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Up-Regulated P21^{CIP1} Expression Is Part of the Regulation Quantitatively Controlling Serum Deprivation-Induced Apoptosis

Atanu Duttaroy,[1,2] **Jing-Fang Qian,**[1,2] **James S. Smith,**[3] **and Eugenia Wang**[1,2]*

[1]The Bloomfield Center for Research in Aging, Lady Davis Institute for Medical Research, Sir Mortimer B. Davis Jewish General Hospital
[2]Department of Medicine, McGill University, Montréal, Québec, Canada
[3]Huffington Center of Aging and Division of Virology, School of Medicine, Baylor College of Medicine, Houston, Texas

Abstract Among the many genes which have been suggested to be required by the molecular mechanism dictating apoptotic death, some have been shown to function as pacemakers to pave the way for cells either to live or to die. Previously we have shown that immediate early gene expressions associated with the G_1 phase of cell cycle traverse are candidates for this function. Here we report that the well-known key regulator for halting cell cycling at the G_1/S border, the p21 protein known as WAF1, Cip1, Pic1, or Sdi1, is also involved in the execution of cells' suicidal death. p21 up-regulation is seen in quiescent mouse 3T3 fibroblasts stimulated to die by serum deprivation, at both message and protein levels, evidenced by increased protein presence in its targeted functional site, the nucleus. In addition, we show that this up-regulation of p21 is functionally related to the operational efficiency of the apoptotic process, in that when cells are stably transfected with an antisense construct to repress the endogenous p21-protein level, death is delayed. Quantitative protection from apoptosis with antisense p21 transfection is relatively proportional to the repressed level of this protein in the cells. Taken together, our results suggest that the apoptosis-dependent additional increase of p21 beyond the base level, seen in serum-deprived quiescent cells, may be involved in the molecular events precipitating a rapid program of cell demise, and that repression of this increase may obstruct the operation of this program and postpone the eventual death. J. Cell. Biochem. 64:434–446.

In general, commitment to programmed cell death (apoptosis) is orchestrated through the activation of specific genetic signal(s). Thus, activation of one or the other of the two opposing genetic programs may lead to either cell death or protecting cells from death. Specific examples of apoptosis-causing genes are *ced-3* in *Caenorhabditis elegans, reaper* in *Drosophila,* ICE and *YAMA/CPP-32* (apopain) in mammalian cells, while examples of apoptosis-protecting genes are *ced-9* in *C. elegans, bcl-2* in mammals, and *crmA* and *p35* genes of viral origin [see reviews by Stellar, 1995; Wyllie, 1995; Vaux et al., 1994]. Activation or deactivation of these genes is under the control of specific external or internal stimuli such as ionizing radiation, receptor-ligand binding, growth factor withdrawal, oxidative stress, calcium ionophores, and intercellular interaction through the extracellular matrix, etc.

Aside from the above-mentioned genes, it has been suggested that genes controlling cell cycle traverse play a decisive role in the program of cell death, most notably p53; its activation in response to ionizing radiation is thought to be the master switch for the events leading to apoptosis. Here, instead of G_1 growth-arrest as a better-known response, p53 functions primarily to eliminate damaged cells with genomic instability, whose persistent presence in the organism could ultimately lead to carcinogenesis [Lowe et al., 1993; Clarke et al., 1993; Slichenmyer et al., 1993]. An obvious proof of this suggestion was obtained in lymphomas by the fact that tumor oncogenesis here is a precipi-

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*Correspondence to: Dr. Eugenia Wang, Lady Davis Institute for Medical Research, Sir Mortimer B. Davis Jewish General Hospital, 3755, chemin de la Côte Ste. Catherine, Montréal, Québec, Canada H3T 1E2.

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tous consequence of the overexpression of *bcl-2* oncogene, which blocks p53-mediated apoptosis after irradiation [Sentman et al., 1991; Strasser et al., 1991]. In addition, specific cellular states such as the unique phase of differentiation or proliferation status, etc., can also influence the regulation of apoptosis. For example, cells of fibroblastic origin are in general more resistant to apoptosis than cytotoxic T lymphocytes [Nishioka and Welsh, 1994]. However, hyperproliferative fibroblasts resulting from overexpression of proto-oncogenes c-*myc* and *E1A* are more susceptible to apoptosis by growth factor withdrawal than their normal counterparts [Evans et al., 1992; Shi et al., 1992; Rao et al., 1992]. It has been suggested that genes regulating check-point controls for cell cycle traverse are equally important to the cellular propensity towards apoptotic cell death. For example, the constitutive overexpression of cyclin D1 is observed in apoptotic neurons [Freeman et al., 1994], and the expressions of c-*fos*, c-*jun*, c-*myc*, *cdc*-2, PCNA, and cyclin-A are observed to be up-regulated during apoptosis in AGF cells when treated with thymidine, and in serum-deprived mouse 3T3 fibroblasts [Gazitt and Erdos, 1994; Pandey and Wang, 1995]. Because many growth-regulating genes may have dual roles in not only controlling the cell cycle checkpoints but also, under certain physiological conditions, affecting the orchestration of successful apoptotic events as well, we decided to investigate the possible cause and effect relationship between the expression of cyclin-dependent kinase inhibitors such as p21$^{Sdi1/Waf1/Cip1}$ and successful operation of the apoptotic process.

Historically, the p21 gene was identified because of three interesting discoveries that a novel protein of 21 kilodalton possesses: (1) inhibitory action on cyclin-dependent kinase (cdk) activity [Harper et al., 1993]; (2) inducible expression under the control of p53 [El-Deiry et al., 1993]; and (3) ability to cause growth arrest in normal young fibroblasts [Noda et al., 1994]. Since the initial discovery, the p21 gene has been further characterized as encoding a 2.1 kb mRNA that is ubiquitously expressed in all embryonic and adult tissues, with the exception of embryonic brain and spinal cord [Harper et al., 1993; Parker et al., 1995]. Treatment with gamma-radiation, serum starvation, or subsequent mitogen stimulation by re-addition of serum can induce p21 expression [Noda et al., 1994; Dulic et al., 1994; El-Deiry et al., 1994]. The G_1-specific function of the p21 gene in arresting growth stems from its specific interaction with the cyclin E-cdk2 complex [Dulic et al., 1994], although other observations suggest that p21 is capable of forming complexes with almost all cyclin-cdk's with varying specificity [Sherr, 1994; Zhang et al., 1994; Xiong et al., 1993]. Besides p21, several other cellular cyclin/cdk inhibitors are also reported: p16$^{INK/MTS1}$ [Serrano et al., 1993], p15^{INK4B} [Hanon and Beach, 1994], p18 [Guan et al., 1994; Hirai et al., 1995]; p19 [Ming-Chang et al., 1995]; p27^{KIP1} [Polyak et al., 1994; Toyoshima and Hunter, 1994], and p57^{KIP2} [Matsuoka et al., 1995; Lee et al., 1995].

Initially, we hypothesized that since the function of specific cell cycle inhibitors is to prevent cells from successfully completing the cell cycle traverse, and since the G_1 phase of this traverse seems to be part of the initiation events for apoptosis, prevention of the G_1-phase experience might also hinder cells from entering the apoptotic experience [Pandey and Wang, 1994; Wang and Pandey, 1995]. We further hypothesize that in order for cells to initiate the apoptotic process by experiencing the G_1-phase event, they must be released from growth arrest, and thus may exhibit down-regulated expression of cyclin-dependent kinase inhibitors (CKIs). To test this hypothesis that cyclin-cdk inhibitors may also have dual inhibitory action on the initiation of apoptosis, we chose to investigate the role of p21 function in cellular self-demise, because: (1) of all the cyclin-cdk inhibitors, the biology of p21 is best understood; (2) of the identified CDK inhibitors, p21 is the only target under the control of p53; (3) unlike the other cyclin-cdk inhibitors, p15, p16, p18, and p19, which are specific inhibitors for the cyclin D-cdk4 complex, p21 can interact with almost all cyclin-cdks [Xiong et al., 1993]; and (4) our model system for inducing apoptosis utilizes the mode of serum deprivation, and the regulation of p21 expression is most sensitive to changes of serum concentration in cultures [Noda et al., 1994].

Contrary to our expectation of the possible down-regulation of CKI expression, our results reported here show that the amount of p21 protein increases steadily while mouse 3T3 fibroblasts undergo apoptosis, subsequent to the activation of death via withdrawing serum completely from the cultures. Parallel experiments

examining the message and protein levels show that this increase is transcriptionally regulated. The increase in p21 protein is primarily localized in the nucleus, which is the prime functional site of this protein. Furthermore, when p21 expression is partially suppressed in these cells by stable transfection with an antisense p21 construct, the survival rate of the stable 3T3 transfectants is enhanced to almost double that of controls subjected to the same apoptotic insult. Taken together, these results suggest that p21 expression is needed to facilitate the process of apoptosis, and when the protein's presence is partially repressed, as in the case of stable transfection, the speed of death is slowed down and final demise is delayed.

MATERIALS AND METHODS

Cells and Induction of Apoptosis

For all experiments described in this report, the balb/c strain of mouse 3T3 fibroblasts was used. We have cloned a specific cell line which possesses the growth property of contact inhibition-induced arrest of proliferation. Therefore, upon reaching confluency, these fibroblasts assume nonproliferative properties such as little or no detectable level of DNA synthesis, and absence of any immediate early gene expression such as c-*fos*, c-*myc*, PCNA or RB phosphorylation. In addition, the presence of a nonproliferation-specific protein, statin, is observed in every cell [Wang and Pandey, 1995]. Apoptosis can be induced in confluent cultures of these mouse 3T3 fibroblasts by total removal of serum; the death of the entire culture is observed within 5 days afterwards. For all experiments, mouse 3T3 fibroblasts were grown and maintained in Dulbecco's minimal essential medium (DMEM) containing 10% fetal calf serum, in a humid atmosphere of 5% CO_2 at 37°C.

SDS-PAGE and Immunoblotting

SDS-PAGE was used to separate proteins electrophoretically on polyacrylamide gel, as previously described [Laemmli, 1970]. Cell samples were collected from cultures treated with various experimental manipulations, and boiled in a sample buffer containing 4.6% (W/V) SDS, 10% β-mercaptoethanol, 20% glycerol, 95.2 mM Tris · Cl at pH 6.8, and 0.01% bromophenol blue for 5–10 min; 100 µg protein samples were loaded onto each lane of a gel containing 12.5% polyacrylamide. The separated proteins on the SDS-PAGE gel were transferred to nitrocellulose paper, as described by Towbin et al. [1979], and nitrocellulose blots bearing the protein samples were incubated overnight at 4°C with p21 polyclonal antibody (Pharmingen, San Diego, CA). Control blots were prepared by eliminating the step of primary antibody incubation in the reaction process. Detection of positive reaction signal was accomplished by incubating the blots, subsequent to the initial incubation with the p21 antibody, with goat anti-rabbit immunoglobulin (IgG; Cappel Organon, Teknika, NY) at room temperature for 1 h, followed by further incubation with 4-chloronaphthol (Sigma Chemicals, St. Louis, MO) and H_2O_2 as the peroxidase substrate for the final revelation of the band position of antigen and antibody reaction.

Immunofluorescence Microscopy

Mouse 3T3 cells were grown to confluency on poly-L-lysine-coated No. 1 glass coverslips, and subjected to serum deprivation as described above to activate apoptosis. Coverslips were removed from the cultureware at designated time points, and were fixed in a solution containing methanol/acetone at 1:1 ratio at −20°C for 10 min. Afterwards, the coverslip specimens were allowed to air dry, rehydrated in phosphate-buffered saline (PBS) at pH 7.2 for 10 min, and then further incubated to reduce nonspecific background staining in PBS containing 10% normal goat serum for 30 min at room temperature. Following this step, the coverslip specimens were incubated with p21 rabbit polyclonal antibody diluted in PBS containing 10% normal goat serum. This step of primary antibody incubation was done overnight at room temperature in a humidified chamber. The next morning the coverslip specimens were washed in PBS three times for 10 min each to remove unbound antibody, and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin (IgG) (CAPPEL, Organon Teknika, Scarborough, ON, Canada) diluted in PBS for 1 h at room temperature. After further rinsing in PBS, the coverslip specimens were prepared for morphological characterization of chromosomal phenotypes by further incubation with propidium iodide (ICN Biomedicals, Costa Mesa, CA) for 10 min at room temperature. Finally, the coverslip specimens were rinsed again in PBS and mounted in the same buffered

solution containing 50% glycerol. Microscopic observation was performed under a Nikon Labophot microscope equipped with epi-illumination for both fluorescein wavelength, for antibody staining reaction, and rhodamine wavelength, for the propidium iodide reaction.

In Situ TUNEL Staining

DNA fragmentation, an apoptosis hallmark, was examined in individual cells by labelling nuclei containing nicked DNA, as described [Gavrieli et al., 1992]. Coverslip specimens were prepared and fixed as described above for the procedure of immunofluorescence microscopy, except that after fixation and rehydration in phosphate-buffered saline, they were rinsed in double-distilled water. Subsequently, these specimens were incubated in a buffer containing 30 mM Tris-HCl, pH 6.8, 100 mM sodium cacodylate, 5 mM cobalt chloride, 0.5 mM dithiothreitol (DTT), 0.1 mg/ml bovine serum albumin (BSA), terminal deoxynucleotide transferase (TdT) (0.3 U/ml) (Pharmacia Biotech, Baie d'Urfé, Quebec, Canada), and biotin-16-dUTP (Boehringer-Mannheim, Laval, QC, Canada) at a final concentration of 10 mM. This reaction was carried out at 37°C for 1 h in a humidified chamber, and terminated by transferring the coverslip specimens to a solution containing 300 mM NaCl and 30 mM sodium citrate, where they were further incubated for 15 min, followed by further rinsing in distilled water. Prior to the final detection reaction, coverslip specimens were incubated for 10 min in 2% BSA and then in fluorescein isothiocyanate (FITC)-conjugated streptavidin for 30 min at room temperature, followed by sequential rinses in PBS and distilled water. These coverslip specimens were finally mounted in 50% glycerol and examined.

Analysis of DNA Fragmentation

DNA fragmentation was analyzed in terms of a "ladder" pattern appearing in agarose gels of both pre-apoptotic and apoptotic cells. For this purpose apoptotic cells, usually detached from the substratum, were harvested from the culture medium by centrifugation, while pre-apoptotic cells were collected by scraping the adherent monolayer cultures into 1.5 ml of media, and then spun down for 5 min at 14,000 rpm. Cell pellets of both types of cells were first resuspended into 300 µl of buffer [10 mM Tris HCl (pH 8.0), 100 mM NaCl, 25 mM EDTA, 0.5% SDS, and 0.5 mg/ml proteinase K], incubated overnight, and then precipitated with 100% ethanol. DNA pellets were then resuspended in 200 µl Tris-EDTA buffer (pH 7.5) containing 50 µl/ml RNase A, and incubated at 37°C for 2 h. After RNase treatment, the samples were spun at 12,000*g* for 15 min, and the supernatant containing fragmented DNA was collected and further analyzed by electrophoresis in a 2% agarose gel.

p21 cDNA Probes and Construction of Antisense Plasmids

The p21 probe was generated after digesting the mouse pACT p21^{CIP1} plasmid with Xho1 restriction enzyme to release a 0.9 kb fragment from the p21 cDNA [Noda et al., 1994]. ^{32}P-dCTP-labelled probes were generated from this 0.9 kb fragment by random primed reaction with a commercially available kit (Boehringer-Mannheim). The 18S control RNA probe was produced by reverse transcription reaction according to the procedure of Lehoux et al. [1987]. For Northern blotting and RNA hybridization, the procedure of Noonberg et al. [1994] was followed.

To construct the antisense p21, the 0.9 kb Xho1 fragment from pACT *Sdi1* was subcloned into the Xho1 site of a mammalian expression vector, pBK-CMV, containing the cytomegalovirus (CMV) promoter and neomycin-resistant gene (Strategene, La Jolla, CA). To verify the crucial point of insert orientation in pBK-CMV, the pBK-CMV CIP1 plasmid was cut first with Acc1; in its sense orientation, CIP1 shows a 350 bp fragment in pBK-CMV background, while in its antisense orientation a 650 bp fragment is expected. One of the three analyzed subclones showed the expected 650 bp Acc1 fragment, and the antisense orientation of p21 insert in pBK-CMV was finally confirmed by partial sequencing of the plasmid.

Transfection Procedure

Cells were grown to 70% confluency and then transfected by calcium phosphate precipitation [Graham and Eb, 1973]; approximately 1 µg of p21 antisense (AS) plasmid was used per transfection reaction. Forty-eight hours after transfection, the medium was replaced with DMEM carrying G418 (Geniticin; Canadian Life Technology, Burlington, Ontario) at a concentration of 800 µg/ml. G418 selection is possible because the original plasmid carries a neomycin-resistant gene. The selection was continued for 3

weeks by replacing the medium every 5 days. During this period of selection, surviving clones bearing neomycin-resistance were picked up and grown under G418, after which they were frozen before further analysis. Control experiments were performed with CMV-constructs containing the neomycin-resistant gene but without the p21 inserts, or cultures with calcium phosphate added alone.

Cell Viability Assays

Determination of cell viability was done by Trypan Blue exclusion assay. In brief, cells were grown in 6-well plates in DMEM containing 10% fetal bovine serum (FBS). After reaching confluency, cells were washed extensively three times in FBS-free DMEM, and kept in the same medium. For viability counting, culture populations containing both dead and live cells from each well were collected, centrifuged, and resuspended in 0.5 ml FBS-free DMEM. An aliquot of 0.1 ml was taken out and incubated with Trypan Blue dye for 5 min. Both live (unstained) and dead (blue) cells were counted from the same randomly selected fields. Viability was expressed as a percentage of cells that did not take up the Trypan Blue dye in the total cell population, composed of both live and dead cells.

RESULTS

Characterization of the Culture Model for Apoptosis

Confluent cultures of the Balb/C strain of mouse 3T3 fibroblasts are contact-inhibited for growth, and remain quiescent in medium containing 10% fetal bovine serum (FBS) for approximately 1 week. Finally, this monolayer culture detaches itself en mass from the substratum of the cultureware. However, before this detachment, if fetal calf serum is totally removed from the culture, cell death occurs rapidly in most cells during the initial 24 h, and is evident by the presence of a large number of floating cells in the culture medium. Subsequent to this initial precipitous loss of viability, a steady decline of viability continues until death is observed in the entire culture, at about 120 h after withdrawing serum (Fig. 1A). The floating cell population is identified as apoptotic cells because the presence of oligonucleosomes in these cells is apparent from the DNA fragmentation pattern (Fig. 1B). With these detached cells, kinetics of DNA fragmentation follow a predictable reduction in the size of fragmented DNA with increasing length of serum deprivation. Conversely, the cells remaining adherent in the same culture show no sign of DNA fragmentation until 72 h. By 96 h, fragmentation of DNA is seen at equal levels, with predominant lower-size oligonucleosomes in both adherent and floating cell populations. From these experiments, it appears that the adherent cell population collected at earlier time points (24, 48, and 72 h) are those that will eventually die in the subsequent time period, and therefore are termed here "pre-apoptotic" (Fig. 1B). For this and all following experiments, samples were only collected up to 96 h; afterwards total degeneration occurs, and most protein or RNA specimens collected are degraded and cannot be evaluated meaningfully.

Increasing programmed cell death after serum is deprived from the culture was also determined by examining chromosomal morphology by staining with propidium iodide. As shown in Figure 2, compaction of chromatin is apparent in numerous cells at 48 and 96 h of serum deprivation; this nuclear deformity is further verified by the detection of nicked DNA in individual cells via in situ TUNEL staining, as shown in Figure 2. Here, identical cells with compacted nuclei seen by propidium iodide staining are also positive for TUNEL staining. TUNEL-positive cells are seen to accumulate in number as the time of serum deprivation increases to 48 and 96 h (Fig. 2). This phenotypic characterization of DNA fragmentation establishes the fact that the cell death seen in confluent cultures of mouse 3T3 fibroblasts after serum deprivation is comparable to known stimuli of programmed cell death working in culture, and therefore the death seen here is apoptotic.

$P21^{CIP1/WAF1/Sdi1}$ Is Up-regulated in Pre-Apoptotic Cells

By the above characterization, the cell population remaining adherent to the substratum after serum deprivation is mostly pre-apoptotic, and will die subsequently. Measurements of message level are assayed by extracting total RNA from these cultures, composed mostly of pre-apoptotic cells, at 24-h intervals starting at the time when serum is withdrawn till 96 h. Northern analysis with $p21^{Cip1/WAF1/Sdi1}$ probe shows that the p21 mRNA level increases dramatically in this cell population as a consequence of serum deprivation (Fig. 3A). Quantitative determination of this increase in ratio by

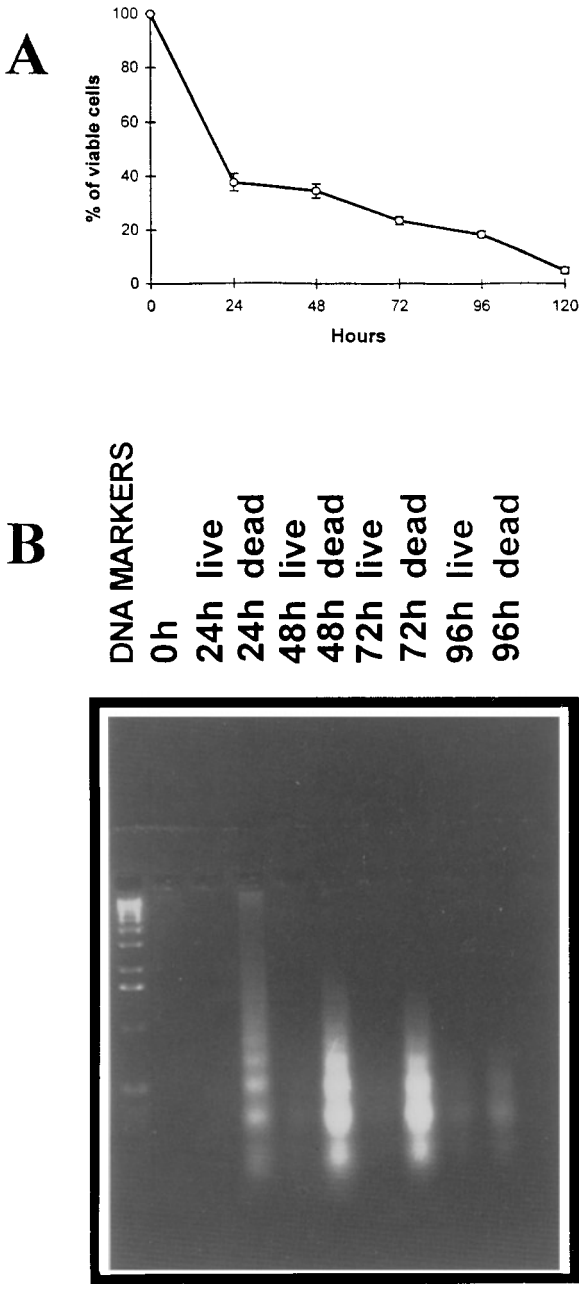

Fig. 1. Typical apoptotic response induced by serum deprivation in confluent cultures of mouse 3T3 fibroblasts. **A:** Trypan-blue viability assays for percentage of viable cells in cultures harvested at 0, 24, 48, 72, 96, and 120 h after serum is removed from the cultures. Notice the precipitous fall during the initial 24 h, followed by steady decrease to almost zero survivability in the subsequent hours. **B:** Oligonucleosome ladders are formed in apoptotic cells within 24 h of serum deprivation. DNA specimens were harvested from the floating cells (dead) and adherent cell population (live), and processed for agarose gel electrophoresis as described in Materials and Methods. Oligonucleosomes in ladder formation were not seen in the 0 h preparation, but mostly in the dead cell fractions with gradual decrease in size at the later time points. The adherent cell populations (live), on the other hand, show very little DNA fragmentation until 72 h after serum withdrawal, and by 96 h both live and dead cell specimens show equal levels and degrees of DNA fragmentation.

phospho-image analysis shows that the increased kinetics of p21 mRNA expression is a function of the duration of serum deprivation (Fig. 3B,C). This linear increase is observed up to 96 h, when there is 4 times as much p21 message as in the control contact-inhibited confluent cultures.

The increase of p21 message is further verified by examining whether there is an accompanying elevation of p21 protein level. Cell extracts from similar cell populations are used for protein extraction; as shown in Figure 3A, the protein level of p21 also shows a steady quantitative increase. As with the RNA level, the maximum quantity of p21 protein is also found in the cell population at 96 h after serum deprivation (Fig. 4). This observation confirms that p21 is up-regulated in pre-apoptotic cells. It is interesting to note that two p21 bands appear at 72 and 96 h of serum deprivation (Fig. 4). We suspect that at this time, i.e., 72 h after serum deprivation, there is a general debility in cellular components, as has been reported earlier during TNF-induced apoptosis [Voelkel-Johnson et al., 1995], which may contribute to the activation of an additional antibody-reactive band below the 21 kDa position.

In order to determine whether the increase in p21 up-regulation eventually reaches its targeted functional site, the nucleus, we studied the subcellular localization of p21 protein in the adherent cells by immunofluorescence microscopic analysis. Confluent cultures of 3T3 cells show low levels of p21 protein in the nucleus, as we detect a basal level presence of this protein in the quiescent state. However, as shown in Figure 5, at 48 h after serum deprivation, the presence of p21 protein is easily detectable in most cells of the monolayer cultures, and intense nuclear staining continues to be observed in cultures till 96 h. This accumulation of p21 protein in the nucleus in cells en route to final apoptotic death is intriguing, and is similar to the nuclear accumulation of p21 observed in p53-mediated G_1 arrest [El-Deiry et al., 1994].

Stable Transfection With Antisense p21 and Characterization of the Consequential Delay in Apoptosis

Three clones of mouse 3T3 fibroblasts stably transfected with antisense p21 were established according to the transfection regimen described in Materials and Methods. Parallel transfection experiments with the vector alone bearing no p21 inserts were established by the

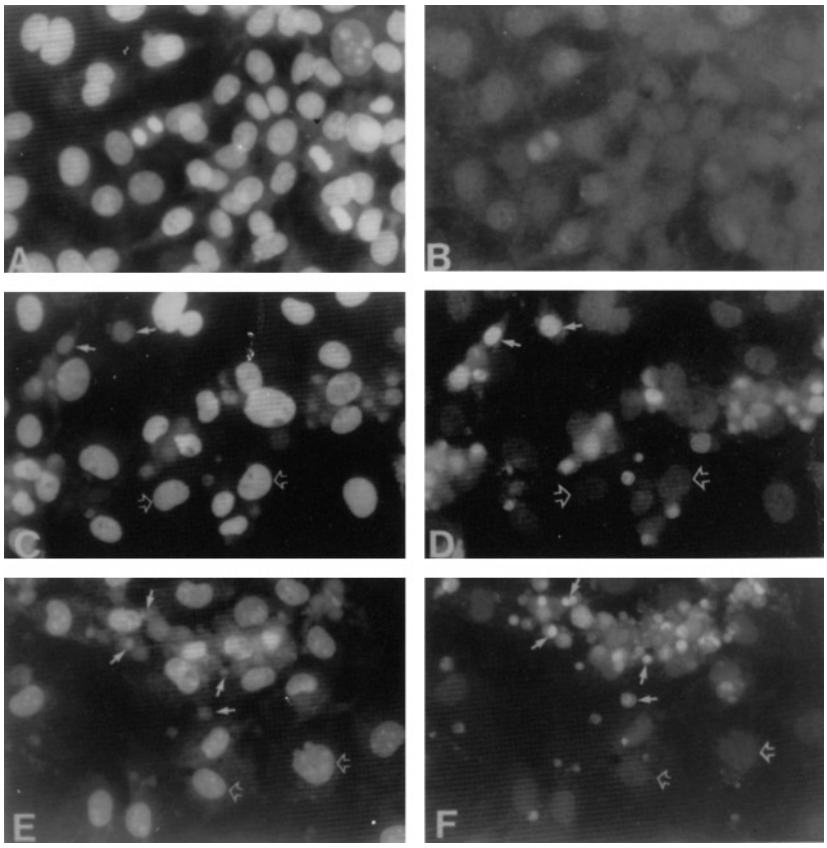

Fig. 2. In situ DNA fragmentation analysis with TUNEL staining shows a maximum number of cells with positive activity for nicked DNA. Illustration of the same fields showing propidium iodide staining **(A,C,E)** and TUNEL staining **(B,D,F)** at 0 (A,B), 48 (C,D), and 96 (E,F) h after serum deprivation. Notice the increased number of TUNEL-positive cells at later time points, as identified by both PI and TUNEL staining.

same regimen, selected and used as controls. Two of the three stably-transfected clones were processed for determination of the antisense effect by examining the p21 protein level. As shown in Figure 6A, the two antisense-p21 clones show reduced expression of p21 protein with respect to the control cultures which are stably transfected with vector alone, and the wild type untransfected confluent cultures of mouse 3T3 fibroblasts. Since all lanes are loaded with equal amounts of protein extracts from the various examined samples, the repression of p21 protein level seen here with the stable transfectant reflects reaction per mg total protein. Quantitative image analysis reveals that in the antisense line AS-1, more than 50% (and over 60% in AS-2) reduction is observed in the level of p21 protein amount with respect to the controls (Fig. 6B).

Based on the Western blot analysis, the two antisense-p21 stable transfectant clones and controls (transfected with vector alone), as well as untransfected wild-type cultures, were examined further for their survival potential after apoptotic insult via total removal of serum. Comparisons of viability between AS-1, AS-2, and control cultures after serum deprivation show detectable differences in survival potential (Table I). At 24 h after serum deprivation, 67.46 $\pm$ 4.8% of AS-1 cultures, and 57.83 $\pm$ 1.02% of AS-2, remain viable as determined by Trypan-blue exclusion test, while in control cells, 37.7 $\pm$ 1.05% cells are found to remain viable (Table I). This difference in viability indicates that in the initial 24 h, the stable transfectants have been protected from apoptotic death in ratios of 1.7 or 1.53 to 1 over the control cells. A similar ratio of protection from apoptotic death is seen at each subsequent time point, and at every time point until 96 h, on the average, about twice as many antisense transfectants are protected from serum deprivation-induced apoptosis (Fig. 7). This increase in survival rate in the stable transfectants of antisense p21 is

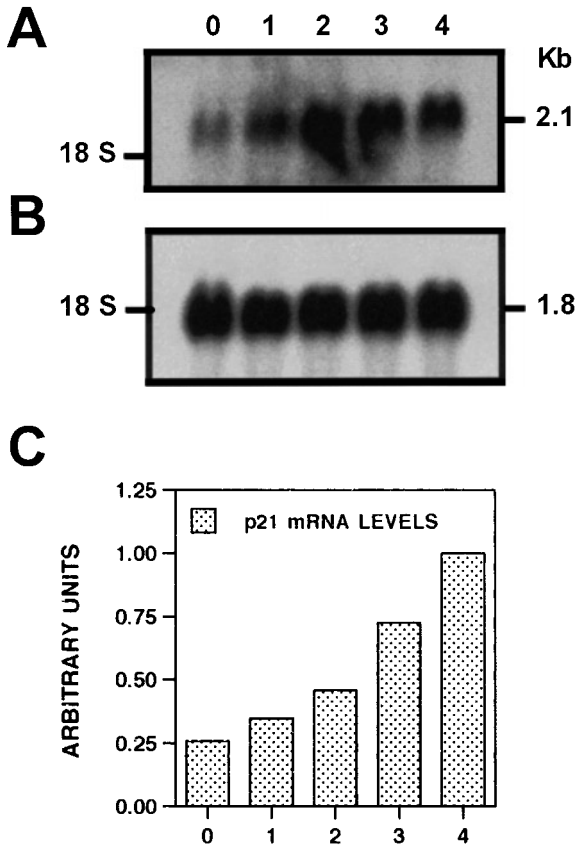

Fig. 3. Northern analysis showing increased p21 message level in mouse 3T3 fibroblasts stimulated to die by serum deprivation. **A:** Increased expression of p21 mRNA in cells harvested at 0 **(lane 0),** 24 **(lane 1),** 48 **(lane 2),** 72 **(lane 3),** and 96 **4)** h after serum deprivation. **B:** 18S RNA probe to ensure equal loading in all lanes shown above. **C:** Quantitative phosphoimage analysis of the ratio of intensity of p21 message as compared with the control, using the same blot shown in A and B. The quantity of p21 message increases steadily, reaching up to 4 times that of the 0 h level at 96 h of serum deprivation.

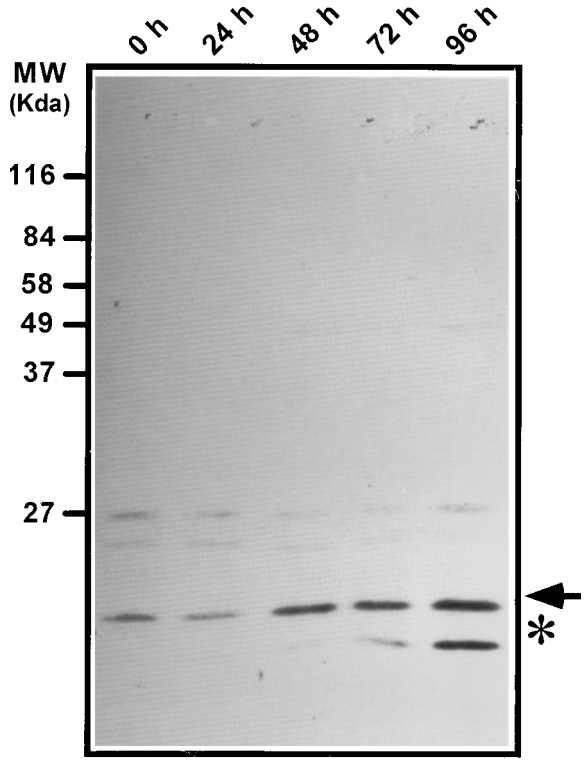

Fig. 4. Up-regulation of p21 protein level as revealed by Western blotting analysis. One hundred micrograms of protein extracts harvested 0, 24, 48, 72, and 96 h after serum deprivation were loaded onto each lane, and processed for SDS-PAGE and Western blotting with p21-specific antibody, as described in Materials and Methods. Steady increase beyond the basal level of p21 protein level (arrow) per mg protein was seen; and by 72 and 96 h, an additional band of lower molecular weight was also seen (*), possibly reflecting general proteolysis at these later stages of apoptosis.

inversely related to the reduction in p21 protein presence. Comparing Figures 6 and 7 shows that the approximate 2-fold protection from death is correlated with a 50% reduction in p21 protein level in AS-1 cells.

DISCUSSION

We report here, in mouse 3T3 fibroblasts, a novel association between up-regulation of p21 gene and rapid induction of cell death. This observation led us to examine two possible scenarios: (1) p21 up-regulation is due to the effect of serum withdrawal and exerts no consequential impact on apoptosis, or (2) p21 up-regulation is a part of the molecular machinery orchestrating apoptotic death. We reason here that if the former notion is true, then down-regulation of p21 should have no consequences on the speed or frequency of serum deprivation-induced apoptotic death. On the other hand, if the latter interpretation is valid, then down-regulation of p21 should alter the apoptotic potential of these cells. Results of our antisense-p21 experiments show that stably transfected cells carrying the antisense p21 construct display repression of p21 protein, in direct association with an increased ability to survive. Here, we must emphasize that this protection from apoptosis is quantitative; in terms of time and rate, the number of cells dying of apoptosis seen at any given time during our experiments is reduced close to half. Therefore, the protection from apoptotic death seen with the repression of p21 by the antisense transfection is a quantitative one, and the functional impact could be more precisely described as a delay in cell death, or protection from rapid apoptotic demise.

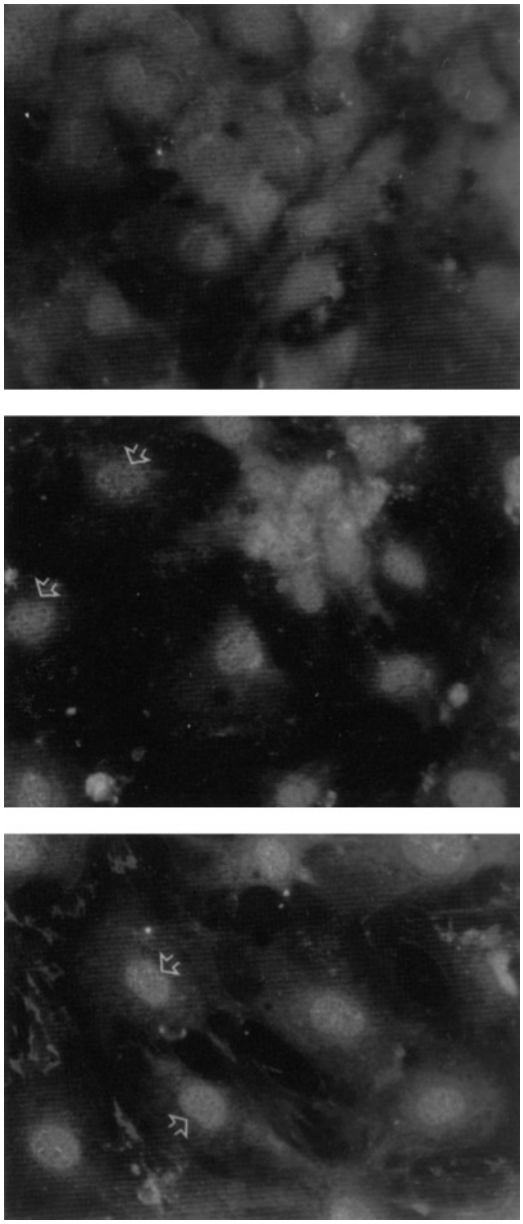

Fig. 5. Immunofluorescence microscopy of the increased presence of p21 in mouse 3T3 fibroblasts after serum deprivation. **Top:** Minimal level of p21 presence in the nuclei of these contact-inhibited, growth-arrested quiescent cells. **Middle:** Increased presence of p21 in the same cells, by the distinct antibody staining of the nuclei (empty arrows) in cells after serum is withdrawn for 48 h. **Bottom:** Same increase in cells fixed at 96 h after serum deprivation. This nuclear staining has been previously observed in cells for the localization of p21 protein [El-Deiry et al., 1994].

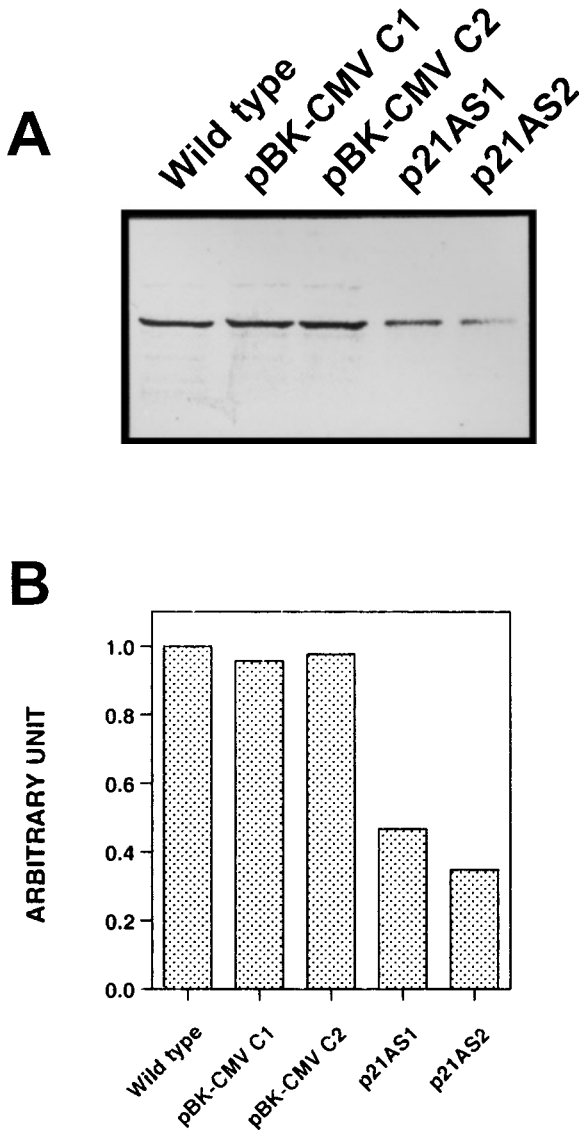

Fig. 6. Immunoblotting analysis showing the repression of p21 protein level in mouse 3T3 fibroblasts that have been stably transfected with antisense p21 construct. One hundred micrograms protein from: control untransfected cells (3T3); two control clones (C1 and C2) stably transfected with pCMV neo vector alone and selected by neomycin-resistance; and two clones (p21 AS1 and p21 AS2) selected after stable transfection with pCMV-neo-p21 antisense construct and neomycin selection; lanes are marked in the figure. **A:** Per mg p21 presence is lower in the stable transfectants, but not in wild-type untransfected cells or control cells without the p21 antisense insert. **B:** Quantitative analysis of this repression is at the level of 50% or less.

These observations suggest that up-regulation of a negative growth regulatory gene, p21, plays a pivotal role during apoptotic cell death in mouse 3T3 cells. Transcriptional activation of p21 gene is controlled under certain conditions by a cellular transcription factor, p53, by virtue of one or more p53-binding sites located in the upstream regulatory region of p21 gene [El-Deiry et al., 1993; Macleod et al., 1995]. More recently, another control element has been mapped in the upstream regulatory region of

TABLE I. Trypan-Blue Exclusion Assay for Viability in Control and p21-Antisense Stable Transfectants*

Length of serum deprivation (h)	% Viability			Wild-type mouse 3T3
	AS-1	AS-2	pBK-CMV	
0	99.80 ± 0.01 (0.00)	99.80 ± 0.01 (0.00)	99.9 ± 0.02	99.1 ± 0.05
24	67.46 ± 4.80 (1.78)	57.83 ± 1.02 (1.53)	37.7 ± 1.05	38.26 ± 1.67
48	50.76 ± 2.50 (1.95)	47.13 ± 2.26 (1.8)	25.9 ± 1.13	30.63 ± 4.24
72	35.46 ± 3.30 (2.05)	32.0 ± 1.98 (1.8)	17.2 ± 0.21	23.6 ± 0.63
96	30.96 ± 2.89 (2.17)	27.0 ± 2.28 (1.9)	14.26 ± 0.60	18.3 ± 1.68

*Numbers in parentheses indicate the ratio of increase in survival rate in comparing AS1 or AS-2 compared with pBK-CMV.

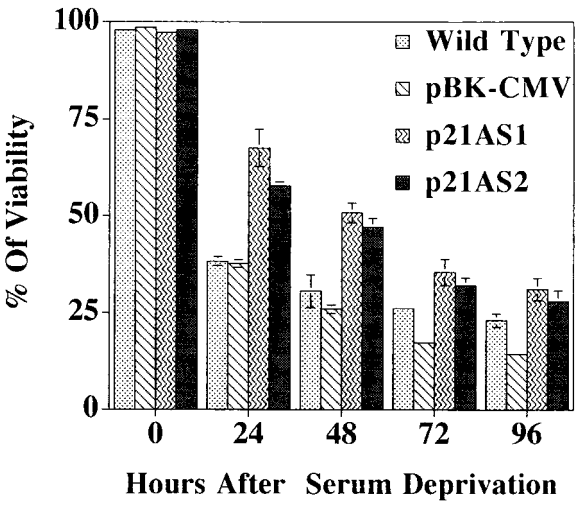

Fig. 7. Viability comparison between antisense p21 cell clones (AS1 and AS2), control cells (empty plasmid) (pBK-CMV), and untransfected cells (3T3). Trypan-blue exclusion assays were done every 24 h for up to 96 h after serum deprivation. All three antisense lines show increased survival from apoptosis with respect to the control line. The protection of survival ability at any given time point is about 50%, as numerically illustrated in Table I.

the p21 gene; it responds under both serum starvation and/or serum re-stimulation situations, as observed in various reporter construct assays [Macleod et al., 1995]. The magnitude of serum-inducibility of this p21 promoter element is weaker than the inducible action of p53. DNA damage-induced apoptosis calls for the induction of p53 gene expression in many cell types [Merlo et al., 1995]. Is this up-regulation of p53 gene expression in serum deprivation-induced apoptosis associated with known effects on targeted genes such as p21, as seen in cell cycle control systems [El-Deiry et al., 1994; Di Leonardo et al., 1994]? The answer is "possibly." As to the present observation on the up-regulation of p21 gene expression in serum-deprived apoptotic cells, we ask the question again, "Is this up-regulation of p21 expression *via* p53 control?" Future experiments measuring p53 levels in serum-deprived apoptotic 3T3 cells will provide the answer. However, studies with serum starvation/re-stimulation show that in $p53^{-/-}$ cells, p21 expression can still be induced [Macleod et al., 1995]. Our own observation with fibroblasts derived from p53 nullizygous mice shows that these cells can undergo serum deprivation-induced cell death with relative ease (E. Wang's lab, unpublished observation). Taken together, these results suggest that the serum-deprivation effect on p21 expression may be a p53-independent process; however, only future experiments on p21 promoter regulation analysis in apoptotic cells can provide a final verdict on the p53-independence reported in various other contexts [Clarke et al., 1995; Parker et al., 1995; Johnson et al., 1994].

The negative growth regulatory action of p21 derives from its ability to form an inactive complex with all G_1-specific cyclin-cdks [Gu et al., 1993; Xiong et al., 1993; Harper et al., 1993] and an essential DNA replication factor, PCNA [Li et al., 1994]. Furthermore, mapping studies confirm the existence of distinct domains in the p21 protein capable of binding with cyclin-cdks and with PCNA separately [Chen et al., 1995; Nakanishi et al., 1995b; Luo et al., 1995; Waga et al., 1994]. In mitogen-stimulated cells, p21 expression peaks in early and mid-G_1, but as the cell progresses towards the G_1/S boundary, the level of p21 goes down with a concomitant activation of the cyclin-cdks, thereby allowing more and more cells to enter into cell cycle traverse. If the p21 increase continues or maintains a steady high level, the protein will enforce persistent inhibition of cyclin-cdks, as the result of an intracellular titration effect between p21 and cyclin-cdks, and growth arrest eventuates because of excess p21. We are now

examining whether the same partner relationships between p21 and cyclin-cdks and between p21 and PCNA hold true in apoptotic mouse 3T3 fibroblasts.

Since our earlier observation shows that the initiation of apoptosis requires expression of immediate early genes such as c-*fos*, c-*myc*, c-*jun*, PCNA and RB phosphorylation, it is thought that prevention of entry into G_1 phase will protect cells from apoptotic death. Along this line, we reason that when p21 is up-regulated, cells should be protected from death, based on the knowledge that p21's predominant function is halting cells from proliferating. Surprisingly, our results show the contrary; i.e., p21 up-regulation, rather than down-regulation, is associated with apoptosis. Down-regulation, not up-regulation, of p21 protects the cells from death. Why is there this dichotomy? The answer may lie in the quantitative "threshold" effect. The burst of p21 increase seen in the apoptotic cells is above and beyond the level already present in quiescent mouse 3T3 fibroblasts. Since all experiments were performed with contact-inhibited cells as the starting materials for apoptotic assays, the observed increase in p21 indicates the added intracellular presence of p21. If pre-existing p21 in quiescent mouse 3T3 cells already functions to inhibit cyclin-cdks, what does the excess p21 accomplish then? We are in the process of examining whether the multiple functions of p21 in complexing with PCNA or DNA-repair enzyme GADD45 take place in apoptotic cells, in addition to the well-known relationship with cyclin-cdks kinases. p21 may completely abolish cells' ability to return to the cell cycle, since readdition of serum cannot prevent cell death.

Increased or decreased expression of p21 is reported in relation to a variety of growth conditions. For example, under regular serum conditions (10% FBS), senescent fibroblasts show a permanently up-regulated p21 gene [Noda et al., 1994], whereas in the case of young dividing cells, the level of p21 is inversely related to cell growth rate [Noda et al., 1994]. In low serum conditions (0.5% serum), p21 up-regulation is evident in quiescent, young cells [Noda et al., 1994]. Finally, when cells are kept under low serum conditions and then restimulated with serum for growth, a transient up-regulation in p21 expression is noticed which returns to the basal level after 1 to 2 h [Noda et al., 1994; Macleod et al., 1995]. p21 expression has been claimed as a pre-requisite for cell growth arrest leading to quiescent, senescent, and terminal differentiation states [Harper et al., 1995; Noda et al., 1994; Halevy et al., 1995]. Conversely, decreasing or abolishing p21 expression by mitogen stimulation, antisense RNA, or p21 gene knock-outs facilitates re-entry into the S phase (with the exception of senescent fibroblasts) [El-Deiry et al., 1993; Noda et al., 1994; Nakanishi et al., 1995a; Deng et al., 1995; Brugarolas et al., 1995]. What is actually causing this modulation in p21 expression when different serum conditions are used? Macleod et al. [1995] have reported that transcription of p21 RNA goes up in the presence of cycloheximide. This observation points out that cells are probably carrying a repressor that helps to keep p21 RNA at a basal level in growing cells. Moreover, this hypothetical repressor may be quite labile, and require activation by serum. It is possible that manipulation of serum concentrations would affect the expression and/or activation of this putative repressor, and in serum-deprived apoptotic cells, the repression of p21 may be relieved, allowing the increase of this protein to be continuous, whereas in mitogenic stimulated cells, the de-repression of p21 is transient and the increase of p21 is abruptly cut off immediately prior to the entry to S-phase. The continuous increase in p21 expression in apoptotic cells may then set them apart from the up-regulation seen in mitogenic stimulated cells. Ultimately, this persistent up-regulation of p21, possibly functioning as a permanent block to DNA repair, contributes to the total molecular mechanism leading to apoptotic death.

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