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Hsp25 Regulates the Expression of p21(Waf1/Cip1/Sdi1) through Multiple Mechanisms

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Exposure of cells to external stresses leads to the induction or activation of certain proteins. Expression of heat shock proteins (Hsps) is induced in response to these stresses. Hsps are known to have molecular chaperone activities; but recent studies have shown that Hsps have a variety of functions such as the triggering of proliferation, differentiation, and apoptosis of cells. Previously, we found that overexpression of a 25 kDa Hsp (Hsp25) induced expression of cell cycle inhibitory protein p21 (Waf1/Cip1/Sdi1) in murine fibroblastoid L929 cells. However, the mechanisms underlying the induction of p21 by Hsp25 are unknown. In the present study, we investigated the mechanisms underlying the regulation of p21 expression by Hsp25 in these cells. The introduction of Hsp25 cDNA stimulated the accumulation of p21 transcripts through transcriptional but not posttranscriptional regulation in these cells. We also found that overexpression of Hsp25 markedly increased the translational rate of p21 and stabilized the protein. Studies involving proteasome inhibitors and Western blot analysis for ubiquitination of p21 demonstrated that the stabilization of p21 is regulated through a ubiquitin-independent pathway. However, no direct association of Hsp25 with p21 was observed. These findings suggest that Hsp25 induces p21 expression through multiple mechanisms, and that transcriptional, translational, and post-translational regulation are important in the regulation of p21.

Key words: Hsp25, overexpression, p21, translation, ubiquitination.

Upon exposure to stresses including heat shock, irradiation, and inflammation, cells induce or activate proteins that protect them from these external insults. Heat shock proteins (Hsps) belong to one class of such proteins and were determined to be inducible in response to elevated temperatures (*1*). Hsps belong to a conserved and ubiquitous protein family found in all organisms, and the expression of Hsps is also induced at elevated levels in response to physiological stresses such as ischemia and infection (*1*). On the other hand, Hsps are constitutively expressed in cells where they may have essential actions (*2*). Hsps have been classified into six major families according to their molecular size in eukaryotes; small heat shock proteins (sHsps) with molecular masses of between 15 and 30 kDa (*3, 4*) have been identified. Their levels increase drastically on exposure to heat shock stress (*5*). These proteins are characterized by complex oligomeric properties and phosphorylation in response to stresses (*4, 6*). It has also been suggested that phosphorylation-induced changes in the ultrastructure may regulate the biological activities of sHsps. Prior studies on artificially manipulated expression of sHsps revealed that sHsps modulate cell survival during stress and apoptosis mediated by the Fas/APO1 receptor. Furthermore, sHsps also regulate the growth rate or differentiation and microfilament organization in response to a growth factor or stress (*7–9*). sHsps also exhibit molecular chaperone activities by preventing unfolded proteins from undergoing irreversible aggregation (*10*). Further studies have shown that sHsps have a number of different, seemingly unrelated functions such as RNA stabilization and elastase inhibition (*11, 12*).

One of the cell cycle inhibitory proteins, p21 (Waf1/Cip1/Sdi1) (cdkn1a), was first discovered as a cyclin-dependent kinase 2 (CDK2)–associated protein, which is regulated by p53 (*13, 14*). Further study revealed that the p21 protein is overexpressed in senescent cells (*15*). p21 has p53-binding sites on the promoter, and is thought to be a key downstream mediator of the p53-induced cell cycle arrest and apoptosis (*14, 16*). This protein also plays an important role in cellular differentiation (*15, 17*). On the other hand, p53-independent pathways for p21 expression have been shown: the activation of a variety of factors, including AP2, BRCA1, STATs, C/EBP-α, C/EBP-β, Sp1, and Sp3, can induce p21 transcription (*18–24*). Moreover, TGF-β, TNF-α, UV radiation, and hypoxia also induce p21 expression in a p53-independent manner (*25–28*).

There is an increasing number of reports on the important role that Hsp25 plays in cell growth. Overexpression of Hsp27 (the human homologue of murine Hsp25) suppresses cell growth in both melanoma A375 and epidermoid carcinoma A431 cell lines (*29*). In addition, the constitutively increased level of Hsp25 is associated with reduced growth rates of Ehrlich ascite tumor cells and hu-

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man B lymphocytes (*30, 31*). Furthermore, several studies have revealed that Hsp27 accumulates in the early stages of differentiation with a concomitant decreased rate of cell proliferation (*29, 30*). Previously, we found that the overexpression of Hsp25 retarded cell growth and changed the cell morphology with a concomitantly increased level of p21 protein in murine fibroblastoid L929 cells, which do not express Hsp25 (*32*). Furthermore, overexpression of Hsp25 did not affect the expression of other Hsp family proteins (*32*). On the other hand, the mechanisms underlying the induction of p21 expression in cells overexpressing Hsp25 are not understood. In the present study, we investigated the mechanisms underlying the regulation of p21 expression by Hsp25. We show that Hsp25 upregulates p21 expression through (i) transcriptional and (ii) translational regulation and (iii) protein stability.

MATERIALS AND METHODS

Chemicals—All the proteasome inhibitors, MG132 (*N*-carbobenzoxyl-leu-leu-leu-H; Calbiochem, La Jolla, CA), MG115 (*N*-carbobenzoxyl-leu-leu-norvalinal-H; Calbiochem-Novabiochem Corporation, San Diego, CA), and LLnN (*N*-acetyl-leu-leu-norleucinal-H; Sigma, St. Louis, MO), were dissolved in DMSO, and diluted at least 1/1,000 in cell culture medium.

Cell Culture—Hsp25-stable transfectants derived from murine fibroblastoid L929 cells (a kind gift from Dr. Y.M. Park, Inchon University College of Natural Sciences, Inchon, Korea) were maintained in α-MEM (Cosmo Bio, Tokyo) containing 7% fetal calf serum (Mitsubishi Kasei, Tokyo) and antibiotics at 37°C under a 5% CO_2 humidified atmosphere.

SDS-PAGE and Western Blot Analysis—After solubilization of cells with lysis buffer (40 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.1% NP-40) containing a protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany), equal amounts of protein (50–100 μg) were analyzed on an SDS–polyacrylamide gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA), and processed for immunoblotting using anti-Hsp25 or -p21 polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). For the detection of ubiquitin, the membranes were autoclaved in water for 20 min prior to staining with anti-ubiquitin polyclonal antibodies (Santa Cruz Biotechnology) as described previously (*33*). Specific bands were visualized using enhanced chemiluminesence (ECL; Amersham Pharmacia Biotech, Piscataway, NJ). The relative densities of the bands in different lanes were scanned with an FLA3000 (Fujix, Tokyo).

DNA Probes—The mouse p21 full-length cDNA was a kind gift from Dr. T. Tokino (Sapporo Medical University, Sapporo). The human Hsp27 full-length cDNA was provided by Dr. Carper (University of Nevada, Las Vegas, Nevada). The 0.68 kb DNA fragment (*Eco*RI/*Eco*RV) from the murine p21 cDNA and the DNA fragment (0.76 kb, *Eco*RI) from the human Hsp27 cDNA were used as probes. These probes were ^{32}P-labeled using a random priming method (*34*). The specific activity of each probe was greater than 2×10^8 cpm/μg of DNA.

RNA Isolation and Northern Blot Analysis—Total RNA was obtained by the guanidinium-hot phenol method as described previously (*35*). Cells were lysed in a guanidinium isothiocyanate mixture (4 M guanidinium isothiocynate, 50 mM Tris-HCl, pH 7.0, 20 mM EDTA, 2% sodium lauryl sarcosinate, 140 mM β-mercaptoethanol). The lysed cells were treated with proteinase K, and then their total RNA was extracted by the phenol/chloroform method. After denaturation at 65°C, the RNA was electrophoresed in a 1% (w/v) formaldehyde-agarose gel and then transferred to a nylon membrane filter (Hybond-N; Amersham Pharmacia Biotech). The filters were hybridized with a ^{32}P-labeled probe for p21 or Hsp25 cDNA for 16–24 h at 42°C in a solution containing 50% formamide, 2× SSC (where SSC is 150 mM NaCl, 15 mM sodium citrate), 5× Denhardt's solution, 0.1% SDS, 10% (w/v) dextran sulfate, and 100 μg/ml salmon sperm DNA. The relative densities of the bands in different lanes were scanned with an FLA3000.

Transcriptional Run-On Assay—Nuclei were isolated by suspending cells in ice-cold hypotonic buffer (10 mM Tris-HCl, pH 7.4, 10 mM KCl, 3 mM $MgCl_2$), and then lysed in the same buffer containing 0.5% NP-40. The nuclei were harvested and washed in a hypotonic buffer containing 0.5% NP-40, and then resuspended in nuclear storage buffer (40% glycerol, 50 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$, 0.1 mM EDTA). The nuclei were incubated for 30 min at 30°C in a reaction buffer containing 150 mM KCl, 3 mM $MgCl_2$, 5 mM Tris-HCl, pH 8.0, 0.25 mM ATP, 0.25 mM GTP, 0.25 mM CTP, and 200 μCi of [α-^{32}P]UTP (3,000 Ci/mM). The reaction was terminated by adding DNase for 10 min at 30°C. The reaction mixture was digested with 400 μg/ml of proteinase K in a solution containing 10 mM EDTA and 1% (w/v) SDS, followed by extraction with phenol/chloroform. The aqueous phase was precipitated at −70°C with 50% (v/v) isopropyl alcohol in the presence of 0.3 M sodium acetate, and the precipitate was collected by centrifugation and then dissolved in TE buffer. After denaturation in ice-cold 0.3 M NaOH and then neutralization in 0.25 M HEPES, the nuclear RNA was run through a Sephadex G50 spin column to remove unincorporated [^{32}P]-UTP. Plasmid DNA containing the cDNA coding inserts was denatured by heat and alkali treatment (0.3 M NaOH). In addition to the murine p21 cDNA, a full-length mouse β-actin and pTracer-CMV2 were used as controls. Denatured plasmids (10 μg for p21, 5 μg for pTracer-PMV2, and 5 μg for β-actin) were bonded to nylon membranes (Hybond-N) with Bio-Dot SF (Bio-Rad), and immobilized using a UV cross-linker. Newly elongated nuclear RNA was hybridized to the filters containing plasmids. Hybridization was performed with 10^7 cpm of ^{32}P-labeled RNA/ ml in a buffer containing 3× SSC, 5 mM EDTA, 0.1% SDS, 10× Denhardt's solution, 50% (v/v) formamide, 200 μg/ml yeast tRNA, 10 mM NaH_2PO_4, pH 7.0, and 100 μg/ml of salmon sperm DNA for 3 days at 42°C. After hybridization, the filters were rinsed in 2× SSC at room temperature, and then in 2× SSC and 0.1× SSC at 42°C. The filters were then exposed to X-ray film (RX; Fuji Photo Film, Kanagawa) and autoradiograms were developed at different exposures. The relative densities of the bands in different lanes were scanned with an FLA3000.

Pulse Labeling Experiments on Protein Synthesis of p21—Cells were pulse-labeled with 150 μCi/ml of Trans-[^{35}S]-Label™ (ICN, Irvine, CA) in methionine-free DMEM (GIBCO/BRL, Gaithersburg, MD) for 2 or 4 h following methionine starvation for 12 h. Immunoprecipitation of the p21 protein was performed with an anti-p21 polyclonal

antibody (Santa Cruz Biotechnology) and protein A-Sepharose (Amersham Pharmacia Biotech). The resulting precipitates were subjected to SDS-PAGE on a 12% gel, autoradiography, and analysis with an FLA3000.

RESULTS

Overexpression of Hsp25 Increases the p21 Protein Level—Previously, we found that the introduction of Hsp25 cDNA resulted in an increased level of p21 in L929 cells (*32*). Figure 1 shows the levels of Hsp25 and p21 in control vector- (pcDNA3) and Hsp25-transfectants (Hsp25) used in the present study on Western blot analysis. The Hsp25 protein was not detected in vector-transfectants. However, the level of p21, which was constitutively expressed in control transfectants, was 6-fold higher in the Hsp25-transfectants compared to in the control transfectants.

Hsp25 Increases p21 at the mRNA Level—To determine whether or not Hsp25 also regulates p21 expression at the mRNA level, we performed Northern blot analysis using a ^{32}P-labeled Hsp25 or p21 cDNA probe. As shown in Fig. 2, control vector cells showed no constitutive expression of Hsp25 mRNA. In contrast, transcripts of p21 (2.1 kb) were constitutively expressed in control cells and the level of p21 mRNA was about 2~3-fold higher in Hsp25-transfectants.

p21 Is Up-Regulated at the Transcriptional but Not the Post-Transcriptional Level in Hsp25-Transfectants—The ac-

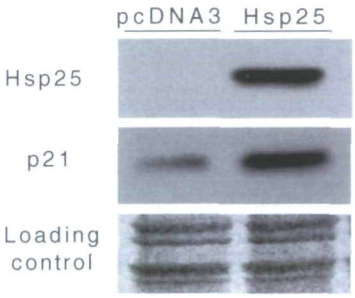

Fig. 1. **Increased steady-state levels of p21 protein in Hsp25-transfected L929 cells.** Whole cell lysates were prepared from control vector- (pcDNA3) and Hsp25-transfectants (Hsp25). Western blot analysis was performed with anti-Hsp25 or anti-p21 polyclonal antibodies. As a loading control, membranes were stained with a Ponceau S solution prior to Western blot analysis.

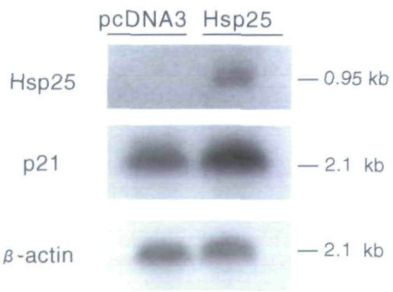

Fig. 2. **Levels of Hsp25 and p21 mRNA in Hsp25-transfectants.** Total RNA (30 μg/lane) was prepared as described under "MATERIALS AND METHODS," analyzed by formaldehyde-agarose gel electrophoresis, and then transferred to a nylon membrane. Hybridization was performed with ^{32}P-labeled Hsp25 cDNA, p21 cDNA and β-actin cDNA.

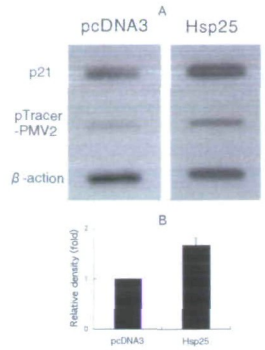

Fig. 3. **Transcriptional run-on analysis of p21.** A: Nuclei were isolated from cells as described under "MATERIALS AND METHODS," and the newly elongated ^{32}P-labeled transcripts were hybridized to the plasmid containing the insert of either p21 or β-actin cDNA, or the control plasmid pTracer-PMV2. The results are typical of three independent experiments. B: Non-specific hybridization observed in pTracer-PMV2 bands was subtracted and the relative densities were calculated according to the ratio of p21 to β-actin. The results are the means ± SD for three independent experiments. pcDNA3 and Hsp25; $p < 0.05$.

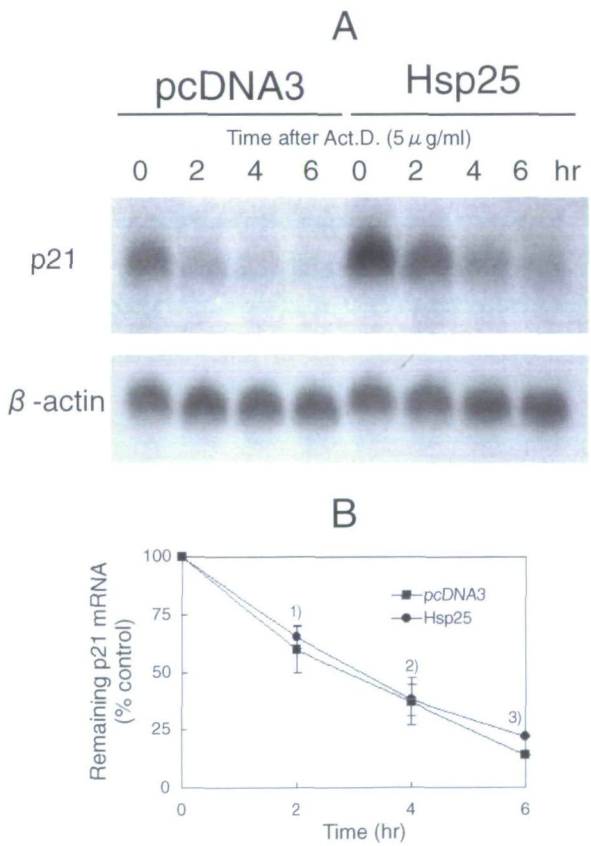

Fig. 4. **Stability of p21 mRNA in Hsp25-transfectants.** A: Cells were incubated in the presence of 5 μg/ml of actinomycin D (Act. D) and harvested at the indicated times. Total RNA was isolated and subjected to Northern blot analysis for p21. The results are typical of two or three independent experiments. B: The p21 levels in panel A were determined by densitometry and plotted. The level in untreated cells was taken as 100%. The results are for two or three independent experiments. [1] [2]Not significant (three experiments), [3]mean of two experiments.

cumulation of mRNA is regulated through both its transcription and degradation. We examined the transcriptional rate and mRNA stability of p21 in these cells. To determine whether or not the induction of p21 by Hsp25 is transcriptionally regulated, we performed transcriptional run-on assays (Fig. 3A). The transcription of p21 increased by 1.67 ± 0.15 fold in Hsp25-transfectants compared to that in the control vector cells ($p < 0.05$) (Fig. 3B). Studies on the transcriptional rate with the luciferase assay using a reporter construct containing the 3.6 kb promoter of p21 were also performed. The results obtained were almost identical with these described above (data not shown).

We also examined the stability of p21 mRNA. Cells were incubated in the presence of actinomycin D (5 μg/ml), and the p21 mRNA level was determined at various times (Fig. 4A). The p21 mRNA decay was plotted as a graph (Fig. 4B). Whereas the initial levels were different between control cells and Hsp25-transfectants, the transcripts decayed almost identically (Fig. 4B). These results suggest that the increased level of p21 mRNA in Hsp25-transfectants occurs at the transcriptional but not the post-transcriptional level.

Hsp25 Increases p21 Protein De Novo Synthesis—To determine the other mechanism for the increased level of p21 in Hsp25-transfectants, we examined the translational rate of p21 in these cells. Cells were pulse-labeled with [^{35}S]-methionine for 2 or 4 h and then cell lysates were subjected to immunoprecipitation analysis with anti-p21 polyclonal antibodies. Faint bands were detected at 2 and 4 h for the control cells (Fig. 5A). In contrast, a band was observed more clearly at 2 h for the Hsp25-transfectants and these cells showed an almost 10-fold-higher level at 4 h compared to that in the control cells.

Hsp25 Regulates p21 Protein Stability—The accumulation of a protein is also regulated through its synthesis and degradation. We examined the stability of the p21 protein in Hsp25-transfectants. Cells were incubated with cycloheximide (10 μg/ml) to inhibit any *de novo* protein synthesis, and then the p21 protein level was determined at various times (Fig. 6). The half-life of the steady state p21 protein in control vector cells was 30 min, while that in Hsp25-transfectants was 1 h. Moreover, almost 30% of the p21 protein remained in Hsp25-transfectants treated with cycloheximide for 4 h, but the control cells retained only 10% of the untreated level. Thus, degradation of the p21 protein was slower in the Hsp25-transfectants than in the control cells.

p21 and Hsp27 Are Induced in Differentiated HL60 Cells—12-*O*-Tetradecanoyl phorbol 13 acetate (TPA) is a potent inducer of the differentiation of leukemic cells (*36*). TPA has also been shown to induce phosphorylation and accumulation of the Hsp27 protein in HL60 cells (*30, 37*). We examined the induction of p21 expression after treatment with TPA in HL60 cells. HL60 cells were incubated with 100 nM TPA for 24 h. Figure 7A shows the morphological changes of HL60 cells induced by TPA. Upon treatment with TPA, cells became adherent to plastic culture dishes and developed a macrophage-like morphology with long filamentous pseudopods. Western blot analysis showed that TPA induced Hsp27 expression with a concomitant increased level of p21 in HL60 cells (Fig. 7B).

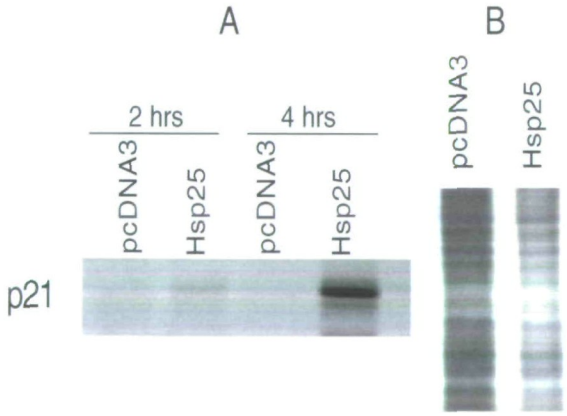

Fig. 5. **Hsp25 increases p21 protein de novo synthesis.** A: Cells were cultured for 12 h in methionine-free medium and then pulse-labeled with [^{35}S]methionine for 2 or 4 h. Immunoprecipitates of p21 were subjected to SDS-PAGE on a 12% polyacrylamide gel. B: Equal amounts of protein extracts at 4 h were subjected to SDS-PAGE and fluorographed. Since the level of newly synthesized total protein at 4 h was identical in the two cells, any artifacts on loading could be excluded. The results are typical of at least three independent experiments.

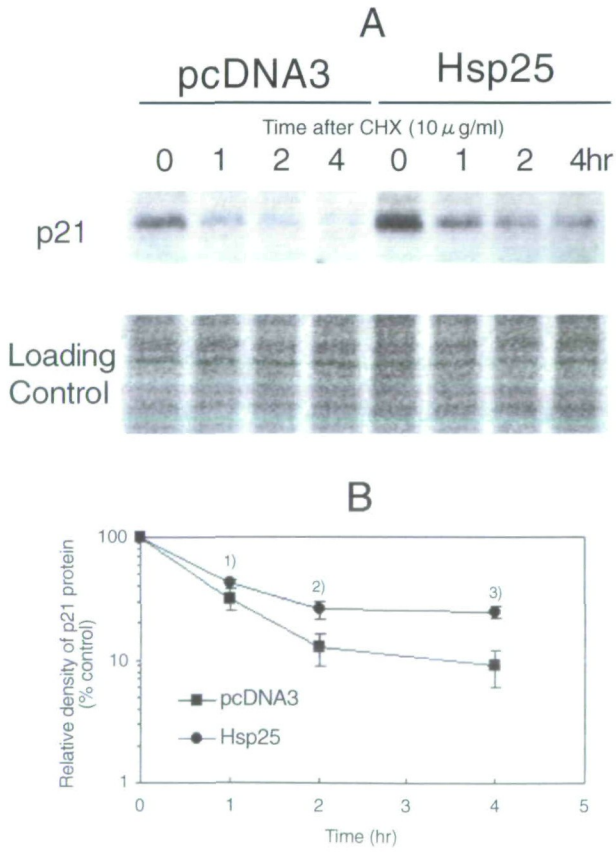

Fig. 6. **Hsp25 stabilizes the p21 protein.** A: Cells were treated with 10 μg/ml of cycloheximide (CHX) and harvested at the indicated times. Cell lysates were prepared and subjected to Western blot analysis for p21. The results are typical of three independent experiments. B: The p21 levels in panel A were determined by densitometry and plotted. The half-life of p21 was extended in Hsp25-transfectants. The level in untreated cells was taken as 100%. The results are the means ± SD for three independent experiments. [1]$p < 0.05$, [2,3]$p < 0.01$.

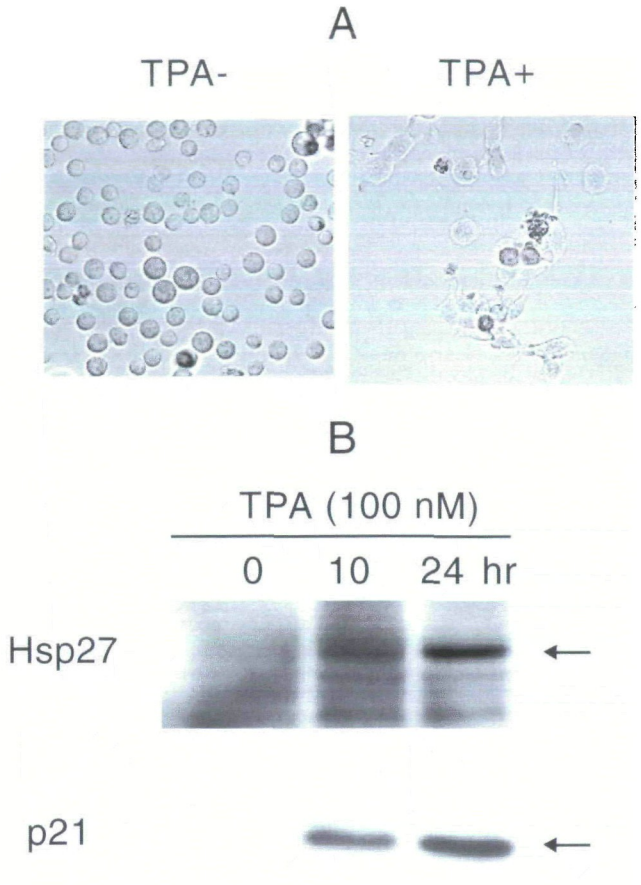

Fig. 7. **Coordinated expression of Hsp27 and p21 in differentiation-induced HL60 cells.** A: HL60 cells were treated with 100 nM TPA for 24 h and then their morphological changes were compared by optical microscopy as to untreated control cells. B: After treatment with TPA, cell lysates were prepared at the indicated times and subjected to Western blot analysis. The results are typical of at least three independent experiments.

DISCUSSION

The expression of heat shock proteins (Hsps) is induced in response to various types of physiological stresses; these proteins then function as molecular chaperones. Recent studies, on the other hand, have shown that Hsps have effects on cell growth and differentiation (*8, 9*). Previously, we showed that Hsp25 is involved in the regulation of p21 expression (*32*). In the present study, we examined the mechanisms of expression of cell-cycle inhibitory protein p21 (Waf1/Cip1/Sdi1) induced by the introduction of Hsp25 cDNA into mouse L929 fibroblasts. We found that Hsp25 induces p21 expression through multiple mechanisms such as transcription, translation, and post-translational control in these cells.

The steady state level of mRNA is dependent on both transcription and degradation. p21 has p53-binding sites on its promoter and its expression is under the transcriptional control of p53 (*14, 16*). On the other hand, there is a growing body of evidence suggesting that the expression of p21 can be induced at the transcriptional level *via* a pathway that does not require p53 (*15, 26*). In the present study, we found that the introduction of Hsp25 cDNA stimulated the accumulation of p21 transcripts through transcriptional but not post-transcriptional regulation in L929 cells. A previous study revealed that p53 regulates the human Hsp70 promoter through a direct protein–protein interaction with CCAAT-binding factor (CBF) (*38*). A recent study revealed the functional p53-dependent induction of Hsp27; adenoviral expression of the wild type of p53 but not mutant p53 induced Hsp27 in prostate cancer cells (*39*). Furthermore, the p53 function is required for G1-arrest after heat shock in normal cells (*40*). L929 cells carry the wild type of p53 but the introduction of Hsp25 cDNA did not affect the level of p53 (*32*). Thus, our results suggest that p53 is unlikely to be involved in the expression of p21 by Hsp25. On the other hand, molecular analyses of Hsps genes revealed the heat shock element (HSE) (5′-nGAAn-3′) on the promoters at various distances upstream of the site of transcription initiation (*41*). Heat shock transcription factor (HSF) binds to HSE transiently and induces the transcription of Hsps. Therefore, we also investigated the involvement of HSF in the regulation of p21 transcription. The expression levels of HSF1 and HSF2 were similar in both control and Hsp25-transfectants at both the protein and mRNA levels (data not shown). However, transcriptional run-on assays and luciferase assays with a reporter construct containing 3.6 kb of the p21 promoter showed that the transcription of p21 was increased in Hsp25-transfectants (not all data shown). Taken together, our results indicate that Hsp25 may either have a direct effect on the transcription of p21 or modify pre-existing transcriptional factors that interact with the p21 promoter. In the present study, on the other hand, we revealed a discrepancy between the magnitudes of the increased levels of p21 mRNA and protein. Therefore, our results suggest that the increased levels of p21 in Hsp25-transfectants are not due entirely to regulation at the mRNA level, whereas it has been reported that p21 mRNA and protein expression are uncoupled in several cell lines; transcriptional and posttranscriptional changes in p21 expression strongly depend on the individual cellular background (*42*).

There has been a report that vitamin D3 regulates p21 expression at the protein level without alteration of the mRNA level, suggesting translational or post-translational regulation (*43*). Therefore, we examined the translational rate of p21 in Hsp25-transfectants. The results of pulse labeling experiments with [^{35}S]methionine demonstrated that the translational rate of p21 was markedly increased in Hsp25-transfectants. We do not know the mechanism responsible for the increased rate of p21 translation in Hsp25-tranfectants. A recent study has shown that Hsp101, which is conserved in bacteria, yeast, and plants, has a translational regulatory function; Hsp101 functions as an RNA binding protein that binds to a poly (CAA) region within the 5′ leader from tobacco mosaic virus (TMV), and mediates the translational enhancement associated with the 5′ leader (*44*). Furthermore, Hsp101 has been shown to enhance the translation from the internal light-regulatory element (iLRE) of ferredoxin (Fed-1)-containing mRNA in yeast (*45*). In mammalian cells, however, the function of translational enhancement of any heat shock protein has not yet been determined, whereas Hsp27 specifically binds eIF4G that is required for the translation of most mRNAs and inhibits eIF4F(cap)-dependent transla-

tion (*46*). Further studies are required.

The stabilization of proteins is an important mechanism for cell cycle regulatory proteins, including p53, p27, c-myc, and c-fos; these proteins must be able to undergo rapid changes in response to the cells being exposed to external insults (*47*). Previously, we reported that protein stabilization is one of the most important mechanisms for the accumulation of p21 (*48*). Our present results show that the introduction of Hsp25 cDNA stabilized the p21 protein in L929 cells. Since one of the mechanisms for the degradation of p21 is ubiquitin-proteasome–mediated proteolysis (*49, 50*), we studied this mechanism as to stabilization of the p21 protein in Hsp25-transfectants. However, studies with inhibitors of proteasome (MG132 and MG115) or calpain (LLnL) showed that the effect of these inhibitors on the expression of p21 was very similar in control cells and Hsp25-transfectants (data not shown). Further studies revealed that there was no difference in the pattern of ubiquitination or the level of ubiquitinated p21 in the two cell lines (data not shown). IκBα degradation is induced by TNFα; or during differentiation of B lymphocytes, it occurs through a pathway that is independent of ubiquitin proteolysis (*51, 52*). Our results suggest that the stabilization of the p21 protein in Hsp25-transfectants may be regulated through a ubiquitin-independent pathway. Our stability analysis of the p21 protein also showed that overexpression of Hsp25 caused only 2-fold stabilization as compared to in the control cells. In contrast, the translational rate of p21 was markedly increased in Hsp25 tranfectants. Taken together, our data strongly suggest that the regulation of p21 occurs mainly at the translational level in these cells.

Hsps are ubiquitous molecular chaperones in prokaryotes and eukaryotes. In mammalians, sHsps are ATP-independent chaperones that complex physically with certain unfolding protein intermediates and thereby limit the extent of their degradation (*10*). Furthermore, there have been many reports suggesting that Hsp25/Hsp27 selectively binds to cellular molecules under physiological conditions (*10, 46*). On the other hand, it has been found that the expression of Hsp25 and/or p21 is involved in differentiation (*9, 17*), and Hsps expression is also modulated by various conditions leading to apoptosis (*53*). Terminal differentiation accompanies cell cycle arrest leading to apoptosis. Indeed, the differentiation induction of human HL60 promyelotic cells toward a monocyte/macrophage lineage led to an increased level of p21 with concomitant accumulation of Hsp27, suggesting that these two proteins may interact. Therefore, we determined the degree of association of Hsp25 with p21 by immunoprecipitation analysis. However, no direct association was observed between Hsp25 and p21 (data not shown). The results of the present study suggest that Hsp25 regulates the p21 level, probably through indirect mechanisms. Further studies are required.

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