ISSN 0026-8933, Molecular Biology, 2007, Vol. 41, No. 5, pp. 790–796. © Pleiades Publishing, Inc., 2007.
Original Russian Text © S.V. Melnikov, A.G. Evstafieva, A.B. Vartapetian, 2007, published in Molekulyarnaya Biologiya, 2007, Vol. 41, No. 5, pp. 868–875.

CELL
MOLECULAR BIOLOGY

UDC 577.112

Interaction with Keap1 Does Not Lead to Ubiquitination and Degradation of Prothymosin α

S. V. Melnikov, A. G. Evstafieva, and A. B. Vartapetian

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

Received October 24, 2006

Accepted for publication November 30, 2006

Abstract—Prothymosin α (ProTα) is highly conserved among vertebrates and has many biological functions, including an enforcement of cell antioxidant defense. This function is due to ProTα interaction with Keap1, a repressor of the Nrf2 transcription factor, activating the expression of several antioxidant protein genes. Keap1 exports Nrf2 from the cell nucleus into the cytoplasm and, acting as an adaptor protein for ubiquitin ligase, facilitates ubiquitination of Nrf2 and its subsequent degradation by the 26S proteasome. ProTα and Nrf2 compete for Keap1 binding. ProTα is capable of displacing Nrf2 from its complex with Keap1, thereby increasing the expression of the Nrf2 target genes. It was found that ProTα remained stable upon its interaction with Keap1. In contrast to Nrf2, ProTα escaped Keap1-dependent ubiquitination, proteasomal degradation, and export from the nucleus. Moreover, ProTα ubiquitination was not detected even when Keap1 and ubiquitin were overproduced. Hence, activation of Nrf2-dependent transcription by ProTα was assumed to result from the increase in free Nrf2 rather than from an increase in total Nrf2.

DOI: 10.1134/S0026893307050123

Key words: prothymosin α, Keap1, ubiquitination, 26S proteasome

INTRODUCTION

Recent studies have revealed a group of proteins lacking secondary- and tertiary-structure elements [1]. Many proteins of this group regulate important processes such as translation, transcription, cell division, and apoptosis. Yet the molecular mechanisms sustaining the function of disordered proteins have, as of yet, not been studied in detail.

Prothymosin α (ProTα) is a typical unfolded protein. ProTα is a small (12 kDa) conserved protein and is found in almost all vertebrate tissues [2, 3]. Human ProTα consists of 109 amino acid residues, of which only seven are hydrophobic and almost a half are Asp and Glu. Owing to this amino acid composition, ProTα is highly hydrophilic and has a low pI (3.5). ProTα is virtually devoid of secondary-structure elements and occurs as a random coil under physiological conditions [4]. Another important feature of ProTα is its high cell content: actively dividing cells, each contain about 17 million ProTα molecules [5].

ProTα has been described as a protein stimulating cell division [3, 6], a negative regulator of apoptosis [7–9], a transcriptional regulator of many genes [10, 11], and a molecular partner of several proteins such as the transcriptional coactivator CBP [12], oncoprotein SET [13], and apoptosis-regulating p8 [8].

We have recently identified KELCH-like ECH-associated protein 1 (Keap1) as a new molecular partner of ProTα [14]. Keap1 plays an important role in the adaptive cell response to oxidative stress. Under normal physiological conditions, when the cell is not exposed to oxidants, Keap1 represses Nrf2. Repression is abolished upon cell exposure to oxidants [15]. In turn, Nrf2 activates transcription of the genes coding for enzymes involved in xenobiotic detoxification and antioxidant biosynthesis [16]. Keap1 consists of two domains, the N-terminal BTB domain and the C-terminal KELCH domain [15]. The BTB domain is responsible for Keap1 dimerization and interaction with cullin 3 (Cul3), a subunit of the ubiquitin ligase complex [17, 18]. The KELCH domain is responsible for Keap1 interaction with ProTα [14] and Nrf2 [15]. Under the normal conditions, Keap1 exports Nrf2 from the nucleus into the cytoplasm and interacts with Cul3, acting as an adaptor to bring Cul3 ubiquitin ligase close to its target Nrf2. As a result, Nrf2 is ubiquitinated and targeted for degradation by the 26S proteasome, its level in the cell decreases, and, eventually, the expression of its target genes becomes less efficient.

ProTα competes with Nrf2 for Keap1 binding and displaces Nrf2 from its complex with Keap1 in vitro. In vivo, ProTα increases the expression of the human hemoxygenase 1 gene (*HO-1*), which is a target of Nrf2. A ProTα mutant incapable of Keap1 binding and Nrf2 displacement from its complex with Keap1 in vitro fails to increase the *HO-1* expression [14].

The objective of this work was to study the consequences of interactions with Keap1 for ProTα. It could be assumed by analogy to the Keap1–Nrf2 interaction that the interaction with Keap1 would reduce the ProTα content and intracellular localization. However, we found that the consequences of interactions with Keap1 strikingly differ between Nrf2 and ProTα, notwithstanding the similarity and competitive character of these interactions.

EXPERIMENTAL

Plasmid construction. To construct pcDNA4/Nrf2ΔHis$_6$, coding for Nrf2 without a hexahistidine tag, the fragment between the *Hin*dIII site and the *Nco*I site closest to the initiator triplet of the Nrf2 cDNA was deleted from pcDNA4/HisMax-Nrf2 [14].

Cell lines and transfection. HeLa and HEK 293 cells were cultured in DMEM supplemented with 10% fetal bovine serum (HyClone). To overproduce Nrf2, Keap1, ProTα, and ubiquitin, cells were transfected with pcDNA4/HisMax-Keap1 [14], pcDNA4/HisMax-Nrf2 [14], pcDNA4-enh-wt ProTα [19], and pHis$_6$-ubi [20] (kindly provided by M. McMahon). Transfection with a mixture of pcDNA4/HisMax-Keap1, pcDNA4/HisMax-Nrf2, and pcDNA4-enh-wt ProTα (1 : 1 : 6) was performed using Lipofectamine 2000 (Invitrogen). Control cells were transfected with a mixture including an equivalent amount of pcDNA4/HisMaxA (Invitrogen) in place of pcDNA4-enh-wt ProTα.

Western blotting, immunoprecipitation, and protein isolation on Ni-NTA-agarose. The contents of Nrf2, Keap1, ubiquitin, and actin were estimated by Western blotting [19]. The ProTα level was estimated by Western blotting with modification, using Hybond N$^+$ membranes (Amersham) [21]. Keap1 was detected with anti-Keap1(308–624) rabbit polyclonal antibodies [14], Nrf2 was detected with rabbit polyclonal antibodies C20 (Santa Cruz Biotechnology), and ProTα was detected with monoclonal antibody 2F11 [19]. The primary antibodies were used at a 1 : 1000 dilution. The proteasome inhibitor MG-132 (Sigma) was added to the culture medium to the final concentration of 10 μM 3 h before cell examination or 2 μM 18 h before cell examination.

Ubiquitinated Nrf2 was detected by protein fractionation on Ni-NTA-agarose and immunoprecipitation. In the former case, HeLa cells were transfected with pHis$_6$-ubi, pcDNA4/HisMax-Nrf2ΔHis$_6$, and pcDNA4/HisMax-Keap1. Cell lysates were resolved on Ni-NTA-agarose as recommended by QIAGEN for protein isolation under nondenaturing conditions. Proteins were eluted with 20 mM EDTA. In the case of immunoprecipitation, HeLa cells were transfected with a mixture of pcDNA4/HisMax-Nrf2, pLA-CMV-Keap1 (kindly provided by A. Khutorenko, Moscow State University, Moscow), pHis$_6$-ubi, and pcDNA4-enh-wt ProTα (1 : 1 : 1 : 3). Control cells were transfected with a mixture containing the empty vector in place of the ProTα-coding plasmid. Immunoprecipitation with anti-ubiquitin monoclonal antibody 4PD1 (Santa Cruz Biotechnology), which was used at a 1 : 100 dilution, was performed as in [22], adding N-enthylmaleimide to the lysis buffer to the final concentration of 10 μM.

Sandwich ELISA was carried out using ProTα-specific monoclonal antibody 2F11 and biotin-conjugated monoclonal antibody 4F4 [19]. Antibody 2F11 was diluted with PBS to the final concentration of 3 μg/ml and applied at 50 μl per well onto a 96-well EIA/RIA high-binding plate (Costar). The plate was incubated at 4°C overnight. The antibody solution was removed and PBS containing 3% skimmed milk was added at 100 μl per well. The plate was incubated at 37°C with continuous agitation for 30 min and washed with PBS containing 0.05% Tween-20 (PBS-T). Test lysates were added in twofold serial dilutions, so that the uppermost row of the plate contained undiluted lysates and the lysate concentration was halved in each subsequent row. To estimate the background absorption, lysates were not added to the two lowermost rows. The plate was incubated at 37°C for 1 h and washed with PBS-T. Biotinylated antibody 4F4 (6 μg/ml) was applied at 50 μl per well, and the plate was incubated at 37°C for 1 h and washed with PBS-T. Horseradish peroxidase-conjugated streptavidin (2 μg/ml) was added at 50 μl per well, and the plate was incubated at 37°C for 30 min and washed with PBS-T and water. Then, 5 ml of 0.4 mg/ml o-phenylenediamine, 0.1 M citrate (pH 5.0) was combined with 30 μl of H_2O_2 and 50 μl of the mixture was added to each well. The plate was incubated in the dark for 5–10 min until an orange color developed. The reaction was terminated by adding 1 M H_2SO_4 at 50 μl per well, and *A*492 was measured using a Stat Fax 2100 96-well plate reader (Awareness Technology).

Immunofluorescent cell staining. Cells were stained 8–24 h after transfection as described previously [14]. To avoid ProTα epitope masking with ubiquitin, ProTα was detected with two monoclonal antibodies, 2F11 and 4F4 [19]. For Keap1, we used anti-Keap1(308–624) rabbit polyclonal antibodies when Keap1 was detected simultaneously with ProTα and monoclonal antibody 2H5 when Keap1 was detected simultaneously with Nrf2 [14]. Nrf2 was detected using rabbit polyclonal antibodies C20 (Santa Cruz Biotechnology). The primary antibodies were used at a 1 : 300 dilution. As secondary antibodies, FITC- or rhodamine-conjugated anti-rabbit Ig and anti-mouse Ig goat polyclonal antibodies (Santa Cruz Biotechnology) were used at a 1 : 400 dilution. Images were obtained with an AxioVert 200M fluorescence microscope equipped with an LSM 510 analyzer (Carl Zeiss).

RESULTS AND DISCUSSION

ProTα Is a Stable Protein That Is Not Degraded by the 26S Proteasome

The effect of proteasome inactivation on the intracellular ProTα pool was studied to check whether ProTα interaction with Keap1 leads to ProTα degradation by the 26S proteasome. HeLa and HEK 293 cells were treated with MG-132 or clasto-lactacystin β-lactone. It is known that a decrease in the proteolytic activity of the proteasome leads to an accumulation of polyubiquitinated proteins in the cell. As Western blotting of cell lysates revealed, cell treatment with MG-132 led to a substantial accumulation of ubiquitinated proteins (Fig. 1a), suggesting a dramatic decrease in proteasome activity. The ProTα level was estimated by two independent methods, ELISA and immunofluorescence microscopy. Analysis of the ProTα concentration in HeLa cell lysates by ELISA showed that MG-132 (Fig. 1b) and clasto-lactacystin β-lactone (data not shown) did not affect the intracellular pool of ProTα. The same conclusion was made on the basis of immunofluorescent staining of HeLa cells. The signal intensity from ProTα was much the same in cells treated with MG-132 and in control cells (Fig. 1c). Similar results were obtained with HEK 293 cells (data not shown).

The above results indicate that ProTα is not subject to degradation by the 26S proteasome. However, ProTα is far more abundant than Keap1 in the cell and it is possible to assume that only a minor fraction of ProTα is degraded and its stabilization by the proteasome inhibitor is undetectable in the total ProTα pool. To check this assumption, we overproduced Keap1 in HeLa cells and studied the effect of overproduction on the intracellular level of ProTα. The Keap1 content significantly increased in cells transfected with a Keap1-coding plasmid (Fig. 2a), but the ProTα content estimated by ELISA did not change (Fig. 2b). Nor did Keap1 change the exclusively nuclear localization of ProTα (Fig. 2c). At the same time, Keap1 overproduction decreased the Nrf2 level (Fig. 2d) and caused a redistribution of Nrf2 from the nucleus into the cytoplasm in parallel experiments (Fig. 2e), which agreed with the published data [15, 23].

Taken together, our findings demonstrate that ProTα is a stable protein, resistant to degradation by the 26S proteasome. This conclusion agrees with the fact that the half-life of ProTα is about 1 day [5], while the half-life of unstable proteins does not exceed a few minutes.

ProTα Is Not Subject to Ubiquitination

The fact that Keap1 exerts no effect on ProTα stability does not necessarily mean that ProTα is not ubiquitinated. Polyubiquitination, targeting the protein to degradation, is not the only possibility: reversible modification with a single ubiquitin molecule does not affect the stability of a protein, but changes its interactions with natural ligands [24].

Further experiments were designed to check whether ProTα is ubiquitinated. HeLa cells were transfected with a plasmid coding for ubiquitin N-tagged with hexahistidine. Cell lysates were fractionated on Ni-NTA-agarose. The bound (i.e., ubiquitinated) protein was analyzed by Western blotting with ProTα-specific antibodies. However, we did not detect a signal differing from the baseline (data not shown). This result suggests that either ProTα is not conjugated with ubiquitin or its conjugates are not abundant enough to be detected by our method. We additionally overproduced Keap1 and ProTα, but a signal distinguishable from the baseline was not detected again (Fig. 3a). Likewise, ubiquitinated ProTα was not observed in cells transfected with Keap1-, ProTα-, and ubiquitin-coding plasmids and treated with MG-132 (Fig. 3a). At the same time, accumulation of ubiquitinated Nrf2 was readily detectable in HeLa cells overproducing Keap1, Nrf2, and ubiquitin upon MG-132 treatment (Fig. 3b). These results indicate that the interaction of ProTα with the ubiquitin ligase adaptor is not accompanied by its ubiquitination.

ProTα Does Not Affect the Intracellular Content and Polyubiquitination of Nrf2

We have previously observed that an increase in ProTα intensifies Nrf2-dependent transcription in human cells [14]. The effect is specific, since it is detectable with the wild-type ProTα, binding with Keap1 but not with a ProTα point mutant incapable of

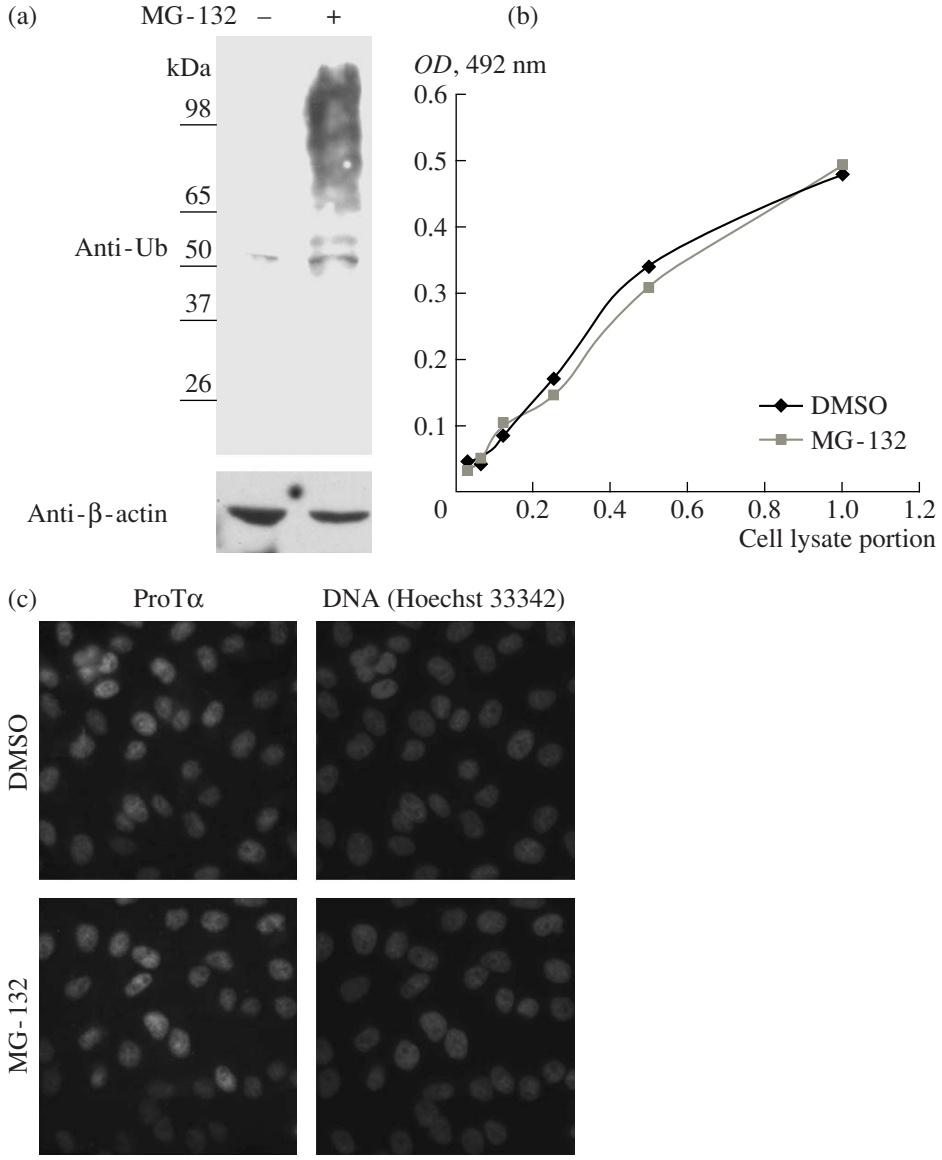

Fig. 1. Proteasome inhibitors do not affect the intracellular ProTα pool. (a) Western blot analysis of polyubiquitinated proteins in HeLa cell lysates. Cells were incubated in the presence of 2 μM MG-132 or an equivalent amount of DMSO for 18 h. An analysis was performed with antibodies against ubiquitin (Ub) and β-actin. (b) Estimation of the ProTα content in cell lysates by sandwich ELISA. (c) Immunofluorescent staining of HeLa cells with monoclonal antibody 2F11, specific for ProTα.

the interaction with Keap1. Two alternative mechanisms can be assumed for the ProTα-induced increase in Nrf2-dependent transcription of target genes. On the one hand, ProTα may increase the total Nrf2 content, which, in turn, allows more efficient expression of the Nrf2 target genes. On the other hand, it is possible that the Nrf2 total pool is unchanged, while the content of free (nonbound with Keap1) Nrf2 increases, as Keap1 interacts with degradation-resistant ProTα.

To decide between these alternatives, we studied the effect of ProTα overproduction on the intracellular level of Nrf2 and the extent of its ubiquitination. HeLa cells were transfected with the Nrf2-, Keap1-, and ProTα-coding plasmids or the empty vector. The protein contents in cell lysates were estimated by Western blotting (Fig. 4a). An increase in ProTα did not affect the Nrf2 pool. In addition, proteins were immunoprecipitated from cell lysates with ubiquitin-specific antibodies and analyzed by Western blotting with Nrf2-specific antibodies. Overproduction of ProTα did not significantly change the extent of Nrf2 ubiquitination (Fig. 4b).

We have previously demonstrated that the Keap1–Nrf2 complex continuously shuttles between the nucleus and cytoplasm and that its visible cytoplasmic

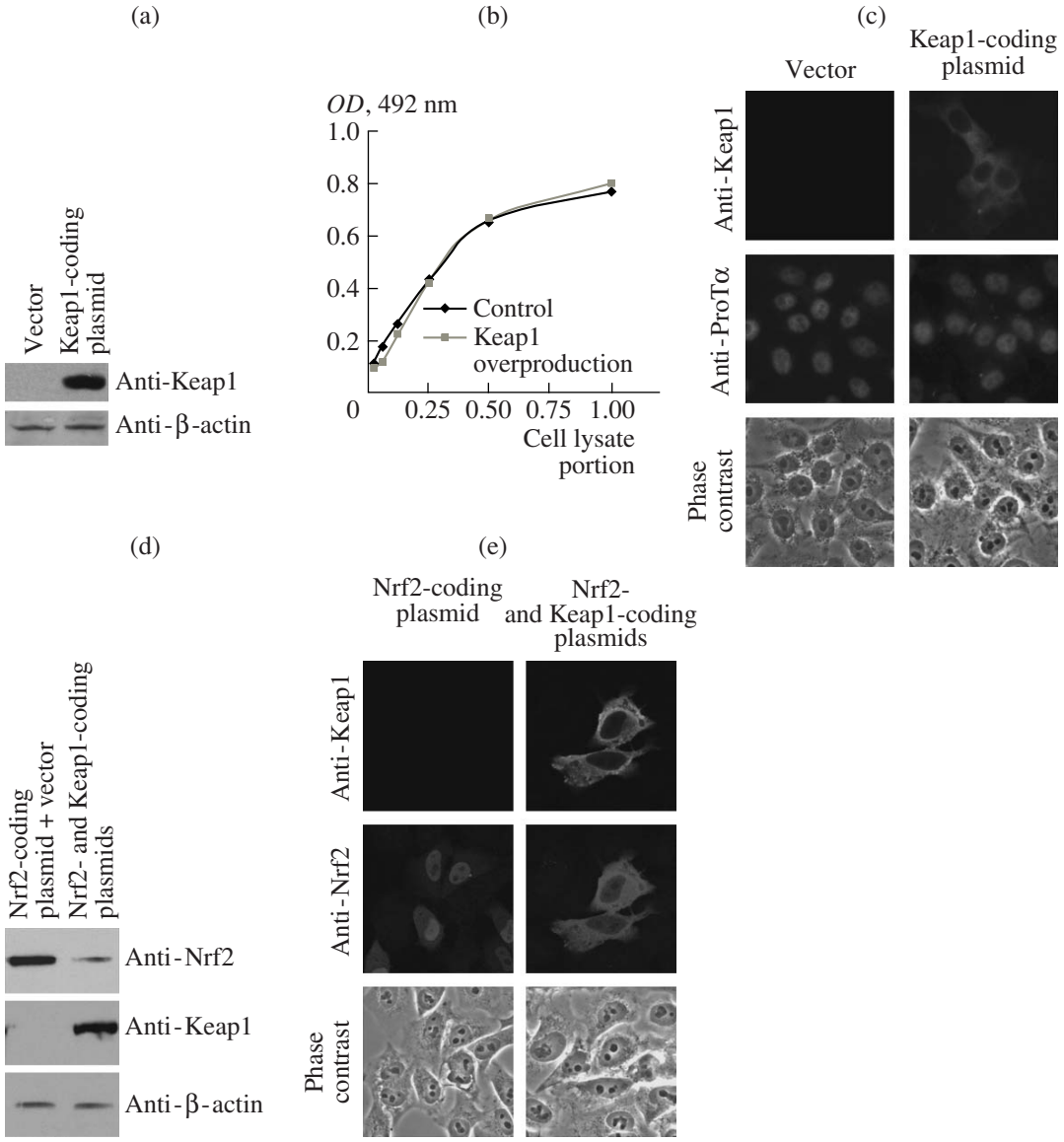

Fig. 2. Overproduction of Keap1 does not affect the content and intracellular localization of ProTα. (a) Western blotting of HeLa cell lysates with antibodies against Keap1 and β-actin. Cells were transfected with a Keap1-coding plasmid or the empty vector (control). (b) ProTα concentration as a function of the Keap1 level in HeLa cells (sandwich ELISA). (c) Immunofluorescent staining of HeLa cells varying in Keap1 content with monoclonal antibody 2F11 against ProTα. (d) Western blotting of lysates of HeLa cells transfected with an Nrf2-coding plasmid together with the empty vector (control) or a Keap1-coding plasmid. (e) Immunofluorescent staining of the same cells with antibodies specific for Keap1 and Nrf2.

location shows that its export from the nucleus prevails over its import into the nucleus [14]. Our results give grounds to assume that, to increase Nrf2-dependent transcription of the genes protecting the cell from oxidative stress, ProTα does not increase the total Nrf2 pool but rather displaces Nrf2 from its complex with Keap1 in the cell nucleus, where Keap1 binds with ProTα. Although Nrf2 and ProTα interact with the same domain of Keap1, the consequences of this interaction differ between the two Keap1 partners. In complex with Keap1, Nrf2 is exported from the nucleus, ubiquitinated, and degraded by the proteasome. None of these processes is characteristic of ProTα. The stability of ProTα can be determined by its nuclear location, since Keap1-dependent degradation occurs in the cytoplasm [25]. It is unclear, however, what the factors are that prevent ProTα from being exported from the nucleus by Keap1. Possibly, a structural difference is responsible for the difference in behavior between the Keap1–ProTα and Keap1–Nrf2 complexes.

As of yet, ProTα is the only natively unfolded protein acting as a partner of the ubiquitin ligase adaptor.

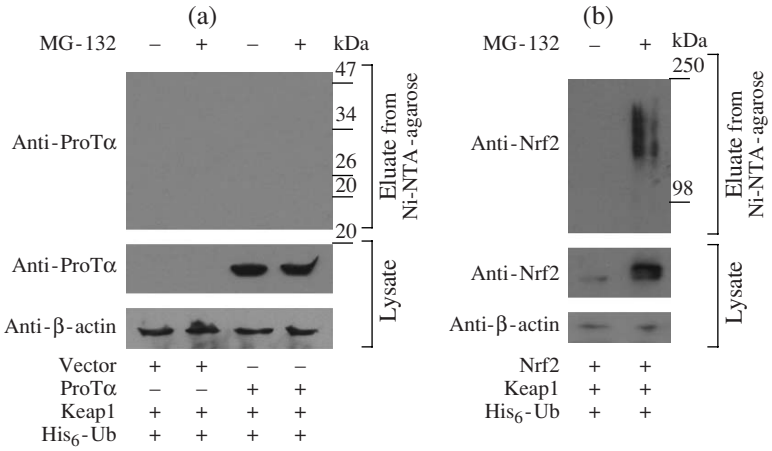

Fig. 3. Western blotting of the proteins that bound to Ni-NTA-agarose from lysates of HeLa cells producing hexahistidine-tagged ubiquitin. (a) ProTα does not form conjugates with ubiquitin. An analysis was performed with monoclonal antibodies specific for β-actin and a monoclonal antibody specific for ProTα. (b) Nrf2 is covalently modified with ubiquitin. An analysis was performed with polyclonal antibodies specific for β-actin and for Nrf2.

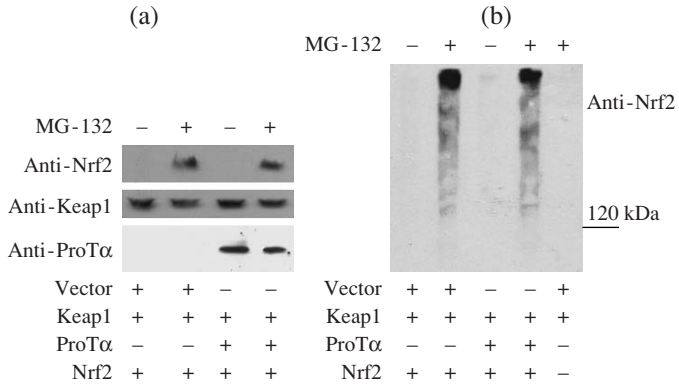

Fig. 4. ProTα does not affect the intracellular content and ubiquitination of Nrf2. (a) Western blotting of lysates of HeLa cells varying in ProTα content. To detect Nrf2 and Keap1, proteins were transferred onto a nitrocellulose membrane; to detect ProTα, proteins were transferred onto a Hybond N(+) membrane. (b) Western blotting of immunoprecipitates with antibodies specific for Nrf2. The immunoprecipitates were obtained from HeLa cell lysates with antibodies specific for ubiquitin.

Our further studies focus on the ProTα resistance to nuclear export and the ubiquitin-transferring system.

ACKNOWLEDGMENTS

We are grateful to M. McMahon (University of Dundee, United Kingdom) for the ubiquitin-coding plasmid and A. Khutornenko (Moscow State University) for pLA-CMV-Keap1.

This work was supported by the Russian Foundation for Basic Research (project nos. 05-04-48491, 05-04-90587-NNS, and 06-04-49126).

REFERENCES

1. Dyson H.J., Wright P.E. 2005. Intrinsically unstructured proteins and their functions. *Nat. Rev. Mol. Cell. Biol*. **6**, 197–208.
2. Haritos A.A., Tsolas O., Horecker B.L. 1984. Distribution of prothymosin α in rat tissues. *Proc. Natl. Acad. Sci. USA*. **81**, 1391–1393.
3. Hannappel E., Huff T. 2003. The thymosins. Prothymosin α, parathymosin, and β-thymosins: Structure and function. *Vitamines Hormones*. **66**, 257–296.
4. Gast K., Damaschun H., Eckert K., Schulze-Forster K., Maurer H.R., Muller-Frohne M., Zirwer D., Czarnecki J., Damaschun G. 1995. Prothymosin α: A biologically active protein with random coil conformation. *Biochemistry*. **34**, 13,211–13,218.
5. Sburlati A.R., De La Rosa A., Batey D.W., Kurys G.L., Manrow R.E., Pannell L.K., Martin B.M., Sheeley D.M., Berger S.L. 1993. Phosphorylation of human and bovine prothymosin α in vivo. *Biochemistry*. **32**, 4587–4596.
6. Conteas C.N., Mutchnick M.G., Palmer K.C., Weller F.E., Luk G.D., Naylor P.H., Erdos M.R., Goldstein A.L., Panneerselvam C., Horecker B.L. 1990. Cellular levels of thymosin immunoreactive peptides are linked to prolif-

erative events: Evidence for a nuclear site of action. *Proc. Natl. Acad. Sci. USA*. **87**, 3269–3273.

7. 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 α in a death regulatory pathway. *Science*. **299**, 223–226.
8. Malicet C., Giroux V., Vasseur S., Dagorn J.C., Neira J.L., Iovanna J.L. 2006. Regulation of apoptosis by the p8/prothymosin α complex. *Proc. Natl. Acad. Sci. USA*. **103**, 2671–2676.
9. 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 α. *Exp. Cell. Res*. **284**, 211–223.
10. Martini P.G., Katzenellenbogen B.S. 2003. Modulation of estrogen receptor activity by selective coregulators. *J. Steroid. Biochem. Mol. Biol*. **85**, 117–122.
11. Kobayashi T., Wang T., Maezawa M., Kobayashi M., Ohnishi S., Hatanaka K., Hige S., Shimizu Y., Kato M., Asaka M., Tanaka J., Imamura M., Hasegawa K., Tanaka Y., Brachmann R.K. 2006. Overexpression of the oncoprotein prothymosin α triggers a p53 response that involves p53 acetylation. *Cancer Res*. **66**, 3137–3144.
12. Karetsou Z., Kretsovali A., Murphy C., Tsolas O., Papamarcaki T. 2002. Prothymosin α interacts with the CREB-binding protein and potentiates transcription. *EMBO Rep*. **3**, 361–366.
13. Karetsou Z., Martic G., Tavoulari S., Christoforidis S., Wilm M., Gruss C., Papamarcaki T. 2004. Prothymosin α associates with the oncoprotein SET and is involved in chromatin decondensation. *FEBS Lett*. **577**, 496–500.
14. 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 α is a partner of Keap1: Implications for expression of oxidative stress-protecting genes. *Mol. Cell. Biol*. **25**, 1089–1099.
15. 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 Dev.* **13**, 76–86.
16. Itoh K., Chiba T., Takahashi S., Ishii T., Igarashi K., Katoh Y., Oyake T., Hayashi N., Satoh K., Hatayama I., Yamamoto M., Nabeshima Y. 1997. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem. Biophys. Res. Commun*. **236**, 313–322.
17. Cullinan S.B., Gordan J.D., Jin J., Harper J.W., Diehl J.A. 2004. The Keap1–BTB protein is an adaptor that bridges Nrf2 to a Cul3-based E3 ligase: Oxidative stress sensing by a Cul3–Keap1 ligase. *Mol. Cell. Biol*. **24**, 8477–8486.
18. McMahon M., Thomas N., Itoh K., Yamamoto M., Hayes J.D. 2006. Dimerization of substrate adaptors can facilitate cullin-mediated ubiquitylation of proteins by a "tethering" mechanism: A two-site interaction model for the Nrf2–Keap1 complex. *J. Biol. Chem*. **281**, 24,756–24,768.
19. 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 α origin, mutations and conformation with monoclonal antibodies. *J. Immunol. Methods*. **266**, 185–196.
20. Treier M., Staszewski L.M., Bohmann D. 1994. Ubiquitin-dependent c-Jun degradation in vivo is mediated by the δ domain. *Cell*. **78**, 787–798.
21. Karey K.P., Sirbasku D.A. 1989. Glutaraldehyde fixation increases retention of low molecular weight proteins (growth factors) transferred to nylon membranes for western blot analysis. *Anal. Biochem*. **178**, 255–259.
22. Buschmann T., Fuchs S.Y., Lee C.G., Pan Z.Q., Ronai Z. 2000. SUMO-1 modification of Mdm2 prevents its self-ubiquitination and increases Mdm2 ability to ubiquitinate p53. *Cell*. **101**, 753–762.
23. 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**, 21,592–21,600.
24. Sigismund S., Polo S., Di Fiore P.P. 2004. Signaling through monoubiquitination. *Curr. Top. Microbiol. Immunol*. **286**, 149–185.
25. Itoh K., Wakabayashi N., Katoh Y., Ishii T., O'Connor T., Yamamoto M. 2003. Keap1 regulates both cytoplasmic–nuclear shuttling and degradation of Nrf2 in response to electrophiles. *Genes Cells*. **8**, 379–391.