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 (FIAsh) and phenylarsine oxide (PAO), were used to probe binding of arsenic to Keap1. Strong fluorescence was observed on binding of FIAsh 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 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, FIAsh, 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. Overexpression 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.

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-Camirero 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).

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ABBREVIATIONS: Nrf2, nuclear factor erythroid 2-related factor 2; Keap1, Kelch-like ECH-associated protein 1; ARE, antioxidant response element; FIAsh, fluorescent biarsenical labeling reagent; PAO, phenylarsine oxide; LR, linker region; tBHQ, *tert*-butylhydroquinone; BAL, 2,3-dimercaptopropanol; 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/bric a brac; CTR, C-terminal region; KelchC, Kelch region plus C terminus of Keap1; GFP, green fluorescence protein; DLG, Asp-Leu-Gly, human Nrf2 peptide ²³LWRODIDLG³¹; ETGE, Gly-Thr-Gly-Glu, human Nrf2 peptide ⁷⁹ETGE⁸².

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 lesions, 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 thiols 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 (As^{3+}), cadmium chloride (Cd^{2+}), *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-dimercaptopropanol (BAL) were from Invitrogen (Carlsbad, CA). MG132 was from BIOMOL Research Laboratories (Plymouth Meeting, PA). Mouse hepa1c1c7 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% CO_2 . 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% CO_2 .

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 NAD(P)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 second 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 $\lambda_{600\text{ nm}}$ 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 Centricon 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 μ l) 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 μ g in 100 μ l) were incubated with As^{3+} or PAO for 30 min. The proteins were mixed with 10 μ g 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 μ l 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 λ_{412} nm.

Quantification and Statistical Analysis. Quantification of fluorescent images was performed using the Optimums 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. FAsH is an arsenic-based fluorophore that fluoresces on binding to protein cysteine thiols. FAsH potentially activates Nrf2 and induces ARE-dependent gene heme oxygenase 1 (Fig. 2A). FAsH 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 FAsH with PBS did not induce detectable fluorescent signals, but incubation with purified Keap1 induced strong fluorescence, indicating binding of FAsH to cysteine thiols (Fig. 2D). Next, Keap1 was incubated with arsenic, PAO, or thiol-reactive BAL for 30 min before incubation with FAsH. Preincubation with these agents totally blocked FAsH fluorescent signals (Fig. 2E). Therefore, FAsH 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 hepa1c1c7 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, hepa1c1c7 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

Fig. 1. Cysteine residues and domain structure of Keap1. A, twenty-five cysteine residues and various domains of Keap1 are schematically illustrated. Kelch, Kelch domain. B, conserved cysteine residues Cys151, Cys273, and Cys288 and their flanking residues among mouse, rat, and human Keap1. All 25 cysteines of mouse Keap1 are conserved in mouse, rat, and human Keap1. Shown are Cys151, Cys273, and Cys288 along with flanking sequences. Boxed are the cysteines and the flanking basic residues.

Fig. 2. Binding of fluorescent arsenic probe to purified Keap1. **A**, activation of Nrf2 by FLAsH. Hepa1c1c7 cells were treated with arsenic (10 μ M) or FLAsH (10 μ M) for 5 h. Activation of Nrf2 and induction of heme oxygenase 1 were measured by immunoblotting. Actin was used as loading control. **B**, purification of Keap1. Keap1 was expressed in bacteria and was purified using nickel affinity column as described under *Materials and Methods*. **C**, immunoblotting. Purified Keap1 was confirmed by immunoblotting, as well as by mass spectrometry (see under *Materials and Methods*). **D**, binding of FLAsH to purified Keap1. Purified Keap1 (10 μ g) was incubated with FLAsH (2 μ M) for 30 min, and fluorescence was measured using a fluorescent spectrophotometer at excitation wavelength of 508 nm. **E**, competition for binding to Keap1. FLAsH (2 μ M) was incubated with Keap1 (10 μ g) or Keap1 preincubated for 30 min with arsenic (50 μ M), PAO (10 μ M), or BAL (50 μ M). Fluorescence was measured as in **D**.

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) (NBTB =

Fig. 3. Binding of PAO to Keap1. A, activation of Nrf2 by PAO. Hepa1c1c7 cells were treated with arsenic (10 µM) or PAO (1 µM) for 5 h. Activation of Nrf2 was determined by immunoblotting. B, binding in vitro. Keap1 was produced using the TNT reticulocyte system and was pulled down by PAO beads. C, specificity of binding. PAO beads were inactivated by incubating with BAL (10 mM) at room temperature for 2 h. TNT Keap1 was incubated with PAO beads or BAL-inactivated PAO beads. Keap1 pulldown was monitored by immunoblotting with anti-Keap1. D, binding of PAO to Keap1 in cells. Hepa1c1c7 cells were treated with MG132 (15 µM) or dimethyl sulfoxide for 2 h. Cell lysate was prepared and incubated with PAO beads. Keap1 pulldown was visualized by immunoblotting with anti-Keap1. E, inhibition of PAO bead pulldown by tBHQ, arsenic, PAO, or cadmium. TNT Keap1 was incubated with tBHQ (30 µM), arsenic (10 µM), PAO (1 µM), or cadmium (10 µM) for 2 h, followed by incubation with PAO beads for overnight. Keap1 pulldown was visualized by immunoblotting with anti-Keap1.

NTR + BTB), the LR, Kelch, and the Kelch and C-terminal region (CTR; KelchC = Kelch + 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, indicating the existence of Nrf2 signaling pathways to the cell (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

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 μ M), arsenic (10 μ M), or PAO (1 μ M) 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 μ M), PAO (1 μ M), or arsenic (10 μ M) 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 λ_{412} nm. *, $P < 0.05$; **, $P < 0.01$.

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. 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.

Fig. 6. Effect of Keap1 cysteine mutations. A, characterization of arsenic response of basal and plasmid-expressed Nrf2 in Cos-7 cells. Cells were transfected with pCMV-HA (vector control) or pCMV-HANrf2 and were treated with arsenic (10 μ M) for 4 h. Total cell lysate was immunoblotted with anti-Nrf2. B, fluorescent microscopy. GFP-Nrf2 and Keap1 or Keap1 mutants were expressed in Cos-7 cells by transfection. Cells were treated with vehicle or arsenic (10 μ M, 4 h). Fluorescent images were taken using a confocal fluorescent microscope to show GFP-Nrf2 (green fluorescence), Keap1 or Keap1 mutant (stained red with anti-V5 and Alexa 594 conjugated second antibody), and nucleus (stained blue with 4,6-diamidino-2-phenylindole). The last micrograph in each panel represents merged images of GFP-Nrf2, Keap1 (Keap1 mutant), and nuclear staining. C, quantification of GFP-Nrf2 fluorescence described in B. **, $p < 0.01$. D, effect of Cys273 and Cys288 of Keap1 on Nrf2 stability. Cos-7 cells were cotransfected with pCMV-HANrf2 and pcDNA3.1HisV5 (vector control) or the vector expressing Keap1, C273A, or C288A. Cells were treated with arsenic (10 μ M) for 4 h before harvesting. Total cell lysate was blotted with anti-Nrf2, anti-V5 (for expression of Keap1 and mutants), or actin (as loading control). E, effect of C151A on Nrf2 stability and arsenic responsiveness. Cos-7 cells were cotransfected with pCMV-HANrf2 and the vector expressing Keap1 or C151A. Cells were treated with arsenic, and total cell lysates were immunoblotted as described above. F, quantification of Nrf2 protein described in E.

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

Fig. 7. Effect of Keap1 cysteine mutations on induction of endogenous *Nqo1*. A, induction of *Nqo1*. Nrf2 or Keap1 and Keap1 mutants were expressed in hep1c7 cells by transfection. Induction of endogenous *Nqo1* by arsenic (10 μ M, 4 h) was examined by Northern blotting. B, quantification of *Nqo1* mRNA described in A.

than the basal expression of the gene (compare lane 11 with lanes 1 and 5) but lower than induced expression of *Nqo1* (compare lane 11 with lanes 6, 8, and 10). It is noteworthy that treatment with arsenic failed to further increase the expression of *Nqo1* mRNA (compare lane 12 with 11). These results reveal that Cys151 is involved in the regulation of *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 cys-

teine 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 FAsH 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 tBHQ, sulforaphane, diethylmaleate, 1,2-dithiole-3-thione, naphthoquinone, and ebselen (Dinkova-Kostova et al., 2002; Zhang and Hannink, 2003; Egger 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 in the C151A mutant Keap1-Nrf2 interaction is weakened but not totally lost in unstressed 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 prevents 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 α -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.

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