signal, recapitulates Nrf2 protein degradation and the responsiveness to electrophiles *in vivo* [44]. Subsequently, we demonstrated that the Neh2 domain mediates the proteasomal degradation of Nrf2. McMahon et al. directly demonstrated that Neh2–Gal4 fusion protein accumulates in response to electrophiles, revealing that Neh2 is a redox-sensitive degradation domain [45]. Using peritoneal macrophages from *Keap1* knockout mice (*Keap1*$^{-/-}$ mice), we demonstrated that Nrf2 protein is constitutively accumulated in *Keap1* knockout macrophages and lacks the response to electrophiles. Moreover, Keap1 enhances Neh2-dependent Nrf2 degradation in transient cotransfection assay in COS1 cells [45]. In the same study, Nrf2 can be constitutively ubiquitinated, in a Keap1- and redox-independent manner, in COS1 cells. The enhancement of Nrf2 degradation by Keap1 under homeostatic conditions, hence, does not necessarily associate with Nrf2 ubiquitination. Conversely, Zhang and Hannink recently demonstrated that Keap1 enhances Nrf2 ubiquitination [46]. They also demonstrated that two cysteines C^{273} and C^{288} of Keap1 are indispensable for Keap1-mediated ubiquitination of Nrf2. Whether unbiquitination is required or not for Keap1-enhanced Nrf2 degradation remains to be clarified in future analysis.

Even under oxidative stress conditions where Nrf2 is liberated from Keap1 repression, Nrf2 is still subjected to proteasomal degradation, indicating the existence of Keap1-independent degradation of Nrf2. From these observations, we proposed two modes of Nrf2 degradation, either homeostatic Keap1-dependent degradation or Keap1-independent degradation under oxidative stress conditions (Fig. 2) [44,45]. Three observations further support the existence of Keap1-independent degradation. First, even under oxidative conditions, where Keap1 repression is largely abolished, Nrf2 is stabilized by the

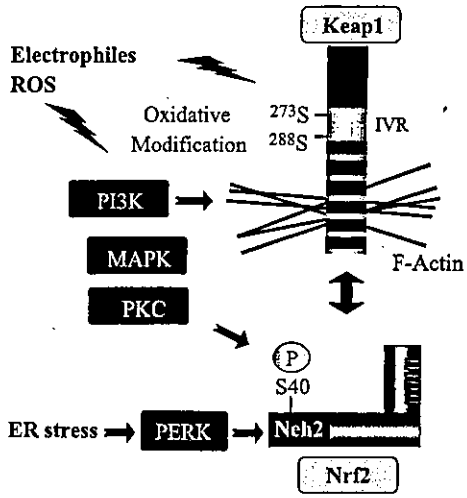

Fig. 3. Mechanisms of Nrf2 liberation from Keap1. Electrophiles provoke Nrf2 phosphorylation via MAPK, PKC, or PI3K or directly interact with Keap1-reactive cysteines. These modified Keap1 and Nrf2 cannot bind with each other as efficiently as under homeostatic conditions. The accumulation of unfolded proteins in endoplasmic reticulum (ER) activates Nrf2 via the direct phosphorylation of Nrf2 by ER-localized PERK independently from redox mechanism.

treatment of proteasome inhibitor, indicating that proteasomal degradation is still occurring in the absence of Keap1 [44]. Second, an Nrf2 mutant that lacks an important binding motif for Nrf2/Keap1 interaction ($Nrf2^{\Delta ETGE}$) can still be degraded with a protein half-life of 30 min [45]. Third, Nrf2 is degraded in a proteasome-dependent manner in *Keap1*$^{-/-}$ mice (see below). We envisaged that Keap1-independent degradation occurs in the nucleus and the Keap1-enhanced mode of degradation occurs in the cytoplasm (Fig. 2) [44]. The clarification of these two modes of degradation mechanism and their relationships are issues worthy of future endeavor.

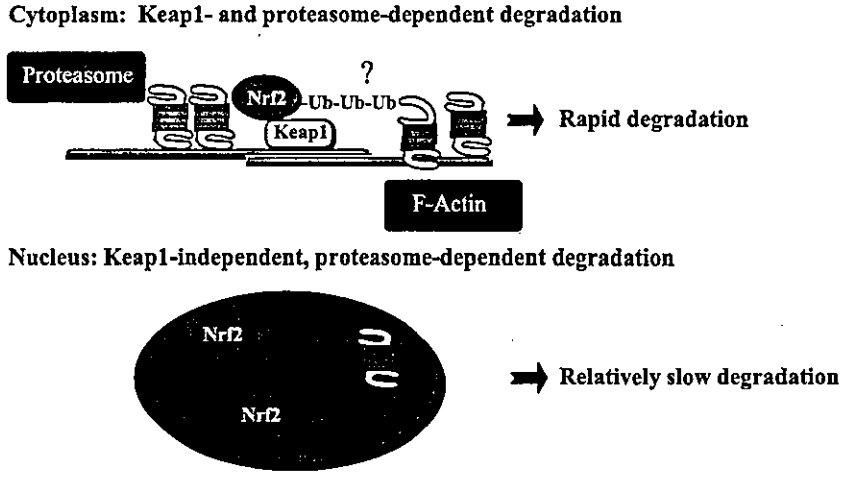

Fig. 2. Two modes of proteasome-dependent Nrf2 degradation. Under homeostatic conditions, Nrf2 is subjected to Keap1-dependent proteasomal degradation in cytoplasm. In cells treated with electrophiles, Nrf2 translocates into the nucleus and is degraded by Keap1-independent proteasomal degradation. The involvement of ubiquitination in these steps remains to be clarified.

The rapid degradation and the requirement of new protein synthesis for Nrf2 activation argue that the cytosolic pool of Nrf2 is quite small and that an entrapment model similar to the NF-κB/IκB system is quite unlikely, although we do not deny the possibility that these occur in a tissue-specific manner or under particular cell culture conditions [46]. We assume that on exposure to electrophiles, newly synthesized Nrf2 protein evade the Keap1 entrapment by still controversial mechanisms to enter the nucleus (Fig. 3) (see the discussion below).

DISRUPTION OF Keap1 REPRESSION IS SUFFICIENT FOR Nrf2 ACTIVATION

To test the hypothesis that Keap1 acts as a negative regulator of Nrf2 *in vivo* and that nullifying Keap1 repression suffices Nrf2 activation, we generated mice bearing mutation in the *Keap1* gene. *Keap1*$^{-/-}$ mice died postnatally from hyperkeratosis in the esophagus and stomach, which led to nutrient obstruction and eventually to severe ulceration of the stomach [47]. In embryonic fibroblasts of *Keap1*$^{-/-}$ mice, Nrf2 was activated in the nucleus and EpRE-regulated genes such as subunits of γ-GCS were constitutively expressed and were not upregulated by electrophiles. In liver of *Keap1*$^{-/-}$ mice, the various subunits of GST were constitutively expressed. These results unequivocally demonstrated that Keap1 acts as a negative regulator of Nrf2 *in vivo* and that the disruption of Keap1 repression is sufficient for provoking Nrf2-mediated gene expression. Bloom and Jaiswal recently reported that overexpression of the Neh2 domain causes Nrf2 nuclear accumulation in Hepa-1 cells [48]. They demonstrated that Nrf2 that accumulated in the nucleus in response to *tert*-butylhydroquinone (tBHQ) was phosphorylated at a serine residue(s), but that Nrf2 accumulated by Neh2 overexpression was not. The latter Nrf2 has as potent transactivation potential as the former, indicating that Nrf2 phosphorylation is neither required for stabilization nor transcriptional activation of Nrf2. Collectively, these results directly demonstrated that modification of Nrf2 is, if any, specifically required for liberation of Nrf2 from Keap1 and that the escape from Keap1 is sufficient for Nrf2 activation.

MECHANISM OF Nrf2 LIBERATION FROM Keap1

Several distinct mechanisms have been proposed for the liberation of Nrf2 from Keap1 repression: direct attack by electrophiles or ROS or indirect actions such as phosphorylation. Several recent investigations argued for the involvement of mitogen-activated protein kinases (MAPKs), protein kinase C (PKC), and phosphoinositol-3-kinase (PI3K) in the activation of Nrf2 [49,50]. For example, Huang et al. proposed that phosphorylation of Nrf2 Ser 40 by PKC disrupts the interaction of Nrf2 with Keap1 in HepG2 cells [51]. They demonstrated that PKC precipitated from tBHQ- or β-naphthoflavone-treated cells showed enhanced activity against Nrf2. Subsequently, Numazawa et al. reported that atypical PKCs are responsible for the phosphorylation of Nrf2 in response to phorone and 4-hydroxy-2,3-nonenal (4-HNE) in human fibroblast WI-38 cells [52]. They reported that activation of atypical PKC leads to Nrf2 activation in a Ser 40-dependent manner. On the other hand, Kang et al. reported that PI3K regulates Nrf2 through actin rearrangement in response to oxidative stress [53]. They demonstrated that the F-actin disruptor cytochalasin B induces nuclear accumulation of Nrf2. With respect to the MAPK pathway, positive and negative regulators of EpRE appear to depend on the signaling context for a particular EpRE or on given circumstances, which have been fully discussed elsewhere [49,50]. Zipper and Mulcahy demonstrated that the disruption of BTB domain-mediated homodimerization, but yet by an unidentified mechanism, is responsible for the dissociation of Nrf2 from Keap1 in response to PDTC in HepG2 cells [38].

An impressive feature of the EpRE is that it responds to nine structurally dissimilar classes of inducers [11]. Therefore, Talalay's laboratory has proposed a common sensor endowed with reactive cysteines that recognize the inducers [12]. These cysteines are predicted to sense the electrophiles even in the presence of the millimolar order of glutathione in cells that neutralizes the inducers. Murine Keap1 contains 25 cysteines that are conserved in human and rat homologs. Some of these are considered reactive cysteines as they are flanked by basic amino acid residues [54]. Dinkova-Kostova et al. demonstrated, using bacterially expressed Keap1 and radiolabeled thiol reactive reagents, that four cysteines in the IVR of Keap1 (C^{257}, C^{273}, C^{288}, C^{297}) are the preferred sites for labeling *in vitro* [55]. They also demonstrated that inducers disrupt the interaction of Keap1 with Neh2 in gel retardation assay using native polyacrylamide gel. As Neh2 does not possess any cysteine residues, the above-mentioned results indicate that the modification of Keap1 leads to the dissociation of Keap1 from Neh2. Several groups subsequently showed that mutation of either C^{273} or C^{288} disrupts the repressive activity of Keap1 against Nrf2, suggesting that modification of these two cysteines is critical for the repression of Nrf2 [46,56,57]. Furthermore, these two cysteines are specifically conserved in the "oxidant sensing" subfamily of Keap1, but not in other Kelch/BTB proteins [58]. The inability of C^{273} or C^{288} mutant to repress Nrf2 activity might be due to the defective ability of these mutants to enhance Nrf2 ubiquitination and degradation [46]. Whether the direct and indirect pathways discussed in this section are used differentially as sensors for distinct chemical classes or

cooperatively used in response to a subset of inducers requires further investigation.

OTHER SIGNALING CUES THAT MODIFY Nrf2–Keap1 INTERACTION

Quite recently, it was shown that the accumulation of unfolded proteins in endoplasmic reticulum (ER) activates Nrf2 via the direct phosphorylation of Nrf2 by ER-localized pancreatic endoplasmic reticulum kinase (PERK) [59]. As discussed above, it is suggested that the disruption or rearrangement of actin cytoskeleton triggers Nrf2 activation [36,53]. These results have raised the interesting possibility that cell signaling other than redox regulation can also upregulate EpRE response. Thus, multiple mechanisms might lead to the Nrf2-mediated cell survival response.

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ELSEVIER

Nrf2–Keap1 defines a physiologically important stress response mechanism

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The transcription factor Nrf2 regulates the basal and inducible expression of numerous detoxifying and antioxidant genes. The cytoplasmic protein Keap1 interacts with Nrf2 and represses its function. Analysis of *keap1*-knockout mice provides solid evidence that Keap1 acts as a negative regulator of Nrf2 and as a sensor of xenobiotic and oxidative stresses. The simultaneous ablation of the *keap1* and *nrf2* genes reversed all apparent phenotypes of the Keap1-deficient mice, suggesting that Nrf2 is a primary target of Keap1. The Nrf2–Keap1 system is now recognized as one of the major cellular defence mechanisms against oxidative and xenobiotic stresses. Furthermore, extensive studies have suggested that the Nrf2–Keap1 system contributes to protection against various pathologies, including carcinogenesis, liver toxicity, respiratory distress and inflammation.

Cellular detoxification is crucial for the maintenance of health by providing protection against the daily exposure to various xenobiotics (Box 1) [1,2]. The common regulatory element found in the 5′-flanking regions of many phase II detoxifying enzyme genes has been designated as the antioxidant responsive elements (ARE) [3]. This motif is similar to the consensus sequence of an erythroid gene regulatory element bound by NF-E2, a heterodimer of p45 and small Maf protein, both of which possess basic region-leucine zipper (b-Zip) motifs. The similarity between the ARE and NF-E2 binding motifs led to the identification of Nrf2 as an indispensable regulator of the coordinated induction of phase II enzyme genes (Box 1, Figure I) [4,5]. Nrf2 belongs to the CNC (cap 'n' collar) family of b-Zip transcription factors, together with p45 NF-E2, Nrf1 and Nrf3, and acts through the formation of a heterodimer with one of the small Maf proteins [6]. The inducible expression of phase II enzyme genes by butylated hydroxyanisole (BHA) was significantly diminished in Nrf2-deficient mice, a fact clearly demonstrating the crucial contribution of Nrf2 to the cellular defence mechanism against xenobiotic stresses [5].

In addition to the classical phase II enzymes, two transporter genes were found to be under Nrf2 regulation. One is the gene encoding the cysteine–glutamate-exchange transporter, which mediates cysteine influx coupled with the efflux of intracellular glutamate [7]. This transporter activity is essential for maintaining the intracellular cysteine concentration and consequently the level of glutathione. The other is *Mrp1*, a member of the multidrug-resistance-associated protein (MRP/ABCC) family [8]. Mrp1 is an ATP-binding cassette transporter that has an important role in the cellular exclusion of conjugated phase II metabolites. Hence, Nrf2 coordinately regulates the xenobiotic conjugation reaction, the supply of intracellular glutathione and the excretion of xenobiotics, enabling efficient detoxification and cytoprotection against xenobiotic toxicity. Indeed, Nrf2-deficient mice are susceptible to xenobiotic stress due to the impaired expression of cytoprotective enzymes [9–13].

Keap1 is an actin-binding cytoplasmic protein that represses the transcriptional activation of Nrf2. Recent studies on Keap1-deficient mice have provided solid *in vivo* evidence for the contention that Keap1 acts as a negative regulator of Nrf2, and suggest that the Nrf2–Keap1 system defines one of the major defence mechanisms [14]. Keap1 is rich in cysteine residues, and this fact has led to the hypothesis that Keap1 is a sensor protein of xenobiotic and oxidative stresses. Therefore, deciphering the function of the Nrf2–Keap1 system will greatly extend our understanding of the molecular basis underlying various common diseases, as well as aging processes.

Nrf2 as a key regulator of phase II detoxifying enzyme genes and antioxidant-responsive genes

The DNA binding domain of Nrf2 is similar to those of the other CNC family members [6]. Therefore, these transcription factors are likely to interact with the ARE, giving rise to elaborate defence regulation against xenobiotic and oxidative stresses. The contribution of the four CNC proteins p45, Nrf1, Nrf2 and Nrf3 to the regulation of ARE-dependent genes was examined *in vivo* by gene targeting (Box 2) [5,15–19]. Germline mutagenesis of the mouse *nrf2* gene and examination of the responsiveness of the resultant mice to electrophilic reagents showed that Nrf2 has a major role in transcriptional activation through the ARE [5].

Although the expression of phase II detoxifying enzyme genes is clearly induced in the wild type and heterozygous

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Box 1. The biotransformation process of xenobiotics and the inducible expression of detoxifying enzymes

Xenobiotics are chemical substances that are foreign to biological systems, including naturally occurring compounds, drugs and environmental agents. A simple model in which to understand their metabolism is to divide the biotransformation process into two consecutive reactions. The Phase I reaction is mediated by cytochrome P450 mono-oxygenase systems, which modify compounds through oxidation and reduction. Phase II enzymes promote the conjugation of phase I products with various hydrophilic moieties, including glutathione and glucuronic acid. Xenobiotics often serve as ligands of the key transcription activators for phase I enzyme genes, whereas the phase II enzyme genes are induced by the metabolites of phase I enzymes, which are often highly electrophilic.

The characterization of the regulatory regions of phase II enzyme genes, such as NAD(P)H:quinone oxidoreductase (NQO-1), glutathione S-transferases (GSTs) and UDP-glucuronosyl transferases (UGTs), revealed that electrophiles transcriptionally activate the expression of these genes through the antioxidant-responsive element (ARE) or electrophile-responsive element (EpRE). The minimum ARE or EpRE sequences that are necessary for transcriptional induction by electrophiles is TGACnnnGC.

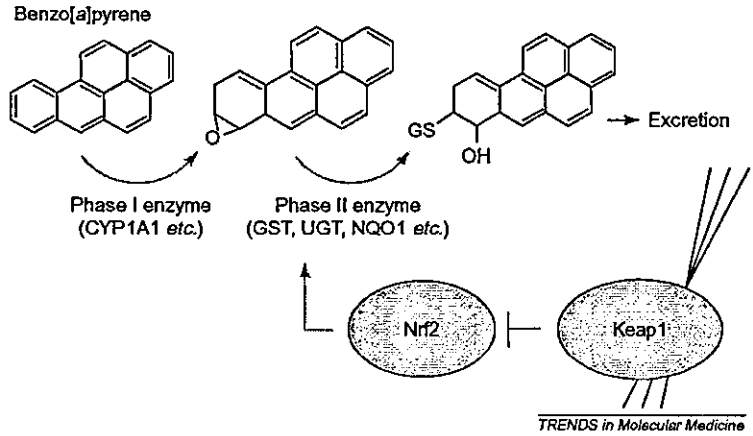

Figure I. The biotransformation of xenobiotics. Xenobiotics are oxidized by the P450 mono-oxygenase system, which includes CYP1A1 and CYP1A2, and is referred to as Phase I. The products of Phase I reactions are often electrophilic and reactive, resulting in the harmful modification of DNA and proteins and, at the same time, the induction of detoxification enzymes required for Phase II reactions. These enzymes, such as glutathione *S*-transferase (GST) and UDP-glucuronosyl-transferase (UGT), promote the conjugation of phase I products with hydrophilic moieties, such as glutathione and glucuronic acid. Nrf2 is essential for the induction of Phase II enzymes, and Keap1 negatively regulates Nrf2 activity until cells are exposed to electrophilic stimuli.

nrf2-knockout mice, the inducible expression of these genes is dramatically reduced in homozygous *nrf2*-knockout mice [20]. In the latter, insufficient induction of cytoprotective enzyme genes results in an increased susceptibility to various xenobiotics, including butylated hydroxytoluene [9], acetaminophen [10,11] and components found in diesel exhaust [12]. Cancer chemoprevention mechanisms are also defective in these Nrf2-deficient mice [13,21]. In the absence of Nrf2, oltipraz and sulforaphane, which are known chemoprotective reagents that act to prevent xenobiotic-induced carcinogenesis, failed to display their chemoprotective effect, indicating that Nrf2 has a crucial role in cancer chemoprevention [13,21].

Box 2. Identification of CNC transcription factors

Studies on the regulation of erythroid-specific gene expression originally identified p45 NF-E2 as a molecule interacting with the NF-E2 (nuclear factor erythroid-2) binding motif. Subsequently, Nrf1, Nrf2 and Nrf3 were identified. All of these factors share a conserved motif located in the N-terminal side of the b-Zip structure. Because this motif is found in the Drosophila cap 'n' collar (CNC) transcription factor, the four factors p45, Nrf1, Nrf2 and Nrf3 are categorized as the CNC family of transcription factors.

Among these four members, Nrf2 is a potent transcriptional activator. Nrf2 is expressed in metabolic and detoxification organs, such as the liver and kidney, and in organs that are continuously exposed to the environment, such as the skin, lung and digestive tract. It was therefore assumed that Nrf2 acts as a transcriptional activator interacting with the antioxidant-responsive element (ARE). Germline mutagenesis of the mouse *nrf2* gene was performed, and Nrf2 turned out to be a key regulator of the majority of Phase II detoxifying and antioxidant enzymes.

The analysis of *nrf1*-null mice and *nrf1*::*nrf2* double-mutant mice showed that Nrf1 also contributes to the regulation of antioxidant genes. Because *nrf1*-null mice are embryonic lethal, cell survival and the regulation of gene expression in response to oxidants were examined in embryonic fibroblasts. In the absence of Nrf1, cells are more sensitive to the toxicity of oxidants. As the induction of antioxidant genes diminishes, intracellular reactive oxygen species (ROS) accumulation is increased. Fibroblasts cultured from *nrf1*::*nrf2* double-mutant embryos showed increased cell death and were rescued by reduced oxygen tension or the addition of antioxidants. An increase in ROS seems to activate p53, resulting in apoptosis and embryonic lethality.

p45 is abundantly expressed in erythroid cells and has also been implicated in the resistance to oxidative stress. A defective elimination of ROS was found in erythrocytes from *p45*-null mice. The deformation of mutant cells under oxidative conditions was remarkably reduced, as was the life span. Considering the fact that erythrocytes are always exposed to oxygen and that p45 is a major CNC protein expressed in erythrocytes, it seems plausible that p45 has a crucial role in the oxidative stress response in erythroid cells. The contribution of Nrf3 is unknown, because the ablation of the *nrf3* gene revealed no obvious phenotypic differences. Furthermore, no additional lethality was observed in *nrf3*::*nrf2* double mutant mice or in *nrf3*::*p45* double-mutant mice.

Nrf2 is also an important regulator of oxidative-stress-inducible genes, including heme oxygenase-1 and peroxiredoxin 1 [22]. Intriguingly, susceptibility to hyperoxia is linked to the *nrf2* locus [23]. A single-nucleotide polymorphism was detected in the promoter region of the *nrf2* gene of the mouse strain C57BL/6J, which is sensitive to hyperoxic stress. Supporting this, *nrf2*-null mutant mice were found to be highly susceptible to hyperoxic lung injury [24].

The impaired defence mechanisms against oxidative stress that are observed in the *nrf2*-null mutant mice could have resulted from the accumulation of reactive oxygen species (ROS) in the absence of Nrf2. A combination of electron paramagnetic resonance (EPR) and spin-probe kinetic analysis confirmed that there is a substantial decrease in the ability of *nrf2*-null mutant liver and kidney to eliminate ROS [25]. This impaired elimination of ROS was exacerbated in aging female animals [25]. Consistent with this result, old female *nrf2*-deficient mice with an ICR genetic background often developed severe lupus-like autoimmune nephritis [26]. Because ROS have a prominent role in the pathogenesis of nephritis, the accumulation of ROS due to Nrf2 deficiency must have exacerbated the mild glomerular lesions that are inherent to the ICR strain of mice.

Transcriptional activation by Nrf2 and its related CNC proteins

Nrf2 contains two activation domains, Neh4 and Neh5, both of which are conserved in various Nrf2 proteins in several species (Figure 1a) [27]. Both Neh4 and Neh5 can bind to the coactivator CBP [cAMP-response-element binding protein (CREB) binding protein] independently, and simultaneous binding of CBP to these two domains synergistically activates the transcription of Nrf2 target genes. We surmise that Nrf2 achieves strong transactivation activity, at least in part, through this mechanism.

Because it is necessary for the CNC factors to form heterodimers for DNA binding, the partner molecule of Nrf2 must have an important role in the function of Nrf2. Considering the similarity between the ARE and Maf recognition elements (MARE), small Maf proteins are expected to serve as heterodimeric partner molecules of Nrf2. MARE consists of a core sequence (TGAGTCA) similar to the 12-0-tetradeconylphorbol-13-acetate (TPA)-responsive element (TRE) flanked by sequences containing GC residues (Figure 1c). The ARE consists of one-half of the core sequence of MARE and the opposite half of the flanking sequence of MARE [3]. The GC residues contained in the MARE flanking regions are recognized by a Maf protein-specific motif called the 'extended homology region' or 'ancillary region', whereas the other bZip proteins, including CNC, Jun and Fos families, do not require these dinucleotides for DNA recognition [6]. Hence, it was expected that the GC contained in the ARE is recognized by small Maf factors.

Because small Maf factors do not possess any canonical transactivation domains, small Maf factors repress transcription as inactive homodimers when in excess, but activate transcription as heterodimers with CNC family members when their availability is in balance with that of the CNC partner molecules [28]. Therefore, whether or not the heterodimer containing a small Maf protein truly serves as a transcriptional activator is controversial [29]. To address this issue, the contribution of small Maf proteins to gene regulation through the ARE requires examination and interpretation under physiological conditions *in vivo*. Recent results from a genetic experiment exploiting small-Maf-factor-knockout mice supported the contention that small Maf proteins serve as functional heterodimeric partner molecules of Nrf2 *in vivo* [30]. Further analyses are necessary for a more comprehensive understanding of the partner factors involved in transcription activation by Nrf2.

Inhibition of Nrf2 activity by the actin-binding protein Keap1

Structure–function analyses of Nrf2 revealed that deletion of the N-terminal region (Neh2 domain) enhances the transcriptional activity of Nrf2 (Figure 1a). Keap1, a novel cytoplasmic protein, was subsequently identified as an Neh2-interacting molecule [31]. Keap1 possesses a BTB (broad complex–tramtrack–bric-a-brac) domain and double glycine repeat (DGR) domain in its N-terminus and C-terminus, respectively (Figure 1b). The DGR domain is important for the interaction with Nrf2 and also for actin binding. When expressed in cultured cells, Keap1 tethered Nrf2 in the cytoplasm and repressed the transactivation activity of Nrf2. An important finding was that the addition of electrophilic reagents to the culture liberated Nrf2 from Keap1, enabling the translocation of the molecule into the nucleus and the activation of target-gene expression (Figure 2a) [31]. ETGE, a stretch of four amino acids within the Neh2 domain, was identified as a crucial motif for the Nrf2–Keap1 interaction. Mutations or deletion of the ETGE motif abolished the interaction of Nrf2 with Keap1 and consequently abolished the repressive effect of Keap1 on Nrf2-mediated gene activation [32,33].

To test the relationship between Nrf2 and Keap1 *in vivo*, germline *keap1*-mutant mice were generated by homologous recombination [14]. Keap1-deficient mice die by the third week after birth as a result of the abnormal hyperkeratosis of the esophagus and forestomach and consequent feeding problems. The expression of phase II detoxifying enzyme genes is increased significantly and constitutively in the livers and embryonic fibroblasts of homozygous *keap1*-knockout animals. These results prove that Keap1 functions as a negative regulator of Nrf2. The simultaneous knockout of the *nrf2* gene completely reversed the apparent phenotypes and lethality observed in *keap1*-null mutant mice, indicating that Nrf2 is the major target protein of Keap1 *in vivo* [14].

The Nrf2–Keap1 system is not only operative in mammals but is also conserved in zebrafish [32], indicating the crucial contribution of this system to the defence mechanisms in animals. Moreover, SKN-1, a CNC homologue protein of *Caenorhabditis elegans*, was shown to regulate phase II detoxifying enzyme genes, conferring resistance to oxidative and xenobiotic stress [34]. The wide inter-species conservation of a CNC protein as a key regulator of the antioxidant response suggests the existence of a *C. elegans* counterpart for Keap1, although it has not yet been identified.

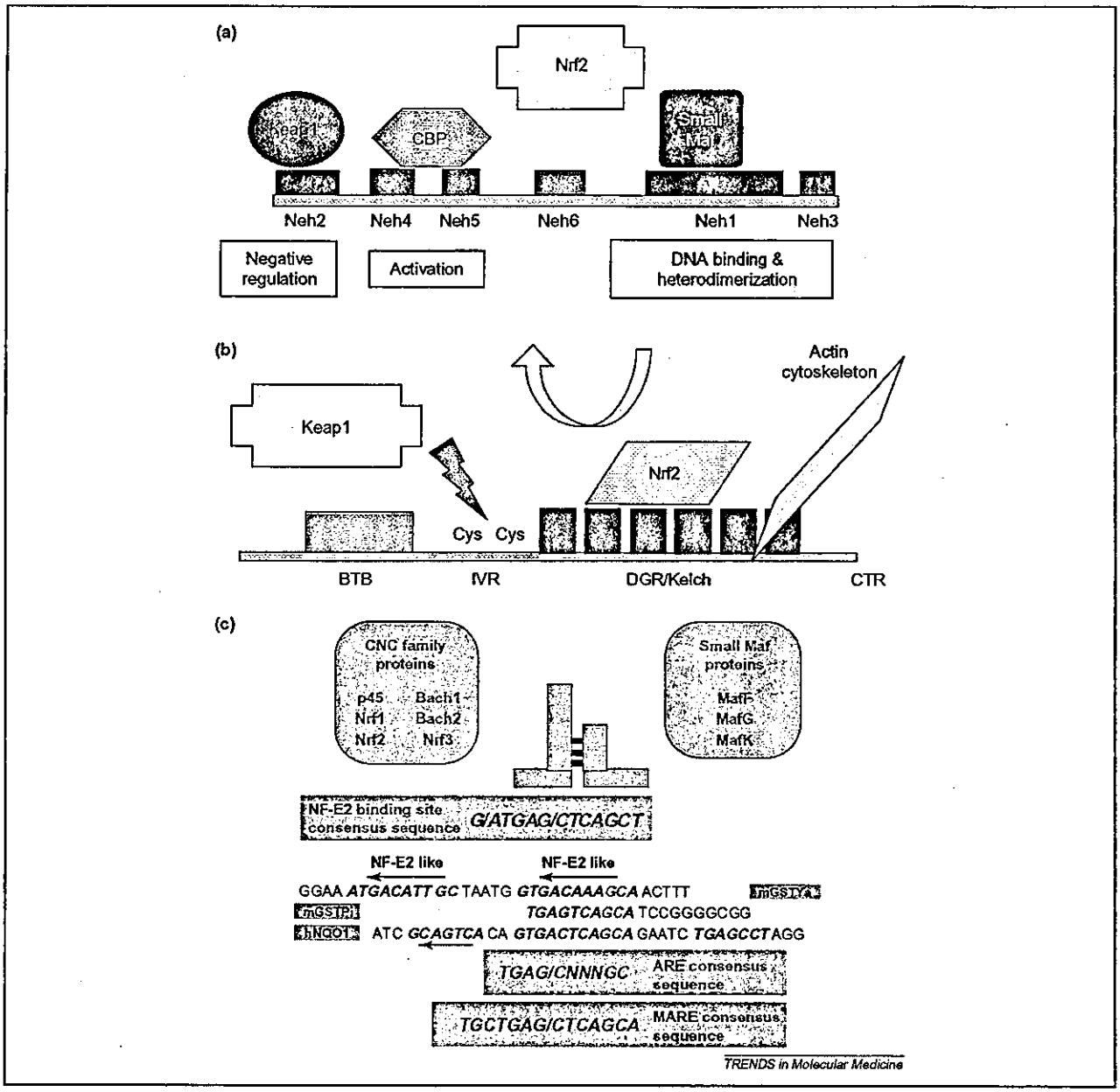

Figure 1. Functional domains identified in Nrf2 and Keap1 molecules and antioxidant-responsive elements (AREs) bound by 'cap-'n'-collar' (CNC)-small Maf heterodimers. (a) Domain structure of Nrf2. Six functional Neh [Nrf2–ECH (erythroid cell-derived protein with CNC homology) homology] units were identified in Nrf2, each well conserved in the Nrf2 molecules of various species. Keap1 interacts with the Neh2 domain and represses the activity of Nrf2. Neh4 and Neh5 interact with CBP [CREB cyclic AMP-response element binding protein (CREB) binding protein] and synergistically contribute to the strong transcriptional activation exerted by Nrf2. Neh1 corresponds to the bZip motif, mediating DNA binding and dimerization with small Maf proteins. (b) Domain structure of Keap1. Keap1 is divided into four domains: BTB (Broad complex–Tramtrack–Bric-a-brac), IVR (intervening region), DGR (double glycine repeat; also called Kelch domain owing to its homology with Drosophila Kelch protein) and CTR (C-terminal region). The DGR/Kelch domain is important for Nrf2 binding and interaction with the actin skeleton. The IVR is important for Keap1 reactivity to electrophilic and oxidative stimuli. Two of the cysteine residues in the IVR are crucial for the repressive activity of Keap1 on Nrf2. The BTB domain is thought to be involved in dimer formation. (c) The consensus sequence of ARE resembles that of the NF-E2 binding site, an important *cis*-regulatory element for erythroid-specific gene regulation. Heterodimers composed of CNC members, including p45 NF-E2, Nrf1, Nrf2 and Nrf3, and small Maf proteins, including MafG, MafK and MafF, interact with these elements. Bach1 and Bach2 form a CNC family subgroup and can also heterodimerize with small Maf proteins. AREs found in the regulatory regions of Phase II enzyme genes; for example, mouse glutathione *S*-transferase Ya (*mGSTYa*), mouse glutathione *S*-transferase Pi (*mGSTPi*) and human NAD(P)H:quinone oxidoreductase 1 (*hNQO1*). The ARE and NF-E2 binding sites are closely related to the MARE (Maf recognition element), containing GC residues (shown in red) that are important for DNA recognition by Maf family proteins.

Molecular mechanisms of Nrf2 activation

Extensive analyses of *nrf2*-null mutant mice have revealed that the inducible expression of detoxifying enzyme genes and antioxidant responsive genes is important for protection against carcinogenesis and the toxicity arising from electrophiles and oxidants. Under basal conditions, Nrf2-mediated transcription is turned off because of the inhibitory effect of Keap1. Keap1 binds to Nrf2 and sequesters the molecule from nuclei, preventing Nrf2 from activating target genes [31]. Recent studies

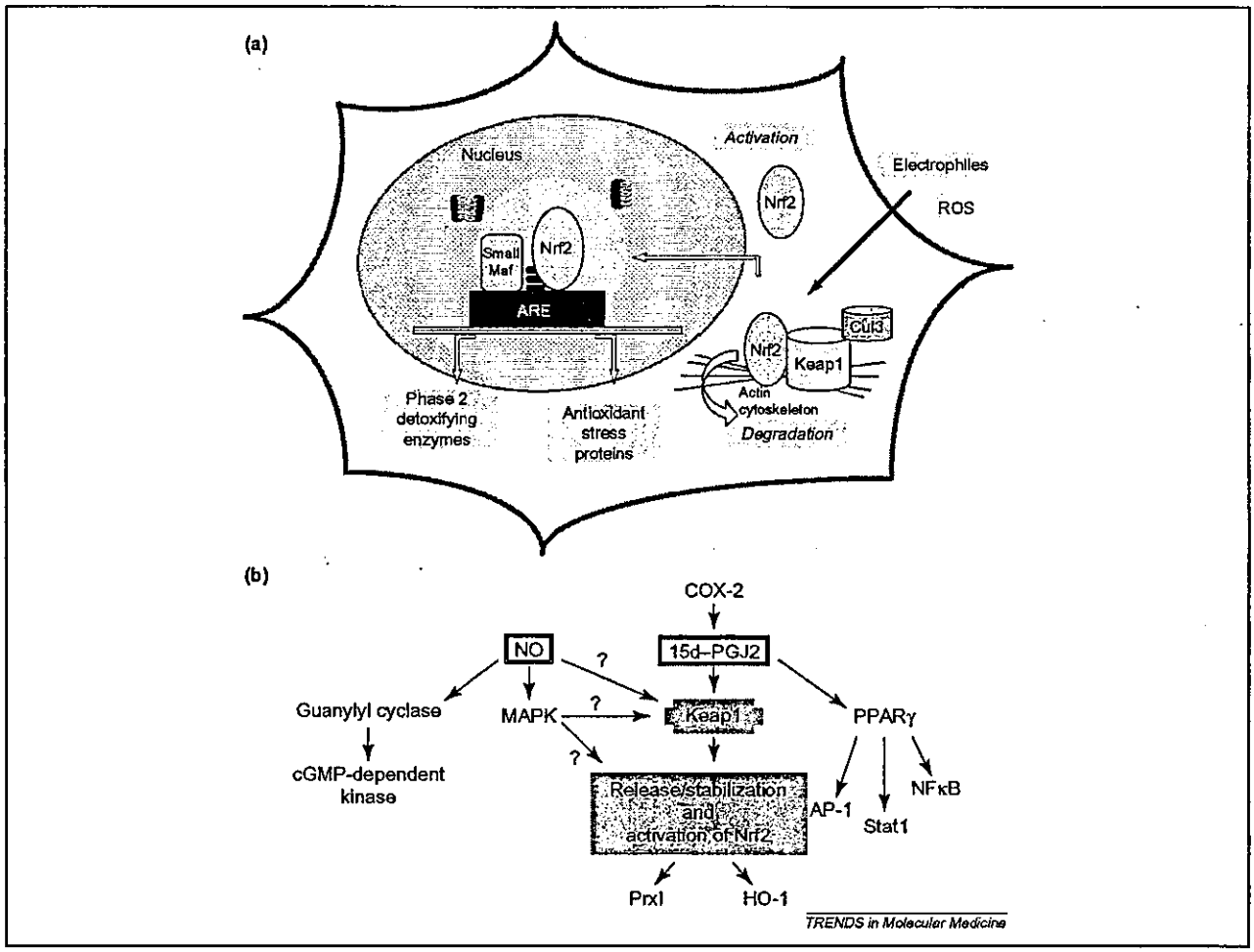

Figure 2. Schematic illustration of the molecular mechanisms of the Nrf2–Keap1 regulatory system and its endogenous activating signals. (a) In unstimulated conditions, Nrf2 is tethered onto actin fibers in the cytoplasm through Keap1 and degraded by proteasomes. Thus, the transcription levels of Nrf2 target genes remain low. Upon exposure to electrophiles and/or oxidative stress (ROS, reactive oxygen species), Nrf2 translocates into nuclei, heterodimerizes with a small Maf protein and binds to the ARE, leading to the transcriptional activation of Phase II enzyme genes and antioxidant stress protein genes. Keap1 interacts with Cullin 3 (Cul3), one of the components of ubiquitin ligase. These reagents seem to attack the sulfhydryl group of Keap1 and interfere with the interaction between Nrf2 and Keap1 or with the integrity of Keap1 and the ubiquitination machinery, resulting in the release of Nrf2 from Keap1 or the shutdown of Nrf2 degradation. (b) 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) covalently binds to Keap1, causing the translocation of Nrf2 into nuclei and the activation of Nrf2 target genes, some of which are found to possess an anti-inflammatory function, such as peroxiredoxin1 (Prx1) and heme oxygenase-1 (HO-1). Because cyclooxygenase-2 (COX-2) is a rate-limiting enzyme for the synthesis of 15d-PGJ_2, COX-2 inhibitors repress the anti-inflammatory response that is mediated by Nrf2. 15d-PGJ_2 is also known to activate the pathway directed by peroxisome proliferator-activated receptor (PPAR)γ. Nitric oxide (NO) also activates Nrf2. NO or its derivatives might affect the redox state of Keap1 or its effect might be indirect, via the MAPK (microtubule-associated protein kinase) signaling pathway. NO typically activates guanylyl cyclase to induce several kinase activities.

further revealed that Nrf2 is rapidly degraded by proteasomes through the interaction with Keap1 [35–38]. In the absence of Keap1, Nrf2 constitutively accumulates in the nucleus, indicating that Keap1 negatively regulates Nrf2 by enhancing its rate of degradation as well as altering its subcellular localization [14].

Based on the analysis of genetically engineered *nrf2*-mutant mice, it can be concluded that the Neh2 domain mediates the response to electrophiles [38]. In these mice, the *nrf2* gene was disrupted by replacing exon V, which encodes the bZip domain, with the *LacZ* gene. The mutant mice thus expressed an Nrf2–LacZ fusion protein consisting of the N-terminal half of Nrf2 with complete β-galactosidase. LacZ activity was detected in the intestine only after electrophilic stimuli, indicating that the N-terminal region, particularly Neh2, confers electrophile sensitivity to Nrf2 and stabilizes Nrf2 [38]. One additional motif involved in the degradation of Nrf2 was also identified in the Neh6 domain located in the central part of the protein (Figure 1a) [33].

The activation of Nrf2 is an important cue for the induction of cytoprotective genes, and disruption of the cytoplasmic complex between Keap1 and Nrf2 represents a major part of the cellular mechanisms for sensing xenobiotic and oxidative stresses. Several models involving the oxidation of redox-sensitive cysteines within Keap1 and the phosphorylation of Nrf2 have been proposed as a molecular basis of Nrf2 activation.

Keap1 is rich in cysteine residues, with 25 cysteines of 624 amino acids, and these cysteines are well conserved among humans, rats and mice. The electrophilic reagent dexamethasone 21 mesylate was used as a probe to alkylate the thiol groups, and five reactive cysteine residues were identified [39], raising the possibility that

Keap1 serves as a primary sensor molecule for oxidative stress. Four of these highly reactive cysteine residues are located in the intervening region (IVR) between the BTB and DGR domains (Figure 1b). Transient transfection assays identified two cysteine residues out of the four as crucial for Keap1-dependent Nrf2 repression [40,41]. Because Nrf2 binds to the DGR domain of Keap1, cysteine residues in the IVR might provoke a conformational change in the DGR or the Keap1 molecule, leading to the dissociation of Nrf2 from Keap1.

A recent study showed that Keap1 interacts with Cullin 3 (Cul3) through the IVR and serves as an adaptor for Cul3-based ubiquitin ligase [42]. This result implies that the domain structure of IVR might be important for Keap1 to form a functional ubiquitin-ligase complex. Actin binding through the DGR motif and dimerization through the BTB domain are also required for the cytoplasmic sequestration of Nrf2 [43,44]. On the contrary, one of the cysteine residues in the BTB domain was suggested to be important for inhibiting Keap1-dependent Nrf2 degradation in response to oxidative stress [40].

Several groups have suggested that Nrf2 phosphorylation is also important. Reports exist demonstrating that protein kinase C is involved in Nrf2 activation by oxidative stress and electrophiles through the phosphorylation of a serine residue located in the N-terminal region [45–48]. Other reports have attempted to address the relationship between microtubule-associated protein (MAP) kinases and ARE activation; however, the roles of MAP kinases in transcriptional activation through the ARE remain controversial [49–52]. The involvement of phosphatidylinositol 3-kinase has also been suggested [53–55]. The significance of Nrf2 phosphorylation in these pathways *in vivo* should be investigated.

Recently, it was reported that Nrf2 phosphorylation is involved in the endoplasmic reticulum (ER) stress response [56]. The activation of one of the transmembrane protein kinases, PERK [RNA-dependant protein kinase (PKR)-like endoplasmic reticulum kinase], is vital for cell survival following the initiation of the ER stress response. Nrf2 is a substrate of PERK and dissociates from Keap1 in a phosphorylation-dependent manner [56]. As expected, Nrf2-deficient cells are vulnerable to the toxicity of the ER stress-inducing reagent tunicamycin, indicating that Nrf2 promotes cell survival against ER stress [56,57].

New perspectives for Nrf2–Keap1

The analyses of *nrf2*-null mutant mice have revealed that the genes regulated by Nrf2 are indispensable components of defence mechanisms against oxidative and xenobiotic stresses. It has been predicted that specific inducers of Nrf2 would make good chemoprotective reagents against ROS and chemical carcinogens. Although the chemopreventive effects of BHA and oltipraz are recognized [13], recent screenings identified many dietary and synthetic compounds that efficiently activate Nrf2 [58,59]. The measurement of redox potentials of various compounds showed a good correlation between the tendency to release electrons and the potency to induce the NAD(P)H:quinone oxidoreductase (*NQO1*) gene, a typical target gene of Nrf2 [60]. Therefore, a compound with a strong electron-donor property is easily oxidized, becomes electrophilic and activates Nrf2. Plant-derived inducers include sulforaphane in broccoli sprouts [21], 6-methylsulfinylhexyl isothiocyanate in Japanese horseradish [61] and curcumin in turmeric powder [62]. Incorporation of these natural constituents into our diet is expected to keep our health in good condition.

In addition to these exogenous inducers, endogenous substances serving as important signaling molecules were found to activate Nrf2. One is 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), which is synthesized, via prostaglandin H_2, from arachidonic acid by the action of cyclooxygenase (COX), and was found to be a potent inducer of phase II genes [63]. 15d-PGJ_2 directly interacts with Keap1 and forms a covalent adduct resulting in the activation of Nrf2 (Figure 2b) [63]. Pleural macrophages that infiltrated after carrageenan (a family of linear sulfated polysaccharides obtained from red seaweeds that can be used to induce inflammation) challenge accumulated 15d-PGJ_2 in abundance at both the earliest stage and resolution stage of inflammation, suggesting that activated Nrf2 regulates the processes of inflammation. A high expression level of COX-2, a major rate-limiting enzyme for 15d-PGJ_2 synthesis, was also observed in the macrophages [63]. In the absence of Nrf2, pleural inflammation induced by carrageenan is prolonged and the resolution process is delayed [63]. The administration of a COX-2 inhibitor to wild-type mice recapitulated the persistence of inflammatory cells that is observed during carrageenan pleurisy, an effect that was canceled by the administration of additional 15d-PGJ_2 [63]. These results suggest that Nrf2 mediates the inflammatory process downstream from 15d-PGJ_2, and provide the new concept of Nrf2 as a mediator of inflammatory resolution. This anti-inflammatory aspect of Nrf2 function might explain the reason why gold-containing compounds activating the Nrf2 pathway are effective for the treatment of rheumatoid arthritis [64].

The involvement of endogenous inducers of Nrf2 has also been suggested in skin wound healing [65]. Nrf2 is strongly expressed in the keratinocytes of hyperproliferative wound epithelium. In *nrf2*-null mutant skin, the expression of proinflammatory cytokines, such as interleukin-1β and tumor necrosis factor α (TNFα), is reduced in the early stage of repair, but higher in the later stage [65]. An explanation for this result seems to be the persistent infiltration of macrophages in the *nrf2*-null skin during wound healing, giving another example of prolonged inflammation in the absence of Nrf2.

Nitric oxide (NO), which is another endogenous inducer of Nrf2, has miscellaneous functions in vasodilation, inflammation and apoptosis, and typically activates soluble guanylyl cyclase resulting in the activation of cGMP-dependent protein kinase and other kinases (Figure 2b) [66,67]. When endothelial [66] and neuroblastoma cells [67] were exposed to NO donors, Nrf2 accumulated in the nucleus and induced its target genes. As a consequence, neuroblastoma cells became resistant to NO-induced apoptosis [67]. These results implicate Nrf2 as having important roles in vascular homeostasis and neuronal cytoprotection from trauma- or ischemia-induced excitotoxicity.

The relationship between Nrf2 and apoptosis has also been described. When Fas-mediated apoptosis was induced by the administration of TNFα or antibodies against Fas, *nrf2*-null thymocytes died easily and *nrf2*-null mice displayed more severe hepatitis compared with wild type [68]. Nrf2 seems to decrease sensitivity to apoptotic signals by maintaining cellular redox homeostasis.

A microarray-based survey unveiled another aspect of Nrf2 function through the identification of proteasome subunit genes as downstream targets of the Nrf2–Keap1 pathway [69]. The proteasome activities in liver homogenates are actually enhanced by 3H-1,2-dithiole-3-thione (D3T), one of the cruciferous vegetable-derived antioxidants. This enhancement was not observed in *nrf2*-null mice. Because the aggregation of abnormal proteins generated by oxidation is considered to be a major cause of neurodegenerative diseases and other oxidative cellular injuries, the enhancement of proteasome activity through an increase in subunit gene expression might represent an important cytoprotective response against oxidative stress.

Concluding remarks

Recent data support the contention that the Nrf2–Keap1 system serves as an indispensable part of the defence mechanisms against various environmental, as well as endogenous, stresses. The activation of Nrf2 is a key initiation step in the cellular response against such insults. Nrf2 deficiency leads to several common pathogenic conditions, including susceptibility to chemical carcinogenesis [13,21], acute hepatotoxicity after medication [10,11], acute respiratory distress following the ingestion of food preservatives [9] and increased DNA-adducts upon exposure to diesel exhaust [12]. The relevance of Nrf2 in the prevention of carcinogenesis and xenobiotic toxicity has been firmly established [59]. The involvement of the Nrf2–Keap1 system for neuroprotection against oxidative insults in the nervous system has also been reported [70].

In addition, Nrf2 seems to make a crucial contribution to the resolution of inflammation at an appropriate stage, which strongly suggests that Nrf2 dysfunction serves as a predisposition to chronic inflammatory diseases. The involvement of ROS in various pathological conditions, including diabetes and aging, has been suggested and implies the importance of redox homeostasis, for which Nrf2 is one of the key regulators. Nrf2 is an important effector for maintaining vascular health and preventing cardiovascular diseases by mediating NO signaling and for reducing the deleterious effects of brain strokes by counteracting NO-induced apoptosis.

Therefore, it is important to analyze the precise mechanisms by which Keap1 either inhibits Nrf2 activity or releases Nrf2 in response to various stress stimuli. The available data suggest that an alteration in the redox state of Keap1 thiol residues and the phosphorylation of Nrf2 might be two crucial events determining Nrf2 activation. The essential steps triggering the Nrf2–Keap1 signaling pathway are Nrf2 stabilization and translocation into the nucleus. Importantly, Keap1 is a component of the ubiquitin ligase that forms a functional linkage between Nrf2 and the degradation machinery, raising two possible target points for the attack by electrophiles. One target might be the disruption of the interaction between Nrf2 and Keap1, with a dissociation of the two molecules resulting in nuclear translocation of Nrf2. A second possible target is a change in, or disruption of, the interaction between Keap1 and the ubiquitination machinery, resulting in the shutdown of Nrf2 degradation. In this case, Keap1 becomes saturated and the newly translated Nrf2 is imported into the nucleus. These possibilities are not mutually exclusive. We still do not understand fully whether these events occur with all Nrf2-activating reagents and stimuli.

It is important to identify the sensor molecules and signaling pathways for each stimulus. Similarly, the degradation mechanisms of Nrf2 need further intensive analysis. Another important question is to elucidate how a functionally impaired Nrf2–Keap1 system relates to various human disorders. Shedding light on the molecular mechanisms governing the functions of the Nrf2–Keap1 pathway will be important to understand the basic strategy for protection against the toxicities of oxygen and xenobiotics in food and the environment. A clear understanding in this area is expected to lead to significant developments both in basic and applied medicine, including cancer chemoprevention, inflammatory lung disease and xenobiotic metabolism.

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Forum Review

Molecular Mechanisms Activating the Nrf2-Keap1 Pathway of Antioxidant Gene Regulation

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ABSTRACT

Several years have passed since NF-E2-related factor 2 (Nrf2) was demonstrated to regulate the induction of genes encoding antioxidant proteins and phase 2 detoxifying enzymes. Following a number of studies, it was realized that Nrf2 is a key factor for cytoprotection in various aspects, such as anticarcinogenicity, neuroprotection, antiinflammatory response, and so forth. These widespread functions of Nrf2 spring from the coordinated actions of various categories of target genes. The activation mechanism of Nrf2 has been studied extensively. Under normal conditions, Nrf2 localizes in the cytoplasm where it interacts with the actin binding protein, Kelch-like ECH associating protein 1 (Keap1), and is rapidly degraded by the ubiquitin-proteasome pathway. Signals from reactive oxygen species or electrophilic insults target the Nrf2-Keap1 complex, dissociating Nrf2 from Keap1. Stabilized Nrf2 then translocates to the nuclei and transactivates its target genes. Interestingly, Keap1 is now assumed to be a substrate-specific adaptor of Cul3-based E3 ubiquitin ligase. Direct participation of Keap1 in the ubiquitination and degradation of Nrf2 is plausible. The Nrf2-Keap1 system is present not only in mammals, but in fish, suggesting that its roles in cellular defense are conserved throughout evolution among vertebrates. This review article recounts recent knowledge of the Nrf2-Keap1 system, focusing especially on the molecular mechanism of Nrf2 regulation. *Antioxid. Redox Signal.* 7, 385–394.

INTRODUCTION

THE ACCUMULATION OF REACTIVE OXYGEN SPECIES (ROS) or electrophilic insults contributes to a wide variety of diseases, including cancer, diabetes, and neurodegenerative diseases. Cytoprotection is provided by the expression of antioxidant proteins and phase 2 detoxifying enzymes that are strongly induced upon exposure to low levels of electrophiles or oxidative stress. For convenience, in this review we have referred to induction as phase 2 induction. Activation of the defense system by phase 2 induction renders cells more resistant to the potential challenges of a subsequent, even greater stress. This coordinated response is regulated through a *cis*-acting element called the antioxidant responsive element (ARE) or electrophile responsive element (EpRE) within the regulatory region of each gene. A number of studies were performed to identify ARE/EpRE binding factors, and NF-E2-related factor 2 (Nrf2) finally got into the limelight as the major contributor to phase 2 induction.

Nrf2 was first isolated as a closely related protein of p45 NF-E2 by an expression cloning procedure using an oligonucleotide containing the NF-E2 site as a probe (37, 65). p45 NF-E2 is the larger subunit of a heterodimer with binding activity at the NF-E2 site (5′-TGCTGAGTCAC-3′), a key *cis*-acting regulator of globin gene expression (5). The smaller subunit was shown to be one of the small Maf proteins, MafK, MafG, or MafF (34). Four members of the p45 NF-E2-related proteins, p45 NF-E2, Nrf1, Nrf2, and Nrf3, have been isolated in mammals and referred to as Cap'n'collar (CNC)-type basic leucine zipper (bZIP) proteins (68). This term was derived from their sequence similarity to *Drosophila* CNC protein. CNC-type bZIP proteins require a member of the small Maf proteins as a heterodimeric partner molecule for DNA binding. Although Nrf2 was assumed to be an important

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regulator of hematopoiesis like p45 NF-E2, Nrf2-deficient mice did not display any abnormality in blood formation (13, 38, 50, 62). Instead, they showed a drastic reduction in the electrophilic-induced gene expression of phase 2 detoxifying enzymes (38). Many subsequent studies demonstrated that most known ARE/EpRE-driven cytoprotective genes, including those encoding antioxidant proteins, are transcriptionally regulated by Nrf2. This shifted the interest of researchers to the regulatory mechanism of Nrf2 activity. As a result, Kelch-like ECH associating protein 1 (Keap1) was isolated and demonstrated to regulate the intracellular localization of Nrf2 by sequestering Nrf2 in the cytoplasm (39). Phase 2 inducers cause the dissociation of Nrf2 from Keap1, allowing for nuclear accumulation of Nrf2 and enhanced expression of its target cytoprotective genes. In this review, we have selected four topics related to the Nrf2-Keap1 system: target genes, roles in the defense mechanism, regulatory mechanism, and evolutional conservation.

TARGET GENES OF Nrf2

When Nrf2 was clarified to be a transcriptional regulator of phase 2 detoxifying enzymes, it was thought to control a relatively small set of genes. However, following various extensive studies, a substantial number of genes are considered to be under Nrf2 regulation. In this section, we have listed Nrf2 target genes, mainly identified through Nrf2-deficient mouse analysis, and classified them into several categories (Fig. 1).

Data from *in vivo* studies using Nrf2-deficient mice clearly implicated Nrf2 as a protein critical in regulating the expression of glutathione *S*-transferases (GSTs) and NAD(P)H quinone oxidoreductase (38). Nrf2 was shown to control genes encoding other phase 2 detoxifying enzymes, such as UDP-glucuronyl transferase 1A6, aflatoxin B1 aldehyde reductase, and microsomal epoxide hydrolase (12, 53). In addition to phase 2 detoxifying enzymes, we demonstrated that induction of antioxidant proteins during oxidative stress depends on Nrf2 activation (35). In this category of genes, heme oxygenase-1, ubiquitin/PKC-ζ-interacting protein A170, peroxiredoxin 1, the heavy and light chain of ferritin, catalase, glutathione peroxidase, superoxide dismutase, and thioredoxin were shown to be regulated by Nrf2 (12, 17, 35, 46, 52, 74).

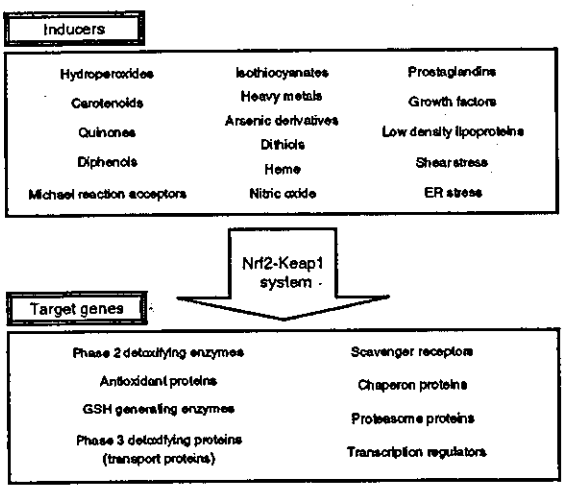

FIG. 1. Inducers and target genes of the Nrf2-Keap1 system.

Glutathione (GSH) is an effective scavenger of electrophiles and ROS that are generated during chemical metabolism within cells. Thus, it is important that the gene expression of γ-glutamylcysteine synthetase (γ-GCS), the rate-limiting enzyme in GSH biosynthesis, is well regulated in order to maintain intracellular levels of GSH. Nrf2 controls both the basal and inducible expression of genes encoding the heavy and light chains of γ-GCS (11, 12, 92). In some cells, cystine/glutamate exchange transport by system X_c^- is crucial for the maintenance of GSH levels. Nrf2 has also been demonstrated to control expression of the gene encoding xCT, one of two protein components of system X_c^- (78).

Chemicals conjugated to GST or similar are actively removed from cells, and factors involved in this elimination process are now designated as phase 3 detoxifying proteins. Multidrug resistance-associated protein 1/ATP-binding cassette transporter C plays an important role in the cellular extrusion of conjugated metabolites and is induced by electrophiles in an Nrf2-dependent manner (29). We recently found that CD36, a gene encoding the scavenger receptor that mediates the uptake of oxidized low-density lipoproteins, is also a target of Nrf2 in vascular smooth muscle cells (36). This result implicates Nrf2 as an important signaling pathway component in atherosclerosis.

Some transcription factors, including regulatory proteins of phase 2 genes, are also regulated by Nrf2. The level of Nrf2 transcription itself is basically unchanged before and after treating cells with phase 2 inducers. However, in keratinocytes, Nrf2 appears to autoregulate its own expression through an ARE/EpRE-like sequence (54). Some oxidative stress was shown to induce the expression levels of small Maf proteins and Keap1 (19, 61, 66, 83, 84). It is suggested that induction of these genes results in a negative feedback regulation of phase 2 induction. Nrf3, another member of CNC-type bZIP proteins, was up-regulated in Nrf2-deficient skin (9).

Finally, several groups have recently tried to identify Nrf2-target genes systematically by use of a microarray-based survey (56, 58, 59, 85). Their results suggested that the Nrf2-Keap1 pathway might modulate in excess of 200 genes. We identified Nrf2-dependent induction of most subunits of the 26S proteasome by antioxidants (56). The promoter of the PSMB5 subunit of the 26S proteasome was analyzed by reporter gene and chromatin immunoprecipitation assays, and its tandem ARE/EpRE sequences were shown to be direct targets for Nrf2 (55). Induction of the 26S proteasome may provide an efficient means for cells to survive conditions of various stresses that collectively enhance the likelihood of chronic disease. Heat shock proteins are also inducible by the Nrf2-dependent pathway (56). Accumulation of unfolded polypeptides following oxidative stress can disturb normal cellular functions and trigger apoptosis. These chaperone proteins, together with the proteasome system, play an essential role in

response to stress by repairing and removing damaged proteins.

ROLES OF Nrf2 IN THE DEFENSE MECHANISM

As Nrf2 regulates various cytoprotective genes, it seems to serve as a key factor in the protection against toxic xenobiotics. Without Nrf2, induction of cytoprotective enzymes is insufficient and the susceptibility of cells to toxic xenobiotics, including acetaminophen, butyrated hydroxytoluene, and diesel exhaust, is increased (6, 12, 25). Moreover, Nrf2 has been implicated in the protection against oxidative damage induced by acute pulmonary injury and hyperoxia (14, 17, 18). Elimination of Nrf2 also enhances the sensitivity of neurons and astrocytes to oxidative stress by reducing both constitutive and inducible gene expression of cytoprotective genes (58, 59). These studies demonstrate that Nrf2 is fundamental to defense against ROS and imply that Nrf2 is involved in the pathogenesis of lung, neural, and other chronic diseases. The redox status of wild-type and Nrf2-deficient mice was measured using a combination of real-time electron paramagnetic resonance imaging and spin probe kinetic analysis (31) and clearly showed that Nrf2 functions in the reduction of ROS *in vivo* (31). Nrf2-deficient mice also form higher levels of DNA adducts following exposure to carcinogens such as aflatoxin B1, diesel particulate matter, and benzo[*a*]pyrene (6, 52, 77). In addition, the effects of cancer chemopreventive reagents such as oltipraz and sulforaphane are abolished in mice deficient in Nrf2 (26, 52, 53, 76, 77). Functions of Nrf2 in cell survival are also clear (20, 58, 59, 67) and thought to be mediated at least partially by inhibition of the FAS pathway (49, 67).

Recently, Nrf2 target genes were suspected to play antiinflammatory roles, and the influence of Nrf2 during acute inflammation was explored. The persistence of inflammatory cells in Nrf2-deficient mice was observed during carrageenan-induced pleurisy (41). In endothelial cells, overexpression of Nrf2 inhibited the tumor necrosis factor-α-mediated induction of vascular cell adhesion molecule-1 gene expression, which is important for monocyte recruitment during the inflammatory response (16). Laminar shear stress, which acts as an anti-inflammatory signal, activated phase 2 genes in an Nrf2-dependent manner. The induced expression of proinflammatory cytokines in wounded skin was delayed in Nrf2-deficient mice (9). Aged Nrf2-deficient female mice developed lupus-like autoimmune nephritis (94). All these results suggest that Nrf2 plays important roles in antiinflammation.

REGULATION OF Nrf2

The activities of Nrf2 in the defense system allowed us to imagine that constitutive expression of Nrf2 causes animals to become more resistant to stress, but this is not the case. Keap1-deficient mice in which Nrf2 is constitutively active die within 3 weeks after birth (90). Therefore, controlled Nrf2 activity is quite important for our health.

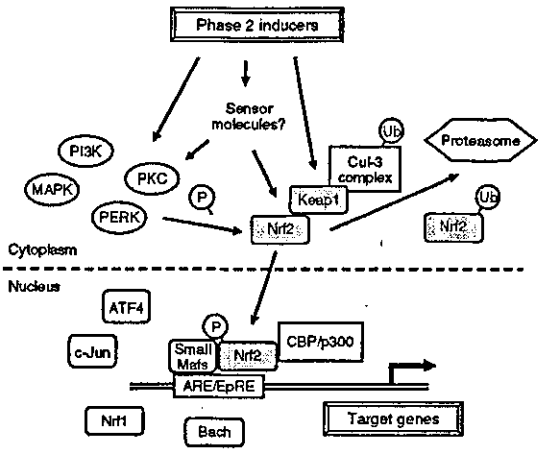

FIG. 2. Model of Nrf2-Keap1 system regulation.

Nrf2 activation is regulated in several steps. Some key features emerged from an extensive study of the molecular mechanism of Nrf2 activation in phase 2 induction. In this section, we discuss current models for Nrf2 regulation (Fig. 2).

DNA binding

The regions homologous between mouse Nrf2 and chicken Nrf2 (ECH) are called Neh (Nrf2-ECH homology) domains. Six Neh domains, Neh1 to Neh6, have been identified (39) (Fig. 3). The Neh1 domain contains a bZIP structure that is required for DNA binding and dimer formation. Nrf2 cannot bind to the ARE/EpRE as a monomer or a homodimer and must heterodimerize with one of the small Maf proteins for DNA binding and transactivation (37, 44, 84). The requirement for a "GC" motif in the ARE/EpRE consensus sequence strongly supports the contention that small Maf proteins serve as the heterodimeric partner molecules for Nrf2 (51). Indeed, we recently demonstrated genetically that small Maf proteins are required for Nrf2 activities *in vivo* using compound mutant mice (69). c-Jun and activating transcription factor 4 (ATF4) were also reported to form heterodimers with Nrf2 *in vitro* and to enhance the activity of ARE/EpRE-driven reporter genes. It is possible that these proteins also act as partner molecules for Nrf2 in some conditions (30, 88).

DNA binding was also controlled through competition with other ARE/EpRE-binding proteins. Among these factors, the roles of the transcriptional repressors Bach1 and Bach2 are the most intriguing, particularly because it has been established that Bach1 antagonizes the function of Nrf2, especially in heme oxygenase-1 gene expression (82), and that oxidative stress induces the nuclear accumulation of Bach2 while reducing ARE/EpRE-dependent reporter gene expression (70). Nrf1 is also fascinating. Chimeric mouse analysis using Nrf1-deficient embryonic stem cells indicated that loss of Nrf1 results in impaired expression of antioxidant genes and increased oxidative stress in the liver (15). Mouse embryonic fibroblasts (MEF) from Nrf1-deficient embryos displayed enhanced sensitivity to oxidative stress and an increased accumulation of free radicals (57). MEF deficient in

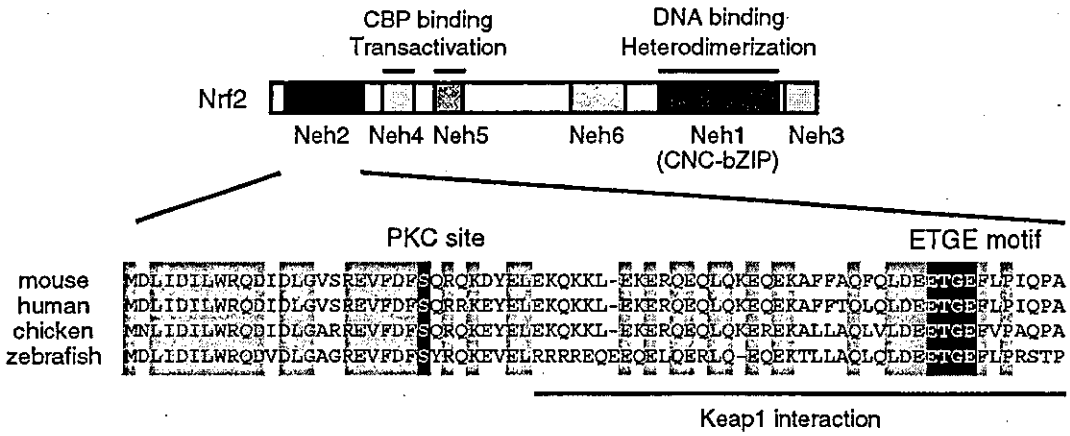

FIG. 3. Neh domains in Nrf2.

both Nrf1 and Nrf2 contained a higher level of intracellular ROS and were more sensitive to oxidative stress than Nrf2-single deficient MEF (60). These results indicate that the functions between Nrf2 and Nrf1 are redundant, especially in liver cells.

Transactivation

Neh4 and Neh5 domains have both been shown to be important for the transactivation activity of Nrf2 (39, 48) (Fig. 3). Neh5 is highly similar to the domain in p45 NF-E2 that is responsible for associating with coactivator CREB binding protein (CBP). The Neh4 domain contains a TRAM binding motif to which CBP and its inhibitor adenovirus E1A protein were shown to interact. Indeed, CBP or p300 was shown to mediate Nrf2 transactivation activity (45, 97). Among CNC-type bZIP family proteins, Nrf2 was found to be the most potent transcriptional activator and typically activates reporter gene transcription by nearly 100-fold (47, 86). The synergistic activity of Neh4-CBP and Neh5-CBP can explain the strong activation potential of Nrf2 (45).

Intracellular localization

Deletion of the N-terminal Neh2 domain enhanced the transcriptional activity of Nrf2 (39) (Fig. 3). This observation suggested that the Neh2 domain recruits a negative regulator of Nrf2. This repressor, Keap1, was identified in a yeast two-hybrid screen using the Neh2 domain as bait (39). Keap1 is a member of the Kelch family of proteins that possess two characteristic domains, the broad complex/tramtrack/bric-a-brac (BTB) domain and the double glycine repeat (DGR) domain (1) (Fig. 4). In common with other Kelch family proteins, Keap1 directly interacts with actin through the DGR domain, thus colocalizing with the actin cytoskeleton in the cytoplasm (43). In the absence of phase 2 inducers, Nrf2 associates with Keap1 in the cytoplasm, but upon the addition of electrophiles, Nrf2 translocates into nuclei and concludes in activation of target gene transcription (22, 39).

As the association and dissociation of the Nrf2-Keap1 complex was considered to be the most significant step for regulating Nrf2 activity, residues essential for the interaction of each protein were analyzed. From this analysis, the ETGE motif in the Neh2 domain was identified as a Keap1-interacting site by a yeast reverse two-hybrid screen (48) (Fig. 3). In the case of Keap1, a point mutation at Ser104 in the BTB domain of Keap1 decreased the association of Keap1 with Nrf2 (99). Keap1 was demonstrated to self-associate, and the mutation at Ser104 disrupts this Keap1 dimerization. In contrast, deletion of the BTB domain did not impair Keap1 activity in our transfection analysis (43). Therefore, the importance of Keap1 dimerization should be elucidated.

The interaction between Nrf2 and Keap1 was also demonstrated at the genetic level (90). Keap1-deficient mice died within 3 weeks after birth due to hyperkeratosis in the esophagus and forestomach. In the liver of these mice, a high steady-state nuclear accumulation of Nrf2 and constitutive expression of phase 2 genes were observed. Importantly, these phenotypes were all rescued in compound Keap1-Nrf2-deficient mice. Our results strongly suggest that Keap1 acts as an indispensable regulator of Nrf2.

Protein stability

Recently, we and other groups demonstrated the rapid degradation of Nrf2 by the ubiquitin-proteasome pathway and the stabilization of Nrf2 by phase 2 inducers (3, 40, 63, 72, 80, 81). By analyzing LacZ or green fluorescent protein fusion proteins, the Neh2 domain was shown to be responsible

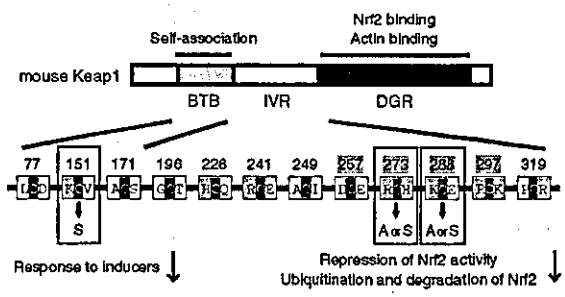

FIG. 4. Critical cysteine residues in mouse Keap1.

for mediating the rapid degradation of Nrf2, in turn suggesting that Keap1 participates in the regulation of Nrf2 degradation (40). Indeed, the addition of Keap1, but not an ETGE motif-deleted mutant, destabilizes Nrf2 (63), and Cys273 and Cys288 in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 (96) (Fig. 4). Interestingly, BTB proteins, including Kelch family proteins, were recently reported to be substrate-specific adaptors of Cul3-based E3 ubiquitin ligase complexes (27, 28, 75, 93). One plausible model is that Keap1 binds to Cul3 and facilitates Nrf2 degradation as an Nrf2-specific adaptor of E3 ubiquitin ligase.*

Sensing inducers

Identifying molecules that sense phase 2 inducers and transduce their signals to Nrf2 have become hot topics. Inducers of phase 2 genes vary as in nine structurally diverse chemical groups (23). Although these inducers share only a few properties, they can all modify sulfhydryl groups by alkylation, oxidation, or reduction. Recognition of these properties suggested that cells possess primary sensors equipped with highly reactive cysteine residues. Interestingly, Keap1 contains 27 cysteine residues, and several of them are reactive, implying that Keap1 might be a direct target of phase 2 inducers. Recently, we showed in a cell-free system that selective cysteine amino acids in Keap1 could react directly with dexamethasone mesylate, a sulfhydryl reactive inducer, and trigger the release of Nrf2 from Keap1 (24). The direct interaction of Keap1 and the phase 2 inducer 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) was also demonstrated (41). The most reactive residues in Keap1 were Cys257, Cys273, Cys288, and Cys297 present in the intervening region (IVR) (24) (Fig. 4). Among them, mutation of Cys273 or Cys288 resulted in the inability of Keap1 to repress Nrf2 activity (91, 96). These cysteine residues were further demonstrated to be required for Keap1-dependent ubiquitination of Nrf2 (96). It is possible that phase 2 inducers directly target these residues, with the resulting modification decreasing ubiquitination activity. The BTB domain may be an alternative target for phase 2 inducers, because Zhang and Hannink (96) further elucidated that a Cys151 mutation in the BTB domain makes Keap1 a constitutive repressor of Nrf2 (Fig. 4).

In addition to Keap1, protein kinases might be candidates as sensor molecules of electrophiles or oxidative stress, because activation of protein kinase C (PKC) (8, 32, 73), extracellular signal-regulated kinases (ERK) (10, 98, 100), p38 mitogen-activated protein kinase (MAPK) (2, 7, 10, 98, 100), MAPK/ERK kinase-1 (79), MEK kinase 1 (95), phosphatidylinositol 3-kinase (PI3K) (42, 71), and PKR-like endoplasmic reticulum kinase (PERK) (20) was observed after treatment with phase 2 inducers. Furthermore, phase 2 gene and ARE/EpRE-driven reporter gene induction was blocked by specific kinase inhibitors. Among the kinases, PKC and PERK are remarkable because both can phosphorylate Nrf2 directly *in vitro* and *in vivo* (20, 32, 33). A coimmunoprecipitation assay revealed that phosphorylation of Nrf2 by PKC promotes its dissociation from Keap1 and that a Ser to Ala mutation at amino acid 40 in Nrf2, which is the target site for PKC, decreased this PKC-dependent dissociation (33) (Fig. 3). On the other hand, PERK-dependent phosphorylation of Nrf2 also triggers dissociation of the Nrf2-Keap1 complex (20). It is possible that PKC and/or PERK or their upstream signaling molecules may be sensors for oxidative stress.

*Specific association of Keap1 with Cul3 has been confirmed during editorial process of this review (101).

EVOLUTIONARY CONSERVATION OF THE Nrf2-Keap1 SYSTEM

The importance of the bZIP protein in cellular defense has been shown in yeast cells (87). The bZIP protein Yap1 in budding yeast and Pap1 in fission yeast regulate the gene expression of various cytoprotective proteins, such as γ-GCS, thioredoxin, the hsp70 family member, NAD(P)H oxidoreductase, glutathione transferase, catalase, and ATP binding cassette-type transporters. Both Yap1 and Pap1 are cytoplasmic in unstressed cells and translocate into nuclei in response to treatment with oxidants, electrophiles, or heavy metals. These characteristics are quite similar to those of Nrf2. The clear difference between the Yap1/Pap1 and Nrf2 systems is the regulatory mechanism of cytoplasmic retention and nuclear translocation. In budding yeast, redox signals promote the formation of disulfide bonds between the intermolecular cysteines of Yap1 that mask the C-terminal nuclear export signal domain, resulting in inhibition of Yap1 nuclear export (21). Cytoplasmic retention molecules such as Keap1 are not required for Yap1, and Nrf2 probably does not contain a nuclear export signal domain as in Yap1.

In nematode, SKN-1 was demonstrated to regulate phase 2 detoxifying genes through constitutive and stress-inducible mechanisms (4). Its binding sites exist in the upstream regions of γ-GCS heavy chain, glutathione synthetase, NADH quinone oxidoreductase, GST, catalase, and superoxide dismutase. SKN-1 mutants are sensitive to oxidative stress and have shortened life spans. Analysis of green fluorescent protein fusion proteins revealed that heat or paraquat treatment induced the nuclear accumulation of SKN-1. Again, the functions of SKN-1 seem to be similar to those of Nrf2. Although SKN-1 shares homology with Nrf2 in both the N-terminal halves of the Neh2 and Neh1 domains, it lacks an ETGE motif or leucine zipper domain. Indeed, homologues for Keap1 or small Maf proteins have not been found in *C. elegans*, implying that the regulatory mechanisms of SKN-1 activation may be different from those for Nrf2.

In fruit fly, CNC protein has homology with Nrf2. CNC was originally identified as a regulatory protein for labral and mandibular development (64). So far, no study has been reported about CNC functions in the defense system. Interestingly, CNCC protein, one of three isoforms of CNC, possesses a Neh2-related region containing an ETGE motif (48). In addition, a Keap1-related gene and a small Maf protein were identified in *Drosophila* (48, 89). In common with Nrf2 in vertebrates, it is possible that CNCC plays important roles in the defense mechanism in fruit flies.

Nrf2 was identified in mouse, human, chicken, and zebrafish and supposedly exists in all other vertebrates (48). Gene knock-down analysis of zebrafish Nrf2 using morpholinophosphorodiamidate-modified antisense oligonucleotide

revealed that Nrf2 is required for phase 2 induction in fish, as it is in mammal. Keap1 also exists in zebrafish and was shown to interact with and repress the activity of zebrafish Nrf2. The molecular mechanism regulating the Nrf2-Keap1 system may be conserved among vertebrates.

CONCLUSION

Recently, Nrf2 has been found to be activated by endogenous products of oxidative stress or other stress generated inside the body, such as 4-hydroxynonenal (36, 73), oxidized low-density lipoproteins (36), heme (2, 46, 71), and nitric oxide (10, 42). In addition, prostaglandin 15d-PGJ_2 (41) and keratinocyte growth factor (9) can induce Nrf2-dependent gene expression. As these agents function as signaling molecules in many systems, the Nrf2-Keap1 system may be considered as a central component of cellular defense networks. Identification of molecules sensing phase 2 inducers and transducing their signals to Nrf2 will greatly contribute to a better understanding of these networks. A number of significant findings were reported in the last couple of years, and the molecular mechanism activating the Nrf2-Keap1 pathway is gradually being unveiled. The complete picture of the Nrf2-Keap1 system should come into view in the near future.

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ABBREVIATIONS

ARE, antioxidant responsive element; ATF4, activating transcription factor 4; BTB, broad complex, tramtrack, and bric-a-brac; bZIP, basic leucine zipper; CBP, CREB binding protein; CNC, Cap'n'collar; DGR, double glycine repeat; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; EpRE, electrophile responsive element; ERK, extracellular signal-regulated kinase; γ-GCS, γ-glutamylcysteine synthetase; GSH, glutathione; GST, glutathione *S*-transferase; IVR, intervening region; Keap1, Kelch-like ECH associating protein 1; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; Neh, Nrf2-ECH homology; Nrf2, NF-E2-related factor 2; PERK, PKR-like endoplasmic reticulum kinase; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; ROS, reactive oxygen species.

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