*BioMetals* **17:** 15–24, 2004.



# Regulation of $p27^{Kip1}$ by intracellular iron levels

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Received 9 December 2002; Accepted 17 February 2003; Published online: June 2003

**Abstract**

Enhanced intracellular iron levels are essential for proliferation of mammalian cells. If cells have entered S phase when iron is limiting, an adequate supply of deoxynucleotides cannot be maintained and the cells arrest with incompletely replicated DNA. In contrast, proliferating cells that are not in S phase, but have low iron pools, arrest in late G1. In this report the mechanism of iron-dependent G1 arrest in normal fibroblasts was investigated. Cells were synchronized in G0 by contact inhibition and serum deprivation. Addition of serum caused the cells to re-enter the cell cycle and enter S phase. However, if the cells were also treated with the iron chelator deferoxamine, S phase entry was blocked. This corresponded to elevated levels of the cyclin dependent kinase inhibitor $p27^{Kip1}$ and inhibition of CDK2 activity. Expression of other cell cycle regulatory proteins was not affected, including the induction of cyclins D1 and E. When the quiescent serum starved cells were supplemented with a readily usable form of iron in the absence of serum or any other growth factors, a significant population of the cells entered S phase. This was associated with downregulation of $p27^{Kip1}$ and increased CDK2 activity. Using an IPTG-responsive construct to artificially raise $p27^{Kip1}$ levels blocked the ability of iron supplementation to promote S phase entry. Thus it appears that $p27^{Kip1}$ is a mediator of G1 arrest in iron depleted Swiss 3T3 fibroblasts. We propose that this is part of an iron-sensitive checkpoint that functions to ensure that cells have sufficient iron pools to support DNA synthesis prior to entry into S phase.

## Introduction

Iron is an essential metal for virtually all living cells. Iron-containing proteins perform numerous functions including energy metabolism and oxygen transport. Mammalian cells obtain iron through transferrin receptors on the cell surface. Transferrin receptors (TR) bind iron-loaded transferrin and deliver the complex to the cell interior through endocytosis. Iron is released from transferrin due the low pH in the endosome and passes through the endosomal membrane into the cytosol. In the cytosol the iron transiently enters the intracellular labile iron pool. This pool is the source of iron for heme synthesis and for iron-requiring enzymes. Excess iron is stored by ferritin.

Due to the reactivity and toxicity of iron, the intracellular pool of iron is highly regulated. High level expression of TR is linked to cell proliferation. Quiescent, nonproliferating cells, in general, have low levels of transferrin receptors on their cell surfaces. However, when cells are mitogenically activated to re-enter the cell cycle, the level of transferrin receptors is markedly elevated leading to increased delivery of iron into the labile pool (Larrick & Cresswell 1979, Trowbridge & Omary 1981, Hamilton 1982, Miskimins *et al.* 1986). The importance of this pathway is emphasized by the fact that depletion of the intracellular labile iron pool either by blocking transferrin receptor function (Trowbridge & Lopez 1982, Neckers & Cossman 1983, Lesley & Schulte 1985, Taetle *et al.* 1986, Yang *et al.* 2001) or by using iron-specific chelators (Hoyes *et al.* 1992, Lucas *et al.* 1992, Lucas *et al.* 1995, Kulp *et al.* 1996, Gao & Richardson 2001, Richardson 2002) leads to cell cycle arrest.

It is believed that proliferating cells require increased iron levels for the production of deoxynucleotides during S phase of the cell cycle. Replication of the genome in a diploid human cell requires approximately $1.2 \times 10^{10}$ deoxynucleotides. Deoxynucleotides are synthesized by the reduction of

ribonucleotides. Compared to the cellular pools of ribonucleotides, the intracellular pools of deoxyribonucleotides are quite small, sufficient to support only a few minutes of DNA replication. Thus, there is a continuing need for the production of deoxynucleotides from ribonucleotides throughout S phase. The enzyme that catalyzes this reaction is ribonucleotide reductase. The expression of this enzyme is cell cycle dependent, being greatly elevated in late G1 and into S phase (Elledge *et al.* 1992). Chemical inhibitors of ribonucleotide reductase, such as hydroxyurea, completely block DNA replication, indicating the fundamental role played by the enzyme in this process. Furthermore, iron is essential for mammalian ribonucleotide reductase activity (Thelander *et al.* 1983). Reduction of ribonucleotides by the enzyme requires the formation of a tyrosyl radical. This tyrosyl radical, which must be regenerated each enzymatic cycle, is stabilized by a dinuclear iron center. An interesting property of the mammalian ribonucleotide reductase enzyme is that the iron center is labile and must be continuously regenerated in a reaction that requires ferrous iron and oxygen (Mann *et al.* 1991, Nyholm *et al.* 1993, Cooper *et al.* 1996, Kauppi *et al.* 1996). The source of iron for this reaction is the labile iron pool (Mann *et al.* 1991, Nyholm *et al.* 1993, Cooper *et al.* 1996). Thus the activity of ribonucleotide reductase is absolutely dependent on the iron status within the cell. If intracellular iron pools are low the activity of the enzyme will be low.

There is evidence that arrest in response to low iron pools occurs primarily at two separate points in the cell cycle (Hoyes *et al.* 1992, Lucas *et al.* 1992, Brodie *et al.* 1993, Lucas *et al.* 1995, Kulp *et al.* 1996). If cells are already in S phase when iron chelators are added they arrest in S phase, presumably because ribonucleotide reductase is inactivated and the supply of deoxyribonucleotides is rapidly depleted.

Interestingly, cycling cells in stages other than S, or quiescent cells that are mitogenically stimulated to re-enter the cell cycle, never enter into S phase if iron levels are depleted but are arrested at a point in late G1 (Hoyes *et al.* 1992, Lucas *et al.* 1992, Brodie *et al.* 1993, Lucas *et al.* 1995, Kulp *et al.* 1996). This is true whether iron pools are depleted by using anti-transferrin receptor monoclonal antibodies that block receptor-mediated iron delivery (Trowbridge & Lopez 1982, Neckers & Cossman 1983, Lesley & Schulte 1985) or by using iron-specific chelators (Hoyes *et al.* 1992, Lucas *et al.* 1992, Brodie *et al.* 1993, Lucas *et al.* 1995, Kulp *et al.* 1996). This suggests that a specific iron-sensitive cell cycle checkpoint exists that is analogous to the DNA damage-sensitive checkpoint. Just as it is important not to replicate a damaged genome, it would be disastrous for the cell to begin DNA replication if there were insufficient resources to complete the process. This situation would exist if cells entered S phase with inadequate iron pools to support deoxynucleotide synthesis by ribonucleotide reductase. Therefore, it would be beneficial to the cell to check cell cycle progression in G1 until sufficient iron levels are attained to support DNA synthesis.

Further evidence for the importance of intracellular iron levels in the control of cell proliferation is given by the fact that the iron storage protein H-ferritin and iron regulatory protein (IRP2) genes are regulated by the proto-oncogene product c-Myc (Wu *et al.* 1999). Expression of IRP2, an RNA-binding protein which enhances the stability of the TR mRNA but inhibits translation of ferritin mRNAs, is enhanced by c-Myc. In contrast, c-Myc represses H-ferritin expression. Both of these processes result in increased availability of iron to cells and this appears to be essential for c-Myc to promote cell proliferation and transformation (Wu *et al.* 1999). In another recent report it was demonstrated that repression of ferritin expression using antisense oligonucleotides caused an increase in the labile iron pool and supported growth stimulation by an oncogenic form of H-ras (Kakhlon *et al.* 2002). Furthermore, repression of ferritin expression was able to block cell cycle arrest by a dominant negative form of H-ras. These findings further substantiate the necessity of increased iron availability for cell proliferation.

Several findings suggest that iron levels may regulate cell cycle progression by influencing cell cycle regulatory proteins. Brodie et al. (Brodie *et al.* 1993) found that deferoxamine (DFO), an iron-specific chelator, decreases the activity of the CDK $p34^{cdc2}$ in neuroblastoma cells. Other investigators have demonstrated that iron chelators lead to inhibition of cyclin E and cyclin A-associated CDK activities (Lucas *et al.* 1995, Kulp *et al.* 1996) and prevention of Rb phosphorylation (Lucas *et al.* 1992), both of which are necessary for the transition from G1 into S phase. These studies suggest that low intracellular iron pools can signal changes in the cell cycle regulatory machinery leading to arrest in late G1 but the mechanisms by which this occurs are unknown.

It is clear that precise regulation of the availability of intracellular iron is essential during the cell cycle. However the interplay between iron levels and

the cell cycle machinery is not well understood. In the present study, we have investigated the effect of iron on progression of the cell cycle in 3T3 fibroblasts. Our results show that depletion of intracellular iron blocks cells in late G1 whereas increased iron availability is able to stimulate quiescent cells into S phase. In both circumstances modulation of cell cycle progression is mediated by changes in the level of the cyclin dependent kinase inhibitor $p27^{Kip1}$.

## Materials and methods

*Cell culture.* Swiss/3T3 and LAP3-p27 (gift of Pradip Raychaudhuri) cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% newborn calf serum, 100 U/ml penicillin and 100 $\mu$g/ml streptomycin. For serum starvation, confluent cultures were incubated in a 1:1 mixture of DMEM and Waymouth's medium for 24–48 h.

*Labeling of S phase cells with bromodeoxyuridine (BrdU).* Quiescent cells were plated on coverslips, grown to confluence, and serum starved. They were then treated with serum in the presence or absence of iron chelators or ferric ammonium citrate (FAC) as indicated in the figures. In addition, 10 $\mu$M BrdU was added to the medium. After 24 h the cells were fixed and cells that had entered S phase were detected using the BrdU Labeling and Detection Kit II (Boehringer Mannheim) following the protocol provided by the manufacturer. Random fields were viewed in a microscope and the number of labeled and unlabeled cells was determined. At least 3 separate fields were analyzed for each coverslip.

*Western blotting.* Equal numbers of cells from each culture were lysed in the same volume of SDS-containing sample buffer [2.5% SDS, 2.5 mM Tris-Cl (pH 6.8), 100 mM DTT, 10% glycerol, 0.025% pyronine Y]. The samples were briefly sonicated to shear DNA, separated by SDS-polyacrylamide gel electrophoresis and transferred to Immobilon P membranes using a semi-dry transfer apparatus. The membranes were blocked with 1–5% non-fat dry milk in Tris-buffered saline [10 mM Tris-Cl (pH 7.5), 150 mM NaCl] containing 0.1% Tween-20 (TBS-T) for 15–60 min at room temperature. The membrane was then incubated with the appropriate antibody in TBS-T containing 1–5% non-fat dry milk for 1 h at room temperature. After extensive washing in TBS-T the membrane was incubated with the appropriate horseradish peroxidase-conjugated secondary antibody. After extensive washing in TBS-T, proteins were detected using the SuperSignal chemiluminescence system (Pierce Chemical Co.). Anti-$p27^{Kip1}$ (K25020) was obtained from Transduction Laboratories. Anti-cyclin D1 (sc-450), anticyclin E (sc-481), anti-CDK2 (sc-163), anti-p27 (M-197), and anti-CDK4 (sc-260) were obtained from Santa Cruz Biotechnology.

*Immunoprecipitation and CDK2 kinase assay.* Cell pellets were washed twice with Dulbecco's phosphate buffered saline and then resuspended in 1 ml cold lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 2.5 mM EGTA, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM $\beta$-glycerophosphate, 1 mM NaF, 1 mM EDTA, 1 mM DTT, 0.1 mM $Na_3VO_4$, 0.1% Tween 20, 10 $\mu$g/ml leupeptin and 10 $\mu$g/ml aprotinin].

After sonicating the sample twice (10 seconds each time) the lysate was centrifuged at full speed in an Eppendorf 5415c microcentrifuge for 10 min at 4°. For pre-clearing, the supernatant was mixed with 20 $\mu$l protein A agarose beads, and incubated for 1 h on a rotator. The lysate was centrifuged in an Eppendorf 5415c microcentrifuge for 5 min at 4° at 4,000 rpm. Thirty $\mu$l anti-CDK2 antibody was added into the supernatant and the sample incubated for 3 h on a rotator at 4°. Thirty $\mu$l protein A agarose beads were added and the sample was incubated for another 2 h on a rotator at 4°. After centrifuging in an Eppendorf 5415c microcentrifuge for 5 min at 4° at 4,000 rpm, the supernatant was discarded and the beads were washed four times in 500 $\mu$l lysis buffer and twice in washing buffer [50 mM HEPES (pH 7.5), 1 mM DTT].

After the final wash the beads were incubated with 30 $\mu$l of kinase assay reaction buffer [5 $\mu$l histone H1 protein (1 $\mu$g/$\mu$l), 150 $\mu$l kinase buffer [50 mM HEPES (pH 7.5), 10 mM $MgCl_2$ , 1 mM DTT, 2.5 mM EGTA, 10 mM $\beta$-glycerophosphate, 0.1 mM $Na_3VO_4$, 1 mM NaF], 1.5 $\mu$l 2 mM ATP and 2.5 $\mu$l ($\gamma$-$^{32}$P) ATP] at 30 °C for 15 min. The reaction was stopped with 30 $\mu$l 2× SDS-sample buffer. After centrifugation, the supernatant was separated on a 10% SDS-polyacrylamide gel. The gel was dried and exposed to film.

## Results

*Iron levels affect entry of serum stimulated 3T3 cells into S phase.* Non-S phase cells arrest in late G1 under conditions of iron deprivation but the mechanisms controlling this process are not well understood. Therefore we were specifically interested in the events that lead to arrest at this stage of the cell cycle and have utilized a population of cells that synchronously pass through G1 and into S phase. For the experiments described below Swiss/3T3 cells were allowed to enter quiescence (G0) due to contact inhibition and serum deprivation. The quiescent cells were then treated with reagents that modulate intracellular iron levels and the effects on entry into S phase were determined.

Quiescent Swiss/3T3 cells were stimulated with 20% serum in the presence or absence of deferoxamine (DFO). DFO is a well characterized iron chelator that specifically affects the cellular labile iron pool (Richardson 2002). Entry into S phase was determined by monitoring the incorporation of BrdU into DNA. The percentage of cells that had entered S phase was determined by directly counting labeled and unlabeled nuclei under a microscope. In the absence of DFO about 60% of the serum stimulated cells entered S phase (Fig. 1A). Addition of DFO inhibited entry into S phase in a dose dependent manner. At 100 $\mu$M, DFO completely blocked serum-stimulated entry into S phase. This inhibition was completely reversed by the addition of 20 $\mu$g/ml ferric ammonium citrate (FAC), a source of iron that is readily available and usable by the cells. These results suggest that the mechanism by which DFO blocks entry into S phase involves an iron-dependent target.

In a separate experiment, quiescent, serum-starved cells were treated with FAC in the absence of serum stimulation (Fig. 1B). Approximately 20% of the cell population entered S phase when treated with FAC in the absence of any mitogens. This is a significant increase in S phase entry over that observed in untreated serum-starved cells, although significantly less than in cells treated with serum. These results suggest that a population of cells in the serum-deprived culture are arrested in G1 because of insufficient intracellular iron levels.

*Iron depletion does not affect the levels of G1 CDKs or cyclins.* Progression from G0 through G1 and into S phase involves the sequential activation of G1 cyclin dependent kinases. First, CDK4 is activated following the induction of its regulatory partner cyclin D1 in mid

**Figure 1A**

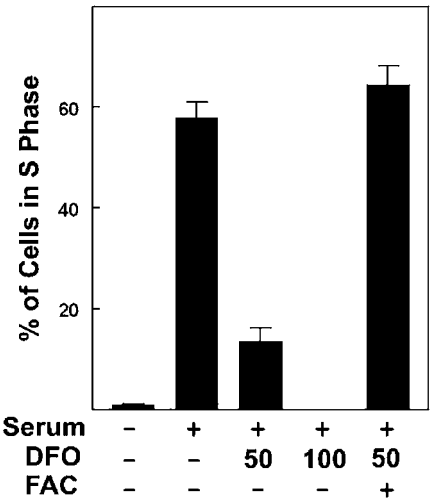


**Figure 1B**

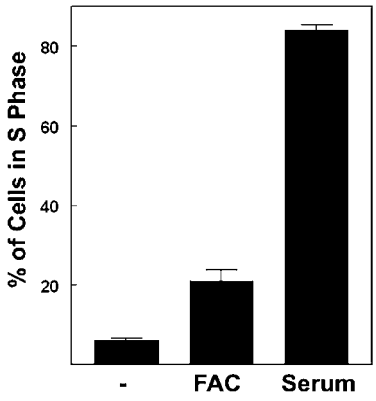


*Figure 1.* Effect of iron and iron chelation on entry of serum stimulated cells into S phase. (A) Swiss 3T3 cells were allowed to enter quiescence following contact inhibition and serum deprivation. They were then treated for 24 h with 20% serum as indicated in either the presence or absence of DFO ($\mu$M) or FAC (20 $\mu$g/ml). The percent of cells that had entered S phase was determined by BrdU labeling as described in experimental procedures. (B) Quiescent cells were treated for 24 h with 160 $\mu$g/ml FAC or 20% serum as indicated and S phase cells were detected by BrdU labeling.

G1. CDK4 activation is important for the subsequent induction of cyclin E expression which leads to activation of CDK2 in late G1. CDK2 activation is essential for entry into S phase. In addition to the availability of their cyclin partners, CDK4 and CDK2 are regulated by phosphorylation and by CDK inhibitor proteins. It is possible that iron levels could affect progression into S phase by affecting the expression of either of the G1 CDKs, their cyclins partners, or inhibitor proteins. CDK4 is expressed at significant levels in quiescent

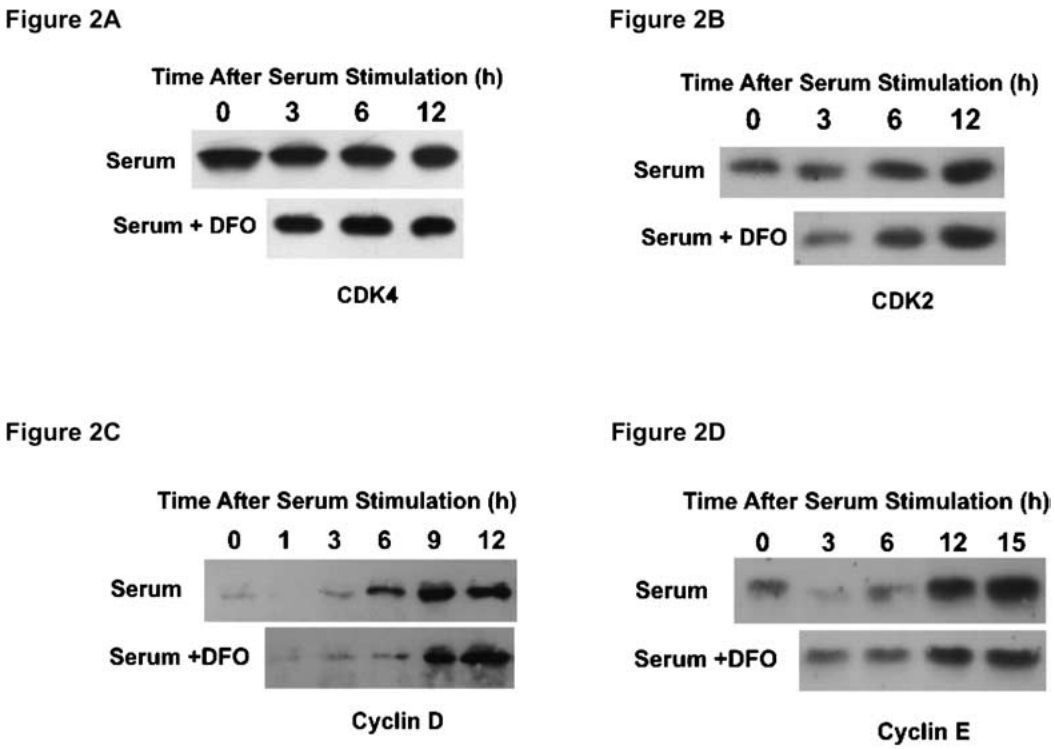


*Figure 2.* Iron chelation does not block serum-induced expression of cyclin D1 or cyclin E or affect the levels of G1 CDK proteins. Swiss 3T3 cells were allowed to enter quiescence following contact inhibition and serum deprivation. They were then treated with 20% serum in either the presence or absence of DFO (100 $\mu$M). At the indicated time the cells were harvested and the levels of CDK4 (A), CDK2 (B), cyclin D1 (C), or cyclin E (D) were determined by western blotting. Each lane represents an equal number of cells.

3T3 cells and this is unaffected by serum stimulation or depletion of iron pools by treatment with DFO (Fig. 2A). CDK2 is also expressed in the quiescent cells and it is increased by serum stimulation but DFO has no effect on its expression (Fig. 2B). The G1 cyclins, cyclin D1 and cyclin E, are present at low levels (Figs. 2C and 2D) in the quiescent cells. When the cells are stimulated with serum to reenter the cell cycle, cyclin D1 expression is enhanced within 6 h and continues to increase until at least 12 h after stimulation. Addition of DFO has almost no effect on the induction of cyclin D1 by serum. Cyclin E expression is also induced by serum stimulation, reaching high levels at 12–15 h after treatment, the time at which the cells are entering into S phase (Ouyang *et al.* 1993). As with cyclin D1, DFO has very little effect on the ability of serum to stimulate cyclin E expression.

From these results it is unlikely that iron modulates G1 progression through effects on the levels of CDK proteins or the signaling mechanisms that lead to induction of cyclin D1 or cyclin E.

*Iron modulates levels of the CDK inhibitor p27*$^{Kip1}$. p27$^{Kip1}$ functions primarily as an inhibitor of CDK2/cyclin E. It is present at high levels in quiescent, contact inhibited cells and must be downregulated for CDK2 to mediate entry into S phase.

Serum stimulation of quiescent 3T3 cells leads to downregulation of the cyclin dependent kinase inhibitor p27$^{Kip1}$ (Fig. 3A). The lowest levels of p27Kip1 are observed 12–15 h after stimulation, the time at which the cells are entering S phase. The iron chelator DFO inhibits the serum-induced loss of p27$^{Kip1}$. During the period of S phase entry (12–15 h after serum addition) p27$^{Kip1}$ levels are substantially higher in the DFO treated cells than in the cells treated with serum alone.

The effect of enhanced iron levels on p27$^{Kip1}$ expression in quiescent cells, in the absence of any other mitogens, was also examined (Fig. 3B). Confluent, serum starved Swiss 3T3 cells were supplemented with FAC for various times and then extracts were prepared for western blotting. There was as substantial decrease in the levels of p27$^{Kip1}$ at the 8 and 15 h

Figure 3A

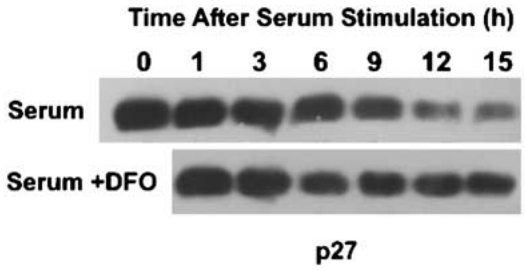


Figure 3B

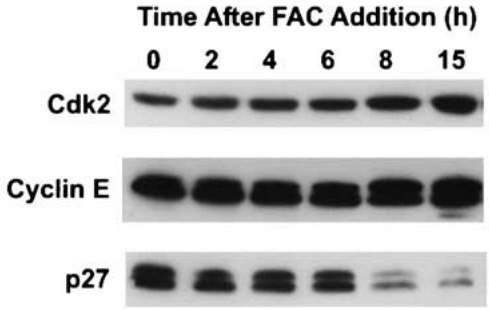


*Figure 3.* Iron modulates expression of the CDK inhibitor $p27^{Kip1}$. (A) Swiss 3T3 cells were allowed to enter quiescence following contact inhibition and serum deprivation. They were then treated with 20% serum in either the presence or absence of DFO (100 $\mu$M). At the indicated time the cells were harvested and the levels of $p27^{Kip1}$ were estimated by western blotting. Each lane represents an equal number of cells. (B) Quiescent cells, in the absence of serum or other growth factors, were treated with 160 $\mu$g/ml FAC. At the times indicated extracts were prepared and used to detect CDK2, cyclin E, or $p27^{Kip1}$ by western blotting. Note that, in this experiment, cyclin E has been resolved into 2 distinct bands because of the length of time that the electrophoresis was performed.

time points. Over the same time period there were only small changes in the levels of CDK2. In this experiment, FAC treatment did not lead to substantial changes in the overall levels of cyclin E. The iron-mediated downregulation of $p27^{Kip1}$ is very similar to that obtained by treating the cells with serum (see Fig. 3A) and is opposite to the effect observed with iron chelators. Thus the intracellular iron pool appears to be an important regulator of $p27^{Kip1}$ expression.

Figure 4A

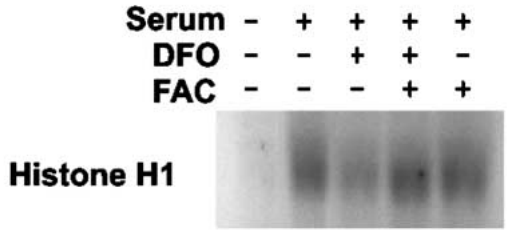


Figure 4B

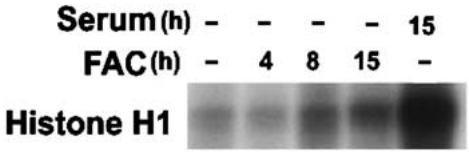


*Figure 4.* Effects of iron levels on CDK2 activity. (A) Quiescent Swiss 3T3 cells were stimulated with serum in the presence of the indicated reagents for 15 h. The cells were then harvested and immunoprecipitation kinase assays of CDK2 activity using histone H1 as a substrate were performed. (B) Quiescent cells were treated with serum or FAC in the absence of serum as shown. At the times indicated the cells were harvested for CDK2 kinases assays as in (A).

*Intracellular iron levels affect CDK2 kinase activity.* Previous experiments, utilizing iron chelators to block proliferation, have demonstrated that G1 arrest occurs in late G1 near the G1/S boundary (Hoyes *et al.* 1992, Brodie *et al.* 1993, Lucas *et al.* 1995). The activity of CDK2 is critical for progression beyond this point in the cell cycle and it is likely that the modulation of $p27^{Kip1}$ by intracellular iron levels impacts this process. Therefore we determined the effect of iron levels on the activity of CDK2 in synchronized 3T3 cells using an immunoprecipitation kinase assay. As expected, very little CDK2 activity was detected in extracts from quiescent cells. However, the CDK2 activity was significantly elevated after serum stimulation (Fig. 4A). The increase in CDK2 activity in response to serum was greatly reduced by the concurrent addition of 50 $\mu$M DFO. The inhibitory effect of DFO was completely reversed by the simultaneous addition of FAC, indicating that the effect of DFO is due to alterations in the cellular iron status.

We also examined the effect of FAC on CDK2 activity in quiescent cells in the absence of serum or any other mitogens. FAC (160 $\mu$g/ml) stimulated an

Figure 5A

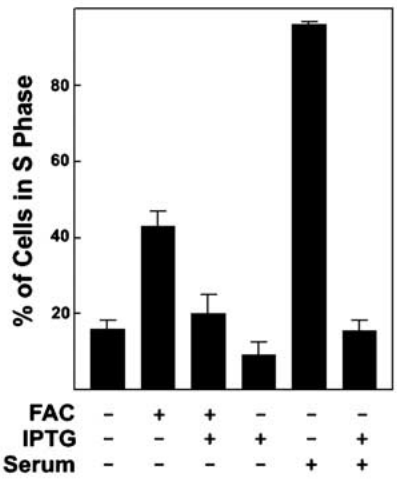


Figure 5B

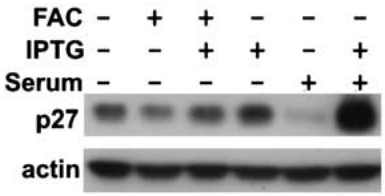


Figure 5C

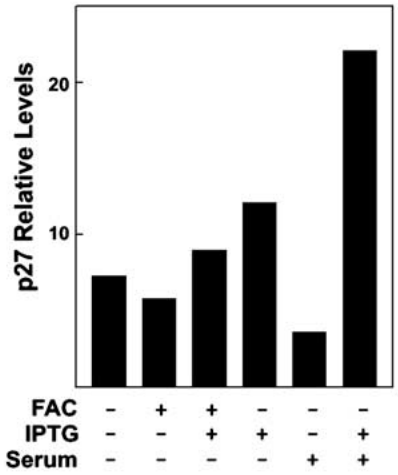


*Figure 5.* Inducible expression of $p27^{Kip1}$ inhibits FAC and serum stimulated S phase entry. (A) Quiescent, serum-starved Lap3-NIH cells, in which $p27^{Kip1}$ expression is responsive to IPTG, were treated with the reagents indicated. Twenty hours after addition of FAC or serum the number of cells that had entered S phase was determined by the BrdU labeling method. (B) Cells were treated as described in (A) except that the cells were harvested for western blot analysis of either $p27^{Kip1}$ or actin. (C) The western blot shown in B was scanned with a densitometer to estimate the relative levels of $p27^{Kip1}$. The levels of actin were used to normalize the $p27^{Kip1}$ data.

increase in CDK2 activity which was maintained for at least 15 h (Fig. 4B). However, the increase mediated by FAC alone (approximately 3 fold) was substantially lower than that observed with serum stimulation. Thus the increase in CDK2 activity following FAC treatment parallels its ability to stimulate entry into S phase (compare Figs. 1B and 4B). Note, however, that the downregulation of $p27^{Kip1}$ by iron (see Fig. 3B) appears to be substantially greater than its ability to enhance CDK2 activity (see Fig. 4B) or entry into S phase (see Fig. 1B). Thus a large proportion of the cells can respond to iron mediated changes in $p27^{Kip1}$ but only a fraction of the contact inhibited, serum starved cells are arrested at a point late in G1. The remainder of the cell population requires additional signals from serum components to progress out of G0 and through G1.

To further examine the relationship between iron levels, $p27^{Kip1}$ expression, and cell cycle progression, the cell line LAP3-p27 was utilized. In this cell line the $p27^{Kip1}$ cDNA can be inducibly expressed by the addition of IPTG (Shiyanov *et al.* 1997). LAP3-p27 cells were derived from the NIH 3T3 cell line which, in terms of cell cycle control, is similar to the Swiss 3T3 line used in the experiments described above. LAP3-p27 cells were allowed to become quiescent by contact inhibition and serum starvation. The cells were then treated with the reagents indicated in Fig. 5A and entry into S phase was monitored by BrdU incorporation. In this cell line greater than 90% of the cells were stimulated to enter S phase by serum. This was completely blocked by addition of IPTG to induce $p27^{Kip1}$ expression. In serum starved cultures treated with FAC alone (no serum) more than 40% of the cells were labeled by BrdU. Again this effect was completely blocked by addition of IPTG to induce $p27^{Kip1}$. The levels of $p27^{Kip1}$ were also monitored under identical conditions (Fig. 5B). The level of $p27^{Kip1}$ is high in the quiescent cells and is greatly diminished by addition of serum. $p27^{Kip1}$ expression is induced to very high levels by treatment with IPTG in serum stimulated cells and this correlates with inhibition of entry into S phase. Addition of IPTG alone to the serum starved cells induces $p27^{Kip1}$ expression but to much lower levels than in the presence of serum. This is most likely due to global effects of serum on nutrient uptake, metabolic processes, and the rates of transcription and translation. Addition of FAC alone also reduces $p27^{Kip1}$ expression but not to the extent observed with serum. When IPTG is added together with FAC, $p27^{Kip1}$ expression is somewhat higher than that

observed in the serum starved cells but lower than with IPTG alone. Thus in every situation the number of S phase cells inversely corresponds to the levels of $p27^{Kip1}$ (compare Fig. 5A and 5C). These results indicate that iron levels control entry into S phase by modulating $p27^{Kip1}$ levels.

## Discussion

Elevated intracellular iron levels are required for cell proliferation. Mitogenic activation of cells leads to enhanced expression of the transferrin receptor and increased uptake of iron (Kwok & Richardson 2002). The oncogene product c-Myc (Wu *et al.* 1999) has been implicated in repression of ferritin expression, leading to release of stored iron and increased intracellular iron pools. In contrast, lowering intracellular iron levels by blocking the transferrin receptor (Trowbridge & Lopez 1982, Neckers & Cossman 1983, Lesley & Schulte 1985, Yang *et al.* 2001), by overexpressing ferritin (Kakhlon *et al.* 2002), or by using iron-specific chelators (Richardson 2002) leads to cell cycle arrest. Depletion of intracellular iron pools can arrest the cell cycle in either S phase or in late G1. S phase arrest is thought to be the result of inhibition of ribonucleotide reductase, the iron-dependent enzyme required for supplying deoxynucleotides for DNA replication. However, the mechanism of G1 arrest in response to low iron levels has not been well characterized. In this report we specifically examined the effect of iron levels on progression through G1 by first arresting 3T3 fibroblasts in G0 by contact inhibition and serum deprivation. When treated with serum the cells synchronously progress through G1 and enter into S phase. However, depletion of iron pools, by the addition of an iron chelator, blocked S phase entry. This was associated with elevated $p27^{Kip1}$ levels and inhibition of CDK2 activity. Other G1-specific events, such as induction of cyclin D1 and cyclin E, were not appreciably affected by the iron chelator. Interestingly, addition of ferric ammonium citrate, a readily usable source of iron, to the serum deprived cells caused a proportion of the cells to enter S phase in the absence of any mitogenic factors. This corresponded to a decrease in $p27^{Kip1}$ levels and an increase in CDK2 activity. These results suggest that some portion of the cells in the serum-starved cultures are arrested in late G1 due to insufficient iron pools and that when the cells are supplemented with iron they are able to enter into S phase.

Based on the findings above we postulate that there is an iron-sensitive G1 checkpoint which functions to ensure that there is sufficient iron to support DNA replication before entry into S phase. This would prevent incomplete DNA replication due to inhibition of ribonucleotide reductase and an inadequate supply of deoxynucleotides.

$p27^{Kip1}$ appears to be an important mediator of the iron-sensitive G1 arrest in 3T3 cells. Although the mechanism by which iron levels modulate $p27^{Kip1}$ expression are not yet understood, there are several possibilities. Treatment of cells with DFO has been shown to activate the hypoxia-responsive transcription factor HIF-1$\alpha$ (Wang & Semenza 1993). It is therefore possible that HIF-1$\alpha$ directly or indirectly mediates iron sensitive changes in $p27^{Kip1}$. Interestingly, $p27^{Kip1}$ has also been shown to be induced by hypoxia (Krtolica *et al.* 1998, Krtolica *et al.* 1999, Gardner *et al.* 2001) but the work of Gardner *et al.* (Gardner *et al.* 2001) indicates that this is independent of HIF-1$\alpha$.

Another DFO-responsive transcription factor, PLAGL2, was recently identified by Furukawa *et al.* (Furukawa *et al.* 2001). PLAGL2 belongs to a family of zinc finger proteins, members of which have been shown to mediate cell cycle arrest (Varrault *et al.* 1998, Furukawa *et al.* 2001) and to be lost in certain types of tumors (Varrault *et al.* 1998). It is unknown if these proteins affect $p27^{Kip1}$ expression but the $p27^{Kip1}$ gene 5′-flanking region contains several GC-rich motifs similar to the PLAGL2 consensus binding site. Another iron-sensitive target is the protein eIF-5A. The function of eIF-5A is not currently understood but it is essential for cell proliferation (Park *et al.* 1997, Caraglia *et al.* 2001). It is the only known protein that is post-translationally modified with hypusine and this modification is essential for eIF-5A activity. The final step in the formation of hypusine is catalyzed by the enzyme deoxyhypusine hydroxylase which is an iron requiring enzyme that is inhibited by DFO (Caraglia *et al.* 1999, Clement *et al.* 2002). Blocking hypusine modification of eIF-5A leads to G1 cell cycle arrest. Thus it is possible that $p27^{Kip1}$ regulation by iron levels lies in a pathway downstream of eIF-5A.

The involvement of these or other mechanisms in the regulation of $p27^{Kip1}$ in response to changes in intracellular iron pools requires further investigation.

At least one other report has described the induction of $p27^{Kip1}$ by DFO. Yoon *et al.* (Yoon *et al.* 2002) found that iron chelation by DFO led to a senescence-like arrest of hepatocyte cell lines that was

associated with enhanced $p27^{Kip1}$ expression. However, this effect required prolonged exposure to the chelator. $p27^{Kip1}$ was not significantly upregulated until at least 3 days after treatment and was dependent on prior induction and expression of TGF-$\beta$1. In addition, the experiments were performed on asynchronous cell cultures. Thus, the experimental approach was significantly different from that described here.

Other investigators have reported changes in the levels of N-Myc, cyclin A, p21, cyclin D1, and GADD45 in response to iron chelation (Lucas *et al.* 1995, Kulp *et al.* 1996, Darnell & Richardson 1999, Fan *et al.* 2001, Gao & Richardson 2001). However, these experiments, again, were generally performed on tumor cell lines and in asynchronous cultures and it is unclear how they relate to the findings reported here. In fact, in asynchronous cell populations it is expected that reduced iron levels of S phase cells will lead to incomplete DNA synthesis. This in turn will activate the S phase checkpoint response which targets several cell cycle regulatory proteins (Bartek & Lukas 2001, Melo & Toczyski 2002). Thus the response to changes in intracellular iron levels may vary with the cell type and will be dependent on the stage of the cell cycle.

The necessity of enhanced iron levels for cell proliferation and the ability of iron chelators to block this process has led to interest in this class of compounds for the treatment of cancer. A number of potent iron chelators have been developed and several are in various stages of clinical trials (reviewed in Richardson 2002). Many types of tumor cells have lost the ability to regulate G1 progression and in most types of cancer there is an inverse correlation between $p27^{Kip1}$ and tumor stage (PhilippStaheli *et al.* 2001). It is therefore possible that many types of cancer cells will not arrest in G1 in response to low iron levels, but will progress into S phase and arrest due to an insufficient supply of deoxynucleotides. This could be an important determinant of whether the cell simply arrests in G1 but remains viable or undergoes apoptosis because of prolonged S phase arrest. It is therefore important to further characterize the cellular processes that respond to changes in iron levels and to understand the molecular mechanisms that mediate these responses.

## Acknowledgements

We thank Pradip Raychaudhuri for the gift of LAP3-p27 cells. This work was supported by NIH grant CA84325.

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