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Prothymosin α interacts with SET, ANP32A and ANP32B and other cytoplasmic and mitochondrial proteins in proliferating cells

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ABSTRACT

Prothymosin α (ProTα) is an acidic protein with a nuclear role related to the chromatin activity through its interaction with histones in mammalian cells. ProTα acts as an anti-apoptotic factor involved in the control of the apoptosome activity in the cytoplasm, however the mechanisms underlying this function are still known. ProTα shares similar biological functions with acidic nuclear-cytoplasmic shuttling proteins included in SET and ANP32 family members. Using affinity chromatography, co-immunoprecipitation and chemical cross-linking, we demonstrate that ProTα interacts with SET, ANP32A and ANP32B proteins. The study by mass spectrometry of the complexes stabilized by chemical cross-linking showed that associations of ProTα consist of six highly acidic ProTα-complexes, which corresponds to differentiated interactions of ProTα either with SET or ANP32 proteins. The presence in the ProTα-complexes of cytoplasmic proteins involved in membrane remodeling and proteins implicated in the mitochondrial permeability, seems to indicate that they could be related to a cytoplasmic-mitochondrial activity. According to the cellular function of the characterized targets of ProTα, and the evolution in the composition of the diverse ProTα-complexes when proliferation activity was reduced or apoptosis induced, leads to hypothesized that ProTα interactions might be related to the proliferation activity and control of the cell survival.

1. Introduction

Prothymosin α (ProTα) is a 109–111 amino acid protein that is widely distributed in mammalian tissues and particularly abundant in lymphoid cells [1–3]. ProTα has a highly conserved sequence, which includes a central acidic region (residues 41–85) comprising Glu and Asp residues [3], a karyophylic signal at its C-terminus [4], a casein kinase consensus motif at its N-terminus [5] and the first 28 amino acids at its N-terminus correspond to the thymosin α_1 ($T\alpha_1$) sequence [6,7]. Cytoplasmic proteolytic processing of ProTα by a lysosomal asparaginyl endopeptidase gives rise to a significant concentration of $T\alpha_1$ [8], which physiological significance remains unknown.

Besides, ProTα is phosphorylated at Thr residues when proliferation of mammalian cells is activated [5]. In lymphocyte primary cultures ProTα is phosphorylated at Thr residues located at positions 7 and 12 or 13 [9], whereas in tumor lymphocytes ProTα is exclusively phosphorylated at Thr residue located at position 7 [10]. The protein kinase responsible for this phosphorylation has been isolated from several mammalian cell types in our laboratory [9] and more recently identified as a phosphorylated form of pyruvate kinase isoform M2 [10,11].

Regarding its biological function, current evidence indicates that ProTα is a multifunctional protein that is essential for cell division [12–14] during which ProTα migrates to the nucleus and thus influences chromatin activity through interactions with transcription factors [15,16], histones [17–19] and other components of the chromatin remodeling process. These components are included in a nuclear complex that together with the core histones, contains helicases, hnRNP proteins, β-actin and acetyltransferase and methyl transferase activities of histones H3 and H4 [19,20].

Extracellular and cytoplasmic actions of ProTα came to expand the multifunctional role of this protein. It has been reported an extracellular neuroprotective effect of ProTα through an anti-necrotic mechanism [21]. As well as a cytoplasmic anti-apoptotic activity of ProTα related to its ability of inhibiting caspase-3 activation by the blockage of the apoptosome function in the caspase-dependent apoptotic pathway [22] This anti-apoptotic effect occurs through a yet unknown mechanism that seems to counteract the pro-apoptotic activity of

Abbreviations: CT, C-terminal; ft, flow-through; FT, formaldehyde treated; NT, N-terminal; ProTα, prothymosin α; $T\alpha_1$, thymosin α_1; STS, staurosporine

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ANP32A protein of promoting the activation of caspases 9 and 3.

The anti-apoptotic activity of ProTα has also been described on diverse cell types where the expression of ProTα was modified. Thus, the inhibition of ProTα gene expression sensitizes cells to apoptosis [23] whereas overexpression of ProTα confers increased resistance to cell death [24]. Together with the peptide p8, ProTα is involved in the regulation of staurosporine-induced apoptosis [25]. Our experiments in which apoptosomic activation of effector caspases were determined in 293T cells transfected with ProTα mutated at the phosphorylation site Thr7 to express non-phosphorylable ProTα, seem to demonstrate that phosphorylation of ProTα is required for its anti-apoptotic activity [26].

According to the present data, we point out the implication of highly acidic proteins, as ProTα, SET, ANP32A and ANP32B, in the control of the apoptotic activity. Apart from the respective anti and pro-apoptotic roles of ProTα and ANP32A in controlling the caspase-dependent apoptotic pathway, it has also been described an anti-apoptotic activity of the SET protein. SET is included in a complex with ANP32A, HMG-2 and Ape-1, and this function, is involved in the regulation of the caspase-independent apoptotic pathway by controlling the activity of DNase NM23-H1 [27]. Moreover, an anti-apoptotic activity of a protein of the ANP32 family: ANP32B, has also been described [28,29].

On the other hand, the reported interaction of ProTα with SET in extracts of HeLa cells [30] reinforces the possibility that interactions between these acidic proteins can be related to the mechanism controlling the cellular activity. This question has led us to carry out the research included in the present work. We aimed to characterize interactions of ProTα with ANP32 proteins, and with SET itself, in order to elucidate the cellular mechanism in which they can be involved.

2. Materials and methods

2.1. Cell culture and subcellular fractionation

Jurkat cells were cultured in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum and 100 U/mL penicillin and 100 μg/mL streptomycin (BioWhittaker), at 37 °C in an humidified atmosphere of 5% CO_2 (v/v) in air.

In order to induce a quiescent state, cells were deprived of serum for 60 h [31]. Cell number and viability were checked by the Trypan-Blue exclusion method, and the absence of apoptotic cells confirmed by DAPI staining.

Apoptosis was induced by treatment with 0.5 μM staurosporine (STS) (Sigma) or actinomycin D (ActD) (Sigma) for 2 h and 4 h. Cells were then suspended (10^8 cells/mL) in lysis buffer [20], submitted to three freeze/thaw cycles and homogenized (10 strokes) in a Potter Teflon glass blender. Whole-cell extracts were obtained by centrifugation at 100,000 × *g* (1 h at 4 °C) and the supernatant were dialyzed against buffer A.

For protein cross-linking, Jurkat cells were pelleted, resuspended in PBS at 5 × 10^6 cells/mL and treated with a final concentrations of 1% formaldehyde. Cells were incubated with mild agitation for 20 min at 37 °C. The reaction was quenched with glycine at a final concentration of 125 mM for 5 min at room temperature [32].

Subcellular fractionation of proliferating and serum-deprived cells was carried out as described [20]. Briefly, nuclei and cytosol were obtained from homogenized cells by differential centrifugation at 2000 × *g* (nuclei) and 100,000 × *g* (cytosolic fraction). The pelleted nuclei were suspended (about 2 × 10^8 nuclei/mL) in 10 mM Tris-HCl pH 7.4 buffer containing 10% sucrose (w/v), 0.1 mM $MgCl_2$, 1 mM DTT, and 1 mM PMSF, supplemented with 80 μg/mL DNase I and 10 μg/mL RNase A, incubated at room temperature for 30 min, and then centrifuged to yield the nucleoplasmic extract. Both the nucleoplasmic and cytosol extracts were dialyzed against 20 mM Tris-HCl pH 7.6, 75 mM NaCl containing 1 mM DTT, 0.5 mM PMSF (buffer A).

2.2. ProTα-affinity chromatography and fractionation of ProTα-affinity proteins

Subcellular fractionates (15 mg) from Jurkat cells were subjected to affinity chromatography on ProTα purified from calf thymus and bound to Sepharose as previously described [20]. Briefly, samples were loaded onto 1 mL of ProTα-bound Sepharose, and retained proteins were eluted using 3 column volumes of 1 M NaCl. Subsequent to elution, flow-through and retained fractions were dialyzed against buffer A, concentrated to 1 mg/mL by centrifugation in Amicon concentrators (10,000 molecular weight cut-off) to be analyzed by Western blotting or separated by anion-exchange chromatography.

Fractionation by anion-exchange chromatography of the ProTα-affinity proteins was carried out on HiTrap Q HP columns (GE Healthcare). Aliquots of 1 mg were loaded onto the columns in 50 mM NH_4HCO_3 pH 7.6, which was sequentially eluted with this buffer containing 0.2 M, 0.5 M or 1 M NaCl, and the different eluated dialyzed against the initial buffer containing 1 mM DTT and 0.5 mM PMSF, concentrated by lyophilization, and aliquots separated by SDS-PAGE either to be analyzed by Western blotting or by Coomassie staining.

2.3. Antibodies and immunoassays

Polyclonal rabbit antibodies against the N-terminal region (NT-ProTα) (residues 2–20) and C-terminal region (CT-ProTα) (residues 95–109) in the ProTα-sequence were custom-produced and affinity-purified as described previously [19]. Rabbit antibodies against SET (sc-25564) and ANP32A/B (sc-292113) and goat antibodies against ANP32A (sc-5652) were from Santa Cruz Biotechnology.

For Western blotting analyses, aliquots of the diverse cellular fractionates or immunoprecipitates were transferred to 0.22 μm nitrocellulose membranes (Schleicher & Schuell Bioscience) following the standard procedure [33]. When specific detection of acidic proteins was required, the gels were equilibrated in 20 mM sodium acetate, pH 4.5 after electrophoresis and electrotransfer performed onto membranes previously activated with glutaraldehyde as described elsewhere [18,19]. The membranes were incubated with primary antibodies (anti-NT-ProTα and anti-CT-ProTα, 1.5 μg/mL; anti-SET, anti-ANP32A/B and anti-ANP32A, 1:1000 dilution), the immunoreactive proteins were detected using the ECL kit (GE Life Science) following the manufacturer's instructions and, when indicated, quantified by densitometric analysis (Quantity One software from Bio-Rad).

Co-immunoprecipitation assays in Jurkat cytoplasmic extracts (2 mg) were performed as described [19] using different primary antibodies as specified in each experiment, and the immunocomplexes analyzed by Western blotting, either in standard or in specific conditions for detection of acidic proteins.

2.4. Identification and purification of ProTα–immunoreactive complexes stabilized by chemical cross-linking with formaldehyde

Aliquots (20 mg) of cytoplasmic extracts of cells treated with formaldehyde, were fractionates by anion-exchange chromatography on HiTrap Q HP columns in the conditions indicated above. The different fractionates were separated by SDS-PAGE either to be analyzed by Western blotting or stained with Coomassie Blue. The stained bands showing a similar electrophoretic mobility to that of the ProTα-immunoreactive products were sliced from the gel, and aliquots of the different slices separated again by SDS-PAGE to be analyzed by Western blotting or Coomassie Blue staining. Proteinic bands with similar mobility to the ProTα-immunoreactive products were sliced from the gels and aliquots of these bands separated again by SDS-PAGE to be analyzed by Western blotting or stained by Coomassie Blue. Finally, aliquots of the ProTα-immunoreactive bands were submitted to heat treatment (90 min, 100 °C) to reverse chemical cross-linking [32] and analyzed by mass spectrometry.