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Modification of Keap1 Cysteine Residues by Sulforaphane

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Abstract

Activation of the transcription factor NF-E2-related factor-2 (Nrf2) through modification of Kelch-like ECH-associated protein 1 (Keap1) cysteines, leading to up-regulation of the antioxidant response element (ARE), is an important mechanism of cellular defense against reactive oxygen species and xenobiotic electrophiles. Sulforaphane, occurring in cruciferous vegetables such as broccoli, is a potent natural ARE activator that functions by modifying Keap1 cysteine residues, but there are conflicting in vitro and in vivo data regarding which of these cysteine residues react. Although most biological data indicate that modification of C151 is essential for sulforaphane action, some recent studies using mass spectrometry have failed to identify C151 as a site of Keap1 sulforaphane reaction. We have reconciled these conflicting data using mass spectrometry with a revised sample preparation protocol and confirmed that C151 is indeed among the most readily modified cysteines of Keap1 by sulforaphane. Previous mass spectrometry-based studies used iodoacetamide during sample preparation to derivatize free cysteine sulfhydryl groups causing loss of sulforaphane from highly reactive and reversible cysteine residues on Keap1 including C151. By omitting iodoacetamide from the protocol and reducing sample preparation time, our mass spectrometry-based studies now confirm previous cell-based studies which showed that sulforaphane reacts with at least four cysteine residues of Keap1 including C151.

Keywords

Keap1; Nrf2; sulforaphane; chemoprevention

Introduction

The Kelch-like ECH-associated protein 1 (Keap1) and its binding partner, transcription factor NF-E2-related factor-2 (Nrf2), are under investigation as chemoprevention targets because of their roles in regulating the antioxidant response element (ARE) in response to oxidative stress and exposure to xenobiotic electrophiles (1). Up-regulation of the ARE causes cells to produce more cytoprotective enzymes such as NAD(P)H-quinone oxidoreductase 1, glutathione S-transferases and heme oxygenase-1. Under basal conditions, the concentration of Nrf2 is low due to constitutive ubiquitination by the Cullin3-based E3-ligase ubiquitination complex (Cul3) and then degradation; this results in low expression of ARE-driven genes (2). Keap1 serves as a bridge between Nrf2 and Cul3. Upon exposure of cells to ARE inducers, Nrf2 ubiquitination is inhibited, and Nrf2 accumulates in the nucleus

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where it dimerizes with a small Maf protein and binds to the ARE to stimulate cytoprotective gene transcription (3).

Although numerous ARE inducers have been identified, they share few structural similarities (4). However, Talalay and coworkers (5-7) have noted that the majority of ARE inducers are electrophilic and can react with cysteine sulfhydryl groups. Since Keap1 binds Nrf2 and contains 27 cysteine residues, Keap1 might be a regulatory sensor for the ARE pathway. Significant effort has been undertaken to identify the critical cysteines in Keap1 and their function. Kobayashi *et al.* (12) have categorized ARE inducers into six classes using a "cysteine code," which defines the preferential target cysteine(s) and distinct biological effects. Most ARE inducers belong to class 1 of the "cysteine code," where C151 is essential for activity.

Found in cruciferous vegetables such as broccoli, sulforaphane (R-1-isothiocyanato-4-methylsulfinylbutane) (Figure 1) has been investigated as a chemopreventive agent using cell culture, animal models and in clinical trials (13-18). Sulforaphane exerts its chemopreventive effects at least in part through the Nrf2-Keap1 signaling pathway with efficacy in the high nanomolar range (19-21). Kobayashi *et al.* (12) found that induction of ARE-regulated genes in zebrafish by sulforaphane is highly dependent on C151 of Keap1 and have categorized sulforaphane as a class 1 ARE inducer. This is consistent with Zhang *et al.* (22) who reported that NIH3T3 cells expressing Keap1 C151S were not responsive to sulforaphane. Recently, we investigated the physico-chemical requirements of modification at position 151 using a series of 12 amino acid substitutions (11). We found that an increased partial molar volume at that position is required and sufficient for Keap1 to lose the ability to repress Nrf2 and target it for ubiquitination in cells. Therefore, modification of Keap1 C151 by an electrophile, at least the size of tryptophan, appears to be sufficient for Nrf2 activation. This result was confirmed in the zebrafish animal model by Kobayashi *et al.* (12) using a mutation to tryptophan.

We have shown that C151 is among the most readily modified cysteine residues of Keap1 toward the model electrophile *N*-iodoacetyl-*N*-biotinylhexylenediamine (8) and the botanical natural products isoliquiritigenin, xanthohumol and 10-shogaol (9). Subsequently, HEK293 cells stably overexpressing Keap1 were used to confirm that C151 reacts with *N*-iodoacetyl-*N*-biotinylhexylenediamine in cells (10). However, LC-MS/MS measurements by Hong *et al.* (23) indicated that sulforaphane did not modify Keap1 C151, rather, it modified Keap1 primarily in the Kelch domain.

Based on these contradictory results regarding Keap1 C151 and sulforaphane, we investigated whether the apparent inability of C151 to react with sulforaphane, as reported by Hong *et al.* (23), was a methodological artifact due to the reversible binding of sulforaphane, an isothiocyanate, to thiols (Figure 1). To address this question, we compared the Keap1 modification pattern by sulforaphane in vitro using two sample preparation methods. One method is that used previously to map cysteines of Keap1 that form stable adducts with electrophiles such as *N*-iodoacetyl-*N*-biotinylhexylenediamine and the natural products xanthohumol, isoliquiritigenin and 10-shogaol (9,25) and includes derivatization of unreacted cysteine residues with iodoacetamide. The other method omits iodoacetamide treatment and is designed to account for the reversible nature of sulforaphane adducts. Our studies indicate that iodoacetamide treatment promotes iodoacetamide competition with sulphoraphane for formation of sulforaphane-cysteine adducts, especially adducts with C151 of Keap1. By eliminating the iodoacetamide treatment step and reducing sample preparation time, we show that C151 is detected as one of the four most readily modified cysteine residues in Keap1 by sulforaphane. These results are consistent with cell studies and in vivo findings.

Experimental Procedures

Modification of Keap1 by sulforaphane

Sulforaphane was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA), and recombinant human Keap1 was expressed and purified as described previously (8). Keap1 (10 μM) was incubated with sulforaphane at a molar ratio of 1:0.5, 1:1, 1:2, 1:5, or 1:10 (Keap1/sulforaphane) in 100 μL 20 mM Tris-HCl buffer (pH 8.0) for 2 h at room temperature. The reaction was quenched by adding 1 mM dithiothreitol (DTT), and the mixture was incubated for an additional 15 min. Samples were either analyzed immediately or (for molar ratios of 1:0.5 and 1:10 only) incubated with 3 mM iodoacetamide for 45 min in the dark followed by addition of 5 mM DTT to remove the excess iodoacetamide before analysis. Mass spectrometry grade trypsin (Promega; Madison, WI) was added to each sample at a trypsin/Keap1 ratio of 1:50 (w/w) and incubated at 37 °C for 1.5 h. The tryptic peptides were analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described below to determine sites of modification by sulforaphane.

Release of sulforaphane from Keap1-sulforaphane adducts

Human Keap1 (10 μM) was treated with sulforaphane at a molar ratio of 1:2 ([Keap1]/[sulforaphane]) in 100 μL 20 mM Tris-HCl buffer (pH 8.0) for 2 h at room temperature. Unbound sulforaphane was separated from the Keap1-sulforaphane adducts using a micro Bio-Spin 6 gel filtration column (Bio-Rad; Hercules, CA). The Keap1-sulforaphane adducts from the gel filtration column were diluted 20-fold with 20 mM Tris-HCl buffer (pH 8.0) containing 3 mM iodoacetamide to test the reversibility of sulforaphane modification of Keap1 or else with buffer containing no iodoacetamide and incubated for 24 h at room temperature. Aliquots of 10 μL each were removed from the incubations at 0.25, 0.5, 1, 2, 3, 4, 5, and 24 h to evaluate the reversibility of Keap1 adducts with sulforaphane. Each aliquot was mixed with 10 μL 500 nM naringenin in 30% methanol as an internal standard and analyzed immediately using LC-MS/MS. The total sulforaphane bound to Keap1 (100%) was determined by measuring the reduction in sulforaphane concentration during a 2 h incubation. The amount of sulforaphane released from Keap1 at each time point was calculated by comparing the amount of free sulforaphane present in the incubation solution with the total sulforaphane bound during the incubation.

DTT quenching time optimization

Human Keap1 (10 μM) was incubated with sulforaphane at a molar ratio of 1:2 ([Keap1]/[sulforaphane]) in 100 μL 20 mM Tris-HCl buffer (pH 8.0). The reaction was carried out at room temperature for 2 h and quenched by adding 1 mM DTT. To determine optimum quenching time while minimizing the loss of Keap1-sulforaphane adducts, sulforaphane concentration was monitored at 5, 15, 30, 45, and 60 min using LC-MS/MS as described above.

LC-MS/MS

Keap1 peptide digests were analyzed on a Thermo Finnigan (San Jose, CA) hybrid linear ion trap Fourier transform ion cyclotron resonance (LTQ-FT ICR) mass spectrometer equipped with a Dionex (Auburn, CA) microcapillary HPLC system. Reversed phase microcapillary HPLC was carried out using an Agilent Zorbax C_{18} column (3.5 μm, 75 μm i.d. × 150 mm) and LC Packings C_{18} PepMap precolumn cartridge (5 μm, 0.3 mm i.d. × 5 mm). The solvent system consisted of a 60 min linear gradient from 5 to 45% solvent B and then from 45 to 80% solvent B over 15 min (solvent A: 95:4.9:0.1; and solvent B: 4.9:95:0.1, water/acetonitrile/formic acid, v/v/v) at a flow rate of 250 nL/min. Positive ion electrospray tandem mass spectra were acquired in a data-dependent mode in which each MS scan was

followed by five MS/MS scans using a normalized collision energy of 35%. Dynamic exclusion was enabled to minimize redundant spectral acquisitions. All LC-MS/MS data were processed using two different search programs to improve confidence levels of identification, BioWorks 3.3.1 (Thermo Finnigan) based on the SEQUEST algorithm, and MassMatrix (26-29). The mass accuracy for precursor ions was set to 10 ppm with up to 2 missed cleavages allowed. Modification was permitted to allow for the detection of the following: methionine oxidation (+15.9949 Da), asparagine/glutamine deamidation (+0.9840 Da), cysteine carbamidomethylation (+57.0214 Da), and sulforaphane adducts (+177.0282 Da). Only peptides with modifications found in both search programs were nominated as potential matches, and the Keap1 modification sites were further validated by manual inspection of the tandem mass spectra. The X_{corr} and ΔC_n scores of the peptide matches from Bioworks were evaluated as described by Peng *et al.* (30), and pp, pp2 and pptag scores from MassMatrix were statistically significant with p values < 0.05 (26).

Sulforaphane released from Keap1-sulforaphane adducts was measured using positive ion electrospray tandem mass spectrometry with collision-induced dissociation and selected reaction monitoring on a Thermo Finnigan TSQ Quantum triple quadrupole mass spectrometer equipped with a Surveyor HPLC system. Sulforaphane and naringenin (internal standard) were separated using a YMC C_{18} reverse phase column (5 μm, 2.0 mm × 50 mm, 120Å) with a 6 min linear gradient from 30 to 100% methanol and a co-solvent of 0.1 % formic acid in water at a flow rate of 0.2 mL/min. Argon was used as the collision gas for collision-induced dissociation at 11 V for sulforaphane and 21 V for naringenin. During selected reaction monitoring, sulforaphane was measured using positive ion electrospray and the ion transition of *m/z* 178 to 114, and naringenin was measured using negative ion electrospray and the ion transition of *m/z* 271 to 151.

Results

Based on the lack of detection of Keap1 C151-sulforaphane adducts by Hong *et al.* (23) and the reversibility of such adducts, we wanted to ascertain if C151-sulforaphane adducts could be detected when adduct stability was maximized during sample preparation. The effect of iodoacetamide competition and sample processing time on the formation and stabilization of Keap1-sulforaphane adducts was assessed by removing unreacted sulforaphane from the reaction mixture using gel filtration chromatography followed by dilution into buffer or buffer containing 3 mM iodoacetamide. The decrease in Keap1-sulphoraphane adduct concentration by the dissociation of free sulphoraphane from the adduct was determined by the observation of an increase in free sulforaphane in the incubation buffer (Figure 2). The concentration of free sulforaphane increased steadily over time and reached a stable concentration after 3 h. At that time, 70% of the sulforaphane had been released from the Keap1-sulforaphane adducts indicating that equilibrium had apparently been reached between adduct formation and disappearance.

When iodoacetamide was added to the dilution buffer, the extent of adduct disappearance increased so that approximately 50% of sulforaphane was released during the first 30 min of reaction instead of only 25% as observed in the incubation without iodoacetamide. Iodoacetamide derivatization of free cysteines effectively blocked, i.e. competed against, free sulforaphane from reacting with Keap1. The loss of Keap1-sulforaphane adducts was 80% complete after 3 h, and no Keap1-sulforaphane adducts were present after 24 h (Figure 2).

To minimize the loss of Keap1-sulforaphane adducts during sample preparation, DTT quenching time was optimized as shown in Figure 3. More than 5 min but no longer than 15 min was needed to quench excess sulforaphane using DTT. By shortening the quenching

time from 30 min used in our previous protocol (9) to 15 min, by eliminating iodoacetamide treatment and by digesting with trypsin for 1.5 h (instead of up to 3 h), the total sample preparation time was reduced to less than 2 h.

We then examined the Keap1 modification pattern obtained using LC-MS/MS following this streamlined sample preparation procedure. During peptide mapping and sequencing using high resolution LC-MS/MS, the protein sequence coverage was at least 90%, and all 27 cysteines were detected and identified among the tryptic peptides. As expected, fewer modified cysteine residues were detected in Keap1-sulforaphane samples that had been treated with iodoacetamide than in samples prepared using the streamlined protocol (Table 1). Treatment with iodoacetamide also resulted in an apparently different modification pattern compared with samples that were untreated. Significantly, no labeling of C151 by sulforaphane was detected in samples treated with iodoacetamide; instead, C151 was observed to be derivatized only by iodoacetamide in these samples (Figure 4). However, in samples that were not treated with iodoacetamide, labeling of C151 was detected at both high (1:10) and low (1:0.5) ratios of Keap1 to sulforaphane (Figure 4). Sulforaphane labeling at C241, C273, C288, C319 in the central linker domain, C395, C406, C434 in the Kelch domain, and at C613, C622 and C624 in the C-terminal domain was also detected only in the samples that were not treated with iodoacetamide (Table 1).

In the iodoacetamide-treated Keap1 that had been incubated with sulforaphane at a ratio of 1:0.5, sulforaphane adducts were detected only at C38, C226, and C368 (Table 1). These three sites of sulforaphane binding were detected in all Keap1 samples incubated with sulforaphane regardless of iodoacetamide treatment or the ratio of Keap1 to sulforaphane. Sulforaphane adducts at C77 and C489 were detected in all samples except the lowest Keap1 to sulforaphane ratio (1:0.5) in iodoacetamide-treated samples. Several other sites of sulforaphane attachment including C23, C171, C196, C513, C518, and C583 were observed in both the iodoacetamide-treated and untreated samples but only at the highest sulforaphane to Keap1 ratio of 10:1.

To determine the relative reactivities of the 27 Keap1 cysteines towards sulforaphane, we used a titration-like process that included five ratios of sulforaphane to Keap1 ranging from 0.5:1 to 10:1 and no iodoacetamide treatment (Table 2). The number of modified Keap1 cysteines increased as the relative sulforaphane concentration was increased, and 22 out of 27 cysteines could be modified by sulforaphane. All of the cysteines in the Kelch, BTB and C-terminal domains (but not all in the *N*-terminal and central linker domains) were modified at the highest ratio of sulforaphane to Keap1. The four most readily modified cysteines of human Keap1 by sulforaphane were C38 in the *N*-terminal domain, C151 in the BTB domain, and C368 and C489 in the Kelch domain. The five cysteines that did not react with sulforaphane were C13 and C14 in the *N*-terminal domain, and C249, C257 and C297 in the central linker domain. The 27 Keap1 cysteines were placed into 7 groups according to their reactivity towards sulforaphane (Table 2). Covalent binding of sulforaphane to C406, which belongs to the third most readily modified cysteine residues in Keap1, has not been reported previously.

Discussion

Previous studies have probed the in vitro modification of Keap1 cysteines by sulphoraphane and its analogs (23, 24) but the results are inconsistent with in vivo observations that demonstrate the importance of C151 in sensing sulforaphane (7,10-12,22,24,31). While investigating these conflicting C151 data for sulforaphane, we found that omitting Keap1 derivatization with iodoacetamide from the sample preparation procedure and shortening the overall procedure time prior to LC-MS/MS analysis allowed the detection of C151 as one of

the most readily modified cysteine residues of Keap1 towards sulforaphane (Table 1). The other most readily modified cysteine residues were C38, C368 and C489. Our modified analytical procedure resulted in an in vitro "cysteine code" for sulforaphane that is consistent with in vivo observations and reconciles previous conflicting data.

It is important to note that we have shown in a previous study that the Keap1 protein preparation we used in the MS studies presented here is fully capable of binding to Cullin3 (expressed from *E. coli* as the full-length Cullin3-RINGBox1 protein complex) and Nrf2 which are its in vivo binding partners (32). Furthermore, we showed that the Cullin3-Rbx1-Keap1 complex is functionally active and can catalyze the ubiquitination of Nrf2, which is the process by which Keap1 regulates cellular levels of Nrf2 in vivo. Since our recombinant human Keap1 has the appropriate tertiary and quaternary protein structure to bind to and form a complex with Cullin3 and Nrf2, and since this is in a biologically suitable and relevant conformation for reaction with sulforaphane.

The ARE is activated in response to modification of Keap1 C151 by an increased amount of Nrf2, a result of decreased Keap1-mediated Nrf2 ubiquitination and degradation (2,11). This decrease in Nrf2 ubiquitination appears to arise from a diminished interaction between Keap1 and Cul3 upon modification of C151, as shown by co-immunopreciptitation experiments in cells for both Keap1 C151W (11) and for sulforaphane (2), as well as for *N*-iodoacetyl-*N*-biotinylhexylenediamine using purified Keap1 and Cul3 (10). Another model for activation of Nrf2 upon modification of Keap1 cysteines is the disruption of the Keap1-Nrf2 interaction. However, we have seen using isothermal titration calorimetry that modification of Keap1 cysteines including C151 by *N*-iodoacetyl-*N*-biotinylhexylenediamine does not alter the affinity of Keap1 and Nrf2 (8). In addition, we find that modification of Keap1 cysteines by sulforaphane does not alter its ability to bind Nrf2, as determined by native electrophoretic mobility shift assays.

The eight most readily modified Keap1 cysteine residues towards sulforaphane (C38, C151, C368, C489, C77, C226, C319, and C434; belonging to the 1^{st} and 2^{nd} most readily modified groups in Table 2) have also been reported to be modified by other electrophilic ARE inducers (8, 9, 25, 33, 34). Reaction of sulforaphane with C406 is reported here for the first time, and reactions with C23, C241, C319, C406, and C622 were not reported previously by Hong *et al.* (23). Although C273 and C288 have been reported to be functionally important (7, 22, 31), these cysteine residues were not readily modified by sulforaphane and appear in the 5^{th} and 6^{th} groups, respectively, in terms of cysteine reactivity (Table 2).

The electrophilic isothiocyanate group of sulforaphane and its analogs reacts rapidly but reversibly with Keap1 cysteine sulfhydryl groups to form dithiocarbamates (6). Since iodoacetamide covalently modifies cysteine sulfhydyl groups through S_N2 reactions to form stable thioethers, treatment of Keap1-sulforaphane adducts with iodoacetamide will result in time-dependent loss of sulforaphane (initially from the most highly modified cysteine residues) and subsequent derivatization of these cysteine sites by iodoacetamide. Natural product ARE inducers including xanthohumol, isoliquiritigenin and 10-shogaol have been reported to modify Keap1 preferentially at C151, C241, C273, C288, C319, C434, and C613 (9). However, these cysteine residues are only detected as targets of sulforaphane when iodoacetamide treatment is not used during sample preparation. This suggests that the binding of sulforaphane to these sites can go undetected due to the inherent reversibility and loss of sulforaphane during sample processing. In contrast, binding of sulforaphane to C38, C226 and C368 of Keap1 has been detected despite treatment with iodoacetamide, which suggests that these cysteine residues are less reversible and form more stable adducts with sulforaphane.

Iodoacetamide derivatization of Keap1 has been used to block unreacted cysteine residues so that they cannot react with each other to form disulfide bonds or continue to react with excess sulforaphane during subsequent sample preparation. Since iodoacetamide derivatization was eliminated from our protocol, dithiothreitol was used to consume unreacted sulforaphane. Therefore, it is unlikely that significant additional reaction could occur between sulforaphane and cysteines during sample processing. Since at least 90% protein coverage was obtained using LC-MS/MS, disulfide bond formation did not interfere with peptide mapping or sequencing and was not a concern using our new method.

Located in the *N*-terminal domain of Keap1 and a target of sulforaphane, C38 is conserved across most species with the exception of the Keap1a isoform in zebrafish (12). Although C38 is readily modified by sulphoraphane, this residue is not observed to be modified to any appreciable extent by other ARE inducers including xanthohumol (9). Sensitive to *S*-glutathionylation in vitro, C23 can also form a disulfide bond with C38 under in vitro conditions of oxidative stress (33), suggesting C23-C38 disulfide bond formation might reduce Keap1 repression of Nrf2. However, a C23Y mutant has been found in breast cancer that results in inhibition of Keap1-directed ubiquitination of Nrf2 (35). Interestingly, a C23A-C38A double mutation did not affect the repressor activity of Keap1 (7). Further studies will be needed to explain the physiological significance of C38 and C23 in Keap1 function and potential regulation through C38 modification by sulforaphane.

Sensitive to both sulforaphane modification and *S*-glutathionylation (33), C368 is located inside the inner barrel of the propeller of the Kelch domain which is the Nrf2-binding region on Keap1. An energy minimization study indicated that modification at C368 could interfere with the Keap1 binding of Nrf2 by inducing a conformational change of the protein (33). Although we did not observe any disruption of the interaction of Keap1 and Nrf2 when using an electrophoretic mobility shift assay after 9 Keap1 cysteines including C368 were modified by sulforaphane (8), modification at C368 might reduce the affinity between Keap1 and Nrf2 to a degree that is undetectable at the protein concentration used in the assay. Another cysteine residue in the Kelch domain, C489 reacts strongly with sulforaphane and has been reported to form adducts with xanthohumol, isoliquiritigenin, 10-shogoal, and *N*-ethylmaleimide (6, 9), but the biological importance of adduct formation at C489 remains uncertain.

In conclusion, we have shown that reaction of Keap1 cysteine sulfhydryl groups with sulforaphane is a highly reversible process. Our studies show that after Keap1 has formed adducts with sulforaphane, treatment with iodoacetamide causes competition between iodoacetamide and the free-sulphoraphane that was formed via dissociation from the Keap1-sulfphoraphane adduct. Iodoacetamide has been routinely used to block unmodified cysteines and prevent disulfide bond formation during tryptic digestion and mass spectrometric analysis. However, we have demonstrated that use of iodoacetamide can obscure results and interpretation since highly reversible, cysteine-sulforaphane adducts at sites such as C151 can readily dissociate and be outcompeted by iodoacetamide thereby making the cysteine-sulphoraphane adducts undetectable. By shortening the DTT quenching time, eliminating treatment with iodoacetamide and minimizing tryptic digestion time, sample preparation is shortened so that even the most reversible cysteine-sulforaphane modified residues such as C151-sulforaphane can be detected. In addition, our methodology shows that one can routinely obtain at least 90% amino acid coverage and detection of all 27 cysteines of Keap1. This approach revealed a Keap1 modification pattern that is considerably different from those reported previously or obtained during this investigation using iodoacetamide in the protocol. Notably, C151 was determined to be one of four cysteine residues preferentially modified by sulforaphane, and these chemical mapping results are consistent with in vivo observations reported by multiple investigators. Future

mapping studies of electrophiles that reversibly react with Keap1 cysteine sulfhydryl groups should also minimize sample preparation time and avoid unnecessary derivatization with iodoacetamide.

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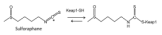

Figure 1.
Reversible reaction of sulforaphane with Keap1 cysteine sulfhydryl groups. The asterisk indicates the isothiocyanate electrophilic carbon.

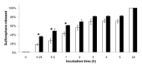

Figure 2.
Sulforaphane released from Keap1-sulforaphane adducts during incubation with and 20 mM Tris buffer (empty bars) or Tris buffer containing 3 mM iodoacetamide (solid bars). Total sulforaphane bound to Keap1 (100%) was determined by measuring the reduction in sulforaphane concentration during a 2 h incubation with Keap1. Free sulforaphane was measured using LC-MS/MS. Data are expressed as the mean ± SD of triplicate experiments. Statistically significant differences ($p \leq 0.05$) are denoted by "*".

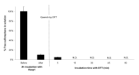

Figure 3.
DTT quench time optimization. Sulforaphane was incubated with Keap1 for 2 h and quenched with 1 mM DTT. The minimum quench time needed to remove free sulforaphane (15 min) was obtained by monitoring free sulforaphane in solution using LC-MS/MS. Data are expressed as the mean ± SD of triplicate experiments and normalized to the initial amount of sulforaphane in the solution.

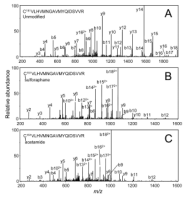

Figure 4.

Product ion tandem mass spectra of Keap1 peptide 151 to 168 obtained during data-dependent LC-MS/MS analysis of tryptic digests of Keap1. Data were acquired using high resolution accurate mass measurement, and mass assignments were within 10 ppm of the theoretical values. Keap1 samples were prepared identically except as follows: A) Control that had not been treated with sulforaphane or iodoacetamide. The $[M+2H]^{2+}$ ion (corresponding to a neutral mass of 2,133.04 u) was used as the precursor for product ion tandem mass spectrometry; B) Keap1 incubated with sulforaphane but not iodoacetamide. The abundant $[M+3H]^{3+}$ ion (corresponding to a neutral mass of 2,310.07) was used as the precursor ion for MS/MS, and an adduct with sulforaphane was detected; C) Keap1 incubated with sulforaphane followed by derivatization with iodoacetamide. The $[M+3H]^{3+}$ ion (corresponding to a neutral mass of 2,190.06 u) was selected for MS/MS. Instead of sulforaphane, iodoacetamide was found to have reacted with the peptide.

Table 1

Detection of Cysteine Residues in Human Keap1 Modified by Sulforaphane with and without Iodoacetamide Treatment

		No iodoacetamide Treatment		Iodoacetamide treatment	
Domain	**Cysteine**	**0.5**[a]	**10**	**0.5**	**10**
N-terminal	C13				
	C14				
	C23		1[b],2,3		2,3
	C38	1,2,3	1,2,3	1,2,3	1,2,3
BTB	C77	1,3	1,2,3		1,2
	C151	1,2,3	1,2,3		
	C171		1,2,3		1,2,3
Central	C196		1,2,3		1
linker	C226	1,2	1,2,3	2,3	1,2
	C241		1,2,3		
	C249				
	C257				
	C273		1,2,3		
	C288		1,3		
	C297				
	C319	1,3	1,2,3		
Kelch	C368	1,2,3	1,2,3	1,2,3	1,2,3
	C395		1		
	C406	3	1,2,3		
	C434	1,3	1,2,3		
	C489	1,2,3	1,2,3		1,2,3
	C513		1,2,3		1,2,3
	C518		1,2,3		1
	C583		1,3		1,2,3
C-terminal	C613	1,3	1,2,3		
	C622		2,3		

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		No iodoacetamide Treatment		Iodoacetamide treatment	
Domain	**Cysteine**	**0.5**[a]	**10**	**0.5**	**10**
	C624		2,3		

[a] Sulforaphane was incubated with Keap1 at a molar ratio of 0.5:1 or 10:1 (sulforaphane/Keap1).

[b] Triplicate experiments were performed. The numbers indicate the experiments in which sulforaphane-modified peptides were detected by using LC-MS/MS.

Table 2

Order of Reactivity of Keap1 Cysteine Residues towards Sulforaphane Measured using High Resolution LC-MS/MS

		[Sulforaphane]/[Keap1]				
Domain	**Cysteine**	**0.5**	**1**	**2**	**5**	**10**
N-terminal	C13					
	C14					
	C23		2[a]	2,3	1,2,3	1,2,3
	C38	1,2,3	1,2,3	1,2,3	1,2,3	1,2,3
BTB	C77	1,3	2,3	1,2,3	1,2,3	1,2,3
	C151	1,2,3	1,2,3	1,2,3	1,2,3	1,2,3
	C171					1,2,3
Central	C196			1	2,3	1,2,3
linker	C226	1,2	1,2,3	1,2,3	1,2,3	1,2,3
	C241				1,3	1,2,3
	C249					
	C257					
	C273			3	1,3	1,2,3
	C288					1,3
	C297					
	C319	1,3	1,2,3	1,2,3	1,2,3	1,2,3
Kelch	C368	1,2,3	1,2,3	1,2,3	1,2,3	1,2,3
	C395			2,3	1,3	1
	C406	3	2,3	2	1,2,3	1,2,3
	C434	1,3	1,2,3	1,2,3	1,2,3	1,2,3
	C489	1,2,3	1,2,3	1,2,3	1,2,3	1,2,3
	C513		2,3	1,2,3	1,2,3	1,2,3
	C518			1,2,3	1,3	1,2,3
	C583				1,3	1,3
C-terminal	C613	1,3	2,3	1,2,3	1,2,3	1,2,3
	C622					2,3
	C624					2,3

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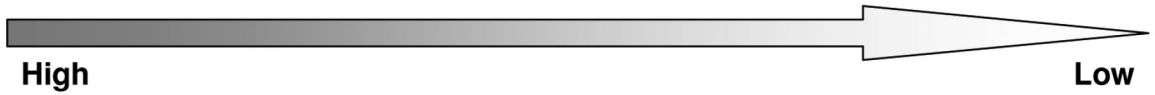
Cysteine reactivity [b]



38, 151, 368, 489	77, 226, 319, 434	406, 513, 613	23, 395, 518	196, 241, 273, 583	171, 288, 622, 624

[a] The numbers indicate the experiments out of three identical replicates in which sulforaphane-modified cysteine residues were detected by using LC-MS/MS.

[b] The order of the Keap1 cysteine reactivity based on triplicate data.