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Prothymosin α fragmentation in apoptosis

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Abstract We observed fragmentation of an essential proliferation-related human nuclear protein prothymosin α in the course of apoptosis induced by various stimuli. Prothymosin α cleavage occurred at the DDVD^{99} motif. In vitro, prothymosin α could be cleaved at D^{99} by caspase-3 and -7. Caspase hydrolysis disrupted the nuclear localization signal of prothymosin α and abrogated the ability of the truncated protein to accumulate inside the nucleus. Prothymosin α fragmentation may therefore be proposed to disable intranuclear proliferation-related function of prothymosin α in two ways: by cleaving off a short peptide containing important determinants, and by preventing active nuclear uptake of the truncated protein.

Key words: Apoptosis; Caspase; Nuclear localization signal; Prothymosin α

1. Introduction

Prothymosin α (ProTα) is a small (109 amino acid residue) highly acidic nuclear protein found in virtually all mammalian tissues, the most abundant source being the thymus [1–3]. Several lines of evidence support the notion that ProTα is an essential protein related to cell proliferation. ProTα is an abundant protein in proliferating cells and, in particular, in cancer cells [4]. A low ProTα mRNA level observed in quiescent cells is increased markedly upon growth stimulation with mitogens or serum [5]. ProTα gene expression is up-regulated by c-Myc [6] due to the presence of a functional E-box in the ProTα gene [7]. Overexpression of ProTα in NIH3T3 and HL-60 cells was shown to stimulate cell division due to shortening of the G1 phase [8,9], suggesting the involvement of ProTα in the G1/S transition or the S phase of the cell cycle. Furthermore, overexpression of ProTα resulted in inhibition of cell differentiation [9]. Finally, inhibition of ProTα synthesis by treatment with ProTα antisense oligonucleotides prevented cell division in a synchronized population of human myeloma cells [10] and induced apoptosis in HL-60 cells [11].

The mechanism of ProTα action is unknown. Subcellular localization pointed to the nucleus as a compartment for accumulation and most probably functioning of ProTα [12–15]. The active nuclear uptake of ProTα is mediated by a bipartite nuclear localization signal (NLS) [16]. Here, we present evidence that ProTα is subjected to caspase-mediated fragmentation in the course of apoptosis. The carboxy terminal caspase cleavage disrupting the NLS of the protein results in a truncated ProTα derivative which fails to accumulate inside the nucleus.

2. Materials and methods

2.1. Plasmid constructions

pHT15A containing the protein-coding region of the human wild-type ProTα cDNA was derived from pHT15 [17] by eliminating the G(75)V mutation by site-directed mutagenesis. The D(99)N mutation was introduced in ProTα sequence by PCR on pHP12 [17] with the mutagenic primer (5′-GATGTCAATACCAAGAAGCAG-3′) to produce pHP-caspN. For constructing pKT1 encoding, in successive order, a His-tag, a thrombin cleavage site, a protein kinase recognition site, and human ProTα as a single fusion protein, the ProTα-encoding DNA fragment from pHT15A was inserted into pET33b(+) vector (Novagen) between the filled-in *Nhe*I site and *Eco*RI site. To obtain pEGFP-ProTα, *Bam*HI-*Eco*RI fragment from pHT15A was inserted into *Bgl*II-*Eco*RI sites of pEGFP-C2 vector (Clontech). cDNA encoding the ProTα (1–99) fragment was obtained in pHT15A by converting the threonine-100 ACC codon into the TAG termination codon. The resultant cDNA was inserted in pEGFP-C2 in analogous fashion, producing the pEGFP-ProTα(1–99) plasmid. The structure of resultant constructs was confirmed by sequencing.

2.2. Cell cultures

A subline of HeLa-S3 cells designated HeLa-B [18] cultivated in DMEM supplemented with 10% of bovine serum was used. Apoptosis was induced in 1 day old cell cultures grown at 2×10^7 cells per roller bottle by incubating the cells in DMEM containing staurosporine (1 μM, Sigma) for 2 or 4 h; cycloheximide (100 μg/ml) for 3.5 h; or cisplatin (20 μg/ml, provided by P. Cheltsov, Institute of General and Inorganic Chemistry, Moscow, Russia) for 15 h. In the latter case, the medium contained 2% bovine serum. In addition, apoptosis was induced by abortive poliovirus type 1 Mahoney strain infection, with cycloheximide (10 μg/ml) added at 1.45 h postinfection [18,19]. Incubation was for 6.45 h at 37°C.

Upon incubation, small aliquots of the cells were suspended in PBS and stained with 5 μg/ml of fluorescent permeable nuclear dye Hoechst-33342 (Sigma). The percentage of apoptotic cells was estimated observing nuclear morphology with epifluorescent Leica DMLS microscope equipped with filter cube A. The bulk of the cells, including those from supernatant and the ones still attached to the bottle, were collected and stored at −80°C for subsequent isolation of ProTα.

Transfection of the HeLa-B cells with 10 μg of pEGFP-ProTα, pEGFP-ProTα(1–99), and pEGFP-C2 (Clontech) was performed by using a modification of the calcium phosphate precipitation method, as described at http://www.sciencexchange.com/sxprotocols/molbiol/phoenx.htm. Two days after transfection localization of the EGFP-ProTα fusion proteins was examined with the epifluorescent micro-

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Abbreviations: GFP, green fluorescent protein; MALDI-MS, matrix assisted laser desorption/ionization mass spectrometry; NLS, nuclear localization signal; ProTα, prothymosin α

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scope equipped with filter cube I3. In parallel, lysates of 10^6 cells from each culture were obtained by boiling in the Laemmli sample buffer followed by brief sonication and subjected to 12% SDS–PAGE and immunoblotting using affinity-purified rabbit anti-GFP antibodies. Detection of EGFP-containing bands was performed by using donkey anti-rabbit Ig conjugated with horseradish peroxidase and ECL Western blotting detection reagents (Amersham Pharmacia).

2.3. Isolation of ProTα and its derivative from HeLa cells

ProTα and its caspase cleavage product were isolated from 5×10^7 HeLa-B cells by the phenol extraction procedure essentially as described [17]. The final high salt/phenol extraction step was applied to the staurosporine- and cisplatin-induced probes only to eliminate the bulk of cellular RNAs from the ProTα preparation. The samples were subjected to 8% PAGE/7 M urea (without SDS), and the ProTα bands were visualized by methylene blue staining as described [17]. For MALDI-MS analysis, the band containing ProTα fragment was cut out basing on its relative electrophoretic mobility without staining the gel. Elution of the ProTα fragment from the gel was performed by addition of 300 μl GES (0.5 M ammonium acetate/1 mM EDTA/0.1% SDS) to excised gel pieces with subsequent vigorous shaking at room temperature overnight. The protein was precipitated with 3 volumes of ethanol, dissolved in water, and loaded on a 50 μl DE-52 column equilibrated with a buffer containing 20 mM Tris–HCl, pH 7.5/100 mM NaCl. After extensive washing, ProTα fragment was eluted with the buffer containing 0.5 M NaCl followed by ethanol precipitation, and was finally dissolved in water.

2.4. MALDI-MS analysis of the ProTα cleavage product

Matrix assisted laser desorption/ionization mass spectrometric (MALDI-MS) analysis was performed on a Reflex-II instrument (Bruker Daltonik, Bremen, Germany) in linear and in reflector mode. MALDI-MS samples were prepared applying the dried drop method on stainless steel targets pre-covered with thin matrix films. 0.6 μl of purified ProTα fragment (~0.3 ng) were mixed with 0.6 μl of saturated sinapinic acid in acetonitrile/0.1% TFA (2:1 v/v) as matrix on the 'scout-26' MALDI-MS target. The dried sample spots were washed by depositing ~4 μl of ice-cold 5% TFA on top of them followed by rapid removal of the wash solution. Horse heart cytochrome *c*, human ubiquitin and bovine insulin were used as external mass standards. Isolated ProTα fragment was also submitted to tryptic digestion using 100 ng sequencing grade modified trypsin (Boehringer Mannheim). Mixture of peptides was purified on C-18 ZipTip micro columns (Millipore, Bedford, MA, USA) and analyzed by MALDI-MS in the presence of α-cyano-4-hydroxy-cinnamic acid as matrix.

2.5. Production and purification of recombinant proteins

The recombinant human wild-type ProTα and ProTα D(99)N mutant were isolated from *Escherichia coli* BL21(DE3)/pHP12 and /pHP-caspN cells as described [17] and additionally purified by DE-52 cellulose column chromatography. To obtain [^{32}P]-end labelled ProTα, the recombinant ProTα derivative was isolated from the lysate of *E. coli* BL21(DE3)/pKT1 cells by means of Ni-NTA agarose (Qiagen) affinity chromatography. The 1 μg ProTα aliquot was subjected to radiolabelling by incubating with 100 μCi [γ-^{32}P]ATP (5000 Ci/mmol) and 1 U protein kinase A (catalytic subunit, Sigma). After ethanol precipitation, the labelled ProTα derivative was treated with 0.1 U thrombin in the thrombin cleavage buffer (Novagen) to eliminate the His-tag. The hydrolysate was fractionated by 8% PAGE/7 M urea, the labelled ProTα was eluted from the gel, ethanol precipitated and dissolved in water. The specific radioactivity of [^{32}P]-labelled ProTα was 10^7 cpm/μg.

2.6. In vitro caspase cleavage assays

Human caspase-3 and caspase-7 and -8 were kindly provided by Y. Lazebnik (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA) and G. Salvesen (The Burnham Institute, La Jolla, CA, USA), respectively. Caspases were preincubated in 25–250 ng aliquots with or without 30 μM zVAD-fmk or 10 μM zDEVD-fmk in 10 μl of a buffer containing 50 mM HEPES, pH 7.5/100 mM NaCl/10% glycerol/10 mM DTT at room temperature for 15 min. The reactions were initiated by the addition of 3 μg unlabelled ProTα or 10 000 cpm [^{32}P]-labelled ProTα in 2–5 μl of the same buffer. After incubation at 37°C for 2 h, the samples were analyzed by 8% PAGE/7 M urea.

3. Results

3.1. ProTα fragmentation in apoptotic cells

Amino acid motifs D-x-x-D conforming to a caspase recognition site are present close to the carboxy terminus of the human ProTα molecule (Fig. 1A). We aimed to determine whether ProTα fragmentation occurs in apoptotic cells.

The integrity of ProTα in control non-infected and poliovirus-infected HeLa cells (non-apoptotic) was compared with that of ProTα isolated from cells triggered to undergo apoptosis by various stimuli: treatment with cycloheximide, cisplatin, staurosporine, and abortive poliovirus infection. In the latter case, a combination of poliovirus infection with cyclo-

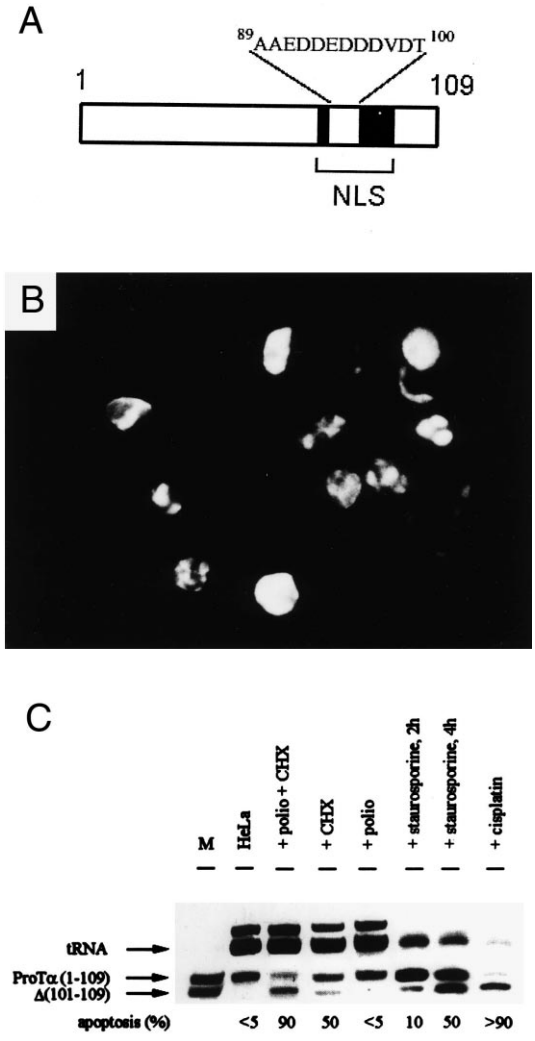
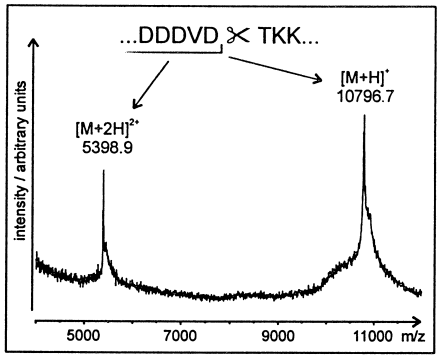


Fig. 1. A: Diagram showing the putative caspase recognition motifs in human ProTα positioned within the spacer region of the bipartite NLS. B: Typical apoptotic morphology of nuclei of HeLa cells treated with staurosporine for 4 h. The cells were stained with Hoechst-33342 and examined by fluorescence microscopy. C: ProTα fragmentation in apoptotic HeLa cells. ProTα was partially purified from HeLa cells induced to undergo apoptosis by various stimuli and from control (non-apoptotic) untreated and poliovirus-infected HeLa cells. The samples were resolved by electrophoresis in an 8% polyacrylamide/7 M urea gel and visualized by methylene blue staining. Lane M, recombinant full-length ProTα and an artificial deletion mutant lacking nine carboxy terminal residues [21] used as markers. The percentage of apoptotic cells in cultures is indicated at the bottom of each lane. CHX, cycloheximide.

A)



B)

m/z of [M+H]+ measured	m/z of [M+H]+ expected	peptide from - to	modification
monoisotopic m/z			
1131.5	1131.5	21-30	
1259.6	1259.6	20-30	
1364.4	1364.5	88-99	
1466.6	1466.7	1-14	N_α-acetyl
average m/z			
6148.4	6146.7	31-87	
6304.5	6302.9	31-88	
7263.4	7259.8	21-87	
7391.4	7388.0	20-87	

Fig. 2. MALDI-MS identification of the ProTα(1–99) fragment generated in apoptotic cells. A: Molecular mass determination of the entire fragment. Mass over charge ratios (*m/z*) of the singly $[M+H]^+$ and doubly $[M+2H]^{2+}$ charged ions comply with the calculated values. The calculated *m/z* of the singly charged ProTα(1–99) fragment corresponds to 10 796.5. B: Tryptic peptide mapping analysis of the same ProTα fragment.

heximide treatment at low concentration of the drug was employed, neither of which alone elicited an appreciable apoptotic response (Agol et al., in preparation). The cells induced to undergo programmed death displayed characteristic morphological features of apoptosis, such as chromatin condensation, fragmentation of the nucleus (Fig. 1B), and cytoplasmic membrane blebbing. ProTα and its putative fragments were isolated from the apoptotic and control cells by a phenol extraction procedure [17,20] which exploits a unique property of highly acidic and hydrophilic ProTα to retain in the aqueous phase, and fractionated by PAGE. As evident from Fig. 1C, ProTα fragmentation occurred in all of the cell cultures induced to undergo apoptosis and in none of the control cell cultures. Electrophoretic mobility of the truncated ProTα was consistent with the predicted cleavage of a short fragment from the protein molecule. The extent of ProTα fragmentation was roughly proportional to the amount of cells undergoing apoptosis. ProTα cleavage has already become evident at 2 h post induction of apoptosis (Fig. 1C) suggesting that ProTα fragmentation is an early event.

To obtain an unequivocal proof that the shifted band observed in apoptotic cell samples corresponds to a ProTα derivative and to determine the site of proteolytic cleavage, MALDI-MS analysis of the truncated protein and its tryptic peptides was performed using the gel-purified material isolated from HeLa cells subjected to cisplatin and to poliovirus+cycloheximide-induced apoptosis. The *m/z* values of the singly and double charged molecule at 10 796.7 and 5398.9, respectively, corresponded to the calculated values for the ProTα(1–99) fragment (Fig. 2A). Furthermore, this

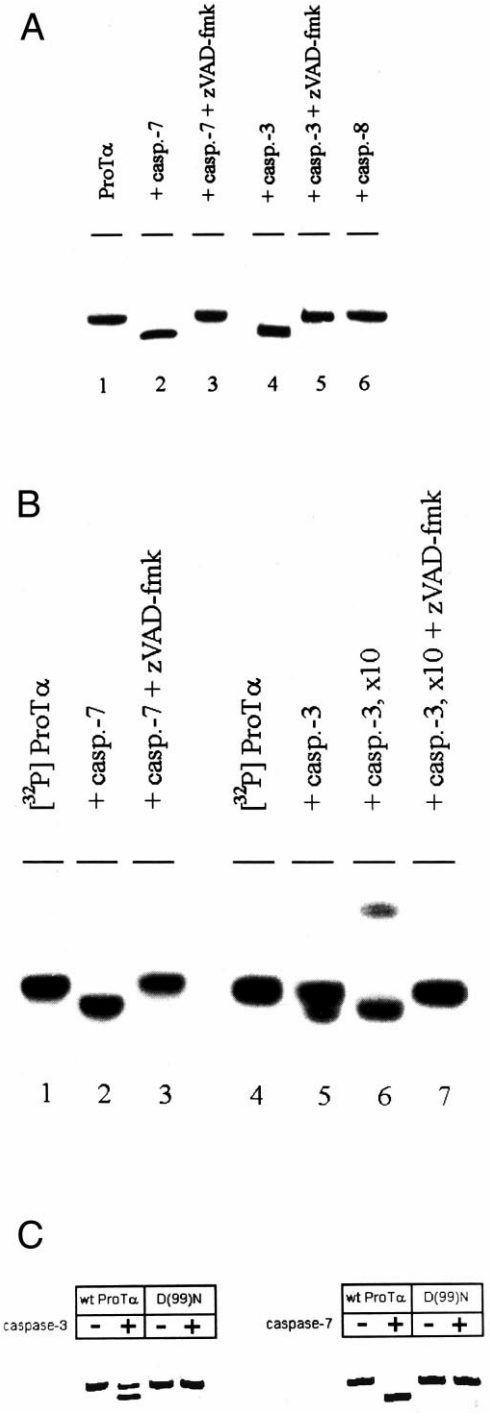


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Fig. 3. ProTα fragmentation in vitro. A: The in vitro treatment of recombinant human ProTα (3 μg per lane) with caspase-3 (180 ng), -7 (75 ng), and -8 (200 ng). B: Cleavage of the [^{32}P]-amino terminally labelled ProTα (10 000 cpm per lane) with caspase-3 (lane 5, 25 ng, and lanes 6, 7, 250 ng) and -7 (75 ng). C: The D(99)N mutation in ProTα abolishes its fragmentation with caspase-3 and -7. Wild-type (wt) ProTα and the ProTα mutant D(99)N (3 μg per lane) were treated with caspase-3 (50 ng) and -7 (40 ng). The samples were fractionated in an 8% polyacrylamide/7 M urea gel and visualized by methylene blue staining (A and C) or by autoradiography (B).

assignment was confirmed by MALDI-MS tryptic peptide mapping analysis of the same fragment, comprising 95% coverage of its sequence including the acetylated amino terminus and the new carboxy terminus generated through the cleavage (Fig. 2B). Thus proteolytic cleavage occurred at the aspartic acid residue-99 in the protein sequence DDVD^{99}.

3.2. Caspase-3 and -7 cleave ProTα in vitro

Recombinant human ProTα was treated with caspase-3, -7, or -8 and fractionated by PAGE. Both caspase-3 and -7 produced a truncated form of ProTα with an electrophoretic mobility similar to that of the ProTα fragment observed in the in vivo samples (Fig. 3A), while treatment with caspase-8 was without any effect. Both a broad spectrum caspase inhibitor zVAD-fmk and the specific caspase group II inhibitor zDEVD-fmk abolished ProTα fragmentation by caspase-3 and -7 (Fig. 3 and data not shown).

An alternative substrate employed for the in vitro ProTα cleavage was represented by a [^{32}P]-end labelled ProTα derivative carrying a protein kinase A recognition motif at its amino terminus. This ProTα substrate provided an increased sensitivity for the cleavage assay and, besides, permitted discrimination between the carboxy and amino terminal cleavage sites in the in vitro reaction. Treatment of [^{32}P]ProTα with caspase-3 and -7 produced a characteristic band with an electrophoretic mobility similar to that of the ProTα(1–99) derivative (Fig. 3B). Preservation of the [^{32}P]-label in this shifted band indicated that the carboxy terminal cleavage has occurred. In this sensitive assay, however, the increased amount of caspase-3 produced a second minor radiolabelled band (Fig. 3B, lane 6). The lower electrophoretic mobility of this newly observed band was most probably due to a decreased negative charge of a short ProTα fragment. Although the nature of this material was not further characterized, its appearance may indicate the existence of an additional minor caspase-3 cleavage site(s) in the amino terminal region of ProTα molecule, at least in vitro.

ProTα fragments generated by caspase-3 and -7 from the recombinant ProTα lacking artificially added sequences (Fig. 3A, lanes 2 and 4), as well as tryptic peptides thereof, were subjected to MALDI-MS analysis. The data obtained confirmed that in both cases the cleavage at D^{99} has occurred (data not shown). To further characterize the caspase cleavage, the ProTα D(99)N mutant was constructed. The D(99)N mutation impaired the caspase-3-, as well as the caspase-7-mediated ProTα cleavage (Fig. 3C). Thus both caspase-3 and -7 utilize the same DDVD^{99} motif for ProTα processing.

3.3. ProTα cleavage product fails to accumulate inside the nucleus

ProTα is a nuclear protein. Nuclear targeting of ProTα is provided by a bipartite NLS consisting of the two short stretches of basic amino acids separated by a spacer region [16]. The identified cleavage site is positioned inside the spacer region of this NLS (Fig. 1A). Therefore, proteolytic cleavage occurring in apoptotic cells generating ProTα(1–99) fragment should lead to elimination of the second basic part of the NLS, which was shown previously to be crucial for the accumulation of ProTα in the nucleus [16].

To compare the subcellular localization of the ProTα(1–99) fragment with that of full-length ProTα, HeLa cells were transfected with plasmids encoding either wild-type ProTα or artificially constructed ProTα(1–99) fragment mimicking the proteolytic cleavage product, fused to the carboxy terminus of GFP. Fluorescence microscopy of the transfected cells revealed that while the wild-type ProTα was exclusively nuclear (Fig. 4A), the ProTα(1–99) fragment lacked the ability to preferentially accumulate inside the nucleus and appeared to be evenly distributed within the cell (Fig. 4B). Western blot analysis of the GFP-ProTα and GFP-ProTα(1–99) from cell lysates using anti-GFP antibodies confirmed that no degradation of the fusion proteins occurred (Fig. 4C). Thus lack of active nuclear uptake is a property of the ProTα(1–99) fragment.

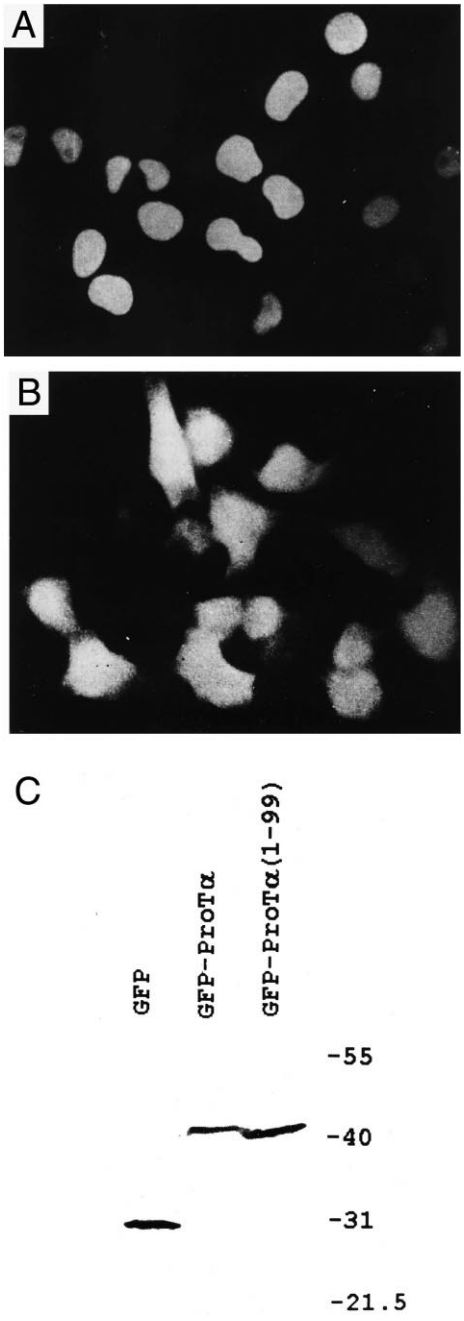

Fig. 4. Subcellular localization of the full-length ProTα (A) and the ProTα(1–99) fragment (B) fused to EGFP. HeLa cells were transfected with the expression constructs and examined by fluorescence microscopy. C: Lysates of HeLa cells producing EGFP, EGFP-ProTα and EGFP-ProTα(1–99) were subjected to 12% SDS–PAGE and immunoblotting with anti-GFP antibodies.

4. Discussion

Our studies add ProTα, an essential nuclear protein involved in cell proliferation, to the expanding yet limited list of the proteins subjected to caspase-mediated degradation in the course of programmed cell death. ProTα cleavage occurs in cells triggered to undergo apoptosis by a variety of cell death inducers and may therefore represent a general feature of apoptotic cell. Caspase fragmentation of ProTα appears to be an early event detectable as early as 2 h post induction of apoptosis. ProTα cleavage occurred close to the carboxy terminus of ProTα at $DDVD^{99}$. ProTα fragmentation at D^{99} was readily observed both in vivo and in vitro. In vitro, caspase-3 and -7, but not caspase-8, could cleave ProTα at D^{99}, and the D(99)N mutation abrogated this cleavage. Caspase-7 is activated independent of caspase-3 [22], each inducing distinct downstream intracellular events. It would be instructive to learn which one of these two enzymes, or both, accomplish the cleavage in vivo.

ProTα fragmentation in apoptosis is likely to impair intranuclear proliferation-related functioning of ProTα through removal of an important determinant within the cleaved-off fragment, as exemplified by the T(105)A loss-of-function mutation [16], as well as through abrogation of the active nuclear import of ProTα. Together with a recent observation indicating that treatment of the cell with ProTα antisense oligonucleotides is able to induce apoptosis [11], our results suggest that ProTα elimination either naturally (by caspase fragmentation) or artificially (by arrest of translation of ProTα mRNA) may promote switching the cells from proliferation to apoptosis. Therefore, a high level of ProTα production in cancer cells may not only maintain their high proliferative status, but also protect these cells from entering into the apoptotic pathway.

Thymus is the organ richest in ProTα, and this observation was commonly associated with the intense thymocyte proliferation. However, thymus appears to be the right place for apoptotic fragmentation of ProTα as well. Indeed, over 90% of immature thymocytes die in the thymus by caspase-mediated apoptosis in the course of positive and negative selection [23,24] and should provide therefore a very significant source of thymic ProTα-derived peptide(s). It is of interest to note that thymosin α_1, the amino terminal peptide of ProTα isolated from the thymus, was reported to be externalized by the cell and activate the cells of the immune system (reviewed in [25]). ProTα fragmentation and relocalization in an apoptotic cell may be proposed to be the first step in converting nuclear protein into an externalized bioactive peptide. It would be instructive to learn whether externalization of the product of caspase fragmentation of ProTα (with or without additional processing) can occur in apoptotic cells, and whether this release may have a physiological significance. Processing of the precursors of interleukin-1β and -18 by caspase-1 and of the pro-IL-16 by caspase-3, prerequisite for activation and secretion of these cytokines [26–28], may provide a precedent for regulation of this type.

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