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Prothymosin α is phosphorylated by casein kinase-2

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Prothymosin α (ProTα) is a 12.5 kDa acidic polypeptide that is considered to have a nuclear function related to cell proliferation. Inspection of its amino acid sequence revealed the presence of sequences that may serve as targets for phosphorylation by casein kinase-2 (CK-2). ProTα isolated from calf thymocytes was phosphorylated in vitro by CK-2. The phosphorylation sites are Ser and Thr residues located among the first 14 amino acid residues in the ProTα sequence. Another site that is theoretically suitable for phosphorylation by CK-2, at the C-terminus of the polypeptide, is not, in fact, phosphorylated. Thymosin α_1 ($T\alpha_1$), a peptide whose sequence corresponds to the first 28 amino acids of ProTα, is also phosphorylated by CK-2 at the same phosphorylation sites as ProTα. In cultured splenic lymphocytes ProTα was phosphorylated at Thr residues located at positions 7, 12 and/or 13. Based on these observations we conclude that CK-2, or another cellular kinase with similar sequence specifity, is responsible for phosphorylation of ProTα in vivo.

Prothymosin α; Thymosin α_1; Casein kinase-2

1. INTRODUCTION

Prothymosin α is a 12.5 kDa acidic polypeptide that includes at its N-terminus the sequences of thymosin α_1 and several related peptides [1]. The primary structures of ProTα from diverse animal tissues are extremely similar [2], suggesting a high degree of conservation in the course of evolution.

Although α-thymosins were originally considered to be involved in the immune response [3], the structure of the ProTα gene [4] and the ubiquitous distribution of their putative precursor, ProTα, in animal tissues [5,6] suggest that ProTα and/or its derived peptides may have a more general cellular function that is still unknown. Recent studies point to a role for ProTα in cell proliferation [6–8], and the presence of the karyophilic signal, TKKQKT, at its C-terminus (Fig. 1) is in keeping with evidence of its interacting with the nucleus [9,10].

Reports concerning the involvement of casein kinase-2, a Ser/Thr kinase using both GTP and ATP as phosphate donors [11], in the phosphorylation of high-mobility group proteins [12] and in protein transport between the nucleus and cytoplasm [13], led us to realize that ProTα from calf thymus and other sources [2] has three sites that are suitable for phosphorylation by CK-2, two at the N-terminus and one at the C-terminus (see the calf thymocyte ProTα sequence shown in Fig. 1). The proven involvement of CK-2 in cell proliferation [14] also relates this enzyme to ProTα.

In view of the above, we have sought phosphoamino acids in the ProTα sequence and investigated the ability of CK-2 to phosphorylate ProTα in vitro. In this paper we show that ProTα from calf thymocytes is phosphorylated in vitro by rat liver CK-2 at Ser and Thr residues located among its first 14 amino residues. In cultured splenic lymphocytes, ProTα was phosphorylated only at Thr residues located in the same N-terminal fragment in which ProTα is phosphorylated by CK-2.

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Abbreviations: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; CK-2, casein kinase-2; ProTα, prothymosin α; $T\alpha_1$, thymosin α_1; HPLC, high performance liquid chromatography.

2. MATERIALS AND METHODS

2.1. *Materials*

The triethylammonium salts of adenosine 5′-[γ-^{32}P]triphosphate ([γ-^{32}P]ATP, 3,000 Ci/mmol) and of guanosine 5′-[γ-^{32}P]triphosphate ([γ-^{32}P]GTP, 6,000 Ci/mmol), and [^{32}P]orthophosphate (1 Ci/mmol) were purchased from Du Pont-New England Nuclear. Poly-L-lysine hydrobromide, dephosphorylated β-casein from bovine milk, alkaline phosphatase from bovine intestinal mucosa (type VII), protamine (grade IV) from salmon, heparin, concanavalin A and trypsin treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone, were purchased from Sigma. Cellulose thin-layer plates, 0.1 mm thick, were from E. Merck. Interleukin-2 was obtained from Boehringer-Mannheim. ProTα was purified from calf thymocytes as described [16]. Rat liver cytosol CK-2 was a gift from Dr. E. Itarte (University of Barcelona, Barcelona, Spain). Synthetic $T\alpha_1$ was a gift from Dr. E.P. Heimer (Hoffmann-La Roche, Nutley, USA). All other reagents and materials were of analytical grade.

2.2. *Phosphorylation of calf thymocyte ProTα and synthetic $T\alpha_1$*

The reaction mixture (25 μl) contained 50 mM Tris-HCl (pH 7.4), 1.6 mM EGTA, 26 mM $MgCl_2$, 0.1 mM [γ-^{32}P]ATP or [γ-^{32}P]GTP, 1.6 mM EDTA, 83 mM β-glycerolphosphate, 3.3 mM dithiothreitol and 150 mM KCl, in the presence or absence of ProTα (5 μg), $T\alpha_1$ (5

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μg) and/or rat liver CK-2 (0.1 U). After 30 min incubation at 37°C, the reaction was stopped with 5 μl of 100 mM ATP or GTP. The components of the reaction mixture were separated by SDS-PAGE following the Laemmli procedure [17], and the gels were stained, dried and autoradiographed.

2.3. *Analysis of phosphopeptides*

[^{32}P]ProTα and [^{32}P]Tα$_1$ were purified from the respective reaction mixtures by reverse-phase HPLC using a Vydac TP200-C18 column (5 μm, 4.6 × 250 mm) as described by Franco et al. [16]. Aliquots (180,000 cpm) of the purified [^{32}P]ProTα or [^{32}P]Tα$_1$ were digested with trypsin for 8 h at 37°C in the presence of 50 μg of unlabeled polypeptides. Separation of tryptic peptides was carried out by reverse-phase HPLC in an Ultrasphere ODS column (5 μm, 4.6 × 250 mm).

2.4. *Phosphoamino acid analysis*

^{32}P-Labeled tryptic peptides derived from [^{32}P]ProTα or [^{32}P]Tα$_1$ purified by HPLC were suspended in 500 μl of 6 N HCl, hydrolysed at 110°C for 2 h, diluted with water, lyophilized and dissolved in 20 μl of 50:5:945 acetic acid/pyridine/water (pH 3.5) (electrophoresis buffer) containing 0.5 μg/μl each of phosphoserine and phosphothreonine. This solution was subjected to one-dimensional thin-layer electrophoresis on cellulose plates for 1 h at 4°C and 1,000 V [18]. After electrophoresis the cellulose plates were dried, stained with ninhydrin and autoradiographed on X-ray film to detect ^{32}P-labeled amino acids.

2.5. *Phosphorylation of ProTα in vivo*

Splenic lymphocytes (14 × 10^7 cells) obtained from 45-day-old female BALB/c mice were cultured for 20 h at 37°C in a humified 5% CO_2 atmosphere in 20 ml of orthophosphate-free RPMI-1640 medium containing 10% of dialysed foetal calf serum, 6 μg/ml of concanavalin A, 10 U/ml of interleukin-2 and 50 μCi/ml of [^{32}P]orthophosphate. Isolation of [^{32}P]ProTα from splenic lymphocyte extracts was carried out using DEAE-cellulose chromatography and reverse-phase HPLC as described [16]. Analysis of phosphopeptides and phosphoamino acids of ProTα phosphorylated in vivo was carried out as indicated above.

3. RESULTS

3.1. *Phosphorylation of calf thymocyte ProTα by CK-2 and identification of the phosphorylation sites*

In view of the presence of possible phosphorylation sites in the ProTα sequence (Fig. 1), we first investigated whether ProTα purified from calf thymocytes without mitogenic stimulation contained phosphoryl groups bound to any of its Ser or Thr residues. Experiments to detect orthophosphate- bound Ser or Thr residues in ProTα treated with base [19] or after acid hydrolysis had negative results (data not shown). The possibility that ProTα might be a substrate for CK-2 was then investigated by incubation of calf thymocyte ProTα with CK-2 purified from rat liver cytosol, which has been found not to contain other protein kinases [15]. The results (Fig. 2) indicate that CK-2 is able to phosphorylate ProTα at a rate that is hardly affected by whether ATP or GTP is the co-substrate (Fig. 2, lanes 1 and 2). Like other authors [11], we found that the CK-2 β-subunit underwent autophosphorylation (Fig. 2, lane 3), but ProTα was not spontaneously phosphorylated (Fig. 2, lane 4). Treatment of the phosphorylated products with alkaline phosphatase removed all [^{32}P]phosphate bound

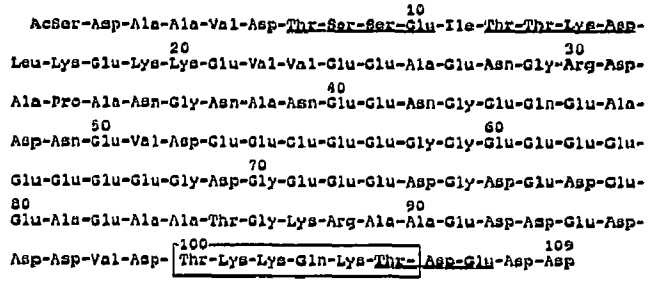

Fig. 1. Amino acid sequence of calf thymus ProTα [2]. Sites theoretically liable to phosphorylation by CK-2 are underlined. The box contains the nuclear targeting signal.

to ProTα and to the β-subunit of CK-2 (Fig. 2, lane 5). ^{32}P-Labeled ProTα purified by reverse-phase HPLC had the same retention time (data not shown) and electrophoretic mobility (Fig. 2) as unphosphorylated ProTα.

In order to determine the phosphorylation sites of ProTα, labeled and unlabeled polypeptide samples were digested with trypsin and the resulting peptides were separated by reverse-phase HPLC. The elution pattern (Fig. 3) showed phosphorylation of the N-terminal peptide (residues 1–14) of [^{32}P]ProTα, but no phosphorylated peptides were obtained from the C-terminal region where the other theoretical phosphorylation site for CK-2 is located (peptides 105–109 and 103–109 in Fig. 3). Thin-layer electrophoretic analysis of the acid hydrolysate of the N-terminal peptide purified by HPLC showed that Ser and Thr residues were phosphorylated by CK-2 (inset A in Fig. 3).

A similar phosphorylation pattern was observed among the tryptic peptides when synthetic Tα$_1$ (the first 28 amino acids of ProTα) was used as the substrate for CK-2 (Fig. 4). Analysis by thin-layer electrophoresis of the acid hydrolysate of the N-terminal peptide (residues 1–14) derived from tryptic digestion of Tα$_1$ indicated

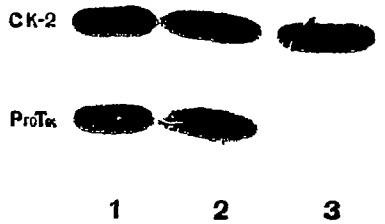

Fig. 2. Autoradiograph of products phosphorylated by CK-2 and separated by SDS-PAGE. Components in the phosphorylation reaction containing [γ-^{32}P]ATP (lanes 1, 3, 4, 5) or [γ-^{32}P]GTP (lane 2) as phosphate donors, lacking ProTα (lane 3) or CK-2 (lane 4), and that indicated in lane 1 after treatment as described [24] with alkaline phosphatase (lane 5), were subjected to SDS-PAGE and autoradiographed as indicated in Materials and Methods. CK-2 and ProTα mark the mobility of the [^{32}P]β-subunit of CK-2 and of calf thymocyte ProTα, respectively.

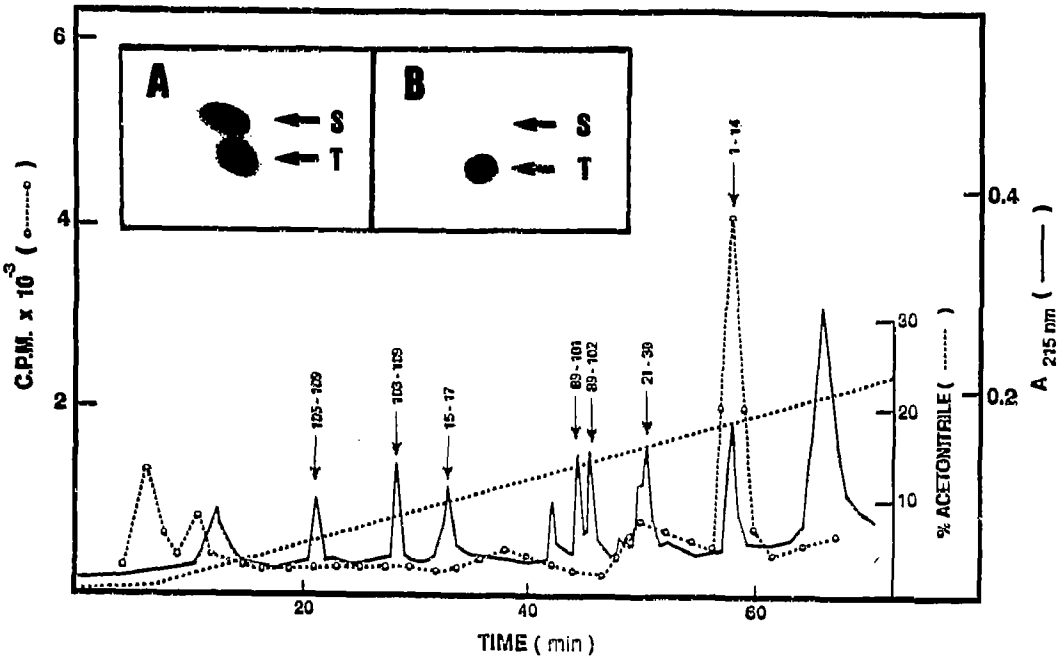

Fig. 3. HPLC of the peptides derived from the tryptic digestion of ^{32}P-labeled ProTα. Peptides from the tryptic digestion of [^{32}P]ProTα (180,000 cpm) and unlabeled ProTα (50 μg) were separated by reverse-phase HPLC as indicated in Materials and Methods. The discontinuous line indicates the programmed gradient of acetonitrile in 0.1% trifluoroacetic acid. Fractions of 0.7 ml were collected every minute and their radioactivity determined. Arrows indicate the sequence of tryptic peptides derived from calf thymus ProTα the structure of which was confirmed by amino acid composition analysis, as described [16]. The inset shows the phosphoamino acid analysis of the N-terminal tryptic peptide (residues 1–14) derived from [^{32}P]ProTα phosphorylated by CK-2 (panel A) and from [^{32}P]ProTα isolated from splenic lymphocytes labeled metabolically with [^{32}P]orthophosphate (panel B). Acid hydrolysates of the N-terminal peptide purified by HPLC (20,000 cpm) were separated by one-dimensional thin-layer electrophoresis and autoradiographed as indicated in Materials and Methods. The positions of phosphoserine (S) and phosphothreonine (T) are marked.

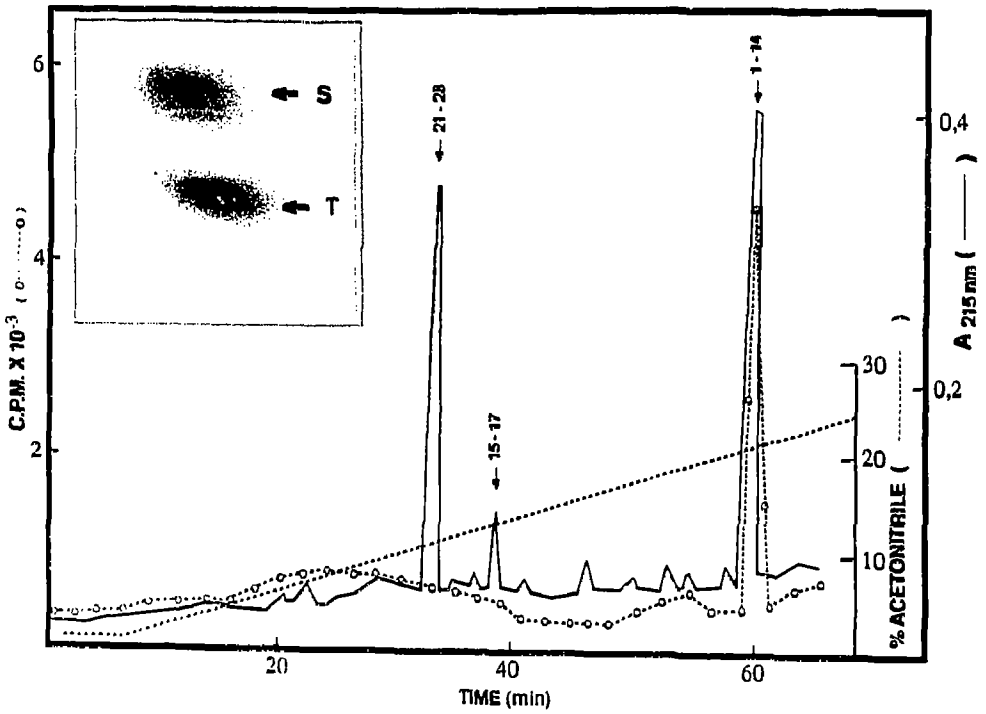

Fig. 4. HPLC of the peptides derived from tryptic digestion of ^{32}P-labeled Tα$_1$. Peptides from the tryptic digestion of [^{32}P]Tα$_1$ (180,000 cpm) and 50 μg of unlabeled Tα$_1$ were separated and analyzed as indicated in the legend of Fig. 3. Arrows indicate the sequence of tryptic peptides derived from synthetic Tα$_1$. The inset shows that the phosphoamino acid analysis of the N-terminal peptide (residues 1–14) of [^{32}P]Tα$_1$ carried out as indicated in Fig. 3. The positions of phosphoserine (S) and phosphothreonine (T) are marked.

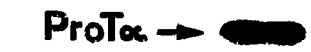

Fig. 5. Isolation of phosphorylated ProTα from cultured splenic lymphocytes. [^{32}P]ProTα was isolated as described in Materials and Methods and analyzed by SDS-PAGE followed by autoradiography.

that, as in ProTα, Ser and Thr residues were phosphorylated (inset in Fig. 4).

3.2. *Effect of activators and inhibitors on the phosphorylation of ProTα*

Since polyamines have been shown to stimulate CK-2, we investigated the effects of protamine and polylysine on the phosphorylation of ProTα by CK-2. Both polycations increased $^{32}P_i$ incorporation 20–30-fold (Table I), in keeping with their effects on other CK-2 substrates. Heparin, which has been reported as an efficient inhibitor of the phosphorylation of casein by CK-2, was not so efficient in inhibiting the labeling of ProTα with $^{32}P_i$ (Table I), since significant inhibition was only achieved by heparin concentrations about 3-times those required to inhibit phosphorylation of casein [11]. Note that heparin concentrations of 3–20 μg/ml have been used to inhibit the phosphorylation by CK-2 of proteins structurally related to ProTα, such as nucleolin [20], nucleoplasmin [21] and P1 [12].

3.3. *Phosphorylation of ProTα in vivo*

In order to ascertain whether ProTα is phosphorylated in vivo, we cultured stimulated splenic lymphocytes in the presence of [^{32}P]orthophosphate, and [^{32}P]ProTα was isolated using DEAE–cellulose chromatography and HPLC (Fig. 5). To determine the phosphorylation sites, labeled and unlabeled polypeptide samples were digested with trypsin and the resulting peptides were separated by reverse-phase HPLC. The pattern of ^{32}P-labeled peptides was identical to the ProTα phosphorylated by CK-2 (Fig. 3). However, thin-layer electrophoresis analysis of the acid hydrolysate of the N-terminal peptide (residues 1–14) purified by HPLC showed that, in vivo, only Thr residues are phosphorylated (inset B in Fig. 3). Identical results were obtained when we analyzed ProTα isolated from subconfluent HeLa cells incubated with [^{32}P]orthophosphate (data not shown).

Table I

Effect of activators and inhibitors on the ProTα phosphorylating activity of casein kinase-2

Addition	Concentration	Casein-kinase activity
None		1
Heparin	0.2 μg/ml	0.99
	0.6 μg/ml	0.47
Protamine	0.04 μg/μl	24.6
	0.08 μg/μl	30.7
Polylysine	4 μM	20.1

ProTα was phosphorylated by CK-2 using [γ-^{32}P]ATP as the co-substrate, and the components of the diverse reaction mixtures were separated and analyzed as indicated in the legend of Fig. 2. CK-2 activity was determined by optical densitometry of the autoradiogram following gel electrophoresis, and expressed relative to activity in the absence of effectors. Results shown are the means of one experiment carried out in duplicate. A second experiment carried out in duplicate gave similar results.

4. DISCUSSION

The above results show that ProTα from calf thymocytes is phosphorylated in vitro by CK-2 with an efficiency that is independent of whether ATP or GTP is the phosphate donor. ProTα was phosphorylated by CK-2 exclusively at Ser and Thr residues located at the N-terminal peptide that include the amino acid residues 1–14. We also found that this peptide was the only one phosphorylated in cultured splenic lymphocytes, but exclusively at Thr residues. Therefore, we can conclude that, in vivo, ProTα is phosphorylated by CK-2 or another cellular kinase with similar sequence specificity.

In our research on the in vivo phosphorylation of ProTα we found that the incorporation of [^{32}P]orthophosphate in the Thr residues of ProTα was highly dependent on cell proliferation activity (unpublished results). This fact is in keeping with our failure to detect phosphoamino acid residues in ProTα obtained from unstimulated calf thymocytes.

The ubiquitous distribution of ProTα in animal cells [5,6] and the evidence for its nuclear targeting [9,10] strongly suggest its having some fundamental function in the nucleus. The finding that ProTα is phosphorylated in vivo supports this hypothesis, inasmuch that it is well documented that phosphorylation is involved in the activity [23] and import into the nucleus [13] of proteins that are structurally related to ProTα. However, additional work is needed to elucidate the physiological significance of the phosphorylation of ProTα.

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