

## Prothymosin $\alpha$ is phosphorylated by casein kinase-2

Miguel G. Barcia, José M. Castro, Cristina D. Jullien, Carlos G. González and Manuel Freire

*Departamento de Bioquímica e Bioloxía Molecular, Facultade de Bioloxía, Universidade de Santiago, Santiago de Compostela, Galicia, Spain*

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Prothymosin  $\alpha$  (ProT $\alpha$ ) 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). ProT $\alpha$  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 ProT $\alpha$  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  $\alpha_1$  (T $\alpha_1$ ), a peptide whose sequence corresponds to the first 28 amino acids of ProT $\alpha$ , is also phosphorylated by CK-2 at the same phosphorylation sites as ProT $\alpha$ . In cultured splenic lymphocytes ProT $\alpha$  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 specificity, is responsible for phosphorylation of ProT $\alpha$  in vivo.

Prothymosin  $\alpha$ ; Thymosin  $\alpha_1$ ; Casein kinase-2

### 1. INTRODUCTION

Prothymosin  $\alpha$  is a 12.5 kDa acidic polypeptide that includes at its N-terminus the sequences of thymosin  $\alpha_1$  and several related peptides [1]. The primary structures of ProT $\alpha$  from diverse animal tissues are extremely similar [2], suggesting a high degree of conservation in the course of evolution.

Although  $\alpha$ -thymosins were originally considered to be involved in the immune response [3], the structure of the ProT $\alpha$  gene [4] and the ubiquitous distribution of their putative precursor, ProT $\alpha$ , in animal tissues [5,6] suggest that ProT $\alpha$  and/or its derived peptides may have a more general cellular function that is still unknown. Recent studies point to a role for ProT $\alpha$  in cell proliferation [6-8], and the presence of the karyophilic signal, TTKQKT, 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 ProT $\alpha$  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 ProT $\alpha$  sequence shown in Fig. 1). The proven involvement of CK-2 in cell proliferation [14] also relates this enzyme to ProT $\alpha$ .

In view of the above, we have sought phosphoamino acids in the ProT $\alpha$  sequence and investigated the ability of CK-2 to phosphorylate ProT $\alpha$  in vitro. In this paper we show that ProT $\alpha$  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, ProT $\alpha$  was phosphorylated only at Thr residues located in the same N-terminal fragment in which ProT $\alpha$  is phosphorylated by CK-2.

### 2. MATERIALS AND METHODS

#### 2.1. Materials

The triethylammonium salts of adenosine 5'-[ $\gamma$ - $^{32}$ P]triphosphate ([ $\gamma$ - $^{32}$ P]ATP, 3,000 Ci/mmol) and of guanosine 5'-[ $\gamma$ - $^{32}$ P]triphosphate ([ $\gamma$ - $^{32}$ 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  $\beta$ -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. ProT $\alpha$  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 ProT $\alpha$ and synthetic T $\alpha_1$

The reaction mixture (25  $\mu$ l) contained 50 mM Tris-HCl (pH 7.4), 1.6 mM EGTA, 26 mM MgCl<sub>2</sub>, 0.1 mM [ $\gamma$ - $^{32}$ P]ATP or [ $\gamma$ - $^{32}$ P]GTP, 1.6 mM EDTA, 83 mM  $\beta$ -glycerolphosphate, 3.3 mM dithiothreitol and 150 mM KCl, in the presence or absence of ProT $\alpha$  (5  $\mu$ g), T $\alpha_1$  (5

*Correspondence address:* M. Freire, Departamento de Bioquímica e Bioloxía Molecular, Facultade de Bioloxía, Universidade de Santiago, Galicia, Spain. Fax: (34) (81) 596 904.

*Abbreviations:* EGTA, [ethylenedis(oxyethylenenitrilo)]tetraacetic acid; CK-2, casein kinase-2; ProT $\alpha$ , prothymosin  $\alpha$ ; T $\alpha_1$ , thymosin  $\alpha_1$ ; HPLC, high performance liquid chromatography.



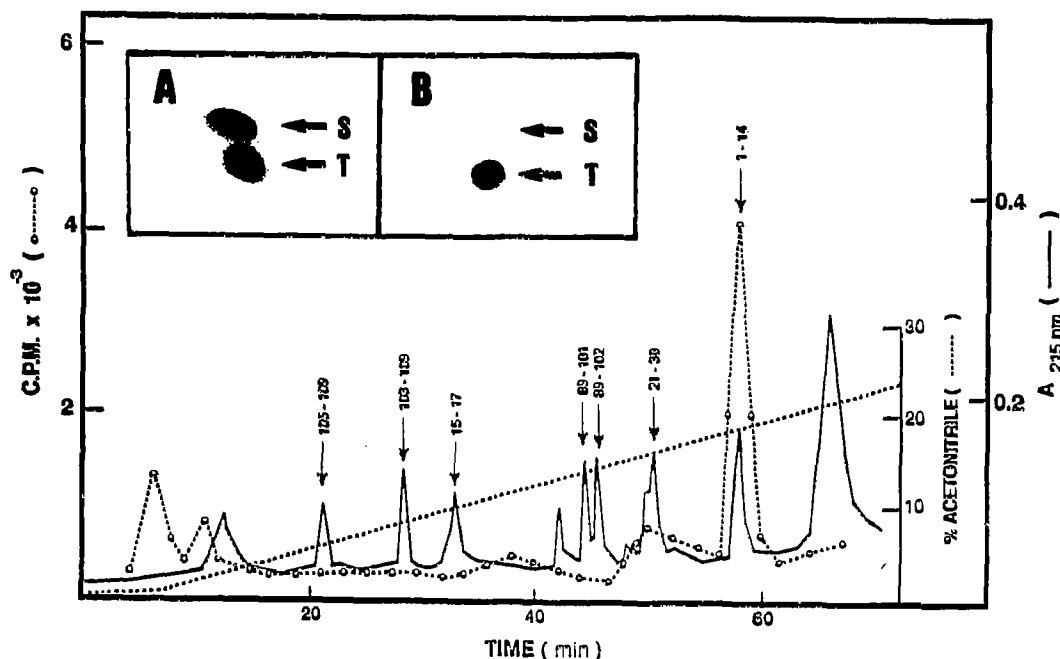


Fig. 3. HPLC of the peptides derived from the tryptic digestion of  $^{32}\text{P}$ -labeled ProT $\alpha$ . Peptides from the tryptic digestion of [ $^{32}\text{P}$ ]ProT $\alpha$  (180,000 cpm) and unlabeled ProT $\alpha$  (50  $\mu\text{g}$ ) were separated by reverse-phase HPLC as indicated in Materials 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 ProT $\alpha$  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 [ $^{32}\text{P}$ ]ProT $\alpha$  phosphorylated by CK-2 (panel A) and from [ $^{32}\text{P}$ ]ProT $\alpha$  isolated from splenic lymphocytes labeled metabolically with [ $^{32}\text{P}$ ]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.

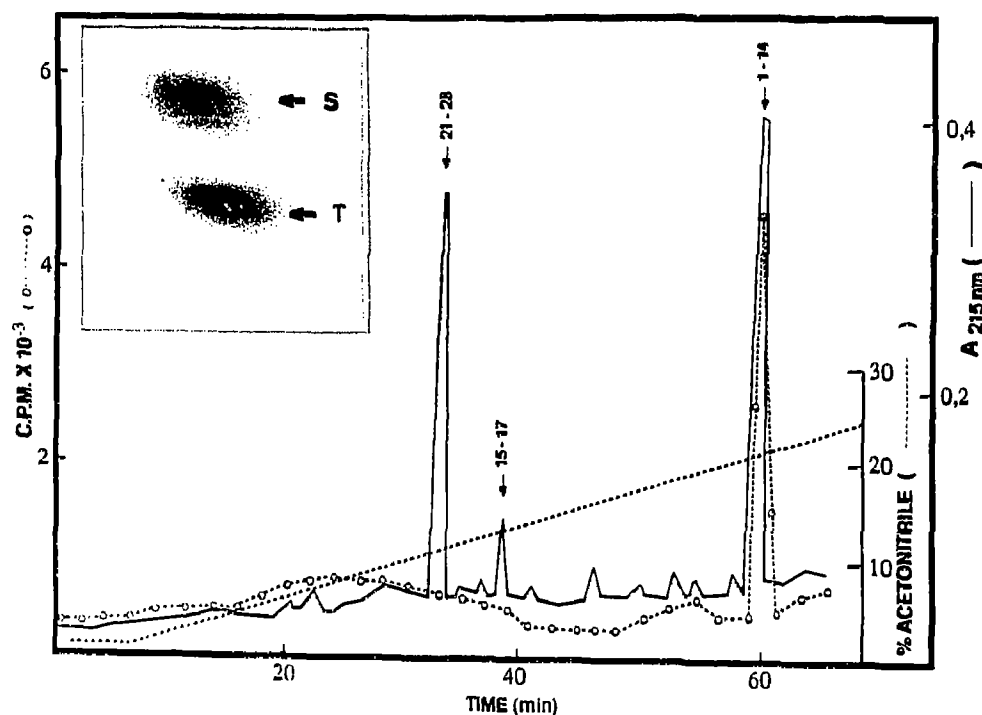


Fig. 4. HPLC of the peptides derived from tryptic digestion of  $^{32}\text{P}$ -labeled T $\alpha_1$ . Peptides from the tryptic digestion of [ $^{32}\text{P}$ ]T $\alpha_1$  (180,000 cpm) and 50  $\mu\text{g}$  of unlabeled T $\alpha_1$  were separated and analyzed as indicated in the legend of Fig. 3. Arrows indicate the sequence of tryptic peptides derived from synthetic T $\alpha_1$ . The inset shows that the phosphoamino acid analysis of the N-terminal peptide (residues 1-14) of [ $^{32}\text{P}$ ]T $\alpha_1$  carried out as indicated in Fig. 3. The positions of phosphoserine (S) and phosphothreonine (T) are marked.

## ProTα →

Fig. 5. Isolation of phosphorylated ProTα from cultured splenic lymphocytes. [<sup>32</sup>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 <sup>32</sup>P<sub>i</sub> 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 <sup>32</sup>P<sub>i</sub> (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 [<sup>32</sup>P]orthophosphate, and [<sup>32</sup>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

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-<sup>32</sup>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.

pattern of <sup>32</sup>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 [<sup>32</sup>P]orthophosphate (data not shown).

## 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 [<sup>32</sup>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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## REFERENCES

- [1] Haritos, A.A., Blacher, R., Stein, S., Calderella, J. and Horecker, B.L. (1985) *Proc. Natl. Acad. Sci. USA* 82, 343–346.
- [2] Frilingos, S., Frangou-Lazaridis, M., Seferiades, K., Hulmes, J.D., Pan, Y.C. and Tsolas, O. (1991) *Mol. Cell. Biochem.* 108, 85–94.
- [3] Low, L.K. and Goldstein, A.L. (1985) *Methods Enzymol.* 116, 213–219.
- [4] Eschenfeldt, W.H., Manrow, R.E., Krug, M.S. and Berger, S.L. (1989) *J. Biol. Chem.* 264, 7546–7555.

- [5] Haritos, A.A., Tsolas, O. and Horecker, B.L. (1984) *Proc. Natl. Acad. Sci. USA* 81, 1391-1393.
- [6] Gomez-Marquez, J., Segade, F., Dosil, M., Pichel, J.G., Bustelo, X.R. and Freire, M. (1989) *J. Biol. Chem.* 264, 8451-8454.
- [7] Eschenfeldt, W.H. and Berger, S.L. (1986) *Proc. Natl. Acad. Sci. USA* 83, 9403-9407.
- [8] Bustelo, X.R., Otero, A., Gomez-Marquez, J. and Freire, M. (1991) *J. Biol. Chem.* 266, 1443-1447.
- [9] Marrow, R.E., Sburlati, A.R., Hanover, J.A. and Berger, S.L. (1991) *J. Biol. Chem.* 266, 3916-3924.
- [10] Clinton, M., Graeve, L., El-Dorry, H., Rodriguez-Boulan, E. and Horecker, B.L. (1991) *Proc. Natl. Acad. Sci. USA* 88, 6608-6612.
- [11] Hathaway, G.M. and Traugh, J.A. (1982) *Curr. Top. Cell. Regul.* 21, 101-127.
- [12] Maelandsmo, G.M., Ostvold, A.C. and Laland, S.G. (1989) *Eur. J. Biochem.* 184, 529-534.
- [13] Rihs, H.P., Jans, D.A., Fan, H. and Peters, R. (1991) *EMBO J.* 10, 633-639.
- [14] Münstermann, U., Fritz, G., Seitz, G., Yiping, L., Schneider, H.R. and Issinger, O.-G. (1990) *Eur. J. Biochem.* 189, 251-257.
- [15] Guasch, M.D., Plana, M., Pena, J.M. and Itarte, E. (1986) *Biochem. J.* 234, 523-526.
- [16] Franco, F.J., Diaz, C., Barcia, M., Arias, P., Gomez-Marquez, J., Soriano, F., Mendez, E. and Freire, M. (1989) *Immunology* 67, 263-268.
- [17] Laemmli, U.K. (1970) *Nature* 227, 680-685.
- [18] Cooper, J.A., Sefton, B.M. and Hunter, T. (1983) *Methods Enzymol.* 99, 387-402.
- [19] Martensen, T.M. (1984) *Methods Enzymol.* 107, 3-23.
- [20] Warrenner, P. and Pethysyn, R. (1991) *Biochem. Biophys. Res. Commun.* 180, 716-723.
- [21] Taylor, A., Allende, C.C., Weinmann, R. and Allende, J.R. (1987) *FEBS Lett.* 226, 109-114.
- [22] Marin, O., Meggio, F., Marchiori, F., Borin, G. and Pinna, L.A. (1986) *Eur. J. Biochem.* 160, 239-244.
- [23] Silver, P.A. (1991) *Cell* 64, 489-497.
- [24] Malek, S.N., Katumuluwa, A.I. and Pasternak, G.R. (1990) *J. Biol. Chem.* 265, 13400-13409.