Specific Patterns of Electrophile Adduction Trigger Keap1 Ubiquitination and Nrf2 Activation* S

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Activation of the transcription factor Nrf2 regulates expression of phase II enzymes and other adaptive responses to electrophile and oxidant stress. Nrf2 concentrations are regulated by the thiol-rich sensor protein Keap1, which is an adaptor protein for Cul3-dependent ubiquitination and degradation of Nrf2. However, the links between site specificity of Keap1 modification by electrophiles and mechanisms of Nrf2 activation are poorly understood. We studied the actions of the prototypical Nrf2 inducer tert-butyldihydroquinone (tBHQ) and two biotin-tagged, thiol-reactive electrophiles, N-iodoacetyl-N-biotinylhexylenediamine (IAB) and 1-biotinamido-4-(4’-maleimidoethyl-cyclohexane)-carboxamidobutane (BMCC). Both IAB and tBHQ induced antioxidant response element (ARE)-directed green fluorescent protein (GFP) expression in ARE/thymidine kinase GFP HepG2 cells, and both initiated nuclear Nrf2 accumulation and induction of heme oxygenase 1 in HEK293 cells. In contrast, BMCC produced none of these effects. Liquid chromatography tandem mass spectrometry (MS-MS) analysis of human Keap1 modified by IAB or BMCC in vitro indicated that IAB adduction occurred primarily in the central linker domain, whereas BMCC modified other Keap1 domains. Treatment of FLAG-Keap1-transfected HEK293 with the Nrf2-activating compounds IAB and tBHQ generated high molecular weight Keap1 forms, which were identified as K-48-linked polyubiquitin conjugates by immunoblotting and liquid chromatography MS-MS. Keap1 polyubiquitination coincided with Nrf2 stabilization and nuclear accumulation. In contrast, BMCC did not induce Keap1 polyubiquitination. Our results suggest that Nrf2 activation is regulated through the polyubiquitination of Keap1, which in turn is triggered by specific patterns of electrophile modification of the Keap1 central linker domain. These results suggest that Keap1 adduction triggers a switching of Cul3-dependent ubiquitination from Nrf2 to Keap1, leading to Nrf2 activation.

Cellular defense against electrophiles and oxidants relies on detoxification by phase II biotransformation enzymes, antioxidan enzymes, and related stress response proteins (1–3). Many of these inducible genes, such as the glutathione S-transferases, NAD(P)H oxidoreductase, heme oxygenase 1 (HO-1), and γ-glutamyl cysteine ligase phase II genes are regulated at the transcriptional level through cis-acting DNA sequences known as antioxidant/electrophile response elements (ARE/EpREs) (4, 5). Inducers of ARE/EpRE-driven genes share the property of electrophilicity (6), which enables them to modify sulfhydryl groups in proteins that regulate signaling pathways involved in toxicity and stress. Oxidative stress induced by tert-butyldihydroquinone (tBHQ) and diethylmaleate also can induce ARE-driven genes to restore redox homeostasis and reduce oxidative damage (5).

A critical regulator of ARE-dependent transcription is the transcription factor Nrf2, which is a member of the basic-leucine zipper NF-E2 family (7, 8). Upon formation of heterodimers with one of the small Maf proteins, Nrf2 activates expression of ARE-driven phase II genes (9–12). Recent studies with Nrf2−/− mice indicate that Nrf2 regulates a variety of genes, including chaperones, antioxidant genes, and genes regulating protein degradation (12–14). Nrf2−/− mice are more susceptible to toxic chemicals and stress (13).

Keap1 is a cysteine-rich cytoplasmic protein that negatively regulates the activation of Nrf2 (15). Keap1 has five domains, an N-terminal domain, a BTB domain, a central linker domain, a Kelch repeat domain, and a C-terminal domain. The Kelch repeat domain is tethered to cytoskeletal actin and binds to Nrf2 directly (15, 16). The central linker domain is the most cysteine-rich domain and is required for cytoplasmic sequestration of Nrf2 (17). Intact cytoskeleton is also critical for allowing Keap1 to bind Nrf2 and prevent its translocation to nuclei (17, 18).

Recent studies have shown that Keap1 functions as a substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex and targets Nrf2 for ubiquitination and proteasomal degradation (19–21). Site-directed mutation of Keap1 led to the hypothesis that the rate-limiting step for Nrf2 stabilization resided at the level of Keap1-directed ubiquitination of Nrf2 (19). Dissociation of Nrf2 from Keap1 may represent a second regulatory step that allows Nrf2 to translocate to the nucleus and activate transcription of ARE-dependent genes.

The mechanism by which electrophiles induce Nrf2-dependent transcription is inadequately understood but appears to involve modification of specific Keap1 cysteine residues. Only

1 The abbreviations used are: HO-1, heme oxygenase 1; ARE/EpRE, antioxidant response element/electrophile response element; BMCC, 1-biotinamido-4-(4’-maleimidoethyl-cyclohexane)-carboxamidobutane; tBHQ, tert-butyldihydroquinone; HMW, high molecular weight; IAB, N-iodoacetyl-N-biotinylhexylenediamine; LC-MS-MS, liquid chromatography-tandem mass spectrometry; SCF, Skp1p-cullin-F-Box protein; HEK, human embryonic kidney; BTB, bricabrac, tramtrack, and broad complex.

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four cysteine residues in murine Keap1 (Cys-257, Cys-273, Cys-288, and Cys-297) have been shown to preferentially react with the prototypical alkylating agent dexamethasone 21-mesy late in vitro (22). Mutation of Keap1 at Cys-273 or Cys-288 impaired the ability of murine Keap1 to repress Nrf2-dependent transcription (23). These studies led to the suggestion that dissociation of Nrf2 from Keap1-Nrf2 may be regulated by the redox status of these specific cysteine residues.

We employed two cell-permeable, thiol-reactive biotin-tagged electrophiles to probe the relationship between Keap1 alkylation and Nrf2 activation. Electrophile-specific adduction patterns were observed in liquid chromatography-tandem mass spectrometry (LC-MS-MS) analyses of purified human Keap1. Electrophile-specific adduction was coincident with electrophile-specific Keap1 ubiquitination, Nrf2 stabilization, nuclear Nrf2 translocation, and ARE/EpRE-dependent gene activation. These novel data provide compelling support for the hypothesis that electrophile-directed Keap1 ubiquitination represents a rate-limiting step for electrophile-directed Nrf2-dependent gene activation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Transfection, and Treatment**—Human embryonic kidney 293 cells were obtained from American Type Culture Collection. Chinese hamster ovary cells (CHO), murine NIH 3T3 cells, and murine L929 cells were obtained from ATCC. Human embryonic kidney 293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin. Human epithelial kidney 293 cells and CHO cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin and containing 1 mg/ml Geneticin. Confluent cells in 100-mm dishes were transfected with pCMV-FLAG-Keap1 using Lipofectamine 2000 reagent at a ratio of 1:6 DNA:Lipofectamine and then were incubated in Dulbecco’s modified Eagle’s medium for 5 hours at 37°C. Medium with 500 μg/ml Geneticin was used to select the single cell colonies stably expressing FLAG-Keap1. Confluent cells in 100-mm plates were washed with phosphate-buffered saline and treated with 100 μl IAB or BMCC (both from Pierce), equal volumes of vehicle (Me2SO at 0.3% of total volume) were delivered in 4 ml of Dulbecco’s modified Eagle’s medium with 5% fetal bovine serum.

**Cell Fractionation and Immunoblot Analyses**—Confluent cells in 75-cm flasks were washed with cold phosphate-buffered saline and lysed in cold nuclear lysis buffer (5 mM Tris, pH 7.4, 50 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40) containing 10 μM protease inhibitor mixture (Sigma, P8340). Lysate was centrifuged at 12,000 rpm for 2 min, and the supernatant was collected as the crude lysate. The crude lysate was washed twice with nuclear lysis buffer to remove cytosolic contaminants, and nuclear pellet was lysed by treatment with all protein lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 10 mM EDTA, 0.1% SDS, 0.5% deoxycholic acid, pH 8.0) containing 5 μM protease inhibitor mixture followed by sonication for 5 cycles. The nuclear lysate was centrifuged at 13,000 rpm for 1 min to remove cell debris. Protein concentration was measured with BCA protein assay kit (Pierce). Cell lysate proteins were diluted 1:1 (v:v) with 5× SDS loading buffer and separated by SDS-polyacrylamide gel electrophoresis in 4–20% Tris-HCl ready gels (Bio-Rad). Resolved proteins were then transferred to polyvinylidene difluoride membranes, which were blocked with 5% milk in TBST buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.1% Tween 20) and then probed with anti-FLAG (Sigma), anti-Ferris), anti-FLAG (Sigma), anti-Keap1 (Santa Cruz), anti-biotin (Zymed Laboratories Inc.), and anti-Nrf2 (Santa Cruz) in 5% milk in TBST buffer. After treatment with the appropriate secondary antibody conjugated to horseradish peroxidase (Santa Cruz), immunostained proteins were detected by enhanced chemiluminescence with Western blotting luminal reagent (Santa Cruz).

**Coyest of Model Electrophiles to His6-Keap1 in Vitro**—Escherichia coli BL21(DE3) was transformed with plasmid pET-15b+HisKeap1, which encodes a full-length cDNA copy of human Keap1 inserted between the NdeI and XhoI sites. His6-Keap1 was expressed by the method developed by Dinkova-Kostova (22), except that LB medium was used to grow bacteria. To prevent protein aggregation and precipitation by disulfide bond formation, 10 mM β-mercaptoethanol was added to lysate buffer. His6-Keap1 protein was then purified by nickel nitrotriacetic acid-agarose affinity chromatography (Qiagen). To maintain Keap1 solubility, the purified protein was dialyzed against 25 mM Tris-HCl, pH 8.4, containing 5 mM EDTA and 5 mM mercaptoethanol before concentration and storage.

Ultratrace-MC low binding regenerated cellulose centrifugal spin filter devices with a 30,000 molecular weight cutoff were obtained from Millipore. Before sample addition, spin filters were sequentially rinsed with 200 μl of methanol and 200 μl of distilled water by centrifugation at 12,000 × g, Keap1 (60–70 μg) was loaded into the upper chamber of the spin filter, and the sample then was centrifuged at 12,000 × g to remove the solution. The proteins on the filter were washed with 200 μl of 1 M ammonium bicarbonate followed by centrifugation. The proteins were suspended in 50 μl of 1 M ammonium bicarbonate, pH 8.4, containing 100 μM IAB or BMCC in 0.3% Me2SO (v/v). After incubation at 37°C in the dark, the reaction was terminated by centrifugation to remove the buffer followed by an additional wash with 1 M NH4HCO3. The filtrates were discarded. The protein on the filter was suspended in 50 μl of 0.1 M ammonium bicarbonate containing 12 μl of 40 μM tris(carboxyethyl)phosphine (Pierce) and incubated at 50°C for 15 min. Then 20 μl of 0.2 M iodoacetamide was added to the sample for 15 min to convert free thiols to carboxamidomethyl derivatives. Modified porcine sequencing grade trypsin (Promega) then was added in a 1:50 protein:trypsin ratio, and the sample was incubated at 37°C for 18–24 h. Tryptic peptides were collected by centrifugation through the filter at 5,000 × g, and the filtrate was acidified with 1 μl of concentrated formic acid for LC-MS-MS analyses.

**Affinity Capture and Digestion of FLAG-Keap1**—Cytosplasmic protein fractions first were incubated with protein G-agarose (Sigma) in batch format for 3 h at 4°C. The sample was centrifuged at 2,000 × g for 2 min, and the supernatant was incubated with anti-FLAG-agarose (Sigma) overnight. After washing with nuclear lysis buffer (5 mM Tris, pH 7.4, 50 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40 and 50 mM Tris-HCl/150 mM NaCl, pH 7.6), the FLAG-Keap1 was eluted with 100 μg/ml FLAG peptide in TBS buffer. Eluted proteins were concentrated with an Amicon ultracentrifugal filter device, 10,000 molecular weight cutoff (Millipore) at 4,000 rpm and then reduced, alkylated, and digested with trypsin as described above.

**In-gel Digestion of Purified FLAG-Keap1 through SDS-PAGE**—FLAG-Keap1 proteins were captured from cytoplasmic fractions as described above and then separated by electrophoresis on 4–20% SDS-polyacrylamide gel (Bio-Rad ready gel) and stained with colloidal Coomassie Blue (Invitrogen). Gel bands then were minced into 1-mm cubes, which were washed with 0.1 M ammonium bicarbonate. Another 50 μl of 0.1 M ammonium bicarbonate was added to cover the gel cubes, then 50 μg of tris(carboxyethyl)phosphine was added, and the samples were incubated at 50°C for 15 min. Finally, 20 μl of 0.2 M iodoacetamide was added to the sample for 15 min in the dark. The liquid was then removed and replaced with 30 μl of 50% acetonitrile, 50 mM ammonium bicarbonate for 15 min. This step was repeated twice to further remove residual stain and reagents. The liquid was removed, and the gel cubes were desiccated in a vacuum centrifuge. The dehydrated gel cubes were then re-swelled in 20 μl of 0.1% puromycin in 25 mM ammonium bicarbonate and incubated at 37°C overnight. The digested peptides were then extracted twice from the gel with 40 μl of 60% acetonitrile, 0.1% formic acid. The extract then was evaporated by vacuum centrifugation, and the peptides were dissolved in 20 μl of 1% formic acid for LC-MS-MS analyses.

**LC-MS-MS Analyses**—Peptide digests were analyzed on a Thermo LTQ linear ion trap instrument equipped with a Thermospray LC system and microelectrospray source (Thermo Electron). LC-MS-MS analyses were done by reverse phase chromatography on an 11-cm fused silica capillary column (100 μm inner diameter) packed with Monitor C-18 (5 μm) (Column Engineering) and eluted first with water/ acetonitrile/formic acid (95:2:0.1, v/v/v) for 5 min. A linear gradient then increased acetonitrile to 50% by 28 min and 80% by 37 min and decreased acetonitrile to 7% by 40 min and then to 1% by 45 min. MS-MS spectra were acquired in data-dependent scanning mode with one full scan followed by one MS-MS scan on the most intense precursor with dynamic exclusion of previously selected precursors for a period of 3 min. MS-MS spectra were analyzed with TurboSequest (Thermo) with specification of carboxamidomethyl (+57 Da) or IAB adducts (+825.2 Da) as variable modifications.

**RESULTS**

**Identification of Keap1 Cysteine Residues Modified by Electrophiles in Vitro**—In ongoing studies of the cellular effects of reactive electrophiles, we have employed biotin-tagged electrophiles.
trophiles (Fig. 1). These compounds display chemistries typical of a number of electrophilic metabolites of drugs and chemical and of endogenous electrophiles. To identify targets for electrophile adduction in Keap1, we used LC-MS-MS to analyze adducts formed with Keap1 in vitro. Treatment of His6-Keap1 with IAB at a molar ratio of 5 to 1 for 2 h at 37 °C yielded a total of 6 IAB-modified cysteines, each of which had a mass increase of 382.5, corresponding to IAB adducts (Table I, Supplemental Fig. 1). All of these cysteines (Cys-196, Cys-226, Cys-241, Cys-257, Cys-288, and Cys-319) are in the central linker domain, which is required for cytoplasmic sequestration of Nrf2 (17). For LC-MS-MS analyses, Keap1 protein was first reduced with tris(carboxyethylphosphine), and reduced cysteine thiols were alkylated with iodoacetamide to produce S-carboxamidomethyl derivatives. LC-MS-MS analyses routinely generated MS-MS spectra corresponding to ~80% of the protein sequence. All of the cysteine-containing tryptic peptides were detected, except for Cys-151, Cys-395, and Cys-406. In analyses of electrophile-treated Keap1, cysteine-containing peptides were detected as S-carboxamidomethylated derivatives (corresponding to unmodified cysteines) or as electrophile-adducted cysteines (Table I). All adducts were characterized by mass shifts to b and/or y ions that confirmed sequence location of the adducts. MS-MS spectra of all adducts are presented as supplemental material (Supplemental Figs. 1–1 to 1–17).

A 2-h treatment of Keap1 with 100 μM IAB at a 5 to 1 molar ratio should be adequate for the critical reactive cysteines to be modified, since in our in vitro studies, Nrf2 was activated, and its downstream biological effects were observed within 2 h of electrophile treatment (see below). Longer exposure to IAB (4 and 6 h) resulted in alkylation of cysteines outside the central linker domain (Table II). These include cysteines in the BTB domain (Cys-77), in the Kelch repeat domain (Cys-368, Cys-489, Cys-518, and Cys-583) and in the C-terminal domain (Cys-622).

The biotin-tagged N-alkylmaleimide BMCC displayed a very different pattern of alkylation than IAB. At 37 °C and at a molar ratio of 5 to 1, BMCC modified 5 cysteines, including Cys-196 and Cys-249 in the central linker domain Cys-77 in the BTB domain and Cys-368 and Cys-489 in the C-terminal Kelch repeat domain (Table I). The only residue attacked by both BMCC and IAB is Cys-196. Thus, the results shown in Table I indicate that Keap1 cysteines exhibit different chemical reactivity toward the two electrophiles.

**IAB and BMCC Produce Differential Nrf2 Activation in Vivo**—IAB was modestly toxic to HEK293 cells, as measured by the lactate dehydrogenase leakage assay, and 100 μM IAB induced <5% lactate dehydrogenase leakage at 4 h. Treatment of ARE/thymidine kinase green fluorescent protein-transfected HepG2 cells with 100 μM IAB for 2 h at 37 °C increased ARE-directed green fluorescent protein expression 2.78-fold (Supplemental Fig. 2), which was similar to the induction by the known ARE activator tBHQ (3.72-fold) and which demonstrated that IAB can induce expression of ARE-driven genes. Furthermore, a 2-h treatment of HEK293 cells with 100 μM IAB induced the accumulation of nuclear Nrf2 and increased the expression of HO-1 protein (Supplemental Fig. 3). Purity of cytosolic and poly(ADP-ribose)polymerase-1, respectively (Supplemental Fig. 3).

To further examine the interplay of electrophiles with the
Under reducing conditions on SDS-PAGE (15 mM β-mercaptoethanol in the loading buffer). Pretreatment of the samples with 8 M urea, reduction with tris(2-carboxyethyl)phosphine, and alkylation of the reduced protein with iodoacetamide before SDS-PAGE failed to alter the migration of the HMW Keap1 products (data not shown). However, these denaturation conditions did result in detection of Cys-151 as the S-carboxamidomethyl derivative (data not shown) and is consistent with the work of Wakabayashi et al. (23), which indicated that Cys-151 does not undergo addition. Treatment of His$_{6}$-Keap1 with IAB in vitro did not generate HMW Keap1 products detectable by immunoblotting (Supplemental Fig. 5).

The formation of HMW Keap1 upon treatment with the prototypical ARE inducer tBHQ was reported previously (17). In FLAG-Keap1-expressing 293 cells, both IAB and tBHQ induced a concentration-dependent formation of HMW Keap1 forms (Fig. 4).

To better understand the nature of the HMW Keap1 forms, we analyzed tryptic digests of the corresponding gel bands by LC-MS-MS. Eight gel sections corresponding to a molecular mass range from 70 kDa and above (indicated in Fig. 3A) were analyzed. The majority of peptides detected in these analyses mapped to Keap1 and ubiquitin, both of which were represented by detection of multiple peptides in different bands. Keap1 proteins from both control and IAB-treated cells were found in multiple bands of 70-kDa molecular mass and higher (Fig. 5). However, the distribution of HMW Keap1 and ubiquitin are different between these samples. In control cells, Keap1 protein with a molecular mass of 70 kDa is the dominant species (Fig. 5A), whereas Keap1 protein was detected primarily in bands corresponding to a molecular mass greater than 150 kDa in the IAB-treated cells (Fig. 5B). Ubiquitin peptides were only detected in band 6 in control samples, which is consistent with the immunoblot result (Fig. 5A). Ubiquitin peptides were found in several bands corresponding to ubiquitin-immunoreactive HMW Keap1 forms from IAB-treated cells (Fig. 5B), especially in band 6, where 6 ubiquitin peptides were detected, corresponding to 68.4% of the ubiquitin sequence. The numbers of Keap1 and ubiquitin peptides detected by LC-MS-MS depends in part on the levels of background contaminant proteins in each gel band. Thus, numbers of peptide identifications for Keap1 and ubiquitin are at best a semiquantitative measure of protein concentration. However, numbers of detected peptides generally coincide with protein levels

Keap1-Nrf2 system, we generated HEK293 cells stably expressing FLAG-Keap1 (Supplemental Fig. 4). Treatment of FLAG-Keap1-transfected cells with 100 μM IAB for 2 h at 37 °C resulted in a robust induction of nuclear Nrf2 as well as increased expression of HO-1 (Fig. 2). TBJQ also induced nuclear Nrf2 accumulation and induction of HO-1 protein. In contrast, neither nuclear Nrf2 accumulation nor expression of HO-1 protein was affected by a 2-h BMCC treatment. This result suggests that IAB but not BMCC can induce Keap1-Nrf2 dissociation, nuclear translocation of Nrf2, and Nrf2-mediated expression of HO-1.

**Different Effects of IAB and BMCC on Formation of High Molecular Weight Keap1 Forms in Vivo**—FLAG-Keap1-transfected cells were treated with 100 μM IAB for 2 h at 37 °C, and FLAG-Keap1 proteins were captured with anti-FLAG antibodies and analyzed on reducing SDS-PAGE gels. The majority of the Keap1 protein from IAB-treated cells migrated in a series of high molecular weight (HMW) bands with a molecular mass of greater than 150 kDa, whereas the Keap1 protein from untreated cells migrated with an observed molecular mass of 70 kDa, which corresponds to the Keap1 monomer (Fig. 3, A and B). Immunoblotting with anti-ubiquitin indicated intense ubiquitin immunoreactivity co-migrating with the HMW Keap1 protein bands from IAB-treated cells but not from controls (Fig. 3C). HMW Keap1 forms were not observed in cells treated with 100 μM BMCC (Fig. 3, D–F) nor was anti-ubiquitin immunoreactivity detected. The HMW Keap1 protein forms were detected under reducing conditions on SDS-PAGE (15 mM β-mercaptoethanol in the loading buffer). Pretreatment of the samples with 8 M urea, reduction with tris(2-carboxyethyl)phosphine, and alkylation of the reduced protein with iodoacetamide before SDS-PAGE failed to alter the migration of the HMW Keap1 products (data not shown). However, these denaturation conditions did result in detection of Cys-151 as the S-carboxamidomethyl derivative (data not shown) and is consistent with the work of Wakabayashi et al. (23), which indicated that Cys-151 does not undergo addition. Treatment of His$_{6}$-Keap1 with IAB in vitro did not generate HMW Keap1 products detectable by immunoblotting (Supplemental Fig. 5).

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![Cellular Localization of Keap1 and Nrf2](http://www.jbc.org/)

**Fig. 2. Effect of IAB, BMCC, and tBHQ on nuclear Nrf2 and cytosolic HO-1 protein levels.** Cells stably expressing FLAG-Keap1 were treated with 100 μM IAB, BMCC, or tBHQ, and nuclear and cytosolic protein fractions were prepared. Nuclear proteins from each sample (20 μg) were analyzed by SDS-PAGE and immunoblotting with anti-Nrf2 antibody. Cytoplasmic proteins (20 μg) were analyzed by SDS-PAGE and immunoblotting with anti-HO1 antibody.

**Fig. 3. Formation of HMW keap1 protein forms in HEK293 cells stably expressing FLAG-Keap1 treated with electrophiles.** FLAG-Keap1 proteins isolated from untreated controls and either IAB treated (A–C) or BMCC-treated (D–F) FLAG-Keap1-transfected HEK293 cells were separated into aliquots to be run on three SDS-PAGE gels and analyzed by Western blotting. Keap1 proteins were detected with anti-Keap1 (A and D), anti-FLAG (B and E), and anti-ubiquitin (C and F).
detected by immunostaining. Lack of detection of ubiquitin peptides in band 8 (Fig. 5B) reflects the presence of contaminating HMW proteins that did not enter the gel.

Trypsin digestion of ubiquitinated proteins leaves a Gly-Gly tag attached to the ε-amino group of the ubiquitin-modified lysine on the target protein, leading to the increased mass shift of 114 on this lysine. One of the ubiquitin peptides was found to be Gly-Gly-modified at the site of Lys-48 (Supplemental Fig. 5), which indicates that the HMW Keap1 proteins contained Lys48-involved polyubiquitin chains (24).

Characterization of Ubiquitination and IAB Adduction Sites on Keap1 in Vivo—To map the sites of IAB-induced modifications on Keap1 in vivo, FLAG-Keap1-transfected cells were treated with IAB, and FLAG-Keap1 protein was captured with an anti-FLAG affinity column. LC-MS-MS analysis of a tryptic digest of FLAG-Keap1 proteins identified a peptide containing a Gly-Gly tag at Lys-298, which is in the central linker domain (Fig. 6).

Further analysis of the LC-MS-MS data also revealed three IAB adduct sites, which were identified as Cys-241 (detected in 3/3 analyses), Cys-257 (2/3), and Cys-288 (3/3). These cysteines are also Keap1 adduction sites after IAB treatment in vitro (see above). Cys-226 was not found to be IAB-modified, which is consistent with our in vitro studies indicating that this residue was found modified in only 1 of 3 samples when Keap1 protein was incubated with IAB for 2 h (see above).

Nrf2 Stabilization Coincides with Electrophile-specific Adduction and Ubiquitination of Keap1 in Vivo—FLAG-Keap1-transfected cells were exposed to 100 µM IAB for 2 h. FLAG-Keap1 proteins then were captured with anti-FLAG antibodies, and associated proteins were analyzed by immunoblotting (Fig. 7). In untreated cells, very little Nrf2 was found associated with Keap1, as detected by immunoblotting. These results are consistent with those reported previously (19) and were interpreted to indicate rapid destabilization of Nrf2 when it is associated with non-adducted Keap1. Treatment with IAB, which resulted in the adduction and ubiquitination of Keap1, also resulted in Nrf2 stabilization (Fig. 7A). IAB treatment-induced Nrf2 stabilization was indicated by elevated cytosolic and nuclear Nrf2 (Fig. 7B).

DISCUSSION

Keap1 serves as a sensor trigger for the activation of Nrf2-regulated genes by electrophiles. Two models have been proposed to account for the role of Keap1. In the first Keap1 sequesters Nrf2 in association with cytoskeletal actin filaments (18). Electrophile modification of Keap1 thiols is proposed to dissociate the Keap1-Nrf2 complex, thus enabling Nrf2 nuclear translocation and activation of Nrf2-regulated genes (22). However, recent work indicates that Keap1 serves as an adaptor for Cul3-dependent Nrf2 ubiquitination (19, 25). This observation suggests that Keap1 does not passively sequester Nrf2 but instead actively directs Nrf2 degradation by facilitating Cul3-dependent Nrf2 ubiquitination. In this model, electrophiles have been shown to block Nrf2 ubiquitination, resulting in Nrf2 stabilization and nuclear translocation (19). However, a major unresolved issue is the mechanism by which electrophiles trigger these events.

Here we have mapped Keap1 modifications by different thiol-reactive electrophiles in vitro and in vivo, and we have demonstrated that site-specific adduction leads to Keap1 ubiquitination and Nrf2 activation. We used two biotin-tagged ele-
trophiles with different thiol-reactive chemistries to probe the relationship between Keap1 modification specificity and Nrf2 activation. The iodoacetamide-containing probe IAB and the N-alkylmaleimide probe BMCC displayed strikingly different specificities for Keap1 alkylation in vitro, and only IAB activated Nrf2 in vivo. Whereas IAB alkylated human Keap1 primarily in the central linker domain, BMCC modified Keap1 primarily in other domains. In our experiments, different electrophiles yielded different adduct maps. IAB alkylated Keap1 preferentially in the central linker domain. Neither IAB nor BMCC modified Cys-273. Our failure to detect adduction at Cys-273 was not due to poor sequence coverage in LC-MS-MS analyses. Indeed, we routinely detected all of the cysteines in question either as adducts or as carboxamidomethylated derivatives after reduction and alkylation of cysteines with iodoacetamide during sample workup. All identifications were based on MS-MS spectra rather than on mass measurements of intact peptides or peptide adducts. The only cysteines not routinely detected in our analyses were Cys-151, Cys-395, and Cys-406. Of these, Cys-151 is of potential interest in Keap1 function (17). In the case of IAB, selective targeting of several central linker domain cysteines correlated with Nrf2 stabilization, nuclear translocation, and Nrf2-directed gene activation.

Previous MS analyses characterized Cys-273 and Cys-288 of murine Keap1 as targets of the electrophile dexamethasone mesylate (22) and led the authors to denote these as the “most reactive residues of Keap1.” In our experiments, human Keap1 is modified at Cys-273 but not Cys-273. Moreover, we found that dexamethasone mesylate modified human Keap1 at several central linker domain Cys residues but not at Cys-273 or Cys-288 (data not shown). Differences between adduction patterns on murine versus human Keap1 may be due in part to sequence differences in the central linker domains, which differ by 12 of 153 residues. Our results suggest that site selectivity is a property not just of the amino acid target but of the electrophile structure and reactivity. Whether alkylation at specific residues in the central linker domain of Keap1 or elsewhere is a general trigger for Nrf2 activation cannot be satisfactorily resolved without further studies with other electrophiles.

Wakabayashi et al. (23) proposed that electrophiles induced dimerization of Keap1 monomers via Cys-273–Cys-288 disulfide linkages of the monomers. This “physical release” mechanism cannot be an obligatory means of Nrf2 activation, as this linkage is not possible in IAB adduction of Keap1, which nevertheless results in Nrf2 activation. Our data and those of Zhang et al. (17, 19) suggest that activation of Nrf2 reflects not merely changes in Keap1 structure but also Keap1 ubiquitination. However, the question of whether Keap1 ubiquitination occurs before or coincident with Nrf2 release will require additional study.

The major physical manifestation of change in Keap1 upon treatment with Nrf2 activators is the formation of HMW Keap1 forms. This was first reported by Zhang et al. (17). In accord with the work of Zhang et al. (17) treatment of Keap1 with either IAB or the prototypical ARE activator tBHQ formed a series of HMW Keap1 bands (Fig. 3). LC-MS-MS analyses of tryptic peptides from in-gel digestion of these HMW Keap1 bands indicated the presence of both Keap1 protein and ubiquitin, including Lys-48-(Gly-Gly)-modified ubiquitin peptides. These HMW Keap1 bands, thus, correspond to a Keap1 dimer and to its polyubiquitinated Keap1 forms. Although the presence of low amounts of a HMW Keap1 form also can be seen in untreated cells, most of the Keap1 migrates...
Recent reports indicated that under basal conditions Keap1 functions as an adaptor protein for Cul3-dependent ubiquitin ligase complex Keap1-Cul3-Rbx1 to target Nrf2 for ubiquitination and thereafter proteasomal degradation (19–21). Inducers of Nrf2-dependent transcription inhibit Keap1-dependent ubiquitination and further imply that adduction triggers a function of thiol reactivity in the protein sequence instead on Lys-298 of the Keap1 protein. This is certainly requires further testing, it is interesting to note that the ubiquitination target site on Keap1 is Lys-298, which lies adjacent to Cys residues in the central linker domain. Zhang et al. (17, 19) showed that substitution of serine at Cys-273 and Cys-288 in murine Keap1 blocked Nrf2 ubiquitination, and they speculated that these thiol reactive residues at central linker domain blocks ubiquitin transfer to Nrf2. On the other hand, electrophiles that modify Keap1 are Nrf2 inducers. Our results indicate that site-specific modifications of Keap1 by electrophiles switches ubiquitin targeting from Nrf2 to Keap1 and that this target switching mechanism governs Nrf2 activation by electrophiles.

Our results also indicate that site-specific adduction triggers Keap1 ubiquitination and further imply that adduction triggers a switching of Cul3-dependent ubiquitination from Nrf2 to Keap1, which results in Nrf2 activation (Fig. 8). Although this hypothesis certainly requires further testing, it is interesting to note that the ubiquitination target site on Keap1 is Lys-298, which lies adjacent to Cys residues in the central linker domain. Zhang et al. (17, 19) showed that substitution of serine at Cys-273 and Cys-288 in murine Keap1 blocked Nrf2 ubiquitination, and they speculated that these thiol reactive residues at central linker domain blocks ubiquitin transfer to Nrf2. The same line of reasoning suggests the possibility that the addition of cysteines in the central linker domain blocks ubiquitin transfer to Nrf2 and causes ubiquitin deposition instead on Lys-298 of the Keap1 protein.
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Specific Patterns of Electrophile Adduction Trigger Keap1 Ubiquitination and Nrf2 Activation

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