An Auto-regulatory Loop between Stress Sensors INrf2 and Nrf2 Controls Their Cellular Abundance*

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Nrf2 and INrf2 are sensors of chemical/radiation stress. Nrf2 dissociates from INrf2 in response to a stress and translocates in the nucleus. This leads to induction of a battery of antioxidant genes that protect cells. Nrf2 is then exported out and degraded. INrf2 functions as an adaptor of ubiquitin ligase for ubiquitination and degradation of Nrf2. Here we demonstrate the presence of a novel feedback autoregulatory loop between INrf2 and Nrf2 that controls cellular abundance of INrf2 and Nrf2. Nrf2 controls its own degradation by regulating expression and induction of the INrf2 gene. The antioxidant treatment of cells led to nuclear localization and stabilization of Nrf2 and induction of INrf2 gene expression. Mutagenesis, transfection, and chromatin immunoprecipitation assays identified an antioxidant-response element in the reverse strand of the proximal INrf2 promoter that binds to Nrf2 and regulates expression and antioxidant induction of the INrf2 gene. In addition, short interfering RNA inhibition or overexpression of Nrf2 led to a respective decrease and increase in INrf2 gene expression. These results implicated Nrf2 in the regulation of expression and induction of INrf2. The induction of INrf2 followed ubiquitination and degradation of Nrf2 and suppression of INrf2 gene expression. In conclusion, Nrf2 regulates INrf2 by controlling its transcription, and INrf2 controls Nrf2 by degrading it.

The endogenous cellular antioxidant defense system plays an important role in protecting cells from various external and internal stresses caused by xenobiotics and drugs (1), inflammation (2), and ionizing radiation (3). The perturbation of these cytoprotective regulations causes the accumulation of reactive oxygen species or electrophilic insults contributing to the pathogenesis of various diseases such as cancer, neurodegenerative disease, and atherosclerosis. Proper detoxification is mediated by the immediate expression of antioxidant proteins and phase 2 detoxifying enzymes through the activation of antioxidant-response element (ARE)\(^2\)-binding transcription factors (4).

The ARE was first identified as cis-element in the upstream regulatory region of the GSTA2 gene (5) and was found in the promoters of detoxifying enzyme genes such as glutathione \(S\)-transferases (6), NAD(P)H:quinone oxidoreductases (NQOs) (7, 8), gastrointestinal glutathione peroxidase (9), and peroxiredoxin 1 (10). The ARE is recognized by a subset of Cap’n’Collar-containing basic leucine zipper proteins, nuclear factor erythroid 2-related factors (Nrf)\(_s\), including Nrf1, Nrf2, and Nrf3. Among the three protein factors, Nrf2 is most potent transcription factor in regulation of basal and induced expression of antioxidant enzyme genes (11). Gene deletion studies also supported the important function of Nrf2 in cellular protection against oxidative stress and neoplasia (12).

Under homeostatic conditions, Nrf2 resides predominantly within the cytoplasm of the cells by an interaction between Nrf2 and actin-bound cytosolic protein, INrf2 (inhibitor of Nrf2) or Keap1 (Kelch-like ECH-associated protein 1) (13–15). Nrf2 functions as a substrate adaptor protein for a Cul3-dependent ubiquitin-protein isopeptide ligase complex to maintain the steady-state levels of Nrf2 (16). It is also believed that Nrf2 is rapidly degraded by INrf2-mediated ubiquitination because Nrf2 is barely detected in the cytoplasm. However, the exposure to oxidative stress leads to dissociation of Nrf2 from INrf2. Nrf2 is stabilized, translocates into the nucleus, and activates transcription of a battery of antioxidant genes. Recently, the mechanisms by which Nrf2 is released from INrf2 under stress have been actively investigated. One mechanism is that antioxidant-induced protein kinase C phosphorylation of serine 40 in Nrf2 leads to dissociation of Nrf2 from INrf2 (17, 18). In addition, several protein kinases, including mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and PKR-like endoplasmic reticulum kinase have also been involved in post-translational modification of Nrf2 and activation of Nrf2 (11, 19). On the other hand, cysteine thiold of INrf2 were shown to function as sensors for oxidative stress that are modified by the chemical inducers, causing formation of disulfide bonds between cysteines of two INrf2 peptides. This results in conformational change that renders INrf2 unable to bind to Nrf2 (20, 21). The free Nrf2 translocates to the nucleus and activate genes leading to cytoprotection.

Several reports suggest that persistent accumulation of Nrf2 in the nucleus is harmful. INrf2-null mice demonstrated persistent accumulation of Nrf2 in the nucleus that led to postnatal death from malnutrition resulting from hyperkeratosis in the esophagus and forestomach (22). Reversed phenotype of INrf2

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2 The abbreviations used are: ARE, antioxidant-response element; Nrf2, inhibitor of Nrf2 also known as Keap1; Nrf2, nuclear transcription factor; NQO1, NAD(P)H:quinone oxidoreductase 1; t-BHQ, tert-butyl hydroquinone; CHIP, chromatin immunoprecipitation; siRNA, short interfering RNA; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA-Ub, hemagglutinin-tagged ubiquitin.
deficiency by breeding to Nrf2-null mice suggested tightly regulated negative feedback might be essential for cell survival (23). The recent systemic analysis of INRF2 genomic locus in human lung cancer patients and cell lines showed that deletion, insertion, and missense mutations in functionally important domains of INRF2 results in reduction of INRF2 affinity for Nrf2 and elevated expression of cytoprotective genes (24, 25). Taken together, unrestrained activation of Nrf2 in cells increases a risk of adverse effects, including tumorigenesis. On the other hand, stress-induced activation of the Nrf2 pathway in normal cells is tightly regulated and confers cytoprotection against oxidative and electrophilic stress and carcinogens. Therefore, it appears that cells contain mechanisms that autoregulate cellular abundance of Nrf2.

In this study, we demonstrate that there is an autoregulatory feedback loop in the Nrf2 pathway. After Nrf2 activation by antioxidant, an increase in Nrf2 expression was detected. Nrf2 promoter and Nrf2 knockdown/overexpression studies show that Nrf2 induces promoter activity of the INrf2 gene through Nrf2 binding to an ARE in the reverse strand of the proximal promoter of the INrf2 gene. The induced Nrf2 protein accelerates ubiquitination of Nrf2 for degradation. Therefore, Nrf2 controls its own degradation by regulating INrf2 levels in the cells.

MATERIALS AND METHODS

Cell Cultures—Human hepatoblastoma (HepG2) and mouse hepatoma (Hepa-1) cells were obtained from the American Type Culture Collection (Manassas, VA). HepG2 cells were grown in minimum essential medium and Hepa-1 in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin (40 units/ml), and streptomycin (40 µg/ml). The cells were grown in monolayer in an incubator at 37 °C in 95% air and 5% CO2.

Generation of Stable Flp-In T-REx HEK293 Cells Expressing Tetracycline-inducible Nrf2—Flp-In T-REx HEK293 cells purchased from Invitrogen were co-transfected with FLAG-Nrf2 cDNA in pcDNA5/FRT/TO and pOG44 plasmids (Invitrogen) by Effectene (Qiagen, Valencia, CA) method and the manufacturer’s instructions. Forty eight hours after transfection, the cells were grown in medium containing 200 µg/ml hygromycin B (Invitrogen). The 293/FRT/FLAG-Nrf2 cells stably expressing tetracycline-inducible N-terminal FLAG-tagged Nrf2 were selected. The stably selected cells were grown and treated with 0.5 µg/ml tetracycline (Sigma) for varying periods of time to follow the overexpression of FLAG-tagged Nrf2.

Plasmid Constructs—Genomic clones (BAC vector) containing mouse Nrf2 loci were purchased from BACPAC Resources Center, Children’s Hospital Oakland Research Institute, Oakland, CA. A 2,706-bp fragment of INrf2 promoter was isolated via PCR using the 5’-CGAGATCTGAGCTCTGAATCTGCAAAGCAGATGTAAGCGAGTGAGG-3’ and 5’-CGAGATCTGAGCTCTGAATCTGCAAAGCAGATGTAAGCGAGTGAGG-3’ primer pairs and high fidelity platinum Taq DNA polymerase (Invitrogen). The PCR-amplified promoter fragments were first subcloned in to pScB- vector (Stratagene, La Jolla, CA) and then subcloned into pGL2-basic luciferase vector (Promega, Madison, WI) using SacI restriction site. The construct was designed as pGL2–2.7 kb (–2,700/-1, +1 is transcription initiation site). The sequence of the PCR forward primers for a series of deletion constructs is as follows: 1.7 kb forward, 5’-CGAGATCTGAGCTCTGAATCTGCAAAGCAGATGTAAGCGAGTGAGG-3’; 1.1 kb forward, 5’-CGAGATCTGAGCTCTGAATCTGCAAAGCAGATGTAAGCGAGTGAGG-3’; the same reverse primer, as used to construct 2.7-kb plasmid, was used. To generate pGL2–0.08 kb deletion construct, pGL2–1.1 kb was digested by Smal and re-ligated after removing −1100/−82 promoter region. pGL2–1.1 and 0.08 kb with mutated ARE were produced by site-directed mutagenesis with the PCR-based QuikChange II XL site-directed mutagenesis kit (Stratagene). Briefly, pGL2–1.1 kb or 0.08 kb was denatured for 1 min at 95 °C in the presence of mutagenic primers as follows: mARE–1 forward, 5’-GGGTG-GGACGGAGGTGTCGATAGCCCGGGAGGATGC-3’; mARE–r1 forward, 5’-GGGTG-GGACGGAGGTGTCGATAGCCCGGGAGGATGC-3’; mARE–r2 forward, 5’-GGGTG-GGACGGAGGTGTCGATAGCCCGGGAGGATGC-3’; mARE–r3 forward, 5’-GGGTG-GGACGGAGGTGTCGATAGCCCGGGAGGATGC-3’. To clarify which ARE has an essential role in Nrf2-induced Nrf2 promoter activity, oligonucleotides containing each ARE sequence were synthesized and cloned into the pGL2 promoter vector. The sequences of oligonucleotides of AREs are as follows: ARE–1, 5’-GGTGGACGGAGGTGTCGACGGCGCCCGCCGATGCGC-3’; ARE–r1, 5’-GGTGGACGGAGGTGTCGACGGCGCCCGCCGATGCGC-3’; ARE–r2, 5’-GGTGGACGGAGGTGTCGACGGCGCCCGCCGATGCGC-3’; ARE–r3, 5’-GGTGGACGGAGGTGTCGACGGCGCCCGCCGATGCGC-3’. The sequence accuracy of all constructs was confirmed by using ABI 3700 capillary sequencer (Applied Biosystems, Foster City, CA). The luciferase plasmid harboring human NQO1 gene ARE is described earlier (13). Plasmids, pcDNA-Nrf2-V5, pCMV-FLAG-INrf2, and HA-Ub are also described previously (26).

RT-PCR and Northern Analysis—Cells were treated with t-BHQ ± actinomycin D as indicated in the figures. The RNA was isolated using the RNeasy mini kit (Qiagen). 250 ng of total RNA was subjected to reverse transcription using a Superscript one-step RT-PCR kit (Invitrogen). After synthesis of cDNA at 50 °C for 30 min, the PCR was performed for 27 cycles consisting of the following steps: denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, and extension at 68 °C for 1 min. The sequences of the forward and reverse primers for RT-PCR were as follows: mouse INrf2, forward 5’-GGCTGGACGAGGTTGAG-3’ and reverse 5’-TCCGCTGGACAGGTTGAG-3’; human Nrf2, forward 5’-CCAGGGTGTGTCTGAGGAGT-3’ and reverse 5’-ACCTGCATGGCGAGGAGT-3’; mouse INrf2, forward 5’-CCAGGGTGTGTCTGAGGAGT-3’ and reverse 5’-ACCTGCATGGCGAGGAGT-3’; human Nrf2, forward 5’-TCCGCTGGACAGGTTGAG-3’ and reverse 5’-ACCTGCATGGCGAGGAGT-3’; mouse INrf2, forward 5’-CCAGGGTGTGTCTGAGGAGT-3’ and reverse 5’-ACCTGCATGGCGAGGAGT-3’; human Nrf2, forward 5’-TCCGCTGGACAGGTTGAG-3’ and reverse 5’-ACCTGCATGGCGAGGAGT-3’; mouse INrf2, forward 5’-CCAGGGTGTGTCTGAGGAGT-3’ and reverse 5’-ACCTGCATGGCGAGGAGT-3’; human Nrf2, forward 5’-TCCGCTGGACAGGTTGAG-3’ and reverse 5’-ACCTGCATGGCGAGGAGT-3’; mouse INrf2, forward 5’-CCAGGGTGTGTCTGAGGAGT-3’ and reverse 5’-ACCTGCATGGCGAGGAGT-3’; human Nrf2, forward 5’-TCCGCTGGACAGGTTGAG-3’ and reverse 5’-ACCTGCATGGCGAGGAGT-3’; mouse INrf2, forward 5’-CCAGGGTGTGTCTGAGGAGT-3’ and reverse 5’-ACCTGCATGGCGAGGAGT-3’; human Nrf2, forward 5’-TCCGCTGGACAGGTTGAG-3’ and reverse 5’-ACCTGCATGGCGAGGAGT-3’; mouse INrf2, forward 5’-CCAGGGTGTGTCTGAGGAGT-3’ and reverse 5’-ACCTGCATGGCGAGGAGT-3’; human Nrf2, forward 5’-TCCGCTGGACAGGTTGAG-3’ and reverse 5’-ACCTGCATGGCGAGGAGT-3’; mouse INrf2, forward 5’-CCAGGGTGTGTCTGAGGAGT-3’ and reverse 5’-ACCTGCATGGCGAGGAGT-3’; human Nrf2, forward 5’-TCCGCTGGACAGGTTGAG-3’ and reverse 5’-ACCTGCATGGCGAGGAGT-3’; mouse INrf2, forward 5’-CCAGGGTGTGTCTGAGGAGT-3’ and reverse 5’-ACCTGCATGGCGAGGAGT-3’; human Nrf2, forward 5’-TCCGCTGGACAGGTTGAG-3’ and reverse 5’-ACCTGCATGGCGAGGAGT-3’.
Ten micrograms of RNA were also analyzed by Northern blotting and hybridization with 32P-labeled mouse INrf2 cDNA. The blot was washed and autoradiographed.

**Western Blot Analysis**—The cells were lysed in ice-cold RIPA-B buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 1% Triton X-100, and protease inhibitor mixture (Roche Applied Science)). Nuclear extracts were made using a nuclear extract kit from Active Motif (Carlsbad, CA) according to manufacturer’s protocol. Thirty micrograms of proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with anti-INrf2 (1:200, Santa Cruz Biotechnology), anti-Nrf2 (1:500, Santa Cruz Biotechnology), anti-FLAG (1:5000, Sigma), or anti-actin (1:5000, Sigma) antibodies, washed, and probed with electrochemiluminescence (Amer sham Biosciences). To confirm the purity of nuclear-cytoplas mic fractionation, the membranes were reprobed with cytoplasm-specific, anti-lactate dehydrogenase (Chemicon) and nuclear specific, anti-lamin B antibodies (Santa Cruz Biotechnology). Protein expression was quantified by using NIH Image program (developed at the National Institutes of Health and available on line at rsb.info.nih.gov/nih-image/). In related experiments, the cells were treated with 50 μM t-BHQ in the absence or presence of 30 μg/ml cycloheximide for different time intervals. The cells were lysed and analyzed by Western blotting and probed with INrf2 antibody. The blot was stripped and reprobed with β-actin antibody.

**Ubiquitination Assay**—Hepa-1 cells were seeded in 100-mm plates and co-transfected with pCMV-FLAG-Nrf2 (1.0 μg) and pCMV-HA-UB (0.5 μg) as described previously (26). The transfected cells were treated with either Me2SO or 50 μM t-BHQ (Sigma) for the indicated time. To check Nrf2 ubiquitination, 1 mg of protein lysate was used to immunoprecipitate with anti-FLAGM2 beads (Sigma). To analyze Nrf2 ubiquitination in cytoplasm and nuclear extracts, Hepa1 cells in 100-mm plates were co-transfected with pDNA-Nrf2-V5 (1.0 μg) with or without plasmids encoding HA-UB (0.15 μg) and pCMV-FLAG-INrf2 (0.5 μg). Nuclear and cytoplasmic extracts were prepared using active motif kit, and 1 mg of extracts was immunoprecipitated with anti-V5 antibody. Immunoprecipitates were resolved on a 10% SDS-PAGE followed by immunoblotting with anti-HA antibody.

**Transient Transfection and Luciferase Assay**—HepG2, Flp-In T-REx HEK293, or 293/FRT/FLAG-Nrf2 cells were plated in 12-well plates at a density of 1 × 105 cells/well 24 h prior to transfection. The cells were transfected with 0.1 μg of the indicated luciferase plasmid using Effectene transfection reagent (Qiagen) according to the manufacturer’s instruction. The pRL-TK plasmid encoding Renilla luciferase (0.01 μg; Promega) was included as an internal control of transfection efficiency. After 32 h, transfected HepG2 cells were stimulated with Me2SO or 50 μM t-BHQ for 16 h. Otherwise, transfected Flp-In T-REx HEK293 or 293/FRT/FLAG-Nrf2 cells were treated with 0.5 μg/ml of tetracycline for 8 or 16 h. The cells were harvested, lysed, and analyzed for luciferase activity using the dual-luciferase reporter assay system (Promega).

**Chromatin Immunoprecipitation (ChIP) Assay**—ChIP assay was performed using a kit from Active Motif as described previously (8). Briefly, 70% confluent Hepa-1 cells were treated with Me2SO or 50 μM t-BHQ for 4 h and then fixed in 1% formaldehyde for 15 min. Cells were lysed and nuclei pelletted by centrifugation. Nuclei were resuspended and sheared using a sonicator (Misonix Inc., Farmingdale, NY) with five pulses of 20 s at 25% of maximum output. Sheared chromatin was immunoprecipitated with 2 μg of anti-Nrf2 or control IgG antibody. The cross-links reversed overnight at 65 °C and deproteinized with 20 μg/ml proteinase K. INrf2-associated INrf2-ARE was detected by PCR amplification with the primers as follows: ARE-r1, forward, 5′-GAGCCCTCGTAGGTGTG-3′, and reverse, 5′-CTGGAAGCGCTCCTACCTAC-3′; ARE-r2, forward, 5′-GGTAGTTGACCGGCAG-3′, and reverse, 5′-GTACGAGGACTCCGAC-3′. The PCR condition used for ChIP assay was 37 cycles of a denaturing step at 94 °C for 30 s, an annealing step at 65 °C for 30 s, and an extension step at 72 °C for 30 s. PCR products (~167 bp with ARE-r1 primers and ~165 with ARE-r2 primers) were separated on 2% agarose gel containing ethidium bromide and imaged using an Eagle Eye System (Stratagene).

**Gel and Supershift Assay**—INrf2 ARE-r2 and ARE-r1 was end-labeled with [γ-32P]ATP and T4 kinase. 100,000 cpm was incubated with 10 μg of HepG2 nuclear extract, and band shift reaction performed as described earlier (13). In the same experiment the gel shift mixture was incubated with 2 μg of control IgG or Nrf2 antibody at 4 °C for 2 h. The mixtures were separated on polyacrylamide gel and autoradiographed.

**Nrf2 siRNA Interference Assay**—Nrf2 siRNA was used to inhibit Nrf2 by a procedure described previously (8). Nrf2 siRNA and control siRNA were purchased from Dharmacon. HepG2 and human 293 kidney cells were transfected with 25 and 50 nM control siRNA or Nrf2 siRNA using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer’s instructions. Twenty four hours later, cells were co-transfected with 0.1 μg of Nrf2 promoter-luciferase and 0.01 μg of pRL-TK Renilla plasmids. Thirty two hours after the second transfection, cells were treated either with Me2SO or with 50 μM t-BHQ for 16 or 4 h. The cells at the end of treatment were harvested and analyzed by measuring luciferase activity, INrf2 and Nrf2 RNA by RT-PCR, and INrf2 and Nrf2 protein by Western blot analysis and probing with Nrf2 and Nrf2 antibodies.

**Statistical Analyses**—The data from luciferase assays were analyzed using a two-tailed Student’s t test. Data are expressed as the mean ± S.D.

**RESULTS**

Antioxidant t-BHQ Up-regulates INrf2 Gene Expression—RT-PCR analysis of mouse Hepa-1 cells treated with t-BHQ showed a concentration- and time-dependent increase in INrf2 gene expression (Fig. 1A, left and middle panels, also see the quantitative densitometry graph below the figure). Precipitation with the transcription inhibitor actinomycin D blocked the t-BHQ-mediated induced expression of INrf2 (Fig. 1A, right panel). Northern analysis also demonstrated the t-BHQ concentration-dependent increase in INrf2 RNA (Fig. 1B). Western blot analysis supported RT-PCR and Northern analysis data (Fig. 1C). The t-BHQ treatment of Hepa-1 cells
Deletion mutagenesis in 2.7-kb INrf2 promoter sequence analysis in HepG2 revealed that 80 bp upstream to the start site of transcription is required for basal expression and induction in response to t-BHQ (Fig. 2A). Mouse INrf2 promoter was analyzed for various putative cis-elements, including Nrf2-binding AREs. Mouse-INrf2 promoter analysis was done using the genomatix web tool, Matinspector, available on line. The binding sites with a matrix similarity score over 0.9 were considered. Analysis of 2.7-kb INrf2 promoter sequence revealed the presence of putative binding sites for transcription factors, hepatic nuclear factor (HNF-1), signal transducer and activator of transcription-6 (STAT-6), CCAAT/enhancer-binding protein, OCT1, aryl hydrocarbon receptor (AhR)/Arnt heterodimers, and hypoxia-inducible factor-binding sites. Interestingly, nucleotide sequence analyses of 2.7-kb INrf2 promoter also revealed the presence of three putative AREs (Fig. 2A). Two of these elements were on the reverse strand at nucleotide position −591 (ARE-r2) and −46 (ARE-r1) from the start site of transcription. The third element was located at nucleotide position −272 on the sense strand. The AREs were individually mutated in 1.1-kb Nqo1 gene promoter-luciferase plasmid. The mutated plasmids were transfected in HepG2 cells and analyzed for luciferase activity in the absence and presence of t-BHQ to determine the role of individual AREs in expression and t-BHQ induction of the luciferase gene (Fig. 2B). An ARE from the human NQO1 gene cloned into luciferase reporter plasmid was used as a positive control for t-BHQ-mediated luciferase gene induction (Fig. 2A, last panel). The results revealed that mutation of the ARE-r1 element in 1.1-kb INrf2 promoter resulted in the significant reduction in basal expression and abrogation of t-BHQ induction as compared with the normal 1.1-kb INrf2 promoter (p < 0.001). The mutation of ARE-r2 had no effect, whereas mutation of ARE-1 had minimal effect on 1.1-kb INrf2 gene expression and induction in response to t-BHQ. Mutation of ARE-r1 in the 0.08-kb INrf2 promoter also resulted in a significant decrease in basal expression and loss of t-BHQ induction of expression as compared with normal 0.08-kb INrf2 promoter (Fig. 2B). The NQO1 gene hARE-luciferase plasmid was used as a positive control for the assay (Fig. 2B). The three AREs were individually cloned in the pGL2 promoter vector, transfected in HepG2, and analyzed for luciferase activity to determine its role in t-BHQ induction of luciferase gene expression.

FIGURE 1. Antioxidant t-BHQ induces INrf2 gene expression. A, RT-PCR analysis of INrf2 gene expression in Hepa-1 cells. Hepa-1 cells were grown in a monolayer and treated with 50 μM t-BHQ for the indicated time intervals (left panel) or with 50, 100, and 200 μM t-BHQ for 16 h (middle panel). A similar experiment Hepa-1 cells were preincubated with 2 μg/ml actinomycin D for 1 h followed by t-BHQ + actinomycin D for the indicated time intervals (right panel). Total RNA was isolated and analyzed by RT-PCR using primers specific for INrf2 mRNA. GAPDH was used as a control. B, Northern analysis. Hepa-1 cells were treated with Me2SO (DMSO, control) or indicated concentrations of t-BHQ for 16 h. RNA was isolated and 10 μg of RNA analyzed by Northern blotting and hybridization with full-length INrf2 cDNA. Northern blot was stripped and reprobed with GAPDH cDNA. C, Western analysis of INrf2 protein in Hepa-1 cells. Total cell lysate from Hepa-1 cells treated with 50 μM t-BHQ alone (left panel) or with 30 μg/ml cycloheximide (CHX, right panel) for the indicated time intervals were analyzed for INRF2 expression by Western blotting and probing with anti-INRF2 antibody. β-Actin was used as a loading control. D, RT-PCR analysis of INRF2 gene expression in HepG2 cells. HepG2 cells were treated with 50 μM t-BHQ for the indicated time (left panel) or with varying concentrations of t-BHQ for 16 h (right panel). RNA was isolated and analyzed for INRF2 expression by RT-PCR. NQO1 and GAPDH were used as positive and loading controls, respectively. NTC, no template control. The relative intensities of amplified bands were quantitated, normalized to GAPDH signal, and plotted as fold induction in transcript versus treatment. All experiments were repeated 3–5 times. The representative results are shown.
INrf2:Nrf2 Auto-regulatory Loop

A

\[
\begin{array}{c}
\text{ARE-r1} \\
-583 \quad \text{GCAGTTCGA} \\
\text{ARE-r2} \\
-272 \quad \text{TGAGCGAGC} \\
\text{ARE-1} \\
-264 \quad \text{GCAGGTTCA} \\
\text{ARE-2} \\
-1109 \quad \text{CGTCCAAGT} \\
\text{ARE-r1} \\
-81 \quad \text{Luc} \\
\end{array}
\]

2.7 kb

1.7 kb

1.1 kb

0.8 kb

B

\[
\begin{array}{c}
\text{Luc} \\
-1109 \\
\text{ARE-1} \\
-1109 \\
\text{ARE-r2} \\
-1109 \\
\text{ARE-r2} \\
-81 \\
\end{array}
\]

DMSO

t-BHQ

\[
\begin{array}{c}
pGL2 \quad 1.1kb \\
1.1 kb-mARE-r1 \\
1.1 kb-mARE-1 \\
1.1 kb-mARE-r2 \\
0.08 kb-mARE-r1 \\
\end{array}
\]

Relative Luciferase Activity

\[
\begin{array}{c}
0 \\
1 \\
2 \\
3 \\
4 \\
5 \\
6 \\
7 \\
\end{array}
\]

P < 0.005

P < 0.005

P < 0.001

P < 0.001

\[
\begin{array}{c}
pGL2 \quad 1.1kb \\
1.1 kb-mARE-r1 \\
1.1kb-mARE-1 \\
1.1 kb-mARE-r2 \\
0.08 kb \\
0.08 kb-mARE-r1 \\
\end{array}
\]

C

\[
\begin{array}{c}
\text{GCTTAGTCA} \\
\text{CGAAATCGT} \\
\text{TGAGCGAGC} \\
\text{ACTGCTGCTG} \\
\text{GCAGGTTCA} \\
\text{CTGCAAGT} \\
\text{pGL2-ARE-r1} \\
\text{pGL2-ARE-1} \\
\text{pGL2-ARE-r2} \\
\end{array}
\]

DMSO

t-BHQ

\[
\begin{array}{c}
pGL2 \quad ARE-r1 \\
\text{pGL2 ARE-r2} \\
\text{ARE-1} \\
\end{array}
\]

Relative Luciferase Activity

\[
\begin{array}{c}
0 \\
1 \\
2 \\
3 \\
4 \\
5 \\
6 \\
7 \\
\end{array}
\]

P < 0.0005

P < 0.0005

P < 0.05

P < 0.05

P < 0.05

P < 0.05

FIGURE 2. ARE sequence in the reverse strand of the proximal promoter regulates expression and antioxidant induction of INrf2 gene. A, deletion mutagenesis and transfection analysis. Serial deletions of mouse INrf2 promoter separately attached to luciferase (Luc) reporter gene were transfected in HepG2 cells, treated with Me$_2$SO (DMSO) or 50 $\mu$M t-BHQ for 16 h, and analyzed for luciferase activity. Three putative ARE sequences, two on the reverse strand (ARE-r1 and ARE-r2) and one ARE (ARE-1) on the sense strand, are shown. B, ARE mutations and transfection analysis. Three putative AREs were individually mutated in 1.1-kb INrf2 gene promoter, transfected in HepG2, and analyzed for luciferase gene expression. In the same experiment ARE-r1 was also mutated in 0.8-kb INrf2 promoter and analyzed for luciferase expression in transfected cells. Human NQO1-ARE luciferase reporter plasmid was also transfected in HepG2 cells as a positive control for t-BHQ-mediated luciferase gene induction. C, ARE-luciferase expression in transfected cells. ARE-r1, ARE-1, and ARE-r2 were separately attached to SV40 basal promoter hooked to luciferase reporter gene by cloning in vector pGL2 promoter, transfected in HepG2 cells, treated with Me$_2$SO or t-BHQ (50 $\mu$M for 16 h), and analyzed for luciferase activity. For all the above experiments, pGL2 empty vector was used as negative control. The results are expressed as fold increase in relative luciferase activity compared with untreated pGL2 transfection. The data shown are mean ± S.D. of three independent transfection experiments in A–C.

through the heterologous promoter (Fig. 2C). The results demonstrated that ARE-r1 and not other two AREs efficiently mediated expression and t-BHQ induction of luciferase gene expression.

Antioxidant Increases in Vivo Binding of Nrf2 to ARE-r1—We performed ChIP assay in Hepa-1 cells using an Nrf2-specific antibody and PCR primers covering the ARE-r1 and ARE-r2 regions in the INrf2 promoter to determine the binding of Nrf2 to ARE-r1 of the INrf2 gene in Me$_2$SO- and t-BHQ-treated Hepa-1 cells. The results demonstrated binding of Nrf2 to the ARE-r1 but not ARE-r2 in INrf2 gene promoter (Fig. 3A). The Nrf2 binding to ARE-r1 was enhanced by 1.7-fold in response to t-BHQ, but there was no Nrf2 bound to ARE-r2 of INrf2 even after t-BHQ treatment (Fig. 3, A and B; $p > 0.05$). These data indicate a specific interaction of Nrf2 to ARE-r1 of the INrf2 gene promoter, which is enhanced upon t-BHQ treatment. INrf2 gene ARE-r1 was also used in a gel/supershift analysis to analyze the specificity of Nrf2 binding (Fig. 3C). The results demonstrate that Nrf2 antibody resulted in a supershifted band indicating the specificity of the interaction (Fig. 3C).

Nrf2 Mediates t-BHQ Induction of INrf2 Gene Expression—Oxidative and electrophilic stresses are known to induce the stability of Nrf2 that leads to nuclear accumulation of Nrf2 resulting in transcriptional activation of antioxidant and phase II drug-metabolizing enzyme genes, including NQO1 gene (11). Therefore, we evaluated the effect of increased and decreased
expression of Nrf2 in regulation of INrf2 gene expression. We used overexpression of Nrf2 and siRNA inhibition of Nrf2 to demonstrate a role of Nrf2 in ARE-r1-mediated expression and t-BHQ induction of INrf2 gene expression (Figs. 4 and 5). We also successfully established the Flp-In T-Rex 293 cell lines (293/FRT/FLAG-Nrf2) that upon stimulation with tetracycline showed a time-dependent increase in FLAG-Nrf2 RNA (Fig. 4A) and protein (Fig. 4B). The RT-PCR analysis also revealed that tetracycline-induced overexpression of FLAG-Nrf2 led to time-dependent increases in INrf2 gene expression. In the same experiment, the Nrf2 downstream gene NQO1 was also induced. The transfection of Flp-In T-Rex 293 or 293/FRT/FLAG-Nrf2 cells with ARE-r1-luciferase plasmid revealed time-dependent increases in ARE-r1-luciferase gene expression upon stimulation with tetracycline (Fig. 4B).

To further explore the role of Nrf2 in the t-BHQ-induced INrf2 gene expression, we co-transfected HepG2 cells with control or Nrf2 siRNA and ARE-r1-Luc plasmids and analyzed for luciferase gene expression (Fig. 5, A and B). Nrf2 siRNA, but not control siRNA, effectively inhibited the expression of Nrf2 (Fig. 5A) and ARE-r1-mediated luciferase gene expression (Fig. 5B). In similar experiments, Nrf2 siRNA also inhibited 1.1-kb normal INrf2 but not ARE-r1 mutant 1.1-kb INrf2 gene-mediated luciferase gene expression and induction in response to t-BHQ (Fig. 5C). RT-PCR analysis showed that siRNA inhibition of Nrf2 in HepG2 cells led to abrogation of t-BHQ induction of INrf2 gene expression (Fig. 5D). The replacement of Hepa-1 with HepG2 cells also demonstrated Nrf2 mediated regulation of INrf2 gene expression (data not shown). Western analysis showed that the transfection of HepG2 cells with Nrf2 siRNA resulted in inhibition of Nrf2 and abrogation of t-BHQ induction of INrf2 (Fig. 5E).

Nrf2-mediated Up-regulation of INrf2 Led to Increased Degradation of Nrf2—The treatment of Hepa-1 cells with antioxidant t-BHQ resulted in stabilization of Nrf2 that started within 0.5 h and peaked at 4 h after treatment (Fig. 6A). The Nrf2 levels declined at 8 and 16 h after t-BHQ treatment. At 16 h, the Nrf2 levels were reduced to almost normal cellular levels. The stabilization of Nrf2 between 0.5 and 4 h led to increased expression of INrf2 starting at 2 h and maximizing at 8 h and then plateauing at 16 h after t-BHQ treatment. The ubiquitination of Nrf2 reduced at 2 and 4 h after t-BHQ treatment and then significantly increased at 8 and 16 h after t-BHQ treatment. In other words, t-BHQ-induced stabilization of Nrf2 was followed by increased expression of INrf2 followed by increased ubiquiti-
nation and degradation of Nrf2. Our earlier published work has suggested that the Nrf2 is mostly degraded in cytoplasm as its degradation could be blocked in the presence of nuclear export inhibitor leptomycin B (27). However, we also found evidence that some of Nrf2 might also be degraded inside the nucleus (27). Next, we analyzed the cellular compartment-specific ubiquitination/degradation of Nrf2. Cytosol and nuclear extracts obtained from Nrf2-V5-transfected Hepa-1 cells were subjected to ubiquitination analysis (Fig. 6B). The results demonstrate that overexpression of FLAG-INrf2 leads to reduced levels of Nrf2 in both cytosol and nucleus confirming INrf2-mediated Nrf2 degradation (Fig. 6B, lower panel). However, enriching the ubiquitinated-Nrf2 indicated that most of the Nrf2 gets ubiquitinated in the cytosolic compartment (Fig. 6B, upper panel). These results are complementary to our earlier published data and together conclude that Nrf2 ubiquitination and degradation mostly takes place in cytosol.

DISCUSSION

The results showed that antioxidant treatment induced the expression of INrf2. This raised an interesting question regarding the mechanism of expression and antioxidant induction of INrf2 and the in vivo role of increased INrf2. The results also demonstrated the presence of a functional ARE on the reverse strand at position −46 of the INrf2 promoter. Nrf2 bound to this ARE on the reverse strand. The increase in expression of Nrf2 resulted in increased INrf2 gene expression. Mutations in ARE at the −46 position and siRNA inhibition of Nrf2 significantly reduced both the expression and antioxidant induction of the INrf2 gene. These experiments concluded that an ARE on the reverse strand at nucleotide position −46 and transcription
factor Nrf2 regulated the expression and antioxidant induction of INrf2 gene expression. The role of other AREs in INrf2 gene promoter was minimal in expression and induction of the INrf2 gene. Further studies showed that increased INrf2 blocked Nrf2 activity by enhancing the ubiquitination and rapid degradation of Nrf2. In other words, Nrf2 induced INrf2 for its own degradation. These results suggested the presence of a novel feedback autoregulatory loop between INrf2 and Nrf2 that controls cellular abundance of INrf2 and Nrf2.

The regulation of the INrf2 gene by the Nrf2 protein has an interesting consequence because INrf2 protein can combine with Nrf2 and modulate down its activity as a transcription factor through degradation of Nrf2 (28, 29). When INrf2 protein is expressed in a cell, it blocks Nrf2 function, which results in less Nrf2 being made. Thus, the activity of Nrf2 and the levels of INrf2 in a cell are kept in balance by the autoregulatory feedback loop. Factors that activate or inactivate either INrf2 or Nrf2 are expected to disrupt the autoregulatory loop with functional consequences. Chemicals and radiation that disrupt this loop by dissociating Nrf2 from INrf2 act to increase Nrf2 activity and Nrf2 downstream antioxidant gene expressions. This leads to protection and cell survival. Factors like mutations in INrf2 leads to inactivate INrf2 resulting in persistent nuclear accumulation of Nrf2 with adverse effects on cell survival. The INrf2-null mice demonstrated persistent accumulation of Nrf2 in the nucleus that led to postnatal death from malnutrition resulting from hyperkeratosis in the esophagus and forestomach (22). In addition, the recent INrf2 genomic locus analysis in human lung cancer patients and cell lines showed that deletion, insertion, and missense mutations in functionally important domains of INrf2 results in reduction of INrf2 affinity for Nrf2 and elevated expression of cytoprotective genes (24, 25).

FIGURE 6. Feedback loop between Nrf2 and INrf2. Activation of Nrf2 increases INrf2 that degrades Nrf2. A, Hepa-1 cells were transfected with HA-Ub plasmid and treated with t-BHQ (50 μM) for the different time intervals. Whole cell lysates (WCL) were prepared and analyzed by Western blotting and probing with INrf2 and Nrf2 antibodies followed by β-actin antibody. In same experiment, whole cell lysates were immunoprecipitated (IP) with anti-Nrf2 antibody. Immunoprecipitates were analyzed by Western blotting and probing with anti-HA antibodies. Right panel, optical densities of Nrf2, INrf2, and ubiquitinated Nrf2 were normalized and plotted by time. The data presented are mean of three independent experiments. B, ubiquitination of Nrf2 in cytosol and nucleus. Hepa-1 cells were transfected with plasmids expressing Nrf2-V5, FLAG-INrf2, and HA-UB in the combinations as displayed. Cytosol and nuclear extracts were subjected to ubiquitination analysis similarly as in A. 50 μg of input extracts were probed with anti-V5, anti-FLAG, anti-lamin B, and anti-lactate dehydrogenase antibodies.

FIGURE 7. Auto-feedback loop between Nrf2 and INrf2. A model that demonstrates autoregulatory loop between INrf2 and Nrf2 is shown. The Nrf2 protein regulates the INrf2 gene at the level of transcription, and the INrf2 protein regulates the Nrf2 protein at the level of its activity. ◆ post-translational modifications of Nrf2.
In summary, Nrf2-INrf2 serves as sensors of chemical- and radiation-induced oxidative/electrophilic stress (Fig. 7). Nrf2 is translocated in the nucleus leading to activation of antioxidant genes that protect cells against adverse effects of chemical/radiation exposure. The Nrf2 is exported out of the nucleus, ubiquitinated, and degraded (30). INrf2 is required for ubiquitination and degradation of Nrf2. INrf2 is also capable of entering inside the nucleus to facilitate degradation of Nrf2 (31). A feedback autoregulatory loop between INrf2 and Nrf2 controls cellular abundance of INrf2 and Nrf2. Nrf2 regulates the expression and induction of INrf2. The induction of INrf2 follows ubiquitination and degradation of Nrf2 and suppression of INrf2 gene expression. In other words, Nrf2 regulates INrf2 by controlling its transcription, and INrf2 controls Nrf2 by facilitating its degradation.

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