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Ubiquitination of Keap1, a BTB-Kelch Substrate Adaptor Protein for Cul3, Targets Keap1 for Degradation by a Proteasome-independent Pathway*

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Keap1 is a BTB-Kelch protein that functions as a substrate adaptor protein for a Cul3-dependent E3 ubiquitin ligase complex. Keap1 targets its substrate, the Nrf2 transcription factor, for ubiquitination and subsequent degradation by the 26 S proteasome. Inhibition of Keap1-dependent ubiquitination of Nrf2 increases steady-state levels of Nrf2 and enables activation of cytoprotective Nrf2-dependent genes. In this report, we demonstrate that Keap1 and three other BTB-Kelch proteins, including GAN1, ENC1, and Sarcosin, are ubiquitinated by a Cul3-dependent complex. Ubiquitination of Keap1 is markedly increased in cells exposed to quinone-induced oxidative stress, occurs in parallel with inhibition of Keap1-dependent ubiquitination of Nrf2, and results in decreased steady-state levels of Keap1, particularly in cells that are unable to synthesize glutathione. Degradation of Keap1 is independent of the 26 S proteasome, because inhibitors of the 26 S proteasome do not prevent loss of Keap1 following exposure of cells to quinone-induced oxidative stress. Our results suggest that a switch from substrate to substrate adaptor ubiquitination is a critical regulatory step that controls steady-state levels of both BTB-Kelch substrate adaptor proteins and their cognate substrates.

Oxidative stress results from an imbalance between the production and removal of reactive oxygen species and has been implicated in numerous pathophysiological settings, including cancer, neurodegeneration, aging, and cardiovascular disease (1–5). The induction of proteins that increase the anti-oxidant capacity of cells and enable restoration of intracellular redox homeostasis is a major protective mechanism against the damaging consequences of oxidative stress. The expression of many anti-oxidant and cytoprotective proteins, including classic phase 2 detoxification enzymes, chaperone proteins, anti-oxidant proteins, and proteins in the proteasomal degradation pathway, is regulated by Nrf2, a bZIP transcription factor (6–9). Induction of Nrf2-dependent genes occurs in response to chemical inducers of oxidative stress, including redox-cycling quinones and heavy metals (9–11). In addition, the cancer-protective properties of structurally diverse natural and synthetic anti-carcinogens derive, in large part, from induction of an Nrf2-dependent transcriptional program (6, 7, 12–14).

Nrf2 is controlled at the post-translational level by the cytoplasmic Keap1 protein (15–17). Keap1 is a member of the large BTB-Kelch protein family, more than 40 of which are encoded by the human genome (18). Recent reports by several groups have demonstrated that Keap1 functions as a substrate adaptor protein for a Cul3-Rbx1 E3[1] ubiquitin ligase complex (19–22). The N-terminal BTB domain and central linker region of Keap1 bind Cul3, whereas the C-terminal Kelch domain of Keap1 binds Nrf2 via residues located within loops that extend out from the bottom of the Kelch domain (20, 21, 23). Under conditions of homeostatic cell growth, Keap1 brings Nrf2 into the Cul3-Rbx1 complex and enables ubiquitin conjugation onto specific lysine residues located within the N-terminal Neh2 domain of Nrf2 (21). However, following exposure of cells to a wide variety of chemical inducers of Nrf2-dependent transcription, Keap1-dependent ubiquitination of Nrf2 is blocked, enabling Nrf2 to accumulate in the nucleus and activate expression of Nrf2-dependent genes (21, 24).

The molecular definition of Keap1 as a substrate adaptor protein for Cul3 provides a conceptual framework for understanding how Keap1-dependent ubiquitination of Nrf2 is regulated. In general, the six cullin proteins encoded by the human genome function as scaffold proteins that bring together a substrate protein and a ubiquitin-charged E2 ubiquitin conjugation (Ubc) protein (25, 26). The E2 protein does not associate directly with the cullin protein but is brought into the complex by the cullin-associated Rbx1 protein. Likewise, the substrate does not typically associate directly with the cullin protein but is brought into the complex by a substrate adaptor protein. Once the entire E3 ubiquitin ligase complex is assembled, the ubiquitin molecule is transferred from a conserved cysteine residue in the Ubc protein to one or more lysine residues in the substrate protein. Cullin-based E3 ubiquitin ligases typically catalyze the addition of a multiubiquitin chain onto the substrate protein and thereby target the substrate protein for proteasome-mediated degradation.

Cullin-based E3 ubiquitin ligase complexes are dynamic complexes that undergo cycles of assembly and disassembly (27, 28). The ability of cullin-based E3 ubiquitin ligase com-

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[1] The abbreviations used are: E3, ubiquitin-protein isopeptide ligase; E2, ubiquitin carrier protein; HA, hemagglutinin; DTT, dithiothreitol; CBD, C-terminal chitin binding domain; tBHQ, *tert*-butylhydroquinone; γGCS, γ-glutamyl cysteine synthase.

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plexes to undergo facile substrate adaptor exchange is a critical functional property, because each cullin protein supports the ability of a large number of substrate adaptor proteins to target their substrates for degradation. Several mechanisms have been suggested to account for facile exchange of substrate adaptor proteins by cullin-based E3 ubiquitin ligase complexes. In yeast, several well characterized F-box-containing substrate adaptor proteins, including Grr1, cdc4p, and Met30, all of which function as substrate adaptors for Cul1, are labile proteins that are subject to auto-ubiquitination by the same Cul1-Rbx1 complex that directs ubiquitination of their substrates (29, 30). Because F-box proteins play critical roles in the cell cycle and in the response of cells to environmental conditions, rapid degradation of a specific substrate adaptor protein may enable timely accumulation of its substrate protein. Rapid degradation of a substrate adaptor protein will also minimize competition for limiting amounts of the cullin-Rbx1 complex and provides a mechanism for substrate adaptor exchange (27). In mammalian cells, which contain numerous substrate adaptor proteins that specifically utilize individual cullin proteins, only two F-box proteins, Skp2 and βTrCP, are known to be regulated at the level of ubiquitination and proteasome-mediated degradation (31–34). Additional mechanisms have been uncovered that contribute to substrate adaptor exchange in mammalian cells. For example, the CAND1 protein has been proposed to displace a substrate adaptor protein from Cul1 following deneddylation of the Cul1 protein by the COP9 signalosome (28, 35, 36).

In this report, we demonstrate that Keap1 is ubiquitinated *in vivo* and *in vitro* by the same Cul3-Rbx1 complex that ubiquitinates its substrate, Nrf2. Other members of the BTB-Kelch protein family are also able to assemble into functional E3 ubiquitin ligase complexes with Cul3 that support ubiquitination of the respective BTB-Kelch protein, providing evidence that the ability to function as a substrate adaptor protein for Cul3 is a conserved property of this large family of proteins. Quinone-induced oxidative stress enhances ubiquitination of Keap1 and decreases steady-state levels of Keap1, resulting in a corresponding increase in steady-state levels of Nrf2. However, in contrast to Nrf2 degradation, which is blocked by inhibitors of the 26 S proteasome, proteasome inhibitors do not block degradation of Keap1. Keap1 ubiquitination and proteasome-independent degradation of Keap1 following exposure to quinone-induced oxidative stress is markedly enhanced in glutathione-deficient cells. The isothiocyanate sulforaphane, a well characterized cancer-preventive inducer of Nrf2-dependent transcription, inhibits Keap1-dependent ubiquitination of Nrf2 but does not induce Keap1 ubiquitination, indicating that Keap1 differentially responds to inducers of Nrf2-dependent transcription. Our results indicate that ubiquitination targets BTB-Kelch proteins for degradation by a proteasome-independent pathway and suggest that a switch from substrate to substrate adaptor ubiquitination is a critical regulatory step that controls steady-state levels of both BTB-Kelch substrate adaptor proteins and their cognate substrates.

MATERIALS AND METHODS

Construction of Recombinant DNA Molecules—Plasmids expressing wild type Keap1, Nrf2, Cul3, Cul3DN, Rbx1, and HA-Ub proteins have been previously described (21). cDNA clones of GAN1, ENC1, and sarcosin were purchased from ATCC (American Type Culture Collection). The CBD-tagged versions of GAN1, ENC1, and sarcosin were generated by insertion of a PCR-generated DNA fragment encoding the chitin binding domain of *Bacillus circulans* chitinase A1 gene upstream of the stop codon. The integrity of all of the plasmids used in this study was confirmed by sequence analysis.

Cell Culture and Transfections—COS-1 and MDA-MB-231 cells were purchased from ATCC. 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. GCS-2 cells were grown in knockout Dulbecco's modified Eagle's medium supplemented with 15% ES-cell qualified bovine serum, 2.5 mM glutathione, and 100 μM β-mercaptoethanol. Transfections were performed with Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions.

Antibodies, Immunoprecipitation, and Immunoblot Analysis—The anti-Keap1 antibody has been described previously (24). Antibodies against Nrf2 and Myc (Santa Cruz Biotechnology), ubiquitin (Sigma), chitin binding domain (New England Biolabs), and HA (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% SDS, 10% glycerol, 100 mM DTT, 0.1% bromphenol blue) at 48 h post-transfection. For immunoprecipitation assays, cells were lysed in radioimmune precipitation assay 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 mixture (Sigma). Cell lysates were pre-cleared 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.

In Vivo Ubiquitination—For detection of ubiquitinated proteins *in vivo*, cells were transfected with expression vectors for HA-ubiquitin and the indicated proteins. Expression vectors for HA-Cul3 and Myc-Rbx1 were included in some of experiments, as indicated. Cells were rapidly 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-protein 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 5-fold in buffer lacking SDS and incubated with anti-Keap1, anti-Nrf2, or anti-CBD antibodies accordingly. Immunoprecipitated proteins were analyzed by immunoblot with antibodies directed against the HA epitope.

In Vitro Ubiquitination—For ubiquitination of the BTB-Kelch proteins *in vitro*, COS-1 cells were transfected with expression vectors for the individual CBD-tagged BTB-Kelch protein, HA-Cul3, and Myc-Rbx1. The transfected cells were lysed in buffer B (15 mM Tris-HCL (pH 7.4), 500 mM NaCl, and 0.25% Nonidet P-40) containing 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture. The lysates were pre-cleared 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% (v/v) glycerol, 1 mM EDTA, 0.01% Nonidet P-40 and 0.1 M NaCl), and twice with reaction buffer (50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM NaF, and 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 3000 × *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 5-fold with buffer lacking SDS prior to immunoprecipitation with anti-CBD antibodies. Immunoprecipitated proteins were subjected to immunoblot analysis with anti-ubiquitin antibodies.

RESULTS

Assembly into a Functional Ubiquitin Ligase Complex Is a Conserved Property of BTB-Kelch Proteins—The major function of substrate adaptor proteins is to target specific substrate proteins for ubiquitination. However, ubiquitination of substrate adaptor proteins may also play an important role in cellular physiology. For example, auto-ubiquitination of several yeast F-box proteins is responsible for their rapid turnover during progression through the cell cycle (29, 30).

To examine the possibility that Keap1 is ubiquitinated by a Cul3-Rbx1 complex *in vitro*, the Keap1-Cul3-Rbx1 complex was purified from COS-1 cells transfected with expression vectors for Keap1, Cul3, and Rbx1. To facilitate purification of Keap1 and Keap1-associated proteins, an expression vector encoding a

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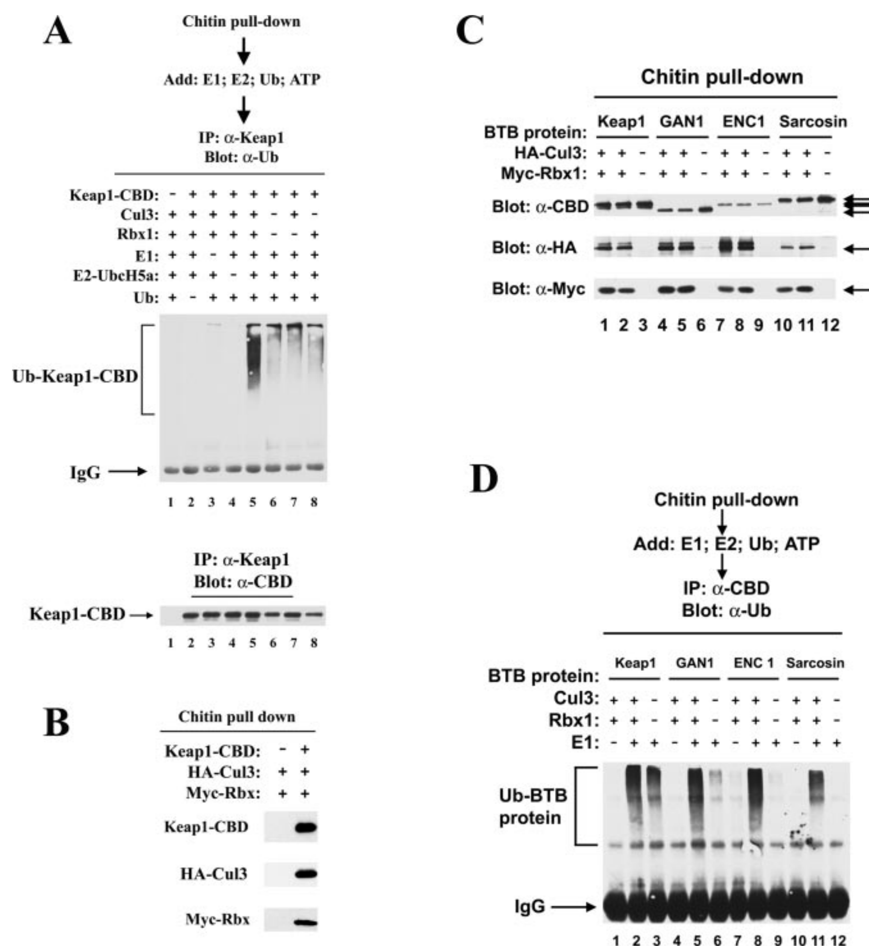

FIG. 1. *A*, cell lysates from COS1 cells transfected with expression vectors for Keap1-CBD, HA-Cul3, and Myc-Rbx1 were incubated with chitin beads. Proteins bound to chitin beads were incubated with E1, E2-UbcH5a, ubiquitin, and ATP. Following the reaction, proteins were eluted from the chitin beads by boiling, and equivalent aliquots of the eluted proteins were either subjected to immunoblot analysis with anti-CBD antibodies (*bottom panel*), or immunoprecipitated with anti-Keap1 antibodies (*top panel*). The anti-Keap1 immunoprecipitates were subject to immunoblot analysis with anti-ubiquitin antibodies. *B*, cell lysates from COS1 cells transfected with expression vectors for Keap1-CBD, HA-Cul3, and Myc-Rbx1 were incubated with chitin beads. Proteins bound to chitin beads were analyzed by immunoblot analysis for Keap1 (*top panel*), HA-Cul3 (*middle panel*), or Myc-Rbx1 (*bottom panel*). *C*, COS1 cells were co-transfected with CBD-tagged expression vectors for Keap1, GAN1, ENC1, or sarcosin, along with expression vectors for HA-Cul3 and Myc-Rbx1. Cell lysates were incubated with chitin beads. Proteins that associated with the chitin beads were eluted and analyzed by immunoblot with anti-CBD, anti-HA, and anti-Myc antibodies. *D*, a portion of the chitin beads from *C* were incubated with E1, E2-UbcH5a, ubiquitin, and ATP. The reactions were terminated by boiling and subjected to immunoprecipitation with anti-CBD antibodies. Anti-CBD immunoprecipitates were analyzed by immunoblot with anti-ubiquitin antibodies.

Keap1 protein containing a C-terminal chitin binding domain (CBD) was utilized in this experiment. Proteins that bound to the chitin beads were incubated with purified E1, E2-UbcH5a, ubiquitin, and ATP. Subsequently, anti-Keap1 immunoprecipitates were prepared under strongly denaturing conditions and subjected to immunoblot analysis using anti-ubiquitin antibodies (Fig. 1*A*, *top panel*). Keap1-ubiquitin conjugates were readily observed in the presence of both Cul3 and Rbx1 (Fig. 1*A*, *top panel*, *lane 5*), and required the addition of ubiquitin, E1, and E2-UbcH5a to the *in vitro* reaction (Fig. 2*C*, *top panel*, *lanes 2–4*). Immunoblot analysis of anti-Keap1 immunoprecipitates confirmed the presence of equivalent levels of Keap1-CBD in all reactions (Fig. 1*A*, *bottom panel*).

Keap1 is one of more than 40 BTB-Kelch proteins that are encoded by the human genome (18, 38). The members of the BTB-Kelch family share both a common domain organization and ~25% sequence identity, when compared on a one-to-one basis with each other. The biochemical functions of this family of proteins are, in general, not known, although the ability of Keap1 to function as a substrate adaptor protein suggests a common function for these proteins. To determine if other BTB-Kelch proteins may function as substrate adaptor proteins for Cul3, the ability of several different BTB-Kelch proteins to associate with Cul3 and Rbx1 to form a functional ubiquitin ligase complex was determined. The three BTB-Kelch proteins used for these experiments, GAN1, ENC1, and sarcosin, were selected because of their known or suspected roles in cancer and neurodegenerative diseases (39–43).

To facilitate purification and detection of these BTB-Kelch proteins, fusion proteins containing a CBD were constructed. Expression vectors for the CBD-tagged BTB-Kelch proteins were transfected into COS-1 cells alone or with expression vectors for HA-Cul3 and Myc-Rbx1. Cell lysates were subjected to affinity purification using chitin beads. Neither Cul3 nor Rbx1 purified with the chitin beads in the absence of a CBD-tagged BTB-Kelch protein (Fig. 1*B*). Each of these BTB-Kelch proteins was able to associate with both Cul3 and Rbx1, as determined by immunoblot analysis of the purified complexes using anti-HA or anti-Myc antibodies (Fig. 1*C*, *middle* and *bottom panels*, respectively).

The ability of the complexes formed between the respective BTB-Kelch proteins, Cul3 and Rbx1, to support ubiquitin conjugation onto the BTB-Kelch protein was determined. The chitin beads containing the purified complexes were incubated with E1, E2-UbcH5a, ubiquitin, and ATP. Subsequently, anti-CBD immunoprecipitates prepared under strongly denaturing conditions were subjected to immunoblot analysis with anti-ubiquitin antibodies. Ubiquitin conjugation, onto each of the BTB-Kelch proteins that was dependent upon the addition of E1 into the *in vitro* reaction, was observed (Fig. 1*D*). The presence of ubiquitin conjugation onto Keap1 and GAN1 in the absence of co-expressed Cul3 and Rbx1 proteins presumably represents co-purification of endogenous Cul3 and Rbx1 proteins with these two BTB-Kelch proteins (Fig. 1*D*, *lanes 3* and *6*).

Keap1 Is Ubiquitinated by a Cul3-Rbx1 Complex in Vivo—To determine if Keap1 is ubiquitinated *in vivo*, MDA-MB-231 cells were co-transfected with expression vectors for HA-ubiquitin, HA-Cul3, and Keap1 and increasing amounts of the expression vector for Myc-Rbx1. Cells were lysed under denaturing conditions and subjected to immunoprecipitation with Keap1 antibodies. The presence of ubiquitin-conjugated Keap1 proteins was assessed by immunoblot analysis of anti-Keap1 immunoprecipitates with anti-HA antibodies. Ubiquitin conjugation

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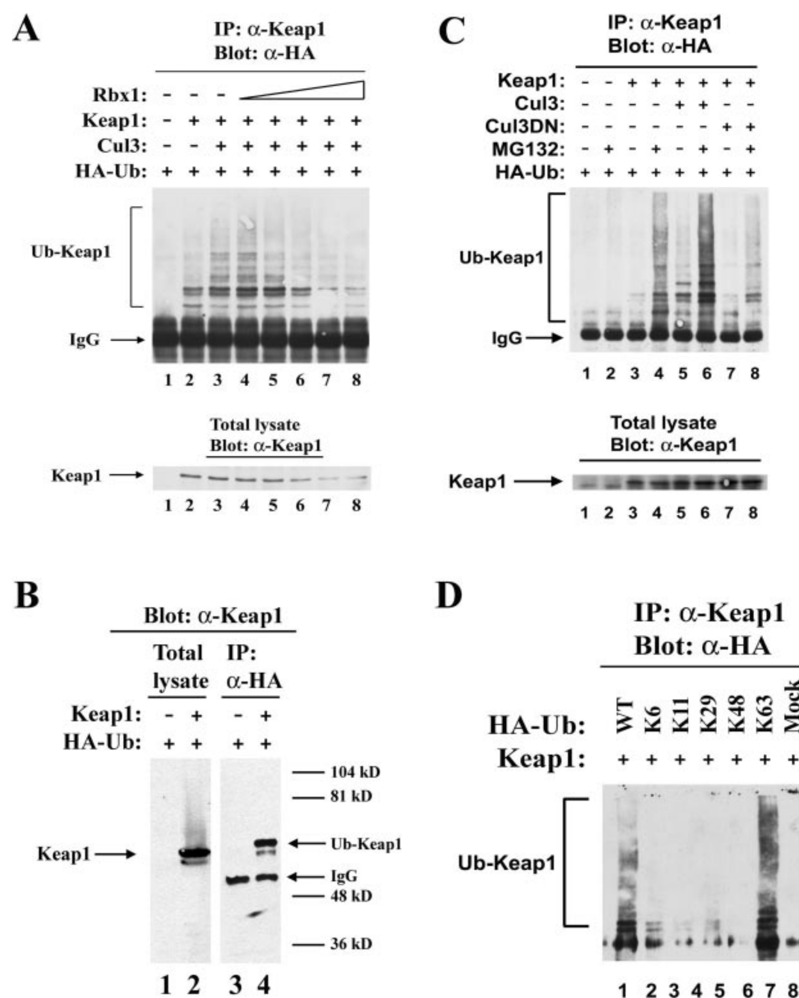

FIG. 2. *A*, MDA-MB-231 cells were transfected with expression vectors for HA-ubiquitin, Keap1, and HA-Cul3 and increasing amounts of an expression vector for Rbx1. Cells were lysed under denaturing conditions and subjected to immunoblot analysis with Keap1 antibodies (*bottom panel*). Equivalent aliquots of each lysates were subjected to immunoprecipitation with anti-Keap1 antibodies. Anti-Keap1 immunoprecipitates were analyzed by immunoblot with anti-HA antibodies (*top panel*). *B*, 293T cells were transfected with expression vectors for HA-ubiquitin and Keap1. Total lysates (*left panel*) or anti-HA immunoprecipitates (*right panel*) were analyzed by immunoblot with anti-Keap1 antibodies. The samples were analyzed in parallel on adjacent lanes. The left panel was exposed for a shorter time than the right panel. *C*, MDA-MB-231 cells were transfected with expression vectors for HA-ubiquitin and Keap1 and the expression vector for either Cul3 or Cul3DN. Cells were either left untreated or treated with 10 μM MG132 for 4 h prior to lysis. Equivalent aliquots of each lysates were analyzed by immunoblot with anti-Keap1 antibodies (*bottom panel*) or subjected to immunoprecipitation with anti-Keap1 antibodies. Anti-Keap1 immunoprecipitates were analyzed by immunoblot with anti-HA antibodies (*top panel*). *D*, cell lysates from MDA-MB-231 cells transfected with expression vectors for Keap1 and the indicated wild-type or mutant HA-ubiquitin proteins were immunoprecipitated with anti-Keap1 antibodies. The immunoprecipitated proteins were analyzed by immunoblot with anti-HA antibodies.

onto ectopically expressed Keap1 was readily observed and was increased by coexpression of Cul3 (Fig. 2*A*, *top panel*, *lanes 1–3*). Low levels of co-expressed Rbx1 slightly increased the levels of ubiquitin conjugation onto Keap1 (Fig. 2*A*, *top panel*, *lane 4*). Increased Rbx1 expression did not further increase Keap1 ubiquitination, but markedly decreased steady-state levels of Keap1 in a dose-dependent manner (Fig. 2*A*, *lanes 5–8*).

To confirm that Keap1 is ubiquitinated *in vivo*, cell lysates from 293T cells transfected with expression vectors for Keap1 and HA-ubiquitin were immunoprecipitated with anti-HA antibodies. The presence of Keap1 in anti-HA immunoprecipitates was confirmed by immunoblot analysis with anti-Keap1 antibodies (Fig. 2*B*).

To define the role of Cul3 in ubiquitination of Keap1 *in vivo*, we determined the ability of a dominant-negative Cul3 protein lacking the C-terminal Rbx1 binding domain to block Keap1 ubiquitination. Cell lysates were collected from MDA-MB-231 cells transfected with expression vectors for Keap1 and either wild-type or dominant-negative Cul3. As expected, co-expression of Keap1 with the wild-type Cul3 protein increased ubiquitination onto Keap1 (Fig. 2*C*, *top panel*, *lanes 3–6*). Co-expression of Keap1 with the dominant-negative Cul3 protein decreased levels of ubiquitin-conjugated Keap1 (Fig. 2*C*, *top panel*, *lanes 7* and *8*). Treatment of the transfected cells with MG132 prior to collection of cell lysates enhanced ubiquitin conjugation onto Keap1 (Fig. 2*C*, *top panel*, compare *even* and *odd lanes*) but did not alter steady-state levels of Keap1 (Fig. 2*C*, *bottom panel*, compare *even* and *odd lanes*). Taken together, these results indicate that ubiquitination of Keap1 is mediated by an E3 ubiquitin ligase complex that contains Cul3 and Rbx1.

A panel of mutant ubiquitin proteins containing a single lysine residue was evaluated for their ability to participate in multiubiquitination onto Keap1. Prominent ubiquitin conjugation onto Keap1 was only observed in the presence of either wild-type ubiquitin (Fig. 2*D*, *lane 1*) or a mutant ubiquitin protein containing only lysine 63 (Fig. 2*D*, *lane 7*).

Increased Ubiquitination Results in Proteasome-independent Degradation of Keap1—In our previous work, we have identified several Keap1 mutants that display increased association with Cul3 (21). To determine if increased association with Cul3 correlates with increased auto-ubiquitination of Keap1, one of these mutant Keap1 proteins, Keap1-125A3, which contains three alanine substitutions for conserved residues located within the BTB domain of Keap1, was further characterized. In MDA-MB-231 cells, ubiquitin conjugation onto the Keap1-125A3 protein was markedly elevated relative to the wild-type Keap1 protein (Fig. 3*A*, *top panel*) and correlated with reduced steady-state levels of the Keap1-125A3 protein (Fig. 3*A*, *bottom panel*). Surprisingly, steady-state levels of the Keap1-125A3 protein were not increased following treatment with MG132, a well characterized proteasome inhibitor. (Fig. 3*A*, *bottom panel*, compare *lanes 4* and *5*). To extend this observation, several other proteasome inhibitors, including clasto-lactacystin β-lactone and epoxomicin, were tested for their ability to increase steady-state levels of the Keap1-125A3 protein. None of these proteasome inhibitors increased steady-state levels of the wild-type or Keap1-125A3 proteins (Fig. 3*B*). Taken together, these observations indicate that ubiquitination of

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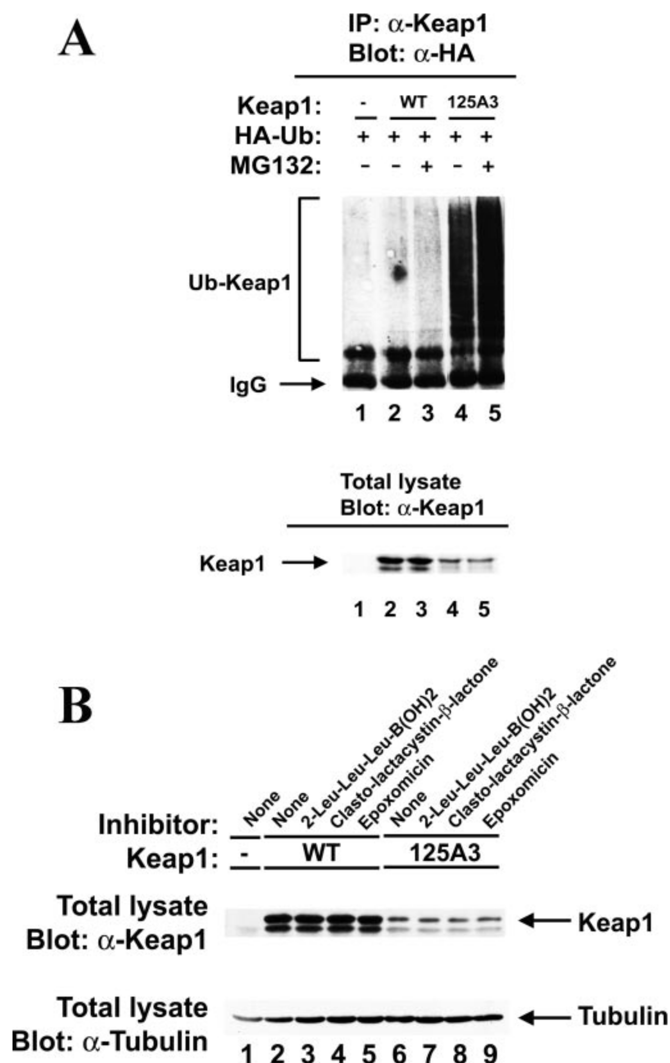

FIG. 3. *A*, MDA-MB-231 cells were transfected with expression vectors for either wild type or mutant Keap1 proteins and expression vectors for HA-ubiquitin. Cells were either left untreated or treated with MG132. Cells were lysed under the denaturing condition, and equivalent aliquots were subjected to immunoblot analysis with anti-Keap1 antibodies (*bottom panel*) or to immunoprecipitation with anti-Keap1 antibodies (*top panel*). Anti-Keap1 immunoprecipitates were analyzed by immunoblot with anti-HA antibodies (*top panel*). *B*, MDA-MB-231 cells transfected with expression vectors for either wild type or mutant Keap1 proteins were treated with Me_2SO, 2-Leu-Leu-Leu-B(OH)2 (1 μM), clasto-lactacystin β-lactone (10 μM), or expoxomicin (1 μM) for 4 h prior to collection of cell lysates. Equivalent amounts of each cell lysate were analyzed by immunoblot with anti-Keap1 antibodies (*top panel*). The blot was stripped and reprobed with anti-tubulin antibodies (*bottom panel*).

Keap1 targets Keap1 for degradation in a proteasome-independent manner.

Quinone-induced Oxidative Stress Increases Ubiquitination and Decreases Steady-state Levels of Keap1—The ability of Keap1 to function as a substrate adaptor protein that targets Nrf2 for ubiquitination is perturbed by chemically diverse compounds, resulting in stabilization of Nrf2 and activation of Nrf2-dependent transcription. For example, both the chemopreventive isothiocyanate sulforaphane and oxidative stress induced by redox cycling of *tert*-butylhydroquinone (tBHQ) inhibit Keap1-dependent ubiquitination of Nrf2 (21, 24). To determine if ubiquitination of Keap1 is altered under conditions that inhibit Keap1-dependent ubiquitination of Nrf2, the presence of ubiquitin-Keap1 conjugates was assessed in MDA-MB-231 cells transfected with an expression vector for Keap1 and exposed to either tBHQ or sulforaphane. Treatment of cells with tBHQ increased ubiquitin conjugation onto Keap1 in a time-dependent manner (Fig. 4*A*, *top panel*, *lanes 4* and *5*). In parallel, decreased steady-state levels of Keap1 were observed in tBHQ-treated cells (Fig. 4*A*, *bottom panel*, *lanes 4* and *5*). In contrast, ubiquitin conjugation onto Keap1 was not increased in sulforaphane-treated cells, with no alteration in steady-state levels of Keap1 (Fig. 4*A*, top and *bottom panels*, *lanes 6* and *7*). These results indicate that quinone-induced oxidative stress, but not sulforaphane, increases ubiquitination onto Keap1, resulting in decreased steady-state levels of Keap1.

To determine if oxidative stress increases ubiquitination conjugation or steady-state levels of the endogenous Keap1 protein, we first determined if ubiquitin conjugation onto the endogenous Keap1 protein could be detected. The endogenous Keap1 protein was immunoprecipitated by anti-Keap1 antibodies conjugated onto Sepharose beads after cells were lysed under strongly denaturing conditions. Anti-Keap1 immunoprecipitates were subjected to immunoblot analysis with anti-ubiquitin antibodies to detect ubiquitin-Keap1 conjugates. Multiple slowly migrating bands representing the ubiquitin-conjugated forms of Keap1 were readily detected in untreated cells (Fig. 4*B*, *top panel*, *lane 2*). The high molecular weight bands were specific to anti-Keap1 immunoprecipitates, as low background levels of cross-reactivity in immunoblot analysis using the anti-ubiquitin antibody was observed with anti-HA immunoprecipitates (Fig. 4*B*, *top panel*, *lane 1*). Anti-Keap1 immunoprecipitates from MDA-MB-231 cells exposed to tBHQ were analyzed in parallel. Comparable amounts of ubiquitin conjugation onto Keap1 were observed in anti-Keap1 immunoprecipitates from either untreated cells or cells treated with tBHQ for 16 h (Fig. 4*B*, *top panel*, compare *lane 2* with *lane 3*). However, the steady-state level of the endogenous Keap1 protein was markedly reduced in lysates from cells exposed to tBHQ (Fig. 4*B*, *bottom panel*, compare *lane 2* with *lane 3*).

A series of Keap1 deletion mutants were used to localize the domains of Keap1 required for ubiquitin conjugation. A Keap1 mutant lacking the N-terminal BTB domain (Keap1-ΔBTB) contained high levels of ubiquitin conjugation that was slightly increased following tBHQ treatment (Fig. 4*C*, *top panel*, *lanes 5* and *6*). Deletion of the central linker domain (Keap1-ΔL) reduced both basal and tBHQ-induced ubiquitination (Fig. 4*C*, *top panel*, *lanes 7* and *8*). Deletion of the C-terminal Kelch domain (Keap1-ΔK) increased both basal and tBHQ-induced ubiquitination (Fig. 4*C*, *top panel*, *lanes 9* and *10*). Finally, deletion of the C-terminal fifteen amino acids of Keap1 (Keap1-ΔC) did not alter basal levels of Keap1 ubiquitination, although tBHQ-induced ubiquitination was increased slightly (Fig. 4*C*, *top panel*, *lanes 11* and *12*). Taken together, these results suggest that one or more of the six lysine residues located in the central linker domain of Keap1 are the likely site(s) of ubiquitin conjugation *in vivo*.

Quinone-induced Degradation of Keap1 Is Enhanced in Cells Deficient in Glutathione Biosynthesis—Intracellular levels of glutathione, a major intracellular reductant, were reduced following exposure of cells to oxidative stress. In an effort to restore an intracellular reducing environment, synthesis of glutathione was enhanced following exposure of eukaryote cells to oxidative stress. The rate-limiting enzyme for glutathione biosynthesis is γ-glutamyl cysteine synthase (γGCS), composed of a large subunit (GCLM), which carries out catalysis, and a small regulatory subunit (GCLC). In vertebrates, Nrf2 regulates transcription of both the GCLM and GCLC genes (44, 45). Thus, oxidative stress, by acting on Keap1, activates an autoregulatory feedback mechanism that counteracts the damaging effects of oxidative stress.

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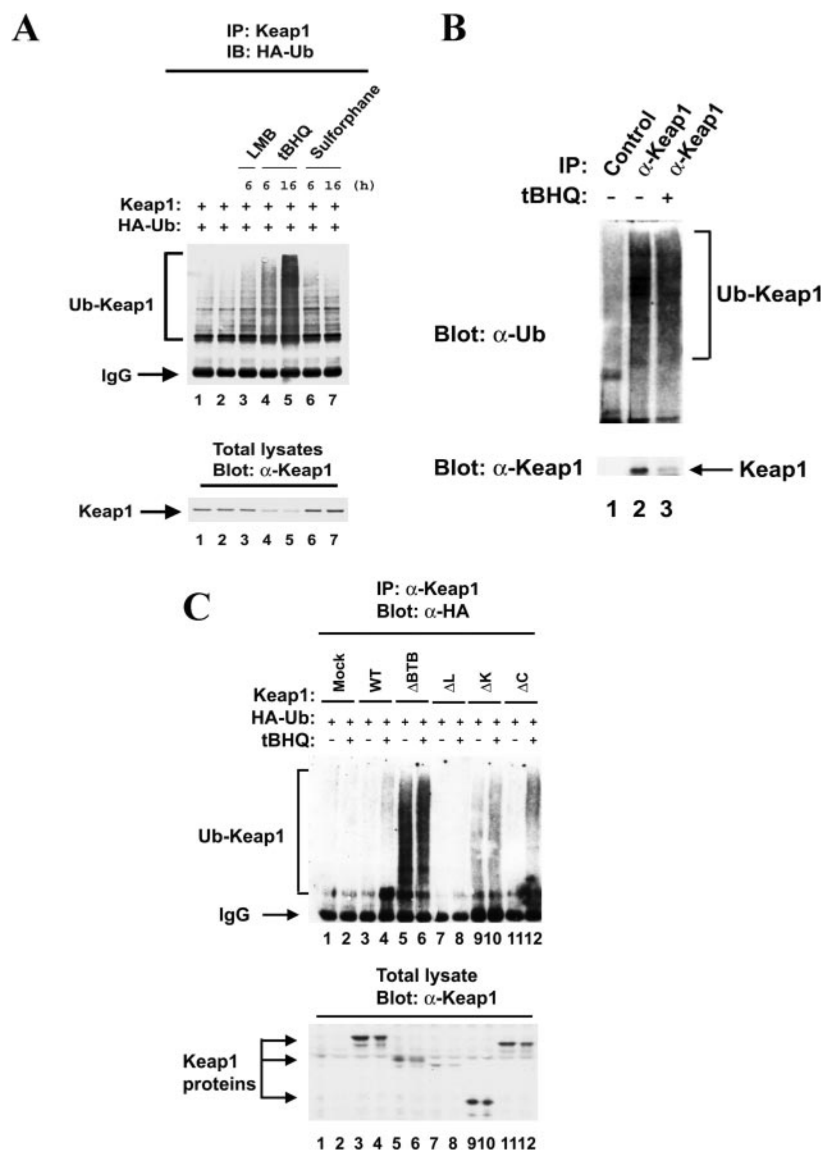

FIG. 4. *A*, MDA-MB-231 cells were transfected with expression vectors for HA-ubiquitin and Keap1. As indicated, the transfected cells were exposed to Me_2SO, 10 μM leptomycin B (*LMB*), 50 μM tBHQ, or 20 μM sulforaphane for 6 or 16 h prior to cell lysis. Equivalent aliquots of cell lysates were subjected to immunoblot analysis with anti-Keap1 antibodies (*bottom panel*) or immunoprecipitated with anti-Keap1 antibodies. Anti-Keap1 immunoprecipitates were analyzed by immunoblot with anti-HA antibodies (*top panel*). *B*, MDA-MB-231 cells were either left untreated or treated with 50 μM tBHQ for 16 h prior to lysis under denaturing conditions. Equivalent amounts of cell lysates were immunoprecipitated with either anti-HA or anti-Keap1 antibodies conjugated to Sepharose beads. The immunoprecipitated proteins were subjected to immunoblot analysis with anti-ubiquitin antibodies for detection of endogenous Keap1-ubiquitin conjugates (*upper panel*). The blot was stripped and re-probed with anti-Keap1 antibodies to detect levels of Keap1 (*bottom panel*). *C*, MDA-MB-231 cells were transfected with expression vectors for HA-ubiquitin and the wild-type or mutant Keap1 proteins. The transfected cells were either left untreated or treated with 50 μM tBHQ overnight prior to lysis under denaturing conditions. Equivalent aliquots of each lysate were subjected to immunoblot analysis with anti-Keap1 antibodies (*bottom panel*) or immunoprecipitation with anti-Keap1 antibodies. Anti-Keap1 immunoprecipitates were analyzed by immunoblot with anti-HA antibodies (*top panel*).

To determine if reduced levels of glutathione would, by itself, be sufficient to inactivate Keap1-dependent ubiquitination of Nrf2, we characterized the ability of Keap1 to regulate Nrf2 in GCS-2 cells that are nullizygous for the large subunit of γGCS (37). GCS-2 cells were derived from blastocysts of mice in which both copies of the GCLM gene were disrupted by homologous recombination. Although mice that lack GCLM are unable to complete embryonic development due to increased apoptosis, GCS-2 cells are able to proliferate in culture when the culture media is supplemented with 2.5 mM glutathione. However, the intracellular glutathione level in the GCS-2 cells grown in the presence of 2.5 mM glutathione is ~2% of that found in normal cells (37).

We first characterized the ability of Keap1 to regulate Nrf2 in GCS-2 cells. Expression of the endogenous Nrf2 or Keap1 proteins could not be detected in GCS-2 cells (data not shown). However, ectopic expression of both Nrf2 and Keap1 in GCS-2 cells was readily accomplished by transient transfection. Despite the reduced levels of glutathione, Keap1 was able to efficiently down-regulate steady-state levels of Nrf2 in GCS-2 cells (Fig. 5*A*, compare *lanes 1* and *2*). Steady-state levels of Nrf2 were restored by treatment with the proteasome inhibitor MG132, confirming the ability of Keap1 to target Nrf2 for proteasome-mediated degradation in GCS-2 cells despite low intracellular levels of glutathione (Fig. 5*B*, *bottom panel*, compare *lanes 1* and *2*). Consistent with this observation, inhibition of γGCS function by buthionine sulfoximine treatment of MDA-MB-231 cells did not increase steady-state levels of Nrf2, either in the absence or presence of co-expressed Keap1 (data not shown). Taken together, these results indicate that reduced levels of glutathione are not sufficient to inhibit the ability of Keap1 to target Nrf2 for ubiquitination and proteasome-mediated degradation.

To determine if chemical inducers of Nrf2-dependent transcription are able to inhibit Keap1-mediated regulation of Nrf2 in GCS-2 cells, GCS-2 cells transfected with expression vectors for Keap1 and HA-Nrf2 were exposed to tBHQ or sulforaphane prior to cell lysis. Steady-state levels of Nrf2 or Keap1 were determined by immunoblot analysis with anti-HA or anti-Keap1 antibodies, respectively. Steady-state levels of Nrf2 were markedly increased when cells were treated with either tBHQ or sulforaphane for 4 h (Fig. 5*B*, *bottom panel*, *lanes 3* and *5*), or for 16 h (Fig. 5*C*, *bottom panel*, *lanes 3* and *5*). As was observed in MDA-MB-231 cells, sulforaphane did not alter the steady-state levels of Keap1 (Figs. 5*B* and 5*C*, *top panels*, *lanes 5* and *6*). In contrast, and in agreement with the previous observations in MDA-MB-231 cells, treatment of GCS-2 cells with tBHQ markedly reduced steady-state levels of Keap1, particularly following prolonged exposure to tBHQ (Figs. 5*B* and 5*C*, *top panels*, *lanes 3* and *4*).

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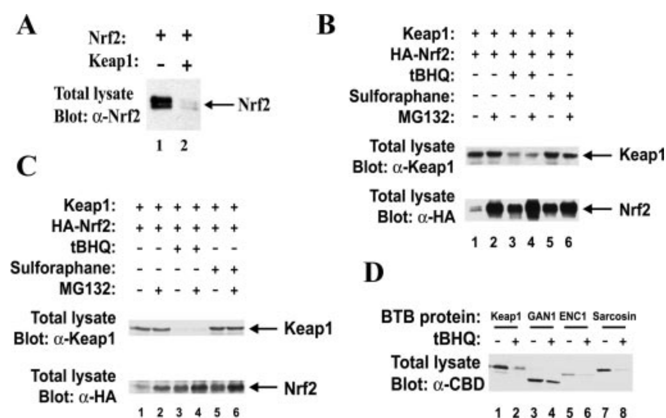

FIG. 5. *A*, cell lysates from GCS-2 cells transfected with expression vectors for Keap1 and HA-Nrf2 were analyzed by immunoblot with anti-Nrf2 antibodies. *B* and *C*, GCS-2 cells transfected with expression vectors for Keap1 and HA-Nrf2 were treated with either Me_2SO, 50 μM tBHQ, or 20 μM sulforaphane for 4 h (*B*) or 16 h (*C*). The cells were either left untreated or treated with 10 μM MG132 for 4 h prior to cell lysis. Equivalent amounts of cell lysates were subjected to immunoblot analysis with anti-Keap1 antibodies (*top panels*) and anti-HA antibodies (*bottom panels*). *D*, GCS-2 cells transfected with expression vectors for Keap1-CBD, GAN1-CBD, ENC1-CBD, or sarcosin-CBD were either left untreated or treated with 50 μM tBHQ for 16 h. Equivalent amounts of cell lysates were subjected to immunoblot analysis with anti-CBD antibodies.

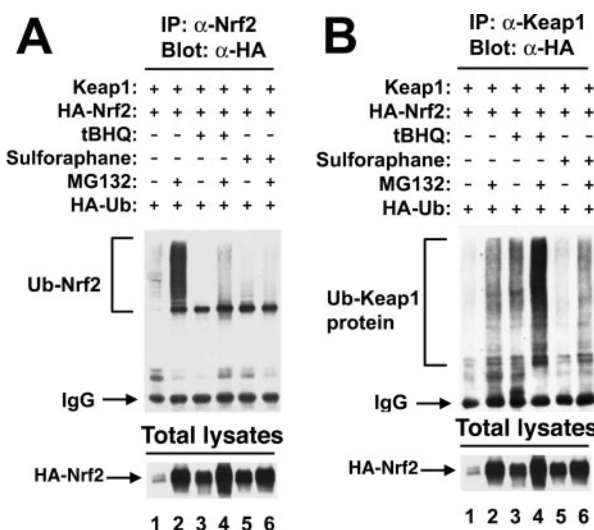

FIG. 6. **GCS-2 cells were transfected with expression vectors for HA-ubiquitin, HA-Nrf2, or Keap1.** The cells were treated with Me_2SO, 50 μM tBHQ, 20 μM sulforaphane or 10 μM MG132 for 4 h prior to cell lysis, as indicated. Lysates were collected under denaturing conditions, and equivalent amounts of each cell lysate were immunoprecipitated with anti-Nrf2 antibodies (*A*) or with anti-Keap1 antibodies (*B*). The anti-Nrf2 and anti-Keap1 immunoprecipitates were analyzed by immunoblot with anti-HA antibodies.

As expected, Keap1-dependent degradation of Nrf2 was blocked by treatment of cells with the proteasome inhibitor MG132 prior to cell lysis (Figs. 5*B* and 5*C*, *bottom panels*, *lanes 1* and *2*). However, MG132 treatment did not restore steady-state levels of Keap1 following exposure of transfected cells to tBHQ (Figs. 5*B* and 5*C*, *top panels*, *lanes 3* and *4*). Several other proteasome inhibitors, including 2-Leu-Leu-Leu-B(OH)2, clasto-lactacystin β-lactone, and epoxomicin were also tested for their ability to block tBHQ-induced degradation of Keap1. None of these inhibitors were able to restore steady-state levels of Keap1 in tBHQ-treated cells (data not shown), indicating that tBHQ-induced degradation of Keap1 is not mediated by the proteasome.

Oxidative Stress Induces Ubiquitination and Degradation of Multiple BTB-Kelch Proteins—The ability of three other BTB-Kelch proteins to associate with Cul3 and Rbx1 and form a functional complex that is competent for ubiquitin conjugation onto the respective BTB-Kelch protein *in vitro* suggests that these BTB-Kelch proteins may function as substrate adaptor proteins in a manner similar to Keap1. To further explore functional similarities between Keap1 and the other BTB-Kelch proteins, the ability of quinone-induced oxidative stress to decrease steady-state levels of these proteins was determined in GCS-2 cells. Reduced levels of Keap1, ENC1, and sarcosin, but not GAN1, were detected in response to tBHQ treatment (Fig. 5*D*, *even-numbered lanes*). As observed for Keap1, quinone-induced oxidative stress increased ubiquitin conjugation onto both ENC1 and sarcosin in GCS-2 cells (data not shown).

Quinone-induced Oxidative Stress Induces a Switch from Substrate to Substrate Adaptor Ubiquitination—To determine the relationship between quinone-induced ubiquitination of Keap1 and the ability of Keap1 to target Nrf2 for ubiquitination, levels of ubiquitin conjugation onto both Keap1 and Nrf2 were measured in GCS-2 cells following exposure to tBHQ. Because prolonged exposure of cells to quinone-induced oxidative stress markedly decreases the steady-state levels of Keap1, levels of ubiquitin conjugation onto both Keap1 and Nrf2 were measured following a 4-h exposure to tBHQ. At this time point, substantial Keap1 protein is still present in tBHQ-treated cells (Fig. 5*B*, *top panel*, *lanes 3* and *4*). Because ubiquitinated Nrf2 proteins are rapidly degraded by the 26 S proteasome, MG132 was added to cells prior to cell lysis to enable detection of ubiquitinated forms of Nrf2 (Fig. 6*A*, compare *lanes 1* and *2*). Exposure of GCS-2 cells to tBHQ for 4 h markedly decreased ubiquitin conjugation onto full-length Nrf2 (Fig. 6*A*, compare *lanes 2* and *4*). In parallel, a marked increase in ubiquitination of Keap1 was observed (Fig. 6*B*, compare *lanes 2* and *4*). In contrast, although sulforaphane completely blocked ubiquitination of Nrf2, no increase in ubiquitin conjugation onto Keap1 was observed (Figs. 6*A* and 6*B*, compare *lanes 2* and *6*). Thus, quinone-induced oxidative stress perturbs the Keap1-Cul3-Rbx1 E3 ubiquitin ligase complex such that Keap1, but not Nrf2, becomes the target for ubiquitin conjugation. Furthermore, this switch in the ubiquitin ligase activity of the Keap1-dependent E3 ubiquitin ligase complex is specific to quinone-induced oxidative stress and not to sulforaphane.

DISCUSSION

BTB-Kelch proteins comprise a large and evolutionarily conserved protein family present in all metazoan organisms, ranging from 5 members in flies to more than 40 family members in humans (18, 38). BTB-Kelch proteins are defined by the presence of an N-terminal BTB domain, a central linker domain, and a C-terminal Kelch domain. The BTB-Kelch proteins share modest sequence identity, ~25%, when compared on a one-to-one basis within the same species. Thus, it has not been clear if these proteins share a common biochemical function or, alternatively, if the conserved domains simply function as protein-protein interaction domains that enable each BTB-Kelch protein to assemble into different macromolecular complexes, each with a unique function. In this report, we demonstrate that four human BTB-Kelch proteins are each able to assemble with Cul3 and Rbx1 into a functional ubiquitin ligase complex capable of auto-ubiquitination of the BTB-Kelch protein. Our results support the hypothesis that BTB-Kelch proteins share a common biochemical function as substrate adaptor proteins that target specific proteins for ubiquitination.

The biological functions of only a small number of BTB-Kelch proteins have been defined. A unifying theme appears to be the

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ability of the BTB-Kelch proteins to regulate the formation of actin filaments or other components of the cytoskeleton. Thus, for example, the Kelch protein of *Drosophila melanogaster*, is required for proper cross-linking of actin filaments at ring canals that enable macromolecular transport between the nurse cells and the oocyte during oogenesis (46, 47). Mutations in the human GAN1 protein, which are the cause of a sensorimotor neuropathy termed giant axonal neuropathy, result in the accumulation of disorganized intermediate filaments in affected neurons (48, 49). Sarcosin associates with a muscle-specific isoform of N-RAP and may play a role in regulation of myofibril assembly and pseudopod formation in fibroblasts (43, 50). In the case of Keap1, an intact actin-based cytoskeleton is required for inhibition of Nrf2-dependent transcription by Keap1 (51). In general, the BTB-Kelch proteins appear to provide critical molecular linkages between cytoskeletal organization and cellular physiology in response to environmental signals. We propose that the ability of BTB-Kelch proteins to function as substrate adaptor proteins for Cul3 is a conserved biochemical function that underlies their diverse biological roles in regulation of the cytoskeleton and cellular physiology.

Under normal conditions of cellular homeostasis, the ability of Keap1 to function as a substrate adaptor for Nrf2 insures that steady-state levels of Nrf2 are maintained at low, nearly undetectable, levels (17). However, perturbation of intracellular homeostasis by a wide variety of chemical compounds, including quinone-induced oxidative stress and sulforaphane, inhibits Keap1-dependent ubiquitination of Nrf2, increases steady-state levels of Nrf2 and activates expression of Nrf2-dependent genes (21). In our previous work, we found that a single cysteine residue in Keap1, Cys-151, is required for inhibition of Keap1-dependent ubiquitination of Nrf2 by both quinone-induced oxidative stress and sulforaphane (21). Furthermore, both agents decreased, but did not abolish, the ability of Keap1 to associate with Cul3 and Rbx1 (21). In these previous experiments, we found that serine substitution of Cys-151 completely abolished responsiveness of Keap1 to sulforaphane. However, the Keap1-C151S protein retained a low level of responsiveness to quinone-induced oxidative stress (24). In our present work, we demonstrate that ubiquitination of Keap1 is increased by quinone-induced oxidative stress but not by sulforaphane. We find that ubiquitination and subsequent degradation of Keap1 is most pronounced following prolonged exposure of cells to oxidative stress, particularly in glutathione-deficient cells that are highly susceptible to oxidative stress. Serine substitution at Cys-151 does not prevent ubiquitination and degradation of Keap1 in response to quinone-induced oxidative stress (data not shown). Taken together, our results are consistent with a model in which the initial event for inhibition of Keap1 by both quinone-induced oxidative stress and sulforaphane is a chemical modification(s) on Cys-151 that decreases efficient assembly of Nrf2-bound Keap1 into a Cul3-dependent ubiquitin ligase complex. However, prolonged oxidative stress, particularly in cells that are depleted of glutathione, may result in other modifications to Keap1 that expose lysine residues within Keap1 for ubiquitination. Ubiquitination and subsequent degradation of Keap1 may be necessary for accumulation of Nrf2 to levels sufficient for mounting a sustained anti-oxidant counter response. Furthermore, our results point to a critical difference in how damaging (quinone-induced oxidative stress) and beneficial (sulforaphane) agents perturb the substrate adaptor function of Keap1 and suggest the possibility of developing novel chemopreventive agents with minimal deleterious side-effects.

Although the major function of substrate adaptor proteins is to bring specific substrates into cullin-based E3 ubiquitin ligase complexes such that the substrate becomes ubiquitinated, ubiquitination of substrate adaptor proteins is also of functional significance. The importance of substrate adaptor ubiquitination was first suggested by the demonstration that several yeast F-box proteins are ubiquitinated by the same Cul1-dependent complex that carries out ubiquitination of their substrates (29, 30). Alternatively, distinct ubiquitin ligase complexes can direct ubiquitination of specific substrate adaptor proteins (32, 33), providing a mechanism for cross-talk between signaling pathways that control different aspects of cellular physiology. In our experiments, we find that ubiquitination of Keap1 is accomplished by the same Cul3-dependent ubiquitin ligase complex that also carries out ubiquitination of Nrf2. Because quinone-induced oxidative stress does not dissociate Nrf2 from Keap1, our results suggest that the activity of the Keap1-Cul3-Rbx1 complex is altered such that Keap1, and not Keap1-associated Nrf2, is targeted for ubiquitination by the E2 enzyme that is recruited into the complex by Rbx1. A switch from substrate to substrate adaptor ubiquitination may be a general mechanism for controlling steady-state levels of both the substrate and substrate adaptor proteins.

A puzzling aspect of our results is the nature of cellular proteases that are responsible for degradation of Keap1. There is compelling evidence that the addition of multiubiquitin chains, linked through Lys-48 of ubiquitin, onto a protein will target it for degradation by the 26 S proteasome (25). Keap1-dependent ubiquitination of Nrf2 results in degradation of Nrf2 by the proteasome, because inhibitors of the proteasome markedly increase steady-state levels of Nrf2 in the presence of Keap1. Indeed, Keap1-dependent multiubiquitination of Nrf2 does occur on Lys-48, although other lysine residues in ubiquitin can also be used (data not shown). In contrast, multiubiquitination onto Keap1 appears to require Lys-63 in ubiquitin. A second difference between Keap1 and Nrf2 is that proteasome inhibitors do not restore steady-state levels of either mutant Keap1 proteins that are highly ubiquitinated or of the wild-type Keap1 protein in cells exposed to oxidative stress. In contrast, proteasome inhibitors are effective inhibitors of Nrf2 degradation. Our results suggest that degradation of Keap1 may not be accomplished by the 26 S proteasome. Eukaryote cells contain a number of other proteolytic systems in addition to the proteasome. For example, a recent report has suggested that ubiquitination of the transcription factor c-Jun may target c-Jun for degradation by the lysosome (52). Our results, which indicate that degradation of ubiquitinated Keap1 is independent of the 26 S proteasome, are consistent with the hypothesis that the nature of the ubiquitin linkage on protein-ubiquitin conjugates can target proteins for proteasome-independent degradative pathways. Our current work is focused on defining the nature of molecular signals that target Keap1 and other BTB-Kelch proteins for proteasome-independent degradation.

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