Identification of Activating Transcription Factor 4 (ATF4) as an Nrf2-interacting Protein

IMPLICATION FOR HEME OXYGENASE-1 GENE REGULATION*

Nrf2 regulates expression of genes encoding enzymes with antioxidant (e.g. heme oxygenase-1 (HO-1)) or xenobiotic detoxification (e.g. NAD(P)H:quinone oxidoreductase, glutathione S-transferase) functions via the stress- or antioxidant-response elements (StRE/ARE). Nrf2 heterodimerizes with small Maf proteins, but the role of such dimers in gene induction is controversial, and other partners may exist. By using the yeast two-hybrid assay, we identified activating transcription factor (ATF) 4 as a potential Nrf2-interacting protein. Association between Nrf2 and ATF4 in mammalian cells was confirmed by co-immunoprecipitation and mammalian two-hybrid assays. Furthermore, Nrf2-ATF4 dimers bound to an StRE sequence from the ho-1 gene. CdCl2, a potent inducer of HO-1, increased expression of ATF4 in mouse hepatoma cells, and detectable induction of ATF4 protein preceded that of HO-1 (30 min versus 2 h). A dominant-negative mutant of ATF4 inhibited basal and CdCl2-stimulated expression of a StRE-dependent/luciferase fusion construct (pE1-luc) in hepatoma cells but only basal expression in mammary epithelial MCF-7 cells. A dominant mutant of Nrf2 was equally inhibitory in both cell types in the presence or absence of CdCl2. These results indicate that ATF4 regulates basal and CdCl2-induced expression of the ho-1 gene in a cell-specific manner and possibly in a complex with Nrf2.

Overproduction of oxygen free radicals, attenuation of antioxidant systems, or both, commonly in response to extracellular stimuli, disturbs the cellular redox status and leads to oxidative stress. Such conditions typically elicit an adaptive response aimed at reversing this imbalance and maintaining redox homeostasis. In part, this adaptive response includes the activation of specific signaling pathways and, ultimately, the coordinate induction of a select set of genes that encode proteins with distinct activities that individually and collectively manifest antioxidant and cytoprotective functions. Central to this induction process are redox-sensitive transcription factors, such as nuclear factor-κB (NF-κB) and activator protein-1, arguably the two most prominent regulators of this cellular response mechanism (reviewed in Refs. 1 and 2).

Recent studies from several laboratories (3–8) have implicated another transcription regulator, Nrf2, with a potentially significant role in the adaptive response to oxidative stress. Nrf2 belongs to the CNC-hZIP subfamily of basic region/leucine zipper (hZIP) transcription factors. CNC-hZIP proteins are distinguished from other hZIP subfamilies, including those composed of Jun, Fos, ATF/CREB, or Maf factors, in that they also contain a Cap’n’Collar (CNC) structural motif homologous to a region within the Drosophila homoeotic selector protein encoded by the cap’n’collar gene (9). hZIP proteins function as obligate dimers; for example, individual Jun-Jun or Jun-Fos dimers are commonly and collectively referred to as activator protein-1 transcription factors. Sequences necessary for both dimerization and DNA binding reside within the bipartite bZIP domain.

Limited but consistent observations (6, 8) suggest that under normal conditions, and as is the case for NF-κB factors, Nrf2 exists in an inactive, cytoplasm-localized state, in part or fully as a consequence of binding to the cytoskeleton-associated protein Keap1. After exposure of cells to electrophiles or oxidative stress-generating agents, the cytoplasmic retention mechanism is inactivated, and Nrf2 is transported to the nucleus by an as yet uncharacterized mechanism(s) but one that may involve protein kinase C-mediated phosphorylation of Nrf2 (8). Within the nucleus, Nrf2 activates transcription of a select set of target genes by binding to distinct but very similar DNA elements, individually or alternatively referred to as the NF-E2-binding site (10), the Maf recognition element (MARE, 11), the stress-response element (12), or the antioxidant-response element (13). Many of the Nrf2 target genes (3–5, 7, 8) encode proteins that play a central role in the adaptive response to oxidative stress. Among others, these include heme oxygenase-1 (HO-1), an enzyme that catalyzes the rate-limiting reaction in heme degradation, a catabolic pathway that leads to the

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The abbreviations used are: NF-κB, nuclear factor-κB; heme, ferriptoporphyrin IX; HO-1, heme oxygenase-1; ATF/CREB, activating transcription factor/cAMP-response element-binding protein; CNC-bZIP, Cap’n’Collar/basic-leucine zipper; Nrf, NF-E2 related factor; NF-E2, nuclear factor-erythroid 2; bZIP, basic region/leucine zipper; Gdbd, Gal4 DNA binding domain; Y2H, yeast two-hybrid; M2H, mammalian two-hybrid; StRE, stress-response element; ARE, antioxidant-response element; MARE, Maf recognition element; EMSA, electrophoretic mobility shift assay; AD, activation domain; aa, amino acid; Hepa, hepatoma.
production of bilirubin, a potent antioxidant; NAD(P)H:quinone oxidoreductase (NQO), which catalyzes two-electron reduction of quinones, preventing the participation of such compounds in redox cycling and oxidative stress; γ-glutamylcysteine synthase, which catalyzes the rate-limiting reaction in glutathione biosynthesis; and glutathione S-transferase, which conjugates hydrophobic electrophiles and reactive oxygen species with glutathione.

Nrf2, like other CNC/bZIP proteins and Fos family members, belongs to a sub-class of bZIP factors with leucine zipper motifs incapable of self-dimerization. Consequently, sequence-specific DNA binding and subsequent induction of target gene transcription requires association of Nrf2 with other transcription factors. In accordance with the paradigm established by NF-E2 (10), the first CNC-bZIP containing mammalian transcription factor isolated, the most prominent dimerization partners of Nrf2 are the small Maf proteins, MafF, MafG and MafK (also referred to as p18 (14)). The precise function of such Nrf2/Maf dimers, however, is controversial, as they have been proposed to function as both positive (5) and negative regulators (15) of ARE-dependent gene transcription. Jun-Nrf2 complexes have also been implicated as positive effectors of ARE-dependent genes (16).

Given our incomplete understanding of Nrf2 function, the propensity of bZIP proteins to form inter- and intra-family dimers (17, 18), and of transcription factors in general to form complexes that tend to provide both diversity to, and discrimination of, genetic responses to extracellular stimuli, we reasoned that additional Nrf2-containing complexes exist intracellularly and that such complexes would likely regulate Nrf2 target gene expression. Accordingly, we have used the yeast two-hybrid screening procedure to identify proteins that associate with Nrf2. Herein, we report the identification of ATF4 as a Nrf2-interacting protein and explore the potential role of ATF4 in the regulation of one Nrf2 target gene, ho-1.

**EXPERIMENTAL PROCEDURES**

**Materials**

Tissue culture media were from Life Technologies, Inc., and fetal bovine serum was obtained from MediTech. Restriction endonucleases and other DNA-modifying enzymes were purchased from either Life Technologies, Inc., or New England Biolabs. Oligonucleotides were synthesized by IDT, Inc. Radiolabeled nucleotides were obtained from PerkinElmer Life Sciences. Reagents for luciferase assays were purchased from Sigma. Anti-mouse Nrf2 was kindly provided by Dr. M. Yamamoto. Antibodies against other transcription factors, including anti-human Nrf2, and HO-1 were obtained from Santa Cruz Biotechnology isolated, the most prominent dimerization partners of Nrf2 are the small Maf proteins, MafF, MafG and MafK (also referred to as p18 (14)). The precise function of such Nrf2/Maf dimers, however, is controversial, as they have been proposed to function as both positive (5) and negative regulators (15) of ARE-dependent gene transcription. Jun-Nrf2 complexes have also been implicated as positive effectors of ARE-dependent genes (16).

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frame with, the hexa-histidine tag in the T7 RNA polymerase-based prokaryotic expression vector series pET30a–c (Novagen Inc.). Recombinant proteins were purified from inclusion bodies by nickel affinity chromatography as per the manufacturer's protocol or according to the protocol of Holzinger et al. (25). Nrf 2M protein (residues 393–581), containing the DNA binding and leucine zipper dimerization domains, was synthesized by coupled in vitro transcription and translation reaction as described previously (26). EMSA was carried out as described previously (23) using a double-stranded oligonucleotide containing the sequence 5'-TTTTCTGCTGAGTCAAGGTCCG-3' (core StRE underlined) as probe. Five μl of Nrf 2M synthesis product and/or 100 ng of recombinant protein were used in EMSA reactions.

Co-immunoprecipitation and Western Blot Analysis

COS-7 cells were transfected 24 h after plating (1 × 10^6/100-mm plate) with a total of 5 μg of either empty vector (pEF/myc/mito), pEF/mATF4, pEF/Nrf2, or a combination of these plasmids using Lipofectin transfection reagent (Life Technologies, Inc.) as specified by the manufacturer. Cells were harvested 36 h after transfection and resuspended in 200 μl of lysis buffer (10 mM Tris-HCl (pH 7.5) containing 0.5% (v/v) Nonidet P-40, 150 mM NaCl, and 1 mM EDTA). Cell lysates were cleared by centrifugation, and immunoprecipitation was carried out with 100 μg of cell lysate using protein G-agarose beads as described (27). Immune complexes were eluted from the beads with 2× SDS-PAGE sample buffer and subjected to denaturing polyacrylamide gel electrophoresis. Western blotting was carried out as described previously (4). All antibodies were used at dilutions recommended by the respective suppliers.

RESULTS

Identification of ATF4 as an Nrf2-interacting Protein—To identify proteins that interact with Nrf2, a cDNA fragment encoding the C-terminal portion of mouse Nrf2 was amplified by PCR and cloned in-frame and downstream of the Gal4 DNA binding domain. The resulting fusion protein was used as “bait” in a yeast two-hybrid screening assay as described under “Experimental Procedures.” From a total of 2 × 10^6 yeast transformants, harboring either rat brain or liver cDNA/Gal4 activation domain fusions, seven independent clones (3 liver and 4 brain) that encoded Nrf2-interacting polypeptides were identified after a series of selection methods.

The nucleotide sequences of the inserts within the positive clones were determined, and the results indicated that five of the cDNAs were derived from the same mRNA. A sequence similarity search using “blastn” revealed significant similarities to sequences encoding mouse ATF4 and human CREB2 (ATF4) (28, 29) suggesting that these clones encoded the rat ATF4 plasmid:

Rat
Mouse
Human

Nrf2 Plasmid:

IP Ab:

BP:

Leucine Zipper II

Basic Region

Leucine Zipper I
homolog of ATF4. Since none of the isolates contained the initiation codon, the full-length ATF4 cDNA was obtained by PCR amplification from a rat brain cDNA library, cloned, and subjected to DNA sequence analysis.

The deduced amino acid sequence of rat ATF4 is aligned with those of mouse and human ATF4 in Fig. 1. Between these three species, ATF4 exhibits 84.4% sequence conservation and the rat protein displays 94.8 and 87.2% sequence identity to mouse and human ATF4 s, respectively. As expected, the highest degree of conservation is observed within the basic region (DNA binding) and the adjacent leucine zipper (dimerization) domains. The second “leucine zipper,” region, to which a function has yet to be assigned, exhibits greater divergence although the repeating leucine (or corresponding) residues at every 7th position are completely conserved in the three proteins.

Interaction between Nrf2 and ATF4 in Mammalian Cells—Association between Nrf2 and ATF4 in mammalian cells was confirmed by co-immunoprecipitation experiments and mammalian two-hybrid assays. For the former, expression plasmids encoding ATF4 or Nrf2 were transfected, individually or in combination, into COS-7 cells; the cells were lysed, and the lysates subjected to immunoprecipitation with anti-ATF4 or anti-Nrf2 antibodies in the presence or absence of the corresponding blocking peptide. Immunoprecipitates were subsequently analyzed by Western blotting. As shown in Fig. 2, Nrf2 was not detected in lysates of cells transfected with an empty vector (lane 3) or the ATF4 expression plasmid (lane 6), but was readily observed in lysates of cells transfected with both ATF4 and Nrf2 expression plasmids (lane 4). More importantly, Nrf2 could be immunoprecipitated with anti-ATF4 antibodies (lane 2), albeit to a lesser extent than that observed with anti-Nrf2 antibodies (lane 1). Immunoprecipitation of Nrf2 by both antibodies was abrogated in the presence of the corresponding blocking peptides (lanes 5 and 7).

The results from co-immunoprecipitation experiments were corroborated by mammalian two-hybrid assays. In these experiments, nearly full-length mouse Nrf2 (aa 13–581; Fig. 3A) or the C-terminal portion of Nrf2 (aa 314–581; Fig. 3B) was fused to the Gdbd, and these fusions served as interaction targets. Sequences encoding test proteins were fused in-frame to an N-terminal region of Nrf2 (amino acids 13–302) that contains a potent transcription activation domain (AD). Gdbd-Nrf2-(13–581) strongly trans-activated a luciferase reporter gene under the control of Gal4-binding sites, pFRhuc. Co-expression of Nrf2 AD further increased luciferase activity by 4–5-fold suggesting self-interaction between Nrf2 proteins. AD fusions containing full-length rat ATF4 or mouse ATF3 exhibited even greater trans-activation capabilities, ~35-fold above control and 7-fold above AD alone. The MafK (p18) fusion served as a positive control and exhibited the highest interaction activity. Gdbd-Nrf2-(314–581) contains the DNA interaction and dimerization (i.e. bZIP) domains but is transcriptionally inactive. Co-expression of AD did not stimulate luciferase activity suggesting that Nrf2 self-interaction is limited to the N-terminal portion of Nrf2. ATF3, ATF4, and p18 interacted with Gdbd-Nrf2-(314–581) with the following rank order: p18 >> ATF4 >> ATF3. Presumably these interactions reflect dimerization between leucine zipper domains. Relative to p18, ATF4 exhibits greater association with Gdbd-Nrf2-(13–581) than with Gdbd-Nrf2 (314–581) (~40% versus 5%), even though the latter is produced at higher levels intracellularly (data not shown).
Fig. 4. The Nrf2-ATF4 heterodimer binds to the StRE. Protein synthesis and EMSA reactions were carried out as described under "Experimental Procedures." EMSA gels were exposed to x-ray film for 16 h. Lanes designated "Nrf2" contained in vitro transcription/translation products from reactions directed by the empty expression vector pGEM2. Nonspecific complexes resulting from the transcription/translation extracts are marked with asterisks.

Fig. 5. ATF4 enhances and p18 represses Nrf2-mediated trans-activation of pE1-luc. Hepa cells were plated (5 x 10^5/well of 6-well plates) 24 h prior to transfection by CaPO_4-DNA co-precipitation. Cells in each well were transfected for 6 h with a DNA mixture consisting of 2 µg of pE1-luc, 1 µg of pCMV-β-Gal, 1 µg of pEF/Nrf2 or pEF/myc-mito, and the indicated amount of pCMV/rATF4 or pEF/p18. Total DNA was equalized with an appropriate empty expression vector. Cells were cultured for an additional 48 h and then lysed. Eight and 4% of the cell extracts were used to measure luciferase and β-galactosidase activities, respectively. Luciferase activity was normalized to β-galactosidase activity in the same extract and is presented as a percentage of activity in cells transfected without pCMV/rATF4 or pEF/p18. Each data point represents the average ± S.E. from three independent experiments. Average fold trans-activation by Nrf2 (in the absence of ATF or p18) was 53.4.
suggested that ATF4 is a stress-response protein (30, 31). As a stimulant, on ATF4 expression as earlier reports had demonstrated the requirement for Nrf 2 in inducer-dependent gene regulation. This potential function was investigated further by examining the ability of ATF4 to trans-activate the ho-1 enhancer, E1, in the reporter construct pE1-luc. In Hepa cells, co-transfection of an ATF4 expression plasmid, up to the maximum level tested, decreased basal pE1-luc expression by 20–25% (Fig. 5). ATF4, however, had a synergetic effect on Nrf2-dependent pE1-luc expression, increasing luciferase activity up to 2-fold. In contrast, co-expression of p18 dramatically inhibited Nrf2-mediated trans-activation of E1. This inhibition may, at least in part, be attributed to p18 homodimers as overexpression of p18 alone also inhibited basal pE1-luc expression.

ATF4 Enhances, whereas p18 Inhibits, Nrf2-mediated Trans-activation of the ho-1 Enhancer—Binding of ATF4-Nrf2 dimers to the StRE suggested a role for ATF4 in ho-1 gene regulation. This potential function was investigated further by examining the ability of ATF4 to trans-activate the ho-1 enhancer, E1, in the reporter construct pE1-luc. In Hepa cells, co-transfection of an ATF4 expression plasmid, up to the maximum level tested, decreased basal pE1-luc expression by 20–25% (Fig. 5). ATF4, however, had a synergetic effect on Nrf2-dependent pE1-luc expression, increasing luciferase activity up to 2-fold. In contrast, co-expression of p18 dramatically inhibited Nrf2-mediated trans-activation of E1. This inhibition may, at least in part, be attributed to p18 homodimers as overexpression of p18 alone also inhibited basal pE1-luc expression.

Cadmium Stimulates ATF4 Expression—Previous studies from our laboratory (4, 26) and other laboratories (7) have demonstrated the requirement for Nrf2 in inducer-dependent ho-1 gene regulation. To determine the role, if any, of ATF4 in this process, we first examined the effect of cadmium, a known HO-1 stimulant, on ATF4 expression as earlier reports had suggested that ATF4 is a stress-response protein (30, 31). As shown in Fig. 6, treatment of Hepa cells with 100 μM CdCl2 increased ATF4 levels in a time-dependent manner to greater than 10-fold above basal values. Of the other transcription factors tested, only expression of ATF3 and c-Jun, both documented stress-responsive proteins (32, 33), was enhanced by cadmium. Interestingly, the temporal profile of c-Jun and ATF3 induction matched that of HO-1 accumulation with the earliest detectable enhancement observed at 2 h post-treatment. Increased expression of ATF4, on the other hand, was detected within 30 min after treatment of cells with CdCl2, even prior to the detectable accumulation of ho-1 mRNA by this agent in Hepa cells (34).

A Dominant-negative Mutant of ATF4 Inhibits Basal and Cadmium-stimulated E1 Activity in Hepa Cells—To establish further the role of ATF4 in ho-1 gene regulation, we generated a dominant-negative mutant of ATF4 and examined its effect on pE1-luc expression. Overexpression of the mutant ATF4 inhibited both basal and cadmium-stimulated luciferase activity in a dose-dependent manner (Fig. 7). This effect was qualitatively and quantitatively similar to that observed with an Nrf2 dominant mutant. Interestingly, an analogous mutant of p18 inhibited cadmium-induced but not basal activity. Mutants of c-Jun or Jun D either enhanced or had no effect on pE1-luc expression.

The ATF4 Dominant-negative Mutant Does Not Inhibit Cadmium-stimulated E1-luc Expression in MCF-7 Cells—We have recently reported that cadmium is a potent activator of the ho-1 gene in MCF-7 human mammary epithelial cells, stimulating ho-1 mRNA accumulation by 300–400-fold, and that Nrf2 is an important regulator of this response (26). Consistent with our previous finding, the Nrf2 mutant significantly inhibited pE1-luc expression in the presence or absence of CdCl2 (Fig. 8). The p18 mutant also inhibited both basal and cadmium-stimulated activities to similar levels. The ATF4 mutant, however, inhibited only basal luciferase activity, revealing cell-dependent differences in the mechanism of ho-1 gene activation by cadmium and the role of ATF4 in this process.

DISCUSSION

In this study we have identified ATF4 as an Nrf2-interacting protein and have provided evidence implicating ATF4 in basal and cadmium-induced regulation of the ho-1 gene, the latter presumably in cooperation with Nrf2. Whereas association between Nrf2 and ATF4/CREB family members has not been previously documented, such an interaction is not necessarily unexpected as both classes of factors belong to the bZIP superfamily. In this regard it is noteworthy that among bZIP protein, ATF4 exhibits relatively promiscuous interaction activity. For instance, ATF4 forms heterodimers with c-Jun, c-Fos, and Fra-1 proteins under conditions where ATF2 and ATF3 heterodimerize only with c-Jun and ATF1 does not form any detectable heterodimers at all (17). In addition, ATF4 also forms heterodimers with CCAAT/enhancer-binding proteins (35), a relatively more distant subfamily (based on comparison of DNA-binding site sequences) than the Jun or Fos subfamilies within the bZIP superfamily.

Although not directly tested, it is not unreasonable to assume that Nrf2 and ATF4 form classical bZIP dimers via their
leucine zipper structures. Indeed, in all likelihood, it is this type of interaction that will most effectively elicit the proper alignment of the basic regions necessary for sequence-specific DNA binding as demonstrated herein. This conclusion is supported by the fact that the C-terminal portion of Nrf2 containing the bZIP domain was used in the original Y2H screening, and the observations that ATF4 exhibits positive interaction with a truncated Nrf2 in M2H assays (Fig. 3) and in EMSA (Fig. 4). Based on the difference in the apparent relative affinities of ATF4 for nearly full-length Nrf2 versus the truncated protein (see Fig. 3) and additional preliminary observations, we cannot rule out the possibility that ATF4 interacts with additional domains within the Nrf2 polypeptide. In this regard, it is interesting that ATF3 interacts very poorly with the truncated Nrf2 but is as effective as ATF4 in binding to the larger Nrf2 protein. At present, the precise location of any of the interaction sites (within Nrf2 or ATF4) is unknown. The functional significance of any non-leucine zipper associations is also not obvious. However, we note that ATF4 is known to associate with non-bZIP factors, including the Tax protein of the human T-cell leukemia virus type 1 (36, 37) and the γ-aminobutyric acid type B receptor (38). Interestingly, in both situations the interacting site within ATF4 was localized to the bZIP domain and, in the case of Tax, the association leads to enhanced trans-activation capacity for the Tax protein, similar to that observed here for Nrf2.

The ho-1 gene is activated by a variety of stress-associated agents including the substrate heme, heavy metals, tumor promoters, UV irradiation, and inflammatory cytokines. Induction of the mouse gene by most stimuli is mediated by two 5′, distal enhancer regions, E1 and E2, each containing multiple StREs. The StREs are sufficient and necessary for inducer-dependent gene activation (reviewed in Ref. 12). Our recent analyses (4, 26) have implicated Nrf2 in the mechanism of ho-1 gene activation by several agents and in particular by cadmium. For instance, stable expression of a dominant-negative mutant of Nrf2, but not of c-Jun, diminishes cadmium-induced ho-1 mRNA accumulation by 75–90% in L929 fibroblasts and MCF-7 cells. Similarly, in MCF-7 cells, overexpression of the Nrf2 mutant inhibits induction of an E1-regulated luciferase reporter gene by cadmium, and mutants of E1 that are not trans-activated by Nrf2 are also unresponsive to cadmium.

Identification of ATF4 as an Nrf2-interacting protein led us to speculate that ATF4, possibly in cooperation with Nrf2, regulates ho-1 gene expression. The transfection experiments demonstrating the inhibitory effects of the ATF4 dominant-

**Fig. 7.** A dominant-negative mutant of ATF4 inhibits basal and cadmium-dependent pE1-luc expression in Hepa cells. Cell were plated and transfected as described in the legend to Fig. 5. The DNA mixtures consisted of 2 μg of pE1-luc, 1 μg of pCMV/βGal, and the indicated amount of the dominant mutant expression plasmid. Total DNA was equalized with an appropriate empty expression vector. Forty hours after transfection, cells were treated with vehicle or 100 μM CdCl2 for 5 h in serum-free medium. Eight and 4% of the cell extract were used to measure luciferase and β-galactosidase activities, respectively. β-Galactosidase-normalized luciferase activity is presented as the percentage of activity in cells transfected without any dominant mutant plasmid. Each data point represents the average of two independent experiments (c-Jun, Jun D) or the average ± S.E. from three experiments.

**Fig. 8.** The ATF4 dominant-negative mutant only inhibits basal pE1-luc expression in MCF-7 cells. Cell were plated (1 × 10⁵/well of a 12-well plate) and transfected 20 h later using Fugene 6 transfection reagent. Each well was transfected with a DNA mixture consisting of 50 ng of pE1-luc, 10 ng of pCMV-βGal and 200 ng of the indicated expression plasmid. Forty hours after transfection, cells were treated with vehicle or 10 μM CdCl2 for 5 h in serum-free medium. Twelve and 6% of the cell extract were used to measure luciferase and β-galactosidase activities, respectively. β-Galactosidase-normalized luciferase activity is presented as the percentage of activity in cells transfected without any dominant mutant plasmid. Each data point represents the average ± S.E. from three experiments.
negative mutant on basal and cadmium-induced E1 activity in Hepa cells provides support for this idea. It is important to point out that transfection studies of this nature, by themselves, do not conclusively demonstrate the role of a specific transcription factor in gene regulation. This limitation arises because of the tendency of bZIP proteins to dimerize with multiple partners. Consequently, inhibition of gene activation observed with a specific dominant mutant can be attributed not only to the corresponding endogenous protein (if capable of homodimerization) but also to any of its dimerization partners, one or more of which may be the actual effector protein(s). Clearly, under such circumstances, it is important to obtain corroborative data for the role of a given factor in gene regulation.

For ATF4, such correlative evidence includes the observations that ATF4-Nrf2 dimers bind to the StRE, that ATF4 expression is enhanced by cadmium, and that ATF4 has a synergistic effect on Nrf2 trans-activation of E1. The latter finding, in particular, distinguishes ATF4 from p18, which exhibits an inhibitory effect similar to that of MafG on Nrf2. Clearly, under such circumstances, it is important to obtain corroborative data for the role of a given factor in gene regulation. For ATF4, such correlative evidence includes the observations that ATF4-Nrf2 dimers bind to the StRE, that ATF4 expression is enhanced by cadmium, and that ATF4 has a synergistic effect on Nrf2 trans-activation of E1. The latter finding, in particular, distinguishes ATF4 from p18, which exhibits an inhibitory effect similar to that of MafG on Nrf2.

In the case of Nrf2, a role in ho-1 gene regulation has been further substantiated with the use of nrf2-targeted mice (39) and cells (7). A similar analysis for ATF4 and p18 would of course be very informative. Our contention that ATF4 in part regulates ho-1 gene expression is consistent with emerging data that indicate ATF4 is a stress-response protein and, consequently, would function as a regulator of the adaptive response to such stress. For example, anoxia, which stimulates HO-1 synthesis, strongly increases the expression and DNA binding activity of ATF4 in fibroblasts (30). Arsenite, another HO-1 inducer, also stimulates ATF4 DNA binding activity in pheochromocytoma PC12 cells (31). In addition, ATF4 levels are increased in endothelial (40, 41) and Jurkat (42) cells in response to homocysteine and the calcium ionophore A23187, respectively, two agents known to cause endoplasmic reticulum stress. Finally, ATF4 expression is increased in cell lines resistant to various DNA-targeting drugs (43).

The difference between Hepa and MCF-7 cells with respect to the ability of the ATF4-dominant mutant to modulate cadmium-dependent E1 activity is puzzling but certainly points to cell-specific differences in the induction mechanism. Perhaps, intracellularly, productive or transcriationally competent interaction between ATF4 and Nrf2 requires an additional cofactor(s) which may be expressed in a cell-specific manner. This cofactor concept is somewhat similar to one postulated by Venugopal and Jaiswal (16) who have proposed that formation of Nrf2-Jun complexes is dependent on one or more presently uncharacterized cytoplasmic proteins. One consequence of this hypothesis is that it apparently precludes a role for Nrf2-ATF4 complexes in cadmium-dependent ho-1 gene activation in MCF-7 cells and, based on our previous studies noted above, requires the use of other Nrf2-containing complexes. Given the tendency for bZIP proteins to form multiple and distinct associations, this requirement is not necessarily insurmountable. Studies are under way to characterize further the role of ATF4, particularly with respect to cell and inducer specificity, in the regulation of ho-1 and other Nrf2 target genes.
Identification of Activating Transcription Factor 4 (ATF4) as an Nrf2-interacting Protein: IMPLICATION FOR HEME OXYGENASE-1 GENE REGULATION
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