Oxidative stress-responsive transcription is regulated in part through cis-active sequences known as antioxidant response elements (ARE). Activation through the ARE involves members of the CNC-subfamily of basic leucine zipper proteins including Nrf1 and Nrf2. In particular, Nrf2 has been shown to coordinate induction of genes encoding antioxidant and phase 2 metabolizing enzymes in response to stimulation with electrophilic compounds and exposure to xenobiotics. Here we show that the 65-kDa isoform of the Nrf1 gene functions as a repressor of Nrf2. Transient expression of p65Nrf1 suppressed Nrf2-mediated activation of ARE-dependent reporter genes in cells. Induction of endogenous ARE-genes is blocked in Hepa1c1c7 cells stably expressing p65Nrf1. Gel shift experiments demonstrated that p65Nrf1 binds the antioxidant response element as a heterodimer with small-Maf protein. Immunoprecipitation studies demonstrated that p65Nrf1 competes with Nrf2 for interaction with small-Maf protein and binding to the antioxidant response element in vivo. Together, these results demonstrate that p65Nrf1 has the potential to play an important role in modulating the response to oxidative stress by functioning as a trans-dominant repressor of Nrf2-mediated activation of ARE-dependent gene transcription.

Basic-leucine-zipper (bZIP) proteins form a large and conserved family of transcription factors that regulate gene expression. There are several distinct subgroups of bZIP proteins: the AP-1, ATF/CREB, MAF, and CNC families (1). The CNC-leucine zipper family consists of 4 closely related members including Nrf1, Nrf2, Nrf3 and p45NFE2, as well as two distantly related factors, Bach1 and Bach2 (2–6). CNC-bZIP proteins are characterized by their highly homologous DNA binding and leucine zipper dimerization domains, as well as a highly conserved 43 amino acid homology region referred to as the “CNC” domain that is named after the Drosophila cap-n-collar protein (7). CNC-bZIP factors function as heterodimers by interacting with the small-Maf family of bZIP proteins, which can form heterodimers as well as homodimers among themselves (8).

Transcription of many oxidative stress inducible genes involved in xenobiotic and electrophile metabolism is regulated in part through cis-acting sequences known as antioxidant response elements (AREs) (9). These elements have been identified in the regulatory regions of genes encoding phase-2 detoxification enzymes and various other cytoprotective proteins such as NQO1 (NAD(P)H:quinone oxidoreductase) that catalyzes reduction of a variety of quinones and quinoid compounds, GST (glutathione S-transferase) enzymes that catalyze the nucleophilic addition of the thiol of glutathione to electrophiles, and GCL (glutamate-cysteine ligase) enzyme consisting of a catalytic subunit GCLC and a modifier subunit GCLM that catalyzes glutathione synthesis in cells. Nrf1 and Nrf2 are expressed in a wide range of tissues and cell types, and both share structural similarities beyond the CNC and bZIP domains (3, 5). Nrf2 is an important activator of AREs in cells by coordinating the expression of antioxidant and phase 2 metabolizing enzymes in response to oxidative stress and xenobiotic exposures (10). For example, genetic and biochemical studies indicate that expression of NQO1, GCLC and ferritin heavy chain are dependent on Nrf2 (11–14). While Nrf2 is dispensable for development in mice, a deficiency of Nrf2 leads to impaired xenobiotic metabolism and various oxidative stress-related pathologies (15). In contrast to Nrf2, loss of Nrf1 leads to embryonic lethality in mice (16, 17). Nrf1 is essential for fetal hematopoiesis, and recent studies indicate a link between Nrf1 and chronic liver disease in mice (18). Cell-based studies indicate that Nrf1 is also important in ARE function. Mouse embryonic fibroblasts derived from Nrf1 mutant embryos have been shown to be susceptible to oxidant-induced cell death, and Nrf1 has been shown to transactivate through the AREs in the promoters of both the catalytic and regulatory subunits of glutamyl-cysteine ligase genes that are involved in glutathione biosynthesis (19, 20). Moreover, fibroblasts deficient in Nrf1 and Nrf2 are severely impaired in expression of ARE-bearing genes and are markedly sensitive to oxidative stress indicating that Nrf1 shares an overlapping role with Nrf2 in the oxidative stress response (21).

The human Nrf1 gene has been shown to encode at least 4 alternatively spliced variants predicted to encode proteins...
ranging in sizes from 728 to 772 amino acids (22). The significance of these alternative mRNA splicing variants is unknown. In addition to these large protein isoforms, an N-terminal-truncated form of Nrf1, consisting of 447 amino acids (also called LCR-F1) has also been described (23). In vitro studies suggest that the N-terminally truncated form of Nrf1, which has an apparent mass of 65-kDa, is generated by alternative translation initiation from highly conserved in-frame start codons downstream from the normal start site present in the Nrf1 transcript (3). The function of 65-kDa Nrf1 (p65Nrf1) in gene activation is not clear. Although p65Nrf1 has been shown to activate globin-reporter gene expression in erythroid cells, transactivation was not observed in other cell types (23). We report here that p65Nrf1 blocked Nrf2-mediated activation of ARE-dependent reporter genes, and enforced expression of p65Nrf1 in Hepa1c1c7 cells inhibited induction of endogenous ARE-genes by oxidative stress. Loss of p65Nrf1 function led to increased induction of ARE-genes, and expression of p65Nrf1 blocked the protective effects of oxidative preconditioning mediated by Nrf2 in fibroblasts. The p65 isoform interacted with small-Maf proteins to bind the ARE in vitro. Chromatin immunoprecipitation (ChiP) assays showed that p65Nrf1 binds to the promoter of the ARE-regulated NQO1 gene in vivo. Overall, these findings suggest that p65Nrf1 can potentially inhibit Nrf2 and may function as an important component modulating ARE-gene expression in response to oxidative stress.

**MATERIALS AND METHODS**

**Reagents**—Tissue culture media, fetal calf serum, media supplements, Lipofectamine, and V5-Tag mouse monoclonal antibody were purchased from Invitrogen (Carlsbad, CA). Horse-radish peroxidase-linked anti-rabbit IgG, anti-mouse IgG, and anti-Myc antibodies were from Cell Signaling (Beverly, MA). Anti-Nrf2 (H200) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A rabbit antibody to detect Nrf1 was previously described (24). Rabbit antibody against MafG was a gift from Dr. V. Blank (McGill University, Montreal, Canada). Chemiluminescent detection system for immunoblot (ECL) was purchased from Amersham Biosciences (Piscataway, NJ). Protease inhibitor mix was purchased from Pierce Biotechnology (Rockford, IL). Restriction enzymes, TaqDNA polymerases, and other modification enzymes were purchased from New England Biolabs (Beverly, MA). Protein G-Sepharose beads, Tert-butyldimethylamine (TBDQ), diethylmaleate (DEM), and all other chemicals were from Sigma-Aldrich.

**Plasmids**—The V5-tagged expression vectors encoding Nrf2 (pEF1Nrf2) were described previously (11, 19). Construction of the p65Nrf1-V5 was generated by PCR amplification using the forward and reverse primers GTGGCAAGAATTCGTCCATCAG and CCAGGCGCCCTTTTCTCCGCTC, respectively, and cloned into the EcoRI and AgeI sites of pBABE to generate pBABE-p65Nrf1. The luciferase reporter driven by the NQO1 ARE was a gift from Dr. J. Johnson (University of Wisconsin, Madison). pNrf1-EGFP expression construct containing an in-frame fusion of the enhanced green fluorescence protein (EGFP) was previously described (24). The p65Nrf1-EGFP plasmid was generated by PCR amplification of Nrf1 using primers CGAATTCTCGAGTATGGGTGAATCCCAATG and CGAATTCTAGGCTCAGTTTTTCTTTTGTA.
containing 1× RT buffer with 1 mM dNTPs, 0.3 μg of random hexamer, 40 units of RNase inhibitor, 250 units of M-MLV reverse transcriptase. Reverse transcription reactions were incubated at 72 °C for 5 min, then 25 °C for 10 min, followed by 42 °C for 60 min, then aliquots of the reaction products were used in PCR reactions. SYBR Green-based Real Time PCR was used to determine cDNA levels. Aliquots of cDNA were amplified in an Option PCR machine (Bio-Rad) using Dynamo Probe PCR reagents (New England Biolabs) in triplicates in 20-μl reaction volumes. Sequences of the PCR primers were previously described (21). PCR cycling conditions consist of 95 °C for 10 min and 40 cycles of 95 °C for 30 s, 60 °C for 30 s, 68 °C for 45 s. Expression levels were calculated relative to RPL10 or 18S rRNA levels as endogenous control. Relative

68 °C for 45 s. Expression levels were calculated relative to

95 °C for 10 min and 40 cycles of 95 °C for 30 s, 60 °C for 30 s,

45 s. Expression levels were calculated relative to RPL10 or 18S rRNA levels as endogenous control. Relative expression was calculated as 2^ΔCt test gene-ΔCt control).

Cell Viability Assay—Viability assays for Hepa1c1c7 cells were carried out in six-well plates. Cells were seeded at a density of 1 × 10^5 cells/well 24 h before hydrogen peroxide treatment. At various times after treatment, floating and attached cells were collected from each well and stained with Trypan Blue. The number of viable and dead cells was counted using a hemocytometer. Viability assays for Nrf1-/- cells were done with CyQuant Kit (Invitrogen) according to the manufacturer’s protocol. Cells were plated in 96-well black wall plates, at a density of 5000 cells/well. For preconditioning, triplicate wells were cultured first in the presence of 20 μM DEM or dimethyl sulfoxide (Me2SO) as the vehicle control. After 24 h, triplicate wells were treated with various concentrations of 1-chloro-2,4-dinitrobenzene (CDNB) or vehicle. After 12 h of treatment at 37 °C, the media were discarded, wells were washed with phosphate-buffered saline, and remaining adherent cells were incubated with the CyQuant dye for 5 min in the dark. Fluorescence was measured on a microplate reader with filters set at 480 nm for excitation and 520 nm for emission. Each experiment was repeated independently three times.

Electrophoretic Mobility Shift Assay—Nrf2, p65Nrf1, and Maf-G were synthesized using the rabbit reticulocyte TnT system from Promega. TNT lysate from vector alone was used as a negative control. Binding reactions were carried out in 20 mM Hepes-KOH pH 7.9, 1 mM EDTA, 20 mM KCl, 5 mM dithiothreitol, 4 mM MgCl2, 1 μg of poly(dI/dC), 4% glycerol containing 32P-labeled double-stranded oligonucleotide probes corresponding to the antioxidant response element of human NQO1 gene promoter, and non-precipitated (input) genomic DNA. The supernatant was pre-cleared with protein-A Sepharose beads and sheared herring sperm DNA. The pre-cleared supernatant was incubated with mouse monoclonal anti-Myc antibody, Nrf2-specific rabbit polyclonal antibody or pre-immune antibody control at 4 °C overnight with 10-fold diluted ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl, pH 8.1, 167 mM NaCl). The DNA-protein complexes were then washed with low salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, 150 mM NaCl), followed by high salt immune complex wash (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, 500 mM NaCl), and then 10 mM Tris, 10 mM EDTA pH 8.0 buffer. DNA was eluted with elution buffer (1% SDS and 0.1 mM NaHCO3) at room temperature for 15 min twice, and then reverse cross-linked by proteinase-K at 65 °C for 16 h with 200 μM sodium chloride followed by phenol/chloroform extraction and ethanol precipitation to purify DNA. Purified DNA was PCR-amplified for 35 cycles (30 s at 95 °C, 30 s at 60 °C, and 45 s at 72 °C) with primers CAGTGCCATGACCCAGGGAA and GCATGCCCTTTTAGCCCTGGCA that flank the ARE site in the NQO1 gene promoter, and non-precipitated (input) genomic DNA was amplified as positive control.

RESULTS

p65Nrf1 Represses Nrf2-mediated Transcriptional Activation of ARE-containing Genes—It was previously observed that the Nrf1 transcript contains several internal AUG codons within a Kozak consensus sequence favorable of supporting alternative translational initiation to generate the 65-kDa form of Nrf1 (Fig. 1a). Consistent with this, immunoblot analysis of whole cell extracts of mouse embryonic fibroblasts detected a band corresponding to the 65-kDa form of Nrf1, which was absent in extracts prepared from Nrf1-/- fibroblasts (Fig. 1a). Western blotting indicated that p65Nrf1 is expressed in various cell lines (Fig. 1a). While we previously demonstrated that the p120-kDa form of Nrf1 is membrane localized, p65Nrf1 expressed as a...
A fusion protein with EGFP was constitutively localized in the nucleus (Fig. 1b). This finding is in accordance with the fact that an ER membrane targeting signal contained within the N-terminal amino acids of the 120-kDa isoform is missing in p65Nrf1 (24). To examine the function of p65Nrf1 in ARE-gene activation, co-transfection studies using p65Nrf1 expression vector and luciferase reporter gene containing an antioxidant response element of the NQO1 gene were done. Transient expression of p65Nrf1 did not activate reporter gene expression in Hepa1c1c7 or COS7 cells, and increasing the levels of p65Nrf1 conferred a progressive decrease in reporter activation instead (Fig. 1c and data not shown). Next, we sought to determine whether p65Nrf1 could function in a dominant negative fashion to inhibit ARE-gene activation. Luciferase reporter gene containing the NQO1-ARE was transfected along with fixed amounts of Nrf2 and increasing amounts of p65Nrf1 expression vector. Transcriptional repression is reported relative to the maximal activation obtained with Nrf2 expression vector alone. In all transfections, Renilla-luciferase plasmid was cotransfected to normalize for different transfection efficiency. The results are means of at least three separate experiments ± S.D. carried out in triplicates. e. immunoblot analysis of Hepa1c1c7 cells transfected with Nrf2-Myc or p65Nrf1-Myc expression vectors. Cells were harvested and analyzed by Western blotting using the anti-Myc antibody 48 h after transfection. f. activation of ARE-containing GCLC and GCLM promoters by Nrf2 is suppressed by p65Nrf1. The GCLC and GCLM reporter genes were cotransfected with Nrf2 and increasing amounts of p65Nrf1 expression vector.
**p65Nrf1 Inhibits Nrf2 Function**

FIGURE 2. Oxidative stress activation of endogenous Nrf2 target genes is suppressed by p65Nrf1.

a, whole cell lysates of Hepa1c1c7 cells transduced with pBabe or pBabe-p65Nrf1 examined for expression of p65Nrf1 and actin (loading control) by Western blotting. b, quantitative RT-PCR analysis of mRNA levels isolated from Hepa1c1c7 cells expressing p65Nrf1, Nrf2DN, or pBabe control treated with TBHQ to induce oxidative stress. Expression levels were determined relative to endogenous 18S levels as an internal reference, and calculated as $2^{-\Delta\Delta C_{T}}$ as described (21). Fold induction was determined from the difference between the averaged expression levels in TBHQ-treated samples relative to expression levels in untreated samples. Mean values ± S.D. of three determinations are shown. c, TBHQ-induction of ARE-luciferase expression is blocked by p65Nrf1. The NQO1-luciferase plasmid was transfected into Hepa1c1c7 cells stably expressing p65Nrf1, Nrf2DN or pBabe and treated with TBHQ or Me$_2$SO (DMSO) vehicle control. Transcriptional activation is reported relative to DMSO-treated cells as control. The results are means of three separate experiments ± S.D. carried out in triplicate.

ent manner (Fig. 1d). Suppression was not observed when a mutant p65Nrf1 with the bZIP domain deleted was cotransfected (Fig. 1d). Immunoblot analysis indicated that expression levels of p65Nrf1 and Nrf2 proteins were comparable in transfected cells (Fig. 1e). Similar repression of reporter activation was observed in COS-7 and Hela cells indicating that the inhibitory effect of p65Nrf1 was not unique to Hepa1c1c7 cells (data not shown). Activation of reporter driven by the GCLC-ARE4 or GCLM-gene promoter was similarly repressed by p65Nrf1 (Fig. 1f). As a control, we examined the effects of p65Nrf1 on luciferase expression driven by the Grp78 promoter, which does not contain antioxidant response elements. Overexpression of p65Nrf1 did not inhibit activation from the Grp78 reporter (data not shown). These results indicate that inhibition by p65Nrf1 is ARE-dependent.

**Expression of Endogenous ARE-genes Is Suppressed by p65Nrf1**—We then investigated whether enforced expression of p65Nrf1 could lead to suppression of endogenous ARE-gene induction by oxidative stress in cells. Hepa1c1c7 cells were transiently transfected with control or p65Nrf1-encoding retrovirus. Hepa1c1c7 cells were chosen because of their responsiveness to electrophilic stimulation of phase 2 gene expression. Overexpression of p65Nrf1 in transduced cells was verified by Western blotting (Fig. 1a). As a positive control, cells stably expressing dominant negative Nrf2 mutant were also generated by retrovirus transduction. Cultures of control, p65Nrf1-, and Nrf2DN-infected lines were treated with TBHQ to induce oxidative stress and analyzed for ARE-gene expression by quantitative RT-PCR. In p65Nrf1-infected cells, TBHQ-induced expression of NQO1 and GCLC genes was severely blunted (Fig. 2b). Interestingly, GCLM induction was not suppressed by p65Nrf1. As expected, expression of Nrf2DN suppressed TBHQ-induction of NQO1, GCLC, and GCLM. To further confirm suppression of ARE-mediated function, we examined NQO1-ARE luciferase reporter expression in p65Nrf1 stable cells. In contrast to pBabe-control cells, reporter gene expression was not induced by TBHQ treatment in p65Nrf1-expressing cells (Fig. 2c). These results suggest that p65Nrf1 acts as repressor of ARE-mediated gene expression.

**Cells Stably Expressing p65Nrf1 Are Hypersensitive to Oxidative Stress**—To determine the functional relevance of the antagonistic effects of p65Nrf1 on ARE-gene expression, we investigated whether cells stably expressing p65Nrf1 are sensitized to oxidative stress. Control Hepa1c1c7 cells, and cells expressing p65Nrf1 were cultured in the presence of hydrogen peroxide and viability was determined. As a positive control, cells expressing Nrf2DN were similarly treated. No difference in viability was observed in untreated cultures of control, p65Nrf1-, and Nrf2DN-expressing Hepa1c1c7 cells (data not shown). Compared with pBABE-control cells, p65Nrf1-expressing cells showed a 2-fold increase in cell death after 6 h of treatment with hydrogen peroxide (Fig. 3). At 12 h after exposure, cell death in p65Nrf1-expressing cultures was 3-fold higher compared with control cells. As expected, Nrf2DN-expressing cells were hypersensitive to hydrogen peroxide treatment (Fig. 3). In addition to hydrogen peroxide, hypersensitivity to TBHQ was similarly observed in Hepa1c1c7 cells expressing p65Nrf1 (data not shown).

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3 W. Wang, A. M. Kwok, and J. Y. Chan, unpublished results.
shown). These results indicate that p65Nrf1 expression sensitizes cells to killing caused by oxidative stress, which is consistent with its ability to down-regulate ARE-gene expression by Nrf2.

**Oxidative Stress Response Is Inhibited by p65Nrf1 in Vivo**

The data presented thus far suggest that p65Nrf1 can inhibit ARE-specific gene expression when expressed at high levels. To begin to determine the physiological significance of this observation, we examined the effects of p65Nrf1-deficiency on ARE-gene expression. To do this, we examined induction of various ARE-genes in Nrf1 knock-out fibroblasts, which lack both p120 and p65Nrf1. Levels for NQO1 and GCLC were ~50% and 2-fold higher, respectively in Nrf1+/− fibroblasts treated with diethyl maleate, which is an electrophilic agent that induces oxidative stress (Fig. 4a). Induction of GSTA2 expression was also higher in DEM-treated Nrf1+/− cells compared with wild-type cells. These results indicate that activation of ARE-gene expression is augmented by the absence of p65Nrf1 in Nrf1+/− cells. To determine the role of p65Nrf1 on oxidative stress response in vivo, we examined the effects of p65Nrf1 on oxidative preconditioning in cells. To assess p65Nrf1 function independent of p120Nrf1 activity, we transduced Nrf1−/−/Nrf2+ fibroblasts with p65Nrf1-encoding retrovirus. As control, stable clones of Nrf1−/− cells transduced with pBabe empty vector were used. Cells were treated with CDNB, which is another electrophilic agent that induces oxidative stress (Fig. 4a). Induction of GSTA2 expression was also higher in DEM-treated Nrf1−/− cells compared with wild-type cells.

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**p65Nrf1 Competes with Nrf2 by Forming Heterodimers with Small-Mafs to Bind the ARE—EMSA analysis was performed using in vitro generated proteins to examine ARE binding by p65Nrf1. A weak complex was detected when binding was done using reticulocyte lysate programmed with either p65Nrf1 or Nrf2 expression plasmid alone, and no protein-DNA complex was observed when control-lysate was used (data not shown). These results are expected based on previous studies indicating that CNC-bZIP factors do not bind AREs as homodimers (26–28). EMSA using lysate programmed with both p65Nrf1 and MafG expression plasmids resulted in formation of a strong complex that was supershifted with MafG or Nrf1 antibodies (Fig. 5a). Similarly, lysate programmed with both Nrf2 and Maf-G expression plasmids also resulted in a strong complex that was supershifted with Nrf2 antibody (Fig. 5a). IgG control antibody had no effect on migration of the complexes (data not shown). To examine whether p65Nrf1 can compete with Nrf2 for DNA binding **in vivo**, EMSA reactions containing increased amounts of p65Nrf1 were subjected to supershifting. Incubation with anti-Nrf1 resulted in a strong supershift, whereas a weak supershift was detected with anti-Nrf2 (Fig. 5a). To further establish the association of p65Nrf1 with Maf-G, coimmunoprecipitation analysis was performed with HEK293 cells expressing Myc-tagged p65Nrf1 and V5-tagged Maf-G proteins. Immunoprecipitation with anti-Myc antibody followed by immunoblotting with anti-V5 antibody, and vice versa showed that p65Nrf1 binds to Maf-G in cells (Fig. 5b). To confirm an interaction between p65Nrf1 and Maf-G, endogenous interaction between p65Nrf1 and Maf-G was also investigated. HEK293 cell lysates were immunoprecipitated with anti-Maf-G antibody to pull-down endogenous Maf-G protein. Immunoblot analysis with anti-Nrf1 antibody detected endogenous p65Nrf1 and p120Nrf1 proteins in the Maf-G precipitate (Fig. 5c). To examine whether p65Nrf1 could compete with Nrf2 for binding to the ARE, coimmunoprecipitation experiments were done on cells transfected with fixed amounts of Nrf2 and V5-tagged Maf-G, and increasing amounts of Myc-tagged p65Nrf1. As shown in Fig. 5d, Nrf2 and Maf-G interactions were readily detected in cells not transfected with p65Nrf1. Co-transfection with increasing concentrations of p65Nrf1 plasmid displaced Nrf2 interactions with Maf-G. Based on these results, we conclude that p65Nrf1 can form a complex with Maf-G to bind the ARE, and p65Nrf1 can compete with Nrf2 for association with Maf-G in vivo.

**p65Nrf1 Binds to the NQO1 Promoter in Vivo**—To extend the findings above, we performed ChIP assays to examine the binding of p65Nrf1 to the NQO1 promoter **in vivo**. We examined binding using Myc-tagged p65Nrf1 as available antibodies to Nrf1 do not distinguish between the p120 and p65 isoforms of the protein. HEK293 cells were transiently transfected with
p65Nrf1 Inhibits Nrf2 Function

**DISCUSSION**

CNC transcription factors are structurally related basic-leucine zipper proteins that play important roles in development and regulation of different cellular processes. The CNC family members include p45NFE2, Nrf1, Nrf2, Nrf3, Bach1, and Bach2. Nrf2 has been demonstrated to be a key regulator of inducible transcription of oxidative stress genes through the antioxidant response element. In addition to Nrf2, Nrf1 has also been shown to regulate oxidative stress response genes. The Nrf1 gene encodes a ubiquitously expressed protein of ~120-kDa in size and a shorter protein of 65-kDa. While p120Nrf1 has been demonstrated to activate ARE-responsive promoters, we investigated the function of p65Nrf1 in ARE-gene expression in this report.

Our current results suggest that p65Nrf1 is a negative regulator of ARE-mediated transcription. We have demonstrated that activation of ARE-dependent reporters by Nrf2 is suppressed by transient expression of p65Nrf1, and oxidative stress activation of endogenous ARE-driven genes including NQO1 and GCLC is also repressed in cells stably expressing p65Nrf1. However, the expression of endogenous GCLM was not inhibited by p65Nrf1. It is interesting to note that genetic activation of Nrf2 function also did not result in GCLM induction suggesting that its expression is not solely under the control of Nrf2 (29). Consistent with its antagonistic function, cells stably expressing p65Nrf1 are sensitized to oxidative stress-induced toxicity. Our results also indicate that the protective effect of oxidative preconditioning in vivo was abolished by p65Nrf1 expression in cells. Blockade of preconditioning is presumably through a dominant negative effect of p65Nrf1 on Nrf2-mediated transactivation of ARE-genes induced by electrophilic agents, as activation of NQO1 and GCLC, both of which are well established Nrf2 target genes, was higher in Nrf1−/− cells, which are deficient in p65Nrf1 compared with wild-type cells. Moreover, induction was blocked in Nrf1−/− cells when p65Nrf1 expression was restored. To determine potential

Myc-tagged p65Nrf1, and ChIP was done with anti-Myc antibody. As shown in Fig. 6a, PCR amplification revealed that p65Nrf1 is bound to the NQO1 promoter within the regions containing the antioxidant response element. As expected, DNA surrounding the antioxidant response element in the NQO1 promoter was amplified by PCR with a polyclonal anti-Nrf2 antibody (Fig. 6a). Precipitation with preimmune serum did not result in an enrichment of the NQO1 promoter. Non-immunoprecipitated cross-linked DNA (input) was used as positive control to confirm the size of the PCR product (Fig. 6a). A significant increase in Nrf2 binding to the NQO1 promoter was seen by ChIP after culturing cells with TBHQ to induce Nrf2 levels (Fig. 6b). The ectopic expression of p65Nrf1 inhibited Nrf2 binding in a dose-dependent manner (Fig. 6b). These results demonstrate that p65Nrf1 binds the NQO1 promoter, and that it can compete with Nrf2 for ARE binding.

DISCUSSION

CNC transcription factors are structurally related basic-leucine zipper proteins that play important roles in development and regulation of different cellular processes. The CNC family members include p45NFE2, Nrf1, Nrf2, Nrf3, Bach1, and Bach2. Nrf2 has been demonstrated to be a key regulator of inducible transcription of oxidative stress genes through the antioxidant response element. In addition to Nrf2, Nrf1 has also been shown to regulate oxidative stress response genes. The Nrf1 gene encodes a ubiquitously expressed protein of ~120-kDa in size and a shorter protein of 65-kDa. While p120Nrf1 has been demonstrated to activate ARE-responsive promoters, we investigated the function of p65Nrf1 in ARE-gene expression in this report.

Our current results suggest that p65Nrf1 is a negative regulator of ARE-mediated transcription. We have demonstrated that activation of ARE-dependent reporters by Nrf2 is suppressed by transient expression of p65Nrf1, and oxidative stress activation of endogenous ARE-driven genes including NQO1 and GCLC is also repressed in cells stably expressing p65Nrf1. However, the expression of endogenous GCLM was not inhibited by p65Nrf1. It is interesting to note that genetic activation of Nrf2 function also did not result in GCLM induction suggesting that its expression is not solely under the control of Nrf2 (29). Consistent with its antagonistic function, cells stably expressing p65Nrf1 are sensitized to oxidative stress-induced toxicity. Our results also indicate that the protective effect of oxidative preconditioning in vivo was abolished by p65Nrf1 expression in cells. Blockade of preconditioning is presumably through a dominant negative effect of p65Nrf1 on Nrf2-mediated transactivation of ARE-genes induced by electrophilic agents, as activation of NQO1 and GCLC, both of which are well established Nrf2 target genes, was higher in Nrf1−/− cells, which are deficient in p65Nrf1 compared with wild-type cells. Moreover, induction was blocked in Nrf1−/− cells when p65Nrf1 expression was restored. To determine potential

**FIGURE 4.** p65Nrf1 negatively regulates oxidative stress response in vivo. a, oxidative stress induction of ARE-genes is augmented by the absence of p65Nrf1 function. Wild type and Nrf1−/− cells were treated with 100 μM diethyl maleate for 6 h to induce Nrf2 activation of ARE-driven genes, and gene expression was analyzed by quantitative RT-PCR. Expression levels were determined relative to endogenous 18S levels as described. The difference between expression levels in treated samples relative to levels in vehicle-treated samples was used to determine fold induction. Mean values ± S.D. of three determinations are shown. b, protective effect of oxidative preconditioning is suppressed by p65Nrf1. Nrf1−/− cells stably expressing p65Nrf1 or pBabe control were cultured in presence or absence of 20 μM DEM for 6 h. Expression levels were determined relative to endogenous 18S levels as described. Fold induction was determined from the difference between treated and untreated samples. Mean values ± S.D. of three determinations are shown. c, induction of ARE-target genes is repressed in Nrf1−/− cells stably expressing p65Nrf1. Quantitative RT-PCR analysis of mRNA from Nrf1−/− cells stably expressing p65Nrf1 or pBabe control treated with 100 μM DEM for 6 h. Expression levels were determined relative to endogenous 18S levels as described. Fold induction was determined from the difference between treated and untreated samples. Mean values ± S.D. of three determinations are shown.

DEM for 6 h. Expression levels were determined relative to endogenous 18S levels as described. Fold induction was determined from the difference between treated and untreated samples. Mean values ± S.D. of three determinations are shown.
mechanisms for the dominant negative influence of p65Nrf1 over Nrf2, we examined p65Nrf1 interaction with small-Maf and DNA binding. Our data shows that p65Nrf1 physically associates with Maf-G and binds to the ARE in the NQO1 promoter, which is a known target for Nrf2. Furthermore, p65Nrf1 competed with Nrf2 for interaction with Maf-G and for binding to the NQO1 promoter region in vivo. As CNC factors function as obligate heterodimers, and experiments have shown small-Maf proteins as major dimerization partners for Nrf2 and Nrf1, these findings are in accord with a model in which p65Nrf1 blocks Nrf2 by competing directly for dimerization with members of the small-Maf family and binding to AREs.

Current models suggest that activation of cytoprotective genes through the ARE involves competitive interplay between activating and inhibitory complexes consisting of Nrf2/small-Maf heterodimers and small-Maf homodimers, respectively (8). Expression of these genes is normally limited due to Keap1-mediated destabilization of Nrf2 (27, 30–32). When cells encounter stress, Keap1 function is blocked leading to nuclear accumulation of Nrf2 and generation of active Nrf2-Maf heterodimers. While small-Maf homodimers function primarily as negative regulators, available evidence indicate that suppression of ARE function may not be mediated solely by small-Maf homodimers. For example, both Bach1 and Bach2 CNC-proteins have been shown to block Nrf2-mediated ARE-gene activation, and genetic studies have also shown that Nrf2-directed transcription of hemeoxygenase-1 is kept low under normal conditions by Bach1 repression (33–36). Moreover, binding affinity studies have also demonstrated that there is preferential binding of AREs to CNC-small-Maf heterodimers over small-Maf homodimers (37). Thus the dominant negative action of p65Nrf1 raises the interesting possibility that one of the roles of p65Nrf1 is to modulate oxidative stress response mediated by Nrf2. Given that p65Nrf1 is widely distributed and localized in the nucleus, it is reasonable to speculate that p65Nrf1 may function to ensure shut-off of antioxidant gene expression when they are not needed under non-stimulated conditions. For example, the sensitivity of different cell types to mounting a response to oxidative stress may be determined by differences in abundance of p65Nrf1. Because p65Nrf1 competes for small-Maf protein binding, p65Nrf1 may also modulate the function of other CNC-factors including the 120-kDa isoform of Nrf1. Thus the dominant negative function of p65Nrf1 may raise a number of questions regarding the phenotype of liver-specific Nrf1 knockout, which develop spontaneous hepatic neoplasm (18). Is the phenotype...
caused by loss of a complex interplay between the repressor function of p65Nrf1 and activator function of Nrf2, and/or p120Nrf1? While activatable expression of antioxidants and detoxifying enzymes by oxidative stress is clearly beneficial, there is also evidence to suggest that their unrestrained expression may have undesirable effects such as promoting proliferation and survival of precancerous cells (38, 39). Thus tumorigenesis in these animals may be driven in part by selective advantage of precancerous hepatocytes caused by up-regulation of certain ARE-genes that are observed in these animals. 3 Although the constitutive nuclear localization of p65Nrf1 would be consistent with the fact that an ER membrane targeting signal contained within the N-terminal amino acids of the 120-kDa isoform is missing in p65Nrf1 (24), whether other mechanisms are also involved in determining p65Nrf1 subcellular localization remains to be determined. Finally, it is also important to note that while p65Nrf1 does not appear to activate gene expression, our study here using ARE-driven reporter does not allow us to exclude the possibility of p65Nrf1 functioning as an activator under certain circumstances. Aside from its antagonistic function then, does p65Nrf1 turn on certain promoters through interactions with other transcription factors or co-activator proteins? Additional studies are required to examine these possibilities.

In summary, we have characterized the function of the 65-kDa form of Nrf1. Data presented in this study demonstrated that p65Nrf1 regulates Nrf2 transcriptional activity in a dominant negative manner. The antagonistic function of p65Nrf1 may serve as another mechanism to limit oxidative stress response mediated by Nrf2, as well as a mechanism to further fine-tune expression of ARE-responsive genes in response to different physiological conditions.

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REFERENCES

1. Pabo, C. O., and Sauer, R. T. (1992) Annu. Rev. Biochem. 61, 1053–1095
2. Andrews, N. C., Erdjument-Bromage, H., Davidson, M. B., Tempst, P., and Orkin, S. H. (1993) Nature 362, 722–728
3. Chan, J. Y., Han, X. L., and Kan, Y. W. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 11371–11375
4. Kobayashi, A., Ito, E., Toki, T., Kogame, K., Takahashi, S., Igarashi, K., Hayashi, N., and Yamamoto, M. (1999) J. Biol. Chem. 274, 6443–6452
5. Moi, P., Chan, K., Asunis, I., Cao, A., and Kan, Y. W. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9926–9930
6. Oyake, T., Itoh, K., Motohashi, H., Hayashi, N., Hoshino, H., Nishizawa, M., Yamamoto, M., and Igarashi, K. (1996) Mol. Cell. Biol. 16, 6083–6095
7. Chan, J. Y., Cheung, M. C., Moi, P., Chan, K., and Kan, Y. W. (1995) Hum. Genet. 95, 265–269
8. Motohashi, H., O’Connor, T., Katsuoka, F., Engel, J. D., and Yamamoto, M. (2002) Gene (Amst.) 294, 1–12
9. Nguyen, T., Sherratt, P. J., and Pickett, C. B. (2003) Annu. Rev. Pharmacol. Toxicol. 43, 233–260
10. Motohashi, H., and Yamamoto, M. (2004) Trends Mol. Med. 10, 549–557
11. Chan, J. Y., and Kwong, M. (2000) Biochim. Biophys. Acta 1517, 19–26
12. Pietsch, E. C., Chan, J. Y., Torti, F. M., and Torti, S. V. (2003) J. Biol. Chem. 278, 2361–2369
13. Venugopal, R., and Jaiswal, A. K. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 14960–14965
14. Wild, A. C., Moinova, H. R., and Mulcahy, R. T. (1999) J. Biol. Chem. 274, 33627–33636
15. Lee, J. M., Li, J., Johnson, D. A., Stein, T. D., Kraft, A. D., Calkins, M. J., Jakel, R. J., and Johnson, I. A. (2005) Faseb J. 19, 1061–1066
16. Chan, J. Y., Kwong, M., Lu, R., Chang, J., Wang, B., Yen, T. S., and Kan, Y. W. (1998) EMBO J. 17, 1779–1787
17. Chan, K., Lu, R., Chang, J. C., and Kan, Y. W. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 13943–13948
18. Xu, Z., Chen, L., Leung, Y., Yen, T. S., Lee, C., and Chan, J. Y. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 4120–4125
19. Kwong, M., Kan, Y. W., and Chan, J. Y. (1999) J. Biol. Chem. 274, 37491–37498
20. Myhrstad, M. C., Husberg, C., Murphy, P., Nordstrom, O., Blomhoff, R., Moskaug, J. O., and Kolsto, A. B. (2001) Biochim. Biophys. Acta 1517, 212–219
21. Leung, L., Kwong, M., Hou, S., Lee, C., and Chan, J. Y. (2003) J. Biol. Chem. 278, 48021–48029
22. Luna, L., Skammelsrud, N., Johnsen, O., Abel, K. J., Weber, B. L., Prydz, H., and Kolsto, A. B. (1995) Genomics 27, 237–244
23. Caterina, J. J., Donze, D., Sun, C. W., Ciavatta, D. J., and Townes, T. M. (1994) Nucleic Acids Res. 22, 2383–2391
24. Wang, W., and Chan, J. Y. (2006) J. Biol. Chem. 281, 19676–19687
25. Morgenstern, J. P., and Land, H. (1990) Nucleic Acids Res. 18, 3578–3596
26. Dhakshinamoorthy, S., and Jaiswal, A. K. (2000) J. Biol. Chem. 275, 40134–40141
27. Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J. D., and Yamamoto, M. (1999) Genes Dev. 13, 76–86
28. Johnsen, O., Skammelsrud, N., Luna, L., Nishizawa, M., Prydz, H., and Kolsto, A. B. (1996) Nucleic Acids Res. 24, 4289–4297
29. Okawa, H., Motohashi, H., Kobayashi, A., Aburatani, H., Kensler, T. W., and Yamamoto, M. (2006) Biochem. Biophys. Res. Commun. 339, 79–88
30. McMahon, M., Thomas, N., Itoh, K., Yamamoto, M., and Hayes, J. D. (2004) J. Biol. Chem. 279, 31556–31567
31. Nguyen, T., Sherratt, P. J., Huang, H. C., Yang, C. S., and Pickett, C. B. (2003) J. Biol. Chem. 278, 4536–4541
32. Zhang, D. D., and Hannink, M. (2003) Mol. Cell. Biol. 23, 8137–8151
33. Dhakshinamoorthy, S., Jain, A. K., Bloom, D. A., and Jaiswal, A. K. (2005) J. Biol. Chem. 280, 16891–16900
34. Hoshino, H., Kobayashi, A., Yoshida, M., Kudo, N., Oyake, T., Motohashi, H., Hayashi, N., Yamamoto, M., and Igarashi, K. (2000) J. Biol. Chem. 275, 15370–15376
35. Igarashi, K., and Sun, J. (2006) Antioxid. Redox Signal. 8, 107–118
36. Sun, J., Hoshino, H., Takaku, K., Nakajima, O., Muto, A., Suzuki, H., Tashiro, S., Takahashi, S., Shibahara, S., Alam, J., Taketo, M., Yamamoto, M., and Igarashi, K. (2002) EMBO J. 21, 5216–5224
37. Yamamoto, T., Kyo, M., Kamiya, T., Tanaka, T., Engel, J. D., Motohashi, H., and Yamamoto, M. (2006) Genes Cells 11, 575–591
38. Motohashi, H., and Yamamoto, M. (2006) Cancer Sci. 98, 135–139
39. Padmanabhan, B., Tong, K. L., Ohta, T., Nakamura, Y., Scharlock, M., Ohtsui, M., Kang, M. I., Kobayashi, A., Yokoyama, S., and Yamamoto, M. (2006) Mol Cell 21, 689–700