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Evidence for new phosphorylation sites for protein kinase C and cyclic AMP-dependent protein kinase in bovine heart 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase

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Bovine heart 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) was phosphorylated by incubation with [γ-^{32}P]MgATP and cyclic AMP-dependent protein kinase (PKA) or protein kinase C (PKC). After digestion with chymotrypsin, the phosphorylation sites for the two protein kinases were identified by peptide mapping, and microsequencing. Evidence for new phosphorylation sites for PKA (Ser-483) and PKC (Ser-84 and Ser-466) was obtained.

6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase; Phosphorylation; Bovine heart

1. INTRODUCTION

The bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) catalyses the synthesis and degradation of fructose 2,6-bisphosphate (Fru-2,6-P_2), which is an important regulator of glycolysis [1,2]. The liver (L-type) and heart (H-type) isozymes, but not the muscle (M-type) isozyme, are substrates of the cyclic AMP-dependent protein kinase (PKA) [3].

Phosphorylation by PKA has different effects on the activities of the L- and H-type isozymes: phosphorylation of the L-type leads to PFK-2 inactivation (increase in K_m^{app} for fructose 6-phosphate (Fru-6-P) and decrease in V_{max}) and FBPase-2 activation (increase in V_{max}) [4,5], whereas phosphorylation of the H-type results in a small activation of PFK-2 (decrease in K_m^{app} for Fru-6-P) with no effect on FBPase-2 activity [6,7]. H-Type PFK-2/FBPase-2 is also a substrate of protein kinase C (PKC), but phosphorylation has no effect on its enzyme activities [7,8].

In rat liver PFK-2/FBPase-2, the phosphorylation site for PKA lies in the N-terminal domain at Ser-32 [9], while for bovine heart PFK-2/FBPase-2 the phosphorylation sites for PKA and PKC are in the C-terminal domain at Ser-466 and Thr-475, respectively [10]. Two forms of PFK-2/FBPase-2 have been identified in bovine heart with subunit M_rs of 58,000 and 54,000 [6,7,11], which result from alternative splicing of the same primary transcript [7]. The 54,000 M_r form of bovine heart PFK-2/FBPase-2 differs from the 58,000 M_r form in that it lacks a C-terminal peptide (residues 451–510), which is encoded by exon 15 in the rat gene [12] and which contains the phosphorylation sites for PKA and PKC; however, since the 54,000 M_r form is also phosphorylated by both PKA and PKC [7], phosphorylation sites other than Ser-466 (PKA) and Thr-475 (PKC) should be present in this form. In this paper we present evidence for new phosphorylation sites for PKA and PKC in bovine heart PFK-2/FBPase-2.

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Abbreviations: FBPase-2, fructose-2,6-bisphosphatase (EC 3.1.3.46); Fru-6-P, fructose 6-phosphate; Fru-2,6-P_2, fructose 2,6-bisphosphate; PFK-2, 6-phosphofructo-2-kinase (EC 2.7.1.105); PKA, cyclic AMP-dependent protein kinase (EC 2.7.1.37); PKC, protein kinase C (EC 2.7.1.37); PTH, phenylthiohydantoin.

2. EXPERIMENTAL

Bovine heart PFK-2/FBPase-2, PKA, and PKC were purified as described previously [7,8]. One unit of enzyme activity is the amount which catalyses the formation of 1 μmol of product/min under the assay conditions.

The bovine heart PFK-2/FBPase-2 preparation (100 μg) was incubated in a final volume of 0.2 ml with 50 μM [γ-^{32}P]MgATP and 1 mU of either PKA or PKC for 60 min as described in [7]. Proteins were precipitated by 10% (w/v) trichloroacetic acid and the mixture was allowed to stand in ice for 30 min. The precipitated proteins were collected by centrifugation (5,000 rpm for 5 min in an Eppendorf microfuge), washed twice with 1 ml of ice-cold acetone and dissolved

in 0.2 ml of 2 M urea, 100 mM NH_4HCO_3, pH 8.0. The phosphorylated proteins were then digested with 2.5 μg of chymotrypsin for 4 h at 35°C.

Peptides were first separated on a Vydac C18 column (0.46 × 25 cm) equilibrated in 0.1% (v/v) trifluoroacetic acid (solvent A) using the following gradient program: 5–100% B in 100 min (where B = 0.1% (v/v) trifluoroacetic acid/70% (v/v) CH_3CN) at a flow rate of 1 ml/min. Fractions (1 ml) were collected and those containing radioactivity were lyophilised. Radiolabelled peptides were dissolved in 200 μl of 0.1% (v/v) trifluoroacetic acid and purified by narrowbore HPLC as described in [7]. Where necessary, radiolabelled peptides were further purified by narrowbore HPLC in which the aqueous phase was 10 mM sodium phosphate, pH 6, and the organic phase was 10 mM sodium phosphate, pH 6, 60% CH_3CN [7]. Purified radiolabelled peptides were sequenced using an Applied Biosystems 477A pulsed liquid-phase sequenator equipped with a 120A PTH analyser.

Peptides, TKASLRISEK [13], KKCSWASYM [13] and YRRQAVKSYK [14], corresponding to residues 17–26, 26–34 and 77–86 of bovine heart PFK-2/FBPase-2, were synthesized as described. The peptides were phosphorylated with PKA and PKC as described in Table II. K_m^{app} values were calculated by non-linear least squares fitting of the data to a hyperbola using a computer program [15].

3. RESULTS AND DISCUSSION

The purified preparation of bovine heart PFK-2/FBPase-2, which contains a mixture of the 58,000 and 54,000 M_r forms, was phosphorylated from [γ^{32}P]MgATP by either PKC or PKA and digested with chymotrypsin. Fig. 1 shows the elution profile of radioactivity obtained by HPLC separation of chymotryptic peptides. PKC treatment led to the labelling of three peaks (peaks I–III) while PKA treatment led to the labelling of two peaks (peaks IV and V). The labelled peaks were further purified by narrowbore HPLC using

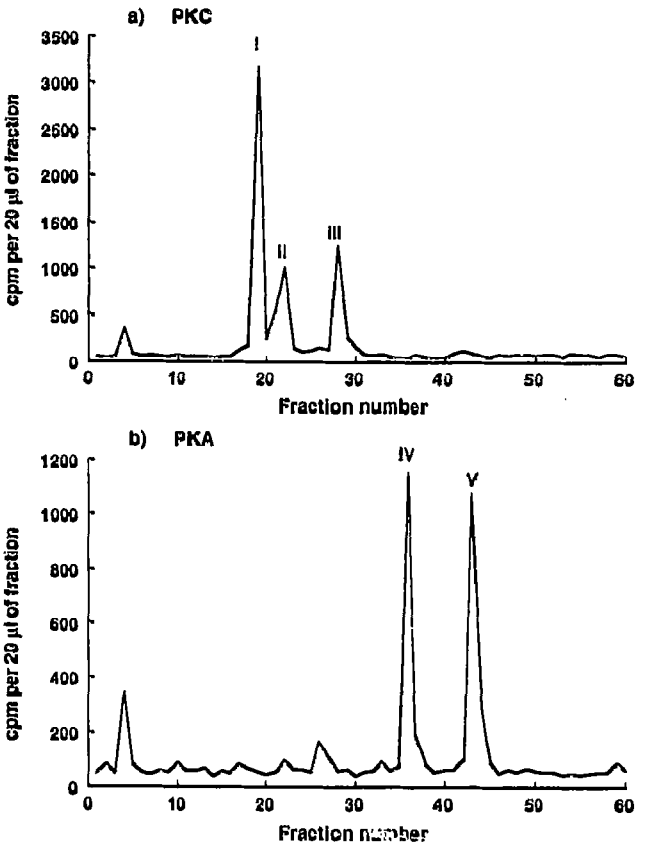

Fig. 1. HPLC of chymotryptic fragments of bovine heart PFK-2/FBPase-2 labelled with [γ-^{32}P]MgATP and either PKC or PKA. Bovine heart PFK-2/FBPase-2 was incubated with [γ-^{32}P]MgATP and either PKC (a) or PKA (b) for HPLC as described in section 2. Fractions (1 ml) were collected and aliquots (20 μl) were counted for radioactivity.

Table 1

Amino acid sequences of phosphorylated peptides purified from bovine heart PFK-2/FBPase-2 incubated with [γ-^{32}P]MgATP and either PKC or PKA

	Peak	Amino acid sequence of purified phosphorylated peptide	Position in bovine heart PFK-2/FBPase-2 sequence
PKC	I	R(19.7), R(13.9), Q(31.1), A(37.9), V(33.6), K(19.3), S(1.7), Y(8.6)	78–85
	ΔS/S	8.0	
	II	X, S(4.4), S(3.2), N(8.6), X, I(7.8), R(2.8), R(3.1), P(6.4), R(2.7), N(5.1), Y(1.8)	471–482
	ΔS/S	1.2 1.6	
	III	R(18 8), R(11.3), N(14.7), S(1.7), F(13.9), T(3.3), P(2.4), L(1.6)	463–470
	ΔS/S	4.7	
PKA	IV	R(2.9), R(3.3), N(12.0), S(0.5), F(19.9), T(5.0), P(5.7), L(3.2)	463–470
	ΔS/S	14.0	
	V	X, V(15.9), G(22.0), S(4.1), R(4.4), P(6.2), L(3.4)	483–489
	ΔS/S	2.0	

^{32}P-Labelled peptides from peaks I–V in Fig. 1 were purified as described for microsequencing. X represents a residue which could not be identified. ΔS/S represents the ratio of dehydroalanine/serine, calculated from the peak heights of their PTH derivatives. A low yield of PTH serine and a high ΔS/S ratio is indicative of a phosphorylated serine residue. PTH amino acid yields in pmol are given in parentheses.

Table II

Phosphorylation of synthetic peptides corresponding to sequences of bovine heart PFK-2/FBPase-2 by PKA and PKC

		Peptide sequence		
		TKASLRISEK	KKCSWASYM	YRRQAVKSYK
K_m^{app} (μM)	PKA	N.M.	640	N.M.
	PKC	810	2910	54
Stoichiometry of phosphorylation (mol/mol)	PKA	0.06	0.73	0.01
	PKC	0.73	0.20	0.35

The synthetic peptides were phosphorylated as described in [7]. For the measurements of K_m^{app}, 0.2 mU of protein kinase was used and the concentration of peptide was varied from 0.01–5 mM. The stoichiometry of peptide phosphorylation was measured with 50 μM peptide and 2 mU of protein kinase. N.M., not measured.

a linear gradient of acetonitrile as described in section 2. Table I shows the sequences of the radiolabelled peptides from peaks I–V. For each peak of radioactivity in Fig. 1 a single labelled peptide was isolated.

Table I shows that, as expected, PKC treatment led to the labelling of a peptide containing Thr-475, the phosphorylation site in the C-terminal domain described by Uyeda and co-workers [6,10]. Thr-475 was not detected in the PTH analyser, because it was phosphorylated. PKC treatment also led to the labelling of a peptide whose sequence was RRNSFTPL containing Ser-466 (Table I), the site previously shown to be phosphorylated by PKA [6,10].

In a previous study, we found that the 54,000 M_r form of bovine heart was phosphorylated by both PKA and PKC, suggesting the existence of other phosphorylation sites, and microsequencing revealed the three following peptides: 19-ASLR-22, 80-QAVKSYK-86 and 57-KLTR-60, but the phosphorylation site could not be identified [7]. Our present results show that treatment of bovine heart PFK-2/FBPase-2 with PKC led to the labelling of a chymotryptic peptide, 78-RRQAVKSY-85 (Table I), which overlaps with the sequence of the tryptic peptide, 80-QAVKSYK-86, previously described [7]. A synthetic peptide containing Ser-84 was phosphorylated by PKC with a low K_m^{app}, whereas a peptide with the sequence ASLR, containing Ser-20, which

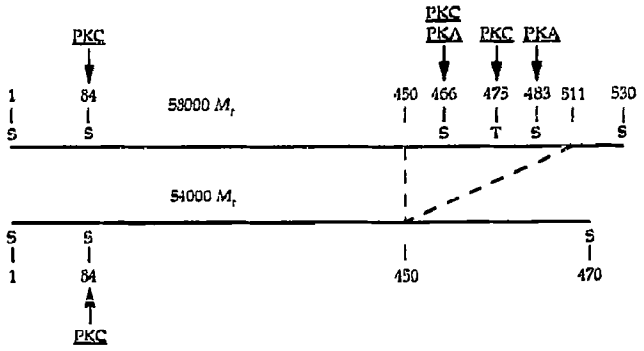

Fig. 2. Schematic representation of the phosphorylation sites in the two forms of bovine heart PFK-2/FBPase-2.

was previously identified as a potential phosphorylation site for PKC [7], was a poor substrate (Table II). Therefore, Ser-84 is the phosphorylation site for PKC in the 54,000 M_r form (Fig. 2).

PKA treatment of bovine heart PFK-2/FBPase-2 led to the labelling of two peptides (Table I), one of which contained Ser-466, the PKA phosphorylation site in the C-terminal domain described by Uyeda and co-workers [6,10]. The sequence of the other labelled peptide was 483-XVGSRPL-489. From the published amino acid sequence, X corresponds to Ser-483 and lies in a favourable consensus sequence (RNYS-483) for phosphorylation by PKA [16]. Both Ser-466 and Ser-483 are located in the C-terminal peptide, which is deleted from the sequence of the 54,000 M_r form of bovine heart PFK-2/FBPase-2. Since, the 54,000 M_r form was phosphorylated by PKA, albeit with low stoichiometry, another phosphorylation site for PKA should exist outside the C-terminal peptide containing Ser-466 and Ser-483 [7]. Ser-29 is a potential phosphorylation site for PKA, since it is flanked on its N-terminal side by two basic lysine residues, however, a synthetic peptide containing Ser-29 was a poor substrate for PKA (Table II). The fact that Ser-29 is a poor substrate might be explained by the presence of the bulky C-adjacent tryptophan residue.

Bovine heart PFK-2/FBPase-2 is a multi-site phosphorylated enzyme (Fig. 2). PKC phosphorylates Thr-475 and Ser-84, whereas PKA phosphorylates Ser-466 and Ser-483. The interactions between the phosphorylation sites are currently being studied.

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