Genetic Evidence that Small Maf Proteins Are Essential for the Activation of Antioxidant Response Element-Dependent Genes

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While small Maf proteins have been suggested to be essential for the Nrf2-mediated activation of antioxidant response element (ARE)-dependent genes, the extent of their requirement remains to be fully documented. To address this issue, we generated *mafG::mafF* double-mutant mice possessing MafK as the single available small Maf. Induction of the NAD(P)H:quinone oxidoreductase 1 (*NQO1*) gene was significantly impaired in double-mutant mice treated with butylated hydroxyanisole, while other ARE-dependent genes were less affected. Similarly, in a *keap1*-null background, where many of the ARE-dependent genes are constitutively activated in an Nrf2-dependent manner, only a subset of ARE-dependent genes, including *NQO1*, were sensitive to a simultaneous deficiency in MafG and MafF. Examination of single and double small *maf* mutant cells revealed that MafK also contributes to the induction of ARE-dependent genes. To obtain decisive evidence, we established *mafG::mafK::mafF* triple-mutant fibroblasts that completely lack small Mafs and turned out to be highly susceptible to oxidative stress. We found that induction in response to diethyl maleate was abolished in a wider range of ARE-dependent genes in the triple-mutant cells. These data explicitly demonstrate that small Mafs play critical roles in the inducible expression of a significant portion of ARE-dependent genes.

Antioxidant or electrophile response elements (AREs or EpREs) are *cis*-regulatory elements vital for the transcription of genes encoding antioxidant and phase II drug-metabolizing enzymes (reviewed in reference 9). In response to oxidative stress, numerous cytoprotective genes are induced in an ARE-dependent manner. The ARE consensus sequence TGA(G/C)nnnGC resembles the Maf recognition element (MARE) TGCTGA(G/C)TCAGCA, which was originally identified as the binding site for the v-maf oncoprotein (8, 15). This similarity originally suggested that small Maf proteins might also be essential coeffectors for transcriptional regulation mediated through the ARE. At present, a consensus of evidence suggests that small Maf proteins form homodimers, various combinations of small Maf heterodimers, heterodimers with transcriptional activator proteins of the CNC family (Nrf1, Nrf2, Nrf3, and p45 NF-E2), and heterodimers with the transcriptional repressor proteins Bach1 and Bach2. Any of these dimeric combinations can then bind to MAREs, as well as to other related sequences, including AREs (for reviews, see references 29 and 30).

The GC residues of the ARE (underlined above), which flank the TRE (TPA-response element) core sequence [TGA(G/C)TCA] of MARE, are critical for the induction of genes in response to oxidative stimuli (38). Furthermore, a recent nuclear magnetic resonance structural analysis suggested that the small Maf proteins contact the GC sequence in the MARE (or ARE) using a unique DNA binding motif termed the extended homology region, which is located N terminal to the basic region-leucine zipper (bZIP) motif (18, 20). Hence, the small Maf proteins are generally regarded as important components of the cellular stress response mediated through the *cis*-acting ARE elements. Since typical MAREs contain a central TRE, bZIP-type transcription factors, including Jun, Fos, and ATF family proteins, may also bind to MAREs and their related sequences and may affect transcriptional responses through these elements (15).

The biological significance of ARE-binding transcription factors has also been examined and confirmed by germ line genetic manipulation. Analysis of *nrf2*-null mutant mice showed that Nrf2 is a central regulator of the induction of many antioxidant-responsive genes and phase II detoxification enzyme genes and that Nrf2 is a key transcriptional activator of AREs (6, 8). Under normal physiological conditions, Nrf2 is captured in the cell cytoplasm by a molecule called Keap1 and turned over rapidly by proteasomal degradation (10, 11, 24, 33, 41). However, in response to oxidative stress, Nrf2 is stabilized, relocates to the nucleus, and binds to and activates target response genes (10, 11). As part of the proof elucidating this pathway, it was shown that Nrf2 accumulates in the nuclei of *keap1*-null mutant mice even in the absence of exogenous stress stimuli, leading to constitutive activation of ARE-dependent genes (43). Analysis of single *nrf1*-null mice and *nrf1::nrf2* compound mutant mice suggests that Nrf1 also contributes to the induction of ARE-dependent genes (22).

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Many studies have attempted to clarify the contribution of the small Maf proteins to ARE-dependent gene regulation. To date, no clear functional differences have been reported among the three small Maf proteins MafG, MafK, and MafF (29). Unlike their CNC and Bach partner molecules, canonical functional domains other than the DNA binding and dimerization motifs have not been identified in these small Maf proteins (29). Published studies have led to the conclusion that the CNC and Bach proteins require small Maf proteins as obligatory partners to promote site-specific ARE/MARE association (1, 2, 7, 19, 25, 37). One interpretation of these data is that the small Maf proteins contribute to transcriptional activation and repression as a consequence of the intrinsic activities of their heterodimeric partners (the CNC and Bach proteins). However, it is well documented that the small Maf proteins form homodimers, which can act as repressive competitors for MAREs with large Maf homodimers, as well as with small Maf-CNC heterodimers (28). In contrast to this view of small Maf contributions to MARE-mediated gene activation, several reports describe evidence that led their authors to conclude that the small Maf proteins contribute exclusively to the repression of ARE-dependent genes and not to their activation (3, 14, 32). In summary, according to divergent interpretations of biochemical, transfection, and in vivo data, it is clear that the contribution of the small Maf proteins to transcriptional activation and repression through ARE elements has not been definitively resolved.

To address the function of the small Maf proteins in vivo, we originally reported their individual germ line disruptions in mice (35, 36, 39). Although $mafF^{-/-}$ (F0) and $mafK^{-/-}$ (K0) mice displayed no apparent mutant phenotypes, $mafG$-null mutant mice displayed mild thrombocytopenia accompanied by a late-onset neurological disorder (39). The neurological abnormality was exacerbated in $mafG^{-/-}::mafK^{+/-}$ (G0K1) compound mutant mice, suggesting that MafG and MafK share compensatory functions in the central nervous system (16). Since several neurodegenerative disorders are suspected to result from a dysregulated oxidative stress response, we analyzed the expression of ARE-dependent genes in the central nervous systems of these symptomatic mutant mice. These studies showed that the heme oxygenase 1 gene (*HO-1*) was induced in G0K1 compound mutant animals, whereas many other ARE-dependent genes were not significantly affected (16). We also found that Bach proteins failed to accumulate in neuronal nuclei in the G0K1 central nervous system, which could explain the observed *HO-1* transcriptional derepression (16, 42). However, these studies did not resolve whether or not the small Maf proteins collaboratively, with a CNC partner, contribute to the activation of ARE-dependent genes. Here, we provide direct and decisive genetic evidence that the small Maf proteins are required for the activation of ARE-dependent gene expression collaboratively with Nrf2 in vivo, thereby excluding the notion that the small Maf proteins contribute merely to ARE-mediated repression.

MATERIALS AND METHODS

Generation of compound mutant mice. Germ line mutagenesis of the mouse *mafG*, *mafK*, *mafF*, *nrf2*, and *keap1* genes has been described previously (8, 35, 36, 39, 43). All mice examined in this study were of a mixed genetic background, with contributions from the 129Sv/J, C57BL/6J, and ICR strains. Genotypes were determined by PCR as described previously (8, 35, 36, 39, 43).

Mouse embryonic fibroblasts (MEFs). MEFs were prepared from individual embryos at embryonic day 11.0 (E11.0) or E13.5. The head and internal organs were removed, and the torso was minced and dispersed in 0.25% trypsin–EDTA. MEFs were maintained in Dulbecco's modified Eagle's medium (Sigma Chemical Co., St. Louis, Mo.) containing 10% fetal bovine serum and antibiotics.

Cell viability analysis. MEFs prepared from $mafG^{+/+}::mafK^{-/-}::mafF^{-/-}$ (G2K0F0) and $mafG^{-/-}::mafK^{-/-}::mafF^{-/-}$ (G0K0F0) embryos were seeded at an initial density of 5×10^3 cells/well in 96-microwell plates. Cells were initially cultured in 25 μM diethyl maleate (DEM). After 24 h, the medium was replaced with medium containing 0, 5, or 10 μM 1-chloro-2,4-dinitrobenzene (CDNB; Wako Pure Chemicals, Osaka, Japan). After 12 h of culturing, a cell viability assay (Cell Counting Kit-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to measure the activities of dehydrogenase enzymes as an indicator of cell viability according to the manufacturer's protocol.

RNA extraction. Total RNA was prepared from mouse liver or mouse embryonic fibroblasts using an Isogen RNA extraction kit (Nippon Gene, Toyama, Japan) and following the manufacturer's protocol.

RNA blot analysis. Purified total RNA was electrophoresed on formaldehyde-agarose gels and transferred onto a nylon membrane (Zeta Probe membrane; Promega). ^{32}P-labeled probes were prepared from the cDNA by random primer labeling. cDNAs encoding *HO-1* and *NQO1* were previously described (6, 8). cDNA that can detect both glutathione *S*-transferase P1 (GSTP1) and GSTP2 mRNAs was a gift from Kimihiko Satoh (Hirosaki University School of Medicine, Hirosaki, Japan) and designated *GSTP1/2*. To generate the probes, cDNA fragments were amplified by PCR using various primer pairs as follows: 5′-CAG TGT TTG AAC GGA ACA GA-3′ and 5′-TAC TTG GTC CAA GAC TTG AC-3′ for the ferritin light chain (*FTL*) gene, 5′-ATG TAT GCA GAT GGC ACC CAG GAC CTG-3′ and 5′-GGA CAA TCC TGA CCA CCT CAA CAT AG-3′ for the *GSTA4* gene, and 5′-GAA GAC CCT AGT AGT TGG TGC ATC-3′ and 5′-AAG GAG TAA ATA CAG TCG TTG GGA C-3′ for the thioredoxin reductase 1 gene (*TXNRD1*). The PCR products were subcloned into the pGEM-T easy vector (Promega).

Administration of butylated hydroxyanisole (BHA) to mice. BHA (Sigma Chemicals) was administered by oral gavage at a dose of 0.6 g/kg dissolved in corn oil. Animals were sacrificed 24 h after treatment, and livers were processed for the purification of RNA. All mice used in this analysis were 10- to 15-week-old females.

cDNA microarray analysis. Isolated total RNA was further purified by RNeasy RNA isolation kit (QIAGEN) by following the manufacturer's protocol. The purified RNA was processed and hybridized to a mouse expression array 430A gene chip (Affymetrix, Santa Clara, CA). Experimental procedures for Gene-Chip were performed according to the Affymetrix Gene Chip Expression Analysis technical manual.

Quantitative real-time PCR. cDNA was synthesized from the isolated RNAs by reverse transcriptase using random hexamer oligonucleotide primers, and real-time PCR was performed using an ABI Prism 7700 (Applied Biosystems, Foster City, CA) as previously described (35). The primer and probe sequences used for detecting NQO1, GSTA4, TXNRD1, glutamate-cysteine ligase catalytic subunit (GCLC), and HO-1 mRNAs are shown in Table 1. The rRNA primers and probes were purchased from Applied Biosystems.

RESULTS

Impaired induction of the *NQO1* gene in *mafG::mafF* double-mutant mice. In order to prove how small Maf proteins contribute to Nrf2-dependent gene regulation, we first exploited $mafG^{-/-}::mafF^{-/-}$ double-mutant (G0F0) mice. BHA was administered to wild-type (WT), G0F0, and *nrf2*-null mutant mice, and the expression of ARE-dependent genes (4, 21, 34, 43) was analyzed by RNA blot analysis. Induction of the *NQO1* gene was severely compromised in the livers of both *nrf2*-null and G0F0 mice compared to that of WT mice (Fig. 1). In contrast, the induction of the other ARE-dependent genes, including *TXNRD1*, *GSTP1/2*, *GSTA4*, and *FTL*, was affected only marginally by the simultaneous deficiency of MafG and MafF, while the expression of these ARE-dependent genes was affected differentially, but more substantially, by the lack of

TABLE 1. Oligonucleotide primers and TaqMan probes used in this study

Gene	Primers (5′ to 3′)	TaqMan probe (5′ to 3′)[a]
NQO1	Sense: AGC TGG AAG CTG CAG ACC TG Antisense: CCT TTC AGA ATG GCT GGC A	FAM-ATT TCA GTT CCC ATT GCA GTG GTT TGG G-TAMRA
GSTA4	Sense: GGG AAC AGT ATG AGA GAT GC AAA A Antisense: CCC ATC GAT TTC AAC CAA GG	FAM-TGG ACA CCT GCT TTT CGG CCA AG-TAMRA
TXNRD1	Sense: AGA AAG TGC TGG TCT TGG ATT TTG Antisense: ACA CGT TCC TCC GAG ACC C	FAM-TCT GGT CCC AAG AGG AGT CGG TGT G-TAMRA
GCLC	Sense: ATC TGC AAA GGC GGC AAC Antisense: ACT CCT CTG CAG CTG GCT C	FAM-ACG GGT GCA GCA AGG CCC A-TAMRA
HO-1	Sense: GTG ATG GAG CGT CCA CAG C Antisense: TTG GTG GCC TCC TTC AAG G	FAM-CGA CAG CAT GCC CCA GGA TTT GTC-TAMRA

[a] FAM, reporter fluorescent dye; TAMRA, quencher fluorescent dye.

Nrf2 (Fig. 1). The induction of GSTP1/2, GSTA4, and FTL mRNAs by BHA was decreased in the *nrf2*-null mouse liver, while the level of *TXNRD1* was not changed much. These results suggest that, in MafG and MafF double-mutant mice, the remaining small Maf protein, MafK, compensated for lack of the small Maf activity required for Nrf2-mediated induction of ARE-dependent genes except *NQO1*. In the case of the *NQO1* gene, the requirement level of small Maf activity may be relatively high, so that MafK could not compensate for lack of MafG and MafF. As for the residual induction by BHA of the *TXNRD1*, *GSTP1/2*, *GSTA4*, and *FTL* genes in Nrf2-null mouse liver, we surmise that there may be important contributions from certain other pathways that are activated by BHA but are independent of Nrf2. Thus, based on these analyses, we conclude that development of a much improved assay system is necessary to evaluate individual contributions of small Maf proteins and Nrf2 to the induction of ARE-dependent genes.

Requirement of small Maf proteins for ARE-dependent gene activation in *keap1*-null mutant mice. To examine the contri-
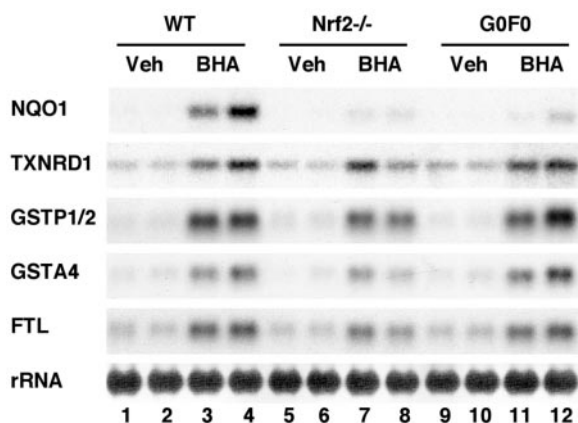

FIG. 1. ARE-dependent gene expression in livers of adult small *maf* mutant mice. RNA blot analysis was performed to examine the expression of *NQO1*, *TXNRD1*, *GSTP1/2*, *GSTA4*, and *FTL*. RNA samples were prepared from the livers of adult female WT controls (lanes 1 to 4), *nrf2*-null mutants (Nrf2−/−; lanes 5 to 8), and *mafG*−/−::*mafF*−/− mutants (G0F0; lanes 9 to 12). BHA (lanes 3, 4, 7, 8, 11, and 12) or corn oil (Veh; lanes 1, 2, 5, 6, 9, and 10) was administered to the mice.

butions of small Maf proteins and Nrf2 to the regulation mediated by the ARE pathway without interference from other (real or postulated) influences, we investigated ARE-dependent activity in *keap1*-null mutant mice. The rationale for this approach is that, in the absence of Keap1, Nrf2 constitutively accumulates in the nucleus and activates ARE-dependent genes (43). Therefore, the *keap1*-null mutant mouse would serve as a powerful means for analyzing Nrf2-dependent gene regulation (27).

We generated two lines of compound *keap1*-null mutant mice, *keap1*−/−::*mafG*−/−::*mafF*−/− (Kp0G0F0) and *keap1*−/−::*nrf2*−/− (Kp0N0). Since *keap1*-null mutant pups die before weaning, the expression of ARE-dependent genes was examined in the livers of mice at postnatal day 10. As previously documented (43), multiple ARE-dependent genes were constitutively induced in the *keap1*-null mutant mice (Kp0) and the induction was fully abrogated in Kp0N0 compound mutant mice (Fig. 2), confirming that ARE-dependent gene activation in the absence of Keap1 is completely dependent on Nrf2. However, in the Kp0G0F0 mutant mice, only *NQO1* and *TXNRD1* induction was partially decreased, while *GSTP1/2* and *FTL* were essentially unaffected by the absence of the small Maf proteins (Fig. 2). Taken together, these results demonstrate that the expression of MafG and/or MafF is indispensable for the induction of the *NQO1* and *TXNRD1* genes through the Nrf2-ARE pathway, while the *GSTP1/2* and *FTL* genes are not sensitive to the loss of both MafG and MafF. Two possibilities were raised regarding the regulation of the *GSTP1/2* and *FTL* genes. One is that the remaining small Maf protein, MafK, is sufficient for the Nrf2-mediated activation of the latter genes. The other possibility is that bZIP proteins apart from small Maf proteins cooperate with Nrf2 and activate these genes.

All three small Maf proteins are functional in the regulation of ARE-dependent gene expression. We next examined the contribution of individual small Maf proteins to the expression of ARE-dependent genes. To this end, we established MEF lines from various mutant mouse embryos, including WT, *mafG*−/− (G0), *mafF*−/− (F0), *mafK*−/− (K0), *mafG*−/−::*mafF*−/− (G0F0), *mafG*−/−::*mafK*−/− (G0K0), and *nrf2*−/− (N0) mutant embryos. These lines of mutant mice have been described in previous studies (27). We treated the fibro-
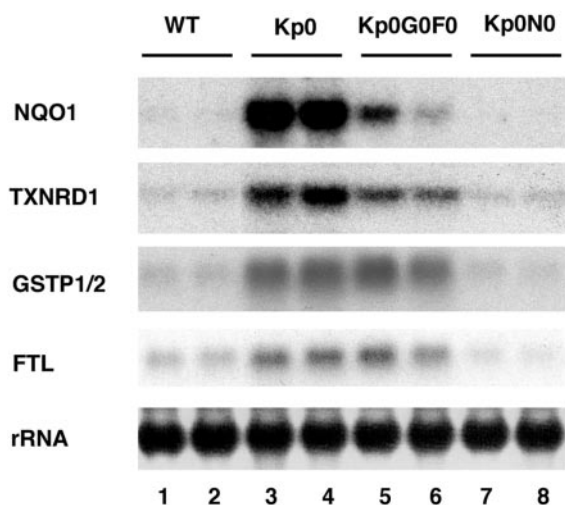
FIG. 2. Induction of *NQO1* and *TXNRD1* in *keap1* mutants is reversed by simultaneous disruption of *nrf2* or *mafG* plus *mafF*. RNA blot analysis was performed to examine the expression of *NQO1*, *GSTP1/2*, *FTL*, and *TXNRD1*. RNA samples were prepared from the postnatal livers of 10-day-old female WT control mice (lanes 1 and 2) and *keap1*$^{-/-}$ (Kp0, lanes 3 and 4), *keap1*$^{-/-}$::*mafG*$^{-/-}$::*mafF*$^{-/-}$ (Kp0G0F0, lanes 5 and 6), and *keap1*$^{-/-}$::*nrf2*$^{-/-}$ (Kp0N0, lanes 7 and 8) mutant mice.
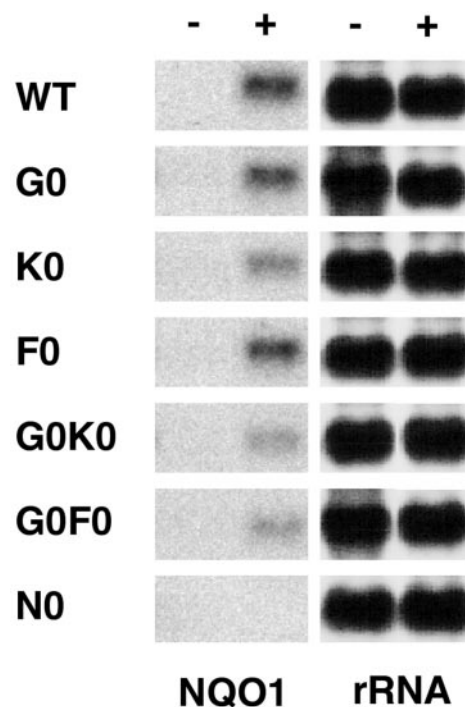

FIG. 3. ARE-dependent gene expression in small *maf* single- and double-mutant embryonic fibroblasts. RNA blot analysis was performed to examine the expression levels of *NQO1*. RNA samples were prepared from the MEFs of WT mice and *mafG*$^{-/-}$ (G0), *mafK*$^{-/-}$ (K0), *mafF*$^{-/-}$ (F0), *mafG*$^{-/-}$::*mafK*$^{-/-}$ (G0K0), *mafG*$^{-/-}$::*mafF*$^{-/-}$ (G0F0), and *nrf2*-null (N0) mutant mice. MEFs were treated with DEM (+) or vehicle (−).

blasts with DEM and examined the NQO1 mRNA level, as the *NQO1* gene is one of the representative Maf-dependent genes (above). Induction of *NQO1* gene expression by DEM was slightly diminished in G0 and F0 MEFs in comparison to WT MEFs (Fig. 3). In contrast, induction of the *NQO1* gene in K0, G0F0, and G0K0 MEFs was significantly impaired (Fig. 3), and the induction was almost abolished in *nrf2*-null mutant MEFs (Fig. 3). These results suggest that all three small Maf proteins are functional in the regulation of ARE-dependent gene expression in collaboration with Nrf2.

Loss of all small Maf proteins renders embryonic fibroblasts insensitive to electrophilic priming against oxidative stress. The above results suggest that the remaining MafK makes a substantial contribution to the induction of ARE-dependent genes in G0F0 mice. So, we finally went on to generate *mafG*$^{-/-}$::*mafK*$^{-/-}$::*mafF*$^{-/-}$ (G0K0F0) triple-mutant mice to see the effect of a complete loss of small Maf proteins. However, the simultaneous disruption of all three small *maf* genes was found to lead mice to embryonic lethality before E11.5 (precise analyses will be published elsewhere). Therefore, we prepared MEFs from the triple small *maf* gene mutant embryos at E11.0. These embryos were reproducibly recovered from *mafG*$^{+/-}$::*mafK*$^{-/-}$::*mafF*$^{-/-}$ (G1K0F0) mouse intercrossing.

We selected a pair of fibroblasts with the G2K0F0 (*mafG*$^{+/+}$::*mafK*$^{-/-}$::*mafF*$^{-/-}$) and G0K0F0 genotypes. To determine whether the G2K0F0 fibroblasts represent an appropriate reference for the gene expression analysis of G0K0F0 cells, we analyzed and compared the sensitivities of these cells to oxidative stress. These cells were incubated for 12 h with 0, 5, and 10 μM CDNB, which is known to act as a strong oxidative stress agent (6). When we recorded an indirect measure of cell viability, the differences in cell viability that

represented sensitivity to CDNB toxicity were insignificant in MEFs with the G2K0F0 or G0K0F0 genotype (Fig. 4).

Therefore, as a second test for the oxidative stress response, we pretreated MEFs for 24 h with 25 μM DEM, a mildly cytotoxic electrophile that has the potential to induce antioxidative stress enzymes. Following DEM treatment, we further treated the cells with CDNB for an additional 12 h. We found that G2K0F0 cells pretreated with DEM were much less sensitive to CDNB compared to cells without DEM pretreatment (Fig. 4). In contrast, there was no significant difference in sensitivity to CDNB between DEM-treated and untreated G0K0F0 cells (Fig. 4). These results allowed us to predict that MafG in G2K0F0 MEFs significantly contributes to the induction of ARE-dependent antioxidant and drug-metabolizing enzyme genes, while these genes were induced only weakly in G0K0F0 cells. Thereby, we concluded that G2K0F0 MEFs could be used as a reference for the gene expression analysis of G0K0F0 MEFs.

Microarray analysis documents the broad contribution of small Maf proteins to ARE-dependent gene transcription. To comprehensively examine the effect of a complete loss of small Maf proteins on gene regulation, we exploited oligonucleotide microarrays and analyzed the expression of genes that are inducible by DEM in MEFs with the genotype G2K0F0 or G0K0F0. These fibroblasts were treated with DEM or a vehicle control, followed by RNA extraction for probe processing. In this analysis, we focused on the genes encoding drug-metabo-
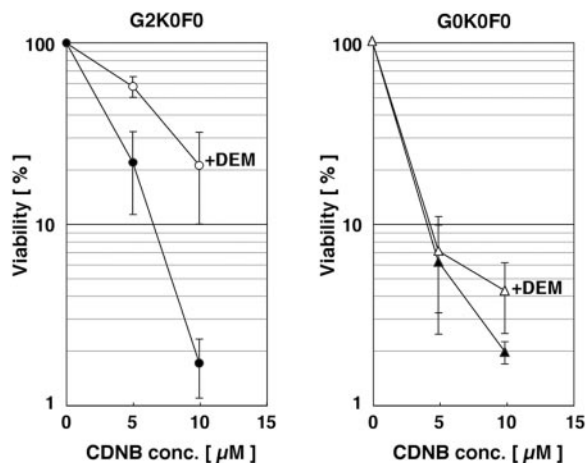
FIG. 4. Small *maf* triple-mutant MEFs are sensitive to an oxidative stress reagent. The viability of MEFs established from $mafG^{+/+}::mafK^{-/-}::mafF^{-/-}$ (G2K0F0; left graph) and $mafG^{-/-}::mafK^{-/-}::mafF^{-/-}$ (G0K0F0; right graph) embryos is shown. MEFs were exposed to increasing concentrations (conc.) of CDNB (0, 5, and 10 μM) with or without DEM pretreatment. Cell viability was examined as described in the text, and the viability of the 0 μM CDNB sample was set to 100%. Closed circles, open circles, closed triangles, and open triangles indicate G2K0F0 MEFs without DEM, G2K0F0 MEFs with DEM, G0K0F0 MEFs without DEM, and G0K0F0 MEFs with DEM, respectively. Error bars indicate standard deviations.

lizing enzymes and antioxidant enzymes, as these genes seem to play important roles in the acquisition of cellular resistance to oxidative stress. We found that in G2K0F0 cells, DEM treatment induced a number of genes coding for chaperone-related proteins, drug-metabolizing enzymes, and antioxidant enzymes (Table 2). A detailed comparison of the results obtained with the previous microarray analyses utilizing *nrf2*-null mice and *nrf2::keap1* compound mutant mice (6, 21) revealed that many of the DEM-inducible genes in G2K0F0 mice were identified as the genes whose expression is heavily dependent on Nrf2 (Table 2).

Several genes that are not regarded as ARE-dependent genes, such as heat shock protein genes and metallothionein genes, were also induced by DEM but less affected by the loss of MafG (Table 2). On the other hand, we found that the induction of ARE-dependent genes was severely impaired in G0K0F0 cells in comparison to that in G2K0F0 fibroblasts (Table 2). The contribution of MafG to basal gene expression was diverse. The *NQO1* and *GSTA4* genes seemed to be strongly dependent on MafG for their basal expression, while the basal expression levels of the other genes were not significantly different between the two genotypes (Table 2). While regulation of *GSTA4* has not been well characterized, accumulating lines of evidence suggest that *GSTA4* is a typical ARE-dependent gene (4, 21). *HO-1* was the only gene whose basal level of expression was increased in the absence of MafG (Table 2). This observation is in very good agreement with our previous analysis showing that the small Maf proteins collaborate with Bach1 and that the Bach1-small Maf heterodimer acts to repress *HO-1* gene transcription in murine neuronal cells (16, 42).

Deficiency of small Maf proteins affects the electrophile-mediated induction of gene expression. To verify the microarray results and to gain further insights into the small Maf contribution to the expression of genes induced by electrophiles, we examined the expression of several pertinent genes by quantitative reverse transcription-PCR analyses. In the analyses, in addition to the triple-null mutant MEFs (G0K0F0), we newly adopted G0K0F1 ($mafG^{-/-}::mafK^{-/-}::mafF^{+/-}$) cells that contain only one copy of MafF, expecting the lowest expression of small Maf activity, and compared the gene expression profile of the cells with that of G2K0F1 ($mafG^{+/+}::mafK^{-/-}::mafF^{+/-}$) cells. Consistent with the microarray data, G0K0F0 cells showed no apparent induction of the *NQO1*, *GSTA4*, *TXNRD1*, or *GCLC* gene (Fig. 5, lower panels). The *HO-1* gene was similarly derepressed in both the basal and induced states, and no induction was observed anymore in G0K0F0 fibroblasts (Fig. 5, lower right panel). In the presence of one *mafF* allele (G0K0F1 cells versus G2K0F1 cells), the loss of MafG did not affect significantly the induction of the *GSTA4*, *GCLC*, and *TXNRD1* genes and decreased and increased moderately the induction of the *NQO1* and *HO-1* genes, respectively (Fig. 5, upper panels). These results strongly support our contention that MafF functionally compensates the lack of MafG and MafK. In summary, we conclude that the small Maf proteins are essential for the induction of multiple ARE-dependent genes encoding xenobiotic-metabolizing enzymes and antioxidant enzymes.

DISCUSSION

The small Maf proteins are unique transcription factors with no recognizable transcriptional regulatory domains other than DNA binding and dimerization motifs. The small Maf proteins have been shown to form heterodimers in combination with CNC and Bach family proteins and can also form homodimers, as well as heterodimers, among themselves, depending on the abundance or activity of each molecule. The physiological concentration of each participant molecule is known to be crucial for the final output of transcriptional activity (28). Therefore, it would be ideal to elucidate the function of the small Maf proteins by a loss-of-function strategy in order to evaluate the contribution of each of these molecules to specific transcriptional responses under physiological conditions. However, since the three small Mafs are expressed in broadly overlapping patterns (35, 39) and since no functional differences among them have been documented, analysis of the single-gene loss-of-function mutants has been limited.

Our group and others have documented that the small Maf proteins act cooperatively with p45 NF-E2 to activate target genes in megakaryocytes (28, 36, 40). Therefore, heterodimers of the other CNC family (Nrf1, Nrf2, and Nrf3) and small Maf proteins were anticipated to act as transcriptional activators as well. However, cell culture studies have shown that small Maf proteins can behave as negative regulators of Nrf2-dependent reporter gene activation (3, 32). To definitively address whether or not the Nrf2 and small Maf proteins act as cooperative transcriptional activators in vivo, we examined the expression of ARE-dependent genes in small Maf and other (*keap1* and *nrf2*) mutant mice whose biochemical pathways hypothetically intersect. The prediction of these experiments

TABLE 2. Effect of small Maf protein deficiency on the regulation of DEM-induced genes in fibroblasts[a]

Gene category and description	G2K0F0-DEM/G2K0F0[b]	G0K0F0-DEM/G0K0F0[c]	G0K0F0/G2K0F0[d]	G0K0F0-DEM/G2K0F0-DEM[e]
Chaperone system/stress response				
Heat shock protein 1A	7.46	8.00	0.71	0.71
Heat shock protein 1B	6.99	7.20	0.79	0.83
HSP40 homolog, subfamily B, member 1	2.56	2.46	0.97	0.90
HSP40 homolog, subfamily B, member 9	2.46	1.32	1.23	0.71
Crystallin, alpha C	2.15	1.28	0.93	0.55
Heat shock protein 1	1.58	1.47	0.80	0.77
Crystallin, alpha B	1.56	1.19	1.04	0.84
DnaJ (HSP40) homolog, subfamily B, member 10	1.52	1.15	0.81	0.62
DnaJ (HSP40) homolog, subfamily A, member 1	1.52	1.32	0.93	0.87
Xenobiotic metabolism				
Metallothionein 2	12.13	10.56	1.07	0.93
Metallothionein 1	9.66	7.84	1.04	1.00
Glutamate-cysteine ligase, modifier subunit	6.06	1.00	0.87	0.16
Glutamate-cysteine ligase, catalytic subunit	3.43	1.00	0.97	0.28
NAD(P)H:quinone oxidoreductase 1	3.25	1.41	0.47	0.18
UDP-glycosyltransferase 1 family, polypeptide A1	3.16	1.52	0.68	0.35
Glutathione *S*-transferase, alpha 4	2.46	1.15	0.13	0.06
Carbonyl reductase 3	2.30	0.93	1.00	0.38
Microsomal glutathione *S*-transferase 1	2.14	1.15	0.66	0.35
Glutathione *S*-transferase, alpha 2	1.87	1.52	1.23	0.62
Glutathione *S*-transferase, mu 1	1.52	1.23	0.81	0.57
P450 (cytochrome) oxidoreductase	1.52	1.32	1.00	0.93
Antioxidant enzymes				
Heme oxygenase 1	10.56	1.23	11.31	1.23
Thioredoxin reductase 1	2.32	1.22	0.77	0.41
Glutathione reductase 1	2.00	1.07	0.71	0.35
Ferritin heavy chain	1.94	1.11	1.11	0.67
Glutaredoxin 1	1.87	1.07	1.00	0.50
Catalase	1.74	0.93	1.11	0.56
Peroxiredoxin 1	1.67	1.09	0.87	0.58
Peroxiredoxin 4	1.62	1.52	0.81	0.54

[a] Each value was calculated from raw data obtained from microarray analysis. The genes categorized into the four classes shown were listed if they were induced by over 1.5-fold after DEM treatment in $mafG^{+/+}::mafK^{-/-}::mafF^{-/-}$ (G2K0F0-DEM/G2K0F0 > 1.5) cells. Gene products in boldface were previously reported to be encoded by Nrf2-dependent genes (4, 6, 21, 34, 43).

[b] Each value indicates the *n*-fold change in the gene expression of G2K0F0-DEM cells relative to that of control G2K0F0 cells.

[c] Each value indicates the *n*-fold change in the gene expression of DEM-treated $mafG^{-/-}::mafK^{-/-}::mafF^{-/-}$ (G0K0F0-DEM) cells relative to that of control $mafG^{-/-}::mafK^{-/-}::mafF^{-/-}$ (G0K0F0) cells.

[d] Each value indicates the *n*-fold change in the gene expression of G0K0F0 cells relative to that of G2K0F0 cells.

[e] Each value indicates the *n*-fold change in the gene expression of G0K0F0-DEM cells relative to that of G2K0F0-DEM cells.

was that if CNC-small Maf heterodimers positively regulate transcription, the induction of Nrf2-dependent genes should be diminished in small *maf* mutant mice, as well as in *nrf2* mutant mice. In contrast, if the small Mafs contribute only to negative regulation through AREs, a loss of small Maf activity would be expected to confer an activation phenotype.

In this study, we first examined G0F0 mice to prove the contribution of small Maf proteins in vivo. We failed to observe any significant effects of small Maf reduction on the abundance of ARE-dependent genes other than *NQO1* and *TXNRD1*. When G0K0F0 fibroblasts lacking all three small Maf proteins were established and examined, we finally observed a complete loss of induction in almost all of the Nrf2-dependent genes induced in this analysis. Intriguingly, many of the ARE-dependent genes, with a few exceptions, responded to electrophilic reagents in G2K0F0 cells and G0K0F1 cells, as

well as in G0F0 (G0K2F0) mouse livers. This implies that each single small Maf protein is more or less capable of supporting Nrf2-mediated transcriptional activation. This is the first report that genetically demonstrates the ability of each small Maf protein to contribute to ARE-dependent gene activation and the essential roles of the family of small Maf proteins in the activation.

Strictly speaking, in this study, we could evaluate the contribution of small Maf proteins only in relation to inducible ARE-dependent genes in fibroblasts. Since *GSTP1/2* and *FTL* were not significantly induced in fibroblasts, it was still possible that in the liver the *GSTP1/2* and *FTL* genes are regulated in an Nrf2-dependent and Maf-independent manner. Indeed, it has been reported that Nrf2 heterodimerizes with c-Jun (13) and ATF4 (5). In contrast, a comprehensive analysis of all possible protein-protein interactions was recently reported us-
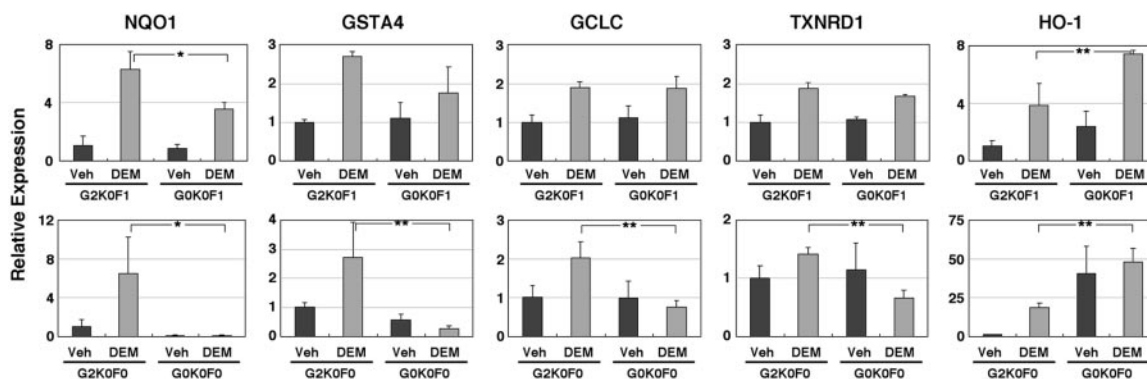
FIG. 5. Deficiency of small Maf proteins affects the electrophile-mediated induction of gene expression. Expression profiles of xenobiotic-metabolizing enzyme and antioxidant enzyme genes were examined by quantitative real-time reverse transcription-PCR. Based on the result of the microarray analysis, five genes were selected. The expression level of each mRNA was normalized to the rRNA abundance. The average values of three independent experiments (upper panels, comparison between G2K0F1 and G0K0F1 cells) and five independent experiments (lower panels, comparison between G2K0F0 and G0K0F0 cells) are shown, with error bars indicating standard deviations. The gene expression levels in vehicle (Veh)-treated G2K0F1 or G2K0F0 cells were set to 1. Asterisks and double asterisks indicate statistically significant differences by unpaired t test (*, $P < 0.05$; **, $P < 0.01$).

ing a protein array that was fabricated with an almost complete set of coiled-coil domains of all the human bZIP transcription factors. The data revealed that the binding affinities between the small Maf and CNC proteins were much stronger than those between CNC proteins and c-Jun or ATF4 (31). This result leads us to believe that small Maf proteins are substantial partners of Nrf2 and that induction of *GSTP1/2* and *FTL* in a G0F0 background was maintained by Nrf2-MafK heterodimer. Nonetheless, it remains an open question whether or not Nrf2–c-Jun or Nrf2-ATF4 heterodimers might be able to functionally compensate for Nrf2-Maf loss in vivo. Simultaneous disruption of *keap1* and c-*jun* or *ATF4* may provide unique insights into this issue. In any case, our present data rejected the notion that the small Maf proteins contribute exclusively to ARE-mediated repression.

Since we here provide genetic evidence that small Maf proteins are essential for the ARE-dependent genes, it might be possible that small Maf proteins are essential for DNA binding of Nrf2 and/or for other processes, including the localization and stabilization of Nrf2. We previously reported that the nuclear localization of Bach proteins is dependent on small Maf proteins (16). Since Bach proteins possess a nuclear export signal (NES), we surmised that small Maf proteins prevent Bach proteins from being exported from nuclei by the NES binding protein CRM1 (exportin 1). Recently, a NES has been identified in the leucine zipper motif of Nrf2 (12, 23), which suggests the possibility that the nuclear accumulation of Nrf2 is also influenced by small Maf proteins.

In conclusion, we have provided evidence that a genetic reduction in the abundance of small Maf proteins has a great influence on the expression of a wide range of ARE-dependent genes. Our in vivo experiments utilizing small *maf* double-mutant mice also implied the differential contribution of small Maf proteins to the expression of ARE-dependent genes. For instance, the *NQO1* gene seems to be more sensitive to the reduction of small Maf proteins compared to several other ARE-dependent genes. We envisage that the differential requirement of small Maf proteins might be attributed to the variation in the ARE and its flanking sequence of each gene,

which might result in the differential affinity for the activator complex containing Nrf2-small Maf heterodimer (34). However, we could not find a clear correlation between the sensitivity to the reduction in small Mafs and the ARE sequence variations. We also surmise that such a differential threshold might enable small Maf proteins, by virtue of a change in their abundance, to differentially regulate specific ARE-dependent genes. Since the abundance of small Maf proteins has been reported to fluctuate in response to oxidative stress (17, 26), the intriguing possibility emerges that the small Maf proteins may generate diversity in Nrf2-mediated gene regulation by changing their abundance.

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