Activation of NRF2 by Nitrosative Agents and H$_2$O$_2$ Involves KEAP1 Disulfide Formation

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The NRF2 transcription factor regulates a major environmental and oxidative stress response. NRF2 is itself negatively regulated by KEAP1, the adaptor of a Cul3-ubiquitin ligase complex that marks NRF2 for proteosomal degradation by ubiquitination. Electrophilic compounds activate NRF2 primarily by inhibiting KEAP1-dependent NRF2 degradation, through alkylation of specific cysteines. We have examined the impact on KEAP1 of reactive oxygen and nitrogen species, which are also NRF2 inducers. We found that in untreated cells, a fraction of KEAP1 carried a long range disulfide linking Cys226 and Cys613. Exposing cells to hydrogen peroxide, to the nitric oxide donor spermine NONOate, to hypochlorous acid, or to S-nitroscysteine further increased this disulfide and promoted formation of a disulfide linking two KEAP1 molecules via Cys151. None of these oxidants, except S-nitroscysteine, caused KEAP1 S-nitrosylation. A cysteine mutant preventing KEAP1 intermolecular disulfide formation also prevented NRF2 stabilization in response to oxidants, whereas those preventing intramolecular disulfide formation were functionally silent. Further, simultaneously inactivating the thioredoxin and glutathione pathways led both to major constitutive KEAP1 oxidation and NRF2 stabilization. We propose that KEAP1 intermolecular disulfide formation via Cys151 underlies the activation of NRF2 by reactive oxygen and nitrogen species.

The Cap’n’collar bZip transcription factor NRF2 regulates an environmental and oxidative stress response of major physiological importance in mammals. NRF2 is activated by reactive oxygen and nitrogen species, electrophilic xenobiotics, and heavy metals and promotes cytoprotection and survival toward these stresses (for a review, see Refs. 1 and 2). Activation of NRF2 is intricate, engaging controls at the level of subcellular distribution, interaction with other proteins, phosphorylation, and protein stability (reviewed in Ref. 2). Among these, protein stability is a major control determinant, involving KEAP1, the adaptor of a Cul3-ubiquitin ligase complex that ubiquitinates NRF2 and marks it for proteosomal degradation (3–6). Stress signals that activate NRF2, herein named NRF2 inducers, are primarily sensed at the level of KEAP1, causing NRF2 protein stabilization (7–9) by inhibiting KEAP1-mediated NRF2 ubiquitination (10, 11).

The large number of NRF2 inducers and their quite different chemical nature have raised the question of how they are specifically sensed by KEAP1. Although NRF2 inducers are chemically very different, they all have electrophilic properties, which has led to the proposal that they must operate by alkyla- tion and/or oxidation of KEAP1 Cys residues (12). The 624-amino acid-long KEAP1 protein has 25 (mouse) or 27 (human) Cys residues and carries a Broad complex, Tramtrack, Bric-à-Brac (BTB)$^2$ dimerization domain, an intervening region (IVR), and a six-Kelch repeat domain (Kelch) (see Fig. 2). It also binds zinc with a 1:1 stoichiometry, possibly through the IVR residues Cys$^{254}$, Cys$^{273}$, Cys$^{288}$, and Cys$^{299}$, as suggested by the 100-fold lower zinc affinity of mutants lacking these residues (13).

Several laboratories have sought to identify in vitro which of the KEAP1 Cys residues are modified by NRF2 inducers, each identifying a different set of Cys residues, with most residues identified at least once (summarized in Refs. 14 and 15). Still the BTB domain Cys$^{151}$ and IVR Cys$^{288}$ came out as the most frequently identified and the most reactive residues. In vivo proofs of the modification of KEAP1 at Cys residues have also been obtained from cells treated with oxidized lipids (16–18), a carcinogenic acid derivative (19), nitric oxide and 8-nitro-cGMP (20, 21), and N-iodoacetyl-N-biotinylhexylenediamine (22), thus corroborating the hypothesis of a Cys residue-based mechanism in KEAP1 regulation. The latter study also mapped modified residues that included IVR cysteines and Cys$^{151}$ (22).

A third and very informative approach to the KEAP1 redox regulatory mechanism has been to evaluate the effect of Cys residue substitution on KEAP1 function. In cells expressing KEAP1 mutants that lack Cys$^{273}$ or Cys$^{288}$, NRF2 is constitutively active (11, 17, 23). Because these residues might contribute to zinc coordination, their substitution (or modification) could alter KEAP1 function through the loss of a structural or a redox regulatory zinc motif. In cells that express a KEAP1 mutant lacking Cys$^{151}$, NRF2 basal activity is in contrast low and cannot be induced by tert-butylhydroquinone (t-BHQ) and many other NRF2 inducers (6, 11, 24–26). Mice transgenic complementation rescue experiments have confirmed the functional importance of KEAP1 Cys$^{273}$, Cys$^{288}$, and Cys$^{151}$ (26). Further, a

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1 The abbreviations used are: BTB, Broad complex, Tramtrack, Bric-à-Brac; Cys-NO, S-nitroscysteine; NEM, N-ethylmaleimide; SpNO, spermine NONOate; t-BHQ, tert-butyl hydroquinone; IVR, intervening region; HA, hemagglutinin; shRNA, small hairpin RNA; YFP, yellow fluorescent protein; TrxR1, thioredoxin reductase 1.

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recent systematic study in zebra fish classified NRF2 inducers into at least two classes, one requiring KEAP1 Cys\textsuperscript{151} and the other requiring Cys\textsuperscript{273}, thus re-emphasizing the functional importance of these residues (27). By concluding that different NRF2 inducers trigger different Cys-based regulatory mechanisms, this study may also explain at least in part why the in vitro searches for reactive Cys residues, which have been conducted by different laboratories using different inducers, have yielded different results.

In the present study, we have examined the mechanism of KEAP1 regulation by H\textsubscript{2}O\textsubscript{2}, NO, and HOCl. These compounds are NRF2 inducers (20, 28 – 32) and are physiologically important as endogenously produced. A negative NRF2 regulation by H\textsubscript{2}O\textsubscript{2} has been described though (33). Although strongly electrophilic, these compounds differ chemically from the NRF2 inducers studied so far, because they are oxidants and not alkylating agents and are thus anticipated to modify Cys residue by oxidation. Despite the many studies of the KEAP1 redox mechanism, it is not known whether its Cys residues could undergo oxidation in vivo. We have thus carefully monitored the effect of H\textsubscript{2}O\textsubscript{2}, the NO releasing agent spermine NONOate (SpNO), and HOCl on the KEAP1 redox state and found that these compounds similarly oxidize KEAP1 with formation of intra- and intermolecular disulfides. Evaluation of the role of the oxidized residues on KEAP1 function suggests that the intermolecular disulfide is important for activation, whereas the intramolecular disulfide could have a structural role. We also show that simultaneous inactivation of the thioredoxin and glutathione systems leads to constitutive KEAP1 oxidation and strong NRF2 stabilization.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents**—HeLa cells were grown at 37 °C, 5% CO\textsubscript{2}, in Dulbecco’s modified Eagle’s medium containing 1 g/liter of glucose, 110 mg/ml sodium pyruvate, 4 mM GlutaMAX (Invitrogen), complemented with 10% fetal calf serum (Sigma). For plasmid transfection, ~5.5 10\textsuperscript{5} cells were incubated 5 h with 3 μg of DNA and Lipofectamine 2000 (Invitrogen) following the supplier’s recommendations, washed, and incubated in fresh medium. The cells were treated as described in the text 24 h after transfection. For the TRxR1 knockdown, ~2.5 10\textsuperscript{5} cells were transfected with 3 μg of DNA, by the same protocol. After 24 h, hygromycin B was added (250 μg/ml) to the culture medium twice every 2 days. The cells were then expanded and kept in the presence of hygromycin B (125 μg/ml). The selection was withdrawn before experiments. N-Ethylmaleimide (NEM), t-BHQ, H\textsubscript{2}O\textsubscript{2}, and cycloheximide were purchased from Sigma, and SpNO was from Cayman Chemical. S-nitrosocysteine (Cys-NO) was prepared by mixing stoichiometric amounts of l-cysteine and sodium nitrite at pH 4, followed by the measure of its concentration by recording the absorbance at 334 nm.

**Plasmids**—Plasmid pcDNA3-HA-mKEAP1 was a gift from Dr. M. Yamamoto, pcCI-HA-mNRF2 from was J. A. Diehl, and peYFP-N1 was purchased from Clontech. Plasmid pcDNA3-Myc-His-mKEAP1 was constructed by PCR-mediated replacement of the HA tag sequence of pcDNA3-HA-mKEAP1 with three Myc tag sequences followed by a stretch of eight His codons. Mutagenesis was done with the Stratagene QuikChange multi kit following the manufacturer’s instructions. For the TRxR1 knockdown, we used TRxR1-specific small hairpin RNA (shRNA) that targeted the following sequences within the open reading frame: GGATTAAGCCAAAC-AATAA (sh1), GCATCAAGCAGCTTTGTTA (sh2), and GCAAGACTTCGAAATTAT (sh3). shRNA sequences were designed with the DSIR program that also operates an exact similarity search algorithm for potential off target detection (34). These shRNA were expressed under the control of the H1 promoter from the Epstein-Barr virus-based replicative plasmid that contains an oriP, the EBNA open reading frame, and a hygromycin B selection cassette (35). Control lines expressed a nonfunctional shRNA as reported (35).

**Redox Western**—The cells were washed on ice with 40 mM NEM in phosphate-buffered saline and lysed in lysis buffer (0.1 M Tris- HCl, pH 8.0, 120 mM NaCl, 0.2% deoxycholic acid, 5% Nonidet P-40, 0.2 mM NaF, 0.2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, Roche-Complete mini protease inhibitor mixture, 40 mM NEM). Centrifuged-cleared lysates were diluted with 2 volumes of 3x loading buffer (0.2 M Tris-HCl, pH 6.8, 45% glycerol, 6% SDS, 0.03% bromphenol blue). Half of the samples were reduced by the addition of β-mercaptoethanol (6% v/v). After heat denaturation, the proteins were separated by SDS-PAGE, transferred onto a nitrocellulose membrane, and immunostained with anti-NRF2 (H300; Santa Cruz), anti-HA (HA11; Covance), anti-Myc (9E10; a kind gift from G. Clément and C. Créminon, France), anti-TRxR1 (a kind gift from A Holmgren, Sweden), or anti-YFP (JL8; Clontech) specific antibodies. Detection was performed after chromophore-coupled secondary antibody staining, using the LICOR Odyssey infrared imager.

**Protein Pulldown Assays**—For pulling down His tag-containing polypeptides, the cells (10\textsuperscript{8}/sample) were washed in ice-cold phosphate-buffered saline containing NEM (40 mM) and lysed in precipitation buffer (0.1 M Tris- HCl, pH 8.0, 1% Nonidet P-40, 2% glycerol, 0.3 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride, Roche-Complete mini protease Inhibitor mixture, 40 mM NEM). Centrifuged-cleared lysates were diluted with 2 volumes of 3x loading buffer (0.2 M Tris- HCl, pH 8.0, 1% Nonidet P-40, 2% glycerol, 0.3 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride, 10 mM NEM, 10 mM imidazole), the proteins were eluted with 2x protein-loading buffer containing 10 mM NEM and separated by SDS-PAGE.

**The Biotin-switch Technique**—The biotin-switch method was performed as described in Ref. 36, with the difference that cells were directly lysed in HEN buffer (250 mM HEPES, pH 7.7, 1 mM EDTA, 0.1 mM neocuproine) containing SDS (2.5%) and the thiol-reactive compound S-methyl methanethiosulfonate (0.1%) to block thiol modification during and after the lysis. Briefly, the proteins were precipitated and washed twice with acetone, then the pellets were dissolved in HEN buffer containing SDS (1%), and reduction and labeling of S-nitrosothiols were achieved by the addition of sodium ascorbate (100 μM) and biotin-HPDP (0.25 mg/ml) upon incubation in the dark at room temperature. For the specific detection of S-nitroso-
KEAP1, the samples were precipitated with acetone, washed with acetone, dissolved in HEN buffer 0.1 M containing SDS (1%). 3 volumes of (v/v) neutralization buffer (25 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100) was added before incubation with a streptavidin-agarose slurry. Elution was performed in 0.1 M HEN buffer containing -mercaptoethanol (1%). Western blots were then performed using anti-Myc antibodies.

RESULTS
Oxidation of KEAP1 in Cells Exposed to H2O2—We evaluated whether H2O2 could oxidize KEAP1 at Cys residues by analyzing the redox state of HA-KEAP1 ectopically expressed in HeLa cells. To prevent Cys residue oxidation, free sulfhydryls were blocked with NEM during sample preparation. HA-KEAP1 from untreated cell lysates that had been reduced by -mercaptoethanol migrated in SDS-PAGE as a single band with an apparent molecular mass of ~70 kDa (Fig. 1A, lower panel). When reduction was omitted, HA-KEAP1 from the same lysates still migrated as a major band of same molecular mass (denoted Red for reduced), but now a second less intense band of faster mobility was observed (denoted OxIM for oxidized intramolecular; see below) (Fig. 1A, upper panel). A 5-min exposure of cells to H2O2 (200 μM) further altered migration of KEAP1 under nonreducing conditions (Fig. 1A, lane 2); the reduced KEAP1 70-kDa band decreased in intensity, whereas OxIM increased and two new bands of slower migration appeared (denoted OxIR1 and OxIR2 for oxidized intermolecular; see below; lane 2). OxIM, OxIR1, and OxIR2 were absent under reducing conditions, indicating that they probably result from disulfide formation. OxIM might correspond to an intramolecular disulfide (see below), the formation of which is predicted to increase SDS-PAGE mobility because of a decrease in the hydrodynamic radius of the SDS-bound polypeptide, especially if its two constitutive Cys residues are far apart in the primary sequence. OxIR1/2 could correspond to intermolecular disulfides between KEAP1 and itself or with another polypeptide. Such oxidative KEAP1 modifications were not seen upon cell exposure to t-BHQ (supplemental Fig. S1A). Time course analysis showed that KEAP1 was maximally oxidized 5 min after exposure to H2O2 and then returned to the redox state of untreated cells 40 min after this treatment (Fig. 1A, lanes 2–7). Such kinetics presumably reflects KEAP1 reduction by endogenous reductases and not de novo protein synthesis, because it was similar in cells treated with the protein synthesis inhibitor cycloheximide (supplemental Fig. S1B). H2O2 dose-response analysis (Fig. 1B) indicated that the basal KEAP1 oxidation seen in lysates from untreated cells

![FIGURE 1. KEAP1 becomes oxidized in cells treated with oxidants. A–C, HeLa cells transfected with pcDNA-HA-KEAP1 were exposed to H2O2 (0.2 mM) for the indicated time (4) or for 5 min in the presence of different concentrations of H2O2 as indicated (B), or to SpNO (1 mM) for the indicated time (C). The cells were lysed in the presence of NEM (40 mM) to block free sulphydryls (see “Experimental Procedures”). The lysates were separated by nonreducing (upper panels) or reducing (lower panels) SDS-PAGE, and KEAP1 was revealed by Western blot using an anti-HA antibody. The arrows indicate the oxidized slow (OxIR1 and OxIR2) and fast (OxIM) and reduced (Red) KEAP1 species. D, HeLa cells expressing pcDNA-HA-KEAP1 were either left untreated or were exposed to H2O2 (0.2 mM for 5 min), to CysNO (0.5 mM) for 10 min, or to SpNO (2 mM) for 45 min. KEAP1 S-nitrosylation was evaluated by the biotin-switch method as described under “Experimental Procedures.” KEAP1 was revealed by anti-HA Western blot of the streptavidin eluate (upper panel) and as control of the corresponding whole cell extracts (lower panel). βME, β-mercaptoethanol.](https://www.jbc.org/content/285/11/8465/F1)

KEAP1-SNO
started to increase at a concentration of 100 μM and was maximal at 200 μM. Higher doses were not tested because of potential toxicity.

**KEAP1 Disulfide Bond Formation Induced by NO Derivatives and HOCl**—We were interested to see whether other oxidants known to activate NRF2, such as NO and derivatives or the potent thiol oxidant hypochlorous acid (HOCl) could also lead to KEAP1 Cys residue oxidation. We used the NO donor SpNO that decomposes in culture medium into two NO equivalents with an approximate half-life of 2 h (not shown). In lysates from HA-KEAP1-expressing HeLa cells exposed for 15 min to SpNO (1 mM), KEAP1 appeared oxidized in nonreduced SDS-PAGE, with a migration very similar to that of H2O2-oxidized KEAP1 (Fig. 1C, compare lanes 1–3). Oxidation was not as important but was maintained up to 3 h versus 40 min with H2O2 (Fig. 1A).

Such a prolonged response probably reflects the 2-h half-life of SpNO-released NO, in contrast to a bolus of H2O2 that is rapidly degraded by cellular consumption. KEAP1 also became oxidized upon cell exposure to HOCl at the low dose of 1 mM, with the formation of oxidized species similar to those seen upon exposure to H2O2 and SpNO (supplemental Fig. S2A). NO can cause protein S-nitrosylation, but we could not detect any modification of KEAP1 by S-nitrosylation when cells were exposed to SpNO (2 mM) for 45 min or to H2O2 for 5 min (Fig. 1D). In contrast, and as a positive control, a 30-min cell exposure to the trans-nitrosylating agent Cys-NO (0.5 mM) led to potent KEAP1 S-nitrosylation. Interestingly, Cys-NO also led to KEAP1 disulfide bond formation (supplemental Fig. S2B).

**Identification of the KEAP1 Cys Residues Engaged in Disulfide Bonds**—We used a mutagenesis approach to identify the KEAP1 Cys residues whose oxidation caused alteration of protein migration. 23 of the 25 Cys residues of murine KEAP1 (Fig. 2A) were substituted by a serine, generating 19 single and two double-Cys residues mutants (C513S/C518S and C622S/C624S). Cys368 and Cys469 were not tested because, as predicted by a KEAP1 Kelch crystallographic structure (37), they are unlikely to participate in redox regulation, as not being solvent-exposed and lacking a proximal Cys residue. These mutants were each expressed in HeLa cells and tested for their oxidation upon a 5-min exposure to H2O2 (200 μM) or to SpNO (2 mM) (Fig. 2B and supplemental Fig. S3). Except KEAP1 Cys151, Cys226, and Cys613, all of the mutants had nonreducing SDS-PAGE migrations similar to that of wild type HA-KEAP1.

KEAP1C151S still formed OxIM, but not OxIR1 and 2 (Fig. 2B, compare lanes 5 and 6 with lanes 9 and 10). A faint and fuzzy band just below OxIR1 was present instead. Note also here a β-mercaptoethanol-insensitive KEAP1 band just above OxIR1 already present in lysates from untreated cells that also disappeared in KEAP1C151S (Fig. 2B) (denoted cov for covalent). This KEAP1 band, which varied in intensity between experiments, was resistant to all reducing agents tested (not shown) and therefore corresponds to a covalent modification of unknown nature engaging Cys151. Disappearance of OxIR1 and 2 in KEAP1C151S indicates that KEAP1 engages Cys151 in an intermolecular disulfide with itself and/or with another protein to account for the two slow migrating β-mercaptoethanol-sensitive species. In the absence of Cys151, illegitimate unstable disulfides might form to account for the faint slow migrating band seen with KEAP1C151S.

Substituting Cys226 or Cys613 had the same effect on KEAP1 migration (Fig. 2B, lanes 3, 4, 7, 8, 11, and 12). These mutants were both unable to form OxIM but still formed the OxIR1 and 2 bands, although their migration was slightly up-shifted. These data indicate that Cys226 and Cys613 are engaged in an intramolecular disulfide linkage spanning across the Kelch domain (Fig. 2A). The presence of this disulfide in the wild type KEAP1 OxIR1/2 complexes would then explain the migration up-shift of these two bands when either Cys226 or Cys613 are mutated. Similarly, the higher intensity of OxIM in KEAP1Cys4151 in oxidant-treated cells reflects the fact that all of the KEAP1 species

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**FIGURE 2. Identification of oxidized Keap1 cysteine residues.** A, domain organization of the human KEAP1 protein. Positions of the KEAP1 cysteine residues are mapped in red. B, HeLa cells transfected with pcDNA expressing wild type (wt) HA-KEAP1 or the cysteine substitution derivatives C151S, C226S, or C613S were left untreated or exposed to H2O2 (0.2 mM) for 5 min, as indicated. The lysates were processed as described for Fig. 1. The arrowed band denoted cov corresponds to a noninducible, redox-insensitive KEAP1 modification (see text).
that carry the intramolecular disulfide are now shifted down to the size of this band. Thus, a fraction of KEAP1 carries an intramolecular Cys\textsuperscript{226–226} disulfide. H\textsubscript{2}O\textsubscript{2} and SpNO further increase this disulfide, also causing formation of an intermolecular KEAP1 disulfide(s). These two disulfides are independent, because one can form in the absence of the other(s).

**KEAP1 Homodimerizes through a Cys\textsuperscript{151–151}‐dependent Disulfide Bond**—We tested whether either one or both of the two KEAP1 intermolecular disulfide‐linked complexes (OxIR1/2) could be the result of KEAP1 dimerization by co‐immunoprecipitation assays, using two versions of KEAP1 that differed by both their size and tag. We thus co‐expressed in HeLa cells wild type KEAP1 but not of KEAP1Cys151S (Fig. 4B, lane 5) and with the monomeric intramolecular disulfide form of HA‐KEAP1 (HA‐OxIM) co‐precipitated with Myc‐His‐KEAP1 (see Fig. 3, lane 8 and supplemental Fig. S4), most probably by virtue of noncovalent dimerization imparted by the BTB domain (4, 5, 38), the inter‐molecular HA‐KEAP1–HA‐KEAP1 disulfide was not co‐precipitated, which suggests that dimerization of KEAP1 through the BTB and through the intermolecular disulfide might exclude each other.

**Functional Consequences of KEAP1 Disulfide Bond Formation**—Stress signals activate NRF2 by inhibiting KEAP1‐dependent NRF2 degradation. To verify the functional significance of KEAP1 oxidation and of identified Cys residues, we monitored NRF2 protein abundance as readout of KEAP1 activity in HeLa cells co‐expressing HA‐NRF2, Myc‐His‐KEAP1 or its Cys mutant derivatives, and YFP as transfection control.

H\textsubscript{2}O\textsubscript{2} induced a slight but reproducible and transient stabilization of NRF2 that closely paralleled KEAP1 oxidation (Fig. 4A). Such a marginal effect of H\textsubscript{2}O\textsubscript{2} might relate to the transient nature of KEAP1 modification induced by this very short‐lived inducer that denied NRF2 build up by neo‐synthesis. HOCl, as a short‐lived oxidant, also slightly stabilized NRF2, with kinetics that paralleled KEAP1 oxidation (supplemental Fig. S2A), whereas Cys‐NO, which had a very potent and extended effect on KEAP1 oxidation, stabilized NRF2 up to 3 h (supplemental Fig. S2B). SpNO was also kinetically different, because stabilization of NRF2 was not detected early in the kinetics (supplemental Fig. S2C) but was significant when measured at 5 h (Fig. 4B, lane 8), consistent with the long half‐life of SpNO causing lower but prolonged KEAP1 oxidation (Fig. 1C) and therefore steady NRF2 accumulation with time.

We took advantage of the potent stabilization of NRF2 by SpNO to evaluate the effect of mutations that affect KEAP1 oxidation. As shown in Fig. 4B, NRF2 levels were elevated in the absence of KEAP1 and decreased upon KEAP1 co‐expression (compare lanes 1 and 2). Under basal conditions, substitution of neither Cys\textsuperscript{151} nor Cys\textsuperscript{226} altered KEAP1 influence on NRF2 steady NRF2 accumulation with time. When we took advantage of the potent stabilization of NRF2 by SpNO to evaluate the effect of mutations that affect KEAP1 oxidation. As shown in Fig. 4B, NRF2 levels were elevated in the absence of KEAP1 and decreased upon KEAP1 co‐expression (compare lanes 1 and 2). Under basal conditions, substitution of neither Cys\textsuperscript{151} nor Cys\textsuperscript{226} altered KEAP1 influence on NRF2 steady NRF2 accumulation with time.
Checking the response to SpNO showed that it was similar to t-BHQ; the potent repression relief caused by SpNO at the presence of wild type KEAP1 was not observed with KEAP1Cys151S (Fig. 4B, lanes 8 and 9); however, KEAP1Cys226S had a wild type response to SpNO-induced derepression of NRF2 (Fig. 4B, lane 10). Therefore, intermolecular disulfide formation via Cys151 is important for relieving KEAP1-mediated NRF2 degradation.

**The Effect of Inactivating Thiol Redox Control on KEAP1 Oxidation and Function**—The transient nature of KEAP1 oxidation by H₂O₂ or SpNO (Figs. 1 and 4) suggests that cellular thiol reductases recycle the protein back to its reduced form. We sought to evaluate the redox state and activity of KEAP1 upon inactivating either one or both of the two thiol-redox control system. To inactivate the GSH pathway, we used buthionine sulfoximine, a specific inhibitor of the GSH rate-limiting biosynthetic enzyme γ-glutamyl cysteine synthase. To inactivate the thioredoxin pathway, we stably expressed in HeLa cells shRNAs targeting the TrxR1 mRNA. Two of the three tested TrxR1 shRNA caused protein level reduction of ~90%, as compared with a TrxR1 unrelated small nonhairpin RNA (Fig. 5A). Buthionine sulfoximine treatment (0.1 mM for 24 h) led to an 80% decrease of the total GSH cellular content as measured by the 5,5'-dithiobis (2-nitrobenzoic acid)-GSSG reductase recycling assay (not shown) but altered neither KEAP1 oxidation nor NRF2 stabilization, only slightly delaying protein reduction (Fig. 5B, compare lanes 1–5 with lanes 6–10). Knockdown of TrxR1 moderately increased the effect of H₂O₂ on KEAP1 oxidation and on
NRF2 stabilization but did not delay reduction (compare lanes 1–5 with lanes 11–15). In contrast, buthionine sulfoximine treatment of TrxR1 knockdown cells caused constitutive KEAP1 oxidation and major NRF2 stabilization. In these cells, H$_2$O$_2$ further increased KEAP1 oxidation up to shifting all of the protein to the OxIR1 and 2 bands but did not further stabilize NRF2 because its levels might have already reached a plateau. Surprisingly 30 min after H$_2$O$_2$ treatment, some reduction of KEAP1 started to occur back to the levels of untreated cells, which indicate the presence of a remnant thiol reductase activity in these cells.

Thus, upon inactivating both thiol redox pathways, low endogenous reactive oxygen species levels cause a build up of KEAP1 oxidation and NRF2 levels with time, further indicating a correlation between KEAP1 oxidation and NRF2 stabilization. These data could also indicate redundancy of the two pathways in KEAP1 reduction, but partial inactivation of these pathways, as indicated by the residual KEAP1 reduction, does not allow such a conclusion.

**DISCUSSION**

In this study we have questioned whether regulation of KEAP1, the inhibitor of the electrophilic and oxidative stress response regulator NRF2, involves disulfide bond formation, a post-translational control mechanism often used by oxidative stress regulators (39). We addressed this question by studying the KEAP1 response to H$_2$O$_2$, the nitric oxide donor SpNO, and HOCI, three chemicals that differ from other inducers by not being alkylating agents but oxidants that modify Cys residues by oxidation or S-nitrosylation. We now show that in untreated cells, a fraction of KEAP1 carried a long range intramolecular disulfide linking Cys$^{226}$ and Cys$^{613}$. Exposing cells to H$_2$O$_2$, SpNO, or HOCI further induced this intramolecular disulfide and also triggered formation of an independent intermolecular disulfide linking two KEAP1 molecules via Cys$^{151}$. Oxidation was transient, with KEAP1 returning to the redox state of untreated cells by endogenous reduction, and this was after variable periods of time, depending on the oxidant. None of these oxidants caused detectable KEAP1 S-nitrosylation, in contrast to the prototypical trans-nitrosative agent S-nitroso-cysteine. Other KEAP1 Cys modifications such as a short range disulfide might have formed but cannot be detected by the techniques used here. Oxidation of KEAP1 at Cys residues in cells exposed to Cys-NO has been reported (40), but neither its nature nor the identity of the Cys residues involved were revealed. Similarly, in vitro reaction of KEAP1 with oxidized GSH caused disulfide formation that included a Cys$^{319}$-based intermolecular disulfide (14). We could not detect this intermolecular disulfide, nor could this study identify the disulfides characterized here, emphasizing the difficulty of extrapolating in vivo results of in vitro studies and vice versa.

Proof for a cause-and-effect relationship between Cys residue modification and regulation is always a difficult task and cannot be definitely established here. Nevertheless, the parallel observed between the kinetics of KEAP1 oxidation and NRF2 stabilization is suggestive of the functional significance of KEAP1 disulfide formation. SpNO, which releases NO with a half-life of ~2 h, had a modest but long lasting effect on KEAP1 oxidation that resulted in a significant increase in NRF2 abundance late in the kinetics, whereas H$_2$O$_2$ and HOCI, which are short-lived, had a temporary effect on oxidation that caused only moderate NRF2 stabilization. Simultaneous impairment of the GSH and thioredoxin pathways caused both constitutive KEAP1 oxidation and NRF2 stabilization, which is further suggestive of a cause-and-effect relationship between the two phenomena, also re-emphasizing that the half-life of the oxidized form, which was in this case perpetuated by defective reduction, is important for efficient NRF2 stabilization.

Significance of disulfide formation was also evaluated by the effect of Cys residue substitution on KEAP1 function. Mutants lacking either Cys$^{226}$ or Cys$^{613}$ were still able to repress NRF2 and to respond to SpNO, indicating that under the experimental conditions used the Cys$^{226}$–Cys$^{613}$ intramolecular disulfide is dispensable for KEAP1 function and regulation. Further studies will be needed to elucidate the role of this long range intramolecular disulfide that spans the entire Kelch domain. Nonetheless, our data suggest that in the fully folded state Cys$^{226}$ and Cys$^{613}$ are close to each other, an unexpected finding based on the current knowledge of KEAP1 structure. In contrast, the mutant lacking Cys$^{151}$ could not be derepressed by SpNO, which indicates that KEAP1 Cys$^{151}$ disulfide-linked homodimer formation is important for derepression in response to oxidants.

Hannink and coworkers (6, 11) initially established the importance of Cys$^{151}$ by showing that cells expressing a KEAP1 mutant lacking this amino acid have low NRF2 basal activity that cannot be derepressed by t-BHQ and sulforaphane. This result was confirmed with many other NRF2 inducers, across species (24, 25, 27), and in mouse transgenic complementation rescue experiments (26). Further, Cys$^{151}$ becomes covalently modified in vivo by biotinylated derivatives of iodoacetamide (22) and has been shown to be consistently modified in vitro using different electrophiles (compiled in Ref. 14 and 15). The data shown here now indicate that KEAP1 Cys$^{151}$ is also required for NRF2 activation by oxidants such as NO and H$_2$O$_2$, which instead of operating by alkylation engage this residue in a disulfide-linked KEAP1 homodimer. How Cys$^{151}$ modification inhibits function is at the heart of KEAP1 regulation.

Several mechanisms have been proposed for how chemical inducers inhibit KEAP1-mediated NRF2 ubiquitination. The initial notion that regulation involves a disruption of the KEAP1-NRF2 interaction (12, 41) was later invalidated by several studies (6, 42, 43). A two-site recognition model has been proposed that predicts a KEAP1-NRF2 2:1 stoichiometry with one of the two Kelch domains of dimeric KEAP1 contacting NRF2 through a strong binding ETGE motif and the other contacting it through a weak binding DLG motif (44, 45). This model suggests that stress signals modify KEAP1 conformation in a way that loosens its interaction at the DLG and places NRF2 in a position unfavorable for ubiquitination but does not decipher the role of Cys$^{151}$. As another regulatory mechanism, electrophiles have been shown to disrupt the KEAP1-Cul3 interaction by modifying Cys$^{151}$, thus also ceasing NRF2 ubiquitination (6, 22, 46). In support of this mechanism, evaluation of a series of substitutions at position 151 of human KEAP1 elegantly established that residues with increasing partial molar
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FIGURE 6. Prediction structure of the KEAP1 BTB domain indicating the location of Cys$^{151}$. The KEAP1 BTB domain structure (delimitations 44–183) was modeled using the Bach1 BTB domain structure (Protein Data Bank code 2Z8H) as template (51). These two BTB sequences share 30% identity with no sequence insertions surrounding KEAP1 Cys$^{151}$ within the alignment, thus ensuring the reliability of the structural model in this region. In this model the Cys$^{151}$ side chain (magenta stick) appears buried by four surrounding positively charged amino acids (labeled in blue: Lys$^{13}$, Arg$^{135}$, Lys$^{150}$ and His$^{154}$). These four residues side chains can be modeled under different conformers, but irrespective of them, the accessibility of Cys$^{151}$ never exceeds 15%, emphasizing the buried character of this residue. The position of Cys$^{151}$ with regard to Cul3 was modeled using the structural similarity between Skp1 and BTB domains and the structure of the Skp1–Cul1 complex (Protein Data Bank code 1LDK) (49). These models show that Cys$^{151}$ is remote from both Cul3 and the BTB interface.

volume decrease the protein affinity for Cul3 and its ability to target NRF2 for ubiquitination and predicted that bulky modification at this position imposes structural effects at the BTB that alter KEAP1–Cul3 binding (47). As shown by Eggler et al. (47) and also in zebrafish KEAP1 by Kobayashi et al. (27), a Cys$^{151}$ substitution by a tryptophan, which has the highest partial molar volume, caused constitutive NRF2 activation.

To gain insight into how Cys$^{151}$ disulfide-linked KEAP1 homodimerization could alter protein function, we have modeled the KEAP1 BTB domain from the Bach1 BTB structure and have positioned Cys$^{151}$ with regard to the BTB dimerization interface and to Cul3 using human LRF1 (48) and the Skp1–Cul1 complex (49) structures (Fig. 6). In this model Cys$^{151}$ appears remote from both the BTB dimerization interface and Cul3. This residue is also buried by four positively charged amino acids, which restrict its accessibility but might also favor its reactivity toward electrophiles and oxidants by stabilization of the deprotonated form. Because of the apparent buried nature of Cys$^{151}$, modification of this residue to the sulfinic acid or S-nitrosylated forms upon reaction with H$_2$O$_2$ or NO-derived reactive nitrogen species, respectively, should necessarily cause some structural editing that might be further augmented upon disulfide formation between the Cys$^{151}$ of two KEAP1 monomers. Such local structural editing could impact the BTB canonical dimerization and Cul3 interaction domains at distance. Co-precipitation experiments (Fig. 3) indeed suggest that nonredox, presumably though the BTB, and disulfide-mediated KEAP1 dimerization are exclusive, supporting the idea that disulfide formation disrupts the BTB dimerization interface. Although we were not able to assay the interaction of KEAP1 with Cul3, such interaction might also be affected by formation of the Cys$^{151}$ intermolecular disulfide, as is the case in the presence of a bulky modification of Cys$^{151}$ (47). The idea of a major conformational editing of KEAP1 following Cys$^{151}$ modification has experimental support. Circular dichroism analysis of recombinant KEAP1 revealed that biotinylated iodoacetamide, which S-alkylates KEAP1 Cys$^{151}$, causes a conformational rearrangement of the protein, an effect that is lost by serine substitution of Cys$^{151}$ (22). Further studies will be needed to establish the impact of KEAP1 intermolecular disulfide formation on its dimerization and association with Cul3, which in both cases, if abrogated, would explain cessation of KEAP1-mediated NRF2 degradation.

Whether oxidation of KEAP1 involves a direct reaction with oxidants or is somehow catalyzed is not known. If direct, both H$_2$O$_2$ and HOCl are expected to proceed via Cys residue sulfenic acid formation, the condensation of which with a proximal Cys residue leads to disulfide formation. The chemistry behind NO-induced disulfide formation is not well understood but is presumably a multistep reactions process involving oxygen-dependent generation of reactive nitrogen species from NO, such as NO$_2$ or N$_2$O$_3$; Cys–NO formation; release of a thyl radical (R–S) via S–N bond homolytic scission or through Cu$^{2+}$-mediated catalysis; and generation of a disulfide with a proximal Cys residue (50). An alternative pathway involving the NO-dependent formation of 8-nitro-cGMP and S-guanylation of KEAP1 has also been suggested (21).

In summary, we have shown here that H$_2$O$_2$, SpNO, and HOCl lead to the oxidation of KEAP1 into two disulfides, one intramolecular and the other intermolecular. We propose that the Cys$^{151}$-based intermolecular disulfide bridging two monomers of KEAP1 represents a novel modification of this Cys residue, which, as proposed for the alkylation of this residue by electrophilic compounds, leads to KEAP1 inactivation and stabilization of NRF2.

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