Covalent Modification at Cys151 Dissociates the Electrophile Sensor Keap1 from the Ubiquitin Ligase CUL3

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Received August 24, 2007

The regulation of cellular stress responses to electrophiles and oxidants is mediated by the transcription factor NF-E2-related factor 2 (Nrf2), which, in turn, is regulated by CUL-E3 (CUL3) ligase-mediated ubiquitylation. The Kelch-like ECH-associated protein 1 (Keap1) serves as an adapter between CUL3 and Nrf2. We used the model electrophile *N*-iodoacetyl-*N*-biotinylhexylenediamine (IAB) to define the relationship among the adduction of Keap1 cysteine residues, structure, and function. Exposure of Keap1 to IAB *in vitro* was accompanied by progressive loss of protein secondary structure, as monitored by CD spectroscopy and a loss of the ability to associate with recombinant CUL3. Dissociation of Keap1 from CUL3 *in vitro* was dependent upon C151 in Keap1. A quantitative mass spectrometry-based kinetic analysis of adduction in HEK293 cells expressing FLAG-Keap1 revealed that Cys151 was one of the most reactive residues *in vivo* and that it was required for IAB-mediated dissociation of the Keap1−CUL3 interaction. These results demonstrate that Cys151 adduction confers a critical alkylation sensor function upon Keap1, making Keap1 unique among BTB CUL3 adapter proteins.

1. Introduction

NF-E2-related factor 2 (Nrf2[1]) is a ubiquitous master transcription factor regulating a complex cellular stress response network that functions to mitigate the progression of a number of diverse diseases and associated pathologies (*1, 2*). It is a member of the CNC (cap 'n' collar) family which contain bZip folds (*3*) that promote heterodimerization. Nrf2 exhibits a proclivity for dimerization with the transcription factors c-Jun, Jun B, Jun D, ATF3, ATF4, and small Mafs (*3–6*). Heterodimeric binding to antioxidant response elements (ARE: 5′-(A/G)TGA (C/G) NNNGC-3′) (*7, 8*) located in the proximal promoter of target genes initiates Nrf2-directed expression of more than 200 gene products, such as those involved in phase II detoxication, antioxidant gene expression, apoptosis, cell growth, and proteasomal activity (*9*) (*2, 10*). Nrf2 regulates both the constitutive and inducible expression of this stress response network and may be considered a node for the integration of stress signaling.

Stressors such as reactive oxygen species, hypoxia, shear stress, or electrophilic stress exhibit significantly different chemistries, yet this diverse information is rapidly integrated into a common biochemical signal: activation of Nrf2. Under homeostatic cellular conditions, Nrf2 levels are maintained at low levels because of E3-ubiquitin ligase mediated ubiquitylation and proteasome-dependent degradation of Nrf2 (*11–13*). Targeting Nrf2 for ubiquitylation proceeds via association of the amino terminal Neh2 domain of Nrf2 with the carboxyl Kelch domain (*14*) of the CUL 3 ligase substrate adaptor protein, Keap1 (*11, 13, 15*). High affinity binding between Keap1 and Nrf2 subjects Nrf2 to rapid degradation (*16*). Stressors, however, rapidly inhibit Nrf2 ubiquitylation, allowing newly synthesized Nrf2 to translocate into the nucleus and induce ARE-directed gene expression.

Ubiquitination proceeds through a coordinated cascade involving an E1 ubiquitin-activating enzyme, an E2-conjugating enzyme, and an E3 ubiquitin ligase (*17*). Of the known E3 ligases, the RING (really interesting new gene) family is the largest. They contain a RING motif that coordinates E2 binding, facilitating the transfer of ubiquitin from the E2 to a lysine of the target protein (*18*). Cullin-RING E3 ligases are multisubunit members of the RING family and utilize 1 of 7 Cullin protein scaffolds for assembling distinct complexes. The amino terminus of a Cullin provides a binding interface for the recruitment of substrate receptor modules, while the C-terminus serves as a platform for E3 RING ligase−E2 interaction (*18–20*). The assembled Cullin−E3 ligase complex imposes architectural constraints on the positioning of the E2 and the target protein that favor ubitquitin transfer (*19*).

Proteins targeted for ubiquitination are captured by a substrate receptor module composed of a substrate recognition protein bound to an adapter protein that regulates association with distinct Cullin−E3 ligase complexes. The modules used to

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[1] Abbreviations: ARE, antioxidant response element; BTB, broad complex tramtrack, bric-a-brac; CD, dircular dichroism; DMSO, dimethylsulfoxide; DTT, dithiothreitol; GST, glutathione-*S*-transferase; HMW, high molecular weight; IAB, *N*-iodoacetyl-*N*-biotinylhexylenediamine; IAM, iodoacetamide; Keap1, Kelch-like ECH-associated protein 1; LC-MS-MS, liquid chromatography-tandem mass spectrometry; Nrf2, NF-E2-related factor 2; RING, really interesting new gene; SDS, sodium dodecyl sulfate; TCEP, tris-carboxyethylphosphine.

10.1021/tx700302s CCC: $40.75
Published on Web 02/06/2008

capture targeted proteins play a passive role: they serve only for protein recognition and positioning in a Cullin−E3 complex. Rather than using two proteins to provide adaptor and recognition functions, CUL3 complexes utilize proteins in which the two functions have coalesced. These proteins contain BTB (Broad complex Tramtrack, Bric-a-brac) domains that bind CUL3 and either Kelch or MATH domains to capture substrates (*21–24*). Keap1 contains an N-terminus BTB domain and a C-terminus Kelch domain (*14*).

Covalent modification of key Keap1 cysteine residues may represent a node for chemical integration of stressor signaling (*25*) and, if proven, would place Keap1 in a regulatory role rather than playing a passive role in substrate ubiquitylation. However, testing of this hypothesis requires demonstration that adduction at key residues *in vivo* alters protein function.

Several reactive cysteine residues have been identified in purified recombinant murine and human Keap1 (*26–28*). Keap1 containing a C273S or C288S mutation is incapable of directing Nrf2 ubiquitylation (*26, 29*). Yet, the biophysical mechanisms responsible for these observations are not well understood. These mutations do not affect the ability of ectopically expressed Keap1 to bind CUL3 (*11*) or associate with Nrf2 *in vivo* (*29*) nor does adduction of these residues *in vitro* inhibit the association between Keap1 and Nrf2 (*28*).

Mutation of C151 to serine in ectopically expressed Keap1 has been shown to inhibit stressor-mediated disassociation between CUL3 and Keap1 *in vivo* as well as stressor-mediated Nrf2 ubiquitylation (*13*). However, it is not known if stressor-mediated disassociation is directly related to Keap1 adduction at C151 *in vivo* or if dissociation is a consequence of upstream events. In this current work, we utilize biochemical and cellular approaches to show that Keap1 cysteine residue 151 regulates stressor-mediated disassociation from CUL3 *in vitro* and *in vivo*. Thus, the adduction of specific key cysteine residues in Keap1 functions to integrate chemical information into biochemical information that regulates the association between Keap1 and CUL3, identifying this relationship as a key regulatory node for the Nrf2 cellular response network.

2. Experimental Procedures

2.1. Construction of Recombinant DNA Molecules. The pCMV2b vector expressing FLAG-tagged human Keap1 and the pET-15b vector expressing His_6 tagged human Keap1 have been described previously (*27*). Mutant Keap1 cDNA containing serine codons in place of specific cysteine codons (C151 and C288) were generated by oligo-directed mutagenesis with standard techniques provided by Quick-change from Stratagene Inc. A pET-15b vector expressing the entire open reading frame of human Cul3 was constructed from pSPORT/Cul3 (I.M.A.G.E. cDNA library) PCR generated fragments that were cloned into pcDNA6/V5-HisA using NotI/XhoI restriction sites. The pCDNA6-Cul3/V5-HisA vector was then digested with PmeI and a Cul3/V5-His_6 fragment ligated into pET15b after NcoI/XhoI digestion.

Mouse Nrf2, a gracious gift from Dr. Michael McMahon (*8*), was PCR amplified and inserted into the pGEX-4T1 vector between *Sal*I and Not I sites. The following primers were used to amplify mNrf2: 5′-CCC GGG TCG ACT CAT GAT GGA CTT GGA GTT GCC ACC G-3′ and 5′-CAG CAG CGG CCG CCT AGT TTT TCT TTG TAT CTG GCT TC-3′. The resulting pGEX-4T1 vectors were used to transform the BL21 (PLyss) strain. Fusion proteins were purified from bacteria using glutathione−agarose resin. The eluted proteins were dialyzed in buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, and 1 mM mercaptoethanol) and stored in solution at 4 °C. An empty vector was used to express glutathione-*S*-transferase for use as a control in the binding assays. All of the genes used in this study were sequenced in the context of their expression vectors.

2.2. Cell Culture and Transfections. COS7 (American type Culture Collection) and human embryonic kidney 293 (HEK293) cells stably transfected with FLAG-tagged human Keap1 (*27*) were maintained in Dulbecco's modified Eagle's medium in the presence of 10% fetal bovine serum (FBS), 2 mM glutamine, 100 IU/mL penicillin, 100 IU/mL streptomycin, and 500 μM/mL Geneticin. Cells were cotransfected with plasmid DNA (5 μg per plasmid) in 100-mm-diameter plates using Lipofectamine-2000 (Invitrogen) according the manufacturer's instructions.

2.3. Keap1 Adduction Kinetics Determination *in Vivo*. Confluent HEK293 cells stably expressing human FLAG-tagged Keap1 were treated in 150 mm dishes with 100 μM IAB or an equal volume of vehicle (DMSO) and incubated at 37 °C in the dark at several time points. Cells were lysed on dishes with 50 mM NaCl, 10 mM EDTA, 25 mM Tris at pH 7.4, 0.5% NP-40, and protease inhibitor cocktail. FLAG-human Keap1 was purified by anti-FLAG agarose affinity chromatography, which was described previously (*27*). Purified FLAG-human Keap1 was concentrated with an ultrafree-MC centrifugal filter unit (Millipore, MWCO 10 K) in 50 mM ammonium bicarbonate at pH 8.0. Following reduction with 10 mM dithiothreitol at 50 °C for 15 min and alkylation with 25 mM iodoacetamide at room temperature in the dark for 15 min, FLAG-human Keap1 was digested by modified porcine sequencing grade trypsin (Promega) at 37 °C for 16–18 h in a 1:50 trypsin/protein ratio. Digestion was stopped, followed by dilution to 1 μg/mL in 0.1% (v/v) formic acid for LC-MS-MS analysis.

2.4. LC-MS-MS Analysis. Tryptic peptides were analyzed by LC-MS-MS with a Thermo LTQ linear ion trap mass spectrometer (Thermo Electron, San Jose, CA) equipped with a Thermo Surveyor HPLC system, nanoelectrospray source, and microautosampler. Peptides were resolved on a fused sillica capillary column packed with C18 resin (5 μm, 300 Å, Jupiter, Phenomenex, Torrance, CA) using a gradient mixture of 0.1% (v/v) formic acid in water (solvent A) and acetonitrile (solvent B). MS-MS spectra were acquired by a multiple reaction monitoring (MRM) method, in which *m*/*z* corresponding to the IAB-adducted peptide–ions are targeted for MS-MS. A window of ± 0.5 *m*/*z* was used for precursor isolation. Quantitation is based on extracted ion current from the MS-MS spectra for the four most abundant *b*- or *y*-ions for each targeted peptide. Peak areas corresponding to the summed product ion signals were measured with the Genesis peak algorithm in Xcalibur (Thermo Electron). To normalize the peak areas for the targeted peptide adducts, four tryptic peptides of Keap1 protein not subject to IAB adduction (EYIYMHFGEVAK, FYVQALLR, LADLQVPR, and LIYTAGGYFR) were chosen as internal standards. Peptide ion *m*/*z* for these standards were also targeted in the same analyses of Keap1 adducts, and the ion current corresponding to the four most abundant b- or y-ions was extracted from the MS-MS spectra, summed, and peak areas integrated as for the adducts. Peak areas for the standards were used to generate normalization factors which enabled comparisons of the adduct-derived peak areas across multiple LC-MS-MS analyses. (The precursor *m*/*z* and the product ion *m*/*z* used for quantitation of the adducts and the internal standard peptides are listed in Table S1, Supporting Information.) Values of k_{obs} were taken from the plots of the normalized adduct peak areas versus time and calculated by one phase exponential association by nonlinear regression using GraphPad Prism 4 software.

2.5. Circular Dichroism (CD) Spectroscopy. Purified human His_6-Keap1 (1.36 μM–36.7 μM Cys residues) was incubated with 10 μM IAB at room temperature. CD spectra were collected in 0.1 cm quartz cuvettes from 260 to 195 at 1 nm intervals with a 20 nm/min scan rate with a Jasco J-810 instrument under a constant nitrogen purge. Secondary structural assignments were calculated using the equation below:

$$[\theta] = 100 \times \text{signal}/\text{cnl}$$

where *c* is the concentration of the protein in mM; *n* is the number of amino acid residues; and *l* is the cell path length in cm.

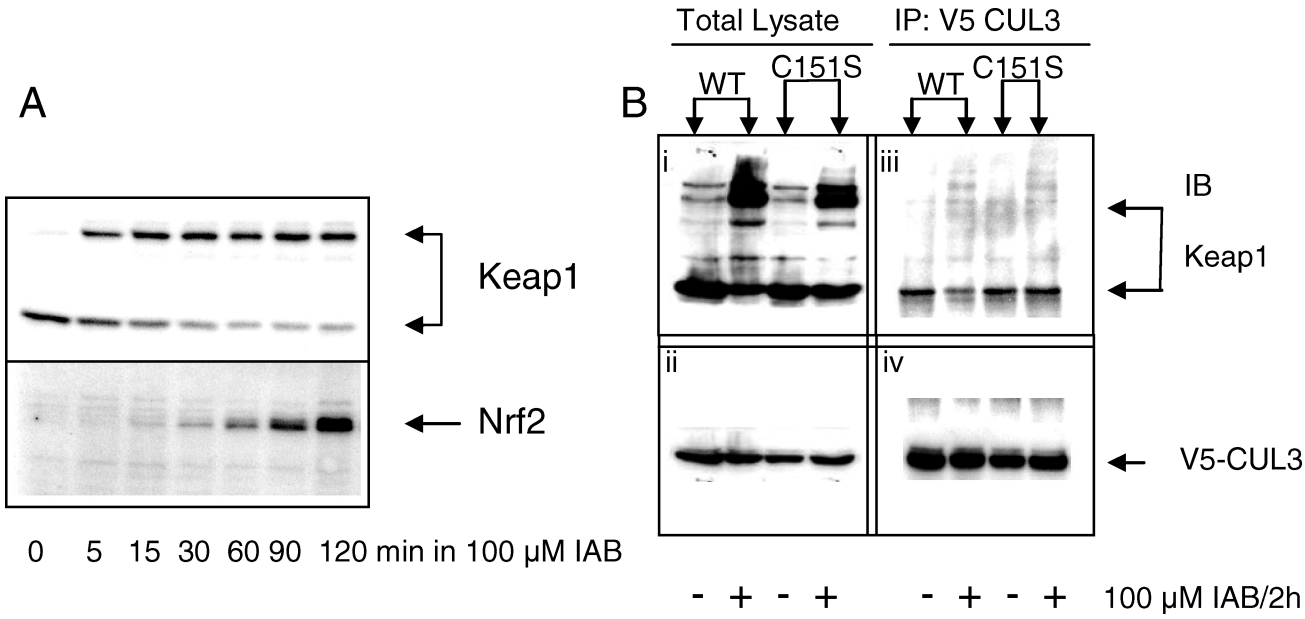

Figure 1. Cys151 is required for IAB-mediated dissociation of the Keap1/CUL3 complex. (A) HEK293 cells stably expressing FLAG-hKeap1 were treated with 100 μM IAB for the indicated times and then immunoblotted for Keap1 and Nrf2. (B) COS7 cells were transiently cotransfected with vectors expressing wild type or mutant (C151S) FLAG-Keap1 and V5-CUL3. After 36 h of transfection, cells were treated with 100 μM IAB for 2 h. Total cell lysate was either directly resolved using 10% SDS−PAGE and immunoblotted for Keap1 (panel i) or V5 (panel ii), or the cell lysate was immunoprecipitated with antibody to V5 proteins, resolved using 10% SDS−PAGE and immunoblotted for Keap1 (pane iii) or V5 (panel iv).

2.6. *In Vitro* Binding Assays. 2.6.1. Keap1/Cul3. Recombinant His_6-human Keap1 protein was expressed from pET15b(+) in *Escherichia coli.* BL21 (DE3) and purified by nickel-nitrilotriacetic acid agarose affinity chromatography (Qiagen), as described previously (*27*). Recombinant V5-His_6 Cul3 was expressed and purified in a similar manner. Purified recombinant Keap1 (25 μg) was treated with IAB (5:1 molar ratio, IAB to Cys residues) or with an equal volume of vehicle (DMSO) for 2.5 h at 37 °C. Recombinant V5-His_6 Cul3 (25 μg) was then added to Keap1, and complexes were incubated at 4 °C for 1.5 h. Keap1/Cul3 complexes were captured using anti-V5 agarose resin (4 °C/1 h), and washed 3 times with 50 mM Tris at pH 8.0 and 150 mM NaCl. Proteins bound to the antiV5 resin were resolved using 10% SDS−PAGE. Protein was visualized by immunoblotting for Keap1 and V5.

Alternatively, purified recombinant human Keap1 (25 μg) was mixed with recombinant V5-Cul3 (25 μg) and the complex incubated at 4 °C for 1.5 h. Complexes were then treated with IAB (5:1 molar ratio) or with an equal volume of vehicle (DMSO) for 2.5 h at 37 °C. Keap1/Cul3 complexes were captured as described above and washed extensively. Proteins bound to the antiV5 resin were resolved using 10% SDS−PAGE. Protein was visualized by immunoblotting for Keap1 and V5.

2.6.2. Keap1/GST-Nrf2. Purified recombinant Keap1 (25 μg) was bound to Ni-NTA agarose beads in 300 μL of NTA buffer (50 mM NaH_2PO_4 at pH 8.0 containing 1 mM TCEP). Purified GST-Nrf2 (25 μg) or GST-protein (25μg) was added and complexes incubated for 1 h at 4 °C. Complexes were washed 2 times with NTA buffer, resuspended in 300 μL of NTN buffer without TCEP and exposed to IAB (5:1 molar ratio) or with an equal volume of vehicle (DMSO) for 2 h at 37 °C. Keap1/GST-Nrf2 complexes were washed 3× with NTA buffer and resolved using 10% SDS−PAGE. Protein was visualized by immunoblotting for Keap1 and Nrf2.

2.6.3. Immunoprecipitation. COS7 cells were lysed in RIPA buffer (10 mM sodium phosphate at pH 8.0, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.2% sodium dodecyl sulfate (SDS) containing 1 mM dithiothreitol (DTT), and protease inhibitor cocktail, (Sigma)). Soluble cell lysates were incubated with 2 μg of affinity-purified V5 antibody for 2.5 h at 4 °C, then protein G plus/protein A-agarose beads were added and incubated at 4 °C for 1.5 h. Unbound proteins were removed by washing four times with RIPA buffer. The immunoprecipitated proteins were resolved using 10% SDS−PAGE and visualized by immunoblotting.

3. Results

3.1. Ability of a Model Stressor to Mediate Disassociation of Keap1 from CUL3 *in Vivo* Is Dependent upon C151. A major challenge in deciphering the consequences of protein alkylation-induced signaling is to define a relationship between adduction and functional biochemical changes. While Keap1 certainly undergoes adduction at multiple sites *in vitro* (*25, 27, 28*) and *in vivo* (*27*), the available evidence does not clearly demonstrate that adduction of Keap1 directly stabilizes or activates Nrf2.

To probe the effects of electrophiles on Keap1 function, we used IAB, a prototypical thiol-reactive electrophile that displays chemistry typical of several environmentally and pharmacologically relevant agents and endogenous electrophiles (discussed in ref *30*). The iodoacetamide group in IAB alkylates thiols by an S_N2 mechanism involving the displacement of the iodide leaving group by the thiol.

IAB has been shown to induce Nrf2 stabilization and Nrf2-directed ARE reporter activity as well as Nrf2-regulated endogenous gene expression (*27*). HEK 293 cells that stably express human FLAG-Keap1 (*27*) were exposed to 100 μM IAB for various lengths of time. Treatment with IAB initiated the rapid appearance of a high molecular weight (HMW) form of Keap1 followed by stabilization of Nrf2 (Figure 1A), consistent with our previous work (*27*) and that of others (*29*). While the composition of HMW Keap1 is not fully understood, we have shown that HMW Keap1 is ubiquitylated at Lys 298 in Keap1 (*27*).

COS 7 cells were transiently cotransfected with V5-CUL3 and either wild type FLAG-Keap1 or FLAG-Keap1 containing a C151S mutation. Thirty-six hours after transfection, cells were exposed to 100 μM IAB for 0 or 2.5 h, and cell lysate was prepared. Total cellular protein was either directly resolved by SDS−PAGE and immunoblotted for Keap1 (panel i) and V5 (panel ii) or subjected to immunoprecipitation with V5 antibody, resolved by SDS−PAGE, and then immunoblotted for Keap1 (panel iii) and V5 (panel iv).

Table 1. Kinetic Rate Constants for the Reaction of IAB with Human FLAG-Keap1 in HEK293 Cells[a]

residue	k_{obs}
Cys13	0.019 ± 0.003
Cys151	0.018 ± 0.005
Cys257	0.008 ± 0.002
Cys288	0.020 ± 0.001
Cys297	0.009 ± 0.000
Cys613	0.018 ± 0.004
Cys622	0.010 ± 0.002

[a] Reported values are k_{obs} measurements in min^{-1} determined as described under Experimental Procedures and represent the mean ± SD for three determinations.

The IAB exposure caused the formation of HMW Keap1 in COS 7 cells transfected with either wild type Keap1 or Keap1 containing a C151S mutation (Figure 1B, panel i). It is clear that there is more HMW Keap1 immuno-reactivity in lanes representing cells exposed to IAB than in lanes representing cells exposed to vehicle control. This is a consistent finding in cells transiently transfected with Keap1 (data not shown). Although the exact reason for this observation is not understood, one interpretation is that cellular exposure to IAB inhibits proteasome-independent degradation (*31*) of adducted Keap1 in this cell system.

Recent work has identified stressor-mediated disassociation of Keap1 from CUL3 as a key regulatory step for Nrf2 stabilization (*13, 32*). The IAB treatment induced wild type Keap1 to dissociate from V5-tagged CUL3 in COS 7 cells in response to IAB treatment (panel iii). In contrast, the C151S mutation in Keap1 prevented stressor-mediated disassociation from CUL3 (panel iii). We did not observe HMW Keap1 C151S in the V5-CUL3 immunoprecipitant, although it was clearly present in the total cell lysate. One interpretation of these observations is that under these experimental conditions, there is a population of Keap1 that is not associated with CUL3 and susceptible to the formation of HMW species. Taken altogether, these data support the hypothesis that C151 in Keap1 regulates IAB-mediated dissociation from CUL3 *in vivo*.

3.2. Cys151 Residue in Keap1 Is Adducted by IAB *in Vivo*. Currently, it is not known whether C151 in Keap1 is adducted *in vivo*. We applied a quantitative mass spectrometry-based kinetic analysis method to determine whether residue C151 in Keap1 is adducted *in vivo*. HEK 293 cells stably expressing wild type human FLAG-Keap1 were exposed to 100 μM IAB. FLAG-Keap1 then was purified from the cellular lysate using an anti-FLAG affinity column. LC-MS-MS analysis of tryptic digests of FLAG-Keap1 protein purified from IAB-treated HEK 293 cells revealed adduction at Cys13, Cys151, Cys257, Cys288, Cys297, Cys613, and Cys622/624 (Table 1 and Figure S1, Supporting Information). To confirm this result, we also measured the kinetics of disappearance of the unmodified Cys151 *in vivo*. The unmodified Cys151 peptide from FLAG-Keap1 was rapidly depleted by IAB *in vivo* in HEK293 cells (Figure 2). It is clear from the data presented in Table 1 and Figure 2 that C151 is rapidly adducted by IAB *in vivo*.

3.3. Exposure to IAB *in Vitro* Alters Keap1 Conformation and Ability to Associate with CUL3. We used CD spectroscopy to determine if IAB induced changes in the secondary structure of Keap1 (Figure 3). Treatment with a 5-fold excess of IAB (IAB concentration to Keap1 Cys residues) caused a rapid loss of secondary structure in wild type Keap1, as indicated by a loss of ellipticity in the range from 220 to 240 nm (Figure 3A). In contrast, the C151S mutation significantly slowed IAB-induced changes (Figure 3B). Observation of changes at lower wavelengths was obscured by strong UV absorbance of the DMSO used as a vehicle for IAB. Adduction of Cys151 was verified by LC-MS-MS analysis of a tryptic digest of the IAB-treated protein (Figure S2, Supporting Information). These data demonstrate that IAB adduction results in concomitant changes in Keap1 secondary structure that are dependent upon C151.

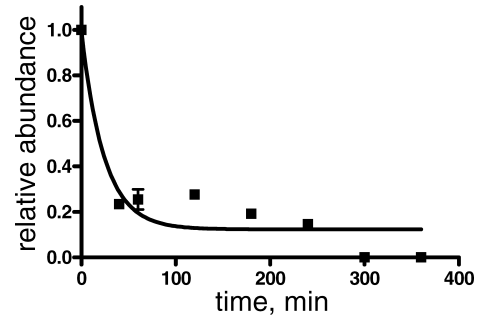

Figure 2. IAB treatment of HEK293 cells expressing FLAG-Keap1 results in the rapid disappearance of unadducted Cys151. HEK 293 cells were exposed to IAB (100 μM) for the indicated times. The relative change in the amount of the unadducted Cys151-containing tryptic Keap1 peptide was quantified by LC-MS-MS as described in Experimental Procedures.

We then assessed the ability of Keap1 to associate with CUL3 and the ability of IAB to induce the dissociation of Keap1 from CUL3. In the first set of experiments, recombinant human His_6-Keap1 was exposed to IAB (2.5 h), resolved by SDS, and then immunoblotted (Figure 4A). In contrast to the *in vivo* situation, we did not observe the formation of HMW recombinant Keap1. Purified, recombinant Keap1 was then exposed to IAB and allowed to bind to purified, recombinant V5-tagged human CUL3. V5 resin was used to capture V5 CUL3. The immunoblot shown in Figure 4B indicates that recombinant Keap1 bound avidly to recombinant CUL3. In contrast, IAB-treated Keap1 lost the ability to bind CUL3. When Keap1 and CUL3 were first allowed to associate and then the complex was exposed to IAB for 2.5 h (5:1 molar ratio), IAB treatment caused Keap1 to dissociate from CUL3 (Figure 4C). However, IAB (5:1 molar ratio) was unable to induce disassociation if recombinant His_6-tagged Keap1 containing a C151S mutation was used (Figure 4D). In contrast, IAB treatment was unable to induce dissociation of a GST-Nrf2 fusion protein from Keap1 (Figure 4E). Taken all together, these experiments demonstrate an IAB-mediated alteration of Keap1 structure *in vitro* and the concomitant loss of association with CUL3 that depends upon C151.

4. Discussion

The biotransformation of drugs and xenobiotics, as well as the formation of endogenous products of cellular oxidative metabolism all have the potential to generate reactive electrophiles that can covalently modify DNA, proteins, and lipids. Electrophile-modified biomolecules represent an important component in the progression of a number of diseases, including asthma, cancer, cardiovascular disease, inflammation, neurodegenerative diseases, and pulmonary fibrosis. To meet this challenge, vertebrates have developed a complex cellular stress response network regulated by heterodimeric binding of the master transcription factor Nrf2 to antioxidant response elements located in the proximal promoter regions of target genes (*4*).

Nrf2-directed gene expression is a consequence of electrophile-mediated post-translational regulation. Nrf2 binding to the CUL 3 substrate adaptor protein, Keap1, subjects Nrf2 to E3-ubiquitin ligase-mediated ubiquitylation and proteasome-dependent degradation (*11–13*). Electrophiles inhibit this process, triggering Nrf2/ARE-directed gene expression.

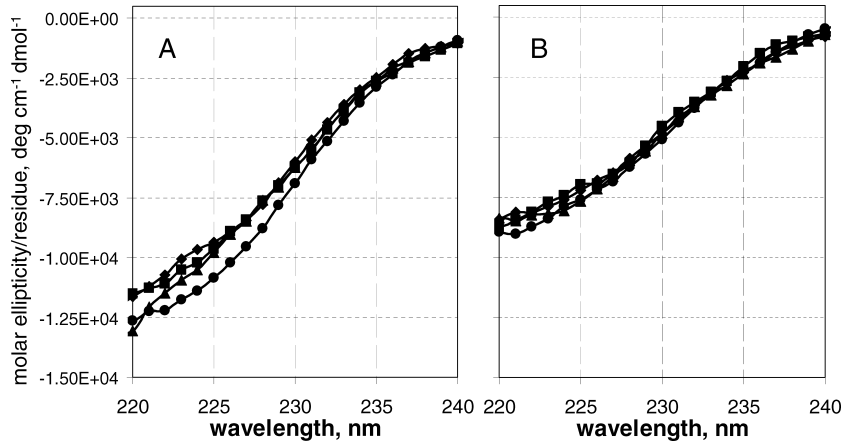

Figure 3. Cys adduction causes recombinant human Keap1 to undergo a rapid change in structural conformation, as measured by CD spectroscopy. One micromolar of purified wild type human His_6-Keap1 (A) or Keap1 containing a C151S mutation (B) was incubated with 20 μM IAB at room temperature. CD spectra were collected at time 0 (●), 1 min (▲), 1 h (■), or 3 h (♦).

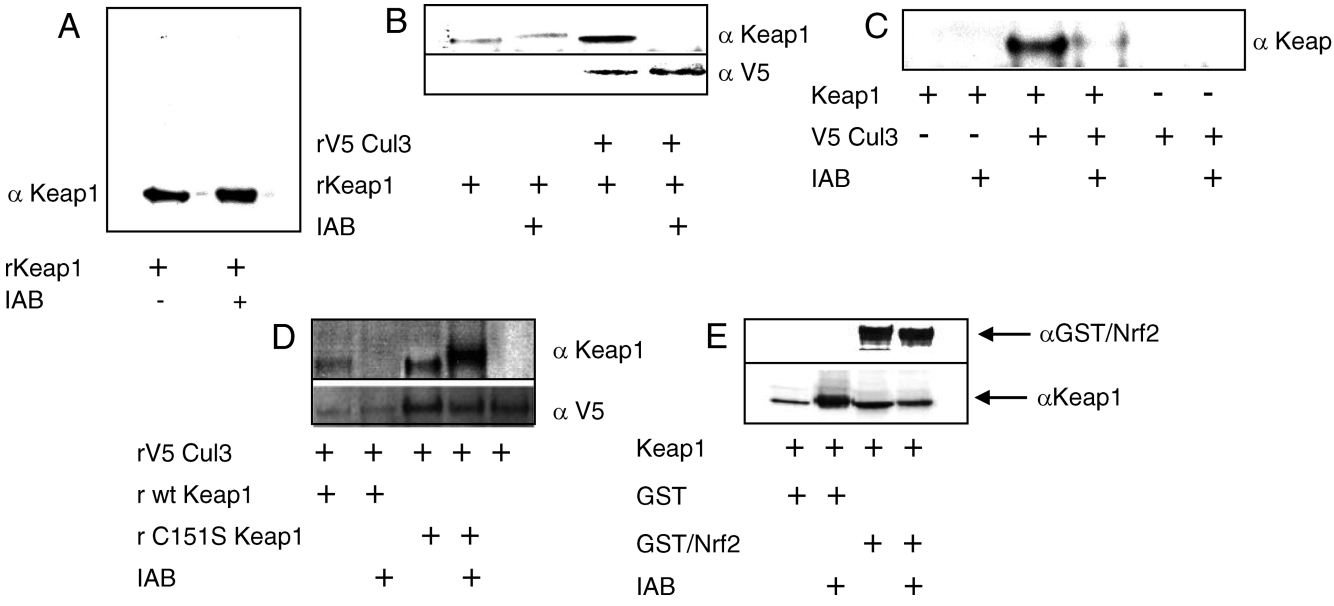

Figure 4. Adduction of Keap1 inhibits the association Keap1 with CUL3. (A) Purified, recombinant Keap1 was adducted with IAB (5:1 molar ratio/2.5 h/37 °C), resolved by SDS−PAGE, and immunoblotted. The immunoblot represents the input for experiments shown in panels B and C. (B) Purified, recombinant Keap1 was adducted with IAB (5:1 molar ratio/2.5 h/37 °C) and then added to V5-His_6-CUL3. V5-His_6-CUL3/Keap1 complexes were captured on V5-agarose beads. Protein complexes were resolved using SDS−PAGE and immunoblotted for V5 and Keap1. (C) Recombinant human Keap1 was first allowed to associate with recombinant V5-His_6-Cul3. The bound complexes were then treated with IAB (5:1 molar ratio/2.5 h/37 °C). V5-His_6-CUL3/Keap1 complexes were captured on V5-agarose beads. Protein complexes were resolved using SDS−PAGE and immunoblotted for V5 and Keap1. (D) Recombinant Keap1 (wild type or Keap1 containing a C151S mutation) was adducted with IAB (5:1 molar ratio/2.5 h) and then added to V5-His_6-CUL3. V5-His_6-CUL3/Keap1 complexes were captured on V5-agarose beads. Protein complexes were resolved using SDS−PAGE and immunoblotted for V5 and Keap1. (E) Purified GST/ Nrf2 or GST-protein was added to recombinant Keap1 bound to Ni-NTA agarose beads. The complexes were exposed to IAB (5:1 molar ratio/2 h/37 °C). Keap1/GST-Nrf2 complexes were then washed 3 times and resolved using SDS−PAGE, and the protein was visualized by immunoblotting.

The Keap1-Nrf2 sensor/transcription factor couple represents perhaps the best-characterized example of a sensor that translates reactive electrophile chemistry into the regulation of critical stress response genes. However, despite several years of intense investigation, the molecular mechanism that governs this system remains incompletely understood, particularly with respect to how chemical modification of the Keap1 protein translates to stabilization and activation of Nrf2. Initial work demonstrated that Keap1 tethered Nrf2 in the cytosol and that electrophilic/oxidative stressors enabled Nrf2 to translocate into the nucleus and direct ARE-regulated gene expression (*14*). Innovative work by Talalay and colleagues (*25, 33*) provided the paradigm for the hypothesis that a sensor protein could function to integrate electrophile chemistry and initiate the Nrf2-directed cellular response network. On the basis of the knowledge that ARE-directed gene expression could be induced by 10 chemically distinct classes and that within individual classes potency mirrored sulfhydryl reactivity, it was hypothesized that induction was regulated by Keap1 cysteine residues (*25, 26*).

Keap1 functions as an adapter protein for Cullin−E3-mediated Nrf2 ubiquitylation (*11, 13, 15*), and recent work has shown that electrophilic stressors can induce disassociation of Keap1-CUL3 complexes *in vivo*, thereby abrogating Nrf2 degradation (*13, 32*). To date, all other cullin substrate–proteins function in a passive role: they serve only for protein recognition and positioning in a Cullin−E3 complex. They do not exhibit regulatory functions. On the basis of the work presented here, it appears that Keap1 is unique among substrate adaptor proteins in that it functions in a regulatory role.

Previous work demonstrated that electrophiles modify several cysteines in Keap1, but no consensus has emerged regarding the mechanism(s) by which adduction of specific residues affected

function (*25, 28*). These studies left a key question unresolved: will adduction of Keap1 directly trigger the dissociation from CUL3, or is disassociation a consequence of an upstream event? The results of our studies demonstrate that exposure of Keap1 to IAB *in vitro* altered protein secondary structure and that a C151S mutant Keap1 was more resistant to IAB-induced structural change, as assessed by CD spectroscopy (Figure 3). These structural changes occurred concomitant with a loss of the ability of Keap1 to associate with CUL3 *in vitro* (Figure 4). Thus, the data demonstrate a direct link between changes in Keap1 structure and function. Furthermore, these studies are the first to show that Cys151 is adducted *in vivo*. We have shown that *in vivo* Cys151 is highly reactive and is clearly required for IAB-mediated dissociation of the Keap1/CUL3 complex (Figure 1). Our results do not rule out some functional significance for the adduction of other Keap1 cysteines, including the highly reactive Cys288. However, it seems clear from our results that Cys151 adduction is an essential trigger for CUL3 dissociation from the complex and thus for Nrf2 stabilization *in vivo*.

Acknowledgment. This work was supported by National Institutes of Health Grants CA104590, ES010056, and ES000267.

Supporting Information Available: Table of MS-MS transitions used for quantitation of Keapl peptides and MS-MS spectra of Keapl Cys151 tryptic peptide from treatment of FLAG-Keapl in HEK293 cells and from treatment of His_6-Keapl with IAB *in vitro*. This material is available free of charge via the Internet at http://pubs.acs.org.

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TX700302S