Identification of a low-M_r acidic nuclear protein as prothymosin α

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We have purified to homogeneity a 15-kDa perchloric acid (PCA)-soluble protein from rat thymus nuclei. This highly acidic protein showed a M_r of ca. 30 kDa in acetic acid/urea gels, probably due to oligomer formation. Sequence analysis of internal tryptic and thermolytic peptides revealed that the purified protein is, in fact, prothymosin α, a very hydrophilic polypeptide, which has been previously classified as a thymic or immunomodulating hormone. We found that prothymosin α is a rather abundant nuclear protein in rat thymus; its concentration is comparable to that of a well-characterized nonhistone protein HMG-14. The subcellular localization and physicochemical properties of prothymosin α suggest that its function is related to those of other long polyacidic regions containing nuclear proteins.

Prothymosin α; Nuclear protein; Protein sequence analysis

1. INTRODUCTION

We have previously fractionated perchloric acid-soluble nuclear proteins from rat tissue nuclei by reversed-phase high performance liquid chromatography (RP-HPLC) into 13 fractions, including 5 histone H1 subtypes, the 4 main high-mobility-group (HMG) proteins (1, 2, 14 and 17), HMG-I, uniquitin (HMG-20) and HMG-18 and -19 together with a protein tentatively identified according to its migration in acetic acid/urea gels as P1 protein [1]. After purifying the protein to homogeneity and analyzing its primary structure by peptide sequencing, it became evident that this nuclear protein is identical to prothymosin α (ProTα) [2].

A group of peptides, named thymosins, were originally isolated from calf thymus tissue, particularly from calf thymosin fraction 5 [3,4], but have since been shown to be widely distributed in mammalian cells and tissues [5-7]. Thymosin α, the first peptide to be isolated from thymosin fraction 5, has been shown to be the N-terminal fragment (amino acid residues 1-28) of a larger polypeptide designated as prothymosin α (111 amino acid residues) [2]. This polypeptide has been suggested to be involved in the regulation of cellular immunity [6] and reported to restore deficient responses in lymphocytes from patients with clinically active multiple sclerosis or systemic lupus erythematosus [8,9]. The complete amino acid sequence of ProTα has been recently deduced from both rat and human spleen cDNAs as well as from human fibroblast cDNA [10-12]. The apparent absence of a signal peptide in the primary translation product of this extremely hydrophilic polypeptide argues against its endocrine/paracrine function. Interestingly, Cómez-Márquez and Segale have recently hypothesized a nuclear site of function for ProTα on the basis of its structural similarity to certain well-characterized nuclear proteins and the presence of a putative nuclear localization signal on the C-terminal region of the molecule [13].

Traditionally, prothymosin α has been extracted and purified from rat thymus using a tedious procedure including powderization of frozen tissue, subsequent boiling of the salt suspension, homogenization of the suspension, desalting, size-exclusion chromatography, and final purification by RP-HPLC [14]. In this paper, we present a rapid and simple RP-HPLC procedure for purifying prothymosin α from perchloric acid extracts of rat thymus nuclei and whole thymus tissue.

2. MATERIALS AND METHODS

Nuclei were purified from one-month-old rat thymus and extracted immediately twice with 6 vols of ice-cold 5% PCA by 10 strokes in a Potter glass teflon homogenizer [1]. PCA-soluble proteins were precipitated with 6-vols of acidified acetone, processed and lyophilized as described [15].

The PCA-soluble nuclear proteins were separated using RP-HPLC on a Bio-Rad RP-304 (C4) column (250 × 4.6 mm) with a water/acetonitrile gradient containing 0.1% (v/v) trifluoroacetic acid (TFA). 2 mg of lyophilized protein (dissolved in 0.1% TFA) was eluted at a flow-rate of 1.0 ml/min at an oven temperature of 37°C using a multistep linear gradient from 7% acetonitrile (buffer A) to 70% acetonitrile (buffer B). The gradient was generated by increasing the percentage of acetonitrile as follows: an increase from 7% to 17.5% over 12 min, from 17.5% to 20.3% over 5 min, from 20.3% to 36.4% over 11 min, and from 36.4% to 70% over 5 min. The eluted proteins were detected by their UV-absorption at 214 nm. The protein peaks were collected, lyophilized in a Hetovac concentrator and

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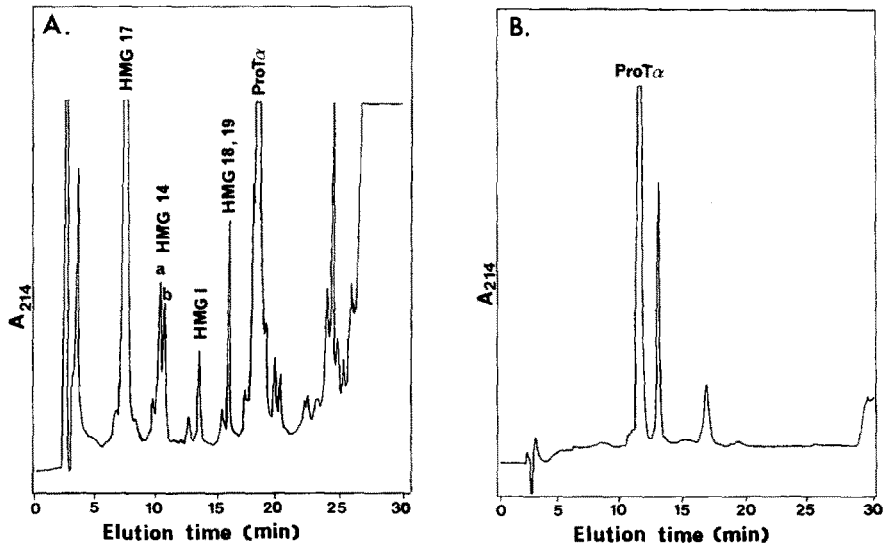

Fig. 1. (A) Fractionation of PCA-soluble nuclear proteins from rat thymus nuclei by reversed-phase HPLC on a C4 column. (B) Further purification of the prothymosin α on a C18 column. Two lyophilized ProTα containing peaks from C4 separations were dissolved in 100 μl 0.1% TFA and fractionated on a Vydac C18 column as described in section 2.

analyzed by acetic acid/urea polyacrylamide gel electrophoresis [16]. Prothymosin α containing peaks were further purified on a Vydac C18 column (250 × 4.6 mm) using buffer A and B and increasing the concentration of acetonitrile as follows: from 10% to 27.4% over 5 min, from 27.4% to 32.9% over 20 min, and from 32.9% to 70% over 2 min. The protein peaks were analyzed as described above and further by SDS-PAGE [17]. Native PAGE and analytical isoelectric focusing were performed using a Phast System according to manufacturer's instructions (Pharmacia LKB Biotechnology).

For proteolytic digestions, the purified ProTα collected from three C18 runs was dissolved in 150 μl of 0.1 M ammonium hydrogen carbonate (pH 8.0). A 50 μl sample was incubated separately either with 1.3 μg thermolysin (Sigma, type X) for 180 min or with 2 μg trypsin (Sigma, type XI) for 120 min at 37°C. The peptides generated by enzymatic digestions were purified on a Vydac C18 column using a linear 0–70% gradient of acetonitrile in 0.1% TFA over 60 min. The lyophilized peptides were dissolved in water and subjected to automated amino acid sequence analysis using an Applied Biosystems 477A Sequencer with a 120 A Analyzer. The search for homologous peptide/protein sequences was performed with a PROSIS sequence software package (Pharmacia LKB Biotechnology).

3. RESULTS

The perchloric acid extract of rat thymus nuclei was subjected to ion-pair RP-HPLC on a C4 column using a multi-step acetonitrile gradient. The system resolved HMG proteins, histone H1, and a peak eluting at 18.1 min previously suspected to contain a nonhistone protein P1 (Fig. 1A). To investigate this heterogeneous protein fraction further, it was separated on a C18 column into three peaks (Fig. 1B). The main component eluting at 11.4 min migrated on 15% acetic acid/urea

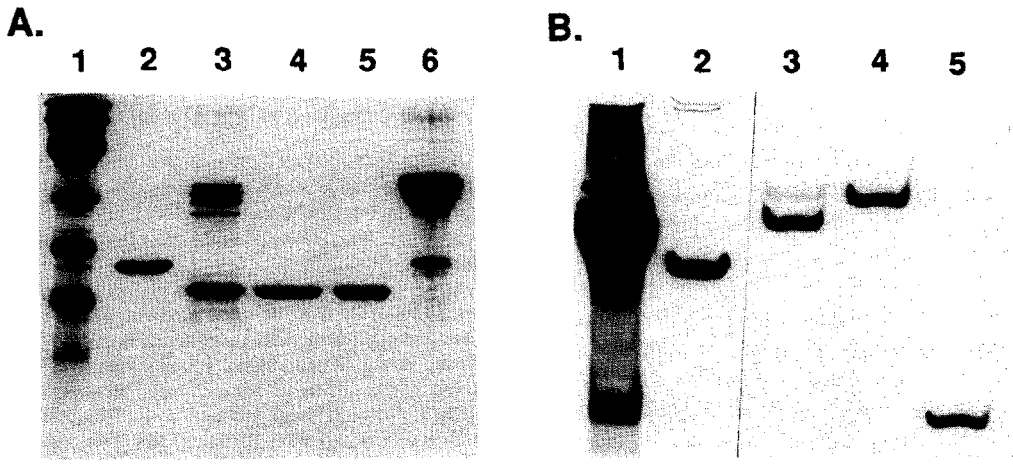

Fig. 2. PAGE analyses of purified ProTα from rat thymus nuclei. (A) 15% SDS-PAGE: (lane 1) low-M_2 markers (Pharmacia LKB Biotechnology); (lane 2) HMG-17; (lane 3) purified ProTα incubated at room temperature in sample buffer containing 1% SDS and 1% 2-mercaptoethanol; (lane 4) purified ProTα incubated in sample buffer containing 2-mercaptoethanol and SDS at 95°C for 5 min; (lane 5) purified ProTα incubated at 95°C in the presence of SDS without 2-mercaptoethanol for 5 min; (lane 6) PCA-soluble nuclear proteins from rat thymus. (B) 15% acetic acid/urea PAGE: (lane 1) PCA-soluble nuclear proteins from rat thymus; (lane 2) histone H1; (lane 3) HMG 2; (lane 4) ProTα (lane 5) HMG-17.

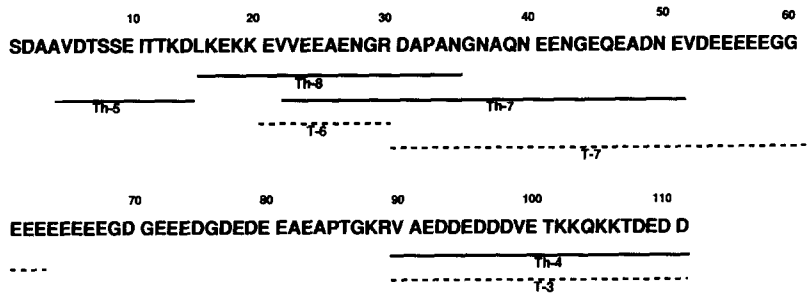

Fig. 3. Amino acid sequences of thermolytic (Th, underlined with solid line) and tryptic peptides (T, dashed line) of rat nuclear prothymosin α aligned with the sequence of rat spleen prothymosin α as deduced from its cDNA [11].

and 15% SDS-PAGE according to M_r 30 000 and 15 000, respectively, and, in both analyses, as a single band (Fig. 2). The protein showed a single band of 32 000 on 10% native-PAGE (not shown). The protein was dissociated completely to its monomer units in the absence of reducing agents in the sample buffer indicating that disulfide bridges were not involved in the oligomer formation (Fig. 2A). In analytical isoelectric focusing, the purified protein yielded a heterogeneous band of p*I* 3.5–3.8 (data not shown). The recovery of this protein from rat thymus nuclei was estimated to be approximately 50 μg/g tissue. The elution profile of PCA-soluble proteins extracted from whole thymus tissue was quantitatively and qualitatively similar to that of proteins extracted from nuclei (not shown).

Automated Edman degradation of the protein did not yield amino acids, indicating that the N-terminal amino acid is blocked. Information on the primary structure was established by sequencing the peptides generated by digestion with trypsin (peptides designated as T) or thermolysin (Th) (Fig. 3). The amino acid sequence of peptide Th-4 was used for a computer-assisted search for homologous protein sequences in the EMBL protein sequence database. The sequence was found to be identical to that of amino acid residues 90–111 of prothymosin α, which has a blocked (acetylated) N-terminus [2,11]. Additionally, prothymosin α contains the amino acid sequences of all the other peptides sequenced (Fig. 3). The automated sequencing was unsuccessful for the regions near the center of the polypeptide chain due to the presence of clusters of glutamic acid residues in this region. ProTα has previously been shown to be a very acidic protein (p*I* 3.55), consistent with an unusually high content of glutamic and aspartic amino acid residues, with a tendency to oligomer formation [18].

4. DISCUSSION

During our characterization of PCA-soluble nuclear proteins, we have identified prothymosin α as a member of this fraction, which contains mainly histone H1 and HMG proteins [19]. In our previous study, ProTα from rat liver, spleen, thymus, and testis nuclei was misidentified as a HMG-like protein P1 due to its anomalous migration on acetic acid/urea PAGE [1]. The results presented in this paper along with our previous data indicate that prothymosin α is a rather abundant nuclear protein in various rat tissues. Prothymosin α is similar in size and amino acid composition to HMG-14 and HMG-17, both nonfolded proteins (reviewed in [20]). These proteins contain no aromatic amino acid residues and very few large hydrophobic amino acid residues. However, the content of basic amino acid residues in HMG-14 and HMG-17 is much higher than that of ProTα, and they do not contain long contiguous stretches of acidic residues like ProTα. Nearly 50% of the amino acid residues of ProTα are acidic and concentrated in the central region of the molecule. Large glutamic or aspartic acid clusters have been recently found in a number of nuclear proteins, such as HMG-1/HMG-2, nucleoplasmin, nucleolin, and many transcription factors (see [21]).

Our results support the hypothesis that ProTα functions also at a nuclear site [13]. Interestingly, using a microinjection technique, Watts et al. showed recently that ProTα purified from whole calf thymus cells migrates into *Xenopus* oocyte nucleus at a rate comparable to that of histone H1 [22]. ProTα remained stable in the nucleus for 48 h. ProTα has also been identified from cytoplasm of mouse cells as a protein covalently linked to a small RNA [23]. No information on the subcellular localization of the RNA/protein linking reaction is available.

Finally, it has been reported that antisera recognizing ProTα stain specifically the nuclei, but not the processes, of astrocytes in human brain [24]. Additionally, ProTα immunoreactivity is present in high amounts in the nuclei of proliferating rat cells [25]. Thus, the function of prothymosin α may be linked to nuclear processes involved in cell proliferation. The method described in this paper provides a fast and simple procedure for purification of ProTα for studies to elucidate its interactions with other nuclear components.

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NOTE ADDED IN PROOF

After submission of this manuscript, Watts et al. [Eur. J. Biochem. (1990) 192, 643–651] reported that both human prothymosin α and rat parathymosin, but not thymosin β_4, migrate into *Xenopus* oocyte nuclei after microinjection. Furthermore, they showed that the C-terminal 21 redisues of ProTα are required for nuclear migration.