Critical Cysteine Residues of Kelch-Like ECH-Associated Protein 1 in Arsenic Sensing and Suppression of Nuclear Factor Erythroid 2-Related Factor 2

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ABSTRACT

Arsenic activates nuclear factor erythroid 2-related factor 2 (Nrf2) to induce phase II and antioxidative genes. Here we analyzed arsenic–Kelch-like ECH-associated protein 1 (Keap1) cysteine thiol interaction in Nrf2 activation. Arsenic-based Nrf2 activators, fluorescent biarsenical labeling reagent (FlAsH) and phenylarsine oxide (PAO), were used to probe binding of arsenic to Keap1. Strong fluorescence was observed on binding of FlAsH to purified Keap1. Pretreatment with arsenic, tert-butylhydroquinone (tBHQ), or 2,3-dimercaptopropanol significantly reduced the fluorescent signal. PAO affinity beads effectively pulled down Keap1 in vitro and from hepatocellular carcinoma 7 (Hepa1c1c7) cells. Arsenic, tBHQ, free PAO, or cadmium blocked Keap1 pull-down. Furthermore, arsenic and free PAO significantly reduced the free thiol contents of purified or endogenous Keap1. Thus, arsenic, FlAsH, and PAO, as well as tBHQ and cadmium, bind to Keap1 cysteine thiols in a similar fashion. All the domains of Keap1 bound PAO, and the linker region exhibited the highest binding activity. The function of arsenic-Keap1 interaction was evaluated in a reconstituted system that mimics endogenous Nrf2 regulation. Mutation of Cys273 or Cys288 in the linker region resulted in high level basal expression of Nrf2 protein. Mutation of Cys151 abolished Nrf2 activation by arsenic. Over-expression of C273A, C288A, or C151A altered the basal and arsenic-induced expression of Nrf2 target genes. The study shows an important role of Cys273 and Cys288 in the suppression of Nrf2 by Keap1 and a critical function of Cys151 in arsenic responsiveness. Our findings support a model in which arsenic binds to different sets of Keap1 cysteine residues to regulate divergent functions in Nrf2 signal transduction.

This work was supported by the Intramural Research Program of the National Institutes of Health National Institute of Occupational Safety and Health. Article, publication date, and citation information can be found at http://jpet.aspetjournals.org. doi:10.1124/jpet.109.160465.

ABBREVIATIONS: Nrf2, nuclear factor erythroid 2-related factor 2; Keap1, Kelch-like ECH-associated protein 1; ARE, antioxidative response element; FlAsH, fluorescent biarsenical labeling reagent; PAO, phenylarsine oxide; BAL, tert-butylhydroquinone; MG132, N-benzoyloxycarbonyl (Z)-Leu-Leu-leucinal; Nqo1, NAD(P)H:quinone oxidoreductase 1; PBS, phosphate-buffered saline; NTR, N-terminal region; BTB, broad complex/tramtrack/bрил a браа; GTP, C-terminal region; KelchC, Kelch region plus C terminus of Keap1; GFP, green fluorescence protein; DLG, Asp-Leu-Gly, human Nrf2 peptide; ETGE, Gly-Thr-Gly-Glu, human Nrf2 peptide 79-ETGE.

Arsenic is an established human carcinogen and causes a broad spectrum of adverse effects in humans (Abernathy et al., 1999; ATSDR, 2005; Rossman, 2007; Liu et al., 2008). Humans are exposed to arsenic from a wide range of occupational and environmental sources. In particular, millions of people are at risk of drinking arsenic-contaminated water, presenting a major public health concern worldwide (Gomez-Caminero et al., 2001; NRC, 2001). On the other hand, arsenic has been used effectively as a therapeutic agent in the treatment of leukemia, psoriasis, and sleeping sickness (Farber, 1992; Carter and Fairlamb, 1993; Wang and Chen, 2008). The pleiotropic nature of arsenic effects implicates multiple protein targets of the soft metal in its diverse biological effects, few of which have been identified.

Arsenic activates the cap ‘n’ collar basic leucine zipper transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) to induce a battery of cytoprotective enzymes/proteins important for cell survival against oxidative and other stresses (He et al., 2006). Nrf2 belongs to a group of specialized transcription factors called xenobiotic-activated receptors. Xenobiotic-activated receptors sense specific chemical changes in the cell and coordinate an array of transcriptional responses to the stimuli and thereby maintain the chemical homeostasis in the cell (Ma, 2008).
In eukaryotes, Nrf2 signaling is highly conserved from fish to chicken, rodents, and humans, reflecting its evolutionary role in defense against endogenous and exogenous chemical insults. Loss of Nrf2 in mice is associated with increased sensitivity to a range of chemical and oxidative insults, such as benzo[a]pyrene-induced cancer, ovotoxicant-induced premature ovarian failure, autoimmune dysfunction and leukoencephalopathy, pulmonary disorders, diabetic cardiomyopathy, and a number of drug-induced toxicities (Chan et al., 2001; Ramos-Gomez et al., 2001; Cho et al., 2002; Leung et al., 2003; Hu et al., 2006; Ma et al., 2006; Hubbs et al., 2007; He et al., 2009). In the case of metals, knockout of Nrf2 substantially increases susceptibility of cells to metal-induced production of reactive oxygen species and cell death (He et al., 2006, 2007, 2008). Consistent with these “loss of function” observations, activation of Nrf2 by chemoprotective agents was shown to be a promising preventive and therapeutic approach against cancer and certain chronic diseases (Dinkova-Kostova et al., 2005; Sussan et al., 2009). These findings provided a solid foundation for a critical role of Nrf2 in the pathogenesis and therapy of cancer and chronic diseases, including metal carcinogenesis and toxicity.

Activation of Nrf2 involves inhibition of Kelch-like ECH-associated protein 1 (Keap1)-mediated ubiquitination and turnover of Nrf2 protein. In unstimulated cells, Keap1 binds and brings Nrf2 into a Cullin 3-dependent E3 complex that ubiquitinates Nrf2, leading to rapid turnover of the protein. Unactivated Nrf2 has a short half-life of ~20 min (He et al., 2006). In the presence of an inducer, Nrf2 protein is stabilized and accumulated in the nucleus, where it dimerizes with a small Maf protein. Nrf2-Maf heterodimer then binds to a DNA recognition sequence called antioxidant response element (ARE) to mediate transcription of target genes.

The molecular events that govern Nrf2 activation by inducers remain largely elusive. Recent studies revealed that a number of inducers interact with the cysteine thiol of Keap1 to inhibit Nrf2 turnover and activate Nrf2 (Dinkova-Kostova et al., 2002; Eggler et al., 2005; Hong et al., 2005a). Mutation and complementation of Keap1 function in cells and in intact animals suggest multiple cysteine codes to which various inducers bind to activate Nrf2 (Yamamoto et al., 2008; Kobayashi et al., 2009). We recently showed that Nrf2 contains evolutionally conserved cysteine residues critical for response to antioxidant and electrophile inducers including arsenic (He and Ma, 2009). Furthermore, Nrf2 cysteines are required for suppression of Keap1-dependent ubiquitination and transcription activation of Nrf2. Therefore, a dual sensor mechanism, in which both Keap1 and Nrf2 recognize inducers, has evolved to ensure a wide range of ligand recognition by Nrf2.

The role of Keap1 cysteine residues in Nrf2 activation by toxic metals has not been well addressed. In this study, we analyzed arsenic-Keap1 cysteine interaction. Our data show that fluorescent biarsenical labeling reagent (FlAsH) and phenylarsine oxide (PAO) (two arsenic-based inducers) bind to Keap1 in vitro and in cells in a similar manner. Mutation and functional analyses reveal that Cys273 and Cys288 in the linker region (LR) are required for suppression of Nrf2 protein expression by Keap1, whereas Cys151 is critical for arsenic sensing and responsiveness. These findings suggest a working model for analyzing metal sensing and signal transduction by the Nrf2/Keap1 system in the toxicity and therapeutic use of metal compounds.

Materials and Methods

Materials. Arsenic chloride (As3+/4), cadmium chloride (Cd2+), tert-butylhydroquinone (tBHQ), and PAO were purchased from Sigma-Aldrich (St. Louis, MO). 4-Amino-PAO was from Toronto Research Chemicals. Inc. (Toronto, ON, Canada). Affigel 10 gel was from Bio-Rad Laboratories (Hercules, CA). Flash and 2,3-dimercaptopropionic acid (BAL) were from Invitrogen (Carlsbad, CA). MG132 was from BIOMOL Research Laboratories (Plymouth Meeting, PA). Mouse hepaticc7 cells were provided by Dr. J. P. Whitlock, Jr. (Stanford University, Stanford, CA). The cells were cultured in α-minimal essential medium with 10% fetal bovine serum and 5% CO2. Cos-7 cells were purchased from American Type Culture Collection (Manassas, VA) and were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 5% CO2.

Plasmid Construction, Site-Directed Mutation, and Cell Transfection. Full-length mouse Nrf2 cDNA coding sequence was cloned into pCMV-HA to generate pCMV-HANrf2. Mouse full-length Keap1 was cloned into pcDNA3.1V5His and pET28a(- +) to generate pcDNA3.1-V5HisKeap1 and pKeap1/PET28a(- +), respectively. Deletion mutants of Keap1 were made by polymerase chain reaction and cloned into pcDNA3.1V5His similarly to the Keap1 construct. Point mutation of Keap1 was obtained by using the Quick-Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). All the plasmid constructs were verified by DNA sequencing. Transfection of the plasmids into cells was performed with Lipofectamine Plus from Invitrogen.

Northern Blotting and Immunoblotting. Total RNA was isolated from cells using the QIAGEN (Valencia, CA) total RNA isolation kit. Samples of 3 μg each were fractionated in 1.2% formaldehyde agarose gel, transferred to a supercharged nylon membrane, and blotted with the digoxigenin-labeled riboprobe prepared with the digoxigenin-labeling reagents (Roche Applied Science, Indianapolis, IN). Probes for NADP(H):quinone oxidoreductase 1 (Nqo1) and Actin were described before (Ma et al., 2004). For immunoblotting, total cell lysates were prepared and fractionated in 10% SDS-polyacrylamide gel as described before (Ma et al., 2000). The proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad Laboratories) and blotted with primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies and visualization with enhanced chemiluminescence detection reagents from GE Healthcare (Piscataway, NJ). Actin was blotted as loading control.

In Vitro Transcription and Translation. The TNT quick-coupled transcription/translation system (Promega, Madison, WI) was used for in vitro transcription and translation of Keap1 and mutant proteins. Proteins were produced with or without biotin tRNA and were detected by blotting with streptavidin-horseradish peroxidase, or specific antibodies followed by chemiluminescence detection.

Purification of Keap1. pKeap1/PET28a(- +) was transformed into bacteria BL21 (DE3) (EMD Chemicals, Gibbstown, NJ). Purification of Keap1 was performed based on modification of published procedures (Hong et al., 2005b). The bacteria were first grown in 10 ml of Luria-Bertani broth medium at 37°C overnight and then transferred to a 4-liter Luria-Bertani broth with absorbance at λ600 nm adjusted to between 0.2 and 0.3. Culture was continued at 37°C with vigorous shaking until optical density at 600 nm reached between 0.5 and 0.6. Isopropyl-β-D-thiogalactoside was added, and culture was continued for another 24 h with gentle agitation at 15°C. Keap1 was purified using the nickel nitrilotriacetic acid-agarose affinity chromatography (QIAGEN). Purified Keap1 was dialyzed against 25 mM Tris-HCl, pH 8.4, containing 5 mM EDTA and 5 mM β-mercaptoethanol, and was then concentrated with Centriprep 30 (Millipore Corporation, Billerica, MA) and confirmed for purity by SDS-polyacrylamide gel electrophoresis and mass spectrometry.
PAO Bead Conjugation and Pulldown. Ten milligrams of 4-amino-phenylarsene oxide was dissolved in 3.05 ml of methanol and mixed with 1.22 ml of Affigel (50% slurry) for 2 h at room temperature. Aminoethanol (100 µl) was then added to block the remaining active binding sites. The mixture was washed three times with methanol and three times with phosphate-buffered saline (PBS) and was then resuspended in 0.6 ml of PBS. Control Affigel was prepared by mixing 1.22 ml of Affigel slurry (50%) with 3.05 ml of methanol and 1 ml of aminoethanol, followed by shaking at room temperature for 2 h.

Measurement of Protein-Free Thiols. Protein-free thiol groups were measured as described previously with modifications (Aitken and Learmonth, 1996; Nishikimi et al., 2001). Purified proteins (10 µg in 100 µl) were incubated with As³⁺ or PAO for 30 min. The proteins were mixed with 10 µg of bovine serum albumin and 10% ice-cold trichloroacetic acid containing 1 mM dithiothreitol. The suspension was centrifuged at 14,000g for 5 min at 4°C. Precipitate was resuspended in 400 µl of Ellman’s reagent or the dithiobis-2-nitrobenzoic acid buffer [0.5 M potassium phosphate buffer, pH 7.4, containing 0.2 mM 5,5'-dithiobis-(2-nitrobenzoic acid) and 5 mM EDTA]. The mixture was incubated at 4°C for 30 min and was centrifuged at 14,000g for 5 min to remove insoluble materials. Absorbance of supernatant was measured at λ₄₁₂ nm.

Quantification and Statistical Analysis. Quantification of fluorescent images was performed using the Optimus version 6.51 software (Media Cybernetics, Inc., Bethesda, MD). Quantification represents mean and S.D. from three experiments. Quantification of protein or mRNA bands in immunoblotting or Northern blotting was performed using the ImageQuant program (GE Healthcare Biosciences). Statistical analysis was performed with one-way analysis of variance followed by t test using Microsoft (Redmond, WA) Excel. A p value of <0.05 was considered statistically significant.

Results

Arsenic Binds to Keap1 Cysteine Thiols. Arsenic is thiol-reactive, and arsenic-protein thiol interaction may mediate the induction of ARE-dependent genes through the Nrf2 signaling pathway. We have recently shown that conserved cysteine residues in Nrf2 play critical roles in Nrf2 activation by arsenic and several other inducers (He and Ma, 2009). In this study, we examined the role of Keap1 cysteines in the activation of Nrf2 by arsenic. Mouse Keap1 contains 25 cysteine residues that disperse throughout the protein (Fig. 1A). Most of the Keap1 cysteines and their flanking residues are highly conserved between the mouse and human proteins (Fig. 1B). It has been postulated that different inducers bind to disparate “cysteine codes” to affect various aspects of Nrf2 signal transduction.

We used sensitive probes to examine whether arsenic directly binds to Keap1 cysteines. FlAsH is an arsenic-based fluorophore that fluoresces on binding to protein cysteine thiols. FlAsH potently activates Nrf2 and induces ARE-dependent gene heme oxygenase 1 (Fig. 2A). FlAsH was used as an arsenic probe for Keap1 binding. Keap1 was expressed in bacteria and was purified (Fig. 2B). Purified Keap1 was sequence-confirmed by mass spectrometry (see under Materials and Methods) and by immunoblotting (Fig. 2C). Incubation of FlAsH with PBS did not induce detectable fluorescent signals, but incubation with purified Keap1 induced strong fluorescence, indicating binding of FlAsH to cysteine thiols (Fig. 2D). Next, Keap1 was incubated with arsenic, PAO, or thiol-reactive BAL for 30 min before incubation with FlAsH. Preincubation with these agents totally blocked FlAsH fluorescent signals (Fig. 2E). Therefore, FlAsH directly binds to Keap1 cysteine thiols, and arsenic, PAO, and BAL bind to Keap1 cysteines in a similar manner.

PAO is an organic arsenic that binds to vicinal protein cysteine residues to form stable structures. PAO activates Nrf2 with a potency that is 10 times higher than that of arsenic (Fig. 3A) (He and Ma, 2009). PAO was used as another probe for arsenic binding to Keap1. Keap1 was produced in vitro using TNT reticulocyte lysates and was pulled down with PAO affinity beads. We found that PAO completely pulled down TNT Keap1 (Fig. 3B). Pulldown was specific for PAO-thiol interaction because inactivated PAO beads, in which the thiol-binding sites were blocked by preincubation with BAL, failed to precipitate Keap1 (Fig. 3C). Furthermore, PAO beads effectively pulled down endogenous Keap1 from hepatic cells in the absence or presence of MG132 (Fig. 3D). MG132 stabilizes Nrf2 but not Keap1; the observation that Keap1 pulldown was similar with or without MG132 indicates that pulldown of endogenous Keap1 is independent of Nrf2. Thus, PAO binds to Keap1 both in vitro and in intact cells by directly binding to vicinal cysteine residues of Keap1. In Fig. 3E, hepatic cells were treated with tBHQ, arsenic, PAO, or cadmium before PAO pulldown. The result showed that tBHQ, arsenic, PAO, and cadmium effectively compete with PAO beads for binding with Keap1, indicating that these chemicals bind to Keap1 cysteine thiols similarly in the cell.

Binding of Keap1 cysteines with inducers reduces the free thiol content of the protein. We further analyzed the interaction between arsenic and Keap1 cysteine thiols by...
measuring Keap1-free thiol content. Incubation of purified Keap1 with arsenic, tBHQ, or PAO significantly reduced the free thiol content of the protein (Fig. 4A). We then expanded this observation to endogenous Keap1. Hepa1c1c7 cells were treated with arsenic, tBHQ, or PAO for 5 h. Keap1 was immunoprecipitated with anti-Keap1. The precipitate was then measured for free thiol contents. The result showed that arsenic, tBHQ, and PAO all significantly reduce the free thiol contents of endogenous Keap1 (Fig. 4B). Taken together, these in vitro and in vivo free thiol measurements support the notion that arsenic, as well as tBHQ and PAO, binds to Keap1 thiol groups both in vitro and in cells.

**LR Confers Strong Binding.** Binding of inducers to Keap1 cysteines may depend on the structure and function of Keap1 domains. Therefore, we examined arsenic-binding activities of Keap1 domains. The N-terminal region (NTR) and broad complex/tramtrack/bric a brac region (BTB) (Fig. 2).
NTR + BTB), the LR, Kelch, and the Kelch and C-terminal region (CTR; KelchC/H11005 Kelch/H11001 CTR) (Fig. 5A) were expressed in Cos-7 cells and were shown by immunoblotting (Fig. 5B, top). All the Keap1 fragments were pulled down by PAO beads to similar levels except LR. Pulldown of LR was significantly more in amount than the other domains even though the input of LR was markedly less than those of the other domains (compare the bottom and top panels in Fig. 5B). The higher molecular mass band of LR pulldown is likely a result of dimerization of LR; aggregation of Keap1 under conditions of SDS-polyacrylamide gel electrophoresis was also observed by others (Hong et al., 2005b). Taken together, all the Keap1 domains bind PAO, but the LR confers significantly stronger binding to arsenic than other domains. Cys273 and Cys288 Mediate Suppression of Nrf2. Among the nine cysteines in the LR, Cys273 and Cys288 were previously shown to be highly reactive toward several Nrf2 activators, such as dexamethasone 21-mesylate, iodoacetyl-N-biotinylhexylenediamine, and biotinylated iodoacetamide. Therefore, we tested whether these two cysteines play a role in arsenic signaling. Cos-7 cells expressed a low level of Nrf2 protein that was inducible by arsenic (Fig. 6A, lanes 1 and 2). Transfecting pCMV-HANrf2 into the cell led to high expression of Nrf2 protein that was slightly induced by arsenic (Fig. 6A, compare lane 3 with lane 1 and lane 4 with lane 3). To analyze Nrf2 regulation, Nrf2 and Keap1 were coexpressed in cells. Reconstitution of Nrf2 regulation was shown by fluorescent microscopy (Fig. 6B). Quantification of expression of green fluorescence protein (GFP)-Nrf2 was shown in Fig. 6C. Nrf2 was expressed well in Cos-7 cells as shown by the green fluorescence of GFP-Nrf2 (Fig. 6, B and C); cell nuclei were stained blue as a control. Expression of GFP-Nrf2 was not significantly affected by arsenic treatment, possibly because of overexpression of the protein (GFP-Nrf2, compare top and bottom panels). Coexpression of GFP-Nrf2 with Keap1 (red fluorescence) reduced the protein level of GFP-Nrf2 markedly, whereas treatment with arsenic significantly increased the amount of GFP-Nrf2 (GFP-Nrf2/Keap1, compare top and bottom panels), indicating suppression of Nrf2 protein level by Keap1 and stabilization of plasmid-expressed Nrf2 by arsenic. In Fig. 6D, coexpression of Keap1 with Nrf2 markedly reduced the protein level of plasmid-expressed Nrf2 as shown by immunoblotting (compare lane 3 with lane 1); treatment with arsenic increased the protein level of Nrf2 (compare lane 4 with lane 3), confirming the regulation of plasmid-expressed Nrf2 by Keap1 and arsenic. Together, the results indicate that coexpression of the two proteins in the cells reconstitutes regulation of Nrf2 by Keap1 and arsenic in a manner that mimics the regulation of endogenous proteins. We examined the effect of Cys273 and Cys288 on the regulation of Nrf2 by mutating the cysteine residues to alanine. Coexpression of C273A or C288A with Nrf2 caused higher expression of Nrf2 protein compared with wild-type Keap1 (Fig. 6D, compare lane 5 or 7 with lane 3), indicating...
that Cys273 and Cys288 are required for complete suppression of Nrf2 protein by Keap1 in the absence of an inducer. Treatment with arsenic further increased Nrf2 protein by ~2-fold (Fig. 6C, compare lanes 6 or 8 with lane 5 or 7, respectively) to the level comparable with that by wild-type Keap1 (compare lanes 6 and 8 with lane 4). The findings suggest that Cys273 and Cys288 are not critical for arsenic-elicited stabilization of Nrf2 protein but are important for Keap1-dependent ubiquitination and degradation of Nrf2 protein under basal conditions.

**Cys151 Is Critical in Arsenic Sensing.** To identify the cysteine residue(s) of Keap1 important in arsenic sensing, we mutated the cysteines and tested for arsenic response. Initial screening suggested that Cys151 is critical for stabilizing of Nrf2 in response to arsenic. As shown in Fig. 6E, plasmid-expressed Keap1 suppressed Nrf2 protein level, and this suppression was reversed by treatment with arsenic. However, coexpression of Nrf2 with Keap1 C151A mutant resulted in a slightly increased protein expression of Nrf2 (compare lane 3 with lane 1), but stabilization of Nrf2 by arsenic was totally lost (compare lane 4 with lane 2). These results revealed that Cys151 may play a minor role in suppression of Nrf2 protein but is required for activation of Nrf2 by arsenic and hence is critical for arsenic responsiveness. Figure 6F shows quantification of Nrf2 protein described in Fig. 6E.

**Effect of Cys273, Cys288, and Cys151 on Induction of Endogenous Nqo1.** We further analyzed the importance of the three cysteine residues of Keap1 by examining the ability of the cysteine mutations to support induction of endogenous Nrf2 target gene *Nqo1*. *Nqo1* was expressed at a low level in hepa1c1c7 cells transfected with vector control (Fig. 7, A and B, lane 1), and arsenic significantly induced *Nqo1* (lane 2).

Fig. 4. Binding of PAO to free thiol groups of Keap1. A, binding of PAO to purified Keap1. Purified Keap1 was incubated with tBHQ (30 μM), arsenic (10 μM), or PAO (1 μM) for 2 h. Keap1 was then precipitated with trichloroacetic acid, and free thiol groups were measured by using Ellman’s reagent with absorbance at 412 nm. B, binding of PAO to Keap1 in cells. Hepa1c1c7 cells were treated with tBHQ (30 μM), PAO (1 μM), or arsenic (10 μM) for 5 h. Keap1 was immunoprecipitated with anti-Keap1. The precipitate was then treated with Ellman’s reagent, and the free thiol groups were detected at λ412 nm. *, P < 0.05; **, P < 0.01.

Fig. 5. Strong binding of PAO to Keap1 LR. A, illustration of deletion mutants of Keap1. B, pulldown of deletion mutants. Deletion mutants of Keap1 were produced in Cos-7 cells and were pulled down by PAO beads. Top, immunoblotting of Keap1 deletion mutants. Bottom, immunoblotting of PAO pulldown of deletion mutants.
Overexpression of Nrf2 increased the basal level of Nqo1 that was further induced by arsenic (compare lane 3 with lane 1 and lane 4 with lane 3). Overexpression of Keap1 alone did not significantly alter the pattern of Nqo1 expression and induction (compare lanes 5 and 6 with lanes 1 and 2). However, overexpression of either C273A or C288A led to higher basal expression of Nqo1 (compare lanes 7 and 9 with lanes 1 and 5), and arsenic further induced the gene (compare lanes 8 and 10 with lanes 7 and 9). The findings indicate that the Cys273 and Cys288 are involved in the regulation of Nrf2 under basal conditions but are not critical for induction by arsenic.

On the other hand, overexpression of C151A resulted in expression of Nqo1 in the absence of arsenic that was higher...
than the basal expression of the gene (compare lane 11 with lanes 1 and 5) but lower than induced expression of \( \text{Nqo1} \) (compare lane 11 with lanes 6, 8, and 10). It is noteworthy that treatment with arsenic failed to further increase the expression of \( \text{Nqo1} \) mRNA (compare lane 12 with 11). These results reveal that Cys151 is involved in the regulation of \( \text{Nqo1} \) expression under basal conditions and is critical for induction of the gene in arsenic response.

**Discussion**

The Nrf2-Keap1 pathway has recently emerged as a major mechanism of cellular defense against oxidative and electrophilic stresses across mammalian species. The pathway controls the transcription of ARE-regulated genes. In light of the diverse spectrum of endogenous and exogenous chemical stimuli that cause oxidative damage in the body, the Nrf2-Keap1 system must have adopted complex and versatile chemical recognition mechanisms to respond to inducers of diverse structures. A common feature of the inducers of ARE-dependent genes is prominent: many of the inducers are thiol-reactive even though they vary in structure. Interaction of the inducers with cysteine thiol groups of Keap1 and/or Nrf2 appear to be a major mechanism of inducer recognition (Dinkova-Kostova et al., 2002; Zhang and Hannink, 2003; Eggler et al., 2005; Hong et al., 2005b; Yamamoto et al., 2008; He and Ma, 2009; Kobayashi et al., 2009). This notion was supported by three lines of evidence: 1) both Keap1 and Nrf2 contain evolutionally conserved cysteine and flanking residues; 2) mass spectrometry and affinity binding assay show that inducers preferentially bind to certain cysteine residues of Keap1 and Nrf2, a finding leading to the concept of cysteine code for inducer recognition by the Nrf2-Keap1 system; and 3) mutation of cysteine residues in Keap1 or Nrf2 alters inducer responsiveness and/or other aspects of Nrf2 signaling in cells and in intact animals.

We have previously reported that human carcinogen arsenic, as well as several other carcinogenic metals including cadmium and chromium, activates Nrf2 and induces ARE genes. Moreover, Nrf2 is critical for survival of cells exposed to the metals at toxic levels because loss of Nrf2 substantially increases the susceptibility of cells to oxidative damage and cell death induced by the metals (He et al., 2006, 2007, 2008). Most toxic metals cause multiple biological effects in animals and humans, but very few of their target proteins have been identified. In this respect, analysis of activation of the Nrf2-Keap1 pathway by arsenic provides a useful model for elucidating toxic metal-macromolecule interaction at a molecular level.

Mouse Keap1 contains 25 cysteine residues, which is more than 3 times greater than Nrf2 (seven cysteines). Several cysteines of Keap1 have been shown highly reactive to thiol-reactive inducers, such as sulforaphane, dexamethasone 21-mesylate, iodoacetyl-N-biotinylhexylenediamine, and biotinylated iodoacetamide. The high reactivity of Keap1 cysteines prompts us to analyze the role of Keap1 cysteine residues in arsenic response of the Nrf2-Keap1 pathway. Our data reveal that arsenic and arsenic-based ARE inducers FlAsH and PAO bind to Keap1 cysteines via metal-thiol interaction. Among the Keap1 domains, the LR has the highest binding activity toward arsenic. Mutational analysis established that Cys273 and Cys288 in the LR are critical for the suppression of Nrf2 protein expression by Keap1, whereas
Cys151 is required for arsenic recognition and responsiveness by Nrf2. Cys151, Cys273, and Cys288 are among the most reactive cysteine residues of Keap1 for a number of structurally diverse inducers. Two factors may contribute to the reactivity of cysteines in a given protein: 1) the basal amino acid residues surrounding the cysteines increase their redox reactivity; and 2) the tertiary structures that contain the cysteines may favorably expose the cysteines to inducers. Indeed, both Cys151 and Cys288 have a lysine residue on their amino side, whereas Cys273 has one arginine and one histidine residue flanking its amino and carboxyl ends, respectively. Although the three-dimensional structures of BTB and LR domains of Keap1 are currently not available, we posit that these three residues are exposed on the surface or have spaces that are large and flexible to permit easy access of inducers with different structures. In this respect, the LR of Keap1 is likely more open and accessible to inducers than most other domains because it exhibits highest binding activities toward arsenic and several other inducers tested.

It has been shown that Cys151 is required for activation of Nrf2 by a number of inducers, including thBHQ, sulforaphane, diethylmaleate, 1,2-dithiole-3-thione, naphthoquinone, and ebselen (Dinkova-Kostova et al., 2002; Zhang and Hannink, 2003; Egliger et al., 2005; Hong et al., 2005b; Kobayashi et al., 2009). We show in this report that Cys151 is critical for Nrf2 responsiveness to arsenic and PAO. Therefore, Cys151 appears to be part of the sensor mechanism for a large number of inducers. In addition, we found that mutation of Cys151 leads to increased expression of Nqo1, indicating that Cys151 also plays a detectable activity in suppression of Nrf2 function in the absence of an inducer. These findings suggest that the C151A mutant Keap1-Nrf2 interaction is weakened but not totally lost in unstimulated conditions to allow a low but detectable level of Nrf2 activity that is higher than the basal function of Nrf2 with wild-type Keap1. In the presence of an inducer, the mutant was unable to change the conformation of Keap1 and/or Nrf2 to dissociate Keap1 and Nrf2 from each other and therefore, lost responsiveness to the inducer. We are currently evaluating whether mutation of Cys151 directly affects Nrf2 function in the toxicity of arsenic and other toxic metals.

It is intriguing that mutation of either Cys273 or Cys288 markedly stabilized Nrf2 and induced Nqo1, indicating a nearly total loss of suppression of Nrf2 by Keap1 under basal conditions. However, both C273A and C288A appeared to retain responsiveness to arsenic and several other inducers. These results imply that the structural entities responsible for Keap1-mediated ubiquitination of Nrf2 in the absence of inducers and for the recognition of inducers can be separated based on the key cysteine residues involved in the functions. Mutation of Cys273 or Cys288 does not affect the binding affinity of Keap1 to Nrf2 (Kobayashi et al., 2004). Therefore, it is likely that in C273A or C288A the Keap1 mutant remains associated with Nrf2, but the mutant takes a conformation that permits effective ubiquitination and/or subsequent Keap1-dependent signaling events in Nrf2 turnover. In this scenario, the high reactivity of Cys273 and Cys288 to arsenic and other inducers does not seem to be relevant to the function of the residues in arsenic sensing. Yamamoto et al. (2008) recently proposed a model in which Nrf2 binds to two molecules of Keap1 at the KelchC region. These interactions are mediated through the DLG and ETGE motifs of Nrf2 located within its Neh2 domain. Binding of both DLG and ETGE to Keap1 is necessary for effective ubiquitination of the central a-helix of Nrf2 between DLG and ETGE (Yamamoto et al., 2008). Binding between ETGE of Nrf2 and KelchC of Keap1 is 2 orders of magnitude higher than binding between DLG of Nrf2 and KelchC, thereby forming a hinge-and-latch mechanism of Nrf2-Keap1 interaction. It is plausible to postulate that mutation of Cys273 or Cys288 disrupts DLG-KelchC binding but not ETGE-KelchC interaction and, therefore, preserves Nrf2-Keap1 binding but blocks ubiquitination and degradation of Nrf2 in the absence of an inducer.

We have recently shown that arsenic binds to conserved cysteine residues of Nrf2, and this metal-cysteine thiol interaction is critical for arsenic sensing, Keap1-dependent ubiquitination/proteasomal degradation of Nrf2, and transcription activation function of Nrf2 (He and Ma, 2009). Thus, Nrf2 cysteines appear to have multiple functions in Nrf2 regulation that include Nrf2 silencing under basal conditions, inducer recognition, and recruiting CBP and other coactivators for gene transcription. Together with the observations of this report, our findings show that arsenic binds to the cysteine residues of both Nrf2 and Keap1. Considering the immensely diverse structures of ARE inducers, it is rational to envisage that a dual sensor mechanism evolved in which cysteine codes in both Keap1 and Nrf2 mediate the recognition of different inducers to activate Nrf2. Certain cysteine residues play additional roles that include integration and switching Nrf2 function from a sensor to a transducer to transduce inducer signals into reproducible and coordinated transcription of ARE-dependent genes. Given the therapeutic potentials of Nrf2 activators in cancer, chronic disease, and chemical toxicity, these findings provide new and significant insights into inducer-Nrf2/Keap1 interactions that will enhance our understanding of the pathogenesis of disease and chemical toxicity and will facilitate the development of ARE inducers as effective therapeutic and protective agents in the future.

Acknowledgments

The findings and conclusions in this article are those of the authors and do not necessarily represent the view of the National Institute for Occupational Safety and Health.

References


Gomez-Caminero A, Howe P, Hughes MF, Lewis DR, Moore M, Ng J, Aitio
He X, Kan H, Cai L, and Ma Q (2009) Nrf2 is critical in defense against high
He X, Chen MG, and Ma Q (2008) Activation of Nrf2 in defense against cadmium-
He X, Lin GX, Chen MG, Zhang JX, and Ma Q (2007) Protection against chromium
He X and Ma Q (2009) Nrf2 cysteine residues are critical for oxidant/electrophile-
Hubbs AF, Benkovic SA, Miller DB, O'Callaghan JP, Battelli L, Schwegler-Berry D,
Ramos-Gomez M, Kwak MK, Dolan PM, Ith K, Yamamoto M, Talalay P, and
Kensler TW (2001) Sensitivity to carcinogenesis is increased and chemoprotective
efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. Proc
National Academy Press, Washington DC.
Ramos-Gomez M, Kwak MK, Dolan PM, Ith K, Yamamoto M, Talalay P, and
Kensler TW (2001) Sensitivity to carcinogenesis is increased and chemoprotective
efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. Proc
National Academy Press, Washington DC.
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specific cysteines of the electrophile-sensing human Keap1 protein is insufficient
to disrupt binding to the Nrf2 domain Neh2. Proc Natl Acad Sci U S A 102:10070–
10075.
Gomez-Caminero A, Howe P, Hughes MF, Kenyon E, Lewis DR, Moore M, Ng J, Aitio
compounds, in International Program on Chemical Safety (Ngi ed), World Health
Organization, Geneva.
oxidoreductase I by disrupting the Nrf2 - Keap1 - Cul3 complex and recruiting
Nrf2 - Maf to the antioxidant response element enhancer. J Biol Chem 281:23620–
23631.
He X, Chen MG, and Ma Q (2008) Activation of Nrf2 in defense against cadmium-
He X, Kan H, Cai L, and Ma Q (2009) Nrf2 is critical in defense against high
He X, Lin GX, Chen MG, Zhang JX, and Ma Q (2007) Protection against chromium
(VI)-induced oxidative stress and apoptosis by Nrf2. Recruiting Nrf2 into the
nucleus and disrupting the nuclear Nrf2/Keap1 association. Toxicol Sci 98:298–
309.
He X and Ma Q (2009) Nrf2 cysteine residues are critical for oxidant/electrophile-
sensing, Keap1-dependent ubiquitination-proteasomal degradation, and transcrip-
Hong F, Freeman ML, and Liebler DC (2005a) Identification of sensor cysteines in
human Keap1 modified by the cancer chemopreventive agent sulforaphane. Chem
Hong F, Sekhar KB, Freeman ML, and Liebler DC (2005b) Specific patterns of
electrophile adduction trigger Keap1 ubiquitination and Nrf2 activation. J Biol
induced by 4-vinyl cyclohexene diepoxy in Nrf2 null mice. Mol Cell Biol 26:940–
954.
Hubbs AF, Benkovic SA, Miller DB, O'Callaghan JP, Battelli L, Schwegler-Berry D,
and Ma Q (2007) Vacular leukoencephalopathy with widespread astroglisis in
Kobayashi A, Kang MI, Okawa H, Ohtsuji M, Zenke Y, Chiba T, Igarashi K, and
Yamamoto M (2004) Oxidative stress sensor Keap1 functions as an adapter for
Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. Mol Cell Biol
24:7130–7139.
Kobayashi M, Li L, Iwamoto N, Nakajima-Takagi Y, Kaneko H, Nakayama Y,
system Keap1-Nrf2 comprises a multiple sensing mechanism for responding to a
Nrf2 transcription factors results in early embryonic lethality and severe oxidative
Doull's Toxicology: The Basic Science of Poisons (Klaassen CD ed) pp 931–979,
Ma Q (2008) Xenobiotic-activated receptors: from transcription to drug metabolism
Ma Q, Battelli L, and Hubbs AP (2006) Multisorgan autoimmune inflammation,
enhanced lymphoproliferation, and impaired homeostasis of reactive oxygen spe-
cies in mice lacking the antioxidant-activated transcription factor Nrf2. Am J Pathol
Ma Q, Kinneer K, Bi Y, Chan JY, and Kan YW (2004) Induction of murine NADPH-
quione oxidoreductase by 2,3,7,8-tetrachlorodibenzo-p-dioxin requires the CNC
(cap 'n' collar) basic leucine zipper transcription factor Nrf2 (nuclear factor ery-
throid 2-related factor 2): cross-interaction between AhR (aryl hydrocarbon recep-
tor) and Nrf2 signal transduction. Biochem J 377:205–213.
Ma Q, Renzelli AJ, Baldwin KT, and Antonini JM (2000) Superinduction of CYP1A1
gene expression. Regulation of 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced degra-
Nrf2/Pex19 interaction with mitochondria and induces cytochrome c release. Bio-
chem J 356:621–626.
NRC (2001) Arsenic in the Drinking Water (update). National Research Council,
National Academy Press, Washington DC.
Ramos-Gomez M, Kwak MK, Dolan PM, Ith K, Yamamoto M, Talalay P, and
Kensler TW (2001) Sensitivity to carcinogenesis is increased and chemoprotective
efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. Proc
Rossman TG (2007) Arsenic, in Environmental and Occupational Medicine (Rom WN
Sussan TE, Rangasamy T, Blake DJ, Malhotra D, El-Haddad H, Bedja D, Yates MS,
Kombairaju P, Yamamoto M, Liby KT, et al. (2009) Targeting Nrf2 with the
triphenylendioxydisilazide attenuates cigarette smoke-induced emphysema and
Wang ZY and Chen Z (2008) Acute promyelocytic leukemia: from highly fatal to
Yamamoto M, Suzuki T, Kobayashi A, Wakabayashi J, Moher J, Motohashi H, and
Yamamoto M (2008) Physiological significance of reactive cysteine residues of
Zhang DD and Hannink M (2003) Distinct cysteine residues in Keap1 are required
for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by che-

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