Redox Regulation of the G₁ to S Phase Transition in the Mouse Embryo Fibroblast Cell Cycle

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ABSTRACT

The hypothesis that intracellular oxidation/reduction (redox) reactions regulate the G₁-G₂ to S-phase transition in the mouse embryonic fibroblast cell cycle was investigated. Intracellular redox state was modulated with a thiol-antioxidant, N-acetyl-L-cysteine (NAC), and cell cycle progression was measured using BrdUrd pulse-chase and flow cytometric analysis. Treatment with NAC for 12 h resulted in an ~6-fold increase in intracellular low-molecular-weight thiols and a decrease in the MFI of an oxidation-sensitive probe, dihydrofluorescein diacetate, indicating a shift in the intracellular redox state toward a more reducing environment. NAC-induced alterations in redox state caused selective delays in progression from G₁-G₂ to S phase in serum-starved cells that were serum stimulated to reenter the cell cycle as well as to inhibit progression from G₁ to S phase in asynchronous cultures with no significant alterations in S phase, and G₂-M transits. NAC treatment also showed a 70% decrease in cyclin D1 protein levels and a 3-4-fold increase in p27 protein levels, which correlated with decreased retinoblastoma protein phosphorylation. Cells released from the NAC treatment showed a transient increase in dihydrofluorescein fluorescence and oxidized glutathione content between 0 and 8 h after release, indicating a shift in intracellular redox state to a more oxidizing environment. These changes in redox state were followed by an increase in cyclin D1, a decrease in p27, retinoblastoma protein hyperphosphorylation and subsequent entry into S phase by 8–12 h after the removal of NAC. These results support the hypothesis that a redox cycle within the mammalian cell cycle might provide a mechanistic link between the metabolic processes early in G₁ and the activation of G₁-regulatory proteins in preparation for the entry of cells into S phase.

INTRODUCTION

In recent years, increasing evidence has suggested that intracellular oxidation-reduction (redox) reactions play a critical role in the regulation of several physiological processes including cell proliferation (1), senescence (2), differentiation (3), and apoptosis (4). ROS potentially includes superoxide, hydrogen peroxide, hydroxyl radical, singlet molecular oxygen, and organic hydroperoxides are believed to be continuously generated intracellularly as byproducts of O₂ metabolism and have traditionally been thought of as unwanted and toxic byproducts of living in an aerobic environment (5, 6). However, results from several studies suggest that the metabolic production of ROS is tightly regulated and serves a physiological function during mitogenic stimulation.

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3 The abbreviations used are: ROS, reactive oxygen species; BrdUrd, bromodeoxyuridine; NAC, N-acetyl-L-cysteine; NPM, N-(1-pyrenyl)maleimide; MFI, mean fluorescence intensity; CDK, cyclin-dependent kinase; Rb, retinoblastoma protein; MEF, mouse embryonic fibroblast; BSO, N-(β-carboxyvinyl)glycine; HPLC, high-performance liquid chromatography; GSH, reduced glutathione; GSSG, oxidized glutathione; γGGS, γ-glutamyl cysteine; DHF-DA, dihydrofluorescein diacetate; PI, propidium iodide; FACS, fluorescence-activated cell sorter.

MATERIALS AND METHODS

Cell Culture and Synchronization. MEF clones [12(1), wild type p53 phenotype] and [10(1), mutant p53 phenotype] were a gracious gift from Dr. Arnold Levine’s laboratory Princeton University, Princeton, New Jersey, USA and were cultured in DMEM supplemented with 10% fetal bovine serum, nonessential amino acids (Life Technologies, Inc.), and antibiotics (penicillin and streptomycin). Cells were cultured at 37°C in a humidified incubator with
were stained with 10 μM H9262 cellular protein extracts prepared from control and NAC-treated cells were used in this study (data not shown). Asynchronously growing cell cultures were incubated simultaneously with NAC and 1 mM BSO, an inhibitor of γ-glutamylcysteine synthase, for the inhibition of glutathione synthesis.

Measurement of Intracellular Glutathione and NAC Levels. Intracellular GSH and GSSG as well as NAC levels were assayed using previously published spectrophotometric and HPLC assays (17, 18). Monolayer cells were scrape-harvested in PBS at 4°C and centrifuged, and the cell pellet was stored frozen at −80°C. Cell pellets were homogenized in 50 mM potassium phosphate buffer (pH 7.8) containing 1.34 mM diethylenetriaminepenta-acetic acid. Total glutathione content was determined in sulfosalicylic acid extracts (5% SSA, w/v) by the method of Anderson (19). GSH and GSSG were distinguished by the addition of 2 μl of a 1:1 mixture of 2-vinylpyridine and ethanol per 30 μl of sample, followed by incubation at room temperature for 1.5 h before the addition of the sulfosalicylic acid. Amounts of individual thiols were normalized per mg protein in whole cell extract.

HPLC (HPLC system; Shimadzu Scientific Instruments, Inc., Columbia, MD) with fluorescent detection was used to determine intracellular low-molecular-weight thiol levels after previously published assays (17). Fifty pmol to 100 nmol of standard thiols (NAC, GSH, γGlc, and cysteine) were derivatized with NPM and resolved using a 15-cm C18 Reliasil column (Column Engineering, Ontario, CA) to generate standard curves. Retention times of individual thiols were determined to be 7.7 min (NAC), 20.1 min (GSH), 24.7 min (γGlc), and 30.9 min (cysteine) using this system. Total cellular protein extracts prepared from control and NAC-treated cells were treated with NPM and were analyzed by HPLC. All of the biochemical determinations were normalized to the protein content of whole-cell homogenates by using the method of Lowry et al. (20).

Measurement of Intracellular Prooxidant Production. MEF cells were trypsinized and pelleted at 200–400 × g for 5 min and the pellets were resuspended in 1 ml of PBS supplemented with 5.5 mM glucose at 37°C. Cells were stained with 10 μM DFH-DA (Molecular Probes, Eugene, OR) for 15 min at 37°C. DFH-DA stock solutions were prepared in DMSO, and cells that were stained with 0.2% DMSO alone were included as a negative control for DFH-DA staining. DFH-DA is a low-molecular-weight nonfluorescent and nonpolar compound that enters cells freely. On entering the cell, the intracellular esterases cleave the diacate bond, resulting in a polar and nonfluorescent compound DFH. Intracellular prooxidants oxidize DFH to the fluorescent compound DF. The intensity of green fluorescence of DF is an indicator of the levels of prooxidant production. Changes in DFH fluorescence were detected using a FACS (Becton Dickinson, San Jose, CA) equipped with a 15-mW and 488-nm argon-ion laser. Excitation was performed at 488 nm and emission at 530 nm and 30-nM band pass filter. Data from 20,000 cells were collected, and geometric mean fluorescence was calculated using the Cell Quest software (Becton Dickinson). MFs of the unstained cell populations were subtracted from the MFs of the stained cell populations, and the variations in geometric MFs were calculated relative to control cells at the time of experimental manipulations. The specificity of prooxidant-mediated changes in DFH fluorescence was determined by staining cells in duplicate dishes with carboxy DCFDA [C-369, 5-(and-6)-carboxy-2′,7′-dichlorofluorescin diacetate (Molecular Probes, Eugene, OR)]. Carboxy DCFDA is an oxidation-insensitive compound that is nearly chemically identical to the oxidation-sensitive compound DFH-DA except that it lacks the oxidation-sensitive site. Thus, it is an appropriate control for changes in DFH-DA uptake, esterase cleavage, or efflux that might result from various experimental manipulations.

DFH fluorescence was also used to measure prooxidant production during the transition of cells from the quiescent phase to the proliferation cycle. Asynchronously growing exponential cultures of MEFs were synchronized by culturing cells in medium containing 0.1% serum for 24 h and serum stimulated to reenter the cell cycle. Cells were harvested at representative times after serum stimulation, stained with DFH-DA, and assayed by flow cytometry as described above. DFH-DA-stained cells were also visualized using a Bio-Rad MRC-600 laser-scanning confocal microscope (Central Microscopy Research and Learning Facility at the University of Iowa).

RESULTS

Redox-sensitive Progression from G1 to S Phase in Exponentially Growing Asynchronous MEFs. A flow cytometry-based BrdUrd pulse-chase assay (Fig. 1) was used to monitor the transit of cells through each phase of the cell cycle. Exponentially growing monolayer cell cultures were pulse-labeled with BrdUrd, an analogue of thymidine, for 30 min and were chased in regular growth medium containing thymidine and cytidine. Thus, cells synthesizing DNA (S-phase cells, BrdUrd positive; Fig. 1A, box I) will incorporate BrdUrd during the...
of cells were identified:

1. **Box 1**, BrdUrd-positive undivided S-phase cells;
2. **Box 2**, BrdUrd-negative G1 cells;
3. **Box 3**, BrdUrd-negative G2 cells;
4. **Box 4**, BrdUrd-positive G1 cells that have completed cell division;
5. **Box 5**, BrdUrd-negative S-phase cells.

The five cell cycle compartments were identified as follows:

- **Box 1**, BrdUrd-positive undivided S-phase cells;
- **Box 2**, BrdUrd-negative G1 cells;
- **Box 3**, BrdUrd-negative G2 cells;
- **Box 4**, BrdUrd-positive G1 cells that have completed cell division;
- **Box 5**, BrdUrd-negative S-phase cells.

Fig. 1. Flow cytometric BrdUrd-pulse-chase assay for measurements of progression through the cell cycle: contour plots of DNA content versus log BrdUrd-FITC histograms of asynchronously growing MEFs and their subdvision into cell cycle compartments. A, exponentially growing, asynchronous population of MEFs analyzed immediately after a 30-min pulse labeling with BrdUrd. B, BrdUrd pulse-labeled cells were followed in culture for 4 h in BrdUrd-free regular growth medium before analysis. Five cell cycle compartments were identified as follows: **Box 1**, BrdUrd-positive undivided S-phase cells; **Box 2**, BrdUrd-negative G1 cells; **Box 3**, BrdUrd-negative G2 cells; **Box 4**, BrdUrd-positive G1 cells that have completed cell division; **Box 5**, BrdUrd-negative S-phase cells.

Entry into BrdUrd-negative S phase

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\text{Number of cells in box 4} = \frac{\text{Mean fluorescence of cells in box 5}}{\text{Mean fluorescence of cells in box 2}} \quad (\text{A})
\]

Transit through S phase

\[
\text{Mean fluorescence of cells in box 1} = -\text{mean fluorescence of cells in box 2} \quad (\text{B})
\]

Transit through S phase, G2, and M

\[
\frac{1}{2} \times \text{number of cells in box 4} \quad (\text{C})
\]

The three parameters were calculated relative to the 0-h untreated control.

Fig. 2 represents HPLC measurements of intracellular low-molecular-weight thiol levels in cells cultured in the absence and presence of NAC. Quantitation of individual thiols (Fig. 2B) was determined by resolving standard solutions of NAC, GSH, γGHC, and cysteine derivatized with NPM and plotting standard curves. Using this method, the amounts of intracellular GSH and cysteine in asynchronously growing exponential cultures of MEFs were determined to be 3.3 ± 0.9 (average ± SD) nmol/mg protein and 0.67 ± 0.16 nmol/mg protein, respectively (Fig. 2C). Intracellular soluble low-molecular-weight thiol levels increased 3–6-fold after a 12-h treatment of exponentially growing MEF cultures with 20 mM NAC (Fig. 2D). NAC-induced increases in intracellular soluble low-molecular-weight thiol levels were calculated to be GSH (11.6 ± 2.1 nmol/mg protein), cysteine (2.6 ± 1.1 nmol/mg protein) and NAC (9.4 ± 0.3 nmol/mg protein). These results show that treatment of MEFs with NAC shifted the intracellular redox state toward a more reducing environment.

In addition, intracellular redox state was also determined by flow cytometric measurements of changes in the fluorescence of a prooxidant-sensitive chemical DFH-DA. Asynchronously growing cultures of MEFs were grown in medium containing 20 mM NAC and were harvested at 4- and 12-h intervals for DFH-DA staining and flow cytometric analysis (Fig. 3). The relative fold change in MFI calculated from the geometric

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\text{Relative Fold MFI} = \frac{\text{MFI of cells cultured in presence of NAC}}{\text{MFI of cells cultured in absence of NAC}} \quad (\text{D})
\]

**Fig. 3.** A decrease in prooxidant-sensitive DFH fluorescence after the exposure of MEFs to NAC. Exponential MEF cultures were treated with or without 20 mM NAC (0, 4, and 12 h) and stained with a prooxidant-sensitive DFH-DA (A) and insensitive C369 (B) probe. Stained cells were analyzed by flow cytometry, and MFI was calculated using CellQuest software. Peak 1 in A, autofluorescence in cells without staining, peaks 2 and 3, fluorescence of cells cultured in absence (−NAC) and presence (+NAC) of NAC for 12 h. **Bottom panels,** means and SDs of relative MFI calculated from three separate experiments. Relative fold-change in MFI in each experiment was calculated relative to 0 h untreated control.

*Transit through S phase or Relative Movement [Begg et al. (24)].*
mean fluorescence of cells grown in the presence of NAC, showed an 
~40% decrease after 12 h of NAC treatment compared with cells grown 
in the absence of NAC (Fig. 3A; P < 0.05). The decrease in DFH 
fluorescence in NAC-treated cells was specific to NAC-induced changes 
in intracellular redox state because staining with the oxidation-insensitive 
C369 compound did not show any significant difference in MFI between 
the cells grown in the absence and the cells grown in the presence of 
NAC (Fig. 3B). Therefore, the NAC-induced decreases in the oxidation 
of the DFH was representative of a shift in intracellular redox state 
toward a more reducing environment and was not caused by changes in 
uptake, ester cleavage, or efflux of the compound.

To determine whether changing the intracellular redox state toward a 
more reducing environment affected progression through G1, S phase, 
and G2+M, a time course study was performed using the BrdUrd 
pulse-chase assay. Asynchronously growing exponential cell cultures of 
MEFs were pulse-labeled with BrdUrd and were chased in regular 
growth medium for 12 h in the absence and presence of NAC. Cell cycle 
phase distributions were analyzed using flow cytometric analysis. Results 
presented in Fig. 4 show that increasing thiol pools during the first 9 h of 
NAC exposure (5, 10, and 20 mM) accelerated entry into S phase by 
~1–1.5 h (Fig. 4A; P < 0.05). However, progression through S phase, 
measured as relative movement (Fig. 4B), was not affected by the 
increase in the thiol pools. Similarly, progression through S phase and 
G2+M, measured as a fraction of BrdUrd-positive cells that have divided 
(Fig. 4C), also showed no difference.

In contrast, continued treatment with NAC for 12 h resulted in a 
significant inhibition of cells entering S phase during both the first 
and second generations (Fig. 4, D–G). In the untreated control group, S 
phase cells at 12 h after BrdUrd labeling in generations 1 and 2 were 
20 ± 0.004% and 28 ± 0.015%, respectively. However, the S-phase 
fraction in NAC-treated cells decreased ~2–3-fold to 7 ± 0.02% in 
generation 1, and 11 ± 0.03% in generation 2 (Fig. 4, F and G). The 
initiation of entry into S phase was also apparent at 24 h of NAC 
treatment (data not shown). These results show that increasing the 
intracellular reduced-thiol pools with NAC did not affect progression 
from late G1 to S phase to G2+M in generation 1. However, progression 
from early G1 in generations 1 and 2 seemed to be inhibited. 
These results suggest that the NAC-sensitive redox signaling event(s) 
initiating progression from G1 to S phase resides early in G1.

To assess whether NAC-induced inhibition in progression from G1 
to S phase is associated with cell death in MEFs, a clonogenic cell 
survival assay was performed. Results from the experiment showed 
that the concentrations of the NAC used in this study did not affect 
cell survival (data not shown), suggesting that the NAC-induced cell 
cycle arrest was not a result of cell death.

To determine whether the NAC-induced G1 arrest could be attributable 
to its thiol antioxidant/radical scavenger properties or its ability 
to increase cellular glutathione content, glutathione synthesis was 
inhibited with 1 mM BSO 1 h before and during the 12-h NAC 
treatment. Cell cycle phase distribution was analyzed by PI staining 
and flow cytometry. Results from this experiment showed that more 
than 75% of the cells reside in G1 in the NAC-treated cells as well as 
cells treated with both BSO and NAC (data not shown). These results 
indicate that NAC-induced G1 arrest is independent of the effect of 
NAC on cellular glutathione levels. These results suggest that the 
antioxidant properties of NAC rather than its effect on glutathione 
levels were the mechanism regulating NAC-induced G1 arrest. Such

![Diagram](image.png)
and NAC levels, cysteine levels decreased from 1.4 ± 0.5 nmol/mg protein at the time of release to 0.2 ± 0.05 nmol/mg protein at 8 h after release. The intracellular cysteine levels decreased from 1.4 ± 0.5 nmol/mg protein at the time of release to approximately control levels of 0.4 ± 0.2 nmol/mg protein at 8 h after release from the NAC-induced G1 block. The percentage of GSSG was 3.4 ± 0.5% at the time of release from the NAC treatment, increased to 11.4 ± 0.5% at 4 h \( (P < 0.05) \) and decreased to 10.3 ± 0.8% at 8 h \( (P < 0.05) \) after release from the NAC-induced G1-arrest (Fig. 6B). The transient increase in percentage GSSG was also associated with an increase in DFH fluorescence. The MFI at the end of the NAC treatment decreased ~55 ± 0.07% compared with control (Fig. 6A, 0 h, \( P < 0.05 \)) and subsequently increased to 78 ± 0.04% of control at 4 h after release from the NAC-induced G1 block (Fig. 6A, 4 h). These results indicate a transient increase in prooxidant production between 2 and 8 h after cells were released from the NAC-induced G1 block. After the transient increase in prooxidant production, ~49 ± 0.06% of the cells entered S phase at 12 h after removal of NAC (Fig. 6, B and C). These results support the earlier finding of redox-regulated event(s) during the entry of G1 cells into S phase (Figs. 4 and 5) and suggest the presence of a prooxidant-stimulated event during progression from G1 to S phase in MEF cultures after release from NAC-induced G1 arrest.

**Redox-regulated Event(s) during G1 Correlates with Changes in G1-Regulatory Protein Levels.** To determine whether the redox-regulated event(s) during G1 impacts on the abundance of G1-regulatory proteins, asynchronously growing exponential cultures of MEFs were cultured in medium containing 20 mM NAC and were harvested at various intervals for immunoblotting. In asynchronously growing control

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**Fig. 5.** Redox-signaling during progression from G1 to S phase is independent of cellular p53 status. A, flow cytometric assay of cell cycle phase distribution in asynchronously growing MEFs with wild-type [clone 12(1), wtp53, left panels] and mutant p53 [clone 10(1), mp53, right panels] phenotypes cultured in absence (top panels) and presence (bottom panels) of 20 mM NAC. Cells were pulse-labeled with BrdUrd at the end of a 12-h NAC treatment and were harvested immediately after labeling for flow cytometric analysis. B, distributions of cell cycle phases in the two cell lines after treatment with the indicated doses (0–20 mM) of NAC. The fraction of G1 in control and 20-mM-NAC-treated cells were as follows: in clone 12(1) wtp53 MEFs, 0.5 ± 0.014 (mean ± SD) and 0.79 ± 0.007, respectively; in clone 10(1) mp53 MEFs, 0.45 ± 0.005 and 0.8 ± 0.009, respectively.

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**Fig. 6.** Prooxidant production after release from NAC-induced G1 arrest is associated with entry of MEFs into S phase. Exponential MEF cultures were treated with 20 mM NAC for 12 h and washed with regular growth medium without NAC. Cells were continued in culture in medium without NAC and were harvested at regular intervals for analysis of glutathione and glutathione disulfide, NAC levels, cysteine levels and DFH fluorescence measurements. Removal of NAC resulted in a decrease in intracellular glutathione levels from 19.7 ± 4 nmol/mg protein at the time of release to 16.2 ± 4 nmol/mg protein at 4 h and 8.9 ± 3.1 nmol/mg protein at 8 h after release. The intracellular cysteine levels decreased from 1.4 ± 0.5 nmol/mg protein at the time of release to approximately control levels of 0.4 ± 0.2 nmol/mg protein at 8 h after release from the NAC-induced G1 block. The percentage of GSSG was 3.4 ± 0.5% at the time of release from the NAC treatment, increased to 11.4 ± 0.5% at 4 h \( (P < 0.05) \) and decreased to 10.3 ± 0.8% at 8 h \( (P < 0.05) \) after release from the NAC-induced G1-arrest (Fig. 6B). The transient increase in percentage GSSG was also associated with an increase in DFH fluorescence. The MFI at the end of the NAC treatment decreased ~55 ± 0.07% compared with control (Fig. 6A, 0 h, \( P < 0.05 \)) and subsequently increased to 78 ± 0.04% of control at 4 h after release from the NAC-induced G1 block (Fig. 6A, 4 h). These results indicate a transient increase in prooxidant production between 2 and 8 h after cells were released from the NAC-induced G1 block. After the transient increase in prooxidant production, ~49 ± 0.06% of the cells entered S phase at 12 h after removal of NAC (Fig. 6, B and C). These results support the earlier finding of redox-regulated event(s) during the entry of G1 cells into S phase (Figs. 4 and 5) and suggest the presence of a prooxidant-stimulated event during progression from G1 to S phase in MEF cultures after release from NAC-induced G1 arrest.

**Redox-regulated Event(s) during G1 Correlates with Changes in G1-Regulatory Protein Levels.** To determine whether the redox-regulated event(s) during G1 impacts on the abundance of G1-regulatory proteins, asynchronously growing exponential cultures of MEFs were cultured in medium containing 20 mM NAC and were harvested at various intervals for immunoblotting. In asynchronously growing control
cells, cyclin D1 protein levels remained high (0 h, Lane 1, Fig. 7A). However, cells grown in the presence of NAC showed approximately a 70% decrease in cyclin D1 protein levels between 6 and 12 h of NAC treatment and remained low at 24 h (Lanes 2–4, Fig. 7A). The decrease in cyclin D1 protein levels in NAC-treated cells at 12 and 24 h was accompanied with an increase in the CDK inhibitor p27 protein levels. Similarly, cyclin D1-dependent Rb phosphorylation, a critical regulatory step in the release of the E2F transcription factor and initiation of S phase, was also differentially regulated in NAC-treated cells. In exponentially growing control cells, hyper- and hypophosphorylated Rb migrated as a diffuse band (0 h, Lane 1, Fig. 7A). In contrast, a faster migrating polypeptide band representative of the hyperphosphorylated form of the Rb protein was observed both at 12 and 24 h in cells grown in presence of NAC (Lanes 3 and 4, Fig. 7A). The alterations in G1-regulatory proteins in cells cultured in the presence of NAC correlated with the inhibition of the prooxidant event in G1 as well as the inhibition in progression from G1 to S phase (Figs. 3 and 4). These results further support the idea that early G1-specific redox-regulated event(s) that are inhibited by NAC might stimulate progression from G1 to S phase in MEF cell cycle.

In a separate experiment, NAC-treated cells were washed with regular cell culture medium and continued in culture in the absence of NAC. Cells were harvested at various intervals after removal of NAC, and G1-regulatory proteins levels were analyzed by immunoblotting. Results presented in Fig. 7B showed that decreases in the thiol pools and transient increases in the oxidation of DFH and percentage of GSSG were followed by an increase in cyclin D1 protein levels between 0 and 10 h after release from the NAC treatment (Lanes 5–7, Fig. 7B). In addition, the protein levels of the CDK inhibitor p27 decreased between 4 and 10 h after release from the NAC-induced G1 block (Lanes 5–7). Consistent with these changes, a slower migrating polypeptide band representing the hyperphosphorylated form of the Rb protein was observed between 4 and 24 h after release from NAC treatment (Lanes 7–8). The reversal of NAC-induced changes in G1-regulatory protein levels accompanies the cells’ subsequent entry into S phase (Fig. 6, B and C). These results clearly suggest that the redox-regulated signaling event(s) during G1 precedes entry into S phase and could impact on the regulation of G1 cell cycle proteins.

The NAC-induced Redox-sensitive Site Responsible for G1 Arrest Resides Early in G1. The NAC-induced redox-sensitive site in progression from G1 to S phase was further mapped in synchronized MEFs by monitoring the G0 to G1 to S-phase transition after mitogenic stimulation of serum-starved cells. Exponentially growing MEFs were reentered into G1 phase by serum starvation for 24 h and then serum stimulated to reenter the cell cycle. Serum-stimulated cells were cultured in the presence or absence of 20 mM NAC. Results presented in Fig. 8 show that ~15% of cells in the control group were synthesizing DNA by 10 h after serum stimulation (Fig. 8A, Control). In contrast, no cells had entered S phase in the NAC-treated group at 10 h after serum stimulation (Fig. 8B, (+) NAC). In the control group, the fraction of cells entering S phase increased to ~38 ± 0.02% and 84 ± 0.03% at 12 and 14 h, respectively (Fig. 8A and C). In contrast, the majority of the cells (~90%) in the NAC-treated group remained in G1 during the same time interval (Fig. 8B and C). These results
support the previous studies using asynchronous cell populations (Fig. 4) and map the NAC-sensitive redox-signaling site to early in G₁ phase. The presence of a redox-signaling event early in G₁ was further evaluated by DFH staining. Cells were synchronized by serum starvation and serum stimulated to reenter the cell cycle. Serum-stimulated cells at various time intervals were stained with DFH-DA for 15 min and assayed by flow cytometry to determine whether changes in proxioxidant production were occurring before entry into S phase. Results presented in Fig. 9A, B, and D, show a transient 1.9 ± 0.12 (mean and SD)-fold increase in DFH fluorescence as measured by MFI of 20,000 cells at 4 h after serum stimulation (Fig. 9A, peak 3) compared with the MFI of cells at the time of serum stimulation (Fig. 9A, peak 2). Interestingly, the transient increase in DFH fluorescence decreased to 1.4 ± 0.12-fold at 6 h after serum stimulation (Fig. 9A, peak 4). DFH fluorescence in serum-stimulated cells was also analyzed by confocal microscopy (Fig. 9C). The frequency of DFH-stained cells was low in cultures collected after 5 min of serum stimulation and stained for 15 min (Fig. 9C, left panel). In contrast, more than 95% of the cells harvested at 4 h after serum stimulation, and stained for 15 min, showed DFH-positive cells (Fig. 9C, right panel). Four to six h after this apparent change in DFH oxidation, ~20–40% cells entered S phase 8–10 h after serum stimulation (Fig. 9D). In contrast, serum-stimulated cells cultured in the presence of NAC did not show any significant changes in DFH fluorescence at 4–6 h after serum stimulation and demonstrated no significant increase in the fraction of S-phase cells at 8–10 h poststimulation (Fig. 9, B and D). These results show that the transient increase in proxioxidant production (as determined by MFI) after serum stimulation is an early event in G₀ to G₁ transition that clearly precedes the entry of the cells into S phase. Furthermore, treatment with a thiol antioxidant, NAC, which suppressed the apparent increase in proxioxidant production, inhibited entry into S phase after serum stimulation. These results suggest a causal relationship between the proxioxidant production during G₁ and progression of the cells into S phase.

**DISCUSSION**

Alterations in intracellular oxidation/reduction (redox) reactions are believed to play a regulatory role in various cellular and molecular events including modulation of protein activities, signal transduction, cell death, and cell proliferation (9, 25–33). Depletion of low-molecular-weight thiols such as L-cysteine and glutathione has been shown to suppress thymidine uptake in natural killer cells (30). Lipid peroxide-induced imbalance of GSH/GSSG in human colon cancer CaCo-2 cells is known to arrest cell proliferation in G₀–G₁ (31). Intracellular glutathione levels have also been reported to vary during the mouse fibroblast cell cycle being maximal in mitotic cells (33). Although these previous studies provide some evidence for a mechanistic link between intracellular redox reactions and cell proliferation, the direct role of intracellular redox state during progression through specific phases of the cell cycle as well as the specific cell-cycle-regulatory processes that may be redox regulated are not completely understood.

In the present study, a membrane-permeate aminothiol, NAC, was used to modulate intracellular redox state and to determine whether such a manipulation affects progression through specific phases of the cell cycle. NAC is a well-known thiol antioxidant as well as a precursor to intracellular glutathione synthesis via its ability to augment cysteine pools. Glutathione is the major low-molecular-weight intracellular thiol that acts in a redox-buffering capacity and in general is used as an indicator of changes in intracellular redox state. Treatment of exponential MEFs with NAC resulted in an increase in intracellular glutathione and cysteine levels, indicating a shift in intracellular redox state toward a more reducing environment. NAC-induced changes in intracellular redox state toward a more reducing environment were also supported by a decrease in relative MFI of a proxioxidant-sensitive compound, DFH-DA. The shift toward a more reducing environment did not affect the transit of cells through S phase, G₂, and M. In fact, cells from mid- to late G₁ appeared to accelerate entry into S phase in the first generation (Fig. 4A). However, cells after the completion of division were unable to progress into S phase and showed an early G₁ arrest in the second generation (Fig. 4, D–G). These results clearly indicate that an oxidation event early in G₁ may be a critical regulatory step in the progression of cells from G₁ to S phase.

The mapping of the redox-signaling event(s) to early in G₁ was further verified in synchronized G₀ cells that were serum stimulated to reenter the cell cycle. Detection of a transient increase in DFH fluorescence in serum-stimulated cells at 4 h poststimulation further...
indicated that the redox-sensitive signaling resides early in G1 (Fig. 9). Inhibiting this redox signaling with NAC inhibited entry into S phase. The presence of a redox-signaling event before S phase entry was also observed in cells released from the NAC-induced G1 arrest. A transient increase in DFH fluorescence and the percentage of GSSG observed between 2 and 8 h after the removal of NAC, and, subsequently, more than 50% cells entered S phase at 12 h after release from the NAC-induced G1 arrest (Fig. 6). These results support the hypothesis that a transient increase in intracellular prooxidant levels early in G1 initiates progression from G1 to S phase and suggest that such a redox-signaling event is cyclic in nature in the MEF cell cycle (Fig. 10). Although a transient increase in prooxidant levels would initiate progression from G1 to S phase, antioxidants would inhibit such a progression. Although the identity of the prooxidant(s) was not investigated in this study, it is possible that ROS (i.e., superoxide and hydrogen peroxide), generated during mitochondrial oxidative phosphorylation, and ATP production could act as a signaling mechanism early in G1 in preparation for entry of the cells into S phase. Under normal conditions of cell growth, cellular antioxidant defense mechanisms, including enzymatic scavenging systems (superoxide dismutases, catalase, glutathione peroxidases, thioredoxin peroxidases) and thiol-reducing buffers (small protein- and nonprotein thiols), would neutralize the transient increase in prooxidant(s) levels providing a redox balance and continuation of the progression of cells from G1 to S phase. In contrast, failure to maintain such a redox balance would halt progression and, depending on the severity of the imbalance, could lead to oxidative stress with deleterious effects. In fact, earlier studies have reported fluctuations in the levels of antioxidant enzymes in proliferating cells compared with cells in the quiescent phase (32, 33). Increased levels of superoxide dismutase enzyme in G1 and variations in intracellular thiol levels during cell division (32, 33) support the idea of a regulatory role of an intracellular redox state during progression through the cell cycle.

In many ways such a redox-sensitive regulation in G1 resembles the “restriction point” (34). Whereas growth factors withdrawal after the restriction point would not affect transit through the remainder of G1, S phase, G2, and M phases, withdrawal of growth factors before the restriction point halts progression from G1 to S phase. Similarly, manipulating the intracellular redox state toward a more reducing environment in cells in which the prooxidant event had already occurred would not be expected to affect cells transit through the remainder of G1, S phase, G2, and M phases of the cell cycle (Fig. 10). In contrast, such a manipulation before the prooxidant event would inhibit progression into S phase. Interestingly, many of the growth factors (e.g., platelet-derived growth factor) are known to generate ROS that are believed to be participating in cell growth (7). Additional mapping experiments are needed to determine whether the timing of the redox signaling event and the restriction point overlap. Furthermore, the periodicity of the redox signaling during the cell cycle (Fig. 10) also supports the idea of a redox cycle within the mammalian cell cycle that could link the activity of crucial metabolic processes early in G1 to the activation of cell-cycle-regulatory proteins necessary for entry into S phase. Such a redox control in G1 could function as an intracellular redox-status-sensitive cell cycle checkpoint pathway. Perhaps loss of such an intracellular redox-status-sensitive cell cycle checkpoint would result in uncontrolled cell growth. In fact a recent study reported a preferential sensitivity of thiol antioxidants toward transformed and tumor-derived human cells (35). Additional study is necessary to determine whether this differential response could be caused by an alteration in the redox-sensitive cell cycle checkpoint pathway.

The mechanism by which NAC affects progression from G1 to S phase is unclear. Because simultaneous treatment of MEFs with BSO and NAC did not reverse the NAC-induced G1 arrest both in this study and in hepatic stellate cells (36), these results suggest that the NAC-mediated increase in glutathione levels per se does not cause the G1 arrest. These results, however, do not rule out the possible role of other intracellular low-molecular-weight thiols (i.e., cysteine, thioredoxin, glutaredoxin) in NAC-induced G1 arrest. Indeed cells released from the NAC-induced G1 arrest showed a gradual decrease in cysteine levels, which was accompanied by a transient increase in percentage of GSSG and MFI before entry into S phase. Furthermore, because the DFH fluorescence decreased after NAC treatment (Figs. 3 and 6), and DFH has been shown to be sensitive to intracellular hydroperoxides, our results indicated that the NAC-induced cell cycle arrest in MEFs was not caused by auto-oxidation of NAC (37) to form greater quantities of hydroperoxides. Therefore, it is possible that the NAC-induced G1 arrest in MEFs could be attributable to the antioxidant properties of NAC. Although, such a possibility was ruled out in hepatic stellate cells (36), it was not clear whether transient fluctuations in peroxide level after the release from the NAC-induced G1-arrest did occur under those experimental systems. Moreover, the addition of peroxide to cells that are arrested in G1 might complicate interpretation of results because of peroxide-mediated toxicity. Our results (Figs. 6 and 9) suggest that the redox event early in G1 is transient in nature and is required for entry into S phase.

The antioxidant property of NAC could be directly inhibiting redox reactions on cellular protein thiols present in the G1-S phase cell-cycle-regulatory proteins (Fig. 10). Thus, target proteins modulated by NAC might contain redox-sensitive cysteine residues that could participate in a thiol-disulfide intra- or intermolecular reaction. Although critical cysteine residues in transcription factors (e.g., activator protein and nuclear factor κB) are known to participate in regulating redox-sensitivity of transcription factor DNA-binding activity, such a thiol-disulfide reaction is yet to be clearly identified in G1-cell-cycle-regulatory proteins. However, a recent study has reported a decrease in Cdc25C protein levels after hydrogen peroxide exposure (38). Cdc25 family of dual specific phosphatase regulates activation of CDK activity by removing inhibitory phosphate groups in CDKs. Whereas Cdc25C acts on the activation of the mitotic cyclin/CDK kinase complexes, Cdc25A activates the G1-cyclin/CDK complexes. The authors in this study have shown that double mutations of cysteines at positions 330 and 377 of Cdc25C protein resulted in resistance of the redox modulation as well as inhibited its degradation (38). In the present study, we did not observe any significant changes in Cdc25A protein levels during the NAC-induced G1 arrest.

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**Fig. 10.** A schematic illustration of a redox cycle within the mammalian cell cycle. Prooxidants would initiate progression from G1 to S phase, whereas antioxidants would inhibit these processes. Once the redox event is initiated, the cells would complete the present cell cycle and repeat the process in G1 of the next cell cycle. Target proteins (cyclin D1, p27, Rb, and so forth), modulated by NAC, may react via the cysteine residues in these proteins that could participate in a thiol-disulfide intra- or intermolecular reaction. CKI, CDK inhibitor; E2F, transcription factor; P, phosphate.
Any possible role of these cysteines in redox regulation of G₁ to S with the cysteines in the Rb B-domain. There are also two cysteine D-type cyclin (39). The Rb protein has 15 cysteine residues, and three one such mechanism could include alterations in the redox status of mechanisms associated with redox signaling early in G₁, it is possible that 3–transient inhibition of cyclin D1 in cell lines shortens G₁, and is sufficient for cells arrested in G₁ to complete the cell cycle. Proc. Natl. Acad. Sci. USA, 91: 8022–8026, 1994.


Induced inhibition in the redox signaling resulted in decreased cyclin D1 protein levels, increased p27 protein levels, and hypophosphorylation of Rb. In contrast, reestablishment of the redox-signaling event by release from NAC led to decreases in p27 protein induction in MFI of oxidation-sensitive probe is much lower (2–9-fold increase; data not shown). This difference in results could be because, although.

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Yamauchi, A., and Bloom, E. T. Control of cell cycle progression in human normal killer cells through redox regulation of expression and phosphorylation of retinoblas-


REFERENCES

1. Shibanuma, M., Kuroki, T., and Nose, K. Induction of DNA replication and expression of proto-oncogenes c-myc and c-fos in quiescent Balb/3T3 cells by xanthine-


2. DeHaan, J. B., Cristiano, F., Lamello, R., Blader, C., Kelner, M. J., and Kola, I. Elevation in the ratio of Cu/Zn superoxide dismutase to glutathione peroxidase activity induces features of cellular senescence and this effect is mediated by hydro-


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