Glutathione Regulates Telomerase Activity in 3T3 Fibroblasts*

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Changes in telomerase activity have been associated either with cancer, when activity is increased, or with cell cycle arrest when it is decreased. We report that glutathione, a physiological antioxidant present at high intracellular concentrations, regulates telomerase activity in cells in culture. Telomerase activity increases in 3T3 fibroblasts before exponential cell growth. The peak of telomerase activity takes place 24 h after plating and coincides with the maximum levels of glutathione in the cells. When cells are treated with buthionine sulfoximine, which decreases glutathione levels in cells, telomerase activity decreases by 60%, and cell growth is delayed. Glutathione depletion inhibits expression of E2F4 and Id2, which regulate the cell cycle. When glutathione levels are restored after incubation with glutathione monoethylester, telomerase activity and the cell cycle-related proteins return to control values. To discover the effect of glutathione redox status on the telomerase multicomplex structure, we incubated protein extracts from fibroblasts with different glutathione redox buffers. Telomerase activity is maximal under reduced conditions i.e. when the reduced/oxidized glutathione ratio is high. Consequently glutathione concentration parallels telomerase activity. These results underscore the main role of glutathione in the control of telomerase activity and of the cell cycle.

The eukaryotic chromosomes are capped by telomeres, which consist of telomeric DNA repeated in tandem, associated with several proteins. These structures play an important role in the stability and the complete replication of the chromosomes. Conventional DNA polymerases cannot fully replicate the 3' end of the lagging strand of linear molecules, and therefore in every cell division telomeric sequences are lost (1).

Telomerase is an important enzyme that ensures the maintenance of normal telomere length. This activity is high in human cancers (2) but virtually absent in normal tissues, except in telomerase activity and of the cell cycle.

Although the role of glutathione in the regulation of the telomerase activity in cells is well-known, it is not known whether the changes in telomerase activity are associated with changes in glutathione levels. We have observed that the peak of telomerase activity takes place 24 h after plating, and coincides with the maximum levels of glutathione in the cells (3). When cells are treated with buthionine sulfoximine, which decreases glutathione levels in cells, telomerase activity decreases by 60%, and cell growth is delayed. Glutathione depletion inhibits expression of E2F4 and Id2, which regulate the cell cycle. When glutathione levels are restored after incubation with glutathione monoethylester, telomerase activity and the cell cycle-related proteins return to control values. To discover the effect of glutathione redox status on the telomerase multicomplex structure, we incubated protein extracts from fibroblasts with different glutathione redox buffers. Telomerase activity is maximal under reduced conditions i.e. when the reduced/oxidized glutathione ratio is high. Consequently glutathione concentration parallels telomerase activity. These results underscore the main role of glutathione in the control of telomerase activity and of the cell cycle.

EXPERIMENTAL PROCEDURES

Cell Culture—3T3 fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin (100 units/ml penicillin, 25 μg/ml streptomycin), and 0.3 μg/ml amphotericin B in 5% CO2 in air at 37 °C in 25 or 75 cm2 flasks. In experiments using BSO to deplete cellular GSH, fibroblasts were cultured with and without BSO (10 μM) for 24 or 48 h. To replenish intracellular GSH levels, we cultured fibroblasts with and without glutathione monoethylester (0.2 mM) for 24 or 48 h.

Flow Cytometric Analysis—Flow cytometric analysis was performed using an Epics Elite cell sorter (Coulter Electronics, Miami, FL). Fluoro-compound were excited with an argon laser tuned to 488 nm. Forward angle and right angle light scattering were measured. Samples were acquired for 15,000 individual cells. Cell cycle phases (S + G1/M + apoptosis) were determined using the fluorescent dye propidium iodide (final concentration, 5 μg/ml) at 630 nm fluorescence emission.

Trypan Blue Dye Exclusion Method—3T3 cell viability was determined using the vital dye trypan blue, which is excluded by living cells but accumulates in dead cells. Cells were counted after they were trypsinized and incubated 1:1 with 0.1% trypan blue.

Determination of Reduced Glutathione— Cultured fibroblasts were harvested, washed, and resuspended in 15% trichloroacetic acid, EDTA 1 mM. GSH was measured spectrophotometrically using the glutathione reductase assay (7).

Quantification of Telomerase Activity—Telomerase activity was determined by a photometric enzyme immunoassay, using the telomeric repeat amplification protocol (8) Roche Diagnostics supplied us the TeloTAGGG Telomerase PCR ELISAPLUS® kit. Results are expressed as relative telomerase activity (RTA) versus a control template that contains eight telomeric repeats.

Immunoblot Analysis of Cell Cycle Proteins Id2, E2F1, and E2F4— Aliquots of cell lysate (40 μg) were immediately boiled for 10 min to inactivate proteases and phosphatases, electrophoresed in SDS-10 or 12.5% polyacrylamide gels, and electoblotted (Bio-Rad) onto an Immobilon-P nylon membrane (Invitrogen). Protein content was determined by a modified Lowry method (9). Membranes were incubated with primary antibodies against Id2, E2F1, or E2F4 (Santa Cruz Biotechnology) overnight at 4 °C. Thereafter, the blots were washed three times with a wash buffer (phosphate-buffered saline, 0.2% Tween 20) for 5 min each time at room temperature and then further incubated for 1 h.
with a secondary horseradish peroxidase-linked anti-rabbit IgG antibody (Cell Signaling Technologies). The blots were washed three times as above and developed by using the LumiGLO® Reagent as specified by the manufacturer (Cell Signaling Technologies). Autoradiographic signals were assessed using a Fujifilm scanning densitometer (Fujifilm LAS-1000 plus).

Statistics—Results are expressed as mean ± S.D. Statistical analysis was performed by the least significant difference test, which consists of two steps. First an analysis of variance was performed. The null hypothesis was accepted for all numbers of those set in which $F$ was non-significant at the level of $p \leq 0.05$. Second, the sets of data in which $F$ was significant were examined by the modified $t$ test using $p \leq 0.05$ as the critical limit.

RESULTS

Telomerase Activity Correlates with GSH Levels in Cells in Culture—Fig. 1 shows that changes in telomerase activity closely parallel those of GSH levels in cultured 3T3 fibroblasts. Both GSH levels and telomerase activity increased after plating, peaking at day 1 after culture. Six days after plating, both GSH and telomerase returned to values similar to day 0.

Manipulation of Glutathione Levels Results in Similar Changes in Telomerase Activity—Buthionine sulfoximine inhibits glutathione synthesis and serves as a useful tool to lower GSH levels in cells (10) GSH does not enter into cells, but its monoethylester does and is converted intracellularly into GSH. Table I shows that incubation with buthionine sulfoximine caused GSH levels to fall to 10% of the controls. This was reversed by incubation with GSH monoethylester. These experimental changes in GSH levels were followed by parallel changes in telomerase activity (Table I). In all cases (see Fig. 1 and Table I) when GSH levels fell, telomerase activity followed. Similarly, restoration of normal GSH levels resulted in an increase in telomerase activity.

Redox Modulation of Telomerase Activity—The results reported above prompted us to determine whether telomerase activity is regulated by changes in redox potential. Thus we incubated cell-free extracts with redox buffers ranging from a GSH/GSSG ratio of 1 up to a ratio of 100. Telomerase activity increased significantly when the cell-free extracts were incubated with glutathione buffers containing solutions of GSH and GSSG at ratios in the physiological range (see Fig. 2). Maximal activity was observed when the incubation medium was reduced by dithiothreitol.

Modulation of Glutathione Levels Affects the Rate of Cell Growth—Fig. 3 shows that manipulation of glutathione levels (and consequently of telomerase activity) resulted in changes in the rate of cell growth. Glutathione depletion caused a significant decrease in cell growth, and its repletion returned it to normal values.

The decrease in cellular proliferation is likely because of a decreased percentage of cells in the S+G2/M phase of the cell cycle. No changes were found in the percentage of cell death (either by apoptosis or necrosis) as shown in Table II.

Glutathione Regulates Cycle-related Proteins—The effect of glutathione on the regulation of telomerase, and subsequently of cell growth, prompted us to determine its role on the regulation of key cell cycle proteins. We chose E2F4 and Id2, because both regulate telomerase activity (11–13). Fig. 4 shows that glutathione depletion by incubation with BSO resulted in a significant decrease in E2F4 and Id2 protein expression. This was prevented when normal glutathione levels were restored by co-incubation with glutathione monoethylester. On the contrary, E2F1, whose activity is inversely related to telomerase expression (12), was increased when cells were depleted of glutathione (controls, 100 ± 7 arbitrary units; GSH-depleted, 150 ± 14 arbitrary units, $p < 0.05$)

DISCUSSION

Telomerase plays a key role in cellular homeostasis, because it maintains the length of the telomeres particularly in germinal cells in which it is necessary to keep a normal telomeric length after many cellular divisions. Thus its activity must be regulated. Long term regulation, particularly at the level of transcription and translation, has been studied (see below). However, little is known about the short term regulation of the activity of this important enzyme. The major aim of this paper was to study the short term regulation of telomerase activity.

The transcriptional control of the expression of telomerase is a major contributor to its regulation in many cell types (14–17). Telomerase is controlled by a number of cell cycle proteins. For instance, Crowe et al. (13) reported that E2F1, a transcription factor, was to study the short term regulation of telomerase activity.

![Fig. 1. Correlation between telomerase activity and glutathione levels in 3T3 fibroblasts in culture. The percentage of RTA versus $T_0$ is shown on the left y axis, and the glutathione levels/10^6 cells are shown on the right y axis. Data are expressed as the means for 10 different experiments. The statistical significance is expressed as $**$, $p < 0.01$ versus values at day 0.]

| Table I |
| Manipulation of glutathione levels (GSH) results in parallel changes in RTA in cultured 3T3 fibroblasts |
| Results for glutathione levels are expressed as mean ± S.D. for five different experiments. RTA is expressed as the percentage versus $T_0$ and was determined as described under "Experimental Procedures." Results are expressed as mean ± S.D. for 4–6 different experiments. |
| | 24 h in culture | 48 h in culture |
| | GSH | RTA | GSH | RTA |
| | nmol/mg protein | | nmol/mg protein | |
| Control | 20 ± 6 | 170 ± 80 | 16 ± 8 | 151 ± 70 |
| 10 μM BSO | 4 ± 2a | 60 ± 19a | 6 ± 3a | 60 ± 7a |
| 10 μM BSO + 0.2 mM GSH ester | 21 ± 18a | 87 ± 6b | 11 ± 1b | 71.4 ± 1.6b |

*a* $p < 0.01$ versus control.  
*b* $p < 0.05$ versus BSO-treated.
factor involved in activation of genes associated with the cell cycle, binds to the human telomerase reverse transcriptase promoter and represses its activity. On the contrary, E2F4 and Id2 are positively related to the activity of telomerase (11, 12). We find here that intracellular glutathione activates both Id2 and E2F4 and that on the contrary it inactivates E2F1. Thus it appears that there is a common regulation between the activity of telomerase and the activity of cell cycle proteins.

We have found that telomerase is regulated by changes in the thiol redox state. There were some indications that this might be the case. Minamino et al. (18), using vascular smooth muscle cells, reported that hypoxia up-regulates telomerase activity. Hypoxia is known to lower oxidative stress and thus to increase levels of glutathione. On the other hand, 2-[3-(trifluoromethyl)phenyl]isothiazolin-3-one, a specific inhibitor of telomerase, acts by reacting with a key cysteine residue, which is essential for telomerase activity and must be kept reduced. Dithiothreitol reverses this inhibition (19). Thus a critical cysteine residue appears essential for telomerase activity. This cysteine must be kept reduced for full telomerase activity. It is likely that the glutathione redox potential may be important to maintain the cysteine residue in a reduced state.

Our results here indicate two facts. Telomerase is regulated by changes in glutathione redox potential at values that are...
similar to those in vivo (20, 21) (see Fig. 2), and changes in telomerase are modulated in coordination with changes in critical cell cycle proteins, particularly Id2 and E2F4. Thus modulation of telomerase occurs in conjunction with changes in cell cycle regulatory proteins.

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