Cellular Iron Depletion and the Mechanisms Involved in the Iron-dependent Regulation of the Growth Arrest and DNA Damage Family of Genes*5

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Iron plays a crucial part in proliferation while iron deficiency results in G1/S arrest, DNA damage, and apoptosis. However, the precise role of iron in cell cycle control remains unclear. We showed that iron depletion using the iron chelators, desferrioxamine (DFO), or 2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazone (311), increased the mRNA levels of the growth arrest and DNA damage 45α gene, GADD45α (Darnell, G. and Richardson, D. R. (1999) Blood 94, 781–792). In this study, we examined the effect of iron depletion on up-regulating GADD family members involved in growth control, including cell cycle arrest, apoptosis, and DNA repair, making them therapeutic targets for tumor suppression. We showed the GADD family members were up-regulated by cellular iron depletion. Further, up-regulation of GADD45α after iron deprivation was independent of hypoxia-inducible factor-1α (HIF-1α), octamer-1 (Oct-1), p53 and early growth response 1 (Egr1). We then analyzed the regulatory elements responsible for iron depletion-mediated regulation of GADD45α and identified the specific transcription factor/s involved. This region was within –117 bp and –81 bp relative to the start codon where the consensus sequences of three transcription factors are located: the CCAAT-binding factor/nuclear factor-Y (NF-Y), the stabilizing molecule v-MYB and the enhancer, CCAAT enhancer-binding protein (CEBPα). Mutation analysis, shRNA studies, Western blotting, and electrophoretic mobility shift assays led to the identification of NF-Y in the transcriptional up-regulation of GADD45α after iron depletion. Furthermore, like GADD45α, NF-YA was up-regulated after iron chelation and down-regulated by iron supplementation. These results are important for understanding the mechanisms of iron depletion-mediated cell cycle arrest, DNA damage repair, and apoptosis.

Iron is vital for proliferation and multiple mechanisms are involved in the anti-tumor activity of iron chelators whose
effect induces cell cycle arrest and apoptosis (1, 2). Iron depletion results in: 1) inhibition of the iron-containing enzyme, ribonucleotide reductase, which is critical for DNA synthesis (1); 2) increased expression of the growth and metastasis suppressor, N-myc downstream-regulated gene 1 (3); 3) up-regulation of p53 (4); 4) decreased cyclin D1 expression (5) that is required for G1/S progression; 5) down-regulation of p21CIP1/WAF1 protein in some cell types (5) that may induce apoptosis; 6) down-regulation of Bcl-2 and up-regulation of pro-apoptotic Bax (6, 7), and 7) up-regulation of cell division cycle 14A, which acts on substrates important for cell cycle progression (e.g. p27kip1 and cyclin E) (8).

The current investigation focuses on five members of the growth arrest and DNA damage (GADD)3 family of genes, namely: GADD34, GADD45α, GADD45β, GADD45γ, and GADD153. The expression of these genes is often increased when cells are subjected to stress such as nutrient deprivation (9–11) or exposure to DNA-damaging agents (12), which may cause cell cycle arrest and/or apoptosis. Our laboratory previously showed that iron depletion mediated by the iron chelators, DFO or 311, caused a pronounced concentration- and time-dependent increase in the expression of GADD45α mRNA in three cell types (13). Similar to the effect observed with iron depletion, GADD45α and GADD153 mRNA are up-regulated during hypoxia (14), suggesting a possible role for hypoxia-inducible factor-1α (HIF-1α), that can be activated by both hypoxia and iron depletion via prolyl-hydroxylases (1).

Products of the GADD45 subgroup of genes encode three structurally related proteins, namely GADD45α, GADD45β, and GADD45γ, that play important roles in the G2/M checkpoint and apoptosis (15). However, only GADD45α has been demonstrated to activate p53-dependent G2/M arrest and inhibit cdc2 kinase (15). Neither GADD45β nor GADD45γ are downstream targets of p53 (15). Interestingly, GADD45α is also known to interact with key cell cycle regulatory molecules, such as p21CIP1/WAF1 (16), cdc2/cyclin B1 (17) and p38 mitogen-activated protein kinase (MAPK) (18). Studies using trans-

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5 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S6.

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2 The abbreviations used are: GADD, growth arrest and DNA damage; 311, 2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazone; Act D, actinomycin D; CCAT-AP, CCAT-binding protein; CEBP, CCAAT enhancer-binding protein; DFO, desferrioxamine; Egr1, early growth response 1; EMSAs, electrophoretic mobility shift assays; ER, endoplasmic reticulum; ERSE, endoplasmic reticulum stress element; FAC, ferric ammonium citrate; HIF-1α, hypoxia-inducible factor-1α; MAPK, mitogen-activated protein kinase; MEFs, murine embryonic fibroblasts; NF-Y, nuclear factor-Y; Oct-1, Octamer-1; TR1, transferrin receptor 1.
fected cells suggest that GADD34 and GADD153 appear to have a direct role in initiating apoptosis rather than inducing cell cycle arrest (19, 20). Overexpression of each GADD gene causes growth inhibition and/or apoptosis, while combined overexpression leads to synergistic or cooperative effects on anti-proliferative activity (21).

The current investigation has demonstrated that members of the GADD gene family are up-regulated in response to iron depletion. Our studies identify the constitutive transcriptional activator, nuclear factor Y (NF-Y), in the transcriptional up-regulation of GADD45a after iron depletion.

**EXPERIMENTAL PROCEDURES**

**Reagents**—DFO was obtained from Novartis (Basel, Switzerland) and 311 was synthesized and characterized as described (22). Actinomycin D and ferric ammonium citrate were from Sigma-Aldrich. (22). Actinomycin D and ferric ammonium citrate were from Sigma-Aldrich. (22). Actinomycin D and ferric ammonium citrate were from Sigma-Aldrich. (22). Actinomycin D and ferric ammonium citrate were from Sigma-Aldrich.

**Cell Culture**—The human MCF7 breast cancer, SK-N-MC neuroepithelioma and SK-Mel-28 melanoma cell-types were obtained from the American Type Culture Collection (Manas-...
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Mutagenesis Analysis—The introduction of specific mutations into the promoter deletion constructs was carried out using the GeneTailor® kit (Invitrogen). Briefly, the modified target sequences were based on the consensus sequences of CCAAT enhancer-binding protein (CEBP), CEBPα, GATA, v-MYB, and NF-Y and then confirmed by sequencing (AGRF). The plasmid carrying the ~117 bp fragment of the GADD45α promoter region was methylated with a DNA methylase enzyme (4 units/μl; Invitrogen) for 1 h/37 °C. Then, a PCR reaction was carried out using a mutagenic and a corresponding complementary primer and the resulting amplified plasmid transformed in One-Shot® MAX Efficiency® DH5α™.TIR competent cells (Invitrogen). Following transformation, the methylated (not mutated) DNA was digested by McrBC and Mrr endonucleases in Escherichia coli (25), leaving only unmethylated mutated products.

shRNA—SureSilencing® shRNA plasmids were purchased from SABioscience (Frederick, MD) and transiently transfected using Lipofectamine™ LTX with PLUS™ Reagent (Invitrogen). RNA and protein were prepared following transfection and incubation of MCF7 cells with either control medium or this medium containing DFO (250 μM) or 311 (25 μM) for 24 h/37 °C.

Chemiluminescent EMSA—Studies examining DNA-protein interactions were carried out using the LightShift™ Chemiluminescent EMSA kit (Thermo Scientific, Rockford, IL) as per the manufacturer’s protocol. The double-stranded biotinylated oligonucleotides were based on the NF-Y consensus sequence in the GADD45 promoter and a mutated sequence, namely 5’-atatgctagctggccaagctg-3’ and 5’-ataagctatggccaagctg-3’, respectively. Briefly, nuclear proteins were extracted using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Scoresby, Australia). The DNA/protein-binding reaction was carried out for 20 min at room temperature. Then equal amounts of protein (2 μg), as determined by the Bio-Rad protein assay reagent, were loaded onto a native 6% polyacrylamide gel in 0.5× TBE and run at 100 V for 1 h at room temperature. The binding reaction was subsequently transferred onto a nylon membrane (GE Healthcare, Australia) at 100 V/30 min at room temperature. To cross-link transferred DNA to the membrane, a 60 s exposure at 120 ml/cm² at room temperature was performed using the UV Stratalinker 1800 (Stratagene, Santa Clara, CA). The biotin-labeled DNA was then detected by chemiluminescence.

Statistics—Results presented are typical of three independent experiments and given as mean ± S.D. Experimental data were compared using Student’s t test. Results were considered statistically significant when p < 0.05.

RESULTS

GADD Family mRNA Levels Are Increased after Iron Depletion—Incubation of cells with DFO or 311 resulted in a slight or no increase in the expression of the GADD family genes. In contrast, 311 caused a significant (p < 0.001) up-regulation of the expression of these genes relative to the control (supplemental Fig. S1). Similar results were also found comparing DFO (250 μM) to the DFO-Fe(III) complex (1:1 ligand-metal ion complex (250 μM); data not shown). Collectively, these data demonstrate that it is the ability of chelators to bind iron that results in up-regulation of the GADD gene family.

Alterations of GADD Family mRNA and Protein Expression after Incubation with Chelators Is Time-dependent—To examine the kinetics of the up-regulation of GADD gene family expression, MCF7 cells were incubated with 311 (25 μM) for 2–24 h and then their mRNA levels assessed (Fig. 1B and supplemental Fig. S2, A–D). In these experiments, 311 was used in preference to DFO due to its rapid permeation of cells to deplete iron pools (13, 26). The effect of hypoxia (1% O2/94% N2/5% CO2) was also assessed (Fig. 1B and supplemental Fig. S2). The effect of hypoxia on expression of the GADD gene family was generally less marked than after incubation with 311. The positive control, Act D, increased the expression of all GADD family members relative to the control (Fig. 1B and supplemental Fig. S2).

Incubation with control medium alone led to either a slight or no increase in the expression of the GADD family genes. In contrast, 311 caused significant (p < 0.001) up-regulation in the expression of all these genes after 24 h, with the initial increase in their mRNA levels being evident after 4–16 h (Fig. 1B and supplemental Fig. S2). These results again suggest coordinate regulation of this family of genes by iron depletion. The effect of hypoxia on expression of the GADD gene family was generally less marked than after incubation with 311. The positive control, Act D, increased the expression of all GADD gene members relative to the control (Fig. 1B and supplemental Fig. S2).

Western blot analysis was then performed to assess protein expression of these members of the GADD gene family. The expression of these proteins remained relatively constant (GADD34) or was increased (GADD45α, -β, -γ, and GADD153) over a 24-h incubation when cells were maintained in control medium alone (supplemental Fig. S3A). It was notable that a double band was sometimes visible for the GADD45α blot that has also been described previously by others and may be due to post-transcriptional processing of the protein (30). Incubation with 311 led to an increase in GADD34, GADD45β, GADD45γ, and GADD153 protein expression after only 2 h relative to the 0 h time point, while for GADD45α, an increase in expression only became evident after 8 h incuba-
Hypoxia increased GADD34, GADD45α, GADD45β, GADD45γ, and GADD153 protein expression after 4 h, while an increase in GADD45α was only found after an 8-h incubation (supplemental Fig. S3C).

Iron Supplementation Decreases GADD mRNA Levels following Iron Chelation—To determine if the observed iron chelator-mediated up-regulation of GADD mRNA was due to cellular iron depletion, we examined the effect of iron repletion by incubating cells with the iron donor, ferric ammonium citrate (FAC) (3, 31). In these studies, after a primary incubation of 24 h with either control medium or medium containing 311 (25 μM) or DFO (250 μM), MCF7 cells were reincubated for a further 24 h (secondary incubation) with either control medium, 311 (25 μM), DFO (250 μM), or FAC (100 μg/ml; [iron] = 280 μM; Fig. 1C and supplemental Fig. S4). These results showed that the mRNA levels of the GADD family members were slightly reduced or significantly (p < 0.05) decreased when cells were incubated with control medium for 24 h and then reincubated with FAC for 24 h when compared with cells incubated with control media for 48 h (Fig.

**FIGURE 1.** Up-regulation of GADD34, GADD45α, GADD45β, GADD45γ, GADD153, p21, and TfR1 mRNA levels after incubation of MCF7 cells with the iron chelators DFO and 311. A, MCF7 breast cancer cells were incubated for 24 h at 37 °C with control medium or this medium containing the iron chelators DFO (250 μM) or 311 (25 μM). Total mRNA was extracted and then RT-PCR performed. B, GADD45α is up-regulated in a time-dependent manner in response to iron chelators, hypoxia, or low concentrations (5 nM) of the DNA-damaging agent, actinomycin D (Act D). MCF7 cells were incubated for 0, 2, 4, 8, 16, or 24 h at 37 °C under standard culture conditions (95% air/5% CO₂) with either: control medium or this medium containing 311 (25 μM) or low concentrations of the DNA-damaging agent, Act D (5 nM), or incubated under hypoxic culture conditions (1% O₂, 94% N₂, 5% CO₂) with control medium only. C, up-regulation of GADD45α and TfR1 (as control) mRNA levels after incubation with DFO or 311 is markedly decreased upon re-incubation with the iron donor, ferric ammonium citrate (FAC, 100 μg/ml). Results shown are typical gels from three experiments, while the densitometry is mean ± S.D. *, p < 0.05; **, p < 0.01; ***, p < 0.001, relative to the control.
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1C and supplemental Fig. S4, lanes 1 and 2). In contrast, when cells were incubated with either DFO or 311 for 24 h to induce iron depletion and then reincubated with either control medium or the chelator, there was a marked increase in the mRNA levels of the GADD family members, or the positive controls for iron depletion, namely p21 or TjRI. However, when cells were first incubated with DFO or 311 and then subsequently reincubated with FAC, there was a marked decrease in the mRNA levels of the GADD members, p21 and TjRI, indicating cellular Fe-repletion. These results indicate that like the TjRI, mRNA expression of the GADD family members was regulated by cellular iron levels.

Deletion Analysis of the GADD45α Promoter Region—Considering the regulation of the GADD gene family by cellular iron levels shown above, further work examined the mechanism involved. In these studies, we focused on GADD45α as it is the best characterized of this group of genes and shows important functions relevant to the response to iron depletion (13). Further, GADD45α activates p53-dependent G2/M arrest and inhibits cdc2 kinase (15). To identify the regulatory element responsible for iron chelator-mediated up-regulation of GADD45α expression, promoter deletion constructs (from the 5′-end of the promoter) of various sizes were joined to a luciferase reporter vector (Fig. 2A). The plasmids were transiently transfected into MCF7 cells, which were then incubated with either control medium or this medium containing 311 (25 μM) for 24 h/37 °C. As shown in Fig. 2A, after incubation with 311 relative to the control, the reporter plasmid containing −234 bp of the GADD45α promoter relative to the transcription start site showed the greatest increase (p < 0.001) in luciferase activity as compared with the empty vector (pGVB2) or to any longer constructs (−817, −1326, and −2256 bp). On the other hand, transfection of smaller fragments of the promoter (−10 and −81 bp) showed almost no luciferase activity (Fig. 2A).

Previous studies using these constructs to demonstrate the transcription factor responsible for up-regulation of GADD45α in response to UV-irradiation identified Oct-1 in the −234 bp region as being responsible (32). However, studies using Oct-1+/− and Oct-1−/− MEFs showed that incubation with 311 (25 μM) could induce GADD45α expression to a similar degree in both cell types (Fig. 2B), demonstrating that Oct-1 was not essential for the up-regulation of GADD45α in response to iron depletion. Similar analysis, using appropriate cell types with or without the molecules of interest, was also extended to assess the role of p53, Egr1, and Hif-1α in the up-regulation of GADD45α after iron depletion (Fig. 2B). Indeed, these transcription factors were considered as potential candidates for transcriptional up-regulation of GADD45α as they are also regulated by intracellular iron levels (33). In addition, the role of p53 and Egr-1 in regulating GADD45α expression has been described (34), whereas the up-regulation of GADD45α mRNA during hypoxia was previously demonstrated (14) and confirmed in the current study (Fig. 1B). In each case, the expression of GADD45α was up-regulated after incubation with 311 in cells with or without p53, Egr1, or Hif-1α, indicating that they were not essential for this effect.

To identify a specific region within the promoter responding to iron depletion, we performed additional promoter truncations (Fig. 2C). The resulting fragments were then cloned into a pGL4 luciferase reporter plasmid which was chosen because the pGVB2 vector used in Fig. 2A was no longer commercially available. Constructs lacking some of the potential transcription factor consensus sequences were generated by sequential deletion of the GADD45α promoter. The truncated constructs were then tested using the luciferase reporter assay in transfected MCF7 cells.

As shown in Fig. 2D, the deletion of the promoter region at base number −234, −143, −124, and −117 bp resulted in a marked and significant (p < 0.001) increase of the luminescence after incubation with 311 or DFO relative to the control. This is possibly due to the removal of extraneous sequence not necessary for transcription, leading to a more compact and efficient transcriptional unit responsible for GADD45α induction after iron deprivation. Further truncation of the promoter region to the −89 bp construct resulted in a 47–48% reduction of luminescence compared with the −117 bp construct, while again the −81 bp construct showed virtually no activity (Fig. 2D). The level of luminescence observed for the −89 bp construct was comparable to that found in the original −234 bp fragment, indicating that the core promoter could be present in the −81 to −89 bp region (Fig. 2D). Taken together, these results indicate that a transcription element is located between −89 and −81 bp, but this element is not sufficient for the highest induction of GADD45α expression. In fact, this can only be achieved by the involvement of a possible enhancer element located between the −89 and −117 bp region.

Mutagenesis Analysis of the GADD45α Promoter Region—The presence of transcription factor binding sites in the promoter region of GADD45α was analyzed using TFBIND and TESS software. Both programs score the highest probability of each potential transcription factor to bind to an appropriate binding site. According to this analysis, the potential consensus sequences with the greatest scores in the region between −89 and −117 bp were: CEBPα (90%), GATA (89%), OCT-1 (85%), and CEBP (81%) (Fig. 3A). Further, in the region between −81 and −89 bp, the potential consensus sequences with the highest scores were: (i) the CCAAT-binding protein (CCATBP), NF-Y (85%); (ii) v-MYB (85%); and (iii) a second NF-Y site (86%; Fig. 3A). Because of the lack of specific restriction enzyme sites in the region between −117 bp and −89 bp, a deletion strategy was not possible. Hence, the site-directed mutagenesis technique was employed to introduce a four base specific substitution into each consensus sequence (Fig. 3B). This technique has been previously shown to prevent transcription factor binding (35).

The introduction of mutations in CEBP, GATA, and the second NF-Y region did not induce a statistically significant reduction of luminescence compared with the non-mutated control plasmid pGL4−117 (Fig. 3C). In contrast, mutation of the CEBPα sequence resulted in a reduction of luciferase gene expression by 46–47% relative to pGL4−117 after treatment of transfected cells with 311 or DFO. Interestingly, the mutations directed against the CCAAT-BP region (i.e. CAAT/NF-Y and NF-Y/v-MYB) almost completely abrogated luminescence rel-
ative to pGL4–117. This mutation not only affects the core of the CCAAT sequence, but also disrupts the binding sequence of two factors potentially involved in the regulation of GADD45/H9251 expression, namely NF-Y and v-MYB (Fig. 3C). In particular, NF-Y has previously been described as a CCAAT-BP (36), while v-MYB may be necessary for stabilization of NF-Y binding to its site (37). Hence, GADD45/H9251 expression can be regulated by the binding of NF-Y and this interaction appeared to receive stabilization by v-MYB and was enhanced by CEBPα.

Abrogation of NF-YA Expression by shRNA Prevents the Iron Chelator-mediated Increase in GADD45α mRNA and Protein Expression—To further examine the involvement of NF-Y in the regulation of GADD45α expression, we transfected MCF7 cells with two plasmids containing sequences for two different shRNAs (i.e. shRNA#1 and shRNA#2) to silence the critical A
subunit of NF-Y (38) or a shRNA-scrambled control plasmid (Scrambled). Subsequently, we incubated the transfected cells with control medium or this medium containing either DFO (250 μM) or 311 (25 μM) for 24 h/37 °C and RT-PCR and Western blot was then performed. When assessing the shRNA scrambled controls, incubation with 311 (25 μM) or DFO (150 μM) significantly (p < 0.001) increased NF-YA and GADD45 mRNA levels compared with control medium (Fig. 4A). Relative to the scrambled shRNA control vector, the transfected NF-YA-shRNA plasmids markedly and significantly (p < 0.001) inhibited NF-YA mRNA in control cells, but also those incubated with DFO or 311 (Fig. 4A). As a consequence of the inhibition of NF-YA expression, GADD45α, GADD45β, and GADD153 (Fig. 4A and supplemental Fig. S5, B and D) mRNA levels were markedly and significantly (p < 0.01–0.001) lower when compared with the scrambled control when cells were incubated with DFO or 311. Hence, abrogation of NF-YA not only affects GADD45α mRNA levels, but also GADD45β and GADD153 mRNA, demonstrating a common mechanism of regulation. In contrast, there was no difference in the expression of GADD34, GADD45γ, and TJRI between the scrambled shRNA control and the NF-YA-shRNA (supplemental Fig. S5, A, C, E). The lack of effect of NF-YA-shRNA on the expression of these latter genes provides appropriate controls for potential off-target effects which could lead to misinterpretation. Expression of TFR1 mRNA which is iron regulated (39), was assessed as a relevant internal control for iron depletion. Again, these results confirm an important role of NF-YA in the chelator-mediated expression of GADD45α and indicate a potential role of this transcription factor in regulating GADD45β and GADD153.

To further examine the role of NF-Y in GADD45α expression, we also performed Western blotting on lysates from NF-YA-shRNA transfected cells (Fig. 4B). These results confirmed that the protein expression of both NF-YA and GADD45α were significantly (p < 0.01–0.001) lower in NF-YA-shRNA-transfected cells relative to the scrambled shRNA-treated cells (Fig. 4B). Taken together, these results demonstrate that NF-YA is necessary for the up-regulation of GADD45α under iron deprivation, as in NF-YA-deficient cells the marked up-regulation of GADD45α expression by iron depletion by DFO and 311 was no longer observed.

![Mutagenesis analysis of the GADD45α promoter](image-url)
NF-YA Protein Expression Is Regulated by Intracellular Iron Levels—To further assess the role of NF-Y in GADD45α up-regulation after iron depletion, we also assessed if NF-Y protein expression was regulated by cellular iron levels (Fig. 4C). In these studies, standard techniques (3, 40) to modify iron levels implementing control medium, DFO (250 μM) or 311 (25 μM), or FAC (100 μg/ml) were used as described for Fig. 1C and supplemental Fig. S4. NF-Y is a hetero-trimeric complex with NF-YA being the main subunit and the one responsible for CCAAT binding (38). Hence, it was chosen as the best representation of the protein complex to assess using Western blot analysis. As shown in Fig. 4C, GADD45α and NF-YA expression was significantly (p < 0.001) elevated in cells incubated with either DFO (250 μM) or 311 (25 μM) for 24 h/37 °C. As a result of the reduced expression of NF-YA, the up-regulation of GADD45α mRNA is compromised after incubation with DFO (250 μM) or 311 (25 μM) for 24 h/37 °C. Consequently, the up-regulation of GADD45α expression is abrogated after incubation with DFO (250 μM) or 311 (25 μM) for 24 h/37 °C. Further examination of the regulation of NF-Y expression by iron levels, MCF7 cells were incubated with either control medium, or this medium containing DFO (250 μM) or 311 (25 μM) for 24 h/37 °C. This medium was then removed and the cells reincubated with either control medium, or this medium containing DFO (250 μM), 311 (25 μM) or the iron donor, ferric ammonium citrate (FAC; 100 μg/ml). Results shown are typical gels from three experiments, while the densitometry is mean ± S.D. **, p < 0.01; ***, p < 0.001, relative to the control.

**FIGURE 4.** Abrogation of NF-YA expression using shRNA markedly decreases GADD45α mRNA and protein expression after incubation with chelators relative to the scrambled shRNA control (Scrambled). In addition, the expression of NF-YA is regulated by cellular iron levels. A, in MCF7 cells transfected with the shRNA plasmids (ID#1–2), the levels of NF-YA and GADD45α mRNA is much lower than in cells transfected with the negative control plasmid (i.e. scrambled shRNA). As a result of the reduced expression of NF-YA, the up-regulation of GADD45α mRNA is compromised after incubation with DFO (250 μM) or 311 (25 μM) for 24 h/37 °C. B, Western analysis confirms the results from RT-PCR in A showing that in MCF7 cells transfected with NF-YA shRNA relative to scrambled shRNA, the expression of NF-YA is markedly reduced. Consequently, the up-regulation of GADD45α expression is abrogated after incubation with DFO (250 μM) or 311 (25 μM) for 24 h/37 °C. C, to further examine the regulation of NF-Y expression by iron levels, MCF7 cells were incubated with either control medium, or this medium containing DFO (250 μM) or 311 (25 μM) for 24 h/37 °C. This medium was then removed and the cells reincubated with either control medium, or this medium containing DFO (250 μM), 311 (25 μM) or the iron donor, ferric ammonium citrate (FAC; 100 μg/ml). Results shown are typical gels from three experiments, while the densitometry is mean ± S.D. **, p < 0.01; ***, p < 0.001, relative to the control.
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Chelators and then down-regulation after control or chelator-treated cells were incubated with FAC.

To assess whether the observed iron-dependent regulation of these genes was specific to MCF7 cells, we assessed NF-YA and GADD45α/H9251 expression in multiple cell-types. Both human SK-N-MC neuroepithelioma and SK-Mel-28 melanoma cell lines showed a very similar pattern of expression as found in MCF7 cells with NF-YA and GADD45α being significantly (p < 0.001) up-regulated by iron chelators and down-regulated by FAC (supplemental Fig. S6). Taken together, these findings indicate that NF-Y is regulated by intracellular iron levels and corroborates the findings above concerning its role in regulating GADD45α expression.

Analysis of the Interaction between NF-Y and the Promoter of GADD45α—To directly demonstrate the ability of NF-Y to bind the CCAAT binding region in the GADD45α promoter, EMSAs were carried out using nuclear protein extracts from cells incubated for 24 h with either control medium or medium containing DFO (250 μM) or 311 (25 μM). Double-strand biotin-labeled oligonucleotides were designed according to the wild-type and mutated GADD45α sequences reported in Fig. 3, A and B. As shown in Fig. 5A, when the binding reaction was carried out in the absence of a nuclear protein extract (lane 1), the only visible band was the excess free biotin-labeled DNA (indicated as free biotin labeled oligo in Fig. 5A). On the other hand, when the nuclear protein extract was added (lane 2), a band-shift occurred due to the binding of transcription factor(s) to this specific sequence. When the same unlabeled oligonucleotide was added in a 50-fold excess to the binding reaction as a specific competitor (lane 3), the shift was prevented. It is notable that the shifted band derived from the nuclear protein extract of cells treated with DFO or 311 was markedly and significantly (p < 0.001) greater than that present in control cells. This indicates that the up-regulation of NF-Y protein expression observed in cells incubated with 311 or DFO (Fig. 4, B and C) was also reflected by the greater binding of this transcription factor to its consequence sequence within the GADD45α promoter (Fig. 5A).

FIGURE 5. Electrophoretic mobility shift assay demonstrating that cellular iron depletion using either DFO or 311 results in increased NF-Y DNA binding activity to the CCAAT-binding sequence present in the promoter region of GADD45α. A, MCF7 cells were incubated with either control medium, or this medium containing DFO (250 μM) or 311 (25 μM) for 24 h/37 °C. Nuclear protein extracts were then prepared and incubated with biotin-labeled oligonucleotide containing the CCAAT binding sequence found in the GADD45α promoter (WILD-TYPE target DNA; see “Experimental Procedures”) in the presence or absence of a 50-fold excess of unlabeled competitor WILD-TYPE target DNA. B, in parallel experiments, MCF7 cells were incubated as in A and nuclear lysates prepared and incubated with a biotin-labeled oligonucleotide containing a mutated CCAAT-binding sequence (MUTATED target DNA; see “Experimental Procedures”) in the presence or absence of a 50-fold excess of unlabeled competitor MUTATED target DNA. Results shown are typical gels from three experiments, while the densitometry is mean ± S.D. *** p < 0.001, relative to the control.
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In the current investigation, a similar potential ERSE was identified within −90 and −70 bp of the GADD45α promoter. As identified in the GADD153 ERSE, the GADD45α response element is also present as a single copy, but maintains the typical GC-rich spacer. It is also notable that the sequence of the domain potentially responsible for the ER-stress inducibility has a substitution of cytosine with thymine (GCA TG instead of GCACG) and that this sequence was identified as a potential NF-Y binding site (Fig. 3A). However, mutagenesis analysis of this particular sequence revealed that it was not necessary for GADD45α up-regulation after iron depletion (Fig. 3C), and thus, it may be responsible for the induction of this gene after ER stress only. Hence, the up-regulation of GADD45α and GADD153 observed in this study may be a response related to iron depletion rather than ER stress.

It is notable that like GADD45α and GADD153, a CCAAT binding sequence is present in the promoter region of GADD45β. Hence, this could explain why the repression of NF-YA was able to markedly prevent GADD45β expression after incubation with DFO or 311 (supplemental Fig. S5B). Interestingly, the CCAAT binding sequence in the promoter region of GADD45γ, does not lead to NF-Y binding since the repression of NF-YA was not able to prevent GADD45γ expression after incubation with DFO or 311 (supplemental Fig. S5C). In contrast, GADD34 does not contain a CCAAT binding sequence, and as such, its expression after iron depletion was not affected by the down-regulation of NF-YA (supplemental Fig. S5A). This indicates that other mechanisms were involved in the up-regulation of GADD34 and GADD45γ under these conditions. In fact, previous studies have indicated that ER stress and hypoxia can activate phosphorylation of the eukaryotic initiation factor 2a to inhibit overall protein synthesis (45). Paradoxically, this also leads to translation of a subset of mRNAs, such as the transcription factor, ATF4, which induces transcription of downstream stress-induced genes such as GADD34 (46). Hence, this mechanism may be responsible for the up-regulation of the latter gene after iron depletion. Collectively, the analysis above indicates a complex response to iron depletion that involves NF-Y and potentially ATF4.

In summary, iron depletion significantly increased the expression of the GADD gene family. Furthermore, the induction of GADD45α expression by iron depletion could be attributed to the binding of NF-Y to the CCAAT-binding motif in the GADD45α promoter with the aid of v-MYB as a stabilizing molecule and with the enhancement role of CEBPα. Together, these results are relevant to understanding the molecular alterations involved in cell cycle arrest during iron deficiency and the mechanism of chelators at inducing anti-proliferative activity and apoptosis.

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