MOLECULAR AND CELLULAR BIOLOGY, Dec. 1996, p. 6654–6660 Vol. 16, No. 12
0270-7306/96/$04.00+0

Protective Role of $p21^{Waf1/Cip1}$ against Prostaglandin A_2-Mediated Apoptosis of Human Colorectal Carcinoma Cells

MYRIAM GOROSPE, XIANTAO WANG, KATHRYN Z. GUYTON, AND NIKKI J. HOLBROOK*

Laboratory of Cellular and Molecular Biology, National Institute on Aging, National Institutes of Health, Baltimore, Maryland 21224

Received 6 June 1996/Returned for modification 2 August 1996/Accepted 9 September 1996

Prostaglandin A_2 (PGA_2) suppresses tumor growth in vivo, is potently antiproliferative in vitro, and is a model drug for the study of the mammalian stress response. Our previous studies using breast carcinoma MCF-7 cells suggested that $p21^{Waf1/Cip1}$ induction enabled cells to survive PGA_2 exposure. Indeed, the marked sensitivity of human colorectal carcinoma RKO cells to the cytotoxicity of PGA_2 is known to be associated with a lack of a PGA_2-mediated increase in $p21^{Waf1/Cip1}$ expression, inhibition of cyclin-dependent kinase activity, and growth arrest. To determine if cell death following exposure to PGA_2 could be prevented by forcing the expression of $p21^{Waf1/Cip1}$ in RKO cells, we utilized an adenoviral vector-based expression system. We demonstrate that ectopic expression of $p21^{Waf1/Cip1}$ largely rescued RKO cells from PGA_2-induced apoptotic cell death, directly implicating $p21^{Waf1/Cip1}$ as a determinant of the cellular outcome (survival versus death) following exposure to PGA_2. To discern whether $p21^{Waf1/Cip1}$-mediated protection operates through the implementation of cellular growth arrest, other growth-inhibitory treatments were studied for the ability to attenuate PGA_2-induced cell death. Neither serum depletion nor suramin (a growth factor receptor antagonist) protected RKO cells against PGA_2 cytotoxicity, and neither induced $p21^{Waf1/Cip1}$ expression. Mimosine, however, enhanced $p21^{Waf1/Cip1}$ expression, completely inhibited RKO cell proliferation, and exerted marked protection against a subsequent PGA_2 challenge. Taken together, our results directly demonstrate a protective role for $p21^{Waf1/Cip1}$ during PGA_2 cellular stress and provide strong evidence that the implementation of cellular growth arrest contributes to this protective influence.

Following exposure to stressful stimuli, mammalian cells activate a number of response mechanisms that ultimately determine cell fate, including senescence, differentiation, death, and resumption of growth after acquisition of mutations or timely repair of damage. Among the model systems that have been developed to study the mammalian stress response is treatment with the cyclopentenone prostaglandin A_2 (PGA_2), which results in the enhanced expression of characteristic stress-related genes (*GADD153*, *HSP70*, *EGR-1*, *GADD45*, *jun*, *fos*, the heme oxygenase gene, etc.) (4, 5, 16). An important component of the stress response is a temporary or permanent alteration in the cell division cycle. Cell cycle progression is driven by the sequential activation of cyclin-dependent kinases (cdks), which are subject to regulation by positive (cyclins) and negative (cdk-inhibitory proteins) effectors (28). One such negative effector is the universal cdk inhibitor $p21^{Waf1/Cip1}$. While $p21^{Waf1/Cip1}$ can be transcriptionally regulated by the p53 tumor suppressor protein (9) and is thus believed to participate in the execution of p53 effects, its induction can also be activated via p53-independent pathways (1, 12, 17, 25, 27, 30).

$p21^{Waf1/Cip1}$ has been found to play an important role in the growth arrest that follows exposure to various stressful insults, presumably because of its cdk-inhibitory function (10, 35). $p21^{Waf1/Cip1}$ has also been postulated to participate in apoptosis, although its involvement in this process remains controversial (12). Indeed, increased $p21^{Waf1/Cip1}$ expression has been correlated with enhanced death in some instances (8, 31), but, more often, its elevated expression has been associated with increased cell survival (3, 21). In this regard, inhibition of $p21^{Waf1/Cip1}$ expression through transfection of $p21^{Waf1/Cip1}$ antisense oligonucleotides was recently demonstrated to block growth factor-induced differentiation of SH-SY5Y neuroblastoma cells and to result in apoptosis (29). Similarly, we have demonstrated that suppression of $p21^{Waf1/Cip1}$ expression through expression of antisense $p21^{Waf1/Cip1}$ transcripts in MCF-7 cells attenuates the growth arrest and $p21^{Waf1/Cip1}$ induction that normally follows treatment with PGA_2 (11) and promotes death (10).

The studies described above suggest that downregulation of $p21^{Waf1/Cip1}$ expression is correlated with decreased cell survival. The focus of the present study was to determine if overexpression of $p21^{Waf1/Cip1}$ could redirect PGA_2-treated RKO cells from a pathway leading to cell death to one resulting in cell survival. To this end, we utilized an adenoviral vector to transiently express high levels of $p21^{Waf1/Cip1}$, thereby allowing us to examine directly the protective influence of $p21^{Waf1/Cip1}$ during stressful conditions that normally result in cell death. Our results indicate that $p21^{Waf1/Cip1}$ overexpression largely protects RKO cells against PGA_2-mediated apoptotic death. This conclusion was further supported by additional experiments in which we examined whether other growth-arresting agents could modulate PGA_2-mediated cell death: growth-arresting agents that elevated $p21^{Waf1/Cip1}$ expression prevented cell death, while growth-arresting agents that did not elevate $p21^{Waf1/Cip1}$ expression failed to exert a protective function. In summary, our findings indicate that $p21^{Waf1/Cip1}$ expression protects against cellular stress and that this protection may be partly, but not solely, due to the implementation of cell cycle arrest.

* Corresponding author. Mailing address: Box 31, Section on Gene Expression and Aging, National Institutes of Health, National Institute on Aging, GRC, 4940 Eastern Ave., Baltimore, MD 21224. Phone: (410) 558-8197. Fax: (410) 558-8335. Electronic mail address: nikkiholbrook@nih.gov.

MATERIALS AND METHODS

Cell culture and treatments. The human colorectal carcinoma cell lines RKOneo (exhibiting wild-type p53 function) and RKOE6 (p53 deficient) (19) were cultured in minimum essential medium (Gibco BRL, Gaithersburg, Md.), and the human breast carcinoma cell line MCF-7 (American Type Culture Collection) was cultured in RPMI 1640 (Gibco BRL). Cell media were supplemented with 10% fetal bovine serum (HyClone, Logan, Utah) and 50 μg of gentamicin (Gibco BRL) per ml, and cell lines were maintained in a humidified atmosphere containing 5% CO_2 in air. RKO cells also received 350 μg of neomycin (Gibco BRL) per ml. PGA_2 (Sigma, St. Louis, Mo.) prepared in ethanol, mimosine (Aldrich Chemical Co., St. Louis, Mo.) prepared in water and neutralized with 10 N NaOH, and suramin prepared in water were added directly into the media to the final concentrations indicated.

Northern (RNA) blot analysis. Total RNA was isolated by the STAT-60 method (Tel-Test "B", Friendswood, Tex.), following the manufacturer's instructions, and 20-μg RNA samples were denatured, size fractionated by electrophoresis in 1.2% agarose-formaldehyde gels, and transferred onto GeneScreen Plus nylon membranes (DuPont/NEN, Boston, Mass.). For the detection of p21$^{Waf1/Cip1}$ mRNA in MCF-7 and RKO cells, the p21$^{Waf1/Cip1}$ cDNA was excised from the pCEP-WAF1 (9) plasmid and labeled with [α-^{32}P]dCTP with a random-primer labeling kit (Boehringer Mannheim, Indianapolis, Ind.). An oligomer complementary to the 18S rRNA (5′-ACGGTATCTGATCGTCTTCGA ACC-3′) (Integrated DNA Technologies, Coralville, Iowa) was 3′ end labeled with [α-^{32}P]dATP by terminal deoxynucleotidyltransferase (Life Technology Laboratories, Gaithersburg, Md.) and was used to normalize for differences in loading and transfer among samples. Hybridization and washes were performed according to the method of Church and Gilbert (6). Incorporation of ^{32}P was visualized with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

Cell counts, flow cytometric cell cycle analysis, and detection of DNA fragmentation. Cell counts were performed either with a hemocytometer or by crystal violet staining (10). Cell cycle distribution was analyzed by flow cytometry as described previously (20). Briefly, 2×10^6 to 5×10^6 cells were trypsinized, washed once with phosphate-buffered saline (PBS), and fixed in 70% ethanol. Fixed cells were washed with PBS, incubated with 1 μg of RNase A per ml for 30 min at 37°C, and stained with propidium iodide (Boehringer Mannheim). The stained cells were analyzed on a FACScan flow cytometer for relative DNA content. The percentages of the cells in the various cell cycle compartments were determined by using the MULTICYCLE software program (Phoenix Flow Systems, San Diego, Calif.). For analysis of DNA fragmentation, high-molecular-weight DNA was removed by centrifugation following cell lysis, whereupon DNA was extracted as described previously (15) and fractionated in 1.5% agarose gels.

Western blot (immunoblot) analysis. Fifty-microgram samples of total cell lysates were size fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes by standard techniques (14). p21$^{Waf1/Cip1}$ protein was detected with the enhanced chemiluminescence system (Amersham, Arlington Heights, Ill.) following incubation with the monoclonal mouse anti-human p21 antibody from Oncogene Science (Uniondale, N.Y.).

Virus preparation, determination of titers, and infection. Adenoviruses either lacking an insert (Ad.null), expressing nucleus-localized β-galactosidase (AdCMV.NLSβgal), or expressing p21$^{Waf1/Cip1}$ (AdWAF1) (18) were amplified and their titers were determined in 293 packaging cells containing the genes for viral replication; purification and storage were performed as previously described (13). Infections were carried out in serum-free Dulbecco modified Eagle medium for 1 h. For determination of infection rates, RKO cultures were infected with AdCMV.NLSβgal at various values of PFU per cell and cultured for 48 h. Following fixation and incubation with 1 μg X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml for 1 to 4 h to detect β-galactosidase activity, the percentages of positive (blue) cells were determined (Table 1). Mock infections were carried out in serum-free Dulbecco modified Eagle medium without viruses.

Immunoprecipitation and kinase activity assays. Cell cultures (60 to 80% confluent) were treated with PGA_2, washed twice with ice-cold PBS, and lysed in buffer containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.4), 50 mM β-glycerophosphate, 1% Triton X-100, 10% glycerol, 2 mM EGTA (ethylene glycol-bis[β-aminoethyl ether]-*N*,*N*,*N*′,*N*′-tetraacetic acid), 1 mM dithiothreitol, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 2 μM leupeptin, 2 μM aprotinin, 2 μM pepstatin A, 1 mM phenylmethylsulfonyl fluoride, and 0.5 μM okadaic acid. Soluble extracts were prepared by centrifugation at 10,000 × *g* for 10 min at 4°C. Following normalization of protein content, endogenous ERK2 or c-Jun terminal kinase 1 (JNK1)/stress-activated protein kinase (SAPK) was immunoprecipitated from the cell extracts by using rabbit polyclonal antibodies against p42^{ERK2} or p46^{JNK1} (Santa Cruz Biotechnology, Santa Cruz, Calif.), respectively. Kinase activity was assayed for 30 min at 37°C in the presence of 6 μg of substrate, 30 μM ATP, and 20 μCi of [γ-^{32}P]ATP in 55 μl of assay buffer (20 mM MOPS [morpholinepropanesulfonic acid] [pH 7.2], 2 mM EGTA, 20 mM $MgCl_2$). Glutathione *S*-transferase–c-Jun-1-135 (22) was used as a substrate for JNK1/SAPK, and myelin basic protein was used for assaying ERK2 activity. After completion of kinase assays, the proteins were resolved by SDS-PAGE and the gels were dried and subjected to autoradiography. The incorporation of ^{32}P was visualized with a PhosphorImager (Molecular Dynamics).

TABLE 1. β-Galactosidase activity and cell viability following infection of RKO cells with AdCMV.NLSβgal

Cell line	Infection level (PFU/cell)	% β-Galactosidase-positive cells[a]	% Cell viability[b]
RKOneo	0	0	>95
	50	2	>95
	100	5	>95
	500	21	>95
	1,000	30	>95
	5,000	62	85
	10,000	85	60
RKOE6	0	0	>95
	50	7	>95
	100	28	>95
	500	80	>95
	1,000	>95	>95
	5,000	>95	80
	10,000	>95	65

[a] Determined 48 h after infection.
[b] Assessed independently by trypan blue dye exclusion.

RESULTS

Apoptotic death of RKO cells by PGA_2 occurs independently of p53. We have previously shown that most human cell lines, including MCF-7 cells (11), respond to PGA_2 treatment with growth arrest and p21$^{Waf1/Cip1}$ induction. However, RKO cells die as a result of PGA_2 treatment (Fig. 1A) (10). That this death occurs via an apoptotic pathway is evidenced by characteristic morphological changes (data not shown) and intranucleosomal degradation of chromosomal DNA (Fig. 1B). Interestingly, these PGA_2 effects were not dependent on functional p53, as similar effects were seen in RKO cells containing wild-type p53 and in cells rendered p53 deficient through constitutive expression of the viral oncoprotein E6 (32) (Fig. 1). In keeping with our earlier findings (10, 11), PGA_2 treatment

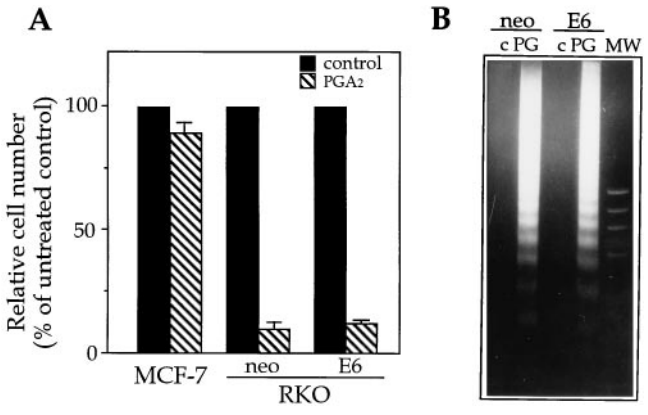

FIG. 1. PGA_2-mediated growth arrest of MCF-7 cells and apoptotic death of RKO cells. (A) MCF-7 cells and RKO cultures with functional p53 (neo) or nonfunctional p53 (E6) were counted at the time of PGA_2 addition (control) and after 48 h of continuous exposure to 36 μM PGA_2. Cell numbers are expressed relative to the number present at the time of addition of PGA_2, and values are means plus standard errors of the means for three independent experiments. (B) PGA_2-induced loss of RKO cell number was due to apoptotic death. Following exposure of parental (neo) and RKOE6 (E6) cells to 36 μM PGA_2 (PG) for 36 h, genomic DNA was extracted and resolved in 1.5% agarose gels. MW, *Hae*III-digested φX174 molecular weight marker; c, untreated controls.

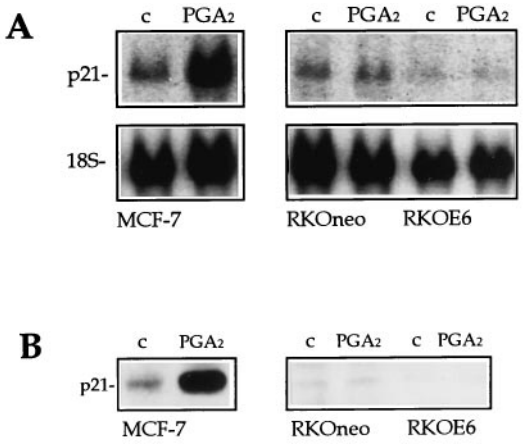

FIG. 2. p21$^{Waf1/Cip1}$ expression in PGA_2-treated MCF-7 and RKO cells. (A) Northern blot analysis for the induction of p21$^{Waf1/Cip1}$ mRNA expression following treatment with 36 μM PGA_2 for 14 h. The 18S rRNA signal is shown as a control for variations in loading and transfer among samples. (B) Western blot analysis of p21$^{Waf1/Cip1}$ expression following a 24-h treatment with 36 μM PGA_2. c, untreated controls.

effectively induced p21$^{Waf1/Cip1}$ mRNA and protein expression in MCF-7 cells; by contrast, neither p21$^{Waf1/Cip1}$ mRNA nor protein was induced by PGA_2 treatment in RKO cells (Fig. 2).

Activation of MAPKs by PGA_2 is impaired in RKO cells. While the precise mechanisms whereby PGA_2 treatment alters the expression of genes such as p21$^{Waf1/Cip1}$ are unclear, we have found that PGA_2 treatment activates the mitogen-activated protein kinase (MAPK) signalling pathways in a variety of cell lines (unpublished data). We have also recently provided evidence that induction of p21$^{Waf1/Cip1}$ in response to growth factors and other signals relies, at least in part, on activation of extracellular signal-regulated kinase (ERK) (25). Therefore, in an effort to understand why RKO cells fail to elevate p21$^{Waf1/Cip1}$ expression, we compared the level of MAPK activation in MCF-7 cells (which show induction of p21$^{Waf1/Cip1}$ by PGA_2) with that in RKO cells. PGA_2-treated MCF-7 cells underwent a prominent activation of both ERK (Fig. 3A) and SAPK/JNK1 (Fig. 3B), exhibiting an 8- to 10-fold

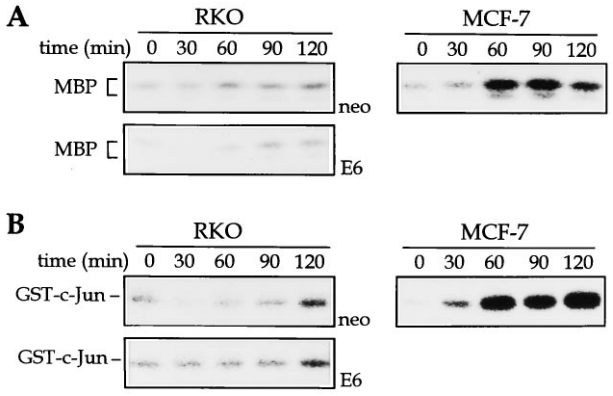

FIG. 3. Kinetics of ERK2 and SAPK/JNK1 kinase activation by PGA_2. MCF-7 and RKO cell lysates were prepared following PGA_2 treatment (36 μM) for the indicated times, and polyclonal anti-ERK2 and anti-SAPK/JNK1 antibodies were used to immunoprecipitate the respective kinases from the soluble fraction of cell lysates. Kinase activity was then assessed by immune complex kinase assay using bovine brain myelin basic protein (MBP) (for ERK2) and glutathione *S*-transferase (GST)–c-Jun (for SAPK/JNK1) as substrates.

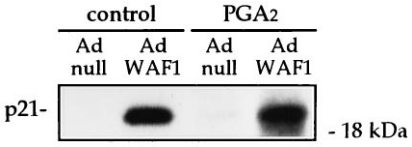

FIG. 4. Western blot analysis of p21$^{Waf1/Cip1}$ expression in RKO cells following adenovirus infection. Twenty-four hours after infection of RKOE6 cells with 1,000 PFU of Ad.null per cell or AdWAF1, cells were either left untreated (control) or treated with 36 μM PGA_2 for an additional 24 h, whereupon expression of p21$^{Waf1/Cip1}$ protein in each treatment group was assessed by Western blot analysis.

activation of these kinases after 2 h of exposure to 36 μM PGA_2. In contrast, neither ERK nor SAPK/JNK1 was significantly activated in PGA_2-treated RKO cells, indicating that perhaps the initial events in the response to PGA_2 are impaired in RKO cells. Thus, a failure to enhance MAPK activity in RKO cells may contribute to the lack of p21$^{Waf1/Cip1}$ induction by PGA_2 in RKO cells. Since attenuation of p21$^{Waf1/Cip1}$ expression in MCF-7 cells diminishes their growth arrest response and enhances the cytotoxicity of PGA_2 treatment (10), we hypothesized that elevation of p21$^{Waf1/Cip1}$ expression in RKO cells would engender protection against the PGA_2 challenge. To determine whether this is the case, we employed an adenoviral vector-based expression system to elevate p21$^{Waf1/Cip1}$ levels in RKO cells, thereby bypassing the requirement for intact signalling cascades.

Ectopic expression of p21$^{Waf1/Cip1}$ protects RKO cells from PGA_2-mediated cell death. To assess whether expression of p21$^{Waf1/Cip1}$ prior to the PGA_2 challenge could alter their sensitivity to the drug, RKO cells were infected with an adenoviral vector capable of expressing high levels of p21$^{Waf1/Cip1}$ (AdWAF1) (18). A control adenoviral vector, AdCMV.NLSβgal (Table 1), was used to assess infection rates. To achieve 95% infection of RKOneo cells, >10,000 PFU per cell was required, which resulted in significant toxicity. However, 1,000 PFU per cell was sufficient to achieve >95% infection rates in RKOE6 cells, and thus RKOE6 cells were chosen for further study when p21$^{Waf1/Cip1}$ was ectopically expressed.

AdWAF1 infection of RKOE6 cells resulted in a significant elevation of p21$^{Waf1/Cip1}$ expression relative to that in RKO cells infected with Ad.null (Fig. 4). The 30-fold elevation observed here is comparable to that seen in other cell lines (e.g., MCF-7 cells, [Fig. 2B]) in response to PGA_2 treatment (10). While PGA_2 stimulated a dose-dependent loss in cell viability after 48 h of continuous treatment in all infection groups, AdWAF1-infected cells were greatly protected against the toxicity of PGA_2 (Fig. 5A). Less than 10% of the mock-infected or Ad.null-infected RKOE6 cells survived treatment with 36 μM PGA_2, while >60% of cells infected with AdWAF1 remained viable. The marked enhancement of cell survival engendered by AdWAF1 infection is further evidenced by a shift in the dose-response curves, which was associated with a >6-fold increase in the concentration resulting in 50% cell death for PGA_2 (Fig. 5A). Resistance of AdWAF1-infected RKOE6 cells to the cytotoxicity of PGA_2 was also readily apparent when time-dependent changes in the cell numbers of AdWAF1- and Ad.null-infected cultures exposed to 36 μM PGA_2 were examined (Fig. 5B). Indeed, the protection conferred by AdWAF1 infection is clearly supported by the fact that, after 48 h of PGA_2 treatment, the number of cells remaining in the Ad.null-infected group was less than that of either the control or the PGA_2-treated AdWAF1-infected groups. Ad.null-infected cells continued to undergo exponential growth over the time period examined, while the Ad.null-infected, PGA_2-

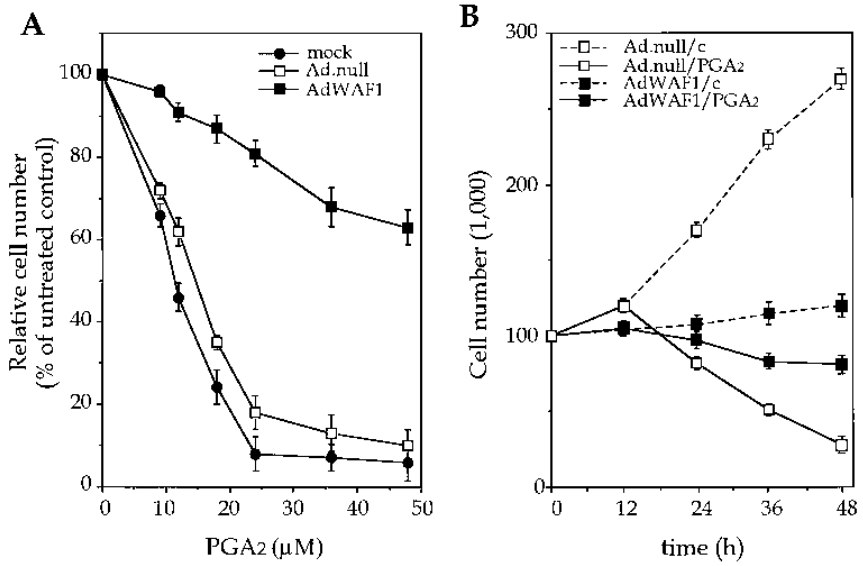

FIG. 5. Ectopic expression of p21$^{Cip1/Waf1}$ protects RKO cells against PGA_2-mediated cell death. (A) p21$^{Cip1/Waf1}$ expression prevents PGA_2-dose-dependent loss of cell viability. Cell viabilities following 48-h treatments of mock-, Ad.null-, and AdWAF1-infected RKOE6 cells with the indicated PGA_2 concentrations were measured by crystal violet staining of 96-well cluster plates (10). Values are means ± standard errors of the means for seven wells. (B) Time-dependent changes in viabilities of RKOE6 cells previously infected with either Ad.null or AdWAF1 adenoviruses for 48 h and subsequently left untreated (c) or treated with PGA_2 (36 μM). Cells were counted with a hemocytometer. Values are means ± standard errors of the means.

treated group underwent a marked loss in cell number (>90% decrease). As expected, AdWAF1 infection mediated growth inhibition of RKOE6 cells, and the cell numbers did not increase. PGA_2 treatment resulted in only a 35% decrease in the number of these cells, significantly less than that observed in the Ad.null-infected cells.

Cell cycle distribution of p21$^{Waf1/Cip1}$-overexpressing RKO cultures. In order to further understand the nature of the growth arrest induced by AdWAF1 infection of RKOE6 cells, cell cycle analysis was undertaken. As shown, p21$^{Waf1/Cip1}$-mediated growth arrest was characterized by a marked decline in S-phase cells (43 to 18%), with a corresponding increase in the G_1 and G_2 compartments (Fig. 6); similar findings were reported by Katayose et al. for breast cancer cells infected with AdWAF1 (18). Interestingly, these results suggest that neither p21$^{Waf1/Cip1}$-mediated growth inhibition nor enhanced survival of RKO cells after exposure to PGA_2 is preferentially associated with arrest in a single cell cycle compartment.

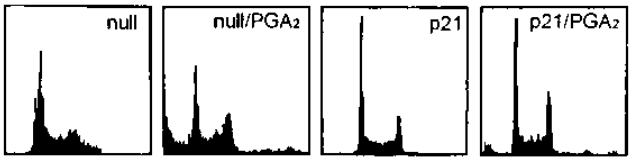

FIG. 6. FACS distribution of p21$^{Waf1/Cip1}$-overexpressing RKOE6 cells in the presence of PGA_2 treatment. Cell cycle analysis of RKOE6 cells that were Ad.null infected (null), Ad.null infected and PGA_2 treated (null/PGA_2), AdWAF1 infected (p21), and AdWAF1 infected and PGA_2 treated (p21/PGA_2) is shown. Infected cells were cultured for 48 h and then were left untreated or treated with PGA_2 for an additional 48 h. Note the apoptotic peak that is evident in the null/PGA_2 treatment group. The percentage of RKOE6 cells in each cell cycle compartment was quantitated and is shown below the histograms.

Effect of growth-arresting agents on proliferation of RKO cells and modulation of PGA_2-mediated cell death. In view of our finding that p21$^{Waf1/Cip1}$ expression exerts a protective influence against PGA_2 cytotoxicity, and given its established role as a growth-inhibitory protein, we questioned whether p21$^{Waf1/Cip1}$-mediated protection is directly achieved through the inhibition of cell growth. If this is the case, other growth-arresting agents should render a similar protection. We therefore investigated whether other general growth-arresting agents would influence the response to PGA_2 in RKO cells and confer a degree of protection similar to that observed for p21$^{Waf1/Cip1}$ overexpression. To this end, we employed serum withdrawal, treatment with the growth factor receptor antagonist suramin, and treatment with the plant amino acid mimosine. These various treatments have been shown to induce cellular arrest through different mechanisms: serum withdrawal induces G_0 or early G_1 arrest in a variety of cell types (11) through depletion of important mitogens in the growth medium, suramin leads to an enrichment in G_1-phase cells through mechanisms involving inhibition of certain growth factor receptors (platelet-derived growth factor, fibroblast growth factor, transforming growth factor β, epidermal growth factor, and interleukin-2 receptors, among others) and inhibition of DNA topoisomerase II (33), and mimosine arrests cells in the late G_1 or early S phase, at least in part by inhibiting DNA replication. Inhibition of DNA replication is believed to occur, at least partly, through interference with thymidylate biosynthesis by an inhibition of serine hydroxymethyltransferase activity (24).

As shown in Fig. 7A, treatment with all three growth-arresting agents resulted in some degree of growth inhibition relative to the exponential growth of untreated RKO cultures. However, only mimosine (300 μM) treatment resulted in complete cessation of growth. Fluorescence-activated cell sorter (FACS) analysis revealed an enrichment in G_1-phase populations of serum-depleted and suramin-treated cells relative to control cultures, whereas mimosine-treated cells underwent a marked

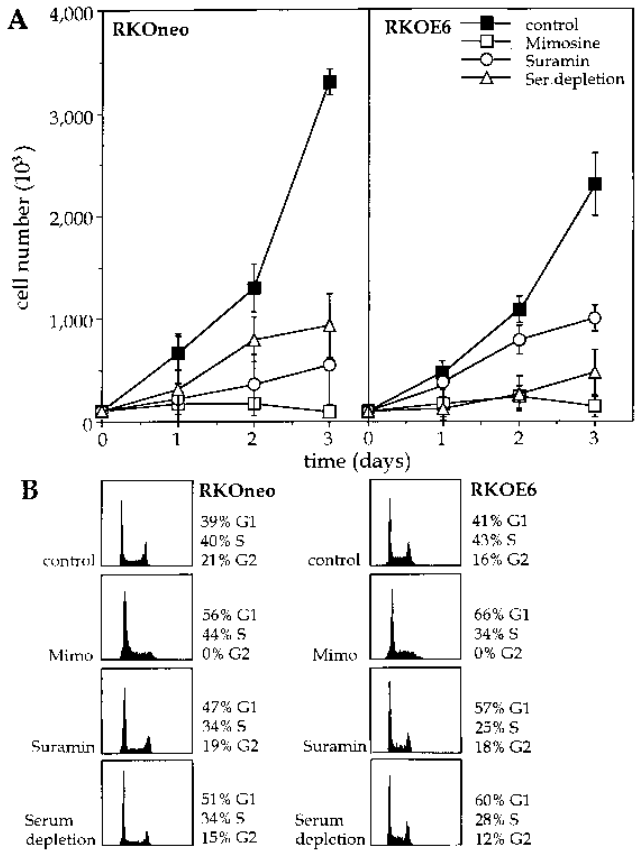

FIG. 7. Effect of growth-inhibitory agents on proliferation of RKO cells. (A) RKO cultures that had been either left untreated (control), serum depleted, or treated with 300 μM mimosine or suramin were counted at 24-h intervals with a hemocytometer. Viability in all treatment groups, determined by trypan blue exclusion, was greater than 95%, with the exception of mimosine, which did exhibit some toxicity (15%) at day 3 of treatment. (B) FACS distribution of RKO cultures that were either left untreated, serum depleted, or treated with 300 μM mimosine or suramin for 24 h, and then fixed and subjected to flow cytometry analysis. Histograms and percentages of cells in G_1, S, and G_2 are shown.

increase in G_1- and S-phase cells and a complete loss of G_2-phase cells (Fig. 7B).

To assess their potential protective effect against PGA_2, 24-h pretreatments of RKO cultures by either serum withdrawal or addition of suramin or mimosine were followed by challenges with 36 μM PGA_2. Cell numbers were monitored for the following 48 h. Neither suramin pretreatment nor serum depletion protected RKO cells against PGA_2-induced cell death. In fact, cell loss was more pronounced compared with that resulting from PGA_2 treatment alone (Fig. 8A). These effects were seen regardless of the cellular p53 status (Fig. 8A). By contrast, mimosine pretreatment markedly protected both RKOneo and RKOE6 cells against PGA_2 cytotoxicity (Fig. 8A); in this treatment group, greater than 65% of cells survived 48 h after PGA_2 addition (relative to the mimosine-alone-treated control), compared with less than 10% cell survival in the other treatment groups.

Mimosine treatment induces $p21^{Waf1/Cip1}$ expression. To determine whether the protection engendered during mimosine-induced growth arrest could be dissociated from $p21^{Waf1/Cip1}$ expression, Northern blot analysis was performed to monitor $p21^{Waf1/Cip1}$ mRNA expression in mimosine-treated RKO cells. As shown in Fig. 8B, mimosine treatment induced $p21^{Waf1/Cip1}$ mRNA expression in RKOneo and RKOE6 cells. However, neither suramin nor serum depletion increased $p21^{Waf1/Cip1}$ mRNA expression in RKO cells; instead, serum depletion generally decreased $p21^{Waf1/Cip1}$ expression. These findings provide further support for the tenet that $p21^{Waf1/Cip1}$ expression plays a protective role during cellular stress.

DISCUSSION

The results of the studies presented here are consistent with the notion that $p21^{Waf1/Cip1}$ exerts a protective influence during cellular stress, blocking PGA_2-induced apoptosis and enhancing cell survival. An important question that arises from these findings centers on the identification of the cellular determinants that enable certain cells to implement a survival response to a given stimulus while other cells exposed to the same stimulus undergo apoptosis. In particular, why do RKO cells

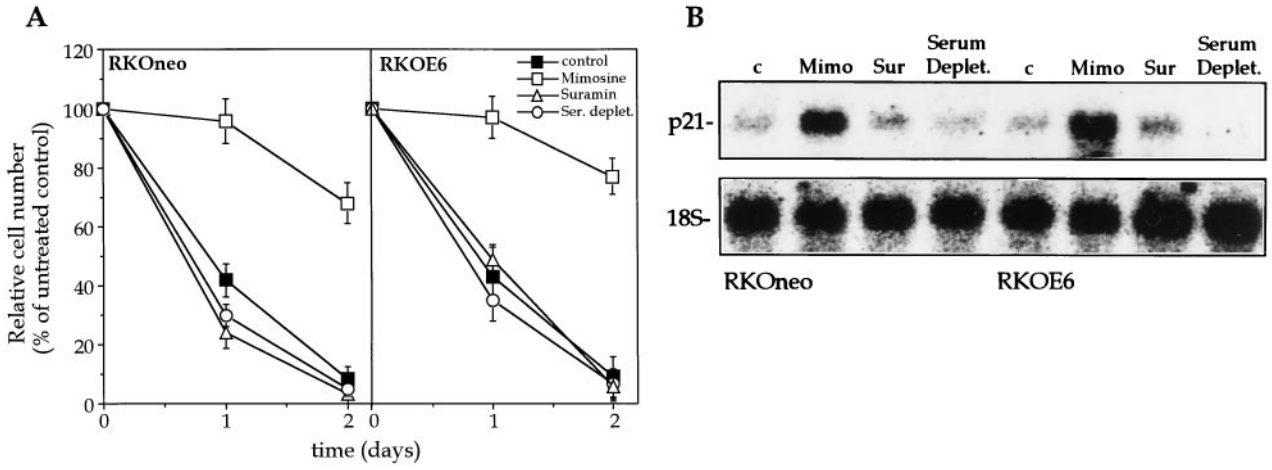

FIG. 8. Effect of growth-inhibitory agents on PGA_2-mediated death and $p21^{Waf1/Cip1}$ expression in RKO cells. (A) Mimosine pretreatment, but not serum depletion or suramin pretreatment, protects against PGA_2-induced dose-dependent loss of cell viability. Following a 24-h period of pretreatment with 300 μM mimosine or suramin or an equivalent period of serum depletion, RKO cells were treated with the indicated PGA_2 concentrations and cell viability was measured by crystal violet staining of 96-well cluster plates (10). Values are expressed as percentages of untreated control and are means ± standard errors of the means for seven wells. (B) $p21^{Waf1/Cip1}$ expression following exposure of RKO cells to growth-inhibitory treatments. After 24 h in serum-depleted medium (Serum Deplet.) or 300 μM suramin (Sur) or mimosine (Mimo), RNA from RKOneo and RKOE6 cells was extracted and subjected to Northern blot analysis to monitor $p21^{Waf1/Cip1}$ mRNA expression. 18S rRNA signals are shown as a control for variation in loading and transfer among samples. c, untreated controls.

fail to induce p21$^{Waf1/Cip1}$ in response to PGA_2 whereas MCF-7 cells clearly show enhanced p21$^{Waf1/Cip1}$ expression with the same treatment? PGA_2 treatment leads to potent activation of ERK and JNK in cells that survive the treatment, such as MCF-7 cells, but fails to activate MAPKs in RKO cells. Thus, the response to PGA_2 stimulation in RKO cells may be blocked at a very early stage in signal transduction processes and perhaps the blockage of the response renders the cells incapable of fully implementing a protective program. MAPK activation by PGA_2 may in fact be directly linked to enhanced p21$^{Waf1/Cip1}$ expression, because we have recently demonstrated that p53-independent transcriptional control of p21$^{Waf1/Cip1}$ is directly regulated via ERK (25).

Although our results clearly implicate p21$^{Waf1/Cip1}$ in the survival response, how p21$^{Waf1/Cip1}$ actually exerts its protective influence remains to be determined. The influence could be directly linked to p21$^{Waf1/Cip1}$'s cdk-inhibitory capacity (35), or it could be dependent on p21$^{Waf1/Cip1}$'s modulatory activity on proliferating cell nuclear antigen function (34). Since these two functions of p21$^{Waf1/Cip1}$ reside in separate domains (inhibitory binding of cdks in the N-terminal portion; inhibitory binding of PCNA in the C-terminal region [26]), it will be interesting to test the effect of different p21$^{Waf1/Cip1}$ deletion constructs in the context of PGA_2-mediated cytotoxicity. The use of the growth inhibitory treatments described here (serum depletion and exposure to suramin or mimosine) provides support for the hypothesis that it is not merely a general inhibition of growth but indeed other p21$^{Waf1/Cip1}$ functions that are required for the implementation of the protective effects. However, the possibility that suramin and serum depletion do not protect because they fail to implement a full arrest remains. In order to further understand whether protection from cellular death is elicited by inhibiting cell cycle progression or whether it requires additional p21$^{Waf1/Cip1}$ functions, we are currently extending these studies to other growth-arresting agents.

While we believe that p21$^{Waf1/Cip1}$ is directly implicated in determining cellular fate (survival versus death) following exposure to many damaging stimuli, it is important to note that p21$^{Waf1/Cip1}$ expression cannot exert protection against all stress and apoptosis paradigms. For example, apoptosis triggered by serum depletion of Rat1 cells overexpressing the proto-oncogene c-*myc* could not be prevented by p21$^{Waf1/Cip1}$ overexpression (unpublished results). Nevertheless, the protective influence of p21$^{Waf1/Cip1}$ is not limited to PGA_2-mediated cell death but also occurs with other stress treatments. For example, our ongoing studies indicate that p21$^{Waf1/Cip1}$ may also play a protective role against p53-dependent apoptosis (unpublished results).

In view of the hypothesis presented here that p21$^{Waf1/Cip1}$ is a survival factor during conditions of stress, it is not surprising that the number of reported cases of loss-of-function mutations of p21$^{Waf1/Cip1}$ in tumor tissues is conspicuously low; rather, its expression was found to be elevated in many tumors analyzed (36). Conversely, enhanced p21$^{Waf1/Cip1}$ expression has been correlated with poor treatment prognosis in myeloid leukemias, while a lower level of p21$^{Waf1/Cip1}$ expression is associated with better responsiveness to chemotherapy. This may be explained by our proposed model whereby a loss of p21$^{Waf1/Cip1}$ function, rather than conferring growth advantage through permissive cdk hyperactivity, in fact leads to a severe growth disadvantage through the loss of important protective functions, such as a loss of DNA repair proficiency (23) and G_1 checkpoint control (2, 7). Taken together with our present findings, these facts suggest that either chemotherapeutic approaches directly aimed at preventing p21$^{Waf1/Cip1}$ expression (for example, through the use of p21$^{Waf1/Cip1}$ antisense oligomers, viruses expressing antisense p21$^{Waf1/Cip1}$ transcripts, etc.) or antagonists of the signalling pathways that lead to p21$^{Waf1/Cip1}$ expression could lead to improved elimination of cancer cells.

ACKNOWLEDGMENTS

We thank P. Seth for providing the AdWAF1 adenovirus, I. Kovesdi for providing the Ad.null adenovirus, and R. G. Crystal for providing the AdCMV.NLSβgal adenovirus. We are grateful to M. B. Kastan for the RKOneo and RKOE6 cells, to F. J. Chrest for the flow cytometry analysis, to K. Inyaku and T. Riccioni for technical assistance, and to D. L. Longo for critical reading of the manuscript.

REFERENCES

1. **Akashi, M., M. Hachiya, Y. Osawa, K. Spirin, G. Suzuki, and H. P. Koeffler.** 1995. Irradiation induces WAF1 expression through a p53-independent pathway in KG-1 cells. J. Biol. Chem. **270:**19181–19187.
2. **Brugarolas, J., C. Chandrasekaran, J. I. Gordon, D. Beach, T. Jacks, and G. Hannon.** 1995. Radiation-induced cell cycle arrest compromised by p21 deficiency. Nature (London) **377:**552–557.
3. **Canman, C. E., T. M. Gilmer, S. B. Coutts, and M. B. Kastan.** 1995. Growth factor modulation of p53-mediated growth arrest versus apoptosis. Genes Dev. **9:**600–611.
4. **Choi, A. M., J. Fargnoli, S. G. Carlson, and N. J. Holbrook.** 1992. Cell growth inhibition by prostaglandin A_2 results in elevated expression of *gadd153* mRNA. Exp. Cell Res. **199:**85–89.
5. **Choi, A. M. K., R. W. Tucker, S. G. Carlson, G. Wiegand, and N. J. Holbrook.** 1994. Calcium mediates expression of stress-response genes in prostaglandin A_2-mediated growth arrest. FASEB J. **8:**1048–1054.
6. **Church, G. M., and W. Gilbert.** 1984. Genomic sequencing. Proc. Natl. Acad. Sci. USA **81:**1991–1995.
7. **Deng, C., P. Zhang, J. W. Harper, S. J. Elledge, and P. Leder.** 1995. Mice lacking p21$^{CIP1/WAF1}$ undergo normal development, but are defective in G1 checkpoint control. Cell **82:**675–684.
8. **El-Deiry, W. S., J. W. Harper, P. M. O'Connor, V. E. Velculescu, C. E. Canman, J. Jackman, J. A. Pietenpol, M. Burrell, D. E. Hill, Y. Wang, K. G. Wiman, W. E. Mercer, M. B. Kastan, K. W. Kohn, S. J. Elledge, K. W. Kinzler, and B. Vogelstein.** 1994. WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. Cancer Res. **54:**1169–1174.
9. **El-Deiry, W. S., T. Tokino, T. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kinzler, and B. Vogelstein.** 1993. WAF1, a potential mediator of p53 tumor suppression. Cell **75:**817–825.
10. **Gorospe, M., and N. J. Holbrook.** 1996. Role of p21 in prostaglandin A_2-mediated cellular arrest and death. Cancer Res. **56:**457–479.
11. **Gorospe, M., Y. Liu, Q. Xu, F. J. Chrest, and N. J. Holbrook.** 1996. Inhibition of G_1 cyclin-dependent kinase activity during growth arrest of human breast carcinoma cells by prostaglandin A_2. Mol. Cell. Biol. **16:**762–770.
12. **Gorospe, M., J. L. Martindale, M. S. Sheikh, A. J. J. Fornace, and N. J. Holbrook.** 1996. Regulation of p21CIP1/WAF1 expression by cellular stress: p53-dependent and p53-independent mechanisms. Mol. Cell. Differ. **4:**47–65.
13. **Graham, F. L., and L. Prevec.** 1991. Manipulation of adenovirus vectors, gene transfer and expression protocols. Humana, Clifton, N.J.
14. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
15. **Hockenberry, D., G. Nunez, C. Milliman, R. D. Schreiber, and S. J. Korsmeyer.** 1990. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature (London) **348:**334–336.
16. **Holbrook, N. J., S. G. Carlson, A. M. K. Choi, and J. Fargnoli.** 1992. Induction of *HSP70* gene expression by the antiproliferative prostaglandin PGA_2: a growth-dependent response mediated by activation of heat shock transcription factor. Mol. Cell. Biol. **12:**1528–1534.
17. **Johnson, M., D. Dimitrov, P. J. Vojta, J. C. Barret, A. Noda, O. M. Pereira-Smith, and J. R. Smith.** 1994. Evidence for a p53-independent pathway for upregulation of SDI1/CIP1/WAF1/p21 RNA in human cells. Mol. Carcinog. **11:**59–64.
18. **Katayose, D., R. Wersto, K. H. Cowan, and P. Seth.** 1995. Effects of a recombinant adenovirus expressing WAF1/Cip1 on cell growth, cell cycle and apoptosis. Cell Growth Differ. **6:**1207–1212.
19. **Kessis, T. D., R. J. Slebos, W. D. Nelson, M. B. Kastan, B. S. Plunkett, S. M. Han, A. T. Lorincz, L. Hedrick, and K. R. Cho.** 1993. Human papilloma virus 16 E6 expression disrupts the p53-mediated cellular response to DNA damage. Proc. Natl. Acad. Sci. USA **90:**3988–3992.
20. **Kim, Y. H., J. J. Proust, M. J. Buchholz, F. J. Chrest, and A. A. Nordin.** 1992. Expression of the murine homologue of the cell cycle control protein p34^{cdc2} in T lymphocytes. J. Immunol. **149:**17–23.
21. **Kobayashi, T., U. Consoli, M. Andreeff, H. Shiku, and W. Zhang.** 1995. Activation of p21WAF1/Cip1 expression by a temperature-sensitive mutant of human p53 does not lead to apoptosis. Oncogene **11:**2311–2316.

22. **Kyriakis, J. M., P. Banerjee, E. Nikolakaki, T. Dai, E. A. Rubie, M. F. Ahmad, J. Avruch, and J. R. Woodgett.** 1994. The stress-activated protein kinase subfamily of c-Jun protein kinases. Nature (London) **369:**156–160.
23. **Li, R., S. Waga, G. J. Hannon, D. Beach, and B. Stillman.** 1996. Differential effects by the p21 CDK inhibitor on PCNA-dependent DNA replication and repair. Nature (London) **371:**534–537.
24. **Lin, H. B., R. Falchetto, P. J. Mosca, J. Shabanowitz, D. F. Hunt, and J. L. Hamlin.** 1996. Mimosine targets serine hydroxymethyltransferase. J. Biol. Chem. **271:**2548–2556.
25. **Liu, Y., J. L. Martindale, M. Gorospe, and N. J. Holbrook.** 1996. Regulation of $p21^{WAF1/CIP1}$ expression through mitogen-activated protein kinase signalling pathway. Cancer Res. **56:**1–5.
26. **Luo, Y., J. Hurwitz, and J. Massague.** 1995. Cell cycle inhibition by independent CDK and PCNA binding domains in $p21^{Cip1}$. Nature (London) **375:**159–161.
27. **Michieli, P., M. Chedid, D. Lin, J. H. Pierce, W. E. Mercer, and D. Givol.** 1994. Induction of WAF1/CIP1 by a p53-independent pathway. Cancer Res. **54:**3391–3395.
28. **Morgan, D. O.** 1995. Principles of CDK regulation. Nature (London) **374:** 131–134.
29. **Poluha, W., D. K. Poluha, B. Chang, N. E. Crosbie, C. M. Schonhoff, D. L. Kilpatrick, and A. H. Ross.** 1996. The cyclin-dependent kinase inhibitor $p21^{WAF1}$ is required for survival of differentiating neuroblastoma cells. Mol. Cell. Biol. **16:**1335–1341.
30. **Russo, T., N. Zambrano, F. Esposito, R. Ammendola, F. Cimino, M. Fiscella, J. Jackman, P. M. O'Connor, C. W. Anderson, and E. Appella.** 1995. A p53-independent pathway for activation of WAF1/CIP1 expression following oxidative stress. J. Biol. Chem. **270:**29386–29391.
31. **Sheikh, M. S., H. Rochefort, and M. Garcia.** 1995. Overexpression of p21WAF1/CIP1 induces growth arrest, giant cell formation and apoptosis in human breast carcinoma cell lines. Oncogene **11:**1899–1905.
32. **Slichenmyer, W. J., W. G. Nelson, R. J. Slebos, and M. B. Kastan.** 1993. Loss of a p53-associated G1 checkpoint does not decrease cell survival following DNA damage. Cancer Res. **53:**4164–4168.
33. **Stein, C. A.** 1993. Suramin: a novel antineoplastic agent with multiple potential mechanisms of action. Cancer Res. **53:**2239–2248.
34. **Waga, S., G. J. Hannon, D. Beach, and B. Stillman.** 1994. The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. Nature (London) **369:**574–578.
35. **Xiong, Y., G. J. Hannon, H. Zhang, D. Casso, R. Kobayashi, and D. Beach.** 1993. p21 is a universal inhibitor of cyclin kinases. Nature (London) **366:** 701–704.
36. **Zhang, W., S. M. Kornblau, T. Kobayashi, A. Gambel, D. Claxton, and A. B. Deisseroth.** 1995. High levels of constitutive WAF1/Cip1 protein are associated with chemoresistance in acute myelogenous leukemia. Clin. Cancer Res. **1:**1051–1057.