Nrf2 (nuclear factor erythroid 2-related factor 2) is a transcription factor that activates transcription of a battery of cytoprotective genes by binding to the ARE (antioxidant response element). Nrf2 is repressed by the cysteine-rich Keap1 (kelch-like ECH-associated protein 1), which targets Nrf2 for ubiquitination and subsequent degradation by a Cul3 (cullin 3)-mediated ubiquitination complex. We find that modification of Cys151 of human Keap1, by mutation to a tryptophan, relieves the repression by Keap1 and allows activation of the ARE by Nrf2. The Keap1 C151W substitution has a decreased affinity for Cul3, and can no longer serve to target Nrf2 for ubiquitination, though it retains its affinity for Nrf2. A series of 12 mutant Keap1 proteins, each containing a different residue at position 151, was constructed to explore the chemistry required for this effect. The series reveals that the extent to which Keap1 loses the ability to target Nrf2 for degradation, and hence the ability to repress ARE activation, correlates well with the partial molar volume of the residue. Other physico-chemical properties do not appear to contribute significantly to the effect. Based on this finding, a structural model is proposed whereby large residues at position 151 cause steric clashes that lead to alteration of the Keap1–Cul3 interaction. This model has significant implications for how electrophiles which modify Cys151, disrupt the repressive function of Keap1.

Key words: antioxidant response element, cysteine, Keap1, Nrf2, partial molar volume, tryptophan.

INTRODUCTION

A promising strategy for prevention of numerous types of diseases is the induction of a battery of cytoprotective enzymes. These enzymes include NQO1 (NAD(P)H:quinone oxidoreductase 1) and GST (glutathione S-transferase), whose induction leads to detoxification and elimination of carcinogens, antioxidant enzymes, such as heme oxygenase-1, and enzymes that regulate the reducing environment of the cell, including the NADPH-regenerating enzyme, glucose 6-phosphate dehydrogenase. The regulatory regions for each of these inducible genes contain an ARE (antioxidant response element) [1], which is activated upon binding of the Nrf2 (nuclear factor erythroid 2-related factor 2) transcription factor protein. The key role of Nrf2 in the transcription of cytoprotective genes, and in disease prevention, has been illustrated in a rapidly increasing number of studies. Some of the disease states that are believed to be mitigated by Nrf2 activation include carcinogenesis, hepatotoxicity, neurodegenerative disorders, sepsis and pulmonary inflammatory diseases, as well as the aging process (reviewed in [2]). Nrf2 is activated by a wide range of molecules, herein termed ARE inducers. A large number of ARE inducers have been identified, and although no canonical structures have been found, most are electrophilic in nature and capable of modifying thiols, including cysteines [3]. Many ARE inducers have been discovered from plant-based sources, including commonly consumed foods [4]. For example, sulforaphane was first isolated from broccoli as a potent inducer of NQO1 in murine hepatoma cells [5], and has since been shown to be effective in animal models of cancer prevention [6–9]. Based on the large number of diseases that could be prevented or attenuated by Nrf2 activation, and the potential to activate Nrf2 through dietary changes, understanding the mechanism of its activation by ARE inducers is of great interest.

The mechanism of Nrf2 regulation and the responses of the regulatory system to ARE inducers are elaborated (reviewed in [4]). Briefly, under basal conditions, Nrf2 resides mainly in the cytoplasm and at low levels overall in the cell, primarily through the interaction of the N-terminal Neh2 domain (approximately the first 100 residues) of Nrf2 with Keap1 (kelch-like ECH-associated protein 1). Keap1 contains a Ccm1 (human exportin 1)-dependent nuclear export sequence, which prevents Nrf2 nuclear localization [10–12], otherwise mediated by a nuclear localization signal in Nrf2 [13]. In addition, Keap1 serves as a bridge between Nrf2 and the Cul3 (cullin 3)-based E3-ligase ubiquitination complex [14–16]. Although details of the overall stoichiometry of the Keap1/Nrf2/Cul3 complex are unknown, there are two binding sites in the Neh2 domain of Nrf2, termed the ETGE and DLG motifs, that each bind to separate Kelch-repeat domains in a Keap1 dimer [17,18]. The binding of each motif to a Kelch domain, proposed as the ‘two-site’ model, is required for ubiquitination of seven lysine residues located between the motifs, as shown by both site-directed mutagenesis experiments and somatic mutations in cancer patients [18,19]. This Nrf2 ubiquitination leads to rapid degradation of Nrf2 by the 26S proteasome and low basal levels of Nrf2 in the cell, contributing to low basal ARE activity.

Abbreviations used: 15d-PGJ2, 15d-deoxy-Δ12,14-prostaglandin J2; ARE, antioxidant response element; As(III), sodium arsenite; BIA, biotinylated iodoacetamide; CBD, chitin binding domain; CBDO-im, 1-[2-cyano-3-,12-dioxo-oleana-1,9(11)-dien-28-oyl]imidazole; Cu13, cullin 3; DTT, dithiothreitol; GSK-3β, glycogen synthase kinase 3 β; HA, haemagglutinin; Keap1, kelch-like ECH-associated protein 1; MMA(III), monomethylarsonous acid; Nrf2, nuclear factor erythroid 2-related factor 2; NOO1, NAD(P)H:quinone oxidoreductase 1; p38 MAPK, p38 mitogen-activated protein kinase; PMV, partial molar volume; tBHQ, tertiary-butyhydroquinone.

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Several phosphorylation events have also been shown to repress Nrf2 activation of the ARE under basal conditions, including phosphorylation of Nrf2 by GSK-3β (glycogen synthase kinase 3β), which leads to Nrf2 cytoplasmic localization [20], and phosphorylation of Nrf2 by p38 MAPK (p38 mitogen-activated protein kinase), which causes enhanced interaction with Keap1 [21].

Numerous mechanisms have been implicated in sensing electrophilic ARE inducers. These can be largely grouped into two categories, modulation of protein kinase pathways and modification of Keap1 cysteines. Whereas many protein kinases have been shown to play a role in Nrf2 activation, only a few of the mechanisms involved have been studied in detail. For example, the phosphatidylinositol 3-kinase pathway, activated by a variety of ARE inducers [22–24], was shown to lead to inactivation of GSK-3β and to Nrf2 nuclear localization [20]. In addition, sulforaphane was shown to inhibit MKK3/6 (mitogen activated protein kinase kinase 3/6), upstream of p38 MAPK, leading to decreased phosphorylation of Nrf2, and subsequent disruption of the Keap1–Nrf2 interaction [21]. However, other groups have found that activation of p38 MAPK, rather than inhibition, leads to ARE induction [25–27]. Phosphorylation of Ser40 in Nrf2 by PKC (protein kinase C) isoforms is also proposed to disrupt the Keap1–Nrf2 interaction [28], leading to Nrf2 activation [29].

Human Keap1 contains 27 cysteine residues, 25 of which are highly conserved among Keap1 homologues. The large number of cysteine residues in Keap1, and the electrophilic nature of the vast majority of the inducers, is suggestive of their importance in sensing ARE inducers. Three Keap1 mutants in particular, C151S, C273S and C288S, have phenotypes that suggest they play a role in sensing inducers. It was previously shown that cells overexpressing Keap1 C151S are much less responsive to ARE inducers tBHQ (tertiary butylhydroquinone) and sulforaphane when compared with wild-type Keap1, although in the absence of inducers, cells expressing the Keap1 C151S protein have the same phenotype as those expressing wild-type Keap1 [30]. Conversely, cells overexpressing Keap1 C228S or C273S have constitutive ARE activation in the absence of ARE inducers, when compared with wild-type Keap1, due to the fact that Nrf2 was no longer ubiquitinated or degraded [30]. More recently, transgenic expression of Keap1 C273A or Keap1 C288A protein in Keap1 null mice confirmed the inability of these Keap1 molecules to target Nrf2 for ubiquitination, whereas mice expressing Keap1 C151S were largely unresponsive to tBHQ, emphasizing the biological importance of Cys151 in sensing ARE inducers [31]. Using MS to detect modification of human Keap1 cysteines in vitro, we previously determined that, although these three cysteines are highly reactive, only Keap1 Cys151 was highly and consistently modified by the molecules tested, including BIA (bionylated iodoacetamide) [32] and BMCC (β-carbanilido-β-cyclohexylethylene-dicyclohexylcarboxamido) butane) [33], as well as the naturally produced ARE inducers xanthohumol from hops, isoquiritigenin from liquorice and 10-shogaol from ginger [33].

An early model suggested that modification of Keap1 cysteine residues by ARE inducers leads to dissociation of Keap1–Nrf2 [34,35]. However, subsequent studies both in vitro and in vivo [16,36,37] indicate the Keap1–Nrf2 interaction is not disrupted upon modification of Keap1 cysteines. In the proposed ‘two-site’ model, the lower affinity DLG site alone would be disrupted, maintaining a Keap1–Nrf2 interaction but preventing Nrf2 ubiquitination [18,38]. Alternatively, modification of Keap1 cysteine residues appears to decrease the interaction of Keap1 and Cul3, leading to downregulation of Nrf2 ubiquitination. Co-purification assays showed a decreased association between Keap1 and Cul3 in transiently transfected mammalian cells [16], and a similar result was observed in vitro using proteins purified from Escherichia coli [39]. In both studies, there was significantly less disruption of the interaction between the Keap1/C151S mutant and Cul3, compared with wild-type Keap1, implying that modification of Cys151 by an ARE inducer is important for the disruption. Cys151 is located in the BTB domain of Keap1, which is known to be important for the Keap1–Cul3 interaction [15,40]. However, it is unknown whether modification of other Keap1 cysteines, in addition to Cys151, is required to alter the Keap1–Cul3 interaction.

To attempt to mimic a modification of Cys151 by an ARE inducer, in this work we substituted Cys151 of Keap1 with the largest natural amino acid, tryptophan. We find that modification of Cys151 to a tryptophan does lead to ARE activation, by altering the Keap1–Cul3 interaction and downregulating Nrf2 ubiquitination. Twelve other amino acids were substituted at position 151 to explore the physico-chemical properties required at this position to alter Keap1’s ability to catalyse Nrf2 ubiquitination. The results offer an insight into how Cys151 modification by an electrophile might alter Keap1–Cul3 binding and hence Nrf2 ubiquitination.

**EXPERIMENTAL**

**Construction of recombinant DNA molecules**

Plasmids expressing wild-type Keap1, tagged with a CBD (chitin binding domain), in pcDNA3, or haemagglutinin (HA)-tagged Cul3 protein in the pCI vector, have been previously described in [16], along with the plasmids expressing HA–Nrf2 in the pCI vector and Gal4–Neh2 protein in pcDNA3 [30] and HA–ubiquitin in the pCI vector [41]. For expression of a non-tagged version of the Keap1 protein, the full-length Keap1 gene [36] was directionally cloned using PCR into the HindIII/Xhol sites of pcDNA3 (Invitrogen). Site-directed mutagenesis was conducted using standard oligo-directed mutagenesis techniques. The entire sequence of each gene in the expression plasmids was verified by dideoxy sequencing by the University of Illinois at Chicago DNA Sequencing Facility.

**Cell culture, transfections, and chemical reagents**

MDA-MB-231 (a human breast cancer cell line) cells were purchased from the ATCC. Cells were maintained in Eagle’s minimal essential medium in the presence of 10% fetal bovine serum. Plasmid DNA transfections were performed with Lipofectamine Plus (Invitrogen), and the CDDO-Im \{1-[2-cyano-3,12-dioxo-oleana-1,9(11)-dien-28-yl]imidazole\} was a gift from Michael Sporn (Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, NH 03755, U.S.A.). 15d-PGJ2 (15d-deoxy-Δ12,14-prostaglandin J2) was obtained from Biomol. DMSO was obtained from Fisher Scientific.

**Reporter gene assays**

The ARE TATA-Inr luciferase reporter plasmid, pARE-Luc, and the Renilla luciferase expression plasmid, pRL-TK, have been previously described in [30,42]. MDA-MB-231 cells, grown on 24-well plates, were transfected with 100 ng of pARE-Luc, 10 ng of pRL-TK reporter plasmid, 100 ng of the Nrf2 expression plasmid, and 50 ng of either the wild-type or mutant Keap1 expression plasmid. The total amount of DNA was maintained at 260 ng with pcDNA3. Both firefly and Renilla luciferase activities were measured 24 h after transfection with the dual luciferase reporter assay system (Promega). Firefly luciferase activity was...
normalized to Renilla luciferase activity to control for sample-to-sample variations in transfection efficiency.

Antibodies, immunoblot analysis and co-immunoprecipitation assays

Primary antibodies against tubulin, Nrf2 and Keap1, and horseradish peroxidase-coupled secondary antibodies, were purchased from Santa Cruz Biotechnology. An antibody against the HA epitope was purchased from Covance.

For detection of protein expression in total cell lysates, cells in 24-well plates were transfected with expression vectors for Nrf2 (100 ng) and either wild-type or mutant Keap1 (50 ng). Cells were washed with 1× PBS and lysed in M-PER buffer (Thermo Scientific) supplemented with Complete Protease Inhibitor Mix (Roche) at 24 h post-transfection. For co-immunoprecipitation assays, cells in 35 mm dishes were transfected with expression vectors for HA–Cul3 (500 ng) and either wild-type or mutant Keap1–CBD (500 ng). Cells were washed with 1× PBS and lysed in M-PER buffer supplemented with Complete Protease Inhibitor Mix, 1 mM DTT (dithiothreitol) and 150 mM NaCl 48 h post-transfection. After centrifugation, cell lysate supernatants were incubated with chitin beads (New England Biolabs) for 4 h at 4°C. Protein complexes on the beads were washed once with M-PER buffer and twice with wash buffer (20 mM Tris/HCl, pH 8.0, 500 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, 1 mM DTT and Complete Protease Inhibitor Mix). Samples were analysed by SDS/PAGE (10% gels), transferred to PVDF membranes, and immunoblotted with the appropriate antibody.

Ubiquitination of Nrf2 in cells

For detection of ubiquitinated Nrf2 in vivo, cells in 35 mm dishes were transfected with expression vectors for HA–ubiquitin (200 ng), HA–Cul3 (100 ng), Gal4–Neh2 (400 ng) and either the wild-type or mutant Keap1 (133 ng). After 48 h, the transfected cells were exposed to 10 μM MG132 (Boston Biochem) for 3 h. Cells were washed with 1× PBS and lysed by boiling in a buffer containing 20 mM Tris/HCl, pH 7.6, 137 mM NaCl, 2% SDS (w/v) and 10 mM N-ethylmaleimide. This procedure inactivates cellular ubiquitin hydrolases, preserving ubiquitin–Nrf2 conjugates. Lysates were incubated on ice to precipitate the SDS, then centrifuged, and supernatants were diluted 5-fold in buffer containing 20 mM Tris/HCl, pH 7.6, 137 mM NaCl and 1% Triton X-100 (v/v). After pre-clearing with A/G beads (Santa Cruz Biotechnology), lysates were incubated with anti-Gal4 antibodies for one hour at 4°C, followed by a 2 h incubation at 4°C with A/G beads. Beads were washed once with buffer containing 20 mM Tris/HCl, pH 7.6, 137 mM NaCl and 0.5 M LiCl, and twice in the same buffer without LiCl. Immunoprecipitated protein was analysed by SDS/PAGE (4–12% gradient gels), transferred to PVDF membranes, and immunoblotted with the anti-HA antibody.

RESULTS

ARE activation by potentially therapeutic ARE inducers is dependent on Keap1 Cys151

Keap1 Cys151 has been shown to be important for ARE activation by several ARE inducers, including sulforaphane (Figure 1A) [30]. We set out to recapitulate this result and to further establish the relevance of Cys151 in sensing other ARE inducers of potential clinical relevance, the Cys151 dependence of ARE activation by CDDO-Im was determined (Figure 1A). Activation of Nrf2 by CDDO-Im has shown promise in prevention or attenuation of disease in several mouse and rat models, including cigarette smoke-induced emphysema [43], acute inflammatory liver injury [44], aflatoxin-induced tumorigenesis [45], and sepsis-induced lethality [46], as well as a preclinical ex vivo study on the LPS (lipopolysaccharide)-induced inflammatory response in sepsis [47]. We also included in the study 15d-PGJ2, a lipid oxidation product that accumulates during acute inflammation. This endogenous molecule has come to be appreciated recently as an electrophilic mediator of cellular signalling [48]. 15d-PGJ2 has been shown to induce nuclear accumulation of Nrf2 [49], and has been shown to bind to native Keap1 in vivo in rat
Substitution of Cys\textsuperscript{151} with a bulky side chain is sufficient for ARE activation

On the basis that various small molecule inducers depend on Keap1 Cys\textsuperscript{151} in order to fully activate the ARE, and the high reactivity of Cys\textsuperscript{151} in response to various ARE inducers \cite{32,33}, it was of interest to see if the sole modification of Cys\textsuperscript{151} is sufficient to activate the ARE response. Due to the large number of cysteine residues in human Keap1, mutation of all 27 cysteines except Cys\textsuperscript{151} was impractical. Furthermore, mutation of Cys\textsuperscript{28} and Cys\textsuperscript{232} to serine or alanine residues results in constitutive ARE activation, by downregulating Nrf2 ubiquitination \cite{30,37}. Therefore we chose to mutate Cys\textsuperscript{151} to the amino acid with the largest partial molar volume (PMV), tryptophan (Figure 2A), in the hope of mimicking sterically the modification of Cys\textsuperscript{151} by electrophilic molecules. The ARE assay in MDA-MB-231 cells was used to determine the level of ARE activation in cells expressing the Keap1 C151W mutant protein. Remarkably, the level of ARE activation by Nrf2 in the presence of Keap1 C151W was substantially higher than that obtained in the presence of wild-type Keap1 (Figure 2B). This result indicates that a tryptophan at position 151 does indeed mimic electrophile modification in that it causes Keap1 to lose its ability to repress Nrf2. As shown in Figure 2B, the effect of the tryptophan mutation is in stark contrast to the effect of the serine mutation, which had a level of repression similar to wild-type Keap1. Interestingly, the level of ARE activation in cells transfected with Keap1 C151W was approximately 5-fold higher than that obtained in cells transfected with wild-type Keap1 and treated with the potent ARE inducer sulforaphane.

The high level of ARE activation in the presence of Keap1 C151W, compared with that seen using sulforaphane, suggests that negative feedback pathways may be activated by sulforaphane that attenuate the amount of ARE activation possible. To test this, cells expressing the Keap1 C151W protein and Nrf2 were treated with sulforaphane. No attenuation of the level of ARE activation was observed (Figure 2B), indicating that negative feedback pathways are not activated by sulforaphane under these conditions.

Modification of Cys\textsuperscript{151} to a tryptophan leads to ARE activation by increasing Nrf2 protein levels, through destabilization of the Keap1–Cul3 interaction and decreased Nrf2 ubiquitination

Previous work comparing the wild-type Keap1 protein with the Keap1 C151S protein showed that Cys\textsuperscript{151} participates in downregulating Nrf2 ubiquitination in response to sulforaphane or tBHQ in cells \cite{30}. The use of the C151S protein in this experiment \cite{30} meant that all cysteine residues in Keap1 except Cys\textsuperscript{151} were available for modification by the ARE inducers, raising the question as to whether modification of other cysteine residues was required for downregulation of Nrf2 ubiquitination. The Keap1 C151W protein allows this question to be addressed directly. The levels of Nrf2 protein and the extent of ubiquitination in the presence of Keap1 C151W were compared with those of wild-type Keap1 in MDA-MB-231 cells in the absence of ARE inducers (Figures 3A and 3B). Co-transfection of wild-type Keap1 and Nrf2 was observed to significantly decrease the level of Nrf2 protein as expected (Figure 3A). Transfection of Keap1 C151W, however, resulted in a level of Nrf2 that was similar to that observed in the absence of overexpressed Keap1. The level of Nrf2 observed in the presence of the Keap1 C151W protein was also compared with that found in the presence of Keap1 C288S. Keap1 protein containing serine or alanine substitutions for Cys\textsuperscript{288} have the opposite phenotype of the C151S protein, in that they are deficient in their ability to target Nrf2 for ubiquitination and to repress Nrf2-dependent gene expression in transfected cells in the absence of inducers \cite{30,52}. Nrf2 protein was stabilized in cells expressing Keap1 C288S, though not to the same extent as cells expressing Keap1 C151W (Figure 3A).

Next, the cellular ubiquitination of Nrf2 in the presence of wild-type or C151W Keap1 was examined. The assay was
Conducted using the Gal4–Neh2 construct. The Neh2 domain of Nrf2 contains the seven lysine residues that are targeted for ubiquitination by Keap1 [16]. MDA-MB-231 cells were transfected with expression vectors for Gal4–Neh2, HA–ubiquitin, Cul3 and either wild-type Keap1 or Keap1 C151W. Gal4–immunoprecipitated proteins were immunoblotted with anti-HA antibodies to determine ubiquitinated forms (Figure 3B). Wild-type Keap1 strongly catalysed the ubiquitination of Nrf2 lysine residues, as shown by the large increase in higher molecular weight species. Keap1 C151W was largely unable to catalyse Nrf2 ubiquitination, with levels similar to those seen in the absence of transfected Keap1.

The mechanism behind the ability of wild-type Keap1, but not Keap1 C151S, to downregulate Nrf2 ubiquitination in response to sulforaphane and tBHQ was shown in MDA-MB-231 cells to involve a decrease of the interaction between Keap1 and Cul3 [16]. A similar result was observed in vitro in a recent study using purified proteins from E. coli in a co-purification assay [39]. The Keap1 C151W protein offers an opportunity to determine whether modification of Cys151 alone leads to a decreased association of Keap1 and Cul3, or whether modification of other cysteine residues in addition to Cys151 by electrophiles is required. A CBD, fused to the C-terminus of Keap1 (Keap1–CBD), was used to enable Keap1 purification, as it is known that the CBD does not alter the ability of Keap1 to associate with either Cul3 or Nrf2, or change the responsiveness of Keap1 to ARE inducers [16]. Expression vectors for Keap1–CBD and HA–Cul3 were transfected into MDA-MB-231 cells, and Keap1 was purified by use of chitin beads. Cul3 co-purified with wild-type Keap1, as shown in Figure 3C. The amount of Cul3 co-purified with Keap1 C151W was observed to be significantly less, indicating that modification of Cys151 alone is sufficient to alter the Keap1–Cul3 interaction. The extent of interaction between Keap1 C151S and Cul3 was determined as a control, and confirms that only modification to tryptophan, and not serine, decreases the interaction.

It is possible that the Keap1 C151W can no longer catalyse Nrf2 ubiquitination due to a disruption of the Keap1–Nrf2 interaction. It seemed unlikely, as we have previously shown using isothermal titration calorimetry that modification of Keap1 Cys151 by BIA does not alter the affinity of Keap1 for Nrf2 [36]. However, to ensure that Keap1 C151W is properly folded and functional within the cell, we examined the ability of Keap1 C151W to bind to Nrf2. The same method described above to detect changes in the cell, we examined the ability of Keap1 C151W to bind to Nrf2. The same method described above to detect changes

Activation of the ARE is dependent on the PMV of the residue at position 151 of the Keap1 sequence

The loss of the ability of Keap1 to repress ARE activation caused by the introduction of a tryptophan at position Cys151 prompted us to examine the effects of introducing residues with different physico-chemical properties at this position on ARE activation. A series of site-directed mutants were therefore created to span the various properties of naturally occurring amino acids such as charge, PMV and hydrophobicity. In addition to serine and tryptophan, eleven mutations were introduced at position Cys151, and the effect on ARE activation was determined (Figure 4A). All of the mutant Keap1 proteins were more effective than the Keap1 C151W protein at repression of ARE-dependent reporter gene activity. The highest repression was seen with the residues of smallest PMV, Asn, Cys, Ser and Asp, and the lowest repression with those of highest PMV, Tyr and Trp. This trend indicates that the PMV at position 151 may be a major determinant of the extent of ARE repression. To further investigate this correlation, ARE activity was plotted against the PMV of each amino acid residue [53], calculated at 310 K, the temperature at which the cells were maintained during the experiment. A good correlation was found between the ARE activity and the PMV of the residue occupying position 151 (Figure 4B). Other physico-chemical properties such as charge or hydrophobicity do not appear to contribute significantly to the extent of ARE activation. For example, the Keap1 C151F mutant had a somewhat
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The PMV of the residue at position 151 in Keap1 determines the extent to which the Keap1 protein can catalyse Nrf2 ubiquitination

The studies above support the idea that repression of ARE activation correlates with the PMV of the residue at position 151 of Keap1, with a tryptophan showing the least repression (Figure 4). The low level of repression by Keap1 C151W observed is due to the inability of the protein to efficiently catalyse the ubiquitination of Nrf2 lysine residues (Figure 3B). In order to verify that the ability of the other Keap1 Cys[151] mutants to catalyse Nrf2 ubiquitination also correlates with the PMV of the residue at position 151, a subset of the thirteen mutants was selected for analysis. In addition to C151W, Keap1 C151D, C151H and C151Y (Figure 5A) were chosen as representative of the spectrum of PMV of the side-chains. Parallel experiments were performed where one experiment determined the extent of ubiquitination of Nrf2 lysine residues as described for Figure 3(B), while the second experiment simultaneously determined the extent of ARE activity associated with co-transfection of each Keap1 mutant along with Nrf2. As shown in Figure 5(B), the ARE repression associated with these Keap1 mutants decreases steadily as the PMV of the residue increases. Correspondingly, the extent of ubiquitination of Nrf2 lysine residues catalysed by Keap1 steadily decreases as the PMV of the Keap1 Cys[151] residue increases (Figure 5C), indicating that the PMV of the residue at position 151 is a major determinant of the ability of Keap1 to catalyse Nrf2 ubiquitination.

DISCUSSION

We find that modification of human Keap1 Cys[151] by mutation to a bulky residue is sufficient to downregulate Nrf2 ubiquitination and significantly activate the ARE in cells. The effect appears to be mediated by altering the interaction between Keap1 and the Cul3 protein, which targets Nrf2 for ubiquitination, as the Keap1 C151W mutation leads to a decrease in the extent of interaction between Keap1 and Cul3. Further, we find that the PMV of the residue at position 151 correlates well with the degree of ARE activation, and the loss of ability of the Keap1 protein to catalyse Nrf2 ubiquitination. No other physico-chemical properties appear to contribute significantly.

No disruption of the Keap1–Nrf2 interaction by the Keap1 C151W mutation is observed. However, it is possible that the C151W mutation alters the interaction of Keap1 with the Nrf2 DLG site, which would disrupt Nrf2 ubiquitination, as illustrated by the two-site model [18,38]. The tryptophan mutation in the Keap1 BTB dimerization domain could alter the conformation of the Keap1 dimer, thereby decreasing the interaction of the Keap1 Kelch domain with the lower affinity DLG binding site on Nrf2. Moreover, the ability of Keap1 C151W to bind to Nrf2 indicates that the C151W mutation causes a specific conformational change in the Keap1 protein affecting its interaction with Cul3, rather than causing a general inactive conformation of Keap1.

The importance of modification of Keap1 Cys[151] for Nrf2 activation agrees well with our previous MS studies of the human proteins, in which Cys151 was the only cysteine residue consistently and highly modified by a variety of ARE inducers [32,33]. In addition, during the preparation of this manuscript, Kobayashi et al. [54] showed in zebrafish embryos that expression of zebrafish Keap1 with a tryptophan mutation at the cysteine position corresponding to Cys[151] in humans leads to constitutive upregulation of an ARE-regulated gene in the absence of ARE inducers. Therefore, across species, modification of this key cysteine residue alone appears to be important and sufficient for ARE activation.

A series of mutations at position 151 of Keap1 reveals that a major contribution to the effects observed is the PMV of the residue at that position. To gain further insight as to how an increased PMV at position 151 could alter the interaction between Keap1 and Cul3, we examined a homology model of the interaction of the BTB domain of Keap1 with Cul3 (Figure 6). In earlier work, we had noted from the model that Cys[151] is not located at the predicted Keap1–Cul3 interface [36]. Keap1 residues 125–127 and 162–164, coloured in dark grey in Figure 6, are predicted to interact with Cul3 based on those required for association of MEI-26, a Caenorhabditis elegans BTB domain protein, with the C. elegans Cul3 protein [16,55]. Mutant Keap1 proteins containing alanine substitutions for either 125–127 or 162–164 had similar phenotypes to the Keap1 C151W mutant in that they were unable to catalyse ubiquitination of the Neh2 domain of Nrf2 [16]. In our earlier study, we noted that Cys[151] is close to the BTB homodimerization interface, and we predicted that modification of Keap1 Cys[151] would perturb the homodimerization interface, thereby disrupting Nrf2 ubiquitination [36]. While a bulky modification at position 151 may alter the protein conformation at the homodimerization interface, the model in Figure 6 instead indicates that a bulky...
Partial molar volume of residue 151 regulates Keap1 activity

Figure 5  Nrf2 ubiquitination catalysed by Keap1 Cys\textsuperscript{151} mutants of increasing PMV

(A) Structures of Cys, Asp, His, Tyr and Trp, shown as a side chain at position 151. (B) Transient transfection reporter gene assays were conducted essentially as described for Figure 1. (C) Gal4–Neh2 ubiquitination assays were performed essentially as described for Figure 3(B).

Figure 6  Stereo view of a model of the putative Keap1 BTB–Cul3 interaction interface and the location of residue 151 of Keap1

A portion of the modelled BTB domain of Keap1 is shown as a ribbon diagram, and a portion of the modelled Cul3 structure is shown in surface representation. Keap1 residues 125–127 and 162–164, in the putative Cul3 binding region, are shown in dark grey. Modification at that position would impose specific structural effects that would alter Keap1–Cul3 binding. In the model, substitution of a tryptophan residue at position 151 causes a significant steric clash of the tryptophan side chain with Lys\textsuperscript{131}. The Arg\textsuperscript{135} residue, depending on its orientation, is also close enough to clash with the tryptophan. These residues reside on an α-helix that forms an α-helix–β-strand–α-helix motif with the α-helix containing Cys\textsuperscript{151}. Importantly, residues Lys\textsuperscript{131} and Arg\textsuperscript{135} are conserved in sequences of Keap1 proteins from divergent species (Figure 7), suggesting their importance to Keap1 function. Interestingly, Lys\textsuperscript{131} is not conserved in the zebrafish Keap1 isoform (Keap1a) that has been shown to have an inactive cysteine at the position corresponding to 151 in human Keap1 [54]. However, Lys\textsuperscript{131} is conserved in the active Keap1b form. We hypothesize that a bulky residue at position 151 alters the interaction between these two helices via a steric clash with Lys\textsuperscript{131}, thereby forcing the α-helix containing Lys\textsuperscript{131} away from position 151. Movement of this helix would then affect the positioning of residues 125–127 relative to Cul3, altering Keap1–Cul3 binding. We predict the positioning of Tyr\textsuperscript{162} of the other binding site, which forms a hydrophobic interaction with Ile\textsuperscript{128}, would be altered as well.

The effect of increasing the PMV of a residue at a key position in a signalling protein has been observed in studies on rhodopsin [56], the photoreceptor molecule of the vertebrate retina and a member of the G-protein-coupled receptor superfamily [57,58]. In these studies, the effect of changing the PMV at position 125 was explored. The original L125R mutation showed impaired signal transduction and an inability to bind 11-cis-retinal [59]. A series of 10 site-directed mutations were introduced at position 125 to study which physico-chemical properties were required for the effect [56]. As we found for position 151 of Keap1, Andrés et al. [56] found that the PMV of the residue at position 125 of rhodopsin was the major determinant of the extent of the effect, with the
residues of greatest PMV showing the greatest impairment in both binding to 11-cis-retinal and downstream activation of transducin. In the case of rhodopsin, the mutations appear to cause a change in protein conformation since the thermal stability of the mutant proteins was reduced. Just as Cys\textsuperscript{151} is located away from the Keap1–Cul3 binding site, the crystal structure of rhodopsin [60] shows that Leu\textsuperscript{125} is located four \(\alpha\)-helix turns away from the residues directly involved in chromophore binding. The data indicate that mutation of Leu\textsuperscript{125} to a bulky residue does not affect the chromophore interaction directly, but rather alters the optimal conformation of the retinal binding pocket.

The linear trend observed in Figure 4, where residues of increasing PMV at Keap1 position 151 lead to a decrease in Keap1’s ability to repress Nrf2, would suggest that electrophiles that are larger than tryptophan would induce at least as much ARE activation as the tryptophan mutant, if not more, upon reacting with Cys\textsuperscript{151}. However, the extent of activation of the ARE by Nrf2 in the presence of Keap1 C151W is much higher than in the presence of wild-type Keap1 and the potent ARE inducers sulforaphane and CDDO-Im (Figures 1 and 2). Comparing the ARE activation by these molecules to that from other Cys\textsuperscript{151} mutations, the extent of activation is less than that observed for ten of the mutants, up to and including the Keap1 C151L protein (Figures 1B and 4A). Therefore, it seems unlikely that the physico-chemical properties of the mutant side chains at position 151 are more suited to cause an effect on the Keap1 protein than modification of Cys\textsuperscript{151} by an electrophile. A more probable explanation is that a significant population of Keap1 molecules adopt a conformation that does not cause a steric clash with Lys\textsuperscript{131} and possibly Arg\textsuperscript{135}, as shown in Figure 6.

One possibility is that 15d-PGJ\textsubscript{2}, when conjugated to Cys\textsuperscript{151}, adopts a conformation that does not cause a steric clash with Lys\textsuperscript{131} or Arg\textsuperscript{135}. Another explanation would be that 15d-PGJ\textsubscript{2} is simply much less reactive towards Cys\textsuperscript{151} than the majority of inducers whose ARE activation is Cys\textsuperscript{151}-dependent, including sulforaphane, CDDO-Im and tBHQ. In support of this possibility, Kobayashi et al. [54] did not detect modification of mouse Keap1 Cys\textsuperscript{151} in vitro by 15d-PGJ\textsubscript{2}, whereas the ARE inducer diethylmaleate was found to be both Cys\textsuperscript{151}-dependent and able to modify Cys\textsuperscript{151} in vitro. Further studies are required to determine whether the trend of Cys\textsuperscript{151} dependence in vivo, reflecting Cys\textsuperscript{151} modification in vitro, is consistent for a larger number of ARE inducers. The mechanism by which the highly reactive Keap1 Cys\textsuperscript{151} is excluded from reacting with electrophiles, such as 15d-PGJ\textsubscript{2}, in vitro is puzzling and of interest to investigate.

In conclusion, we hypothesize that modification of Cys\textsuperscript{151} by an electrophile of sufficient PMV alters the interaction of the modelled \(\alpha\)-helix–\(\beta\)-strand–\(\alpha\)-helix motif of Keap1 through steric clashes with Lys\textsuperscript{131} and possibly Arg\textsuperscript{135}, as shown in Figure 6. These steric clashes would then affect the positioning of the Keap1 residues that interact with Cul3, causing altered Keap1–Cul3 binding and decreased Nrf2 ubiquitination. The Keap1 C151W protein promises to be a useful tool to test effects of Keap1 Cys\textsuperscript{151} modification.

**AUTHOR CONTRIBUTION**

Aimee Egger, Mark Hannink and Andrew Mesecar designed the research. Aimee Egger performed the research. Aimee Egger, Mark Hannink, Evan Small and Andrew Mesecar analysed results and wrote the paper.
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