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Prothymosin α binds to Histone H1 in vitro

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Abstract

Previous studies have shown that prothymosin α (ProTα) is a nuclear acidic protein implicated in cell proliferation. To identify proteins that interact with ProTα we have used ligand-blotting assays. We report here that purified ProTα binds specifically to histone H1 in a dose dependent manner. Polyglutamic acid, an analog of the central acidic domain of ProTα, strongly inhibits the above interaction, suggesting that the binding of ProTα to histone H1 is mediated through its acidic domain.

Key words: Prothymosin α; Thymosin α1; Nucleosome; Histone H1; Ligand-blotting assay

1. Introduction

Prothymosin α (ProTα) is an acidic polypeptide (MW 12.5 kDa), originally isolated as a precursor of thymosin α1 [1], a peptide earlier proposed to function as a 'thymic hormone' promoting lymphocyte maturation [2]. Although the exact function of ProTα has not been defined, its high evolutionary conservation [3,4] and its wide tissue distribution [4–6] suggest that it plays an essential role in the cell. Evidence has been accumulated for a nuclear function [7–11] associated with cell proliferation [12–15]. Treatment of myeloma cells with ProTα antisense oligo deoxyribonucleotides was found to inhibit cell division [16]. Furthermore, it has been demonstrated that the gene of ProTα is activated by the c-myc protein [17] an observation that supports a role for the polypeptide in nuclear functions related to cell proliferation.

Inspection of the amino acid sequence of ProTα reveals that the protein possesses a middle domain containing clusters of aspartic and glutamic acid. The amino- and carboxyterminal domains contain both basic and acidic residues, whereas aromatic and sulfur amino acids are totally absent. This sequence peculiarity creates a highly acidic protein with an isoelectric point of 3.5 [1,12,18].

Acidic regions have been identified in a number of nuclear proteins, such as HMG-1 and -2, nucleoplasmin, c-myc and others [19] and it has been proposed that they are involved in nucleosome assembly and disassembly. This suggestion was based on the finding that polyglutamic acid facilitates the assembly of nucleosome particles in vitro [20]. Evidence to support this notion both in vitro and in vivo has been accumulated from studies on nucleoplasmin, the most abundant protein in the *Xenopus* oocyte nucleus [21] and the non-histone protein HMG-1 [22]. Interestingly, ProTα is similar to both proteins in its acidic domain. Another role for certain anionic regions might be the unfolding of condensed chromatin. This could be a result of electrostatic interactions between the acidic domains with the core histones and/or histone H1 [23].

Several important questions concerning the nuclear function of ProTα are emerging from these findings. To address these questions, we have investigated the potential association of ProTα with nuclear components. We report here, that ProTα binds selectively to H1 in vitro, possibly via its central acidic domain.

2. Materials and methods

ProTα was purified from calf thymus by standard chromatographic methods (manuscript in preparation). Calf thymus nuclei were prepared according to Aaronson and Blobel [24]. Crude mitochodrial fractions were obtained from thymus postnuclear supernatant by centrifuging at 10,000 × *g* for 10 min at 4°C and crude microsomes by pelleting the membranes of a post-mitochondrial fraction at 110,000 × *g* for 60 min. Nucleosomes were isolated according to Zentgraf & Franke [25] and fractionated with respect to histone H1 content.

2.1. Immunochemical techniques

For immunization with the carboxyterminal peptide, the synthetic peptide ct (sequence shown in Fig. 1) described by Barlos et al. [26] was coupled by glutaraldehyde to keyhole limpet hemocyanin (Sigma) and injected to rabbits, as described [27]. Anti-ct antibodies were purified by affinity chromatography, over a ct-Affigel 15 matrix (2 mg of peptide/ml resin) [27].

ProTα dot-blotting was performed by spotting ProTα or peptides α1 and ct diluted in 10 mM sodium acetate, pH 4.5, onto activated nitrocellulose membranes, treated with divinyl sulfone, ethylenediamine and glutaraldehyde [28]. Peptides were attached to the treated membrane by reaction of the amino groups with the free carbonyl, forming covalent bonds. This modification ensures maximal retention of the peptides on the filters during processing and was found to be necessary since ProTα is not immobilized on untreated nitrocellulose membranes (data not shown). The filters were incubated for 2 h with affinity-purified anti-ct diluted 1:2,500 in the blocking buffer TBST–0.1% gelatin (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, 0.1% gelatin, pH 7.4). After washing, the membranes were incubated with the secondary anti-

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Abbreviations: ECL, enhanced chemiluminescence; ProTα, prothymosin α; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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body anti-rabbit IgG-peroxidase, diluted in the blocking buffer (1:5,000), and detected by enhanced chemiluminescence (ECL), according to the manufacturer's instructions (Amersham, Life Science).

2.2. Ligand-blotting assays

Ligand-blotting assays were based on previously reported protocols [27, 29] and were performed as follows:

Proteins were resolved in 13% SDS-PAGE and transferred onto nitrocellulose membranes (30 V, 3 h, RT) in a buffer containing 0.192 M glycine, 0.025 M Tris-HCl, 0.1% SDS, 20% methanol. Blots were washed once with TBST and blocked with TBST–0.1% gelatin for 2–12 h. ProTα solution was dialysed overnight against 0.9 M NaCl, 60 mM Tris-HCl, 2.4% gelatin, 0.6% Tween 20, pH 7.4. Before incubation, the protein was adjusted to 0.05–1.5 μg/ml in 150 mM NaCl, 10 mM Tris-HCl, 0.4% gelatin, 0.1% Tween 20 by appropriate dilution of a concentrated sample. The membranes were then incubated with the ProTα solution for 2 h at room temperature (unless indicated otherwise), washed four times with the blocking solution and probed with anti-ct antibody (1:5,000) and anti-rabbit IgG-peroxidase (1:5,000) as described above. Detection was performed by ECL.

2.3. Other methods

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [30]. Protein concentrations were measured using a protein determination kit (Bio-Rad Laboratories). ProTα concentration was determined by RIA [1,4].

3. Results

3.1. Interactions of ProTα with cellular components

To search for proteins interacting with ProTα, we performed ligand-blotting assays, using as substrates nucleosomes, subcellular fractions from calf thymus (nuclei, mitochondria, microsomes, soluble fraction) and purified ProTα, shown in Fig. 1 and Fig. 2A. Detection of the potential protein-ProTα complexes was performed by the anti-ct antibody raised against the carboxyterminal peptide ct of ProTα (residues 87–109, Fig. 1). To rule out cross-reactions, anti-ct antibody was purified over a ct-agarose column. As depicted in Fig. 2B, the affinity-purified antibody binds to the peptide ct and detects nanogram quantities of ProTα. Moreover, no binding of the antibody is detected with the aminoterminal peptide

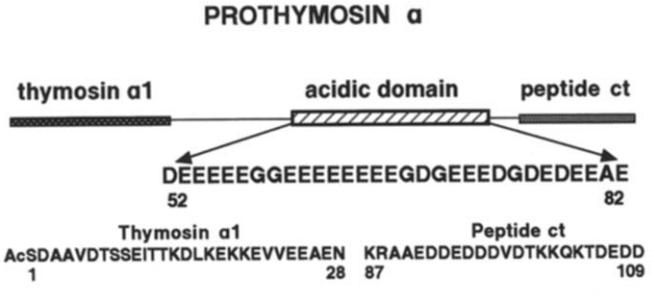


Fig. 1. Prothymosin α and peptides used in this study. In the sequence of bovine thymus ProTα [4,18], the NH_2-terminal 1–28 peptide (thymosin α1) and the COOH-terminal 87–109 peptide (ct) are shown as bars, whereas the acidic middle domain is shown as a shaded box. The peptide ct shown, was used for generating the anti-peptide antibody anti-ct.

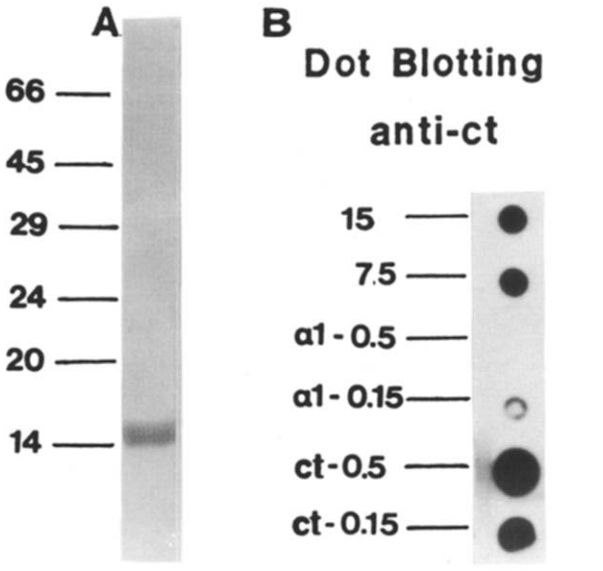


Fig. 2. Purity of ProTα and specificity of anti-ct antibodies. A: 15% SDS-PAGE and Coomassie blue staining of ProTα preparation used in the ligand-blotting assay. Numbers indicate molecular weight markers in kDa. B: dot-blotting, ProTα (15, 7.5 ng) or peptides α1, ct (0.5, 0.15 μg each) diluted in 10 mM sodium acetate, pH 4.5, were immobilized by covalent linkage onto glutaraldehyde-treated nitrocellulose membrane [27] and probed with affinity-purified anti-ct (1:2,500) and anti-rabbit IgG-peroxidase (1:5,000). Detection was performed by ECL in 1 min exposure time.

of ProTα, thymosin α1 (residues 1–28, Fig. 1) demonstrating that the anti-ct antibody is site specific.

Nucleosomes were prepared by micrococcal nuclease digestion of calf thymus chromatin, after sucrose gradient centrifugation (SDS-PAGE pattern shown in Fig. 3).

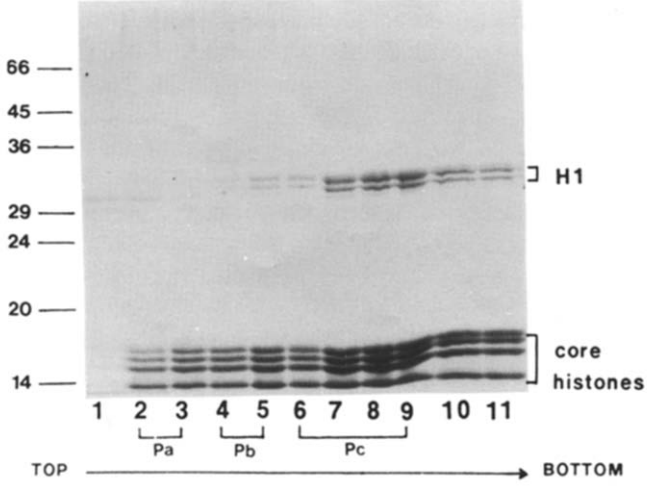


Fig. 3. Preparation of calf thymus nucleosomes. SDS-PAGE and Coomasie blue staining of 15 μl fractions from calf thymus micrococcal-nuclease digested chromatin, after fractionation on 10–50% sucrose gradient. Fractions 2 and 3 (pool A, Pa), fractions 4 and 5 (pool B, Pb) and fractions 6–9 (pool C, Pc) were pooled according to their content of histone H1, and used as described in section 2. Histone H1 and core histones are indicated. Numbers on the left correspond to molecular weight markers in kDa.

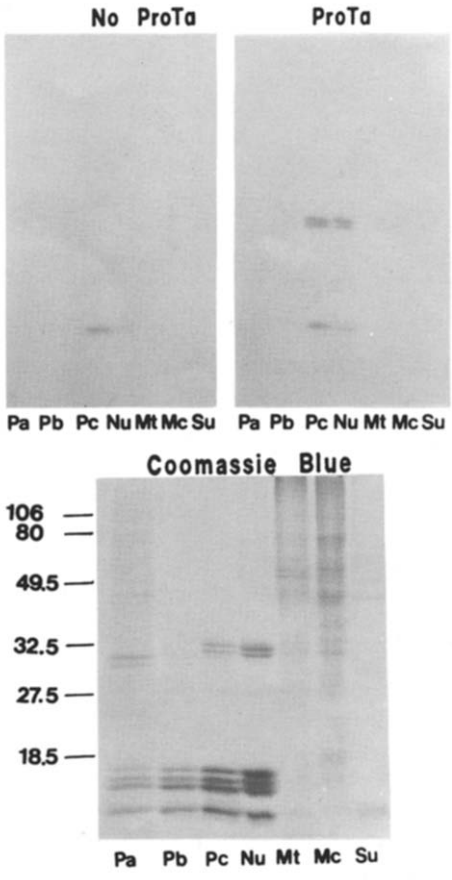


Fig. 4. ProTα interacts with nuclear proteins. Chromatin pools Pa, Pb, Pc and calf thymus subcellular fractions (nuclei, Nu; mitochondria, Mt; microsomes, Mc; post-microsomal fraction, Su), 60 μg each, were electrophoresed by SDS-PAGE and transferred to nitrocellulose. After blocking, the membranes were incubated with blocking buffer (No ProTα) or 1.5 μg/ml ProTα in blocking buffer (ProTα) and then probed with anti-ct antibody and the secondary antibody anti-rabbit IgG-peroxidase. Detection was performed by ECL in 1 min exposure time. Note the binding of ProTα to the chromatin pool C (Pc) and to the nuclear fraction (Nu) (doublet band). A replica gel was stained by Coomassie blue (Coomassie blue). →

The gradient fractions were pooled according to their content to linker histone H1 as follows: fractions 2 and 3, which correspond to chromatin depleted of histone H1(pool A, Pa); fractions 4 and 5 partially depleted from histone H1 (pool B, Pb); and fractions 6–9 which contain the full histone complement of chromatin (pool C, Pc).

The chromatin pools Pa, Pb, Pc and the subcellular fractions from calf thymus nuclei (Nu), mitochondria (Mt), microsomes (Mc), post-microsomal supernatant (Su) were fractionated by SDS-PAGE and transferred to nitrocellulose membranes. The filters were incubated with buffer alone or with 1.5 μg/ml ProTα solution. Subsequently, the membranes were probed with the affinity-purified antibody anti-ct, as described in section 2.

As shown in Fig. 4 (panel ProTα), ProTα binds to the chromatin pool C (Pc) and to the nuclear fraction (lanes Pc, Nu) decorating the same protein duplex. This binding is not detected in the absence of ProTα (Fig. 4, panel No ProTα). The lower band seen in both membranes represents fortuitous binding of the secondary antibody anti-rabbit IgG-peroxidase alone, in the absence of anti-ct, to this overloaded region of the nitrocellulose membrane (data not shown). Interestingly, no binding is observed in the core histone region suggesting that the decoration of the protein duplex represents a specific association of ProTα with these nuclear proteins.

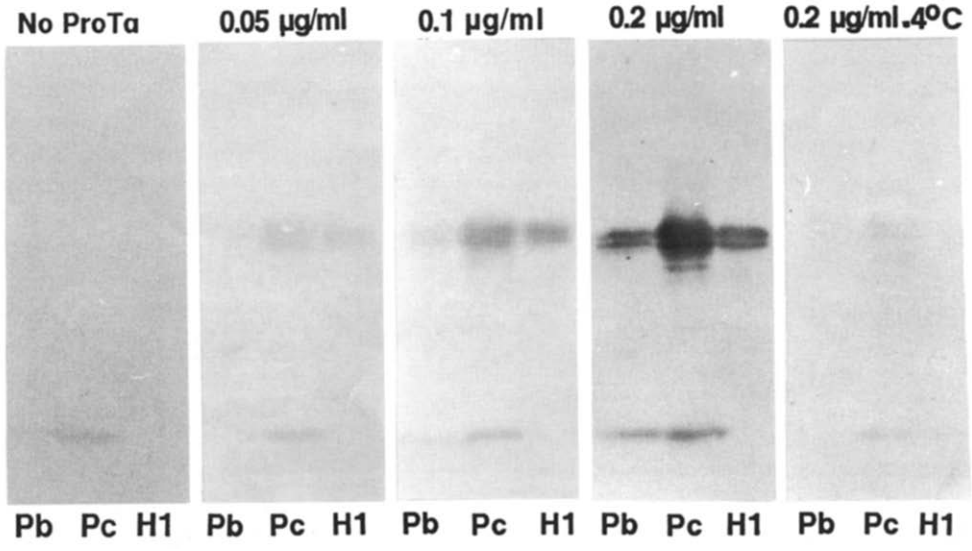


Fig. 5. ProTα binds to histone H1 in a concentration dependent manner. Chromatin pools Pb, Pc, 40 μg each, and 1 μg histone H1 (Boehringer Mannheim), were electrotransferred onto nitrocellulose filters. The membranes were incubated with blocking buffer alone (No ProTα) , 0.05 μg/ml ProTα, 0.1 μg/ml ProTα, 0.2 μg/ml ProTα and 0.2 μg/ml ProTα at 4°C. After appropriate washings, the blots were incubated with affinity-purified antibody anti-ct, anti-rabbit IgG peroxidase and ECL reagents in 2 min exposure time. Note the binding of ProTα to pure histone H1 and the dependance of the decoration on ProTα concentration.

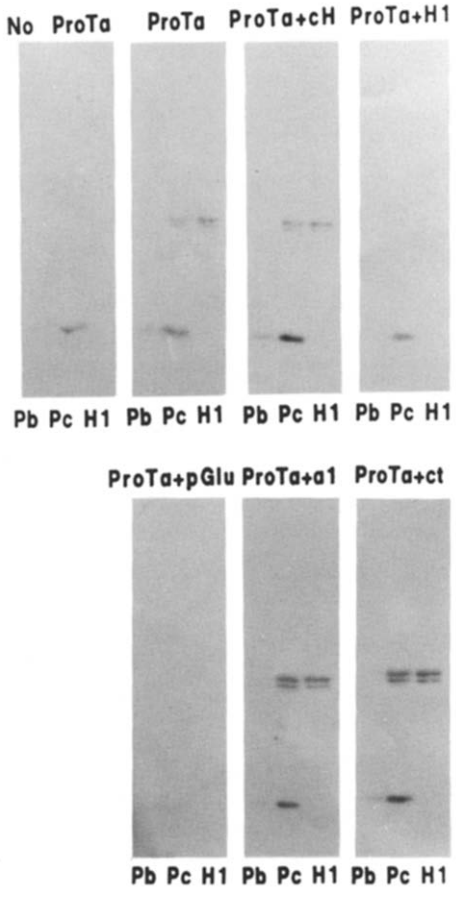


Fig. 6. Effects of core histones, histone H1, polyglutamic acid and peptides thymosin α1, ct on the ProTα-histone H1 interaction. Upper panels: chromatin pools Pb, Pc, 40 μg each, and 1 μg histone H1 (Boehringer Mannheim), were electrotransferred onto nitrocellulose filters. The membranes were incubated with blocking buffer alone (No ProTα), 1 μg/ml ProTα (ProTα), 1 μg/ml ProTα + 5 μg/ml core histones (Boehringer Mannheim) (ProTα + cH) and with 1 μg/ml ProTα + 5 μg/ml histone H1 (ProTα + H1). Lower panels: identical blots as defined in the upper panel, were probed with 1 μg/ml ProTα + 5 μg/ml polyglutamic acid (Sigma, MW (vis) 13.3 kDa) ProTα + pGlu, 1 μg/ml ProTα + 2.5 μg/ml thymosin α1 (ProTα + α1) and (c) 1 μg/ml ProTα + 2.5 μg/ml peptide ct (ProTα + ct). After appropriate washings, the blots were incubated with affinity-purified antibody anti-ct, anti-rabbit IgG peroxidase and ECL reagents. Exposure time was 1 min.

3.2. *ProTα binds to histone H1 in vitro*

Judging from the pattern and the mobility of the decorated protein duplex in the nuclei and nucleosomes, we suspected that histone H1 is a candidate partner of ProTα. Moreover, the discreet binding of ProTα to Pc, which differs from Pa and Pb in the content of histone H1 (Fig. 4, panel Coomassie blue) further strengthens this assumption. To test this prediction, pure calf thymus histone H1 was used as a substrate in the ligand-blotting assay. Fig. 5 (panels 0.05, 0.1, 0.2 μg/ml, lanes Pb, Pc, H1), shows that ProTα binds to pure histone H1 and to pools Pb and Pc in a concentration dependent manner. Performing the ligand-blotting assay at 4°C demonstrated a temperature dependence of the binding. As shown in Fig. 5, panel 0.2 μg/ml, 4°C, reducing the normal incubation temperature of 25°C to 4°C resulted in a decrease of the binding.

3.3. *Specificity of the ProTα–H1 interaction*

To characterize further the interaction of ProTα with histone H1 we repeated the ligand-blotting assay in the presence of soluble core histones (H2A, H2B, H3, H4), histone H1, peptides thymosin α1, ct and polyglutamic acid. Fig. 6, panels ProTα and ProTα + cH shows that soluble core histones do not affect the binding, while in the presence of soluble H1 the binding is strongly decreased (Fig. 6, panel ProTα + H1). Polyglutamic acid, an analog of the central acidic domain of the protein, eliminates the binding of ProTα to histone H1 (Fig. 6, panel ProTα + pGlu). Conversely, in the presence of an excess of the amino- and carboxyterminal peptides the binding was not decreased, but on the contrary, an increase was observed (Fig. 6, panels ProTα + α1 and ProTα + ct). These data suggest the binding of ProTα to histone H1 reflects a specific interaction which is possibly mediated by the central acidic domain of the protein.

4. Discussion

We have attempted to identify nuclear proteins that interact with ProTα in vitro. Using ligand-blotting assays, we obtained evidence for an interaction between ProTα and histone H1. The specificity of this interaction has been demonstrated by several criteria. First, ProTα binds only to the nuclear fraction, and to chromatin fractions which contain histone H1. The decoration is detected exclusively on histone H1 among the plethora of proteins present on the membranes. More importantly, the binding is dependent on the concentration of ProTα and it is affected by temperature. Second, no decoration is detected with the immobilized core histones, although they have size and charge similar to histone H1. Moreover, soluble core histones do not affect the binding of ProTα, even when added in excess, while equivalent amounts of soluble histone H1 diminish the decoration. Furthermore, no binding was detected when porcine thymus HMG-17, a lysine-rich protein, was used as substrate in the ligand-blotting assay (data not shown). Polyglutamic acid, an analog of the acidic region of ProTα, eliminates completely the binding of ProTα to histone H1 while the tail peptides do not inhibit the above interaction, even when added in excess. Taken together, these data suggest that the binding of ProTα to histone H1 reflects a specific interaction between the two ligands and point to a role of the acidic domain in this interaction.

Histone H1 binds to the nucleosome core particle, sealing the entry and exit points of the DNA and is

involved in the higher-order chromatin packing [31]. Several studies have demonstrated the contribution of histone H1 in the generalized repression of gene activity (reviewed in [32]). It has been proposed that reduction of the affinity of histone H1 for the nucleosome, presumably by trans-acting factors, leads to the displacement of histone H1 from the chromatin fiber and results in the derepression of transcription [33]. Considering the role of ProTα in cell proliferation [12–16], one attractive possibility is that ProTα may be among the proteins that modulate histone H1 function in chromatin during transcription or replication. Future studies should substantiate such an interaction under physiological conditions.

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