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Induction of *WAF1/CIP1* by a p53-independent Pathway[1]

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Abstract

The p53-inducible gene *WAF1/CIP1* encodes a M_r 21,000 protein (p21) that has been shown to arrest cell growth by inhibition of cyclin-dependent kinases. Induction of *WAF1/CIP1* in cells undergoing p53-dependent G_1 arrest or apoptosis supports the idea that *WAF1/CIP1* is a critical downstream effector of p53. In the present study, we used embryonic fibroblasts from p53 "knock-out" mice to demonstrate p53-independent induction of *WAF1/CIP1*. We show that serum or individual growth factors such as platelet-derived growth factor, fibroblast growth factor, and epidermal growth factor but not insulin are able to induce *WAF1/CIP1* in quiescent p53-deficient cells as well as in normal cells. The kinetics of this transient induction, which is enhanced by cycloheximide, demonstrates that *WAF1/CIP1* is an immediate-early gene the transcript of which reaches a peak at approximately 2 h following serum or growth factor stimulation. On the other hand, DNA damage elicited by γ-irradiation induces *WAF1/CIP1* in normal human and mouse fibroblasts but does not affect *WAF1/CIP1* expression in p53-deficient cells. These results suggest the existence of two separate pathways for the induction of *WAF1/CIP1*, a p53-dependent one activated by DNA damage and a p53-independent one activated by mitogens at the entry into the cell cycle. The possible function of p21 at this early stage is discussed.

Introduction

Progression of eukaryotic cells through the cell cycle is a complex process that is finely regulated by external stimuli and internal checkpoints. When growth factors interact with their receptors at the cell surface, a cascade of biochemical events is triggered which transduce the mitogenic signal to the nucleus, where the coordinated transcription of both immediate and delayed early response genes is promoted (1). This sequential expression of specific genes, triggered initially by mitogenic signals, is part of a genetic program that leads the cells from a quiescent state into DNA synthesis and subsequently to mitosis. One interesting feature of this program is the existence of internal checkpoints at different stages of the cell cycle, the function of which is to prevent the cell from prematurely entering the next phase before all the necessary macromolecular events have been completed (2).

The Cdks[3] play a particularly relevant role in this process, since their activation and subsequent inactivation has been shown to regulate the orderly flow of the cells from one stage of the cell cycle to the next (3). Another key component of this regulatory mechanism is the tumor suppressor p53, which controls a G_1 checkpoint at which the cell cycle can be arrested prior to the initiation of DNA synthesis (4). Following DNA damage, p53 protein levels rise dramatically, and the entry into S is delayed until the genomic lesions are fully repaired (5–7). When p53 function is lost, cells enter S without appropriate DNA repair, leading to fixation and propagation of genetic alterations (8).

Recently, evidence for a link between the growth suppressing activity of p53 and the inactivation of Cdks has been provided by the cloning of the *WAF1/CIP1* gene, the transcription of which is directly activated by p53 (9, 10). *WAF1/CIP1* encodes a protein of M_r 21,000 (p21), which potently inhibits Cdks *in vitro* (10, 11). Overexpression of *WAF1/CIP1* in mammalian cells inhibits cell growth, suggesting that p21 is a downstream mediator of p53 function (9–11). In addition, it has recently been shown that *WAF1/CIP1* is induced by DNA damaging agents that trigger G_1 arrest or apoptosis in cells with wild-type p53 but not in tumor cells harboring deletions or mutations in the *p53* gene (12).

These recent findings provide a rational model which links tumor suppression with cell cycle regulation; p53 overexpression promotes the transcription of *WAF1/CIP1*, the product of which, p21, causes growth arrest through inhibition of Cdks, which are required for G_1 to S transition. However, to understand the function of *WAF1/CIP1* during normal cell growth, it is essential to determine whether factors other than p53 induce *WAF1/CIP1* expression and at what stages of the cell cycle such an induction may occur. In an effort to identify p53-independent pathways that may induce *WAF1/CIP1*, we analyzed its expression in embryonic fibroblasts from p53 "knock-out" mice. In this study, we demonstrate p53-independent induction of *WAF1/CIP1* by serum or purified growth factors. This induction is transient and independent of protein synthesis, indicating that *WAF1/CIP1* is an immediate-early gene.

Materials and Methods

Cell Culture Conditions and Treatments. Embryonic fibroblasts from normal and p53 knock-out mice, generously provided by Dr. Tyler Jacks (Massachusetts Institute of Technology, Cambridge, MA), as well as the conditions for culturing them, have been described previously (13). For serum or growth factor stimulation, cells were grown until they reached 90% confluence and then were starved in DMEM containing 30 nM Na_2SeO_4 and 5 mg/ml human transferrin (UBI, Lake Placid, NY). After 48 h, cells were stimulated by adding fetal bovine serum to a final concentration of 20% or individual growth factors at the concentrations indicated below. The human glioblastoma cell line GM47.23 was cultured and stimulated as described previously (4, 9, 14). Determination of the labeling index was performed as described previously (4, 14). NIH 3T3 fibroblasts were cultured with DMEM supplemented with 10% fetal calf serum. Human normal foreskin fibroblasts, obtained from the National Institute of Aging Cell Culture Repository, Coriell Institute, Camden, NJ (repository no. AG1523A), were cultured in DMEM supplemented with 10% fetal bovine serum. For both NIH 3T3 and human foreskin cells, stimulation with serum was performed as described for the primary mouse fibroblasts, except that 10% fetal calf serum was used for NIH 3T3. For irradiation experiments, exponentially growing cells were irradiated with 20 Gy (2,000 rad) in a Shepherd Mark I model 68 ^{137}Cs gamma irradiator

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[3] The abbreviations used are: Cdk, cyclin-dependent kinase; DMEM, Dulbecco's modified Eagle's medium; PCR, polymerase chain reaction; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; EGF, epidermal growth factor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; OA, okadaic acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; DEX, dexamethasone.

at a rate of approximately 3.5 Gy/min, incubated for the indicated times at 37°C, and then harvested for total RNA extraction.

Growth Factors and Tumor Promoters. Human recombinant PDGF (BB homodimer), acidic bovine FGF, and human recombinant insulin were obtained from UBI (Lake Placid, NY). Human recombinant EGF was purchased from Peprotech (Rocky Hill, NJ). TPA and OA were obtained from Sigma (St. Louis, MO) and Boehringer (Mannheim, Germany), respectively. The concentrations used in the triggering experiments were 100 ng/ml for PDGF, FGF, TPA, and OA; 200 ng/ml for EGF; and 5 μg/ml for insulin.

Hybridization Probes. Human and mouse *WAF1/CIP1* DNA probes were amplified by PCR (polymerase chain reaction) from genomic DNA and cloned using standard techniques (15). For PCR, the following oligonucleotides within exon 2 of *WAF1/CIP1* were used: mouse sense, 5′-CGGGATCCGGCACCATGTCCAATCCTGG-3′; mouse antisense, 5′-CGGAATTCTGTCAGGCTGGTCTGCCTCC-3′; human sense, 5′-CGGGATCCGGCGCCATGTCAGAACCGGC-3′; and human antisense, 5′-CGGAATTCCATGCTGGTCTGCCGCCGTTTTCG-3′. For both mouse and human, the PCR conditions were denaturation at 94°C for 1 min, annealing/extension at 72°C for 2 min, and a total number of cycles of 35. The identity of the amplified fragment was confirmed by sequencing. The control probe GAPDH (human) was obtained from Clonetech (Palo Alto, CA).

RNA Isolation and Northern Blot Analysis. Total RNA extraction and Northern blot analysis were carried out as described previously using approximately 20 μg RNA per lane (15). Probes were labeled with ^{32}P using the T7 Quickprime kit from Pharmacia (Uppsala, Sweden) according to the manufacturer's instructions. Prehybridization, hybridization, and washing conditions were as described previously (15).

Results

***WAF1/CIP1* Expression in p53-deficient Cells.** Embryonic normal fibroblasts and p53-deficient fibroblasts are derived from sibling embryos obtained by crossing genetically engineered mice that possess only one *p53* allele. Thus, the only genetic difference between the p53 +/+ fibroblasts and the p53 −/− fibroblasts is the presence or absence of a functional *p53* gene. The strategy for generation and analysis of these cells, as well as their biological and genetic properties, have been described previously (13). For this study, we used lines p45.41C (p53 +/+) and p45.41A (p53 −/−), which have been shown to contain wild-type p53 protein and no p53 protein, respectively. Cells were used at early passages when they were documented to have a normal karyotype. Following irradiation, line p45.41A lacked the p53-dependent G_1 arrest observed in line p45.41C and in several other cells containing wild-type p53 (5–7). Consistent with p53 being a growth suppressor, the doubling time of line A is approximately 2-fold shorter than the doubling time of line C. Moreover, as the passage number increases, line C reaches senescence, while line A continues to grow at an unchanged rate. These observations agree with those reported by others (16).

Total RNA was extracted from exponentially growing p45.41C and p45.41A cells at the same early passage, and *WAF1/CIP1* expression was determined by Northern blot analysis. As shown in Fig. 1*A*, the 2.0-kilobase *WAF1/CIP1* transcript is expressed at a much higher level in normal fibroblasts (p53 +/+) than in p53-deficient fibroblasts (p53 −/−), where it is barely detectable. We conclude that the basal expression of *WAF1/CIP1* in mouse embryonic fibroblasts is dependent upon p53 function.

Serum or Purified Growth Factors Induce *WAF1/CIP1* in p53-deficient Cells. These results prompted us to investigate whether *WAF1/CIP1* could be induced in p53-deficient cells. Fig. 1*B* shows that when quiescent p53 −/− fibroblasts are stimulated to reenter the cell cycle with serum, *WAF1/CIP1* transcript levels rise rapidly, reach a peak at about 2 h, and then decrease towards the basal level as the cells progress into the cell cycle. A similar pattern is also observed with the p53 +/+ fibroblasts, the only difference being a higher basal level which is present under both starvation and serum stimulation.

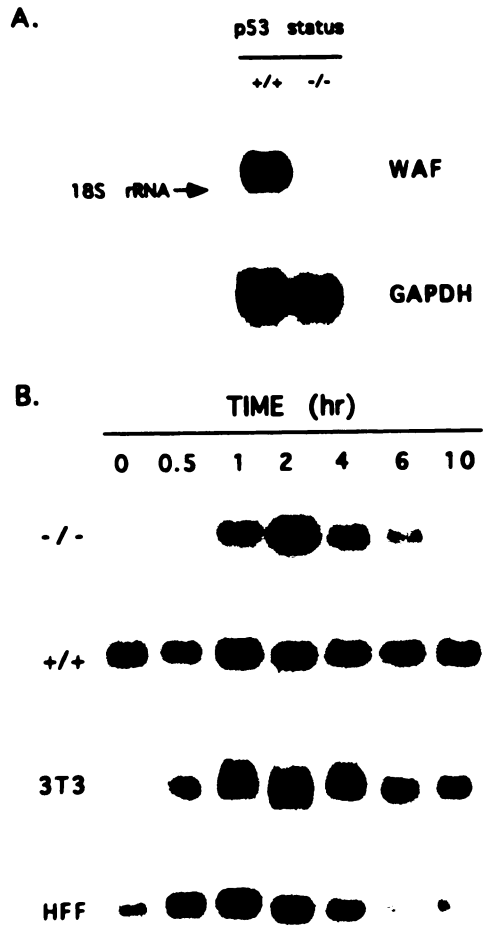

Fig. 1. Expression of *WAF1/CIP1* in normal and p53-deficient cells. *A*, *WAF1/CIP1* expression was determined by Northern blot analysis in exponentially growing embryonic fibroblasts with wild-type p53 (p53 +/+) or without p53 (p53 −/−). The same blot was hybridized with a GAPDH probe to control the amount of RNA loaded. *B*, p53 −/− cells, p53 +/+ cells, NIH 3T3 fibroblasts (*3T3*), and human normal foreskin fibroblasts (*HFF*) were stimulated with serum following 48 h starvation, and *WAF1/CIP1* expression was determined by Northern blot analysis at various times after stimulation. Equal amounts of RNA were loaded in each lane, as determined by ethidium bromide staining (not shown).

NIH 3T3 and normal human foreskin fibroblasts also responded in a similar way following serum stimulation, as shown in the lower panels of Fig. 1*B*.

To dissect the ability of serum to induce *WAF1/CIP1* in p53 knock-out cells, we serum-starved subconfluent p53 −/− fibroblasts for 48 h and then stimulated them with several purified growth factors, alone or in combination with insulin, which act synergistically with growth factors during cell cycle progression (17). After a 2-h incubation time, which corresponds to the peak observed in the serum time course, cells were harvested, and total RNA was extracted. Northern blot analysis revealed that PDGF, FGF, and with lower efficiency, EGF, are all strong inducers of *WAF1/CIP1*. Insulin alone induces *WAF1/CIP1* only slightly but can enhance the induction triggered by PDGF, FGF, and particularly EGF. Interestingly, the tumor promoters TPA and OA, which have been shown to induce several growth-associated genes (1), have a remarkable effect on *WAF1/CIP1* expression (Fig. 2*A*). We conclude that p53-deficient cells as well as normal cells have a p53-independent pathway for *WAF1/CIP1* induction.

***WAF1/CIP1* Is an Immediate-Early Gene.** To determine whether *WAF1/CIP1* induction in early G_1 is dependent upon protein synthesis, we preincubated quiescent p53 "null" cells (p53 −/−) with cycloheximide and subsequently stimulated them with PDGF. In parallel, identical samples were treated with cycloheximide alone or

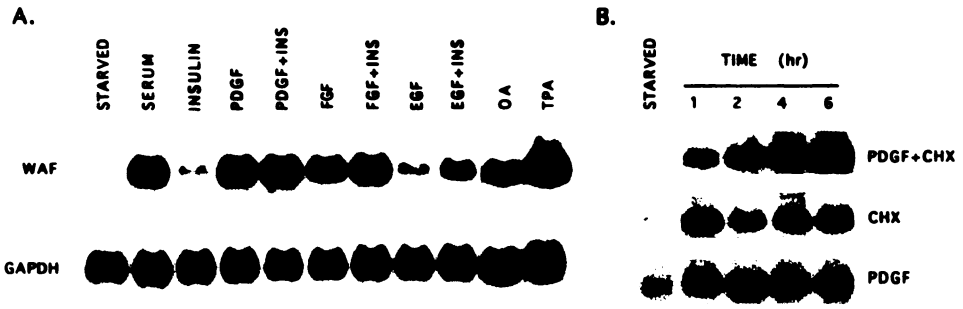

Fig. 2. *WAF1/CIP1* is an immediate-early gene. *A*, p53-deficient cells were starved for 48 h in the absence of serum and then treated with various growth factors and tumor promoters. *WAF1/CIP1* expression was determined by Northern blot analysis 2 h after the treatment. To control the amount of RNA loaded, the same blot was hybridized with a GAPDH probe. *B*, serum-starved p53-deficient cells were preincubated for 2 h with cycloheximide (*CHX*) and then stimulated with PDGF. In the control experiments, cells were either incubated with cycloheximide alone or stimulated with PDGF alone. *WAF1/CIP1* expression was determined by Northern blot analysis at the indicated times after stimulation.

PDGF alone. As shown in Fig. 2*B*, cycloheximide does not block the induction of *WAF1/CIP1* by PDGF; on the contrary, it prolongs and enhances it. This demonstrates that PDGF induces *WAF1/CIP1* independent of protein synthesis, indicating that *WAF1/CIP1* is an immediate-early gene (1). Cycloheximide alone also causes significant *WAF1/CIP1* induction; this may suggest that *WAF1/CIP1* is under the negative control of a rapidly turned-over suppressor, either at the transcriptional or posttranscriptional level. Consistent with a possible role of p21 in regulating early events in the cell cycle, *WAF1/CIP1* induction by PDGF is transient and resembles the kinetics observed with serum stimulation (Fig. 1*B*). Thus, *WAF1/CIP1* is not only a downstream effector of the tumor suppressor p53, but it is also an immediate-early gene whose induction by serum or growth factors is p53-independent.

p53-dependent and -independent *WAF1/CIP1* Induction in GM47.23 Cells. El-Deiry *et al.* (9) cloned *WAF1* from the human glioblastoma cell line GM47.23, which contains a mutant endogenous p53 and a transfected glucocorticoid-inducible wild-type p53 (9). When high levels of wild-type p53 are induced by DEX, *WAF1* expression increases, leading to cell cycle arrest in G_1. To compare p53-dependent with p53-independent pathways, quiescent GM47.23 cells were stimulated with either DEX or serum, as well as with a combination of DEX and serum, and *WAF1/CIP1* expression was determined by Northern blot analysis at different times following stimulation. Simultaneously, in order to correlate *WAF1/CIP1* expression with cell cycle progression, the percentage of cells undergoing DNA synthesis was determined by [^{3}H]thymidine incorporation followed by autoradiography.

As shown in Fig. 3*A*, the *WAF1/CIP1* transcript is barely detectable in starved cells without glucocorticoid stimulation but is dramatically up-regulated following induction of wild-type p53 with DEX. This indicates that, even in the absence of growth factors, wild-type p53 is sufficient to induce *WAF1/CIP1*. Following stimulation with serum alone, *WAF1/CIP1* expression increases transiently, thus resembling the pattern observed in mouse and human fibroblasts (Fig. 1*B*). In contrast, when cells are treated with serum in combination with DEX, a sustained and prolonged induction of *WAF1/CIP1* is elicited. Accordingly, as *WAF1/CIP1* expression returns to basal levels in cells stimulated with serum alone, the labeling index increases, and by 24 h almost 90% of the cells have undergone DNA synthesis (Fig. 3*B*). On the other hand, in the presence of DEX, the percentage of labeled cells does not increase significantly in spite of the mitogenic stimulation, indicating that the cell cycle has been arrested prior to initiation of DNA synthesis.

Thus, both a p53-dependent and a p53-independent induction of *WAF1/CIP1* can occur in GM47.23 cells, although the former is correlated with G_1 arrest and the latter with the entry into the cell cycle. Consistent with p21 being a growth inhibitor, *WAF1/CIP1* expression decreases as the cells enter S but remains high in G_1-arrested cells.

Radiation Is Unable to Induce *WAF1/CIP1* in p53-deficient Cells. While the DEX-treated GM47.23 cells provide an example of p53-dependent induction of *WAF1/CIP1*, they do not represent a physiological up-regulation of p53 protein. To overcome this limitation and to study *WAF1/CIP1* involvement in physiological pathways that are known to activate endogenous p53 function, we irradiated several cell types to induce DNA damage.

As shown in Fig. 4*A*, the *WAF1/CIP1* transcript level rapidly increases in normal human skin fibroblasts irradiated with 20 Gy, and it remains up-regulated for as long as 24 h. When normal mouse embryonic fibroblasts (p53 +/+) were treated with the same dose, a similar induction was observed (Fig. 4*B*). In contrast, irradiated mouse p53 −/− fibroblasts did not show any significant increase in *WAF1/CIP1* in the same time frame (Fig. 4*B*). Thus, *WAF1/CIP1* induction elicited by γ-irradiation is strictly p53-dependent as the experiments with tumor cells containing mutant p53 suggested (12).

Therefore, at least two pathways may lead to increased *WAF1/CIP1* expression. One is triggered by growth factors, is associated with cell growth, and is p53-independent; the second pathway is elicited by DNA damage, leads to growth arrest, and is p53-dependent.

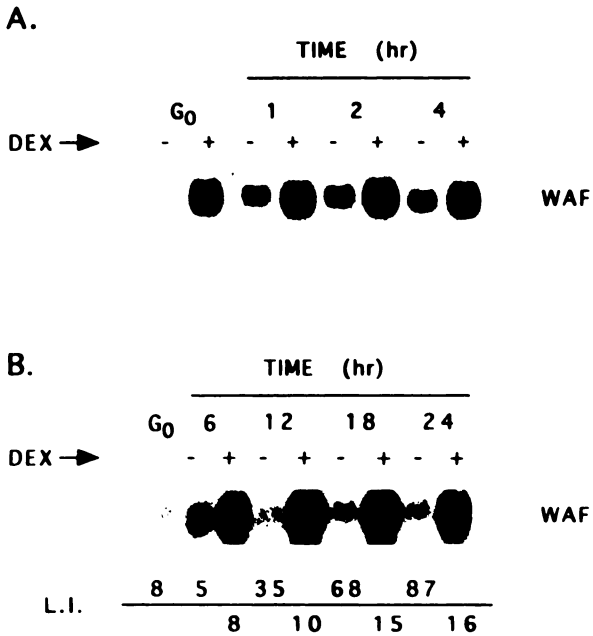

Fig. 3. p53-dependent and -independent *WAF1/CIP1* induction in the GM47.23 glioblastoma cell line. *A*, quiescent GM47.23 cells were either untreated (G_0 −), treated for 2 h with dexamethasone (*DEX*) alone (G_0 +), or stimulated with serum in the presence (+) or absence (−) of DEX, and *WAF1/CIP1* expression was determined by Northern blot analysis at the indicated times. *B*, quiescent GM47.23 cells were stimulated with serum in the presence (+) or absence (−) of DEX, and [^{3}H]thymidine was added to the medium at time 0. *WAF1/CIP1* expression and the labeling index (*L.I.*) were determined at the indicated times by Northern blot analysis and autoradiography, respectively. The basal L.I. of quiescent cells (G_0) was determined following a 24-h incubation with [^{3}H]thymidine.

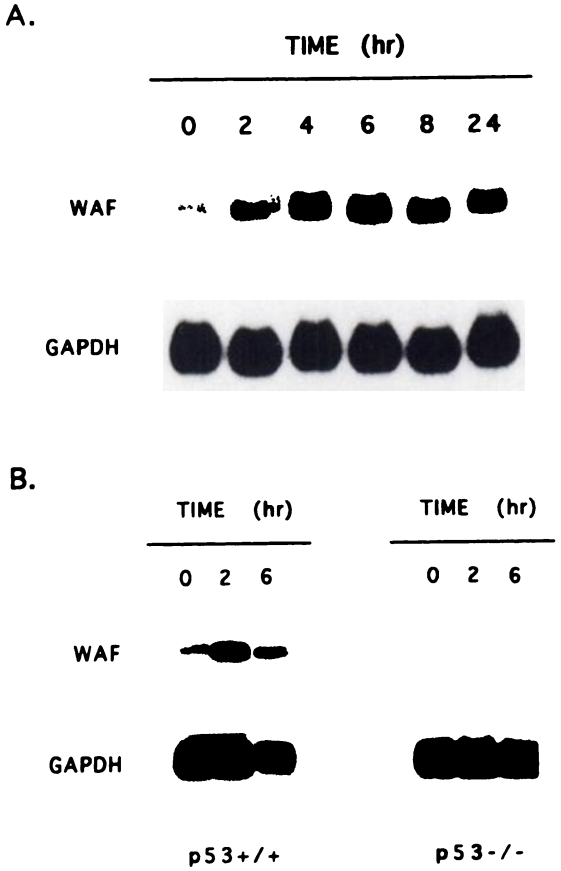

Fig. 4. Induction of *WAF1/CIP1* following irradiation is p53 dependent. *A*, exponentially growing normal human foreskin fibroblasts were irradiated with 20 Gy (2000 rad), and *WAF1/CIP1* expression was determined by Northern blot analysis at the indicated times. *B*, lines p45.41C (p53 +/+) and p45.41A (p53 −/−) mouse embryonic fibroblasts were irradiated as in *A*, and *WAF1/CIP1* expression was determined by Northern blot analysis after 2 and 6 h. To control the amount of RNA loaded, the same blots were hybridized with a GAPDH probe.

Discussion

The recent discovery of p21 as an inhibitor of cyclin-kinase complexes and its ability to be transcriptionally activated by p53 provided a link between tumor suppression and cell cycle regulation (2, 9–11). Exposure of cells to agents which cause DNA damage leads to p53-dependent induction of *WAF1/CIP1* and elevated expression of p21, resulting in growth arrest or apoptosis (12). In this study, we used embryonic fibroblasts from p53 knock-out mice to demonstrate p53-independent induction of *WAF1/CIP1*. We show that treatment of quiescent p53 null cells with serum or individual growth factors results in increased expression of *WAF1/CIP1* mRNA. The kinetics of *WAF1/CIP1* accumulation and decay, as well as the superinduction by cycloheximide, indicates that *WAF1/CIP1* is an immediate-early gene whose induction may play a role during early stages of the cell cycle (1). This p53-independent induction of *WAF1/CIP1* takes place not only in p53-deficient cells but also in normal mouse or human fibroblasts. In the human glioblastoma cell line GM47.23, which contains an endogenous mutant p53 and a glucocorticoid-inducible exogenous wild-type p53, transient induction of *WAF1/CIP1* through the p53-independent pathway is associated with active proliferation, while its p53-activated up-regulation leads to growth arrest in G_1. Following DNA damage elicited by γ-irradiation, mouse embryonic fibroblasts from p53 knock-out mice do not show any augmented expression of *WAF1/CIP1*, which in contrast increases dramatically in the control p53 +/+ cells and in normal human fibroblasts.

Taken together with previous observations (12), these results suggest that *WAF1/CIP1* induction may occur under different physiological situations. When cells are treated with agents that cause DNA damage, such as radiation or chemotherapeutic drugs, *WAF1/CIP1* expression is induced through the p53-dependent pathway. On the other hand, as cells enter the G_1 phase, a transient and p53-independent induction of *WAF1/CIP1* is elicited. The basal expression of *WAF1/CIP1* seems to be largely dependent upon p53 function, since p53 −/− fibroblasts show negligible levels of *WAF1/CIP1* compared to normal fibroblasts. However, this might not be a general rule, since *WAF1/CIP1* expression in other p53 −/− tissues has not been analyzed.

While the up-regulation of p21 in response to growth-arresting signals can be understood, the transient induction of *WAF1/CIP1* following mitogenic stimulation is more puzzling. Why should a gene encoding for a growth inhibitor be induced as an immediate-early gene? This apparent paradox can be explained if we assume that p21 causes growth arrest only when it stoichiometrically exceeds the amount of cyclin-Cdk complexes present in the cell. In early G_1, p21 could function to damp the activity of newly formed cyclin-Cdk complexes, serving as an internal control mechanism which prevents the cell from entering S prematurely (2, 10). This idea is supported by the observation that p21 is associated with cyclin-Cdk complexes in normal growing cells (11) and that p53 −/− fibroblasts, which express an undetectable amount of *WAF1/CIP1*, have a significantly shorter G_1 phase and doubling time (Ref. 16 and this work). In this model, p21 levels represent a threshold that cyclin-Cdk complexes have to overcome before the cell can entry into S. When DNA damage is elicited, p53-dependent *WAF1/CIP1* induction causes the p21 threshold to rise, delaying the initiation of DNA synthesis. In senescent cells where *WAF1/CIP1* expression is high (18), it is likely that a higher p21 threshold is responsible for the inability of the cells to proliferate.

Alternatively, the growth-suppressing activity of p21 in early G_1 could be modulated in some other fashion, *e.g.*, by binding to another molecule that sequesters it and keeps it in an inactive form. A similar model has been recently proposed for another inhibitor of Cdks, p27, which mediates transforming growth factor β ability to arrest the cell cycle in late G_1 and is also present in proliferating cells (19).

In conclusion, we have identified a p53-independent pathway that leads to the induction of *WAF1/CIP1*. As it has been recently suggested, the absence of *WAF1/CIP1* induction following DNA damage in cells that have lost wild-type p53 function may be responsible for the escape of tumor cells from treatment with various drugs or radiotherapy (12). Thus, the knowledge of agents that induce *WAF1/CIP1* through alternative pathways might be relevant to the chemotherapeutic treatment of cancer. Interestingly, we observed that the chemotherapeutic drug Adriamycin, which has been shown to cause apoptosis (20) and *WAF1/CIP1* induction in a p53-dependent manner (12), is indeed able to induce *WAF1/CIP1* in p53 null cells at higher doses (data not shown), therefore providing an example of a drug acting through both p53-dependent and p53-independent pathways. The search for other drugs with such characteristics will be of great applicative value.

Acknowledgments

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