Chemical Research in Toxicology

DECEMBER 2005
VOLUME 18, NUMBER 12

Reviews

The Role of Keap1 in Cellular Protective Responses

Albena T. Dinkova-Kostova,*,† W. David Holtzclaw,† and Thomas W. Kensler†,‡

The Lewis B. and Dorothy Cullman Cancer Chemoprotection Center, Department of Pharmacology and Molecular Sciences, School of Medicine, and Department of Environmental Health Sciences, Bloomberg School of Public Health, The Johns Hopkins University, Baltimore, Maryland 21205

Received August 2, 2005

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1. Introduction

Multiple defense systems have evolved in all multicellular organisms in order to ensure protection against the toxic effects of the plethora of intrinsic and extrinsic oxidants and electrophiles to which they are exposed. In higher eukaryotes, these systems may be divided into four categories: (i) phase 1 enzymes, which introduce functional groups onto largely hydrophobic organic molecules, and are usually cytochrome P450 enzymes — in some instances, these enzymes produce highly reactive products that are toxic to the cell; (ii) phase 2 enzymes, such as glutathione *S*-transferases (GSTs)[1] and UDP-glucuronosyl transferases, which conjugate the products of phase 1 enzymes with hydrophilic groups in order to facilitate their excretion, and also enzymes such as superoxide dismutases, glutathione peroxidase, and catalase, which inactivate reactive oxygen species; (iii) phase 3 efflux transporters that export toxic metabolites acting synergistically with phase 2 enzymes to provide protection against electrophiles and carcinogens; and (iv) thiol-containing molecules such as glutathione and thioredoxin, which function to maintain reducing conditions within the cell and inactivate electrophilic compounds. Often the outcome of the encounter of a cell with a potentially toxic agent is largely determined by the balance between the activities of phase 1 enzymes that activate substrates to reactive intermediates and the activities of phase 2 enzymes that detoxify these reactive species. Normally,

* To whom correspondence should be addressed. Tel: 410-955-3503. Fax: 410-502-6818. E-mail: adkostov@jhmi.edu.
† School of Medicine.
‡ Bloomberg School of Public Health.

[1]Abbreviations: ARE, antioxidant response element; CTR, C-terminal region of Keap1; DEM, diethylmaleate; Dex-mes, dexamethasone 21-mesylate; DGR, double glycine (Kelch) region of Keap1; GFP, green fluorescent protein; GST, glutathione S-transferase; IVR, intervening region of Keap1; Keap1, Kelch-like ECH-associated protein 1; LMB, leptomycin B; NES, nuclear export signal; Nrf2, nuclear factor-erythroid 2-related factor 2; NTR, N-terminal region of Keap1.

10.1021/tx050217c CCC: $30.25
Published on Web 11/16/2005

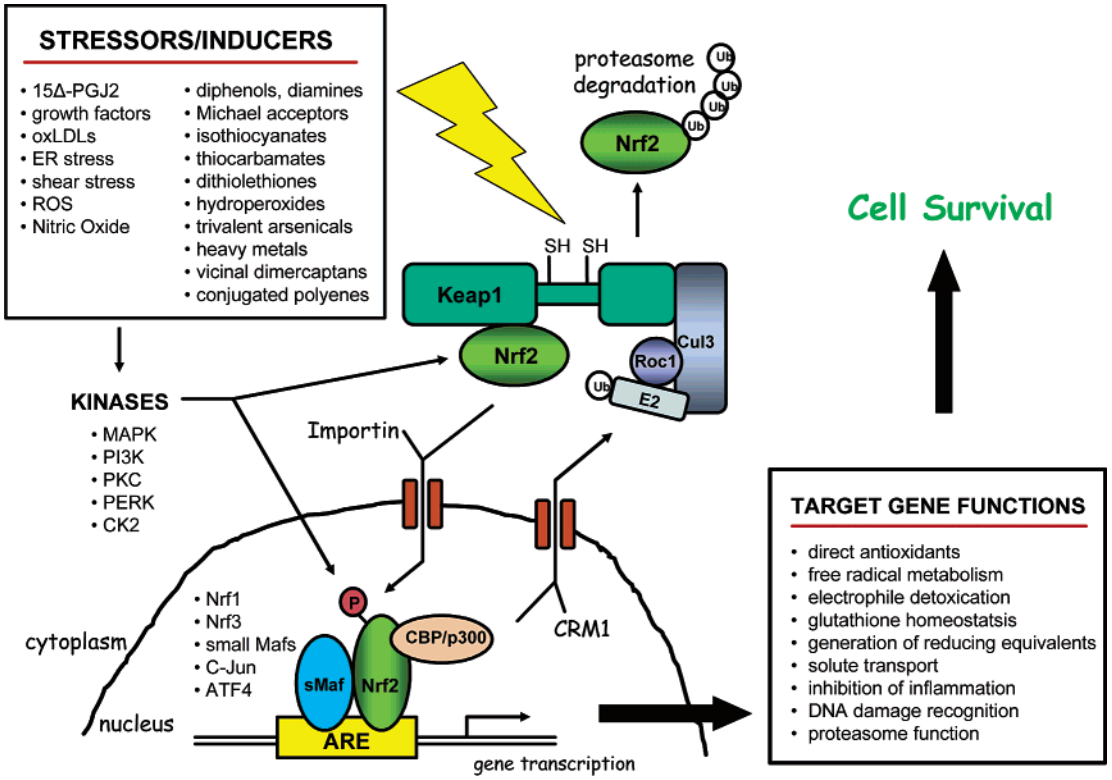


Figure 1. General scheme for the induction of gene expression through the Keap1-Nrf2-ARE signaling pathway. Multiple stresses as well as small molecules of endogenous and exogenous origin lead to activation of Nrf2-regulated genes. In many instances, these processes and agents disrupt the association of Nrf2 with Keap1, leading to diminished rates of proteolysis of Nrf2 and enhanced nuclear accumulation. Phosphorylation of Nrf2 by a series of kinases also affects its fate and distribution. Interaction of Nrf2 with other transcription factors and proteins of the transcriptional complex allows for transactivation of ARE responsive genes. Induction of these genes, which include prototypic phase 2 and antioxidative genes, results in an adaptive response that enhances the resistance of cells to environmental stresses mediated by electrophiles and free radicals.

phase 2 enzymes are not expressed at their maximal capacity but are highly inducible. Importantly, phase 2 enzymes can be induced selectively and independently of phase 1 enzymes, resulting in enhanced cellular protection and survival.

The discovery of the protective effects resulting from selective induction of phase 2 enzymes represents one of the true hallmarks of the principle of chemoprotection and offers a promising strategy for reducing the risk of cancer and other chronic diseases (*1*, *2*). It has become increasingly clear that the induction of phase 2 enzymes protects against various oxidative and electrophilic agents in many ways by (i) providing more direct antioxidants, e.g., bilirubin, and CO through induction of heme oxygenase and biliverdin reductase (*3*, *4*); (ii) increasing levels of enzymes that catalyze direct inactivation of oxidants, e.g., catalase, superoxide dismutase, selenium-dependent glutathione peroxidase, and the glutathione peroxidase function of GSTs (*5*, *6*); (iii) increasing levels of enzymes that catalyze detoxification of electrophiles, e.g., GSTs, epoxide hydrolase, and quinone reductase 1 (NQO1) (*7*); (iv) stimulating glutathione synthesis and regeneration through elevation of the activities of γ-glutamatecysteine ligase, glutathione reductase, and thioredoxin reductase (*8*); (v) stimulating NADPH synthesis (*9*); (vi) enhancing the export of toxic drugs via the multidrug response transporters (*10*); (vii) inhibiting cytokine-mediated inflammation, e.g., via leukotriene B_4 dehydrogenase (*11*); (viii) preventing iron overload and consequently oxidative stress, e.g., by increasing ferritin levels (*4*, *12*, *13*); and (ix) recognition, repair, and removal of damaged proteins (*14*).

As depicted in Figure 1, induction of the phase 2 response requires at least three essential components: (i) antioxidant response elements (AREs) (*15*), upstream regulatory sequences present on each phase 2 gene in either single or multiple copies; (ii) Nrf2, nuclear factor-erythroid 2-related factor 2, the principal transcription factor that heterodimerizes with members of the small Maf family of transcription factors, binds to the ARE, and recruits the general transcriptional machinery for expression of ARE-regulated genes (*16*); and (iii) Keap1, a cytosolic repressor protein that binds to Nrf2, retains it in the cytoplasm, and promotes its proteasomal degradation. Since its initial discovery by Yamamoto and his colleagues (*17*), it has become increasingly clear that Keap1 has multiple partners and plays multiple roles in regulating the phase 2 response as will be discussed in this review.

The development of nrf2 knockout mice provided the first key insights into the toxicological importance of this pathway (*18*, *19*). As examples, *nrf2*-disrupted mice are more sensitive to the hepatotoxicity of acetaminophen (*20*, *21*), the pulmonary toxicity of butylated hydroxytolene (*22*) or hyperoxia (*23*), and the neurotoxicity of 3-nitropropionic acid (*24*, *25*). The carcinogenicity of benzo[*a*]pyrene (*26*) and *N*-nitrosobutyl(4-hydroxybutyl)-amine (*27*) is exacerbated in the forestomach and bladder, respectively, of knockout mice. Chronic exposure of knockout mice to cigarette smoke leads to enhanced development of emphysema in the lungs (*28*). Finally, disruption of *nrf2* increases susceptibility to severe airway inflammation and asthma following an allergen challenge (*29*).

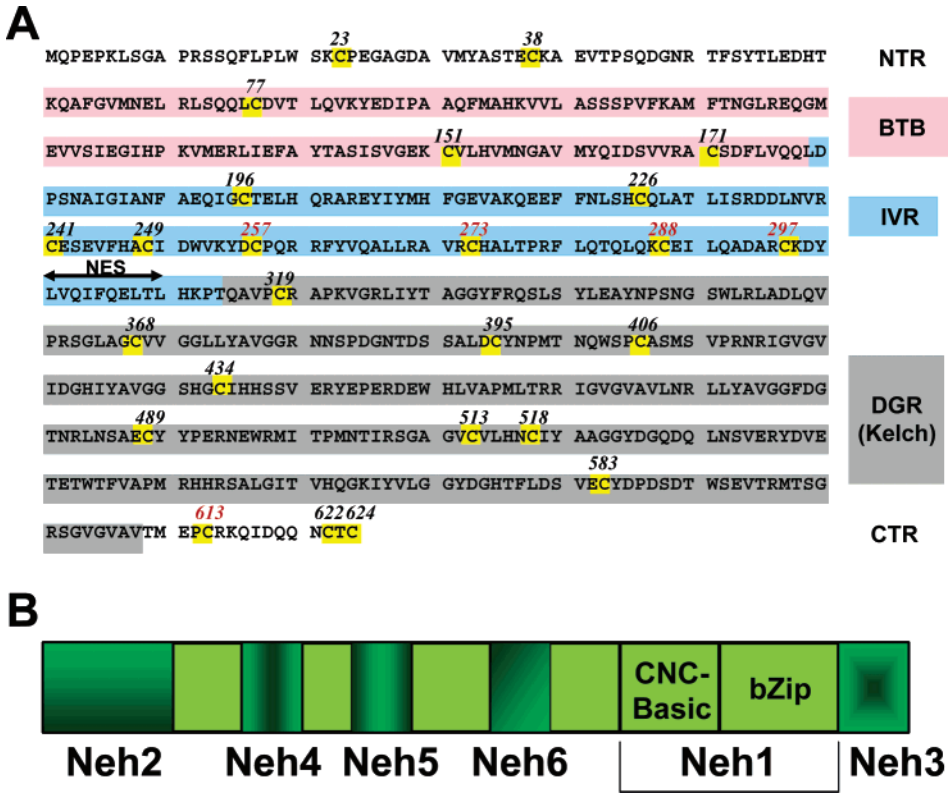


Figure 2. Domain organization of mouse Keap1 (A) and Nrf2 (B). The amino acid sequence of the five domains of mouse Keap1 is shown in panel A. NTR, N-terminal region; BTB, broad complex, tramtrack, bric-a-brac domain; IVR, intervening region; DGR (Kelch), double glycine region; CTR, C-terminal region. The 25 cysteine residues are highlighted in yellow and their positions in the sequence of the protein are indicated. Shown in red are the positions of the most reactive cysteine residues with Dex-mes. NES, nuclear export signal.

The Keap1-Nrf2-ARE signaling pathway can be activated by a number of cellular stresses (endoplasmic reticulum stress, oxidative stress, and shear stress), signaling molecules (nitric oxide, growth factors), and oxidized lipids (15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, oxidized low-density lipoproteins) (*30*−*32*). Of great relevance to disease prevention, this pathway also can be induced by an expanding array of small molecule drugs and natural products. Although inducers of phase 2 enzymes belong to a variety of chemical classes and may be structurally dissimilar, they appear to have a common chemical feature: the ability to react with sulfhydryl groups by oxido-reduction, alkylation, or disulfide interchange (*33*). This common property led Talalay and his colleagues to propose that the sensor for inducers must be a molecule (perhaps a protein) equipped with highly reactive cysteine thiols that are chemically modified upon encounter with an inducer (*34*). This proposition gained further strength when it was found that many inducers are substrates for GSTs (*35*), and the potency of inducers to elevate NQO1 paralleled their reactivity with nucleophilic donors such as mercaptans (*36*). After the discovery of Keap1, interest became focused on its cysteine residues and their potential reactivity with electrophiles. Examination of its sequence revealed, perhaps not surprisingly, that it is extremely cysteine-rich with 25 and 27 cysteine residues among the 624 amino acids of murine and human Keap1, respectively (Figure 2A). This fact was consistent with the idea that the sensor protein would be a thiol-containing target for electrophiles, and so, Keap1 emerged as "the perfect candidate" for the long-sought sensor for inducers.

2. Discovery of Keap1

The unequivocal establishment that Nrf2, a member of the Cap'n'Collar (CNC) subfamily of bZIP transcription factors, is the principal transcription factor responsible for phase 2 gene induction (*19*) raised the question—How is the signal from inducers transmitted to this DNA-binding nuclear protein? A detailed examination of the function of the six domains of Nrf2 (Neh1−Neh6) revealed that the Neh1 domain contains the bZIP region through which it participates in heterodimerization with transcription factors of the small Maf subfamily and in subsequent DNA binding (Figure 2B). Neh4 and Neh5 are independent activation domains that act cooperatively to bind the CREB-binding protein and thereby recruit the general transcriptional machinery (*37*). In contrast, the Neh2 domain (the N-terminal, ~100 amino acids) is a negative regulatory domain (*17*). Itoh et al. (*17*) showed that cells transfected with DNA coding for Nrf2 that lacks the Neh2 domain expressed a much higher activity for a luciferase reporter gene than cells transfected with a construct encoding full length Nrf2. This result suggested the existence of a cellular repressor that binds to the Neh2 domain of Nrf2 and thus prevents its transactivation function. To address this possibility, a construct encoding for a Gal4−Neh2 fusion protein was used as bait in the yeast two-hybrid system. This approach led to the isolation of a single protein that was named Keap1 (Kelch-like ECH-associated protein 1) because of its sequence homology with the Drosophila actin-associated protein Kelch (*38*). Under basal conditions, Keap1 binds Nrf2, retains it in the cytoplasm, and targets it for proteasomal degradation. In the presence

of phase 2 gene inducers, however, Nrf2, perhaps in the presence of Keap1, undergoes nuclear translocation and activates the transcription of phase 2 genes (*17*).

3. Structure of Keap1

The complete amino acid sequences of Keap1 from eight species have been published as follows: mouse, rat, pig, human (KIAA0132), Xenopus, zebrafish, Drosophila, and rice (*17*, *39*, *40*). The four mammalian proteins exhibit a high degree of homology.

Keap1 belongs to the superfamily of BTB-Kelch proteins that occur in the Metazoa. The murine protein has 624 amino acids and a molecular weight of 69552. On the basis of its sequence, Keap1 consists of five distinct domains (Figure 2A). The first 60 amino acids comprise the amino terminal region (NTR). Amino acids 61−179 form the BTB/POZ domain (bric-a-brac, tramtrack, broad complex/Poxvirus zinc finger). This is an evolutionary conserved domain also found in actin-binding proteins, zinc finger transcription factors, and substrate specific adaptor proteins in Cullin3 (Cul3)-based E3 ubiquitin ligase complexes (*41*−*43*). In many cases, this is a protein−protein interaction domain that mediates dimerization (*44*). Indeed, Keap1 forms a homodimer through the BTB domain and dimerization is required for binding to Nrf2 (*45*). Interestingly, T'Jampens et al. (*46*) have shown that Keap1 can bind ATP (most likely through its BTB domain), but the significance of this finding has not been pursued and remains unclear. On the basis of the fact that several BTB domain-containing proteins serve as substrate adaptor molecules in Cul3-dependent protein ubiquitination systems and that Keap1 has been shown to form such a functional complex that targets Nrf2 for ubiquitination and subsequent degradation through the 26S proteasome pathway (see below), it is tempting to speculate that the binding of ATP could be involved in the mechanism of Keap1-mediated Nrf2 degradation.

The intervening region (IVR) of Keap1 spans amino acids 180−314 and is particularly cysteine-rich containing eight cysteine residues among 134 amino acids. Furthermore, it is the domain in which highly reactive cysteine residues reside. Because of its particularly important role in the sensing of inducers and in the regulation of the interaction between Keap1 and Nrf2, it will be discussed separately (see below).

The double glycine repeat (DGR), also known as the Kelch domain, encompasses amino acids 315−608. This is another evolutionary conserved multiple protein−protein interaction domain consisting of six repeating Kelch motifs that form a β-propeller structure with multiple potential protein−protein interaction sites (*47*). At least 70 Kelch repeat proteins have been identified in the human genome, three-quarters of them containing both BTB and Kelch domains (*48*). It is this domain through which Keap1 binds to the Neh2 domain of Nrf2.

The crystal structure of the Kelch domain of human Keap1 was recently determined by X-ray crystallography to a resolution of 1.85 Å (*49*). The Kelch domain is a monomer and is comprised of six Kelch repeats that form a symmetric, six-bladed β-propeller structure. The structure reveals that the Kelch repeat motif is defined by highly conserved glycine, tyrosine, and tryptophan residues. There are eight cysteine residues, none of them engaged in disulfide bonds. Interestingly, the first blade of the propeller consists of three strands from the N terminus of the protein and one strand from the C terminus, thus bringing the carboxy terminal region of Keap1 into close proximity to the IVR.

Amino acids 609−624 comprise the short carboxy terminal domain (CTR). This region is of interest because it ends with a −CTC tripeptide, which is conserved in the other three mammalian proteins. This motif is very similar to the −CXXC− sequence found in the active site of protein disulfide isomerase. Indeed, Woycechowsky and Raines (*50*) have reported that the tripeptide CGC−NH_2 has a disulfide reduction potential similar to that of protein disulfide isomerase and even possesses disulfide isomerization activity. Whether the −CTC sequence in Keap1 has any disulfide reduction/isomerization activity has not yet been determined.

4. Keap1, a Zinc Metalloprotein

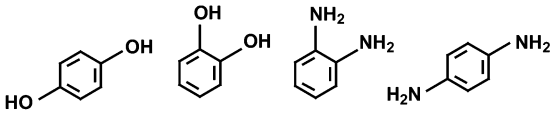
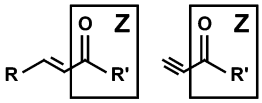
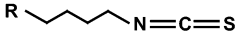
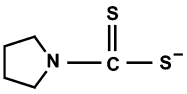
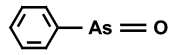
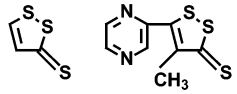
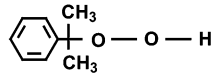
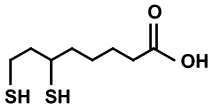
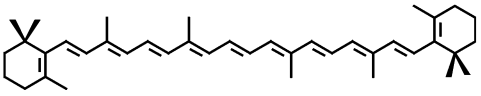
Recombinant murine Keap1 is a dimeric metalloprotein in which each monomer binds stoichiometric amounts of zinc with an association constant of 1.02×10^{11} M^{-1} (*51*). As is the case with many other metalloproteins, Keap1 can also accommodate cobalt (although with lower affinity) in its metal-binding site both during protein synthesis in *Escherichia coli* as well as in vitro. Titration of increasing amounts of $CoCl_2$ into metal deficient Keap1 leads to the appearance and increase of a characteristic absorption spectrum for cobalt complexes that have a tetrahedral geometry until stoichiometric amounts of $CoCl_2$ are added and no further change occurs in the presence of excess cobalt. Overall, the optical spectrum of cobalt-saturated Keap1 has an absorbance maximum at 310 nm, a shoulder at 360 nm, and a maximum at 640 nm with $a_m = 420$ M^{-1} cm^{-1}. Titration of $ZnCl_2$ into cobalt-saturated Keap1 leads to a decrease in the absorbance at 640 nm indicating that zinc can displace cobalt from its binding site in Keap1 and that the binding affinity for zinc is higher than for cobalt.

The identity of the ligands involved in the coordination of the metal is still unknown, but recombinant mutant Keap1 in which the reactive cysteine residues in the IVR (C257, C273, C288, and C297) were mutated to alanine residues has nearly 100-fold lower zinc-binding affinity as compared to its wild-type counterpart. The precise role of zinc in the function or structure of Keap1 has not been established. Reaction with inducers of various types leads to removal of the metal, but zinc is not required, at least in vitro, for the ability of Keap1 to react with inducers or to bind to Nrf2. Furthermore, Keap1 is more reactive, at least in vitro, with inducers in the presence of known zinc chelators (EDTA, EGTA, or TPEN), but not the Fe^{3+}-chelator deferoxamine. It is tempting to speculate that the metal plays a role in the "fine-tuning" of the reaction with inducers: It protects its ligands from "overreacting" under basal conditions yet keeps them poised for immediate response upon inducer sensing.

5. Keap1 as a Sensor for Phase 2 Gene Inducers

Inducers of phase 2 genes belong to 10 chemically distinct classes (*52*−*54*): (i) oxidizable diphenols, phenylenediamines, and quinones; (ii) Michael acceptors (olefins or acetylenes conjugated to electron-withdrawing groups); (iii) isothiocyanates; (iv) thiocarbamates; (v)

Table 1. Classes of Inducers of the Phase 2 Response

Inducer Class	Examples
Oxidizable diphenols, phenylenediamines, quinones	
Michael acceptors	 $Z = NO_2 > COAr > CHO > COCH_3 > CO_2CH_3$
Isothiocyanates	 $R = C_2H_5$, HOOC, CH_3OOC, CH_3SCO, CH_3CO
Dithiocarbamates	
Trivalent arsenicals	
1,2-Dithiole-3-thiones	
Hydroperoxides	
Dimercaptans	
Heavy metals	$Hg^{2+} > Cd^{2+} > Zn^{2+}$
Polyenes	

trivalent arsenicals; (vi) dithiolethiones; (vii) hydroperoxides; (viii) vicinal dimercaptans; (ix) heavy metals; and (x) polyenes (Table 1). Within the class of Michael acceptors, the order of inducer potency parallels the order of reactivity with nucleophiles in the Michael reaction (*33*). Similarly, the order of inducer potency in the class of heavy metals parallels the order of their affinity for sulfhydryl groups ($Hg^{2+} > Cd^{2+} > Zn^{2+}$). Inducers were found to be GST substrates, and known GST substrates were found to be phase 2 gene inducers. Moreover, the inducer potency was closely correlated with the electron-withdrawing power of the substituents at the para position within a series of methyl cinnamate analogues (*35*) and with the rate of reactivity with sulfhydryl reagents within a large series of phenolic mono and double Michael reaction acceptors (*36*). Thus, compelling evidence has accumulated to support the idea that the cellular sensor for inducers must be endowed with highly reactive sulfhydryl groups, most likely two cysteine residues in close spacial proximity to account for the extremely high inducer potency of trivalent arsenicals that are classically known to react with vicinal thiols leading to the formation of stable cyclic thioarsenites.

The high cysteine content of Keap1 suggested that it would be an excellent candidate as the sensor for inducers. Murine Keap1 contains 25 cysteine residues (Figure 2A), whereas its human homologue has 27. In the primary sequence of murine Keap1, nine (C23, C38, C151, C241, C273, C288, C297, C319, and C613) of the 25 cysteines are flanked by basic amino acid residues, a phenomenon that is known to decrease their pK_a values thus increasing their reactivity (*55*). To determine which cysteine residues are targets for phase 2 gene inducers, Keap1 was overexpressed in *E. coli* and the recombinant protein was purified to homogeneity. It was demonstrated spectroscopically that Keap1 reacted directly with inducers of many different types (Figure 3), e.g., the isothiocyanate sulforaphane, the double Michael reaction acceptor bis(2-hydroxybenzylidene)acetone, the thiol reagents 2,2′- and 4,4′-dipyridyl disulfide (*56*), and more recently, with a synthetic electrophilic triterpenoid, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-onitrile (*57*). In addition, a number of inducers that belong to different classes were used in a competition assay for binding to Keap1 with the steroid dexamethasone 21-mesylate (Dex-mes) (Figure 3), an inducer that alkylates cysteine residues irreversibly (*56*). The order of potency of the tested compounds as inhibitors in this assay closely paralleled their order of potency as phase 2 inducers, i.e., phenylarsenoxide > 1-nitrocyclohexene > 1-chloro-2,4-dinitrobenzene > 2-cyclohexanone > α-methylene-γ-butyrolactone. A similar correlation was also found with three triterpenoid analogues that are very closely related in structure but have very different inducer potencies (*57*).

Using 4,4′-dipyridyl disulfide, an inducer that reacts quantitatively and unidirectionally with thiols, a hierarchy of reactive cysteine residues in Keap1 was identified (*56*). The unexpected finding that addition of one equivalent of dipyridyl disulfide to Keap1 resulted in the formation of two molecules of pyridinethione suggested for the first time that as soon as the first Keap1-pyridyl mixed disulfide is formed, this bond is attacked by

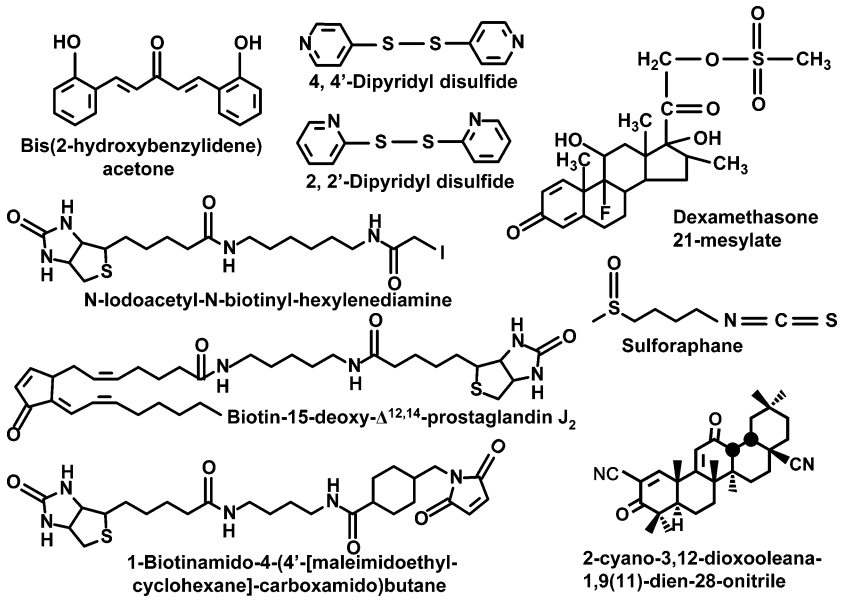


Figure 3. Some inducers that have been used in reactions with cysteine residues of Keap1.

another cysteine thiolate ion on the protein giving rise to either an intra- or intermolecular protein disulfide. In addition, the formation of higher molecular weight species has been observed when recombinant Keap1 was treated with inducers of different types and then subjected to nonreducing SDS−PAGE. Subsequent experiments established the formation of disulfide-linked dimers of Keap1 when mammalian cells overexpressing Keap1 were exposed to various inducers, e.g., sulforaphane, 1,2-dithiole-3-thione, and bis(2-hydroxybenzylidene)acetone (*58*). At the end of the exposure period, cell-free extracts were prepared and analyzed by two-dimensional SDS−PAGE. The first dimension was carried out under nonreducing condition in tube gels. Following incubation in 2-mercaptoethanol-containing buffer, each tube was inserted into the well of a slab gel and subjected to SDS−PAGE under reducing conditions in the second dimension. Western blot analysis was then used to localize Keap1. The anti-Keap1 antibody recognized only a single immunoreactive product corresponding to monomeric Keap1 in extracts from control cells, but it detected two products in extracts from induced cells corresponding to a monomer and an intermolecular disulfide-linked dimer. This experiment provided evidence that one outcome of the encounter of an inducer with Keap1 in cells is the formation of intermolecular dimers and suggested that Keap1 could operate as a redox switch.

Dex-mes was used as a probe in an attempt to identify the reactive cysteine residues of murine Keap1. Titration experiments with [^{3}H]Dex-mes revealed that 3−4 cysteine residues were the most reactive. Reaction of Keap1 with a limited amount of Dex-mes followed by a large excess of *N*-ethylmaleimide, subsequent tryptic digestion of the labeled protein, separation of the tryptic peptides, and MALDI-TOF mass spectrometric analysis revealed that C257, C273, C288, and C297, all residing in the IVR domain, were consistently labeled with the steroid mesylate. In addition, C613 from the CTR was also labeled, probably due to its predicted close spacial proximity to the IVR cysteines and its high reactivity due to a neighboring basic amino acid.

This finding suggested that C257, C273, C288, and C297 were candidates for the actual targets for inducers. Single and multiple mutations of these and other cysteine residues of Keap1 established that modification of C273 and C288, either individually or in combination, but not of any other cysteine residues, abrogated the capacity of Keap1 to repress the activity of Nrf2 as promoter of transcription of ARE-dependent genes (*58*−*60*). This finding is in agreement with the conclusions drawn from a phylogenetic sequence comparison of Keap1 from several species, which showed that C273 and C288 are conserved only in members of the "oxidant sensing" Keap1 homologues. C297, however, is almost invariably present in all Keap1-related homologues suggesting a structural rather than a "sensor" role (*61*).

The finding that individual or simultaneous substitution(s) of C273 and C288 with alanine or serine renders Keap1 unable to repress Nrf2 was somewhat unexpected, since (i) C273 and C288 are located in the intervening domain of Keap1, yet Keap1 interacts with Nrf2 via its Kelch domain (*17*), and (ii) given the fact that Keap1 requires a reducing environment in order to bind Nrf2 (*56*). Because C273 and C288 are critical for this interaction and exposure to inducers leads to the formation of disulfide-linked dimers of Keap1 (*58*), the inability of C273 and C288 to form a disulfide bond is expected to lock Keap1 permanently bound to Nrf2. Instead, it was found that any kind of modification of C273 and C288 (e.g., alkylation with inducers, participation in disulfide linkages and possibly higher orders of oxidation states, or even amino acid substitution) leads to the inability of Keap1 to repress Nrf2. Furthermore, such mutant proteins are not able to target Nrf2 for ubiquitin-dependent proteasomal degradation (*59*) (see sections 6 and 7 below). Thus, C273 and C288 appear critical not only for the inducer sensing function of Keap1 but also for its repressor activity.

Cysteine-to-serine mutagenesis within the BTB domain of human Keap1 revealed that substitution of C151 with a serine residue resulted in constitutive repression of the Nrf2-dependent transcriptional activity both under basal conditions as well as upon exposure to inducers (sulforaphane or tBHQ) (*59*). Although tBHQ treatment of cells transfected with wild-type Keap1 led to the formation of high molecular weight species of Keap1, this

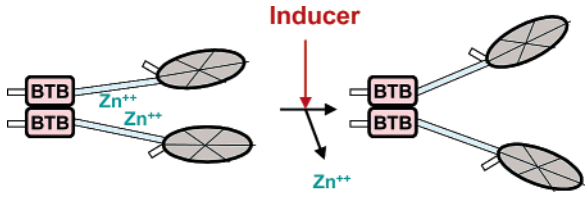


Figure 4. Inducer binding leads to zinc release and a conformational change in Keap1. It is proposed that this conformational change renders the Keap1 dimer unable to repress Nrf2.

did not occur in transfected cells expressing the C151S mutant protein, suggesting that this cysteine residue could also be a target site for modification by certain inducers.

Initial characterization of interactions of inducers with human Keap1 suggests a similar, but nonetheless distinct, mode of interaction. Incubation of His-tagged recombinant human Keap1 with N-iodoacetyl-N-biotinylhexylenediamine (Figure 3), an inducer of ARE-dependent transcription, yielded adducts with C196, C226, C241, C257, C288, and C319, all of which are within the IVR domain (*63*), while 1-biotinamido-4-(4′-[maleimidoethyl-cyclohexane]carboxamido)butane labeled C77, C196, C249, C368, and C489 (*63*). In another study, the reaction of a His-tagged recombinant human Keap1 with biotinylated iodoacetamide labeled primarily C151, C288, and C297 and to a lesser extent C257, C273, and C613 (*62*). It is noteworthy that the pattern of cysteine modifications by biotinylated iodoacetamide is similar to the modification pattern by Dex-mes, both reagents being phase 2 inducers, while 1-biotinamido-4-(4′-[maleimidoethyl-cyclohexane]carboxamido)butane, which did not induce the phase 2 response, labeled a completely different set of cysteine residues. It is possible, however, that the system is much more finely tuned in vivo under conditions in which the concentrations of both proteins and inducers are expected to be much lower than those used for in vitro studies. Furthermore, sites of sulfydryl adduction or modification may vary among different classes of inducers and across phylogenic species. It might be possible that different reagents and reaction conditions could be "sensed" by different cysteines. Such flexibility is in agreement with the fact that a plethora of conditions can trigger the phase 2 response.

Reaction with inducers of various structural types leads to zinc release and a conformational change in murine Keap1 (Figure 4) (*51*). This can be followed by monitoring the intrinsic tryptophan fluorescence of Keap1 because six of its eight tryptophan residues are located in the Nrf2-binding Kelch domain. Native Keap1 has an emission maximum at 337 nm with four times higher fluorescent intensity than either an equimolar amount of tryptophan alone, equimolar amounts of the mixture of all amino acids comprising Keap1, or denatured Keap1, all of which have an emission maximum at 350 nm. These spectral characteristics indicate that some of the tryptophan residues of the native protein are located in a hydrophobic environment that is not accessible to fluorescence quenching by the buffer and are in agreement with the crystal structure of the Kelch domain in which the tryptophan residues are part of a hydrophobic core at the interface between two blades of the propeller (*49*). Reaction with inducers leads to substantial quenching of the tryptophan fluorescence with a blue shift in the emission maximum to 344 nm, suggesting the occurrence of a conformational change in the protein and a decrease in its hydrophobicity. An identical conclusion was also reached when a reaction of Keap1 with an inducer (4,4′-dipyridyl disulfide) was carried out in the presence of a hydrophobicity probe (bis-ANS), whose fluorescence was markedly decreased in a manner that was linearly dependent on the concentration of the inducer. Taken together, these experiments indicate that upon reaction with inducers Keap1 undergoes a profound conformational change that subsequently allows Nrf2 to activate the expression of phase 2 cytoprotective genes.

6. Binding of Keap1 to Nrf2

The discovery of Keap1 as the cytosolic repressor of Nrf2 was followed by establishing that the binding between Keap1 and Nrf2 occurred through their Kelch and Neh2 domains, respectively (*17*). Deletion of the Neh2 domain of Nrf2 or of the Kelch domain of Keap1 enhances the transcriptional activity of Nrf2 (*17*, *38*) indicating that such mutant proteins are not able to interact. Analysis by native gel electrophoresis revealed the formation of a complex when Keap1 was incubated in vitro with the Neh2 domain of Nrf2 (*56*). The phylogenetically conserved acidic ETGE sequence motif at the C terminus of the Neh2 domain was shown to be critical for this interaction, and point mutations in this sequence abolished the ability of Keap1 to repress Nrf2 (*40*). This affinity may be explained by the fact that in the crystallographic structure of the Kelch domain of Keap1 the bottom face of the β-propeller is positively charged due to the presence of a highly conserved arginine residue in each of the six blades of the propeller, and it has been suggested that electrostatic interactions with the ETGE motif of Nrf2 could contribute to this binding (*49*).

In addition to the ETGE motif that is essential for binding of Nrf2 to Keap1, the sequence LxxQDxDLG (called the DLG motif) in the Neh2 domain was recently found to be important for the interaction between the two proteins (*64*). The DLG motif is highly conserved among members of the CNC bZIP family members that also have the ETGE motif. Deletion or mutations in this region decrease the binding affinity of Nrf2 to Keap1 and consequently abolish Keap1-mediated Nrf2 proteasomal degradation in the cytoplasm with no significant effect on the Keap1-independent proteasomal degradation of Nrf2 in the nucleus. The authors hypothesized that binding of Keap1 to the ETGE motif of Neh2 occurs first followed by binding to the DLG motif and these cooperative interactions contribute collectively to the overall stability of the Keap1−Nrf2 complex.

7. Role of Keap1 in Regulating the Stability of Nrf2

Keap1 binds to Nrf2 and targets it for ubiquitin-dependent degradation through the 26S proteasome, thus playing a role not only in the cytosolic sequestration of the transcription factor but also in regulating its steady state level in the cell (*15*, *59*, *65*−*67*). Exposure to inducers causes nuclear translocation of Nrf2 and also an increase in the total cellular content of Nrf2 as a result of both enhanced transcription and inhibition of its proteasomal degradation (*15*, *67*−*69*). This finding is consistent with a previous report of Sekhar et al. (*70*)

who observed that proteasome inhibitors activate gene expression through the ARE in HepG2 cells and that inducers increase the protein stability of Nrf2 in transfected cells (*15*, *59*, *67*). McMahon et al. (*66*) and Itoh et al. (*65*) demonstrated that Nrf2 undergoes Keap1-independent and Keap1-dependent proteasomal degradation. Consistent with this finding is the demonstration that Keap1 targets Nrf2 for ubiquitination (*59*, *66*). Under homeostatic conditions, Keap1 binds Nrf2 and targets it for ubiquitination and proteasomal degradation and is thus responsible for the very short half-life of the transcription factor. Upon exposure to inducers, the half-life of Nrf2 is substantially extended.

As is the case for the nuclear translocation of Nrf2, the redox status of Keap1 is critically important for the ubiquitination and subsequent proteasomal degradation. C273 and C288 are required for Keap1-dependent ubiquitination of Nrf2 (*59*), and C151 was identified to be an essential residue for inhibition of the Keap1-dependent degradation of Nrf2. It was proposed that Keap1 is a component of an E3 ubiquitin ligase complex that is inhibited by exposure to inducers. A critical element in the Neh2 domain of Nrf2 (DIDLID, amino acids 17−32) is indispensable for the Keap1-dependent destabilization of Nrf2 and its rapid turnover under homeostatic conditions (*71*). Removal of this element enhances the stability of the transcription factor; apparently, it functions to recruit the ubiquitin ligase. Subsequent studies in four different laboratories demonstrated that Keap1 associates with Cul3 to form an E3 ubiquitin ligase complex that targets Nrf2 for ubiquitination (*72*−*75*).

Because BTB domain-containing proteins have been reported to function as substrate adaptors for Cul3-dependent ubiquitin ligase complexes (*43*, *76*), it was hypothesized that Keap1 may associate with Cul3 through its BTB domain and thus promote ubiquitination of Nrf2. Immunoprecipitation analysis detected Cul3 in immunocomplexes of Keap1 (*72*−*74*). In vitro translated Nrf2 was able to bind to Cul3 only in the presence of Keap1, and the association between Cul3 and Nrf2 was markedly decreased in cells in which Keap1 was knocked down (*73*). These results indicated that Keap1 provides a bridge between Cul3 and Nrf2.

Although the data presented from these laboratories have agreed that Keap1 is a component of an E3-ubiquitin ligase system, one major point of controversy still remains. The laboratory of Yamamoto made the surprising finding that a BTB deletion mutant of murine Keap1 was still able to associate with Cul3, while an IVR deletion mutant was not (*72*). This observation is consistent with the findings that inducers modify primarily highly reactive cysteine residues that reside in the IVR domain of Keap1 and that exposure of cells to inducers leads to inhibition of the proteasomal degradation (increased protein stability) of Nrf2 and its subsequent nuclear translocation. In contrast, Cullinan et al. (*73*) and Furukawa and Xiong (*75*) showed that a BTB deletion mutant of human Keap1 did not coprecipitate with Cul3. Zhang et al. (*74*) found that although the BTB domain of human Keap1 was necessary for binding to Cul3, it was not sufficient. Whether this discrepancy is due to species differences or to the various experimental conditions that all utilize different deletion strategies and forced overexpression is not clear at present.

In a recent study, Eggler et al. reported that electrophilic modifications of cysteine residues of Keap1 by several inducers (i.e, sulforaphane, isoliquiritigenin, 15-deoxy-Δ-12,14-prostaglandin J_2, 1-Cl-2,4-dinitrobenzene, and biotinylated iodoacetamide) did not lead to dissociation of the Keap1−Nrf2 complex, and when human Keap1 was preincubated with inducers, it was still able to bind the Neh2 domain of Nrf2 in vitro (*62*). In addition, the laboratory of Hannink showed (*74*) that exposure of cells to sulforaphane or tBHQ: (i) decreased the levels of ubiquitinated Gal4-Neh2 fusion protein—ubiquitination of this fusion protein was also decreased in cells transfected with expression vectors for Keap1 and Cul3; (ii) decreased the level of Cul3 that copurified with Keap1; and (iii) increased the level of Keap1-associated Nrf2. On the basis of these results, it was hypothesized that inducers disrupt the ability of Keap1 to assemble into E3 ubiquitin ligase complex, decrease the ubiqutination of Nrf2, and increase its steady state levels.

Interestingly, certain mutations in the BTB domain of human Keap1 resulted in an increased association with Cul3 and an increased level of ubiquitination of Keap1 but a decrease in ubiquitination of Nrf2 (*74*). Treatment of HEK293 cells transfected with FLAG-tagged human Keap1 with tBHQ or N-iodoacetyl-N-biotinylhexylenediamine (Figure 3) resulted in polyubiquitination of Keap1 that coincided with increased stability and nuclear accumulation of Nrf2 (*63*). Similarly, exposure to tBHQ causes Keap1 ubiquitination by a Cul3-dependent E3 ubiquitin ligase complex coincidentally with a decrease in Nrf2 ubiquitination (*77*). Interestingly, exposure to sulforaphane, although inhibiting ubiquitination of Nrf2, does not promote ubiquitination of Keap1. Furthermore, in contrast to the Nrf2 degradation that is dependent on a functional proteasome, degradation of Keap1 is not. Analysis of deletion mutants of Keap1 suggested that lysine residues residing in the IVR domain of Keap1 are the likely sites of ubiquitination (*77*). Indeed, LC-MS-MS analysis of tryptic peptides derived from in-gel digestion of Keap1 bands from cell lysates of FLAG-Keap1 expressing 293 cells treated with N-iodoacetyl-N-biotinylhexylenediamine revealed ubiquitination at lysine 298 (*63*). Both research groups have proposed a model according to which inducer binding to Keap1 triggers a switch in ubiquitination from Nrf2 to Keap1. In addition, Eggler et al. (*62*) suggested that this switch in ubiquitination becomes possible due to a conformational change in Keap1 triggered by its reaction with electrophiles. Although the ubiquitination switch hypothesis is certainly viable, especially for inducers that modify Keap1 irreversibly (e.g., alkylating agents), regeneration of reversibly modified Keap1 (e.g., by disulfide bond formation or thiol-disulfide interchange) remains an attractive possibility given the fact that many components of the systems for glutathione and thioredoxin biosynthesis and regeneration are elevated as part of the phase 2 response.

8. Nuclear-Cytoplasmic Shuttling of Keap1

A recent paper by Karapetian and co-workers (*78*) has called into question the current view of Keap1 as an exclusively cytoplasmic protein that releases Nrf2 for nuclear translocation upon exposure to inducers. Furthermore, they also have introduced a new protein that appears to interact with the Keap1−Nrf2 complex, prothymosin α (ProTα).

ProTα is a small (12 kDa), highly acidic, apparently nuclear protein originally isolated from rat thymus but

also occurs in numerous other tissues (*79*). To identify proteins that interacted with ProTα, a yeast two-hybrid screen was carried out using cDNA libraries from human brain and bone marrow. Ultimately, four positive clones were obtained that, upon sequencing of the inserts from the rescued library plasmids, revealed that they all encoded for portions of the same protein, Keap1. Further experiments using deletion mutants of ProTα identified the region of this protein that interacted with Keap1 as amino acids 32−52. This region contains the sequence −ENGE− that presumably is analogous to the −ETGE− sequence in Nrf2 that is required for binding to Keap1 (*40*). Similarly, the region of Keap1 to which ProTα binds was localized to the carboxy half of the protein or Kelch domain to which Nrf2 also binds. An association between Keap1 and ProTα was demonstrated in cell extracts by immunoprecipitation techniques. Further experiments uncovered a nuclear export signal (NES) sequence of the "leucine-rich" type (*80*) near the end of the IVR region of Keap1. In HeLa cells transfected with Keap1, Keap1-green fluorescent protein (GFP), or just containing the endogenous protein, Keap1 was found by immunofluorescent staining to be localized in the cytoplasm under normal conditions. However, in the presence of leptomycin B (LMB), an inhibitor of the CRM1 nuclear export protein (*81*), Keap1 was relocated to the nucleus. In cells transfected with both wild-type Keap1 and Nrf2, colocalization of both proteins in the cytoplasm was observed, but in the presence of LMB or using a Keap1 with a mutated NES, both proteins were found in the nucleus. Finally, in HeLa cells overexpressing wild-type ProTα, upregulation of an Nrf2-dependent gene mRNA (heme oxygenase-1) was demonstrated by Northern blot hybridization. On the other hand, no upregulation was seen if a mutant ProTα with an altered Keap1 recognition sequence (E44A, E50A) was used. In conclusion, these authors presented a model of the phase 2 response in which the Keap1−Nrf2 complex shuttles between the cytoplasm and the nucleus. After entry into the nucleus, the much higher concentration of ProTα relative to the complex is able to displace some to the Nrf2 resulting in basal level expression of Nrf2-controlled genes. Inducers of phase 2 genes destabilize the Keap1−Nrf2 association and permit greater displacement of Nrf2 by ProTα, and this results in upregulation of gene expression.

The nuclear-cytoplasmic shuttle model has received additional support from a recent paper by Velichkova and Hasson (*82*) who also noted the NES sequence at the end of the IVR region of Keap1 (Figure 2A). To examine the function of this NES, a series of GFP−Keap1 fusion constructs were created. Transfection of NIH 3T3 cells with those constructs that contained the NES resulted in expression of fusion proteins that were localized in the cytoplasm. In contrast, the ones lacking the NES were seen predominantly in the nucleus, and this is consistent with the finding of Zhang and Hannink (*59*) that overexpression of a Keap1 deletion mutant that lacked the IVR was mislocalized to the nucleus and was unable to sequester overexpressed Nrf2 in the cytoplasm. In the presence of LMB, even constructs containing the NES sequence were found in the nucleus. More detailed analysis suggested that a minimal CRM1-binding site is located between amino acids 272 and 315. Further characterization of the NES was achieved by the introduction of point mutations in this region. Mutation of the hydrophobic amino acids L301, L304, L308, and L310 to alanine residues resulted in nuclear localization of the fusion proteins, while mutation of C273, C288, and C297 to serine residues had no effect on the subcellular distribution of the resulting proteins. These results demonstrated the critical role of the leucine residues. Although the cysteine residues appear not to be critical for the function of the NES, the possibility that their modification could disrupt the functional integrity of the NES cannot be excluded. In addition to their impact on the subcellular distribution of Keap1, mutations of the NES also affected the subcellular localization of Nrf2, which was now predominantly nuclear. A similar result was obtained by treatment with either LMB or diethylmaleate (DEM) that led to nuclear accumulation of endogenous Keap1 and Nrf2. However, the expression of an ARE target gene, GSTα, was much higher in cells treated with DEM as compared to those treated with LMB, suggesting that nuclear trapping of Keap1 and Nrf2 is not sufficient for maximal induction of phase 2 genes. This prompted the examination of the effect of phosphorylation of Nrf2 by a protein kinase C activator, phorbol 12-myristate 13-acetate. While this treatment had no effect on the nuclear distribution of Keap1 and Nrf2, it elevated the expression of GSTα to a level similar to that of LMB. Simultaneous treatment with LMB and phorbol ester was necessary to achieve induction comparable to that caused by DEM. Further studies using immunostaining techniques discerned three pools of Keap1 within the cell: cytoskeletal, cytoplasmic, and nuclear. Interestingly, Nrf2 was not collocalized with the cytoskeletal fraction but instead with the cytoplasmic Keap1. Treatment of cells with the inducer DEM caused a dissociation of Keap1 from the cytoskeleton, and much like endogenous Nrf2, it underwent a nuclear translocation. This result is in contrast with the observation that in the same cell line overexpressed Keap1−GFP fusion protein does not translocate to the nucleus upon inducer treatment, sending out a warning against drawing conclusions under conditions of forced overexpression. A model for Keap1 and Nrf2 function was proposed that shares similarities with the one suggested by Karapetian and co-workers (*78*). Most of the cytoplasmic Keap1−Nrf2 complex is associated with proteasomes, but some can translocate to the nucleus because of the NLS on Nrf2. However, most of this complex is rapidly exported due to the dominant function of the NES of Keap1. Some dissociation of the complex within the nucleus may occur resulting in basal level expression of ARE-controlled genes. In the presence of phase 2 inducers, the critical cysteine residues of Keap1 are modified, and the protein undergoes a conformational change that has two effects: (i) It brings about a dissociation of Keap1−Nrf2 from the proteasomal apparatus and Keap1 from the cytoskeleton, and (ii) it masks the NES of Keap1. The free Keap1−Nrf2 and Keap1 can now accumulate in the nucleus. Dissociation of Nrf2 from Keap1, possibly through the action of protein kinase C, leads to induced levels of expression of phase 2 genes.

In a recent report, the Pickett laboratory has suggested that Nrf2 is primarily a nuclear protein (*83*). The authors have proposed a model according to which Nrf2 immediately after synthesis undergoes nuclear translocation activating expression of phase 2 genes and thus accounting for their basal expression. The transiently shuttling Keap1 then binds to Nrf2 in the nucleus and targets it for degradation. The presence of inducers

interferes with the ability of Keap1 to target Nrf2 for degradation, leading to its stabilization, which, together with its de novo protein synthesis, accounts for the nuclear accumulation of Nrf2 and increased transcription of phase 2 genes. The nuclear-cytoplasmic shuttle models have not only added a new dimension to the traditional view of Keap1 as an exclusively cytoplasmic, actin-bound protein but also have questioned the presence of Nrf2 in the cytoplasm. However, better reagents for monitoring the trafficking of endogenous Keap1 and Nrf2 will be required to reveal the full significance of this model.

9. Keap1 and the FAC1 Protein

In addition to ProTα, discussed in the previous section, another recent paper, also employing a yeast two-hybrid screen, identified the Fetal ALZ-50 Clone 1 (FAC1) protein, a transcriptional regulator prominent in neural tissue, as a partner of human Keap1 (*84*). Using GST pull down assays, hKeap1 binding to GST:FAC1 was localized to amino acids 501−610, which contains a PEST domain. In addition, the Kelch domain of Keap1 was identified as the region to which FAC1 bound. Immunofluorescent staining of PT67 murine fibroblast cells transfected with epitope-taged FAC1 showed that FAC1 colocalized with Keap1 and with a subset of F-actin in the cytoplasm. Neither protein was detected in the nucleus. In vitro GST pull down assays demonstrated that Nrf2 could compete with GST:FAC1 (501−610) for binding to Keap1. In the presence of DEM, an inducer of phase 2 enzymes, this competition was impaired. However, DEM did not affect the affinity of GST:FAC1 (501−610) for Keap1. The physiological significance of this interaction between FAC1 and Keap1 is not known at present but may relate to Keap1-directed proteasomal degradation of FAC1.

10. Keap1 in Adhesion Junctions

A yeast two-hybrid screen was also used to identify Keap1 as a protein interacting with the SH3 domain of the molecular motor myosin-VIIa, an unconventional myosin that has an essential role in the development and function of the eye and the ear (*85*). Myosin-VIIa is also found in Sertoli cells, specifically in specialized adhesion structures within the testis termed ectoplasmic specialization that are subjected to continuing remodeling during spermatogenesis. Although Keap1 is associated with myosin-VIIa within the ectoplasmic specialization, the presence of myosin-VIIa is not required for proper Keap1 localization, which appears normal in mice lacking myosin-VIIa (known as *shaker-1* mice). In many epithelial cell types, Keap1 is an integral component of focal adhesions, the sites of attachment of the cell membrane to the extracellular matrix, and is similar to the ectoplasmic specialization. They are also dynamic structures that undergo continuous remodeling. Subsequent studies revealed that Keap1 is present only in peripheral focal adhesions and assembling adherens junctions and does not collocalize entirely with any markers that are typical for these structures, suggesting its recruitment to specific subregions in a subset of adhesion structures, probably those that undergo constant dynamic structural rearrangements (*86*). It was hypothesized that Keap1 functions to bundle F-actin within these cell adhesion components, but the underlying mechanism still remains an unanswered question. Because cell adhesion complexes interact with the extracellular environment, thus playing an essential role in signal tranduction and cellular communications, it will be important to investigate whether Keap1 modifications (e.g., caused by phase 2 inducers) have any impact on the assembly/disassembly of these structures.

11. Targeted Deletion of Keap1: The Consequences

The Yamamoto laboratory has generated mice in which the *keap1* gene is disrupted (*87*). *Keap1*-null pups initially are indistinguishable from their wild-type counterparts. However, at about postnatal day 4 (P4), they begin to show signs of growth retardation with progressively increasing severity, such that none of the mutant mice survive beyond P21, with the cause of death being asthenia. In addition, these mice display a scaling skin phenotype. Histological analysis of skin sections revealed a thicker stratum corneum but a similar number of hair follicles as compared to the wild-type genotype. The levels of the squamous cell differentiation markers keratin K1 and flaggrin did not differ between genotypes, but the terminal differentiation marker loricrin was more abundant in the skin of *keap1* knockout mice. Consistent with the observed scaling of the skin, there were multiple cornified layers on the inner walls of the esophagus and forestomach and large masses in the lumens of these organs. Histological analyses demonstrated markedly increased levels of loricrin and keratin K6 and decreased levels of involucrin in the esophagus. This excessive hyperkeratosis leads to gastric obstruction that is probably the primary cause of premature death of these animals. These observations also suggested that some of the squamous differentiation markers could be under the direct negative control of Keap1. Indeed, reporter assays confirmed that loricrin and keratin K6 have functional AREs that, in the absence of Keap1, are under the control of the constitutively active Nrf2. The ultimate proof of the hypothesis that the scaling skin and hyperkeratotic esophageal phenotype of the *keap1* knockout mouse and the squamous cell differentiation markers associated with them were entirely due to improper hyperactivity of Nrf2 was achieved by generating the Keap1/Nrf2 double knockout mouse whose rescued phenotype was indistinguishable from that of wild-type mice (*87*). Furthermore, a complementation rescue assay indicated that the lethality of the *keap1*-null mice could be reversed by transgenic expression of wild-type Keap1 but not C273A/C288A mutant Keap1 (*88*).

Keap1 knockout mice have high levels of phase 2 enzymes indicating that Keap1 is not required for nuclear import and accumulation of Nrf2. Gene expression levels of GSTπ and NQO1 are much higher in livers and mouse embryo fibroblast cells prepared from *keap1* knockout mice as compared to their wild-type counterparts (*87*). In contrast to wild-type cells in which the phase 2 response is induced by DEM, 1,2-dithiole-3-thiones, or sulforaphane, no induction is observed in cells from *keap1* knockout, *nrf2* knockout, or *keap1*/*nrf2* double knockout mice (*58*, *87*, *89*).

Among the first experiments to demonstrate a role of Keap1−Nrf2 signaling as a stress response pathway in vivo were the studies of Enomoto et al. (*21*) and Chan et al. (*20*). Both groups described the enhanced sensitivity of *nrf2* knockout mice to the hepatotoxicity of acetami-

nophen and related this outcome to lowered cellular thiol levels and diminished capacity for conjugation reactions. Taking an opposite experimental approach, Okawa et al. (*90*) have observed recently that hepatocyte specific disruption of the *keap1* gene confers striking resistance of mice toward acetaminophen-induced hepatic injury. Because of the juvenile lethality of simple *keap1* knockout mice, hepatocyte specific *keap1*-null mice were generated using the Cre-loxP system regulated by the albumin promoter, allowing for viable mice with high constitutive expression of hepatic phase 2 enzymes. Thus, Nrf2 activation through a genetic approach leads to acetaminophen resistance. Consistent with these genetic models, it has been long established that pharmacological inducers (e.g., oltipraz, BHA) of the phase 2 response are effective inhibitors of acetaminophen hepatotoxicity (*91*).

Small interfering RNA (siRNA) strategy was recently used to knockdown Keap1 in the human keratinocyte cell line HaCaT (*92*). Transfection of siRNA into these cells reduced the steady state levels of Keap1 mRNA to ~30% of the levels in control cells, and the knockdown was maintained up to 72 h posttransfection. The levels of Keap1 protein in treated cells were below the limit of detection. In contrast, Nrf2 protein levels were increased and this correlated with increased *nqo1*-ARE-reporter gene activity as well as with increased expression of endogenous ARE-driven phase 2 genes, i.e., NQO1, aldo-keto reductase 1C1/2, γ-glutamatecysteine ligase catalytic and modifier subunits, and cellular glutathione 48 h after transfection. The extent of increase was comparable to that achieved by induction with sulforaphane. Similar to the knockout strategy, the knockdown strategy also unequivocally demonstrated that Keap1 functions to control the expression of phase 2 genes through negative regulation of the transcription factor Nrf2 and that the absence of Keap1 is sufficient for the constitutive activation of Nrf2 and high expression of phase 2 genes.

12. Conclusions

Since the discovery of Keap1 was reported in 1999, steady progress has been made in elucidating its cellular function. Initial research focused on its role in phase 2 enzyme induction and suggested that it acted as a cytoplasmic anchor for the transcription factor Nrf2. In the presence of inducers, the association between the two proteins was disrupted allowing Nrf2 to migrate to the nucleus and activate the transcription of ARE-controlled genes. Recent studies, however, have expanded this picture of Keap1. Evidence has accumulated that it is one component of an E3-ubiquitin ligase system that actively targets Nrf2 for degradation by the 26S proteasomes. The discovery of a NES in the IVR region led to studies revealing nuclear-cytoplasmic shuttling of at least some Keap1−Nrf2 complexes. Immunostaining experiments have distinguished three cellular pools of Keap1, cytoskeletal, cytoplasmic, and nuclear, and the cytoskeletal fraction is apparently not associated with Nrf2. Several other proteins have been found to interact with Keap1, and one of them, ProTα, may play a role in the regulation of phase 2 enzymes. Taken together, these results suggest that Keap1 may have a more complex function within the cell than was originally envisioned. Further work will be needed before a complete understanding of the role of Keap1 in cellular protective responses can be achieved. Nonetheless, it is clear that Keap1 plays a central role in regulating adaptive responses to electrophilic and oxidative stresses.

Acknowledgment. Studies from the authors' laboratories were supported by grants from the National Cancer Institute, Department of Health and Human Services (CA39416, CA94076, and CA93780), the American Institute for Cancer Research, as well as by generous gifts from the Lewis B. and Dorothy Cullman Foundation, the Barbara Lubin Goldsmith Foundation, and the McMullan Family Fund. We are extremely grateful to our colleagues P. Talalay, N. Wakabayashi, and M. K. Kwak for their critical contributions to the experimental design and performance.

References

(1) Talalay, P. (2000) Chemoprotection against cancer by induction of phase 2 enzymes. *Biofactors 12*, 5−11.
(2) Talalay, P. (1999) The war against cancer: New hope. *Proc. Am. Philos. Soc. 143*, 52−72.
(3) Prestera, T., Talalay, P., Alam, J., Ahn, Y. I., Lee, P. J., and Choi, A. M. (1995) Parallel induction of heme oxygenase-1 and chemoprotective phase 2 enzymes by electrophiles and antioxidants: Regulation by upstream antioxidant-responsive elements (ARE). *Mol. Med. 1*, 827−837.
(4) Primiano, T., Kensler, T. W., Kuppusamy, P., Zweier, J. L., and Sutter, T. R. (1996) Induction of hepatic heme oxygenase-1 and ferritin in rats by cancer chemopreventive dithiolethiones. *Carcinogenesis 17*, 2291−2296.
(5) Hayes, J. D., and Pulford, D. J. (1995) The glutathione S-transferase supergene family: Regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. *Crit. Rev. Biochem. Mol. Biol. 30*, 445−600.
(6) Hayes, J. D., Flanagan, J. U., and Jowsey, I. R. (2005) Glutathione transferases. *Annu. Rev. Pharmacol. Toxicol. 45*, 51−88.
(7) Talalay, P. (1989) Mechanisms of induction of enzymes that protect against chemical carcinogenesis. *Adv. Enzyme Regul. 28*, 237−250.
(8) Moinova, H. R., and Mulcahy, R. T. (1988) An electrophile responsive element (EpRE) regulates beta-naphthoflavone induction of the human gamma-glutamylcysteine synthetase regulatory subunit gene. Constitutive expression is mediated by an adjacent AP-1 site. *J. Biol. Chem. 273*, 14683−14689.
(9) Thimmulappa, R. K., Mai, K. H., Srisuma, S., Kensler, T. W., Yamamoto, M., and Biswal, S. (2002) Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. *Cancer Res. 62*, 5196−5203.
(10) Hayes, J. D., and McLellan, L. I. (1999) Glutathione and glutathione-dependent enzymes represent a coordinately regulated defence against oxidative stress. *Free Radical Res. 31*, 273−300.
(11) Primiano, T., Li, Y., Kensler, T. W., Trush, M. A., and Sutter, T. R. (1998) Identification of dithiolethione-inducible gene-1 as a leukotriene B4 12-hydroxydehydrogenase: Implications for chemoprevention. *Carcinogenesis 19*, 999−1005.
(12) Torti, F. M., and Torti, S. V. (2002) Regulation of ferritin genes and protein. *Blood 99*, 3505−3516.
(13) Wilkinson, J., 4th, Pietsch, E. C., Torti, S. V., and Torti, F. M. (2003) Ferritin regulation by oxidants and chemopreventive xenobiotics. *Adv. Enzyme Regul. 43*, 135−151.
(14) Kwak, M. K., Wakabayashi, N., Itoh, K., Motohashi, H., Yamamoto, M., and Kensler, T. W. (2003) Modulation of gene expression by cancer chemopreventive dithiolethiones through the Keap1-Nrf2 pathway. Identification of novel gene clusters for cell survival. *J. Biol. Chem. 278*, 8135−8145.
(15) Nguyen, T., Sherratt, P. J., and Pickett, C. B. (2003) Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. *Annu. Rev. Pharmacol. Toxicol. 43*, 233−260.
(16) Motohashi, H., O'Connor, T., Katsuoka, F., Engel, J. D., and Yamamoto, M. (2002) Integration and diversity of the regulatory network composed of Maf and CNC families of transcription factors. *Gene 294*, 1−12.
(17) Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J. D., and Yamamoto, M. (1999) Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev. 13*, 76−86.
(18) Chan, K., Lu, R., Chang, J. C., and Kan, Y. W. (1996) NRF2, a member of the NFE2 family of transcription factors, is not

essential for murine erthropoiesis, growth, and development. *Proc. Natl. Acad. Sci. U.S.A. 93*, 13943−13948.

(19) Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., Oyake, T., Hayashi, N., Satoh, K., Hatayama, I., Yamamoto, M., and Nabeshima, Y. (1997) An Nrf2/small maf heterodimer mediates induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem. Biophys. Res. Commun. 236*, 313−322.

(20) Chan, K., Han, X.-D., and Kan, Y. W. (2001) An important function of Nrf2 in combating oxidative stress: Detoxification of acetaminophen. *Proc. Natl. Acad. Sci. U.S.A. 98*, 4611−4616.

(21) Enomoto, A., Itoh, K., Nagayoshi, E., Haruta, J., Kimura, J., Kimura, T., O'Connor, T., Harada, T., and Yamamoto, M. (2001) High sensitivity of Nrf2 knockout mice to acetaminophen hepatotoxicity associated with decreased expression of ARE-regulated drug metabolizing enzymes and antioxidant genes. *Toxicol. Sci. 59*, 169−177.

(22) Chan, K., and Kan, Y. W. (1999) Nrf2 is essential for protection against acute pulmonary injury in mice. *Proc. Natl. Acad. Sci. U.S.A. 96*, 12731−12736.

(23) Cho, H.-Y., Jedlicka, A. E., Reddy, S. P. M., Kensler, T. W., Yamamoto, M., Zhang, L.-Y., and Kleeberger, S. R. (2002) Role of Nrf2 in protection against hyperoxic lung injury in mice. *Am. J. Respir. Cell Mol. Biol. 26*, 175−182.

(24) Calkins, M. J., Jakel, R. J., Johnson, D. A., Chan, K., Kan, Y. W., and Johnson, J. A. (2005) Protection from mitochondrial complex II inhibition in vitro and in vivo by Nrf2-mediated transcription. *Proc. Natl. Acad. Sci. U.S.A. 102*, 244−249.

(25) Shih, A. Y., Imbeault, S., Barakauskas, V., Erb, H., Jiang, L., Li, P., and Murphy, T. H. (2005) Induction of the Nrf2-driven antioxidant response confers neuroprotection during mitochondrial stress in vivo. *J. Biol. Chem. 280*, 22925−22936.

(26) Ramos-Gomez, M., Kwak, M.-K., Dolan, P. M., Itoh, K., Yamamoto, M., Talalay, P., and Kensler, T. W. (2001) Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in *nrf2* transcription factor deficient mice. *Proc. Natl. Acad. Sci. U.S.A. 98*, 3410−3415.

(27) Iida, K., Itoh, K., Kumagai, Y., Oyasu, R., Hattori, K., Kawai. K., Shimazui, T., Akaza, H., and Yamamoto, M. (2004) Nrf2 is essential for the chemopreventive efficacy of oltipraz against urinary bladder carcinogenesis. *Cancer Res. 64*, 6424−6431.

(28) Rangasamy, T., Cho, C. Y., Thimmulappa, R. K., Zhen, L., Srisuma, S. S., Kensler, T. W., Yamamoto, M., Petrache, I., Tuder, R. M., and Biswal, S. (2004) Genetic ablation of Nrf2 enhances susceptibility to cigarette smoke-induced emphysema in mice. *J. Clin. Invest. 114*, 1248−1259.

(29) Rangasamy, T., Guo, G., Mitzner, W. A., Roman, J., Singh, A., Fryer, A. D., Yamamoto, M., Kensler, T. W., Ruder, R. M., Georas, S. N., and Biswal, S. (2005) Disruption of *Nrf2* enhances susceptibility to severe airway inflammation and asthma in mice. *J. Exp. Med. 202*, 47−59.

(30) Cullinan, S. B., and Diehl, J. A. (2004) PERK-dependent activation of Nrf2 contributes to redox homeostasis and cell survival following endoplasmic reticulum stress. *J. Biol. Chem. 279*, 20108−20117.

(31) Itoh, K., Mochizuki, M., Ishii, Y., Ishii, T., Shibata, T., Kawamoto, Y., Kelly, V., Sekizawa, K., Uchida, K., and Yamamoto, M. (2004) Transcription factor Nrf2 regulates inflammation by mediating the effect of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2. *Mol. Cell. Biol. 24*, 36−45.

(32) Hosoya, T., Maruyama, A., Kang, M.-I., Kawatani, Y., Shibata, T., Uchida, K., Itoh, K., and Yamamoto, M. (2005) Differential responses of the Nrf2-Keap1 system to laminar and oscillatory shear stresses in endothelial cells. *J. Biol. Chem. 280*, 27244−27250.

(33) Talalay, P., De Long, M. J., and Prochaska, H. J. (1988) Identification of a common chemical signal regulating the induction of enzymes that protect against chemical carcinogenesis. *Proc. Natl. Acad. Sci. U.S.A. 85*, 8261−8265.

(34) Talalay, P., Fahey, J. W., Holtzclaw, W. D., Prestera, T., and Zhang, Y. (1995) Chemoprotection against cancer by phase 2 enzyme induction. *Toxicol. Lett. 82*−*83*, 173−179.

(35) Spencer, S. R., Xue, L. A., Klenz, E. M., and Talalay, P. (1991) The potency of inducers of NAD(P)H:(quinone-acceptor) oxidoreductase parallels their efficiency as substrates for glutathione transferases. Structural and electronic correlations. *Biochem. J. 273*, 711−717.

(36) Dinkova-Kostova, A. T., Massiah, M. A., Bozak, R. E., Hicks, R. J., and Talalay, P. (2001) Potency of Michael reaction acceptors as inducers of enzymes that protect against carcinogenesis depends on their reactivity with sulfhydryl groups. *Proc. Natl. Acad. Sci. U.S.A. 98*, 3404−3409.

(37) Katoh, Y., Itoh, K., Yoshida, E., Miyagishi, M., Fukamizu, A., and Yamamoto, M. (2001) Two domains of Nrf2 cooperatively bind CBP, a CREB binding protein, and synergistically activate transcription. *Genes Cells 6*, 857−868.

(38) Xue, F., and Cooley, L. (1993) Kelch encodes a component of intracellular bridges in Drosophila egg chambers. *Cell 72*, 681−693.

(39) Nagase, T., Seki, N., Tanaka, A., Ishikawa, K., and Nomura, N. (1995) Prediction of the coding sequences of unidentified human genes, IV. The coding sequences of 40 new genes (KIAA0121-KIAA0160) deduced by analysis of cDNA clones from human cell line KG-1. *DNA Res. 2*, 164−174.

(40) Kobayashi, M., Itoh, K., Suzuki, T., Osanai, H., Nishikawa, K., Katoh, Y., Takagi, Y., and Yamamoto, M. (2002) Identification of the interactive interface and phylogenic conservation of the Nrf2-Keap1 system. *Genes Cells 7*, 807−820.

(41) Bardwell, V. J., and Treisman, R. (1994) The POZ domain: a conserved protein−protein interaction motif. *Genes Dev. 8*, 1664−1677.

(42) Stogios, P. J., and Privé, G. G. (2004) The BACK domain in BTB-kelch proteins. *Trends Biochem. Sci. 29*, 634−637.

(43) Pintard, L., Willems, A., and Peter, M. (2004) Cullin-based ubiquitin ligases: Cul3-BTB complexes join the family. *EMBO J. 23*, 1681−1687.

(44) Ahmad, K. F., Engel, C. K., and Privé, G. G. (1998) Crystal structure of the BTB domain from PLZF. *Proc. Natl. Acad. Sci. U.S.A. 95*, 12123−12128.

(45) Zipper, L. M., and Mulcahy, R. T. (2002) The Keap1 BTB/POZ dimerization function is required to sequester Nrf2 in cytoplasm. *J. Biol. Chem. 277*, 36544−36552.

(46) T'Jampens, D., Devriendt, L., De Corte, V., Vandekerckhove, J., and Gettemans, J. (2002) Selected BTB/POZ-kelch proteins bind ATP. *FEBS Lett. 516*, 20−26.

(47) Adams, J., Kelso, R., and Cooley, L. (2000) The kelch repeat superfamily of proteins: propellers of cell function. *Trends Cell. Biol. 10*, 17−24.

(48) Prag, S., and Adams, J. C. (2003) Molecular phylogeny of the kelch-repeat superfamily reveals an expansion of BTB/kelch proteins in animals. *BMC Bioinformatics 4*, 42.

(49) Li, X., Zhang, D., Hannink, M., and Beamer, L. J. (2004) Crystal structure of the Kelch domain of human Keap1. *J. Biol. Chem. 279*, 54750−54758.

(50) Woycechowsky, K. J., and Raines, R. T. (2003) The CXC motif: a functional mimic of protein disulfide isomerase. *Biochemistry 42*, 5387−5394.

(51) Dinkova-Kostova, A. T., Holtzclaw, W. D., and Wakabayashi, N. (2005) Keap1, the sensor for electrophiles and oxidants that regulates the phase 2 response, is a zinc metalloprotein. *Biochemistry 44*, 6889−6899.

(52) Prestera, T., Holtzclaw, W. D., Zhang, Y., and Talalay, P. (1993) Chemical and molecular regulation of enzymes that detoxify carcinogens. *Proc. Natl. Acad. Sci. U.S.A. 90*, 2965−2969.

(53) Prestera, T., Zhang, Y., Spencer, S. R., Wilczak, C. A., and Talalay, P. (1993) The electrophile counterattack response: Protection against neoplasia and toxicity. *Adv. Enzyme Regul. 33*, 281−296.

(54) Holtzclaw, W. D., Dinkova-Kostova, A. T., and Talalay, P. (2004) Protection against electrophile and oxidative stress by induction of phase 2 genes: The quest for the elusive sensor that responds to inducers. *Adv. Enzyme Regul. 44*, 335−367.

(55) Snyder, G. H., Cennerazzo, M. J., Karalis, A. J., and Field, D. (1981) Electrostatic influence of local cysteine environments on disulfide exchange kinetics. *Biochemistry 20*, 6509−6519.

(56) Dinkova-Kostova, A. T., Holtzclaw, W. D., Cole, R. N., Itoh, K., Wakabayashi, N., Katoh, Y., Yamamoto, M., and Talalay, P. (2002) Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proc. Natl. Acad. Sci. U.S.A. 99*, 11908−11913.

(57) Dinkova-Kostova, A. T., Liby, K. T., Stephenson, K. K., Holtzclaw, W. D., Gao, X., Suh, N., Williams, C., Risingsong, R., Honda, T., Gribble, G. W., Sporn, M. B., and Talalay, P. (2005) Extremely potent triterpenoid inducers of the phase 2 response: Correlations of protection against oxidant and inflammatory stress. *Proc. Natl. Acad. Sci. U.S.A. 102*, 4584−4589.

(58) Wakabayashi, N., Dinkova-Kostova, A. T., Holtzclaw, W. D., Kang, M. I., Kobayashi, A., Yamamoto, M., Kensler, T. W., and Talalay, P. (2004) Protection against electrophile and oxidant stress by induction of the phase 2 response: Fate of cysteines of the Keap1 sensor modified by inducers. *Proc. Natl. Acad. Sci. U.S.A. 101*, 2040−2045.

(59) Zhang, D. D., and Hannink, M. (2003) Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2

and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. *Mol. Cell. Biol. 23*, 8137−8151.
(60) Levonen, A. L., Landar, A., Ramachandran, A., Ceaser, E. K., Dickinson, D. A., Zanoni, G., Morrow, J. D., and Darley-Usmar, V. M. (2004) Cellular mechanisms of redox cell signaling: Role of cysteine modification in controlling antioxidant defences in response to electrophilic lipid oxidation products. *Biochem. J. 378*, 373−382.
(61) Zhang, X., Garfinkel, M., and Ruden, D. (2003) Phylogenetic conservation of the Nrf2-Keap1 signaling system. In *Signal Transduction by Reactive Oxygen and Nitrogen Species: Pathways and Chemical Principles*, pp 256−274, Kluwer Academic Publishers, Dordrecht.
(62) Eggler, A. L., Liu, G., Pezzuto, J. M., van Breemen, R. B., and Mesecar, A. D. (2005) Modifying 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.
(63) Hong, F., Sekhar, K. R., Freeman, M. L., and Liebler, D. C. (2005) Specific patterns of electrophile adduction trigger Keap1 ubiquitination and Nrf2 activation. *J. Biol Chem. 280*, 31768−31775.
(64) Katoh, Y., Iida, K., Kang, M. I., Kobayashi, A., Mizukami, M., Tong, K. I., McMahon, M., Hayes, J. D., Itoh, K., and Yamamoto, M. (2005) Evolutionary conserved N-terminal domain of Nrf2 is essential for the Keap1-mediated degradation of the protein by proteasome. *Arch. Biochem. Biophys. 433*, 342−350.
(65) Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., O'Connor, T., and Yamamoto, M. (2003) Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles. *Genes Cells 8*, 379−391.
(66) McMahon, M., Itoh, K., Yamamoto, M., and Hayes, J. D. (2003) Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of antioxidant response element-driven gene expression. *J. Biol. Chem. 278*, 21592−21600.
(67) Stewart, D., Killeen, E., Naquin, R., Alam, S., and Alam, J. (2003) Degradation of transcription factor Nrf2 via the ubiquitin-proteasome pathway and stabilization by cadmium. *J. Biol. Chem. 278*, 2396−2402.
(68) Kwak, M. K., Itoh, K., Yamamoto, M., Sutter, T. R., and Kensler, T. W. (2001) Role of transcription factor Nrf2 in the induction of hepatic phase 2 and antioxidative enzymes in vivo by the cancer chemoprotective agent, 3H-1, 2-dimethiole-3-thione. *Mol. Med. 7*, 135−145.
(69) Kwak, M. K., Itoh, K., Yamamoto, M., and Kensler, T. W. (2002) Enhanced expression of the transcription factor Nrf2 by cancer chemopreventive agents: role of antioxidant response element-like sequences in the nrf2 promoter. *Mol. Cell. Biol. 22*, 2883−2892.
(70) Sekhar, K. R., Soltaninassab, S. R., Borrelli, M. J., Xu, Z. Q., Meredith, M. J., Domann, F. E., and Freeman, M. L. (2000) Inhibition of the 26S proteasome induces expression of GLCLC, the catalytic subunit for gamma-glutamylcysteine synthetase. *Biochem. Biophys. Res. Commun. 270*, 311−317.
(71) McMahon, M., Thomas, N., Itoh, K., Yamamoto, M., and Hayes, J. D. (2004) Redox-regulated turnover of Nrf2 is determined by at least two separate protein domains, the redox-sensitive Neh2 degron and the redox-insensitive Neh6 degron. *J. Biol. Chem. 279*, 31556−31567.
(72) Kobayashi, A., Kang, M. I., Okawa, H., Ohtsuji, M., Zenke, Y., Chiba, T., Igarashi, K., and Yamamoto, M. (2004) Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. *Mol. Cell. Biol. 24*, 7130−7139.
(73) Cullinan, S. B., Gordan, J. D., Jin, J., Harper, J. W., and Diehl, J. A. (2004) The Keap1-BTB protein is an adaptor that bridges Nrf2 to a Cul3-based E3 ligase: oxidative stress sensing by a Cul3-Keap1 ligase. *Mol. Cell. Biol. 24*, 8477−8486.
(74) Zhang, D. D., Lo, S. C., Cross, J. V., Templeton, D. J., and Hannink, M. (2004) Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex. *Mol. Cell. Biol. 24*, 10941−10953.
(75) Furukawa, M., and Xiong, Y. (2005) BTB protein Keap1 targets antioxidant transcription factor Nrf2 for ubiquitination by the Cullin 3-Roc1 ligase. *Mol. Cell. Biol. 25*, 162−171.
(76) van den Heuvel, S. (2004) Protein degradation: CUL-3 and BTB−Partners in proteolysis. *Curr. Biol. 14*, R59−R61.
(77) Zhang, D. D., Lo, S. C., Sun, Z., Habib, G. M., Lieberman, M. W., and Hannink, M. (2005) Ubiquitination of Keap1, a BTB-Kelch substrate adaptor protein for Cul3, targets Keap1 for degradation by a proteasome-independent pathway. *J. Biol. Chem. 280*, 30091−30099.
(78) Karapetian, R. N., Evstafieva, A. G., Abaeva, I. S., Chichkova, N. V., Filonov, G. S., Rubtsov, Y. P., Sukhacheva, E. A., Melnikov, S. V., Schneider, U., Wanker, E. E., and Vartapetian, A. B. (2005) Nuclear oncoprotein prothymosin α is a partner of Keap1: implications for expression of oxidative stress-protecting genes. *Mol. Cell. Biol. 25*, 1089−1099.
(79) Piñeiro, A., Cordero, O. J., and Nogueira, M. (2000) Fifteen years of prothymosin alpha: Contradictory past and new horizons. *Peptides 21*, 1433−1446.
(80) Fabbro, M., and Henderson, B. R. (2003) Regulation of tumor suppressors by nuclear-cytoplasmic shuttling. *Exp. Cell Res. 282*, 59−69.
(81) Kudo, N., Matsumori, N., Taoka, H., Fujiwara, D., Schreiner, E. P., Wolff, B., Yoshida, M., and Horinouchi, S. (1999) Leptomycin B inactivates CRM1/Exportin1 by covalent modification at a cysteine residue in the central conserved region. *Proc. Natl. Acad. Sci. U.S.A. 96*, 9112−9117.
(82) Velichkova, M., and Hasson, T. (2005) Keap1 regulates the oxidation-sensitive shuttling of Nrf2 in to and out of the nucleus via a Crm1-dependent nuclear export mechanism. *Mol. Cell. Biol. 25*, 4501−4513.
(83) Nguyen, T., Sherratt, P. J., Nioi, P., Yang, C. S., and Pickett, C. B. (2005) Nfr2 controls constitutive and inducible expression of ARE-driven genes through a dynamic pathway involving nucleo-cytoplasmic shuttling by Keap1. *J. Biol. Chem. 280*, 32485−32492.
(84) Strachan, G. D., Morgan, K. L., Otis, L. L., Caltagarone, J., Gittis, A., Bowser, R., and Jordan-Sciutto, K. L. (2004) Fetal Alz-50 clone 1 interacts with the human orthologue of the Kelch-like Ech-associated protein. *Biochemistry 43*, 12113−12122.
(85) Velichkova, M., Guttman, J., Warren, C., Eng, L., Kline, K., Vogl, A. W., and Hasson, T. (2002) A human homologue of Drosophila kelch associates with myosin-VIIa in specialized adhesion junctions. *Cell Motil. Cytoskeleton 51*, 147−164.
(86) Velichkova, M., and Hasson, T. (2003) Keap1 in adhesion complexes. *Cell Motil. Cytoskeleton 56*, 109−119.
(87) Wakabayashi, N., Itoh, K., Wakabayashi, J., Motohashi, H., Noda, S., Takahashi, S., Imakado, S., Kotsuji, T., Otsuka, F., Roop, D. R., Harada, T., Engel, J. D., and Yamamoto, M. (2003) Keap1-null mutation leads to postnatal lethality due to constitutive Nrf2 activation. *Nat. Genet. 35*, 238−245.
(88) Yamamoto, T., Kobayashi, A., Wakabayashi, J., Motohashi, H., and Yamamoto, M. (2005) In vivo analysis of Keap1 functional domains using transgenic complementation rescue approach. *Proc. Mol. Mech. Environ. Response Food Oxygen*, p 46, Tsukuba, Japan.
(89) Kwak, M. K., Ramos-Gomez, M., Wakabayashi, N., and Kensler, T. W. (2004) Chemoprevention by 1,2-dithiole-3-thiones through induction of NQO1 and other phase 2 enzymes. *Methods Enzymol. 382*, 414−423.
(90) Okawa, H., Motohashi, H., Kobayashi, A., Aburatani, A., Kensler, T. W., and Yamamoto, M. (2005) Hepatocyte-specific disruption of *keap1* gene confers potent resistance against acute drug toxicity. *Biochem. Biophys. Res. Commun. 339*, 79−88.
(91) Ansher, S. S., Dolan, P., and Bueding, E. (1983) Chemoprotective effects of two dithiolthiones and of butylhydroxyanisole against carbon tetrachloride and acetaminophen toxicity. *Hepatology 3*, 932−935.
(92) Devling, T. W., Lindsay, C. D., McLellan, L. I., McMahon, M., and Hayes, J. D. (2005) Utility of siRNA against Keap1 as a strategy to stimulate a cancer chemopreventive phenotype. *Proc. Natl. Acad. Sci. U.S.A. 102*, 7280−7285.

TX050217C