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# Effect of Prothymosin α and Its Mutants on the Activity of the p53 Tumor Suppressor

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**Abstract**—The nuclear oncoprotein prothymosin α (ProTα) was tested for the ability to regulate the p53 activity with the use of a reporter gene controlled by a p53-responsive promoter. Overexpression of the ProTα gene stimulated the p53 activity, while downregulation of the endogenous ProTα level via RNA interference suppressed transcription of the reporter gene. An increase in ProTα activated p53-dependent transcription and increased the intracellular p53 content in human HeLa, but not HCT116, cells. N-terminal deletions had almost no effect on the ability of ProTα to activate p53-dependent transcription, while deletions from the central region and C-terminal mutations distorting the active transport of ProTα into the cell nucleus prevented its transactivating effect. Mutations affecting Keap1 binding did not impair the ProTα ability to activate the p53-responsive reporter gene. Based on the results, stimulation of p53-dependent transcription was ascribed to the central acidic region of ProTα. The conclusion was supported by the fact that parathymosin, another protein containing an extended acidic region, was also capable of activating p53.

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*Key words*: prothymosin α, Keap1, parathymosin, p53 tumor suppressor, reporter gene expression, deletion and point mutagenesis

## INTRODUCTION

Human prothymosin α (ProTα) is a small, highly acidic multicopy nuclear protein of 109 amino acid residues. ProTα is almost completely devoid of secondary structure elements and occurs as a random coil under physiological conditions [1, 2]. Relatively simple as it is, ProTα is a multifunctional protein that is involved in various processes sustaining cell life and death.

One of the most important ProTα functions is associated with cell proliferation [2, 3]. Upregulation of the ProTα gene has been observed in tumors originating from various organs and tissues [1–3]. Overproduction of ProTα increases the proliferation rate, allows cells to grow at a lower serum content in the medium, impairs contact inhibition, and results in substrate-independent cell growth [4]. These findings have made it possible to consider ProTα to be an oncoprotein.

We have previously shown that ProTα is hydrolyzed by caspase 3 in apoptotic cells, which inactivates its nuclear localization signal (NLS) [5, 6]. A high level of ProTα protects human cells from apoptosis [6, 7]. Antiapoptotic activity of ProTα agrees with its oncoprotein functions, since a block of programmed cell death is an essential prerequisite to tumor progression.

ProTα interacts with histones and induces structural rearrangements of chromatin [1, 2]. Several other proteins have also been identified as interaction partners of ProTα, including the transcriptional coactivator CBP [8] and histone acetyltransferase p300 [9]. We have found that ProTα is involved in regulating the cell response to oxidative stress [10]. ProTα displaces the Nrf2 transcription factor from its complex with the Keap1 repressor, thus activating Keap1 and allowing its transport into the nucleus to induce transcription of the genes for antioxidant proteins. ProTα directly (without any third protein) and specifically interacts with Keap1.

The p53–Mdm2 transcription system is similar to the Nf2–Keap1 system. The p53 tumor suppressor is activated in response to genotoxic stress, arresting the cell cycle or triggering apoptosis [11, 12]. Acting as a transcription factor, p53 binds to specific nucleotide sequences in the promoter regions of target genes and activates or suppresses transcription. The key role in stabilizing p53 and enhancing its transcriptional activity is played by distortion of its interaction with inhibitory proteins, such as Mdm2 [13].

The p53–Mdm2 and Nrf2–Keap1 systems share several major features. Both of the inhibitors (Mdm2 and Keap1) continuously migrate between the nucleus and cytoplasm and, in stress-free conditions, ensure the export of the corresponding transcription factor (p53 and Nrf2) from the nucleus. Both of the inhibitors are components of the E3–ubiquitin ligase complex, which promotes proteasomal degradation of transcription factors. Stress weakens the inhibitor–transcription factor interaction, and the factor is consequently stabilized, is redistributed to the nucleus, and interacts with DNA.

We assumed that ProTα regulates p53 activity, as a similar effect is known for the Nrf2–Keap1 system, involved in the cell response to oxidative stress. To check this assumption, we studied the effect of changes in ProTα concentration on the transcriptional activity of p53 in human cells.

We found that ectopic expression of the ProTα gene stimulated transcription of a p53-responsive reporter gene, while downregulation of ProTα via RNA interference suppressed its transcription. An elevated ProTα level increased, not only the expression of p53-dependent genes, but also the intracellular p53 content in HeLa human cervical carcinoma cells (but not in HCT116 human colorectal carcinoma cells). HeLa cells are defective in p53 regulation because of the expression of the *E6* oncogene of human papillomavirus type 18 (HPV18) [14]; consequently, the p53 level in HeLa cells is decreased and is independent of Mdm2.

In addition, we studied the effect of point and deletion mutants of ProTα on transcription of the p53-responsive reporter gene and identified the ProTα structural elements essential for activation of p53-dependent gene expression.

## EXPERIMENTAL

**Plasmids.** To construct the p53RE-luc reporter plasmid, the regulatory cassette containing eight p53-binding sites and the cytomegalovirus (CMV) promoter was obtained as a *Xho*I–*Hin*dIII fragment of the lentiviral vector pLC5 [15] and inserted into the *Xho*I–*Hin*dIII sites in pGL3-Promoter (Promega) in place of the SV40 minimal promoter upstream of the luciferase gene (*luc*). The pLC5 and pCMV-p53 plasmids were kindly provided by P.M. Chumakov. As another reporter plasmid, we used pHM-LacZ (pcDNA4/HisMax/LacZ, Invitrogen).

Ectopic expression of ProTα and its several mutants was achieved with pHM-ProTα (pcDNA4-enh-ProTα) wt, (1–99), K87E [6], and E44.50G [10] as described previously. To obtain pHM-ProTα(32–109), the *Eco*RI–*Kpn*I fragment of pYeGFP-ProTα V [16] was cloned in pcDNA4/HisMax/C (Invitrogen). To obtain pHM-ProTαΔ(33–81), the *Bam*HI–*Eco*RI fragment of pUC-VIII [16] was cloned in pcDNA4/HisMaxA (Invitrogen).

To construct pHM-ProTαΔ(33–43), we ligated three DNA fragments: pcDNA4/HisMaxA digested with *Hin*dIII and *Eco*RI; the *Hin*dIII–*Acy*I fragment of pHM-ProTα wt, which contained a translational enhancer and the Pro(Tα1–32)-coding sequence; and the fragment coding for ProTα(44–109), which was amplified from pHT15A [5] with primers 5'-AAG ACG CCG AGC AGG AGG CTG AC-3' and M13 reverse and digested with *Acy*I and *Eco*RI.

To construct pHM-ProTα**Δ**(44–51), we ligated three DNA fragments: pcDNA4/HisMaxA digested with *Hin*dIII and *Eco*RI; the *Acc*I–*Eco*RI fragment (180 bp) of pHT15A [5], which coded for ProTα(52–109); and the 300-bp fragment obtained via partial hydrolysis of pUC-enh-ProTα(1–43) with *Hin*dIII and *Acc*I. To obtain pUC-enh-ProTα (1–43), a fragment containing the translational enhancer and the ProTα(1–43)-coding sequence was PCR-amplified from pUC-enh-ProTα(1–99) [6] with primers 5'-TT GTC TAC CCC ATT TTC CTC ATT AG-3' and M13 reverse and ligated with pUC19 digested with *Sma*I–*Hin*dIII.

To construct pHM-ProTα**Δ**(53–67), we ligated three DNA fragments: pcDNA4/HisMaxA digested with *Hin*dIII and *Eco*RI; the 340-bp fragment that was obtained via partial hydrolysis of pHM-ProTα wt with *Hin*dIII and *Acc*I and contained the translational enhancer and the ProTα(1–52)-coding sequence; and the 140-bp *Acc*I–*Eco*RI fragment of pBluescript $SK^+$/ProTα(68–109). To obtain the last plasmid, a fragment of pHT15A [5] was PCR-amplified with primers 5'-AA GTA GAC GGT GAT GGT GAG GAA G-3' and M13 forward and ligated with pBluescript II $SK^+$ digested with *Sma*I.

To construct pHM-parathymosin, the *Eco*RI–*Sal*I fragment of pBluescript II $KS^-$/parathymosin [17] was inserted in pcDNA4/HisMax/C.

To construct pHM-TNLS-ProTα(1-99) and pHM-TNLS-ProTα, the 75-bp fragment coding for the SV40 NLS was obtained from pGAD424 (Clontech) with *Kpn*I + *Hin*dIII/Klenow and inserted in pUC-enh-6 [6] treated with *Kpn*I + *Nco*I/Klenow. The *Hin*dIII–*Kpn*I fragment containing the translational enhancer and the SV40 NLS-coding sequence was excised from the resulting plasmid and cloned in pcDNA4/HisMax/C carrying the ProTα(1-99) or wild-type ProTα cDNA, which was preliminarily inserted into the *Bam*HI–*Pst*I sites.

The structures of all mutants were verified by sequencing.

To construct pLS-siRNA$_{33–53}$, which coded for the ProTα-specific siRNA (fragment 33–53), the oligonucleotides 5'-GATCCCCTCACCACCA AGGACTTA-AATTCAAGAGATTTAAGTCCTTGGTGGTGATT-

TTTGGAAA-3' and 5'-AGCTTTTCCAAAAATCA-CCACCAAGGACTTAAATCTCTTGAATTAGTCC-TTGGTGGTG AGGG-3' (3 μg of each oligonucleotide) were dissolved in 50 μl of an annealing buffer (100 mM $CH_3COOK$, 30 mM HEPES-KOH (pH 7.4), 2 mM $Mg(CH_3COO)_2$). The mixture was incubated at 95°C for 4 min and at 70°C for 10 min and slowly chilled at room temperature. The annealing product was phosphorylated with T4 polynucleotide kinase. The resulting minigene was cloned in pUC19 (New England Biolabs) digested with *Bam*HI and *Hin*dIII, sequenced, and cloned in the lentiviral vector pLS-Lpw [15]. The plasmids for the lentiviral system were kindly provided by Chumakov.

**Cell cultures, transfection, and infection.** HeLa-B, HEK293, HCT116, and HCT116 p53–/– cells were grown in DMEM containing 10% fetal bovine serum (HyClone) at 37°C in an atmosphere containing 5% $CO_2$. Transfection was carried out with Lipofectamine 2000 or ExGen 500 as recommended by Invitrogen or Fermentas, respectively. The transfection mixture contained the p53RE-luc and pHM-lacZ reporter plasmids (10% each) and other plasmids (the amounts are indicated in the figures). As a control vector, we used pcDNA4/HisMax/B (Invitrogen, pHM-B). Lentivirus particles were obtained and used to infect HeLa cells according to a published protocol [15]. Cells infected with pLS-$siRNA_{33–53}$ were selected in the presence of 1 μg/ml puromycin for 7 days.

**Tests for β-galactosidase and luciferase activities.** Cells were grown for 40–48 h after transfection and lysed in a reporter lysis buffer (RLB, Promega). A cell lysate (50 μl) was combined with an equal volume of 1.33 mg/ml o-nitrophenyl-β-D-galactopyranoside (Sigma) in 200 mM Na-phosphate (pH 7.3), 2 mM $MgSO_4$, 100 mM β-mercaptoethanol. The mixture was incubated at 37°C until an intense yellow color developed, and the reaction was terminated by adding 0.5 ml of 1 M $Na_2CO_3$. The optical density was measured at 420 nm. The luciferase activity of a cell lysate (15 μl) was measured with a $20/20^n$ luminometer (Turner BioSystems) and the substrate of a luciferase assay system (Promega) as recommended by the manufacturer. Luciferase activity was normalized by the β-galactosidase activity of the same lysate.

**ProTα** was isolated from mammalian cells as described previously [6] and analyzed by PAGE in 8% gel containing 7 M urea. Gels were stained with 0.2% Methylene Blue in 0.04 M ammonium-acetate (pH 5.0) for 5 min and washed with water.

**Immunoblotting.** A cell lysate (30 μg of total protein) was separated by SDS-PAGE in 12% gel, and proteins were transferred onto a Protran BA83 membrane (Schleicher & Schuell) according to a standard protocol. Antibodies DO-1 against p53 and C-2 against β-actin (Santa Cruz Biotechnology) were used. Proteins were detected via enhanced luminescence with an ECL kit (Amersham Pharmacia) by a standard method.

To analyze the ProTα mutants, a cell lysate (50 μg of total protein) was separated by SDS-PAGE in 18% gel. After blotting, the membrane was treated with 0.5% glutaraldehyde [17] for covalent fixation of low-molecular-weight proteins. The mutants were detected with monoclonal antibody 2F11, specific for the N-terminal region of ProTα [17].

## RESULTS

### Expression of a p53-Responsive Reporter Gene Is Stimulated by Overproduction of ProTα and Inhibited by ProTα-Specific RNA Interference

The effect of the intracellular ProTα level on the expression of p53-dependent genes was studied using p53RE-luc, carrying the firefly luciferase gene (*luc*) under the control of a p53-responsive promoter (Fig. 1a). To construct p53RE-luc, a regulatory cassette containing a minimal CMV promoter under the control of eight p53-responsive elements (six elements from the promoter region of the cyclin-dependent protein kinase inhibitor p21, the ConA consensus sequence, and the p53-responsive element of the ribosomal gene cluster [15]) was inserted in pGL3-Promoter (Promega) upstream of *luc*.

To check whether the reporter construct was functional, HeLa cells were transfected with increasing amounts of pCMV-p53, coding for p53. Immunoblotting revealed a proportional increase in p53 as compared with the endogenous *p53* level (Fig. 1b, lower panel). Ectopic expression of the p53 gene increased the luciferase activity in a gene dosage-dependent manner (Fig. 1b, upper panel).

The endogenous p53 level was increased by treating HeLa cells with doxorubicin (Dox), which is known to stabilize p53 and to increase its pool in the cell (Fig. 1c, lower panel). Dox substantially increased the *luc* activity (Fig. 1c, upper panel). Thus, p53RE-luc adequately responded to an increase in ectopic or endogenous p53 in HeLa cells.

To study the effect of the intracellular ProTα level on the transactivating activity of p53, human ProTα was produced in HeLa, HEK293, and HCT116 cells. The intracellular concentration of ProTα was estimated, taking advantage of the fact that ProTα remains in the aqueous phase upon phenol extraction [18]. Partly purified ProTα preparations obtained from transfected cells were analyzed electrophoretically (Fig. 2a). The amount of cell tRNA copurified with ProTα served as an internal control for the cell number. The ProTα level in transfected cells was substantially higher than the level of the endogenous protein (Fig. 2a, *1* and *5*).

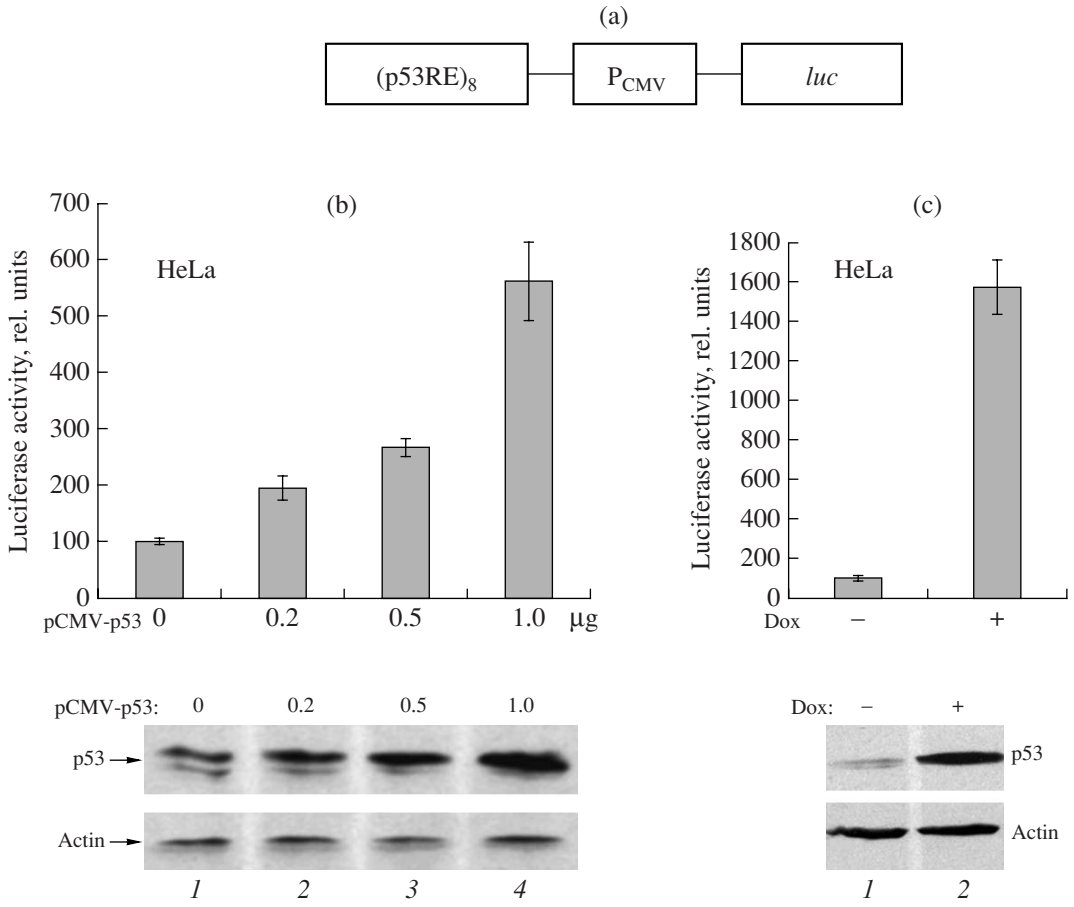


**Fig. 1.** (a) Scheme of the p53-responsive reporter gene and (b, c) its functional testing. (a) $P_{CMV}$, cytomegalovirus promoter; *luc*, firefly luciferase gene; p53RE, p53-binding site. (b) Luciferase activity of HeLa cell lysates 40 h after transfection with a mixture of the reporter plasmids (p53RE-luc and pHM-lacZ) and pCMV-p53 and/or the control vector (upper panel). Here and in Figs. 2, 4, and 5, the luciferase activity was normalized by the β-galactosidase activity. Cell lysates were examined by immunoblotting with antibodies against p53 and actin (lower panel). (c) Luciferase activity of HeLa cell lysates 40 h after transfection with a mixture of p53RE-luc and pHM-lacZ. A part of cells were treated with 2 μg/ml Dox 20 h after transfection. Cell lysates were examined by immunoblotting with antibodies against p53 and actin (lower panel).

Overproduction of ProTα in HeLa cells stimulated the expression of the p53-responsive reporter gene in a dose-dependent manner (Fig. 2b). The expression of the control *lac*Z reporter gene, controlled by the constitutive CMV promoter, remained unchanged. ProTα similarly stimulated the expression of the p53-responsive reporter gene in HEK293 cells, while this effect was not observed in HCT116 cells (data not shown).

To decrease the intracellular level of ProTα, HeLa cells were infected by the lentiviral vector coding for a ProTα-specific siRNA. Partly purified ProTα preparations obtained from infected cells were analyzed by electrophoresis. The ProTα level in infected cells was lower than in control cells (Fig. 3a). The expression of the p53-responsive reporter gene decreased as well (Fig. 3b). The effect was relatively weak but statistically significant.

Thus, the experiments with ProTα overproduction and RNA interference showed that the expression of the p53-responsive reporter gene depended on the intracellular ProTα level in HeLa cells.

## The C-Terminal Nuclear Localization Signal of ProTα Is Essential for Stimulation of p53-Dependent Gene Expression

To map the ProTα region responsible for stimulation of p53-dependent transcription, deletion and point ProTα mutants were produced in HeLa cells (Figs. 4a, 5a). Deletion of the ten C-terminal residues (ProTα(1–99) substantially reduced the activation of p53-dependent transcription (Fig. 4b). Note that ProTα(1–99) was synthesized as efficiently as the wild-type ProTα (Fig. 4c). Since the deletion eliminated the C-terminal fragment containing $KKQK^{104}$, a component of the bipartite NLS [5, 6], the observed effect was probably explained by a distorted active transport of ProTα into the cell nucleus.

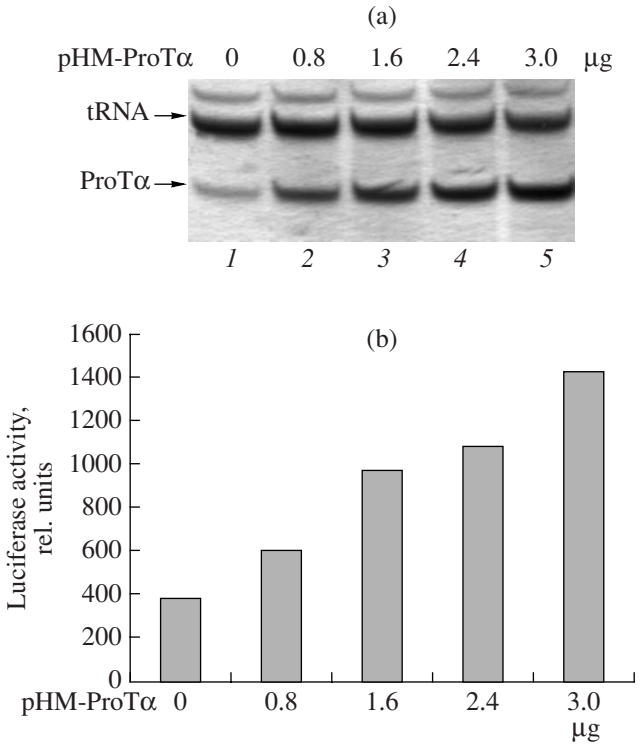


**Fig. 2.** Overproduction of ProTα stimulates the expression of the p53-responsive reporter gene. HeLa cells were transfected with 4 μg of a mixture containing p53RE-luc (0.4 μg), pHM-lacZ (0.6 μg), empty pHM-B, and pHM-ProTα (as indicated). ProTα was partly purified 40 h after transfection and analyzed by denaturing PAGE in 8% gel. (a) Gel staining with Methylene Blue. The positions of cell tRNA and ProTα are indicated with arrows. (b) Luciferase activity of cell lysates.

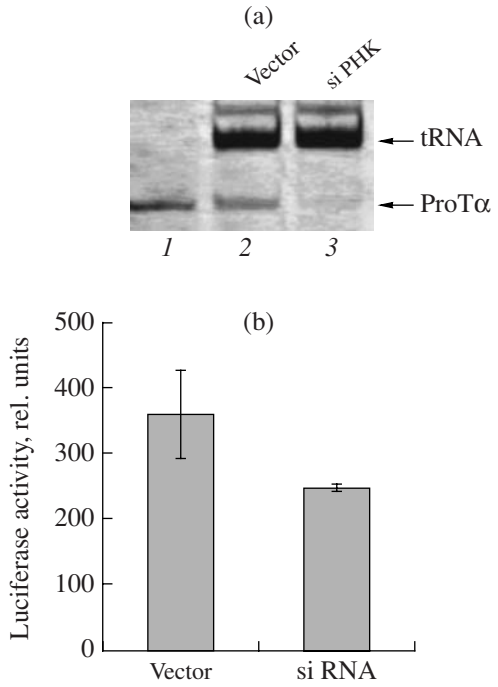


**Fig. 3.** ProTα-specific RNA interference inhibits the expression of the p53-responsive reporter gene. (a) Electrophoresis of ProTα partly purified from HeLa cells infected with (*2*) the empty lentiviral vector pLS-Lpw or (*3*) pLS-siRNA$_{33–53}$. Lane *1*, recombinant ProTα (0.5 μg). Proteins were separated by PAGE in 8% gel containing 7 M urea and stained with Methylene Blue. (b) HeLa cells infected with pLS-Lpw (Vector) or pLS-siRNA$_{33–53}$ (siRNA) were transfected with the reporter plasmids p53RE-luc and pHM-LacZ. The luciferase activity of cell lysates was measured 40 h after transfection.

Two experiments confirmed this assumption. First, HeLa cells were used to produce the ProTα K87E mutant, which had a mutation affecting K$^{87}$R, the other component of the bipartite NLS. The active transport of the K87E mutant into the nucleus is distorted [6], while its C-terminal peptide (100–109) is the same as in the wild-type ProTα. The K87E mutation reduced the ability of ProTα to activate p53-dependent transcription (Fig. 4b), although the mutant was synthesized as efficiently as the wild-type ProTα (Fig. 4c). Thus, the C-terminal region of ProTα harbors a determinant that is essential for stimulating the p53-dependent gene expression, and this determinant is the NLS.

Second, we produced ProTα(1–99) N-fused with the NLS of the SV40 T antigen. The chimeric protein activated p53-responsive reporter gene almost as efficiently as the control full-size ProTα N-fused with the SV40 T-antigen NLS (Fig. 4b). This finding indicates that the C end of ProTα harbors only one determinant important for the activation of p53-dependent transcription, namely, the NLS.

## Interaction of ProTα with Keap1 Is Not Essential for p53-Dependent Transcription

As shown recently, expression of a siRNA specific for the Keap1 mRNA activates p53-dependent transcription in HeLa cells [15]. Hence, it is possible to assume that Keap1 acts as a p53 inhibitor, for instance, owing to its ability to target proteins for proteasomal degradation. Since Keap1 interacts with ProTα, an increase in ProTα can be expected to reduce the free Keap1 pool and, consequently, to activate p53. To check whether this mechanism underlies the observed stimulatory effect of ProTα on the expression of the p53-responsive reporter gene, the ProTα E44,50G double mutant was produced in HeLa cells. As we have found previously, ProTα containing Gly in place of Glu44 and Glu50 is incapable of interacting with Keap1 [10]. It was observed, however, that the E44,50G mutations did not impair the ProTα ability to stimulate the p53-responsive reporter gene expression (Fig. 4b). Thus, the interaction of ProTα with Keap1 was insignificant for the observed stimulation of p53-dependent transcription.

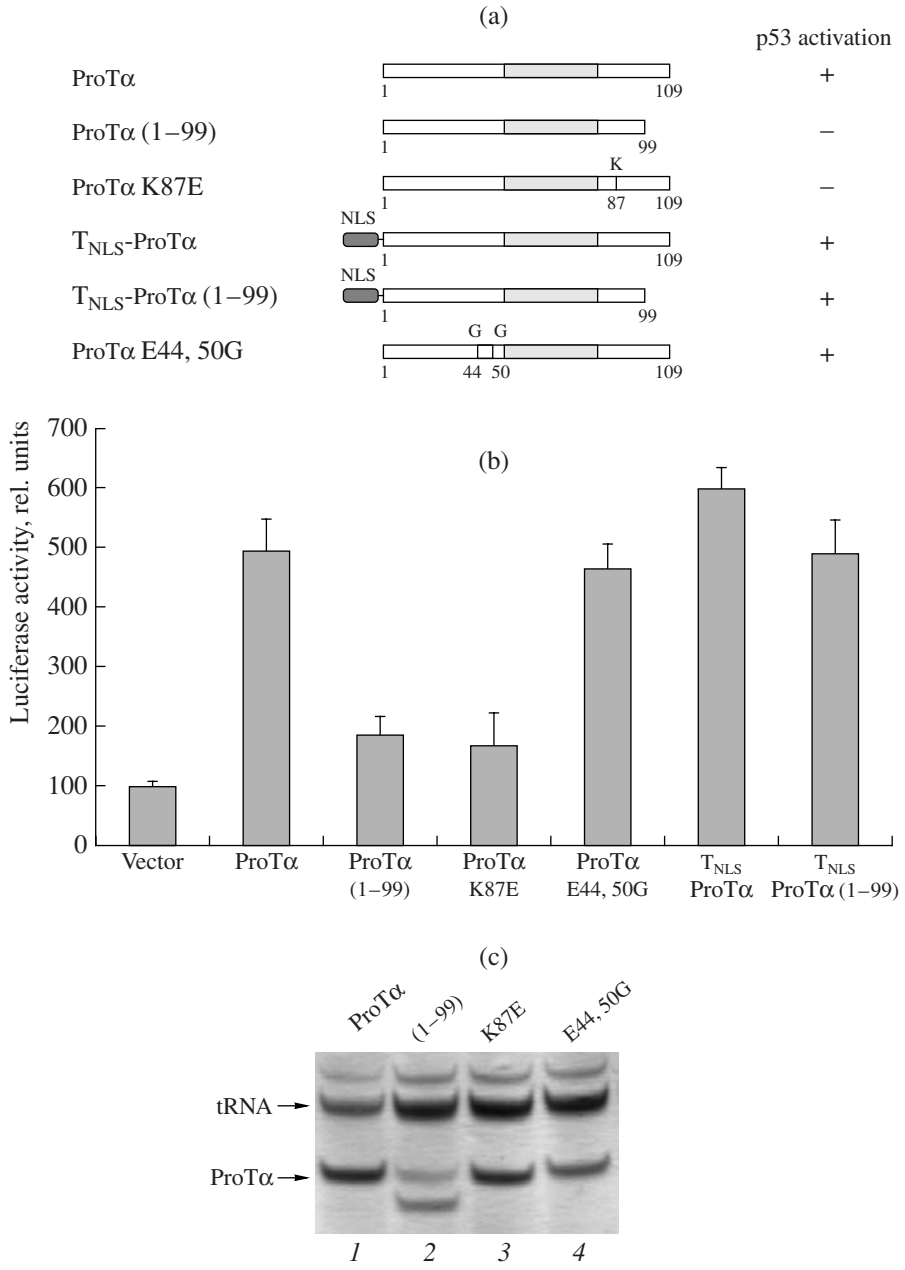


**Fig. 4.** Effect of C-terminal deletions and point mutations on the ability of ProTα to activate the p53-responsive reporter gene. (a) Schemes of the ProTα mutants. Gray bar, the ProTα region enriched in acidic residues; NLS, the NLS of the SV40 large T antigen. The extent of p53 activation was (+) more than 75% or (–) less than 25% of that determined by the full-size ProTα. (b) Luciferase activity of cell lysates 40 h after transfection of HeLa cells with a mixture of p53RE-luc (0.15 μg), pHM-lacZ (0.25 μg), and a plasmid coding for a ProTα mutant (1.6 μg). Empty pHM-B (Vector) was used as a negative control. (c) Electrophoretic analysis of the production of some proteins.

## Deletion of the N-Terminal Half Does Not Affect the Ability of ProTα to Activate p53-Dependent Transcription

To study the contribution of the N-terminal half into the stimulatory effect of ProTα on p53-dependent transcription, ProTα mutants with N-terminal and internal deletions were produced in HeLa cells (Fig. 5a). In contrast to C-terminal deletions, deletion of the N-terminal region did not prevent ProTα from activating p53-dependent transcription. For instance, ProTα(32–109), devoid of the 31 N-terminal residues, remained capable of activating the expression of the p53-responsive reporter gene (Fig. 5b). This mutant had a hexahistidine tag and the vector-encoded X-press epitope in place of the N-terminal peptide. Hence, the effect of ectopic expression on the reporter

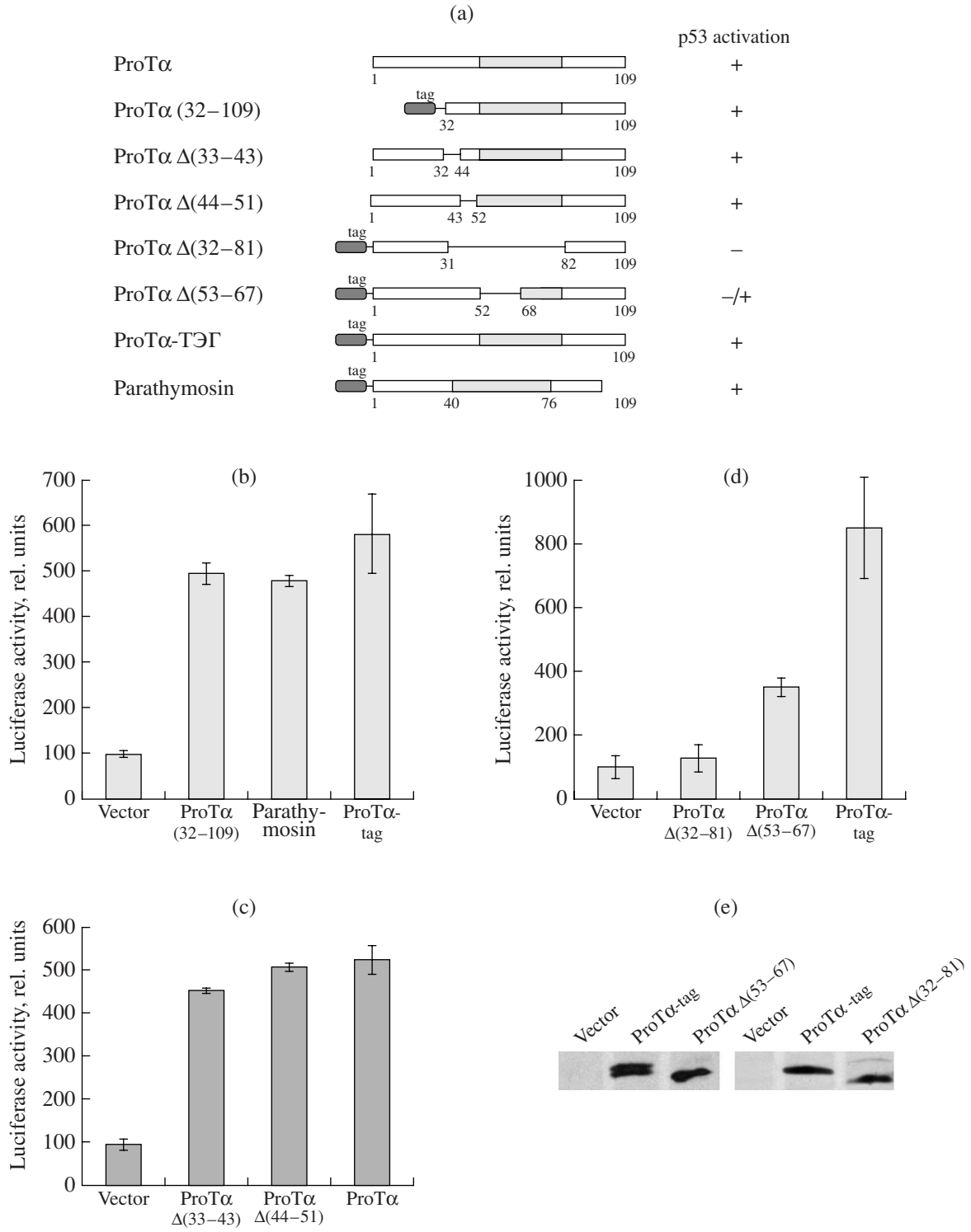


**Fig. 5.** Effect of deletions from the N-terminal and central region on the ability of ProTα to activate the p53-responsive reporter gene. (a) Schemes of the ProTα mutants. Gray bar, the ProTα and parathymosin regions enriched in acidic residues; black bar, the hexahistidine tag and the X-press epitope, encoded by the vector. The extent of p53 activation was (+) more than 75%, (–) less than 25%, or (+/–) 25–75% of that determined by the full-size ProTα. (b–d) Luciferase activity of cell lysates 40 after transfection of HeLa cells with a mixture of p53RE-luc (0.15 μg), pHM-lacZ (0.25 μg), and a plasmid coding for the protein indicated (1.6 μg). Empty pHM-B (Vector) was used as a negative control. (e) Immunoblotting of the lysates of HeLa cells producing ProTα Δ(53–67), ProTα Δ(32–81), and ProTα-tag (positive control) with antibody 2F11, specific for the N-terminal region of ProTα.

gene was compared for ProTα(32–109) and the full-size ProTα N-tagged with the same additional sequences (ProTα-tag).

We failed to produce detectable amounts of ProTα mutants with larger deletions in HeLa cells, possibly, because such mutants were unstable. The role of ProTα region 32–51 in activating p53 was studied with two mutants having short internal deletions from this region, ProTα Δ(33–43) and ProTα Δ(44–51). The mutants did not have an additional peptide or tag, and their effects on reporter gene expression was compared with that of the full-size ProTα. The two mutants retained the capability of activating the expression of the p53-responsive reporter gene (Fig. 5c). Thus, deletions from the N-terminal half did not affect the ProTα ability to activate p53-dependent transcription.

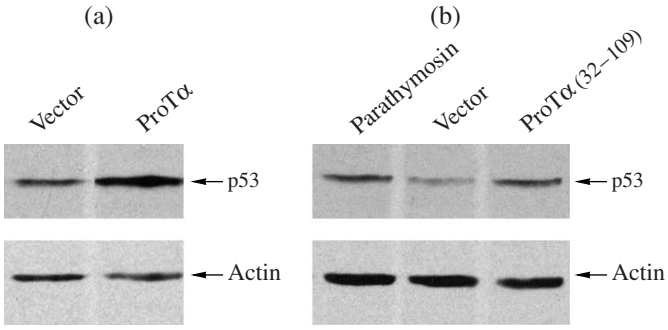


**Fig. 6.** Overproduction of (a) ProTα, (b) ProTα(32–109), and parathymosin increases the intracellular level of p53 in HeLa cells. The lysates of HeLa cells transfected as described in Figs. 4 and 5 were analyzed by immunoblotting with antibodies against p53 (upper panel) and actin (lower panel).

### The Central Region of ProTα Is Responsible for Stimulation of p53-Dependent Transcription

A deletion of 15 central amino acid residues (Δ(53–67)) partly reduced the stimulatory effect of ProTα on the expression of the p53-responsive reporter gene, and a deletion of the entire central acidic region (Δ(32–81)) completely abolished the stimulatory effect (Fig. 5d). The intracellular levels of ProTα Δ(53–67) and ProTα Δ(32–81) were comparable with that of the full-size ProTα (Fig. 5e).

It is possible to assume on the basis of these findings that the central region, along with the C-terminal bipartite NLS, is responsible for the ProTα ability to stimulate p53-dependent transcription. The central region of ProTα harbors large clusters of negatively charged Glu and Asp residues (region 52–82). To check whether p53 transcription can be activated by clusters of acidic residues, HeLa cells were used to produce parathymosin, which contains similar acidic clusters and a bipartite NLS. Parathymosin activated the expression of the p53-responsive reporter gene to the same extent as ProTα (Fig. 5b). The results demonstrate that the central acidic region and C-terminal NLS are responsible for the ability of ProTα to stimulate p53-dependent transcription.

### Overproduction of ProTα Increases the Intracellular Level of p53 in HeLa Cells

The effect of ProTα on the intracellular level of p53 was studied by immunoblotting with a monoclonal antibody against p53 (Fig. 6). Ectopic expression of ProTα proved to increase the p53 level in HeLa cells, as compared with the level observed in control cells (Fig. 6a). Thus, an increase in ProTα stabilizes p53, rather than merely stimulating its transcriptional activity. It should be noted, however, that the increase in p53 level upon ProTα production was lower than in the case of genotoxic stress caused by Dox (Figs. 1c, 6a). Ectopic expression of the genes for parathymosin and the ProTα mutant with an N-terminal deletion similarly increased the p53 level (Fig. 6b).

The effect of ProTα on the intracellular level of p53 was not observed in HCT116 and HEK293 cells. We think that the difference is probably associated with specifics of p53 regulation in HeLa cells.

## DISCUSSION

We found that ProTα stimulates the expression of the p53-responsive reporter gene in HeLa cells in a dose-dependent manner. To study the mechanism of this effect, we mapped the ProTα region responsible for stimulation. Deletions of residues 1–31, 33–43, and 44–51 from the N-terminal half did not prevent ProTα from stimulating p53-dependent transcription. The N-terminal region harbors the 28 residues that belong to thymosin α1, an immunostimulating peptide isolated from the thymus [1, 2]. In addition, this region contains the LKEKK cluster of positively charged residues, which is presumably responsible for the competition of ProTα and interferon α for the interaction with surface receptors of fibroblasts [19], and Thr7 and Thr12/13, which can be phosphorylated in ProTα [20]. These functional domains of ProTα and the region of ProTα interaction with Keap1 [10] proved to be inessential for stimulation of p53-dependent transcription.

In total, the N-terminal half (residues 1–51) of ProTα has a 31% content of acidic amino acid residues and a 10% content of positively charged residues, thus being far less acidic than the central region (52–82), which consists of acidic residues to the extent of 80%. According to our deletion analysis, it is the acidic region that is responsible for the ProTα ability to stimulate p53-dependent transcription. This

conclusion is supported by the fact that parathymosin, a small acidic protein that is similar in properties to ProTα, activates the expression of the p53-responsive reporter gene as well as ProTα does. Parathymosin has several regions of high homology to ProTα [21]. Two peptides, $^{14}$KDLKEKKE$^{21}$ and $^{19}$KKEVVEE$^{25}$, common for the two proteins and located in the N-terminal region of ProTα, are inessential for stimulation of p53-dependent transcription. The other regions of high homology to ProTα are in the extended acidic region (39–76) of parathymosin (compare with acidic region 52–82 or ProTα): the parathymosin region is EE$^{40}$ EEQGAEEEEE$^{50}$ ETAEDGEEED$^{60}$ EGEEEDDEEE$^{70}$ EEEDDE$^{76}$, and the ProTα region is DEEEEEGGE$^{60}$ EEEEEEEGDG$^{70}$ EEEDGDEDEE$^{80}$ AE$^{82}$.

We think that the large clusters of acidic residues are responsible for the ability of the two proteins to stimulate p53-dependent transcription. Deletion of E$^{53}$EEEEGGE EEEEEEE$^{67}$ from the acidic region substantially reduced the stimulating effect of ProTα on the expression of the p53-responsive reporter gene, and deletion of the entire central region (**Δ**(33–81)) completely abolished this effect.

In addition, activation of p53 required an intact NLS, which consists of two clusters of positively charged residues, K$^{87}$R and KKQK$^{104}$, in ProTα. The K87E mutation, affecting the first cluster, or deletion of the KKQK$^{104}$ cluster substantially impaired the ability of ProTα to activate p53-dependent transcription. In the case of the KKQK$^{104}$ deletion, fusion with the heterologous NLS of the SV40 large T antigen restored the ability of ProTα(1–99) to activate the expression of the p53-responsive reporter gene. This indicates that the C-terminal peptide of ProTα lacks any determinant important for p53 activation apart from the NLS. It should be noted that the C-terminal region of parathymosin also harbors a bipartite NLS (K$^{80}$R……KRNK$^{95}$) [21].

The central acidic region of ProTα is responsible for its interactions with histones and histone acetyltransferases. It is through that ProTα modulates the chromatin structure by interacting with histone H1 [1]. Polyglutamic acid, an analog of the central acidic domain of ProTα, abolishes this interaction, suggesting its electrostatic nature [22]. Gene expression is regulated at the level of chromatin structure via histone acetylation by histone acetyltransferases. ProTα interacts with homologous histone acetyltransferases p300 and CBP and enhances their stimulating effect on transcription from several promoters [8, 9]. The ProTα region interacting with CBP has been mapped to central domain 52–69, which consists almost completely of acidic residues [8].

Therefore, our finding that the acidic region of ProTα plays a key role in stimulating p53-dependent transcription in HeLa cells suggests that the mechanism of this effect involves a recruitment of histone acetyltransferases to the target promoters with consequent histone acetylation and chromatin decondensation. Similar data have been reported for parathymosin [23]. Parathymosin stimulates the hormone-dependent transcriptional activity of the glucocorticoid receptor, and its central acidic domain and NLS are essential for this effect. Hormone-dependent coimmunoprecipitation with parathymosin has been observed for the glucocorticoid receptor, p300, and CBP, suggesting that parathymosin is a component of a coactivation complex containing these proteins [23]. However, both parathymosin and ProTα activate transcription by recruiting p300 and/or CBP on some, rather than all, promoters. For instance, parathymosin increases the transcriptional activity of the glucocorticoid, but not estrogen, receptor [23]. ProTα affects p53-dependent transcription and exerts no effect on E2F- or Myc-dependent transcription [24]. The mechanism sustaining the selective effect of ProTα remains unclear.

Our results agree with published data that ectopic expression of the ProTα gene stimulates p53-dependent transcription in several cells lines: H1299, Saos-2, HepG2, A549, HEK293, and U2OS [24]. Ectopic expression of the ProTα gene has been found to stimulate p53 acetylation at Lys373, Lys382, and Lys320 [24], which seems to mediate the increase in transcription of p53-dependent genes. Stimulation of p53-dependent transcription has not been observed in HCT116 cells, which agrees with our results. HeLa cells have not been examined in [24].

Overproduction of ProTα in HeLa cells increased, not only the transactivating activity, but also the intracellular level of p53, which has not been observed in other cell lines [24]. We think that ProTα utilizes an additional mechanism to stabilize and activate p53 in HeLa cells and, possibly, other HPV-positive cervical carcinoma cells.

A distinctive feature of HeLa cells is that p53 regulation is distorted as a result of the expression of the HPV18 *E6* oncogene. It is thought that E6 acts in complex with cell ubiquitin ligase E6-AP (E6-associated protein) to promote p53 degradation by the ubiquitin-dependent proteolytic system [25]. Consequently, the p53 level in HeLa cells is substantially reduced and is independent of Mdm2. To explain the stabilization of p53 upon ProTα overproduction, it is possible to assume that ProTα interacts with E6 or E6-AP, which regulate the p53 level in HeLa cells, rather than with Mdm2, as we believed earlier.

Similar systems are known. For instance, Pitx2a binds with E6 and this binding inhibits E6/E6-AP-dependent degradation of p53 and stimulates its transactivating activity in HeLa cells but not in HPV-negative HCT116 cells [26].

Is there any contradiction in the fact that ProTα, acting as an oncoprotein, stimulates transcription of the target genes of p53, a tumor suppressor? Such dual properties are known for several oncoproteins and tumor suppressors. For instance, the cyclin-dependent kinase inhibitor p21 suppresses apoptosis (oncogenic activity) or arrests the cell cycle (antioncogenic activity) in different systems [27, 28]. The well-known oncoproteins Myc and E2F-1 promote apoptosis (antioncogenic activity) or stimulate cell proliferation (oncogenic activity) in different systems [29]. Hence, it is not surprising that ProTα, an oncoprotein, stimulates cell proliferation in some systems and, in some others, activates p53-responsive genes, which may lead to a cell cycle arrest. ProTα-dependent transcriptional activation of p53-responsive genes is possibly a cell response to an inadequate proliferation stimulus and is similar to p53 activation in response to an increase in the intracellular pool of other known oncoproteins [28].

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