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Oxidative and Electrophilic Stresses Activate Nrf2 through Inhibition of Ubiquitination Activity of Keap1†

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The Keap1-Nrf2 system is the major regulatory pathway of cytoprotective gene expression against oxidative and/or electrophilic stresses. Keap1 acts as a stress sensor protein in this system. While Keap1 constitutively suppresses Nrf2 activity under unstressed conditions, oxidants or electrophiles provoke the repression of Keap1 activity, inducing the Nrf2 activation. However, the precise molecular mechanisms behind the liberation of Nrf2 from Keap1 repression in the presence of stress remain to be elucidated. We hypothesized that oxidative and electrophilic stresses induce the nuclear accumulation of Nrf2 by affecting the Keap1-mediated rapid turnover of Nrf2, since such accumulation was diminished by the protein synthesis inhibitor cycloheximide. While both the Cys273 and Cys288 residues of Keap1 are required for suppressing Nrf2 nuclear accumulation, treatment of cells with electrophiles or mutation of these cysteine residues to alanine did not affect the association of Keap1 with Nrf2 either in vivo or in vitro. Rather, these treatments impaired the Keap1-mediated proteasomal degradation of Nrf2. These results support the contention that Nrf2 protein synthesized de novo after exposure to stress accumulates in the nucleus by bypassing the Keap1 gate and that the sensory mechanism of oxidative and electrophilic stresses is closely linked to the degradation mechanism of Nrf2.

The cellular response to stresses originating from the environment is controlled by the coordinated function of multiple cellular regulatory factors, providing animals with an important means of protection against environmental insults. This response against environmental stresses or the "environmental response" can be divided into three steps. First, a cellular protein acts as a sensor and detects signals from the environmental changes. Second, the sensor transduces the signal to the gene expression machinery. In the third step, the transduced signal activates transcription factors, which induce the expression of a set of stress-responsive genes involved in cellular protection. These processes must be tightly regulated and precisely coordinated in order to sustain cellular homeostasis (reviewed in references 13, 20, 28, and 35).

Among environmental stresses, oxidative and xenobiotic (or electrophilic) stresses are known to be one of the causes of complex human diseases such as cancer, diabetes, and atherosclerosis. In defense, vertebrates have developed multiple stress response systems, including the system regulated by the Nrf2-Keap1 pathway (13, 21, 23, 24, 28, 29). Nrf2 is a transcription factor important for the stress-dependent expression of a set of cytoprotective genes, such as those for NAD(P)H-quinone oxidoreductase 1 (NQO1) and glutathione *S*-transferase (GST). Nrf2 activates the expression of these genes through a *cis*-acting element called the antioxidant/electrophile responsive element (ARE/EpRE) (13, 21, 23, 24, 28, 29).

Recent studies unveiled intriguing aspects of the Nrf2-Keap1 regulatory pathway. Under normal, unstressed conditions, Keap1 represses Nrf2 transactivation activity. In this situation, Keap1 appears to have two functional operations: it acts as a sensor molecule of oxidative and electrophilic stresses, and it accelerates Nrf2 degradation through a direct association. Nrf2 turns over rapidly through the proteasome protein degradation system with a half-life of less than 20 min (17). This rapid turnover of Nrf2 prevents the unnecessary expression of Nrf2 target genes (16, 27, 30, 38). Thus, when Keap1 detects oxidative or electrophilic stresses, Nrf2 is liberated from Keap1-mediated repression and accumulates in the nucleus, which in turn robustly induces the expression of a set of cytoprotective genes.

As for the molecular mechanisms of oxidative and electrophilic stress detection, it has been proposed that various inducers of Nrf2 have the common chemical property of reacting with the sulfhydryl groups of cysteine residues (7). Indeed, Keap1 contains 25 cysteine residues that are conserved between human and mouse Keap1 molecules (8, 15). The electrophile dexamethasone mesylate has been found to directly modify five cysteine residues of Keap1 in vitro (8). Extensive mutation studies in which the cysteine residues were mutated to alanine in a cell culture system demonstrated that Cys273 and Cys288 in the intervening region (IVR) of Keap1 are crucial for its repression activity (25, 39, 43). The functional significance of these cysteine residues has also been verified in vivo (our unpublished observation). These broad observations

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support our contention that the cysteine residues are essential constituents of the Keap1-based oxidative and electrophilic stress sensor machinery.

Proteasomal protein degradation plays critical roles in various biological processes, including signal transduction, cell cycle progression, and transcription (12, 32). Ubiquitin conjugation to a substrate protein proceeds through the sequential reaction of three enzymes (31, 41), the ubiquitin-activating enzymes (E1), the ubiquitin-conjugation enzymes (E2), and the ubiquitin ligases (E3). E3 ligases have two distinct functions: one is to target the substrate protein and the other is to catalyze ubiquitin conjugation to the substrate protein. The Cullin (Cul) protein family has emerged as one subtype of E3 ligases. There are seven Cullin members in mammals: Cul1, Cul2, Cul3, Cul4A/B, Cul5, and Cul7 (3, 31, 41). A key feature of Cul-type E3 ligases is that each Cul can assemble with numerous substrate-specific adaptors. Quite recently, four teams independently revealed that Keap1 functions as an adaptor for Cullin 3 (Cul3)-based E3 ligase to regulate Nrf2 stability (6, 11, 22, 44). Cul3 is a scaffold protein that forms the E3 ligase complex with Roc1/Rbx1/Hrt1 and recruits a cognate E2 enzyme. Intriguingly, the critical stress response factors IκB, Hif1α, and Nrf2 have been shown to share a common feature that is repressed by the ubiquitin-proteasome system under normal, unstressed conditions, with IκB, Hif1α, and Nrf2 exploiting the specific Cul-type E3 ligases Cul1, Cul2, and Cul3, respectively (reviewed in references 31 and 41).

While extensive analyses have been carried out to elucidate the molecular basis of the Keap1-Nrf2 function, there still remain many unanswered questions. Of the important questions left, we are very much interested in addressing how oxidative and electrophilic stresses provoke the nuclear accumulation of Nrf2 through the modification of two critical cysteine residues. One explanation is that modification of these cysteine residues causes a dynamic conformational change in Keap1, thereby provoking the dissociation of Nrf2 from Keap1. Although many lines of evidence currently available are consistent with this explanation (8, 9, 39), one datum that we recently obtained does not support this modification-dissociation hypothesis. We found that a Keap1 mutant lacking the IVR domain (where the important reactive cysteine residues reside) lost the ability to repress the activity of Nrf2 in a luciferase reporter analysis, while the mutant Keap1 was able to sequester an Nrf2 model protein (Neh2-green fluorescent protein [GFP]) in the cytoplasm (18). Based on this result, we hypothesized an alternative model in which the oxidative modification or mutation of the Keap1 cysteine residues affects the rapid turnover process of Nrf2.

To address the molecular mechanisms regulating Nrf2 activation following exposure to oxidative and electrophilic stresses, we analyzed the molecular interaction between Keap1 and Nrf2 as well as among Keap1 and electrophiles. We found that the nuclear accumulation of Nrf2 by electrophiles requires de novo protein synthesis and that the Cys273 and Cys288 residues of Keap1 are involved in the ubiquitin-proteasomal degradation machinery of Nrf2. Importantly, these cysteine residues did not modulate the association or dissociation of Nrf2 and Keap1. These results support our contention that electrophiles and oxidants activate Nrf2 by impairing the Keap1-mediated Nrf2 degradation pathway, which opens the Keap1 gate for Nrf2 protein synthesized de novo after an electrophile or oxidant challenge.

MATERIALS AND METHODS

Chemical reagents. MG132, *tert*-butyl hydroquinone (tBHQ), 15d-prostaglandin J_2 (15d-PGJ_2), and cycloheximide (CHX) were purchased from Peptide Institute Inc., Sigma, Cayman, and Wako Chemicals, respectively. Biotinylated 15d-PGJ_2 was prepared as described previously (36).

Plasmid construction. Expression plasmids for the Keap1 cysteine mutants and IVR deletion mutant (ΔIVR) were constructed as described previously (18, 39). Expression plasmids of maltose binding protein (MBP)-fused Keap1-IDC (MBP-Keap1-IDC; corresponds to amino acids 180 to 624 of mouse Keap1) and Keap1-IDC-C273&288A, harboring two alanine substitution mutations, were constructed by subcloning PCR-amplified fragments into the blunt-ended EcoRI and XbaI sites of pMalc-2 (New England Biolabs). The expression plasmid of the GST-fused Neh2 domain (amino acids 1 to 89) of mouse Nrf2 was generated by inserting a SmaI-BamHI fragment harboring the mouse Neh2 domain into the blunt-ended BamHI site of pGEX-2T (Pharmacia). All constructs were confirmed by sequencing.

Protein expression and purification. MBP-Keap1-IDC and MBP-Keap1-IDC-C273&288A were expressed in *Escherichia coli* BL21(DE3) cells (Novagen) and purified as described in the instruction manual of the pMAL protein fusion and purification system (New England Biolabs). GST-mNeh2 was also expressed in BL21(DE3) cells. The cells were lysed with column buffer (phosphate-buffered saline [PBS], 1% Triton X-100, 10 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) and subjected to centrifugation. Cell extracts were applied to glutathione Sepharose 4B column chromatography (Pharmacia). The column was washed three times with 10 bed volumes of PBS. GST-mNeh2 proteins were eluted with elution buffer (1 M Tris-HCl [pH 9.6], 100 mM glutathione, and 1 M dithiothreitol). The eluate was neutralized with 200 mM Tris-HCl (pH 6.8).

BIAcore assay. The surface plasmon resonance measurements were performed on a BIAcore 2000 instrument (Biacore AB, Uppsala, Sweden). Anti-GST antibody was immobilized to the surface of a CM5 sensor chip with the GST capturing kit (Biacore AB). GST or GST-Neh2 was bound to the immobilized GST antibody on the CM5 sensor chip in PBS. The association among recombinant proteins was examined with different concentrations of MBP-Keap1-IDC and MBP-Keap1-IDC-C273&288A at 25°C. The dissociation constant (K_d) was calculated with BIAevaluation (version 3.0) by the nonlinear fitting method following the manufacturer's recommendation.

Cell culture, transfection, and luciferase reporter analysis. NIH 3T3, 293T, and Cos7 cells were maintained in Dulbecco's modified Eagle medium (Sigma) supplemented with 10% fetal bovine serum (Gibco), 4,500 mg of glucose per liter, 40 μg of streptomycin per ml, and 40 U of penicillin per ml. Wild-type and Nrf2-deficient mouse embryonic fibroblast (MEF) cells were maintained in Iscove's modified Dulbecco's medium (Sigma) supplemented with 10% fetal bovine serum, 40 μg of streptomycin per ml, and 40 U of penicillin per ml. DNA transfection was performed with Fugene 6 (Roche) and Lipofectamine Plus (Invitrogen). The luciferase reporter analysis was performed as described previously (18).

Immunoprecipitation assay. Expression plasmids for Flag-tagged Nrf2 and/or Keap1 were transfected into 293T cells. At 24 h after transfection, cells were treated with tBHQ (final concentration, 100 μM) and subsequently cultured for 12 h. The cells were lysed with RIPA buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.1% deoxycholate, 0.1% sodium dodecyl sulfate, and protease inhibitor [Roche]). Whole-cell extracts were subjected to an immunoprecipitation assay using anti-Flag antibody beads (Sigma). The immune complex was visualized by immunoblot analysis using anti-Keap1 and anti-Nrf2 antibodies. For the in vitro tBHQ treatment experiment, whole-cell extracts expressing Flag-tagged Nrf2 and Keap1 were treated with tBHQ at several final concentrations (10, 50, and 500 μM) for 4 h at 4°C. Subsequently, the extracts were subjected to immunoprecipitation and immunoblot analysis as described above.

The in vivo degradation and ubiquitination assay. Full-length Keap1 and several mutants were expressed in Cos7 cells along with enhanced green fluorescent protein (EGFP) as an internal control to verify the transfection efficiency. At 36 h after transfection, the cells were directly lysed and boiled in Laemmli sample buffer supplemented with β-mercaptoethanol (final concentration, 2%). Cell extracts were subjected to immunoblot analysis with an anti-Nrf2 antibody (C-20; Santa Cruz) and an anti-EGFP antibody (Molecular Probes). An in vivo ubiquitination assay was performed as described previously (22).

Immunohistochemical staining. The expression plasmids for Nrf2 and Flag-tagged Keap1 were transfected with Fugene 6 (Roche) into Cos7 cells on glass slides (Falcon). At 24 h after transfection, the cells were treated with tBHQ (final

FIG. 1. Oxidative/electrophilic stress provokes the nuclear accumulation of de novo Nrf2 protein. Wild-type MEF cells were treated with tBHQ (lanes 2 to 9; final concentration, 100 μM) or DMSO (lanes 1 and 10 to 13) in the presence (lanes 6 to 13) or absence (lanes 1 to 5) of the protein synthesis inhibitor cycloheximide (10 μM) for different time points as indicated in the figure. Nuclear extracts were prepared and subjected to immunoblot analysis using anti-Nrf2 and anti-lamin B antibodies (top and bottom panels).

concentration, 100 μM) and subsequently cultured for 12 h. The cells were washed with PBS, fixed with 4% paraformaldehyde for 10 min and 100% acetone (−20°C) for 1 min, and washed three times with PBS. Cells were blocked with 2% goat serum in PBS at room temperature for 30 min and then treated with anti-Nrf2 (100-fold dilution) and anti-Flag antibody (Sigma, 100-fold dilution) for 1 h. After being washed with PBS, cells were incubated with goat anti-rabbit immunoglobulin G antibody conjugated with fluorescein-5-isothiocyanate (Zymed) and goat anti-mouse immunoglobulin G antibody conjugated with tetramethyl rhodamine B isothiocyanate (Zymed) for 1 h. Nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI). After they were washed with PBS, a drop of fluorescent mounting medium (DAKO) was placed on the slides.

Cell fractionation. At 36 h after transfection, cells were lysed in buffer A (20 mM HEPES-KOH [pH 8.0], 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, and protease inhibitor [Roche]). After cell centrifugation, supernatants were saved as the cytoplasmic fractions and the nucleus pellets were washed with buffer A three times. These nuclei were lysed and boiled in Laemmli sample buffer supplemented with β-mercaptoethanol (final concentration, 2%). The supernatants of these nuclear lysates were saved as nuclear extract fractions. The cytoplasmic and nuclear extract fractions were subjected to immunoblot analysis using anti-Nrf2, anti-lamin B (a nuclear fraction marker; Santa Cruz Biotechnology), and anti-α-tubulin antibodies (the cytoplasmic fraction marker; Sigma).

RESULTS

Oxidative/electrophilic stress provokes the nuclear accumulation of de novo-synthesized Nrf2 protein. It has been shown previously that Keap1 binds to Nrf2 and represses its activity (15, 16, 18) and that Keap1 promotes the rapid turnover of Nrf2 through the ubiquitin-proteasome pathway utilizing Cul3-based E3 ligase (6, 11, 22, 44). However, how Nrf2 accumulates in the nucleus in response to the oxidative and electrophilic stresses is not understood. The data obtained to date led us to hypothesize that Nrf2 accumulates in the nucleus through protein stabilization caused by oxidative and electrophilic stresses. To address this hypothesis, we first examined the nuclear accumulation of endogenous Nrf2 in wild-type MEF cells after treatment with the protein synthesis inhibitor CHX. At several time points after CHX treatment, nuclear extracts were prepared and subjected to immunoblot analysis with an anti-Nrf2 antibody. While the nuclear accumulation of Nrf2 was induced 1 h after tBHQ treatment (Fig. 1, lanes 1 to 5), it was severely inhibited by the concomitant treatment of CHX (lanes 6 to 9). Similar results were observed by using Cos7 and 293T cells (data not shown). These results suggest that Nrf2 protein synthesized de novo, rather than that liberated from Keap1, accumulates in the nucleus in response to oxidative and electrophilic stresses.

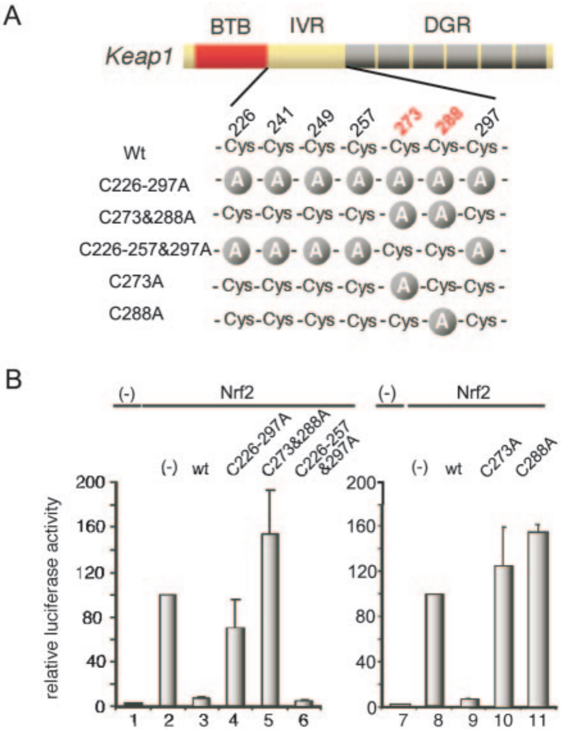

FIG. 2. Keap1 requires Cys273 and Cys288 to suppress Nrf2 activity. (A) Schematic structures of the cysteine mutants of the IVR domain. (B) Keap1 repression activity was measured by luciferase assay. Expression plasmids for Nrf2 and Keap1 wild type (lanes 3 and 9) or cysteine mutants (lanes 4 to 6, 10, and 11) (90 ng and 10 ng, respectively) were transfected into NIH 3T3 cells (2×10^4) along with pNQO1-ARE reporter plasmid (50 ng) and pRL-TK (50 ng) as an internal control. At 36 h after transfection, the luciferase activity was measured according to the manufacturer's instructions. Assays were performed twice in triplicate.

Keap1 requires both Cys273 and Cys288 to suppress Nrf2 activity. Two reactive cysteines, Cys273 and Cys288, of Keap1 have been identified as suppressing the transactivation activity of Nrf2 in MEFs (39). Since we wished to perform molecular biological analyses on the function of Keap1, MEFs did not fully meet our experimental requirements, as we required more stably proliferating cells. In addition, we wished to confirm the functional significance of these cysteine residues in other types of cells. Therefore, we carried out an extensive reporter cotransfection analysis exploiting several established cell lines. The various Keap1 cysteine mutants are depicted in Fig. 2A. The results of the experiments with cotransfection of Nrf2 and/or Keap1 mutants into NIH 3T3 cells along with the luciferase reporter plasmid pNQO1-ARE are shown in Fig. 2B.

Since this reporter harbors a single ARE, we analyzed the repression activity of Keap1 and its mutants by evaluating the Nrf2-mediated luciferase activity (Fig. 2B). Nrf2 alone markedly activated the reporter expression (compare lanes 1 and 2 and lanes 7 and 8), but the concomitant expression of Keap1 severely suppressed the transactivation activity of Nrf2 (lanes 3 and 9). Mutation of all seven cysteine residues abrogated the repression activity of Keap1 on Nrf2 (lane 4). A single or double mutation of Cys273 and Cys288 (i.e., C273A, C288A, or C273&288A) also eliminated the repression activity of Keap1 (lanes 5, 10, and 11). These observations were reproducible in the experiments using Nrf2-deficient MEF cells (see Fig. S1 in

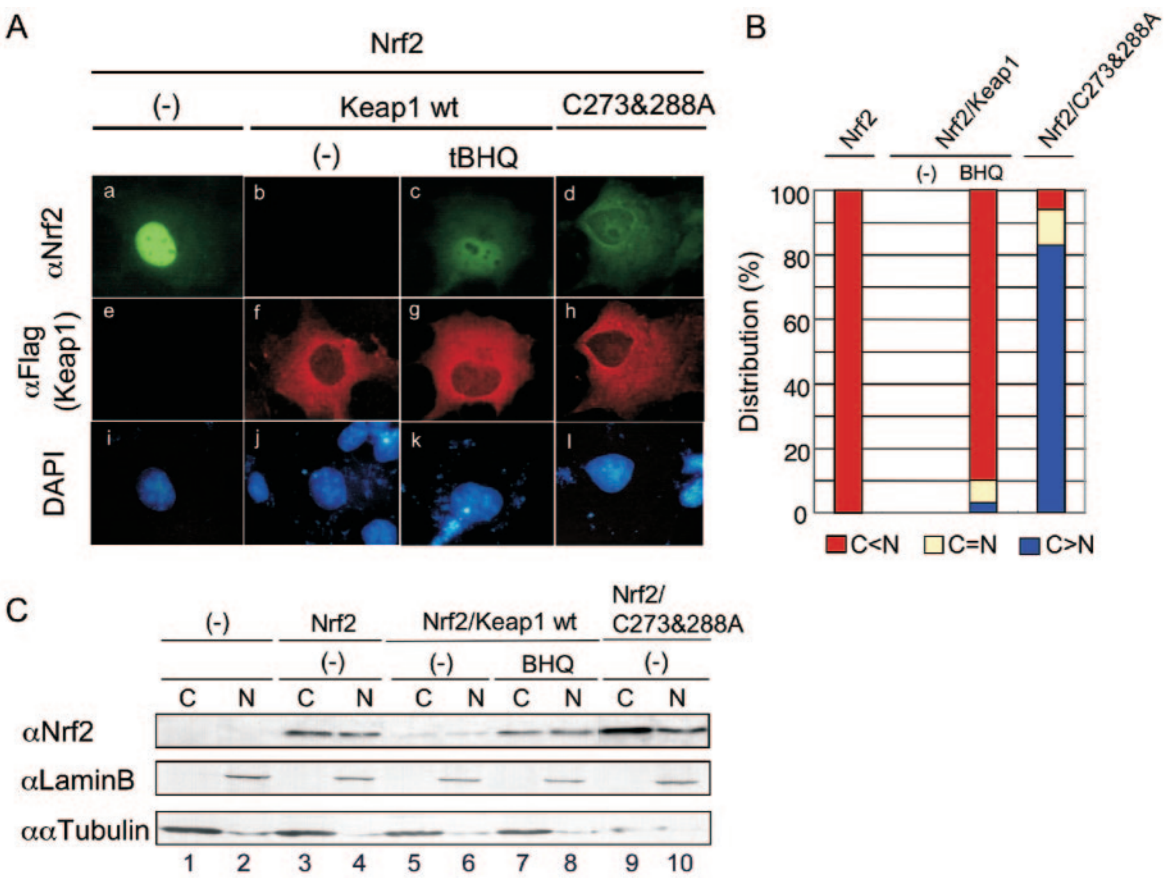

FIG. 3. The Keap1-C273&288A mutant sequestrates but does not degrade Nrf2 in the cytoplasm. (A) Expression plasmids for Nrf2 and Flag-tagged Keap1 or Keap1-C273&288A mutant were transfected into Cos7 cells. At 36 h after transfection, the cells were subjected to immunohistochemical staining using anti-Nrf2 (a to d) and anti-Flag (M2) (e to h) antibodies. Nuclei were stained with DAPI (i to l). (B) The subcellular localization of Nrf2 was classified into three categories: predominantly cytoplasmic localization ($C > N$, blue bars), roughly equal localization between the cytoplasmic and nuclear compartments ($C = N$, yellow bars), and predominantly nuclear localization ($C < N$, red bars). The subcellular localization within 100 cells is shown. (C) Immunoblot analysis using fractionated cell extracts of transfected cells. The transfection method was performed as described above. The cells were subjected to cell fractionation into cytoplasmic (C) and nuclear (N) fractions and subsequent immunoblot analysis. Anti-lamin B and anti-α-tubulin antibodies were used as markers for the nuclear and cytoplasmic extracts, respectively.

the supplemental material). These results thus indicate that the two reactive cysteine residues Cys273 and Cys288 are crucial for the activity of Keap1.

The C273&288A mutant of Keap1 associates with Nrf2. To clarify mechanisms pertaining to the effect of a C273&288A mutation on the repression activity of Keap1, we examined whether such a mutant associates with Nrf2. We determined the K_d of Keap1 and Nrf2 by the BIAcore interaction assay. MBP-fused Keap1-IDC protein containing the *I*VR, *D*GR, and *C*TR domains of Keap1 (amino acids 180 to 624; MBP-Keap1-IDC) and its mutant harboring C273&288A substitutions (MBP-Keap1-IDC-C273&288A) were expressed in *E. coli* and purified using affinity beads. We also prepared a GST-fused Neh2 domain of mouse Nrf2 (GST-mNeh2), which contains the association surface for Keap1. In the BIAcore assay, we immobilized GST-Neh2 on the sensor chip, applied MBP-fused Keap1 protein to the mobilizing solution, and measured the K_d value. Remarkably, the K_d value of the Keap1 C273&288A mutant and Neh2 was almost similar to that of wild-type Keap1 and Neh2 (2.7×10^{-6} and 3.1×10^{-6}, respectively). Furthermore, we also performed the BIAcore assay using full-length recombinant and mutant Keap1 proteins (data not shown). We observed no significant difference in their K_d values, while their values are close to 10^{-9}. These results thus indicate that the alanine substitution mutations of residues Cys273 and Cys288 do not affect the ability of Keap1 to associate with Nrf2, despite the fact that the mutation does abolish Keap1 repression of Nrf2 activity in the reporter cotransfection analysis.

The C273&288A mutant impairs Keap1-mediated degradation of Nrf2. To examine how the C273&288A mutation of Keap1 affects the subcellular localization of Nrf2, we carried out immunohistochemical analyses using Cos7 cells. The expression plasmid for Flag-tagged wild-type Keap1 or C273&288A mutant Keap1 was transfected into Cos7 cells along with an Nrf2 expression plasmid, and the cellular localization of Nrf2 and Keap1 was examined with anti-Nrf2 and anti-Flag antibodies, respectively (Fig. 3A and B). For a quantitative measurement, we also performed immunoblot analysis with the nuclear and cytoplasmic extracts of these cells (Fig.

3C, top panel). Anti-lamin B and anti-α-tubulin antibodies were used in the immunoblot analysis as nuclear and cytoplasmic protein markers, respectively (middle and bottom panels).

Transfection of Nrf2 alone resulted in the localization of Nrf2 predominantly in the nucleus (Fig. 3A, panels a, e, and i), although immunoblot analysis showed the presence of Nrf2 in both cytoplasmic and nuclear extracts (Fig. 3C, lanes 3 and 4). Since anti-lamin B antibody gave rise to signals exclusively in the nuclear extract, we judged that practically no cross-contamination of nuclear proteins into the cytoplasmic extract had occurred (Fig. 3C, middle panel). Therefore, the discrepancy between these two analyses remains to be resolved. One plausible explanation is a diffuse but low-level accumulation of Nrf2 in the cytoplasm that is detectable by immunoblot analysis but not by immunohistochemistry. Simultaneous expression of wild-type Keap1 diminished expression of Nrf2 (Fig. 3A, panels b, f, and j; Fig. 3C, lanes 5 and 6), most likely because Keap1 accelerates the Nrf2 degradation. Consequently, we did not identify cells coexpressing Keap1 and Nrf2 (Fig. 3B). Treatment of Cos7 cells with tBHQ in this condition caused the nuclear accumulation of Nrf2 (Fig. 3A, panels c, g, and k; Fig. 3C, lanes 7 and 8).

To our surprise, Keap1-C273&288A mutant allowed Nrf2 to accumulate in the cytoplasm (Fig. 3A, panels d, h, and l; Fig. 3C, lanes 9 and 10) and partially in the nucleus (Fig. 3C, lane 10). More than 80% of Cos7 cells transfected with the C273&288A mutant showed accumulation of Nrf2 in the cytoplasm, in clear contrast to cells transfected with wild-type Keap1 and treated with tBHQ (Fig. 3B). These results thus demonstrate that Nrf2 was not liberated from Keap1 by the substitution of Cys273 and Cys288 with alanine, but that the substitution mutations abrogated the activity of Keap1 that leads Nrf2 to rapid degradation through the proteasome system.

Treatment with tBHQ did not recover the weakened nuclear entry of Nrf2 caused by the C273&288A Keap1 mutation (data not shown). This observation further supports the notion that Cys273 and Cys288 contribute to the cellular response to oxidative and electrophilic stresses by regulating the rapid degradation of Nrf2.

Cys273 and Cys288 are crucial for the Keap1-mediated degradation of Nrf2. To assess the contribution of Cys273 and Cys288 to the Keap1-mediated degradation of Nrf2, we carried out an in vivo protein degradation assay. Nrf2 was transiently expressed in Cos7 cells along with wild-type or C273&288A mutant Keap1, and the protein stability of Nrf2 was monitored in whole-cell extracts by immunoblot analysis using anti-Nrf2 antibody (Fig. 4A, top panel). A GFP expression plasmid was also transfected into the cells as an internal control (Fig. 4A, bottom panel). Nrf2 was detected in the cells upon transfection of Nrf2 alone (lane 2). However, the concomitant expression of Keap1 significantly reduced the Nrf2 expression level (lane 3), in very good agreement with the contention that Keap1 promotes the proteasomal degradation of Nrf2 (6, 11, 22, 44). In contrast, oxidative modification by tBHQ or alanine substitution mutations of Cys273 and Cys288 (C273&288A) abrogated this Nrf2 degradation and Nrf2 accumulated in the cells (lanes 4 and 5). Thus, Cys273 and Cys288 are indispensable for the Keap1-mediated degradation of Nrf2.

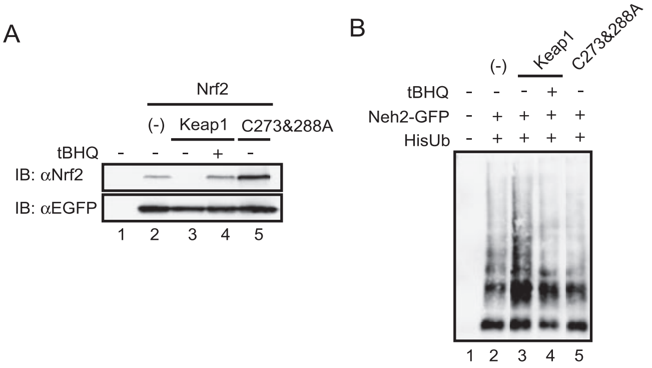

FIG. 4. Reduced Cys273 and Cys288 are crucial for the ubiquitin-dependent degradation of Nrf2. (A) Alanine mutation or oxidation of Cys273 and Cys288 impairs Keap1-mediated degradation of Nrf2. The degradation activity of Keap1 was monitored by an in vivo degradation assay. The expression plasmids for Nrf2 and wild type or C273&288A mutant Keap1 (2 μg and 1.5 μg, respectively) were transfected into Cos7 cells, as indicated in the figure. EGFP plasmid (50 ng) was cotransfected to verify the transfection efficiency. At 24 h after transfection, cells were treated with DMSO (lanes 1 to 3 and 5) or tBHQ (lane 4; final concentration, 100 μM) for 12 h. Whole-cell extracts were prepared and subjected to immunoblot analysis using anti-Nrf2 and anti-GFP antibodies (top and bottom panels, respectively). (B) Alanine mutation or oxidation of Cys273 and Cys288 impairs Keap1-mediated ubiquitination of the Neh2 domain. An in vivo ubiquitination assay was performed. A GFP-fused Neh2 domain that harbors the Keap1-dependent ubiquitination site was used in this assay (Neh2-GFP). The expression plasmids for Neh2-GFP and wild-type Keap1 or Keap1-C273&288A mutant were transfected into 293T cells, as indicated in the figure, along with a His-tagged ubiquitin (HisUb) plasmid. At 24 h after transfection, cells were treated with MG132 (final concentration, 2 μM) in the absence (lanes 1 to 3 and 5) or presence (lane 4; final concentration, 100 μM) of tBHQ for 12 h. Whole-cell extracts were prepared and subjected to Ni^{2+} affinity purification. Precipitates were visualized by immunoblot analysis with anti-Nrf2 antibody.

Cys273 and Cys288 are crucial for Keap1-mediated Nrf2 ubiquitination. Since Keap1 promotes the polyubiquitination of Nrf2 through the bridging of Nrf2 and Cul3-based E3 ligase, the data in the previous section imply that oxidative and electrophilic stresses may affect this process directly within the cells. To examine whether tBHQ treatment or a C273&288A substitution affects the Keap1-mediated ubiquitination of Nrf2, we carried out an in vivo ubiquitination assay (Fig. 4B). Since the ubiquitination site of Nrf2 seems to be located in the Neh2 domain (19, 26, 27, 43), we transfected a GFP-fused Neh2 domain (Neh2-GFP) expression plasmid into the 293T cells. His-tagged ubiquitin (HisUb) was also transfected in order to purify the ubiquitinated Neh2-GFP by nickel affinity beads. At 24 h after transfection, cells were treated with a proteasome inhibitor, MG132, for 12 h to inhibit the degradation of Neh2-GFP. Whole-cell extracts were prepared and subjected to affinity purification and immunoblot analysis with anti-Nrf2 antibody. Expression of Neh2-GFP alone gave a smeared migration pattern, suggesting that it was conjugated with ubiquitin chains in various patterns (lane 2). The concomitant expression of Keap1 significantly promoted modification of the Neh2 domain (lane 3). In contrast, tBHQ treatment or a C273&288A mutation significantly reduced this modification (lanes 4 and 5). These results demonstrate that oxidative/elec-

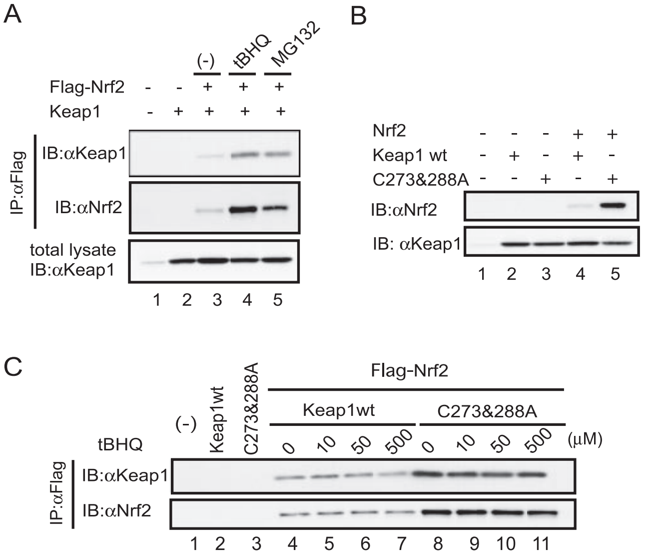

FIG. 5. Oxidative/electrophilic stress does not cause dissociation of the Keap1-Nrf2 complex. (A) The effects of in vivo tBHQ treatment on the association between Keap1 and Nrf2 were examined by immunoprecipitation. Expression plasmids for Flag-tagged Nrf2 and Keap1 (2 μg and 1.5 μg, respectively) were transfected into 293T cells. At 24 h after transfection, cells were treated with tBHQ (lane 4, 100 μM final concentration) or the proteasome inhibitor MG132 (lane 5, 2 μM final concentration) for 12 h. Whole-cell extracts were prepared and subjected to immunoprecipitation experiments using anti-Flag antibody beads. Immunoprecipitates were visualized by immunoblot analysis with anti-Keap1 and anti-Nrf2 antibodies (top and middle panels, respectively). The expression levels of Keap1 in whole-cell extracts were monitored by immunoblot analysis with an anti-Keap1 antibody (bottom panel). (B and C) In vitro tBHQ treatment does not cause dissociation of the Keap1-Nrf2 complex. Transfection was performed as described above, and whole-cell extracts were prepared from the transfected 293T cells. The expression levels of Nrf2 and Keap1 mutant were monitored by immunoblot analysis using anti-Nrf2 and anti-Keap1 antibodies (B, top and bottom panels, respectively). Whole-cell extracts were treated with DMSO (C, lanes 4 and 8) or tBHQ (lanes 5 and 9, 10 μM final concentration; lanes 6 and 10, 50 μM final concentration; lanes 7 and 11, 500 μM final concentration) for 4 h at 4°C and subjected to immunoprecipitation with anti-Flag antibody beads. Precipitates were visualized by immunoblot analysis using anti-Keap1 and anti-Nrf2 antibodies (top and bottom panels, respectively). Lanes 1 to 3 were loaded with the immunoprecipitates of cells transfected with empty, wild-type Keap1, and C273&288A mutant Keap1 expression vectors, respectively.

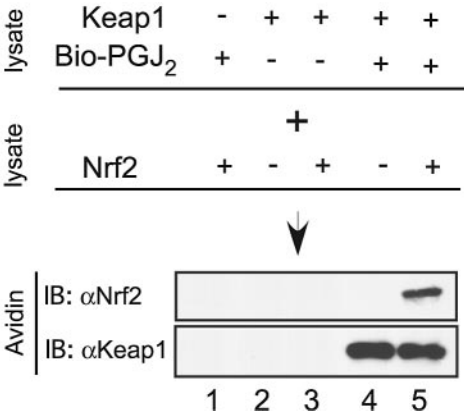

FIG. 6. Oxidative/electrophilic stress does not affect formation of the Keap1-Nrf2 complex. Electrophilic modification of Keap1 with biotinylated 15d-PGJ$_2$ does not affect formation of the Keap1-Nrf2 complex. 293T cells expressing Keap1 were treated with biotinylated 15d-PGJ$_2$ (Bio-PGJ$_2$, lanes 1, 4, and 5; 10 μM final concentration) for 1 h. Whole-cell extracts were prepared and mixed with whole-cell extracts of Nrf2-expressing (lanes 1, 3, and 5) or wild-type (lanes 2 and 4) cells. Biotinylated Keap1 was precipitated from the mixed cell extracts using avidin beads, and precipitates were subjected to immunoblot analysis with anti-Nrf2 and anti-Keap1 antibodies (top and bottom panels, respectively).

trophilic stress transmits signals to the cysteine residues and affects Keap1-mediated ubiquitin conjugation.

Keap1-Nrf2 complex in the presence of oxidative/electrophilic stress. Thus, our model is that oxidative and electrophilic stresses provoke the nuclear accumulation of Nrf2 by enfeebling the cellular Nrf2 degradation machinery. To test the validity of this model, we performed a series of experiments exploiting immunoprecipitation procedures. At first, 293T cells expressing Keap1 and Flag-tagged Nrf2 were treated with dimethyl sulfoxide (DMSO) (Fig. 5A, lane 3), tBHQ (100 μM; lane 4), or MG132 (lane 5), and whole-cell extracts were subjected to immunoprecipitation using anti-Flag antibody beads. The precipitates were visualized by immunoblot analysis with anti-Keap1 and anti-Nrf2 antibodies (top and middle panels). Keap1 coprecipitated with Flag-Nrf2 (lane 3), indicating an association between Keap1 and Nrf2 within the cells. Importantly, the tBHQ treatment did not cause dissociation of the complex but instead increased the amount of Nrf2 protein (middle panel, compare lanes 3 and 4), with more Keap1 protein being precipitated (top panel, lane 4). Treatment of the cells with MG132 showed similar effects (top and middle panels, lane 5).

We next examined the effect of in vitro tBHQ treatment on this association. Whole-cell extracts derived from 293T cells expressing Flag-Nrf2 and wild-type Keap1 or C273&288A mutant Keap1 (Fig. 5B) were treated with several concentrations of tBHQ (10, 50, and 500 μM) for 4 h and subjected to immunoprecipitation and immunoblot analyses (Fig. 5C). In this in vitro experimental condition, the tBHQ treatment did not dissociate the Keap1-Nrf2 complex into components, so we could still obtain coprecipitation of Nrf2 and Keap1 (Fig. 5C). Based on these observations, we conclude that oxidative/electrophilic stresses practically do not dissociate Nrf2 from Keap1.

Oxidative/electrophilic stress does not affect formation of the Keap1-Nrf2 complex. One remaining possibility is that oxidative insults may affect the formation, but not dissociation, of the Keap1-Nrf2 complex. To address this possibility, we utilized biotinylated 15d-PGJ$_2$ (17, 36). 15d-PGJ$_2$ is an endogenous electrophile that affects the activity of Keap1, and biotinylated 15d-PGJ$_2$ has been shown to directly modify the cysteine residues of Keap1 (17). Therefore, biotinylated 15d-PGJ$_2$ enables us to specifically isolate the oxidized form of Keap1 with avidin beads. Purified Keap1 was subjected to an in vitro investigation to ascertain whether oxidized Keap1 associates with Nrf2 in vitro. We transfected Keap1 or Nrf2 expression plasmid into 293T cells, and whole-cell extracts were prepared. Before the whole-cell extracts were prepared, Keap1-expressing cells were treated with DMSO (Fig. 6, lanes 2 and 3) or

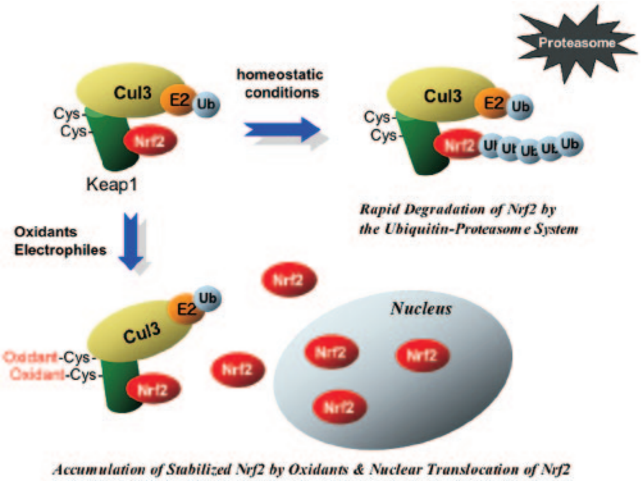

FIG. 7. Schematic model of the Nrf2 activation mechanism that is tightly coupled to Keap1-mediated degradation of Nrf2. Under homeostatic conditions, Nrf2 is sequestered in the cytoplasm by the Keap1-Cul3 complex and rapidly degraded in the ubiquitin-proteasome dependent manner. This Keap1-mediated degradation activity requires two reactive cysteine residues (Cys273 and Cys288) of Keap1. After the oxidative/electrophilic stress challenge, modification of these cysteine residues of Keap1 inhibits ubiquitin conjugation to Nrf2 by the Keap1-Cul3 complex, thereby provoking opening of the Keap1 gate and resulting in the nuclear accumulation of Nrf2.

biotinylated 15d-PGJ_2 (lanes 4 and 5) for 1 h. The cell extracts were mixed with extracts of wild-type cells or Nrf2-expressing cells to form the Keap1-Nrf2 complex, followed by precipitation using avidin beads. Precipitates were visualized by immunoblot analysis with anti-Nrf2 and anti-Keap1 antibodies. Intriguingly, the Keap1 molecule modified with biotinylated 15d-PGJ_2 significantly coprecipitated with Nrf2 (Fig. 6, lane 5), suggesting that both the oxidized and nonoxidized forms of Keap1 can associate with Nrf2. These results suggest that, once trapped by Keap1, Nrf2 is not liberated from Keap1 even in the presence of oxidative/electrophilic stresses.

DISCUSSION

In this study, we deciphered how Nrf2 accumulates in the nucleus through evasion of the cytoplasmic Keap1 gate after exposure of cells to electrophiles and oxidants. We found that, while Cys273 and Cys288 in the IVR domain of Keap1 modulate the proteasomal degradation of Nrf2, mutation or modification of these two cysteine residues does not cause dissociation of Nrf2 from Keap1 or impairment of the association between Nrf2 and Keap1. Modification of these cysteine residues rather inhibits ubiquitin conjugation to Nrf2 by the Keap1-Cul3 complex, provoking opening of the Keap1 gate and resulting in the nuclear accumulation of Nrf2. Consistent with this notion, we found that the accumulated nuclear Nrf2 was supplied mainly from the Nrf2 protein pool synthesized de novo after the electrophilic exposure. Thus, we conclude that the sensor for the oxidative/electrophilic stress is closely linked to the Nrf2 degradation machinery. Our schematic model in Fig. 7 summarizes the Nrf2 activation mechanism that is tightly coupled with the Keap1-mediated degradation of Nrf2.

One conventional model for the nuclear accumulation of Nrf2 in response to electrophiles and oxidants is that oxidative or electrophilic modification of the Cys273 and Cys288 residues dissociates Nrf2 from Keap1, for example by eliciting conformational changes in Keap1, thereby allowing Nrf2 to migrate into the nucleus (reviewed in reference 39). In this study, we propose an alternative model in which the oxidative or electrophilic agent affects the Keap1-mediated degradation of Nrf2. In fact, mutation of the Cys273 and Cys288 residues or electrophilic treatment did not affect the formation of the Keap1-Nrf2 complex either in vivo or in vitro, but the alanine substitution (C273&288A) or tBHQ treatment stabilized the Nrf2 protein. This observation is consistent with the recent observation reported by Zhang and Hannink in that electrophile treatment or serine substitution for either Cys273 or Cys288 impairs the Keap1-mediated degradation of Nrf2 (43). Furthermore, it was reported that the addition of proteasome inhibitor induced the gene expression of *GCL*, a target gene of Nrf2 (34), implying that inhibition of Nrf2 degradation results in its nuclear accumulation and the subsequent induction of cytoprotective genes.

The latter model gives rise to an alternative question as to how oxidants and electrophiles inhibit the Keap1-mediated degradation of Nrf2 and subsequently induce cytoprotective gene expression. While we do not have the immediate answer to this important question, we believe that the following three lines of evidence or thoughts are pertinent in this regard.

First, modification of the cysteine residues may induce conformational changes within the Keap1-Cul3 E3 ligase complex, thereby impairing the ubiquitin conjugation reaction. It has been reported that the ring finger protein Mdm2, which is a known component of the E3 ligase for p53, requires integrity of the protein structure through the coordination of zinc ions with cysteine residues for its ubiquitin ligase activity (10). Indeed, Dinkova-Kostova and colleagues recently identified Keap1 as a zinc-containing protein, and Cys273 and Cys288 are crucial for zinc coordination (9). So, the zinc coordination in Keap1 may contribute to the formation of the crucial tertiary structure needed for the ubiquitin ligase activity of the Keap1-Cul3 E3 ligase complex, implying that oxidative modification of cysteine residues may cause zinc release and loss of the Keap1 activity. This may be the mechanism of stress sensing by Keap1. The model structure of the Skp1-Cul1-F-box protein (SCF) E3 ligase complex provides excellent insights into this hypothesis (3, 31, 47). The model shows that different protein subunits fit together into a single rigid C-shaped superstructure with a distance of approximately 59 Å of space between the adaptor protein and the E2 enzyme. This size of space is presumably suitable for a substrate protein to enter and catch ubiquitin molecules from the E2 enzyme. It seems plausible that modification of the cysteine residues of Keap1 affects this pocket size through conformational changes of the Keap1-Cul3 complex, resulting in the loss of ubiquitin-conjugation function.

Second, it also seems possible that oxidative/electrophilic stress may induce the dissociation of Keap1 from Cul3 and prevent Nrf2 from ubiquitin conjugation. One such example has been reported for the yeast transcription factor Met4, which regulates biosynthesis of the sulfur-containing amino acids methionine and cysteine. Met4 is degraded by Met30 containing E3 ligase in an unstressed condition, but cadmium exposure disassembles the Met30-E3 ligase complex and stabilizes Met4 (2). Alternatively, in this model, we can assume the presence of an interfering protein in the formation of the Keap1-Cul3 complex. Indeed, in the case of Cul1, CAND1 disrupts the assembly of Cul1, Skp1, and F-box protein and inhibits the Cul1-based E3 ligase activity (46). In our current experimental condition, we were not able to observe dissociation of Keap1 from Cul3 by the electrophile treatment of or alanine substitution for critical cysteine residues (22; data not shown). Nonetheless, it is still important to explore this hypothesis.

Third, there is a possibility that oxidative stress may directly regulate the Cul3 activity, for example through NEDD8 modification. The ubiquitin-like protein NEDD8 regulates the E3 ligase activity of Cul3 by covalent modification, and this modification is essential for the association of Cul3 with the E2 enzyme (14, 33, 42). However, we feel that this last possibility is less likely than the previous two, as several proteins, including cyclin E, the topoisomerase-DNA complex, and RhoBTB, have already been identified as substrates for Cul3-type E3 ligase (37, 40, 45), such that oxidative/electrophilic stress would affect simultaneous divergent biological functions in this last model.

It has been reported that IκB and Hif1α, two prototype transcription factors important for the environmental response, are also degraded rapidly through the proteasome-dependent protein degradation pathway exploiting Cul1- and Cul2-type E3 ligases, respectively (1, 20). Therefore, repression by rapid protein degradation and derepression from the repression must be one of the common mechanisms for the regulation of cellular defense against environmental stresses. In the case of IκB and Hif1α, Cul-dependent degradation requires modification of substrate proteins, either phosphorylation of IκB or proline hydroxylation of Hif1α, and these modifications cause association of the substrate protein with the Cul-type E3 ligase. Importantly, these modifications are rigorously regulated in a stress-dependent manner. Meanwhile, the oxidative modification of Keap1 does not seem to contribute to either the association or dissociation between Nrf2 and Keap1-Cul3 E3 ligase complex but instead affects the ubiquitination activity of this complex. Thus, the stress-mediated activation mechanism for the Nrf2-Keap1 system appears to be quite different from those for the IκB and Hif1α systems in this regard.

In light of present progress, we have evaluated our initial hypothesis that the dissociation of Nrf2 from Keap1 contributes to the stress response as still valid. It has been reported that both endoplasmic reticulum stress-dependent kinase and protein kinase C phosphorylate Nrf2, resulting in the dissociation of Nrf2 from Keap1 (4, 5, 29). This suggests the possibility that posttranslational modification of Nrf2 may be one of the triggers for the activation of Nrf2. It also implies that, in addition to oxidative and electrophilic stresses, some alternative signal transduction pathways may activate Nrf2 through the modification of Nrf2.

Keap1 possesses a BTB domain, a well-known protein-protein interaction domain. The BTB domain of Keap1 was reported to contribute to the homodimerization of Keap1, which in turn promotes cytoplasmic sequestration of Nrf2 (48). In addition, it was recently reported that serine substitution for Cys151 in the BTB domain renders Keap1 unable to liberate Nrf2 even in the presence of electrophilic stress, suggesting that Cys151 might function as an alternative sensor for oxidants (43). We and other groups observed that deletion or point mutations of the BTB domain abrogate Keap1-mediated degradation or ubiquitination of Nrf2 (6, 11, 22, 44). These results thus suggest that the BTB domain of Keap1 may also play a crucial role in the oxidative stress response mechanism.

In summary, our study revealed that oxidative and electrophilic stresses impair the Keap1-mediated proteasomal degradation of Nrf2. This impairment enables the Nrf2 protein synthesized de novo after exposure to the stress to accumulate in the nucleus by bypassing the Keap1 gate. Based on these observations, we conclude that the sensing mechanism for oxidative and electrophilic stresses is closely linked to the degradation system of Nrf2. Thus, we are now one step closer to understanding Nrf2-Keap1 function in vivo.

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