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Regulatory Mechanisms of Cellular Response to Oxidative Stress

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An antioxidant responsive element (ARE) or electrophile responsive element (EpRE) mediates the transcriptional activation of genes encoding phase II drug metabolizing enzymes. The ARE consensus sequence shows high similarity to an erythroid gene regulatory element, and based on the observation, we have recently found that transcription factor Nrf2 is essential for the coordinate induction of phase II detoxifying enzymes. The expression of anti-oxidative stress enzyme genes is also regulated by Nrf2. Detailed analysis of the regulatory mechanisms of Nrf2 activity has ultimately led us to the identification of a new protein, which we have named Keap1, that suppresses Nrf2 activity by specific binding to its evolutionarily-conserved N-terminal Neh2 regulatory domain.

Keywords: ARE, EpRE, Nrf2, phase II enzyme

Xenobiotics present in the modern environment are metabolized by a group of detoxifying enzymes which convert them into substances that are more readily soluble in water. This biotransformation process is divided into two reactive phases; phase I reactions are catalyzed by the cytochrome P-450 mono-oxygenase system and phase II reactions are catalyzed by a group of enzymes, such as glutathione S-transferase (GST) and NAD(P)H: quinone oxidoreductase (NQO1). The phenolic antioxidant butylated hydroxyanisole (BHA) is known to prevent tumor formation in mice after exposure to various carcinogens.[1] While the precise mechanism of BHA action in this tumor preventative process is unclear at present, currently available data suggest that the BHA action is largely attributable to the induction of phase II enzymes.

In the mouse liver and intestine, BHA markedly increases the expression of GST and NQO1 genes at the transcriptional level. An antioxidant responsive element (ARE)[2] or electrophile responsive element (EpRE)[3] has been found to be responsible for this induction. Recent analysis has demonstrated that ARE is also important in the inducible expression of a set of antioxidant enzyme genes by electrophiles or reactive oxygen species (ROS),[4,5] indicating that ARE regulates a wide-ranging metabolic response to oxidative stress. We found that transcription factor Nrf2 is essential for the coordinate induction of phase II

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detoxifying enzymes and antioxidant enzymes, both of which are under ARE regulation.[6,7]

Nrf2-SMALL Maf HETERODIMER BINDS TO NF-E2 SEQUENCE

An NF-E2 binding site was first identified in the gene regulatory region for porphobilinogen deaminase.[8] A site was also found in the ferrochelatase gene regulatory region,[9] as well as in the DNase I hypersensitive sites of the β-globin locus control region (LCR).[10,11] Subsequent study revealed that NF-E2 is a heterodimer of basic leucine zipper (bZip) transcription factor p45 and MafK, one of the small Maf proteins.[12–14] The small Maf proteins appear to be members of the Maf family transcription factors, which are characterized by their specific bZip domain structure.[15] To date, this family consists of four large Maf proteins (c-Maf, MafB, NRL and L-Maf) and three small Maf proteins (MafF, MafG and MafK).[16–20]

The small Maf proteins can form heterodimers with any of the CNC family transcription factors. The founding member of the CNC family in vertebrates is p45.[12] CNC was named after the similarity of the bZip domain structure of p45 to that of the Drosophila Cap'n'Color (CNC) protein.[21] The CNC family members have now expanded to six, i.e. p45, Nrf1, Nrf2, Nrf3, Bach1 and Bach2.[12,22–26] Since the small Maf proteins lack a canonical transcription activation domain, CNC factors provide a transcriptional regulatory function for the heterodimer. In contrast, the small Maf subunit is required for the high affinity, sequence-specific DNA binding of the CNC–small Maf heterodimer.[14] In addition, each small Maf protein can form a homodimer, and the homodimer can bind to most NF-E2 binding sequences (such as the NF-E2 site in the chicken β-globin enhancer). Overexpression of the small Maf proteins represses transcription from the NF-E2 binding sequence in a DNA transfection assay.[14] Thus, while the CNC–small Maf heterodimer is a transcriptional activator (or repressor, see Ref. [25,27]) of the NF-E2 site, the homodimer of the small Maf protein acts as a repressor of the same site.

SIMILARITY OF ARE CONSENSUS SEQUENCE TO NF-E2 BINDING SITE

Since the ARE sequence (RTGACNNNGC) shows significant sequence similarity to TRE (TPA-responsive element; TGAC/GTCA),[3–4] transcription factor AP-1 has been believed to be responsible for the inducible expression of phase II enzyme genes through ARE. However, the GC nucleotides (*italic*) outside the core TRE sequence are shown to be essential for enzyme induction, indicating that a transcription factor other than AP-1 should be involved in phase II enzyme induction.[2,4] This binding sequence specificity shows excellent coincidence with that of NF-E2, as the corresponding **C** nucleotide of the NF-E2 binding sequence (TGAG/CTCAGCA) has been reported to be indispensable for NF-E2 binding.[28] Furthermore, Kerppola *et al*. reported that the consensus binding sequence of the c-Fos–c-Maf heterodimer is TGACNNNGCA (note that the right half is recognized by c-Maf).[29] Also, both c-Fos–small Maf and CNC–small Maf heterodimers were reported to recognize similar binding sequences.[30] These observations suggest that the CNC–small Maf heterodimer binds to ARE and regulates the expression of phase II detoxifying enzyme genes.

REGULATION BY Nrf2 OF THE EXPRESSION OF PHASE II DETOXIFYING ENZYME GENES

The expression level of Nrf2 is particularly high in tissues where phase II enzymes are either highly inducible or abundantly expressed.[6,27,31] This observation suggests that Nrf2 might interact with ARE and induce the expression of phase II

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drug metabolizing enzyme genes. To test this hypothesis, we carried out targeted disruption of the *nrf2* gene in mouse.[6]

We examined the inducible expression of phase II enzyme genes by BHA in an *nrf2*-deficient mouse. The induction of phase II enzymes such as GSTs, NQO1 and epoxide hydrolase by BHA was significantly affected in the *nrf2*-null mouse, demonstrating that Nrf2 regulates coordinately the inducible expression of phase II enzyme genes in mouse (Ref. [6] and our unpublished observation). Nrf2 can bind ARE in the regulatory region of these genes by forming a heterodimer with one of the small Maf proteins. This suggests that the Nrf2–small Maf heterodimer directly activates the transcription of these genes.

Recently, ARE has also been implicated in the regulation of anti-oxidative stress enzymes, including heme oxygenase-1 (HO-1), peroxyredoxin Msp23 and γ-glutamylcysteine synthetase.[4,5] Using a primary culture of peritoneal macrophages from Nrf2-deficient mice, we found that the induction of HO-1 and Msp23 by electrophilic agents or ROS was affected in the *nrf2*-null peritoneal macrophages.[7] These results thus demonstrate that Nrf2 functions in a wide-ranging metabolic response to oxidative stress (Figure 1).

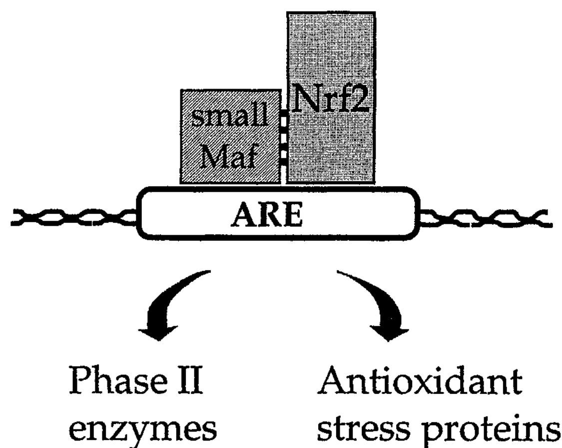

FIGURE 1 Nrf2 regulates the induction of phase II enzymes and antioxidant proteins via ARE. Nrf2 forms a heterodimer with a small Maf protein and interacts with ARE. Nrf2 is a general regulator of the defense genes against oxidative stress and the phase II detoxifying enzyme genes.

Neh2 IS AN EVOLUTIONARILY CONSERVED REGULATORY DOMAIN OF Nrf2

Although the binding activity of Nrf2 to ARE of the HO-1 gene was markedly increased by electrophilic agents or ROS, the Nrf2 mRNA level did not increase.[7] This result implies that electrophilic agents or ROS activate Nrf2 at a post-transcriptional step in the macrophages.

To understand how Nrf2 activity is regulated, we dissected the domain structure/function of Nrf2.[32] Comparing the chicken Nrf2 (ECH)[33] structure with that of human Nrf2, we identified six conserved domains in the Nrf2 proteins. These are referred to as Neh (Nrf2-ECH homology) domains. Of the six Neh domains, the N-terminal Neh2 domain shows the most striking homology among species (Figure 2). An interesting observation here is that the Neh2 domain can be divided into two subdomains.[32] While the N-terminal subdomain (16–40) contains hydrophobic amino acid residues, the C-terminal subdomain is rich in hydrophilic residues.

In this regard, the assignment of the translation initiation methionine (Met) codon in mammalian *nrf2* genes has been confusing. The translation initiation Met of mouse Nrf2[31] was reported to be at a similar position to that of chicken Nrf2 (ECH).[33] We have confirmed the presence of a termination codon upstream of the Met codon in chicken Nrf2 cDNA (isolated from a λ phage cDNA library[33]), and this assignment was confirmed by a 5′RACE (rapid amplification of the cDNA end) analysis (unpublished observation). However, the initiation Met of mouse Nrf2 was recently reported to be at a position 16 residues more extended than the previously assigned Met[31] (Figure 2). We therefore sequenced our mouse Nrf2 cDNA that was isolated independently by a phage cDNA library screening, and

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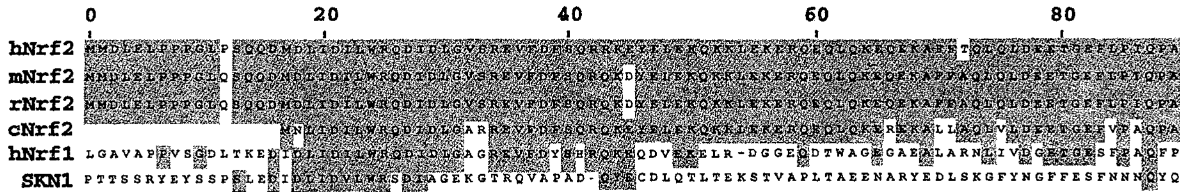

FIGURE 2 Sequence homology in Neh2 domain. The 25 amino acid residues (in mouse Nrf2; amino acid (a.a.) 16–40) are rich in hydrophobic amino acid residues and conserved within Nrf2, Nrf1 and Skn1. In contrast, C-terminal portion of Neh2 domain (a.a. 41–90) is rich in hydrophilic residues and specifically conserved between cross-species Nrf2 molecules.

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found that the sequence of our mouse Nrf2 cDNA matches the latter. This assignment also matches the rat[34] and human Nrf2[31] structure (Figure 2). Since all of our expression vector constructs utilize cDNA containing the extended portion (to 5′) of the *nrf2* gene, results of the transfection analysis described below and also in Ref. [32] were unaffected by this change in assignment of translation initiation Met.

Deletion of the Neh2 domain resulted in a marked increase of Nrf2 activity in a co-transfection/transactivation assay into an HD3 chicken erythroid cell line, indicating that Neh2 works as a negative regulatory domain of Nrf2. The negative regulatory activity of Neh2 was nullified by the simultaneous expression of Neh2–GBD fusion (decoy) protein with wild type Nrf2 in HD3 cells.[32] This shows that the Neh2 domain negatively regulates Nrf2 activity by interacting with an unknown repressor protein.

Keap1 NEGATIVELY REGULATES Nrf2

The result described above prompted us to search for the molecular identity of the negative regulatory activity in the HD3 cells. To this end, we used a yeast two-hybrid screening system, and identified a new protein, Keap1, which interacts with Nrf2 and negatively regulates Nrf2 activity in a reporter co-transfection/transactivation assay.[32] Keap1 shows structural similarity to the *Drosophila* protein Kelch, which has two canonical protein interaction motifs. One of the motifs is the Kelch (double glycine repeat or DGR) domain and the other is the BTB domain. When overexpressed in fibroblasts or 293T cells, Keap1 co-localizes in the cytoplasm with Nrf2, which otherwise accumulates in the nucleus. An important finding here is that this co-localization of Keap1 and Nrf2 was counteracted by the addition of diethylmaleate to the culture media. The electrophilic agent allowed Nrf2 to translocate into the nucleus even in the presence of co-transfected Keap1. These results thus suggest that Keap1 and Nrf2 constitute a cytoplasmic sensor for electrophiles.[32]

CONCLUSION

The finding that the ARE response is defective in *nrf2* knockout mice, concomitant with the progress in the analysis of the gene regulatory element of each phase II enzyme, has led to the elucidation of the molecular basis for the phase II enzyme and antioxidant gene induction. We have presented evidence that Nrf2 is the key molecule in this response, and that other CNC proteins cannot fully compensate for a lack of Nrf2 activity. Keap1 is a negative regulator of Nrf2 and seems to constitute one of the cytoplasmic sensors for electrophilic agents. Further studies are required to clarify how electrophilic agents regulate Nrf2–Keap1 activity *in vivo*.

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