Glutathione Is Recruited into the Nucleus in Early Phases of Cell Proliferation*

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We have studied the possible correlation between nuclear glutathione distribution and the progression of the cell cycle. The former was studied by confocal microscopy using 5-chloromethyl fluorescein diacetate and the latter by flow cytometry and protein expression of Id2 and p107. In proliferating cells, when 41% of them were in the S+G2/M phase of the cell cycle GSH was located mainly in the nucleus. When cells reached confluence (G0/G1) GSH was localized in the cytoplasm with a perinuclear distribution. The nucleus/cytoplasm fluorescence ratio for GSH reached a maximal mean value of 4.2 ± 0.8 at 6 h after cell plating. A ratio higher than 2 was maintained during exponential cell growth. In the G0/G1 phase of the cell cycle, the nucleus/cytoplasm GSH ratio decreased to values close to 1. We report here that cells concentrate GSH in the nucleus in the early phases of cell growth, when most of the cells are in an active division phase, and that GSH redistributes uniformly between the nucleus and the cytoplasm when cells reach confluence.

Glutathione (GSH) is the most abundant non-protein thiol in mammalian cells and performs many physiological functions (1). We have reported that cellular glutathione decreases in apoptosis (2).

Although the role of nuclear GSH in the synthesis of DNA (3) and in protection against oxidative damage or ionizing radiation (4) is well established, little is known about the concentration of GSH in the nucleus and its regulation. This is due to two main factors. The first is methodological: it is impossible to determine the nuclear concentration of GSH using standard cell fractionation and analytical approaches (for a review see So¨derdahl et al. (5). In view of this problem, we used confocal microscopy.

The second factor is that, if not all, of the reports share the common view of nuclear GSH distribution in a static situation. Cells are usually studied under steady state conditions i.e. when they are confluent (G0/G1 phase of the cell cycle). The nucleus changes dramatically during the different phases of the cell cycle. Thus, studies addressed to determining the nuclear GSH distribution must take cell cycle physiology into account.

To our knowledge there is a lack of information about the cellular distribution of glutathione during the different phases of the cell cycle and the possible correlation between cellular growth and nuclear GSH levels. We report here that GSH concentrates in the nucleus in the early phases of cell growth, when most of the cells are in an active division phase, and it redistributes uniformly between nucleus and cytoplasm when cells reach confluence. Nuclear Bcl-2 may be responsible for this change, as its expression changes in parallel with glutathione levels in nuclei.

EXPERIMENTAL PROCEDURES

Cell Culture

3T3 fibroblasts were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics (25 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.

Enzymatic Determination of Reduced Glutathione

Cultured fibroblasts were washed with phosphate-buffered saline, and acid GSH extracts were obtained in 6% perchloric acid containing 1 mM EDTA. GSH was measured spectrophotometrically using the glutathione-S-transferase assay (6).

Determination of GSSG/GSH

Determination of reduced (GSH) and oxidized glutathione (GSSG) was carried out using the high-performance liquid chromatography method with UV-visible detection, which we developed to measure GSSG in the presence of a large excess of GSH (7). The essence of this method consists of minimizing GSH oxidation, which otherwise would result in a large increase in GSSG.

Flow Cytometric Analysis

Analyses were performed using an Epics Elite cell sorter (Coulter Electronics, Miami). Fluorochromes were excited with an argon laser tuned at 488 nm. Forward angle and right angle light scattering were measured. Samples were acquired for 15,000 individual cells. Cell cycle phases were determined using the fluorescent DNA dye propidium iodide (final concentration, 5 μg/ml) at 630 nm fluorescence emission (8).
**Fluorescence Analyses**

The distributions of green CMFDA fluorescence (GSH levels) and blue Hoechst fluorescence (nuclei localization) were analyzed by profile and by area as follows.

**Fluorescence Profile Analysis**—A cross-section line of 200 ± 20 µm was drawn through a cell field to compare the intensity and distribution of Hoechst (DNA) and CMFDA (GSH) fluorescence. The graphs obtained from the quantification were overlaid using the following color code: blue for nuclei localization (Hoechst fluorescence) and green for GSH distribution (CMFDA fluorescence).

**Fluorescence Area Analysis**—Perimeters were drawn around the nucleus (according to the area marked with Hoechst) and around the entire cell excluding nucleus area (according to transmission image obtained by light microscopy). The nucleus/cytoplasm ratio for GSH in every cell analyzed was established by dividing the mean of green CMFDA fluorescence of nuclear area by the mean of CMFDA fluorescence in cytoplasmic area. We calculated the nucleus/cytoplasm ratio (n/c) using CMFDA fluorescence in three separate experiments (100 cells/condition).

**Immunoblot Analysis of Cell Cycle Proteins Id2 and p107**

Aliquots of cell lysates (40 µg) were immediately boiled for 10 min to inactivate proteases and phosphatases, electrophoresed in SDS-10 or 12.5% polyacrylamide gels, and electroblotted (Bio-Rad) onto Immobilon-P nylon membranes (Invitrogen). Protein content was determined by a modified Lowry method (10). Membranes were blocked with 0.05g/ml skimmed milk in TBS-0.2% Tween 20 (TBST), washed three times at room temperature, and incubated with primary antibodies against Id2 (1:1000) and p107 (1:1000) (both from Santa Cruz Biotechnology) in TBST with 0.05g/ml bovine serum albumin overnight at 4 °C. Thereafter, the blots were washed again with TBST and further incubated for 1h with a secondary horseradish peroxidase-linked anti-rabbit IgG antibody (1:2000) (Cell Signaling Technologies). After washing with TBST as above, blots were developed by using the LumiGLO® reagent as specified by the manufacturer (Cell Signaling Technologies).

**Immunoblot Analysis of Nuclear Proteins**

3T3 cells were subjected to nuclear protein extraction for the determination of protein oxidation and protein glutathiolation following the instructions for the nuclear extract kit (Active Motif North America). The efficiency of nuclear extraction was determined by measuring the relative activity of a cytosolic enzyme, lactate dehydrogenase, in the nuclei and in whole cell. Nuclear lactate dehydrogenase activity was less than 1.2% of the total cellular activity.

To measure the level of nuclear protein oxidation, samples were lysed and derivatized, and Western blotting was performed according to the recommendation of the manufacturer. The level of nuclear protein oxidation was determined by the Oxy Blot protein oxidation detection kit (Chemicon International), which detects carbonylated proteins.

**Nuclear Protein Glutathiolation Level**

Nuclear lysates were obtained in the absence of reductive agents. Western blotting procedure was performed as described previously, and the membrane was probed against anti-glutathione antibody (1:1000; Virogen) at 4 °C overnight.

**Presence of Bcl-2 and γ-Glutamylcysteine Synthetase (GCS) in the Nuclear Lysate**

Nuclear lysates were obtained, and Western blotting procedure was performed as described previously. Membrane was probed against anti-bcl2-antibody (1:1000; rabbit polyclonal IgG, Santa Cruz Biotechnology) or, alternatively, against anti-GCS antibody (1:750; NeoMarkers, rabbit polyclonal antibody). Autoradiographic signals were assessed using a Fujifilm scanning densitometer (Fujifilm LAS-1000 plus).

**ATP Depletion by Mitochondrial Uncoupling Agent**

Cells were incubated for 30 min to 2 h with freshly prepared 20 µM p-trifluoromethoxy carbonyl cyanide phenylhydrazonel.4 The abbreviations used are: PI, propidium iodide; CMFDA, 5-chloromethylfluorescein diacetate; TBS, Tris-buffered saline; TBST, TBS with Tween 20; FCCP, p-trifluoromethoxy carbonyl cyanide phenylhydrazone; GCS, γ-glutamylcysteine synthetase.
drazone (FCCP; Sigma), loaded with CMFDA, PI, and Hoechst, and analyzed by confocal microscopy as described above.

Statistics
Results are expressed as mean ± S.D. The statistical analysis was performed using the least significant difference test with analysis of variance. The null hypothesis is accepted for all numbers of those set in which $F$ is nonsignificant at the level of $p \leq 0.05$.

RESULTS
We assessed the changes in nuclear GSH compartmentalization in different phases of the cell cycle. Twenty-four hours after plating, 3T3 fibroblasts begun to grow exponentially (Fig. 1); at 24 and 48 h after plating the percentage of cells at the S/M/G2 phase increased significantly (41 and 35%, respectively). At 72 h or later, cell growth slowed, and 6 days after plating, when the cells reached confluence, most of the cells (81%) were in the G0/G1 phase.

GSH concentration in whole cells was maximal (20 nmol/10⁶ cells) at 24 h after plating (Fig. 2A) when a high percentage of cells (41%) was entering or preparing for division. However when the cell population reached confluence (day 6) and most cells were quiescent, GSH concentration was at a low level (11 nmol/10⁶ cells). We determined the GSH/GSSG ratio in cells during the cell cycle and found that it does not change significantly (see Fig. 2B).

In view of these results, we studied whether nuclear distribution of GSH might have a role in the progression of the cell cycle during the early phases of cell culture. Fig. 3 shows the cell cycle-dependent GSH distribution in 3T3 fibroblasts. Six hours after plating, few cells could be seen on the plate (see Fig. 3, a and b). The blue staining of the nuclei with Hoechst 33342 is shown in Fig. 3b, and green (glutathione) staining is shown in panel c. Magnified detail of glutathione staining (Fig. 3d) shows that the highest fluorescence is located in the nucleus. We drew a white line throughout the cell field. The number of cells that the line crosses shows a quantification of the fluorescence per each cell. Fig. 3e shows that the nuclei blue staining (blue peaks in the graph) co-localizes with the green glutathione staining (green peaks in the graph) at the beginning of the culture. At 12 and 24 h of culture, cells continued to populate the plate. Cellular glutathione (CMFDA green fluorescence intensity) was maximal at 24 h. Analysis of the blue/green fluorescence (Fig. 3, b and d, respectively) distribution and intensity showed a maximum correlation of blue/green fluorescence at 24 h. When cells were confluent after 72 h or 5 days in culture, no correlation among the peaks of green and blue fluorescence could be found.

The increase over culture time in total cellular staining for CMFDA correlated with the peak of glutathione concentration (20 nmol/10⁶ cells) determined spectrophotometrically (see Fig. 2). As expected, green (GSH) fluorescence clearly decreased at 72 h and 5 days in culture when cell culture reached confluence, cell-to-cell growth inhibition took place, and as a consequence the cell population entered quiescence (70–80% G0/G1). At these stages no significant difference could be seen between the nucleus and cytoplasm, and there was a homogenous green (CMFDA) pattern on the plates. Thus, when cells started to grow, GSH was located mainly in the cell nucleus. However, when cell growth slowed, GSH distribution was homogenous throughout the cell.
The capacity of the cell to concentrate GSH in the nucleus was estimated by measuring the nuclear/cytoplasmic ratio, which was calculated after measuring the ratio in 100 cells one by one in each experiment. The results in Fig. 4 show a distribution pattern where the maximal nuclear/cytoplasmic ratio (4.2 ± 0.8) was found very early after plating (6 h). Although a high nuclear/cytoplasmic ratio for GSH was maintained during cell growth, the culture progressed, a steady decrease in the nucleus/cytoplasm ratio was present. Once the cells reached confluence and most of them were in the G_0/G_1 phase, the nuclear/cytoplasmic ratio for GSH was homogeneous, and no difference could be seen between the nucleus and the cytoplasm.

To verify that most cells at 24 and 48 h in culture were in the S/M + G_2 phase of the cell cycle, we studied Id2 and p107 expression patterns during cell growth. These proteins are overexpressed when cell division is active. The results in Fig. 5 show high Id2 and p107 expression at 24 and 48 h in culture, demonstrating that the cells divided at 24 and 48 h in culture. Expression of Id2 and p107 at 72 h of culture or later was very low.

Mitochondrial GSH was not responsible for the areas of high CMFDA fluorescence. To test this, we incubated fibroblasts with Hoechst to stain genomic DNA (in blue), CMFDA for GSH (green), and MitoTracker for mitochondrial staining (red) (see Fig. 6). A selection of images obtained along the z axis starting from the cell apex is shown in Fig. 6 (a representative experiment of three). The nuclear shape is clearly visible as is the presence of GSH in the nucleus. Mitochondrial distribution was perinuclear.

To determine whether glutathione concentrates in the nucleus via an ATP-dependent mechanism, we incubated the cells with FCCP, a mitochondrial uncoupling agent. The results shown in Fig. 7 suggest that glutathione concentrates in the nucleus by an ATP-independent process, because the glutathione distribution was similar in both treated and untreated cells.

An additional possible explanation for the reported nuclear increase in GSH during the early phases of cell cycle would be the nuclear GSH synthesis. We studied the presence of the enzymes for GSH synthesis GCS and GSH synthetase. Fig. 8A shows a lack of expression of the rate-limiting enzyme GCS (11) in nuclear extracts, whereas samples from whole cells showed a marked expression. Thus, under our experimental conditions, the nuclear synthesis of GSH seems improbable.

Fig. 8B shows the nuclear presence of Bcl-2, an anti-apoptotic protein that has been reported to increase nuclear glutathione (12). Our result points to a correlation between
the expression of Bcl-2 during the cell cycle and the distribution of cellular GSH. When cells are dividing (6 and 24 h of culture), the presence of nuclear Bcl-2 is high, but it decreases later (48 h and 5 days of culture) when cell growth stops.

If nuclear GSH is high during the early phases of cell cycle and low when cells are confluent, nuclear oxidized proteins are likely to be higher during cell cycle arrest than at 6 h after culture. This is indeed the case as is shown in Fig. 9.

If GSH is consumed by the reactive oxygen species in the nucleus, it will be converted into GSSG or other mixed disulfides and will not diffuse back to the cytoplasm as free GSH. Indeed, Western blot analysis of nuclear extracts showed the differences in the glutathiolation of nuclear proteins (Fig. 10); it was higher at 6 h after culture, when nuclear compartmentalization reached its maximum. As expected, when cells reached confluence and nuclear/cytoplasmic distribution was homogeneous, glutathiolation of nuclear proteins decreases.

**DISCUSSION**

We reported previously that GSH regulates telomerase activity in cells in culture (8). GSH increases in 3T3 fibroblasts before exponential cell growth. Peak telomerase activity takes place 24 h after plating and coincides with the maximal levels of GSH in cells. Thus, telomerase activity is maximal under reduced conditions. In view of these results and because telomerase exerts its activity in the nucleus, we decided to study GSH distribution in the cell nucleus before, during, and after exponential cell growth.

Mercury orange, monochlorobimane, and CMFDA are the most commonly used probes for GSH determination, but the results obtained by these methods are conflicting. Pioneer work by Bellomo et al. (13) using monochlorobimane-GSH conjugation showed a 3:1 nucleus:cytoplasm ratio. However, more recent reports by Briviba et al. (14) have shown that the high nuclear fluorescence was because of an influx of the fluorescent bimane-GSH adduct into the nucleus. Thomas et al. (15) used fractionation techniques and flow cytometry with mercury orange, as this probe readily forms fluorescent adducts with GSH and other non-protein sulfhydrys, reacting much more slowly with protein sulfhydrys. Contrary to the previous reports, they found lower GSH levels in the nucleus than in the cytoplasm, with a mean nuclear/cytoplasmic ratio of 0.57 ± 0.05 (15). They suggested that there is a distinct pool of GSH in the nucleus because in their experiments GSH was partially resistant to buthionine sulfoximine depletion compared with the cytoplasm (15). More recently, Söderdahl et al. (5) showed the highest GSH staining in a perinuclear mitochondria-rich compartment and low nuclear GSH staining using mercury orange and a specific GSH antibody. Finally, Voehringer et al. (12) using CMFDA (which binds in 95% to GSH, see “Experimental Procedures” and Ref. 9) showed that GSH is distributed mainly in the cytoplasm, although Bcl-2 overexpression is able to increase nuclear GSH levels.

In recent years new methods have become available to measure the nuclear redox state. These have been reviewed recently by Hansen et al. (16). Two forms of the redox blot have been developed to separate on the basis of different charge- or mass-oxidized nuclear proteins. Other techniques include antibodies that bind only to the oxidized form of the protein (17) and mass spectrometry (18). Another recent approach to determining
the nuclear redox state is the use of redox-sensitive green fluorescent proteins. Although it is a promising step, until now no physiological oxidative treatment has been able to cause detectable changes in the excitation ratios of redox-sensitive green fluorescent proteins (17). Although the nuclear redox state (mainly thioredoxins) can be determined, the measurement of nuclear GSH concentrations is limited, and no methods are available to measure nuclear GSSG (17).

To study glutathione compartmentalization in 3T3 fibroblasts during cell growth, we used a triple staining (CMFDA-Hoechst-propidium iodide) or (CMFDA-Hoechst-MitoTracker) by confocal microscopy analysis. Figs. 3E and 6 show that GSH co-localized with the nuclei at 6, 12, and 24 h after plating. However, when cells were confluent, at 3 and 5 days after plating, no differences between the nucleus and cytoplasm could be seen. The images obtained at 3 or 5 days in culture are similar to those reported previously by Söderdahl et al. (5) using GSH antibody and mercury orange. Thus, our results may explain the apparent discrepancies in the GSH distribution. It is very important to take into account the moment of the cell cycle in which glutathione distribution is studied. We show here that cells that are preparing to divide have high nuclear GSH levels. Quiescent cells have similar (or even lower) GSH levels in the nucleus than the cytoplasm. Fig. 3 shows higher GSH levels in the nucleus of the cells early after plating.

The availability of GSH in cellular compartments is determined by a complex equilibrium between utilization, transport, synthesis, glutathiolation of proteins, and reduction of glutathione disulfide (GSSG) and GS-SX GSSG plus mixed disulfides. The regulation of such interactions is unclear. According to Smith et al. (19), the possible biochemical mechanisms responsible for the turnover of nuclear GSH are: 1) GSH may be taken up from the cytoplasm into the nuclei either passively or through energy dependent processes; 2) GSH may be synthesized de novo in the nucleus by the enzymes γ-glutamylcysteine synthetase and GSH synthetase; 3) GSH may function to transport γ-Glu-Cys-Cys. Ho and Guenthner (20), using nuclear fractions from liver, concluded that GSH is taken up by the nucleus by passive diffusion, and no evidence for an ATP-dependent mechanism for GSH concentration was observed. According to Ho and Guenthner (20) γ-glutamylcysteine synthetase and GSH synthetase activities were found in nuclei. About 4–8% of the GSH synthetic activity of the cell was found in the nucleus, maintaining nuclear GSH levels (20).

The role of ATP-dependent mechanisms in maintaining the nuclear/cytoplasmic GSH concentration was studied by Bellomo et al. (13, 22) using culture hepatocytes pretreated with the uncoupler protonophore CCCP. After 20 min of incubation the nuclear/cytoplasmic GSH gradient disappeared, but the total GSH content remain unchanged.

In fibroblasts, we did not find an ATP-dependent mechanism or γ-glutamylcysteine synthetase in the cell nucleus. Different authors reported that overexpression of Bcl-2 leads to higher cellular levels of GSH (12, 23–28). Hoetelmans et al. (28)
reported that high nuclear Bcl-2 expression correlates with higher nuclear GSH levels in rat CC531 colorectal cancer cells. Thus, we suggest that during the changes in the nuclear membrane that precede the cell division, nuclear Bcl-2 could allow the specific translocation of selected substances, among them glutathione, to the cell nucleus. It has been reported that Bcl-2 is associated with nuclear pore complexes (29). Thus Bcl-2 has been suggested as an ideal candidate for catching proteins as they cross the nuclear envelope (30).

A number of physiopathological situations unravel the importance of nuclear GSH. Atzori et al. (31) demonstrated variations in the amount and redox state of cellular thiols, particularly reduced glutathione, supporting a role for thiols in the regulation of growth and squamous differentiation of human bronchial epithelial cells. In a similar fashion, depletion of nuclear GSH to 50–60% of its initial value prior to irradiation (400 centigray) resulted in nuclear DNA fragmentation and apoptosis, suggesting that GSH plays a critical protective role in maintaining nuclear functional integrity and in determining the intrinsic radiosensitivity of cells (32).

Thalidomide became sadly famous in the 1950s because of its ability to cause human birth defects, mainly limb reduction malformations (phocomelia) (33). A rat thalidomide-resistant and a rabbit thalidomide-sensitive species were used to compare potential differences among limb bud cells. Confocal microscopy revealed that glutathione distribution determined by CMFDA staining was different in these cell types. Thalidomide induced cytosolic GSH depletion in both cell lines; however, nuclear GSH levels remained high in the rat thalidomide-resistant cells but not in the rabbit thalidomide-sensitive cells. Hansen et al. suggest that a redox shift in the nucleus may result in the misregulation of interactions between transcription factors and DNA causing defective growth and development (34). Thalidomide is used today as an inhibitor of angiogenesis and tumor growth.

Chen et al. (35) reported the specific role of nuclear GSH in preventing apoptosis and its importance in estrogen action. An important number of nuclear proteins, including transcription factors, require a reduced environment to bind to DNA. More than 62 proteins are involved directly in transcription, nucleotide metabolism, (de)phosphorylation, or (de)ubiquitinylation, which are all essential processes for cell cycle progression (36). According to Jang and Surh (37) nuclear GSH may act as a transcriptional regulator of NF-κB, AP-1, and p53 by altering their nuclear redox state. Toledano et al. (21) found a redox-dependent shift of oxyR-DNA contacts along genomic DNA, suggesting a mechanism for differential promoter selection. For a
review of the importance of a reduced nucleus and the role of nuclear GSH, see Conour et al. (36).

In conclusion, we have shown that cells need a reduced nuclear environment provided by glutathione to proliferate (see Fig. 11). Indeed, the nucleus/cytoplasm ratio is high several hours after plating. Thus, glutathione translocates to the nucleus before exponential cell growth. When cells reach confluence, at 3 and 5 days after plating, GSH distribution is similar in both of the cell compartments, nucleus and cytoplasm.

The phase of the cell cycle must be taken into account when studying cellular and/or nuclear GSH levels. Thus our results demonstrate that the cell nucleus suffers dramatic changes in its oxidative status and underscore the role of nuclear glutathione in the physiology of the cell cycle.

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FIGURE 9. Oxidized nuclear proteins during cell growth. 3T3 cells were subjected to nuclear protein extraction, for the determination of protein oxidation. Samples were lysed and derivatized, and Western blotting was performed according to the recommendation of the manufacturer. The level of nuclear protein oxidation was determined by detecting the carbonylation level with the Oxy Blot protein oxidation detection Kit (Chemicon International).

FIGURE 10. Glutathiolated nuclear proteins during cell growth. Nuclear lysates of 3T3 cells were obtained in the absence of reductive agents. Western blotting procedure was performed as described under “Experimental Procedures,” and the membrane was probed against anti-glutathione antibody (1:1000) (Virogen) at 4 °C overnight.

FIGURE 11. Correlation between nuclear GSH distribution and cell growth. The number of cells x 10^6 is shown on the right y axis. The nucleus/cytoplasm ratio is shown on the left y axis. Data are expressed as the mean of ten different experiments.
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