Post-transcriptional Modulation of Iron Homeostasis during p53-dependent Growth Arrest*

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Iron plays an essential role in cell proliferation and is a required cofactor for a number of critical cellular enzymes. In this report we investigate changes in proteins of iron metabolism during p53-mediated replicative arrest. Following the induction of p53 in H1299 lung cancer cells containing a doxycycline-inducible p53, an increase in both H and L subunits of ferritin protein was observed. To determine the mechanism of this effect, we investigated the ability of p53 to regulate ferritin. Real time reverse transcription-PCR demonstrated no difference in levels of ferritin H mRNA in the presence and absence of p53. Because these results suggested that transcriptional mechanisms were not responsible for the p53-dependent increase in ferritin, we tested whether a post-transcriptional mechanism was involved. RNA bandshift assays revealed that induction of p53 decreased iron regulatory protein binding. Consistent with this observation, Western blot analysis revealed a decline in transferrin receptor 1 protein levels following induction of p53. Collectively, these results suggest that p53 may induce cell cycle arrest not only by well described mechanisms involving the induction of cyclin-dependent kinase inhibitors but also by the recruitment of pathways that reduce the availability of intracellular iron.

The p53 tumor suppressor protein is a critical mediator of cell cycle arrest and apoptosis. p53 is a nuclear transcription factor that is activated by cellular stress, including DNA damage, hypoxia, ribosomal stress, and loss of adhesion (reviewed in Ref. 1). Following its induction, p53 transcriptionally induces a set of genes that promote growth arrest, cell death, or senescence, including p21<sup>wd1</sup>, bax, fas, and KILLER/DR5 (2). p53 can also function as a transcriptional repressor (3). Whether a cell undergoes cell cycle arrest or apoptosis in response to p53 depends on several factors, including the presence of extracellular survival factors, the presence of other oncogenic alterations, and the availability of additional transcription factors or cofactors, as well as the character and magnitude of p53 induction (4–6). In addition to its role as a transcription factor, p53 exhibits transcription-independent functions in the cytosol that augment its pro-apoptotic activity (1). Although an increase in reactive oxygen species may contribute to p53-mediated apoptosis (7), p53 can also play a role as an antioxidant (8) by inducing genes that reduce reactive oxygen species, such as TIGAR (9), sestrins (10), and ALDH4 (11).

Iron is critically important for cell growth and proliferation. Iron is required for the activity of ribonucleotide reductase, the rate-limiting enzyme for <i>de novo</i> DNA synthesis (12). It is also an important cofactor of the G1 phase cyclins E/cdk2, D/cdk5 (13), D1 (14), and the S phase cyclin A/cdk2 (15), as well as numerous proteins involved in respiration and energy metabolism. Accordingly, iron deprivation inhibits proliferation. For example, small molecule iron chelators that sequester iron can inhibit cell proliferation and/or induce apoptosis (16–18).

Ferritin is a 480-kDa ubiquitous and highly conserved protein that can store up to 4500 atoms of iron (19). It is composed of 24 subunits of two types, termed H (heavy or heart) and L (light or liver). By sequestering iron and preventing the participation of iron in reactions that generate oxygen free radicals, ferritin plays a key role in the protection against oxidant stress (20, 21). Regulation of iron levels by ferritin has also been shown to affect cell growth (22–24). Ferritin synthesis is decreased in cells stimulated by some oncogenes (25–27). For example, the E1A adenoviral oncogene represses ferritin at a transcriptional level (25), and the c-myec proto-oncogene both represses ferritin H and stimulates IRP2 (26). These events decrease iron storage and increase the availability of intracellular iron for cell proliferation (26). Similarly, an increase in the labile iron pool caused by ferritin repression enhances growth induced by H-ras and reverts growth arrest induced by dominant negative H-ras (28). In keeping with these findings, overexpression of ferritin H can inhibit cell proliferation (22).

The iron regulatory proteins are central regulators of ferritin and other proteins of iron metabolism. Translation of both H and L subunits of ferritin are coordinately regulated through the interaction of RNA-binding proteins (iron regulatory proteins (IRPs))<sup>5</sup> with the iron-responsive element (IRE) in the 5′-untranslated region of ferritin H and L mRNAs (29). The IRPs also regulate the stability of transferrin receptor 1 mRNA.

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IRE binding of the IRPs is in turn modulated by iron, as well as by oxidative stress (30), nitrogen radicals (31), and phosphorylation (32, 33). Under conditions of iron deprivation, the IRP proteins are activated to repress ferritin translation and stabilize transferrin receptor 1 mRNA, leading to decreased iron storage in ferritin and increased iron uptake by transferrin receptor 1; conversely, when iron is abundant, ferritin translation is induced and transferrin receptor 1 mRNA is degraded, leading to enhanced iron storage and reduced iron uptake (19). This elegant mechanism is a critical component of the maintenance of intracellular iron homeostasis.

In this manuscript, we tested whether p53 impinges on this iron regulatory network. Specifically, we hypothesized that p53 might induce ferritin and thus limit iron availability during growth arrest. We report here that ferritin protein is indeed up-regulated by p53. However, this effect is not mediated by transcriptional activation of ferritin but rather by a p53-dependent reduction in IRP binding. Consistent with these findings, TfR1 protein was decreased by p53. These p53-mediated changes in ferritin and transferrin receptor 1 may contribute to growth arrest and support a growing set of observations that iron availability is critical to proliferative regulation during malignant change (18, 26, 34).

MATERIALS AND METHODS

Reagents—All of the chemicals and reagents were purchased from Sigma unless noted otherwise. The rainbow molecular weight marker and ECL system were obtained from Amersham Biosciences. Goat anti-rabbit IgG-horseradish peroxidase-conjugated antibody and 30 and 40% acrylamide/bis-acrylamide solutions were purchased from Bio-Rad. Anti-p21 antibody was purchased from Roche Applied Science. Immobilon-P transfer membranes (polyvinylidene difluoride) were purchased from Millipore Corp. (Bedford, MA). Cell Culture—p53.ind and p53 – cells were generated by stable transfection of wild type p53 or empty vector into the H1299 human non-small cell lung carcinoma cell line containing a tet-on transactivator, and were a generous gift of Dr. C. Koumenis (Wake Forest University Health Sciences), p53.ind and empty vector (p53 –) cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% Tet system approved fetal bovine serum, 100 μg/ml streptomycin G

sodium, and 100 μg/ml streptomycin sulfate. Empty vector transfected p53 – cells were treated with doxycycline to control for effects of doxycycline itself, because doxycycline has weak chelating activity (14) and thus might influence iron metabolism. However, at the concentrations used in these experiments, there were no effects of doxycycline itself on any parameter measured (data not shown). HCT 116 human colon carcinoma TP53 wild type (TP53+/+) and TP53 knock-out (TP53–/) cell lines were generous gifts from Dr. B. Vogelstein (Johns Hopkins University, Baltimore, MD). These cell lines were maintained in McCoy’s 5A medium supplemented with 10% fetal bovine serum.

Western Immunoblot—To assess p53, p21, ferritin H, ferritin L, TfR1, ferroportin, and β-actin protein levels, cytosolic extracts were prepared as previously described (35). 50 μg of protein (except in the case of TfR1 detection, where 10 μg of protein was used) was fractionated on 12% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, blocked with 5% nonfat milk in Tris base, pH 7.6, NaCl, 0.1% Tween 20 buffer, washed, and incubated with 1:2000 dilution of radish peroxidase.

Real Time RT-PCR—Real time RT-PCR was performed as described previously (36). The following primers were used: for hepcidin, forward, 5′-CTGCAACCCAGAACAGAGG-3′, and reverse, 5′-GAAATAAAAAAGGAAGGGGAGG3′-3′ (37); and for ferritin H, forward, 5′-CTTTGACCAGTAGATGGGGTTT-3′, and reverse, 5′-TTTGCAGTGGCCAGTTT-GACGAGA3′. Scrambled siRNA was used as a negative control.

IRE Binding Assays—RNA band shift assays were performed as previously described (36). Briefly, the cells (2 × 10^5) were lysed in 50 μl of extraction buffer (10 mM HEPES, 10 mM KCl, 2 mM MgCl₂, 40 mM NaCl, 5% glycerol, 1 mM dithiothreitol, and 0.2% Nonidet P-40). Samples of cytoplasmic extract were diluted to 2 μg/μl in lysis buffer without Nonidet P-40, and 10 μg of protein from each sample was analyzed for IRP binding by incubating with 32P-labeled IRE probe. The ferritin H IRE probe was prepared from in vivo transcription of BamHI-linearized pST18 plasmid (a generous gift of Dr. P. Ponka, McGill University, Montreal, Canada). To form RNA-protein com
plexes, cytoplasmic extracts were incubated for 10 min at room temperature with $3 \times 10^5$ cpm probe. Heparin (5 mg/ml) and RNase T1 were added for another 10 min, and RNA-protein complexes were separated on 6% nondenaturing polyacrylamide gels.

Statistical Analysis—All of the experiments were repeated at least three times. Unpaired Student’s t tests were used for experiments in which two groups were being compared. For all studies involving more than two groups, differences among groups were analyzed by one-way analysis of variance. Significance was accepted at $p < 0.05$.

RESULTS

Ferritin Protein Is Increased by p53—To assess the effect of p53 on intracellular iron homeostasis, we used H1299 p53 null human non-small cell lung carcinoma cells stably transfected with doxycycline-inducible p53 (these cells are termed p53.ind cells throughout this manuscript). Preliminary experiments indicated that optimal induction of p53 protein in these cells was attained using 1 µg/ml doxycycline (data not shown). As depicted in Fig. 1A, p53 protein was induced following doxycycline treatment in these cells.

To assess changes in proteins of iron metabolism following induction of p53, we first assessed whether ferritin protein levels changed after p53 induction. As shown in Fig. 1B, treatment of p53.ind cells with doxycycline led to an increase in both ferritin H and ferritin L protein. Control p53-H1299 cells transfected with empty vector exhibited no change in levels of ferritin in response to doxycycline, indicating that doxycycline itself did not contribute to the change in ferritin in these cells.

To confirm the ability of p53 to induce ferritin protein, we compared ferritin levels in HCT 116 human colon carcinoma cells with doxycycline for 24 h and subjected to Western blot analysis using antibodies specific to ferritin H and β-actin. For clarity, the lanes from the same transfer and exposure have been rearranged. A, p53.ind and p53− cells were incubated with 1 µg/ml doxycycline for 36 h and subjected to Western analysis using antibodies specific to ferritin H, ferritin L, and β-actin. The experiments in this figure were repeated at least three times with similar results.

IRE Binding of IRPs Is Decreased by p53—Because these results suggested that transcriptional mechanisms were not responsible for the p53-dependent increase in ferritin, we tested whether a post-transcriptional mechanism was involved. Because the IRPs are central regulators of the translation of ferritin, we examined effects of p53 on IRE binding of IRPs. p53.ind H1299 cells were treated with doxycycline to induce p53 for 24 h, and IRE binding was assessed by bandshift assays. As depicted in Fig. 4, left panels, induction of p53 decreased IRE binding compared with controls. Because p53 can function as a transcripational repressor, we tested whether p53 inhibited IRP through direct transcriptional repression. IRP mRNA levels were assessed by real time RT-PCR in cells treated with or without doxycycline for 24 h. mRNA levels of GAPDH were used as a control. There was no change in mRNA levels for either IRP1 or IRP2 under these conditions (Table 1).

To confirm these results, we examined the levels of endogenous ferritin mRNA in the presence and absence of p53. p53.ind cells were treated with doxycycline for 24 h to induce p53, and the levels of ferritin H mRNA were compared with untreated cells by real time RT-PCR. As shown in Fig. 3, the levels of ferritin H mRNA were not increased in response to p53.
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p53 +/+ cells compared with HCT116 p53−/− cells (Fig. 4, right panels).

IRP1 Plays an Important Role in p53-mediated Induction of Ferritin—To determine whether p53-mediated induction of ferritin is attributable to IRP1, IRP2, or both, we assessed the effect of knockdown of IRPs on p53-dependent ferritin H induction. siRNA was used to knock down IRP1, IRP2, or both in H1299 cells. Scrambled siRNA was used as a negative control. As assessed by real time RT-PCR, in IRP1 knockdown cells, IRP1 was knocked down to 58 ± 8% of control (means ± S.D.). In IRP2 knockdown cells, IRP2 was knocked down to 51 ± 12%. In cells in which IRP1 and IRP2 were simultaneously knockdown, IRP1 was knocked down to 53 ± 5%, and IRP2 was knocked down to 47 ± 7%. The cells were then treated with doxycycline to induce p53, and ferritin H protein measured by Western blotting. As shown in Fig. 5, p53-mediated ferritin induction was preserved in cells transfected with scrambled siRNA (compare lane 2 with lane 1). In contrast, knockdown of IRP1 or the combination of IRP1 and IRP2 abolished ferritin induction by p53 (compare lane 4 with lane 3 and lane 8 with lane 7). Induction of ferritin was preserved following knockdown of IRP2, although the magnitude of the effect was diminished (compare lane 5 with lane 6). There was no apparent effect of IRP1 knockdown on basal levels of ferritin, perhaps because of the relatively modest levels of knockdown attained in these cells. These results demonstrate that p53 exerts its effects on ferritin through IRPs, with IRP1 playing a prominent role.

Effects of p53 on Ferritin Are Not Mediated through a Regulatory Loop Involving Hepcidin and Ferroportin—p53 has recently been shown to induce hepcidin transcription (39). Hepcidin is a peptide hormone that binds directly to ferroportin, an iron efflux pump, and triggers its degradation (40). Thus an increase in hepcidin would be expected to decrease iron efflux, potentially leading to an increase in cellular iron, an increase in ferritin, and a decrease in IRP binding. To test whether p53 mediates its effects on iron homeostasis via a pathway involving hepcidin and ferroportin, we induced p53 in H1299 cells and assessed its effects on hepcidin mRNA and ferroportin protein. Consistent with previous results, we observed that hepcidin mRNA was modestly induced by p53 (Fig. 6A), although the basal level of hepcidin mRNA was low in this cell line. However, the increase in hepcidin was insufficient to alter the levels of ferroportin protein, as measured by Western blotting (Fig. 6B).

TfR1 Protein Expression Decreases Following the Decline in IRP Binding—TfR1 plays a prominent role in cellular iron uptake and is another important contributor to overall cellular iron status that is regulated by IRPs. To determine whether the effects of p53 on proteins of iron metabolism were more broadly based, we measured TfR1 protein levels by Western blot analysis following induction of p53. As depicted in Fig. 7, TfR1 levels declined following induction of p53. This result is consistent with the decrease in IRP binding seen in Fig. 4, because a reduction in IRP destabilizes TfR1 mRNA (29).
Changes in Ferritin and TfR1 Occur Following Induction of p53 and Are Associated with Cell Cycle Arrest—To determine whether these changes in proteins of iron metabolism are associated with cell cycle arrest, we assessed expression of p21 following induction of p53. As shown in Fig. 8A, p53 was induced at 4 h following doxycycline addition, reached a peak at 24 h, and remained at maximal levels until ~48 h. After 48 h, the levels of p53 began to decline as expected, because of the known ability of p53 to transcriptionally induce mdm2, an ubiquitin ligase that targets p53 for degradation (41). As anticipated from the ability of p53 to transcriptionally activate p21 (7), p21 levels increased following p53 induction (Fig. 8A). To determine the effect of p53 on cell proliferation, growth rate and viability were measured. As shown in Fig. 8B, cell replication was arrested in cells induced to express p53. Growth of control cells was not affected by doxycycline. There was no evidence of increased cell death following p53 induction as measured by trypan blue exclusion, suggesting that under these conditions p53 induced cell cycle arrest rather than apoptosis. Thus these cells exhibit p53-dependent growth arrest. Time course analysis revealed that the decrease in TfR1 protein was coordinate with the induction of ferritin H and ferritin L and occurred following the increase in p53 and p21 (Fig. 9). These p53-dependent changes in the proteins of iron metabolism are incorporated into a model of p53-dependent growth arrest in Fig. 10.

DISCUSSION

The intracellular availability of iron has been linked to cell proliferation. Oncogenes, including E1A (25), c-myc (26), and H-ras (28), repress ferritin and increase the pool of iron available for proliferation (the labile iron pool). Conversely, forced overexpression of ferritin increases the sequestration of iron, reduces the labile iron pool, and reduces growth rate (22). The negative effect of ferritin H on cell growth is dependent on the ferroxidase activity of ferritin and is secondary to the iron deficiency induced by it (22).

In this report we demonstrate that p53-induced growth arrest triggers multiple changes in iron homeostasis. Following the addition of doxycycline to cells expressing doxycycline-inducible p53, we observed an early increase in p53 protein levels (Figs. 8 and 9). This was followed by an increase in p21 and growth arrest by 24 h (Fig. 8). At the 24-time point, IRP proteins exhibited a decrease in binding (Fig. 4). Because IRP proteins bind to ferritin mRNA and inhibit its translation and also bind to TfR1 mRNA to stabilize the TfR1 message, a reduction in IRP binding would be expected to both increase ferritin protein and decrease levels of the transferrin receptor. These changes were observed after induction of p53 (Figs. 1, 7, and 9). These responses are expected to decrease the size of the labile iron pool (24). We attempted to test whether changes in IRP binding were accompanied by changes in the labile iron pool. However, although we were readily able to detect changes in the labile iron pool in HeLa cells, the small size of the iron pool in both p53.ind and p53-H1299 cells prevented us from accurately assessing p53-mediated changes in the labile iron pool.6 Overall, however, our results support a model in which p53

6 F. Zhang and J. Buss, unpublished observations.
induces changes in proteins of iron metabolism that operate to restrict the amount of iron available for proliferation. We speculate that iron limitation may collaborate with other p53-dependent mechanisms, such as the induction of the cyclin-dependent kinase inhibitor p21, to facilitate growth arrest (Fig. 10).

Our results demonstrate that IRP binding is repressed in cells induced to express p53 (Fig. 4). Because induction of p53 by doxycycline did not affect levels of either IRP1 or IRP2 mRNA, this is not due to direct transcriptional repression of IRP mRNA in H1299 cells (Table 1). We speculate that p53 may regulate IRP binding at a post-transcriptional level, perhaps through the activity of one or more of the downstream targets of p53. For example, oxidative stress, hypoxia, and protein kinases are known modulators of IRP binding (32); all of these are directly or indirectly affected by p53 and its target genes (1, 41). Interestingly, our results suggest that IRP1 may be a more important target of p53 than IRP2, because knockdown of IRP1 completely abrogated p53-mediated ferritin H induction, whereas knockdown of IRP2 had only a modest effect (Fig. 5).
Following induction of p53, both ferritin H and L subunits were increased, consistent with the involvement of IRPs, which translationally regulate both ferritin subunits (19). Transcriptional mechanisms that target both ferritin H and L are also known (for example, oxidative stress and chemopreventive agents induce both ferritin subunits (21, 23, 30, 42); however, induction of p53 did not induce ferritin transcription. In fact, reporter assays indicated that ferritin H promoter activity was slightly repressed by p53 (data not shown), consistent with results reported by others (43). This may reflect interaction of p53 with sequences present in the luciferase plasmid used for creation of ferritin reporter constructs or an artifactual effect on the relatively short (1 kb) ferritin H promoter sequence used in these studies, because we found no effect of p53 on the endogenous ferritin H gene as assessed by real time RT-PCR (Fig. 3) or Northern blot analyses (data not shown).

Overall, our results suggest a model in which restriction of available iron contributes to p53-dependent cell cycle arrest (Fig. 10). Extensive work from other laboratories has shown that p53 transcriptionally activates genes such as p21, 14-3-3o, GADD45, and others to inhibit cell cycle progression (1). Our results suggest that by inducing ferritin and repressing TfR1, p53 simultaneously recruits pathways that restrict the availability of intracellular iron as an additional mechanism to limit cell cycle progression. This is consistent with studies of small molecule iron chelators, which have shown that iron depletion facilitates growth arrest by inhibiting ribonucleotide reductase, an iron-dependent enzyme that catalyzes the rate-limiting step in DNA synthesis (12), as well as by inhibiting cyclins D, A, and B and cdk2 (34, 44).

In addition to triggering growth arrest, p53 can also induce apoptosis, particularly in response to chemotherapeutic drugs (45). Depletion of the labile iron pool by p53-dependent inactivation of IRPs and induction of ferritin H may also contribute to resistance to such agents, particularly those that bind iron, such as the anthracyclines (46).

We have previously demonstrated that the adenoviral oncogene E1A represses ferritin (30). The oncogene c-myc also represses ferritin and activates IRP2, expanding the labile iron pool and facilitating proliferation (26). Similarly, growth stimulated by activated H-ras is facilitated by ferritin repression and retarded by ferritin overexpression (24). These results indicate that oncogenes may expand the labile iron pool as part of their pro-proliferative activity. In this report we examine events that ensue following p53-mediated cell cycle arrest. Our results suggest that the tumor suppressor p53 triggers events opposite to those induced by oncogenes, including inactivation of IRP, induction of ferritin, and reduction of TfR1. Our data suggest that restricting iron availability is a possible additional pathway that contributes to p53-mediated growth arrest.

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