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FORUM REVIEW ARTICLE

Discovery of the Negative Regulator of Nrf2, Keap1: A Historical Overview

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Abstract

An antioxidant response element (ARE) or an electrophile responsive element (EpRE) regulate the transcriptional induction of a battery of drug-detoxifying enzymes that are protective against electrophiles. Based on the high similarity of the ARE consensus sequence to an erythroid gene regulatory element NF-E2 binding site, we have found that the transcription factor Nrf2 is indispensable for the ARE-mediated induction of drug-metabolizing enzymes. Recent genome-wide analysis demonstrated that Nrf2 regulates hundreds of genes that are involved in the cytoprotective response against oxidative stress. In-depth analysis of Nrf2 regulatory mechanisms has led us to the discovery of a novel protein, which we have named Keap1. Keap1 suppresses Nrf2 activity by specifically binding to its evolutionarily conserved N-terminal Neh2 regulatory domain. In this review article, we summarize the findings and observations that have lead to the discovery of the Nrf2–Keap1 system. Furthermore, we briefly discuss the function of the Nrf2–Keap1 system under the regulation of the endogenous electrophilic compound 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2. We propose that Nrf2–Keap1 plays a significant physiological role in the response to endogenous, environmental, and pharmacological electrophiles. *Antioxid. Redox Signal.* 13, 1665–1678.

Introduction

XENOBIOTICS OCCURRING IN THE MODERN ENVIRONMENT are metabolized by a battery of detoxifying enzymes (13). This biotransformation process is conventionally divided into phase I and phase II reactions (Fig. 1) (13). Phase I reactions functionalize compounds by oxidation, reduction, and hydrolysis reactions; these reactions typically involve cytochrome P450 monooxygenases (CYPs). On the other hand, phase II reactions are conjugation reactions of phase I metabolites with endogenous ligands, such as glutathione, glucuronic acid, and sulfate, and are catalyzed by glutathione *S*-transferases (GSTs), UDP-glucuronosyl transferases (UGTs), and sulfotransferases, respectively (120). In phase II reactions, phase I metabolites are converted into more readily excretable or less pharmacologically active compounds. Therefore, phase I reactions often act as the activation steps of xenobiotic metabolism, as seen in the metabolism of carcinogens such as dimethylbenzanthracene (DMBA). Phase II reactions are generally true detoxification steps (Fig. 1).

Electrophiles are substances that possess electron-deficient centers and thereby make adducts with nucleophilic cellular proteins, DNA, and other substances. Therefore, certain electrophiles act as mutagens or cause acute injury when encountered in large amounts (67, 72). Electrophiles, or the substances that are converted into electrophiles by phase I reactions, are mainly conjugated with glutathione by GSTs, which include seven classes of cytosolic and one class of microsomal subunits, and only one mitochondrial subunit in mammalian species (25). Therefore, glutathione conjugation reactions play a central role in the detoxification of electrophiles. In general, detoxification enzymes, such as GSTs in animals, recognize nonpolar substrates with promiscuous specificity and low affinity, but they compensate for this low affinity and specificity by expressing large amounts of the enzymes (43). For example, the GSTs, which have a relatively low catalytic efficiency (*i.e.*, around 500 $\mu M\ min^{-1}\ mg^{-1}$) even for one of the best substrates (*e.g.*, 1-chloro-2,4-dinitrobenzene), compose up to 10% of cytosolic proteins in some organs. This seemingly inefficient system enables animals to cope with a nearly infinite number of structurally divergent chemicals that animals eat from plants or other species and inadvertently uptake from the environment. Each of the GST reactions in itself is a low-cost reaction because it does not require high-energy substrates, such as 5′-phosphoadenosine-3′-phosphosulfate and UDP-glucuronic acid. The other important feature of the animal detoxification system lies in its inducibility by substrates, as described below.

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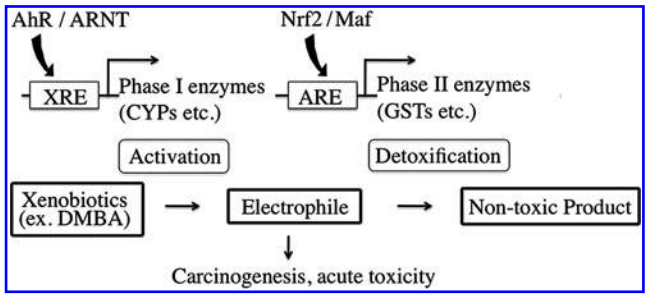

FIG. 1. Sequential detoxification of carcinogens by phase I and phase II detoxification enzymes. Xenobiotics such as DMBA are metabolized to electrophilic intermediates by CYPs and then conjugated with GSH by GSTs. DMBA induces the expression of CYPs via the AhR–XRE pathway, and electrophiles induce GSTs by the Nrf2–ARE pathway. Enzyme induction contributes to the efficient detoxification of the original compound, DMBA. Nrf2 is known to induce many subunits of GSTs (6, 38, 64), as well as a small fraction of UGTs (30) and a phenol sulfotransferase (118). Please note that the phase I-phase II classification paradigm has its limitations. as discussed in Reference 12.

Discovery of an Inducible Cytoprotective System that Detoxifies Electrophiles

A wide variety of chemicals protect rodents against neoplastic, mutagenic, and other toxic effects of carcinogens. In the early 1970s, Wattenberg and colleagues established that the phenolic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), which are widely used in food additives in the USA, prevent tumor formation in mice after exposure to various carcinogens of distinct chemical classes (114). Prevention is observed especially when antioxidants are administered prior to carcinogen challenge. The phenomenon of one chemical agent preventing the action of multiple carcinogens is called "chemoprotection" rather than chemoprevention (103, 104). While the precise mechanism of BHA action in the tumor preventative process is unclear at present, available data suggest that BHA action is largely indirect and attributable to the induction of phase II enzymes that enhance the metabolism and deposition of the reactive intermediates of the toxic compounds, such as electrophiles and reactive oxygen species (ROS). Indeed, in the mouse and rat liver, as well as intestine, BHA markedly increases the expression of GSTs and other detoxification enzymes at the transcriptional level (82, 85).

Transcriptional Regulation of Detoxifying Enzymes

Polycyclic aromatic hydrocarbons (*e.g.*, benzo[*a*]pyrene), β-naphthoflavone, and 2′3′7′8′-tetrachlorodibenzo-*p*-dioxin (TCDD) increase both phase I and phase II enzymes (88). In contrast, monofunctional inducers, such as diphenols and the Michael reaction acceptor diethylmaleate (DEM), primarily increase phase II enzymes. The former are called bifunctional inducers and the latter monofunction inducers. Talalay and colleagues first demonstrated from the analysis of diphenols and diamines that the monofunctional inducers are redox-labile chemicals or electrophiles (*i.e.*, 1,2-diphenol and 1,4-diphenol are the inducers, but 1,3-diphenol is not), indicating the involvement of redox chemistry in phase II enzyme induction (87). In contrast, bifunctional inducers first increase phase I enzymes via the action of aromatic hydrocarbon receptor (AhR) to generate electrophilic metabolites, similar to monofunctional inducers, and then promote the induction of phase II enzymes (88). Later, it was found that the AhR regulates the induction of *Cyp1A1* by TCDD, polycyclic aromatic hydrocarbons, and β-naphthoflavone through xenobiotic responsive elements (XRE) (99).

Discoveries of Responsive Elements for Electrophiles

In 1990, Pickett and colleagues first discovered a regulatory element distinct from XRE that is responsive to β-naphthoflavone and *t*-butylhydroquinone (*t*-BHQ) in the promoter region of rat *Gst-Ya*. They named it the antioxidant responsive element (ARE) (93). The responsive element was further characterized in the same laboratory by point-mutation analysis, and the core sequence was RGTGACNNNGC, where R represents purine and N represents any base (Fig. 2A) (94). Similar responsive elements were also found in human and mouse (20, 60). In the case of the mouse, the responsive element was called the electrophile responsive element (EpRE) (20). Hereafter, we will use the more prevalent name, ARE, in this article. Because BHA is supposedly metabolized into the potent electrophile *t*-BHQ *in vivo*, BHA induction of a battery of detoxification enzymes is considered a cytoprotective response against electrophiles (86). Subsequent analyses have demonstrated that ARE is also important for the inducible expression of a set of antioxidant enzymes by electrophiles or ROS (84, 85), indicating that ARE regulates a wide-ranging metabolic response to oxidative stress. ARE-regulated detoxification enzymes are not always phase II detoxification enzymes. NAD(P)H:quinone oxidoreductase 1 (NQO1) and microsomal epoxide hydrolase (EH-1) are phase I enzymes, but they are cytoprotective against and inducible by electrophiles. In this respect, Talalay *et al*. called ARE-regulated and electrophile-inducible cytoprotective genes "phase 2" enzymes (103). Hereafter, we use the Arabic-numerical "phase 2" enzyme to refer to the Talalay's meaning of a "phase 2" enzyme and the Roman-numerical "phase II" enzyme designation to refer to the classical conjugating enzymes of the detoxification pathway.

Talalay and colleagues examined the structure–function relationship of phase 2 inducers in the ARE response. To systemically analyze the inducer property, they employed a reporter construct in which the mouse *Gst-Ya* ARE is linked to a growth hormone reporter gene (83). They measured the concentration of the inducers that doubled the reporter gene expression and simultaneously measured the concentrations that doubled the endogenous NQO1 activity, and they ranked the potencies of the inducers. By this method and subsequent analysis, phase 2 inducers are now classified into 10 chemically distinct classes: I) oxidizable diphenols, phenylendiamines, and quinones; II) Michael reaction acceptors; III) isothiocyanates; IV) heavy metals; V) trivalent arsenicals; VI) dithiolethiones; VII) hydroperoxide; VIII) vicinal dimercaptans; IX) thiocarbamates; and X) polyenes (16). They concluded that the only common feature in this diverse class of chemical inducers lies in their reactivities with sulfhydryls. From these observations, they predicted the existence of a common sensor molecule with highly reactive cysteines that responds to the phase 2 enzyme inducers.

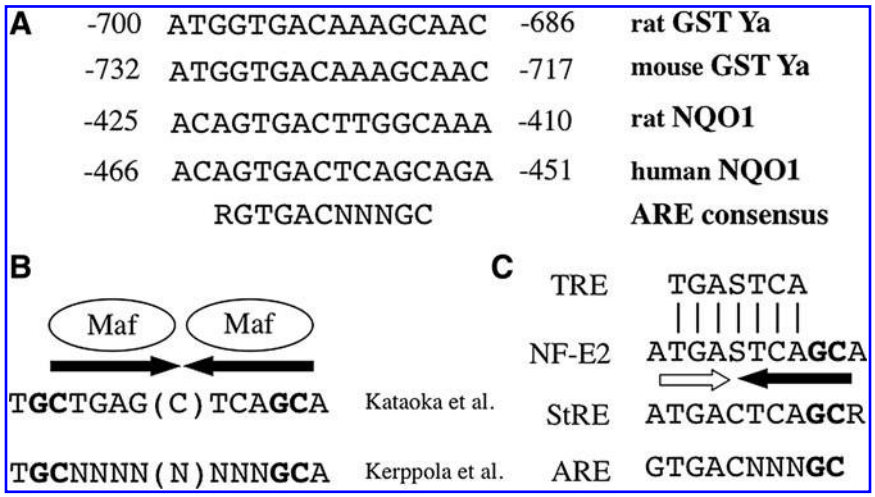

FIG. 2. Similarity of AREs to NF-E2 and MARE. (**A**) AREs identified in the regulatory regions of GSTA1 and NQO1 from several species are aligned. Regulatory sequences of each gene are quoted from the original paper as follows: Rat GST Ya (94), mouse GST Ya (20), rat NQO1 (94), and human NQO1 (60). The ARE consensus sequence originally proposed by Rushmore *et al*. (94) is shown as RGTGACNNNGC (R: A or G, N: any nucleotide), but a functional ARE consensus sequence (TMAnnRTGAYnnnGCRwwww; M: A or C, Y: C or T, W: A or T) (112) or variant ARE (RTKAYnnnGCR; K: G or T) (19) were more recently proposed. (**B**) Sequence of the Maf recognition element (MARE). Kataoka *et al*. reported the MARE as the palindromic TGCTGA GTCAGCA (TRE-type MARE) or TGCTGAGCTCAGCA (CRE-type MARE), and Kerppola *et al*. reported the Maf binding sequence as $TGC(N)_{7\text{-}8}$ GCA. The GC dinucleotides are written in bold to emphasize the importance of these bases for Maf binding. (**C**) Similarity between TRE, NF-E2, StRE, and ARE. The *filled arrow* indicates the half-site recognized by MafK, and the *open arrow* indicates the half-site recognized by NF-E2p45 when NF-E2 binds to the NF-E2 binding sequence. The GC dinucleotides are written in bold to emphasize the importance of these bases for Maf binding. R, purine nucleotides; S, a guanine or cytosine nucleotide; TRE, TPA-responsive element. This figure is modified from Reference 36.

CNC–Small Maf Heterodimers Bind to the Erythroid Regulatory Element NF-E2 Site

In mouse erythroleukemia cells, NF-E2 interacts with the NF-E2 binding site, which was first identified in the gene regulatory region of the porphobilinogen deaminase gene (66). An NF-E2 binding site was also found in the ferrochelatase gene regulatory region and the DNase I hypersensitive sites of the β-globin locus control region (73, 102). Subsequent biochemical purification revealed that NF-E2 is a heterodimer of basic leucine zipper (bZip) transcription factors composed of 45-kDa and 18-kDa subunits, the former being NF-E2p45 and the latter MafK, one of the small Maf proteins (sMafs) (2, 3, 29). The sMafs are members of the Maf family of transcription factors that are characterized by their specific bZip domain structure (Fig. 3) (71). The founding member of the Maf protein family is the v-Maf oncogene that was discovered as the transforming component of the avian masculoaponeurotic fibrosarcoma virus AS42 (77). c-Maf was identified as the cellular counterpart of v-Maf. Maf family proteins are divided into two subgroups: the large Maf proteins, c-Maf, MafA, MafB, and NRL (117), all of which contain a distinctive acidic domain that enables transcriptional activation; and the sMafs, which include MafF, MafG, MafK, and recently identified teleost MafT (Fig. 3) (71, 101).

The sMafs can heterodimerize with any of the CNC family transcription factors (70), Fos, and FosB (46, 98). The CNC family was named after the similarity of the bZip domain structure in this family of proteins to that of the Drosophila Cap'n'Color (CNC) protein (70). The CNC family includes NF-E2p45, Nrf1 (NF-E2–related factor 1), Nrf2, Nrf3, Bach1 (BTB and CNC homolog 1), and Bach2 (Fig. 3) (2, 7, 54, 69, 80). Given that the sMafs lack a canonical transcription activation domain, CNC factors provide the heterodimer with a transcriptional regulatory function. The sMaf subunit is required for the high-affinity, sequence-specific DNA-binding activity of the CNC–sMaf heterodimer (70). In addition, each sMaf subunit can form a homodimer, and the homodimer can bind to certain NF-E2 binding sequences (such as the NF-E2 site in the chicken β-globin enhancer), albeit at a lower affinity (29, 39).

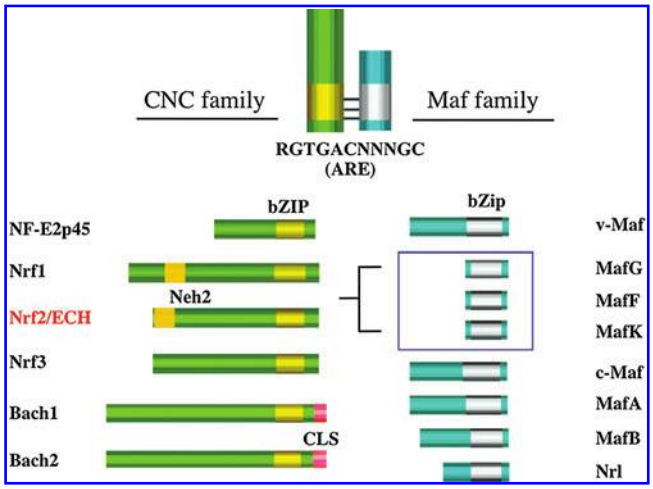

FIG. 3. Members of the Maf and CNC transcription factor family. Maf transcription factors are classified into large Maf proteins, consisting of c-Maf, MafA, MafB, and NRL; and small Maf proteins, comprising MafF, MafG, MafK and MafT. The CNC family comprises NF-E2p45, Nrf1-3, Bach1, and Bach 2. In the figure, only mammalian family members are shown. Nrf factors generally act as activators of transcription, and the Bach family act as repressors of transcription (70). CLS: cytoplasmic localization signal. This figure is modified from Reference 36. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

Small Mafs Play a Pivotal Role in ARE-Mediated Gene Regulation

The ARE core sequence (RGTGACNNNGC) shows significant sequence similarity to that of NF-E2 (TGAG/CT CAGCA), and the function of both elements requires the GC nucleotide outside of the core AP-1 or AP-1–like elements (Fig. 2) (66, 94). In the case of the heme oxygenase 1 (HO-1) gene (*Hmox1*), the upstream enhancer regions (*i.e.,* E1 and E2 enhancers) that are responsive to a variety of stresses, including oxidative stress, contain multiple responsive elements called stress responsive elements (StREs) (1). These StREs more closely resemble NF-E2 binding sequences than AREs (*i.e.,* core sequence conservation) (Fig. 2). Again, the GC dinucleotide is required for StREs to be responsive to most of the HO-1 inducers, including electrophiles (32, 84). These results suggest that factors other than AP-1 may regulate the cytoprotective responses involving AREs or StREs (32, 84).

Kataoka *et al*. determined the consensus recognition sequence for the Maf transcription factor and reported it to be a palindromic TGCTGACTCAGCA or TGCTGACGTCAGCA using the v-Maf bZip domain by a PCR-based selection method (Fig. 2B) (45). However, in their analysis, the core sequence is less stringently conserved compared to the outside GC sequence. Kerppola *et al*. used a similar method to determine that the consensus binding sequence for the Maf homodimer is $TGC(N)_{7\text{-}8}GCA$ and for the Fos-Maf heterodimer is $TGAC(N)_{3\text{-}4}GCA$ (51). These results indicate that members of the Maf family of transcription factors recognize the GC half-side of NF-E2 and that the core sequence is less important for Mafs to bind DNA. Considering these observations, we hypothesized that the heterodimeric transcription factors containing sMafs should regulate the ARE response. Given that sMafs mainly heterodimerize with CNC family transcription factors, these observations suggest that the heterodimer consisting of a CNC factor and a sMaf binds to the ARE and regulates the expression of phase 2 genes, pinpointing the pivotal roles of sMafs in ARE-mediated gene regulation. Recent crystal structure analysis of the MafG homodimer bound to DNA revealed the precise molecular mechanism by which Maf factors preferentially recognize adjacent GC residues instead of the core ARE sequence (57).

Regulation by Nrf2–sMaf Heterodimer of Phase 2 Gene Expression

Nrf2 was first identified in humans as a protein that recognizes the NF-E2 binding site of human β-globin genes (69). Its structure is shown in Figure 4. We independently cloned chicken Nrf2 (ECH; erythroid cell–derived protein with CNC homology) by sequence homology with the mouse NF-E2p45 from an anemic chicken peripheral blood cDNA library (39). The first indication of the involvement of Nrf2 in ARE response came from the finding of the Jaiswal group that Nrf transcription factors Nrf1 and Nrf2 positively regulated ARE-mediated gene expression in a co-transfection assay, whereas c-Fos and Fra1 negatively regulated this activity (109). Among the CNC family proteins, the expression level of Nrf2 is particularly high in the detoxification organs or tissues facing the environment, such as intestine, lung, and choroid plexus of the brain in mouse embryo (9). Chicken Nrf2 is also highly expressed in the kidney and intestine (39). Furthermore, chicken Nrf2 possesses markedly more potent transactivation activity against the NF-E2 binding site than NF-E2p45 in the quail fibroblast cell line QT6 (39). These observations suggest that Nrf2 might be a key player in phase 2 enzyme induction. To test this hypothesis, we carried out *Nrf2* gene targeting in mice.

The Phenotype of *Nrf2*-Knockout Mice

The *Nrf2*-KO mice develop normally and are fertile (9, 38). The only apparent phenotype is the decolorization of the upper teeth due to the iron transport defect of the enamel in mice on an ICR/129sv-mixed background (116). In older female mice on an ICR/129sv background, *Nrf2*-KO mice develop lupus-like nephritis (119). *Nrf2*-KO on a 129sv background develop more severe multiorgan autoimmune inflammation and vacuolar leukoencephalopathy (27, 62). Therefore, the phenotypes of *Nrf2*-KO mice differ from strain to strain.

We examined phase 2 enzyme induction by BHA in *Nrf2*-KO mice. The induction of phase 2 enzymes, such as GSTs, NQO1, and EH-1, by BHA in the liver and intestine was markedly attenuated in *Nrf2*-KO mice, demonstrating that Nrf2 coordinately regulates the inducible expression of phase 2 enzyme genes in mice (ref. 38 and our unpublished observation). Electrophoretic mobility shift assays showed that Nrf2 binds the ARE in the regulatory region of mouse *Gst-Ya* or *Nqo1* genes by forming a heterodimer with MafK (38). These results demonstrated that the Nrf2–small Maf heterodimer directly binds to AREs and activates the transcription of these genes *in vivo*.

The ARE has also been implicated in the regulation of antioxidative stress enzyme genes, including HO-1 and γ-glutamylcysteine synthetase (γGCS) (84, 85). It was previously reported that the expression levels of cystine/glutamate exchange transporter xCT and peroxiredoxin (Prx) I are inducible by electrophiles in mouse peritoneal macrophages, as well as in other tissues (Fig. 5) (33, 95, 113). Using a primary culture of peritoneal macrophages, we found that the induction of HO-1, xCT, and Prx I by electrophilic agents or ROS was affected in the *Nrf2*-KO macrophages, indicating that Nrf2 regulates a wide-ranging metabolic response to oxidative stress (34). Furthermore, Nrf2 regulates both basal and inducible expression of phase 2 enzyme genes in mouse liver and intestine (6, 24, 64). Recent microarray studies have identified hundreds of genes that are regulated in an electrophile- and Nrf2-dependent manner (Fig. 5) (23, 53, 58, 59, 61, 79, 90, 96).

Nrf2-KO mice are susceptible to chemical-induced carcinogenesis in the form of benzo[*a*]pyrene-induced forestomach tumors and BBN (*N*-nitrosobutyl (4-hydroxybutyl)amine)-induced urinary bladder tumors (30, 89). These tumors are mainly attributable to decreased expression of Nrf2-regulated drug-detoxifying enzymes in *Nrf2*-KO mice. Furthermore, the efficacy of the phase 2 inducer oltipraz for the prevention of benzo[*a*]pyrene-induced forestomach tumors and BBN-induced urinary bladder tumors was abrogated in *Nrf2*-KO mice (30, 89). These results suggest that tumor protection by phase 2 inducers is largely dependent on Nrf2. *Nrf2*-KO mice are also susceptible to oxidative stress–related diseases, such as hyperoxia- or BHT-induced acute lung injury (8, 10), cigarette smoke–induced emphysema, and acetaminophen-

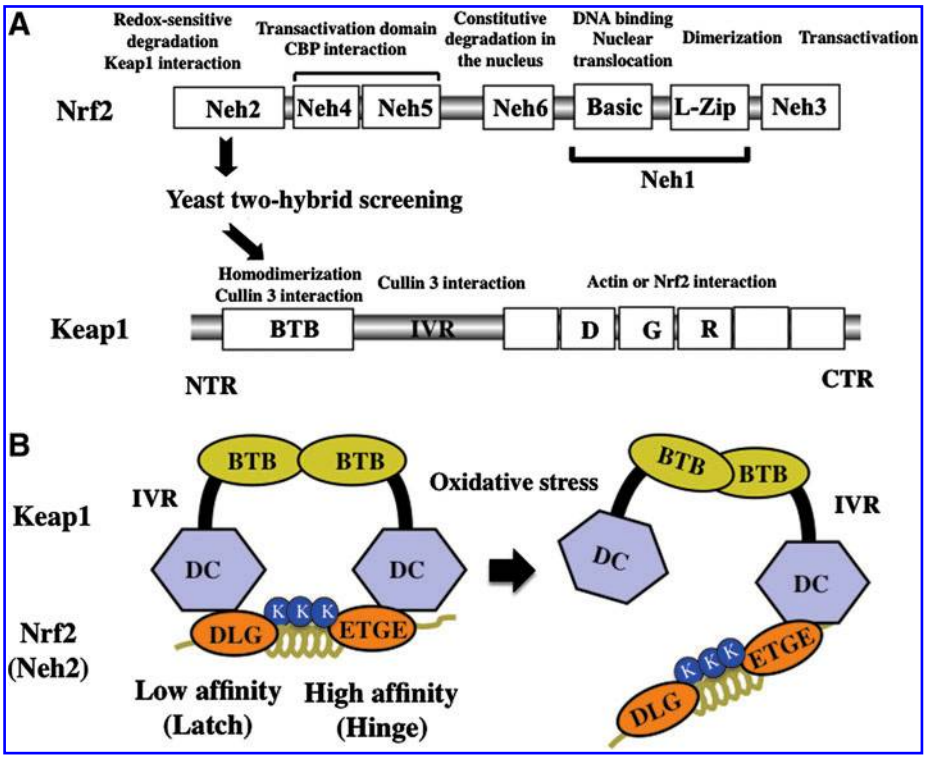

FIG. 4. Domain structures of Nrf2 and Keap1. (**A**) Nrf2 possesses six evolutionarily conserved domains called Neh1–6. Neh1 serves as the DNA binding and heterodimerization domain with small Maf proteins. Neh6 functions as a degron in the nucleus. The transactivation activity of Nrf2 lies in Neh4 and Neh5, and this activity can be transposed to the heterologous Gal4 DNA binding domain. Neh4 and Neh5 cooperatively bind CBP (48), and Neh5 is required for binding to BRG1 (121, 122). Neh3 is reportedly required for Nrf2 transactivation activity via interaction with CHD6 (76). Keap1 interacts with F-actin in fibroblasts (44). Dimerization through the BTB domain is required for Keap1 to repress Nrf2 (123). See text for details. This figure is modified from Reference 36. Basic, basic region; CTR, C-terminal region; IVR, intervening region; L-Zip, leucine zipper domain; NTR, N-terminal region. (**B**) Schematic representation of the two-site recognition model. The Keap1 DGR and CTR together comprise a six-bladed β propeller structure, shown as DC (DGR, CTR). Keap1 homodimerizes via its BTB domain and binds to the ETGE and DLG motifs of the Nrf2 Neh2. The ETGE motif is a high-affinity and the DLG motif is a low-affinity binding site for Keap1. The lysine residues that are ubiquitinated localize to one side of the intervening α-helix, enhancing Keap1-mediated ubiquitination. After oxidative stress, only the binding via the low-affinity site is disrupted by yet-unidentified mechanisms, and Nrf2 ubiquitination is inhibited. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

induced liver injury (18, 31). The above-mentioned phenotypes can be largely explained by the decreases in both basal and inducible expression of phase 2 enzymes. *Nrf2*-KO mice are also susceptible to drug-induced neurodegenerative disorders (5, 97) or inflammation, such as endotoxin shock (105), carrageenin- and elastase-induced lung inflammation (35, 40, 68) and dextran sulfate sodium-induced colitis (52). A more comprehensive review of these factors is available elsewhere (50).

Regulatory Mechanism of Nrf2: Neh2 Is an Evolutionally Conserved Domain that Regulates Nrf2 Activity

Although the Nrf2 DNA-binding activity to the StRE of *Hmox1* is markedly increased by treatment with electrophiles, ROS and cadmium in mouse peritoneal macrophages, these agents do not affect *Nrf2* mRNA levels (34). This result indicates that electrophilic agents or ROS activates Nrf2 at a post-transcriptional step in mouse macrophages.

To understand how Nrf2 activity is regulated, we performed a domain structure/function analysis of Nrf2. Comparison of human Nrf2 protein with chicken Nrf2 identified six highly conserved domains, which have been termed Neh (Nrf2-ECH homology) domains (Fig. 4) (41). Of the six Neh domains, the N-terminal Neh2 domain and Neh1 domain that encodes the bZip region show the most striking similarity among species. The Neh2 domain can be divided into two subdomains (47, 56). While the N-terminal subdomain (residues 16–31) contains hydrophobic amino acid residues that can potentially form an amphipathic α-helix (47, 106), the central portion of the domain is rich in hydrophilic residues that may also adopt an α-helical structure (106) (Fig. 6A).

Interestingly, we noted that deletion of the Neh2 domain resulted in a marked increase of Nrf2 activity in a co-transfection assay using a HD3 chicken erythroid cell line, indicating that Neh2 is a negative regulatory domain of Nrf2 (Figs. 6B and 6C) (41). The negative regulatory activity of the Neh2 domain was counteracted by simultaneous expression of the Neh2–GBD (GAL4 DNA binding domain) fusion protein as a decoy in HD3 cells (41). This result shows that Nrf2 activity is negatively regulated by the Neh2 domain through an interaction with an unknown repressor protein.

Discovery of Keap1

The results described above led us to search for the titratable negative regulatory activity of the HD3 cells. To this end, we performed a yeast two-hybrid screen using Neh2 as bait and identified a new protein, Keap1 (Kelch-like ECH-associated protein 1), which shows similarity to the human protein KIAA0132 of unknown function (41). Jaiswal's group cloned the rat homologue of Keap1 through the purification of Nrf2-interacting proteins from rat liver extracts and named it inhibitor of Nrf2 (INrf2) (14).

Keap1 shows structural similarity to the Drosophila protein Kelch and has two canonical protein interaction domains, BTB (bric-a-brac, tramtrack, broad complex) and Kelch (also called double glycine repeat or DGR) (Fig. 4A). The Keap1 DGR and

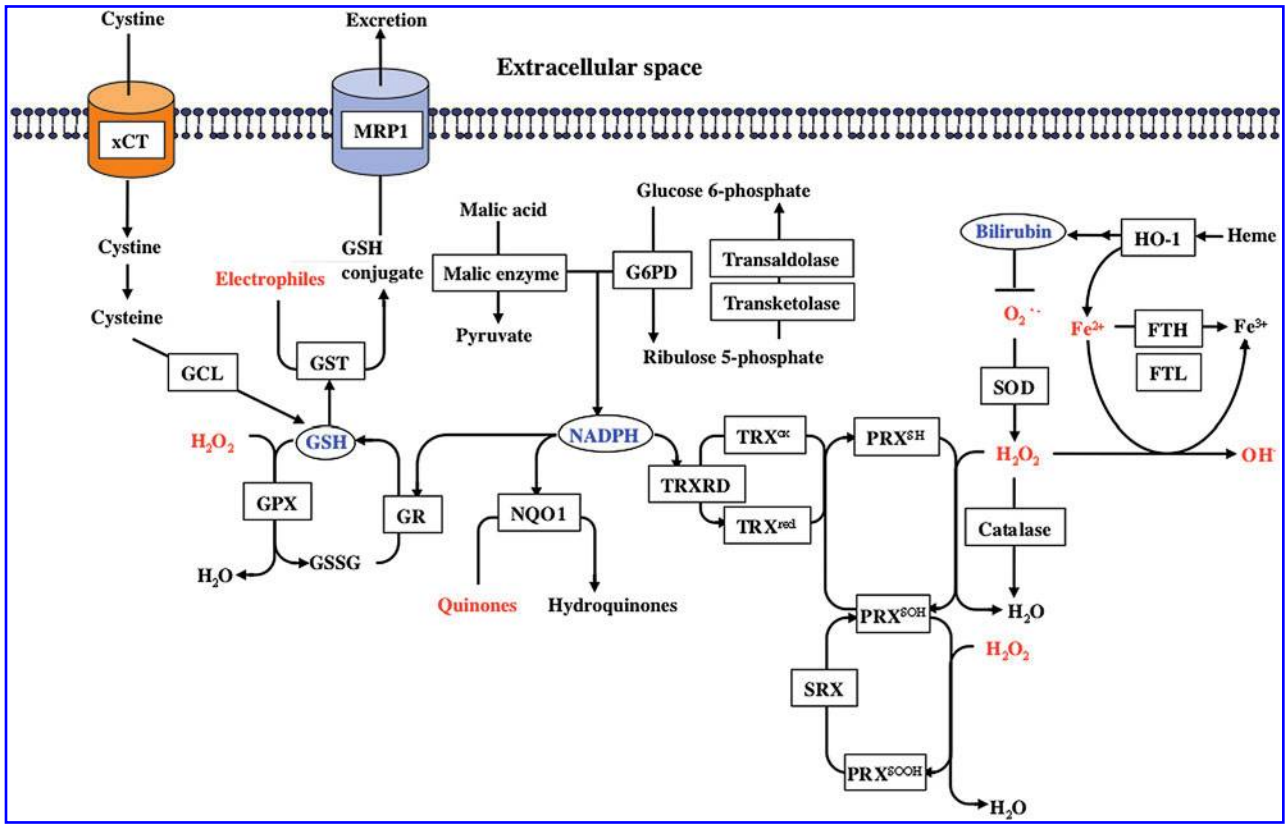

FIG. 5. Schematic representation of phase 2 genes. Nrf2 coordinately regulates the cytoprotective genes against electrophiles (phase 2 genes). The *boxed* genes indicate the genes that have been experimentally shown to be inducible by electrophiles in an Nrf2-dependent manner. Small-molecule antioxidants are written in *blue letters* and reactive intermediates in *red.* Nrf2 regulates multiple subunits of GSTs (6, 38, 64), and PRX I and PRX VI (11, 34). Although not indicated in the Figure, peroxiredoxin and glutathione peroxidase may also catalyze the two-electron reduction of peroxynitrite (108). xCT, cystine/glutamate transporter; GCL, glutamate–cysteine ligase; G6PD,: glucose-6-phosphate dehydrogenase; GPX, glutathione peroxidase; GR, glutathione reductase; GSTs, glutathione S-transferases; HO-1, heme oxygenase-1; MRP1, multidrug resistance–associated protein 1; NQO1, NAD(P)H quinone oxidoreductase 1; PRX, peroxiredoxin; SOD, superoxide dismutase; SRX, sulfiredoxin; TRX: thioredoxin; TXNRD: thioredoxin reductase. This figure is modified from Reference 36. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

CTR (C-terminal region) together comprise six-bladed β propeller structure (81) and directly interacts with two Nrf2 Neh2 motifs (*i.e.*, DLG and ETGE motifs), such that the Keap1 dimer recognizes one molecule of Nrf2 (two-site recognition model) (Figs. 4B and 6A) (106). The BTB domain is required for Keap1 homodimerization and therefore for repression of Nrf2 (123). On the other hand, Keap1 possesses 25 and 27 cysteines in human and mouse, respectively (41). Several of these cysteines are highly reactive cysteines with adjacent basic amino acids, consistent with the idea that Keap1 is the direct sensor molecule for electrophiles. Indeed, Talalay and colleagues subsequently demonstrated that the potencies of the chemicals to act as phase 2 inducers *in vivo* parallels their potencies to bind to recombinant Keap1 protein *in vitro*, indicating that Keap1 is a long-sought sensor molecule for phase 2 inducers (15).

Liberation of Nrf2 from Keap1 Repression Initiates the ARE Response

Keap1 negatively regulated Nrf2 activity in a reporter cotransfection transactivation assay (41). When both proteins were overexpressed in 293T cells, Keap1 co-localized in the cytoplasm with Nrf2, which otherwise accumulated in the nucleus. Importantly, this co-localization of Keap1 and Nrf2 was disrupted by the treatment of the cells with DEM. DEM allowed Nrf2 to accumulate in the nucleus even in the presence of co-transfected Keap1. These results suggest that Keap1 constitutively represses Nrf2 and that liberation from Keap1 repression leads to Nrf2 activation.

Another clue indicating that Keap1 is a key molecule for phase 2 induction came from the analysis of *Keap1*-KO mice (110). *Keap1*-KO mice die within 7–10 days of birth from hyperkeratosis in the esophagus and stomach, which leads to nutrient obstruction and eventually to severe ulceration of the stomach (110). In embryonic fibroblasts of *Keap1*-KO mice, Nrf2 is constitutively stabilized in the nucleus, and phase 2 genes, such as subunits of γGCS, are constitutively induced and are not further upregulated by electrophiles. In the liver of *Keap1*-KO mice, several subunits of GSTs are constitutively expressed. In general, these results unequivocally demonstrate that Keap1 acts as a critical negative regulator of Nrf2 *in vivo*.

A more detailed analysis was performed using hepatocyte-specific knockdown of *Keap1* by an albumin–Cre-loxP system (78, 79). The hepatocyte-specific conditional knockout mice

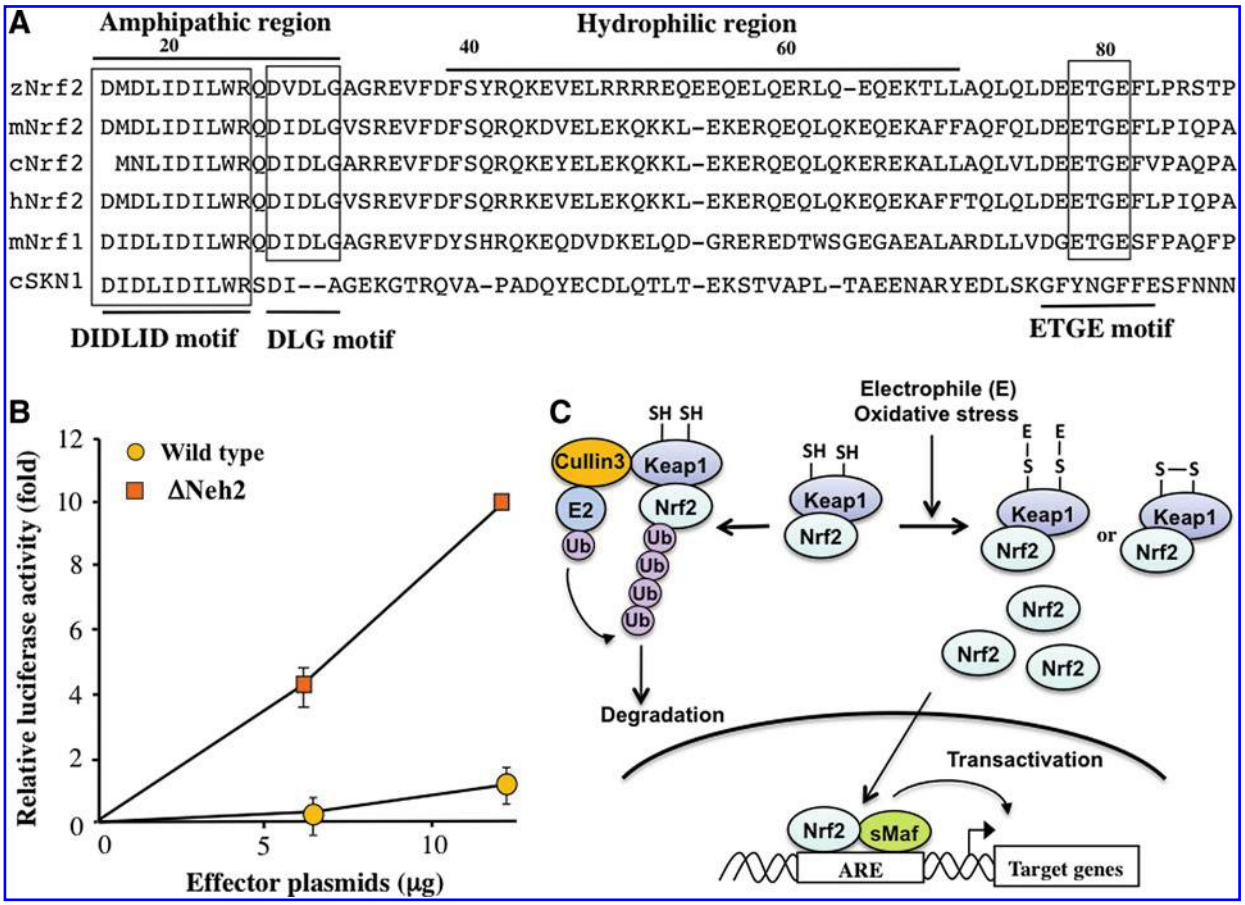

FIG. 6. Sequence identity of the Neh2 domain. (**A**) The 25 amino acid residues (in mouse Nrf2; residues 16–40) are rich in hydrophobic amino acids and conserved within Nrf2, Nrf1, and SKN1. In contrast, the C-terminal portion of the Neh2 domain (residues 41–90) is rich in hydrophilic residues and specifically conserved between cross-species Nrf2 molecules. The ETGE motif was discovered from a genetic screen in yeast that detected Neh2 mutations that disrupted the binding to Keap1 (56). The DIDLID motif acts as an activation domain in the *C. elegans* Nrf2 homologue SKN1 (111). cNrf2, chicken Nrf2; hNrf2, human Nrf2; mNrf1, mouse Nrf1; mNrf2, mouse Nrf2; cSKN1, *C. elegans* SKN1; zNrf2; zebra fish Nrf2. (**B**) Incremental amounts of the wild-type and ΔNeh2 Nrf2 expression plasmids were transfected into HD3 erythroblasts together with the pRBGP2 reporter plasmid containing triplicate NF-E2 binding sites from the chicken *β*-globin enhancer. Luciferase activity with the ΔNeh2 mutant at its maximum dose was set at 100%. Mean values of three independent experiments, each carried out in duplicate, are shown with standard error of means. (**C**) Model of Nrf2 activation mechanism. In nonstressed conditions, Nrf2 is constitutively ubiquitinated through the Keap1/Cullin3 ubiquitin ligase complex. Upon exposure to electrophile (E) or oxidative stress, Keap1 is modified at its regulatory cysteine residues and inactivated. Keap1 inactivation enhances Nrf2 nuclear accumulation and ARE-dependent transcription. See manuscript for details. This figure is modified from Reference 36. E2, ubiquitin-conjugating enzyme. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

are viable and resistant to acetaminophen hepatotoxicity (79). However, subsequent analysis showed that mice homozygous for the floxed *Keap1* allele have decreased expression of *Keap1*, leading to increased activation of Nrf2 in multiple organs lacking transgenic expression of albumin promoter–driven CRE recombinase (hereafter, we will refer to these mice as *Keap1*-knockdown (*Keap1*-KD) mice) (78). Therefore, Nrf2 accumulation increases in the liver in the *Keap1*-KD mice by 2-fold (90). Microarray analysis demonstrated that most of the prototypical phase 2 genes, such as *Gsts* and *Nqo1*, are upregulated in the liver of *Keap1*-KD mice (90). However, several genes, including antioxidant enzymes, such as superoxide dismutase and catalase, and EH-1, are less induced or not induced at all. Therefore, these results suggest that the induction of some Nrf2 target genes might require phase 2 inducer–dependent post-translational modification of Nrf2 and/or Nrf2-associated proteins. On the other hand, the expression of mouse *Hmox1* gene appears to be under more complex regulation. In both the complete *Keap1*-knockout and the *Keap1*-KD mice, *Hmox1* expression is not increased and seems to require liberation from Bach1 repression (28, 79, 90 and our unpublished observation). This effect can be explained by the specific and dominant repressive action of Bach1 over mouse *Hmox1* gene expression (17).

Overexpression of the Neh2 domain promotes Nrf2 nuclear accumulation in Hepa-1 cells (4). Those authors demonstrated that Nrf2 that accumulates in the nucleus after *t*-BHQ exposure is phosphorylated at a serine residue(s) but that Nrf2 that accumulates following Neh2 overexpression is not phosphorylated. The transactivation potential is similar for both

the former and latter Nrf2 proteins, indicating that Nrf2 phosphorylation is required neither for Nrf2 stabilization nor transcriptional activation by Nrf2. Therefore, these results demonstrate that post-translational modification of Nrf2 by electrophiles may be required for liberation of Nrf2 from Keap1 repression, but this modification plays a limited role in the transcription of the target genes. These results are consistent with the finding that overexpression of Nrf2 causes Nrf2 target gene induction in human HEK293 cells in the absence of oxidative stress (53, 121).

The Regulation of Nrf2 Activity by Keap1-Dependent and -Independent Degradation

In nonstressed conditions, Keap1 acts as an adaptor molecule for the Cul3 E3 ligase complex and leads to Nrf2 degradation through the ubiquitin–proteasome pathway (Fig. 6C) (55). The mechanism by which electrophiles or oxidative stress inhibit Keap1-mediated Nrf2 repression is still an open question. The inhibition may require post-translational modification of Keap1 and/or Nrf2 and may involve degradation and/or nuclear shuttling of both proteins (74, 75). Electrophiles and reactive oxygen species have been proposed to decrease the ability of Keap1 to repress Nrf2 by an incompletely understood mechanism that involves oxidative modification of several reactive cysteines of Keap1 (107), leading to Nrf2 stabilization and accumulation in the nucleus (Fig. 6C).

Regulation of Keap1-dependent degradation of Nrf2 can be recapitulated using a LacZ reporter gene embedded in the Nrf2 genomic locus (Fig. 7A) (42). Importantly, β-galactosidase is not detectable in intestinal epithelial cells but can be detected after treatment with BHA (Fig. 7B). A similar pattern can be observed in the peritoneal macrophages of LacZ-embedded *Nrf2*-KO mice (Fig. 7C). These results are consistent with the hypothesis that the Keap1–Neh2 interaction regulates Nrf2 stability in response to oxidative stress *in vivo*.

Even under oxidative stress conditions, where Nrf2 is liberated from Keap1 repression, Nrf2 is subjected to proteasomal degradation, indicating the existence of Keap1-independent degradation of Nrf2 (42). From these observations, we previously proposed two modes of Nrf2 degradation, homeostatic Keap1-dependent degradation or Keap1-independent degradation in oxidative stress conditions (Fig. 7D) (42). McMahon *et al.* demonstrated that Keap1-independent degradation occurs through the Neh6 domain, but the dependency of the Neh6 degron on the ubiquitin proteasome pathway is not clear at present (65). We argue that Keap1-independent degradation occurs in the nucleus (42) and envisage that the Keap1-enhanced mode of degradation occurs in the cytoplasm (Fig. 7D). Indeed, Sun *et al.* showed that Cul3 localizes in the cytoplasm and that Keap1-dependent degradation occurs in the cytoplasm (100). Nguyen *et al.* argued that Nrf2 is a constitutively nuclear protein and that Nrf2 degradation might occur in the nucleus (74). Our previous report also demonstrated that constitutive Nrf2 expression is detectable in the

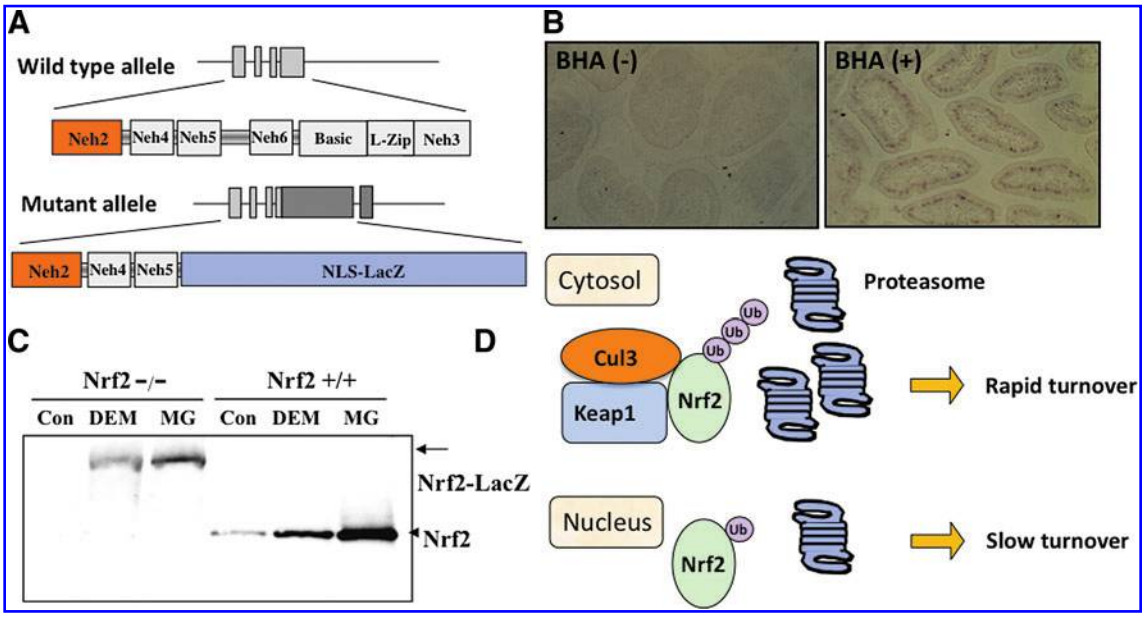

FIG. 7. The Nrf2 N-terminal region containing Neh2, Neh4, and Neh5 confers electrophile responsiveness on the heterologous protein. (**A**) Schematic representation of the LacZ knockin–knockout strategy for the generation of *Nrf2*-KO mice. As a result of homologous recombination, an NLS–β-galactosidase fusion protein linked N-terminally to the Nrf2 N-terminal region containing Neh2, Neh4, and Neh5 is expressed. (**B**) Immunohistochemical staining of BHA-treated or -untreated mice using anti-β-galactosidase antibody. *Nrf2*-KO mice were fed a diet supplemented without (*left panel*) or with (*right panel*) 0.7% BHA for 3 days, and tissue sections of intestine were analyzed by staining with anti-β-galactosidase antibody. (**C**) In the peritoneal macrophages obtained from *Nrf2*-KO mice, the Nrf2–β-galactosidase fusion protein is inducible by both DEM and proteasome inhibitor MG132 (MG). In wild-type macrophages, native Nrf2 is similarly inducible by both DEM and MG132. The same amounts of proteins were loaded in each lane. (**D**) Schematic representation of Keap1-dependent and -independent degradation of Nrf2 in distinct subcellular compartments. In the cytoplasm, Nrf2 is bound by Keap1 and subjected to rapid proteasomal degradation by the Keap1/Cul3 ubiquitin ligase system. In the nucleus, Nrf2 is relatively stabilized by the lack of Keap1. This figure is modified from Reference 36. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

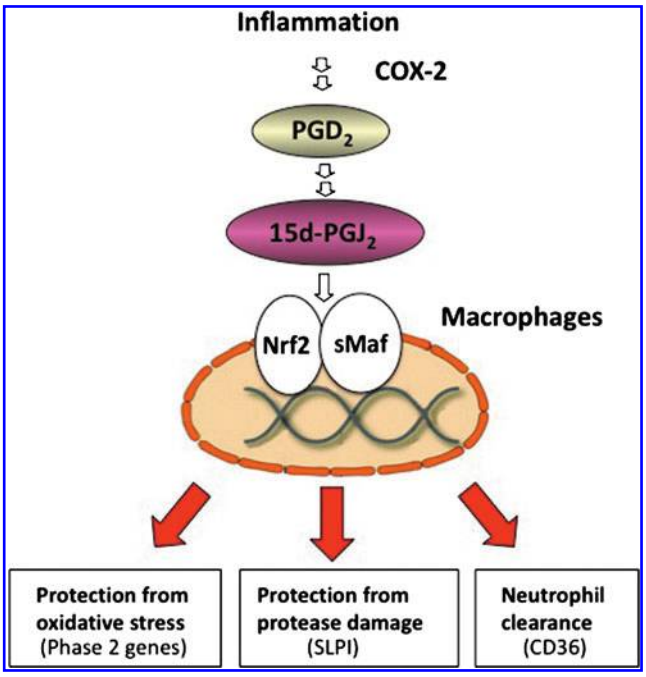

FIG. 8. Nrf2 protects against inflammation through multiple mechanisms. 15d-PGJ_2 is generated in macrophages via upregulation of COX-2 and activates the Keap1–Nrf2 pathway. Through gene regulation in lung macrophages, Nrf2 protects against inflammation by regulating not only redox balance but also protease/antiprotease balance and neutrophilic inflammation by upregulating SLPI (secretory leukoprotease inhibitor) and CD36, respectively (31, 35, 63). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

nucleus but not in the cytoplasm of peritoneal macrophages (42). However, constitutive Nrf2 nuclear localization can be explained by other mechanisms. Provided that some fraction of newly synthesized Nrf2 translocates to the nucleus, the faster Nrf2 degradation in the cytoplasm compared to that in the nucleus (Fig. 7D) may simply explain the apparent constitutive localization of Nrf2 in the nucleus. Alternatively, there might be a weak oxidative stress or endogenous Nrf2-activating signals in the cells. Further studies are required to clarify the mechanism by which Nrf2 constitutively localizes to the nucleus in nonstressed conditions in certain cell types.

The 15d-PGJ_2–Mediated Nrf2 Pathway Exerts Anti-inflammatory Effects in Lung Inflammation

Kawamoto *et al.* established a screening system for the inducers of GST activity in rat liver epithelial cells (RL34) and showed that J-series prostaglandins (PGs), such as PGJ_2 and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2), are inducers of GST (49). Cyclopentenone PGs (cyPGs), including 15d-PGJ_2 and PGA_1, possess a reactive α,β-unsaturated carbonyl moiety in their cyclopentenone ring that confers on the molecule the capability of Michael adduct formation with nucleophilic cellular molecules. Indeed, we have demonstrated that cyPGs, including 15d-PGJ_2 and Δ^{12}-PGJ_2, directly bind to Keap1 via the cysteines of the Keap1 intervening region (26, 40) and activate Nrf2 in mouse peritoneal macrophages (40). Gong *et al.* reported that 15d-PGJ_2 increases HO-1 expression through the StRE-Nrf2 pathway (22). These results suggest that Nrf2 may contribute, at least in part, to the known anti-inflammatory function of 15d-PGJ_2.

15d-PGJ_2 exerts anti-inflammatory effects via inhibition of the NF-κB pathway and activation of PPARγ (91, 92). Furthermore, it is reported that 15d-PGJ_2 accumulates in the inflammatory pleural fluid and that 15d-PGJ_2 accumulation is associated with inducible expression of HO-1 in rat carrageenin-induced pleurisy model (21, 115). Therefore, we generated a reversible acute pleurisy model by intratracheal injection of carrageenin, an inducer of cyclooxygenase-2 (COX-2), to clarify the roles of Nrf2 in inflammation (40). We observed that acute carrageenin-induced pleurisy was exacerbated in *Nrf2*-KO mice compared with wild-type mice. Analysis of pleural lavage fluids demonstrated that the magnitude and duration of inflammation, measured by the albumin concentration and number of neutrophils, were significantly exacerbated in the *Nrf2*-KO mice. Treatment of wild-type mice with the selective COX-2 inhibitor NS-398 significantly exacerbated acute pleurisy to a level comparable to that observed in *Nrf2*-KO mice. In the lungs of NS-398-treated wild-type mice, both accumulation of 15d-PGJ_2 and Nrf2 target gene induction were significantly decreased. Administration of 15d-PGJ_2 into the pleural cavity rescued the degenerative effects of NS-398 and the induction of antioxidant genes. However, the therapeutic effect of 15d-PGJ_2 was lost in *Nrf2*-KO mice. These results demonstrate that 15d-PGJ_2 plays a protective role against acute pleurisy by activating the Nrf2-mediated transcriptional pathway (Fig. 8). Similar phenotypes to those of the *Nrf2*-KO mice were observed in our carrageenin-induced lung injury model (68). Therefore, we propose that 15d-PGJ_2 induced by the upregulation of COX-2 activates the Nrf2 pathway and protects against inflammation in the lung (37).

Summary and Perspectives

The finding that phase 2 enzyme induction is defective in *Nrf2*-KO mice, in parallel with the analysis of gene regulatory elements of each phase 2 enzyme, led to elucidation of the molecular basis for phase 2 enzyme gene induction. We have presented genetic evidence that Nrf2 is the key molecule involved in this response and that other CNC proteins cannot fully compensate for the lack of Nrf2 activity. Keap1 was discovered to be a negative regulator of Nrf2 and seems to act as the cytoplasmic sensor for electrophilic agents. However, the actual sensing mechanism for electrophiles and the precise mechanism for Nrf2 activation have yet to be clarified. The next decade will witness how electrophilic agents regulate Nrf2–Keap1 activity *in vivo*.

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Abbreviations Used

15d-PGJ_2 = 15-deoxy-$\Delta^{12,14}$-PGJ_2
AhR = aromatic hydrocarbon receptor
ARE = antioxidant response element
Bach = BTB and CNC homolog
BBN = *N*-nitrosobutyl (4-hydroxybutyl)amine
BHA = butylated hydroxyanisole
BHT = butylated hydroxytoluene
BTB = bric-a-brac, tramtrack, broad complex
CNC = Cap'n'Color
COX-2 = cyclooxygenase-2
CYP = cytochrome P450 monooxygenase
cyPGs = cyclopentenone prostaglandins
DEM = diethylmaleate
DMBA = dimethylbenzanthracene
EH-1 = microsomal epoxide hydrolase
EpRE = electrophile responsive element
γGCS = γ-glutamylcysteine synthetase
GST = glutathione *S*-transferases
HO-1 = heme oxygenase 1
Keap1 = Kelch-like ECH-associated protein 1
MARE = Maf recognition element
Neh = Nrf2-ECH homology
NQO1 = NAD(P)H:quinone oxidoreductase 1
Nrf = NF-E2–related factor
ROS = reactive oxygen species
sMafs = small Maf proteins
StREs = stress responsive elements
TCDD = 2′3′7′8′-tetrachlorodibenzo-*p*-dioxin
UGT = UDP-glucuronosyl transferases
XRE = xenobiotic responsive element

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