

## Nuclear Oncoprotein Prothymosin $\alpha$ Is a Partner of Keap1: Implications for Expression of Oxidative Stress-Protecting Genes

Ruben N. Karapetian,<sup>1</sup> Alexandra G. Evstafieva,<sup>1</sup> Irina S. Abaeva,<sup>1</sup> Nina V. Chichkova,<sup>1</sup> Grigoriy S. Filonov,<sup>1</sup> Yuri P. Rubtsov,<sup>1</sup> Elena A. Sukhacheva,<sup>2</sup> Sergey V. Melnikov,<sup>1</sup> Ulrich Schneider,<sup>3</sup> Erich E. Wanker,<sup>4</sup> and Andrey B. Vartapetian<sup>1\*</sup>

*Belozersky Institute of Physico-Chemical Biology, Moscow State University,<sup>1</sup> and Shemiakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences,<sup>2</sup> Moscow, Russia, and Max Planck Institute for Molecular Genetics<sup>3</sup> and Max Delbrueck Center for Molecular Medicine,<sup>4</sup> Berlin, Germany*

Received 8 June 2004/Returned for modification 9 August 2004/Accepted 25 October 2004

**Animal cells counteract oxidative stress and electrophilic attack through coordinated expression of a set of detoxifying and antioxidant enzyme genes mediated by transcription factor Nrf2. In unstressed cells, Nrf2 appears to be sequestered in the cytoplasm via association with an inhibitor protein, Keap1. Here, by using the yeast two-hybrid screen, human Keap1 has been identified as a partner of the nuclear protein prothymosin  $\alpha$ . The in vivo and in vitro data indicated that the prothymosin  $\alpha$ -Keap1 interaction is direct, highly specific, and functionally relevant. Furthermore, we showed that Keap1 is a nuclear-cytoplasmic shuttling protein equipped with a nuclear export signal that is important for its inhibitory action. Prothymosin  $\alpha$  was able to liberate Nrf2 from the Nrf2-Keap1 inhibitory complex in vitro through competition with Nrf2 for binding to the same domain of Keap1. In vivo, the level of Nrf2-dependent transcription was correlated with the intracellular level of prothymosin  $\alpha$  by using prothymosin  $\alpha$  overproduction and mRNA interference approaches. Our data attribute to prothymosin  $\alpha$  the role of intranuclear dissociator of the Nrf2-Keap1 complex, thus revealing a novel function for prothymosin  $\alpha$  and adding a new dimension to the molecular mechanisms underlying expression of oxidative stress-protecting genes.**

The defense against oxidative stress and electrophilic attack is mediated in animal cells by activation of a battery of genes encoding detoxification enzymes [such as glutathione *S*-transferase (GST) and NAD(P)H-quinone oxidoreductase-1 (NQO1)] and antioxidant proteins [such as  $\gamma$ -glutamylcysteine synthetase and heme oxygenase-1 (HO-1)] (for a review, see reference 15). Coordinated up-regulation of expression of these genes is attained through the presence of *cis*-acting antioxidant response elements (AREs), which are recognized by the Nrf2 transcription factor in their regulatory regions (35). Heterodimerization of Nrf2 with small Maf proteins may be required for efficient DNA binding and transcription activation (18, 29). Nrf2 has been demonstrated to regulate both the basal and induced expression of many ARE-responsive genes (27), as the response to electrophilic and reactive oxygen species-producing agents is profoundly impaired in Nrf2-deficient cells (17). Accordingly, *nrf2*-deficient mice displayed elevated sensitivity to chemical carcinogenesis and oxidative stress (31).

The ability of Nrf2 to direct transcription of the target genes is subject to regulation through an interaction with an ubiquitously expressed inhibitor protein, Keap1 (19). Because Keap1 displays similarity to a *Drosophila* Kelch protein, which is an actin-binding protein (44), Keap1 was proposed to bridge Nrf2 to the cytoskeleton in the cytoplasm of nonstressed cells, thus mediating its inhibitory action (8, 19, 22). Recently, Keap1 was

also reported to target Nrf2 for cytoplasmic ubiquitination and degradation by the proteasome (28, 45). Induction of oxidative stress and treatment of cells with chemopreventive agents enable Nrf2 to escape Keap1-dependent cytoplasmic sequestration and degradation, leading to stabilization of Nrf2, increased nuclear accumulation of Nrf2, and activation of Nrf2-dependent cytoprotective genes (19, 28, 45). The importance of Keap1-mediated regulation of Nrf2 activity is emphasized by the observations that in cells lacking Keap1, Nrf2 is constitutively accumulated in the nucleus (20) and that mice with constitutively activated Nrf2 due to the absence of Keap1 died postnatally, with the phenotype being reversed in *keap1 nrf2* double mutants (40).

Attempts to identify how the stress signals are transduced to the target genes point to the constituents of the Nrf2-Keap1 complex as well. In particular, Nrf2 has been demonstrated to be a protein kinase C and a PERK substrate, and in both cases Nrf2 phosphorylation led to destabilization of the Nrf2-Keap1 complex in stress-induced cells and promoted Nrf2 nuclear accumulation and transcriptional activity (3, 7, 16). On the other hand, Keap1 was also reported to be a sensor of oxidative and electrophilic stress due to the presence of several reactive Cys residues. Keap1 Cys273 and Cys288 mutants were impaired in their ability to repress Nrf2-dependent transcriptional activation under basal conditions and in Keap1-dependent ubiquitination and degradation of Nrf2 (24, 41, 45). In vitro, exposure to electrophiles disrupted the interaction of Keap1 with the Neh2 domain of Nrf2 (9).

Thus, the cytoplasmic Nrf2-Keap1 complex emerged as the critical regulator of ARE-dependent transcription. Here, we

\* Corresponding author. Mailing address: Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119992, Russia. Phone: (7) (095) 939-4125. Fax: (7) (095) 939-3181. E-mail: varta@genebee.msu.su.

report the identification of a novel Keap1 partner which, quite unexpectedly, turned out to be the nuclear protein prothymosin  $\alpha$  (ProT $\alpha$ ). We demonstrate that ProT $\alpha$  releases Nrf2 from the Nrf2-Keap1 complex in vitro and contributes to Nrf2-dependent gene expression in vivo. To lend mechanistic support for the involvement of ProT $\alpha$  in the regulation of ARE-dependent transcription, we provide evidence that Keap1 is a nuclear-cytoplasmic shuttling protein equipped with a functional nuclear export signal (NES), which apparently confers nuclear-cytoplasmic shuttling to the Nrf2-Keap1 complex.

ProT $\alpha$  is a ubiquitously and abundantly expressed small nuclear protein (6, 11, 25) that is involved in proliferation of mammalian cells (14) and in their protection against apoptosis (13, 21). Overexpression of ProT $\alpha$  in NIH 3T3 and HL-60 cells was shown to accelerate proliferation (32, 43), whereas inhibition of ProT $\alpha$  synthesis prevented cell division (36) and induced apoptosis in HL-60 cells (33). Overexpression of ProT $\alpha$  in a rat fibroblast cell line resulted in loss of contact inhibition, anchorage-independent growth, and decreased serum dependence (30). Consistent with its properties, ProT $\alpha$  is particularly abundant in tumor cells (10, 38). The antiapoptotic activity of ProT $\alpha$  likely arises from its ability to inhibit apoptosome formation (21), whereas the mechanism of ProT $\alpha$  action in stimulating proliferation is not clearly established. Mounting evidence suggests ProT $\alpha$  involvement in transcription regulation (23, 26, 39). The newly identified ProT $\alpha$ -Keap1 interaction may have important implications for the mechanism(s) of Nrf2-dependent gene expression.

#### MATERIALS AND METHODS

**Yeast two-hybrid screen.** Manipulations with yeast cells were performed essentially as described previously (Yeast protocols handbook, Clontech, Palo Alto, Calif., 2000). *Saccharomyces cerevisiae* strain L40ccU containing pBTM117c-ProT $\alpha$  was transformed with GAL4 cDNA human brain (HB) and human bone marrow (HBM) libraries (Matchmaker; Clontech). Totals of  $6 \times 10^6$  and  $4 \times 10^6$  independent transformants, respectively, were plated on a minimal medium lacking tryptophan, leucine, histidine, and uracil. After incubation at 30°C for 4 to 8 days, a total of 47 colonies formed were picked to SD plates lacking Trp and Leu. After incubation at 30°C for 2 days,  $\beta$ -galactosidase ( $\beta$ -Gal) activity was tested.  $\beta$ -Gal-positive clones were picked to SD plates lacking Leu and containing canavanine to eliminate the bait, and  $\beta$ -Gal activity was retested after incubation at 30°C for 2 days. Total DNA was prepared from  $\beta$ -Gal-negative clones and transformed into *Escherichia coli* JM109. To check for true positives, isolated plasmids were transformed into L40ccU harboring pBTM117c, pBTM117c-ProT $\alpha$ , pBTM117c-Sim1, or pBTM117c-Rev and tested for selective growth on SD plates lacking Trp, Leu, His, and Ura and for  $\beta$ -Gal activity as described above. The inserts of library plasmids of positive clones, pACT2-HBM and pACT2-HB, were sequenced.

**Human cells and fluorescence microscopy.** HeLa and HepG2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (ICN). Transfections were done with Lipofectamine 2000 (Invitrogen), using 2 to 6  $\mu$ g of plasmid DNA, according to the manufacturer's protocol. At 24 h after transfection, cells were fixed and processed for microscopy as described previously (13). When indicated, cells were treated with 2 nM leptomycin B (LMB) (Sigma) for 4 h prior to fixation. Protein fusions with enhanced green fluorescent protein (EGFP) were intracellularly localized as described previously (12). For immunolocalization, anti-Keap1 2H5 and 2E4 monoclonal antibodies and rabbit anti-Nrf2 polyclonal antibodies (C-20; Santa Cruz Biotechnology) at a dilution of 1:300 were used as primary antibodies. For Keap1 competition assay, the 2H5 antibody (0.64  $\mu$ g) was preincubated with 12.8  $\mu$ g of recombinant GST-Keap1(308-624) expressed in and purified from *E. coli* or with 6.4  $\mu$ g of GST at 4°C for 1 h prior to its addition to fixed cells. Fluorescein isothiocyanate-conjugated goat anti-mouse antibodies and rhodamine-conjugated goat anti-rabbit antibodies (Santa Cruz Biotechnology) were used as secondary antibodies at a dilution of 1:400. Images were acquired through an Axiovert 200 M fluorescence microscope equipped with an LSM510 confocal laser scanning module or with an AxioCam digital camera (Carl Zeiss).

**Plasmids.** pBTM117c-ProT $\alpha$  and its derivatives encoding wild-type human ProT $\alpha$  and various deletion mutants thereof fused to LexA were constructed by in-frame ligation of the ProT $\alpha$ -encoding DNA fragments derived from pHT15A (12) downstream from the LexA-encoding sequence in pBTM117c (42). pHT15A(44,50) was obtained by replacing the 110-bp StyI-AccI DNA fragment of pHT15A with that containing the E44G E50G double mutation (34). The MProT $\alpha$ (44,50)-encoding sequence was then used to replace the wild-type ProT $\alpha$ -encoding sequence in pHP12A (37), pKTL1 (5), pcDNA4-enh-wt ProT $\alpha$  (13), and pBTM117c-ProT $\alpha$ .

To obtain the complete ORF of human Keap1, the missing N-terminal Keap1-encoding DNA fragment was amplified by PCR from a SW480 cDNA library by using primers 5'-GGAACCGCATGCAGCCAGAT-3' and 5'-GTGTAGGCGAATTCAATGAG-3'. The SphI-EcoRI-digested PCR product and the EcoRI-XhoI DNA fragment from pACT2-HBM were then ligated in frame into the SphI-SalI-digested pQE32 (Qiagen) to produce pQE32-Keap1. pACT2-Keap1 was generated by inserting the BamHI-EcoRI DNA fragment from pQE32-Keap1 into pACT2-HBM. The complete Keap1 ORF in pACT2-Keap1 was then replaced with DNA fragments encoding different domains of Keap1. pQE-Keap1(1-139) was constructed by deleting the Keap1-encoding region downstream from the EcoRI site in pQE32-Keap1. pGEX-Keap1(308-624) was obtained by inserting the EcoRI-XhoI DNA fragment from pACT2-HB into pGEX-4T-2 (Amersham Pharmacia Biotech).

To produce Keap1 and its mutants as EGFP fusions in human cells, the Keap1-encoding DNA fragments were excised from pACT2-Keap1 and ligated in frame into the polylinker regions of the pEGFP-C series of vectors (Clontech). The L(308,310)A double mutation was introduced in Keap1 by PCR with a 5'-TCGAGGAGgcCACGgcGCACAAGC-3' mutagenic primer (lowercase letters indicate the nucleotide substitutions) and the pUC19 direct and reverse sequencing primers. The structure of the cloned PCR product was verified by sequencing. The Keap1mNES-encoding sequence was inserted into pQE32 and then transferred, as the BamHI-HindIII and BamHI-XhoI DNA fragments, into pEGFP-C2 and pcDNA4/HisMaxC (Invitrogen) vectors, respectively.

pcDNA4/HisMax-Keap1 was constructed by transferring the BamHI-XhoI fragment from pACT2-Keap1 into pcDNA4/HisMaxA (Invitrogen). For overproduction of zz-Keap1 in yeast, the zz-encoding DNA fragment from pQEzz59 (5) and the Keap1-encoding DNA fragment from pACT2-Keap1 were inserted in frame between the EcoRI and BamHI sites of the pYEDP1/8-2 yeast shuttle vector (34).

The cDNA fragment encoding human Nrf2 was amplified by PCR from a HeLa cDNA library (Clontech) with primers 5'-CGGGATCCCATGGATTGATTGACATA-3' and 5'-CCGCTCGAGTCTAGTTTTTCTTAACATCT-3'. The PCR product was digested with BamHI and XhoI, inserted into pcDNA4/HisMaxB (Invitrogen), and then transferred into the BamHI-SalI-digested pQE31 (Qiagen).

pARE/luc was constructed by inserting a synthetic ARE of the human NOO1 gene (5'-GTACCGCAGTCACAGTGACTACGAGAATCGCTAG-3') between the NheI and KpnI sites of pGL3-Promoter (Promega).

pSuper-RNAi was constructed by ligating annealed double-stranded oligonucleotide 5'-CCCGAGACGCCCCCTGTAACGGTTCAAGAGACCGTTAGCAGGGGCGTCTCTTTTGGAAA-3' with 5' overhangs GATC (sense strand) and AGCT (antisense strand) into the BglII-HindIII-digested pSuper (4) (kindly provided by R. Agami, The Netherlands Cancer Institute, Amsterdam, The Netherlands). The structure of the resulting construct was confirmed by sequencing.

**Antibodies and Western blotting analysis.** Anti-ProT $\alpha$  monoclonal antibodies 2F11 and 4F4 and anti-GFP monoclonal antibody 3A9 were described previously (37). Anti-Nrf2 (C-20), anti-I $\kappa$ B $\alpha$  (C-21), anti-histone H1 (AE-4), antiactin (C-2), and anti-LexA (N-19) antibodies were from Santa Cruz Biotechnology. Anti-caspase-3 antibodies were kindly provided by Y. Lazebnik (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). Mouse Keap1-specific monoclonal antibodies 2H5 and 2E4 and rabbit anti-Keap1(1-139) and anti-Keap1(308-624) polyclonal antibodies were generated essentially as described previously (37), using recombinant GST-Keap1(308-624) [for 2H5], His<sub>6</sub>-Keap1(1-139) [for 2E4 and anti-Keap1(1-139)] and His<sub>6</sub>-Keap1(308-624), respectively, as immunogens. Antibodies were affinity purified from ascitic fluids and sera by protein A-Sepharose (Amersham Pharmacia Biotech) chromatography, and polyclonal antibodies were additionally purified with membrane-immobilized Keap1 fragments. Specificity of the antibodies was confirmed by enzyme-linked immunosorbent assay, immunoblotting, and immunoprecipitation techniques. Notably, anti-Keap1 polyclonal antibodies and the 2E4 monoclonal antibody could bind both native and denatured Keap1, whereas the 2H5 monoclonal antibody could recognize nondenatured Keap1 only.

Immunoprecipitations of ProT $\alpha$  and Western blot analyses were performed as

described previously (37). Detection of protein bands was performed by using sheep anti-mouse immunoglobulin (Ig) or goat anti-rabbit Ig conjugated with horseradish peroxidase as secondary antibodies and ECL Western blotting detection reagents (Amersham Pharmacia Biotech) or Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences). For coimmunoprecipitation of endogenous Keap1,  $3 \times 10^6$  HeLa cells, either nonstressed or treated with 100  $\mu$ M diethyl maleate (DEM) (Sigma) for 4 h, or HepG2 cells were lysed for 40 min on ice in 200  $\mu$ l of EBC buffer (50 mM Tris-HCl [pH 8.0], 120 mM NaCl, 0.5% NP-40, 1 mM EDTA) supplemented with 1 mM phenylmethylsulfonyl fluoride. The cleared lysates were preincubated with 20  $\mu$ g of control mouse IgG and protein A-Sepharose at 4°C for 2 h. After centrifugation for 5 min, the supernatants were incubated with 20  $\mu$ g of 2F11 anti-ProT $\alpha$  antibody or with control mouse IgG at 4°C overnight. Protein A-Sepharose was added, and incubation was continued for 1 h. The beads were collected by brief centrifugation, washed two times with EBC buffer and two times with EBC buffer containing 0.5 M NaCl and 0.1% sodium dodecyl sulfate (SDS) at 0°C, and boiled in Laemmli sample buffer for 3 min. The samples were subjected to Western blot analysis with affinity-purified rabbit anti-Keap1(1–139) or anti-Keap1(308–624) polyclonal antibodies.

**Protein purification and in vitro protein binding assays.** Recombinant human ProT $\alpha$  and its derivatives were isolated from *E. coli* BL21(DE3) cells transformed with appropriate plasmids by a phenol extraction procedure and further purified by DEAE-chromatography (12).  $^{32}$ P labeling of the ProT $\alpha$  derivatives containing a protein kinase recognition site was as described by Chichkova et al. (5). The specific radioactivity of  $^{32}$ P-ProT $\alpha$  thus obtained was  $10^7$  cpm/ $\mu$ g. Recombinant His $_6$ -Nrf2, His $_6$ -Keap1(1–139), His $_6$ -Keap(308–624), and GST-Keap1(308–624) were overproduced in *E. coli* JM109 carrying an appropriate plasmid and isolated with Ni-nitrilotriacetic acid agarose (Qiagen) and glutathione-Sepharose 4B (Amersham Pharmacia Biotech) chromatography, respectively. zz-Keap1 was isolated from galactose-induced *S. cerevisiae* 2805 cells transformed with pYeDPI/8-2/zzKeap1 by cell disruption with glass beads at 0°C for 10 min and absorption of the recombinant protein onto IgG-Sepharose (Amersham Pharmacia Biotech).

Interaction of  $^{32}$ P-ProT $\alpha$  (30,000 cpm per sample) with IgG-Sepharose-immobilized zz-Keap1 (150 ng of fusion protein per sample) or zz alone (30 ng) was assessed in binding buffer (20 mM HEPES [pH 6.8], 100 mM NaCl, 0.1% Tween 20). When indicated, the binding buffer was supplemented with 100  $\mu$ M ZnSO $_4$ , CaCl $_2$ , or MgCl $_2$ . After 1 h of incubation at 0°C with shaking, the resin was washed with the same buffer, and the amounts of  $^{32}$ P-ProT $\alpha$  in pulled-down fractions and in supernatants were determined.

For competition assays, aliquots of IgG-Sepharose with immobilized zz-Keap1 (50 ng of fusion protein per sample) were incubated in binding buffer containing 100  $\mu$ M ZnSO $_4$  with either  $^{32}$ P-ProT $\alpha$  (30,000 cpm) or Nrf2 (250 ng) at 0°C for 1 h with shaking. The resin was washed with binding buffer and then incubated in the same buffer containing 250 ng of Nrf2 (for the Keap1-ProT $\alpha$  complex) and with the indicated amounts of wild-type ProT $\alpha$  or MProT $\alpha$ (44,50) mutant (for the Keap1-Nrf2 complex) at 0°C. Incubations with buffer alone served as controls. At the indicated time intervals, the amounts of  $^{32}$ P-ProT $\alpha$  and Nrf2 in pulled-down fractions and in supernatants were measured by radioactivity counting and by immunoblotting with anti-Nrf2 antibodies, respectively.

To assess binding of endogenous Keap1 in the lysates of HeLa and HepG2 cells to matrix-immobilized ProT $\alpha$ , wild-type ProT $\alpha$  and its MProT $\alpha$ (44,50) mutant (1 mg each) were coupled in parallel to 1 ml of CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) according to the manufacturer's protocol. The beads were then preincubated with 5 mg of bovine serum albumin per ml in buffer A (50 mM Tris-HCl [pH 8.0], 120 mM NaCl, 1 mM EDTA, and 0.3% Tween 20) at 0°C for 15 min and washed with the same buffer. Whole-cell lysates of  $1.5 \times 10^6$  cells were incubated with 10- $\mu$ l aliquots of ProT $\alpha$ -Sepharose beads in 200  $\mu$ l of buffer A at 0°C overnight, the beads were washed with the same buffer, and the bound proteins were eluted with 1% SDS in buffer A at 37°C for 10 min. Eluates were subjected to SDS–8% polyacrylamide gel electrophoresis (PAGE) and analyzed by Western blotting with anti-Keap1(1–139) and anti-Keap1(308–624) polyclonal antibodies.

**Reporter gene assays.** Luciferase and  $\beta$ -Gal were used as reporters. Cotransfection with  $\beta$ -Gal-expressing plasmid pcDNA4/HisMax/lacZ (Invitrogen) was employed to normalize for transfection efficiencies. Cells were lysed, 36 h after transfection, in reporter lysis buffer (Promega), and luciferase and  $\beta$ -Gal assays were performed with the Luciferase Assay System (Promega) according to the protocol supplied. All experiments were performed in triplicate.

**RNA isolation and Northern blotting.** Total cellular RNA was isolated from  $5 \times 10^5$  transfected HeLa cells with the RNAqueous total RNA isolation kit (Ambion) according to the protocol supplied by the manufacturer. RNA samples, in 20- $\mu$ g aliquots, were electrophoresed on 1.5% agarose–2.2% formalde-

hyde gels, transferred onto Hybond N+ membranes (Amersham Pharmacia Biotech), and hybridized sequentially with [ $^{32}$ P]DNA probes obtained by random-priming labeling of the indicated cDNAs. Quantification was performed with ImageQuant software after exposure of the membranes to a PhosphorImager (Molecular Dynamics).

**ProT $\alpha$  overproduction and small interfering RNA (siRNA) knockdown in human cells.** ProT $\alpha$  lacking artificially added sequences was overproduced in HeLa cells transfected with pcDNA4-enh-wt ProT $\alpha$  and pcDNA4-enh-MProT $\alpha$ (44,50) as described previously (13). Cells were treated with 100  $\mu$ M DEM or with diluent (dimethyl sulfoxide) only for 22 h prior to harvesting. For ProT $\alpha$  mRNA interference experiments, HeLa cells were grown on 35-mm-diameter dishes to 80% confluence and transfected twice, at a 2-day interval, with 6  $\mu$ g of pSuper or pSuper-RNAi. Total RNA was isolated from cells 48 h after the last transfection step and subjected to Northern blot analysis. Aliquots of the same cells were used for Western blot analysis with the indicated antibodies and for partial purification of ProT $\alpha$  followed by 7 M urea–8% PAGE (13).

## RESULTS

**Yeast two-hybrid screen for ProT $\alpha$ -interacting proteins.** A search for human proteins interacting with ProT $\alpha$  was performed with the yeast two-hybrid system. cDNA libraries from HB and HBM cloned downstream from the Gal4 activation domain (AD)-coding sequence served as prey, whereas full-length human ProT $\alpha$  fused to LexA served as bait. The bait construct alone failed to activate transcription of the *lacZ*, *HIS3*, and *URA3* reporter genes when introduced into *S. cerevisiae* L40ccU cells despite the synthesis of easily detectable amounts of the LexA-ProT $\alpha$  protein (data not shown). Yeast L40ccU cells cotransformed with the bait and the library prey constructs were selected for the gained ability to grow in the absence of uracil and histidine and were further screened with the  $\beta$ -galactosidase filter assay. Of  $10^7$  transformants tested, three positive clones from the HB library (with identical inserts) and one positive clone from the HBM library (with a larger insert) were identified. The library plasmids rescued from these clones encoded putative ProT $\alpha$  interactors, as they were able, upon the retransformation into the yeast strain, to activate expression of the reporter genes in the presence of the bait construct encoding LexA-ProT $\alpha$  but not in the absence of the bait or in the presence of unrelated proteins fused to LexA. Sequencing of the inserts from the rescued library plasmids (one plasmid for each library) revealed that they encode portions of the same protein, known as Keap1 (KIAA0132), a 624-amino-acid protein inhibitor of the transcription factor Nrf2. One plasmid (pACT2-HB) contained the Keap1 ORF starting from residue 308, and another (pACT2-HBM) contained the Keap1 ORF starting from residue 50. We then restored the complete ORF of human Keap1 and demonstrated that, in the yeast two-hybrid assays, full-length Keap1 binds ProT $\alpha$  efficiently (data not shown).

**Delineating a Keap1-binding region in ProT $\alpha$ .** To learn which region in ProT $\alpha$  is responsible for binding to Keap1, a set of ProT $\alpha$  deletion mutants was constructed (Fig. 1A). The ability of these mutants to bind full-length Keap1 was assessed with the yeast two-hybrid system. A short central region of ProT $\alpha$  (residues 32 to 52) turned out to be both necessary and sufficient to provide the interaction (Fig. 1A). Furthermore, testing the ProT $\alpha$  E(44)G,E(50)G double mutant [MProT $\alpha$  (44,50)] from our collection (34) in the yeast two-hybrid assay demonstrated the loss of interaction of this ProT $\alpha$  mutant with Keap1 (Fig. 1A). The effect was not due to instability of either the ProT $\alpha$  or Keap1 derivatives in yeast cells (Fig. 1B and C)

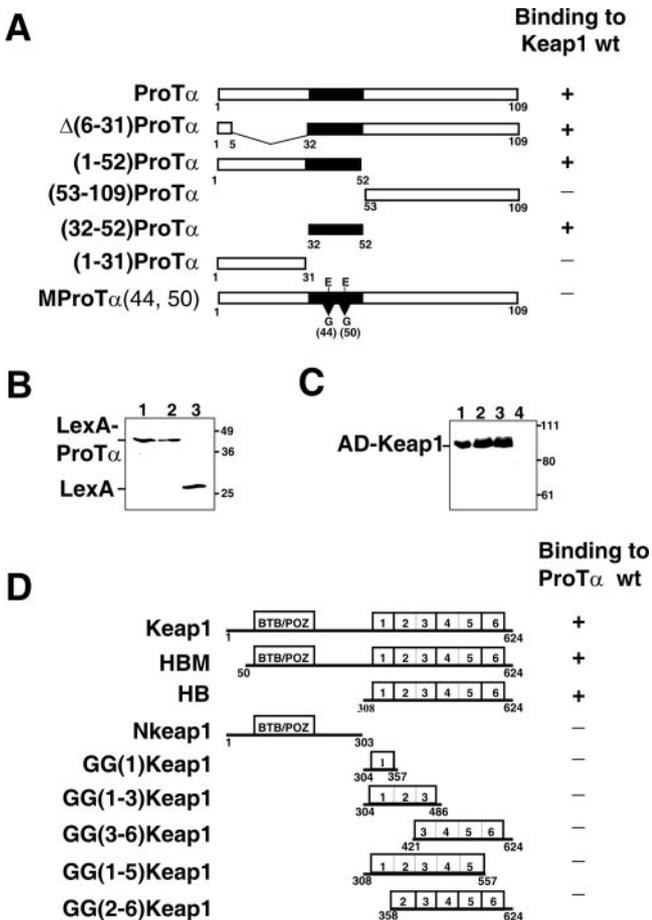


FIG. 1. Keap1 is a ProT $\alpha$  interactor: evidence from yeast two-hybrid analysis. (A) Identification of a Keap1-binding region (black) in ProT $\alpha$ . + and -, relative  $\beta$ -Gal levels above 300 and below 10 U, respectively. wt, wild type. (B and C) Lack of detectable interaction between the MProT $\alpha$ (44,50) mutant and Keap1 is not due to instability of either protein. Shown is Western blot analysis of lysates of yeast cells producing the following: lanes 1, LexA-ProT $\alpha$  plus AD-Keap1; lanes 2, LexA-MProT $\alpha$ (44,50) plus AD-Keap1; lanes 3, LexA plus AD-Keap1; lane 4, LexA-MProT $\alpha$ (44,50) plus AD. The primary antibodies used were anti-LexA (B) and anti-Keap1 (C). (D) An intact Kelch (diglycine, GG) repeat domain of Keap1 is required for ProT $\alpha$  binding. Individual repeats are numbered from 1 to 6. BTB/POZ, BTB/POZ domain of Keap1.

and provides a further argument for high specificity of the ProT $\alpha$ -Keap1 binding.

**Domain in Keap1 involved in binding to ProT $\alpha$ .** We then identified a region in Keap1 that is involved in the interaction with ProT $\alpha$ . A set of Keap1 deletion mutants was constructed (Fig. 1D) and assayed for ProT $\alpha$  binding by using the yeast two-hybrid system. The carboxyl-terminal half of Keap1 turned out to be the shortest Keap1 fragment capable of ProT $\alpha$  binding (Fig. 1D). This area of Keap1 comprises six Kelch repeats which together form a tertiary structure known as the  $\beta$ -propeller (1). Deletion of even one Kelch repeat in Keap1 resulted in the elimination of ProT $\alpha$  binding (Fig. 1D), suggesting that the intact  $\beta$ -propeller structure in Keap1 is essential for ProT $\alpha$  recognition.

**Evidence for the ProT $\alpha$ -Keap1 interaction in human cell lysates.** To learn whether Keap1 could bind to ProT $\alpha$  in human cells, a whole-cell lysate of HeLa cells ectopically expressing full-length Keap1 was treated with anti-ProT $\alpha$  2F11 monoclonal antibody or with control mouse IgG, and the antigen-antibody complex was collected with protein A-Sepharose and analyzed by Western blotting with anti-Keap1 polyclonal antibodies. Figure 2A demonstrates that Keap1 coprecipitated with endogenous ProT $\alpha$  when anti-ProT $\alpha$  antibody was used (lane 1), whereas control mouse IgGs were inactive (lane 2). The possibility that anti-ProT $\alpha$  antibody could recognize Keap1 directly was excluded by Western blot analysis of Keap1 with anti-ProT $\alpha$  2F11 monoclonal antibody (data not shown). Coimmunoprecipitation of the ectopically produced Keap1 with ProT $\alpha$  was then confirmed with lysates of HepG2 cells (data not shown).

Likewise, endogenous Keap1 could be coimmunoprecipitated with endogenous ProT $\alpha$  from whole-cell lysates of HeLa and HepG2 cells, as determined by Western blot analysis with two different anti-Keap1 polyclonal antibodies (Fig. 2B, left panel). Furthermore, even larger amounts of ProT $\alpha$ -associated Keap1 were detectable in HeLa cells treated with DEM, an oxidative stress inducer, relative to the nonstressed sample (Fig. 2B, right panel). Of note, Keap1 coimmunoprecipitated from the DEM-treated cell sample displayed a slightly lower electrophoretic mobility and appeared to be more heterogeneous (Fig. 2B, right panel, lane 3 versus lane 2), probably due to posttranslational modification of the protein.

Therefore, the ProT $\alpha$ -Keap1 interaction may occur in the homologous cell system, under both normal and oxidative stress conditions.

To further substantiate this conclusion and to check for the specificity of the interaction, recombinant human ProT $\alpha$  and its MProT $\alpha$ (44,50) mutant were immobilized on BrCN-activated Sepharose and used for isolation of endogenous Keap1 from HeLa and HepG2 cell lysates. The amount of Keap1 bound to each resin was evaluated by Western blotting with two different types of anti-Keap1 antibodies. Binding of Keap1 to wild-type ProT $\alpha$  was observed (Fig. 2C), whereas only trace amounts of Keap1 could bind to the MProT $\alpha$ (44,50) mutant, thus confirming our data on the specificity of the ProT $\alpha$ -Keap1 interaction obtained with the use of the yeast two-hybrid system.

**Binding of Keap1 to ProT $\alpha$  is direct.** To test whether the interaction between ProT $\alpha$  and Keap1 is direct or is mediated by some "bridging" component of cell lysates, a pull-down assay with purified recombinant human ProT $\alpha$  and Keap1 proteins was employed. Human Keap1 was overproduced as a fusion with the zz domain (z is an IgG-binding domain of staphylococcal protein A) and immobilized on IgG-Sepharose. For accurate and quantitative determination of ProT $\alpha$  binding to this affinity matrix, human wild-type ProT $\alpha$  and MProT $\alpha$ (44,50) were fused at their N termini to a short peptide comprising the protein kinase A recognition sequence and  $^{32}$ P radiolabeled. Binding of the labeled wild-type ProT $\alpha$  to the immobilized zz-Keap1 was observed in this *in vitro* system (Fig. 2D), whereas binding of ProT $\alpha$  to the zz domain alone was negligible. Recombinant unlabeled ProT $\alpha$  lacking artificially added sequences could compete with the binding of the radiolabeled probe (data not shown), indicating that binding to

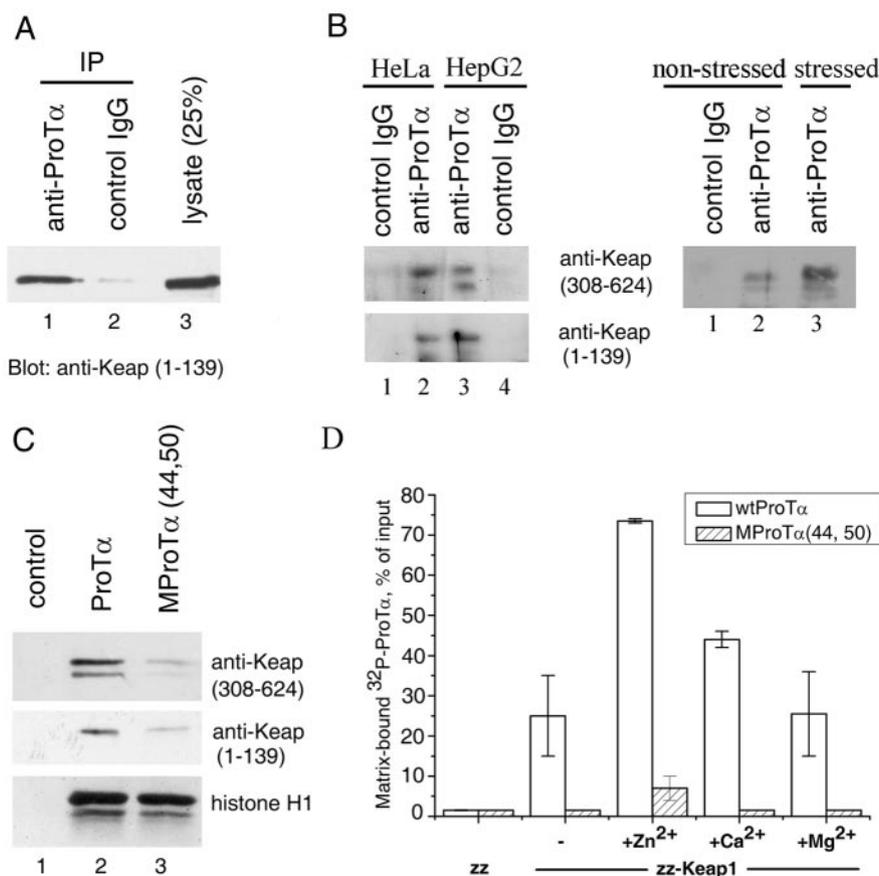


FIG. 2. Keap1 interacts with ProT $\alpha$  in human cell lysates (A, B, and C) and in vitro (D). (A) Coimmunoprecipitation of the ectopically produced Keap1 with ProT $\alpha$  from a whole-cell lysate of HeLa cells. Precipitation was performed with anti-ProT $\alpha$  2F11 monoclonal antibody (lane 1) or with control mouse IgG (lane 2). Lane 3, whole-cell lysate. Detection of Keap1 was performed by Western blot analysis with anti-Keap1 polyclonal antibodies. (B) Endogenous Keap1 associates with ProT $\alpha$ . Left panel: Coprecipitation of endogenous Keap1 with ProT $\alpha$  from whole-cell lysates of HeLa (lanes 1 and 2) and HepG2 (lanes 3 and 4) cells. Precipitation and detection were performed as described for (A). Right panel: Coprecipitation of endogenous Keap1 from lysates of HeLa cells either untreated (lanes 1 and 2) or treated with 100  $\mu$ M DEM for 4 h (lane 3). Note that in panels A and B the membranes were exposed to X-ray film for 30 s and 30 min, respectively. (C) Binding of endogenous Keap1 to Sepharose-immobilized ProT $\alpha$  is impaired by the (44,50) mutation in ProT $\alpha$ . Lane 1, resin without ProT $\alpha$ ; lane 2, wild-type ProT $\alpha$ ; lane 3, MProT $\alpha$ (44,50) mutant. The amount of Keap1 from HeLa whole-cell lysates bound to each resin was evaluated as described for panel A with two different anti-Keap1 antibodies. Bottom row: histone H1 binding to the same affinity resins, verifying equal amounts of immobilized ProT $\alpha$ . (D) Interaction of the purified recombinant ProT $\alpha$  and Keap1 in vitro. IgG-Sepharose-immobilized zz-Keap1 (150 ng) or zz alone (30 ng) was incubated with <sup>32</sup>P-radiolabeled wild-type (wt) ProT $\alpha$  or the MProT $\alpha$ (44,50) mutant, either in the absence or in the presence of the indicated metal cations (100  $\mu$ M). After incubation, the unbound ProT $\alpha$  was removed by washing the resin, and the amount of <sup>32</sup>P-ProT $\alpha$  bound to each affinity resin was determined by measuring the radioactivity. Error bars indicate standard deviations.

Keap1 is specific for the ProT $\alpha$  sequence. Furthermore, the MProT $\alpha$ (44,50) mutant failed to bind Keap1 (Fig. 2D).

Previously, we have demonstrated that ProT $\alpha$  binds Zn<sup>2+</sup> and Ca<sup>2+</sup> (but not Mg<sup>2+</sup>) (5). Therefore, we tested whether these divalent cations could affect ProT $\alpha$  binding to Keap1. In the presence of 100  $\mu$ M Zn<sup>2+</sup> or Ca<sup>2+</sup>, this binding was markedly enhanced (Fig. 2D). Substitution of Mg<sup>2+</sup> for these cations did not result in ProT $\alpha$  binding over the level seen in the absence of divalent cations (Fig. 2D).

Thus, ProT $\alpha$  interacts with Keap1 directly and specifically, and the divalent cations that bind to ProT $\alpha$  significantly enhance the ProT $\alpha$ -Keap1 interaction.

**How can ProT $\alpha$  and Keap1 meet each other?** The in vivo and in vitro data presented above seem to strongly suggest that Keap1 might be a genuine partner of ProT $\alpha$ . One major objection to this idea is that ProT $\alpha$  and Keap1 have been thought

to be localized to different subcellular compartments. While ProT $\alpha$  is known to be an exclusively nuclear protein, Keap1 was reported to be cytoplasmic and excluded from the nucleus. Moreover, such a localization is consistent with the postulated role of Keap1 as a cytoplasmic anchor for the Nrf2 transcription factor.

To test an idea that Keap1 might be a shuttling protein, we localized Keap1 in HeLa cells before and after treatment with LMB, a known and specific inhibitor of nuclear export of proteins possessing leucine-rich NESs. HeLa cells ectopically expressing Keap1 or EGFP-Keap1 fusion protein or containing endogenous Keap1 only were examined by fluorescence microscopy with anti-Keap1 monoclonal antibodies or the intrinsic fluorescence of EGFP. In the absence of LMB, both the endogenous and ectopically produced Keap1 were predominantly cytoplasmic (Fig. 3A to C). Upon treatment with LMB,

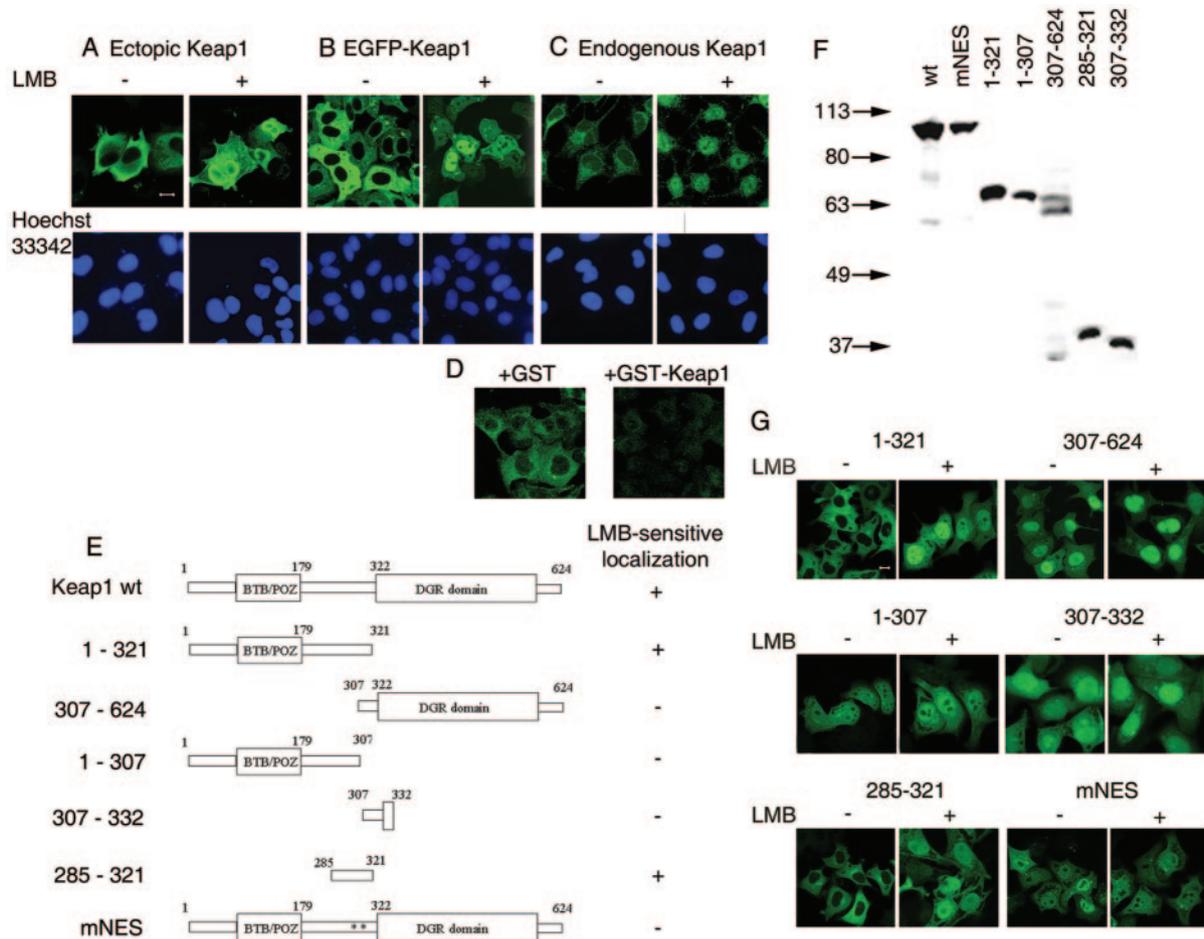


FIG. 3. Keap1 is a NES-containing nuclear-cytoplasmic shuttling protein. (A to C) Treatment of HeLa cells with LMB induces nuclear translocation of the ectopically produced full-length Keap1 (A), of the EGFP-Keap1 fusion protein (B), and of endogenous Keap1 (C). Confocal images were obtained by using anti-Keap1 2H5 monoclonal antibody (A and C) or intrinsic fluorescence of EGFP (B). (D) Recognition of endogenous Keap1 in HeLa cells is prevented by preincubation of the anti-Keap1 2H5 monoclonal antibody with recombinant GST-Keap1(308–624) (+GST-Keap1 panel) but not with GST alone (+GST panel). Images were acquired with identical confocal microscope settings. (E) Schematic representation of the Keap1 mutants used for the identification of the NES of human Keap1. DGR, diglycine repeat; wt, wild type. The indicated proteins were fused to the C terminus of EGFP. (F) Western blot analysis of the production of the EGFP-fused Keap1 mutants shown in panel E in transfected HeLa cells. Anti-EGFP antibody was used for detection of the respective proteins. (G) Confocal images showing intracellular localization of the Keap1 mutants fused to EGFP in HeLa cells either untreated or treated with LMB for 4 h. The results presented are also summarized in panel E, right column. Bars, 10  $\mu$ m.

a dramatic relocation of all three proteins from the cytoplasm to the nucleus occurred (Fig. 3A to C). The specificity of detection of endogenous Keap1 with the anti-Keap1 2H5 monoclonal antibody was confirmed by using an exogenously added GST-Keap1 competition assay (Fig. 3D) and a different anti-Keap1 monoclonal antibody, 2E4 (data not shown). A very similar nuclear translocation of Keap1 in response to LMB treatment was observed in another human cell line, HepG2 (data not shown). Our data thus imply that Keap1 is indeed a shuttling protein likely equipped with a leucine-rich NES. Importantly, an ability of Keap1 to enter the nucleus lends further support to our conclusion that Keap1 may be a true ProT $\alpha$  interactor.

**Identification of a nuclear export signal in Keap1.** To identify a putative NES of Keap1, a set of deletion mutants of Keap1 fused to the C terminus of EGFP reporter protein was constructed (Fig. 3E). HeLa cells transfected with the respec-

tive plasmids and producing EGFP-Keap1 derivatives of the expected size (Fig. 3F) were inspected for intracellular localization of the fusion proteins by fluorescence microscopy both before and after LMB treatment (Fig. 3G). Only the Keap1 derivatives encompassing the central “intervening” region of the protein displayed LMB-sensitive subcellular localization, being excluded from the nucleus in the absence of LMB and relocating into the nucleus after LMB treatment [Keap1(1–321) and Keap1(285–321)] (Fig. 3G).

Within the Keap1(285–321) region, there is the amino acid sequence L<sup>301</sup>VKIFEELTL<sup>310</sup>, which conforms to the consensus of the leucine-rich NES, H-2/3X-H-2X-H-X-H (where H stands for hydrophobic [frequently Leu] residues). Within this putative Keap1 NES, we replaced two leucine residues (L<sup>308</sup> and L<sup>310</sup>) that are known to be the most essential for NES function with alanines to produce an EGFP-linked full-length Keap1 mutant (Keap1mNES). In contrast to the wild-type

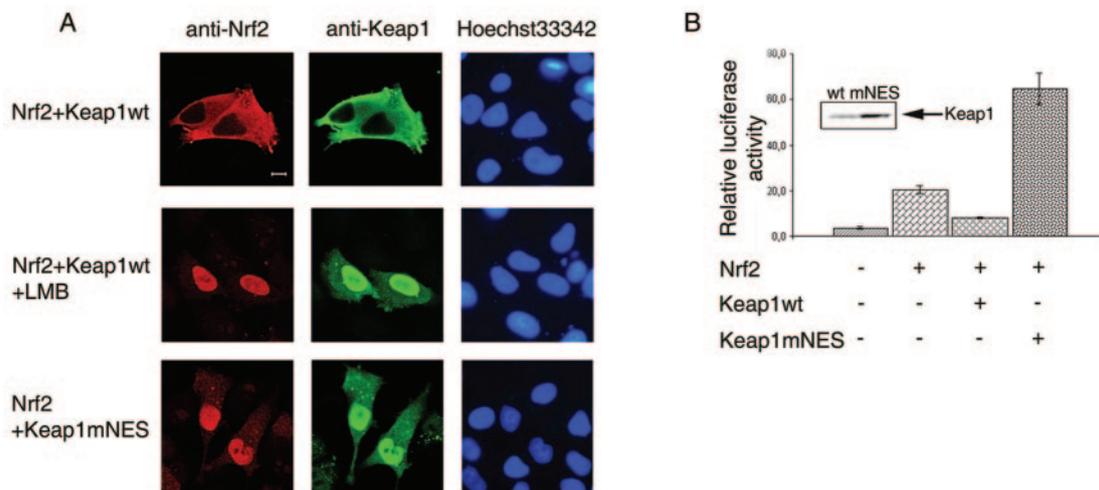


FIG. 4. Impairment of the Keap1 NES function results in coordinated nuclear translocation of Keap1 and Nrf2 (A) and in transcriptional activation (B). (A) HeLa cells were cotransfected with the Nrf2- and either with the wild-type (wt) Keap1- or Keap1mNES-encoding plasmids. In the former case, transfected cells were either treated with 2 nM LMB for 4 h or left untreated. For simultaneous detection of Keap1 and Nrf2, fixed cells were incubated with mouse anti-Keap1 and rabbit anti-Nrf2 antibodies, followed by treatment with fluorescein isothiocyanate-labeled anti-mouse and Texas Red-labeled anti-rabbit secondary antibodies. The microscope settings used excluded cross-fluorescence between the red and green channels. Right column, nuclei stained with Hoechst 33342. Bar, 10  $\mu$ m. (B) The pARE/luc reporter plasmid was transfected into HepG2 cells together with expression plasmids encoding Nrf2, wt Keap1, or Keap1mNES, as indicated. A  $\beta$ -Gal-expressing plasmid was used as a transfection control. The total amount of DNA used for transfection was normalized with an empty vector. Cells were harvested at 48 h after transfection, and the luciferase activity of each sample was measured and normalized for  $\beta$ -Gal activity. The intactness of wt Keap1 and Keap1mNES was verified by Western blotting analysis of the lysates with anti-Keap1 antibody and is shown in the inset. Error bars indicate standard deviations.

Keap1 (Fig. 3B), the mutant protein was no longer excluded from the nuclei of HeLa (Fig. 3G, mNES) and HepG2 (data not shown) cells in the absence of the LMB treatment. Since our results indicate that the identified sequence is both necessary and sufficient for the LMB-sensitive nuclear exclusion of Keap1, we conclude that it represents a genuine NES in Keap1 and that Keap1 is equipped with a single NES.

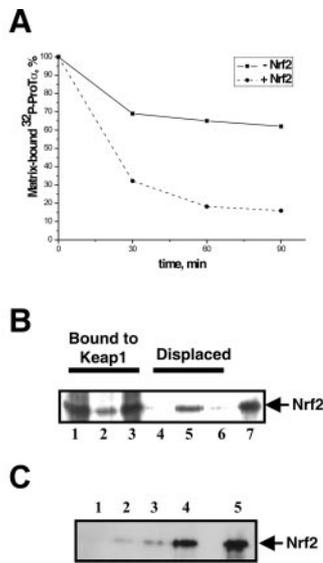
**Coordinated nuclear-cytoplasmic shuttling of Nrf2 with Keap1.** To learn whether shuttling of Keap1 between the nucleus and cytoplasm may be of functional significance, an effect of dysfunction of the NES of Keap1 on the subcellular localization of Nrf2 was studied by confocal laser scanning microscopy. In HeLa cells cotransfected with the Nrf2- and the wild-type Keap1-encoding plasmids, both proteins were essentially cytoplasmic (Fig. 4A, top row). LMB treatment of these cells resulted in the expected nuclear translocation of Keap1, similar to that observed without Nrf2 overproduction, which was mimicked by nuclear accumulation of Nrf2 (Fig. 4A, middle row). Alternatively, in cells cotransfected with the Nrf2- and Keap1mNES-encoding plasmids efficient nuclear accumulation of both proteins was observed even in the absence of LMB (Fig. 4A, bottom row). Thus, nuclear translocation of Nrf2 triggered by the dysfunction of the Keap1 NES is indicative of the nuclear-cytoplasmic shuttling of the Nrf2-Keap1 complex.

**NES-less Keap1 is an activator, rather than an inhibitor, of Nrf2-mediated transcription.** Alteration of the subcellular localization of Nrf2 and Keap1 due to destruction of Keap1's NES might be expected to influence Nrf2-mediated transcriptional activation. To monitor Nrf2-dependent transcription, the reporter plasmid pARE/luc was constructed by inserting a synthetic Nrf2-responsive ARE of the human NQO1 gene into

the luciferase-expressing pGL3-Promoter plasmid. Expression of this luciferase reporter construct, upon transfection into HepG2 cells, was confirmed to be up-regulated by ectopic production of Nrf2 and down-regulated by the coexpression of wild-type Keap1 together with Nrf2, as expected (Fig. 4B). However, coexpression of the Keap1mNES mutant together with Nrf2 not only failed to suppress luciferase gene expression but resulted in a very pronounced enhancement of Nrf2-driven transcription (Fig. 4B). This activating effect was reproduced in HeLa cells (not shown). Thus, destruction of the NES by point mutations converted Keap1 from an inhibitor to an activator of Nrf2-dependent gene expression.

**ProT $\alpha$  competes with Nrf2 for binding to Keap1.** As demonstrated above, ProT $\alpha$  binds to the C-terminal half of Keap1 comprising six Kelch (diglycine) repeats. Nrf2 was shown previously to interact with the C-terminal half of Keap1 as well (19). To test whether ProT $\alpha$  and Nrf2 could compete with each other for Keap1 binding, an *in vitro* system utilizing purified recombinant proteins was employed. A Keap1-ProT $\alpha$  complex was preformed by binding  $^{32}$ P-ProT $\alpha$  to zz-Keap1 immobilized on IgG-Sepharose. This complex was challenged with recombinant human Nrf2. A parallel sample was treated with buffer lacking Nrf2 to serve as a control. The amounts of  $^{32}$ P-ProT $\alpha$  shifted from the matrix-bound complex to the supernatant were determined at several time points. As shown in Fig. 5A, addition of Nrf2 resulted in the efficient dissociation of ProT $\alpha$  from Keap1. In the absence of Nrf2, dissociation of the Keap1-ProT $\alpha$  complex was much slower (Fig. 5A), indicating that it was Nrf2 that forced ProT $\alpha$  displacement from Keap1.

To verify the competition, a reciprocal experiment was performed. IgG-Sepharose-immobilized zz-Keap1 was charged



**FIG. 5.** ProT $\alpha$  competes with Nrf2 for binding to Keap1 in vitro. (A) Recombinant zz-Keap1 (50 ng) was immobilized on IgG-Sepharose and charged with  $^{32}$ P-ProT $\alpha$ . Unbound ProT $\alpha$  was removed, and the immobilized Keap1-ProT $\alpha$  complex was incubated with buffer alone (solid line) or with the same buffer containing recombinant Nrf2 (250 ng) (dotted line). At the indicated time intervals, the amount of  $^{32}$ P-ProT $\alpha$  in the pulled-down fractions was determined. The amount of matrix-bound ProT $\alpha$  at zero time was taken as 100%. (B) Recombinant zz-Keap1 (50 ng) immobilized on IgG-Sepharose was charged with recombinant Nrf2 (250 ng). Unbound Nrf2 was removed, and the immobilized Keap1-Nrf2 complex was incubated in parallel with recombinant wild-type ProT $\alpha$  (lanes 2 and 5), with the MProT $\alpha$ (44,50) mutant (lanes 3 and 6) (50  $\mu$ g each), or with buffer alone (lanes 1 and 4) for 1 h. The amount of Nrf2 in the pulled-down (Bound to Keap1, lanes 1 to 3) and supernatant (Displaced, lanes 4 to 6) fractions was determined by Western blotting analysis of each fraction with anti-Nrf2 antibody. Lane 7, Nrf2 used as a marker. (C) Displacement of Nrf2 from the Nrf2-Keap1 complex by ProT $\alpha$  is dose dependent. zz-Keap1-Nrf2 complex was formed as in panel B and challenged with increasing amounts of wild-type ProT $\alpha$  for 1 h. Amounts of displaced Nrf2 were determined by immunoblotting as in panel B. Lanes 1 to 4, 0.05, 0.5, 5.0, and 50  $\mu$ g of ProT $\alpha$ , respectively. Lane 5, Nrf2 used as a marker.

with Nrf2 and challenged, in three experiments performed in parallel, with either wild-type ProT $\alpha$ , the MProT $\alpha$ (44,50) mutant that fails to bind Keap1, or buffer alone. After 1 h of incubation, the amounts of Nrf2, both displaced from the complex with immobilized Keap1 and remaining bound after this treatment, were monitored by immunoblotting with anti-Nrf2 antibodies. Displacement of Nrf2 from its complex with Keap1 occurred upon challenge with wild-type ProT $\alpha$ , with a concomitant decrease in the amount of Nrf2 remaining bound to Keap1 (Fig. 5B, lanes 2 and 5). Only trace amounts of displaced Nrf2 were observed when the Keap1-Nrf2 complex was challenged with the MProT $\alpha$ (44,50) mutant or with buffer alone (Fig. 5B, lanes 4 and 6 versus lanes 1 and 3). Displacement of Nrf2 by wild-type ProT $\alpha$  was dose dependent (Fig. 5C). These findings indicate that ProT $\alpha$ , when competent for Keap1 binding, is able to specifically liberate Nrf2 from its complex with Keap1. It should be noted, however, that despite the use of a high molar excess of the competitor, ProT $\alpha$  was

able to displace Nrf2 only partially (Fig. 5B, lane 5 versus lane 2).

**ProT $\alpha$  contributes to Nrf2-dependent gene expression.** We then assessed the physiological relevance of the ProT $\alpha$ -Keap1 interaction. Binding of ProT $\alpha$  to Keap1 may indicate involvement of ProT $\alpha$  in regulation of the Nrf2-mediated transcription of the stress-protective genes. Because ProT $\alpha$  was able to liberate Nrf2 from its complex with Keap1, the latter being an inhibitor of the transcription factor, we proposed a role for ProT $\alpha$  that consists of dissociation of the Nrf2-Keap1 complex and thus of up-regulation of the expression of the Nrf2-dependent genes. If this was true, then alteration of the intracellular ProT $\alpha$  level should influence the Nrf2 transcriptional activity.

To test this idea, the effect of ProT $\alpha$  overproduction on Nrf2-driven transcription was assessed first. RNA samples from HeLa cells transfected with either the wild-type ProT $\alpha$  or the MProT $\alpha$ (44,50)-encoding plasmid or with an empty vector were analyzed by Northern blot hybridization with an HO-1 probe. The HO-1 gene contains an ARE, and its expression is known to be regulated in an Nrf2-dependent fashion (2, 17). A  $\beta$ -actin probe served as a control. Overproduction of the wild-type ProT $\alpha$  (Fig. 6A, top panel) resulted in a concomitant increase in the HO-1 mRNA level, relative to the empty vector control (Fig. 6A, middle panel), both in nonstressed cells (lanes 1 to 3) and in cells treated with DEM to induce oxidative stress (lanes 4 to 6). The  $\beta$ -actin mRNA level remained unaffected (Fig. 6A, bottom panel). Importantly, overproduction of the MProT $\alpha$ (44,50) mutant, which has an impaired ability to bind Keap1 in vivo and in vitro and to liberate Nrf2 from the Nrf2-Keap1 complex (Fig. 5B), failed to up-regulate HO-1 gene expression (Fig. 6A). Thus, in agreement with our model, an increase in the ProT $\alpha$  level could stimulate transcription of an Nrf2-dependent gene, and this effect appeared to be specifically mediated by the ProT $\alpha$  binding to Keap1 (see Fig. 6B for quantification).

To further verify our model, a reciprocal, ProT $\alpha$  mRNA interference approach was taken. Plasmid-driven production of a hairpin siRNA targeted to the ProT $\alpha$  mRNA region encoding amino acid residues 30 to 36 of the protein (ProT $\alpha$  siRNA) led to a significant reduction in the intracellular ProT $\alpha$  level relative to the vector-transfected sample, as revealed by PAGE upon partial purification of the protein (Fig. 6C, top panel) and confirmed by sandwich ELISA with crude cell lysates and a pair of anti-ProT $\alpha$  monoclonal antibodies (data not shown). No difference from the control sample was observed when the lysates were probed for actin, histone H1, I $\kappa$ B $\alpha$ , and procaspase-3 contents by Western blotting (Fig. 6C, bottom panels), indicating that the effect of RNA interference was ProT $\alpha$  specific.

At the RNA level, expression of the ProT $\alpha$  siRNA resulted in a marked decrease in the amount of ProT $\alpha$  mRNA detected, relative to the empty vector control (Fig. 6D, top panel), while it had no effect on the level of  $\beta$ -actin mRNA (Fig. 6D, middle panel). To determine whether the reduction in the ProT $\alpha$  level could influence Nrf2-dependent gene expression, the RNA blot depicted in Fig. 6D was rehybridized with the HO-1 probe. Down-regulation of the expression of HO-1 mRNA was observed in cells expressing ProT $\alpha$  siRNA (Fig. 6D, bottom panel; see also Fig. 6E for quantification). Thus, the level of Nrf2-dependent gene expression was corre-

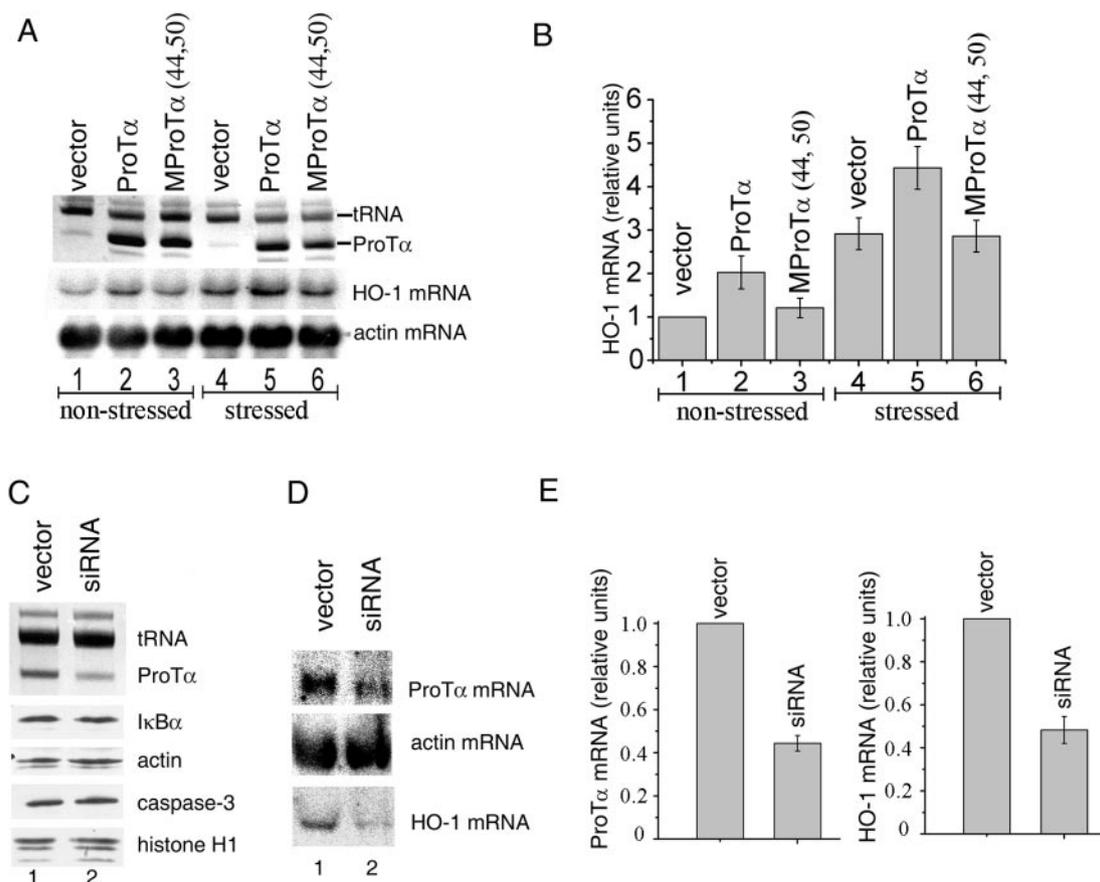


FIG. 6. HO-1 gene expression in HeLa cells is directly proportional to the ProT $\alpha$  level. (A) ProT $\alpha$  overproduction enhances HO-1 transcription. Lysates of HeLa cells transfected with the wild-type ProT $\alpha$ - or with the MProT $\alpha$ (44,50)-encoding plasmid or with vector alone were analyzed 48 h after transfection for the ProT $\alpha$  content (top panel) and for relative levels of expression of HO-1 mRNA (Nrf2 dependent, middle panel), and  $\beta$ -actin mRNA (Nrf2 independent, bottom panel). Lanes 1 to 3, nonstressed cells; lanes 4 to 6, cells treated with 100  $\mu$ M DEM for 22 h prior to harvesting. Top panel: Partially purified ProT $\alpha$  was fractionated in a 7 M urea-8% polyacrylamide gel and visualized by methylene blue staining. tRNA present in the partially purified ProT $\alpha$  samples verifies equal loading. Middle and bottom panels: Northern blot analysis of lysates with HO-1 and  $\beta$ -actin probes, respectively. The same membrane was used with both probes. (B) Quantitative analysis of the data presented in panel A. In panels B and E, all transfection experiments were performed a minimum of four times before calculating means and standard deviations. The amount of the corresponding mRNA from vector-transfected cells was taken as 1.0. panel C Down-regulation of ProT $\alpha$  level through ProT $\alpha$  mRNA interference. HeLa cells were transfected with either ProT $\alpha$  siRNA-expressing plasmids or with empty vector, as described in Materials and Methods. Two days after the last transfection step, cell lysates were analyzed for the ProT $\alpha$  content (top panel) as described for panel A. In parallel, the lysates were analyzed for the levels of several unrelated proteins by Western blotting with antibodies to actin, histone H1, I $\kappa$ B $\alpha$ , and procaspase-3 (bottom panels). (D) ProT $\alpha$  mRNA interference down-regulates HO-1 gene expression. Lysates of HeLa cells transfected as described for panel C were analyzed by Northern blotting with ProT $\alpha$ , HO-1, and  $\beta$ -actin probes. (E) Quantitative analysis of the data presented in panel D.

lated with the intracellular level of ProT $\alpha$  by using both up- and down-regulation of ProT $\alpha$  synthesis.

## DISCUSSION

Interaction of the Nrf2 transcription factor with its inhibitor Keap1 is central in regulation of expression of the genes defending cells against oxidative stress and electrophilic attack. According to current thinking, in the absence of stress, actin-bound Keap1 serves as a cytoplasmic anchor for Nrf2, preventing nuclear uptake of the transcription factor and activation of expression of Nrf2-dependent genes and targeting Nrf2 for proteasome-mediated degradation. Upon induction of stress, the Nrf2-Keap1 interaction is destabilized, most probably due

to posttranslational modification of Nrf2, Keap1, or perhaps both constituents, thus liberating Nrf2 from sequestration and permitting its nuclear translocation to induce expression of stress-preventing genes.

In this study, we searched for protein partners of the small nuclear protein ProT $\alpha$ . ProT $\alpha$  is an essential protein involved in the proliferation of mammalian cells and in their protection against apoptosis. We provided *in vivo* and *in vitro* evidence that Keap1 is a genuine partner of ProT $\alpha$  and that ProT $\alpha$  contributes to Nrf2-dependent gene expression. The notion that ProT $\alpha$  might be involved in up-regulation of expression of genes protecting cells from oxidative stress was not anticipated earlier and illuminates a novel function of this small but evidently multifunctional protein. However, our results appear to

provide clues to the functioning of the Nrf2-Keap1 system as well.

First, we demonstrated that Keap1 is a shuttling protein that migrates dynamically between the nucleus and the cytoplasm. Nuclear export of Keap1 is Crm1 (exportin-1) dependent and is mediated by a functional leucine-rich NES positioned in the central intervening region of Keap1 (residues 301 to 310). Besides, because Keap1, even when overproduced, is rapidly translocated to the nucleus upon LMB- and point mutation-mediated NES dysfunction, Keap1 is expected to possess a nuclear localization signal as well. Mutating the NES in Keap1 results in a profound nuclear accumulation of both Keap1 and Nrf2, implying that, besides shuttling by itself, Keap1 confers nuclear-cytoplasmic shuttling on the Nrf2-Keap1 complex. The shuttling model proposed here may have several advantages over the accepted cytoplasmic anchoring model, as follows. (i) The shuttling Nrf2-Keap1 complex could gain an ability to sense stress conditions in both compartments, nuclear and cytoplasmic. (ii) The appearance of Nrf2, albeit in complex with Keap1, in the nuclei of nonstressed cells is in line with the occurrence of the basal ARE-mediated transcription, which is not readily explicable in terms of the cytoplasmic anchoring model. (iii) Shuttling of Keap1 could provide a mechanism for termination of the induced expression of the stress-protective genes by withdrawing Nrf2 from the nucleus when the insult is surmounted.

Second, our *in vivo* and *in vitro* data indicate that interaction of ProT $\alpha$  with Keap1 is highly specific (e.g., suppressed by point mutations in ProT $\alpha$ ) and functionally relevant [Nrf2-dependent transcription was found to be directly proportional to the intracellular level of wild-type ProT $\alpha$  but not to that of MProT $\alpha$ (44,50)]. Clues to the mechanism of ProT $\alpha$  involvement in Nrf2-Keap1 functioning came from the identification of the ProT $\alpha$ -binding domain in Keap1. We showed that ProT $\alpha$  binds to the C-terminal half of Keap1 comprising six Kelch repeats, as Nrf2 does. Consistent with this finding, ProT $\alpha$  competed with Nrf2 for binding to Keap1. Furthermore, challenging the Nrf2-Keap1 complex with ProT $\alpha$  leads to displacement of Nrf2. Significantly, the ProT $\alpha$  mutant with an impaired ability to bind Keap1 failed to liberate Nrf2. A high molar excess of ProT $\alpha$  required for partial displacement of Nrf2 may, of course, be considered to represent a limitation of the *in vitro* assay. However, we think that this situation is close to the natural one, as ProT $\alpha$  is a very abundant nuclear protein. Limited release of Nrf2 triggered by ProT $\alpha$  is likely to provide basal ARE-mediated transcription. Upon stress induction, destabilization of the Nrf2-Keap1 complex should facilitate its dissociation by ProT $\alpha$  and result in a far larger amount of liberated Nrf2, leading to enhanced expression of the ARE-containing genes.

Our results attribute a role to ProT $\alpha$  as the intranuclear dissociator of the Nrf2-Keap1 complex. According to our model, the Nrf2-Keap1 complex shuttles constantly between the cytoplasm and the nucleus. Once in the nucleus, the complex is attacked by ProT $\alpha$  present in large excess, resulting in ProT $\alpha$  binding to Keap1 and concomitant displacement of Nrf2, with the latter being directed to the target genes to activate their expression. This liberation of Nrf2 is partial in nonstressed cells, providing a basal level of transcription, and is far more pronounced in stress-induced cells due to prede-

stabilization of the Nrf2-Keap1 complex. After completion of stress, an excess of free Nrf2 is withdrawn from the nucleus by binding to the shuttling Keap1 because dissociation of the stabilized (nonmodified) Nrf2-Keap1 complex by ProT $\alpha$  again becomes inefficient.

In conclusion, our data have revealed an unexpected function of ProT $\alpha$  and contribute to understanding of molecular mechanisms of expression of oxidative stress-protecting genes.

#### ACKNOWLEDGMENTS

We thank R. Agami for providing pSuper and Y. Lazebnik for providing anti-caspase-3 antibodies. We are grateful to Tatyana Pestova and Christopher Hellen for their constant support.

This work was supported in part by the Ludwig Institute for Cancer Research (to A.B.V.) and by grants from the Russian Foundation for Basic Research (to N.V.C., A.G.E., and A.B.V.), the U.S. Civilian Research and Development Foundation for the Independent States of the Former Soviet Union (CRDF) (to A.B.V.), and the Russian Frontiers in Genetics Program (to A.B.V.).

#### REFERENCES

- Adams, J., R. Kelso, and L. Cooley. 2000. The kelch repeat superfamily of proteins: spellers of cell function. *Trends Cell Biol.* **10**:17–24.
- Alam, J., D. Stewart, C. Touchard, S. Boinapally, A. M. Choi, and J. L. Cook. 1999. Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. *J. Biol. Chem.* **274**:26071–26078.
- Bloom, D. A., and A. K. Jaiswal. 2003. Phosphorylation of Nrf2 at Ser40 by protein kinase C in response to antioxidants leads to the release of Nrf2 from I $\kappa$ Nrf2, but is not required for Nrf2 stabilization/accumulation in the nucleus and transcriptional activation of antioxidant response element-mediated NAD(P)H:quinone oxidoreductase-1 gene expression. *J. Biol. Chem.* **278**:44675–44682.
- Brummelkamp, T. R., R. Bernards, and R. Agami. 2002. A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296**:550–553.
- Chichkova, N. V., A. G. Evstafieva, I. G. Lyakhov, A. S. Tsvetkov, T. A. Smirnova, R. N. Karapetian, E. M. Karger, and A. B. Vartapetian. 2000. Divalent metal cation binding properties of human prothymosin alpha. *Eur. J. Biochem.* **267**:4745–4752.
- Clinton, M., L. Graeve, H. el-Dorry, E. Rodriguez-Boulan, and B. L. Horecker. 1991. Evidence for nuclear targeting of prothymosin and parathyromin synthesized *in situ*. *Proc. Natl. Acad. Sci. USA* **88**:6608–6612.
- Cullinan, S. B., D. Zhang, M. Hannink, E. Arvisais, R. J. Kaufman, and J. A. Diehl. 2003. Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. *Mol. Cell Biol.* **23**:7198–7209.
- Dhakshinamoorthy, S., and A. K. Jaiswal. 2001. Functional characterization and role of I $\kappa$ Nrf2 in antioxidant response element-mediated expression and antioxidant induction of NAD(P)H:quinone oxidoreductase1 gene. *Oncogene* **20**:3906–3917.
- Dinkova-Kostova, A. T., W. D. Holtzclaw, R. N. Cole, K. Itoh, N. Wakabayashi, Y. Katoh, M. Yamamoto, and P. Talalay. 2002. Direct evidence that sulphydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proc. Natl. Acad. Sci. USA* **99**:11908–11913.
- Dominguez, F., C. Magdalena, E. Cancio, E. Roson, J. Paredes, L. Loidi, J. Zalvide, M. Fraga, J. Forteza, B. J. Regueiro, and J. L. Puente. 1993. Tissue concentrations of prothymosin alpha: a novel proliferation index of primary breast cancer. *Eur. J. Cancer* **29A**:893–897.
- Eschenfeldt, W. H., and S. L. Berger. 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.
- Evstafieva, A. G., G. A. Belov, M. Kalkum, N. V. Chichkova, A. A. Bogdanov, V. I. Agol, and A. B. Vartapetian. 2000. Prothymosin alpha fragmentation in apoptosis. *FEBS Lett.* **467**:150–154.
- Evstafieva, A. G., G. A. Belov, Y. P. Rubtsov, M. Kalkum, B. Joseph, N. V. Chichkova, E. A. Sukhacheva, A. A. Bogdanov, R. F. Petterson, V. I. Agol, and A. B. Vartapetian. 2003. Apoptosis-related fragmentation, translocation, and properties of human prothymosin alpha. *Exp. Cell Res.* **284**:211–223.
- Gomez-Marquez, J., F. Segade, M. Dosil, J. G. Pichel, X. R. Bustelo, and M. Freire. 1989. The expression of prothymosin alpha gene in T lymphocytes and leukemic lymphoid cells is tied to lymphocyte proliferation. *J. Biol. Chem.* **264**:8451–8454.
- Hayes, J. D., and M. McMahon. 2001. Molecular basis for the contribution of the antioxidant responsive element to cancer chemoprevention. *Cancer Lett.* **174**:103–113.
- Huang, H. C., T. Nguyen, and C. B. Pickett. 2002. Phosphorylation of Nrf2

- at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription. *J. Biol. Chem.* **277**:42769–42774.
17. **Ishii, T., K. Itoh, S. Takahashi, H. Sato, T. Yanagawa, Y. Katoh, S. Bannai, and M. Yamamoto.** 2000. Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. *J. Biol. Chem.* **275**:16023–16029.
  18. **Itoh, K., T. Chiba, S. Takahashi, T. Ishii, K. Igarashi, Y. Katoh, T. Oyake, N. Hayashi, K. Satoh, I. Hatayama, M. Yamamoto, and Y. Nabeshima.** 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.
  19. **Itoh, K., N. Wakabayashi, Y. Katoh, T. Ishii, K. Igarashi, J. D. Engel, and M. Yamamoto.** 1999. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev.* **13**:76–86.
  20. **Itoh, K., N. Wakabayashi, Y. Katoh, T. Ishii, T. O'Connor, and M. Yamamoto.** 2003. Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles. *Genes Cells* **8**:379–391.
  21. **Jiang, X., H. E. Kim, H. Shu, Y. Zhao, H. Zhang, J. Kofron, J. Donnelly, D. Burns, S. C. Ng, S. Rosenberg, and X. Wang.** 2003. Distinctive roles of PHAP proteins and prothymosin- $\alpha$  in a death regulatory pathway. *Science* **299**:223–226.
  22. **Kang, M. I., A. Kobayashi, N. Wakabayashi, S. G. Kim, and M. Yamamoto.** 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.
  23. **Karetsov, Z., A. Kretsovali, C. Murphy, O. Tsolas, and T. Papamarcaki.** 2002. Prothymosin  $\alpha$  interacts with the CREB-binding protein and potentiates transcription. *EMBO Rep.* **3**:361–366.
  24. **Levonen, A. L., A. Landar, A. Ramachandran, E. K. Ceaser, D. A. Dickinson, G. Zanon, J. D. Morrow, and V. M. Darley-Usmar.** 2004. Cellular mechanisms of redox cell signaling: the role of cysteine modification in controlling antioxidant defenses in response to electrophilic lipid oxidation products. *Biochem. J.* **378**:373–382.
  25. **Manrow, R. E., A. R. Sburlati, J. A. Hanover, and S. L. Berger.** 1991. Nuclear targeting of prothymosin  $\alpha$ . *J. Biol. Chem.* **266**:3916–3924.
  26. **Martini, P. G., R. Delage-Mourroux, D. M. Kraichely, and B. S. Katzenellenbogen.** 2000. Prothymosin  $\alpha$  selectively enhances estrogen receptor transcriptional activity by interacting with a repressor of estrogen receptor activity. *Mol. Cell. Biol.* **20**:6224–6232.
  27. **McMahon, M., K. Itoh, M. Yamamoto, S. A. Chanas, C. J. Henderson, L. I. McLellan, C. R. Wolf, C. Cavin, and J. D. Hayes.** 2001. The Cap'n'Collar basic leucine zipper transcription factor Nrf2 (NF-E2 p45-related factor 2) controls both constitutive and inducible expression of intestinal detoxification and glutathione biosynthetic enzymes. *Cancer Res.* **61**:3299–3307.
  28. **McMahon, M., K. Itoh, M. Yamamoto, and J. D. Hayes.** 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.
  29. **Motohashi, H., F. Katsuoka, J. D. Engel, and M. Yamamoto.** 2004. Small Maf proteins serve as transcriptional cofactors for keratinocyte differentiation in the Keap1-Nrf2 regulatory pathway. *Proc. Natl. Acad. Sci. USA* **101**:6379–6384.
  30. **Orre, R. S., M. A. Cotter II, C. Subramanian, and E. S. Robertson.** 2001. Prothymosin  $\alpha$  functions as a cellular oncoprotein by inducing transformation of rodent fibroblasts in vitro. *J. Biol. Chem.* **276**:1794–1799.
  31. **Ramos-Gomez, M., M. K. Kwak, P. M. Dolan, K. Itoh, M. Yamamoto, P. Talalay, and T. W. Kensler.** 2001. Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. *Proc. Natl. Acad. Sci. USA* **98**:3410–3415.
  32. **Rodriguez, P., J. E. Vinuela, L. Alvarez-Fernandez, M. Buceta, A. Vidal, F. Dominguez, and J. Gomez-Marquez.** 1998. Overexpression of prothymosin  $\alpha$  accelerates proliferation and retards differentiation in HL-60 cells. *Biochem. J.* **331**:753–761.
  33. **Rodriguez, P., J. E. Vinuela, L. Alvarez-Fernandez, and J. Gomez-Marquez.** 1999. Prothymosin  $\alpha$  antisense oligonucleotides induce apoptosis in HL-60 cells. *Cell Death Differ.* **6**:3–5.
  34. **Rubtsov, Y. P., A. S. Zolotukhin, I. A. Vorobjev, N. V. Chichkova, N. A. Pavlov, E. M. Karger, A. G. Evstafieva, B. K. Felber, and A. B. Vartapetian.** 1997. Mutational analysis of human prothymosin  $\alpha$  reveals a bipartite nuclear localization signal. *FEBS Lett.* **413**:135–141.
  35. **Rushmore, T. H., M. R. Morton, and C. B. Pickett.** 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.
  36. **Sburlati, A. R., R. E. Manrow, and S. L. Berger.** 1991. Prothymosin  $\alpha$  antisense oligomers inhibit myeloma cell division. *Proc. Natl. Acad. Sci. USA* **88**:253–257.
  37. **Sukhacheva, E. A., A. G. Evstafieva, T. V. Fateeva, V. R. Shakulov, N. A. Efimova, R. N. Karapetian, Y. P. Rubtsov, and A. B. Vartapetian.** 2002. Sensing prothymosin  $\alpha$  origin, mutations and conformation with monoclonal antibodies. *J. Immunol. Methods* **266**:185–196.
  38. **Tsitsiloni, O. E., J. Stiakakis, A. Koutselini, J. Gogas, C. Markopoulos, P. Yialouris, S. Bekris, D. Panoussopoulos, V. Kiortsis, W. Voelter, and A. A. Haritos.** 1993. Expression of  $\alpha$ -thymosin in human tissues in normal and abnormal growth. *Proc. Natl. Acad. Sci. USA* **90**:9504–9507.
  39. **Vareli, K., M. Frangou-Lazaridis, I. van der Kraan, O. Tsolas, and R. van Driel.** 2000. Nuclear distribution of prothymosin  $\alpha$  and parathymosin: evidence that prothymosin  $\alpha$  is associated with RNA synthesis processing and parathymosin with early DNA replication. *Exp. Cell Res.* **257**:152–161.
  40. **Wakabayashi, N., K. Itoh, J. Wakabayashi, H. Motohashi, S. Noda, S. Takahashi, S. Imakado, T. Kotsuji, F. Otsuka, D. R. Roop, T. Harada, J. D. Engel, and M. Yamamoto.** 2003. Keap1-null mutation leads to postnatal lethality due to constitutive Nrf2 activation. *Nat. Genet.* **35**:238–245.
  41. **Wakabayashi, N., A. T. Dinkova-Kostova, W. D. Holtzclaw, M. I. Kang, A. Kobayashi, M. Yamamoto, T. W. Kensler, and P. Talalay.** 2004. Protection against electrophile and oxidant stress by induction of the phase 2 response: fate of cysteines of the Keap1 sensor modified by inducers. *Proc. Natl. Acad. Sci. USA* **101**:2040–2045.
  42. **Wanker, E. E., C. Rovira, E. Scherzinger, R. Hasenbank, S. Walter, D. Tait, J. Colicelli, and H. Lehrach.** 1997. HIP-1: a huntingtin interacting protein isolated by the yeast two-hybrid system. *Hum. Mol. Genet.* **6**:487–495.
  43. **Wu, C. L., A. L. Shiau, and C. S. Lin.** 1997. Prothymosin  $\alpha$  promotes cell proliferation in NIH3T3 cells. *Life Sci.* **61**:2091–2101.
  44. **Xue, F., and L. Cooley.** 1993. kelch encodes a component of intercellular bridges in *Drosophila* egg chambers. *Cell* **72**:681–693.
  45. **Zhang, D. D., and M. Hannink.** 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.