Small stress proteins are developmentally regulated and linked to cell growth and differentiation. The early phase of murine embryonic stem (ES) cell differentiation, characterized by a gradual growth arrest, is accompanied by hsp27 transient accumulation. This differentiation process also correlated with changes in hsp27 phosphorylation and oligomerization. The role of hsp27 was investigated in ES clones stably transfected with murine or human hsp27 genes, placed in sense or antisense orientation. Several clones were obtained that either underexpressed endogenous murine hsp27 or overexpressed murine or human hsp27. Maintained undifferentiated, these clones showed similar growth rates. We report here that hsp27 constitutive overexpression enhanced the differentiation-mediated decreased rate of ES cell proliferation but did not alter morphological changes. In contrast, hsp27 underexpression, which attenuated cell growth arrest, induced differentiation and some morphological changes. Recently, we showed that hsp27 interfered with cell death probably because of its ability to modulate intracellular glutathione. hsp27 accumulation during ES cell differentiation was also correlated with an increase in glutathione, which was attenuated by hsp27 down-expression. Hence, hsp27 transient expression seems essential for preventing differentiating ES cells from undergoing apoptosis, a switch that may be redox regulated.

Mammalian hsp27 belongs to the family of small heat shock proteins (SHSP)† that are characterized by a strong homology to lens α-crystallin (reviewed in Ref. 1). SHSP share the ability to form oligomeric structures (2, 3) and are often detected as phosphoproteins (4, 5). Many stimuli, such as serum, oxidative injury, thermal stress, inflammatory cytokines (tumor necrosis factor-α, interleukin-1), and retinoic acid have been described as potent modulators of mammalian hsp27 phosphorylation and oligomerization (reviewed in Ref. 1). SHSP expression was shown to protect against cell necrosis induced by stimuli such as hyperthermia (6, 7), anti-cancerous drugs, oxidative stress (7–10), and inflammatory cytokines (9, 11). Recently, we reported that SHSP are also negative regulators of apoptosis that counteract Fas/APO-1 or staurosporine-induced programmed cell death (12).

To explain the protective activity of SHSP, it has been proposed that these proteins act as molecular chaperones (13) or actin capping/decapping enzymes (14, 15). In addition, we recently reported that the expression of SHSP from different species induced an increase in glutathione that resulted in a decreased level of intracellular reactive oxygen species (ROS) (10). This conserved property was found to be essential for the protective activity of SHSP against oxidative stress- or tumor necrosis factor-α-induced cell death.

An interesting feature of SHSP concerns their transient expression during development and cell differentiation. This was first observed in Drosophila (reviewed in Ref. 16), and studies performed in other organisms revealed the ubiquitous nature of this phenomenon (reviewed in Refs. 1 and 17). Remarkably, during Drosophila development, Dhsp27 accumulates during the differentiation of imaginal discs, suggesting that this protein plays a role in this process (18). Recent studies have strengthened the hypothesis that the mammalian small stress protein hsp27 is linked to the differentiation process. Indeed, this protein is transiently expressed and/or phosphorylated during the early differentiation of several mammalian cells including embryonal carcinoma and stem cells (19), Ehrlich ascites cells (20), normal B and B lymphoma cells (21), osteoblasts, promyelocytic leukemia cells (22–25), and normal T cells (26). hsp27 accumulation usually occurs concomitantly with the differentiation-mediated decrease of cellular proliferation (23–25).

In this study, we have analyzed the expression and function of hsp27 during the in vitro pluripotential differentiation of embryonic CGR8 embryonic stem cells obtained through leukemia inhibitory factor (LIF) withdrawal. We show that the early phase of the differentiation process is accompanied by a transient accumulation of hsp27, which occurs concomitantly with a decreased rate of cellular proliferation. hsp27 accumulation was preceded by an increase in the level of the mRNA encoding this protein. The transient increase in hsp27 level was also time correlated with an increased oligomerization of this protein, a phenomenon that was preceded by hsp27 dephosphorylation. As an approach toward understanding the role of hsp27 during cell differentiation, we have analyzed CGR8 cells that either under- or overexpressed endogenous murine hsp27 or overexpressed human hsp27. The different
cell lines obtained showed similar growth rates in the presence of LIF. However, following LIF withdrawal, the differentiation-mediated decreased rate of cell proliferation was inversely proportional to the level of hsp27 present within the cell. Although murine or human hsp27 overexpression did not seem to modify the differentiation-mediated morphological changes, the underepression of endogenous hsp27 provoked an abortion of the differentiation process because of an overall cell death by apoptosis. Remarkably, during CGR8 cell differentiation, the raise in hsp27 level correlated with an increase in glutathione, a redox modulator described as being essential for the differentiation process (27). The raise in glutathione was less intense in cells that underexpress hsp27. These results are discussed in view of a role of hsp27 as switch that allows differentiating cells to escape from apoptosis through a redox-dependent mechanism.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures, Induction of Differentiation**—The murine embryonic stem cell CGR8 was obtained from A. Smith (Center of Genome Research, University of Edinburgh, UK). Undifferentiated CGR8 cells were grown on gelatinized flasks in BHK 21 medium (Life Technologies, Inc.). Supplemented with LIF (1 000 conditioned medium from p10-68 DIA-LIF transfected COS cell line) (28), 0.05 mM β-mercaptoethanol, 2 mM l-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 1 x minimal essential medium, and 10% fetal calf serum (Life Technologies, Inc.). To induce differentiation, CGR8 cells were dissociated by trypsinization and seeded at 3 x 10^6 cells/ml on bacterial grade Petri dishes (Bibby Sterilin Ltd., Stone, UK) in LIF-devoid growth medium.

**Vectors and Reagents**—The mammalian expression vector psvK3 (Pharmacia, Upsalla, Sweden) bearing or not human (psvhp/PV27; Ref. 9) or murine (psvWT; Ref. 29) hsp27 cDNAs under the control of the early promoter of SV40 virus were used. To construct antisense expression vectors EcoRI-EcoRI or SacI-Sacl, DNA fragments containing the entire human or murine hsp27 coding sequences, respectively, were subcloned in reverse orientation in the corresponding site of psvK3 polylinker; these expression vectors were denoted psvant-hsp27 (human hsp27) and psvant-mhsp27 (murine hsp27). Anti-hsp70 serum was from Amersham International (Buckinghamshire, UK). The specificity of anti-human hsp27 and anti-murine hsp27 antibodies was as previously described (2, 9).

**Transfection Experiment**—DNA transfection was performed by electroporation using a Bio-Rad gene pulser (Bio-Rad). 5 x 10^6 CGR8 cells were resuspended in Opti-MEM medium (Boehringer, Mannheim, Germany) and incubated 10 min at room temperature in the presence of DNA made of 10 μg of pMC1-neopoly(A) plasmid (30) and 50 μg of psvK3, psvnant-hsp27, psvnant-mhsp27, psvhp or psvWT vector. After electroporation (500 microfarads, 250 V), cells were centrifuged for 3 min at 200 g and resuspended for 30 min at room temperature and then reseeded on gelatin-treated flasks in BHK-21 medium. 250 μg/ml G418 were added 48 h after electroporation, and resistant clones were isolated 10 days later.

**Cellular Proliferation**—Cellular proliferation was monitored by counting cells using a hemocytometer chamber and a Nikon TMS inverted photomicroscope equipped with phase-contrast equipment. Cells were also labeled for 1 h with 1 μCi of 3H-thymidine (Amersham International) as described by Mehlen and Arrigo (3).

**Cell Cycle Analysis**—Cell cycle analysis was performed essentially as described by Suzuki et al. (31). At different times before and following LIF removal, cells were washed, fixed with ethanol, resuspended in phosphate-buffered saline, and treated with RNase I. 50 μg/ml protease K, 0.5 μg/ml propidium iodide were added, and cells were analyzed by flow cytometry (Facscalibur, Becton Dickinson, Belgium).

**RNA Isolation**—Total RNA from undifferentiated CGR8 cells or CGR8 cells derived from embryoid bodies were prepared with RNAzol™B (Bioprobe-Interchim, Montluchon, France) according to the manufacturer’s instructions.

**RT-PCR**—For RT-PCR analyses, 1 μg of total RNA was denatured for 10 min at 65 °C and reverse-transcribed for 1 h at 37 °C in a medium containing 50 mM Tris-HCl, pH 8.3, 10 mM diithiothreitol (Life Technologies, Inc.), 1 unit/μl RNAsin (Promega), 0.5 μM random hexamers (Pharmacia, St-Quentin-Yvelines, France), 0.5 μM of each dNTP (Pharmacia), 75 mM KC1, 5 mM MgCl2, and 20 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.). Reactions were stopped by incubating the mixtures 5 min at 95 °C. For PCR reactions, 5 μl of the RT reactions were transferred in a medium containing 20 mM Tris-HCl, pH 8.3, 50 mM KC1, 1.5 mM MgCl2, 0.2 mM of each dNTP, 0.2 μM of each primer, and 1 unit of Taq DNA polymerase (Life Technology Inc.). HPRT-, collagen IV-, or β-major globin-specific primers were identical to those described by Keller et al. (32). PCR reactions were carried out with a Perkin Elmer thermal cycler for 30 cycles with a regimen of 94 °C for 1.5 min, 50 °C (for HPRT) or 55 °C (for collagen IV and β-major globin) for 1.5 min, and 72 °C for 2 min, followed by 72 °C for 10 min. Aliquots (10 μl) of each PCR reaction were analyzed by gel electrophoresis (2% agarose Nu sieve; Tebu, France) in Tris-borate-EDTA buffer.

**Northern Blot Analysis**—RNA (10 μg) was analyzed in 1% agarose formaldehyde gel and transferred to Hybond C extra membrane (Amersham International); hybridization was then performed at 65 °C (33). The murine hsp27 probe was a 0.6-kilobase SacI cDNA fragment of the psvWT plasmid (29).

**Gel Filtration Analysis**—At different times before and following LIF removal, CGR8 cells were washed in phosphate-buffered saline and lysed at 4 °C in a buffer containing 20 mM Tris, pH 7.4, 20 mM NaCl, 5 mM MgCl2, 0.1 mM EDTA, and 0.1% Triton X-100. The 20,000 x g supernatants were then applied to a Sepharose 6B gel filtration column (1 x 100 cm) (Pharmacia, Sweden), equilibrated, and developed in the lysis buffer devoid of Triton X-100. The presence of hsp27 in the fraction eluted of the column was detected by one-dimensional immunoblot analysis using anti-hsp27 serum. Molecular mass markers used to mark the gel filtration column included blue dextran (>2,000,000 Da), thyroglobulin (669,000 Da), apoferritin (440,000 Da), β-amylase (200,000 Da), and carbonic anhydrase (29,000 Da).

**Gel Electrophoresis and Immunoblotting**—One- or two-dimensional gel electrophoresis and immunoblots using hsp27 or hsp70 antisera were performed as already described (9, 11, 34) and revealed with the ECL kit from Amersham Corp. Autoradiographs were recorded on X-Omat AR films (Eastman Kodak Co.). A Bioprofil system (Vibher Lourmat, France) was used for quantification. The analysis was performed within the range of proportionality of the film. The level of hsp27 expressed in CGR8 cells was compared with serial dilutions of the purified protein (StressGen Corp., Victoria, British Columbia, Canada).

**Cell Death Analysis**—The vital dye Trypan blue (Sigma, St-Quentin, France) was used to monitor cell death (9). DNA fragmentation was analyzed essentially as described by Hockenbery et al. (35). Briefly, cells were lysed for 20 min 4 °C in a medium containing 5 mM Tris buffer, pH 7.4, 0.5% Triton X-100, 20 mM EDTA. After centrifugation at 20,000 x g for 15 min, the supernatants were extracted with phenol-chloroform, and nucleic acids were precipitated in ethanol before being analyzed by gel electrophoresis (1.5% agarose; Nu sieve, Tebu, France). Thereafter, the gel was incubated for at least 3 h at 37 °C in the presence of 20 μg/ml RNase A before being stained with ethidium bromide.

**Determination of Intracellular Glutathione Levels**—Total cellular content glutathione from undifferentiated CGR8 cells (6 x 10^6 cells) or CGR8 cells derived from embryoid bodies was determined by the Bioxytech GSH-400 enzymatic method from OXIS International (Bonneuil-sur-Marne, France) according to the manufacturer’s instructions.

**RESULTS**

Immediately following LIF removal, CGR8 cells gradually decreased their growth rate and underwent pluripotential differentiation. The accumulation of markers, which are specific for different tissues, was used to follow the differentiation of these cells (32). This was assessed by specific RT-PCR analysis that was performed before and at different times following LIF withdrawal. As seen in Fig. 1A, the presence of collagen IV mRNA, a marker specific of endodermic cells, began to be detectable 4 days after LIF removal. 1 day later, the mRNA encoding the β-major globin precursor, a marker of hematopoietic lineage, was observed. As a control, the level of the ubiquitous HPRT mRNA was measured, as detectable markers of monocyte differentiation process (Fig. 1A). Cell growth inhibition was monitored by 3H-thymidine incorporation and cell number. Fig. 1B demonstrates that, 12 h after LIF removal, 3H-thymidine incorporation decreased by a factor of almost 2 compared with the value determined in cells kept in the presence of LIF. After 24 h of differentiation, a 6-fold decreased 3H-thymidine incorporation was observed. This phenomenon reflected the gradual cell
division inhibition of differentiating CGR8 cells, which is illustrated in Fig. 1C by cell numeration. FAC-scan analysis of the different phases of the cell cycle revealed that the early differentiation of CGR8 cells is accompanied with an increased number of cells in G1 phase (not shown and Ref. 36).

Since hsp27 has been increasingly linked to cell differentiation, we investigated the level of this protein during the differentiation of CGR8 cells. This was assessed by analyzing the level of this protein before and at different times following LIF withdrawal. The immunoblot analysis presented in Fig. 2A shows that hsp27, which is already expressed in undifferentiated CGR8 cells, displayed a transient increased level in cells incubated in the absence of LIF. The maximal increase of hsp27 level (2.5-fold) was observed 24 h after LIF removal. By 72 h of differentiation, the level of this protein was below that observed in control cells (see Fig. 4A for a quantitative analysis of this phenomenon). A similar observation has been previously reported when ES cells were launched to differentiate as a consequence of retinoic acid treatment (19). Moreover, the accumulation of hsp27 in response to LIF withdrawal did not result in a stress response because the level of the major stress protein hsp70 was not significantly altered (not shown). Analysis performed at the mRNA level showed, already 6 h after LIF removal, an increased accumulation of the mRNA encoding hsp27 (Fig. 2, B and C). This phenomenon lasted for about 12 h; thereafter, the level of hsp27 mRNA rapidly declined.

Because changes in hsp27 phosphorylation and oligomerization have been reported to occur during HL60 cell differentiation (24, 25), we have investigated these different properties of hsp27 during the differentiation of CGR8 cells. Phosphorylation analysis was assessed by two-dimensional immunobLOTS as already described (11, 34) and was performed before and at different times following LIF withdrawal. As seen in Fig. 3A, in cells kept in the presence of LIF, hsp27 was resolved as two major isoforms: “a” and “b.” The “a” isoform is the non-phosphorylated form, whereas the “b” isoform is the major phosphorylated form of the protein (1, 11, 34). Because similar levels of the “a” and “b” isoforms are detected in cells kept in the presence of LIF, this means that hsp27 is strongly phosphorylated in undifferentiated CGR8 cells. 1 h after LIF withdrawal, the level of the “b” isoform had declined, and this isoform was no more detectable later, particularly during the transient accumulation of this protein (between 12 and 48 h). These results therefore suggest that ES differentiation is characterized by a drastic dephosphorylation of hsp27. In addition of being a phosphoprotein, hsp27 also undergoes changes in its oligomerization state (1, 11, 37). To study this particular property of hsp27 during ES differentiation, CGR8 cells were harvested and lysed at various time points before and at different times following LIF withdrawal. Lysates were subjected to sizing chromatography on a Sepharose 6B column, and the proteins present in the different fractions were analyzed in immunoblot probed with anti-hsp27 antibody as described under “Experimental Procedures.” It is seen in Fig. 3B that in undifferentiated CGR8 cells, hsp27 is in the form of small oligomers whose molecular masses are comprised between 30 and 150 kDa. Remarkably, 24 h after LIF withdrawal, and concomitantly with hsp27 accumulation, hsp27 oligomers drastically shifted toward high molecular masses (100–600 kDa). By 48 h, the reverse phenomenon occurred because the distribution of hsp27 oligomers was again observed in the range of 30–150 kDa. These results therefore indicate that a drastic dephosphorylation and a transient increase in the oligomerization state of hsp27 occur during ES differentiation.

As an approach toward a better understanding of the role played by hsp27 during ES cell differentiation, we have undergone experiments aimed at modulating the level of this protein. This was assessed by transfecting CGR8 cells with psvK3 vec-
The level of endogenous hsp27 during differentiation of the different CGR8 cell lines described above was investigated by immunoblot analysis as described in Figs. 2 and 3. At different time periods, before and after LIF withdrawal, the level of murine hsp27 in CGR8 clones that either underexpresses (ES-ant-hsp27-2) or overexpresses (ES-hsp27-5) murine hsp27 was compared with the level of this protein in control cells (ES-control-1). A quantitative analysis of the immunoblots is presented in Fig. 4C. This figure clearly shows that the level of endogenous hsp27 was strongly reduced (by 3-fold to almost 5-fold, depending on the time period) during the early differentiation of CGR8 cells that express the antisense construct (ES-ant-hsp27-2). An intriguing observation came from the analysis of ES-hsp27-5 cells, which constitutively overexpress murine hsp27. In these cells, the level of murine hsp27 increased during the first 24 h of differentiation, and from then on it resembled that observed in control cells. This suggests a mechanism that inhibits a too high production of hsp27 during CGR8 cell differentiation.

We next investigated whether changes in the level of endogenous hsp27 and/or a high level of human hsp27 could alter the growth of CGR8 cells maintained or not in the presence of LIF. The doubling time (approximately 8 h) of CGR8 cells maintained undifferentiated in the presence of LIF was not found to be altered by variations in the level of murine hsp27 or by the presence of human hsp27 (not shown). In contrast, perturbations in the level of endogenous hsp27 or the presence of human hsp27 induced drastic effects when CGR8 cells were launched to differentiate as a consequence of LIF withdrawal. Differentiating CGR8 cells are characterized by a transient cell growth inhibition, as determined by counting the total cell number during this differentiation process (Figs. 1C and 5). Hence, the time required to observe a 2-fold increase in the number of differentiating CGR8 cells was about 40 h. After the same time period in the absence of LIF, only a 130% (±5%) increase in the number of ES-hsp27-5 cells, which express the highest level of murine hsp27 (see Fig. 4), was observed. High levels of human hsp27 expression induced a similar effect. In ES-ant-hsp27-2 cells, which express the lowest level of murine hsp27 (see Fig. 4), the cell population doubling time after LIF withdrawal was only 28 h instead of the 40 h that were necessary for control cells. Analysis of the other CGR8 cell lines revealed that the cell doubling time in the absence of LIF was inversely proportional to the level of hsp27 (Fig. 5). Hence, by interfering with the transient accumulation of hsp27, a modulation of the dif-
expression of murine hsp27 did not significantly interfere with the morphological changes mediated by LIF withdrawal. A similar result was observed in cells that overexpress human hsp27 (not shown). A completely different result was observed when ES-ant-hsp27-2 cells that underexpress hsp27 were analyzed. In this case, the classical morphology of differentiating CGR8 cells was no longer observed, and the differentiation process was arrested at the level of young embryoid bodies (Fig. 6C). Moreover, the presence of specific markers, such as collagen IV or the β-major globin precursor, was not observed (not shown). Similar observations were made when the other CGR8 cells that underexpress murine hsp27 were analyzed (not shown). Hence, the inhibition of the transient hsp27 increase resulted in differentiation abortion.

The differentiation abortion of CGR8 cells induced by hsp27 underexpression was further characterized at the level of cell viability. This was assessed by Trypan blue staining of cells present in 8-day embryoid bodies (see "Experimental Procedures"). This analysis revealed that all the cells present in these bodies were Trypan blue-positive, suggesting that they were dying or already dead. A kinetic analysis of this phenomenon revealed that, 5 days after LIF withdrawal, all the hsp27 underexpressing cells (clone ES-ant-hsp27-2) were dead, whereas at the same time period more than 70% of control CGR8 cells were still alive (Fig. 7A). Remarkably, hsp27 downexpression, which slowed down the differentiation-mediated inhibition of cell division (see also Fig. 5), led these cells toward death. We then analyzed whether this phenomenon resulted in an apoptotic phenomenon. Apoptosis is usually characterized by DNA breakages leading to the accumulation of oligonucleotides with defined lengths. These fragments are detectable by analyzing the nucleic acids present in the supernatant of cells lysed with non-ionic detergent (12, 35). This procedure requires a drastic RNase treatment of the agarose gels to eliminate the cytoplasmic RNA, which interferes with the visualization of the DNA fragments (see "Experimental Procedures"). Similar loading of the different samples was verified by staining the gel before the RNase treatment (not shown). It is seen in Fig. 7B that, in control CGR8 cells, a weak level of fragmented DNA was detectable 48 h after LIF withdrawal, indicating that, during normal differentiation, some CGR8 cells were undergoing apoptosis. Of interest is the fact that, in CGR8 cells that

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**Fig. 4.** Analysis of hsp27 levels in the different CGR8 cell lines. A, immunoblot analysis of the different clones. CGR8 cells were transfected with either the control (cont.) or hsp27 expression vectors. Vectors carrying either antisense (ant-hsp27) or sense (hsp27) hsp27 gene (murine or human) constructions were used (see "Experimental Procedures"). Stable cell lines were obtained, and the level of hsp27 was determined in immunoblot analysis of total cellular proteins probed with an antiserum that recognizes both human and murine hsp27. B, control immunoblot probed with anti-hsp70 antiserum. Autoradiographs of ECL-revealed immunoblots are presented. Note the drastic decreased level of the endogenous murine hsp27 in clone ES-ant-hsp27-2 and ES-ant-hsp27-11. Murine hsp27 is slightly increased in clone ES-hsp27-11 and strongly increased in clone ES-hsp27-5. Both resulted in a transfection with the murine sense construct. A slight increase in ES-hsp27-2 and ES-hsp27-3 clones is also observed, but in this case, the transfection was performed with the human sense construct. C, kinetics of endogenous hsp27 accumulation during differentiation of either control CGR8 cells or CGR8 cells that underexpress or overexpress murine hsp27. Total cellular proteins were isolated from ES-control-1, ES-hsp27-5, or ES-ant-hsp27-2 cells following LIF withdrawal and analyzed in immunoblots probed with an antiserum specific for murine hsp27. In each case, the level of hsp27 was estimated by densitometry as described under "Experimental Procedures." Results are in the form of percentage of hsp27 level calculated as the ratio between the level of hsp27 determined for the different samples to that obtained in ES-control-1 cells maintained in the presence of LIF. Percentage of hsp27 level is presented as a function of the duration of cell culture in the absence of LIF.

The effect mediated by hsp27 overexpression or underexpression on the morphological changes that characterize differentiating CGR8 cells has been analyzed. To this end, 8 days after LIF withdrawal, cell morphology was analyzed by phase contrast microscopy. It is seen in Fig. 6, A and B, that the overexpression of murine hsp27 did not significantly interfere with the morphological changes that characterize differentiation-mediated decreased cellular growth was induced.

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**Fig. 5.** Cell growth analysis of differentiating CGR8 cells that express different levels of hsp27. Control cells and cells that either underexpress (ant-hsp27-2, ant-hsp27-11) or overexpress (hsp27-2, hsp27-5) hsp27 growing in the presence of LIF were trypsinized and plated (3 × 10⁵ cells/100-mm dish) in a medium devoid of LIF. Cultures were then counted daily for 72 h. The percentage of growing cells was calculated by dividing the number of cells observed to that originally plated (3 × 10⁵ cells). A typical experiment is presented. Standard deviations are indicated (n = 3).
underexpress hsp27, DNA fragmentation was already detectable 12 h after LIF removal and was very intense 36 h later. Hence, our results suggest that hsp27 down-regulation drives differentiating cells toward cell death through a drastic amplification of the normally occurring apoptotic process.

We recently reported that in murine cells, such as L929 or NIH-3T3-ras, the expression of different SHSP decreased the intracellular level of ROS as a consequence of a raise in total glutathione (10). Because glutathione is an important regulator of cell differentiation and apoptosis (27, 38), we investigated the level of this compound during the early differentiation of CGR8 cells. The result, presented in Fig. 8, clearly shows that the level of total glutathione increased by a factor of 3.4 during the early differentiation of CGR8 cells. This phenomenon was transient and peaked 24 h after LIF withdrawal at a time period when hsp27 level was also maximal (see above and Figs. 2 and 4). Only a 2.4-fold increase in glutathione was observed in LIF-depleted CGR8 cells that underexpress hsp27. This attenuated increase in glutathione was also maximal 24 h after LIF withdrawal and at a time period when cells were still Trypan blue negative (see Fig. 7A). This suggests that this phenomenon is related to hsp27 down-regulation and is not a direct consequence of cell death. Hence, redox parameters are probably altered by hsp27 depletion in differentiating CGR8 cells.

**DISCUSSION**

hsp27 transiently accumulates during the early differentiation of murine CGR8 embryonic stem cells. This phenomenon, which peaks 24 h after LIF withdrawal, is preceded by an increase in hsp27 mRNA, suggesting that it is transcriptionally regulated. A regulation of hsp27 stability may also occur because, after 24 h of differentiation, the levels of endogenous and...
Differentiation is a fundamental cellular program that limits the clonal expansion of cell populations because, in general, differentiation and cell growth are mutually exclusive. The early differentiation steps are therefore often accompanied by a decreased rate of cellular proliferation (42). In CGR8 cells, this phenomenon is characterized by a gradual increase in the number of cells in G1 phase (36). Several studies performed in different cell systems have revealed that hsp27 accumulates during early differentiation concomitantly with the decrease in cell proliferation (23–25). Using CGR8 cells that underexpress hsp27, we report here that a decreased level of hsp27 interfered with the reduced cellular proliferation, which occurs during the early differentiation of these cells. The reverse effect was induced by the constitutive overexpression of either murine or human hsp27. A similar effect was observed in HL-60 cells induced to differentiate in granulocytes (25). Hsp27 accumulation in the course of various differentiation processes may therefore be interpreted as a phenomenon driving to reduced cell growth. It is not yet clear whether the modulations of cellular proliferation described above resulted in a direct action of hsp27 toward the cell division apparatus of differentiating cells or were a consequence of an erroneous differentiation program.

We show here that the inhibition of hsp27 accumulation during the early differentiation of CGR8 cells is sufficient to abort the differentiation program because of an overall death of the cells by apoptosis. Hence, it is interesting to note that hsp27 down-expression, which slowed down cell growth arrest during the process of differentiation, also lead to the death of these dividing cells. In contrast, the constitutive over-expression of hsp27 had no effect, except for the enhanced decrease in cell proliferation described above. Thus, it can be concluded that the level of hsp27 has to be increased during the early phase of the differentiation program to allow differentiating cells to escape from apoptosis. Moreover, the rapid disappearance of hsp27 after 48 h of differentiation suggests that the hsp27-controlled switch between apoptosis and differentiation is an early event that is concomitant with the gradual decrease of cell growth. This may also reflect that this protein is of no use, or toxic, for the later stages of the differentiation process. In that sense, hsp27 accumulation during early differentiation could be considered as an essential event that is indispensable to the normal development of this process. Our results therefore strongly suggest that hsp27 is a key protein that plays a role at the level of the pivotal control between cell division, apoptosis, and differentiation and confirms that these three events are intimately linked.

The results presented here extend our previous observations that overexpression of human hsp27 and related proteins from the same family can counteract apoptotic cell death induced by Fas/APO-1 or staurosporine (12). This leads to the conclusion that the naturally occurring transient accumulation of hsp27 in differentiating cells may represent a barrier against cell death, a phenomenon that is inherent to the process of differentiation. Interestingly, another anti-apoptotic protein, Bcl-2 (35, 43) also modulates differentiation. For example, down-regulation of Bcl-2 was reported to enhance apoptosis and further epidermal differentiation of Pam212 keratinocytes (44). It was also reported that the constitutive overexpression of Bcl-2 interfered with cell death that accompanies differentiated HL-60 cells (45) and that neuronal differentiation of PC12 cells resulted in prevention of cell death by this protein (46). It is also interesting to mention that down-regulation of the c-fes protooncogene, which displays an enhanced expression during the granulocytic differentiation of HL-60 cells, was found to inhibit differentiation and to induce apoptosis (47). However, in contrast to

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2. X. Preville, H. Schultz, U. Knauf, M. Gaestel, and A.-P. Arrigo, submitted for publication.

3. Mehlen, P., Hickey, E., Weber, L., and Arrigo, A.-P. (1997) Biochem. Biophys. Res. Commun., in press.
hsp27, Bcl-2 or c-fes has not yet been reported to display an enhanced expression during all types of cell differentiation that have been tested so far. Hence, hsp27 may represent the first example of a ubiquitously induced anti-apoptotic protein during early cell differentiation.

It has been proposed that the molecular mechanism that allows hsp27 to block cell death could result in its chaperone activity (13) toward actin (48) or some proteins that play a role in the cell death process. However, the capacity of hsp27 to block necrosis or apoptosis induced by a wide range of different stimuli suggests an intervention at the level of a general and early mediator of cell death. A possible target could be ROS (49) stimuli suggests an intervention at the level of a general and block necrosis or apoptosis induced by a wide range of different in the cell death process. However, the capacity of hsp27 protective activity against tumor necrosis factor-α or hydrogen peroxide (10), but this is probably not the case when apoptosis is considered. Indeed, attempts to consistently detect a requirement for ROS in apoptosis have been inconclusive (49–52). Recently, evidence has converged toward the importance of the glutathione redox state in the control of apoptosis. For example, in cells induced to undergo apoptosis, a rapid efflux of reduced glutathione and/or accumulation of oxidized glutathione has been reported, which thereby leads to an enhanced oxidative stress independently of ROS production (38, 53–55). In addition, an increase in the cellular content of reduced glutathione was found to delay the apoptotic process (38). Moreover, a 150% increase in glutathione was observed in a T leukemia cell line that is resistant to Fas/APO-1-mediated apoptosis (56). Taken together, these observations favor the hypothesis that the protective activity of hsp27 against apoptosis is related to the high level of glutathione, which is generated when this protein is overexpressed (10). In this respect, it should be noted that Bcl-2 overexpression also leads to an increase in glutathione (57). Finally, we show here that during the early differentiation of CGR8 cells, the normally occurring transient accumulation of hsp27 correlated with an increase in glutathione. Such a glutathione increase during early differentiation has already been observed in several other cell systems (27, 58, 59), and its inhibition resulted in differentiation abortion (27). Of interest, we show here that the differentiation-mediated increase in glutathione was less intense in CGR8 cells that contain reduced levels of hsp27 and that thereafter died by apoptosis. Glutathione is believed to modulate some transcription factors, such as AP-1 and Egr-1, that have repressed-dependent activation and considerable importance in the process of differentiation (27, 60, 61). Glutathione accumulation may also protect against redox modulations induced by the change of metabolism linked to the differentiation process.

The mechanism by which hsp27 modulates glutathione is still unknown. Since reduced levels of hsp27 did not decrease the basal level of glutathione in non-differentiating CGR8 cells, this protein may not be directly involved in the mechanism that controls the basal metabolism of glutathione. Rather, hsp27 may interfere with this mechanism when this protein is overexpressed, as for example during early differentiation or after heat shock. Another explanation may be that hsp27 is inactive in undifferentiated cells and is only able to modulate glutathione during the differentiation of CGR8 when it is in the form of large oligomers. In that sense, preliminary results using mutants that interfere with hsp27 oligomerization favor the hypothesis that increased glutathione levels are observed only when hsp27 is in the form of large oligomers. 2,3

The significance of the transient increase in hsp27 oligomerization during CGR8 cell differentiation is unknown but may be important for the function of this protein. Since hsp27 displays in vitro chaperone activity (13), its increased native size during early differentiation may reflect its interaction with proteins. This hypothesis has been recently strengthened by the fact that, during heat shock, the increased oligomerization of different types of SHSP (2) results in their interaction with non-native proteins (63, 64). This phenomenon probably creates a reservoir of folding intermediates that prevents further aggregation of non-native proteins and will allow their refolding by other chaperones, such as hsp70. Of interest, during cell differentiation, hsp27 expression and oligomerization occur concomitantly with cell growth inhibition (Ref. 25 and this study). One function of hsp27 may be therefore to prevent the aggregation of proteins that played an active role during cell growth but that are unnecessary during differentiation, thus ensuring and accelerating their proteolysis. hsp27 may also bind and protect crucial proteins that regulate glutathione metabolism, hence inducing a beneficial pro-reducing state that will interfere with aberrant protein folding.

Taken together, these observations suggest that hsp27, probably in connection with other elements, participates in the control of redox parameters during early differentiation. Erroneous control of one of these parameters, for example hsp27 level, could then lead to aberrant cell growth, resulting in differentiation abortion because of uncontrolled cell death.

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