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Localization of Prothymosin α in the Nucleus

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Prothymosin α is a widely distributed acidic protein to which an immunological function that involved its secretion was initially asigned. However, recent experiments suggest that it is a nuclear protein related to cell proliferation. To study the subcellular distribution of prothymosin α we have used a polyclonal antibody raised against its C-terminus. Using indirect immunofluorescence prothymosin α was located in the nucleus of HeLa, L929, IT-45R1, and NIH3T3 cells, mouse thymocytes, and human thymic stromal cells. The levels of prothymosin α mRNA were increased when the proliferation of IT-45R1 cells was induced, as has been described by others in thymocytes and NIH3T3 cells. These results show that prothymosin α is a nuclear protein related to cell proliferation.

Prothymosin α (ProTα) is a 12.5 kDa acidic protein of unknown function [1]. Some authors have considered ProTα as the putative precursor for several peptides secreted by the thymus for the purpose of promoting maturation of lymphocytes [2,3]. Among these are thymosin α_1 (Tα_1) and thymosin α_{11} (Tα_{11}) , whose sequences of 28 and 35 amino acids respectively are identical to the N-terminus of ProTα [4]. However, others believe that ProTα is the protein from which Tα_1 and Tα_{11} are originated by proteolytic modification during the tissue extraction procedure [1,4]. Since ProTα was reported to protect mice against oportunistic infection [5] and to restore deficient responses in lymphocytes from patients with clinically active multiple sclerosis [6], it was regarded as a regulatory protein of cell immunity with a function involving its secretion. Nevertheless, the absence of a N-terminal signal peptide, as deduced from its cDNA [7], and the wide distribution of ProTα in mammalian tissues [8] make this function unlikely.

The finding that ProTα mRNA rises sharply in stimulated peripheral blood mononuclear cells and NIH3T3 fibroblasts led to the proposal of an alternative role for this protein in cell proliferation [9]. On the basis of the presence of a nuclear localization signal near the C-terminus of ProTα it was proposed that it might function as a nuclear protein [10]. In relation to this hypothesis, microinjection of ProTα into the cytoplasm of *Xenopus* oocytes showed that this protein migrates to the nucleus [11]. Furthermore, chimeric proteins with the ProTα sequence were located in the nucleus of HeLa and COS cells [12,13]. In addition, ProTα has been isolated from rat thymus nuclei [6]. However, in other reports ProTα has been found covalently linked to a small cytoplasmic RNA [14] and it was characterized in the medium in which calf and rat thymocytes had been cultured [15]. On the other hand, localization of ProTα using antibodies against Tα_1 has given rise to contradictory data on its subcellular distribution, indicating either nuclear [16,17] or cytoplasmic localization [18,19,20].

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Abbreviations: ProTα, prothymosin α; Tα_1, thymosin α_1; Tα_{11}, thymosin α_{11}; DPBS, Dulbecco's phosphate buffered saline; Tβ_4, thymosin β_4; KLH, keyhole limpet hemocyanin.

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In the present work, the variation of ProTα mRNA levels during cellular proliferation and the localization of this protein in the nucleus of several cellular types are described.

MATERIALS AND METHODS

Materials. Fluorescein-conjugated goat anti-rabbit IgG and peroxidase-conjugated goat anti-rabbit IgG were from *Pasteur Diagnostics.* ProTα was purified from calf thymus as described [15]. Tα_1 and Tα_{11} were synthesized by Dr. E. P. Heimer (Hoffman-La Roche, Nutley, USA). All other reagents and materials were of analytical grade.

Cell culture and antibodies. HeLa, IT-45R1 and NIH3T3 cells were grown at 37°C in 7.5% CO_2 in Dulbecco's modified Eagle's medium with high glucose, supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 mg/ml streptomycin. IT-45R1 is an epithelial cell line from rat thymus [21]. L929 cells were cultured in the same medium, except that 5% fetal calf serum was used. Thymocytes were obtained by free diffusion from BALB/c mouse thymus fragments followed by cytocentrifugation. Human thymic stromal cells (fibroblasts and epithelial cells) came from a monolayer of adherent cells originated from human thymus explants essentially as described [20].

Monoclonal anti-Tα_1 antibodies [22] and rabbit polyclonal anti-ProTα antibody were provided generously by Dr. J. C. Monier, Department of Immunology, Alexis Carrel Faculty of Medicine (Lyon, France). Rabbit polyclonal anti-ProTα antibody was obtained by Dr. A. A. Haritos, Zoological Laboratory, Faculty of Science (Athens, Greece) and was raised against a KLH conjugated peptide corresponding to amino acids 90-109 of the human ProTα sequence. Rabbit polyclonal anti-Tα_1 antibodies were produced essentially as described [23].

RNA analysis. RNA preparation, dot blots and hybridization analysis were carried out as previously described [24]. After hybridization, the blots were washed twice in 2× SSPE (1× = 0.18 M ClNa, 10 mM NaH_2PO_4, pH 7.4, and 1 mM EDTA), 0.5% sodium dodecyl sulphate at room temperature for 10 min each and three times in 0.1× SSPE, 0.5% sodium dodecyl sulphate at 65°C for 30 min each. The filters were air-dried and exposed to X-ray films at −70 °C in the presence of intensifying screens. Exposure times of autoradiographs were different for each probe. The intensity of dots was quantified by scanning densitometry (λ = 595) of different autoradiographic exposures of each filter. The probes were a 1.15 kb *EcoRI* fragment containing the human ProTα cDNA [25], a 1.12 kb *BamHI* fragment containing the human cyclin cDNA [26] and a 0.5 kb *BamHI* fragment containing the rat Tβ_4 cDNA [27].

Indirect immunofluorescence. For immunofluorescence studies cells were grown in eight-compartment tissue culture Lab-Tek chamber slides (Nunc). Cells were subjected to reagents for immunofluorescence at room temperature. After 48-72 h cells were rinsed with Dulbecco's phosphate buffered saline (DPBS), fixed by incubation with 2.5% formaldehyde in DPBS for 15 min, incubated for 5 min in each of three changes of DPBS, permeabilized with methanol for 2 min, washed with three changes of DPBS and incubated with 100 ml of rabbit polyclonal anti-ProTα antibody (diluted 1:50 in DPBS) for 1 h. After three washes with DPBS cells were incubated with 100 ml of fluorescein-conjugated goat anti-rabbit IgG using the conditions described for the primary antibody. Subsequently, slides were washed three times with DPBS, mounted in 90% glycerol/10% DPBS and examinated with a Universal Zeiss epifluorescence microscope. Photographs were prepared with Kodak Ektachrome 400 ASA films. Monolayers of thymocytes obtained by cytocentrifugation were dried in air for 15 min followed by incubation with formaldehyde as described above. From this point, all steps were carried out in the same way as with adherent cells.

Western blotting. Protein extracts were obtained from subconfluent HeLa cells by freezing and thawing four times in PBS containing 1 mM phenylmethylsufonyl fluoride. Extracts were subjected to SDS-PAGE on 15% acrylamide gels and, as ProTα did not bind to nitrocellulose, they were transferred to nylon GeneScreen*Plus* membranes (Du Pont New England Nuclear) according to the manufacturer's manual. Immediately after transfer, filters were treated with glutaraldehyde as described [28] and were blocked with 5% nonfat dry milk in PBS for 12 h at 4 °C. The membranes were cut and each strip incubated with primary antiserum diluted 1:100 in MTP (1% nonfat dry milk, 0.05% Tween 20 in PBS) for 2 h. After three washes with PBS containing 0.05% Tween 20, the membranes were incubated with the second antiserum (peroxidase-conjugated goat anti-rabbit IgG) diluted 1:200 in MTP for 2 h. Membranes were washed four times with water and specifically bound antibodies were localized with an enzyme substrate solution containing 3.3 mM 4-chloro-1-naphthol, 20% methanol and 3 mM H_2O_2.

RESULTS

Analysis of ProTα mRNA levels during the proliferation of IT-45R1 cells. It has been reported that ProTα mRNA levels of NIH3T3 serum deprived cells and thymocytes increase during cellular proliferation [9,29]. However, NIH3T3 cells that were synchronized at different stages of the cycle, by means of mitotic shake-off after nocodazole arrest or double thymidine block, did not show variations in the levels of ProTα mRNA when progressed through the cycle [30]. To confirm the variation of ProTα mRNA levels during proliferation we used IT-45R1 cells. Cellular proliferation was induced by trypsinization of monolayers and subconfluent culture of the cells. As a control for proliferation it was used the cDNA of proliferating cell nuclear antigen/cyclin (PCNA), the

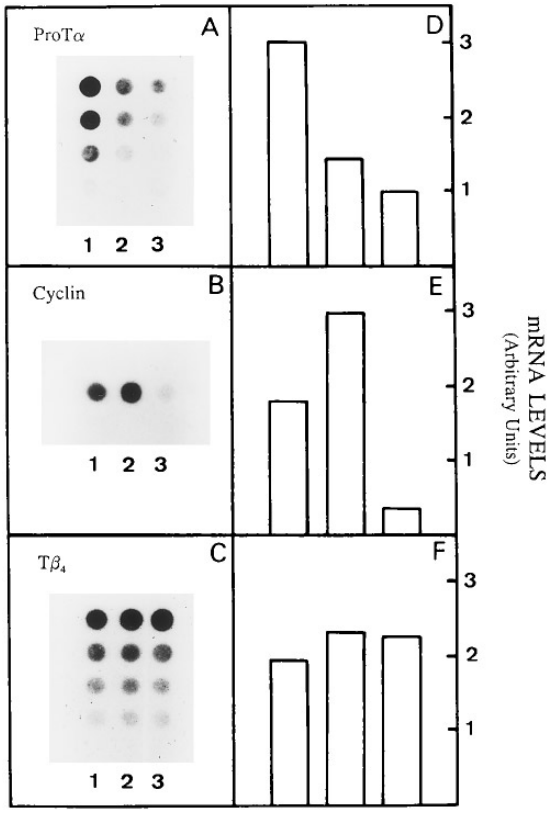

FIG. 1. Analysis of ProTα mRNA levels during the cellular proliferation of IT-45R1 cells. Total RNA was transferred by dot blot to nitrocellulose. Filters were hybridized with cDNAs of ProTα (A), cyclin (B), and Tβ_4 (C). RNA was obtained at 48 h after trypsinization (lane 1), 72 h (lane 2) and three days after formation of the monolayer (lane 3). For hybridization with ProTα and Tβ_4 cDNAs aliquots of 6 μg, 3μg, 1.5 μg, and 0.75 μg of RNA were used and aliquots of 7 μg for hybridization with cyclin cDNA. On the right are shown the mean values of scanning densitometry of the autoradiographic exposures that belong to each filter.

polymerase δ auxiliary factor whose mRNA levels are directly related to the proliferative activity of the cells [31]. The analysis showed that after 48 h in culture (about 30% of confluence, Figs. 1B and 1E, lane 1) mRNA levels of cyclin increased four times when compared with those in a confluent monolayer (lane 3) and the maximal expression was reached after 72 h (about 65%-70% of confluence, lane 2).

When the levels of ProTα mRNA were studied, a similar result to the cyclin was obtained (Figs. 1A and 1D), but the highest expression was found at 48 h (lane 1). When a monolayer was formed the levels came down three times compared with the maximum (lane 3). The control hybridization with the cDNA of thymosin β_4 (Tβ_4), a cytoplasmic actin sequestering peptide [32], showed that its mRNA levels remain constant independent of the proliferative activity (Figs. 1C and 1F).

Localization of ProTα. The localization of ProTα using polyclonal and monoclonal antibodies against Tα_1 has shown contradictory results. Some authors have found immunoreactive peptides in the nucleus [16,17] and others in the cytoplasm [18,19,20]. All these works have in common that none of them has characterized the proteins recognized by the antibodies.

Using two polyclonal and two monoclonal antibodies against Tα_1, by indirect immunofluorescence we have found immunoreactive peptides in the nucleus of IT-45R1, HeLa, NIH3T3

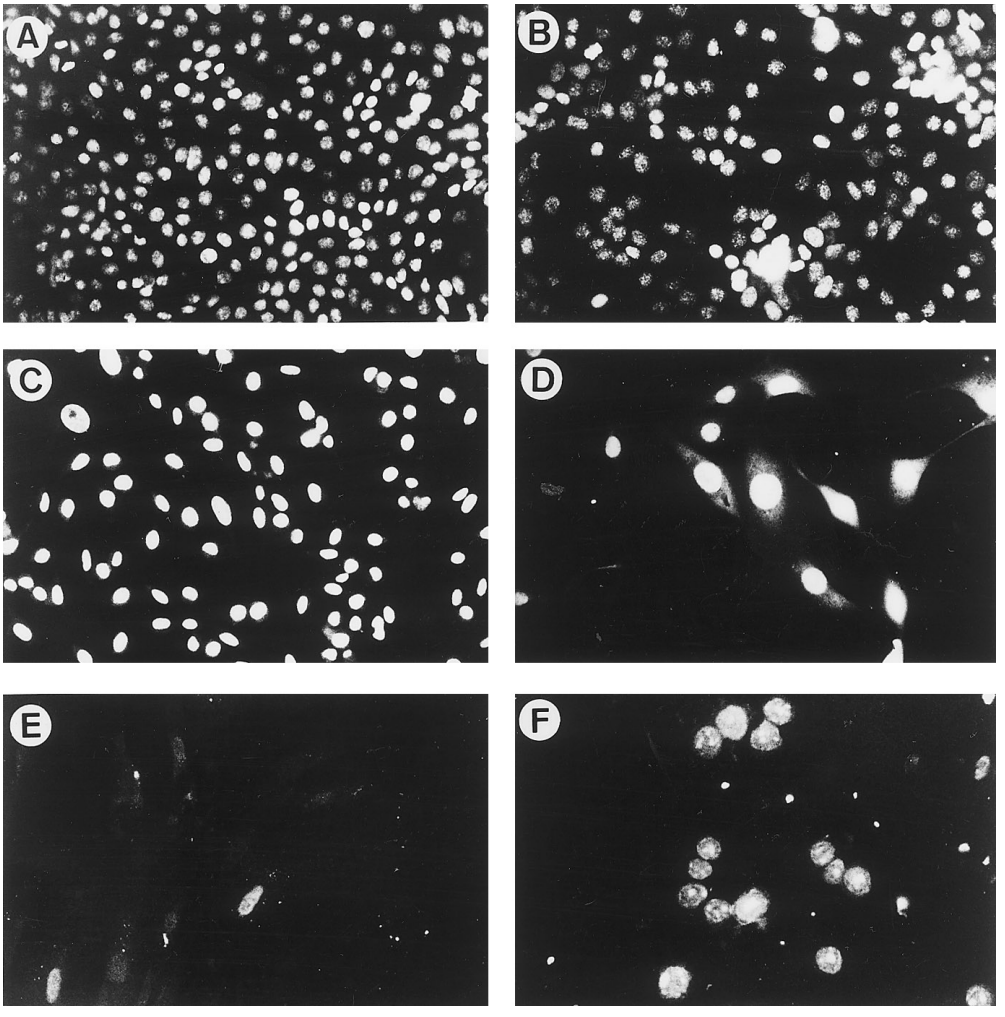

FIG. 2. Localization of ProTα in the nucleus. Cells were processed for indirect immunofluorescence using the polyclonal antibody against the C-terminus of ProTα. (A) IT-45R1 cells, (B) HeLa cells, (C) L929 cells, (D) NIH3T3 cells, (E) thymic stromal cells, and (F) thymocytes.

and L929 cells, mouse thymocytes and human thymic stromal cells (data not shown). However, when the peptides recognized by the antibodies were studied, by western blotting on extracts of HeLa and NIH3T3 cells, the monoclonal antibodies reacted with two 29 kDa proteins, as was described in mouse tissues [33], and the polyclonal antibodies reacted with the same proteins and also with ProTα (data not shown). In ELISA the monoclonal antibodies recognized only Tα_1 and the polyclonal antibodies Tα_1, Tα_{11} and ProTα. Therefore, we can conclude that the antibodies against Tα_1 detect other nuclear proteins besides ProTα and they are not adequate to localize it.

ProTα is a weakly antigenic protein and it is difficult to obtain antibodies with enough specificity. To avoid this problem we have used a rabbit antiserum raised against its C-terminus. In ELISA, it reacted specifically with ProTα but not with Tα_1, Tα_{11} and the homologous protein parathymosin α. Using indirect immunofluorescence with IT-45R1, HeLa, NIH3T3, L929 cells, mouse thymocytes and human thymic stromal cells, ProTα was located exclusively

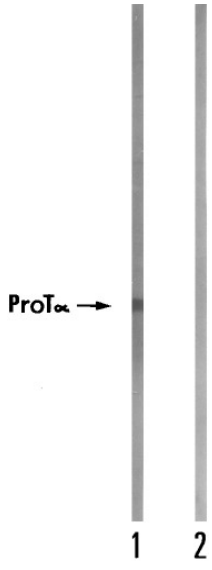

FIG. 3. Specificity of the polyclonal antibody against ProTα by western blotting. Whole-cell extracts were prepared from HeLa cells. Proteins were resolved in 15% SDS-polyacrylamide gels and transferred to nylon membranes. The filters were incubated with the anti-ProTα antibody (lane 1) or only with the second antibody (lane 2). The electrophoretic mobility of ProTα is indicated by an arrow.

in the nucleus (Fig. 2). Only NIH3T3 cells showed slight reactivity in the cytoplasm (Fig. 2D). On the other hand, what we see stained in cytocentrifuged thymocytes is only the nucleus (Fig. 2F), as was confirmed incubating cells with anti-DNA topoisomerase I antibodies (data not shown). This result is in agreement with the isolation of ProTα from rat thymus nuclei together with high mobility group (HMG) proteins [6].

The antiserum specificity was assayed by western blotting of HeLa cells extracts. The antibody recognized specifically a protein with the same electrophoretic mobility as ProTα (Fig. 3, lane 1).

DISCUSSION

Tsitsiloni et al., using a polyclonal antibody to Tα_1 in radioimmunoassay, located the immunoreactive peptides mainly in the cytoplasm from liver and thymic cell extracts [34]. However, in this work the integrity of the isolated nucleus was not controlled and the antigens recognized by the antibodies were not characterized. Moreover, it is well known that many nuclear proteins escape from the nucleus during cellular homogenization [35].

Franco et al. have reported the *apparent* secretion of ProTα from calf and rat thymocytes [15]. However, these results have been also interpreted as a general loss of protein from the cells rather than a specific secretion of ProTα (Horecker, B. L., personal communication). Furthermore, we must consider that the cells had a mean viability of 85% and in neither case could cellular debris or dying cells be excluded as the source of the extracellular proteins.

Makarova et al. have found ProTα covalently linked to a small RNA in the cytoplasm of mouse Krebs II ascites carcinoma cells [14]. Other authors using preparations of ProTα from calf thymus saw no NMR evidence of RNA in the bulk of the protein [36]. Therefore, it is possible that there is a very low proportion of ProTα linked to RNA [37]. Moreover, Makarova et al. employed in the extraction buffer SDS as detergent and it has been demonstrated that in COS and human myeloma cells ProTα and other nuclear proteins

escape easily from the nucleus during the isolation procedure, independently of the detergent concentration used [13,38].

In regard to the possible function of ProTα in the nucleus, it has been reported that ProTα binds specifically to histone H1 *in vitro.* This fact was tested by transferring histone H1 onto nitrocellulose membranes and subsequently incubating with ProTα [39]. Nevertheless, using the same assay with a concentration of ProTα 30 times higher than the one previously used (50 μg/ml), it has been found that ProTα binds to all histones (H1, H2A, H2B, H3 and H4). This result has been confirmed by affinity chromatography of histones on ProTα bound to Sepharose and eluent analysis on SDS-polyacrylamide gels (Diaz, C., paper submitted).

Searching homologous proteins to ProTα in the MIPSX database, we have found that the nuclear high mobility group-1 protein (HMG-1) has a 35%-42% of homology with ProTα, the percentage depending on species. The homologous region corresponds to the series of acidic residues identified as the histone-binding domain of the HMG-1 [40]. Moreover, it has been reported that incubation of HMG-1 with the four core histones favours histone association primarily into tetramers and that the assembly of pre-formed histone-HMG-1 complexes with DNA yields nucleosome-like subunits [41]. Therefore, it will be interesting to investigate the role of ProTα in the constitution of nucleosomes.

The initial characterization of possible ProTα receptors on peripheral blood mononuclear cells has been recently published [42]. However, phytohemaglutinin or periodate can stimulate lymphocytes to divide and a panoply of apparently unrelated substances ranging from viruses to poly(I)-poly(C) to tilorone can induce interferon. No one would suggest that these pharmacologically active substances are part of the cell's machinery for producing a physiological effect [43]. In this case, we believe that ProTα is acting as a xenobiotic in the studied cells and that it is a nuclear protein that could be interacting with membrane receptors non-specifically. This interpretation is supported by the fact that ProTα presents homology with interferon α_2 in the sequence Leu-Lys-Glu-Lys-Lys (residues 16-20 in ProTα) and the synthetic fragment Leu-Lys-Glu-Lys-Lys-Tyr-Ser-Pro produces a regulatory effect on cellular and humoral types of immune response [44]. Furthermore, it has been found that this sequence is involved in the competition of interferon α_2, ProTα and cholera toxin B subunit for common receptors on human fibroblasts and mouse thymocytes [45].

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