Induction of WAF1/CIP1 by a p53-independent Pathway

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Abstract

The p53-inducible gene WAF1/CIP1 encodes a Mr 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 G1 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 (p21) 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 G1 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 Mr 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 G1 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 G1 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 p53 knockout 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 mM Na2SeO4 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 GM4723 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 137Cs gamma irradiator...
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 were used: mouse sense, 5'-CCGATCCTCG-3'; mouse antisense, 5'-CGGAATCTGTCAAGCTAG-3'; human sense, 5'-CCGATCCTCGGCACTGT-CAGAACCGGC-3'; and human antisense, 5'-CGGAATTCTGTCTGTCG-3'. Both for mouse and human probes, 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 32P 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 G1 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. 1A, 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. 1B 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. 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. 2B, 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. 1B). 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 G1. 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 [3H]thymidine incorporation followed by autoradiography.

As shown in Fig. 3A, 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. 1B). 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. 3B). 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 G1 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 G1-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. 4A, 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. 4B). In contrast, irradiated mouse p53 −/− fibroblasts did not show any significant increase in WAF1/CIP1 in the same time frame (Fig. 4B). 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.
While its p53-activated up-regulation leads to growth arrest in G1. were irradiated as in A, and WAF1/CIP1 expression was determined by Northern blot times. B, lines p45.41C (p53 +/+ ) and p45.41A (p53 −/−) mouse embryonic fibroblasts contains an endogenous mutant p53 and a glucocorticoid-inducible plexes and its ability to be transcriptionally activated by p53 provided

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 G1. 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 G1 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 grow arrest only when it stoichiometrically exceeds the amount of cyclin-Cdk complexes present in the cell. In early G1, 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 G1 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 enter 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 G1 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 Cdns, p27, which mediates transforming growth factor β ability to arrest the cell cycle in late G1, 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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