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 thiol 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 G₀-G₁ 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 transitions. 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). ROS¹ including 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 stimula-}

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

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¹ 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 fibroblasts; BSO, broad spectrum of sulfhydryl inhibitors; HPLC, high-performance liquid chromatography; GSH, reduced glutathione; GSSG, oxidized glutathione; γGCG, γ-glutamyl cysteine; DHF-DA, dihydrofluorescein diacetate; PI, propidium iodide; FACS, fluorescence-activated cell sorter.

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5% CO₂. Stock solutions of NAC (Sigma Chemicals) were adjusted to pH 7.0 with sodium bicarbonate, and appropriate aliquots were added to the cell culture medium. The toxicity of NAC was determined by single-cell colony-forming assay, and no toxicity was observed in the concentration ranges (1–20 mM) 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, γGCC, 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 (γGCC), 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 diacete bond, resulting in a polar and nonfluorescent 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 15-mW and 488-nm excitation wavelengths. Red fluorescence from PI was detected through a 640-nm long pass filter, and green fluorescence from FITC was detected through a 525-nm band pass filter. Data from a minimum of 20,000 nuclei were acquired in list mode and were processed using Cytomation software (Cytomation, Ft. Collins, CO). The acquired data were displayed as dual-parameter PI versus log-FITC histograms and three compartments (BDU-negative S phase cells, BDU-negative G₁, and BDU-negative G₂ phases) identified using Cytomation software. The fraction of cells in G₁ and G₂ phases were measured as a measurement of G₁ and G₂ transit time. Transit through S phase was measured by determining relative movement as described earlier by Begg et al. (24). Relative movement was calculated from the mean DNA content of undivided BDU-positive cell population normalized to mean DNA content of G₁ and G₂ populations. The fraction of BDU-positive cells that had divided was also calculated for measurements of the transit of the cells through S phase, G₁, G₂, and G₃.

For staining with PI only, cells were harvested by trypsinization and fixed in 70% ethanol. Approximately 1 × 10⁶ cells were incubated with 100 μl of RNase A for 30 min at room temperature followed by staining with PI (30 μg/ml) for 60 min. Samples were analyzed by flow cytometry, and cell cycle phase distribution was determined using CellQuest software (Becton Dickinson, Immunocytemetry Systems).

Immunoblotting. Total cellular proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (BIORAD Labs.). Blots were incubated with antibodies to cyclin D1 (G124–326; PharMingen), p27 (sc-1641; Santa Cruz Biotechnology), and Rb (G3–245; PharMingen). Immunoreactive polypeptide was visualized using horseradish peroxidase-conjugated secondary antibodies and enhanced-chromiluminescence detection reagents (Amersham Pharmacia Biotech.) following manufacturer-supplied protocols. Blots were reprobed with antibodies to actin (sc-1615; Santa Cruz Biotechnology) for comparison of results.

Data Analysis. Experiments were repeated two or three times, and data were calculated as means, SDs, and SE. Statistical comparisons were carried out using two-tailed Student’s t test and ANOVA. Ps were calculated with 95% confidence intervals.

RESULTS

Redox-sensitive Progression from G₁ to S Phase in Exponentially Growing Asynchronous MEFs. A flow cytometry-based BDU 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 BDU, 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, BDU positive; Fig. 1A, box I) will incorporate BDU during the
of cells were identified:

**Box 4**
- **Transit through S phase**: G2, and M

**Box 5**
- **Transit through S phase**: G1, positive S-phase cells; Box 4, BrdUrd-negative G1 cells entering S phase. Using this assay, we determined three parameters (23, 24) by measuring the progression of cells through different phases of the cell cycle (see Fig. 1B):

**Entry into BrdUrd-negative S phase**

\[
\text{Mean fluorescence of cells in box 5} = \frac{\text{Mean fluorescence of cells in box 2}}{\text{Mean fluorescence of cells in box 2}}
\]

**Transit through S phase**

\[
\text{Mean fluorescence of cells in box 1} = \frac{\text{Mean fluorescence of cells in box 2}}{\text{Mean fluorescence of cells in box 3}}
\]

**Transit through S phase, G2, and M**

\[
\frac{1}{2} \times \text{Number of cells in box 4} = \text{Number of cells in box 1} + \frac{1}{2} (\text{number of cells in box 4}) - \text{mean fluorescence of cells in box 2}
\]

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, γGCG, 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 ~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 labeling period, whereas both G1 (Fig. 1A, box 2) and G2 (Fig. 1A, box 3) phases will show BrdUrd-negative staining. Fig. 1B shows a representative histogram 4 h after the BrdUrd-labeling. Two additional groups of cells were identified: box 4 (Fig. 1B), BrdUrd-positive cells with G1 DNA content that have completed cell division; and box 5 (Fig. 1B), BrdUrd-negative G1 cells entering S phase. Using this assay, we determined three parameters (23, 24) by measuring the progression of cells through different phases of the cell cycle (see Fig. 1B):

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\]

**Transit through S phase**

\[
\text{Mean fluorescence of cells in box 1} = \frac{\text{Mean fluorescence of cells in box 2}}{\text{Mean fluorescence of cells in box 3}}
\]

**Transit through S phase, G2, and M**

\[
\frac{1}{2} \times \text{Number of cells in box 4} = \text{Number of cells in box 1} + \frac{1}{2} (\text{number of cells in box 4}) - \text{mean fluorescence of cells in box 2}
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The three parameters were calculated relative to the 0-h untreated control.

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

![Image](cancerres.aacrjournals.org)
Similarly, the fraction of G1 in control cells were 0.45 and increased to 0.6 in 20 mM NAC-treated cells were as follows: in clone 12(1) wtp53 MEFs, 0.5 ± 0.02 and 0.8% after exposure to 5 and 20 mM NAC, respectively. 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, A 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 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, A 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.

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- 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.

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.

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 DFH staining (A), thiol assays (B), and measurements of S-phase distribution (C). A, relative fold change in MFI of DHF-stained cells at the time of release (0 h) and 4 h after the removal of NAC was calculated relative to the untreated control. B, percentage GSSG and fraction of S phase measured in cells at various intervals after the removal of NAC. The changes in percentage of GSSG was statistically significant as determined by two-tailed Student’s t test (P < 0.05). C, representative PI versus log BrdUrd-FITC histograms of cells at the end of the NAC treatment (0 h, left histogram), and 12 h after removal of NAC (right histogram); arrowhead, position of S-phase cells.

an antioxidant property of NAC could be directed in the inhibition of redox reactions occurring on cellular protein thiols.

Redox-sensitive Progression from G1 to S Phase in Exponentially Growing MEFs Is Independent of Cellular p53 Status. To determine whether the redox-sensitive progression from G1 to S phase depends on p53 status, asynchronously growing exponential cultures of MEFs [clone 12(1), wild type p53; and clone 10(1) containing mutant p53] were treated with 1–20 mM NAC for 12 h and were pulse-labeled with BrdUrd before harvest. Flow cytometric analysis of cell cycle phase distribution showed that asynchronous cells in the absence of NAC had a normal cell cycle distribution with 34 ± 0.014% and 39 ± 0.007% S-phase cells in the wild-type and mutant p53 cells, respectively (Fig. 5A, upper panels, and B, open bar graphs). However, both cell lines showed less than 5% S phase cells after 12 h of 20-mM NAC treatment, which indicated a G1 delay in both cell lines (Fig. 5A, lower panels, and B). The fraction of G1 in both cell lines increased with 5 mM of NAC and became maximal (~0.7–8.8%) with 20 mM of NAC (Fig. 5B). The fraction of G1 in control 12(1) cells was 0.5 ± 0.014 and increased to 0.7 ± 0.02 and 0.79 ± 0.007 after exposure to 5 and 20 mM NAC, respectively. Similarly, the fraction of G1 in control 10(1) cells were 0.45 ± 0.005 and increased to 0.6 ± 0.002 and 0.8 ± 0.009 after incubation with 5 and 20 mM NAC, respectively. These results indicate that the NAC-sensitive progression from G1 to S phase in MEF cell cycle is independent of cellular p53 status.

Prooxidant Production after Release from NAC-induced G1 Block Is Associated with MEFs Entry into S Phase. To further investigate the presence of a NAC-sensitive redox event(s) before S phase entry, exponentially growing asynchronous cultures of MEFs were incubated with 20 mM NAC for 12 h and were released from the NAC-induced G1 block by washing the monolayer with regular cell culture medium without NAC. Cells were continued in culture in the absence of 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, A 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.

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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, cyclin D1 protein levels remained high (0 h, Lane 1, Fig. 7A). In contrast, a faster migrating polypeptide band representing 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 recruited into G0 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. 8, A and C). In contrast, the majority of the cells (~90%) in the NAC-treated group remained in G1 during the same time interval (Fig. 8, B 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. 9, A 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 G₁ to G₂ 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 was 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 phosphatase 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.
NAC-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 levels, increases in cyclin D1 protein levels, and an increase in Rb phosphorylation with subsequent entry into S phase. Whereas additional studies are needed to understand the precise molecular mechanisms associated with redox signaling early in G1, it is possible that one such mechanism could include alterations in the redox status of redox-sensitive thiol-disulfide reactions at critical cysteine residues in cyclin D1, p27, and Rb proteins. In fact the B domain of the Rb protein is a site critical for binding to the LXCXE motif found in the D-type cyclin (39). The Rb protein has 15 cysteine residues, and three of these cysteines are located within the B domain (GeneBank accession no. M33647 J02994). It is not clear whether cysteine in the LXCXE motif of cyclin D1 forms an intermolecular disulfide linkage with the cysteines in the Rb B-domain. There are also two cysteine residues within the cyclin A binding domain of p27 (40). Although any possible role of these cysteines in redox regulation of G1 to S phase is speculative at present, it is reasonable to suggest that thiol-redox reactions could act as “sulfhydryl switches” that reversibly modulate cell-cycle-regulatory protein activity.

In summary, results from this study indicate the presence of a redox-signaling event early in G1, which is required for entry into S phase. Although shifting of the intracellular redox state toward a more reducing environment selectively inhibited the entry of early G1 cells into S phase, a shift toward an oxidizing environment seemed to stimulate entry into S phase. The periodicity of such a redox cycle within the mammalian cell cycle may provide a mechanistic link between metabolic processes early in G1 and the activation of G1 regulatory proteins in preparation for the entry of cells into S phase.

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