Nuclear Oncoprotein Prothymosin α Is a Partner of Keap1: Implications for Expression of Oxidative Stress-Protecting Genes

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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 α. The in vivo and in vitro data indicated that the prothymosin α-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 α 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 α by using prothymosin α overproduction and mRNA interference approaches. Our data attribute to prothymosin α the role of intranuclear dissociator of the Nrf2-Keap1 complex, thus revealing a novel function for prothymosin α 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-quione oxidoreductase-1 (NQO1)] and antioxidant proteins [such as γ-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
report the identification of a novel Keap1 partner which, quite unexpectedly, turned out to be the nuclear protein prothymosin α (ProTα). We demonstrate that ProTα releases Nfr2 from the Nfr2-Keap1 complex in vitro and contributes to Nfr2-dependent gene expression in vivo. To lend mechanistic support for the involvement of ProTα 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 Nfr2-Keap1 complex.

ProTα 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 (15, 21). Overexpression of ProTα in NIH 3T3 and HL-60 cells was shown to accelerate proliferation (32, 43), whereas inhibition of ProTα synthesis prevented cell division (36) and induced apoptosis in HL-60 cells (33). Overexpression of ProTα in a rat fibroblast cell line resulted in loss of contact inhibition, anchorage-independent growth, and decreased serum dependence (30). Consistent with its properties, ProTα is particularly abundant in tumor cells (10, 38). The antiapoptotic activity of ProTα likely arises from its ability to inhibit apoptosome formation (21), whereas the mechanism of ProTα action in stimulating proliferation is not clearly established. Mounting evidence suggests ProTα involvement in transcription regulation (26, 29, 36). The newly identified ProTα-Keap1 interaction may have important implications for the mechanism(s) of Nfr2-dependent gene expression.

**MATERIALS AND METHODS**

**Yeast two-hybrid screen.** Mannipulations with yeast cells were essentially as described previously (Yeast protocols handbook, Clontech, Palo Alto, Calif., 2000). Saccharomyces cerevisiae strain L40ccU containing pBTM117c-ProTα was transformed with GAL4 cDNA human brain (HB) and human bone marrow (HBM) libraries (Matchmaker; Clontech). Totals of 6 × 10⁴ and 4 × 10⁴ 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, β-galactosidase (β-Gal) activity was tested, β-Gal-positive clones were picked to SD plates lacking Leu and containing canavanine to eliminate the bait, and β-Gal activity was restated after incubation at 30°C for 2 days. Total DNA was prepared from β-Gal-negative clones and transformed into Escherichia coli JM109. To check for true positives, isolated plasmids were transformed into L40ccU harboring pBTM117c, pBTM117c-ProTα, pBTM117c-Sim1, or pBTM117c-Rev and tested for selective growth on SD plates lacking Trp, Leu, His, and Ura and for β-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 µg 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 GFP were intracellularly localized as described previously (12). For immunolocalization, anti-Keap1 2H5 and 2E4 monoclonal antibodies and rabbit anti-Nfr2 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 µg) was preincubated with 12.8 µg of recombinant GST-Keap1(308-624) expressed in and purified from E. coli containing pGEX-4T-2 (Amersham Pharmacia Biotech). Plasmids, pBTM117c-ProTα and its derivatives encoding wild-type human ProTα and various deletion mutants thereof fused to LexA were constructed by in-frame ligation of the ProTα-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 Styl-AccI DNA fragment of pHT15A with that containing the E4G4 E9O0 double mutation (34). The MProTα(44,50)-encoding sequence was then used to replace the wild-type ProTα-encoding sequence in pPH12A (37), pKT15 (3), pcdNA4-hnt-wt ProTα (13), and pBTM117c-ProTα.

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′-GGACCCCGCATGCAAGCAATG-3′ and 5′-GGTTAGGCGG ATTCAATTAG-3′. The Spl-ECori-digested PCR product and the EcoRl-HpaI DNA fragment from pACT2-HBM were then ligated in frame into the Spl-HpaI-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 pJ32-Keap1. pGEX-Keap1(308-624) was obtained by inserting the EcoRI-XhoI DNA fragment from pACT2-HB into pGEX-K-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 L3(308,310)A double mutation was introduced in Keap1 by PCR with a 5′-TCGAGGAGGACGACgCCgCgAGAgCCG-3′ mutagenic primer (lowercase letters indicate the nucleotide substitutions) and the pUC19 direct and reverse DNA sequencing primers. The structure of the cloned PCR product was verified by sequencing. The Keap1MNES-encoding sequence was then cloned 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/HisMaxC (Invitrogen). For overproduction of zz-Keap1 in yeast, the zz-encoding DNA fragment from pQ2E2S5 (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 Nfr2 was amplified by PCR from a HeLa cDNA library (Clontech) with primers 5′-CCGGATCCCAATGGATTTGATTCAATGAG-3′ and 5′-CCGCTCGAGGACTGCTAGTATTTCATCAACATC-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/lec was constructed by inserting a synthetic ARE of the human NQO1 gene (5′-GTACCGAGTCATACCTAGTCGAGAATTCAATGAG-3′) downstream from the LexA-encoding sequence in pBTM117c (42). The PCR product was digested with BamHI and XhoI, inserted into pcDNA4/HisMaxB (Invitrogen), and then transferred into the BamHI-SalI-digested pQE32 (Qiagen). pARE/lec was constructed by inserting a synthetic ARE of the human NQO1 gene (5′-GTACCGAGTCATACCTAGTCGAGAATTCAATGAG-3′) downstream from the LexA-encoding sequence in pBTM117c (42). 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/lec was constructed by inserting a synthetic ARE of the human NQO1 gene (5′-GTACCGAGTCATACCTAGTCGAGAATTCAATGAG-3′) downstream from the LexA-encoding sequence in pBTM117c (42). 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/lec was constructed by inserting a synthetic ARE of the human NQO1 gene (5′-GTACCGAGTCATACCTAGTCGAGAATTCAATGAG-3′) downstream from the LexA-encoding sequence in pBTM117c (42). 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/lec was constructed by inserting a synthetic ARE of the human NQO1 gene (5′-GTACCGAGTCATACCTAGTCGAGAATTCAATGAG-3′) downstream from the LexA-encoding sequence in pBTM117c (42). 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/lec was constructed by inserting a synthetic ARE of the human NQO1 gene (5′-GTACCGAGTCATACCTAGTCGAGAATTCAATGAG-3′) downstream from the LexA-encoding sequence in pBTM117c (42). The PCR product was digested with BamHI and XhoI, inserted into pcDNA4/HisMaxB (Invitrogen), and then transferred into the BamHI-SalI-digested pQE31 (Qiagen).
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 communoprecipitation of endogenous Keap1, 3 × 10^6 HeLa cells, either nonstressed or treated with 100 μM dithyl maleate (DEM) (Sigma) for 4 h, or HeP2 cells, were lysed for 40 min on ice in 200 μl 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 precipitated with 20 μg 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 μg of 2F11 anti-ProT 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 twice 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 5 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 ProTα 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). 32P labeling of the ProTα derivatives containing a protein kinase recognition site was described by Chichikova et al. (5). The specific radioactivity of 32P-ProTα thus obtained was 10^7 cpm/μg. Recombinant His6-Nrf2, His6-Keap1(1–139), His6-Keap1(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 protein G-Sepharose. Protein A-Sepharose 4B (Amersham Pharmacia Biotech) chromatography, respectively. zz-Keap1 was isolated from galactose-induced S. cerevisiae 2805 cells transformed with pYDPI/8/22/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 32P-ProTα (30,000 cpm per sample) with IgG-Sepharose-immobilized Keap1 (150 μg 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 μM ZnSO4, CaCl2, or MgCl2. After 1 h of incubation at 0°C with shaking, the resin was washed with the same buffer, and the amounts of 32P-ProTα in pull-down fractions and in supernatants were determined.

For competition assays, aliquots of IgG-Sepharose with immobilized zz-Keap1 (150 μg of fusion protein per sample) or zz alone (30 ng) was assessed in binding buffer containing 100 μM ZnSO4 with either 32P-ProTα (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-ProTα complex) and with the indicated amounts of M ProTα or M ProTα(44,50) mutant (for the Keap1-Nrf2 complex) at 0°C. Incubations with buffer alone served as controls. As described in the indicated time intervals, the amounts of 32P-ProTα and Nrf2 in pull-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 HeG2 cells to matrix-immobilized ProTα, wild-type ProTα and its M ProTα(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 precipitated 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 × 10^6 cells were incubated with 10-μl aliquots of ProTα-Sepharose beads in 200 μl 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 B at 57°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 β-Gal were used as reporters. Cotransfection with β-Gal-expressing plasmid pCNA/HisMax/IIa2 (Invitrogen) was employed to normalize for transfection efficiencies. Cells were lysed, 36 h after transfection, in reporter lysis buffer (Promega), and luciferase and β-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 × 10^6 transfected HeLa cells with the RNAqueous total RNA isolation kit (Ambion) according to the protocol supplied by the manufacturer. RNA samples, in 20-μg aliquots, were electrophoresed on 1.5% agarose–2.2% formaldehyde–polyacrylamide gels, transferred onto Hybond N+ membranes (Amersham Pharmacia Biotech), and hybridized sequentially with [32P]DNA probes obtained by random-primer labeling of the indicated cDNAs. Quantification was performed with ImageQuant software after exposure of the membranes to a PhosphorImager (Molecular Dynamics).

**ProTα overproduction and small interfering RNA (siRNA) knockdown in human cells.** ProTα lacking artificially added sequences was overproduced in HeLa cells transfected with pCDNA4-ehn-wt ProTα and pCDNA4-ehn-MProTα(44,50) as described previously (13). Cells were treated with 100 μM DEM or with diluent (dimethyl sulfoxide) only for 22 h prior to harvesting. For ProTα 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 μg 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 ProTα followed by 7 M urea–8% PAGE (13).

**RESULTS**

**Yeast two-hybrid screen for ProTα-interacting proteins.** A search for human proteins interacting with ProTα 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 ProTα 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 L40cEU cells despite the synthesis of easily detectable amounts of the LexA-ProTα protein (data not shown). Yeast L40cEU 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 β-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 ProTα 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-ProTα 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 ProTα efficiently (data not shown).

**Delineating a Keap1-binding region in ProTα.** To learn which region in ProTα is responsible for binding to Keap1, a set of ProTα 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 ProTα (residues 32 to 52) turned out to be both necessary and sufficient to provide the interaction (Fig. 1A). Furthermore, testing the ProTα E(44)G,E(50)G double mutant [M-ProTα(44,50)] from our collection (34) in the yeast two-hybrid assay demonstrated the loss of interaction of this ProTα mutant with Keap1 (Fig. 1A). The effect was not due to instability of either the ProTα or Keap1 derivatives in yeast cells (Fig. 1B and C).
and provides a further argument for high specificity of the ProTα-Keap1 binding.

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

![Diagram](http://mcb.asm.org/)

**FIG. 1.** Keap1 is a ProTα interactor: evidence from yeast two-hybrid analysis. (A) Identification of a Keap1-binding region (black) in ProTα. + and −, relative β-Gal levels above 300 and below 10 U, respectively. wt, wild type. (B and C) Lack of detectable interaction between the MProTα(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-ProTα plus AD-Keap1; lanes 2, LexA-MProTα(44,50) plus AD-Keap1; lanes 3, LexA plus AD-Keap1; lane 4, LexA-MProTα(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 ProTα binding. Individual repeats are numbered from 1 to 6. BTB/POZ, BTB/POZ domain of Keap1.

Evidence for the ProTα-Keap1 interaction in human cell lysates. To learn whether Keap1 could bind to ProTα in human cells, a whole-cell lysate of HeLa cells ectopically expressing full-length Keap1 was treated with anti-ProTα 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 ProTα when anti-ProTα antibody was used (lane 1), whereas control mouse IgGs were inactive (lane 2). The possibility that anti-ProTα antibody could recognize Keap1 directly was excluded by Western blot analysis of Keap1 with anti-ProTα 2F11 monoclonal antibody (data not shown). Coimmunoprecipitation of the ectopically produced Keap1 with ProTα was then confirmed with lysates of HepG2 cells (data not shown).

Likewise, endogenous Keap1 could be communoprecipitated with endogenous ProTα 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 ProTα-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 ProTα-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 ProTα and its MProTα(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 ProTα was observed (Fig. 2C), whereas only trace amounts of Keap1 could bind to the MProTα(44,50) mutant, thus confirming our data on the specificity of the ProTα-Keap1 interaction obtained with the use of the yeast two-hybrid system.

**Binding of Keap1 to ProTα is direct.** To test whether the interaction between ProTα and Keap1 is direct or is mediated by some “bridging” component of cell lysates, a pull-down assay with purified recombinant human ProTα 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 ProTα binding to this affinity matrix, human wild-type ProTα and MProTα (44,50) were fused at their N termini to a short peptide comprising the protein kinase A recognition sequence and 32P radiolabeled. Binding of the labeled wild-type ProTα to the immobilized zz-Keap1 was observed in this in vitro system (Fig. 2D), whereas binding of ProTα to the zz domain alone was negligible. Recombinant unlabeled ProTα lacking artificially added sequences could compete with the binding of the radiolabeled probe (data not shown), indicating that binding to
Keap1 is specific for the ProTα sequence. Furthermore, the MProTα(44,50) mutant failed to bind Keap1 (Fig. 2D). Previously, we have demonstrated that ProTα binds Zn\(^{2+}\) and Ca\(^{2+}\) (but not Mg\(^{2+}\)) (5). Therefore, we tested whether these divalent cations could affect ProTα binding to Keap1. In the presence of 100 μM Zn\(^{2+}\) or Ca\(^{2+}\), this binding was markedly enhanced (Fig. 2D). Substitution of Mg\(^{2+}\) for these cations did not result in ProTα binding over the level seen in the absence of divalent cations (Fig. 2D).

Thus, ProTα interacts with Keap1 directly and specifically, and the divalent cations that bind to ProTα significantly enhance the ProTα-Keap1 interaction.

How can ProTα 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 ProTα. One major objection to this idea is that ProTα and Keap1 have been thought to be localized to different subcellular compartments. While ProTα 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,
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 (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 μm.

**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 μm.
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.

**ProTx competes with Nrf2 for binding to Keap1.** As demonstrated above, ProTx 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 ProTx and Nrf2 could compete with each other for Keap1 binding, an in vitro system utilizing purified recombinant proteins was employed. A Keap1-ProTx complex was preformed by binding 32P-ProTx 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 32P-ProTx 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 ProTx from Keap1. In the absence of Nrf2, dissociation of the Keap1-ProTx complex was much slower (Fig. 5A), indicating that it was Nrf2 that forced ProTx displacement from Keap1.

To verify the competition, a reciprocal experiment was performed. IgG-Sepharose-immobilized zz-Keap1 was charged...
with Nrf2 and challenged, in three experiments performed in parallel, with either wild-type ProTα, the MProTα(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 the complex with Keap1-2-Keap1 complex by ProTα is dose dependent. zz-Keap1–Nrf2 complex was formed as in panel B and challenged with increasing amounts of wild-type ProTα 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 μg of ProTα, respectively. Lane 5, Nrf2 used as a marker.

FIG. 5. ProTα competes with Nrf2 for binding to Keap1 in vitro. (A) Recombinant zz-Keap1 (50 ng) was immobilized on IgG-Sepharose and charged with [γ-32P]-ProTα. Unbound ProTα was removed, and the immobilized Keap1-ProTα 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 [γ-32P]-ProTα in the pulled-down fractions was determined. The amount of matrix-bound ProTα 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 ProTα (lanes 2 and 5), with the MProTα(44,50) mutant (lanes 3 and 6) (50 μg 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 ProTα is dose dependent. zz-Keap1–Nrf2 complex was formed as in panel B and challenged with increasing amounts of wild-type ProTα 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 μg of ProTα, respectively. Lane 5, Nrf2 used as a marker.

ProTα contributes to Nrf2-dependent gene expression. We then assessed the physiological relevance of the ProTα–Keap1 interaction. Binding of ProTα to Keap1 may indicate involvement of ProTα in regulation of the Nrf2-mediated transcription of the stress-protective genes. Because ProTα was able to liberate Nrf2 from its complex with Keap1, the latter being an inhibitor of the transcription factor, we proposed a role for ProTα 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 ProTα level should influence the Nrf2 transcriptional activity.

To test this idea, the effect of ProTα overproduction on Nrf2-driven transcription was assessed first. RNA samples from HeLa cells transfected with either the wild-type ProTα or the MProTα(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 β-actin probe served as a control. Overproduction of the wild-type ProTα (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 DEX to induce oxidative stress (lanes 4 to 6). The β-actin mRNA level remained unaffected (Fig. 6A, bottom panel). Importantly, overproduction of the MProTα(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 ProTα level could stimulate transcription of an Nrf2-dependent gene, and this effect appeared to be specifically mediated by the ProTα binding to Keap1 (see Fig. 6B for quantification).

To further verify our model, a reciprocal, ProTα mRNA interference approach was taken. Plasmid-driven production of a hairpin siRNA targeted to the ProTα mRNA region encoding amino acid residues 30 to 36 of the protein (ProTα siRNA) led to a significant reduction in the intracellular ProTα 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-ProTα monoclonal antibodies (data not shown). No difference from the control sample was observed when the lysates were probed for actin, histone H1, IκBα, and procaspase-3 contents by Western blotting (Fig. 6C, bottom panels), indicating that the effect of RNA interference was ProTα specific.

At the RNA level, expression of the ProTα siRNA resulted in a marked decrease in the amount of ProTα mRNA detected, relative to the empty vector control (Fig. 6D, top panel), while it had no effect on the level of β-actin mRNA (Fig. 6D, middle panel). To determine whether the reduction in the ProTα 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 ProTα siRNA (Fig. 6D, bottom panel; see also Fig. 6E for quantification). Thus, the level of Nrf2-dependent gene expression was corre-
lated with the intracellular level of ProT/H9251 by using both up- and down-regulation of ProTα 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 proteosome-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 ProTα. ProTα 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 ProTα and that ProTα contributes to Nrf2-dependent gene expression. The notion that ProTα 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

FIG. 6. HO-1 gene expression in HeLa cells is directly proportional to the ProTα level. (A) ProTα overproduction enhances HO-1 transcription. Lysates of HeLa cells transfected with the wild-type ProTα- or with the MProTα(44,50)-encoding plasmid or with vector alone were analyzed 48 h after transfection for the ProTα content (top panel) and for relative levels of expression of HO-1 mRNA (Nrf2 dependent, middle panel), and β-actin mRNA (Nrf2 independent, bottom panel). Lanes 1 to 3, nonstressed cells; lanes 4 to 6, cells treated with 100 μM DEM for 22 h prior to harvesting. Top panel: Partially purified ProTα was fractionated in a 7 M urea-8% polyacrylamide gel and visualized by methylene blue staining. tRNA present in the partially purified ProTα samples verifies equal loading. Middle and bottom panels: Northern blot analysis of lysates with HO-1 and β-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 ProTα level through ProTα mRNA interference. HeLa cells were transfected with either ProTα 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 ProTα 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, IκBα, and procaspase-3 (bottom panels). (D) ProTα mRNA interference down-regulates HO-1 gene expression. Lysates of HeLa cells transfected as described for panel C were analyzed by Northern blotting with ProTα, HO-1, and β-actin probes. (E) Quantitative analysis of the data presented in panel D.
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 resulted 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 ProTα with Keap1 is highly specific (e.g., suppressed by point mutations in ProTα) and functionally relevant [Nrf2-dependent transcription was found to be directly proportional to the intracellular level of wild-type ProTα but not to that of MProTα(44,50)]. Clues to the mechanism of ProTα involvement in Nrf2-Keap1 functioning came from the identification of the ProTα-binding domain in Keap1. We showed that ProTα binds to the C-terminal half of Keap1 comprising six Kelch repeats, as Nrf2 does. Consistent with this finding, ProTα competed with Nrf2 for binding to Keap1. Furthermore, challenging the Nrf2-Keap1 complex with ProTα leads to displacement of Nrf2. Significantly, the ProTα mutant with an impaired ability to bind Keap1 failed to liberate Nrf2. A high molar excess of ProTα 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 ProTα is a very abundant nuclear protein. Limited release of Nrf2 triggered by ProTα is likely to provide basal ARE-mediated transcription. Upon stress induction, destabilization of the Nrf2-Keap1 complex should facilitate its dissociation by ProTα 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 ProTα 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 ProTα present in large excess, resulting in ProTα 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 ProTα again becomes inefficient.

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

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