Molecular Biology, Vol. 39, No. 5, 2005, pp. 631–645. Translated from Molekulyarnaya Biologiya, Vol. 39, No. 5, 2005, pp. 729–745.

REVIEWS

UDC 577.122.2;577.214.6;577.218

This review is dedicated to A.A. Bogdanov, our teacher and a true gentleman who is held in high esteem by all who know him

New Functions of a Well-Known Protein: Prothymosin α Is Involved in Protecting Cells from Apoptosis and Oxidative Stress

A. G. Evstafieva, R. N. Karapetian, Yu. P. Rubtsov, G. S. Filonov, I. S. Abaeva, T. V. Fateeva, S. V. Melnikov, N. V. Chichkova, and A. B. Vartapetian

Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow, 119992 Russia
e-mail: evstaf@genebee.msu.ru

Received April 20, 2005

Abstract—Recent studies have revealed two new functions of prothymosin α (ProTα), a well-known protein and a subject of intense research. In addition to acting as an immunomodulator and stimulating cell proliferation, ProTα is involved in protecting the cell from apoptosis and regulating the expression of oxidative stress defense genes. The review considers the methods and approaches used to demonstrate the two new functions of ProTα.

Key words: prothymosin α, nuclear localization signal, apoptosis, caspases 3 and 7, oxidative stress, Keap1, Nrf2, RNA interference

INTRODUCTION

To study new functions of cell proteins and elucidate the mechanisms of their action are among the most topical and intricate problems of molecular biology in the era of genomics and proteomics. The function of a protein in the cell is rather difficult to understand even when everything or almost everything is known about the protein structure. Studies performed in the past few years have revealed two new functions for prothymosin α (ProTα), which was identified 20 years ago and has been actively studied since then. It was found that ProTα not only acts as an immunomodulator and stimulates cell proliferation, but it also protects cells from apoptosis and oxidative stress. The methods and approaches used to demonstrate these two new functions of ProTα are the focus of this review.

TRADITIONAL FUNCTIONS OF PROTHYMOSIN α

ProTα is a small (12.6 kDa), extremely acidic (pI = 3.5) protein and has no elements of regular structure under physiological conditions [1–6] (Fig. 1). Relatively simple as it is, ProTα performs several functions in the cell. ProTα was initially identified as a precursor of the thymus hormone thymosin α1, an immunostimulating peptide consisting of the 28 N-terminal residues of ProTα [1]. An immunostimulating effect was also observed for ProTα (for review, see [7]). Found in serum and in the thymus, ProTα was classified as a hormone involved in the normal functioning of the mammalian immune system (for review, see [6, 7]).

More recent data has questioned the hormonal nature of ProTα. There is still no evidence for ProTα secretion. ProTα lacks a signal sequence for export from the cell and is synthesized on free, rather than membrane-associated, polysomes [8]. Moreover, ProTα proved to be a nuclear protein [9–12]. A nuclear localization signal (NLS) was found in the C-terminal region of ProTα [12], suggesting a function in the cell nucleus for this protein.

Ample experimental data implicate ProTα in cell proliferation (for review, see [6]). For instance, the levels of ProTα and its mRNA correlate with the proliferation status of the cell. ProTα accumulates in tissues with intense cell proliferation. The maximal content is characteristic of the thymus and the spleen, but ProTα is also abundant in nonlymphoid tissues. While cells actively proliferate during embryonic and early postnatal development, ProTα occurs at a high content in all tissues. The ProTα level is elevated in regenerating tissues.

0026-8933/05/3905-0631

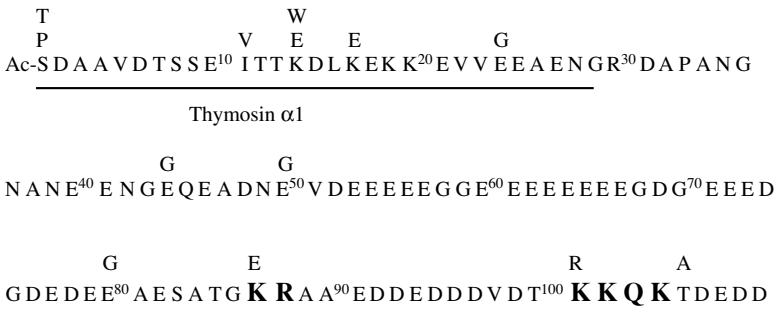

Fig. 1. Primary structure of human ProTα. The N-terminal 28-mer peptide, corresponding to thymosin α1, is underlined. Mutations considered in the text are shown at the top of the wild-type ProTα sequence. The C-terminal region of ProTα contains two blocks of basic amino acid residues, which represent a bipartite NLS (boldfaced).

The role of ProTα in processes associated with cell division is confirmed by the fact that cells defective in ProTα synthesis stop proliferating until the synthesis is restored [13]. Moreover, cell treatment with antisense oligonucleotide directed to the ProTα mRNA induces apoptosis [14]. An increase in ProTα production thereby accelerates proliferation by reducing G_1 and retards cell differentiation [15].

The activation of ProTα gene expression was observed in colonic, enteric, hepatic, lung, and breast carcinoma cells (for review, see [6]). Robertson and colleagues [16] have recently shown that an overproduction of ProTα not only affects cell proliferation, but also allows Rat-1 cells to grow in a serum-depleted medium *in vitro*, prevents contact inhibition, and stimulates the growth of nonattached cells. Cells with an elevated content of ProTα are similar in properties to cells with a high-level production of the well-known Ras oncoprotein. Thus, ProTα possesses some characteristics of oncoproteins.

IDENTIFICATION OF FUNCTIONALLY IMPORTANT DETERMINANTS OF PROTHYMOSIN α

Random mutagenesis often makes it possible to identify functionally important regions of a protein, provided that an adequate system is available for screening mutations inactivating the protein of interest. A system for the *in vivo* selection of functionally important mutations of ProTα has been developed in our laboratory [12], taking advantage of the fact that human ProTα suppresses cell division when overproduced in yeast *Saccharomyces cerevisiae* cells [17].

Experiments were designed as follows. Mutations were introduced at random in the ProTα cDNA. Mutant cDNAs were cloned in a yeast expression vector under the control of an inducible promoter. When transformed cells were grown in the presence of the corresponding inducer, only cells that produced functionally inactivated mutant ProTα grew and formed colonies. When functionally important domains of ProTα were not affected by mutations, ProTα production suppressed the growth of yeast cells and no colonies were formed. Plasmid DNAs were isolated from growing colonies and sequenced. Some clones carried the ProTα cDNA harboring several mutations. In some of these cases, individual mutations were isolated by subcloning. Each plasmid containing the ProTα cDNA with one (or several) mutation was used to transform *S. cerevisiae* cells, and the growth curves of transformants were analyzed.

This system made it possible to identify several functionally important regions in ProTα and to obtain some useful point mutations inactivating the protein. Several regions were mapped in the amino acid sequence of ProTα (Fig. 1). All mutations of the N-terminal fragment, corresponding to thymosin α1—S1P, S1T, I11V, K14W, K17E, and E24G—had no effect on the growth suppression of yeast cells by ProTα. The effect of the C-terminal mutations K87E and T105A was maximal; the E80G and K101R mutations alleviated growth suppression. Experiments with the mutants made it possible to identify a bipartite NLS in ProTα (see below). The double mutation E(44,50)G also alleviated growth suppression. The role of this mutation was determined by studying the interaction of ProTα with one of its molecular partners.

Identification of a Bipartite NLS in ProTα

Primary structure analysis of the C-terminal domain of ProTα and identification of the mutations inactivating ProTα made it possible to assume that two blocks of basic amino acid residues, KR^{88} and $KKQK^{104}$, form a so-called bipartite NLS. To verify this assumption, yeast cells were transformed with chimeric constructs allowing production of the green fluorescent protein (GFP) fused with the wild-type ProTα or its mutants with the above amino acid substitutions. The intracellular localization of the chimeric proteins was studied by fluorescence microscopy [12]. The K87E and K101R mutations each

changed the intracellular localization of ProTα from nuclear to nuclear–cytoplasmic; i.e., ProTα lost the capacity to be efficiently transported into the nucleus. Similar changes in intracellular localization were observed for GFP-PorTα(1–100), which lacked the nine C-terminal residues of ProTα, including the second basic block $KKQK^{104}$. Since yeast cells are heterologous for human ProTα, similar experiments were carried out with a homologous system of human cells [12]. The chimeric proteins were overproduced in HEK293 human embryonic kidney cells, and the results obtained with yeast cells were fully reproduced. Thus, the two blocks of basic amino acid residues, KR^{88} and $KKQK^{104}$, are necessary for the nuclear localization of ProTα. To check whether the blocks are sufficient for nuclear localization, HEK293 cells were used to overproduce chimeric GFP-ProTα(82–109), which contained a short fragment of ProTα, including both KR^{88} and $KKQK^{104}$ blocks. The chimeric protein was localized exclusively in the nucleus. The results (table) showed that NLS is in the region 82–109 of ProTα and consists of two parts.

Thus, the alleviation of the suppressor effect of human ProTα on yeast cell proliferation is probably explained by the fact that the K87E and K101R mutations of the C-terminal domain of ProTα inactivate its NLS; as a result, mutant ProTα is redistributed from the nucleus into the cytoplasm. However, this mechanism does not apply to all C-terminal mutations. For instance, ProTα with the T105A mutation does not affect yeast cell proliferation, notwithstanding its nuclear localization. This finding suggests another functional determinant within this region of ProTα, but its role remains unclear.

Analysis of ProTα NLS

ProTα	Suppression of yeast cell growth	Intracellular localization
Wild type	+	Nuclear
K87E	–	Nuclear-cytoplasmic
K101R	+/–	Nuclear-cytoplasmic
T105A	–	Nuclear
Δ(101–109)	–	Cytoplasmic
NLS(82–109)	nd	Nuclear

Note: Suppression of yeast cell growth by human ProTα (+) was or (–) was not observed or (nd) was not determined. The intracellular localization of the proteins added to the C terminus of GFP was studied in HEK293 cells.

FRAGMENTATION OF PROTHYMOSIN α DURING APOPTOSIS

The main effectors of programmed cell death (apoptosis) are caspases, cysteine proteases that are activated in apoptosis and cleave a limited set (1–3%) of key cell proteins at specific sites after Asp. ProTα is an extremely acidic protein containing 19 Asp residues. Its C-terminal region harbors several DxxD motifs (Fig. 1), which are consensus sites recognized by group 2 caspases, including the main effector caspases of the cell. On the other hand, ProTα is essential for cell division [6, 13–15]. Thus, ProTα can be regarded as a potential natural substrate of caspases.

To check this hypothesis, the integrity of ProTα was studied in HeLa cells during apoptosis induced with cisplatin, staurosporine, the tumor necrosis factor (TNF) combined with emetine, UV irradiation, or abortive poliovirus infection [18, 19]. A procedure was developed for the rapid isolation of ProTα and its fragments from HeLa cells, taking advantage of the unique property of ProTα to remain in the aqueous phase upon phenol extraction owing to its extraordinary hydrophilicity [20]. It was found that apoptotic, but not normal, cells contain a shortened ProTα that is similar in electrophoretic mobility to marker ProTα (1–100) and occurs in an amount that is directly associated with the proportion of apoptotic cells (Fig. 2a).

Fragmentation of ProTα becomes detectable as early as 2 h after the induction of apoptosis and, consequently, is an early apoptotic event. Mass spectrometry (MALDI-MS) of the shortened protein showed that ProTα is cleaved after D^{99} belonging to the motif $DDVD^{99}$ [18].

In Vitro Cleavage of ProTα by Caspases 3 and 7

The $DDVD^{99}$ site of ProTα cleavage in apoptotic cells corresponds to the consensus site DxxD recognized by group 2 caspases, that is, by caspases 3 and 7. To determine which of these enzymes is responsible for ProTα hydrolysis, recombinant ProTα was digested with each of them.

Caspase 7 cleaved ProTα at a single site, after D^{99} [18]. The wide-range caspase inhibitor carbobenzoxy-Val-Ala-Asp fluoromethyl ketone (zVAD-fmk) prevented the cleavage. A similar effect was observed for the D99N mutation of the recombinant protein (Fig. 2b).

In contrast, caspase 3 cleaved ProTα into several fragments [19]. As the stringency of digestion with caspase 3 was increased, PAGE consecutively revealed three bands: a, b, and c (Fig. 2c), which corresponded respectively to the ProTα fragments 1–99, 7–99, and 32–99 on the evidence of mass spectrometry. Preincubation of caspase 3 with the specific inhibitor carbobenzoxy-Asp-Glu-Val-Asp fluoromethyl ketone (zDEVD-fmk) prevented the generation of all three products, suggesting the specificity of hydrolysis. The results agree with the presence of three cleavage sites for caspase 3 in ProTα: one major site is after $DDVD^{99}$ and two minor ones are after $AAVD^{6}$ and $NGRD^{31}$.

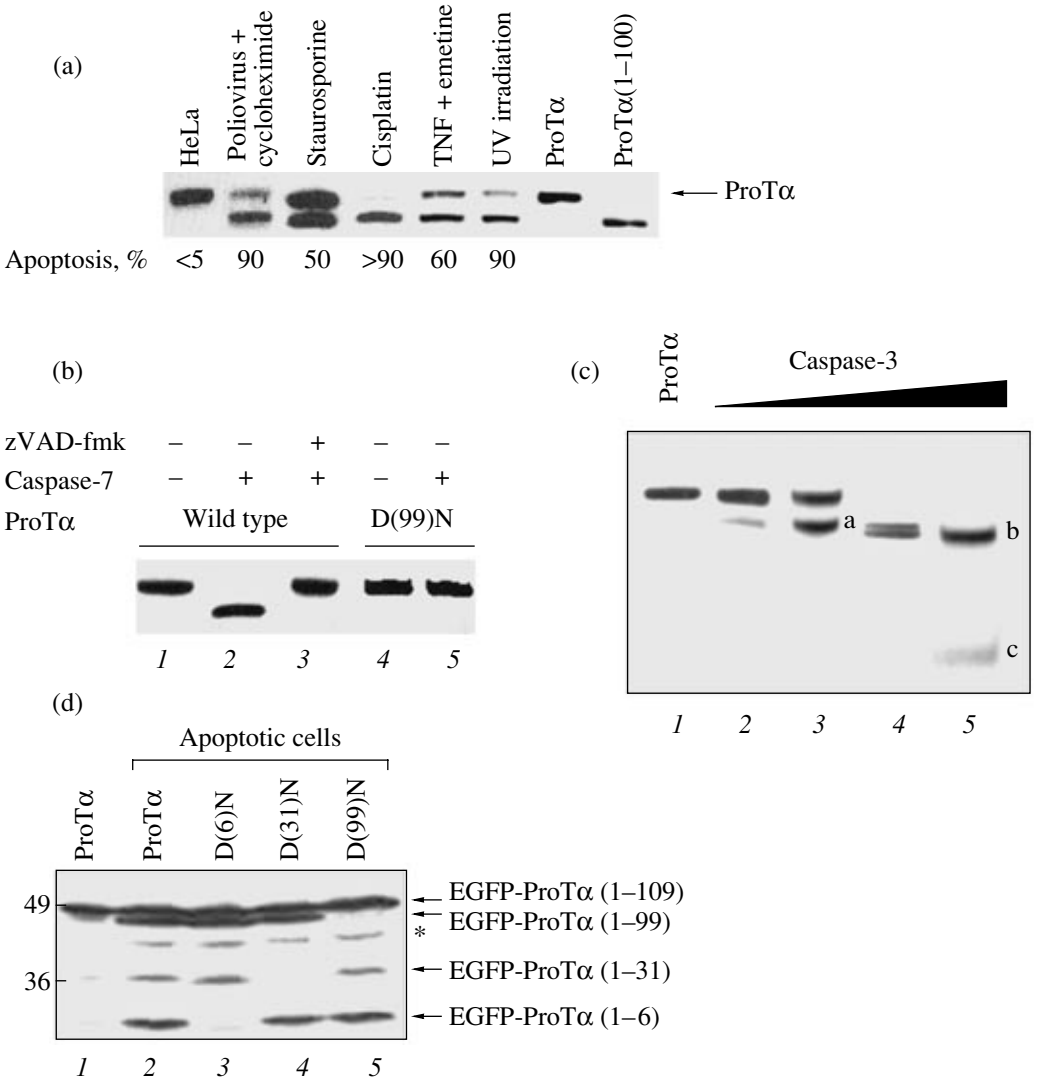

Fig. 2. Fragmentation of ProTα and its mutants by caspases *in vivo* and *in vitro.* (a) ProTα fragmentation in apoptotic HeLa cells. ProTα was isolated from control (HeLa) cells and cells treated with various proapoptotic agents (indicated at the top). Recombinant ProTα and its 1–100 fragment were used as markers (the two rightmost lanes). The percent of apoptotic cells in each culture is indicated at the bottom. Here and in (b, c), proteins were analyzed by denaturing PAGE in 8% gel and stained with Methylene Blue. (b) Hydrolysis of (*1–3*) recombinant ProTα and (*4, 5*) its D99N mutant by caspase 7 *in vitro.* Digestion with caspase 7 was carried out in the (*2, 5*) absence or (*3*) presence of the caspase inhibitor zVAD-fmk. (c) Incubation of recombinant ProTα (*1*) without or (*2–5*) with increasing amounts of caspase 3 *in vitro.* Bands a, b, and c correspond to ProTα hydrolysis products. (d) Fragmentation of EGFP-ProTα and its mutants in apoptotic HeLa cells. HeLa cells overproducing the wild-type ProTα or its mutants D6N, D31N, and D99N fused with the C end of EGFP were lyzed (*1*) before or (*2–5*) after treatment with TNF and emetine. The lysates were analyzed by denaturing PAGE in 12% gel with subsequent immunoblotting with monoclonal antibodies against EGFP. The band presumably corresponding to the product of ProTα hydrolysis after D^{69} is indicated with an asterisk.

To verify this conclusion and to check whether some ProTα fragments are lost during purification, a ProTα digest by caspase 3 was examined by mass spectrometry without preliminary purification [19]. As early as 3 min after adding caspase 3, the peptide bonds after D^{99} and D^{69} were cleaved to yield the fragments 1–99 and 1–69. ProTα was cleaved after D^{6} and D^{31} during further digestion, and 3-h incubation lead to the accumulation of the fragments 1–99, 7–99, and 32–99. It is of interest that the fragment 1–69 was detectable only in early digestion and was virtually absent from the reaction mixture at later stages. To explain this finding, it is possible to assume that the fragments 1–99, 7–99, and 32–99 are cleaved after D^{69} with an extremely low efficiency in contrast to the full-size ProTα, while the fragment 1–69 is efficiently cleaved after D^{31} by caspase 3.

ProTα Sites Cleaved by Caspases *in vivo*

The ProTα fragment 1–99 was the only product of caspase hydrolysis that was isolated from apoptotic

cells. The shorter fragments 7–99 and 32–99, which accumulate during ProTα digestion with caspase 3 *in vitro*, were not detected in apoptotic cells [18, 19]. Two explanations of this finding are possible: either the short fragments of ProTα are unstable intermediates and are further degraded *in vivo* or ProTα cleavage after D^6 and D^{31} by caspase 3 is an artifact of the *in vitro* system.

Which of the two alternatives is true? In other words, is ProTα cleaved after D^6 and D^{31} in apoptotic cells? The following experiment was designed to answer this question. Assuming that the ProTα fragments 7–99 and 32–99 are indeed unstable, an attempt could be made to detect the corresponding fragments 1–6 and 1–31. To allow detection, reporter EGFP was added to the N terminus of ProTα. Chimeric EGFP-ProTα was overproduced in HeLa cells, and apoptosis was induced using TNF and emetine. Fragmentation of the chimeric protein was assayed by immunoblotting with antibodies against EGFP. A specific set of ProTα fragments fused with EGFP was observed in apoptotic, but not in normal, cells (Fig. 2d). It was assumed that the bands revealed correspond to the expected fragments 1–6, 1–31, and 1–99 of EGFP-ProTα. To verify this, Asn was substituted for Asp in each of the putative cleavage sites (the D6N, D31N, and D99N mutations). Each mutation led to the disappearance of the corresponding band from the set of cleavage products of the chimeric protein. Thus, caspases proved to cleave ProTα after D^6 and D^{31} in apoptotic cells *in vivo.* It should be noted that another, minor band was observed along with the three above bands of the EGFP-ProTα hydrolysis products, and this additional band corresponded in electrophoretic mobility to the EGFP-ProTα fragment 1–69, suggesting ProTα cleavage after D^{69} *in vivo.*

To estimate the contribution of caspase 3 to ProTα cleavage *in vivo*, similar experiments were carried out with MCF-7 human breast cancer cells, which do not exhibit caspase 3 activity [19]. Chimeric EGFP-ProTα was not cleaved in MCF-7 cells treated with TNF and emetine, although such treatment induces the processing of procaspase 7. When intact procaspase 3 was overproduced in MCF-7 cells, apoptotic cells again displayed the characteristic set of EGFP-ProTα hydrolysis products. The mutations affecting D^6, D^{31}, and D^{99} led to a disappearance of the corresponding bands.

The above data demonstrate that caspase 3 is mostly responsible for ProTα fragmentation in apoptotic cells *in vivo.*

Changes in Intracellular Localization of ProTα in Apoptotic Cells

In apoptotic cells, ProTα is cleaved predominantly at the major $DDVD^{99}$ site in the spacer of bipartite NLS. As a result, ProTα loses the ten C-terminal amino acid residues, including the $KKQK^{104}$ block, essential for the accumulation of ProTα in the nucleus. Hence, redistribution from the nucleus into the cytoplasm can be expected for ProTα cleaved by caspases.

To check this assumption, the intracellular localization was studied for truncated ProTα(1–99), which mimics the product of ProTα cleavage by caspase at the major site. Chimeric EGFP-ProTα(1–99) was overproduced in HeLa cells, and its intracellular location was determined by fluorescence microscopy [18]. As expected, EGFP-ProTα(1–99) was less efficiently transported into the nucleus and was evenly distributed between the nucleus and the cytoplasm. The wild-type EGFP-ProTα was detected exclusively in the nucleus (Fig. 3a).

Redistribution of endogenous ProTα from the nucleus into the cytoplasm in apoptotic cells was demonstrated with monoclonal antibodies highly specific to ProTα [21]. Hep2 cells were treated with TNF and emetine to induce apoptosis and prevent the *de novo* synthesis of ProTα. Fluorescence microscopy of fixed permeabilized cells showed that ProTα occurs exclusively in the nucleus in normal cells (Fig. 3b), with the exception of a mitotic cell (indicated with an arrowhead). In apoptotic cells, ProTα found its way into the cytoplasm, which was detectable as early as 2 h after the induction of apoptosis. Thus, caspase hydrolysis of ProTα correlates with its redistribution from the nucleus into the cytoplasm.

The extracellular function of ProtTα-specific peptides has already been discussed [22, 23], though in the absence of any data on the mechanisms of their production and secretion. To determine whether the caspase-dependent fragmentation and release of ProTα from the nucleus in apoptotic cells contribute to its hypothetical externalization, fluorescence microscopy was used to monitor the expression of endogenous ProTα epitopes on the surface of normal and apoptotic cells [19]. Intense staining of the cell surface was observed for apoptotic, but not normal, nonpermealized Hep2 and HeLa cells (Fig. 3c). Detection of ProTα epitopes on the surface of apoptotic cells was protein-specific, because the interaction with the cell surface was observed neither for control antibodies against endogenous glyceraldenyde 3'-phosphate dehydrogenase and β-actin nor for antibodies against ectopically produced EGFP and Keap1(1–139). These findings suggest specific mechanisms of ProTα transport onto the surface of apoptotic cells.

A question arises as to the biological significance of caspase cleavage of ProTα and its exposure on the surface of apoptotic cells. Such cells are recognized and quickly eliminated by macrophages. Since ProTα and its peptides exert an immunostimulating effect

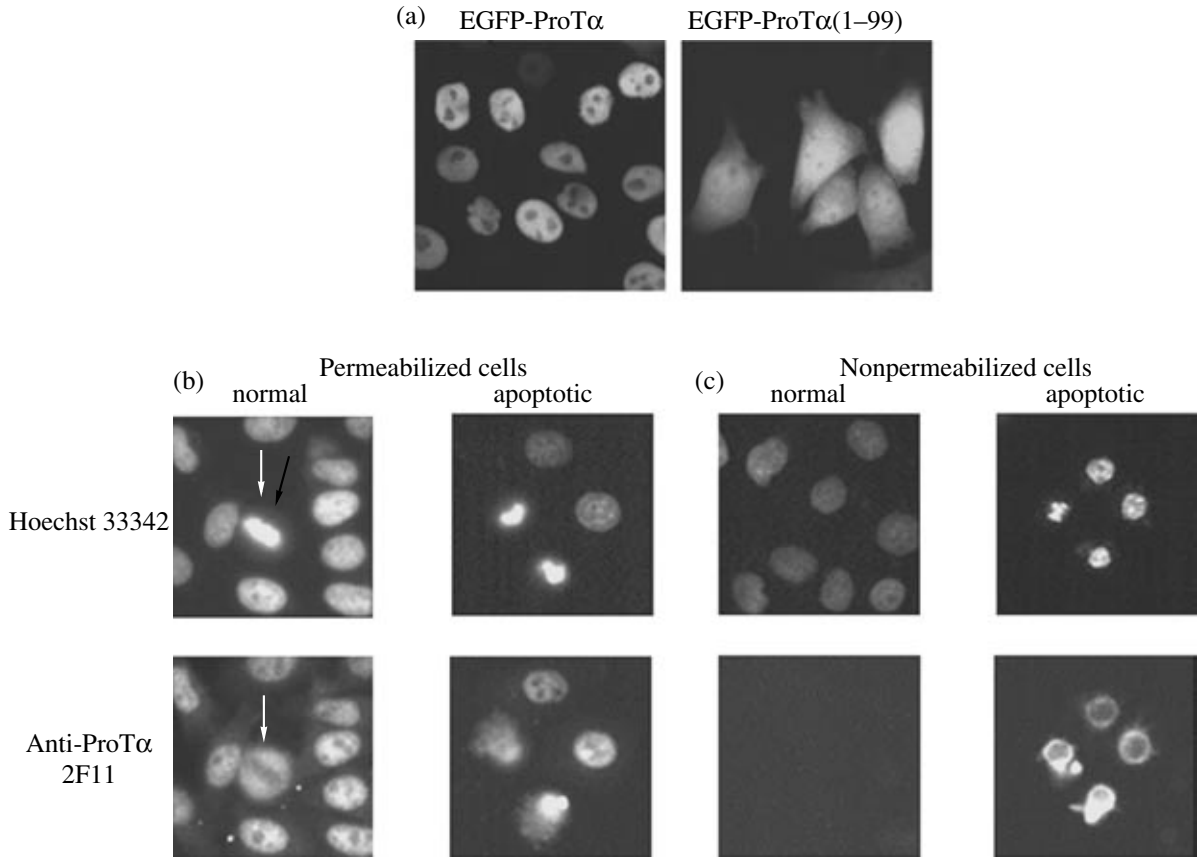

Fig. 3. Apoptosis-associated changes in the intracellular localization of ProTα. (a) To study the subcellular localization of ProTα and its fragment 1–99, each fused with EGFP, HeLa cells were transfected with the corresponding expression plasmids and examined by fluorescence microscopy. (b, c) The intracellular localization of endogenous ProTα in Hep2 cells changed upon treatment with TNF and emetine. Cells fixed with 4% formaldehyde (b) were permeabilized with 0.2% Triton X-100 to study the intracellular localization of ProTα or (c) were not permeabilized to detect ProTα epitopes on the cell surface. In the upper row, cells were stained with Hoechst 33342. The lower row shows the same fields stained with monoclonal antibody 2F11 against ProTα and secondary antibodies conjugated with Cy3. A mitotic cell is indicated with an arrowhead.

and activate macrophages [6, 7, 20], it is possible to assume that the exposure of a ProTα fragment alone or in complex with other proteins on the surface of an apoptotic cell provides a signal for its recognition and elimination by macrophages.

OVERPRODUCTION OF PROTHYMOSIN α AND ITS MUTANTS PROTECTS CELLS FROM APOPTOSIS

Fragmentation of ProTα in apoptosis suggests that ProTα must be inactivated to allow for the realization of the apoptosis program; i.e., ProTα is a potential antiapoptotic protein. To check this assumption, we studied whether the overproduction of ProTα and its mutants varying in intracellular localization affects apoptosis of HeLa cells [19]. Cells were transfected with plasmids directing synthesis of the wild-type ProTα, the D99N mutant resistant to caspase cleavage of the C-terminal region, the K87E mutant with partly inactivated NLS, and ProTα(1–99) simulating the major product of caspase cleavage. The first two proteins were exclusively nuclear; the other two occurred both in the nucleus and in the cytoplasm. Each ectopic protein was produced to a level ten times higher than that of endogenous ProTα.

Two parameters were used to estimate the sensitivity of transfected cells to apoptosis induced by TNF in combination with emetine: the activation of caspases, which was inferred from the intensity of cell staining with the fluorescent caspase inhibitor FITC-VAD-fmk, and chromatin condensation, which was visualized using the DNA-specific dye Hoechst 33342. Flow cytometry of control cells, transfected with the empty vector, was performed 0, 3, and 6 h after the induction of apoptosis and revealed a gradual increase in caspase activity. Ectopic production of the wild-type ProTα and its mutants dramatically suppressed caspase activation in apoptotic cells. This effect was most clearly seen after 6 h (Figs. 4a–4e). The cell subpopulation corresponding to the peak with the highest fluorescence (the interval M3) was major in a normal

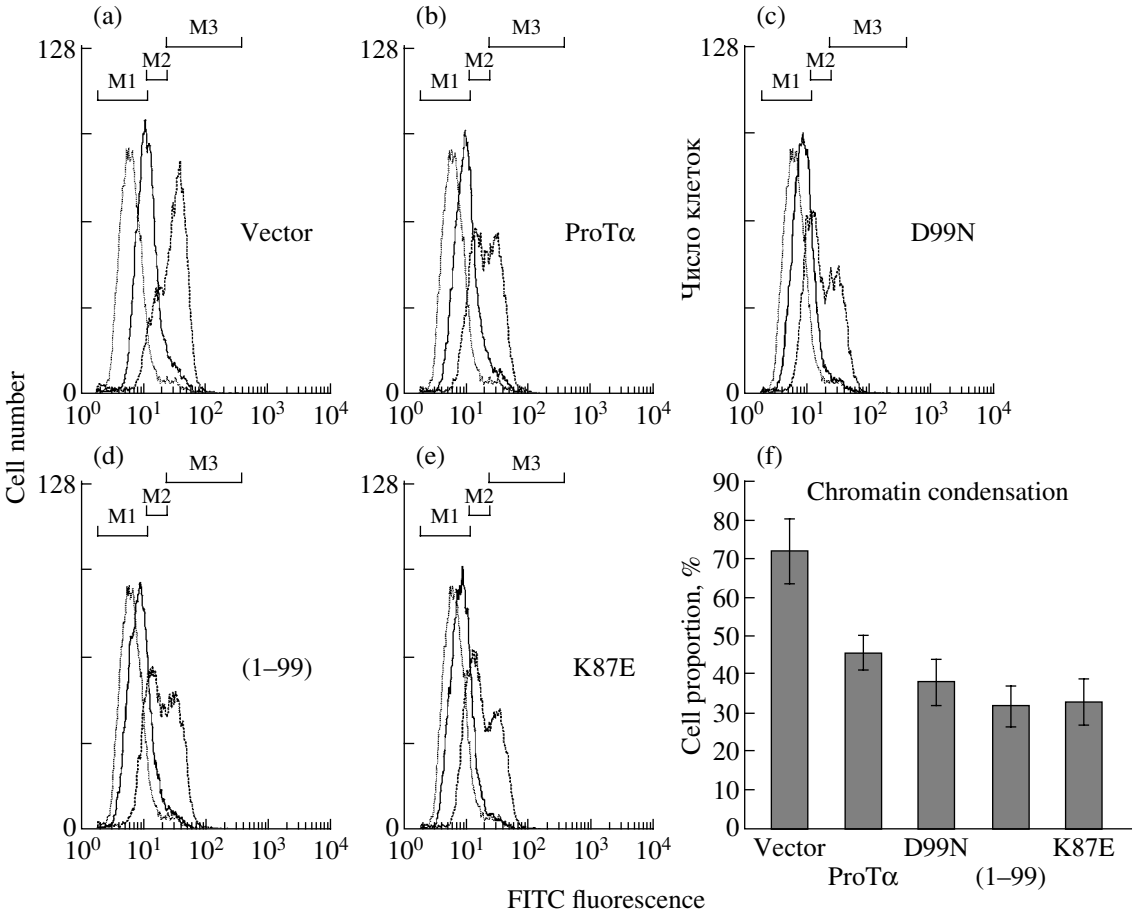

Fig. 4. ProTα protects cells from apoptosis. HeLa cells overproducing the wild-type ProTα, truncated ProTα(1–99), or the D99N or K87E mutant and control cells, transfected with the empty vector, were treated with TNF and emetine. (a–e) Caspase activation 3 (solid line) and 6 (line dashed) h after induction of apoptosis. Cells were treated with FITC-VAD-fmk and analyzed by flow cytometry. Cells of the M1 fluorescence interval were considered to be normal. Cells of this interval accounted for 90% of the control culture, which was not treated with TNF and emetine (dotted line). The M2/M3 boundary between the fluorescence intensities of cells with a high or a lower extent of caspase activation was set so that the two overlapping peaks of 6-h cultures were separated. At least 10^4 cells were scored in each case. (f) Chromatin condensation in HeLa cells cotransfected with the same plasmids as in (a–e) and pEGFP-C1 (Clontech). Staining with Hoechst 33342 was performed 6 h after the induction of apoptosis, and the proportion of EGFP-positive cells with condensed chromatin was estimated by fluorescence microscopy. The results were averaged over four independent transfections.

culture but did not dominate in transfected cultures producing wild-type ProTα or its mutants. A decrease in the M3 peak correlated with an increase in the peak with a lower fluorescence (the M2 interval), which corresponded to cells with a lower degree of caspase activation.

Similar results were obtained by fluorescence microscopy of cells stained with Hoechst 33342. The proportion of apoptotic cells was estimated as the proportion of cells with condensed chromatin 6 h after the induction of apoptosis. Ectopic expression of ProTα decreased the proportion of cells with condensed chromatin in HeLa cell cultures treated with TNF and emetine (Fig. 4f).

The antiapoptotic effect of ProTα, which was observed in the above experiments, agrees with the recent data indicating that ProTα negatively regulates caspase activation by suppressing apoptosome formation [24]. The apoptosome is an intricate cytoplasmic complex in which seven subunits of apoptotic protease-activating factor 1 (Apaf-1) interact with cytochrome *c*, an important mediator of apoptosis [25, 26]. When cells are exposed to apoptotic stimuli, cytochrome *c* migrates from mitochondria into the cytoplasm and, acting together with dATP, induces apoptosome formation. The apoptosome binds initiator procaspase 9 and induces its activation. Active caspase 9 triggers the protease cascade by activating effector caspases of the cell.

An apparent contradiction is that ProTα is a nuclear protein, while the apoptosome is formed in the cytoplasm. The above data on ProTα cleavage by

caspases and on the redistribution of the truncated protein into the cytoplasm resolve this contradiction. In view of this, it is of particular interest that, unexpectedly, the ProTα mutants incapable of accumulating in the nucleus exert the same, if not a greater, antiapoptotic effect as the wild-type ProTα (Fig. 4). A possible explanation for this is that the ProTα mutants localized in the cytoplasm are more efficient in inhibiting the formation of the apoptosome. The mechanism of this phenomenon is obscure. We recently observed, however, that ProTα(1–99), which simulates the major product of caspase cleavage of ProTα, interacts with cytochrome *c in vitro* [27]. The interaction of cytochrome *c*, which migrates from mitochondria into the cytoplasm, with truncated ProTα, released from the nucleus into the cytoplasm, is a possible mechanism whereby ProTα inhibits apoptosome formation and protects the cell from apoptosis.

SEARCH FOR MOLECULAR PARTNERS OF PROTHYMOSIN α

One way to determine the mechanism of action for a protein is to identify its intracellular partners. There is evidence that ProTα utilizes its central domain, which consists of extended blocks of dicarboxylic amino acids, to interact with histones, in particular, histone H1 [28] and induces structural changes in chromatin (for review, see [6]). In addition, ProTα was shown to interact with several other proteins, including the repressor of estrogen receptor activity [29]; the CREB-binding protein (CBP), which acts as a transcriptional coactivator; histone acetyltransferase p300, which is highly homologous to CBP [30–32]; Rev of the human immunodeficiency virus type 1 [34]; the SET oncoprotein [35]; and the abovementioned cytochrome *c* [27]. The interactions with these proteins suggest that ProTα plays a role in gene expression regulation. For instance, ProTα may contribute to the structural remodeling of chromatin, modulate the activities of transcription factors, or play a role in nucleocytoplasmic transport.

One of the few general approaches to the mechanisms of protein function is to identify the molecular partners of a protein in the yeast two-hybrid system [36]. The identification of new proteins interacting with the protein of interest may suggest its absolutely new function.

The yeast two-hybrid system was used to identify the human proteins that interact with ProTα [37]. We screened brain and bone marrow human cDNA libraries with inserts cloned in the polylinker of a yeast expression vector downstream of the nucleotide sequence coding for the activation domain of the yeast transcriptional activator Gal4 (AD_{Gal4}). The full-size ProTα fused with the bacterial DNA-binding protein LexA was used as a bait. Yeast *S. cerevisiae* cells with the *lac*Z, *HIS3*, and *URA3* reporter genes controlled by LexA-binding sequences and with inactivated chromosomal *HIS3* and *URA3* were cotransformed with a plasmid coding for the LexA-ProTα bait and the libraries coding for the AD_{Gal4}-ΣY_i proteins. Cells were selected according to their capacity to grow in the absence of histidine and uracil, and growing clones were screened for β-galactosidase activity. Screening of about 10^7 transformants yielded four positive clones. Each of the four plasmids isolated from these clones proved to code for the C-terminal fragment of Keap1 (KIAA0132), an inhibitor of the transcription factor Nrf2, which activates genes involved in the cell response to oxidative stress [38]. The Keap1 cDNA was amplified, and the full-size Keap1 was shown to efficiently bind with ProTα in the yeast two-hybrid system.

Identification of Protein Regions Involved in the Interaction of ProTα and Keap1

To identify the ProTα region responsible for the binding with Keap1, a series of ProTα deletion mutants was constructed and tested for its capacity to interact with Keap1 in the two-hybrid system [37]. A short fragment of the central region of ProTα (amino acid residues 32–52) was necessary and sufficient for the interaction. It is of interest that E(44,50)G, a double mutation of this region, had already been observed in one mutant from the above collection of ProTα variants with functionally important mutations. The double mutation abolished the ProTα–Keap1 interaction in the two-hybrid system, suggesting the specificity of interaction and providing a reliable control for further experiments.

To identify the Keap1 region interacting with ProTα, a series of Keap1 deletion mutants was constructed and tested for the interaction with ProTα in the yeast two-hybrid system [37]. The interaction proved to require the total C-terminal half of Keap1 (amino acid residues 304–624), which contains six so-called Kelch repeats forming a tertiary structure known as a β-propeller [38]. The deletion of at least one Kelch repeat prevented the interaction with ProTα, suggesting the necessity of the intact β-propeller. It should be noted that the same region of Keap1 interacts with the transcription factor Nrf2; i.e., ProTα and Nrf2 possibly compete for the binding with Keap1.

Interaction of ProTα and Keap1 *in vitro*

The interaction in the yeast two-hybrid system reports only candidate molecular partners of the protein of interest and must be verified in further tests. For instance, it is necessary to check the interaction in a homologous cell system by coimmunoprecipitation

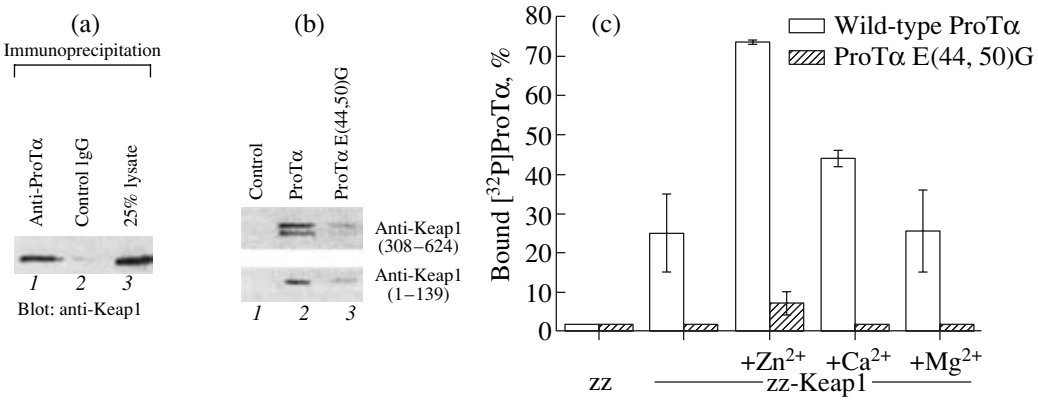

Fig. 5. Keap1 interacts with ProTα (a, b) in human cell lysates and (c) *in vitro.* (a) Coimmunoprecipitation of ectopically produced Keap1 with ProTα from HeLa cell lysates. Precipitation was carried out with (*1*) monoclonal antibodies against ProTα or (*2*) control mouse IgG; lane *3*, total cell lysate. Keap1 was detected by immunoblotting with specific polyclonal antibodies. (b) Binding of endogenous Keap1 from HeLa cell lysates to (*2*) the wild-type ProTα and (*3*) its E(44,50)G mutant immobilized on Sepharose. Lane *1*, binding to control Sepharose without ProTα. Keap1 was detected by immunoblotting with polyclonal antibodies against its C- and N-terminal fragments. (c) Interaction of recombinant ProTα and Keap1 *in vitro.* IgG-Sepharose with immobilized zz-Keap1 or zz (z is the IgG-binding domain of *Staphylococcus* protein A) was incubated with ^{32}P-labeled ProTα (open columns) or its E(44,50)G mutant (filled columns) in the presence of 100 μM cations (indicated metal ions). Nonbound [^{32}P]ProTα was removed, and the radioactivity of each Sepharose sample was counted.

from cell lysates and *in vitro* with purified proteins and, what is most important, to obtain evidence for the biological significance of the interaction.

To study whether ProTα and Keap1 do interact in human cells, immunoprecipitation with monoclonal antibodies against ProTα was performed with lysates of HeLa and HepG2 cells producing ectopic Keap1 [37]. Ectopic Keap1 coprecipitated with endogenous ProTα (Fig. 5a). Similar experiments showed that endogenous Keap1 coprecipitates with ProTα. Thus, ProTα and Keap1 interact in a homologous cell system.

To verify this conclusion and to check the specificity of the interaction, endogenous Keap1 of HeLa and HepG2 cell lysates was tested for the binding with recombinant ProTα and its E(44,50)G mutant immobilized on BrCN-activated Sepharose [37]. Endogenous Keap1 efficiently bound with the wild-type ProTα and only weakly interacted with the mutant (Fig. 5b). This result confirmed the specificity of the ProTα–Keap1 interaction observed in the two-hybrid system.

Tests for the binding of purified recombinant proteins *in vitro* make it possible to determine whether the proteins directly interact with each other or their interaction is mediated by another component of a cell lysate. Keap1 was obtained as a fusion with the zz domain (z is the IgG-binding domain of *Staphylococcus* protein A) and immobilized on IgG-Sepharose. To allow for the quantitation of the binding, the N terminus of ProTα was modified with a short peptide containing a recognition site for protein kinase A and labeled with ^{32}P. The labeled wild-type ProTα efficiently bound with immobilized zz-Keap1 and showed almost no binding with the immobilized zz domain [37]. Tests for the competitive binding of labeled and cold recombinant ProTα containing no additional peptide showed that the binding is specific for the ProTα amino acid sequence. Moreover, the E(44,50)G mutant was not able to interact with Keap1 (Fig. 5c).

It was shown earlier that ProTα binds Zn^{2+} and Ca^{2+} but not Mg^{2+} [39]. We studied whether divalent cations affect the interaction of ProTα with Keap1. The ProTα–Keap1 binding considerably increased in the presence of 100 μM Zn^{2+} or Ca^{2+} but not Mg^{2+} (Fig. 5c). In the presence of Mg^{2+}, ProTα binds with Keap1 as efficiently as in the absence of divalent cations.

Thus, ProTα directly and specifically interacts with Keap1. Divalent cations binding with ProTα substantially strengthen their interaction.

MODEL OF REGULATION OF THE CELL RESPONSE TO OXIDATIVE STRESS

Here, it is appropriate to consider the function of the Nrf2–Keap1 system in more detail. Animal cell defense against oxidative stress and the effects of electrophilic agents is due to the activation of genes coding for detoxification enzymes and antioxidants, such as glutathione S-transferase, NAD(P)H quinone oxireductase 1, γ-glutamylcysteine synthase, and hemoxygenase 1 (HO-1) (for review, see [40]). The concerted induction of these genes is due to the binding of Nfr2, acting as a heterodimer with small Maf proteins, to a regulatory DNA region known as the Antioxidant Response Element (ARE) [41]. Nfr2 reg-

ulates both the basal and induced transcription of ARE-dependent genes.

Transcription regulation depends on the interaction of Nrf2 with its inhibitor Keap1 [38]. Keap1 persists in the cytoplasm, as does Nrf2 in the absence of oxidative stress. Since Keap1 is homologous to the actin-binding Kelch protein of *Drosophila* [42], it was assumed that, in the absence of oxidative stress, Keap1 exerts an inhibitory effect by anchoring Nrf2 in the cytoplasm as a result of simultaneous interactions with Nrf2 and actin elements of the cytoskeleton [38, 43]. In addition, the interaction with Keap1 was shown to facilitate the ubiquitination and subsequent proteasomal degradation of Nrf2 [44, 45].

When oxidative stress is induced, Nrf2 finds it way from the cytoplasm into the nucleus and binds to DNA, dissociating from Keap1. The model that has so far been accepted assumed that, after the dissociation of the Nrf2–Kea1 complex in the cytoplasm, Nrf2 is transferred into the nucleus, binds to DNA, and activates the transcription of ARE-dependent genes [38, 46].

How Does ProTα Find Keap1 in the Cell?

The results of *in vivo* and *in vitro* experiments suggest with a high probability that Keap1 is a molecular partner of ProTα. Yet this conclusion seems to disagree with the fact that ProTα and Keap1 are in different cell compartments: ProTα is a nuclear protein, whereas Keap1 occurs in the cytoplasm and is excluded from the nucleus [38, 46]. The localization of Keap1 is consistent with its presumable role as an anchor that renders Nrf2 inactive in the cytoplasm.

To act as a molecular partner of ProTα, Keap1 has to enter the nucleus, that is, to function as a shuttle protein migrating between the nucleus and the cytoplasm. Such shuttle proteins usually contain NLS and a nuclear export signal (NES). When NES is more potent than NLS, the protein seems to be cytoplasmic, because it occurs in the cytoplasm most of the time. However, such proteins accumulate in the nucleus when nuclear export is inhibited. To check whether this is the case with Keap1, its localization in HeLa and HepG2 cells was determined before and after cell treatment with leptomycin B (LMB), which specifically inhibits the nuclear export of proteins containing leucine-rich NES [37]. Fluorescence microscopy was employed in studying the intracellular localization of fused EGFP-Keap1, taking advantage of internal fluorescence of EGFP, and of ectopic and endogenous Keap1, using anti-Keap1 monoclonal antibodies. In the absence of LMB, endogenous and ectopic Keap1 and EGFP-Keap1 occurred mostly in the cytoplasm. In the presence of LMB, all three proteins were redistributed to a great extent from the cytoplasm into the nucleus (Fig. 6).

These data testify that Keap1 is a shuttle protein migrating between the nucleus and the cytoplasm and probably containing NLS and leucine-rich NES. The latter was indeed identified in the central region of Keap1 [37]. The sequence L^{301}VKIFEELTL^{310} proved to be necessary and sufficient for the LMB-responsive export of Keap1 from the nucleus, and the L(308,310)A double mutation of this sequence changed the localization of Keap1 from cytoplasmic to nuclear–cytoplasmic. These findings support the hypothesis that Keap1 is a molecular partner of ProTα and suggest an alternative mechanism for the function of the Nrf2–Keap1 system, assuming that Keap1 continuously migrates between the nucleus and the cytoplasm in complex with Nrf2 under normal conditions. In oxidative stress, Keap1 dissociates from Nrf2 in the nucleus, which leads to Nrf2 activation and triggers ARE-dependent transcription.

Coordinate migration of Nrf2 and Keap1 between the nucleus and the cytoplasm has been demonstrated experimentally [37]. When ectopically coproduced in HeLa cells, the wild-type Nrf2 and Keap1 both occurred in the cytoplasm. When cells were treated with LMB, the two proteins were simultaneously redistributed into the nucleus. The same effect was observed for the inactivation of Keap1 NES by point mutations: Nrf2 was relocated into the nucleus as well as Keap1. Thus, Nrf2 and Keap1 are transported into or from the nucleus as a single complex.

The above shuttle model has several advantages over the currently accepted model of the anchoring of the Nrf2–Keap1 complex in the cytoplasm. First, the Nrf2–Keap1 complex migrating between the nucleus and the cytoplasm is sensitive to oxidative stress in both compartments. Second, the assumption that Nrf2 periodically comes into the nucleus, even if in complex with Keap1, explains the basal transcription of ARE-responsive genes in the absence of oxidative stress. Finally, the shuttle migration of Keap1 provides a mechanism that terminates the induced expression of the oxidative stress defense genes: Keap1 binds with Nrf2 and removes it from the nucleus as the stress factor ceases to affect the cell.

ProTα Competes with Nrf2 for the Binding with Keap1

As already mentioned, ProTα and Nrf2 interact with the same Keap1 region, the C-terminal half with six Kelch repeats forming a β-propeller. It is logical to assume that ProTα and Nrf2 compete with each other for the binding with Keap1. To check this assumption, we studied the effect of Nrf2 on the dissociation of the complex formed by [^{32}P]ProTα with zz-Keap1 immobilized on IgG-Sepharose [37]. Nrf2 increased the efficiency of dissociation of ProTα from its complex

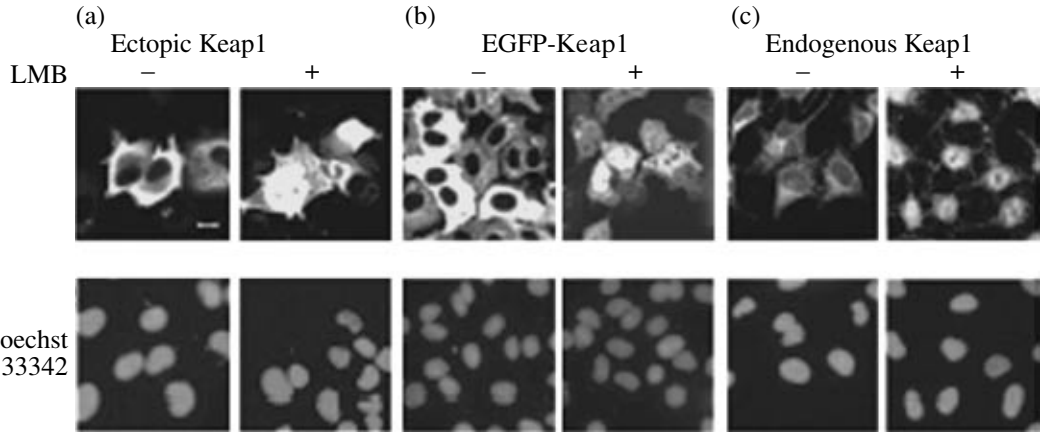

Fig. 6. LMB induces translocation of ectopic (a) Keap1 and (b) EGFP-Keap1 and (c) endogenous Keap1 into the nuclei of HeLa cells. Microphotographs were obtained by confocal fluorescence microscopy. In the upper row, (a, c) cells were stained with Keap1-specific monoclonal antibody 2H5 and FITC-conjugated secondary antibodies or (b) EGFP fluorescence was detected. The lower row shows the same fields stained with Hoechst 33342.

with Keap1 (Fig. 7a). In the absence of Nrf2, the complex dissociated at a considerably lower rate.

In another experiment, we studied the effect of an excess of recombinant ProTα on the dissociation of the Nrf2 complex with zz-Keap1 immobilized on IgG-Sepharose. The eluted and bound Nrf2 fractions were quantitated by immunoblotting with Nrf2-specific antibodies. Nrf2 dissociated from its complex with Keap1 in the presence of the wild-type ProTα but not of its E(44,50)G mutant (Fig. 7b). It should be noted that ProTα only partly displaced Nrf2 even when used in a great molar excess.

ProTα Affects Nrf2-Dependent Gene Expression

The above data suggest a mechanism sustaining the role of ProTα in the function of the Nrf2–Keap1 system. ProTα presumably acts as a nuclear factor stimulating the dissociation of the Nrf2–Keap1 complex. ProTα displaces Nrf2 from its complex with Keap1, but only when present in a great excess. It is known that the ProTα concentration in the cell nucleus is several orders of magnitude higher than the concentrations of Nrf2 and Keap1. According to the above model [37], the partial dissociation of the Nrf2–Keap1 complex in the presence of an excess of ProTα in the nucleus is responsible for the basal level of ARE-dependent transcription. Oxidative stress destabilizes the Nrf2–Keap1 complex. The molecular mechanism weakening the Nrf2–Keap1 interaction in oxidative stress is unclear. This effect is variously ascribed to Nrf2 phosphorylation [47–49] or cysteine modification in Keap1 [45, 50, 51]. Whichever is the case, the model suggests that oxidative stress stimulates the dissociation of the complex by ProTα; increases the content of free Nrf2, which is capable of binding to DNA; and thereby enhances the expression of ARE-dependent genes.

The model allowed several predictions that could be verified experimentally. For instance, changes in the intracellular concentration of ProTα were

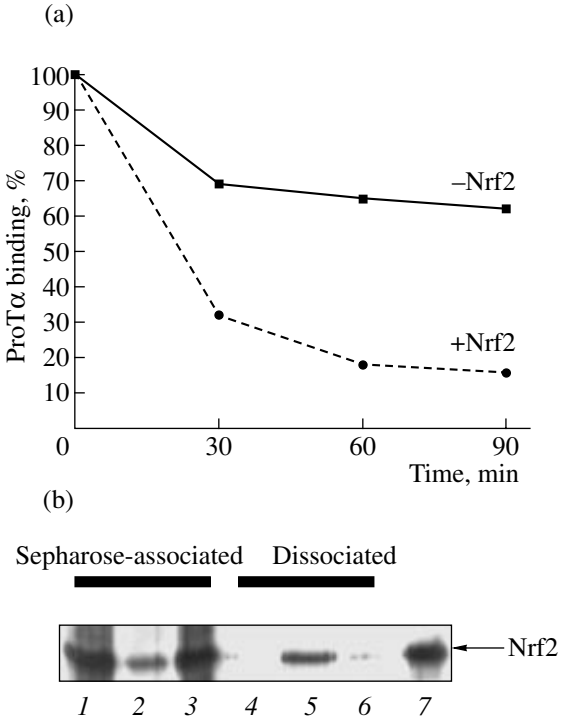

Fig. 7. ProTα competes with Nrf2 for the interaction with Keap1 *in vitro*. (a) A complex of [^{32}P]ProTα with recombinant zz-Keap1 immobilized on IgG-Sepharose was incubated with (dashed line) or without (solid line) recombinant Nrf2. Sepharose-associated radioactivity was measured at several time intervals. (b) A complex of Nrf2 with recombinant zz-Keap1 immobilized on IgG-Sepharose was incubated for 1 h (*1, 4*) without or with (*2, 5*) recombinant ProTα or (*3, 6*) its mutant E(44,50)G. The proportions of Nrf2 (*1–3*) associated with Sepharose and (*4–6*) dissociated were estimated by immunoblotting with polyclonal antibodies against Nrf2. Lane *7*, marker Nrf2.

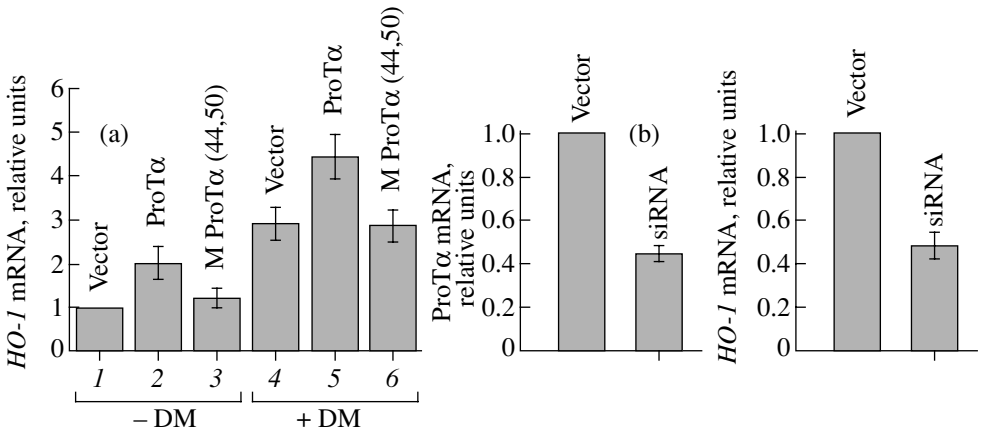

Fig. 8. Expression of the *HO-1* gene in HeLa cells correlates with the level of ProTα. (a) Overproduction of ProTα increases the transcription of the *HO-1* gene. HeLa cells (*1, 4*) transfected with the empty vector or overproducing (*2, 5*) the wild-type ProTα or (*3, 6*) its E(44,50) mutant were examined by RNA blot hybridization with probes directed to the *HO-1* mRNA (a product of Nrf2-dependent transcription) or the β-actin mRNA (a product of Nrf2-independent transcription). The content of the *HO-1* mRNA in each sample was normalized with respect to the β-actin mRNA. Cells were examined (*1–3*) before and (*4–6*) after treatment with 100 μM diethyl maleate (DM) for 22 h. Mean values and standard deviations were calculated with four independent transfections. (b) Decrease in ProTα as a result of RNA interference inhibits the expression of the *HO-1* gene. HeLa cells were transfected with a plasmid coding for the siRNA or with the empty vector and examined by RNA blot hybridization with probes directed to the ProTα, HO-1, and β-actin mRNAs. The contents of the ProTα and *HO-1* mRNAs were normalized with respect to the β-actin mRNA and averaged over four independent transfections.

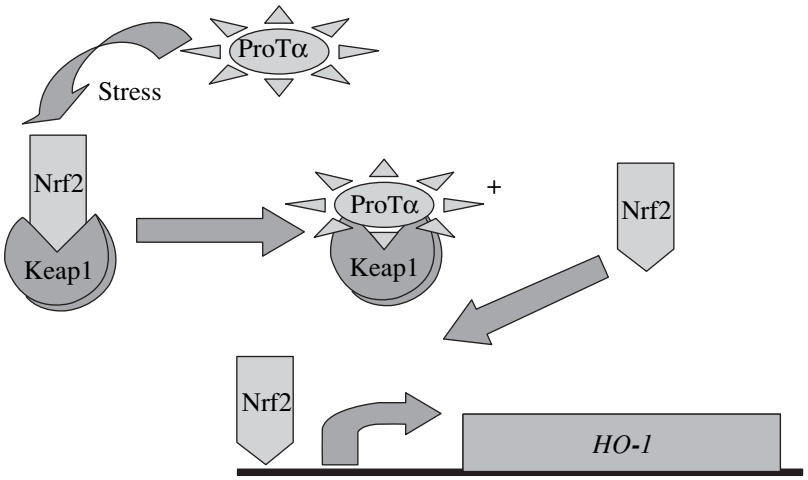

Fig. 9. Model of the involvement of ProTα in the cell response to oxidative stress. ProTα acts as a nuclear factor that facilitates the dissociation of the Nrf2–Keap1 complex, which continuously migrates between the nucleus and the cytoplasm. Partial dissociation of the Nrf2–Keap1 complex in the presence of an excess of ProTα in the nucleus is responsible for the basal level of ARE-dependent transcription. Oxidative stress destabilizes the complex; expedites its dissociation in the presence of ProTα; increases the concentration of free Nrf2, which is capable of binding to DNA; and thereby stimulates the expression of ARE-dependent genes.

assumed to similarly change the level of ARE-dependent gene expression. To check this assumption, we used the ectopic expression of ProTα to increase its concentration in HeLa cells and RNA interference to reduce it [37]. As an ARE-dependent reporter, we used the endogenous HO-1 gene, whose expression is regulated by Nrf2 [52, 53].

First, total RNAs isolated from HeLa cells overproducing the wild-type ProTα or its E(44,50)G mutant and from cells transfected with the vector (control) were tested by RNA blot hybridization with probes specific to the *HO-1* and actin genes. Overproduction of ProTα increased the level of the *HO-1* mRNA both in normal cells and in cells treated with the oxidative stress inductor diethyl maleate (Fig. 8a). The level of the control actin mRNA was not affected by the overproduction of ProTα. It is important that the E(44,50)G mutant, which is incapable of binding with Keap1 and displacing Nrf2 from the Nrf2–Keap1 complex, also failed to affect the expression of the *HO-1* gene.

Thus, in agreement with our model, the overproduction of ProTα stimulated the transcription of an Nrf2-dependent gene. This effect was mediated by the ProTα–Keap1 interaction, as followed from the results of the experiment with the mutant ProTα. To further test the model, we complemented the experiments on the overproduction of ProTα by reducing its concentration with the use of RNA interference.

In RNA interference, short double-stranded RNAs identical in nucleotide sequence to a fragment of the target gene are introduced in the cell and cause a selective degradation of the target gene mRNA [54, 55]. Such RNAs, known as small interfering RNAs (siRNAs), can be synthesized chemically and introduced into the cell. Alternatively, a plasmid can be used to express a hairpin RNA precursor, whose processing by cell nucleases yields the siRNA of interest.

A genetic knock-out in ProTα was achieved with an expression plasmid directing the production of the siRNA specific to the region 90–110 of the ProTα mRNA [37]. Transfection with the plasmid dramatically reduced the ProTα content in HeLa cells as demonstrated by the electrophoretic analysis of partly purified ProTα and ELISA of cell lysates with monoclonal antibodies against two different epitopes of ProTα. The content of several other endogenous proteins was unchanged, suggesting the specificity of the siRNA effect.

As RNA blot hybridization showed, expression of the ProTα-specific siRNA considerably reduced the level of the ProTα mRNA (as compared with cells transfected with the vector) and had no effect on the level of the β-actin mRNA. To check whether a decrease in ProTα affects Nrf2-dependent gene expression, the same RNA blot was hybridized with a probe specific for the *HO-1* gene. The content of the *HO-1* mRNA proved to decrease in cells expressing the ProTα-specific siRNA (Fig. 8b).

Thus, the experiments on ProTα overproduction and RNA interference showed that Nrf2-dependent gene expression correlates with the intracellular content of ProTα. The predictions based on the model of the Nrf2–Keap1 function received experimental support. The results provide evidence in favor of our model (Fig. 9) and testify again that ProTα plays the new role of protecting the cell from oxidative stress.

To summarize, the identification of molecular partners suggests an action mechanism for a protein. Here, we described an arduous path from detecting the interaction of two proteins in the two-hybrid system to concluding with confidence that the interaction does take place in the cell and is biologically significant. Analysis of the ProTα–Keap1 interaction revealed the new ProTα function of protecting the cell from oxidative stress and suggested its mechanism. Shuttle migration between the nucleus and the cytoplasm was demonstrated for the Nrf2–Keap1 complex, which made it possible to revise the existing model of the system that regulates the activation of oxidative stress defense genes in the cell.

We observed that ProTα is fragmented during apoptosis and finds its way to the surface of apoptotic cells, while its normal localization is nuclear. These findings led to the identification of another new function of ProTα: its role in protecting the cell from apoptosis. This protective role is characteristic both of the nuclear ProTα and its mutants that are incapable of accumulating in the nucleus, suggesting that several mechanisms sustain this function. Further studies will detect new interactions of ProTα and elucidate the well-known interactions. This is the only way to achieve an understanding of the ProTα function in the cell.

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research (project nos. 02-04-48428, 03-04-48534, 05-04-48491) and the Ludwig Institute of Cancer.

REFERENCES

1. Haritos A.A., Goodall G.J., Horecker B.L. 1984. Prothymosin alpha: Isolation and properties of the major immunoreactive form of thymosin alpha 1 in rat thymus. *Proc. Natl. Acad. Sci. USA*, **81**, 1008–1011.
2. Haritos A.A., Blacher R., Stein S., Caldarella J., Horecker B.L. 1985. Primary structure of rat thymus prothymosin alpha. *Proc. Natl. Acad. Sci. USA*. **82**, 343–346.
3. Eschenfeldt W.H., Berger S.L. 1986. The human prothymosin alpha gene is polymorphic and induced upon growth stimulation: Evidence using a cloned cDNA. *Proc. Natl. Acad. Sci. USA*. **83**, 9403–9407.
4. Gast K., Damaschun H., Eckert K., Schulze-Forster K., Maurer H.R., Muller-Frohne M., Zirwer D., Czarnecki J., Damaschun G. 1995. Prothymosin alpha: A biologically active protein with random coil conformation. *Biochemistry.* **34**, 13211–13218.
5. Uversky V.N.,.Gillespie J.R, Millett I.S., Khodyakova A.V., Vasiliev A.M., Chernovskaya T.V., Vasilenko R.N., Kozlovskaya G.D., Dolgikh D.A., Fink A.L., Doniach S., Abramov V.M. 1999. Natively unfolded human prothymosin alpha adopts partially folded collapsed conformation at acidic pH. *Biochemistry.* **38**, 15009–15016.
6. Vartapetian A.B., Uversky V.N. 2003. Prothymosin alpha: A simple yet mysterious protein. In: *Protein Structures: Kaleidoscope of Structural Properties and Functions*. India: Research Signpost.
7. Pineiro A., Cordero O.J., Nogueira M. 2000. Fifteen years of prothymosin alpha: Contradictory past and new horizons. *Peptides*. **21**, 1433.
8. Eschenfeldt W.H., Manrow R.E., Krug M.S., Berger S.L. 1989. Isolation and partial sequencing of the human prothymosin alpha gene family. Evidence against export of the gene products. *J. Biol. Chem*. **264**, 7546–7555.

9. Watts J.D., Cary P.D., Crane-Robinson C. 1989. Prothymosin alpha is a nuclear protein. *FEBS Lett*. **245**, 17–20.
10. Manrow R.E., Sburlati A.R., Hanover J.A., Berger S.L. 1991. Nuclear targeting of prothymosin alpha. *J. Biol. Chem*. **266**, 3916–3924.
11. Clinton M., Graeve L., Dorry H., Rodrigues-Boulan E., Horecker B.L. 1991. Evidence for nuclear targeting of prothymosin a and parathymosin synthesized *in situ. Proc. Natl. Acad. Sci. USA*. **88**, 6608–6612.
12. Rubtsov Y.P., Zolotukhin A.S., Vorobjev I.A., Chichkova N.V., Pavlov N.A., Karger E.M., Evstafieva A.G., Felber B.K., Vartapetian A.B. 1997. Mutational analysis of human prothymosin alpha reveals a bipartite nuclear localization signal. *FEBS Lett*. **413**, 135–141.
13. Sburlati A.R., Manrow R.E., Berger S.L. 1991. Prothymosin alpha antisense oligomers inhibit myeloma cell division. *Proc. Natl. Acad. Sci. USA*. **88**, 253–257.
14. Rodriguez P., Vinuela J.E., Alvarez-Fernandez L., Gomez-Marquez J. 1999. Prothymosin alpha antisense oligonucleotides induce apoptosis in HL-60 cells. *Cell Death Differ.* **6**, 3–5.
15. Rodriguez P., Vinuela J.E., Alvarez-Fernandez L., Buceta M., Vidal A., Dominguez F., Gomez-Marquez J. 1998. Overexpression of prothymosin a accelerates proliferation and retards differentiation in HL-60 cells. *Biochem. J*. **331**, 753–761.
16. Orre R.S., Cotter M.A., Subramanian C., Robertson E.S. 2000. Prothymosin alpha functions as a cellular oncoprotein by inducing transformation of rodent fibroblasts *in vitro. J. Biol. Chem*. **276**, 1794–1799.
17. Pavlov N., Evstafieva A., Rubtsov Y., Vartapetian A. 1995. Human prothymosin alpha inhibits division in yeast *Saccharomyces cerevisiae* cells, while its mutant lacking nuclear localization signal does not. *FEBS Lett*. **366**, 43–46.
18. Evstafieva A.G., Belov G.A., Kalkum M., Chichkova N.V., Bogdanov A.A., Agol V.I., Vartapetian A.B. 2000. Prothymosin alpha fragmentation in apoptosis. *FEBS Lett*. **467**, 150–154.
19. Evstafieva A.G., Belov G.A., Rubtsov Y.P., Kalkum M., Joseph B., Chichkova N.V., Sukhacheva E.A., Bogdanov A.A., Pettersson R.F., Agol V.I., Vartapetian A.B. 2003. Apoptosis-related fragmentation, translocation, and properties of human prothymosin alpha. *Exp. Cell Res*. **284**, 211–223.
20. Evstafieva A.G., Chichkova N.V., Makarova T.N., Vartapetian A.B., Vasilenko A.V., Abramov V.M., Bogdanov A.A. 1995. Overproduction in *Escherichia coli*, purification and properties of human prothymosin alpha. *Eur. J. Biochem*. **231**, 639–643.
21. Sukhacheva E.A., Evstafieva A.G., Fateeva T.V., Shakulov V.R., Efimova N.A., Karapetian R.N., Rubtsov Y.P., Vartapetian A.B. 2002. Sensing prothymosin alpha origin, mutations and conformation with monoclonal antibodies. *J. Immunol. Meth*. **266**, 185–196.
22. Freire M., Rey-Mendez M., Gomez-Marquez J., Arias P. 1985. Evidence for the synthesis of thymosin alpha 1 by calf thymocytes and the production of this peptide by natural processing. *Arch. Biochem. Biophys*. **239**, 480–485.
23. Naylor P.N., Friedman-Kien A., Hersh E., Erdos M., Goldstein A.L. 1986. Thymosin alpha 1 and thymosin beta 4 in serum: Comparison of normal, cord, homosexual and AIDS serum. *Int. J. Immunopharmacol*. **8**, 667–676.
24. Jiang X., Kim H.E., Shu H., Zhao Y., Zhang H., Kofron J., Donnelly J., Burns D., Ng S.C., Rosenberg S., Wang X. 2003. Distinctive roles of PHAP proteins and prothymosin-alpha in a death regulatory pathway. *Science*. **299**, 223–226.
25. Acehan D., Jiang X., Morgan D.G., Heuser J.E., Wang X., Akey C.W. 2002. Three-dimentional structure of the apoptosome: Implications for assembly, procaspase-9 binding and activation. *Mol. Cell*. **9**, 423–432.
26. Adrain C., Martin S.J. 2001. The mitochondrial apoptosome: A killer unleashed by the cytochrome seas. *Trends Biochem. Sci*. **26**, 390–397.
27. Markova O.V., Evstafieva A.G., Mansurova S.E., Moussine S.S., Palamarchuk L.A., Pereverzev M.O., Vartapetian A.B., Skulachev V.P. 2003. Cytochrome *c* is transformed from anti- to pro-oxidant when interacting with truncated oncoprotein prothymosin alpha. *Biochim. Biophys. Acta*. **1557**, 109–117.
28. Papamarcaki T., Tsolas O. 1994. Prothymosin alpha binds to histone H1 *in vitro. FEBS Lett*. **345**, 71–75.
29. Martini P.G., Delage-Mourroux R., Kraichely D.M., Katzenellenbogen B.S. 2000. Prothymosin alpha selectively enhances estrogen receptor transcriptional activity by interacting with a repressor of estrogen receptor activity. *Mol. Cell Biol*. **17**, 6224–6232.
30. Karetsou Z., Kretsovali A., Murphy C., Tsolas O., Papamarcaki T. 2002. Prothymosin alpha interacts with the CREB-binding protein and potentiates transcription. *EMBO Rep*. **3**, 361–366.
31. Cotter M.A., Robertson E.S. 2000. Modulation of histone acetyltransferase activity through interaction of Epstein–Barr nuclear antigen 3C with prothymosin alpha. *Mol. Cell Biol*. **20**, 5722–5735.
32. Subramanian C., Hasan S., Rowe M., Hottiger M., Orre R., Robertson E.S. 2002. Epstein–Barr virus nuclear antigen 3C and prothymosin alpha interact with the p300 transcriptional coactivator at the CH1 and CH3/HAT domains and cooperate in regulation of transcription and histone acetylation. *J. Virol*. **76**, 4699–4708.
33. Yang C.H., Murti A., Baker S.J., Frangou-Lazaridis M., Vartapetian A.B., Murti K.G., Pfeffer L.M. 2004. Interferon induces the interaction of prothymosin-alpha with STAT3 and results in the nuclear translocation of the complex. *Exp. Cell Res*. **298**, 197–206.
34. Kubota S., Adachi Y., Copeland T.D., Oroszlan S. 1995. Binding of human prothymosin alpha to the leucine-motif/activation domains of HTLV-I Rex and HIV-1 Rev. *Eur. J. Biochem*. **233**, 48–54.
35. Karetsou Z., Martic G., Tavoulari S., Christoforidis S., Wilm M., Gruss C., Papamarcaki T. 2004. Prothymosin alpha associates with the oncoprotein SET and is involved in chromatin decondensation. *FEBS Lett*. **577**, 496–500.
36. Fields S., Song O. 1989. A novel genetic system to detect protein–protein interactions. *Nature*. **340**, 245–247.
37. Karapetian R.N., Evstafieva A.G., Abaeva I.S., Chichkova N.V., Filonov G.S., Rubtsov Y.P., Sukhacheva E.A., Melnikov S.V., Schneider U., Wanker E.E., Vartapetian A.B. 2005. Nuclear oncoprotein prothymosin alpha is a partner of Keap1: Implications for expression of oxida-

tive stress-protecting genes. *Mol. Cell Biol*. **25**, 1089–1099.

38. Itoh K., Wakabayashi N., Katoh Y., Ishii T., Igarashi K., Engel J.D., Yamamoto M. 1999. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Develop*. **13**, 76–86.
39. Chichkova N.V., Evstafieva A.G., Lyakhov I.G., Tsvetkov A.S., Smirnova T.A., Karapetian R.N., Karger E.M., Vartapetian A.B. 2000. Divalent metal cation binding properties of human prothymosin alpha. *Eur. J. Biochem*. **267**, 4745–4752.
40. Hayes J.D., McMahon M. 2001. Molecular basis for the contribution of the antioxidant response element to cancer chemoprevention. *Cancer Lett*. **174**, 103–113.
41. Rushmore T.N., Morton M.R., Pickett C.B. 1991. The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. *J. Biol. Chem*. **266**, 11632–11639.
42. Xue F., Cooley L. 1993. Kelch encodes a component of intercellular bridges in *Drosophila* egg chambers. *Cell*. **72**, 681–693.
43. Kang M.L., Kobayashi A., Wakabayashi N., Kim S.G., Yamamoto M. 2004. Scaffolding of Keap1 to the actin cytoskeleton controls the function of Nrf2 as key regulator of cytoprotective phase 2 genes. *Proc. Natl. Acad. Sci. USA*. **101**, 2046–2051.
44. McMahon M., Itoh K., Yamamoto M., Hayes J.D. 2003. Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of antioxidant response element-driven gene expression. *J. Biol. Chem*. **278**, 21592–21600.
45. Zhang D.D., Hannink M. 2003. Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. *Mol. Cell. Biol*. **23**, 8137–8151.
46. Kobayashi M., Yamamoto M. 2005. Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation. *Antioxid. Redox Signal*. **7**, 385–394
47. Huang H.C., Nguyen T., Pickett C.B. 2002. Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription. *J. Biol. Chem*. **277**, 42769–42774.
48. Cullinan S.B., Zhang D.D., Hannink M., Arvisais E., Kaufman R.J., Diehl J.L. 2003. Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. *Mol. Cell. Biol*. **23**, 7198–7209.
49. Bloom D.A., Jaiswal A.K. 2003. Phosphorylation of Nrf2 at Ser-40 by protein kinase C in response to antioxidants leads to the release of Nrf2 from INrf2, but is not required for Nrf2 stabilization/accumulation in the nucleus and transcriptional activation of antioxidant response element-mediated NAD(P)H:quinone oxyreductase-1 gene expression. *J. Biol. Chem*. **278**, 44675–44682.
50. Levonen A.L., Landar A., Ramachandran A., Ceaser E.K., Dickinson D.A., Zanoni G., Morrow J.D., Darley-Usmar V.M. 2004. Cellular mechanisms of redox cell signaling: The role of cysteine modification in controlling antioxidant defences in response to electrophilic lipid oxidation products. *Biochem. J*. **378**, 373–382.
51. Wakabayashi N., Dinkova-Kostova A.T., Holtzclaw W.D., Kang M.I., Kobayashi A, Yamamoto M., Kensler T.W., Talalay P. 2004. Protection against electrophilic and oxidant stress by induction of of the phase 2 response: fate of cysteins of the Keap1 sensor modified by inducers. *Proc. Natl. Acad. Sci. USA*. **101**, 2040–2045.
52. Alam J., Stewart D., Touchard C., Boinapally S., Choi A.M., Cook J.L. 1999. Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. *J. Biol. Chem*. **274**, 26071–26078.
53. Ishii T., Itoh K., Takahashi S., Sato H., Yanagawa T., Katoh Y., Bannai S., Yamamoto M. 2000. Transcription factor Nrf2 coordinataly regulates a group of oxidative stress-inducible genes in macrophages. *J. Biol. Chem*. **275**, 16023–16029.
54. Caplen N.J., Parrish S., Imani F., Fire A., Morgan R.A. 2001. Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proc. Natl. Acad. Sci. USA*. **98**, 9742–9747.
55. Elbashir S.M., Harborth J., Lendeckel W., Yalcin A., Weber K., Tuschl T. 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature*. **411**, 494–498.