[CANCER RESEARCH 54, 1169–1174, March 1, 1994]

Advances in Brief

WAF1/CIP1 Is Induced in *p53*-mediated G_1 Arrest and Apoptosis[1]

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Abstract

The tumor growth suppressor *WAF1/CIP1* was recently shown to be induced by *p53* and to be a potent inhibitor of cyclin-dependent kinases. In the present studies, we sought to determine the relationship between the expression of *WAF1/CIP1* and endogenous regulation of *p53* function. WAF1/CIP1 protein was first localized to the nucleus of cells containing wild-type p53 and undergoing G_1 arrest. *WAF1/CIP1* was induced in wild-type p53-containing cells by exposure to DNA damaging agents, but not in mutant *p53*-containing cells. The induction of WAF1/CIP1 protein occurred in cells undergoing either *p53*-associated G_1 arrest or apoptosis but not in cells induced to arrest in G_1 or to undergo apoptosis through *p53*-independent mechanisms. DNA damage led to increased levels of WAF1/CIP1 in cyclin E-containing complexes and to an associated decrease in cyclin-dependent kinase activity. These results support the idea that WAF1/CIP1 is a critical downstream effector in the *p53*-specific pathway of growth control in mammalian cells.

Introduction

The tumor suppressor *p53* is a transcription factor (reviewed in Ref. 1) which has been identified as a participant in the cellular DNA damage response resulting in either G_1 arrest (2, 3) or apoptosis (4, 5). The mechanism by which p53 induction results in growth suppression is not firmly established, but a potential explanation was provided by the demonstration that p53 transcriptionally activates the production of a M_r 21,000 protein [WAF1 (6)] which was simultaneously discovered as a potent inhibitor of cyclin-dependent kinases [CIP1 (7)]. In normal diploid fibroblasts, WAF1/CIP1 is associated with every cyclin-kinase complex examined, including those containing A-, B-, D-, and E-type cyclins and Cdc2, Cdk2, Cdk4, and Cdk5 (7, 8). In contrast, cyclin immune complexes from fibroblasts transformed by SV40 or cells containing germ line *p53* mutations contain nearly undetectable levels of WAF1/CIP1 (9). *WAF1/CIP1* inhibits growth of both human tumor cell lines (6) and normal diploid fibroblasts (7) when introduced via transfection, and this effect is largely reversed by expression of T-antigen in normal fibroblasts (7). Together, these data suggest a direct link between p53, G_1 arrest, and negative regulation of the cell cycle kinases required for the G_1-S transition.

Molecular details of the programmed cell death pathway are still poorly defined (10), but recent studies indicate that there are at least two pathways which mediate apoptosis in hematopoietic cell lineages (4, 5). One pathway can be triggered by exposure of mouse thymocytes to dexamethasone and is independent of *p53*. The second pathway is dependent upon the induction of p53 and follows exposure to ionizing radiation or other agents associated with DNA damage (11). Several cellular proteins, including bcl-2 (12), adenoviral E1B (13), and bcr-abl (14), have been identified as inhibitors of apoptosis, whereas other proteins, including c-myc (15), adenoviral E1A (16, 17), and bcl-2-associated proteins (18, 19), have been identified as inducers of apoptosis. The interactions between these inhibitors and the mediators of apoptosis are beginning to reveal a complex regulation of cell death (20).

In the present studies, we sought to determine whether *WAF1/CIP1* induction occurs in the endogenous DNA damage response pathways leading to either cell cycle block in G_1 or apoptosis. We further analyzed the induction of G_1 arrest or apoptosis by *p53*-independent pathways to determine if *WAF1/CIP1* is a generally induced growth inhibitor or a more specific stress/damage-induced mediator of *p53* function.

Materials and Methods

Cell Culture Conditions and Treatments. The v-myc retrovirus-induced murine T-cell lymphoma parental line J3D and the temperature-sensitive murine Val135 p53 mutant-transfected cell line M3 have been described previously (21). A fourth passage of the WI38 human lung fibroblast cell line was obtained from R-W. C. Yen and S. B. Baylin. The murine hematopoietic (pre-B-cell) cell line BAF3, which undergoes apoptosis when the growth factor IL3[2] is withdrawn, was obtained from A. Bedi and R. Jones (22). Cells were seeded 24 h before drug or radiation treatment and were 50–70% confluent at the time of such treatment. Radiation treatments of 2–8 Gy were delivered by a ^{137}Cs γ-irradiator at approximately 1 Gy/min. Cells were treated with the chemotherapeutic agent doxorubicin (Adriamycin) at a concentration of 0.2 μg/ml for 14 or 28 h at 37°C.

Immunocytochemistry and Western Blot Analysis. Cells were fixed and stained with mouse anti-human WAF1 polyclonal antibodies using minor modifications of previously described methods (23). Cell lysates were harvested in SDS/PAGE sample-loading buffer and Western blot analysis was performed using pAb1801 (Ab2; Oncogene Science) to detect human p53, Ab3 (Oncogene Science) to detect murine p53, or mouse anti-human WAF1 polyclonal sera to detect either human or mouse WAF1/CIP1 protein. The mouse WAF1 polyclonal sera was obtained following immunization of mice with an *Escherichia coli*-expressed GST-WAF1 fusion protein (WAF1/CIP1 residues 1 to 164). Mice were immunized by i.p. injection with 7.5 μg of electroeluted GST-WAF1 protein at weeks 0, 4, 7, and 15, and serum was obtained from the intraorbital plexus on weeks 9 and 16. Serum titers were measured in an enzyme-linked immunosorbent assay using soluble, purified GST-WAF1 essentially as described (23). Specificity towards WAF1/CIP1 was determined by immunoblot using a 1:500 dilution of serum on purified GST-WAF1 and on lysates of GM cells expressing or not expressing WAF1/CIP1 (6). Rabbit

Received 12/28/93; accepted 1/20/94.

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[1] This work was supported by the Preuss Foundation, the Clayton Fund, the National Foundation for Cancer Research, the Glaxo Research Institute, Inc. (M. B. K. and C. E. C.), the Council for Tobacco Research (M. B. K. and C. E. C.), and NIH Grants GM07309, GM07184, ES05777 (M. B. K.), CA-09071, CA-55541 (W. E. M.), AG-11085 (S. J. E., and J. W. H.), and CA-43460. B. V. is an American Cancer Society research professor.

[2] The abbreviations used are: IL3, interleukin 3; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; FBS, fetal bovine serum.

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polyclonal antibodies against cyclin E, used for immunoblotting, were generously provided by J. M. Roberts (24).

Cell Cycle, Thymidine Incorporation, and Northern Blot Analysis. Cell cycle parameters were assessed by pulsing the cells with bromodeoxyuridine and then staining with a fluorescein isothiocyanate-conjugated anti-bromodeoxyuridine antibody and propidium iodide (3, 25). Thymidine incorporation in GM cells, which contain an endogenous mutant *p53* and an exogenous dexamethasone-inducible wild-type p53, was carried out as described previously (26). G_1 arrest of GM cells (in the absence of wild-type p53 induction) was carried out using the plant amino acid mimosine as described (27, 28) except that confluent cells were first serum starved (1% serum) for 3 days and split into media containing either 10% FBS, 10% FBS plus 0.5 mM mimosine, 10% FBS plus 1 μM dexamethasone, or 10% FBS plus both 0.5 mM mimosine and 1 μM dexamethasone. After an 18-h incubation at 37°C, cells were pulsed for 2 h with [^{3}H]thymidine and total RNA was isolated for Northern blot analysis, performed as described (6).

Cell Viability and DNA Fragmentation Analysis. The viable fraction of J3D or M3 cells was determined at various time points (up to 37 h) of incubation at 37°C or 31°C using trypan blue exclusion as an indicator of cell viability (29). Total genomic DNA was extracted from BAF3 cells, which were incubated either in the presence or absence of IL3 and either untreated or treated with 4 Gy at 5, 8, and 15 h following treatment. The integrity of genomic DNA was evaluated by agarose gel electrophoresis and ethidium bromide staining.

***In Vitro* Cyclin E Kinase Assays.** Approximately 10 million cells were lysed in 0.3 ml containing 50 mM Tris (pH 8.0); 120 mM NaCl; 50 mM NaF; 0.1 mM sodium vanadate; 2 mM EDTA; 10 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin A (all from Sigma); 2 μg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride (Calbiochem); and 0.4% Nonidet P-40. The extracts were clarified by centrifugation at 14,000 rpm for 15 min at 4°C. For each condition, triplicate plates were lysed and assayed independently. Lysates were incubated for 1.5 h at 4°C with either a monoclonal antibody against cyclin E (HE111, provided by Drs. Emma Lees and Ed Harlow) or a monoclonal antibody against phage T7 gene 10 (Novagen) as a negative control. Immune complexes were collected using 20 μl of protein G-Sepharose and washed 3 times with 1.0 ml of lysis buffer and once with 1 ml of 20 mM Tris (pH 7.5)-10 mM $MgCl_2$. Histone H1 kinase assays were performed using 0.1 ml (one-tenth of the immune complex) as described previously (7). The beads were pelleted and 90 μl of supernatant were removed. The remaining 10 μl of beads were mixed with 15 μl of a kinase reaction mix containing 2 μg of histone H1 and [γ-^{32}P]ATP. After 30 min, 25 μl of 2 $\times$ SDS-PAGE buffer were added and 20 μl were analyzed by SDS-PAGE and autoradiography and quantitated on a Betagen scanner.

Results

WAF1/CIP1 Localizes to the Nucleus of Cells Blocked in G_1 following p53 Induction. In order to determine the subcellular location of WAF1/CIP1 in cells undergoing G_1 arrest, GM cells (containing an endogenous mutant *p53* and an exogenous wild-type p53 which is dexamethasone inducible) were induced with dexamethasone as reported previously (26). Fig. 1 shows that GM cells treated with dexamethasone produce a nuclear antigen recognized by mouse anti-human WAF1 polyclonal antibodies (Fig. 1*B*), an antigen not found in untreated cells (Fig. 1*A*). We note that dexamethasone treatment alone, in the absence of wild-type p53, did not induce *WAF1/CIP1* gene expression (Ref. 6 and data not shown). Fig. 1*C* shows that cells were indeed cell cycle arrested as demonstrated by their decreased incorporation of thymidine compared either to untreated cells or to a control cell line (DEL, containing a dexamethasone-inducible mutant *p53*). No WAF1/CIP1 was detectable in the nucleus or cytoplasm of GM cells not undergoing G_1 arrest. These results demonstrate that WAF1/CIP1 is located in the nucleus of cells undergoing G_1 arrest mediated by p53 and predict a nuclear localization for the various cyclin-Cdk-WAF1/CIP1 complexes which function to prevent cell cycle progression.

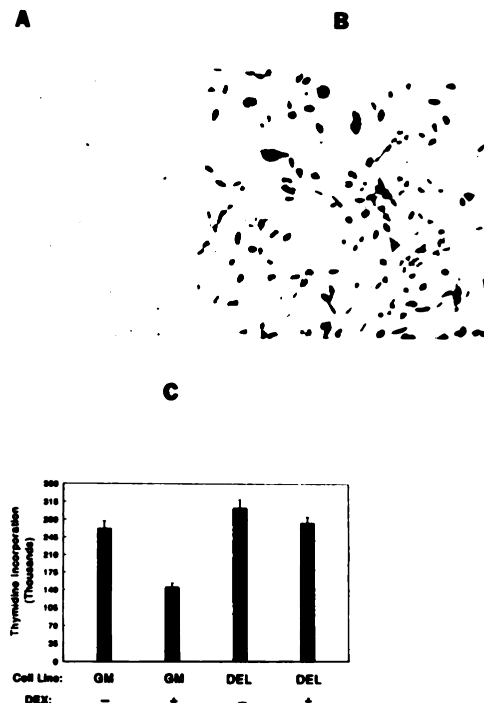

Fig. 1. WAF1/CIP1 localizes to the nucleus of cells undergoing G_1 arrest. Anti-WAF1 antibodies were used to immunocytochemically stain GM cells, either uninduced (*A*) or induced (*B*) to produce WAF1/CIP1 by dexamethasone (*DEX*). *C*, inhibition of DNA synthesis as a result of dexamethasone treatment.

Basal *WAF1/CIP1* Expression and Induction following DNA Damage Correlates with p53 Status. Previous studies have shown that exogenous wild-type p53 was capable of inducing *WAF1/CIP1* expression (6). The present studies addressed the question of whether *WAF1/CIP1* expression could be induced by endogenous wild-type p53 in cells undergoing G_1 arrest or apoptosis. A survey of WAF1/CIP1 levels in "normal" and tumor cell lines was performed prior to and following treatment with various DNA-damaging agents. The agents tested included ionizing radiation, UV radiation, treatment with various chemical agents including etoposide, 5-fluorouracil, Adriamycin, genistein, hydrogen peroxide, and methylmethane sulfonate. These treatments induced cellular p53 protein to varying extents, in both a dose- and time-dependent fashion, as has been observed by others (2, 3, 11, 30, 31). The chemotherapeutic drug Adriamycin appeared to be one of the strongest and most reproducible inducers of p53 protein and was therefore used for most of our studies. A variety of cell lines containing exogenous or endogenous wild-type or mutant *p53* genes were analyzed (Table 1). All cell lines tested which contained wild-type p53 protein (N = 8) expressed WAF1/CIP1, whereas cell lines with mutant *p53* (N = 7) expressed low or undetectable levels of WAF1/CIP1 (Figs. 2 and 3 and data not shown). Treatment of cell lines with Adriamycin induced both p53 and *WAF1/CIP1* expression only in the cell lines which contained wild-type p53 protein, as noted below.

WAF1/CIP1 Accumulation Is Associated with Ionizing Radiation-induced G_1 Arrest. We investigated the relationship between WAF1/CIP1 levels and G_1 arrest induced by ionizing radiation in lymphoid cell lines in which *p53* allele status and G_1 arrest capacity has been characterized (25). As shown in Fig. 2, WAF1/CIP1 was detected in unirradiated cells that contained wild-type p53 (WMN, FWL, and PC119) but not mutant *p53* (CA46, RAMOS, and ST486). The WMN and PC119 cells exhibited higher basal levels of wild-type p53 and WAF1/CIP1 than FWL cells (Fig. 2). In two of the three cell lines expressing wild-type p53 (WMN and FWL), accumulation of

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WAF1/CIP1 occurred when the cells were induced to arrest in G_1 by ionizing radiation (Fig. 2). No induction of WAF1/CIP1 was observed in lymphoid cells containing mutant *p53* (CA46, RAMOS, and ST486), which do not arrest following radiation (25). Of particular interest were the PC119 cells. These cells contain wild-type p53 but do not arrest in G_1 following ionizing radiation (25). No induction of p53 protein (25) or WAF1/CIP1 protein (Fig. 2) was observed following irradiation of these cells. These results demonstrate a close correlation between WAF1/CIP1 accumulation and radiation-induced G_1 arrest in lymphoid cells.

WAF1/CIP1 Induction and Apoptosis. The v-myc retrovirus-induced T-cell lymphoma line J3D expresses no endogenous *p53* due to loss of one allele coupled with the insertion of a murine leukemia provirus in the second allele. The M3 derivative of J3D contains a stably integrated murine Val 135 temperature-sensitive *p53* gene expressed at high levels (21). A shift from 37°C to 32°C results in loss of viability and apoptosis induction only in the M3 line. We chose this system to determine whether *WAF1/CIP1* would be induced in the pathway leading to apoptosis. Fig. 3*A* shows significant loss of viability at 32°C in the M3 line but not in the J3D parent line. The results in Fig. 3*B* confirmed the absence of p53 in the parental J3D line as well as the absence of detectable WAF1/CIP1 (*Lanes 1–4*). The M3 line expressed murine p53 under all the experimental conditions, but WAF1/CIP1 induction was observed only at the temperature resulting in a wild-type p53 conformation and subsequent apoptosis induction (*Lanes 5–8*).

The BAF3 murine hematopoietic cell line undergoes apoptosis following withdrawal of the growth factor IL3 (22). Treatment of this cell line with ionizing radiation results in G_1 arrest in the presence of IL3, and a much more rapid apoptosis following irradiation in the absence of IL3 (22).[3] We sought to determine whether *WAF1/CIP1* expression was induced in BAF3 cells undergoing either G_1 arrest or apoptosis. Fig. 4*A*, *top*, shows that BAF3 cells treated with 4 Gy in the presence of IL3 underwent G_1 arrest (shown here at 14 h following irradiation). This was preceded by induction of p53 and *WAF1/CIP1* expression (shown here at 2 h following irradiation; Fig. 4*A*, *bottom*). After IL3 withdrawal, BAF3 cells underwent apoptosis, with DNA fragmentation evident 15 h later (Fig. 4*B*, *top*). IL3-starved BAF3 cells treated with 4 Gy underwent a more rapid DNA fragmentation associated with induction of both p53 and *WAF1/CIP1* expression (Fig. 4*B*, *bottom*).

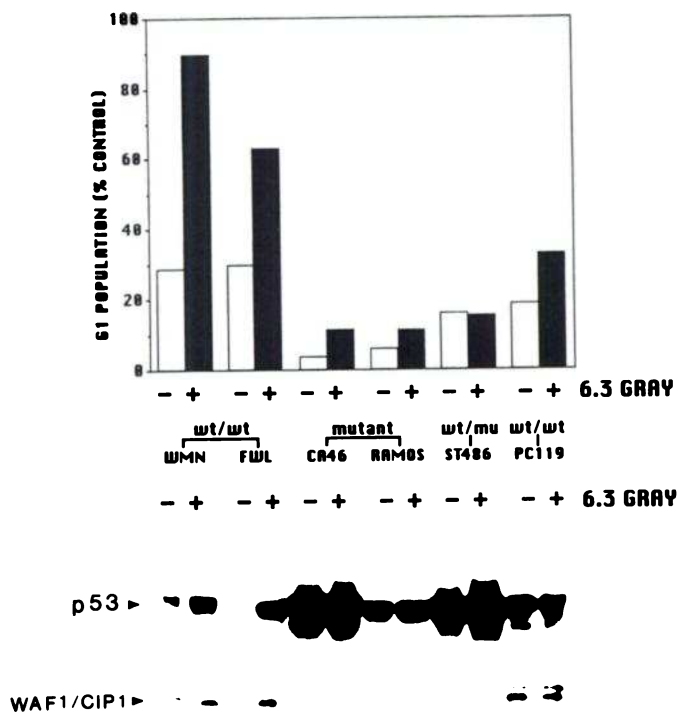
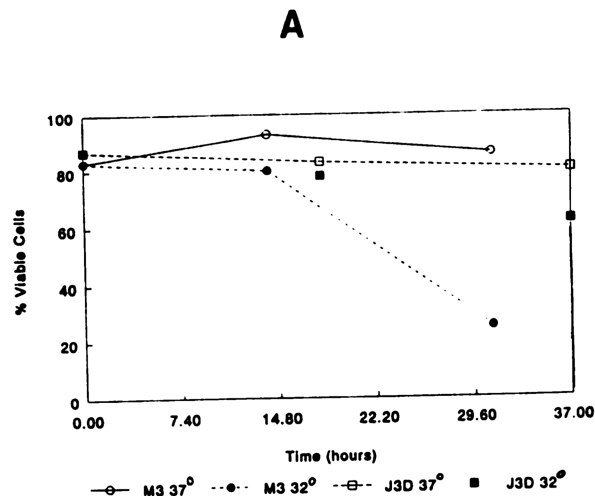
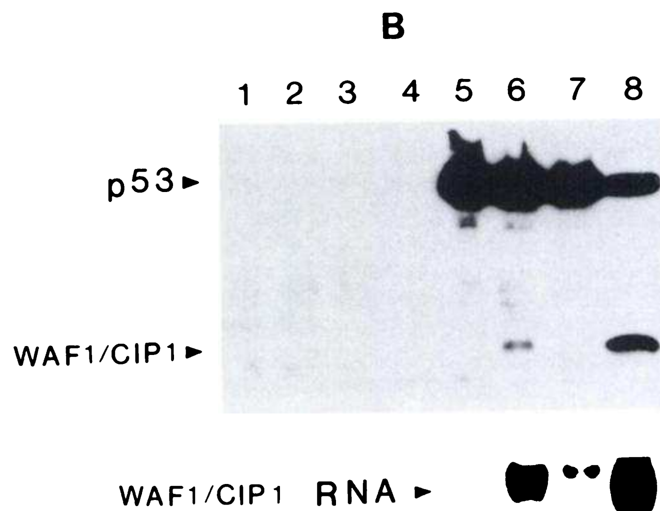


Fig. 2. Relationship among irradiation-induced G_1 arrest, p53, and WAF1/CIP1 in Burkitt's lymphoma cell lines. *Top*, percentage of the original G_1 population that remained in G_1 16 h after addition of the microtubule inhibitor, nocodazole (□, 0.4 μg/ml) or exposure to 6.3 Gy [gamma]-radiation plus nocodozole (■). Nocodozole was included to prevent cells from entering G_1 of the next cell cycle. The cell cycle distribution was measured by flow cytometry at 16 h following irradiation. Western blot analysis of p53 and WAF1/CIP1 was performed on cells 4 h after irradiation. *wt*, wild-type; *mu*, mutant.





WAF1/CIP1 RNA ►

Fig. 3. *WAF1/CIP1* is induced in p53-mediated apoptosis. In *A*, the viability of parental J3D and daughter M3 (containing an exogenous temperature sensitive Val 135 p53 mutant) T-cell lymphoma cell lines is shown as a function of incubation time at 37°C or 32°C. The plotted values represent the mean of triplicate determinations, with a range within 10% of the mean. *B*, Western blot analysis of J3D cells (*Lanes 1*, *2* at 17 h and *Lanes 3*, *4* at 37 h) and M3 cells (*Lanes 5*, *6* at 14 h and *Lanes 7*, *8* at 31 h), correlating *WAF1/CIP1* expression with apoptosis induction at 32°C (*Lanes 2*, *4*, *6*, *8*) or the control temperature (*Lanes 1*, *3*, *5*, *7*). Northern blot analysis (*bottom*) was performed on lysates obtained from M3 cells, demonstrating that *WAF1/CIP1* induction was regulated at the mRNA level.

[3] C. E. Canman and M. B. Kastan, unpublished observations.

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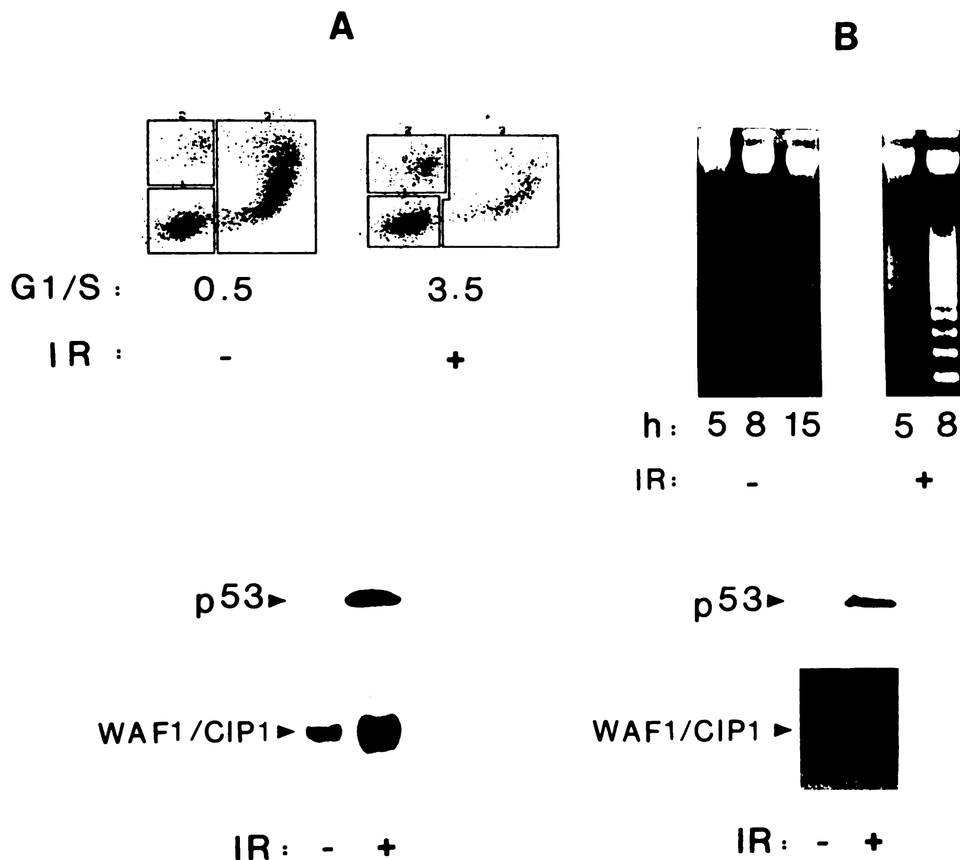

Fig. 4. *WAF1/CIP1* expression is not induced in p53-independent apoptosis. *A* (*top*), cell cycle analysis at 14 h following exposure of BAF3 cells to 4 Gy [gamma]-radiation (*IR*) in the presence of IL3. For cell cycle fluorescence-activated cell sorter analysis, the *lower left box*, *upper left box*, and *box* to the *right* represent G_1, G_2/M and S phase populations, respectively. The ratio of G_1-S-phase populations is indicated beneath the fluorescence-activated cell sorter analysis. p53 protein (*middle*) and *WAF1/CIP1* mRNA (*bottom*) levels were increased 2 h postirradiation. *B* (*top*), DNA fragmentation analysis of BAF3 cells at intervals (h) following IL3 withdrawal, with or without exposure to 4 Gy ionizing radiation at time zero. p53 protein (*middle*) and *WAF1/CIP1* mRNA (*bottom*) levels were increased at 2 h following IL3 withdrawal only in the irradiated BAF3 cells. In the absence of IL3, unirradiated cells undergoing apoptosis did not exhibit higher levels of either p53 or *WAF1/CIP1* expression.

***WAF1/CIP1* Expression Is Not Induced in Cells Which Undergo G_1 Arrest or Apoptosis through *p53*-independent Pathways.** To determine whether *WAF1/CIP1* expression is induced in cells undergoing *p53*-independent G_1 arrest or apoptosis, two experimental systems were used. In the first, the glioblastoma cell line GM [expressing only mutant *p53* in the absence of dexamethasone (Table 1)] was induced to arrest in G_1 by treatment with the plant amino acid mimosine (27, 28). Under these conditions, there was no measurable increase in *WAF1/CIP1* gene expression (Fig. 5). However, when G_1 arrest was caused by dexamethasone induction of exogenous wild-type p53, *WAF1/CIP1* mRNA was expressed, even in the presence of mimosine (Fig. 5). Additionally, no *WAF1/CIP1* induction was observed in GM cells arrested in G_0 by serum starvation in the absence of dexamethasone (not shown).

The BAF3 murine hematopoietic cell line undergoes apoptosis following withdrawal of the growth factor IL3, but no induction of p53 occurs.[3] Under these conditions, there was no associated induction of *WAF1/CIP1* gene expression (Fig. 4*B*; data not shown). When the same cells were treated with ionizing radiation both *p53* and *WAF1/CIP1* expression were induced (Fig. 4*B*).

Table 1 *WAF1/CIP1 induction correlates with p53 status*

Cell line/species	Cell type	p53 status		*WAF1/CIP1* induction			
		Endo[a]	Exo	Adriamycin	Radiation	Dex	Temp
WI38/H	Lung fibroblast	wt/wt	–	+	+		
BAF3/M	Pre-B cell line	wt/wt	–		+		
RKO/H	Colorectal carcinoma	wt/wt	–	+	+		
HCT-116/H	Colorectal carcinoma	wt/wt	–	+	+		
WMN/H	Burkitt's lymphoma	wt/wt	–		+		
FWL/H	Lymphoblastoid	wt/wt	–		+		
M3/M	T-cell lymphoma	mu/–	ts Val 135				+
GM/H	Glioblastoma	mu/–	Dex ind wt			+	
SW480/H	Colorectal carcinoma	mu/–	–	–	–		
DEL/H	Glioblastoma	mu/–	Dex ind mu	–	–	–	
J3D/M	T-cell lymphoma	mu/–	–				–
CA46/H	Burkitt's lymphoma	mu/–	–		–		
RAMOS/H	Burkitt's lymphoma	mu/–	–		–		
ST486/H	Burkitt's lymphoma	wt/mu	–		–		
PC119/H	Burkitt's lymphoma	wt/wt[b]	–		–		

[a] Endo and Exo, endogenous and exogenous p53 status in the cell lines; +, significant expression was induced following the indicated treatment; –, little or no induction of expression was detected; Dex, treatment of cells with 1 μM dexamethasone to induce the exogenous p53; Temp, temperature shift to 32°C. wt and mu, wild-type and mutant *p53* alleles, respectively. H and M, human and mouse origin, respectively. *WAF1/CIP1* expression was measured at the protein level, and in some cases also at the mRNA level as indicated in Figs. 3–5.
[b] Genotypically wild-type p53 but did not undergo G_1 arrest or express higher levels of p53 after radiation (see text).

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Induction of WAF1/CIP1 Correlates with Inhibition of Cyclin E-associated Kinase Activity. The results described above led to two predictions. (*a*) that there would be an increase in cyclin associated WAF1/CIP1 following induction of endogenous *p53*. (*b*) that cyclin-associated complexes would have reduced activity following such induction. These predictions were tested following induction of endogenous wild-type p53 in the wild-type p53 containing RKO and HCT-116 cell lines. Antibodies to cyclin E were used to immunoprecipitate cell extracts following Adriamycin treatment, and the immunoprecipitates were assessed for WAF1/CIP1 association. Cyclin E associates with Cdk2 during G_1 (24) and the activity of this complex is thought to be required for the G_1 to S transition. WAF1/CIP1 was indeed induced by the drug (Fig. 6*A*, *Lanes 1–4*), and there was a corresponding increase in the amount of WAF1/CIP1 associated with cyclin E (Fig. 6*B*, *Lanes 1–4*). No WAF1/CIP1 was found in immunoprecipitates or total cell extracts of the mutant *p53* containing tumor cell lines SW480 and DEL (Fig. 6, *A* and *B*, *Lanes 5–8*). The immunoprecipitates from RKO and HCT-116 also had significantly reduced H1 kinase activity following Adriamycin treatment (Fig. 6*C*, *Lanes 1–4*). There was little or no decrease in the cyclin E-associated H1 kinase activity of the *p53* mutant SW480 and DEL tumor cell lines (Fig. 6*C*, *Lanes 5–8*) when normalized to the amount of cyclin E in the immunoprecipitates. In the case of SW480, the apparent increase in H1 kinase activity following Adriamycin treatment was likely the result of an apparent increase in cyclin E in the immunoprecipitate (Fig. 6*B*, *Lanes 5* and *6*).

Discussion

The results presented here support the following model. When exposed to agents which cause DNA damage, cells which contain endogenous wild-type p53 are stimulated to produce higher levels of the protein. This induced p53 transcriptionally activates *WAF1/CIP1* expression by directly interacting with its regulatory elements. Induction of WAF1/CIP1 protein and its transport to the nucleus result in association and inhibition of the function of cyclin-dependent kinase complexes. Inhibition of these kinases in turn results in a failure of cells to exit G_1. This G_1 arrest following induction of p53 allows "normal cells" to check their growth. In tumors, loss of wild-type p53 function prevents the activation of this growth control pathway. This failure to induce transcriptionally active p53 may play a role in the unregulated growth of the tumors and also in the failure to respond to chemotherapeutic agents which normally trigger p53-regulated cell arrest or death.

The induction of wild-type p53 can occur in cells which are undergoing either G_1 arrest or apoptosis. It seems unlikely that p53 itself is the determinant of whether apoptosis occurs or not. Other factors, including cell type, developmental stage, additional pathways triggered by the p53-inducing signal, growth factors, and the induction of other proteins which modulate cell death may play important roles in the cell death decision. Moreover, it is clear that both G_1 arrest and apoptosis can occur without activation of either p53 or *WAF1/CIP1*. Thus, cells blocked in G_0 by serum starvation, blocked in G_1 by mimosine, or undergoing apoptosis after IL3 withdrawal did not express higher levels of WAF1/CIP1. These observations suggest a rationale for the design of anticancer therapy. For the treatment of tumors with an intact p53-regulated DNA damage response, it may be advantageous to combine chemotherapeutic agents which induce G_1 arrest or apoptosis independently of p53 with agents which activate the p53-*WAF1/CIP1* growth control pathway.

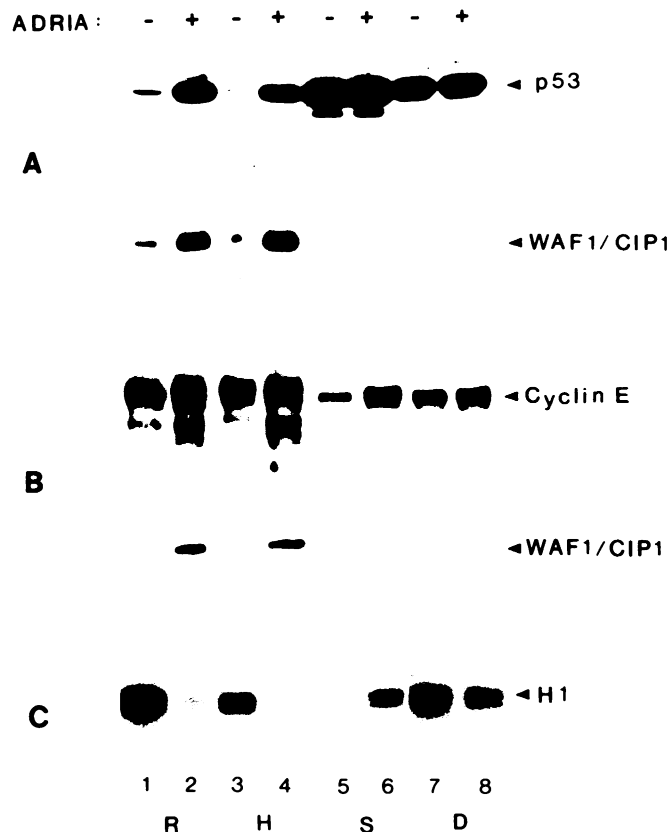
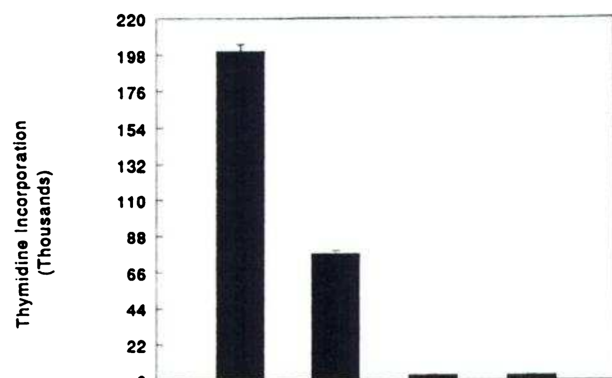


Fig. 6. DNA damage-induced WAF1/CIP1 associates with and inhibits the kinase activity of the cyclin E complexes. The tumor cell lines, labelled as *R*, *H*, *S*, or *D* to indicate RKO, HCT-116, SW480, or DEL, respectively, were either untreated (–) or treated (+) with Adriamycin and immunoprecipitated as described in "Materials and Methods." *A* and *B*, Western blot analysis of p53 and WAF1/CIP1 in total cell lysate or cyclin E immunoprecipitates, respectively. *C*, Cyclin E-associated H1 kinase activity.



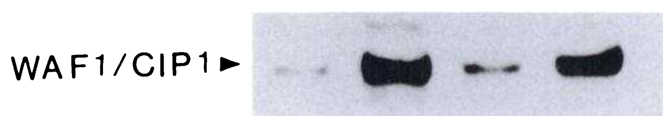


Fig. 5. *WAF1/CIP1* expression is not induced in p53-independent G_1 arrest. *Top*, inhibition of thymidine incorporation in GM cells (with mutant *p53*) treated with mimosine. Northern blot analysis, shown below, revealed that the p53-independent mimosine-induced cell cycle block does not involve induction of *WAF1/CIP1* expression. *DEX*, dexamethasone.

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Acknowledgments

The authors thank Bob Fay for animal work.

References

1. Vogelstein, B., and Kinzler, K. W. *p53* function and dysfunction. Cell, *70:* 523–526, 1992.
2. Kastan, M. B., Onyekwere, O., Sidransky, D., Vogelstein, B., and Craig, R. W. Participation of p53 protein in the cellular response to DNA damage. Cancer Res., *51:* 6304–6311, 1991.
3. Kastan, M. B., Zhan, Q., El-Deiry, W. S., Carrier, F., Jacks, T., Walsh, W. V., Plunkett, B. S., Vogelstein, B., and Fornace, A. J., Jr. A mammalian cell cycle checkpoint pathway utilizing *p53* and *GADD45* is defective in ataxia-telangiectasia. Cell, *71:* 587–597, 1992.
4. Lowe, S. W., Schmitt, E. M., Smith, S. W., Osborne, B. A., and Jacks, T. *p53* is required for radiation-induced apoptosis in mouse thymocytes. Nature (Lond.), *362:* 847–849, 1993.
5. Clarke, A. R., Purdie, C. A., Harrison, D. J., Morris, R. G., Bird, C. C., Hooper, M. L., and Wyllie, A. H. Thymocyte apoptosis induced by *p53*-dependent and independent pathways. Nature (Lond.), *362:* 849–852, 1993.
6. El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. *WAF1*, a potential mediator of *p53* tumor suppression. Cell, *75:* 817–825, 1993.
7. Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K., and Elledge, S. J. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell, *75:* 805–816, 1993.
8. Xiong, Y., Zhang, H., and Beach, D. D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. Cell, *71:* 505–514, 1992.
9. Xiong, Y., Zhang, H., and Beach, D. Subunit rearrangement of the cyclin-dependent kinases is associated with cellular transformation. Genes Dev., *7:* 1572–1583, 1993.
10. White, E. Death-defying acts: a meeting review on apoptosis. Genes Dev., *7:* 2277–2284, 1993.
11. Lowe, S. W., Ruley, H. E., Jacks, T., and Housman, D. E. *p53*-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell, *74:* 957–967, 1993.
12. Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R. D., and Korsmeyer, S. J. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature (Lond.), *348:* 334–336, 1990.
13. White, E., Sabbatini, P., Debbas, M., Wold, W. S. M., Kusher, D. I., and Gooding, L. The 19-kilodalton adenovirus E1B transforming protein inhibits programmed cell death and prevents cytolysis by tumor necrosis factor α. Mol. Cell. Biol., *12:* 2570–2580, 1992.
14. Evans, C. A., Owen-Lynch, P. J., Whetton, A. D., and Dive, C. Activation of the Abelson tyrosine kinase activity is associated with suppression of apoptosis in hematopoietic cells. Cancer Res., *53:* 1735–1738, 1993.
15. Fanidi, A., Harrington, E. A., and Evan, G. I. Cooperative interaction between c-*myc* and *bcl-2* proto-oncogenes. Nature (Lond.), *359:* 554–556, 1992.
16. Rao, L., Debbas, M., Sabbatini, P., Hockenbery, D., Korsmeyer, S., and White, E. The adenovirus E1A proteins induce apoptosis which is inhibited by the E1B 19K and Bcl-2 proteins. Proc. Natl. Acad. Sci. USA, *89:* 7742–7746, 1992.
17. Debbas, M., and White, E. Wild-type *p53* mediates apoptosis by E1A, which is inhibited by E1B. Genes Dev., *7:* 546–554, 1993.
18. Boise, L. H., Gonzalez-Garcia, M., Postema, C. E., Ding, L., Lindsten, T., Turka, L. A., Mao, X., Nunez, G., and Thompson, C. B. *bcl-x*, a *bcl-2*-related gene that functions as a dominant regulator of apoptotic cell death. Cell, *74:* 597–608, 1993.
19. Oltvai, Z. N., Milliman, C. L., and Korsmeyer, S. J. *Bcl-2* heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. Cell, *74:* 609–619, 1993.
20. Williams, G. T., and Smith, C. A. Molecular regulation of apoptosis: genetic controls on cell death. Cell, *74:* 777–779, 1993.
21. Wang, Y., Ramqvist, T., Szekely, L., Axelson, H., Klein, G., and Wiman, K. G. Reconstitution of wild-type p53 expression triggers apoptosis in a p53-negative v-myc retrovirus-induced T-cell lymphoma line. Cell Growth & Differ., *4:* 467–473, 1993.
22. Collins, M. K. L., Marvel, J., Malde, P., and Lopez-Rivas, A. Interleukin 3 protects murine bone marrow cells from apoptosis induced by DNA damaging agents. J. Exp. Med., *176:* 1043–1051, 1992.
23. Smith, K. J., Johnson, K. A., Bryan, T. M., Hill, D. E., Markowitz, S., Willson, J. K. V., Paraskeva, C., Peterson, G. M., Hamilton, S. R., Vogelstein, B., and Kinzler, K. W. The *APC* gene product in normal and tumor cells. Proc. Natl. Acad. Sci. USA, *90:* 2846–2850, 1993.
24. Koff, A., Giordano, A., Desai, D., Yamashita, K., Harper, J. W., Elledge, S. J., Nishimoto, T., Morgan, D. O., Franza, B. R., Jr., and Roberts, J. M. Formation and activation of a cyclin E-cdk2 complex during the G_1 phase of the human cell cycle. Science (Washington DC), *257:* 1689–1694, 1992.
25. O'Connor, P. M., Jackman, J., Jondle, D., Bhatia, K., Magrath, I., and Kohn, K. W. Role of the *p53* tumor suppressor gene in cell cycle arrest and radiosensitivity of Burkitt's lymphoma cell lines. Cancer Res., *53:* 4776–4780, 1993.
26. Mercer, W. E., Shields, M. T., Amin, M., Sauve, G. J., Appella, E., Romano, J. W., and Ullrich, S. J. Negative growth regulation in a glioblastoma tumor cell line that conditionally expresses human wild-type p53. Proc. Natl. Acad. Sci. USA, *87:* 6166–6170, 1990.
27. Dijkwel, P. A., and Hamlin, J. L. Initiation of DNA replication in the dihydrofolate reductase locus is confined to the early S period in CHO cells synchronized with the plant amino acid mimosine. Mol. Cell. Biol., *12:* 3715–3722, 1992.
28. Mosca, P. J., Dijkwel, P. A., and Hamlin, J. L. The plant amino acid mimosine may inhibit initiation at origins of replication in Chinese hamster cells. Mol. Cell. Biol., *12:* 4375–4383, 1992.
29. Coligan, J. E., Kruisbeek, A. M., Margulies, D. H., Shevach, E. M., and Strober, W. (eds.). Current Protocols in Immunology: Trypan Blue Exclusion Test of Cell Viability. Appendix 3B, A.3.3, New York: Green Pub. Assoc. and Wiley-Interscience, 1992.
30. Maltzman, W., and Czyzyk, L. UV irradiation stimulates levels of p53 cellular tumor antigen in nontransformed mouse cells. Mol. Cell. Biol., *4:* 1689–1694, 1984.
31. Lu, X., and Lane, D. P. Differential induction of transcriptionally active p53 following uv or ionizing radiation: defects in chromosome instability syndromes? Cell, *75:* 765–778, 1993.

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Cancer Res 1994;54:1169-1174.

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