Molecular Basis Distinguishing the DNA Binding Profile of Nrf2-Maf Heterodimer from That of Maf Homodimer*

Momoko Kimura†‡, Tae Yamamoto‡, Jianyong Zhang‡, Ken Itoh‡, Motoki Kyo†, Terue Kamiya‡, Hiroyuki Aburatani‡, Fumiki Katsuoka‡, Hirofumi Kurokawa‡, Toshiyuki Tanaka§, Hozumi Motohashi‡, and Masayuki Yamamoto‡

From the †Graduate School of Comprehensive Human Sciences and Center for Tsukuba Advanced Research Alliance and ‡Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba 305-8572, †Department of Medical Biochemistry, Tohoku University Graduate School of Medicine, 2-1 Seiryo-cho, Aoba-ku, Sendai 980-8575, ‡Department of Stress Response Science, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, TOYOBO Co. Ltd. Biotechnology Frontier Project, 10-24 Toyo-Cho, Tsuruga, 914-0047, **Research Center for Advance Science and Technology, University of Tokyo, Tokyo 153-8904, §§Institute of Multidisciplinary Research for Advanced Materials, Tohoku University, Katahira 2-1-1, Aoba-ku, Sendai 980-8577, and ¶¶Environmental Response Project, Exploratory Research for Advanced Technology-Japan Science and Technology Corp. (ERATO-JST), Tohoku University Graduate School of Medicine, 2-1 Seiryo-cho, Aoba-ku, Sendai 980-8575, Japan

Received for publication, August 17, 2007, and in revised form, September 17, 2007. Published, JBC Papers in Press, September 17, 2007 DOI 10.1074/jbc.M706863200

Nrf2-small Maf heterodimer activates the transcription of many cytoprotective genes through the antioxidant response element and serves as a key factor in xenobiotic and oxidative stress responses. Our surface plasmon resonance-microarray binding analysis revealed that both Nrf2-MafG heterodimer and MafG homodimer bind to the consensus Maf recognition element with high affinity but bind differentially to the suboptimal binding sequences degenerated from the consensus. We examined the molecular basis distinguishing the binding profile of Nrf2-MafG heterodimer from that of MafG homodimer and found that the Ala-502 residue in the basic region of Nrf2 is a critical determinant of its binding specificity. In Maf proteins, a tyrosine resides in the position corresponding to Ala-502 in Nrf2. We prepared a mutant Nrf2 molecule in which Ala-502 was replaced with tyrosine. In surface plasmon resonance-microarray analysis, heterodimer of Nrf2(A502Y) and MafG displayed a binding specificity similar to that of MafG homodimer. The target genes activated by mutant Nrf2(A502Y)-small Maf heterodimer were largely different, albeit with some overlap, from those activated by wild-type Nrf2-small Maf, indicating that the array of target genes regulated by Nrf2-small Maf heterodimer differs substantially from those activated by wild-type Nrf2-small Maf (2). The Maf recognition element (MARE) was defined as a 13-bp (TGCTGA(G/C)TCAGCA) or 14-bp (TGCTGA(G/C)TCAGCA) palindromic sequence through the systematic evolution of ligands by exponential enrichment. The transcriptional level in response to electrophiles and oxidative stress. The antioxidant response element (ARE) was identified as a critical cis-regulatory element for the inducible expression of these cytoprotective genes (1). A sequence common to individual regulatory elements was extracted and TGA(G/C)NNNGC was defined as the ARE consensus sequence.

Maf family proteins share a conserved basic region and leucine zipper (bZip) motif and are divided into two groups, large Maf (c-Maf, MafA, MafB, and Nrl) with an activation domain and small Maf (MafG, MafK, and MafF) without an activation domain (2). The Maf recognition element (MARE) was defined as a 13-bp (TGCTGA(G/C)TCAGCA) or 14-bp (TGCTGA(G/C)TCAGCA) palindromic sequence through the systematic evolution of ligands by exponential enrichment (3, 4) that identifies a sequence with the highest affinity to the transcription factor as its consensus sequence. The MARE core contains a 12-O-tetradecanoylphorbol-13-acetate-responsive element (TGA(G/C)TCA) or a cAMP-responsive element (TGA(G/C)TCA), which is the binding sequence of Jun/Fos family proteins or CREB (cAMP-response element-binding protein)/ATF family proteins, respectively. In addition, the MARE core is flanked on both sides by extended elements (5′-}

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TGC-(12-O-tetradecanoylphorbol-13-acetate-responsive element/cAMP-responsive element)-GCA-3′, a unique feature of the binding site of Maf family proteins. A core sequence on one side and a flanking sequence on the other side of the MARE were found to comprise the ARE consensus sequence. This striking similarity suggested the involvement of Maf proteins in ARE-dependent gene regulation (2, 5).

Smaller Maf and Nrf2, a member of the Cap’n’Collar (CNC) family of transcription factors, form a heterodimer that serves as a key regulator for the inducible expression of cytoprotective genes (6–8). Other Maf-containing dimers also interact with the MARE and its related sequences, including the ARE. Large Maf homodimers and heterodimers of small Maf and a CNC family member (NF-E2 p45, Nrf1, Nrf2, and Nrf3) are MARE-dependent activators, whereas small Maf homodimers and het-
erodimers of small Maf with a Bach family member (Bach1 and Bach2) are MARE-dependent repressors (2, 9–12). Considering that all these dimers bind to similar target sites, the question is how each Maf-containing dimer elicits its specific biological function.

The individual MARE-related sequences in the regulatory regions of endogenous genes have often diverged from the consensus MARE. We surmised that such divergence has enabled the differential binding of distinct Maf-containing dimers and generated the diversity in MARE-dependent gene regulation. To test our hypothesis, we performed a comprehensive analysis on the binding affinities of Maf homodimer and Nrf2-Maf heterodimer to MARE-related sequences generated by systematic base alterations using the surface plasmon resonance (SPR) microarray imaging technique (13, 14). We found that Maf homodimer and Nrf2-Maf heterodimer bind similarly to the consensus MARE with high affinity but bind differentially to the suboptimal binding sequences degenerated from the MARE consensus. The biological significance of the latter has not been clarified.

Because Nrf2-MafG heterodimer was more sensitive to a core region mutation than MafG homodimer, we suspected that Nrf2 mainly recognizes the core region of the MARE, which is a characteristic common to other bZip transcription factors, including the Jun and Fos family. On the contrary, Maf family DNA recognition has been considered unique due to the requirement of flanking regions within the MARE (3, 15). The structural characteristics accounting for the unique DNA recognition by Maf family proteins reside in the basic region and the extended homology region (EHR), a unique stretch of 24 amino acids just in front of the basic region (Fig. 1A). We found that the EHR partially supports the Maf-specific binding mode (16), and the critical contribution of the basic region was also suggested (17).

In this study, we found that Ala-502 in the basic region of Nrf2 is critical in determining the binding specificity of Nrf2-small Maf heterodimer. Ala-502 of Nrf2 corresponds to the well conserved alanine residue in the basic region of all bZip transcription factors, except for Maf family proteins that possess a tyrosine residue instead. We generated a mutant Nrf2(A502Y) molecule by replacing the Ala-502 residue with tyrosine. Nrf2(A502Y)-MafG heterodimer and MafG homodimer displayed similar binding specificities in an SPR microarray analysis. Using the Nrf2(A502Y) mutant with its altered specificity, we examined whether the distinct profiles of suboptimal binding of Nrf2-MafG heterodimer and MafG homodimer prove significant in the induction of Nrf2 target genes in vivo. Compared with those of wild type, the target genes activated by Nrf2(A502Y)-small Maf heterodimer were fundamentally different, although somewhat overlapping. This advocates that a significantly different set of target genes is regulated by Nrf2-small Maf heterodimer compared with those associated with Maf homodimer. These results suggest that the distinct DNA binding profile of Nrf2-small Maf heterodimer is essential for Nrf2 to function as an activator of cytoprotective genes. Our results also support our contention that the differential DNA binding specificity between Maf homodimers and CNC-Maf heterodimers forms a strong foundation for differential gene regulation by dimer-forming bZip transcription factors.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Previously constructed pET15b-Nrf2CT (Nrf2 fragment containing a CNC domain and bZip motif spanning from alanine 318 to the C-terminal end asparagine 597) was used to prepare the Nrf2 bZip domain for analyzing heterodimer binding (14). pET15b-Nrf2CT(A502Y) was generated by replacing the alanine at position 502 with tyrosine. Previously constructed pLM-His-MafG-XB (18) and pcDNA3.1-3xFLAG-Nrf2 (19) were used for the transient mammalian expressions of full-length MafG and Nrf2, respectively. pcDNA3.1-3xFLAG-Nrf2(A502Y) was generated by replacing the alanine 502 with tyrosine. An earlier construct, pcDNA5/FRT/TO-FLAG-Nrf2 was used for stable mammalian expression of Nrf2 (19). For stable expression of Nrf2(A502Y), the cDNA fragment encoding FLAG-tagged Nrf2(A502Y) of pcDNA3.1-3xFLAG-Nrf2(A502Y) was inserted into pcDNA5/FRT/TO (Invitrogen) between EcoRV and ApaI to generate pcDNA5/FRT/TO-FLAG-Nrf2(A502Y). Among the five kinds of luciferase reporter genes used, four had been constructed previously (14), and one was generated by replacing the MluI-NheI fragment of pRBGP2 (20) with a triplicate consensus MARE sequence (5′-CGGAAT-TGCTGAGTCAGCA-TTACTC-3′).

Protein Preparation and SPR Microarray Imaging Analysis—Fragments of MafG, Nrf2, and Nrf2(A502Y) containing the bZip motifs MafG-(1–123), Nrf2CT, and Nrf2CT(A502Y), respectively, were expressed in bacteria and purified as described previously (14). SPR microarray imaging analysis was performed as outlined before (14). For detecting homodimer binding, 250 nM MafG-(1–123) was applied to the array surface in SPR buffer containing 20 mM HEPES (pH 7.2), 300 mM NaCl, 4 mM MgCl2, 10 mM EDTA, 0.005% Tween 20, and 1 mM phosphate. For detecting heterodimer binding, Nrf2CT (1 μM)/MafG-(1–123) (100 nM) and Nrf2CT(A502Y) (1 μM)/MafG-(1–123) (100 nM) were applied to the array surface in SPR buffer containing 20 mM HEPES (pH 7.9), 250 mM NaCl, 4 mM MgCl2, 10 mM EDTA, 0.005% Tween 20, and 1 mM phosphate. A 10 molar excess of Nrf2CT or Nrf2CT(A502Y) compared with MafG-(1–123) was used, since essentially all MafG-(1–123) molecules were expected to exist in heterodimeric form under this condition based on our titration study (14). Association rate constants (k+a) and dissociation rate constants (k−a)
were obtained from the SPR measurement. The association constants ($K_a$) were determined from the ratio of $k_a$ and $k_d$ ($K_a = k_a / k_d$).

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was performed as described previously (13, 14). The binding activity was examined using 75 or 300 nM MafG-(1–123) and 75 nM–7.5 μM of Nrf2CT or Nrf2CT(A502Y).

Cell Culture and Reporter Assay—293T cells were maintained in DMEM (Sigma) with 10% fetal bovine serum and 1% penicillin-streptomycin. Reporter plasmids were introduced into 293T cells along with Nrf2 and Nrf2(A502Y) expression vectors using FuGENE6 transfection reagent (Roche). Luciferase activity was measured 24 h after transfection using a Dual Reporter Assay System (Promega) and a luminometer (Berthold Japan, Tokyo). All samples were prepared in triplicate and means and standard deviations were calculated.

Establishment of Stable Cell Lines—Stable cell lines were established as instructed earlier (21). pcDNA5/FRT/TO-FLAG-Nrf2 and pcDNA5/FRT/TO-FLAG-Nrf2(A502Y) were transfected into a parental FlpIn TREx 293 cell line (Invitrogen) with pOG44 (Invitrogen) that encodes Flp recombinase. The parental cells contain a single FLP recombinase target (FRT) site located at a transcriptionally active genomic locus to which the gene of interest can be targeted. Transfectants were selected using 75 or 300 nM MafG-(1–123) and 75 nM–7.5 μM of Nrf2CT or Nrf2CT(A502Y).

Microarray Analysis—Before and 6 h after tetracycline treatment, total RNA samples were extracted from 293/FLAG-Nrf2 and 293/FLAG-Nrf2 A502Y cells using IsoGen (Nippon Gene). Isolated RNA was purified using an RNeasy Mini kit (Qiagen), then processed and hybridized to a human expression array Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix). Experimental procedures for Gene Chip were performed according to the Affymetrix Technical Manual.

Quantitative Real Time Reverse Transcription-PCR—cDNAs were synthesized from total RNAs prepared from 293/FLAG-Nrf2 and 293/FLAG-Nrf2 A502Y cells using random hexamers. Real time PCR was performed using an ABI7700 or ABI7300 sequence detection system. The reaction was carried out for 40 cycles of 30 s at 95 °C and 1 min at 60 °C (HO-1, NQO1, and ribosomal RNA) or 55 °C (GLIPR1 and TPM4) using qPCR Mastermix (Eurogentec). Ribosomal RNA control reagents (Applied Biosystems) were used as an internal control. The probes and primers used for the quantitative real time PCR are described in Table 1.

RESULTS

Replacing an Alanine Residue in the Basic Region of Nrf2 with the Corresponding Tyrosine Residue of MafG Converts the Specificity of Heterodimeric Binding to That of Homodimeric Binding—Our previous SPR-microarray analysis demonstrated the differential DNA binding profiles of Nrf2-MafG heterodimer and MafG homodimer, which seemed to be attributed to the distinct DNA recognition modes of Nrf2 and MafG, mainly recognizing the core and the flanking regions of the MARE, respectively (14). Chief recognition of the core is the DNA binding feature typical of dimer-forming bZip transcription factors, whereas Maf proteins uniquely recognize the flanking regions. One of the structural features distinguishing between the Maf family and the rest of the bZip factors are the alanine residue at the corresponding position. The importance of tyrosine for Maf-specific DNA recognition was suggested by analysis of Maf proteins with tyrosine mutations (17). Replacement with alanine converted sequence recognition from the flanking regions to the core, whereas replacement with phenyl-

### Table 1: Oligonucleotides for real time reverse transcription—PCR

| Name        | Sequence                   |
|-------------|----------------------------|
| HO-1 F      | 5’-CCA GCA ACA AAG TGC AAG ATT C-3’ |
| HO-1 R      | 5’-TCA CAT GGC ATG TAG ACC TAC AG-3’ |
| HO-1 P      | 5’-FAM-TCT CGG ATG GCT TAC ACT CAG CCT TCC TAMRA-3’ |
| NQO1 F      | 5’-AGT TGG TCA CCA AAT TCG GGA AC-3’ |
| NQO1 R      | 5’-FAM-CTG CAT GTA GCT TTT TGG GCA CAC TCC AG TAMRA-3’ |
| NQO1 P      | 5’-AGT TGG TCA CCA AAT TCG GGA AC-3’ |
| GLIPR1 F    | 5’-AAC CTG GAC TAG GAT GTG TG-3’ |
| GLIPR1 R    | 5’-AAC CTG GAC TAG GAT GTG TG-3’ |
| TPM4 F      | 5’-FAM-AGA TGG TAA GAT GAA AAG AT-3’ |
| TPM4 R      | 5’-FAM-AGA TGG TAA GAT GAA AAG AT-3’ |
| TPM4 P      | 5’-FAM-AGA TGG TAA GAT GAA AAG AT-3’ |
| PRKAR2A F   | 5’-FAM-CAG AGG TTC AAC CCA CAC CT TAMRA-3’ |
| PRKAR2A R   | 5’-FAM-CAG AGG TTC AAC CCA CAC CT TAMRA-3’ |

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FIGURE 1. Identification of the alanine residue in the basic region of Nrf2 as a critical determinant of the binding specificity. A, amino acid sequence alignment of mouse bZip family members, *Saccharomyces cerevisiae* GCN4, and *Drosophila* CNC. Basic regions, Maf-specific extended homology regions, and CNC domains characteristic for the CNC family proteins (p45, Nrf1, Nrf2, and Nrf3) are boxed. Alanine residues conserved in non-Maf family proteins and tyrosine residues conserved in Maf family proteins are indicated by an arrow.

B, relative $K_A$ values of the Nrf2(A502Y) mutant molecule calculated from the rate constants obtained in the SPR-microarray analysis. MafG homodimer, Nrf2-MafG heterodimer, and Nrf2(A502Y)-MafG heterodimer were applied to an SPR microarray system to examine their binding affinities to various MARE-related sequences. The sequences of oligonucleotides assembled in the DNA microarray are shown on the left-hand side. The uppercase and lowercase letters indicate conserved and mutated bases, respectively, in the consensus MAREs (CEN-C and CEN-G), respectively. Relative $K_A$ values against the one for CEN-C were shown in bar graphs. Red, blue, orange, and green bars indicate the relative $K_A$ values for consensus MAREs, homodimer MAREs, dual MAREs, and heterodimer MAREs, respectively. Dotted lines indicate the level of $K_A$ values for CEN-C. The SPR was measured three times independently and the representative set of data is shown.
lalanine generally reduced the binding affinity. From this, we suspected that an alanine residue might be essential for recognition of the core. To test our hypothesis, we replaced alanine with tyrosine in the basic region of Nrf2 and generated an Nrf2(A502Y) mutant molecule.

We first performed comprehensive binding analysis using the SPR microarray imaging technique (13). Protein fragments of MafG, Nrf2, and Nrf2(A502Y) containing bZip domains were expressed in bacteria and purified. The DNA microarrays were assembled using double-stranded oligonucleotides containing MARE variants generated by systematic base alterations as described previously (Fig. 1B, Ref. 14). MafG homodimer, Nrf2-MafG heterodimer, and Nrf2(A502Y)-MafG heterodimer were applied to the DNA microarray, and SPR signals were obtained.

MafG homodimer bound to the consensus MARE (CEN-C and CEN-G, see Fig. 1B) with $K_a$ values (m$^{-1}$) of 2.1 $\times$ 10^8 and 3.2 $\times$ 10^8 for CEN-C and CEN-G, respectively. The $K_a$ values of Nrf2-MafG heterodimer for CEN-C and CEN-G were 5.3 $\times$ 10^7 and 6.5 $\times$ 10^7, respectively. Those of Nrf2(A502Y)-MafG heterodimer were 8.4 $\times$ 10^7 and 9.1 $\times$ 10^7, respectively. Nrf2-MafG and Nrf2(A502Y)-MafG heterodimers displayed similar affinities for the consensus MAREs.

A calculation of relative $K_a$ values against the one for CEN-C revealed that the binding profile of Nrf2(A502Y)-MafG heterodimer mimicked that of MafG homodimer. Nrf2-MafG heterodimer did not bind to a group of MARE variants with symmetric base alterations (e.g., CO-S1CG, CO-S1TA, CO-S2AT, CO-S3AT, CO-S3CG, FL-S4TA, FL-S5AT, and FL-S5CG, indicated by blue bars in Fig. 1B), unlike MafG homodimer. Nrf2(A502Y)-MafG heterodimer partially acquired the ability to bind to these sequences (FL-S4TA, FL-S5AT, and FL-S5CG). Nrf2-MafG heterodimer bound with a higher affinity to a group of MARE variants with base alterations at position 4 (FL-S4AT, FL-A4C, and FL-A4T, indicated by green bars in Fig. 1B) than to consensus MAREs (CEN-C and CEN-G, indicated by red bars in Fig. 1B). In contrast, Nrf2(A502Y)-MafG heterodimer showed lower affinities to MARE variants with base alterations at position 4 than to consensus MAREs, resembling the binding profile of MafG homodimer.

**Binding Specificity of Nrf2(A502Y)-MafG Heterodimer Resembles That of MafG Homodimer**—We performed EMSA to address the changes in the binding specificity of the Nrf2(A502Y) mutant molecule (Fig. 2). For this assay, four well characterized MARE sequences present in endogenous gene regulatory regions were used as probes (Table 2, Ref. 14). Nrf2-MafG and Nrf2(A502Y)-MafG heterodimers interacted similarly with the MAREs in probes A and F (dual MAREs). Nrf2-MafG heterodimer bound strongly to the MARE variants in probes B and C (heterodimer MAREs) and weakly to those in probes D and E (homodimer MAREs). Nrf2(A502Y)-MafG heterodimer displayed weaker binding to the heterodimer MAREs in probes B and C and stronger binding to the homodimer MAREs in probe D and E. The binding specificity of Nrf2(A502Y)-MafG heterodimer resembled that of MafG homodimer. These results with MAREs in endogenous gene regulatory regions are consistent with the results obtained from the comprehensive SPR-microarray analysis.
erase reporter gene. The assay was not supplemented with MafG, as endogenous small Maf was expected to be sufficiently abundant for heterodimerization with Nrf2 and its mutant molecule. Luciferase activities were measured 24 h after transfection. When the luciferase gene was driven by the consensus MARE (Fig. 3A), reporter gene activation was comparable between Nrf2 and Nrf2(A502Y). The nuclear accumulation of Nrf2 and Nrf2(A502Y) was also analogous (Fig. 3F). These results and the comparable binding affinities of Nrf2 and Nrf2(A502Y) to the consensus MARE (see Fig. 2A) suggested that the Ala to Tyr mutation in the basic region did not affect the transcriptional activity of Nrf2.

When the reporter gene was driven by heterodimer MARE (Fig. 3, B and C), Nrf2 elicited a stronger activation than Nrf2(A502Y). On the contrary, Nrf2(A502Y) elicited a stronger activation than Nrf2 when the reporter gene was driven by homodimer MARE (Fig. 3, D and E). These findings show that the difference between the binding profiles of Nrf2-MafG and Nrf2(A502Y)-MafG heterodimers was faithfully reflected in the differential activation of reporter genes driven by different subtypes of MARE variants.

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Endogenous Genes Respond Differentially to Nrf2 and Nrf2(A502Y)—The effects of Nrf2 and Nrf2(A502Y) on the expression levels of endogenous genes using 293/FLAG-Nrf2 and 293/FLAG-Nrf2(A502Y) cells were examined. Total RNAs were prepared from each cell line before and after induction and subjected to transcriptome analysis. 255 genes and 126 genes were induced by over 1.4-fold after tetracycline using anti-FLAG antibody and enrichment of DNA fragments containing HO-1 E1 enhancer (containing both HO-1 MARE-1 and -2), or NQO1 MARE was examined by PCR (Fig. 4, D–F). Nrf2 and Nrf2(A502Y) bound similarly to the HO-1 E1 enhancer (Fig. 4D), whereas Nrf2(A502Y) bound to the NQO1 MARE less efficiently than Nrf2 (Fig. 4E). Anti-Nrf2 antibody gave a similar result (data not shown). These outcomes suggested that Nrf2(A502Y) could interact with the E1 enhancer, since HO-1 MARE-2 should allow its binding and NQO-1 MARE, with its preference for the heterodimer, did not strongly recruit Nrf2(A502Y). The third exon fragment of the HO-1 gene was detected as a negative control (Fig. 4F). Thus, the different binding specificities between Nrf2 and Nrf2(A502Y) were significant in vivo.

Endogenous Regulatory Region—To verify whether the binding specificity alteration of Nrf2(A502Y) mutant is actually operating in vivo, we examined the binding of Nrf2 and Nrf2(A502Y) in vivo by a ChIP analysis through establishing stable cell lines expressing FLAG-tagged Nrf2 or Nrf2(A502Y), which is inducible by tetracycline (293/FLAG-Nrf2 and 293/FLAG-Nrf2(A502Y) cells). The nuclear accumulation of Nrf2 and Nrf2(A502Y) after the tetracycline induction was comparable between the two cell lines (Fig. 4A).

In this analysis we chose two Nrf2 target genes with well characterized regulatory regions, hemeoxygenase-1 (HO-1) and NADP(H):quinone oxidoreductase 1 (NQO1). Two tandem MAREs, referred to as the E1 distal enhancer (21), are located 4 kbp upstream from the transcription initiation site of the human HO-1 gene (Fig. 4B). HO-1 MARE-1 and HO-1 MARE-2 have the characteristics of heterodimer MARE and homodimer MARE, respectively. A single MARE with characteristics of heterodimer MARE is located 400 bp upstream from the transcription initiation site of the human NQO1 gene (Fig. 4C). A ChIP assay was performed with the cells before and after induction by tetracycline using anti-FLAG antibody and enrichment of DNA fragments containing HO-1 E1 enhancer (containing both HO-1 MARE-1 and -2), or NQO1 MARE was examined by PCR (Fig. 4, D–F). Nrf2 and Nrf2(A502Y) bound similarly to the HO-1 E1 enhancer (Fig. 4D), whereas Nrf2(A502Y) bound to the NQO1 MARE less efficiently than Nrf2 (Fig. 4E). Anti-Nrf2 antibody gave a similar result (data not shown). These outcomes suggested that Nrf2(A502Y) could interact with the E1 enhancer, since HO-1 MARE-2 should allow its binding and NQO-1 MARE, with its preference for the heterodimer, did not strongly recruit Nrf2(A502Y). The third exon fragment of the HO-1 gene was detected as a negative control (Fig. 4F). Thus, the different binding specificities between Nrf2 and Nrf2(A502Y) were significant in vivo.

Endogenous Genes Respond Differentially to Nrf2 and Nrf2(A502Y)—The effects of Nrf2 and Nrf2(A502Y) on the expression levels of endogenous genes using 293/FLAG-Nrf2 and 293/FLAG-Nrf2(A502Y) cells were examined. Total RNAs were prepared from each cell line before and after induction and subjected to transcriptome analysis. 255 genes and 126 genes were induced by over 1.4-fold after tetracycline
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**FIGURE 4.** In vivo DNA binding of Nrf2 and Nrf2(A502Y). A, immunoblot analysis of 293/FLAG-Nrf2 or 293/FLAG-Nrf2(A502Y) cell lines. After treatment with tetracycline (Tet) for the times indicated, nuclear extracts of 293/FLAG-Nrf2 and 293/FLAG-Nrf2(A502Y) cells were subjected to immunoblot blot analysis with anti-Nrf2 (upper panel) or anti-tubulin antibodies (lower panel). B, MARE variants located in the regulatory region (E1 distal enhancer) of the human HO-1 gene. The PCR primer set used for the ChIP assay is indicated by arrows. C, a MARE variant located in the promoter region of the human NQO1 gene. The PCR primer set used for the ChIP assay is indicated by arrows. D–F, 293/FLAG-Nrf2 and 293/FLAG-Nrf2(A502Y) cells were treated with tetracycline for the times indicated, and ChIP assays were performed using antibodies against FLAG. Enrichment of the regulatory regions of the HO-1 (D) and NQO-1 (E) genes are shown. The third exon of the HO-1 gene was detected as a negative control (F).

**FIGURE 5.** Activation of endogenous genes by Nrf2 and Nrf2(A502Y). A, the number of genes induced by Nrf2, Nrf2(A502Y), or both. Microarray analysis was performed using RNA samples taken before and 6 h after tetracycline treatment. A gene was counted if it was induced by >1.4-fold and its signal level after induction was >80.8-fold induction ratios of the representative genes were examined by quantitative real time reverse transcription-PCR. 293/FLAG-Nrf2 and 293/FLAG-Nrf2(A502Y) cells were treated with tetracycline and harvested after 6 h. The black and gray bars show the average-fold induction ratios of triplicate samples of Nrf2 and Nrf2(A502Y), respectively. Error bars indicate S.D. These results are representative of three independent experiments.

**DISCUSSION**

In this study we found that a heterodimer of MafG and Nrf2(A502Y), the basic region mutant of Nrf2, displayed a DNA...
binding specificity similar to that of MaF homodimer. Our microarray analysis utilizing the cell line stably expressing the Nrf2(A502Y) mutant molecule revealed the substantial difference between the gene expression profiles activated by Nrf2-small Maf and Nrf2(A502Y)-small Maf heterodimers. These results strongly argue that subtle changes accumulated in the binding sequences for the Maf and CNC family of transcription factors during molecular evolution give rise to the functionally discrete cis-elements categorized as homodimer MAREs or heterodimer MAREs (e.g. ARE), which prefers MaF homodimer binding and MaF-CNC heterodimer binding, respectively. This study further clarified that one amino acid substitution in the basic region of the CNC and Maf family transcription factors serves as part of the molecular basis for this specific matching of cis-elements and transcription factors. Thus, the biological significance of differential binding by MaF homodimer and CNC-Maf heterodimer should be interpreted based on the accumulation of base changes in the cis-acting elements and evolution of the transcription factors acquiring activities recognizing the deviation.

Several previous reports suggested a possible heterogeneity in MARE-related sequences; those bound by MaF homodimers and those bound by CNC-Maf heterodimers. The target sequences of large MaF homodimers have been deduced from promoter analysis of the target genes and were found to have well conserved flanking regions rather than core regions (3, 14). The target sequences of CNC-Maf heterodimers were reported to be asymmetric, where the flanking region was conserved on one side of the MARE, e.g. the NF-E2 binding site for NF-E2 homodimer (TGCTGACTCAT; Ref. 23) and the ARE site for Nrf2-small Maf heterodimer (GCGNNTCTCA; Ref. 24). Consistent with these observations, we found that the gene profile activated by Nrf2-small Maf heterodimer substantially differed from that activated by Nrf2(A502Y)-small Maf heterodimer that mimicked MaF homodimer. The present results suggest that the distinct DNA binding specificity of Nrf2-small Maf heterodimer is essential for Nrf2 to function as a key regulator of cytoprotective genes, as most of these genes were ineffectively activated by Nrf2(A502Y). The partially overlapping activated gene profiles of Nrf2-Maf and Nrf2(A502Y)-Maf heterodimers implicated that several genes are co-regulated by MaF homodimer and Nrf2-small Maf heterodimer.

X-ray crystallography revealed the structure of typical dimeric bZip domains in complex with DNA (25, 26). The homodimeric bZip domain of GCN4 and heterodimeric bZip domains of c-Jun and c-Fos were crystallized in the presence of palindromic target DNA molecules. In both cases each bZip domain was found to form a continuous α-helix and to recog-
nize the DNA sequence corresponding to the core region of the MARE. Compared with these typical bZip factors, the absolute difference in DNA recognition by Maf homodimers is the requirement of the flanking region of the MARE. One previous report showed that glycine and tyrosine residues in the basic region of Maf proteins are critical determinants of their unique binding property (17) and proposed the model that these residues break the α-helix and position the EHR closer to the flanking region of the MARE. Mutation of alanine to tyrosine in Nrf2 might have bent the CNC domain (see Fig. 1A) so that it can interact with the flanking region of the MARE. Our previous analysis on the NMR structure of the EHR of MafG revealed that it resembles the CNC domain of Skn-1 (16). The conformational similarity between the EHR of MafG and the CNC domain of Nrf2 might have enabled Nrf2(A502Y) to recognize the flanking region of the MARE with its CNC domain and to recapitulate the binding specificity of Maf.

Genes activated by tetracycline-induced Nrf2 in 293 cells included typical Nrf2 target genes involved in cytoprotection (e.g. thioreredoxin reductase 1, glutamate-cysteine ligase modifier subunit, and NQO1). Several genes induced by exposure to electrophiles in an Nrf2-dependent manner were not activated in this experiment (e.g. glutathione S-transferases). One reason for this might be the increase in Nrf2 protein in the absence of electrophilic or oxidative stresses in this system. As far as the canonical Nrf2 target genes induced under this condition were concerned, cross-talk between Nrf2-small Maf heterodimer and large Maf homodimer was less probable since Nrf2(A502Y) did not activate these genes. We missed out several genes expected to have been induced in the physiological stress response in this study. Therefore, a rational extension of this study would be to generate a knock-in mouse harboring an A502Y mutation in the study would be to generate a knock-in mouse harboring an A502Y mutation in the Nrf2 locus and examine the function of Nrf2(A502Y) in mice with an Nrf2-null background.

Small Maf homodimers lacking canonical activation domains are expected to repress transcription when competing with active Nrf2-small Maf heterodimer for the MARE (9). However, most of the cytoprotective genes regulated by Nrf2 were not activated by the Nrf2(A502Y) mutant molecule, suggesting that Nrf2(A502Y)-small Maf heterodimer has a lower binding affinity to the MAREs in the regulatory regions of typical Nrf2 target genes. Because Nrf2(A502Y)-MafG heterodimer displayed a binding profile similar to that of MafG homodimer, this result implies that small Maf homodimer has a lower binding affinity to the MAREs in the regulatory regions of Nrf2 target genes and may not be an efficient repressor of the Nrf2 target genes, assuming that comparable amounts of small Maf homodimer and Nrf2-small Maf heterodimer are present. Therefore, small Maf in excess of Nrf2 would be required for small Maf homodimer to elicit repression of cytoprotective genes.

The genes activated by Nrf2(A502Y) might include some of the large Maf target genes. Expressed under the control of the viral promoter, c-Maf was reported to efficiently trans- form fibroblasts (27), but the target genes responsible for this transformation have not been pinpointed. Several genes (e.g. ribosomal protein S6 kinase, 90 kDa, polypeptide 4, and serine/threonine/tyrosine kinase 1) reported to be involved in tumorigenesis and cell proliferation (28, 29) could be such targets.

Some of the genes induced by both Nrf2 and Nrf2(A502Y), which were interpreted as those co-regulated by Nrf2-small Maf heterodimer and large Maf homodimer, might also be involved in tumorigenesis (e.g. GLIPR1, Ref. 30). A previous study suggested that the cis-regulatory sequence recognized by Jun homodimer is necessary for Maf-induced transformation (31). Although there are several possibilities, we favor the interpretation that the MARE sequence shared by AP-1 (Jun/Fos) and Maf, which should behave as a dual MARE, is involved in Maf-induced transformation. We surmise that Jun/Fos and Maf family proteins may be able to exert their oncogenic activities by regulating common genes through the dual MARE.

An intriguing revelation from our study is that the artificially selected consensus sequence with the highest affinity does not necessarily represent the majority of significant cis-acting elements targeted by a transcription factor in vivo. Both Nrf2 and Nrf2(A502Y) bound to the consensus MARE with high affinity, yet the activated gene profiles in vivo were quite different from each other. The sequences that diverged from the consensus, sometimes with lower affinities, seemed to confer the differential specificities required for diverse gene regulation by related family members of transcription factors.

Acknowledgment—We greatly appreciate the assistance of Y. Meguro for the microarray analysis.

REFERENCES

1. Itoh, K., Ishii, T., Wakabayashi, N., and Yamamoto, M. (1999) Free Radic. Res. 31, 319–324
2. Motohashi, H., O’Connor, T., Katsuoka, F., Engel, D.J., and Yamamoto, M. (2002) Gene (Amst.) 294, 1–12
3. Kerppola, T. K., and Curran, T. (1994) Oncogene 9, 3149–3158
4. Kataoka, K., Noda, M., and Nishizawa, M. (1994) Mol. Cell. Biol. 14, 700–712
5. Motohashi, M., and Yamamoto, M. (2004) Trends Mol. Med. 10, 549–557
6. Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., Oyake, T., Hayashi, N., Satoh, K., Hatakeyama, I., Yamamoto, M., and Nabeshima, Y. (1997) Biochem. Biophys. Res. Commun. 236, 4184–4193
7. Motohashi, H., Katsuoka, F., Engel, J. D., and Yamamoto, M. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 6379–6384
8. Katsuoka, F., Motohashi, H., Ishii, T., Aburatani, H., Engel, J. D., and Yamamoto, M. (2005) Mol. Cell. Biol. 25, 8044–8051
9. Motohashi, H., Katsuoka, F., Shavit, J. A., Engel, J. D., and Yamamoto, M. (2000) Cell 103, 865–875
10. Sun, J., Hoshino, H., Takaku, K., Nakajima, O., Muto, A., Suzuki, H., Tashiro, S., Takahashi, S., Shibahara, S., Alam, J., Taketo, M. M., Yamamoto, M., and Igarashi, K. (2002) EMBO J. 21, 5216–5224
11. Katsuoka, F., Motohashi, H., Tamagawa, Y., Kure, S., Igarashi, K., Engel, J. D., and Yamamoto, M. (2003) Mol. Cell. Biol. 23, 1163–1174
12. Brand, M., Ranish, J. A., Kummer, N. T., Hamilton, J., Igarashi, K., Francel, C., Chi, T. H., Crabtree, G. R., Aebersold, R., and Groudine, M. (2004) Nat. Struct. Mol. Biol. 11, 73–80
13. Kyo, M., Yamamoto, T., Motohashi, H., Kamiya, T., Kuroita, T., Tanaka, T., Engel, J. D., Kawakami, B., and Yamamoto, M. (2004) Genes Cells 9, 153–164
14. Yamamoto, T., Kyo, M., Kamiya, T., Tanaka, T., Engel, J. D., Motohashi, H., and Yamamoto, M. (2006) Genes Cells 11, 575–591
15. Blank, V., and Andrews, N. C. (1997) Trends Biochem. Sci. 22, 437–441
16. Kosunoki, H., Motohashi, H., Katsuoka, F., Morohashi, A. Y., Yamamoto, M., and Tanaka, T. (2002) Nat. Struct. Biol. 9, 252–256
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17. Đlakic, M., Grinberg, A. V., Leonard, D. A., and Kerppola, T. K. (2001) EMBO J. 20, 828–840
18. Motohashi, H., Katsuoka, F., Miyoshi, C., Uchimura, Y., Saitoh, H., Francastel, C., Engel, J. D., and Yamamoto, M. (2006) Mol. Cell. Biol. 26, 4652–4663
19. Zhang, J., Hosoya, T., Maruyama, A., Nishikawa, K., Maher, J. M., Ohta, T., Motohashi, H., Fukamizu, A., Shibahara, S., Itoh, K., and Yamamoto, M. (2007) Biochem. J. 404, 459–466
20. Igarashi, K., Kataoka, K., Itoh, K., Hayashi, N., Nishizawa, M., and Yamamoto, M. (1994) Nature 367, 568–572
21. Nioi, P., Nguyen, T., Sherratt, P. J., and Pickett, C. B. (2005) Mol. Cell. Biol. 25, 10895–10906
22. Zhang, J., Ohta, T., Maruyama, A., Hosoya, T., Nishikawa, K., Maher, J. M., Shibahara, S., Itoh, K., and Yamamoto, M. (2006) Mol. Cell. Biol. 26, 7942–7952
23. Ney, P. A., Sorrentino, B. P., Lowrey, C. H., and Nienhuis, A. W. (1990) Nucleic Acids Res. 18, 6011–6017
24. Rushmore, T. H., Morton, M. R., and Pickett, C. B. (1991) J. Biol. Chem. 266, 11632–11639
25. Ellenberger, T. E., Brandl, C. I., Struhl, K., and Harrison, S. C. (1992) Cell 71, 1223–1237
26. Glover, J. N. M., and Harrison, S. C. (1995) Nature 373, 257–261
27. Kataoka, K., Nishizawa, M., and Kawai, S. (1993) J. Virol. 67, 2133–2141
28. Shamji, A. F., Nghiem, P., and Schreiber, S. L. (2003) Mol. Cell 12, 271–280
29. Liu, L., Yu, X. Z., Li, T. S., Song, L. X., Chen, P. L., Suo, T. L., Li, Y. H., Wang, S. D., Chen, Y., Ren, Y. M., Zhang, S. P., Chang, Z. J., and Fu, X. Y. (2004) Cancer Res. 64, 3491–3499
30. Rosenzweig, T., Ziv-Av, A., Xiang, C., Lu, W., Cazacu, S., Taler, D., Miller, C. G., Reich, R., Shoshan, Y., Anikster, Y., Kazimirsky, G., Sarid, R., and Brodie, C. (2006) Cancer Res. 66, 4139–4148
31. Kataoka, K., Shioda, S., Yoshitomo-Nakagawa, K., Handa, H., and Nishizawa, M. (2001) J. Biol. Chem. 276, 36849–36856