ture. Fig. 2C presents the α-helical wheel for the Neh2-NT subdomain. When we plotted the amino acids that comprise the DLG motif out according to the predicted α-helical wheel, we found that the conserved residues of LxxQDxDLG sequence are confined to one side of the helix as indicated by asterisks (Fig. 2C). We also noticed that three leucine residues are clustered on the same side of the surface (Fig. 2C, shadowed). Amongst these leucine amino acids, two out of the three residues contribute to the DLG motif. The other residue (L19) has also been highly conserved during molecular evolution, with the exception that in CncC it has been replaced by an isoleucine.

DLG motif is dispensable for the nuclear degradation of native Nrf2

The fact that the Neh2-NT subdomain mediates degradation of Nrf2 in the nucleus prompted us to examine how the DLG motif might contribute to this process. We first sought to define a role for the DLG motif in Neh2-mediated degradation of EGFP. To define roles of DLG motif play in the nuclear degradation of Nrf2, we first attempted to assess contribution of the motif to the Neh2-mediated EGFP degradation. Substitution of all three leucine residues (L19, L23, and L30) with alanine in Neh2-NT-EGFP completely blocked degradation of the chimeric protein, a finding implicating the DLG motif as an essential structure for the Neh2-mediated degradation in the nucleus. We therefore examined the effect of DLG mutation in the context of native Nrf2 in the nucleus. For this purpose, DLG mutation was introduced into the native Nrf2 protein (Nrf2-LA; see Fig. 3A). Expression plasmids for Nrf2-LA mutant and native Nrf2 were transfected into Cos7 cells and the cells were subsequently treated with 100 μM diethylmaleate (DEM). Under this experimental condition, the half-life of wild-type Nrf2 and the Nrf2-LA mutant was 24.7 and 30.6 min, respectively (Table 1). This difference is statistically insignificant. This result suggests that DLG motif makes a minimal contribution to the degradation of native Nrf2 in the nucleus. Although the Neh2-NT subdomain directs degradation of Nrf2 in the nucleus when fused to EGFP, this degradation activity of Neh2-NT may not contribute substantially to the nuclear degradation of Nrf2.

Table 1
Half-life of Nrf2 in the presence of Keap1

Treatment	Half-life (min)	
	WT	Nrf2 LA
Keap1 (−)/DEM (−)	20.2 ± 5.0	31.7 ± 5.6
Keap1 (−)/DEM (+)	24.7 ± 4.7	30.6 ± 1.9
Keap1 (+)/DEM (−)	11.5 ± 2.4	39.2 ± 11.1

Cos7 cells were transfected with expression plasmids of mutant or wild-type Nrf2 in the absence or presence of Keap1. As an internal control, EGFP expression plasmid was transfected concomitantly. The cells were treated with cycloheximide and Nrf2 protein levels were determined by immunoblot analysis. Intensity of the bands was measured by densitometry analysis. In each case, intensity of the Nrf2 band was divided with that of EGFP, and resulting figures were plotted against time. Mean time of calculated half-lives obtained from three independent experiments is shown with SEM.

DLG motif is essential for Keap1-mediated proteasomal degradation of Nrf2

Since the presence of DLG and ETGE motifs shows tight linkage during molecular evolution, one hypothesis is that the DLG motif may contribute to the Nrf2 degradation that is dependent on Keap1. Therefore, we examined the effect of exogenous Keap1 on the nuclear accumulation of Nrf2 in the Cos7 cell system because we have found previously that in this cell line ectopic expression of Keap1 can enhance the degradation of Nrf2 both dose-dependently and in an ETGE motif-dependent manner [28].

As shown in Fig. 3B, Keap1 markedly decreased the accumulation of wild-type Nrf2 (lane 3), and this decrease was largely canceled by the treatment with MG132, a proteasome inhibitor (lane 4). In contrast, Keap1 did not affect the accumulation of either Nrf2-LA or Nrf2-ΔNeh2-NT that lacks 5–44 a.a. of Neh2 (lanes 5–12; see also Fig. 3A). The Nrf2-LA protein migrated slower than the native Nrf2 protein in this gel electrophoresis (lanes 10–12) for a reason unknown at present. These results thus suggest that the DLG motif contributes to the Keap1-dependent proteasomal degradation of Nrf2 in the cytoplasm.

We then determined the half-life of Nrf2 in the presence of Keap1 and under normal homeostatic conditions. For this purpose, Cos7 cells were transfected with wild-type and Nrf2-LA expression plasmids, and their half-lives were determined following treatment with CHX. As shown in Table 1, co-expression of Keap1 decreased the half-life of wild-type Nrf2 from 20.2 to 11.5 min in this system. In contrast, Keap1 did not diminish the half-life of Nrf2-LA. Thus, these results indicate that the DLG motif is indispensable for the Keap1-mediated degradation of Nrf2 in the cytoplasm. Available data further suggest that this function of the DLG motif may be achieved in collaboration with the ETGE motif as will be discussed below.

Binding affinity to Keap1 is decreased in Neh2-NT mutants

To address further the mechanism underlying the resistance of Neh2-NT mutants to degradation, we examined how Keap1 affects the transactivation activity of Nrf2 mutants in quail fibroblast cell line QT6. We assumed that the repression by Keap1 of the Nrf2

transactivation activity is correlated tightly and solely to the binding affinity of Nrf2 to Keap1 in this system [29]. In the absence of exogenous Keap1, Nrf2, Nrf2-ΔNeh2-NT, and Nrf2-LA all activated reporter gene expression and this activation depended on the amount of plasmid transfected (Fig. 4A). Transactivation activity of Nrf2-ΔNeh2-NT was lower than that of wild-type Nrf2, but that of Nrf2-LA was comparable to wild-type Nrf2. We also confirmed that exogenous expression of Keap1 did not enhance the Nrf2 degradation in this cell line (data not shown).

We then co-transfected Keap1 with the Nrf2 mutants and luciferase reporter. While Keap1 repressed the transactivation activity of wild-type Nrf2 dose-dependently, the repressive effect of Keap1 was weakened when the Neh2-NT subdomain was mutated; the activity of Nrf2-ΔNeh2-NT and Nrf2-LA was only mildly diminished upon incremental addition of Keap1 plasmid when compared with the decrease in activity of wild-type Nrf2 affected by Keap1 (Fig. 4B). Repression by Keap1 was stronger for Nrf2-LA than for Nrf2-ΔNeh2-NT. E82G mutation in the ETGE motif, which is known to abolish the binding affinity of Nrf2 to Keap1 [29], produced a protein that was completely immune to repression by Keap1 (Fig. 4B). These results thus suggest that the Neh2-NT mutants retain weakened binding affinity for Keap1.

We therefore examined affinity of these Nrf2 mutants for Keap1 experimentally through an immunoprecipitation experiment. In this analysis, immunoprecipitation with anti-Keap1 antibody was followed by immunoblotting with anti-Nrf2 antibody. The analysis revealed that while wild-type Nrf2 was co-precipitated with Keap1 efficiently, Nrf2-ΔNeh2-NT and Nrf2-LA mutants were not effectively co-precipitated (Fig. 4C). Thus, these results indicate that the binding affinity of Nrf2-ΔNeh2-NT and Nrf2-LA to Keap1 is considerably weakened compared to that of the wild-type Nrf2, and this may be the reason for the decrease in Keap1-dependent protein degradation.

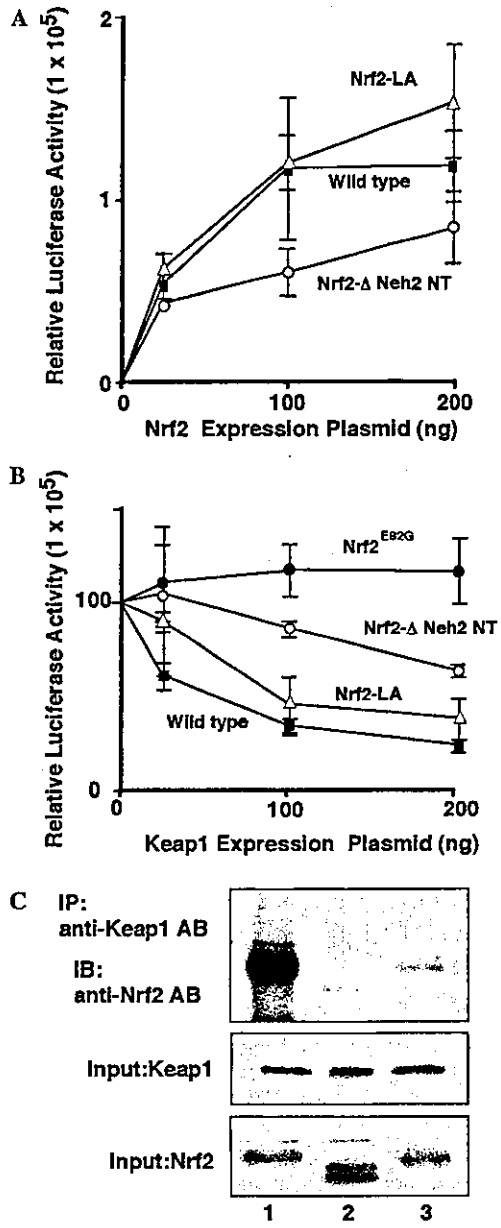

Fig. 4. Neh2-NT and leucine residues in the predicted α-helix are important for stable binding of Nrf2 with Keap1. (A) pRBGP2 reporter construct was transfected into QT6 fibroblasts along with wild-type Nrf2, Nrf2-ΔNeh2-NT or Nrf2-LA expression vector. Luciferase activity in the absence of DEM was set at 100, and results of three independent experiments each carried out in duplicates are shown with SEM. (B) pRBGP2 reporter construct was transfected into QT6 fibroblasts along with wild-type Nrf2, Nrf2-ΔNeh2-NT, Nrf2-LA or $Nrf2^{E82G}$ expression vector in the presence of increasing amounts of Keap1 expression plasmid. (C) 293T cells were transfected with wild-type Nrf2 (lane 1), Nrf2-ΔNeh2-NT (lane 2), and Nrf2-LA (lane 3) expression vectors along with HA-tagged Keap1 expression plasmid. Subsequently, cells lysates were immunoprecipitated with anti-Keap1 antibody and subjected to immunoblot analysis by anti-Nrf2 antibody (top panel). Input fraction was also subjected to immunoblot analysis by anti-Keap1 antibody (middle panel) or anti-Nrf2 antibody (bottom panel).

Ubiquitination of Neh2 and its subdomains

To assess whether ubiquitination contributes to the Neh2-mediated proteasomal degradation, we transfected HEK 293T cells with expression plasmids for both Neh2-EGFP and HA-tagged-ubiquitin and examined how Neh2 domain is ubiquitinated in the immunoprecipitate generated by anti-EGFP antibody. We used CL-1 fused EGFP, which was shown previously to degrade through the ubiquitin–proteasome pathway [34], as a positive control of ubiquitination in this study. The immunoblot analysis by anti-HA antibody and its densitometric analysis revealed that immunoprecipitates obtained from Neh2-EGFP transfected cells contain a higher amount of ubiquitinated protein compared with that from EGFP transfected cells confirming previous reports, demonstrating that Neh2 mediates ubiquitination (Figs. 5A and B) [26]. A similar analysis using Neh2-EGFP deletion mutants showed that Neh2-NT and

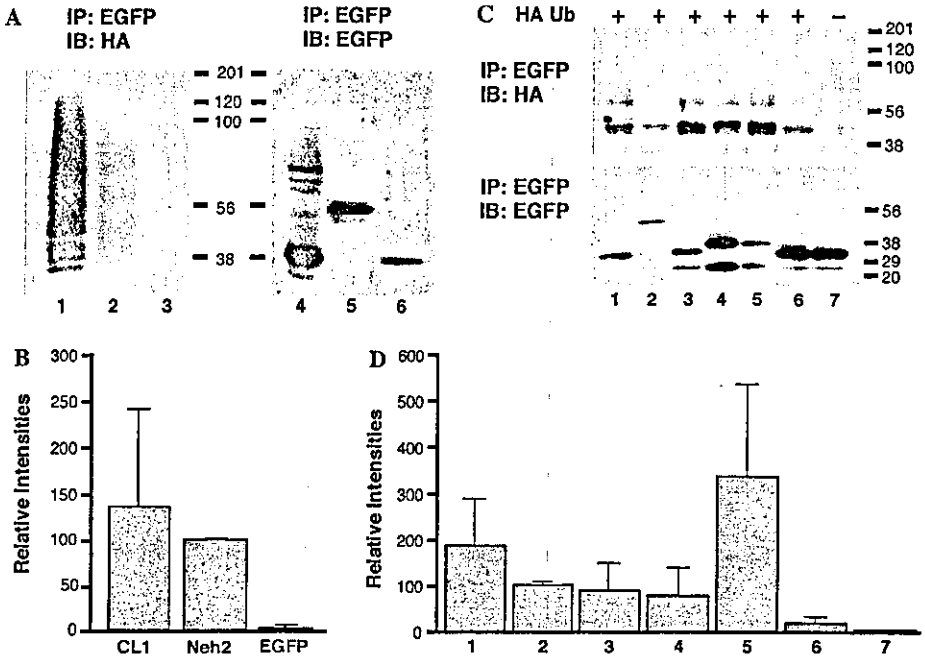

Fig. 5. Neh2-NT mediates ubiquitination. (A) 293T cells were transiently transfected with CL1-EGFP (lanes 1 and 4), Neh2-EGFP (lanes 2 and 5) or EGFP (lanes 3 and 6) expression plasmids along with HA-tagged ubiquitin expression vector. Cell lysates were immunoprecipitated by anti-EGFP antibody. Subsequently, precipitated fractions were subjected to immunoblot analysis by anti-HA antibody (left panel) or anti-EGFP antibody (right panel). (B) Band intensity of (A) was quantitated by densitometric analysis. Band intensity obtained by anti-HA antibody was divided by those obtained by anti-EGFP antibody. The relative mean values of three independent experiments were presented with standard error of means (SEM). The mean value of Neh2-EGFP was set as 100. (C and D) 293T cells were transiently transfected with expression plasmids for CL1-EGFP (lane 1), Neh2-EGFP (lane 2), Neh2-NT-EGFP (lane 3), Neh2-CT-EGFP (lane 4), Neh2-NT-LA-EGFP (lane 5) or EGFP (lanes 6 and 7) with (lanes 1–6) or without (lane 7) HA-tagged ubiquitin expression vector. Data were treated as in (A and B).

Neh2-CT were ubiquitinated to the same extent as wild-type Neh2-EGFP (Figs. 5C and D). In contrast, the ubiquitination of Neh2-NT-LA-EGFP, which was shown to have a half-life >8 h, was markedly enhanced (Figs. 5C and D). Therefore, we failed to correlate the extent of ubiquitination with the degradation activity of Neh2.

Discussion

In this study, we have identified a functionally important conservation of amino acids in Neh2-NT that is distinct from the previously characterized DIDLID element. We have called this newly identified element the DLG motif, and report that it is conserved in a subset of CNC factors that possesses ETGE motif for the Keap1 binding. We found that both deletion of Neh2-NT and mutation of the DLG motif largely abolished Keap1-mediated Nrf2 degradation and at the same time decreased the binding affinity of Nrf2 for Keap1. On the other hand, when fused to a reporter protein, Neh2-NT directed the degradation in the nucleus in a DLG motif-dependent, but Keap1-independent, manner. However, the mutation of DLG sequence did not largely affect the half-life of native Nrf2 in the nucleus under oxidative stress. These results

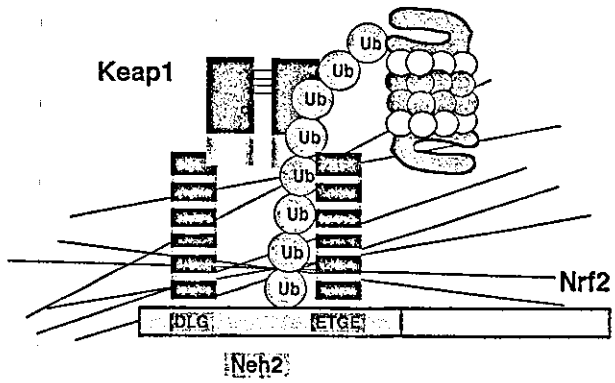

Fig. 6. Schematic model of Keap1-mediated Nrf2 degradation. Keap1 dimerization through BTB domain is essential to repress Nrf2 activity [37,38]. Both the DLG sequence and ETGE motif are necessary to cooperatively bind to Keap1 and therefore, are required for Keap1-mediated proteasomal degradation of Nrf2.

demonstrated that Neh2-NT, via the DLG motif, plays critical roles in the Keap1-mediated, proteasomal degradation of Nrf2 in the cytoplasm, but is dispensable for Nrf2 degradation in the nucleus.

At the amino terminus of Neh2, a conserved amino acid region called the DIDLID element was previously identified in a subset of CNC-related proteins, including Nrf1, Nrf2, and SKN-1 (Fig. 2B) [29,32,33]. DIDLID element of SKN-1 was previously shown to act as the

transactivation domain of SKN-1 [35]. Our sequence comparisons have however, shown that the DIDLID element is not conserved in *Drosophila* CncC, but that the region we refer to as the DLG motif is highly conserved among CNC bZIP factors which also possess an ETGE motif (Fig. 2A). Interestingly, on one side of the α-helix that DLG is predicted to form we have found a preponderance of leucine residues (Fig. 2C). The requirement of a cluster of leucine residues is reminiscent of short helical protein–protein interaction modules, such as LxxLL motif characterized in nuclear receptor co-activators [36]. Thus, it is likely that the ETGE and DLG motifs may work cooperatively in Keap1-mediated function in a subset of CNC factors.

We previously demonstrated that the C-terminal region of Neh2 is essential for the Nrf2 binding to Keap1 in a yeast two-hybrid assay [19]. Furthermore, a reverse two-hybrid screening executed in yeast identified ETGE motif as an indispensable Keap1 binding motif in Nrf2 [29]. Our present results argue that in addition to ETGE, the DLG motif is another important element for the stable interaction between Nrf2 and Keap1. We envisage that the DLG and ETGE motifs cooperatively bind to Keap1 (Fig. 6). In this hypothesis, the ETGE motif may serve as a primary Keap1 binding site, whereas the DLG motif as a secondary Keap1 binding site. Hence after the binding of Neh2 to the primary ETGE site of Keap1, the second step of the binding will initiate and Neh2 DLG binds to Keap1. Consequently, this cooperative binding secures a stable Keap1–Nrf2 interaction. This speculation may help to explain why $Nrf2^{E82G}$ mutant was not repressed at all by Keap1 in the assay tested. Furthermore, Nrf2-ΔNeh2-NT and Nrf2-LA, with an intact EGTE motif, coprecipitated with Keap1 albeit in a weaker manner than wild-type Nrf2. It has been shown that homodimerization of Keap1 is required to stably repress Nrf2 activity [37,38]. While the mechanism is not clear at present, it is intriguing to elucidate the relationship between Keap1 homodimerization and stable binding of Keap1 to Nrf2 (Fig. 6).

Because Neh2-NT mediates Keap1-independent proteasomal degradation in the nucleus when linked to EGFP in a DLG sequence-dependent manner, it is surprising that DLG sequence is dispensable for Nrf2 degradation in the nucleus under oxidative condition. Quite recently, McMahon et al. [39] reported that Neh6 domain can compensate for the Nrf2 degradation in the nucleus in the absence of Neh2.

Although our results argue for the hypothesis that Neh2-NT is essential for the cooperative binding of Nrf2 to Keap1, we cannot absolutely exclude the possibility that Neh2-NT may recruit some ubiquitin ligase activity and thereby cooperate in the Keap1-enhanced Nrf2 degradation. Actually, Neh2-NT can recruit ubiquitin ligase activity when expressed as a chimeric protein in the nucleus (Figs. 5C and D). However, in this case, ubiquitination does not occur on lysine residues as neither Neh2-NT nor EGFP contains this amino acid. Ubiquitination might be achieved through the N-terminus-dependent ubiquitination pathway [40]. In this respect, it is of note that Keap1 enhances Nrf2 degradation by post-ubiquitination mechanism in Cos1 [26] and Cos7 cells (data not shown). Therefore, Neh2-NT might regulate ubiquitination independently from Keap1 that will work cooperatively with a Keap1-mediated Nrf2 degradation mechanism. Although Neh2 mediates ubiquitination in the chimeric protein, the DLG motif is not required for ubiquitination in our assay conditions (Figs. 5C and D). Therefore, another region besides the DLG motif might be involved in the regulation of ubiquitination. It is apparent that further analysis is required to provide a full understanding of the role of Neh2-NT in Keap1-enhanced degradation of Nrf2.

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Serial Review: EpRE and Its Signaling Pathway
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MOLECULAR MECHANISM ACTIVATING Nrf2–Keap1 PATHWAY IN REGULATION OF ADAPTIVE RESPONSE TO ELECTROPHILES

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Abstract—Electrophile responsive element (EpRE)-mediated gene induction is a pivotal mechanism of cellular defense against the toxicity of electrophiles and reactive oxygen species (ROS). Nrf2, which belongs to the cap'-n'-collar family of basic region-leucine zipper transcription factors, has emerged as an essential component of an EpRE-binding transcriptional complex. Detailed analysis of the regulatory mechanism governing Nrf2 activity led to the identification of Keap1, which represses Nrf2 activity by directly binding to the N-terminal Neh2 domain. Keap1 interaction with Neh2 leads to the sequestration of Nrf2 in the cytoplasm and to the enhancement of Nrf2 degradation by proteasomes conferring tight regulation on the response. Electrophiles act to counteract sequestration of Nrf2 by Keap1 and provoke Nrf2 activation. Constitutive activation of Nrf2-regulated transcription in *Keap1* knockout mice clearly demonstrated that the disruption of Keap1 repression is sufficient for the activation of Nrf2. These observations indicated that the mechanism that modulates Nrf2–Keap1 interaction is pivotal for the cellular sensing mechanism for electrophiles. Recent analyses argue that the redox mechanism that modifies cysteine residues of Keap1 governs the Keap1–Nrf2 interaction and therefore is critical for sensing of electrophiles.

Keywords—Electrophile responsive element, NF-E2-related factor 2, Keap1, Proteasome, Phosphorylation, Free radicals

INTRODUCTION

Reactive electrophiles generated during food metabolism or in the pathological process directly or indirectly disturb the physiological function of cellular macromolecules such as DNA, protein, or lipids and contribute to the pathogenesis of various diseases including cancer, neurodegenerative diseases, atherosclerosis, and aging [1–3]. To counteract these insults, cells have acquired, during evolution, an intricate mechanism of defense against this toxicity. A battery of genes encoding detoxifying and antioxidative stress enzymes/proteins are coordinately induced on exposure to electrophiles and reactive oxygen species (ROS) [4,5]. 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 target genes [6,7]. Genes encoding a subset of drug metabolizing enzymes, such as glutathione *S*-transferases (GSTs) [6] and NAD(P)H-quinone oxidoreductase 1 (NQO1) [7], have been shown to be under ARE/EpRE regulation, along with a subset of antioxidant genes, such as heme oxygenase 1 (HO-1) [8], the subunits of γ-glutamylcysteine synthetase (γ-GCS) [9], and thioredoxin [10].

A variety of chemicals activating the EpRE pathway are classified into nine structurally dissimilar inducers that include, for example, Michael reaction acceptor, isothiocyanates, and hydroperoxides [11]. Considering this great structural diversity among inducers, a mechanism of action requiring interaction with a structurally complementary receptor seemed unlikely. The only

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apparent universal attribute of these inducers is their ability to react with thiol/disulfide groups by alkylation, oxidation, reduction, or thiol interchange [12]. These observations point to the hypothesis that highly reactive cysteines in a protein(s) are sensors to these chemicals [12].

Nrf2/ECH (NF-E2-related factor 2 [13] or chicken erythroid-derived CNC-homology factor [14]) was recently identified as the major regulator of ARE-mediated gene expression [15–17]. Nrf2/ECH belongs to the cap'-n'-collar (CNC) family of transcription factors that share a highly conserved basic region-leucine zipper (bZIP) structure (Fig. 1) [18]. Nrf2 requires a member of the small Maf proteins as an obligatory partner molecule for binding to their cognate DNA sequence [14]. Through *Nrf2* gene targeting analysis, we demonstrated that Nrf2 coordinately regulates a battery of genes encoding drug-metabolizing enzymes and antioxidant proteins [15,17]. Recent analysis in *Nrf2* knockout mice ($Nrf2^{-/-}$ mice) has expanded the range of Nrf2 target genes to include NADPH-generating enzymes such as malic enzymes and glucose-6-phosphate dehydrogenase [19,20], phase 3 detoxifying enzymes such as MRP1 [21], and a group of 26 S proteasome subunits [22,23]. Because of the lack of this coordinated stress response, $Nrf2^{-/-}$ mice are highly susceptible to the acute toxicity generated by acetaminophen [24,25], butylated hydroxytoluene [26], or hyperoxia [27] and to carcinogenesis induced by benz[*a*]pyrene [28]. $Nrf2^{-/-}$ mice experience higher levels of DNA adduct formation provoked by diesel exhaust particle [29], aflatoxin [30], and benz[*a*]pyrene [31].

Detailed analysis of Nrf2 activity and structure revealed that the Neh2 domain of Nrf2 is an evolutionary conserved regulatory domain of Nrf2 [32]. Subsequently, we identified Keap1 (Kelch-like ECH-associating protein 1) as a direct binding partner of Neh2 through a yeast interaction screen (Fig. 1) [33].

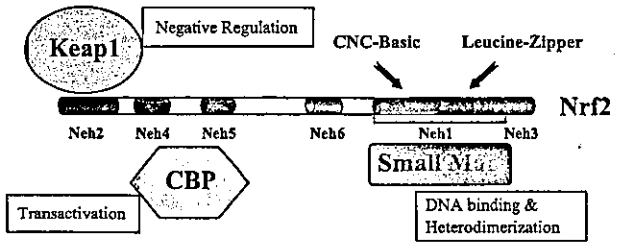

Fig. 1. Nrf2 regulatory network. Nrf2 has six highly conserved protein regions called Neh1 to Neh6 (Nrf2-ECH homology). At its C terminus, Nrf2 protein has a basic leucine zipper structure for dimerization with small Maf proteins and for binding to the ARE. At its N terminus, Nrf2 has a Neh2 domain, which is most highly conserved among species, and two activation domains, Neh4 and Neh5. Keap1 binds Neh2, whereas Neh4 and Neh5 cooperatively bind CBP to activate transcription.

DOMAIN ARCHITECTURE OF Keap1

Murine Keap1, a 624-amino-acid polypeptide, comprises five domains: (1) the N-terminal region (NTR); (2) the BTB domain, an evolutionarily conserved protein–protein interaction domain found in actin-binding proteins and zinc finger transcription factors; (3) the intervening region (IVR); (4) the double glycine repeat (DGR) or Kelch domain; and (5) the C-terminal region (CTR). Keap1 DGR domain possesses six double glycine repeats and is predicted to form a six-bladed β-propeller structure [34]. This protein family, with a similar structural composition, is growing and there are at least 71 BTB/Kelch proteins encoded in the human genome [35]. Keap1 binds Neh2 and actin through the DGR domain [33,36]. The subcellular localization of endogenous Keap1 is not well understood, but it appears to localize at the cytoskeleton in the nuclear periphery, colocalizing with F-actin [36]. Velichkova and Hasson reported that human Keap1 localizes in adhesion complexes [37]. On the other hand, Zipper and Mulcahy reported that homodimerization of Keap1 BTB domain is required to sequester Nrf2 in cytoplasm [38].

Keap1 IS A SATURATABLE REPRESSOR OF Nrf2

Several key features have emerged from an extensive study of the molecular mechanisms of Nrf2 activation by electrophiles and ROS. In the cotransfection analysis in cell culture, the concomitant expression of Keap1 sequesters Nrf2 in the cytoplasm and represses Nrf2 transactivation activity [33]. Treatment of the cells with electrophiles liberates Nrf2 from Keap1 repression with subsequent translocation of Nrf2 into the nucleus and activation of transcription. On the other hand, forced overexpression of Nrf2 alone gave rise to ARE-reporter gene transcription [14] or switched on endogenous target genes in zebrafish [39]. These results suggest that the overexpression of Nrf2 can saturate the repressive activity of endogenous Keap1 and activate transcription.

Keap1 ENHANCES PROTEASOMAL Nrf2 DEGRADATION UNDER HOMEOSTATIC CONDITIONS

Accumulating evidence has shown that activation of Nrf2 accompanies the Nrf2 accumulation in total cell lysates [40–45]. Furthermore, Nrf2 nuclear accumulation and upregulation of Nrf2 target genes require new protein synthesis [44]. Based on these observations, several laboratories have demonstrated that Nrf2 turns over rapidly by proteasome [40–45]. Treatment of the cells with electrophiles significantly prolongs the half-life of Nrf2 [42–45]. Using Nrf2–LacZ knock-in mice, we demonstrated that the N-terminal region of Nrf2 (i.e., 1–317 amino acids), in combination with a nuclear localization

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

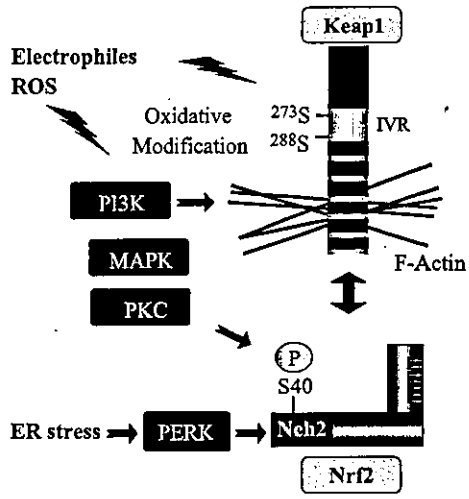

Fig. 3. Mechanisms of Nrf2 liberation from Keap1. Electrophiles provoke Nrf2 phosphorylation via MAPK, PKC, or PI3K or directly interact with Keap1-reactive cysteines. These modified Keap1 and Nrf2 cannot bind with each other as efficiently as under homeostatic conditions. The accumulation of unfolded 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.

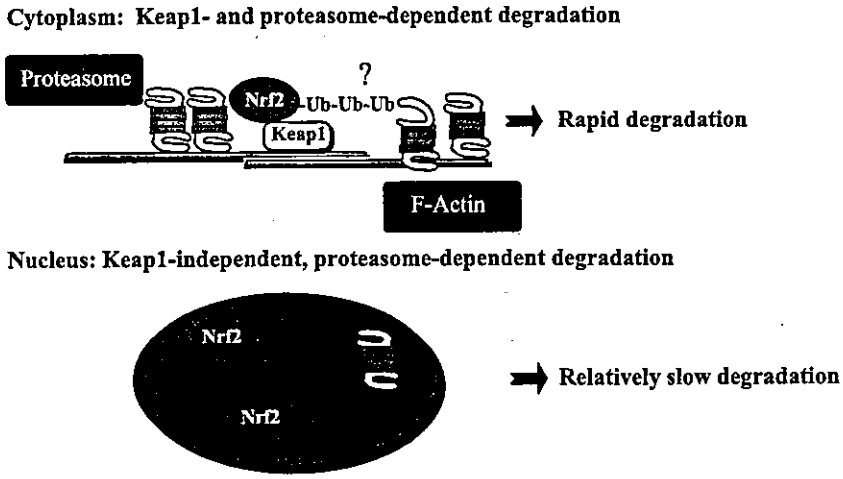

Fig. 2. Two modes of proteasome-dependent Nrf2 degradation. Under homeostatic conditions, Nrf2 is subjected to Keap1-dependent proteasomal degradation in cytoplasm. In cells treated with electrophiles, Nrf2 translocates into the nucleus and is degraded by Keap1-independent proteasomal degradation. The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

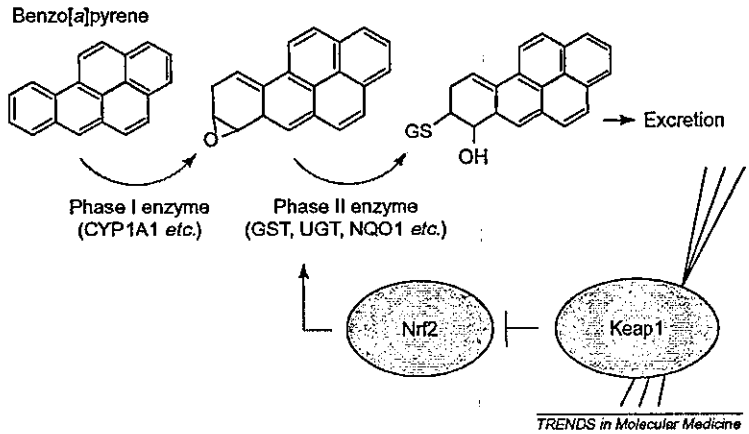

Figure I. The biotransformation of xenobiotics. Xenobiotics are oxidized by the P450 mono-oxygenase system, which includes CYP1A1 and CYP1A2, and is referred to as Phase I. The products of Phase I reactions are often electrophilic and reactive, resulting in the harmful modification of DNA and proteins 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.

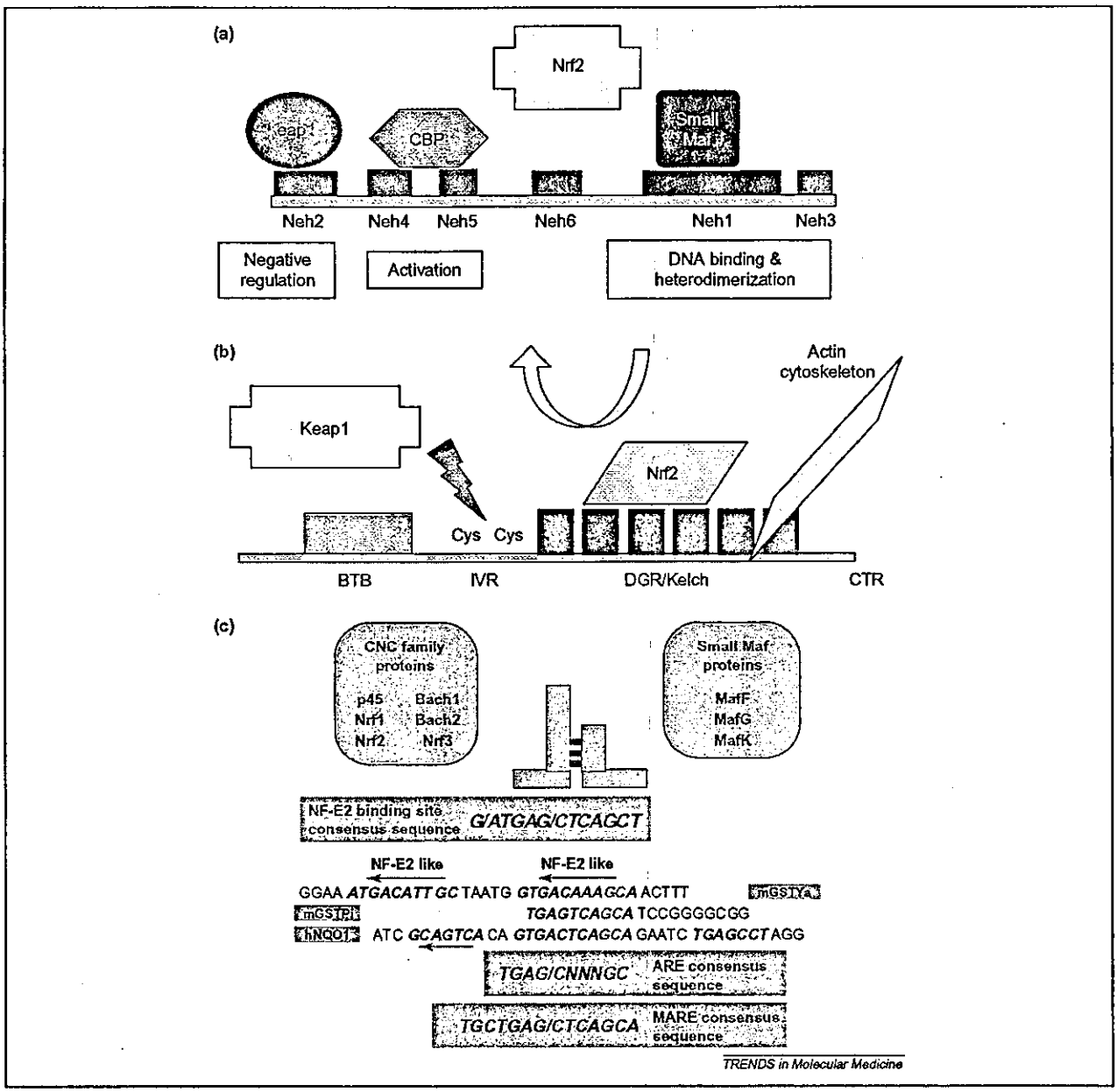

Figure 1. Functional domains identified in Nrf2 and Keap1 molecules and antioxidant-responsive elements (AREs) bound by 'cap-'n'-collar' (CNC)-small Maf heterodimers. (a) Domain structure of Nrf2. Six functional Neh [Nrf2–ECH (erythroid cell-derived protein with CNC homology) homology] units were identified 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

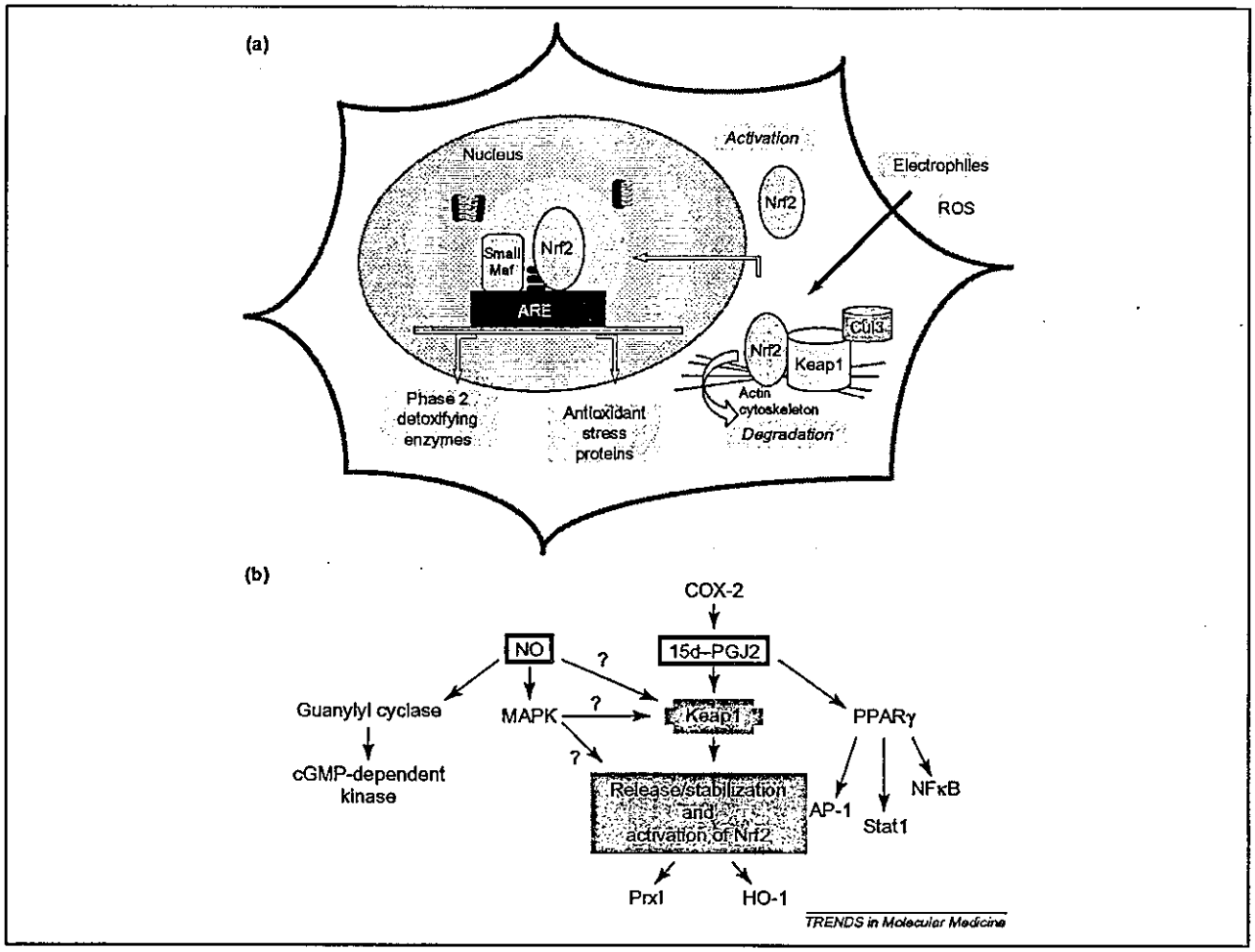

Figure 2. Schematic illustration of the molecular mechanisms of the Nrf2–Keap1 regulatory system and its endogenous activating signals. (a) In unstimulated conditions, Nrf2 is tethered onto actin fibers in the cytoplasm through Keap1 and degraded by proteasomes. Thus, the transcription levels of Nrf2 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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