Keap1-dependent Proteasomal Degradation of Transcription Factor Nrf2 Contributes to the Negative Regulation of Antioxidant Response Element-driven Gene Expression

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Keap1 is a negative regulator of Nrf2, a bZIP transcription factor that mediates adaptation to oxidative stress. Previous studies suggested this negative regulation is a consequence of Keap1 controlling the subcellular distribution of Nrf2. We now report that Keap1 also controls the total cellular level of Nrf2 protein. In the RL34 non-transformed rat liver cell line, Nrf2 was found to accumulate rapidly in response to oxidative stress caused by treatment with sulforaphane, and the accumulation resulted from inhibition of proteasomal-mediated degradation of the bZIP protein. By heterologously expressing in COS1 cells epitope-tagged Nrf2 and an Nrf2ARTECH mutant lacking the Keap1-binding site, in both the presence and absence of Keap1 we demonstrate that Nrf2 is subject to ubiquitination and proteasomal degradation independently of both Keap1 and the redox environment of the cell. In oxidatively stressed cells, this is the sole mechanism responsible for Nrf2 degradation. However, under homeostatic conditions Nrf2 is subject to a substantially more rapid mode of proteasomal degradation than it is in oxidatively stressed cells, and this rapid turnover of Nrf2 requires it to interact with Keap1. Within Nrf2, the N-terminal Neh2 domain is identified as the redox-sensitive degron. These data suggest that Keap1 negatively regulates Nrf2 by both enhancing its rate of proteasomal degradation and altering its subcellular distribution.

Under homeostatic conditions, cells maintain their redox environment at a higher reducing potential than their surroundings (1). This is essential for correct biological function, and changes in the redox environment of the cell toward a more oxidized potential is referred to as oxidative stress. This can arise from exposure to pro-oxidant psychochemicals such as isothiocyanates, which react with GSH, and quinones, which can decouple mitochondrial electron transfer reactions, generating the superoxide anion (2, 3). Regardless of the source, oxidative stress, if prolonged, results in perturbation of cellular biochemistry and aberrant covalent modification of macromolecules, leading to tissue damage and DNA mutations.

Being disteleological, cells sense oxidative stress and respond by transcriptionally up-regulating numerous cytoprotective genes. These encode proteins with a variety of functions. For example, glutamate cysteine ligase catalytic and modifier subunits, glutathione reductase, malic enzyme 1, glucose-6-phosphate dehydrogenase, thioredoxin reductase, thioredoxin, peroxiredoxin MSP23, heme oxygenase 1, and the cysteine/glutamyl exchange transport system (4–9) act directly to replenish the cells major reductants. In addition, detoxication enzymes such as glutathione S-transferases, UDP-glucuronosyltransferases, aldo-keto reductases, and NADP(H:quinone oxidoreductase 1 (NQO1) act indirectly by metabolizing and excreting the causative agents and byproducts of oxidative stress (10–12). Ferritin L chain (12) is induced and inhibits the ability of iron to catalyze the formation of reactive oxygen species. Genes encoding DNA damage repair enzymes might also be induced (8). Thus, in response to oxidants, the cell alters its biochemistry in many ways to reduce the potential for further oxidative stress, restore its redox balance, and repair damage accumulated during the stress. The antioxidant response element (ARE), a cis-acting element present in the promoters of many of the genes encoding the proteins listed above, provides a mechanism to explain their coordinated transcriptional regulation (for review, see Ref. 13).

Nrf2, a member of the “nc” subfamily of bZIP transcription factors (for review, see Ref. 14), is an essential transactivator of genes containing an ARE, as evidenced by the marked impairment in the expression of genes encoding a number of detoxication enzymes and GSH biosynthetic enzymes in the liver and gastrointestinal tract of Nrf2−/− mice (11, 15, 16). Nrf2 is highly conserved from mammalian species to chicken and Zebra fish, particularly within six regions designated the Neh1–6 (Nrf2-ECH homology) domains (17). The Neh1 domain contains the cnc-bZIP region, which dictates dimerization partners and confers DNA binding specificity. The Neh4 and Neh5 domains act cooperatively to bind the coactivator CREB-binding protein, thereby activating transcription (18). Of particular interest is the N-terminal region of −100 amino acids, called the Neh2 domain, which negatively regulates Nrf2 function under homeostatic conditions (19) by mechanisms that are not fully understood.

1 The abbreviations used are: NQO1, NAD(P)H:quinone oxidoreductase 1; ARE, antioxidant response element; Neh, Nrf2-ECH homology; Keap1, Kelch-like ECH-associated protein 1; HA, hemagglutinin; Sul, sulforaphane; CAT, chloramphenicol acetyltransferase; CBX, cycloheximide; 2-ME, 2-mercaptoethanol; CREB, cAMP-response element-binding protein; CMV, cytomegalovirus.
The negative regulation of Nrf2 requires the interaction of the Neh2 domain with an actin-bound cytosolic protein, Keap1 (Kelch-like ECH-associated protein 1) (19). This appears to be a direct interaction as bacterially expressed and purified mKeap1 interacts in vitro with the Neh2 domain of mNrf2 (20). A tetrapeptide motif, ETGE, that resides close to the C-terminal boundary of Neh2 is critical for this phylogenetically conserved interaction (17). Itoh et al. (19) proposed that Keap1 negatively regulates Nrf2 function by controlling its subcellular localization. According to this model, Keap1 sequesters the βZIP protein in the cytoplasm under homeostatic conditions, leading to low expression of ARE-driven genes. During oxidative stress, a signal that involves phosphorylation and/or redox modification is transduced to the Keap1-Nrf2 complex (19–21), leading to its disruption and nuclear translocation of Nrf2. The increased level of nuclear Nrf2 results in enhanced occupancy of ARE enhancers by Nrf2/small Maf heterodimers, recruitment of CREB-binding protein, and transactivation of cytoprotective genes (18).

A drawback of this model is that it does not account for the apparent accumulation of total cellular Nrf2 in response to oxidative stress (22–25). Two research groups demonstrated that the accumulation of Nrf2 results from inhibition of its degradation by the 26 S proteasome (24, 25), a feature common to many transcription factors including p53, c-myc, c-Jun, and β-catenin (26). In this study, we confirm that endogenous Nrf2 is subject to 26 S proteasome-dependent degradation and that its rate of degradation depends upon the redox environment of the cell. Importantly, we demonstrate that although Nrf2 is constitutively ubiquitinated and degraded by the proteasome, a direct interaction between the transcription factor and Keap1 confers redox sensitivity upon its half-life. Thus, under homeostatic conditions, Keap1 interacts with Nrf2 and increases its rate of proteasome-mediated degradation, leading to a reduction in the cellular levels of the βZIP protein. Oxidative stress, by antagonizing this interaction, stabilizes Nrf2, leading to its rapid accumulation within the cell. We identify the Neh2 domain of Nrf2 as the redox-sensitive degron.

EXPERIMENTAL PROCEDURES

Plasmids

Mouse Nrf2-expressing Plasmids—pcDNA3.1/V5HisBmNrf2 was generated by PCR amplification of the murine Nrf2 coding sequence and 50 nucleotides of 5′-untranslated region (27) using the primer pair 5′-CACAGGTCGCCCCATCACGTG-3′ and 5′-GTTTTTCTCTTGTACGTGGCCTCTTGCC-3′. The product was ligated into EcoRI-digested pcDNA3.1/V5HisB (Invitrogen), allowing expression of mNrf2-V5-his fusion protein from a CMV promoter. The pcDNA3.1/V5mNrf2, encoding mNrf2-V5, was generated from pcDNA3.1/V5HisBmNrf2 by site-directed mutagenesis using the primer 5′-TCTGGCTTCTTGC-3′ and 50 nucleotides of 5′-untranslated region (27) using the primer pair 5′-CACAGGTCGCCCCATCACGTG-3′ and 5′-GTTTTTCTCTTGTACGTGGCCTCTTGCC-3′. The product was ligated into EcoRI-digested pcDNA3.1/V5HisB (Invitrogen), allowing expression of mNrf2-V5-his fusion protein from a CMV promoter. The pcDNA3.1/V5mNrf2, encoding mNrf2-V5, was generated from pcDNA3.1/V5HisBmNrf2 by site-directed mutagenesis using the primer 5′-TCTGGCTTCTTGC-3′ and 50 nucleotides of 5′-untranslated region (27) using the primer pair 5′-CACAGGTCGCCCCATCACGTG-3′ and 5′-GTTTTTCTCTTGTACGTGGCCTCTTGCC-3′. The product was ligated into EcoRI-digested pcDNA3.1/V5HisB (Invitrogen), allowing expression of mNrf2-V5-his fusion protein from a CMV promoter. The pcDNA3.1/V5mNrf2, encoding mNrf2-V5, was generated from pcDNA3.1/V5HisBmNrf2 by site-directed mutagenesis using the primer 5′-TCTGGCTTCTTGC-3′ and 50 nucleotides of 5′-untranslated region (27) using the primer pair 5′-CACAGGTCGCCCCATCACGTG-3′ and 5′-GTTTTTCTCTTGTACGTGGCCTCTTGCC-3′.
Cell Extracts, Subcellular Fractionation, and Immunoblots

For immunoblots, whole-cell lysates were prepared by scraping cell monolayers into ice-cold radioummune precipitation assay buffer (50 mm Tris-Cl, pH 7.4, 150 mm NaCl, 1% (v/v) Nonidet P-40, 0.5% (w/v) deoxycholic acid, 0.1% (w/v) SDS) supplemented with Complete, EDTA-free protease inhibitor mixture (Roche Applied Science). Lysates were clarified by centrifugation (16,000 × g, 15 min, 4 °C).

Nuclear and 1000 × g supernatant fractions were prepared as follows: Monolayers in 60-mm dishes were trypsinized, and the cell suspension was washed with 3 volumes of ice-cold phosphate-buffered saline by repeated centrifugation (500 × g, 2 min, 4 °C). The cell pellet was gently resuspended in 300 μl of STM-N buffer (250 mm sucrose, 50 mm Tris-Cl, pH 7.5, 5 mm MgCl₂, 10 mm iodoacetamide, 0.5% (v/v) Nonidet P-40 supplemented with Complete, EDTA-free protease inhibitor mixture). After 5 min on ice, the nuclei were pelleted (1000 × g, 5 min, 4 °C). The resulting supernatant was harvested and is referred to as the 1000 × g supernatant. The nuclei were gently resuspended in 1 ml of STM buffer (STM-N, but lacking the Nonidet P-40) and layered onto 200 μl of a sucrose cushion (40% (w/v) sucrose, 10 mm HEPES, pH 7.5, 10 mm KCl, 1.5 mm MgCl₂, and 1 mm dithiothreitol) Nuclei were pelleted (1000 × g, 15 min, 4 °C) and lysed in 100 μl of ice-cold radioummune precipitation assay buffer on ice for 15 min. The lysate was clarified by centrifugation (16,000 × g, 15 min, 4 °C), and the supernatant was harvested and is referred to as the nuclear fraction. SDS/polyacrylamide-gel electrophoresis, immunoblotting, and protein determination were carried out as previously described (11).

Relative Quantitation of rNQO1 and rNrf2 mRNA

This was carried out by TaqMan® chemistry. Total RNA was isolated from RL34 cells using Trizol reagent (Invitrogen). Approximately 1.5 μg of total RNA was reverse-transcribed to cDNA using random hexamers (Promega) and the SuperScript™ II RT kit (Invitrogen) according to the manufacturer’s instructions.

For the real-time PCR analysis, the following matching oligonucleotide primers and probes, designed using PrimerExpress™ software (PerkinElmer Life Sciences), were used as follows: rNQO1, forward primer, 5′-GGGTGGTGAAGACTGGGTGTGAGTGCTTGGCTGAGATATATG-3′, and reverse primer, 5′-GGTGAGGAGAAGAAGGTCCTCTC-3′; Probe 5′-(6-carboxyfluorescein)AGATGACCATGACCCATGTATG-3′; rNrf2, forward primer, 5′-TTTGGGAGAGCATCCTCCATT-3′, and reverse primer, 5′-GGTGTTGGAAAGCTTGAGCTGAGCTCTC-3′; Probe 5′-(6-carboxyfluorescein)AGAGGTAGCCATGAGTGTCCTGGCTC-3′. The PCR mix was prepared and amplified as described previously (16). The expression of 18S RNA was used as an internal control and was quantitated by TaqMan® chemistry using the TaqMan® ribosomal RNA control reagent (PerkinElmer Life Sciences).

Data acquisition and analysis utilized the ABI PRISM® 7700 sequence detection system (PerkinElmer Life Sciences). The relative gene expression levels in different samples was calculated using the Comparative Ct Method as outlined in the ABI PRISM® 7700 Sequence Detection System User Bulletin 2.

In Vivo Ubiquitination Assay

This was carried out using the method of Treier et al. (30). COS1 cells were transfected with pHfusUb along with either pCMV3.1/Nrf2 or pCMV3.1/NQO1 and other expression vectors as described in Fig. 7. Approximately 24 h later, the transfected cells were treated with 10 μm MG132 for 2 h before the monolayers were washed with pre-warmed phosphate-buffered saline and scraped into 0.36 ml of phosphate-buffered saline. A whole-cell lysate was prepared from 50 μl of the cell suspension and is referred to as the “input” fraction. His-tagged protein was purified from the remainder of the cell suspension as follows; the cell suspension was lysed by the addition to 2.2 ml of buffer A (6 μg guanidine, 10 mm Tris, 0.1 m phosphate buffer, pH 8.0) supplemented with 5 μm iodoacetamide. The resulting lysate was sonicated to reduce viscosity before 50 μl of Ni-NTA® resin (Sigma) was added, and the mixture was rotated overnight at 4 °C. Thereafter, the beads were washed sequentially with buffer A supplemented with 10 mm 2-mercaptoethanol (2-ME), buffer B (8 μl urea, 10 mm Tris, 0.1 m phosphate buffer, pH 8.0) supplemented with 10 mm 2-ME, and 15 min, 4 °C) and lysed by the addition to 2.2 ml of modified Laemmli sample buffer (20 mm Tris-Cl, pH 6.8, 10% (w/v) glycerol, 0.8% (v/v) SDS, 0.1% (w/v) bromphenol blue, 0.72 M 2-ME, and 300 mM imidazole) followed by boiling for 4 min. The suspension was centrifuged (16,000 × g, 1 min, 20 °C), and the resulting supernatant was collected and is referred to as the “pull-down” fraction.

RESULTS

Sulforaphane Transcriptionally Activates rNQO1 in RL34 Cells—Previous workers have shown that NQO1 genes are regulated by agents that cause oxidative stress, and evidence suggests that this is mediated by Nrf2 through an ARE (11, 33, 34). In the present study, we sought to provide further information about how the function of Nrf2 is controlled by oxidative stress. To this end, the isothiocyanate Sul, which causes oxidative stress by reacting with GSH to form dithiocarbamates, (35), was chosen as the oxidative stressor. As a model system we chose the non-transformed rat RL34 cell line over the more commonly utilized human HepG2 and mouse Hepa1c1c7 cell lines because its phenotypic response to Sul more closely recapitulates that observed in rodent liver. Specifically, untreated RL34 cells express an undetectable level of rNQO1 protein that is highly inducible by Sul treatment (Fig. 1A). By contrast, HepG2 and Hepa1c1c7 cell lines both express high basal levels of NQO1 with little or no induction observed after treatment with Sul (data not shown). The accumulation of rNQO1 protein in the RL34 cell line is paralleled by a rapid (significantly increased after 1 h, p < 0.05) elevation in the level of its cognate mRNA (Fig. 1B). As CAT activity expressed from the pARE-164CAT construct, but not p-164CAT, is induced by Sul (Fig. 1C), the increase in NQO1 protein is a...
Fig. 2. Sulforaphane causes rapid nuclear accumulation of rNrf2 in RL34 cells. A, whole-cell lysates prepared from RL34 cells treated with 15 μM Sul for the indicated periods of time were electrophoresed through a 6% resolving gel and probed with rabbit anti-mNrf2. A 1.4-ng portion of immunogen was used as a standard. M, markers are indicated to the left of the blot. A cross-reacting band, which comigrates with the rNrf2 band in 10% resolving gels, is indicated by an asterisk. B, whole-cell lysates were prepared from RL34 cells treated with 15 μM Sul (+) or vehicle (−) for the indicated periods of time and blotted with rabbit anti-mNrf2. C, whole-cell lysates were prepared from duplicate dishes of untreated RL34 cells (lanes 1). Nuclear and 1000 × g supernatant fractions were prepared from duplicate dishes of untreated RL34 cells (lanes 2) or cells treated for 2 h with vehicle (lanes 3) or 15 μM Sul (lanes 4). 12 μg (whole-cell lysates), 12 μg (1000 × g supernatant fractions), and 6 μg (nuclear fractions) of protein were separated through a 10% resolving gel and probed with antibodies raised against the proteins indicated on the left. The band developed by the anti-mNrf2 antiserum in nuclear fractions from untreated and vehicle-treated cells (nuclear fraction lanes 2 and 3) does not represent rNrf2, but the band indicated by an asterisk in A, UBF, upstream binding factor; MnSOD, manganese superoxide dismutase; CPR, cytochrome P450 reductase; MEK, mitogen-activated protein kinase-extra-cellular regulated kinase. D, RL34 cells were treated with 15 μM Sul for 2 h before the addition of CHX to a final concentration of 10 μM. Subsequently, whole-cell lysates were prepared at different time points and probed with rabbit anti-mNrf2. The graph depicts the natural logarithm of the relative expression of rNrf2 protein (quantitated by densitometry) as a function of CHX-chase time (mean ± S.D. of three independent experiments). The best-fit line, 95% confidence limits (broken lines), and the derived half-life (τ 1/2) are presented.

Sulforaphane Causes a Rapid Nuclear Accumulation of rNrf2 Protein.—To determine how Sul controls rNrf2 activity in RL34 cells, whole-cell lysates were prepared at different times after Sul treatment and blotted with a rabbit anti-mNrf2 antiserum (Fig. 2A). An immunochemically cross-reacting electrophoretic band representing rNrf2 was identified by virtue of its comigration with the immunogen. Treatment of cells with Sul, but not vehicle, led to a rapid and sustained elevation in the level of this protein (Fig. 2, A and B). The Nrf2 protein could not be unequivocally identified in untreated RL34 cells but was readily detected 15 min after treatment with Sul. Its level appeared to peak between 2 and 4 h after Sul treatment and remained elevated for at least 24 h. Subcellular fractionation revealed that, whenever detectable, the majority of rNrf2 was associated with the nuclear fraction (Fig. 2C). The procedure provided a highly pure nuclear fraction with no evidence of cytoplasmic, mitochondrial, or endoplasmic reticulum contamination, as assessed by the absence of MEK (mitogen-activated protein kinase-extra-cellular regulated kinase), manganese superoxide dismutase, or cytochrome P450 reductase marker proteins, respectively.

Being undetectable in homeostatic RL34 cells, the stability of rNrf2 under such conditions remains a matter of conjecture. CHX-chase experiments revealed, however, that after 2 h of Sul treatment, degradation of rNrf2 followed first-order kinetics with an approximate half-life of 30 min (Fig. 2D).

Sulforaphane Antagonizes Proteasome-dependent Degradation of rNrf2—The amount of protein expressed in mammalian cells is controlled primarily by regulating its rate of degradation and/or by altering the transcriptional activation of its gene. Evidence that rNrf2 might be transcriptionally activated by oxidative stress was presented by Kwak et al. (22, 23). Although such a mechanism could play some role in the later response to Sul, it is unlikely to account for the large increase in rNrf2 protein within the first 2 h. To discount the possibility that induction of rNrf2 contributes to the accumulation of Nrf2 protein, we measured the level of mRNA for the protein after exposure of RL34 cells to Sul. Real-time PCR showed no statistically significant change in the expression of rNrf2 mRNA at any time. By contrast, rNQO1 mRNA levels were elevated 20-fold within 8 h (Fig. 3). We therefore conclude that Sul does not cause any significant transcriptional activation of rNrf2.

Failure of Sul to affect induction of rNrf2 mRNA suggested that the increase in rNrf2 protein observed after treatment with the isothiocyanate might be wholly due to a reduction in its rate of degradation. In eukaryotic cells, regulatory proteolysis of intracellular proteins is in most cases accomplished by the proteasome (36–38). The notion that Nrf2 protein levels might be controlled by Sul through a proteasome-based mechanism is supported by the work of Sekhar et al. (39) who reported that proteasome inhibition activated ARE-driven gene expression in HepG2 cells. More recently, it has been reported that tert-butylhydroquinone can stabilize hNrf2 in HepG2 cells, and CdCl2 can stabilize mNrf2 protein in Hepa cells (24, 25). To evaluate the role of the proteasome in regulation of rNrf2, we treated RL34 cells with three structurally dissimilar proteasome inhibitors, epoxomicin, clasto-lactacystin-β-lactone (Fig. 4A), and MG132 (data not shown). All resulted in the accumulation of rNrf2, demonstrating that it is subject to proteasome-dependent degradation. In fact, after MG132-treatment of RL34 cells, CHX-chase experiments failed to reveal any
degradation of Nrf2 during a chase period of 60 min (Fig. 4B).

This finding suggested that the major proteolytic activities responsible for rNrf2 degradation are proteasomal in nature. In addition, because rNrf2 was calculated to have a half-life of 31 min in Sul-treated cells (Fig. 2D), the bZIP protein appears to be subject to proteasome-mediated degradation even during oxidative stress.

Because rNrf2 protein cannot be unequivocally identified in homeostatic RL34 cells, we could not determine directly whether under such circumstances it is subject to a greater rate of proteasome-dependent degradation than when treated with Sul. To investigate this point, the fact that MG132-mediated inhibition of the proteasome is reversible was exploited to generate a measurable pool of rNrf2 in RL34 cells before either returning them to normal conditions or subjecting them to oxidative stress. RL34 cells were pretreated with MG132 for 2 h before the proteasome inhibitor was removed and replaced with media containing CHX and either Sul or vehicle. When cotreated with CHX and vehicle, a notable reduction in the expression of rNrf2 was observed after a chase period of 60 min (Fig. 4C), indicative of proteasomal-mediated degradation. Most importantly, this degradation was clearly inhibited by cotreatment with Sul. Overall, these data suggest that Nrf2 is subject to proteasome-dependent degradation under both homeostatic and oxidative stress conditions but that the rate of degradation is reduced significantly during oxidative stress. Redox-regulated proteasomal degradation is likely to be a universal feature of Nrf2 regulation, at least in mammals.
treatment for 60 and 120 min. This interpretation is incorrect, and the difference in the mNrf2-V5-his signal merely results from the fact that the protein was blotted onto two different membranes (albeit at the same time). No experimental evidence has been obtained to support a difference in the expression of mNrf2-V5-his after 2 h of sulforaphane treatment regardless of the presence or absence of coexpressed mKeap1 (data not shown).

The above findings suggest that under homeostatic conditions Keap1 increases the rate of proteasomal degradation of Nrf2 through a direct interaction and that during oxidative stress this interaction is antagonized, exposing Nrf2 to a reduced rate of proteasomal degradation. To test this model we compared the biological properties of mNrf2-V5-his coexpressed with mKeap1 with the mutant Nrf2, mNrf2<sup>ΔETGE</sup>-V5-his, coexpressed with mKeap1. Mutant mNrf2<sup>ΔETGE</sup>-V5-his was found to be subject to proteasomal degradation, judged by the fact its half-life in homeostatic COS1 cells was 29 min (Fig. 6D), but when treated with MG132, its half-life increased to at least 3 h (Fig. 6A, panel ii). Thus, mutant Nrf2 protein accumulated in response to treatment with MG132 (Fig. 6B, panel ii). As predicted by the model, the level of mNrf2<sup>ΔETGE</sup>-V5-his protein, which could not interact with mKeap1, was not influenced by treatment with Sul (Fig. 6C, panel ii), indicating that its half-life is independent of the redox environment. mNrf2-V5-his was also subject to proteasomal degradation (Fig. 6A,
in the presence of V5-tagged proteins by immunoblot analysis (Fig. 7). His-tagged proteins were affinity-purified and assayed for the indicated in Fig. 7. After 24 h, cells were treated with 10 μM MG132 for 2 h with or without cotreatment with 15 μM Sul before both a whole-cell lysate (input) and affinity-purified His-tagged protein fraction (pull-down) were prepared from each dish of transfected cells and blotted with anti-V5. M₉ markers are indicated to the left of each blot.

panel i, and B, panel i), but two major differences were observed between it and mNrf2²ETGE-V5-his. First, under homeostatic conditions mNrf2-V5-his was less stable than mNrf2²ETGE-V5-his. Although we could not model precisely its kinetics of degradation, a CHX-chase period of between 7.5 and 15 min was sufficient to reduce mNrf2-V5-his expression by half (Fig. 6E). This compares with a half-life of 29 min for mNrf2²ETGE-V5-his (Fig. 6D). Not surprisingly therefore, mNrf2-V5-his was present at lower levels than was the mutant protein under homeostatic conditions (Fig. 6C). Second, mNrf2-V5-his was found to accumulate rapidly in COS1 cells after exposure to Sul, demonstrating that its half-life was increased by oxidative stress (Fig. 6C). As a consequence, the level of wild-type Nrf2 protein approached that of mutant Nrf2 protein after Sul-treatment.

These results demonstrate that Nrf2 is subject to at least two different modes of proteolysis. First, there exists a Keap1-independent, redox-insensitive proteasomal degradation responsible for the short half-life of mNrf2 in oxidatively stressed RL34 cells (31 min) and for the half-life of mNrf2²ETGE-V5-his in COS1 cells (29 min). Second, Nrf2 is subject to a substantially more rapid Keap1-dependent, redox-dependent proteasomal degradation. Thus, Keap1 appears to function as a trans-activating factor that stimulates the rate of proteasomal degradation of Nrf2. This activity entails a physical interaction between Keap1 and Nrf2 that occurs under homeostatic conditions but not during oxidative stress (18).

Nrf2 Is Ubiquitinated in Vivo in a Keap1- and Redox-independent Fashion—Many substrates of the 26 S proteasome require polyubiquitination to target them for degradation (40). In light of the data in Fig. 6, we were interested in determining whether Nrf2 is subject to ubiquitination and, if so, whether the ubiquitination is modulated by Keap1 and/or Sul treatment. To determine the ubiquitination status of Nrf2, COS1 cells were transfected with vectors expressing the proteins indicated in Fig. 7. After 24 h, cells were treated with MG132 with or without Sul cotreatment as indicated. Subsequently, His-tagged proteins were affinity-purified and assayed for the presence of V5-tagged proteins by immunoblot analysis (Fig. 7). A smear of high molecular mass V5-tagged protein was evident in the “pull-down” fraction of COS1 cells cotransfected with mNrf2-V5, mKeap1, and Ub-his (lanes 5 and 6). Because no V5-tagged protein was detected if the vector expressing either mNrf2-V5 (lanes 9 and 10) or Ub-his (lanes 11 and 12) was omitted from the transfection, this smear represents polyubiquitinated mNrf2-V5. In addition, Fig. 7 also demonstrated that polyubiquitination of Nrf2 occurs in a Keap1-independent fashion; in the absence of heterologously expressed mKeap1, mNrf2-V5 was still ubiquitinated (lanes 1 and 2), and heterologously expressed mNrf2²ETGE-V5 (lanes 3 and 4) was successfully polyubiquitinated in this assay. Finally, oxidative stress caused by treatment with Sul did not impair the ubiquitination of mNrf2-V5 (compare lanes 5 and 6 with lanes 7 and 8). It is, therefore, concluded that Nrf2 can be ubiquitinated by a mechanism that is independent of both Keap1 and the redox environment of the cell.

The Neh2 Domain of Nrf2 Constitutes a Redox-sensitive Degron—Although it was evident that the ETGE tetrapeptide motif of Nrf2 is essential for its Keap1-dependent degradation, it was unclear what cis-acting sequences within the transcription factor were sufficient to target it for redox-sensitive degradation by the proteasome. We postulated that its Neh2 do-
main, which is highly conserved across species and contains the ETGE tetrapeptide motif, might serve as a redox-sensitive "degron" (a protein sequence sufficient to confer metabolic instability; see Varshavsky (41)). To evaluate this hypothesis, constructs expressing either the mNeh2 domain or mNeh2ETGE fused to the C terminus of a HA-tagged GAL4 DNA binding domain were generated.

An initial experiment revealed that during heterologous coexpression of either GAL4(HA)mNeh2 or GAL4(HA) mNeh2ETGE with mKeap1, the level of neither fusion protein was altered when the COS1 cells were exposed to Sul (data not shown). One explanation considered for the failure of mKeap1 to confer redox sensitivity upon the expression of Gal4(HA)mNeh2 protein was that the level of expression of the N-terminal fusion protein might be sufficiently great as to saturate the binding capacity of the heterologously expressed mKeap1. To reduce the expressed levels of these fusion proteins, we transfected reduced amounts of the relevant expression constructs into COS1 cells. In line with the above interpretation, when lower amounts of the fusion proteins were expressed, the expression of Gal4(HA)mNeh2 became Sul-dependent, in contrast to the expression of Gal4(HA) mNeh2ETGE (Fig. 8A). Next, we sought to show that the half-life of the HA-tagged GAL4 DNA binding domain was reduced by fusing the mNeh2 domain to its C terminus. Because of a low signal-to-noise ratio, it was not possible to calculate or estimate by immunoblot analysis the half-life of Gal4(HA) mNeh2 or Gal4(HA)mNeh2ETGE when they were expressed at the low levels required to confer redox sensitivity upon the expression level of the wild-type fusion protein. Nonetheless, when expressed at higher levels, CHX-chase experiments revealed that the half-lives of both Gal4(HA)mNeh2 (33 min) and Gal4(HA)mNeh2ETGE (29 min) were much shorter than that of Gal4(HA) (500 min) (Fig. 8B). The similar half-life of both fusion proteins is complete in accordance with the observation that, under these transfection conditions, the expression of Gal4(HA)mNeh2 protein was unaltered by sulforaphane treatment. The calculated half-lives for both fusion proteins are close to those calculated for mNrf2V5-his in COS1 cells (Fig. 6D), suggesting that the Neh2 domain of Nrf2 is the sole cis-acting determinant of Nrf2 stability. Overall, these data suggest that the Neh2 region of Nrf2 constitutes a redox-sensitive degron.

DISCUSSION

This paper contains the first report that Nrf2 is subject to constitutive, Keap1-independent polyubiquitination and degradation by the proteasome. During oxidative stress, this appears to be the sole mechanism accounting for degradation of the bZIP transcription factor. It confers a half-life of 31 min upon rNrf2 in oxidatively stressed RL34 cells and a half-life of 29 min upon mNrf2ETGE-V5-his heterologously expressed in COS1 cells (cf. Figs. 2D and 6D). The ubiquitin-conjugating enzymes involved in the process remain unidentified, but the Neh2 domain of Nrf2 constitutes the relevant degron.

Under homeostatic conditions, the amount of Nrf2 protein in the RL34 cell line (this paper), other cell lines (23–25), and in murine liver (22) is significantly lower than in cells subjected to oxidative stress. This reduction is a consequence of a second more rapid mode of proteasomal degradation of Nrf2 that operates in non-stressed cells and requires Keap1 as an essential trans-acting factor (Figs. 5 and 6). Keap1 increases the homeostatic rate of proteasomal degradation of Nrf2 by directly interacting with it via the ETGE tetrapeptide motif present between residues 79 and 82 in the Neh2 domain of the bZIP protein (Figs. 5 and 6). The fact that this interaction is antagonized by oxidative stress (19) explains why this mode of degradation is restricted to homeostatic conditions. The half-life of endogenous rNrf2 in non-stressed RL34 cells could not be calculated because it was undetectable, but the half-life of mNrf2V5-his heterologously coexpressed with mKeap1 in homeostatic COS1 cells was between 7.5 and 15 min. By contrast, the half-life of mNrf2ETGE-V5-his heterologously expressed under the same conditions was ~30 min (Fig. 8).

Although the Neh2 domain of Nrf2 constitutes a degron common to both Keap1-independent and -dependent degradation of Nrf2 (Fig. 8), the mechanism of Keap1-dependent degradation and its relationship to Keap1-independent degradation remains a matter of conjecture. It is not known whether the Keap1-dependent degradation of Nrf2 involves a direct enhancement of the Keap1-independent degradation or constitutes a distinct parallel pathway. It is not even clear whether the Keap1-dependent pathway requires polyubiquitination of Nrf2. In yeast, the efficient degradation of many proteins, such as Mato2 and Far1, requires their localization to discrete cellular compartments, apparently due to the non-uniform distribution of components of the ubiquitin system and the proteasome (42, 43). In mammalian cells, the proteasome and ubiquitin-conjugating enzymes also appear to be non-uniformly distributed throughout the cytoplasm and the nucleus (38, 44–46). In common with many other Kelch-repeat proteins (for review, see Ref. 47), Keap1 has a restricted cellular distribution, being localized to the cytoskeleton (19, 48, 49). Thus, Keap1 may recruit Nrf2 in a redox-dependent manner to regions of the cytoskeleton to facilitate proteasomal degradation. Certainly, proteasomes have been observed localized to the cytoskeleton in mammalian cells (Ref. 45 and references therein). It is even possible that Keap1 might actively recruit Nrf2 to the proteasome, a notion supported by the functional similarity between Keap1 and the recently identified yeast protein Cic1 (50). Like Keap1, Cic1 increases the rate of proteasomal degradation of specific substrates (the SCF subunits Cdc4 and Grr1) but has no effect on the global rate of proteasomal degradation. Cic1 was originally isolated in a yeast two-hybrid screen for proteins interacting with the α4 subunit of the yeast proteasome and appears to act as an adaptor between the relevant SCF complexes and the proteasome, thereby ensuring the preferential proteasomal degradation of important regulatory proteins over the general population of ubiquitinated substrates (50). The existence in mammalian cells of proteins with characteristic similar to Cic1 or "extrinsic factors that assist in the recognition of certain substrates" (40), has been predicted. Keap1 may act as such a factor by a similar mechanism to that observed for Cic1. Keap1 appears to function as a dimer (49), with each monomer containing six Kelch-repeat motifs (19). Because this motif represents a general protein-protein interaction module (47), Keap1 possesses the characteristics required to bind and bring together multiple proteins.

It is curious that reports to date on Keap1 function have emphasized its sequestration of Nrf2 in the cytosol without noting an enhanced rate of degradation of Nrf2 (19, 48). It should be borne in mind that although heterologous expression of Nrf2 and Keap1 in cells is sufficient to observe an interaction between the two proteins, enhanced degradation of Nrf2 might require other conditions to be fulfilled. Thus, we have shown that Keap1 is essential to enhance the rate of degradation of Nrf2 in homeostatic cells but have not demonstrated that it is sufficient for this purpose. It is likely that the consequence of the Keap1-Nrf2 interaction will depend upon the amounts of heterologous proteins expressed and the cell line studied. It is intriguing to note that mouse liver (22) and two non-transformed cell lines (RL34 (this study) and PE cells (23)) contain no detectable Nrf2 protein under homeostatic conditions. By
contrast, two of three transformed cell lines for which data are available (HepG2 and H4IEC3 (24)) contain detectable levels of Nrf2 proteins under homeostatic conditions, and treatment of these cell lines with oxidative stressors only modestly increases the half-lives and levels of the bZIP proteins. It has been argued that up-regulation of GSH-dependent enzymes might contribute to the transformed phenotype by allowing cancerous cells to better withstand oxidative stress (51, 52), and it is conceivable that in certain transformed cell lines Keap1 function is partly disabled, and it does not efficiently degrade Nrf2. This might be one explanation for the high expression of NQO1 in HepG2 cells. In cell lines emphasizing degradation Nrf2. This might be one explanation for the high expression of NQO1 in HepG2 cells. In cell lines emphasizing degradation Nrf2. This might be one explanation for the high expression of NQO1 in HepG2 cells. In cell lines emphasizing degradation Nrf2. This might be one explanation for the high expression of NQO1 in HepG2 cells. In cell lines emphasizing degradation Nrf2. This might be one explanation for the high expression of NQO1 in HepG2 cells. In cell lines emphasizing degradation Nrf2. This might be one explanation for the high expression of NQO1 in HepG2 cells. In cell lines emphasizing degradation Nrf2. This might be one explanation for the high expression of NQO1 in HepG2 cells. In cell lines emphasizing degradation Nrf2. This might be one explanation for the high expression of NQO1 in HepG2 cells. In cell lines emphasizing degradation Nrf2. This might be one explanation for the high expression of NQO1 in HepG2 cells. In cell lines emphasizing degradation Nrf2. This might be one explanation for the high expression of NQO1 in HepG2 cells.
