# Posttranscriptional Stabilization Underlies $p53$-independent Induction of $p21^{\text{WAF1/CIP1/SDI1}}$ in Differentiating Human Leukemic Cells

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## Abstract

$p21^{\text{WAF1/CIP1/SDI1}}$ is a recently identified gene expressed in cells harboring wild-type but not mutant $p53$ gene. It encodes a nuclear protein of 21 kD which inhibits cyclin-dependent kinase activity. Constitutive $p21^{\text{WAF1/CIP1/SDI1}}$ mRNA expression was detected in neoplastic cells from patients with various hematological malignancies as well as in normal bone marrow mononuclear cells and in myeloid and lymphoid cell lines independent of their $p53$ status. Induced differentiation of the $p53$-deficient promyelocytic HL-60 cells along the monocytic lineage by phorbol ester or $1\alpha,25$ dihydroxyvitamin $D_3$ resulted in a marked increase of both $p21^{\text{WAF1/CIP1/SDI1}}$ mRNA and protein expression due to enhanced mRNA stability. Differentiation towards the granulocytic lineage by all-*trans* retinoic acid or dimethylsulfoxide failed to produce this effect. $p21^{\text{WAF1/CIP1/SDI1}}$ is an immediate early gene since its upregulation occurred independently of de novo protein synthesis. The induction of $p21^{\text{WAF1/CIP1/SDI1}}$ expression and its regulation in $p53$-deficient differentiating leukemic cells support the idea of an additional, $p53$-independent role of $p21^{\text{WAF1/CIP1/SDI1}}$ in human hematopoiesis. (*J. Clin. Invest.* 1995. 95:973–979.) Key words: $p21^{\text{WAF1/CIP1/SDI1}}$ • leukemia • myeloid differentiation • immediate early gene • posttranscriptional stabilization

## Introduction

The $p53$ gene is one of the most frequently altered genes in human cancer. This nuclear phosphoprotein is inactivated in a variety of human tumors by mutation; $p53$ can also be disabled through complex formation with either viral oncogene products or by binding to a cellular protein encoded by the $mdm-2$ oncogene (1). Wild-type (wt)[1]-$p53$ suppresses oncogene-mediated cell transformation and decreases the tumorigenicity of human tumor cell lines. The physiological role of $wt$-$p53$ probably is to maintain the integrity of the normal genome. In response to exposure to DNA-damaging agents (gamma or ultraviolet irradiation), the level of $wt$-$p53$ protein increases and acts as a checkpoint control by blocking the cell cycle in the $G_1$ phase, which in turn results either in a delay in progress through the cell cycle to permit repair processes or in the initiation of programmed cell death (apoptosis) (2, 3). Although the exact molecular mechanisms to explain the function of $wt$-$p53$ have not yet been elucidated, $wt$-$p53$ can both transactivate and suppress gene expression (4). In vitro, $wt$-$p53$ protein represses the activity of several gene promotors (e.g., c-$fos$, c-$jun$, β-actin, and IL-6) by interaction with the TATA binding protein (5). By binding to $p53$ sequence specific DNA consensus sites, $wt$-$p53$ can transactivate the expression of $GADD45$, $mdm-2$ and $WAF1$ ($wt$-$p53$-activated fragment) gene (6–8).

$WAF1$ is a recently identified gene induced in wild-type but not in mutant $p53$ gene–expressing cells (8). A $p53$ DNA-binding site was found 2.4 kb upstream of the coding sequences of $WAF-1$. In vitro growth of human brain, lung, and colon tumor cells is suppressed when a $WAF1$ expression vector is transduced into these cells. The $WAF1$ gene is localized to human chromosome 6p21.2 and encodes a nuclear protein of 21 kD ($p21^{\text{WAF1}}$). The same protein was simultaneously discovered as a cyclin-dependent kinase-interacting protein ($CIP1$) with potent $G_1$–cyclin-dependent kinase (Cdk) inhibitory activity (9). In normal human diploid fibroblasts, $p21^{\text{WAF1/CIP1}}$ forms complexes with cyclins (A, B, D, and E classes) associated with Cdks and proliferating nuclear antigen (PCNA) (10, 11). In SV-40 transformed cells, Cdk4 dissociates from the quaternary complex and associates with a protein of 16 kD (p16) which inhibits Cdk4/cyclin D enzyme activity (12, 13). Moreover, $p21^{\text{WAF1/CIP1}}$ directly inhibits PCNA-dependent DNA replication in the absence of cyclin/Cdk (14). In fibroblasts from Li-Fraumeni patients with $p53$ germline mutations and secondary loss of the $wt$-$p53$ allele through continuated passage, both PCNA and $p21^{\text{WAF1/CIP1}}$ protein were absent and not replaced by p16 (12). Furthermore, a cDNA isolated from senescent human fibroblasts exhibiting DNA synthesis inhibitory activity when introduced into young cycling cells, referred to as senescent cell–derived inhibitor ($SDI1$), also encodes for $p21^{\text{WAF1/CIP1/SDI1}}$ (15). The induction of $p21^{\text{WAF1/CIP1/SDI1}}$ expression in $wt$-$p53$ but not in mutant $p53$-containing cells by exposure to DNA-damaging agents provided further support for the notion that $p21^{\text{WAF1/CIP1/SDI1}}$ might be a critical effector of $p53$-mediated growth control (16). In addition, in response to γ-irradiation, $p21^{\text{WAF1/CIP1/SDI1}}$ inactivated cyclin E–Cdk2 complexes in human diploid fibroblasts resulting in a $G_1$ arrest of the cell cycle (17).

Involvement of the $p53$ gene in human leukemias has been well documented. In contrast to many lymphoid leukemic cell

lines which overexpress *p53*, myeloid leukemic cell lines frequently fail to express *wt-p53* (18–20). Mutations in the *p53* coding sequence are found in lymphoid and myeloid tumor cell lines, aggressive non-Hodgkin's lymphomas (NHL) and in at least 25% of chronic myeloid leukemia (CML) cases in blast crisis (21–25).

In this study, we examined the expression of $p21^{WAF1/CIP1/SDI1}$ in neoplastic cells from patients with various hematological malignancies and in several leukemia/lymphoma cell lines. By using the HL-60 promyelocytic cells which lack *p53* expression because of a large deletion of the *p53* gene (18, 26), we show that $p21^{WAF1/CIP1/SDI1}$ is expressed independently of *p53* and that its upregulation during myeloid differentiation is due to stabilization of the $p21^{WAF1/CIP1/SDI1}$ mRNA.

## Methods

*Reagents.* All-*trans* retinoic acid (RA) (Sigma Chemical Co., St. Louis, MO) and $1\alpha,25$ dihydroxyvitamin $D_3$ (Vit $D_3$; kindly provided by Dr. A. Kaiser, Hoffmann-La Roche, Basel, Switzerland) were dissolved in 95% ethanol to stock concentrations of 1 mM and stored at $-20°C$. Phorbol-12-myristate-13-acetate (PMA; Sigma Chemical Co.) was dissolved in acetone to a stock solution of 1 mM and stored at $-20°C$. Actinomycin D (ADM; Fluka Chemical Co., Buchs, Switzerland) was kept as stock solution (2 mM) dissolved in dimethyl sulfoxide (DMSO; Merck Chemical Co., Darmstadt, Germany). Final concentrations of DMSO never exceeded 0.5% (vol/vol).

*Cell lines.* The following human cell lines were cultured in McCoy's medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (Nabi, Miami, FL) in a humidified atmosphere containing 5% $CO_2$ at 37°C, HL-60 (promyelocytic, passages 25–40), KG-1 (myeloblastic) and KCL-22 (myeloblastic, Ph⁺), U-937 (monoblastic), HEL and K-562 (erythroid/myeloblastic, Ph⁺), DAMI (megakaryoblastic), MOLT-4 (T lymphoblastic), RAJI, DAUDI, RPMI 8866, and NC-37 (B lymphoblastic). All cell lines were obtained from the American Type Culture Collection (Rockville, MD), except for RPMI 8866, which was provided by Dr. R. Meier (Laboratory for Clinical and Experimental Research, University of Berne, Switzerland). In addition, normal diploid human lung fibroblasts (WI-38) were used. Differentiation of the induced cells was assessed by morphology and esterase staining. After exposure to RA and DMSO or Vit $D_3$ and PMA, > 85% of HL-60 cells displayed granulocytic or monocytic features, respectively.

*Patients and samples.* Fresh bone marrow or peripheral blood cells were obtained at diagnosis from 22 acute myeloid leukemias (AML, cases 1–22), 3 CML (1 blast crisis; cases 23–25), and 10 acute lymphoid leukemias (ALL, 9 B-ALL, cases 26–34; 1 T-ALL, case 35), 1 B cell prolymphocytic leukemia (B-PLL, case 36), 2 chronic lymphocytic leukemias of B cell type (B-CLL, cases 37 and 38), and 1 patient with high-grade B cell NHL in leukemic phase (B-NHL, case 39). Informed consent was obtained from all patients. The diagnosis of acute leukemia was established according to the French-American-British (FAB) classification and by immunophenotyping. AML M0 was defined as a leukemia with < 3% blasts positive for Sudan Black B or peroxidase by light microscopy and by demonstration of CD13 and CD33 expression. Mononuclear cells from the leukemic samples were separated by Ficoll-Hypaque density gradients (1.077 g/liter; Nyegaard Co., Oslo, Norway) and cryopreserved in liquid nitrogen until use. All leukemic mononuclear cell samples were composed of > 95% blast cells as shown by morphology and specific esterase staining on cytospin preparations. We also analyzed lymph node biopsies from six patients with NHL (two B cell low-grade [cases 40 and 41] and four B cell high-grade NHL [cases 42–45]). Furthermore, Ficoll-Hypaque density gradient–separated mononuclear bone marrow cells from five healthy donors (cases 46–50) were investigated.

*Northern blot analysis.* Total RNA was extracted by the single-step acid guanidinium thioacetate phenol-chloroform method. RNA samples (10 μg) were size-separated by an agarose-formaldehyde gel (1% wt/vol) and transferred to nylon membrane (Hybond-N; Amersham International, Buckinghamshire, United Kingdom). Hybridization with random-primer $^{32}P$-labeled probes (1–2 × 10⁶ cpm/ml hybridization solution) was performed for 16–24 h at 42°C. Filters were washed to a final stringency of 0.25 % SSC at 65°C and exposed for 6–96 h at $-70°C$ to XM-films (3M; Trimax, Ferrania, Italy). Some filters were deliberately overexposed with respect to the positive controls to detect low levels of mRNA. Purified inserts were used as human cDNA probes: *wt-p53* (1.1 kb, BamHI/EcoRI) from pcDNAI (27), and $p21^{WAF1/CIP1/SDI1}$ (2.1 kb, BamHI/HindIII) from pCEP (8), myeloperoxidase (*MPO*; PstI fragment) from pUC8 (28), and interleukin 8 (*IL-8*; 0.85 kb BamHI) from p(NAP)6T3 (29). Normal human diploid fibroblasts WI-38, known to express *wt-p53* and $p21^{WAF1/CIP1/SDI1}$ were used as positive controls (11). In the half-life experiments, autoradiograms were quantified by optical scanning using the IMAGEQUANT® software (Version 3.3, Molecular Dynamics, Inc., Sunnyvale, CA) on a personal computer.

*Nuclear run-on assay.* Nuclear run-on assays were performed as described previously (30) with some modifications. Briefly, nuclei were prepared by lysis of HL-60 cells in 10 mM Tris/HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP-40. 6 × 10⁷ nuclei, suspended in 100 μl of buffer consisting of 50 mM Tris/HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA, were added to 100 μl transcription buffer (20% glycerol, 100 mM KCl, 5 mM $MgCl_2$, 1 mM each of ATP, GTP, and CTP, 5 mM dithiothreitol, 100 μCi [$\alpha$-$^{32}P$]UTP (400 Ci/mmol), and 100 U RNasin). After incubation for 20 min at 30°C, the elongated transcripts were extracted and purified by the acid guanidinium thiocyanate phenol-chloroform procedure. The RNA was finally dissolved in 2 ml of hybridization buffer and hybridized to denatured plasmid DNAs ($p21^{WAF1/CIP1/SDI1}$, *p53*, *MPO*, *IL-8*, and *pUC*18) immobilized on nitrocellulose membranes (5 μg/dot) at 42°C for 72 h. Membranes were washed for 20 min at 65°C in 0.2 × SSC/0.1% SDS and in 0.1 × SSC/0.1% SDS. After treatment with 10 μg/ml RNase A at 37°C for 1 h, filters were washed in 2 × SSC for 20 min at room temperature and then exposed to x-ray film at $-70°C$.

*Western blot analysis.* Total cellular protein (10 μg) was separated on a 14% SDS-polyacrylamide gel. After transfer to nitrocellulose, $p21^{WAF1/CIP1/SDI1}$ protein was detected by enhanced chemiluminescence following the manufacturer's instructions (Amersham International). Nonspecific binding was blocked with 0.5% gelatin. A polyclonal rabbit anti–human $p21^{WAF1/CIP1/SDI1}$ specific antibody (PharMingen, San Diego, CA) was used at a dilution of 1:1,000.

## Results

$p21^{WAF1/CIP1/SDI1}$ *mRNA expression in cells from patients with leukemias and lymphomas and in hematological cell lines.* Northern blot analysis of total RNA from purified blast cells from 22 AML, 3 CML, 9 B-ALL, 1 T-ALL, 2 B-CLL, 1 B-PLL, and 1 B-NHL (in leukemic phase), and in lymph node biopsies from 6 patients with B-NHL of different subtypes was performed by using a $p21^{WAF1/CIP1/SDI1}$ cDNA probe that detects a 2.1-kb mRNA transcript. Various levels of $p21^{WAF1/CIP1/SDI1}$ transcripts were detected in all but two investigated samples (case 2, AML M0; case 23, CML in blast crisis). No aberrant transcripts were seen. There was no correlation between the FAB type and the level of $p21^{WAF1/CIP1/SDI1}$ mRNA expression. $p21^{WAF1/CIP1/SDI1}$ transcripts were also found in specimens of mononuclear bone marrow cells from five healthy volunteers. These data indicate that most normal and leukemic hematopoietic cells constitutively express $p21^{WAF1/CIP1/SDI1}$ mRNA.

We also examined $p21^{WAF1/CIP1/SDI1}$ expression in seven myeloid (HL-60, K-562, KG-1, KCL-22, U-937, HEL, DAMI) and five lymphoid (MOLT-4, RAJI, DAUDI, NC-37, RPMI 8866) cell lines. Constitutive expression of $p21^{WAF1/CIP1/SDI1}$
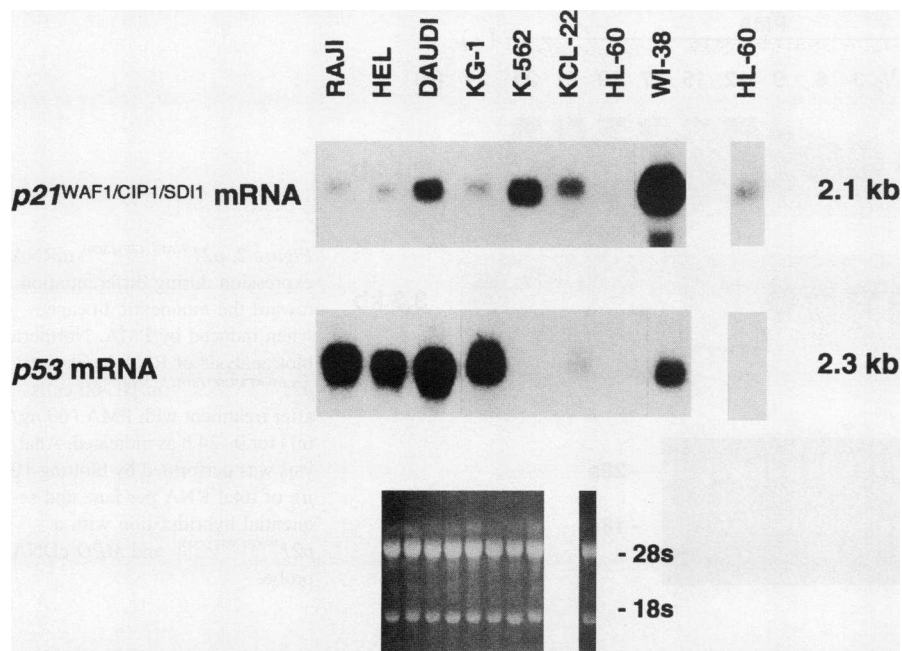
*Figure 1.* Expression of $p21^{WAF1/CIP1/SDI1}$ and $p53$ mRNA in human hematopoietic cell lines. Northern blot analysis of total RNA (10 μg/lane) from myeloid (HL-60, KCL-22, KG-1), biphenotypic (K-562, HEL), and lymphoid (RAJI, DAUDI) cell lines. The blot was sequentially hybridized with $^{32}$P-labeled cDNA probes for $p21^{WAF1/CIP1/SDI1}$ and $p53$. WI-38 cells (human diploid lung fibroblasts) were used as positive control for $p21^{WAF1/CIP1/SDI1}$ mRNA expression. Exposure time of the autoradiograms were ≤ 3 d. The left small top panel shows $p21^{WAF1/CIP1/SDI1}$ mRNA expression of HL-60 cells when the autoradiogram was exposed for 8 d. In all Northern blots shown, the small bottom panel shows the ethidium bromide–stained Northern gel with 28s and 18s rRNA demonstrating equivalent RNA-loading per lane.

mRNA was observed in all 12 cell lines with levels varying from low (RAJI, HEL, KG-1, HL-60, U-937, DAMI) to high (DAUDI, K-562, KCL-22, MOLT-4, NC-37, RPMI 8866) (Fig. 1). In HL-60, KG-1, and U-937 cells, the low levels of $p21^{WAF1/CIP1/SDI1}$ mRNA were detected after exposure of the autoradiograms for ≥ 3 d. No aberrant transcripts were detected in any of the cell lines. The Northern blots of leukemic patients and the cell lines were rehybridized with a *wt-p53* cDNA probe detecting a 2.3-kb mRNA. *p53* mRNA was seen in 37 out of 39 leukemia cases and in 9 out of 11 cell lines examined (KG-1, KCL-22, HEL, DAMI, MOLT-4, RAJI, DAUDI, NC-37, RPMI 8866). There was no correlation between levels for $p21^{WAF1/CIP1/SDI1}$ and $p53$ transcripts.

HL-60 and K-562 cells do not express $p53$ mRNA and protein (18, 31), and yet they did express $p21^{WAF1/CIP1/SDI1}$ mRNA. The HL-60 cells used in our experiments contained a previously reported $p53$ deletion as assessed by Southern blot and PCR analysis (our own results [not shown], and references 18, 26, and 32). RT-PCR experiments furthermore confirmed that no $p53$ mRNA was transcribed of the 5' undeleted region of the $p53$ gene in both uninduced and PMA-induced HL-60 cells when using primers covering exons 1–4 and 4–5. These results show that leukemic cells can display constitutive expression of $p21^{WAF1/CIP1/SDI1}$ mRNA in the absence of $p53$.

*Differentiation of HL-60 cells toward monocytes but not to granulocytes is associated with an increase of $p21^{WAF1/CIP1/SDI1}$ mRNA expression.* Low constitutive expression of $p21^{WAF1/CIP1/SDI1}$ mRNA was observed in HL-60 cells when autoradiograms were exposed for ≥ 3 d despite the lack of $p53$ transcripts (Fig. 1). To examine whether $p21^{WAF1/CIP1/SDI1}$ mRNA levels can be altered in the absence of $p53$ during myeloid cell differentiation, experiments were performed with HL-60 cells induced to differentiate toward either monocytic or granulocytic cells. Both PMA and Vit $D_3$ were used separately to induce monocyte/macrophage differentiation, and RA and DMSO were used separately to induce granulocytic differentiation (33). Exposure to PMA (60 ng/ml) resulted in a marked increase of $p21^{WAF1/CIP1/SDI1}$ mRNA levels. Transcripts for $p21^{WAF1/CIP1/SDI1}$ were very

rapidly induced after 30–90 min of exposure to PMA, and plateau levels were reached by 9–12 h (Fig. 2). Further experiments revealed that high levels of $p21^{WAF1/CIP1/SDI1}$ mRNA persisted for at least 72 h. Rehybridization of the blot with a *MPO* cDNA probe showed a marked decrease of *MPO* mRNA expression, as described previously (34). When compared with the PMA treatment, a delayed and less pronounced increase of $p21^{WAF1/CIP1/SDI1}$ mRNA expression was seen when HL-60 were cultured with Vit $D_3$ ($10^{-7}$ mol/liter) (Fig. 3). Plateau levels were reached after 72–96 h of exposure to this agent. In contrast to the monocytic pathway of differentiation, induction of HL-60 cells toward granulocytes by either RA ($10^{-6}$ mol/liter, 0–96 h) or DMSO (1.25%, 0–96 h) did not result in an increase of $p21^{WAF1/CIP1/SDI1}$ mRNA expression.

To assess whether the increase of $p21^{WAF1/CIP1/SDI1}$ mRNA during phorbol-ester–induced differentiation of HL-60 cells toward the monocytic lineage is associated with an increase in $p21^{WAF1/CIP1/SDI1}$ protein expression, Western blot experiments were performed. As shown in Fig. 4, no $p21^{WAF1/CIP1/SDI1}$ protein (21 kD) could be detected in unstimulated HL-60 cells. Similarly to the $p21^{WAF1/CIP1/SDI1}$ mRNA, $p21^{WAF1/CIP1/SDI1}$ protein levels increased and became detectable after 3 h of PMA treatment. Peak level of $p21^{WAF1/CIP1/SDI1}$ protein was seen after 24 h and remained elevated for at least 72 h of PMA exposure. Taken together, these data show that differentiation of HL-60 cells toward the monocytic lineage, but not to the granulocytic lineage, is associated with a sustained increase of both $p21^{WAF1/CIP1/SDI1}$ mRNA and protein.

*Regulation of $p21^{WAF1/CIP1/SDI1}$ mRNA expression in phorbol ester–induced HL-60 cells.* To examine whether de novo protein synthesis is required for the PMA-induced increase in $p21^{WAF1/CIP1/SDI1}$ expression in leukemic cells, experiments were performed with cycloheximide (CHX, 40 μg/ml). In HL-60 cells, CHX at 40 μg/ml blocked > 95% of protein synthesis as determined by [$^{35}$S]methionine incorporation. Cells were preincubated for 1 h with CHX and then exposed for 30 min to 24 h to PMA. As shown in Fig. 5, levels of $p21^{WAF1/CIP1/SDI1}$ transcripts increased in a manner that paralleled those observed
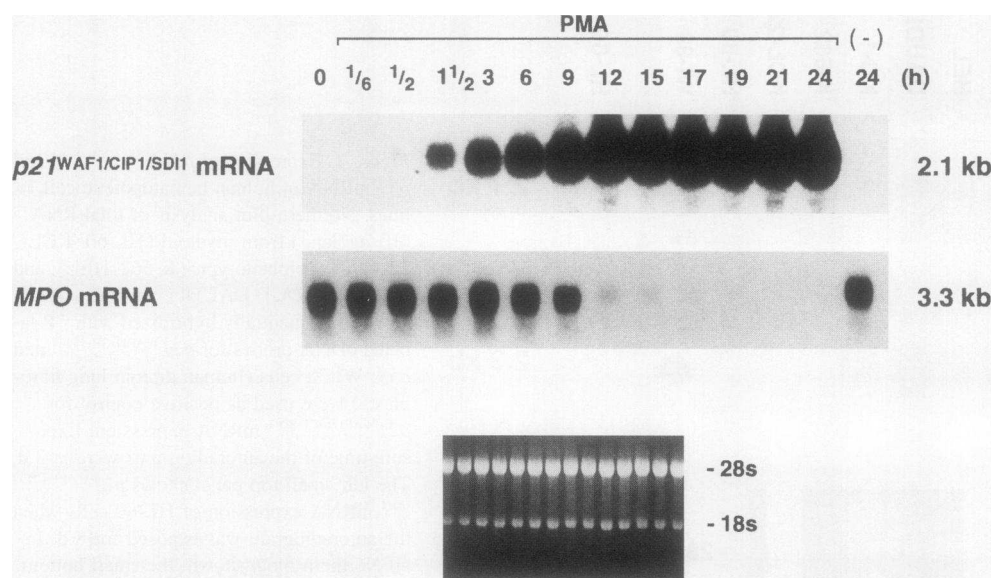
Figure 2. $p21^{WAF1/CIP1/SDI1}$ mRNA expression during differentiation toward the monocytic lineage when induced by PMA. Northern blot analysis of RNA levels for $p21^{WAF1/CIP1/SDI1}$ in HL-60 cells after treatment with PMA (60 ng/ml) for 0–24 h as indicated. Analysis was performed by blotting 10 $\mu$g of total RNA per lane and sequential hybridization with a $p21^{WAF1/CIP1/SDI1}$ and MPO cDNA probe.

in the absence of CHX (Fig. 2). This indicates that upregulation of $p21^{WAF1/CIP1/SDI1}$ expression occurs independently of intermediary proteins and that $p21^{WAF1/CIP1/SDI1}$ belongs to the set of primary response genes.

The increase of steady state $p21^{WAF1/CIP1/SDI1}$ mRNA levels could be due either to an enhanced rate of transcription or stabilization of previously transcribed $p21^{WAF1/CIP1/SDI1}$ mRNA or to a combination of both mechanisms. To determine whether stabilization of $p21^{WAF1/CIP1/SDI1}$ transcripts might be enhanced after PMA stimulation, half-life $(t_{1/2})$ studies of $p21^{WAF1/CIP1/SDI1}$ mRNA were performed by blocking overall transcription with ADM (10 $\mu$g/ml) in uninduced and PMA-induced HL-60 cells. Total RNA was extracted at each experimental point (0–8 h), and the decay of $p21^{WAF1/CIP1/SDI1}$ mRNA was determined by Northern blot analysis. In the absence of PMA, $p21^{WAF1/CIP1/SDI1}$ mRNA was barely measurable due to the low constitutive expression level. Nevertheless, a long exposure of autoradiograms and blotting of 20 $\mu$g RNA in each lane showed a rapid decay of $p21^{WAF1/CIP1/SDI1}$ mRNA with an estimated $t_{1/2}$ of 45 min. To assess $p21^{WAF1/CIP1/SDI1}$ mRNA stability in PMA-induced cells and to examine whether it might be different at early compared with later stages of induction, the cells were exposed for 90 min, 12 h, and 21 h to PMA, and then to ADM for 0–8 h. As shown in Fig. 6, 12 h of exposure to PMA markedly increased $p21^{WAF1/CIP1/SDI1}$ mRNA $t_{1/2}$ from 45 min to 4.5 h. PMA exposure of the cells for 90 min or 21 h increased the $t_{1/2}$ to 4.5 and 5.5 h, respectively. This suggests that levels of $p21^{WAF1/CIP1/SDI1}$ mRNA are regulated at the posttranscriptional level. To determine whether a change in transcription rate of $p21^{WAF1/CIP1/SDI1}$ might contribute to the increased $p21^{WAF1/CIP1/SDI1}$ expression after PMA stimulation, nuclear run-on experiments were performed. In unstimulated cells, the rate of $p21^{WAF1/CIP1/SDI1}$ transcription was very low and no increase was seen after 12 h of PMA stimulation. MPO and IL-8, which were used as internal controls, were markedly down- and upregulated, respectively, upon PMA stimulation, as described (34, 35). Consistent with the results of our RT-PCR experiments, no transcription of $p53$ was detected in both uninduced and induced HL-60 cells. pUC plasmid containing no insert was used as negative control.
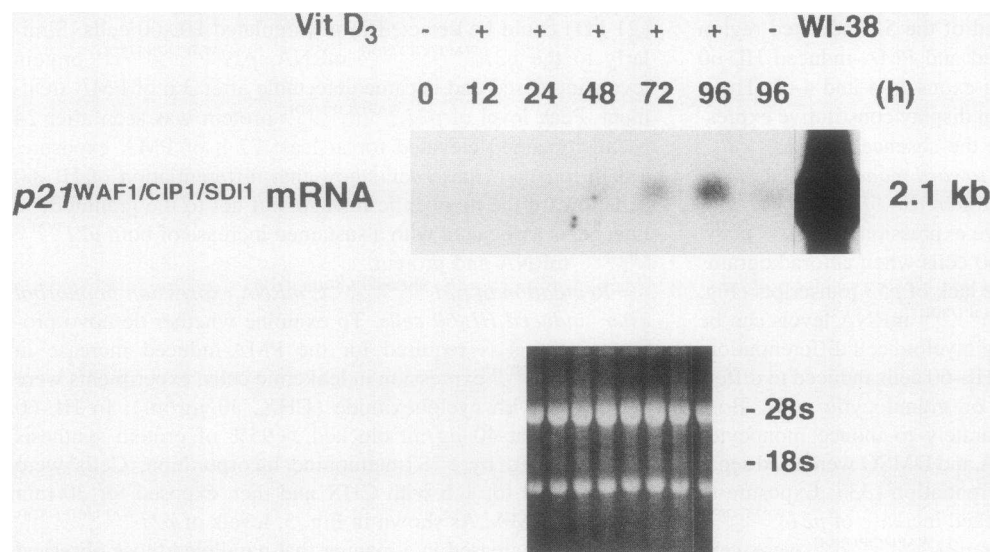


Figure 3. $p21^{WAF1/CIP1/SDI1}$ mRNA expression in HL-60 cells induced to differentiate toward the monocytic lineage with Vit $D_3$. HL-60 cells were treated with Vit $D_3$ ($10^{-7}$ mol/liter) for 0–96 h. Total RNA (10 $\mu$g/lane) was blotted and hybridized with a $p21^{WAF1/CIP1/SDI1}$ cDNA probe.
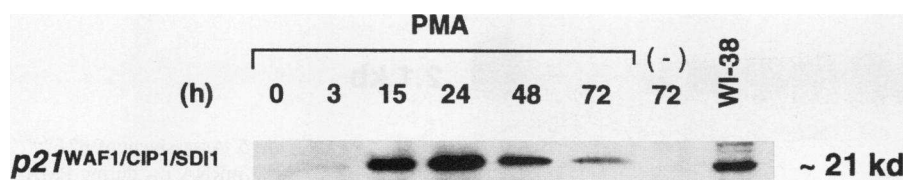
*Figure 4.* Assessment of p21WAFI/CIPI/SDII protein levels during PMA-induced differentiation of HL-60 cells. Total cellular protein (10 μg) from HL-60 cells treated for 3–72 h with PMA (60 ng/ml) was separated on a 14% SDS-polyacrylamide gel and transferred to a nitrocellulose filter. p21WAFI/CIPI/SDII protein (~21 kD) was visualized by incubating the filter with a polyclonal p21WAFI/CIPI/SDII _ specific antibody as described in Methods. WI-38 cells served as positive control.

In addition, we determined the $t_{1/2}$ of p21WAFI/CIPI/SDII mRNA in cultured blast cells from two AML patients. As shown in Fig. 7, the higher p21WAFI/CIPI/SDII steady state transcript levels found in AML case 11 (AML M2) compared with that of AML case 16 (AML M4) might reflect the higher mRNA stability in AML case 11 ($t_{1/2}$ 2 h) compared with case 16 ($t_{1/2}$ 1 h). Taken together, these results indicate that p21WAFI/CIPI/SDII mRNA expression in human myeloid leukemia is primarily regulated at the posttranscriptional level.

## Discussion

Our finding of constitutive expression of p21WAFI/CIPI/SDII in normal human hematopoietic cells and in a broad range of hematological malignancies suggests that p21WAFI/CIPI/SDII might exert a common basic cellular function. All of the 12 investigated myeloid and lymphoid cell lines constitutively expressed p21WAFI/CIPI/SDII. Most of these cell lines contain *p53* mutations. K-562 cells, which are early biphenotypic (myeloid/ erythroid) blast cells derived from a Ph$^+$ CML patient, contain a frameshift mutation in exon 5 and do not express *p53* (25, 31). In the promyelocytic HL-60 cell line, which was established from a patient with AML M2, most of the *p53* gene is deleted with some residual sequences mapping to the 5' region (26, 32). Although these two cell lines lack *p53* mRNA and protein expression, we found that both constitutively expressed p21WAFI/CIPI/SDII mRNA. These data indicate that p21WAFI/CIPI/ SDII is constitutively expressed in human leukemic cells independent of *p53*. These findings are in agreement with a very recent study showing expression of p21WAFI/CIPI/SDII in embryonal fibroblasts from *p53*-deficient mice (36).

Induction of differentiation of the HL-60 cells with PMA or Vit D₃ was associated with an increase of p21WAFI/CIPI/SDII expression. Phorbol ester–induced differentiation of human HL-60 myeloid leukemic cells results in a loss of proliferative capacity, adherence, and increased expression of monocyte surface markers (37, 38). In addition, the cells become arrested in $G_0/G_1$ of the cell cycle and show internucleosomal DNA cleavage, suggesting that induction of differentiation involves at least one characteristic feature of apoptosis (39, 40). In contrast, the HL-60 cells treated with either RA or DMSO acquire morphological and functional characteristics of mature granulocytes (33) and similar to phorbol ester–induced monocytic differentiation, the cells also become growth arrested in a $G_0/G_1$ state of the cell cycle (41, 42). The lack of detection of p21WAFI/ CIPI/SDII transcripts during granulocytic differentiation as opposed to its increase during monocytic/macrophage differentiation suggests that p21WAFI/CIPI/SDII expression is not necessarily dependent on the $G_0/G_1$ growth arrest associated with differentiation. Furthermore, even though formal proof is lacking, induction of p21WAFI/CIPI/SDII is unlikely to represent a direct result of exposure to either PMA or Vit D₃. The former stimulates the activation of protein kinase C and the latter binds to the vitamin D receptor and transactivates genes through the vitamin D response elements (43, 44). The induction of p21WAFI/CIPI/ SDII expression by Vit D₃ was weaker than by PMA, which suggests that Vit D₃ is not a particularly good stabilizer of the p21WAFI/CIPI/SDII message, despite cell differentiation. The finding of increased levels of p21WAFI/CIPI/SDII associated with differentiation of hematopoietic cells toward monocytes supports the hypothesis that p21WAFI/CIPI/SDII could have functions apart from its known proliferation inhibitory properties.

Our experiments addressed the mechanism by which p21WAFI/CIPI/SDII mRNA accumulates and whether the regulation of p21WAFI/CIPI/SDII mRNA expression might occur at the transcriptional or posttranscriptional level. Similar to *p53*-deficient
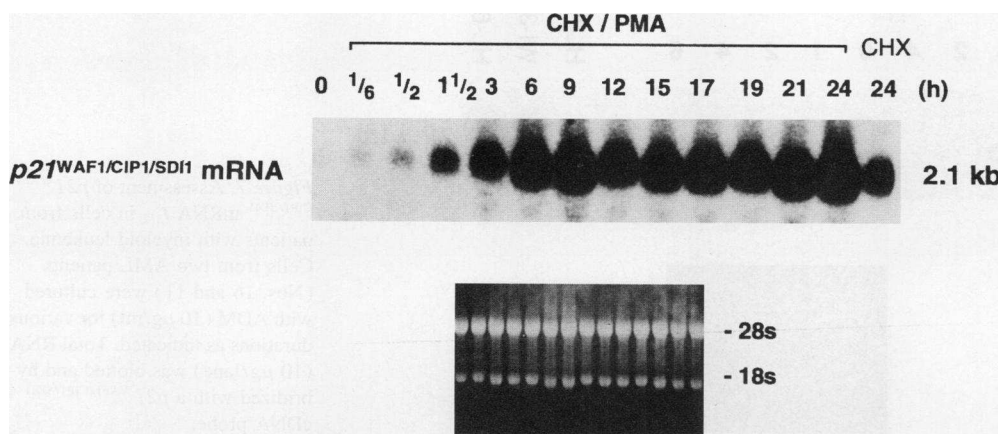


*Figure 5.* Influence of ongoing protein synthesis on the PMA-induced expression of p21WAFI/CIPI/ SDII mRNA. Northern blot analysis in HL-60 cells which were pretreated for 1 h with the protein synthesis inhibitor CHX (40 μg/ ml) and then with PMA (60 ng/ ml) as indicated. Analysis was performed by blotting 10 μg total RNA per lane and hybridization with a p21WAFI/CIPI/SDII cDNA probe.
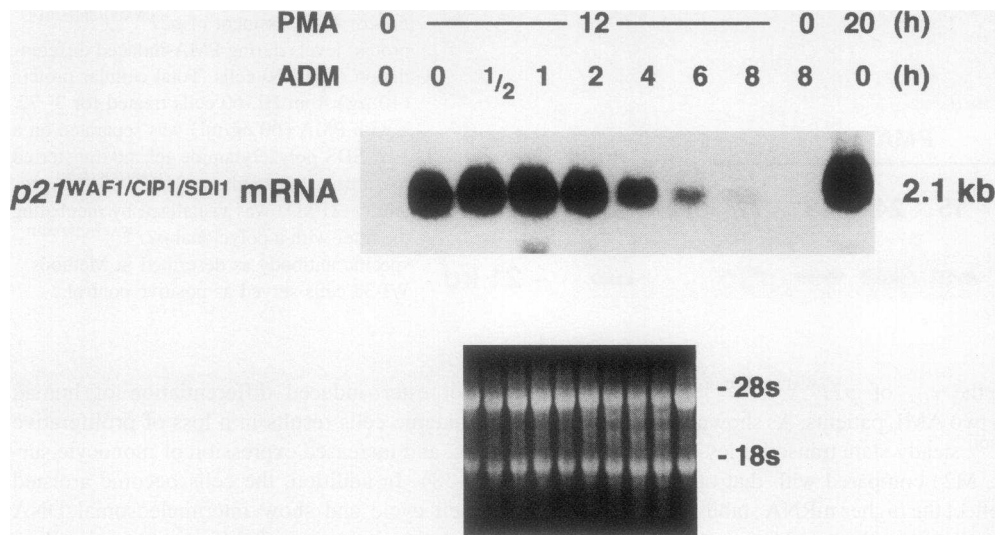
Figure 6. Assessment of $p21^{WAF1/CIP1/SDI1}$ mRNA $t_{1/2}$ during PMA-induced differentiation of HL-60 cells. HL-60 cells were induced for 12 h with PMA (60 ng /ml) and then ADM (10 μg/ml) was added for various durations as indicated. Analysis was performed by blotting of 10 μg total RNA per lane and hybridization with a $p21^{WAF1/CIP1/SDI1}$ cDNA probe.

embryonal fibroblasts treated by various growth factors (36), the expression of $p21^{WAF1/CIP1/SDI1}$ mRNA in phorbol ester–induced HL-60 cells was not dependent on newly synthesized proteins. Independence of intermediary protein synthesis together with the requirement of modification of preexisting transcriptional modulators is a characteristic feature for primary response genes also called immediate early genes (45).

Our experiments revealed that $p21^{WAF1/CIP1/SDI1}$ mRNA expression is regulated at the posttranscriptional level. The $t_{1/2}$ of $p21^{WAF1/CIP1/SDI1}$ mRNA in unstimulated HL-60 cells was estimated to be < 45 min. This is only an approximation because a long exposure of autoradiograms was necessary to detect the low constitutive levels of $p21^{WAF1/CIP1/SDI1}$ mRNA. Clearly, no mRNA was seen after 1.5 h of exposure to ADM. Monocytic differentiation induced by PMA increased the stability of the $p21^{WAF1/CIP1/SDI1}$ transcripts approximately sixfold. This increase in stability was seen in early as well as in later stages of induction of differentiation. The exposure of the cells to PMA had no detectable effect on $p21^{WAF1/CIP1/SDI1}$ transcription rate, sug-

gesting that changes in RNA transcription do not account for the increase in $p21^{WAF1/CIP1/SDI1}$ mRNA accumulation. The mRNAs of some immediate early genes (e.g., c-myc, c-fos) are specifically targeted for rapid degradation (45). The AU-rich sequences in their 3' untranslated region often containing multiple copies of the AUUUA sequence motif have been shown to be able to direct rapid mRNA decay (46). Since studies have clearly shown that posttranscriptional stabilization is an important mechanism for accumulation of labile mRNAs of cytokines and oncogenes in phorbol ester–treated cells (47), perhaps the three copies of the AUUUA motif present in the 3' untranslated region of the $p21^{WAF1/CIP1/SDI1}$ mRNA are involved in the regulation of its stability (8).

Our study demonstrates that in HL-60 cells $p21^{WAF1/CIP1/SDI1}$ is a p53-independent immediate early gene induced during monocytic/macrophage but not granulocytic differentiation due to posttranscriptional stabilization. This supports the idea of an additional role of $p21^{WAF1/CIP1/SDI1}$ in hematopoiesis and perhaps in other tissues.
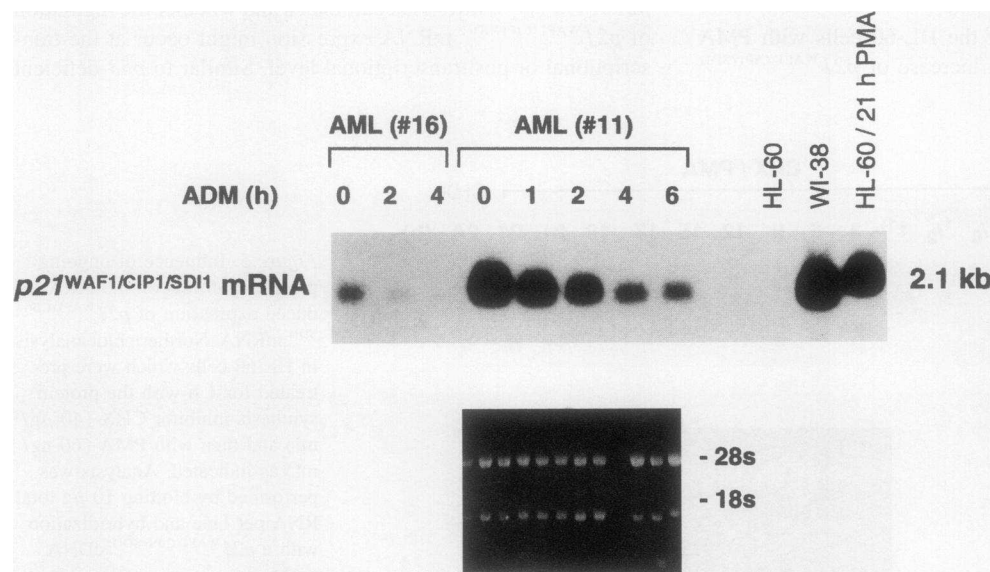


Figure 7. Assessment of $p21^{WAF1/CIP1/SDI1}$ mRNA $t_{1/2}$ in cells from patients with myeloid leukemia. Cells from two AML patients (Nos. 16 and 11) were cultured with ADM (10 μg/ml) for various durations as indicated. Total RNA (10 μg/lane) was blotted and hybridized with a $p21^{WAF1/CIP1/SDI1}$ cDNA probe.

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