Chemistry of the Cysteine Sensors in Kelch-Like ECH-Associated Protein 1

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Abstract

The protein Kelch-like ECH-associated protein 1 (Keap1) is a cysteine-rich regulatory and scaffold protein. Human Keap1 contains 27 cysteines. Some of these cysteines are believed to mediate derepression of the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2), which subsequently upregulates phase 2 enzymes, in response to electrophilic/oxidative assault. Some current models depict a highly select group of two and possibly a few more cysteine residues as key sensors. The assumptions and approaches undergirding these models are commented upon. The chemical reactivity of the cysteines of Keap1 toward an array of electrophiles and one oxidant is reviewed. A number of reports in the recent literature of molecules that putatively modify cysteines of Keap1 are also included. Insights into the current molecular basis of electrophile/oxidant activation of the Nrf2 pathway *via* reaction at cysteines of Keap1 are discussed. Finally, important knowns and unknowns are summarized. *Antioxid. Redox Signal.* 13, 1749–1761.

Introduction

THE PROTEIN KELCH-LIKE ECH-ASSOCIATED PROTEIN 1 (Keap1) plays a central role in the compartmentalization and trafficking of nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a transcription factor that mediates upregulation of the expression of a large array of cytoprotective enzymes in response to electrophilic and oxidative assaults (6, 26, 57). Keap1 is a ~70-kDa protein that is proposed to function as a homodimer (7, 40, 58, 65), binds an equivalent of Zn²⁺ per subunit, and contains a number of functional domains, as indicated by the green linear schematic at the bottom of Figure 1 (3, 7, 21, 22). These domains include the bric-a-brac, tram track, broad complex (BTB) dimerization region, toward the N-terminus; the Kelch repeat domain, which has been demonstrated to bind a peptide fragment from the Neh2 (Kelch binding domain of Nrf2) (40) and is also an actin-binding domain; and the central linker region (CLR) that conjoins the former two domains. Keap1 facilitates ubiquitylation of Nrf2, targeting it for proteasomal degradation by serving as an adapter protein for cullin 3 (Cul3) protein, an E3 ubiquitin ligase (11, 32, 64). The Cul3 binding domain of Keap1 spans adjacent regions of the BTB and CLR domains. Keap1 contains a nuclear export signal, originating in the C-terminal end of the CLR, which is hypothesized to assist in the escort of Nrf2 from the nucleus (56).

There is a divergence of opinion about the detailed mechanism and manner of the derepression of Nrf2 by Keap1 (15, 33, 42). Human Keap1 contains 27 cysteine residues, some of which are thought to act as sensors of electrophilic/oxidative assault (8). A number of reports attest to a general stabilization of Nrf2 as a result of electrophilic/oxidative assault (15, 33, 42). Attendant with this is the formation of high-molecular-weight forms of Keap1 (HMWK) (49, 63), which are ubiquitylated and possibly oxidatively crosslinked, and in some cases, a decrease in levels of Keap1 generally and an increase in nuclear localization of Nrf2. There are also reports indicating a comigration of Keap1 and Nrf2 to the nucleus under stress, whereas this is not observed under other conditions (16, 42, 59). Keap1 has been proposed to tether Nrf2 to actin filaments in the cytoplasm from where it may be released upon electrophilic/oxidative assault if it is not first ubiquitylated and transported to the proteasome (24, 60). It has also been reported that Nrf2 ubiquitylation by Keap1 occurs in the nucleus and it is the disruption of this complex in the nucleus by electrophiles/oxidants that leads to phase 2 enzyme upregulation (42). There are reports that some electrophiles/oxidants mediate dissociation of Nrf2 from Keap1 (35, 44), whereas others report that electrophiles induce Cul3 dissociation from Keap1, thereby preventing Nrf2 ubiquitylation (9, 12, 49). Others suggest that Keap1-bound cytoplasmic Nrf2 is irretrievably destined for destruction (60).

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FIG. 1. Cysteine reactivity map of Keap1. Domains of Keap1 and other functional elements are described on the green linear map at the bottom where *small sequence numbers* define the domain limits and the *horizontal dashed line* below indicates the nuclear export sequence. *Large vertical bold numbers* across the bottom and top are numbers for the cysteine residues. Numbers of the cysteine residues are indicated at the bottom and top of the (main) central portion; *vertical dashed lines* are for clarity. Classes of electrophiles are defined in the leftmost column (see text). Each electrophile is identified with a number, second left column, corresponding to the structures in Figure 3. A single compound about which there is more than one report is so identified by its number and an additional letter. References for

each compound are reported in the text. Reactivity is denoted by color with the most reactive, intermediate reactive, and less reactive indicated by cherry, azure, and canary, respectively. Cysteines unreactive toward a given electrophile are indicated by the absence of a symbol in the row for that electrophile. Assignment of reactivity order is described in Ref. (17). *Solid circles* indicate cysteine residues of human Keap1; *shaded circles* are for experiments with mouse Keap1. Gray symbols indicate detectable reactivity but no basis for assigning relative reactivity (see text). For S-S bis-glutathione disulfide (class E), *solid circles* represent type 1 (Pr-cys-SG) disulfides (see text), while *connectors* indicate type 2 (Protein cys-cys) disulfides formed from the residues connected. The *bold horizontal lines* separate the electrophiles/oxidant into chemical classes (see text, Discussion section). Keap1, Kelch-like ECH-associated protein 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

The general Figure 2 is presented not as representation of established/agreed facts, but rather as generally not inconsistent with most of the above models to make a point relevant to a main thrust of this review. Active Keap1 is represented in Figure 2 as a rectangle. It may bind to Nrf2 on its own, or as a part of a ubiquitylating complex, and when it is fully assembled as an Nrf2-loaded ubiquitylating complex, it can actively target Nrf2 for destruction. As such, there are multiple points at which electrophiles/oxidants might react with the cysteine residues of Keap1 (red lightning bolts in Fig. 2), leading to its inactivation—in the form of the trapezoidal Keap1 (Fig. 2). The exact nature of the inactivated “trapezoidal” Keap1 is not elaborated here—it may be ubiquitylated or have many partners and may or may not release Nrf2, but its inactivation leads to a net “stabilization” of Nrf2, which can then proceed to replace repressor Bach1 and stimulate phase 2 enzyme induction (Fig. 2). Figure 2 is entirely general in that it may be placed in the context of the cytoplasm or the nucleus or may straddle these two locations. The main point to bear in mind is that the form of Keap1 that actually acts as a sensor of environmental stress is presently unknown and the possibility exists that multiple forms of Keap1 complexes may act as detectors.

The purpose of this review is to set forth what is known about the chemistry of the cysteines of Keap1 as possible sensors of electrophilic/oxidative assault and to examine recent reports of agents purported to react with the cysteines of

Keap1. At present, there are some relatively detailed notions of the involvement of a very select group of cysteine residues as transducers of electrophilic/oxidative stress and it is important to understand the limitations of the approaches on which these models are based. Thus, we will proceed with a critique of the bases of some current models, with an intent toward taking a broader view of what may be the cysteine sensors of Keap1.

A Critique Concerning Conclusions Based on Site-Directed Mutagenesis and Related Approaches

The current thinking about the key cysteine residues of Keap1 that mediate the upregulation of phase 2 enzymes by Nrf2 is anchored in the experimental approach of site-directed mutagenesis. The preponderance of literature reports using this approach entails measurement of enzyme or message expression in cell cultures transfected with, and over-expressing protein from, plasmids containing the Keap1 gene with one or a few mutations at cysteine(s) that convert this residue to ala or ser. A molecule that has been demonstrated to be a phase 2 enzyme inducer in wild-type (WT) cells and that fails to induce enzymes in the mutant-expressing cells has been taken, inappropriately (*vide infra*), as a *prima facie* evidence that the cysteine that was mutated is an electrophile/oxidant target that mediates the signal transduction. Mainly, on the basis of the above approach, the field is focused

FIG. 2. General scheme depicting protein interactivity in the Keap1-Nrf2 system. Active Keap1 is indicated as a rectangle. Assembly into the active Nrf2 ubiquitylating complex involving Cul3, rbx, and Nrf2 may take place by any of the routes indicated by curved arrows. Deactivation of Keap1, indicated by the red lightning bolts, yields the trapezoidal Keap1 and may occur at any of several complexes, or with free Keap1. The nature of trapezoidal Keap1 and its associations is not specified other than its formation leads to the depression of Nrf2 that can subsequently upregulate phase 2 enzyme induction at the ARE. Associated small Maf proteins are not indicated. Activities summarized by this figure may transpire in the cytoplasm, in the nucleus, or in a combination of the two. ARE, antioxidant response element; Cul3, cullin 3; ER, endoplasmic reticulum; Keap1, Kelch-like ECH-associated protein 1; Nrf2, nuclear factor (erythroid-derived 2)-like 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

on cys151, cys273, and cys288, though very recently this focus has been further narrowed (*vide infra*).

A fundamental requirement of the site-directed mutagenesis approach is that an attempt be made to determine that the single-point mutation introduced causes no or minimal “detectable” alteration in protein structure so that the mere absence of the amino acid functional group replaced, as opposed to some general structural alteration, can be ascribed as causal to the observed altered activity measurement. In the absence of the determination of minimal structural alteration, conclusions from such experiments can be based on little more than faith, the enemy of knowledge.

The current literature concerning site-directed mutagenesis approaches toward identifying cysteine residues important for phase 2 enzyme upregulation is devoid of any structural characterization of the mutant Keap1 proteins generated, with a single exception that should give considerable pause. Recently, a partial circular dichroism spectrum of molar ellipticity against wavelength for WT Keap1 and the C151S mutant was published in an attempt to measure differences in structural changes in response to the alkylator biotinylated iodoacetamide (IAB) (49). Unremarked upon, but of special interest here, are the two spectra at $t = 0$ [Fig. 3 in Ref. (49)], which demonstrate a marked change in the molar ellipticity spectrum of the C151S mutant. Changes in this region of the spectrum typically reflect alterations in α -helical or β -sheet

content and possibly tertiary structure. This significant change means that the failure of a C151S mutant to respond to a particular electrophile by upregulation of phase 2 enzymes may be due to (1) the possibility that cys151 is required for signal transduction, (2) the possibility that the observed conformational alterations alter accessibility of other reactive cysteine residues that mediate signal transduction of that electrophile, or (3) the possibility that the observed conformational alterations alter the ability of the protein to transduce the signal from a particular electrophile/oxidant, independent of the cysteine at which it reacts. None of the above is any more probable than the other. It has been clearly established that, at least in transfection experiments, the C151S mutant is capable of repressing upregulation of antioxidant response element (ARE)-mediated gene expression by Nrf2 and ubiquitylating Nrf2, but none of this eliminates the uncertainties of the three competing interpretations outlined above (49, 62).

The preponderance of the mutagenesis literature on which current thinking about key sensor cysteines is based involves the above approach—transfection of cultured cells, overexpression of mutant Keap1, and electrophile/oxidant assault—however, the validity of the conclusions from a number of these “in transfecto” experiments have recently been indicted by a leading contributor in the field (33, 62). An alternative *in vivo* approach, employing transgenic (Tg) complementation rescue in mice, involves creation of strains of mice in which the Keap1^{-/-} genotype is crossed with mice bearing mutant Keap1 proteins and analysis of gene expression in tissues derived from the mice. In contrast to conclusions of earlier “in transfecto” experiments (24, 63), deletion of the BTB domain in Keap1 is lethal, with mice dying at 3 weeks and analysis of the messenger ribonucleic acid (mRNA) from the forestomachs revealing that the deletion mutant fails to repress NAD(P)H dehydrogenase [quinone] 1 (NQO1) transcription, unlike WT Keap1. Other “in transfecto” experiments demonstrated that loss of Keap1 repression of Nrf2 is imbued by C273A (containing normal C288) or C288A (containing normal C273) mutations, whereas double transfections with plasmids of both mutants demonstrated significant recovery of repression (58). This observation of “complementation” led to a model in which intermolecular interactions, within the Keap1 dimer, between C273 of one molecule and C288 of the other molecule, were essential for Keap1 function. Consistent with the inactivity of the single-mutant proteins above, mice containing either Tg complementation for C273A or C288A failed to survive or repress Nrf2-mediated gene expression. However, in contrast to the “in transfecto” results with cells expressing both mutant proteins, mice resulting from double Tg complementations, expressing both C273A and C288A proteins, failed to survive and failed to repress expression of NQO1 implying the absolute requirement for both cys273 and cys288 in a single Keap1 monomer to accomplish Nrf2 repression (62). A second *in vivo*, as opposed to “in transfecto,” Tg rescue approach contradicts one of the above Tg complementation results—C288S mutant Keap1 fails to repress Nrf2-mediated expression of *gstp* (33). This has led to a recent model in which C151 and C273 are the chief sensors of most electrophiles with some other undetermined sensors residing in other proteins or other forms of Keap1-containing complexes (33).

The Tg complementation results and “in transfecto” experiments are not uniformly discordant—the C151S actively

represses Nrf2—but the frank lack of concordance in some cases has led to the suggestion that “in transfecto” approaches may be misleading, undercutting the validity of conclusions so derived (9, 33, 49, 62). It was opined that overexpression in cells in culture enables proteins to “overcome functional impairment” (33, 62). It was further suggested that cells in culture are oxidatively stressed compared with most *in vivo* environments and that they may thus be already activated for cytoprotective enzyme expression to some degree. The latter at least is an important general consideration. However, how those factors explain the experimental facts of, for example, loss of function of C288A mutants, or, recovery of function by coexpression of both C273A and C288A in “in transfecto” regimens was not addressed.

Finally, even the interpretation of some of the Tg complementation experimental approaches appears problematic (62). It was recently concluded that C151 is the key sensor for *tert*-butylhydroquinone (tBHQ)-initiated Nrf2-mediated phase 2 enzyme induction based on experiments with a Keap1^{-/-}TgC151S. This conclusion is at odds with the experimental observations. It is an experimental fact [Fig. 9C, lanes 7 and 8 in Ref. (62)] that the Tg mouse Keap1^{-/-}TgC151S exhibits nuclear accumulation of Nrf2 in response to tBHQ. It is a further experimental fact [Fig. 9D, rightmost pair of bars in Ref. (62)] that tBHQ more than doubles the expression of NQO1 mRNA. This enhancement, on a percentage basis, is larger than that observed in the case of the Keap1^{-/-}TgKeap1, containing the WT rescue. From these facts, the conclusion is inescapable that tBHQ-initiated nuclear accumulation and message induction are independent of C151.

It is true that, upon addition of tBHQ, there is less total nuclear accumulation of Nrf2 with Keap1^{-/-}TgC151S than with Keap1^{-/-}TgKeap1, and there is less total basal NQO1 message and less total tBHQ-inducible NQO1 message in Keap1^{-/-}TgC151S. There is also less Keap1 expression in the Keap1^{-/-}TgC151S [Fig. 9B, compare lanes 5/6 to 7/8 in Ref. (62)]. Whether there is some feedback between Keap1 and Nrf2 levels that may be at the root of the differences in absolute amounts of the measured quantities is uncertain. However, the facts of both appreciable tBHQ-stimulated Nrf2 accumulation and a larger fold induction of NQO1 message expression in the Keap1^{-/-}TgC151S leads unavoidably to the conclusion italicized in the preceding paragraph. Consistent with this is the observation that tBHQ initiates destruction of C151S mutant Keap1 (44), which has been shown to be concurrent with nuclear accumulation of Nrf2. We shall have more to say about the important, but not exclusive, sensor C151 in a later section.

Agents Reacting with Cysteines of Keap1

Figure 3 summarizes the structures of molecules that have been demonstrated, through the development of peptide maps using liquid chromatography/mass spectrometry, to react with specific cysteine residues of Keap1. The reactive molecules have been grouped into classes on the basis of the differences in the types of chemical reactions that they undergo. These include class A, simple second-order nucleophilic substitution (S_N2) alkylating agents (5, 8, 20, 37, 49); class B, a bifunctional molecule containing an S_N2 alkylator, the epoxide, and a Michael acceptor (4); class C, the pure Michael acceptors (15, 33, 42); class D, the thiocarbamoylating

agent sulforaphane (19); and class E, the oxidant S-S bis-glutathione disulfide (GSSG) (17). It is noted that in the case of 3, the exact structure is not certain (4). The likely reactive electrophilic atoms of each molecule are labeled with an asterisk in Figure 3. In all but one case, reactions of these electrophiles have been carried out with purified Keap1, so target cysteines of Keap1 engaged in the multiprotein complexes depicted in Figure 2 are unknown (but see the caveat below).

In most cases, by varying electrophile/oxidant concentration or measuring the frequency in multiple trials with which a specific residue was modified, it has been possible to assign a spectrum of reactivity of the different cysteines and this is summarized in Figure 1 (17). At the bottom of Figure 1 is a green linear schematic of Keap1 with the various domains identified. The vertically displayed numbers above are those of the sequence numbers of cysteine residues of human Keap1. The various classes and compounds are identified in the leftmost columns of Figure 1. Reactivity of an electrophile/oxidant with a particular cysteine is indicated by a circle above the residue number. The three reactivity groups that represent high, intermediate, and lower reactivity are represented by the colors cherry, azure, and canary, respectively. The absence of a circle indicates no reaction was detected.

Figure 1 indicates some differences between different groups of investigators studying the same electrophile: compare 1a (5) and 1b (37) (Fig. 1), the data for compound 1 in two reports; and compare 2a (20), 2b (8), and 2c (49), the data for compound 2 in three reports. The first report of the reaction of mouse Keap1 with any electrophile involved compound 1 (Fig. 1, row 1a) and identified five most reactive cysteines. A series of associations, which have been discussed (17), led to the conclusion that the four cysteines in the CLR were the key electrophile sensors. Subsequent experiments failed to validate the same selectivity (37) (see row 1b in Fig. 1) and the claim that these modifications initiated Nrf2 dissociation (8). In the case of compound 2, initial disagreement of the first two reports (rows 2a and 2b in Fig. 1) was found to revolve around a methodological problem in the initial method and a subsequent study (row 2c in Fig. 1) established reasonable agreement with the second study.

A general conclusion from Figure 1 is that there appear to be cysteines that are reactive toward a range of electrophiles/oxidants across the entire Keap1 primary sequence, though there may be regions of higher reactivity toward different types of electrophiles. The two classes of electrophiles that comprise the largest numbers of experiments are the class A (S_N2) and class C (Michael acceptors) reagents. We arbitrarily assigned numerical values to the colors in Figure 1 (3 for cherry, 2 for azure, and 1 for canary) and generated a “weighted frequency” by summing the values from all the experiments for each cysteine in its reaction to the two types of agents. The resulting plots are summarized in Figure 4. They appear to indeed indicate some significant differences in the limited dataset. These class A agents generally tend to be unreactive to the Kelch repeat domain while tending to target the CLR. At the same time, the class C agents seem to more evenly react across the length of Keap1 with a clear preference for cys151 and a predilection as well for the Kelch repeat domain and cys613.

Unique among the agents in Figures 1 and 3 is 14, GSSG, which was demonstrated to form glutathionylated cysteines

FIG. 3. Structures of the electrophiles demonstrated to react with specific cysteine residues of Kelch-like ECH-associated protein 1. Structure numbers correspond to the numbers in Figure 1. Classes indicate electrophiles/oxidants with common electrophilic chemical functionalities. Asterisks indicate atoms at which nucleophilic cysteines may attach.

(type 1 disulfides; solid circles in Fig. 1), as well as initiating formation of cysteine–cysteine protein disulfides (type 2 disulfides), indicated as connectors in Figure 1, the termini of which identify the cysteines crosslinked (17). Oxidative assault can give rise to a temporary imbalance in the reduced glutathione (GSH)/GSSG redox couple and has been demonstrated to generate protein disulfides and glutathionylated proteins (13, 29–31, 47, 53). The modification of Keap1 was studied in a range of ratios of GSH/GSSG that span the physiologically relevant oxidation potential. The cysteine reactivities in the case of the reaction with GSSG were based on the largest ratio of GSH/GSSG at which modifications were observed—all of those listed in Figure 1 are within the range of physiologically relevant oxidation potential. The most readily formed type 2 disulfides are notable. The cys319–cys319 crosslink represents a homodimeric crosslink that is far from the BTB “dimerization” domain, suggesting that there is considerable conformational flexibility during oxidative assault. The cys257–cys297 occurs in the CLR, which has been the focus of numerous models that attempt to explain derepression of Nrf2 by electrophilic assault on Keap1. Both of these disulfides about the nuclear export signal that has been proposed to assist nuclear exportation of Nrf2 by Keap1. Finally, the cys23–cys38 disulfide involves a cysteine for which a mutant, C23Y, was recently discovered in breast cancer cells (43). This mutant is unable to mediate Nrf2 ubiquitylation, so

the transient formation of a cys23–cys38 might possibly serve as control mechanism for Nrf2 stabilization. The other modifications have been discussed in further detail (17), and we reserve some discussion of those in the Kelch repeat domain for a later section. In sum though, this reagent, like most of the others in Figure 1, targets cysteine residues across the Keap1 sequence.

The initial report summarizing a limited set of reactive cysteines, while temptingly simple, appears incomplete in the context of all of the data in Figure 1. A legitimate criticism of Figure 1 is that most of the data derive from reactions with purified protein, and Figure 2 emphasizes that there may be substantial complexity in the biologically relevant forms of Keap1 that undergo electrophile/oxidant modification. These forms could have significantly altered reactive cysteine profiles. The single entry, row 2c (49), derived from reactions in cells overexpressing FLAG (the polypeptide ^NDYKDDDDK^C-tagged Keap1 seems to concur with the general conclusion from Figure 1—cysteine residues spanning the sequence, most certainly not grouped exclusively in the CLR, are reactive toward this electrophile.

Finally, a caveat needs to be included regarding care that is needed in thinking more broadly on the basis of Figure 1. Although all these agents covalently modify Keap1, it is not necessarily the case that the mechanism by which they upregulate phase 2 enzymes involves the formation of these

FIG. 4. Plot of weighted electrophile/oxidant modification frequencies as a function of cysteine residue position for reactions. (A) Class A electrophiles; (B) class C electrophiles.

adducts. For example, a number of these agents, **10** and **11**, and the well-known isothiocyanate sulforaphane, **13**, are known to stimulate the formation of reactive oxygen species (ROS) that could act as the agents that ultimately modify Keap1 and lead to enzyme induction (23, 28, 45, 54, 55). Such a mechanism has recently been generalized for cancer chemopreventive dithiolethiones (18).

Agents That Putatively Act on Cysteines of Keap1

Class A compounds

Myrosinase-catalyzed hydrolysis of vinyl glucosinolates generates epithionitriles (**15**) in addition to the better known isothiocyanates (**25**) (Fig. 5). These would appear to be a novel example of a naturally derived S_N2 agent. A number of these compounds upregulate cytoprotective enzymes in cell cul-

ture, and they induce enzyme expression through the ARE, as indicated by reporter assays. There is no effect of these agents in Keap^{-/-} cells, which already express high levels of NQO1. Both WT Keap1 or zebrafish Keap1a knock-ins show diminished NQO1 expression and are inducible by **15**. The zebrafish Keap1a lacks a cys at the site analogous to human/mouse cys273, so it is argued that this is not a target that signals electrophilic assault. A functional cys could be in the intervening region, or elsewhere of course. It may be of great interest to locate the key sensors as these agents have plant-derived chemopreventive potential.

Likely/possible class C compounds

All of the compounds **16–25** (1, 12, 34, 38, 39, 46, 51, 52, 61) have been demonstrated to induce reporter gene constructs with cis-acting ARE sequences, in some cases stimulate nuclear localization of Nrf2, and in a number of cases upregulate protein and/or message levels of phase 2 enzymes.

Compounds **17** and **18** (12), derived from the oxidation of omega-3 fatty acids, cinnamaldehyde and cinnamic acid derivatives, **19** (61), zerumbone, **23** (46), perillaldehyde, **24** (39), all derived from plant extracts, and the oxidized extracts of lycopene **25** (38) all contain α - β unsaturated carbonyls, the classic Michael acceptor by which they might interact with Keap1 cys residues. Direct interaction of some compounds **17/18** with Keap1 is affirmed by the change in circular dichroism spectrum of Keap1 when presented with mixtures of **17/18**. Pull-down of Cul 3, from COS-7 (African green monkey kidney cell line) cells overexpressing Keap1 and tagged Cul3 that were treated with **17/18**, and analysis by Western blots demonstrates significantly less Keap1 associated with Cul3 in drug-treated, compared with untreated, cells. Oxidized omega-3 fatty acid eicosapentaenoic acid (EPA) competes with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) for Keap1 cys and it has been suggested that this is consistent with the original (5) disulfide closure mechanism of Keap1-mediated phase 2 enzyme induction. This is highly unlikely if structures such as **17** and **18** are the activators, but it is unknown whether oxidants that could mediate disulfide closure are also components of the oxidized mixtures. Direct binding of **23** to Keap1 has been detected in extracts of cells passed through a column containing covalently bound **23**, subsequent to alkaline elution. Both free **23** and *N*-ethylmaleimide (NEM) compete with binding to the solid matrix. The ability of the aldehyde **24** to enhance expression of an ARE-luciferase (*luc*) construct is inhibited by overexpression of WT Keap1 but not by R272A/K287A or C273A/C288A Keap1 mutants. The former mutant is presumed to deactivate the adjacent cys residues at each site by elevating their pK_a values, whereas the nucleophilicity of the latter mutants is ablated at the indicated positions. On this basis it is suggested to affirm the original (5), now discarded, mechanism of signal transduction by Keap1. The caveats (*vide supra*) to these types of analyses apply and a significant range of compounds now establish (Fig. 1) that residues cys273 and cys288 have no exalted affinity for Michael acceptors—though it cannot strictly be excluded that these sites are transducers in these particular cases.

Compounds **16** (34) and **20–22** (1, 51, 52) have been included in the class C because they have the potential, in their oxidized quinone forms, to act as Michael acceptors. It must

FIG. 5. Structures of the electrophiles that putatively react with specific cysteine residues of Kelch-like ECH-associated protein 1. Classes are grouped with designation identical with Figure 3. In the case of new classes, see text.

be born in mind that structures of this type, like the prototypical tBHQ, also have the potential to engage in redox cycling, thus creating ROS, such as hydrogen peroxide that could act upon the cysteine residues of Keap1. The ability of compounds **16** to induce luciferase from an ARE-luc reporter construct correlates linearly with E_{HOMO} , a measure of the ease of their oxidation. On the basis of a slightly better correlation with the inclusion of a parameter describing their Van der Waals volumes, it has been argued that mechanism of induction likely involves covalent attachment of the oxidized forms of compounds **16** to Keap1. This, rather than reaction of intermediary ROS with Keap1, might be expected to encounter some steric limitations. However, adducts of oxidized **16** with Keap1 have not been directly detected, and it is often the case that proliferation of variable parameters in a fitting routine will improve the correlation without a direct causal implication. Similarly, the potency of inducers **21** and **22** (1, 52) correlates linearly with their potential to undergo one electron oxidation, and although this is discussed ultimately

in terms of reaction of quinones with Keap1, the relative impact of redox cycling as a competing mechanism is unknown. The binding of presumably oxidized (quinone form) biotinylated **20** (the biotin being attached at the carboxylic acid functionality) to Keap1 has been investigated with deletion mutants of Keap1. Cells overexpressing deletion mutant hemagglutinin (HA)-tagged Keap1 proteins containing deletions— ΔBTB or ΔCLR or ΔDGR domains—were treated with biotinylated **20** followed by streptavidin immunoprecipitation and Western blotting with anti-HA. No signal was observed for the ΔBTB Keap1, whereas an intermediate and darker band was detected for the ΔIVR and ΔDGR Keap1 proteins, respectively. The absence of signal from the ΔBTB mutant may indicate an “absolute requirement” for the BTB domain as claimed, but then the observation of an intermediate signal for the ΔIVR mutant requires the conclusion that this mutant, at least, undergoes a substantial structural alteration that diminishes the reactivity of the remaining BTB domain. Uniformly, the possible major structural changes in

the large deletion mutants and the impact that this might have on reactivity of the cysteines remaining in the protein are not considered.

Class D compounds

Many compounds of the structure **26** are known activators of the Nrf2 pathway and more have been reported recently (14, 27, 48). The compound 4-(3-isothiocyanatopropyl)morpholine demonstrated upregulation of heme oxygenase (HO-1) protein, NQO1, and 1A1 form of uridine diphosphate glucuronyltransferase (UGT1A1). This compound causes nuclear accumulation of Nrf2 and a decrease in Keap1 levels. Direct interaction with Keap1, unlike sulforaphane, has not been demonstrated, although it seems reasonable based on the precedent. Study of the morpholino derivative demonstrates a decrease, followed by a subsequent reestablishment, of GSH levels as has been seen with isocyanates in general. The activity of compounds of this structure is affected by cellular GSH levels, although interpretation of these types of experiments is problematic. The possible role of ROS as a mediator deserves some emphasis.

Class E compounds

Admittedly there is some arbitrariness on our part in grouping these compounds in a single class, but they likely act as agents, or through agents, that accrue with oxidative stress and inflammation. Lipoic acid, **27**, acts, albeit at approximately millimolar concentrations, to increase glutathione peroxidase, glutathione reductase, and NQO1 and elicits a decrease in cellular Keap1 and nuclear accumulation of Nrf2 (10). Although **27** could presumably act to close a cysteine disulfide switch on Keap1, the mechanism of action on Keap1 is unknown.

Nitric oxide (NO), **28**, has been shown to upregulate cytoprotective enzymes. The NO donors **29** and **30** have been shown to oxidatively modify HA-Keap1 in HEK293H cells by using a biotin switch assay (2). This assay involves cell lysis, subsequent to drug treatment, with media containing NEM, which blocks all remaining free thiols, followed by treatment with DTT. This reductant is powerful enough to expose free cysteines from cysSNO, cys-cys, and cysSOH moieties. Subsequent treatment with a biotinylated NEM reagent traps these remaining cys permitting Keap1 pull-down and identification with horseradish peroxidase (HRP)-linked avidin. Compound **30** was the most active in modifying Keap1 and treatment with **30** was accompanied by nuclear accumulation of both Nrf2 and Keap1, with qualitatively similar kinetics, while cytosolic Keap1 was dissipated. Direct treatment of cells with NO leads to upregulation of HO-1 and NQO1 and likely nitrosylated cysteine of Keap1 (36). The biotin switch assay used in this study employed the much weaker reductant ascorbate that does not reduce disulfides.

Class F compounds

These compounds are grouped together only in so far as they contain elements below the third row of the periodic table. Ebselen, **31**, has variously been reported as a phase 2 enzyme inducer that acts through the Nrf2 pathway (50). It has been shown to decrease basal ROS and the flux induced by 4-hydroxynonenal. It oxidizes thiols and protein thiols,

making Keap1 a presumptive target. Ebselen causes nuclear localization of Nrf2 and stimulates the formation of an HMWK that is associated with activation of Nrf2. Cells overexpressing Keap1 mutants C273S or C288S form HMWK on treatment with ebselen, whereas mutant C151S does not. This establishes that C151 is necessary for ebselen-stimulated formation of HMWK, and presumably Nrf2 activation, but it does not establish that it is sufficient for activation and does not require that C151 is covalently modified by ebselen, in contrast to subsequent claims (33). The metals **32–35** all act to induce through the Nrf2 pathway. Arsenic trioxide, **32**, is claimed to act directly on Keap1 as opposed to acting through the generation of ROS (41). Although **32** stimulates an increase in dihydroethidine fluorescence, diminution of the fluorescence by various antioxidants does not alter NQO1 induction by **32**. Arsenite, **33**, and methylarsonous acid, **34**, activate transcription from an ARE-*luc* construct, **34** being the more potent (59). Both **33** and **34** stabilize Nrf2 without an accompanying increase in Nrf2 mRNA. Neither compound appears to cause marked dissociation of the Keap1/Nrf2 complex, like sulforaphane and tBHQ. An additional difference is that **33** and **34** are able to block Keap1-mediated ubiquitination even with the Keap1 mutant C151S, indicating that C151 is not required for Nrf2 activation by **33** and **34**. The cysteine residues of Keap1 that are targeted by **33** and **34**, or any messengers derived therefrom, are unknown. The cadmium salt **35** increases levels of NQO1 in Nrf2^{+/+} cells but not in Nrf2^{-/-} cells (16). There is, in Nrf2^{+/+} cells, a stabilization of Nrf2 upon treatment with **35**, and an increase in both cytoplasmic and nuclear Keap1/Nrf2 complex in pull-down experiments. It is claimed that the complex migrates as such to the nucleus and subsequently dissociates, but the mechanism of this process, the involvement of cysteines of Keap1, and the possible role of ROS that are known to be generated by cadmium salts are unclear. Similar results have been obtained for arsenic and chromium. The avidity of these metals for thiols is well documented.

Molecular Basis of Keap1 Cysteine-Mediated Activation

Despite the large amount of study of the reactivity of Keap1 cysteines and mutant Keap1 proteins with electrophiles, there is surprisingly little definitive that is known about which are crucial for the initiation of Nrf2-mediated gene upregulation. In part this is because of an unstated notion on the part of many that only a very few of the 27 cysteines in human Keap1 are the “key” sensors of oxidative/electrophilic assault. This certainly derives from the original report of four “highly reactive” cysteines in the IVR, to the exclusion of the fifth equally reactive distant residue, cys613, in the C-terminal region, and the fabrication of a model involving a pair of cysteine disulfide closures by cysteines 257, 273, 288, and 297 (5). The illusory logic upon which this model was based has been discussed (17). In any case, with time, the focus has shifted dramatically, a most recent model dispenses with three of these four cysteine residues, retaining only cys273 and one other, cys151, as the “key” sensors for most electrophiles (33). So, while the identity of the players has shifted, the focus has narrowed. It is posited here that this focus is overly narrow and ignores the broader perspective from which one should consider the problem. Over evolutionary time, human Keap1 retains 27 cysteines. Although a number of these may be

engaged in structurally critical disulfide linkages and so are unresponsive to electrophile/oxidant assault, a significant number of the likely remaining cysteines are relatively more reactive to the electrophiles/oxidants that have been tested (Fig. 1, cherry and azure circles). Among the 14 experiments to date, there is but a single example in which one and only one cysteine is claimed to react with an electrophile (4), and a single other example in which only two appear to react (33). Although many of the experiments summarized in Figure 1 utilize purified Keap1 protein, and we have emphasized that the nature and association status of Keap1 as an electrophile/oxidant target is unknown (Fig. 2), analysis of cysteine reactivity in cultured cells suggests a similar reactivity picture—multiple approximately equi-reactive cysteines across the length of the protein (49). These considerations argue against a narrow focus on one or two reactive residues. The multiplicity of manifestly reactive cysteines (Fig. 1) probably derives from the complex role of Keap1 as a scaffold for a multiprotein complex, the range of chemical reactivity of the electrophiles/oxidants against which the Nrf2 transcriptional system has evolved to defend, and the multiple functions that electrophile/oxidant-modified cysteines must mediate to manifest stabilization of Nrf2 in the most biologically economical way. There is evidence suggestive of this even in the case of the single elaborated case of a key sensor cysteine in human Keap1, as described below.

Two pieces of evidence point to cys151 as a key sensor. cys151 in WT Keap1 is the target of a wide range of phase 2 enzyme inducers (Fig. 1). Additionally, it has been recently observed that an increase in the partial molar volume of amino acid side chains at position 151 of mutant human Keap1 proteins correlates with the level of constitutive activity of an ARE reporter construct in cultured cells and correlates inversely with the ability of the mutant protein to ubiquitylate Nrf2 (9). Increase in size alone at position 151 is sufficient for Nrf2 stabilization and so it is more than reasonable to presume that formation of suitably sized covalent adducts of cys151 is sufficient for Nrf2 stabilization and gene upregulation in these systems.

A number of pieces of evidence suggest, or frankly require, that other sensors must exist. Even in the study establishing the importance of size at position 151 (9), it was a demonstrated fact [Fig. 1B in Ref. (9)] that a range of electrophiles activate an ARE-luc reporter construct in cells overexpressing mutant Keap1 C151S. The activation is less than that in cells expressing only WT Keap1, but generally it is only a factor of 2 less. This requires that there are other cysteine residues that can respond appropriately to electrophile assault. Recall too the caveat to experiments with mutant proteins—they may respond less efficiently because of structural alterations; in this case, known alterations (*vide supra*) that have nothing whatever to do with the chemical reactivity of cys151. It has been demonstrated that C151S mutant undergoes destruction much more rapidly than WT Keap1 in cells treated with tBHQ and this decrease in Keap1 attends Nrf2 stabilization (44). Experiments with the alkylating inducer IAB demonstrate that a Cul3 pull-down and probe for Keap1 shows qualitatively more Keap1 bound to Cul3 than would be expected on the basis of the rate constants measured for Keap1 cys151 reaction with IAB in cells—if simple adduction at cys151 is all that is required for Cul3 dissociation (49). This, and other results, suggests that under these experimental conditions the

formation of adducts at cys151 may be necessary but not sufficient, requiring the reactions of other cysteine residues, for Nrf2 stabilization. Surprisingly, in the same study (49), the mutant C151S forms massive amounts of HMWK, a form that is generally considered to attend Nrf2 stabilization. Finally, a number of inducers are known to lack the requirement for cys151 (33, 44, 59). The evidence above emphasizes caution regarding the “cult of personality” of cys151 as the exclusive sentinel of electrophilic/oxidant assault.

It is essential to bear in mind the data summarized in Figure 1 that indicate that, for any given electrophile, with a single exception, modification of any purported “key” cysteine sensor is very likely contemporaneous with modification of a cadre of other cysteines of similar reactivity in the population of Keap1 molecules in the cell.

In silico evidence suggests that other adducts could engender major changes in Keap1 structure that might alter its binding properties (17). It was demonstrated that cys368 and cys434, in the Kelch repeat domain that is the binding interface with Nrf2, undergo glutathionylation in *in vitro* experiments in which the GSH/GSSG ratio was varied over a physiologically relevant range of oxidation potential. The potential structural consequences of these modifications on the Kelch repeat domain, the crystal structure of which was recently solved, were elaborated. The docking of a glutathionyl moiety first to cys434, which lies near the Keap1–Neh2 domain interface, was carried out followed by energy minimizations. The process was repeated with glutathione moieties docked to both cys434 and cys368, which lies in the core of the Kelch repeat domain barrel, followed by energy minimizations. Figure 6 indicates that the energy-minimized structures for both modifications exhibit a marked contraction of the binding domain of Keap1 for Neh2. This contraction appears to be enforced by a hydrogen-bonding network involving the glutathionyl moiety at cys434 and a number of residues that are known to be essential for Neh2 binding. It is possible that under oxidative assault and transient disturbance of the cellular GSH/GSSG redox couple, glutathionylation of Keap1 at cys434 and cys368 could result in Nrf2 release or prevent Keap1 association with, or ubiquitylation of, Nrf2. Interestingly, the redox cycling agent tBHQ is reported to elicit the release of Nrf2-bound Keap1 (44). The above mechanism, and obviously alternatives, could effect this release.

Finally, there are a small amount of data that demonstrate that Keap1 does undergo structural alterations upon reaction with electrophiles. The circular dichroism spectrum of Keap1 undergoes marked change upon reaction with the alkylator IAB, **2** (Fig. 3) (49) and Michael acceptors, **17** and **18** (Fig. 5) (12), derived from fatty acid oxidation, indicating detectable changes in secondary and tertiary structure. The parts of the protein from which these changes emanate are not known, but these electrophiles appear to effect dissociation of the Keap1–Cul3 complex.

Knowns and Unknowns

Knowns

1. For a broad range of electrophiles, Figure 1 summarizes that, with a single exception, these compounds target multiple essentially equi-reactive cysteines (cherry) as well as some that are slightly less reactive (azure). A

population of Keap1 molecules undergoing assault by these electrophiles will likely contain members modified at one or several of these sites. Simple alkylating agents (class A) appear to target the CLR (Fig. 3), whereas the largest group, Michael acceptors (class C),

target cysteine residues that span the entire length of the protein. Of these, cys151 is most heavily targeted, but there are an appreciable number of residues in the Kelch repeat domain that are quite sensitive. A single biologically relevant oxidant, GSSG, targets a number of pairs of cysteines for closure to disulfides at physiologically relevant oxidation potentials. Other cysteines undergo glutathionylation at the same or similar potentials.

2. The residue cys151 is a presumptive sensor. Experimental data establish that other cysteine residues also mediate the fate of Keap1 and phase 2 enzyme upregulation.

Unknowns

There are a number of important unknowns that hinder the development of a reasonable mechanism or set of mechanisms whereby electrophiles/oxidants elicit phase 2 enzyme induction through Keap1. Some of these apply specifically to modification of the cysteines of Keap1 and some of these apply to interpretation of experiments in the extant literature.

1. We often do not know the nature of the species that is modifying Keap1 in any moderately complex milieu. A number of the classes of compounds in Figures 3 and 5, particularly members of classes C, D, and F, can react through multiple modes including direct covalent modification and redox cycling, thereby generating ROS that may modify Keap1. A general caveat beyond this is that all electrophiles/oxidants have the capacity to shift the redox balance of the cell through reaction with GSH, which would thereby give rise to an oxidative burst that likely can modify Keap1 cysteines.
2. We do not know the forms of Keap1 upon which electrophile/oxidant assault will yield detectable phase 2 enzyme induction. This is emphasized by the multiple lightening bolts in Figure 2. Layered atop this uncertainty is whether these events are nuclear or cytoplasmic or both. Certainly, foundational progress in this area that can be made with purified proteins would be edifying, although this would need to be followed with particularly challenging analytical approaches in the cellular milieu.
3. We do not know which of the 27 cysteines are most important for sensing assault. cys151 is clearly impor-

FIG. 6. Structures of the Kelch repeat domain after energy minimization. (A) Minimized structure of unmodified Keap1 starting from the coordinates of the crystal structure in the protein data bank (PDB). (B) Minimized structure containing a type 1 disulfide at cys434, starting from the lowest energy structure of Keap1 docked to noncovalently bound GSH. (C) Minimized structure containing type 1 disulfides at cys434 and cys368. Orange indicates cys434 and cys368, the GSH fragment (B, C) is in red, green residues are those that have been shown to contact a peptide fragment of the Neh2 domain of Nrf2 in Keap1-peptide cocrystals, blue indicates his436, and mauve indicates gly364 and gly430. GSH, reduced glutathione; Keap1, Kelch-like ECH-associated protein 1; Neh2, Keap1-binding domain of Nrf2; Nrf2, nuclear factor (erythroid-derived 2)-like 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

tant, but the extant evidence requires that others exist. Within this unknown is the further obscurity of the possibility that specific cysteine modifications may have specific functional roles in the dismantling of Keap1 complexes (Fig. 2), or disfigurement that inhibits assembly/activity, or dispatch by ubiquitylation.

4. We do not know the extent to which cellular Keap1 must be disabled by a given electrophile/oxidant assault to give rise to sufficient Nrf2 stabilization to permit detectable phase 2 enzyme induction. This is highly important but little appreciated. Eggler and colleagues (9) demonstrated that cys151 mutant Keap1 proteins containing the bulkiest amino acids were much more active in an ARE-luc reporter assay than WT Keap1 treated with powerful inducers. This is, as indicated (9), a consequence of the likelihood that far <100% of the WT Keap1 molecules were modified by the inducer. Until the threshold is known for the extent of the population of Keap1 molecules that is required to be modified to elicit signal transduction, it is difficult to place much stock in the extensive literature using pull-down and Western blotting approaches to detect changes, or apparent lack thereof, in Keap1 interactions. In general, there is little attempt in this literature toward quantitation, and changes of $\pm 20\%$ are probably the limits of detection for changes and this may well bracket the threshold required for detectable enzyme induction, leading potentially to spurious conclusions.
5. We do not know the structural consequences either of modification of the cysteines of Keap1 by electrophiles/oxidants or the structural consequences of mutations of the cysteines of Keap1. In the former case we know that changes occur or that they are likely on the basis of computational methods. But a more detailed understanding will elucidate what is likely to be multiple mechanisms of Keap1 inactivation and the roles of various target cysteines in its disabling/dismantling/disassembly, in determining its multiple associations, and in its fate and/or functions. In the case of the mutants of Keap1 the use of site-directed mutagenesis approaches can have a powerful role in identifying target Keap1 cysteines, but definitive conclusions await structural confirmation that the mutations are structurally passive relative to WT. This appears not to be the case in the one instance in which it has been indirectly elaborated.

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Abbreviations Used

ARE = antioxidant response element
 BTB = bric-a-brac, tram track, broad complex
 CLR = central linker region
 COS = African green monkey kidney cell line
 Cul3 = cullin 3
 DTNB = 5,5'-dithiobis(2-nitrobenzoic acid)
 EPA = eicosapentaenoic acid
 FLAG = the polypeptide ^NDYKDDDDK^C
 GSH = reduced glutathione
 GSSG = S-S bis-glutathione disulfide
 HA = hemagglutinin
 HMWK = high-molecular-weight forms of Keap1
 HO-1 = heme oxygenase
 HRP = horseradish peroxidase
 IAB = biotinylated iodoacetamide
 Keap1 = Kelch-like ECH-associated protein 1
 luc = luciferase
 mRNA = messenger ribonucleic acid
 Neh2 = Keap1-binding domain of Nrf2
 NEM = N-ethylmaleimide
 NO = nitric oxide
 NQO1 = NAD(P)H dehydrogenase [quinone] 1
 Nrf2 = nuclear factor (erythroid-derived 2)-like 2
 PDB = protein data bank
 ROS = reactive oxygen species
 S_N2 = second-order nucleophilic substitution
 tBHQ = *tert*-butylhydroquinone
 Tg = transgenic
 UGT1A1 = 1A1 form of uridine diphosphate glucuronyltransferase
 WT = wild type
 Δ = deletion