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Phosphorylation of the phosphatase modulator subunit (inhibitor-2) by casein kinase-1

Identification of the phosphorylation sites

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The isolated modulator subunit of the inactive protein phosphatase-1 is phosphorylated in vitro by casein kinase-1 at two different sites: Ser-86 and Ser-174. The Ser-86 site is a common target for casein kinase-1 and casein kinase-2, but is preferentially phosphorylated by the former enzyme. The Ser-174 site seems to be specific for casein kinase-1, and is phosphorylated at a slower rate. These results give a new insight into the in vitro phosphorylation pattern of the modulator subunit of the phosphatase and provides additional data on the specificity of casein kinase-1.

Phosphatase modulator; Inhibitor-2; Casein kinase-1; Casein kinase-2; Phosphorylation site

1. INTRODUCTION

A cytosolic inactive form of protein phosphatase-1 results from the association of the 37 kDa catalytic subunit (C) with a 23 kDa modulator subunit (M), which is identical to the heat-stable phosphatase inhibitor-2. The activation of this enzyme, which is also referred to as the ATP,Mg-dependent protein phosphatase (F_CM), is triggered by the phosphorylation of the M subunit by the kinase F_A/GSK_3 at Thr-72 (reviewed in [1–3]). This process is potentiated by a prephosphorylation of the M subunit on Ser residues by casein kinase-2 (CK-2) [4,5], a pleiotropic protein kinase independent of second messengers which is involved in a variety of cellular functions [6]. Three Ser residues in the modulator were found to be phosphorylated in vivo [7]: Ser-86, which is rather poorly phosphorylated by CK-2 in vitro, and the two adjacent amino acid residues Ser-120 and Ser-121, which are the preferred CK-2 targets [8].

Casein kinase-1 (CK-1), an ubiquitous enzyme [9] belonging to a rather atypical branch of the protein kinase family [10], readily phosphorylates the M subunit in vitro and this phosphorylation also promotes the subsequent phosphorylation of Thr-72 by the kinase F_A/GSK_3 [11]. Surprisingly, the CK-1-mediated phosphorylation of the F_CM enzyme prevents the subsequent activation of the phosphatase by the kinase F_A/GSK_3 [11], in sharp contrast to the stimulating effect that CK-2 has on this process. This led us to speculate that CK-1 and CK-2 work synergistically with the kinase F_A/GSK_3 by phosphorylating a common site in the M protein, while CK-1 must also affect residue(s) that are not recognized by CK-2 and the phosphorylation of which is detrimental to the activation of the phosphatase [1,11].

The present report confirms this hypothesis by showing that two serine residues of the modulator are phosphorylated by CK-1: Ser-86 and Ser-174. The Ser-174 site is not a target for any other known protein kinase.

Abbreviations: CK-1, casein kinase-1; CK-2, casein kinase-2; kinase F_A/GSK_3, kinase F_A/glycogen synthase kinase 3; M, modulator subunit; (F_CM), inactive ATP, Mg-dependent protein phosphatase (protein phosphatase 1I); PTH, phenylthiohydantoin; PTC, phenylthiocarbamoyl.

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2. EXPERIMENTAL

2.1. *Materials*

The M subunit was purified from rabbit skeletal muscle as described in [12]. CK-1 was obtained either from rat liver or porcine spleen cytosols according to [13] with the exception that a further phosvitin-Sepharose 4B column was included as a final purification step in the enzyme preparation from porcine spleen. CK-2 was also purified from porcine spleen cytosol essentially as in [13] with the inclusion of a final ion-exchange chromatography on a Mono Q FPLC column (Pharmacia). CK-1 and CK-2 had a specific activity of about 360 U/mg and 880 U/mg, respectively; one unit of casein kinase activity incorporates 1 nmol phosphate per min in casein (2 mg/ml) at 37°C. [γ-^{32}P]ATP was purchased from Amersham International, Dowex 1-X8 ion-exchange resin from Bio-Rad and trypsin from Fluka.

2.2. *Phosphorylation of the modulator*

The modulator (0.05–0.5 mg) was phosphorylated for the indicated time, by either 0.57 U of CK-1 or 0.54 U of CK-2 in the presence of 100 μM [γ-^{32}P]ATP (specific activity 100–500 cpm/pmol) as described [11]. The reaction was stopped by adding 30% (v/v) acetic acid (final concentration) and the [γ-^{32}P]ATP separated from the labelled modulator through 1 ml of Dowex 1-X8 anion exchanger equilibrated in 30% acetic acid according to [14]. Alternatively, for the determination of the kinetic parameters of CK-1 phosphorylation, after 15 min of incubation at 30°C, 25 μl out of 30 μl of the phosphorylation mixture, were spotted onto P-81 (Whatman) papers and processed as described in [15].

2.3. *Proteolytic digestion of the ^{32}P-labelled modulator and purification of the peptides by HPLC*

^{32}P-labelled M phosphorylated by CK-1 (5 or 60 min) or CK-2 (60 min), was digested with tosylphenylchloromethyl ketone-treated trypsin in 0.2–0.4 ml of 0.1 M NH_4HCO_3 pH 7.8 at 37°C for 12 h at a weight ratio trypsin/modulator of 1:15 and then for another 4 h at a weight ratio trypsin/modulator of 1:5. The digests were dried in a vacuum concentrator, dissolved at 4°C overnight in 0.1 ml of 0.1% (v/v) trifluoroacetic acid (HPLC purity grade) and fractionated on a SuperPac Pep-S (Pharmacia) C_2/C_{18} reverse-phase column (4 × 250 mm, 5 μm particle size) connected to a Perkin-Elmer 410 LC BIO HPLC system. The column was eluted with a 200 min linear gradient from 0% to 50% B (A: 0.1% (v/v) trifluoroacetic acid in water, B: 0.08% (v/v) trifluoroacetic acid in acetonitrile) with a flow rate of 1 ml/min. Peptides were detected at 220 nm and the radioactivity determined in an aliquot of the fraction.

2.4. *Amino acid- and sequence analysis*

The peptides were sequenced on an Applied Biosystems (Foster City, CA, USA) 470A gas phase sequencer equipped with the 120A analyser for on line PTH-amino acid detection. All reagents were supplied by Applied Biosystems. Amino acid analysis was performed with the 420A derivatizer/hydrolyser with the 130A analyser for PTC-amino acid detection. Phosphorylated Ser was identified by a drop in the yield of PTH-Ser. The amino acid analysis of the peptides indicated that they had been sequenced through to the end. The peptide K1B was probably produced by a residual chymotryptic activity contaminating the trypsin.

3. RESULTS

The isolated modulator is an excellent substrate for CK-1, exhibiting an apparent K_m value of 3 μM and incorporating between 1 and 2 mol phosphate per mol of M, exclusively on Ser residues (not shown).

After 60 min of phosphorylation in the presence of [γ-^{32}P]ATP, the radiolabelled modulator (1 mol ^{32}P/mol M) was digested with trypsin and the resulting peptides were resolved by reverse-phase HPLC. As shown in Fig. 1A, equal amounts of two major radioactive peptides termed K1A and K1B, were detected in the fractions eluting at retention times of 65 and 80 min, respectively. The K1B site is phosphorylated faster than K1A fragment since at shorter incubation times (5 min), most of the radioactive label was found to be associated with this peak (Fig. 1B). The elution of K1B coincides with K2B, one of the two major radiolabelled tryptic peptides generated from M phosphorylated by CK-2 (Fig. 1C); the other CK-2 generated phosphopeptide (K2A) which elutes earlier than K1A, was not radiolabelled to any detectable extent by CK-1.

In order to identify the amino acid residues phosphorylated by CK-1, the K1A and K1B peptides were subjected to automated microsequencing. The amino acid sequence of K1A (Table 1A) corresponds exactly to that of the 35 residues of the predicted tryptic fragment encompassing residues 163 to 197 of M (including 5 Ser residues). The dramatic drop in the yield of PTH-serine specifically occurring at the 12th cycle indicates that Ser-174 is phosphorylated. The acidic cluster upstream of Ser-174, representing a known con-

Table 1

Amino acid sequence of the tryptic phosphopeptide K1A and K1B from the modulator phosphorylated by CK-1

(A) The peptide K1A isolated by reverse-phase HPLC (see Fig. 1A) was analyzed for its amino acid sequence as described in the experimental section. An asterisk denotes the phosphorylated residue as deduced by the drop in the yield of the PTH-amino acid derivative

Cycle	Amino acid	Yield (pmol)	Cycle	Amino acid	Yield (pmol)
1	Asp	83	19	Ser	6
2	Leu	155	20	Met	12
3	His	70	21	Asn	9
4	Asp	86	22	Thr	5
5	Asp	93	23	Glu	13
6	Glu	59	24	Glu	16
7	Glu	90	25	Ser	4
8	Asp	57	26	Asn	7
9	Glu	69	27	Gln	7
10	Glu	84	28	Gly	21
11	Met	44	29	Ser	4
12	Ser*	5	30	Thr	3
13	Glu	39	31	Pro	5
14	Thr	11	32	Ser	3
15	Ala	23	33	Asp	4
16	Asp	19	34	Gln	5
17	Gly	34	35	Arg	1
18	Glu	21			

(B) The peptide K1B isolated by reverse-phase HPLC (see Fig. 1A) was analyzed for its amino acid sequence.

Cycle	Amino acid	Yield (pmol)	Cycle	Amino acid	Yield (pmol)
1	His	55	14	Thr	6
2	Ser	29	15	Glu	6
3	Met	37	16	Thr	6
4	Ile	35	17	Thr	6
5	Gly	40	18	Glu	6
6	Asp	22	19	Ala	6
7	Asp	29	20	Met	3
8	Asp	29	21	Thr	2
9	Asp	29	22	Pro	7
10	Ala	20	23	Asp	4
11	Tyr	14	24	Thr	2
12	Ser*	2	25	Leu	6
13	Asp	11	26	Ala	3
			27	Lys	--

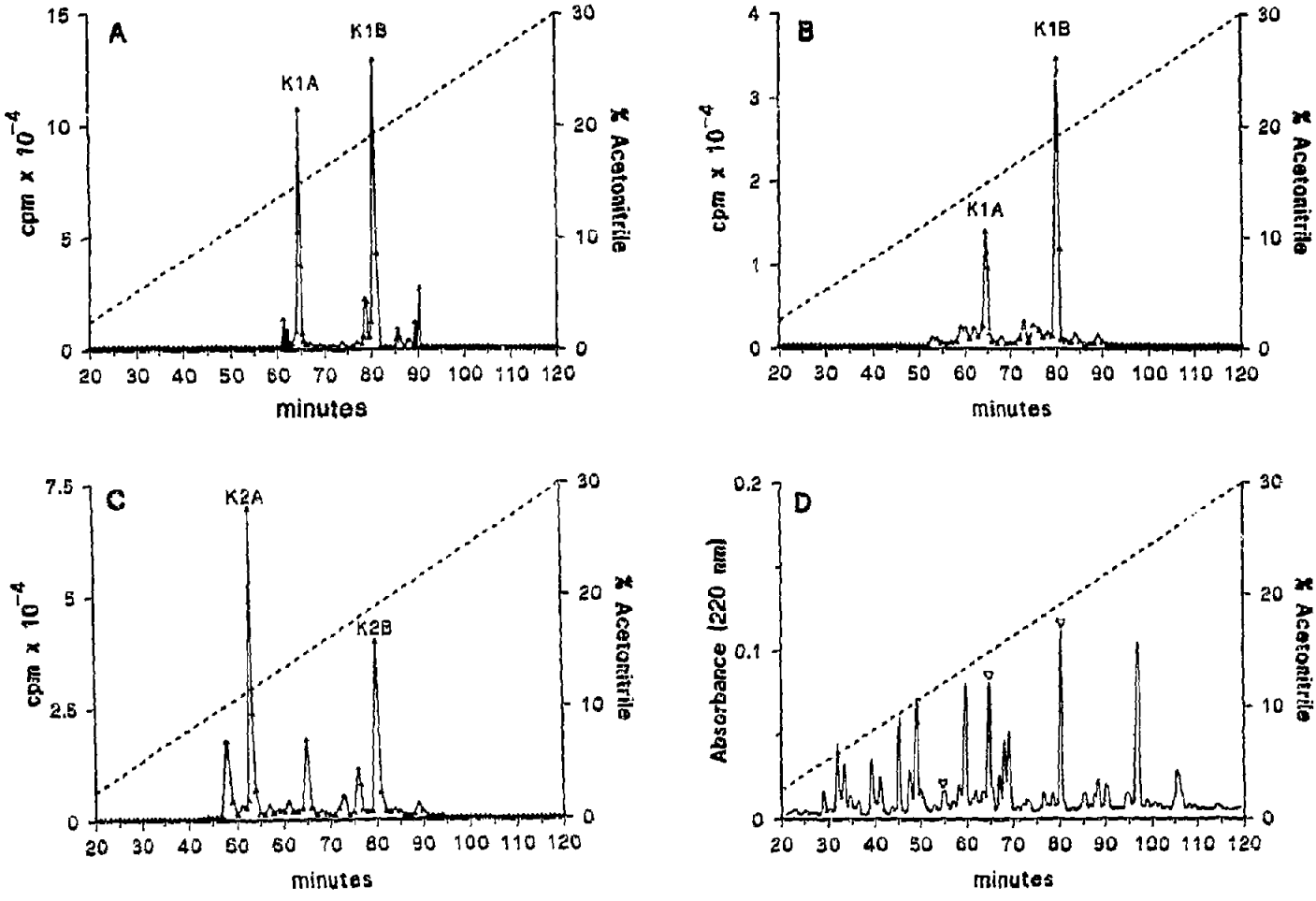

Fig. 1. Comparative separation by reverse-phase HPLC of the tryptic peptides from ^{32}P-labelled modulator. The column (SuperPac Pep-S C_2/C_{18} reverse phase, Pharmacia) was eluted with a linear acetonitrile gradient (broken line) as described in section 2. (A–C) ^{32}P-labelled peptides. (A) ^{32}P-labelled modulator (24 μM) phosphorylated by CK-1 for 60 min. An identical chromatogram was obtained with a modulator concentration of 11 μM; (B) ^{32}P-labelled modulator (11 μM) phosphorylated by CK-1 for 5 min, or (C) phosphorylated by CK-2 for 60 min; (D) full line shows absorbance at 220 nm. The elution position of the corresponding ^{32}P-labelled peptides is indicated by an inverted triangle.

sensus sequence for CK-1 [16] corroborates these results.

The sequence of K1B (Table 1B) overlaps perfectly with the segment 75–102 of M and contains the Ser-86 which is also a target for CK-2 [8]. The same peptide is also present in the CK-2 phosphorylated M digest (K2B). The drop in the yield of PTH-Ser at the 12th cycle supports the notion that Ser-86 is indeed also phosphorylated by CK-1.

Partial sequencing of the most prominent peptide radiolabelled by CK-2 (K2A) is quite consistent with its expected identification as the fragment 117–133, containing Ser-120 and Ser-121 which are the preferred in vitro targets for CK-2 [8]. This peptide was not radiolabelled by CK-1. Both serines seem to be phosphorylated by CK-2 as judged from the disappearance of PTH derivatives at the two degradation cycles (3rd and 4th) involving Ser-120 and Ser-121 (data not shown).

4. DISCUSSION

In conclusion, the results presented clearly show that two residues of the modulator are extensively phosphorylated by CK-1: Ser-86 which is also a target for CK-2, and the more C-terminal Ser-174, which is not appreciably phosphorylated by CK-2. The in vitro activation of the ATP,Mg-dependent phosphatase is strictly dependent upon the phosphorylation of Thr-72 of the M subunit by the kinase F_A/GSK$_3$ [1–3]. This Thr-72 phosphorylation is facilitated by a prephosphorylation of M by either CK-1 or CK-2, presumably at the Ser-86 site [1,4,5,11]. This would confirm an earlier report by DePaoli-Roach et al. who showed that site-directed mutagenesis of M, which eliminated the Ser-120/121 phosphorylation sites, resulted in a functional modulator subunit for the phosphatase, which still exhibited the synergistic phosphorylation by CK-2

and the kinase F_A/GSK_3 [17]. It is presently not known which kinase is responsible for the in vivo phosphorylation of the enzyme at this regulatory site, but its fast in vitro phosphorylation by CK-1, together with the observation that CK-2 preferentially phosphorylates the Ser-120/121 residues [8], raises the question as to whether CK-1, rather than CK-2, may be producing the synergistic effect with the kinase F_A/GSK_3 in the phosphorylation of the Thr-72 site of M, leading to the activation of the F_CM enzyme. The blocking of the activation of the F_CM enzyme by the kinase F_A/GSK_3 reported in [1,11] could be related to the slower but specific phosphorylation by CK-1 of a second site, at the more C-terminal Ser-174. There is presently nothing known about the possible effects that the phosphorylation of the Ser-120/121 (exclusive CK-2 sites) may have on the activity or the regulation of the phosphatase.

The identification of two new phosphorylation sites for CK-1 provides additional information about the specificity determinants for this enzyme. Both the Ser-86 and the Ser-174 sites feature a cluster of acidic amino acid residues close, albeit not adjacent, to the N terminal side of the target residue. In the case of Ser-86, four consecutive Asp residues encompass positions −6 to −3, while as many as seven acidic residues (3 Asp and 4 Glu) form an uninterrupted cluster between positions −8 and −2 of Ser-174. These observations corroborate the concept that the consensus sequence for CK-1 is generated by N-terminally located acidic amino acid residues [16]. An interesting feature of the modulator CK-1 sites however is the total absence of potentially phosphorylatable residues upstream of the target serine. PhosphoSer and/or -Thr residues at position −3 (or, less effectively −4) has been shown to play a crucial role in determining the efficiency of phosphorylation of peptide substrates by CK-1 [18–20]. The replacement of these phosphodeterminants with carboxyl groups invariably results in a dramatic drop in phosphorylation efficiency, as reflected by K_m values in the mM range [19–21]. The apparent K_m value of CK-1 for M (3 μM) is lower than for any peptide/protein CK-1 substrate known so far. The modulator protein could therefore prove to be an ideal model for deciphering the structural or conformational features that determine the efficient targeting of CK-1 to non-phosphorylated consensus sequences.

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