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Keap1 Is a Redox-Regulated Substrate Adaptor Protein for a Cul3-Dependent Ubiquitin Ligase Complex

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The bZIP transcription factor Nrf2 controls a genetic program that protects cells from oxidative damage and maintains cellular redox homeostasis. Keap1, a BTB-Kelch protein, is the major upstream regulator of Nrf2 and controls both the subcellular localization and steady-state levels of Nrf2. In this report, we demonstrate that Keap1 functions as a substrate adaptor protein for a Cul3-dependent E3 ubiquitin ligase complex. Keap1 assembles into a functional E3 ubiquitin ligase complex with Cul3 and Rbx1 that targets multiple lysine residues located in the N-terminal Neh2 domain of Nrf2 for ubiquitin conjugation both in vivo and in vitro. Keap1-dependent ubiquitination of Nrf2 is inhibited following exposure of cells to quinone-induced oxidative stress and sulforaphane, a cancer-preventive isothiocyanate. A mutant Keap1 protein containing a single cysteine-to-serine substitution at residue 151 within the BTB domain of Keap1 is markedly resistant to inhibition by either quinone-induced oxidative stress or sulforaphane. Inhibition of Keap1-dependent ubiquitination of Nrf2 correlates with decreased association of Keap1 with Cul3. Neither quinone-induced oxidative stress nor sulforaphane disrupts association between Keap1 and Nrf2. Our results suggest that the ability of Keap1 to assemble into a functional E3 ubiquitin ligase complex is the critical determinant that controls steady-state levels of Nrf2 in response to cancer-preventive compounds and oxidative stress.

Eukaryote cells are exposed to both intrinsic and extrinsic sources of reactive oxygen species and other chemically reactive molecules that can damage biological macromolecules, including DNA, proteins, and lipids (22). Oxidative damage to biological macromolecules can have profound effects on cellular functions and has been implicated in cancer, inflammation, cardiovascular and neurodegenerative diseases, and aging (2, 3, 9, 21, 39, 45). Eukaryote cells have evolved multiple mechanisms to provide protection from oxidative damage. One major mechanism for protection against oxidative damage involves the coordinated induction of a group of cytoprotective genes that enable cells to neutralize reactive molecules and restore cellular redox homeostasis (14, 41). These cytoprotective genes, which include classical phase 2 genes, such as the glutathione *S*-transferases, NAD(P)H oxidoreductase (NQO1), and γ-glutamyl cysteine synthase, are regulated at the transcriptional level by *cis*-acting DNA sequences termed antioxidant response elements (AREs) or electrophilic response elements (14, 41, 54).

The bZIP transcription factor Nrf2 is the major regulator of the cytoprotective ARE-dependent transcriptional program (27, 41). Nrf2 is a member of the Cap n' Collar (CNC) subclass of bZIP proteins, which share a conserved dimerization and DNA binding domain (38). Nrf2 binds DNA as a heterodimer with one of several small Maf proteins and is a potent activator of ARE-dependent transcription. The absence of Nrf2 results in impaired basal and inducible expression of genes that encode a diverse array of proteins, including the classical phase 2 proteins, protein chaperones, antioxidant enzymes, and proteins involved in ubiquitin-dependent proteolysis, cell growth, and apoptosis (23, 33, 34, 36, 51). The absence of Nrf2 does not impair normal development in mice but results in increased sensitivity to chemical-induced stresses (4, 10, 13, 18), hyperoxia (11), autoimmune-like nephritis (59), and chemical-induced carcinogenesis (44). Conversely, increased expression of Nrf2 protects against neurotoxic agents (35, 47). Activation of Nrf2-dependent gene expression by plant-derived phytochemicals contributes to the gene products' cancer-preventive properties (50).

Genetic and biochemical evidence has implicated the Keap1 protein as the major upstream regulator of Nrf2. Keap1 was first identified in a yeast two-hybrid screen by virtue of its ability to bind the N-terminal Neh2 regulatory domain of Nrf2 (15, 25). Mice that lack Keap1 develop normally during embryogenesis but die soon after birth due to hyperkeratotic constrictions of the esophagus and forestomach (53). The absence of Keap1 results in constitutive activation of Nrf2-dependent genes, while the postnatal lethality seen in Keap1-deficient mice is reversed in mice that lack both Nrf2 and Keap1 (53).

The cytoplasmic Keap1 protein is a potent repressor of Nrf2-dependent transcription (25). Keap1 contains an N-terminal BTB domain, a conserved linker domain, and a C-terminal Kelch domain. The Kelch domain of Keap1 binds to the Neh2 domain of Nrf2 and, together with sequences located within the linker domain, enables Keap1 to sequester Nrf2 in the cytoplasm (25, 60). The Kelch domain of Keap1 is also able to bind actin, and an intact actin-based cytoskeleton is required for cytoplasmic sequestration of Nrf2 by Keap1 (29).

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Keap1 also regulates steady-state levels of Nrf2. Consistent with several reports that Nrf2 is degraded by the proteasome in a Keap1-dependent manner (26, 37, 40, 49, 60), we have previously demonstrated that Keap1 is able to target Nrf2 for ubiquitination (60). In this report, we demonstrate that Keap1 associates with Cul3 and Rbx1 to form a functional E3 ubiquitin ligase complex that targets Nrf2 for ubiquitination both in vivo and in vitro. Our results provide the first direct evidence that a human BTB-Kelch protein can function as a substrate adaptor protein for a Cul3-dependent E3 ubiquitin ligase complex. Our results support the emerging view that proteins with BTB domains can function as substrate adaptors for Cul3-dependent E3 ubiquitin ligase complexes (19, 20, 43, 55, 58).

The ability of increased Nrf2 expression to protect cells from oxidative damage suggests that small molecules that increase Nrf2-dependent gene expression may have broad therapeutic benefits. Indeed, chemical inducers of Nrf2-dependent gene expression are promising cancer-preventive agents (32, 50). A large number of small-molecule inducers of Nrf2-dependent gene expression, of both natural and synthetic origins, have been identified, and the ability of these structurally diverse molecules to react with thiols correlates with their ability to activate Nrf2-dependent transcription (17). In this report, we demonstrate that two well-characterized inducers of Nrf2-dependent transcription, quinone-induced oxidative stress and sulforaphane, a plant-derived isothiocyanate with chemopreventive properties, inhibit Keap1-dependent ubiquitination of Nrf2. A single cysteine-to-serine substitution at position 151 in the BTB domain of Keap1 confers significant resistance to inhibition by either oxidative stress or sulforaphane. Exposure of cells to these chemical inducers decreased association of Keap1 with Cul3. However, these chemical inducers do not disrupt association between Keap1 and Nrf2. Regulation of ubiquitin ligase activity by a single redox-sensitive amino acid of the substrate adaptor protein represents a novel paradigm for regulation of cullin-dependent E3 ubiquitin ligases.

MATERIALS AND METHODS

Construction of recombinant DNA molecules. Plasmids expressing wild-type Keap1, Nrf2, or Gal4-Neh2 proteins have been previously described (60). The CBD-tagged version of wild-type Keap1 was generated by insertion of a PCR-generated DNA fragment encoding the chitin binding domain of the *Bacillus circulans* chitinase A1 gene upstream of the stop codon for Keap1. The Keap1 mutants described in this study were generated by oligonucleotide-directed mutagenesis (46). Plasmids containing the individual cullin cDNAs were purchased from the American Type Culture Collection. A PCR-generated DNA containing the coding region of each cullin protein was cloned into the SmaI/NotI sites of a hemagglutinin (HA)-tagged pCI vector (Clontech). A Cul3 cDNA containing a stop codon at amino acid 381 was constructed by a PCR-based approach. The Myc-Rbx1 expression vector was obtained from Joan Conaway (28). The integrity of all of the plasmids used in this study was confirmed by sequence analysis.

Cell culture, transfections, indirect immunofluorescence, and reporter gene assays. COS1 and MDA-MB-231 cells were purchased from the American Type Culture Collection. Cells were maintained in either Dulbecco's modified Eagle's medium or Eagle's minimal essential medium in the presence of 10% fetal bovine serum. Transfections were performed with Lipofectamine Plus (Gibco BRL) according to the manufacturer's instructions. The DNA amounts in each transfection were kept constant by addition of empty pcDNA3 plasmid. The ARE TATA-Inr luciferase reporter plasmid pARE-Luc was obtained from Bill Fahl (54). A plasmid encoding *Renilla* luciferase was included in all samples to control for transfection efficiency. Reporter assays were performed using the Promega Dual-Light assay system as previously described (60).

Antibodies, immunoprecipitation, and immunoblot analysis. The anti-Keap1 antibody has been described previously (60). Antibodies against Nrf2 (Santa Cruz), Gal4 (Santa Cruz), ubiquitin (Sigma), chitin binding domain (New England Biolabs), and the Myc and HA epitopes (Covance) were purchased from commercial sources.

For detection of protein expression in total cell lysates, cells were lysed in sample buffer (50 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% glycerol, 100 mM dithiothreitol [DTT], 0.1% bromophenol blue) at 48 h post-transfection. For immunoprecipitation assays, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (10 mM sodium phosphate [pH 8.0], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Sigma). Cell lysates were precleared with protein A beads and incubated with 2 μg of affinity-purified antibodies for 2 h at 4°C, followed by incubation at 4°C with protein A-agarose beads for 2 h. Immunoprecipitated complexes were washed four times with RIPA buffer and eluted in sample buffer by boiling for 4 min, electrophoresed through SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and subjected to immunoblot analysis.

Pulse-chase analyses. Transfected MDA-MB-231 cells in 35-mm-diameter dishes were labeled with Dulbecco's modified Eagle's medium containing 100 μCi of [^{35}S]methionine and [^{35}S]cysteine supplemented with 10% dialyzed fetal bovine serum for 15 min. Either the labeled cells were collected in RIPA buffer, or the labeling medium was replaced with complete growth medium. Cell lysates were collected in RIPA buffer following the indicated chase periods and subjected to immunoprecipitation with anti-HA antibodies. The immunoprecipitated proteins were electrophoresed through a 7.5% SDS-polyacrylamide gel and visualized by fluorography. The relative intensities of immunoprecipitated Nrf2 were quantified by phosphorimager analysis (FXImager; Bio-Rad).

Ubiquitination of Nrf2. For detection of ubiquitinated Nrf2 in vivo, cells were transfected with expression vectors for HA-ubiquitin, HA-Cul3, Myc-Rbx1, Keap1, and Gal4-Neh2. The transfected cells were exposed to MG132 (Boston Biochem) for 5 h. Cells were lysed by boiling in a buffer containing 2% SDS, 150 mM NaCl, 10 mM Tris-HCl, and 1 mM DTT. This rapid lysis procedure inactivates cellular ubiquitin hydrolases and therefore preserves ubiquitin-Nrf2 conjugates present in cells prior to lysis. Protein-protein interactions, including association of Nrf2 with Keap1, are also disrupted by this lysis procedure. For immunoprecipitation, these lysates were diluted fivefold in buffer lacking SDS and incubated with anti-Gal4 antibodies (8). Immunoprecipitated proteins were analyzed by immunoblotting with antibodies directed against the HA epitope.

For ubiquitination of Nrf2 in vitro, COS1 cells were transfected with expression vectors for HA-Nrf2, Keap1-CBD, HA-Cul3, and Myc-Rbx1. The transfected cells were lysed in buffer B (15 mM Tris-HCl [pH 7.4], 500 mM NaCl, 0.25% NP-40) containing 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail. The lysates were precleared with protein A beads prior to incubation with chitin beads (New England Biolabs) for 4 h at 4°C. Chitin beads were washed twice with buffer B, twice with buffer A (25 mM Tris-HCl [pH 7.5], 10% [vol/vol] glycerol, 1 mM EDTA, 0.01% NP-40, 0.1 M NaCl), and twice with reaction buffer (50 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 2 mM NaF, 0.6 mM DTT). The pellets were incubated with ubiquitin (300 pmol), E1 (2 pmol), E2-UbcH5a (10 pmol), and ATP (2 mM) in 1× reaction buffer in a total volume of 30 μl for 1 h at 37°C. Ubiquitin, E1, and E2-UbcH5a were purchased from Boston Biochem. The chitin beads were centrifuged at 3,000 × *g*; resuspended in 2% SDS, 150 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM DTT; and boiled for 5 min to release bound proteins, inactivate any contaminating ubiquitin hydrolases, and disrupt protein-protein interactions. The supernatant was diluted fivefold with buffer lacking SDS prior to immunoprecipitation with anti-Nrf2 antibodies. Immunoprecipitated proteins were subjected to immunoblot analysis with antiubiquitin antibodies.

RESULTS

Association of Keap1 with Cul3 mediates Keap1-dependent ubiquitination of Nrf2. The N-terminal BTB domain of Keap1 is required for efficient repression of Nrf2-dependent gene expression (Fig. 1) (29, 60). The well-characterized Skp1 protein, which bridges the Cul1 protein and F-box-containing substrate adaptor proteins, contains a BTB-like fold (61). Several BTB-domain-containing proteins have been reported to function as substrate adaptor proteins for Cul3-dependent E3 ubiquitin ligase complexes (19, 20, 43, 58). To determine whether Keap1 is able to associate with one or more of the cullin proteins, expression vectors for several HA-tagged cullin pro-

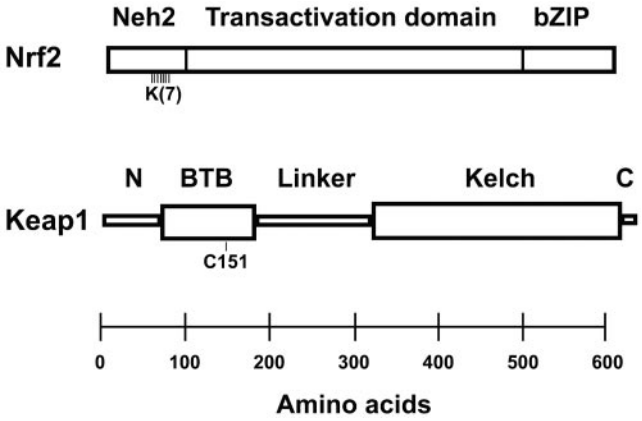

FIG. 1. Domain structures of Nrf2 and Keap1. Nrf2 contains three discrete domains, including an N-terminal Neh2 domain, a central transactivation domain, and a C-terminal bZIP domain. The N-terminal Neh2 domain interacts with the Kelch domain of Keap1. The locations of seven lysine residues that are candidate sites for ubiquitination within the Neh2 domain are indicated. Keap1 contains five discrete domains that are designated as N, BTB, Linker, Kelch, and C. The location of Cys 151 in the BTB domain of Keap1 is indicated.

teins were constructed and transfected into COS1 cells along with an expression vector for Keap1. Expression of Keap1 and the individual cullin proteins was confirmed by immunoblot analysis of total cell lysates (Fig. 2A, top and middle panels). Equivalent amounts of cell lysates were immunoprecipitated with anti-Keap1 antibodies, and the presence of the HA-tagged cullin proteins in the anti-Keap1 immunoprecipitates was determined by immunoblot analysis. In agreement with a recent report by Yamamoto and coworkers (31), both Cul2 and Cul3 were able to associate with Keap1 (Fig. 2A, bottom panel).

To examine the functional relevance of association between the cullin proteins and Keap1, the ability of the cullin proteins to increase Keap1-dependent ubiquitination of Nrf2 was determined. For these experiments, a Gal4-Neh2 fusion protein containing the N-terminal Neh2 domain of Nrf2 was coexpressed in MDA-MB-231 cells along with HA-ubiquitin, Keap1, and the cullin proteins. Consistent with our previous results (60), the Neh2 domain of Nrf2 is efficiently ubiquitinated by Keap1 in a dose-dependent manner (Fig. 2B, lanes 2 to 5). Expression of Cul3, but neither Cul1 nor Cul2, increased Keap1-dependent ubiquitination of Gal4-Neh2 in a dose-dependent manner (Fig. 2B, lanes 6 to 17). Ectopic expression of Cul3 was most effective at increasing Keap1-dependent ubiquitination of Gal4-Neh2 when Keap1 was expressed at submaximal levels (Fig. 2B and data not shown), consistent with the notion that Keap1 competes with other BTB-domain-containing proteins for Cul3.

To confirm that Keap1 utilizes Cul3 to target Nrf2 for ubiquitination, the ability of a dominant-negative Cul3 protein to block Keap1-dependent degradation of Nrf2 was determined. A stop codon was introduced at amino acid 381 in Cul3 to construct a truncated protein lacking the C-terminal Rbx1-binding domain of Cul3. Expression of this dominant-negative Cul3 protein in MDA-MB-231 cells inhibited the ability of Keap1 to both target the Gal4-Neh2 protein for ubiquitination (Fig. 2C) and decrease the steady-state levels of the full-length Nrf2 protein (Fig. 2D) in a dose-dependent manner.

Keap1 functions as a substrate adaptor protein for a Cul3/Rbx1 E3 ubiquitin ligase complex. Cullin proteins function as molecular scaffolds to bring together a substrate adaptor protein and the RING protein, Rbx1 (42). A specific substrate(s) is brought into the complex by the substrate adaptor protein, while the Rbx1 protein recruits a ubiquitin-charged E2 protein. To further define the role of Cul3 in Keap1-dependent ubiquitination of Nrf2, we first determined whether Keap1 is able to assemble into a ternary complex with Cul3 and Rbx1. To facilitate purification of Keap1, a chitin binding domain was fused to the C terminus of Keap1 (Keap1-CBD). The presence of the C-terminal chitin binding domain did not alter the ability of Keap1 to associate with either Cul3 or Nrf2 or alter the responsiveness of Keap1 to chemical inducers of Nrf2 (data not shown). Expression vectors for Keap1-CBD, Cul3, and Rbx1 were transfected into COS1 cells, and Keap1 was purified by use of chitin beads. Both Cul3 and Rbx1 were copurified with Keap1, as determined by either immunoblot analysis (Fig. 3A) or silver staining (data not shown). Copurification of Rbx1 with Keap1-CBD was markedly enhanced in the presence of coexpressed Cul3 (Fig. 3A, lane 4) consistent with the notion that Cul3 functions as a molecular scaffold to assemble both Keap1 and Rbx1 into an E3 ubiquitin ligase complex.

The ability of the Keap1-Cul3-Rbx1 complex to target Nrf2 for ubiquitination and subsequent proteosome-mediated degradation was determined. In one set of experiments, steady-state levels of Nrf2 were determined in MDA-MB-231 cells transfected with expression vectors for Nrf2, Keap1, Rbx1, and one of several cullin proteins. The input amounts of the various plasmid DNAs were carefully titrated so that submaximal repression of Nrf2 steady-state levels by expression of Keap1 alone was achieved. Under these conditions, a marked reduction in steady-state levels of Nrf2 was achieved by coexpression of Cul3 and Rbx1 (Fig. 3B, lane 5). Treatment of the transfected cells with MG132 prior to cell lysis restored steady-state levels of Nrf2 (Fig. 3B, lane 6). Coexpression of Cul3 and Rbx1 also markedly increased Keap1-dependent ubiquitination of the Gal4-Neh2 fusion protein (Fig. 3C, compare lanes 2 and 4). In contrast, coexpression of Rbx1 with either Cul1 or Cul2 did not alter steady-state levels of Nrf2 (Fig. 3B, lanes 7 and 8) or Keap1-dependent ubiquitination of Gal4-Neh2 (data not shown).

To confirm that the Keap1-Cul3-Rbx1 complex assembles into an active E3 ubiquitin ligase complex, the ability of the Keap1-Cul3-Rbx1 complex to target Nrf2 for ubiquitination in vitro was determined. Expression vectors for Nrf2, Keap-CBD, Cul3, and Rbx1 were transfected into COS1 cells, and the complex was purified by use of chitin beads (Fig. 3D, lower panel). As expected, coexpression of Cul3 and Rbx1 along with Keap1 resulted in decreased levels of Nrf2 that were restored by treatment of the transfected cells with MG132 prior to cell lysis (Fig. 3D, bottom panel, compare lanes 2 and 3). The chitin beads were mixed with purified E1, E2-UbcH5a, and ubiquitin in the presence of ATP. The ubiquitination reactions were terminated by boiling to release proteins from the chitin beads. The samples were immunoprecipitated under stringent denaturing conditions using anti-Nrf2 antibodies. The immunoprecipitated proteins were subjected to immunoblot analysis

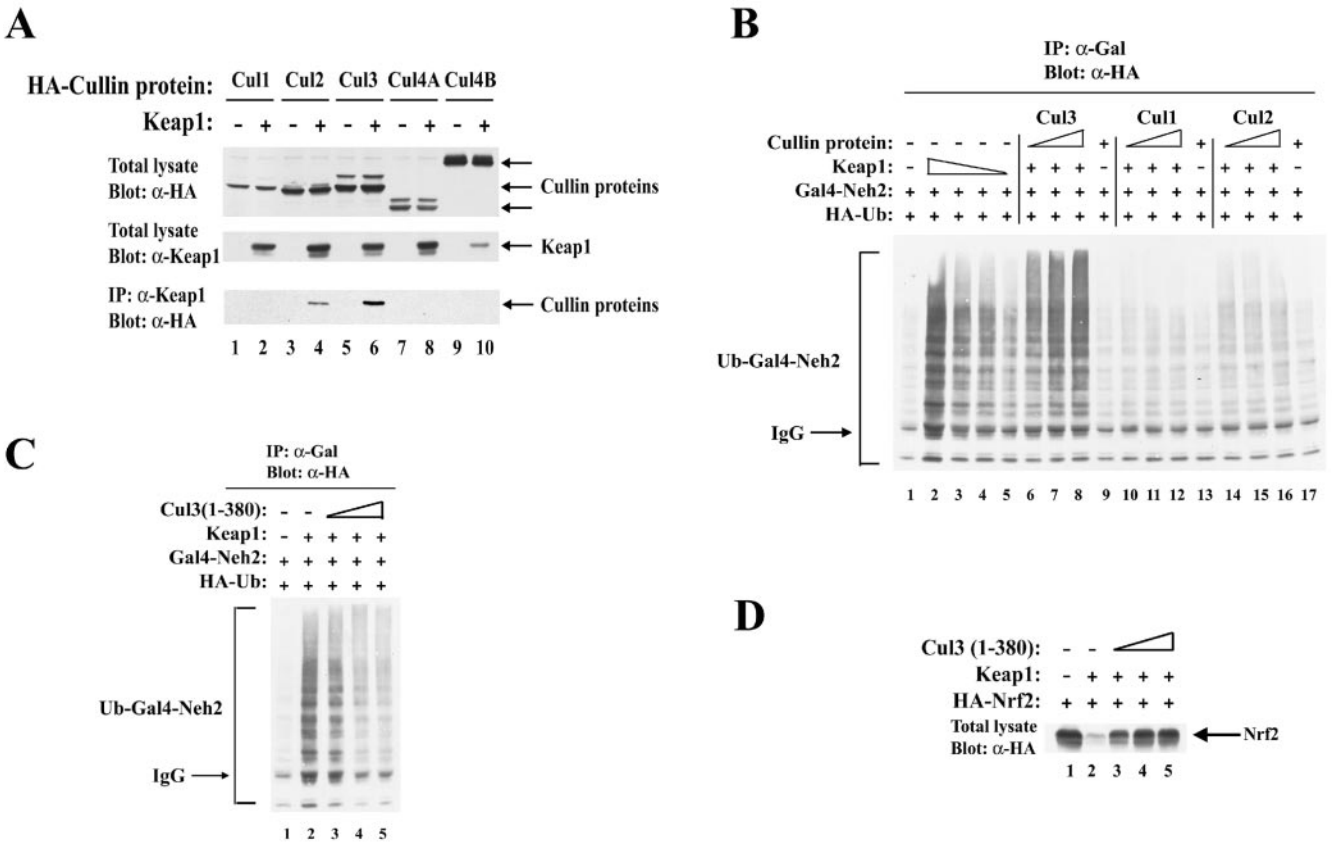

FIG. 2. (A) COS1 cells were cotransfected with expression vectors for wild-type Keap1 and the indicated cullin proteins. Total cell lysates were immunoblotted with anti-HA antibodies (top panel) and anti-Keap1 antibodies (middle panel). Anti-Keap1 immunoprecipitates (IP) were subjected to immunoblot analysis using anti-HA antibodies (lower panel). (B) Sixty-millimeter-diameter dishes of MDA-MB-231 cells were transfected with expression vectors for HA-ubiquitin (Ub), Gal4-Neh2, Keap1, and each of the cullin proteins as indicated. Constant amounts of expression vectors for HA-ubiquitin (0.6 μg) and Gal4-Neh2 (0.6 μg) were included in all samples. The Keap1 expression vector was either omitted from some samples (lanes 1, 9, 13, and 17), titrated down from 0.6 to 0.038 μg (lanes 2 to 5), or kept constant at 0.038 μg per dish (lanes 6 to 8, 10 to 12, and 14 to 16). Increasing amounts of each cullin expression vector, from 0.1 to 0.6 μg, were added to some samples (lanes 6 to 17). Anti-Gal4 immunoprecipitates were analyzed by immunoblot analysis with anti-HA antibodies. IgG, immunoglobulin G. (C) Sixty-millimeter-diameter dishes of MDA-MB-231 cells were transfected with expression vectors for HA-Ub (0.6 μg), Gal4-Neh2 (0.6 μg), Keap1 (0.3 μg, lanes 2 to 5), and Cul3(1-380) (from 0.1 to 0.5 μg, lanes 3 to 5). Anti-Gal4 immunoprecipitates were analyzed by immunoblot analysis with anti-HA antibodies. (D). Twenty-four-well plates of MDA-MB-231 cells were transfected with expression vectors for HA-Nrf2 (0.18 μg, lanes 1 to 5), Keap1 (0.02 μg, lanes 2 to 5), and Cul3(1-380) (from 0.02 to 0.18 μg, lanes 3 to 5). Total cell lysates were subjected to immunoblot analysis with anti-HA antibodies.

using antiubiquitin antibodies. Ubiquitination of Nrf2 was markedly enhanced in the presence of both Cul3 and Rbx1 (Fig. 3D, compare lanes 1 and 3). Importantly, very low levels of ubiquitinated Nrf2 proteins were observed when purified E1 was not included in the reaction (Fig. 3D, lane 4), confirming that conjugation of ubiquitin onto Nrf2 occurred in vitro. Although the Keap1-Cul3-Rbx1 complex is also able to target Nrf2 for ubiquitination in vivo (Fig. 3C, lane 4), the ubiquitin-Nrf2 conjugates formed in vivo are likely removed by ubiquitin hydrolases during cell lysis and purification of the complex. Alternatively, ubiquitination of Nrf2 by the purified Keap1-Cul3-Rbx1 complex may simply be more efficient in vitro, perhaps due to the absence of proteins that compete with Keap1 for binding to Cul3.

Mutations within the BTB domain of Keap1 decrease ubiquitination of Nrf2 but increase ubiquitination of Keap1. Molecular contacts between the cullin proteins and their cognate BTB domain-containing substrate adaptor proteins are highly conserved, as the crystal structure of the Skp1-Cul1-Rbx1 complex has been used to predict amino acids required for association of MEI-26 with the *Caenorhabditis elegans* Cul3 protein (58, 61). The corresponding residues in Keap1 are residues 123 to 127 and 160 to 164. Two mutant Keap1 proteins, containing alanine substitutions for amino acids 125 to 127 (Keap1-125A3) and 162 to 164 (Keap1-162A3), were constructed and characterized for their ability to assemble with Cul3 and Rbx1 into a functional E3 complex that targets Nrf2 for ubiquitination. The mutant Keap1 proteins were not impaired in their ability to associate with Nrf2 (data not shown). In the presence of ectopic Cul3 and Rbx1, the mutant Keap1 proteins were expressed at reduced levels in COS1 cells compared to the wild-type Keap1 protein (Fig. 4A, top panel). Therefore, to compare the ability of the wild-type and mutant Keap1 proteins to associate with Cul3 and Rbx1, the amount of cell lysate used in the affinity purification experiments was normalized such that the input levels of the wild-type and mutant Keap1

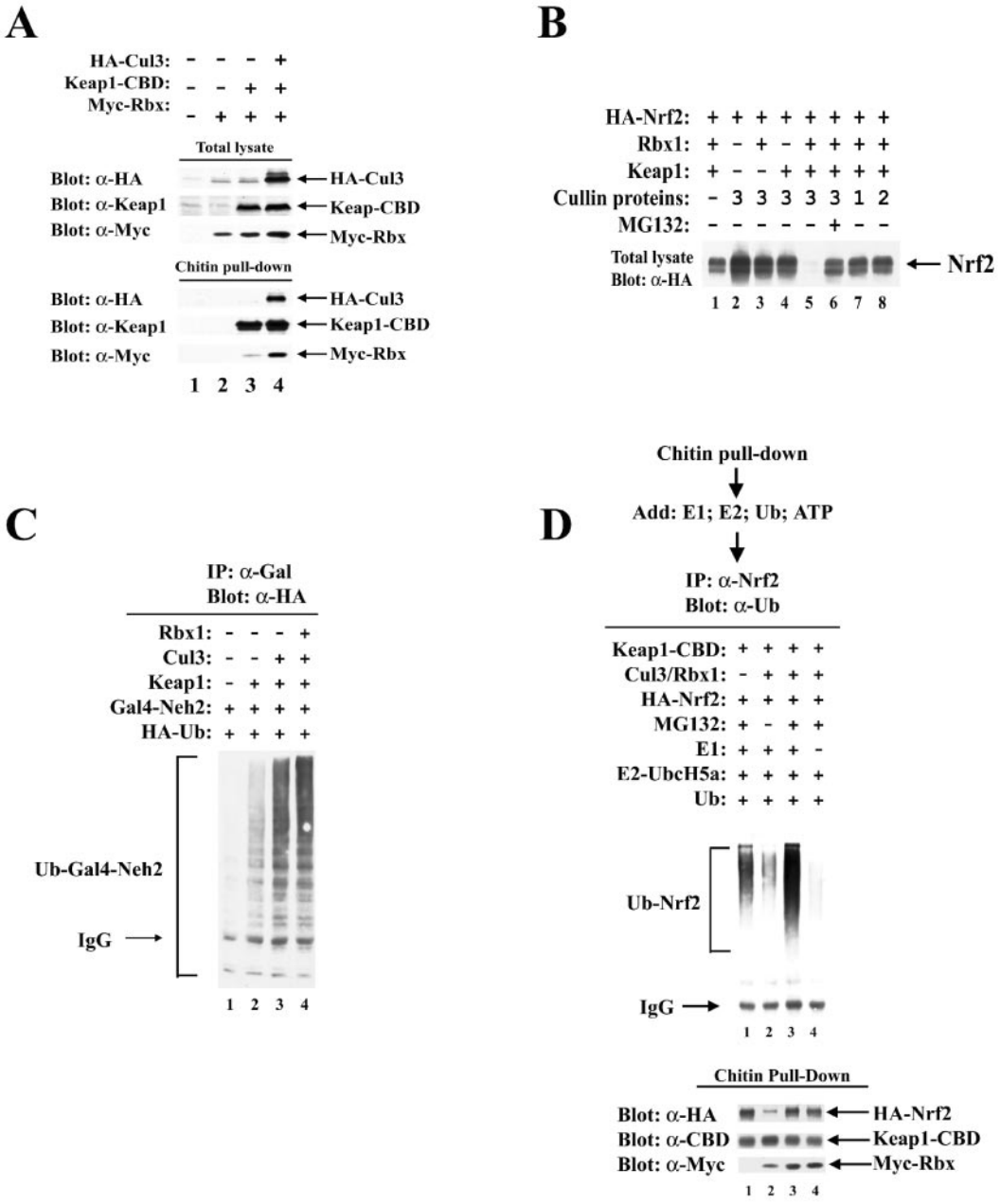

FIG. 3. (A) Sixty-millimeter-diameter dishes of COS1 cells were mock transfected (lane 1) or cotransfected with 0.7 μg each of expression vectors for Myc-Rbx1 (lanes 2 to 4), Keap1 (lanes 3 and 4), and HA-Cul3 (lane 4). Total cell lysates (7 μg) were analyzed by immunoblotting with anti-HA, anti-Myc, and anti-Keap1 antibodies (upper three panels). The lysates (700 μg) were incubated with chitin beads, pelleted by centrifugation (3,000 × *g*), and washed three times in RIPA buffer. Proteins that remained associated with the chitin beads were analyzed by immunoblotting with anti-HA, anti-Myc, and anti-Keap1 antibodies (lower three panels). (B) Twenty-four-well plates of MDA-MB-231 cells were cotransfected with expression vectors for HA-Nrf2 (0.15 μg), Myc-Rbx1 (0.118 μg; lanes 1, 3, and 5 to 8), Keap1 (0.015 μg, lanes 1 and 4 to 8), and each of the cullin proteins (0.117 μg, lanes 2 to 8) as indicated. The cells were either untreated (lanes 1 to 5, 7, and 8) or treated with 10 μM MG132 (lane 6) for 5 h prior to analysis of total cell lysates by immunoblotting with anti-HA antibodies. (C) Thirty-five-millimeter-diameter dishes of MDA-MB-231 cells were transfected with expression vectors for HA-ubiquitin (Ub) (0.3 μg), Gal4-Neh2 (0.35 μg), Keap1 (0.05 μg, lanes 2 to 4), Cul3 (0.15 μg, lanes 3 to 4), and the Myc-Rbx1 expression plasmid (0.02 μg, lane 4). Anti-Gal4 immunoprecipitates (IP) were analyzed by immunoblot analysis with anti-HA antibodies. IgG, immunoglobulin G. (D) Sixty-millimeter-diameter dishes of COS1 cells were transfected with expression vectors for Nrf2 (0.4 μg, lanes 1 to 4), Keap1-CBD (0.4 μg, lanes 1 to 4), HA-Cul3 (0.4 μg, lanes 2 to 4), and Myc-Rbx1 (0.4 μg, lanes 2 to 4). Lysates from three 60-mm-diameter dishes were pooled for each sample and incubated with chitin beads. After washing, the chitin beads were incubated with E1, E2-UbcH5a, ubiquitin, and ATP. Subsequently, the chitin beads were pelleted and washed, and proteins that were eluted from the beads after boiling were split into two sets of samples. One set was immunoprecipitated with anti-Nrf2 antibodies and then analyzed by immunoblotting with anti-ubiquitin antibodies (top panel). The other set was subjected to immunoblot analysis using anti-HA, anti-CBD, and anti-Myc antibodies (bottom three panels).

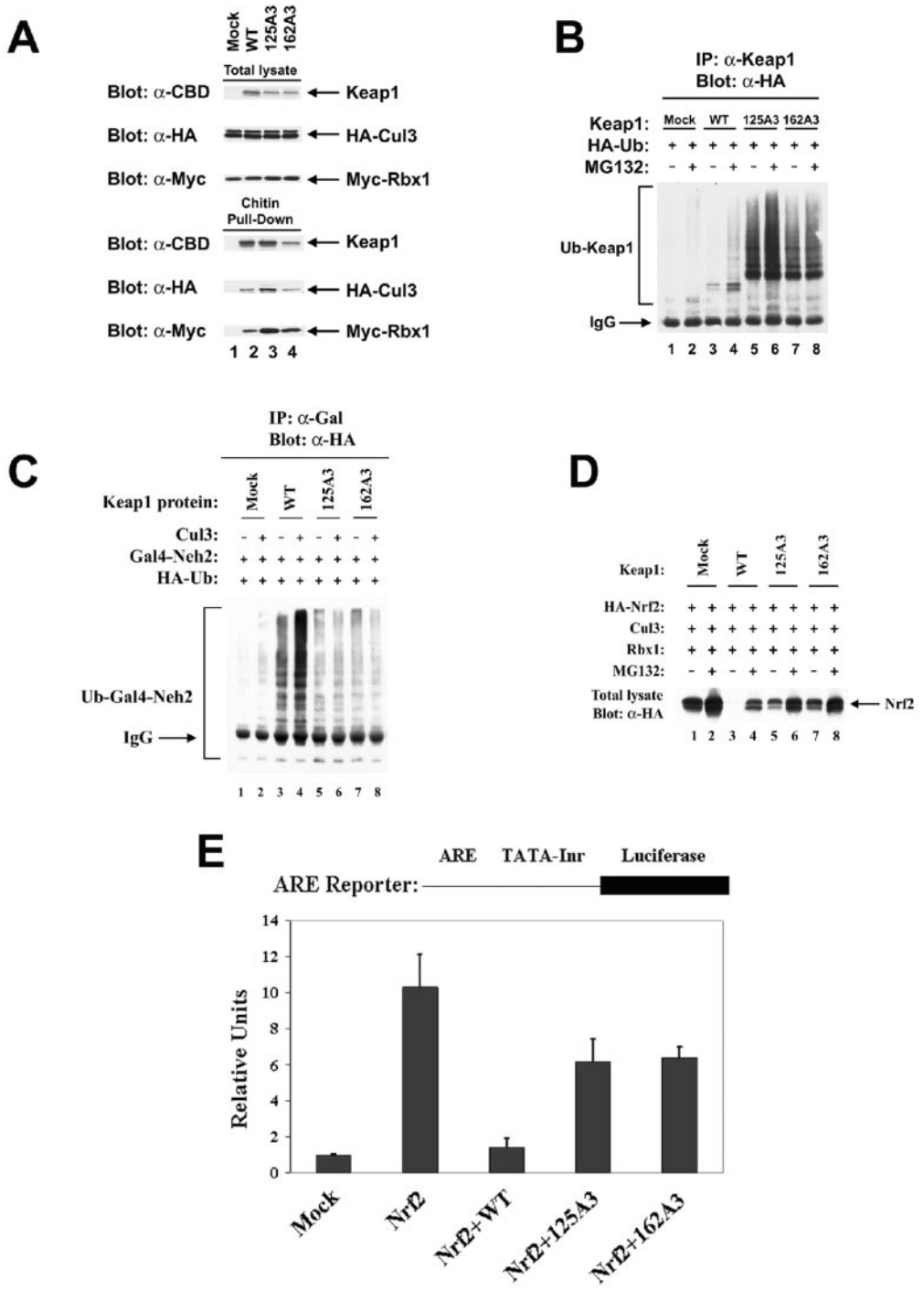

FIG. 4. (A) Sixty-millimeter-diameter dishes of COS1 cells were transfected with equal amounts of expression vectors for the indicated Keap1-CBD proteins, HA-Cul3, and Myc-Rbx1. Cell lysates were collected and immunoblotted with the indicated antibodies (top three panels) or incubated with chitin beads. Proteins that remained bound to the chitin beads after extensive washing were analyzed by immunoblotting with the indicated antibodies (bottom three panels). WT, wild type. (B) Thirty-five-millimeter-diameter dishes of MDA-MB-231 cells were transfected with expression vectors for HA-ubiquitin (Ub; 0.5 μg) and the indicated Keap1 proteins (0.5 μg) and either left untreated (odd-numbered lanes) or treated with MG132 for 5 h (even-numbered lanes) prior to collection of cell lysates. Anti-Keap1 immunoprecipitates (IP) were analyzed by immunoblotting with anti-HA antibodies. IgG, immunoglobulin G. (C) Thirty-five-millimeter-diameter dishes of MDA-MB-231 cells were transfected with expression vectors for HA-Ub (0.31 μg), Gal4-Neh2 (0.36 μg), Cul3 (0.15 μg, even-numbered lanes), and the wild-type or mutant Keap1 proteins (0.18 μg), as indicated in lanes 3 to 8. Anti-Gal4 immunoprecipitates were analyzed by immunoblot analysis with anti-HA antibodies. (D) Twenty-four-well plates of MDA-MB-231 cells were transfected with expression vectors for HA-Nrf2 (0.2 μg), Cul3 (0.02 μg), Myc-Rbx1 (0.02 μg), and the wild-type or mutant Keap1 proteins (0.1 μg), as indicated in lanes 3 to 8. The transfected cells were either untreated (odd-numbered lanes) or treated with MG132 for 5 h (even-numbered lanes) prior to collection of cell lysates and determination of HA-Nrf2 levels by immunoblot analysis. (E) Twenty-four-well plates of MDA-MB-231 cells were transfected in duplicate with an ARE-dependent firefly luciferase reporter gene construct (100 ng) and expression plasmids for Nrf2 (100 ng) and the wild-type or mutant Keap1 proteins (50 ng). A plasmid encoding *Renilla* luciferase (10 ng) was included as a control for transfection efficiency. Lysates were collected at 48 h posttransfection, and both firefly and *Renilla* luciferase activities in cell lysates were analyzed. The data shown represent the means and standard errors of results from three independent experiments.

proteins were approximately equivalent (Fig. 4A, fourth panel from top). To our surprise, association of Cul3 with the mutant Keap1 proteins was not reduced. Rather, increased levels of Cul3 and Rbx1 were observed to be copurified with the mutant Keap1 proteins compared to those with the wild-type protein (Fig. 4A, bottom three panels, compare lane 1 with lanes 2 and 3). Increased association of the mutant Keap1 proteins with Cul3 and Rbx1 was reflected in increased ubiquitination onto the mutant Keap1 proteins (Fig. 4B, compare lanes 3 and 4 with lanes 5 to 8). However, these mutant Keap1 proteins were unable to cooperate with Cul3 for ubiquitination of the Gal4-Neh2 protein (Fig. 4C, compare lane 4 with lanes 6 and 8) and repression of steady-state levels of Nrf2 (Fig. 4D). In addition, the mutant Keap1 proteins were impaired in their ability to down-regulate Nrf2-dependent gene expression (Fig. 4E). Thus, contrary to our expectation based on published reports (58, 61), mutation of conserved residues within the BTB domain of Keap1 did not disrupt association with Cul3. Nevertheless, these mutations disrupt the ability of the Keap1-Cul3-Rbx1 complex to efficiently target Nrf2 for ubiquitination and subsequent proteosome-mediated degradation.

Identification of lysine residues in Nrf2 targeted for ubiquitination by Keap1. The N-terminal Neh2 domain of Nrf2 contains seven lysine residues (Fig. 5A). The full-length Nrf2 protein contains an additional 32 lysine residues that are potential candidates for Keap1-dependent ubiquitination. To determine whether the seven lysine residues in the Neh2 domain are the major determinants of Keap1-dependent ubiquitination, a mutant Nrf2 protein was constructed in which these lysine residues were replaced by arginine residues (Nrf2-R7). The presence of these seven arginine substitutions did not alter the ability of the Nrf2-R7 protein to coimmunoprecipitate with Keap1 from cotransfected COS1 cells or associate with Keap1 in vitro (data not shown). However, in the presence of coexpressed Keap1, steady-state levels of the Nrf2-R7 protein were markedly increased relative to those of the wild-type Nrf2 protein (Fig. 5B). To confirm that the observed difference in steady-state levels reflected differences in the stability of the wild-type and mutant Nrf2 proteins, the half-lives of the respective Nrf2 proteins were determined by pulse-chase analysis. The half-life of the wild-type Nrf2 protein in the presence of coexpressed Keap1 was slightly more than 1 h, while the half-life of the Nrf2-R7 protein in the presence of coexpressed Keap1 was nearly 3 h (Table 1). Furthermore, ubiquitination of the Gal4-Neh2-R7 protein in the presence of coexpressed Keap1 was markedly reduced relative to that of the wild-type Gal4-Neh2 protein, both in vivo (Fig. 5C, lane 11) and in vitro (data not shown). Taken together, these experiments demonstrate that one or more lysine residues in the Neh2 domain of Nrf2 are the major determinants of Keap1-dependent ubiquitination of Nrf2.

The Nrf2-R7 cDNA was used to construct "add-back" mutant Nrf2 proteins containing single lysine residues within the Neh2 domain. The steady-state levels of these mutant Nrf2 proteins in the presence of Keap1 were intermediate between the wild-type Nrf2 and the Nrf2-R7 proteins (Fig. 5B). Pulse-chase analysis confirmed that the half-lives of the Nrf2 proteins containing single lysine residues were intermediate between that of the wild-type and Nrf2-R7 proteins (data not shown). The ability of Keap1 to target these add-back mutant Nrf2 proteins for ubiquitination in vivo was also determined. In all cases, the presence of a single lysine residue within the Neh2 domain resulted in increased levels of ubiquitination of the Gal4-Neh2 fusion protein compared to the Gal4-Neh2-R7 fusion protein, particularly for lysine residues 52 and 53 (Fig. 5C, lanes 6 and 7). These results indicate that Keap1 targets multiple lysine residues within the Neh2 domain of Nrf2 for ubiquitination.

Keap1-dependent ubiquitination of Nrf2 is inhibited by oxidative stress. The ability of Keap1 to function as a substrate adaptor protein for Cul3 and thereby target Nrf2 for ubiquitin conjugation provides an efficient mechanism for repression of Nrf2 steady-state levels and of Nrf2-dependent transcription. However, exposure of cells to a wide variety of thiol-reactive chemicals results in elevated steady-state levels of Nrf2 and transcriptional activation of the cytoprotective Nrf2-dependent genetic program (1, 26, 49, 60). For example, we have previously demonstrated that both quinone-induced oxidative stress and sulforaphane, a chemopreventive isothiocyanate, increase the stability of Nrf2 and activate Nrf2-dependent gene expression (60). Furthermore, members of our laboratory have previously reported that a single cysteine-to-serine substitution at position 151, located within the BTB domain of Keap1, specifically blocked activation of Nrf2-dependent transcription in response to both quinone-induced oxidative stress and sulforaphane (60).

To determine whether oxidative stress and sulforaphane inhibit the ability of Keap1 to target Nrf2 for ubiquitination, levels of Keap1-dependent ubiquitin conjugation onto the Gal4-Neh2 protein were determined in cells exposed to either *t*-butylhydroquinone (tBHQ) or sulforaphane prior to cell lysis. Exposure of cells to either tBHQ or sulforaphane markedly decreased levels of ubiquitinated Gal4-Neh2 protein (Fig. 6A, lanes 1 to 3). A 4-h exposure of cells to tBHQ resulted in maximal inhibition of Keap1-dependent ubiquitination of Gal4-Neh2 (data not shown). In contrast, the ability of the Keap1-C151S protein to target the Gal4-Neh2 protein for ubiquitination was not inhibited by either tBHQ or sulforaphane (Fig. 6A, lanes 4 to 6).

To confirm that tBHQ and sulforaphane inhibit the ability of Keap1 to function as a substrate adaptor protein for Cul3, ubiquitination onto the Gal4-Neh2 protein was examined in the presence of both Keap1 and Cul3. Input ratios of Keap1 and Cul3 expression vectors were carefully titrated to achieve maximal ubiquitination onto the Gal4-Neh2 protein in the presence of both Cul3 and Keap1 (Fig. 6B, lanes 1 to 3). Exposure of the transfected cells to either sulforaphane or tBHQ decreased ubiquitination onto the Gal4-Neh2 protein in the presence of Cul3 and the wild-type Keap1 protein (Fig. 6B, lanes 4 and 5). The Keap1-C151S protein was largely resistant to inhibition by either sulforaphane or tBHQ, although a slight decrease in ubiquitination of the Gal4-Neh2 protein was observed following sulforaphane treatment (Fig. 6B, lanes 8 and 9).

The ability of Keap1 to associate with Cul3 was assessed following treatment of cells with either tBHQ or sulforaphane. Treatment of cells with either tBHQ or sulforaphane decreased the level of Cul3 that was copurified with the wild-type Keap1-CBD protein following affinity purification (Fig. 6C, lanes 2 to 4). Association of the Keap1-C151S protein with

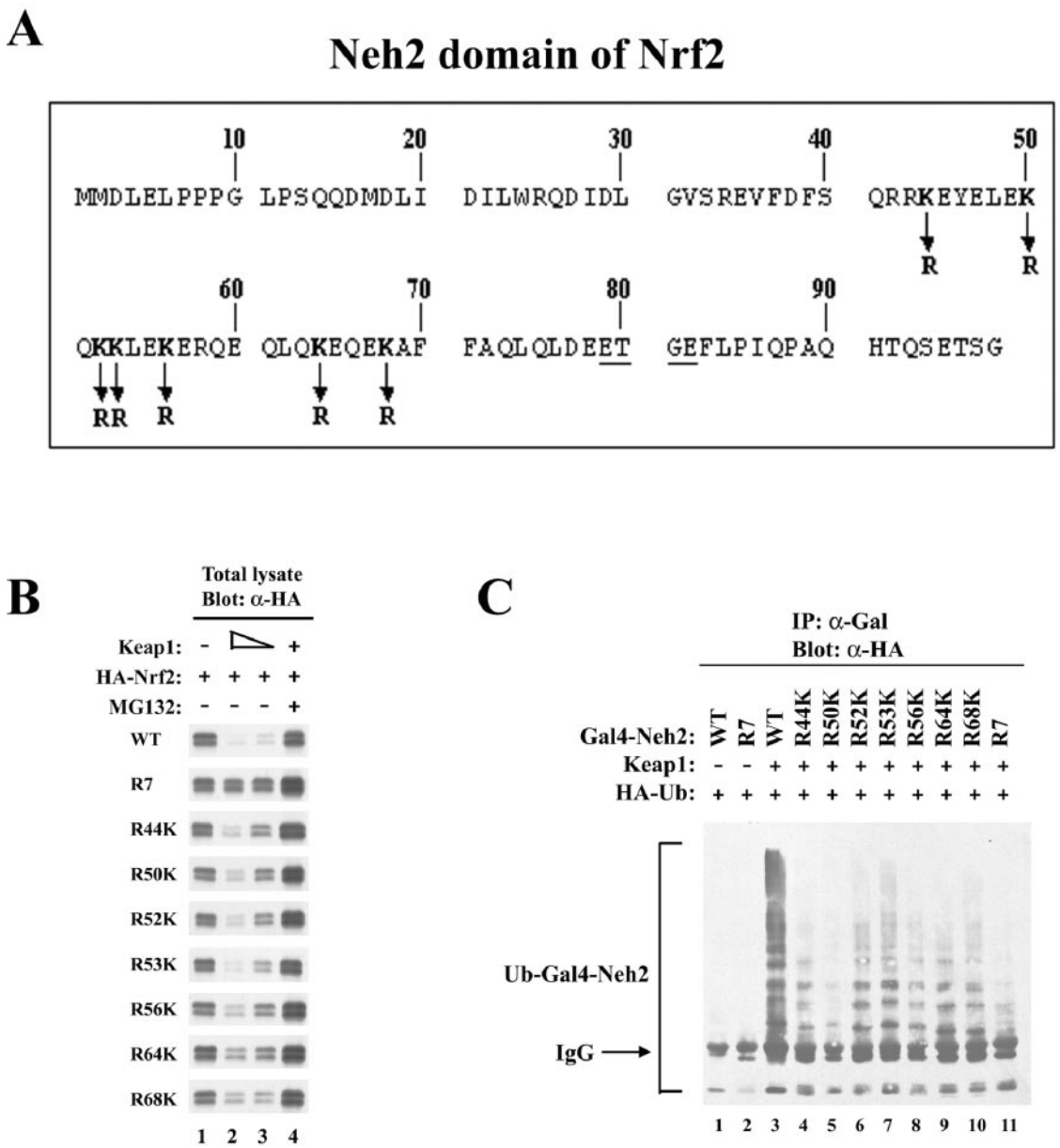

FIG. 5. (A) Seven lysine residues in the Neh2 domain of Nrf2 (boldface) were substituted with arginine residues. A conserved ETGE motif (underlined) is required for association of Nrf2 with Keap1. (B) Twenty-four-well plates of MDA-MB-231 cells were transfected with expression vectors for wild-type (WT) or mutant HA-Nrf2 proteins (0.2 μg each, lane 1) and with different amounts of the expression vector for Keap1 (0.1 μg, lanes 2 and 4, or 0.025 μg, lane 3). The transfected cells were either untreated or treated with 10 μM MG132 (lane 4) for 4 h prior to analysis by immunoblotting using anti-HA antibodies. (C) Sixty-millimeter-diameter dishes of MDA-MB-231 cells were transfected with expression vectors for HA-ubiquitin (Ub; 0.6 μg), either wild-type Nrf2 or each of the mutant Nrf2 proteins as indicated (0.7 μg), and Keap1 (0.7 μg, lanes 3 to 11). The cells were treated with 10 μM MG132 for 4 h prior to lysis. Anti-Gal4 immunoprecipitates (IP) were analyzed by immunoblotting with anti-HA antibodies. IgG, immunoglobulin G.

Cul3 was not significantly affected by either tBHQ or sulforaphane treatment (Fig. 6C, lanes 5 to 7). Importantly, the amount of Cul3 that was copurified with the Keap1-C151S protein was greater than the amount of Cul3 that was copurified with the wild-type Keap1 protein following treatment with either tBHQ or sulforaphane (Fig, 6C, compare lanes 3 and 6 and lanes 4 and 7), in agreement with the increased ability of the Keap1-C151S protein to target Nrf2 for ubiquitination in cells exposed to either tBHQ or sulforaphane (Fig. 6A and B).

TABLE 1. Half-lives of Nrf2 proteins

Nrf2 protein[a]	$t_{1/2}$ (h)[b]
Wild-type Nrf2	1.23
R7-Nrf2	2.96

[a] MDA-MB-231 cells were transfected with expression vectors for wild-type Keap1 and either wild-type Nrf2 or R7-Nrf2. The ratio of expression vectors for Keap1 and Nrf2 in the transfection was 1:4.

[b] The half-life of HA-Nrf2 was determined by pulse-chase labeling.

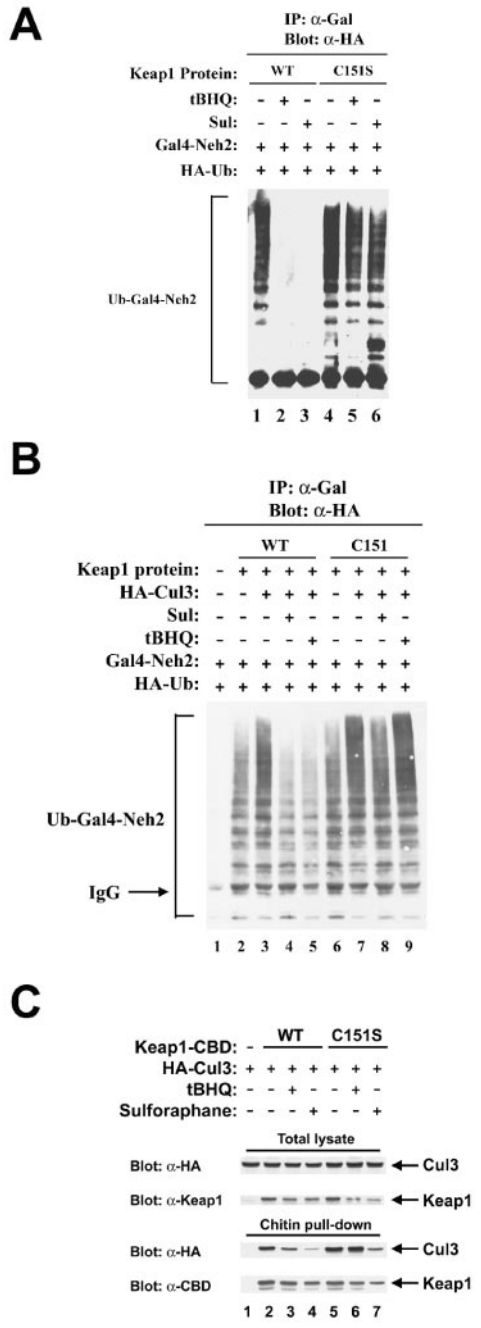

FIG. 6. (A) Sixty-millimeter-diameter dishes of MDA-MB-231 cells were transfected with expression vectors for HA-ubiquitin (Ub; 0.6 μg) and Gal4-Neh2 (0.7 μg) and for either wild-type (WT) Keap1 (0.7 μg, lanes 1 to 3) or Keap1-C151S (0.7 μg, lanes 4 to 6). The cells were either untreated (lanes 1 and 4) or treated with 50 μM tBHQ (lanes 2 and 5) or 20 μM sulforaphane (Sul, lanes 3 and 6) for 4 h prior to cell lysis. Anti-Gal4 immunoprecipitates (IP) were analyzed by immunoblot analysis with anti-HA antibodies. (B) Sixty-millimeter-diameter dishes of MDA-MB-231 cells were transfected with expression vectors for HA-Ub (0.6 μg), Gal4-Neh2 (0.7 μg), Cul3 (0.3 μg, lanes 3 to 5 and 7 to 9), and either wild-type Keap1 (0.4 μg, lanes 2 to 5) or Keap1-C151S (0.4 μg, lanes 6 to 9). The cells were either untreated (lanes 1 to 3, 6, and 7) or treated with 50 μM tBHQ (lanes 5 and 9) or 20 μM sulforaphane (lanes 4 and 8) for 4 h prior to cell lysis. Anti-Gal4 immunoprecipitates were analyzed by immunoblot analysis with anti-HA antibodies. IgG, immunoglobulin G. (C) Thirty-five-millimeterdiameter dishes of MDA-MB-231 cells were transfected with expression vectors for HA-Cul3 (0.5 μg) and either CBD-tagged wild-type Keap1 (0.5 μg, lanes 2 to 4) or CBD-tagged Keap1-C151S (0.5 μg, lanes 5 to 7). The cells were either left untreated (lanes 1, 2, and 5) or treated with 50 μM tBHQ (lanes 3 and 6) or 25 μM sulforaphane (lanes 4 and 7) for 5 h. Cell lysates were immunoblotted with the indicated antibodies (top two panels) or incubated with chitin beads. Proteins that remained bound to the chitin beads after extensive washing were analyzed by immunoblotting with the indicated antibodies (bottom two panels).

The ability of Nrf2 to associate with Keap1 was assessed following exposure of cells to either tBHQ or sulforaphane. In one experiment, MDA-MB-231 cells transfected with expression vectors for Keap1-CBD and HA-Nrf2 were treated with either tBHQ or sulforaphane for 5 h, and the level of Keap1-associated HA-Nrf2 was assessed by immunoblot analysis following affinity purification using chitin beads. The level of Keap1-associated HA-Nrf2 following treatment with either tBHQ or sulforaphane was markedly increased, in parallel with increased levels of HA-Nrf2 in total cell lysates (Fig. 7A, lanes 2 to 4, compare top and middle panels). In a second experiment, cells were transfected with an expression vector for Keap1-CBD, and levels of endogenous Nrf2 protein associated with Keap1-CBD were measured following affinity purification of Keap1-CBD (Fig. 7B). As expected, endogenous Nrf2 was copurified with Keap1 from MG132-treated cells (Fig. 7B, middle panel, lane 3). Endogenous Nrf2 was also copurified with Keap1 from both tBHQ and sulforaphane-treated cells (Fig. 7B, middle panel, lanes 4 and 5). In a third experiment, the level of endogenous Nrf2 associated with the endogenous Keap1 protein was determined by immunoblot analysis following immunoprecipitation with anti-Keap1 antibodies. As expected, elevated levels of Nrf2 in total lysates were observed in cells treated with tBHQ, sulforaphane, or MG132, either individually or in combination (Fig. 7C, upper panel). The level of endogenous Nrf2 present in anti-Keap1 immunoprecipitates was markedly increased in cells treated with tBHQ, sulforaphane, or MG132 (Fig. 7C, lower panel, lanes 3 to 8). Taken together, these results suggest that the ability of Keap1 to assemble into a functional E3 ubiquitin ligase complex, not the ability of Nrf2 to associate with Keap1, is the critical factor that is perturbed by these chemical inducers of Nrf2-dependent transcription.

DISCUSSION

In this report, we demonstrate that Keap1 functions as a substrate adaptor protein for a Cul3-dependent E3 ubiquitin ligase complex to target Nrf2 for proteosome-mediated degradation under normal culture conditions. Upon exposure to chemical inducers of Nrf2-dependent transcription, Keap1-dependent ubiquitination of Nrf2 is inhibited, leading to accumulation of Nrf2 and allowing subsequent activation of Nrf2-dependent transcription. Mutation of a single cysteine residue in the BTB domain of Keap1 markedly reduces inhibition of Keap1-dependent ubiquitination of Nrf2 by oxidative stress or sulforaphane. The ability of Keap1 to function as a redox-sensitive substrate adaptor protein for an E3 ubiquitin ligase

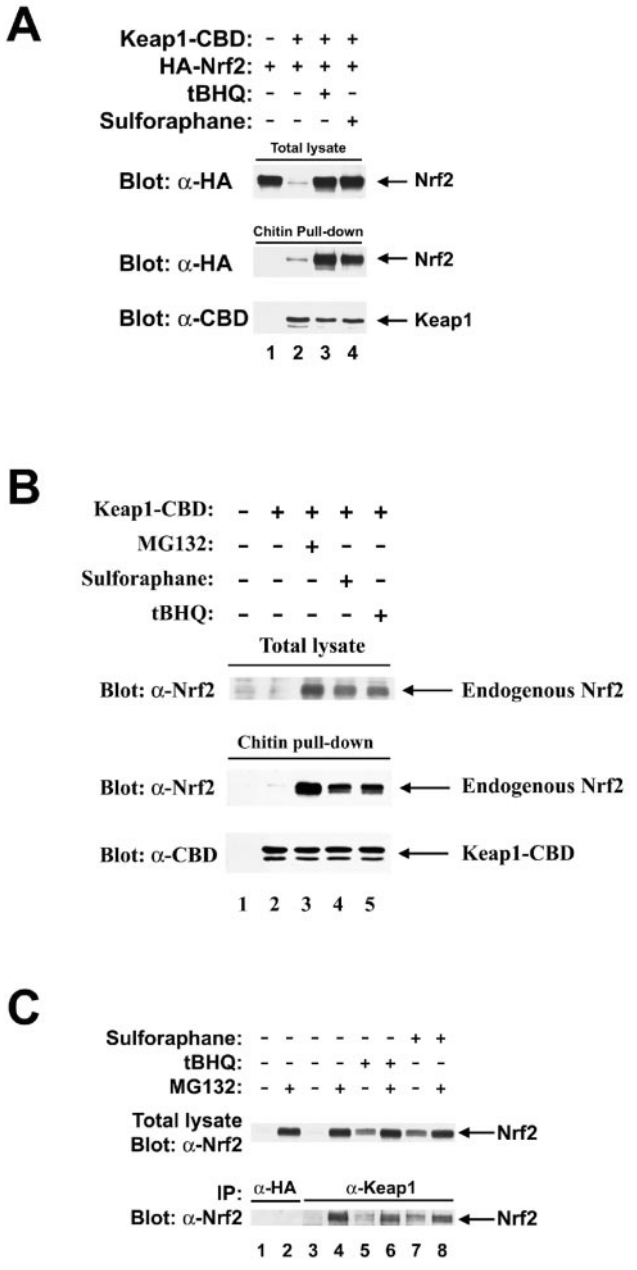

FIG. 7. (A) Sixty-millimeter-diameter dishes of MDA-MB-231 cells were transfected with expression vectors for HA-Nrf2 (0.32 μg, lanes 1 to 4) and Keap1-CBD (0.16 μg, lanes 2 to 4). The cells were either left untreated (lanes 1 and 2) or treated with 50 μM tBHQ (lane 3) or 25 μM sulforaphane (lane 4) for 5 h. Cell lysates were immunoblotted with anti-HA antibodies (top panel) or incubated with chitin beads. Proteins that remained bound to the chitin beads after extensive washing were analyzed by immunoblotting with either anti-HA (middle panel) or anti-CBD (bottom panel) antibodies. (B) Sixty-millimeter-diameter dishes of MDA-MB-231 cells were transfected with an expression vector for Keap1-CBD (2 μg, lanes 2 to 5) and treated with 10 μM MG132 (lane 3), 20 μM sulforaphane (lane 4), or 50 μM tBHQ (lane 5) for 5 h. Total cell lysates were subjected to immunoblot analysis with anti-Nrf2 antibodies (top panels) or incubated with chitin beads (middle and bottom panels). Proteins that remained bound to the chitin beads after extensive washing were analyzed by immunoblot analysis using either anti-Nrf2 antibodies (middle panel) or anti-CBD antibodies (bottom panel). (C) One hundred-millimeter-diameter dishes of MDA-MB-231 cells were either untreated (lanes 1 and 3) or treated for 5 h with 10 μM MG132 (even-numbered lanes), 50 μM tBHQ (lanes 5 and 6), or 25 μM sulforaphane (lanes 7 and 8). Total cell lysates were collected, and 2% of the cell lysate was subjected to immunoblot analysis with anti-Nrf2 antibodies (top panel). The remainder of the lysate was subjected to immunoprecipitation with anti-HA antibodies (bottom panel, lanes 1 and 2) or affinity-purified anti-Keap1 antibodies (bottom panel, lanes 3 to 8). The immunoprecipitated proteins (IP) were analyzed by immunoblotting with anti-Nrf2 antibodies.

complex constitutes a novel mechanism by which cells are able to sense and respond to electrophilic chemicals and oxidative stress.

Keap1 is one of more than 50 human proteins that share an N-terminal BTB domain, a central linker domain, and a C-terminal Kelch domain. BTB-Kelch proteins appear to have diverse biological roles in the regulation of the cytoskeleton (7, 30, 48). In *Drosophila melanogaster*, the Kelch protein, which is the founding member of the BTB-Kelch family, binds actin and regulates the cross-linking of actin filaments at ring canals that form between cells in the *D. melanogaster* oocyte (30). In humans, mutations within the GAN1 gene cause giant axonal neuropathy, an autosomal recessive disease characterized by defects in intermediate filament organization in sensorimotor neurons (6). Our results provide direct biochemical evidence that Keap1 assembles into a functional E3 ubiquitin ligase complex with Cul3 and Rbx1. Several other BTB-Kelch proteins are able to associate with Cul3, including GAN1 (19). Furthermore, residues within the BTB domain of Keap1 that are conserved in other BTB-Kelch proteins, including a highly conserved serine residue within the BTB domain, S104, are required for ubiquitination of Nrf2 (S.-C. Lo and M. Hannink, unpublished data) and repression of Nrf2-dependent transcription (62). A glycine substitution at the corresponding residue in GAN1 has been reported in a patient with giant axonal neuropathy (6). We suggest that the ability of BTB-Kelch proteins to function as substrate adaptors for Cul3-dependent E3 ubiquitin ligase complexes reflects a conserved biochemical function that underlies their diverse biological functions.

The Skp1 protein, which functions as a linker between Cul1 and F-box substrate adaptor proteins, contains a BTB domain fold (61). The crystal structure of the Skp1-Cul1-Rbx1 complex has been used to predict amino acids required for association of MEI-26, a *C. elegans* BTB domain protein, with the *C. elegans* Cul3 protein (58, 61). Therefore, we constructed two mutant Keap1 proteins that contained alanine substitutions in place of the corresponding residues in Keap1. To our surprise, these mutant Keap1 proteins displayed an increased ability to associate with Cul3 and Rbx1. Our results suggest that Keap1 interacts with Cul3 in a manner distinct from the way in which Skp1 interacts with Cul1. In other experiments, we find that the BTB domain of Keap1 is not sufficient to associate with Cul3 (data not shown), in agreement with a recent report by Yamamoto and coworkers suggesting that the linker domain of Keap1 is critically required for association with Cul3 (31). Nevertheless, the BTB domain of Keap1 is required for efficient down-regulation of steady-state levels of Nrf2 (31, 60). Furthermore, mutations within the BTB domain of Keap1, as reported in the present study, can result in increased association with Cul3 and increased levels of autoubiquitination. Im-

portantly, these mutant Keap1 proteins are impaired in their ability to efficiently target Nrf2 for ubiquitination and subsequent proteosome-mediated degradation. These results suggest that the balance between ubiquitination of substrate (Nrf2) and substrate adaptor (Keap1) may contribute to regulation of the Keap1-Cul3-Rbx1 E3 ubiquitin ligase complex, perhaps by regulating steady-state levels of Keap1.

Substrate ubiquitination by cullin-dependent E3 ubiquitin ligase complexes is often tightly regulated by changes in cell physiology induced by environmental signals or cell cycle progression. For example, the well-characterized F-box protein βTrCP, which functions as a substrate adaptor protein for Cul1, only recognizes substrate proteins that are phosphorylated on two serine residues embedded within a conserved sequence motif of DSGϕXS (57). As a result, the activity of the $SCF1^{\beta TrCP}$ E3 ubiquitin ligase towards its substrates, which include IκBα and β-catenin, is regulated at the level of substrate binding. We find that inducers of Nrf2, including sulforaphane and quinone-induced oxidative stress, result in accumulation of Nrf2 but do not abolish the ability of Nrf2 to bind to Keap1. Thus, the Keap1-dependent E3 ubiquitin ligase complex, in contrast to other cullin-dependent E3 ubiquitin ligase complexes typified by the $SCF1^{\beta TrCP}$ complex, is not regulated at the level of substrate binding.

Recent experiments suggest that E3 ubiquitin ligase complexes that assemble around cullin scaffolds undergo cycles of assembly and disassembly that enable exchange of the core cullin-Rbx1 complex between different substrate adaptor proteins (12, 56). Substrate adaptor exchange is likely to be an important mechanism by which a new substrate molecule is brought into the complex. This substrate adaptor exchange model provides an attractive paradigm for understanding how Keap1-dependent ubiquitination of Nrf2 is regulated by oxidative stress or sulforaphane. Our results demonstrate that both sulforaphane and quinone-induced oxidative stress result in reduced association between Keap1 and Cul3. In contrast, association between Cul3 and the Keap1-C151S mutant protein, which is markedly resistant to inhibition by both sulforaphane and quinone-induced oxidative stress, is not significantly perturbed by either inducer of Nrf2. We propose that exposure of cells to sulforaphane or oxidative stress, by altering the redox state of Cys 151, reduces the ability of Nrf2-bound Keap1 proteins to associate with the Cul3-Rbx1 core complex. As a result, fewer Nrf2 molecules will be targeted for ubiquitination and subsequent degradation, leading to increased accumulation of Nrf2.

Nrf2 must also escape Keap1-mediated cytoplasmic sequestration in order to accumulate in the nucleus and activate gene expression. Keap1 binds to actin via its Kelch repeat domain and pharmacological disruption of the actin cytoskeleton enables Nrf2 to escape Keap1-mediated sequestration in the cytoplasm (29). Inducers of Nrf2 may perturb the ability of Keap1 to associate with the actin cytoskeleton and thus enable release of Nrf2 into the nucleus. However, our results indicate that neither sulforaphane nor quinone-induced oxidative stress results in quantitative release of Nrf2 from Keap1. An alternative possibility is suggested by the observation that ongoing protein synthesis is required for accumulation of Nrf2 in the nucleus (24, 26). We propose that, under normal conditions, a single Keap1 protein is able to target multiple Nrf2 proteins for destruction. However, when the ability of Keap1 to efficiently target Nrf2 proteins for degradation is inhibited, each Keap1 protein (or Keap1 dimer) (62), is only able to sequester a single Nrf2 protein. Thus, newly synthesized Nrf2 proteins will no longer be bound by Keap1 proteins and, instead, accumulate in the nucleus following transport from the cytoplasm. Consistent with this saturation model, overexpression of a Neh-GFP fusion protein enables nuclear accumulation of endogenous Nrf2 (5).

The ability of structurally diverse chemicals to activate Nrf2-dependent gene expression correlates closely with their reactivity toward thiols (17). Talalay and coworkers have identified four cysteine residues in Keap1 (Cys 257, Cys 273, Cys 288, and Cys 297) that are preferentially labeled following in vitro exposure of purified Keap1 to a cysteine-reactive alkylating agent (16, 52). Mutant Keap1 proteins containing serine substitutions for two of these residues (Cys 273 and Cys 288) are impaired in their ability to target Nrf2 for ubiquitination and to repress Nrf2-dependent gene expression in transfected cells (52, 60). In a previous study, members of our laboratory demonstrated that Cys 151 is required for both a novel redox-dependent alteration in Keap1 in cells exposed to oxidative stress and the ability of oxidative stress to activate Nrf2-dependent gene expression (60). In the present work, we demonstrate that Cys 151 is required for inhibition of Keap1-dependent ubiquitination of Nrf2 by both sulforaphane and oxidative stress. Taken together, our results and those reported by Talalay's group suggest that multiple cysteine residues in Keap1 are capable of undergoing redox-dependent alterations. Identification of redox-dependent biochemical modifications that occur on the endogenous Keap1 protein will further our understanding of how cells sense the presence of reactive molecules and activate an Nrf2-dependent transcription program that protects sensitive biological molecules from chemical and oxidative damage.

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REFERENCES

1. **Alam, J., E. Killeen, P. Gong, R. Naquin, B. Hu, D. Stewart, J. R. Ingelfinger, and K. A. Nath.** 2003. Heme activates the heme oxygenase-1 gene in renal epithelial cells by stabilizing Nrf2. Am. J. Physiol. Renal Physiol. **284:**F743–F752.
2. **Ames, B. N., and M. K. Shigenaga.** 1993. DNA and free radicals, p. 1–15. *In* B. Halliwell and O. I. Aruoma (ed.), Oxidants are a major contributor to cancer and aging. Ellis Horwood, New York, N.Y.
3. **Andreassi, M. G.** 2003. Coronary atherosclerosis and somatic mutations: an overview of the contributive factors for oxidative DNA damage. Mutat. Res. **543:**67–86.
4. **Aoki, Y., H. Sato, N. Nishimura, S. Takahashi, K. Itoh, and M. Yamamoto.** 2001. Accelerated DNA adduct formation in the lung of the Nrf2 knockout mouse exposed to diesel exhaust. Toxicol. Appl. Pharmacol. **173:**154–160.
5. **Bloom, D. A., and A. K. Jaiswal.** 2003. Phosphorylation of Nrf2 at Ser^{40} by protein kinase C in response to antioxidants leads to the release of Nrf2 from INrf2, 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.
6. **Bomont, P., L. Cavalier, F. Blondeau, C. Ben Hamida, S. Belal, M. Tazir, E. Demir, H. Topaloglu, R. Korinthenberg, B. Tuysuz, P. Landrieu, F. Hentati, and M. Koenig.** 2000. The gene encoding gigaxonin, a new member of the cytoskeletal BTB/kelch repeat family, is mutated in giant axonal neuropathy. Nat. Genet. **26:**370–374.
7. **Bomont, P., and M. Koenig.** 2003. Intermediate filament aggregation in fibroblasts of giant axonal neuropathy patients is aggravated in non dividing cells and by microtubule destabilization. Hum. Mol. Genet. **12:**813–822.
8. **Buschmann, T., S. Y. Fuchs, C. G. Lee, Z. Q. Pan, and Z. Ronai.** 2000. SUMO-1 modification of Mdm2 prevents its self-ubiquitination and increases Mdm2 ability to ubiquitinate p53. Cell **101:**753–762.
9. **Butterfield, D. A., B. J. Howard, and M. A. LaFontaine.** 2001. Brain oxidative stress in animal models of accelerated aging and the age-related neurodegenerative disorders, Alzheimer's disease and Huntington's disease. Curr. Med. Chem. **8:**815–828.
10. **Chan, K., and Y. W. Kan.** 1999. Nrf2 is essential for protection against acute pulmonary injury in mice. Proc. Natl. Acad. Sci. USA **96:**12731–12736.
11. **Cho, H. Y., A. E. Jedlicka, S. P. Reddy, T. W. Kensler, M. Yamamoto, L. Y. Zhang, and S. R. Kleeberger.** 2002. Role of NRF2 in protection against hyperoxic lung injury in mice. Am. J. Respir. Cell Mol. Biol. **26:**175–182.
12. **Cope, G. A., and R. J. Deshaies.** 2003. COP9 signalosome: a multifunctional regulator of SCF and other cullin-based ubiquitin ligases. Cell **114:**663–671.
13. **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.
14. **Dalton, T. P., H. G. Shertzer, and A. Puga.** 1999. Regulation of gene expression by reactive oxygen. Annu. Rev. Pharmacol. Toxicol. **39:**67–101.
15. **Dhakshinamoorthy, S., and A. K. Jaiswal.** 2001. Functional characterization and role of INrf2 in antioxidant response element-mediated expression and antioxidant induction of NAD(P)H:quinone oxidoreductase1 gene. Oncogene **20:**3906–3917.
16. **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 sulfhydryl 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.
17. **Dinkova-Kostova, A. T., M. A. Massiah, R. E. Bozak, R. J. Hicks, and P. Talalay.** 2001. Potency of Michael reaction acceptors as inducers of enzymes that protect against carcinogenesis depends on their reactivity with sulfhydryl groups. Proc. Natl. Acad. Sci. USA **98:**3404–3409.
18. **Enomoto, A., K. Itoh, E. Nagayoshi, J. Haruta, T. Kimura, T. O'Connor, T. Harada, and M. Yamamoto.** 2001. High sensitivity of Nrf2 knockout mice to acetaminophen hepatotoxicity associated with decreased expression of ARE-regulated drug metabolizing enzymes and antioxidant genes. Toxicol. Sci. **59:**169–177.
19. **Furukawa, M., Y. J. He, C. Borchers, and Y. Xiong.** 2003. Targeting of protein ubiquitination by BTB-Cullin 3-Roc1 ubiquitin ligases. Nat. Cell Biol. **5:**1001–1007.
20. **Geyer, R., S. Wee, S. Anderson, J. Yates, and D. A. Wolf.** 2003. BTB/POZ domain proteins are putative substrate adaptors for cullin 3 ubiquitin ligases. Mol. Cell **12:**783–790.
21. **Golden, T. R., D. A. Hinerfeld, and S. Melov.** 2002. Oxidative stress and aging: beyond correlation. Aging Cell **1:**117–123.
22. **Imlay, J. A.** 2003. Pathways of oxidative damage. Annu. Rev. Microbiol. **57:**395–418.
23. **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.
24. **Itoh, K., K. I. Tong, and M. Yamamoto.** 2004. Molecular mechanism activating nrf2-keap1 pathway in regulation of adaptive response to electrophiles. Free Radic. Biol. Med. **36:**1208–1213.
25. **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.
26. **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.
27. **Jaiswal, A. K.** 2004. Nrf2 signaling in coordinated activation of antioxidant gene expression. Free Radic. Biol. Med. **36:**1199–1207.
28. **Kamura, T., D. M. Koepp, M. N. Conrad, D. Skowyra, R. J. Moreland, O. Iliopoulos, W. S. Lane, W. G. Kaelin, Jr., S. J. Elledge, R. C. Conaway, J. W. Harper, and J. W. Conaway.** 1999. Rbx1, a component of the VHL tumor suppressor complex and SCF ubiquitin ligase. Science **284:**657–661.
29. **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.
30. **Kelso, R. J., A. M. Hudson, and L. Cooley.** 2002. Drosophila Kelch regulates actin organization via Src64-dependent tyrosine phosphorylation. J. Cell Biol. **156:**703–713.
31. **Kobayashi, A., M. I. Kang, H. Okawa, M. Ohtsuji, Y. Zenke, T. Chiba, K. Igarashi, and M. Yamamoto.** 2004. Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. Mol. Cell. Biol. **24:**7130–7139.
32. **Kwak, M. K., P. A. Egner, P. M. Dolan, M. Ramos-Gomez, J. D. Groopman, K. Itoh, M. Yamamoto, and T. W. Kensler.** 2001. Role of phase 2 enzyme induction in chemoprotection by dithiolethiones. Mutat. Res. **480–481:**305–315.
33. **Kwak, M. K., N. Wakabayashi, K. Itoh, H. Motohashi, M. Yamamoto, and T. W. Kensler.** 2003. Modulation of gene expression by cancer chemopreventive dithiolethiones through the Keap1-Nrf2 pathway. Identification of novel gene clusters for cell survival. J. Biol. Chem. **278:**8135–8145.
34. **Lee, J. M., M. J. Calkins, K. Chan, Y. W. Kan, and J. A. Johnson.** 2003. Identification of the NF-E2-related factor-2-dependent genes conferring protection against oxidative stress in primary cortical astrocytes using oligonucleotide microarray analysis. J. Biol. Chem. **278:**12029–12038.
35. **Lee, J. M., A. Y. Shih, T. H. Murphy, and J. A. Johnson.** 2003. NF-E2-related factor-2 mediates neuroprotection against mitochondrial complex I inhibitors and increased concentrations of intracellular calcium in primary cortical neurons. J. Biol. Chem. **278:**37948–37956.
36. **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.
37. **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.
38. **Motohashi, H., T. O'Connor, F. Katsuoka, J. D. Engel, and M. Yamamoto.** 2002. Integration and diversity of the regulatory network composed of Maf and CNC families of transcription factors. Gene **294:**1–12.
39. **Nathan, C.** 2002. Points of control in inflammation. Nature **420:**846–852.
40. **Nguyen, T., P. J. Sherratt, H. C. Huang, C. S. Yang, and C. B. Pickett.** 2003. Increased protein stability as a mechanism that enhances Nrf2-mediated transcriptional activation of the antioxidant response element. Degradation of Nrf2 by the 26 S proteasome. J. Biol. Chem. **278:**4536–4541.
41. **Nguyen, T., P. J. Sherratt, and C. B. Pickett.** 2003. Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. Annu. Rev. Pharmacol. Toxicol. **43:**233–260.
42. **Pickart, C. M.** 2001. Mechanisms underlying ubiquitination. Annu. Rev. Biochem. **70:**503–533.
43. **Pintard, L., J. H. Willis, A. Willems, J. L. Johnson, M. Srayko, T. Kurz, S. Glaser, P. E. Mains, M. Tyers, B. Bowerman, and M. Peter.** 2003. The BTB protein MEL-26 is a substrate-specific adaptor of the CUL-3 ubiquitin-ligase. Nature **425:**311–316.
44. **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 chemopreventive efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. Proc. Natl. Acad. Sci. USA **98:**3410–3415.
45. **Rao, A. V., and B. Balachandran.** 2002. Role of oxidative stress and antioxidants in neurodegenerative diseases. Nutr. Neurosci. **5:**291–309.
46. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
47. **Shih, A. Y., D. A. Johnson, G. Wong, A. D. Kraft, L. Jiang, H. Erb, J. A. Johnson, and T. H. Murphy.** 2003. Coordinate regulation of glutathione biosynthesis and release by Nrf2-expressing glia potently protects neurons from oxidative stress. J. Neurosci. **23:**3394–3406.
48. **Spence, H. J., I. Johnston, K. Ewart, S. J. Buchanan, U. Fitzgerald, and B. W. Ozanne.** 2000. Krp1, a novel kelch related protein that is involved in pseudopod elongation in transformed cells. Oncogene **19:**1266–1276.
49. **Stewart, D., E. Killeen, R. Naquin, S. Alam, and J. Alam.** 2003. Degradation of transcription factor Nrf2 via the ubiquitin-proteasome pathway and stabilization by cadmium. J. Biol. Chem. **278:**2396–2402.
50. **Talalay, P., and J. W. Fahey.** 2001. Phytochemicals from cruciferous plants protect against cancer by modulating carcinogen metabolism. J. Nutr. **131:** 3027S–3033S.
51. **Thimmulappa, R. K., K. H. Mai, S. Srisuma, T. W. Kensler, M. Yamamoto, and S. Biswal.** 2002. Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. Cancer Res. **62:**5196–5203.
52. **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.
53. **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.

54. **Wasserman, W. W., and W. E. Fahl.** 1997. Functional antioxidant responsive elements. Proc. Natl. Acad. Sci. USA **94:**5361–5366.
55. **Wilkins, A., Q. Ping, and C. L. Carpenter.** 2004. RhoBTB2 is a substrate of the mammalian Cul3 ubiquitin ligase complex. Genes Dev. **18:**856–861.
56. **Wolf, D. A., C. Zhou, and S. Wee.** 2003. The COP9 signalosome: an assembly and maintenance platform for cullin ubiquitin ligases? Nat. Cell Biol. **5:**1029–1033.
57. **Wu, G., G. Xu, B. A. Schulman, P. D. Jeffrey, J. W. Harper, and N. P. Pavletich.** 2003. Structure of a beta-TrCP1-Skp1-beta-catenin complex: destruction motif binding and lysine specificity of the SCF(beta-TrCP1) ubiquitin ligase. Mol. Cell **11:**1445–1456.
58. **Xu, L., Y. Wei, J. Reboul, P. Vaglio, T. H. Shin, M. Vidal, S. J. Elledge, and J. W. Harper.** 2003. BTB proteins are substrate-specific adaptors in an SCF-like modular ubiquitin ligase containing CUL-3. Nature **425:**316–321.
59. **Yoh, K., K. Itoh, A. Enomoto, A. Hirayama, N. Yamaguchi, M. Kobayashi, N. Morito, A. Koyama, M. Yamamoto, and S. Takahashi.** 2001. Nrf2-deficient female mice develop lupus-like autoimmune nephritis. Kidney Int. **60:**1343–1353.
60. **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.
61. **Zheng, N., B. A. Schulman, L. Song, J. J. Miller, P. D. Jeffrey, P. Wang, C. Chu, D. M. Koepp, S. J. Elledge, M. Pagano, R. C. Conaway, J. W. Conaway, J. W. Harper, and N. P. Pavletich.** 2002. Structure of the Cul1-Rbx1-Skp1-F boxSkp2 SCF ubiquitin ligase complex. Nature **416:**703–709.
62. **Zipper, L. M., and R. T. Mulcahy.** 2002. The Keap1 BTB/POZ dimerization function is required to sequester Nrf2 in cytoplasm. J. Biol. Chem. **277:** 36544–36552.