Superfine grade, Pharmacia), which resolved the ^{32}P-radioactivity associated with the 'marker' peptides into 3 major peaks, termed A, B and C (fig.1). Peak A was established in separate studies (not shown) to comprise residues 1–32 of the regulatory domain (section 1) and contained sites 1, 3a and 3b. It resulted from the failure of trypsin to cleave the Arg-Arg bond in the sequence Ser(site 3b)-Arg-Arg-Gly-Ser(site 1), when both site 3b and site 1 were phosphorylated. Peak B (fig.1) contained 90% of the tryptic peptide containing site 1 (residues 14/15–32, section 1), while peak C contained the site 2 tryptic peptide (residues 33/34–48), the tryptic peptide containing sites 3a and 3b (residues 1–13) and traces of the site 1 tryptic peptide. Peaks A, B and C were pooled (fig.1), the volume reduced to ≈0.5 ml in a vacuum concentrator (avoiding compete drying which leads to the loss of the site 2 peptide [3,6]), made 1% (by vol.) in 0.1% trifluoroacetic acid and chromatographed on the C_{18} column equilibrated at pH 1.9.

The ^{32}P-radioactivity in peak A derived from the 'marker' peptide eluted from the C_{18} column as a single peak at 23.3% acetonitrile, but no 214 nm absorbing peak eluted at this position. Amino acid analysis of the same region, using preparations isolated from animals injected with either propranolol or adrenaline, confirmed the lack of material corresponding to this peptide.

Table 1

In vivo phosphorylation stoichiometries of the G-subunit after injection of propranolol or adrenaline

Preparation	Injection	site 1	site 2	site 3a	site 3b
1	propranolol	0.76 ± 0.01	0.27 ± 0.03	0.23 ± 0.01	0
2	propranolol	0.58 ± 0.03	0.13 ± 0.01	0.23 ± 0.01	0
Ave. of 1 and 2	propranolol	0.67 ± 0.09	0.20 ± 0.07	0.23 ± 0.01	0
3	adrenaline	0.88 ± 0.01	0.72 ± 0.01	0.21 ± 0.02	0
4	adrenaline	0.91 ± 0.03	0.71 ± 0.02	0.24 ± 0.03	0
Ave. of 3 and 3	adrenaline	0.90 ± 0.02	0.72 ± 0.01	0.23 ± 0.02	0

Phosphorylation stoichiometries were calculated as a ratio of the amount of phosphopeptide to (dephosphopeptide + phosphopeptide). This was determined firstly by quantitative amino acid analysis of the separated phospho- and dephospho-peptides, and secondly by comparison of the areas under the 214 nm absorbance peaks corresponding to the phospho- and dephosphopeptides (see figs.2 and 3). Values obtained by each procedure were then averaged to yield the values shown

The ^{32}P-radioactivity in peak B eluted from the C_{18} column at 17.3% acetonitrile, and FABMS analysis (not shown) demonstrated that it contained both the site 1 phosphopeptide RGS(P)ESSEEVYVHTASSGGR ($[M+H]^+ = 2075$) and a dephosphorylated derivative GSESSEEVYVHTASSGGR ($[M+H]^+ = 1839$), which were resolved by a further chromatography at pH 6.5 as reported previously [5]. Phosphorylation stoichiometries presented in table 1 were similar to the values reported previously [5].

Chromatography of peak C on the C_{18} column, resolved the tryptic peptides containing sites (3a + 3b) and site 2. The ^{32}P-labelled 'marker' corresponding to the sites (3a + 3b) tryptic peptide (residues 1–13, see section 1) eluted at 21% acetonitrile and FABMS analysis of this region revealed molecular ions ($[M + H]^+$) of 1512 and 1432 corresponding to monophosphorylated and dephosphorylated derivatives. No diphosphorylated derivtive ($[M + H^+]$ = 1592) was detectable in earlier eluting fractions by either FABMS or amino acid analysis. The region containing the phosphopeptide and dephosphopeptide (21.0–22.6% acetonitrile) was pooled, dried and rechromatographed at pH 6.5. This procedure resolved and purified the two derivatives (fig.2) which were identified by FABMS. Phosphorylation stoichiometries are presented in table 1.

The ^{32}P-labelled 'marker' corresponding to the site 2 tryptic phosphopeptide eluted at 30.7% acetonitrile. FABMS analysis revealed than the phosphopeptide RVS(P)FADNFGFNLVSVK ($[M + H]^+ = 1880$) coeluted with the dephosphopeptide VSFADNFGFNLVSVK ($[M + H]^+ = 1644$), an analogous result to that obtained with the site 1 tryptic peptide [5]. A second form of the dephosphorylated peptide was also present ($[M + H]^+ = 1800$), which contained an additional arginine at its N-terminus, and eluted later at 32.0% acetonitrile. The entire region from 30.7–32.0% acetonitrile was pooled, reduced to ≈ 0.2 ml and chromatographed at pH 6.5. This procedure resolved and purified the phosphopeptide and both dephosphopeptides (fig.3), which were identified by FABMS. Phosphorylation stoichiometries are presented in table 1.

The tryptic phosphopeptide containing site 1 is phosphorylated in vivo [5] on the same serine that is phosphorylated in vitro by PKA [4]. In order to determine which serines in the site 2 and site (3a + 3b) tryptic peptides were phosphorylated, phosphoserine

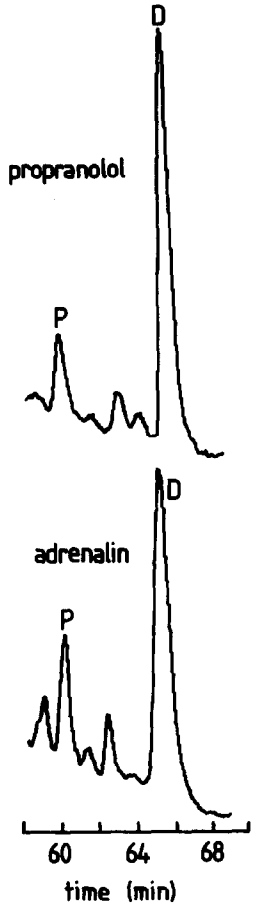

Fig.2. Reverse-phase chromatography at pH 6.5 of the tryptic peptides containing sites 3a and 3b. The peptide from propranolol (upper trace) or adrenaline (lower trace) treated animals were resolved into phospho- (P) and dephospho- (D) forms (detected by monitoring absorbance at 214 nm) which were eluted at 20.3% and 22.2% acetonitrile, respectively. Further details are given in sections 2.3 and 3.1. The unmarked peaks are unrelated peptides.

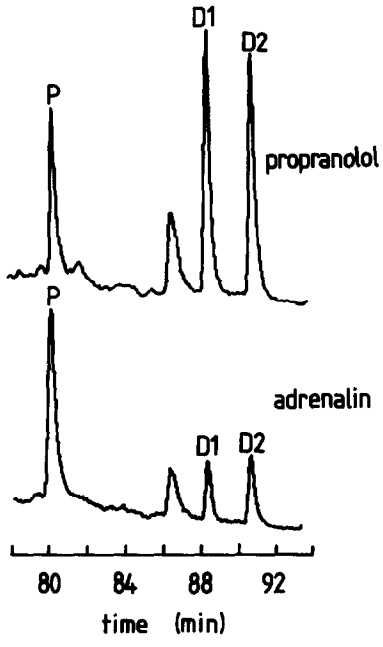

Fig.3. Reverse-phase chromatography at pH 6.5 of the tryptic peptides containing site 2. The peptides from propranolol (upper trace) or adrenaline (lower trace) treated animals was resolved into 3 components. The monophosphorylated derivative (P) and two dephosphorylated peptides D1 and D2 (detected by monitoring absorbance at 214 nm), were eluted at 26.7%, 29.3% and 30.2% acetonitrile, respectively. Peptide D2 contains an additional arginine residue at its N-terminus compared to D1. Further details are given in sections 2.3 and 3.1. The unmarked peaks are unrelated peptides.

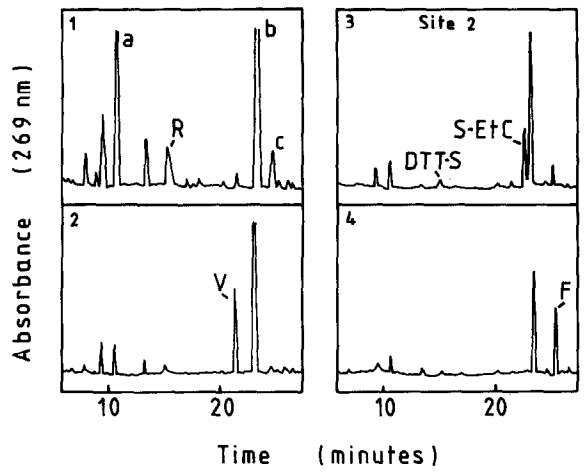

Fig.4. Sequence analysis of the monophosphorylated site 2 tryptic peptide after incubation with NaOH and ethanethiol (section 2.3). The peptide (residues 33–48, section 1) isolated from propranolol-treated animals was incubated for 1 h with ethanethiol, dried and the residue washed twice with water, redissolved in 0.1% (v/v) trifluoroacetic acid containing 50% acetonitrile and analysed on the gas phase sequencer. The figure shows reverse-phase chromatograms of Pth derivatives corresponding to the first 4 cycles. The amount applied to the sequencer was 275 pmol. The absorbance scale is 0–0.03. The phenylthiohydantoin derivative of serine was detected as its dithiothreitol adduct (DTT-S). Other abbreviations: R, Pth-arginine; V, Pth-valine; S-EtC, Pth-S-ethylcysteine; F, Pth-phenylalanine; a,b and c are dimethylphenylthiourea, diphenylthiourea and diphenylurea, respectively.

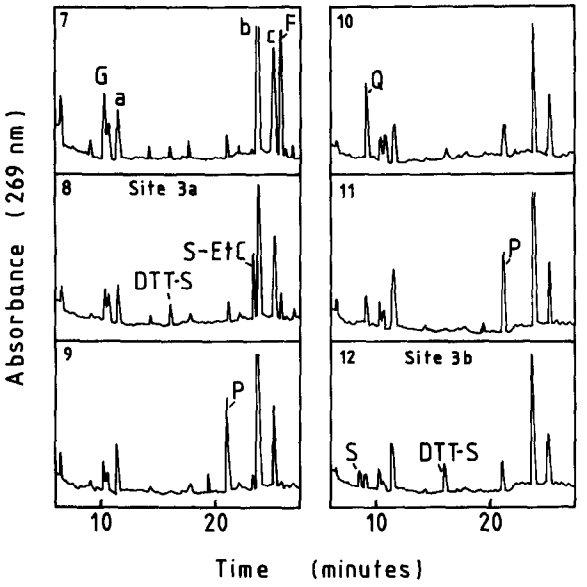

Fig.5. Sequence analysis of the monophosphorylated tryptic peptide containing sites 3a and 3b after incubation with NaOH and ethanethiol (section 2.3). The peptide (residues 1–13, section 1) isolated from propranolol-treated animals was incubated for 6 h with ethanethiol. The figure shows reverse-phase chromatograms of Pth derivatives corresponding to residues 7–12. The amount applied to the sequencer was 600 pmol and the absorbance scale is 0–0.005. Abbreviations: G, Pth-glycine; P, Pth-proline; Q, Pth-glutamine; S,Pth-serine. The glycine present at residue 7 is carryover from the previous cycle. Further details and other abbreviations are given in the legend to fig.4.

residues in the monophosphorylated derivatives (figs.2,3) were converted to *S*-ethylcysteine [12,13] and sequenced. After the third cycle of Edman degradation the site 2 peptide from propranolol (fig.4) and adrenaline (not shown) treated animals revealed the presence of Pth-*S*-ethylcysteine, while only traces of Pth-Ser (dithiothreitol adduct), no higher than at the previous cycle, were present (fig.4). At the 14th cycle Pth-Ser and its dithiothreitol adduct were present but no Pth-*S*-ethylcysteine was found (not shown). Similar analysis of the tryptic peptide containing sites 3a and 3b, established that only site 3a was phosphorylated (fig.5). The phosphoserine residue at site 3a was very resistant to β-elimination of the phosphate moiety, and a 6 h (instead of the usual 1 h) reaction was required for conversion to *S*-ethylcysteine explaining why small amounts of Pth-Ser (dithiothreitol adduct) were observed at this cycle. The resistance of phosphoseryl-proline bonds to alkaline hydrolysis has been noted previously [14].

4. DISCUSSION

A procedure has been devised that allows the in vivo phosphorylation states of 4 potential phosphoserine residues on the G-subunit to be quantitated. Phosphopeptides and their dephosphorylated counterparts are copurified through gel filtration at pH 8 and chromatography on a C_{18} column at pH 1.9, and finally resolved by chromatography on the C_{18} column at pH 6.5. The phosphorylation stoichiometries (table 1) assume that recoveries of phosphopeptides are identical to dephosphopeptides through all steps of purification and that the presence of fluoride and inorganic pyrophosphate prevents any dephosphorylation of the G-subunit during its isolation [5]. The latter assumption is clearly valid, at least for site 1 and site 2, since near stoichiometric phosphorylation of these serine residues is observed in response to adrenaline (table 1).

Recent in vitro studies have established that phosphorylation of the G-subunit by PKA is accompanied by dissociation of the C-subunit, the extent of dissociation correlating with the state of phosphorylation of site 2, but not site 1 [3]. Intravenous injection of adrenaline promotes dissociation of the G and C subunits in vivo, increasing (from 35 to 70%) the proportion of PP1 activity remaining in the cytosol after sedimentation of glycogen particles [5,15]. The proportion of PP1 in cytosol correlates well with the in vivo phosphorylation state of site 2, but not site 1 (table 1). A perfect correlation would not be expected since 100% of the $PP1_G$ in muscle extracts does not sediment with glycogen [5], and small amounts of other active forms of PP1, distinct from $PP1_G$, might also be present in the extracts. The free C subunit has $\approx$5-fold lower activity than $PP1_G$ towards glycogen phosphorylase and glycogen synthase when assayed at physiological ionic

strength and under conditions where both $PP1_G$ and these two substrates are bound to glycogen [16]. Thus subunit dissociation is a mechanism for preventing the dephosphorylation of glycogen-bound substrates in response to adrenaline [16].

Although phosphorylation of site 2 triggers subunit dissociation, a role for site 1 phosphorylation in this process is not yet excluded [6]. Site 1 has a further function in permitting phosphorylation of sites 3a and 3b by GSK3 in vitro [9,10]. The present study has established that one of the serines phosphorylated by GSK3 (site 3a) is also phosphorylated in vivo, but the stoichiometry seems to be low and does not alter in response to adrenaline, despite a high level of site 1 phosphorylation [5], table 1). The high phosphorylation of site 1 in the absence of adrenaline may be explained by its resistance to dephosphorylation [3] and/or phosphorylation by a protein kinase distinct from PKA. We are now studying the effects of site 1, 3a and 3b phosphorylation in vitro on activity and how insulin alters their phosphorylation in vivo.

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