Transforming Growth Factor β1 Can Induce CIP1/WAF1 Expression Independent of the p53 Pathway in Ovarian Cancer Cells

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Abstract

Transforming growth factor β (TGFβ) is an important regulator of cellular proliferation. In normal ovarian epithelial cells, TGFβ acts to inhibit growth. However, in ovarian cancer cell lines, this effect is usually lost. Although the regulatory pathway of TGFβ remains unclear, TGFβ-treated cells arrest late in G1. This inhibition appears to involve blocking of the cyclin-dependent kinase phosphorylation of the retinoblastoma protein. Recently, a general inhibitor of cyclin-dependent kinases, CIP1/WAF1/p21, was identified. Expression of CIP1 is positively regulated by binding of wild-type p53 to a consensus response element upstream of the CIP1 gene. Overexpression of the CIP1 protein causes growth suppression, analogous to TGFβ and wild-type p53. We have examined the induction of CIP1 by TGFβ1 in ovarian cancer cell lines that have been previously characterized for their proliferative response to TGFβ1. OVCA420, a cell line that is dramatically growth inhibited by TGFβ1, significantly induced CIP1 expression in response to TGFβ1. CIP1 induction was accompanied by a decrease in cdk2 kinase activity and cdk2 protein levels. In three other cell lines that respond weakly to TGFβ1, CIP1 expression was not induced. To determine if TGFβ1 induction occurs via p53, regulation of p53 RNA and protein was examined. No differences in p53 transcription, steady-state protein level, de novo synthesis, phosphorylation, or subcellular accumulation were noted. Furthermore, TGFβ1 could not induce transcription from a consensus p53 DNA binding site in the TGFβ1-responsive cell line. Our results suggest that CIP1 expression is an important mediator of TGFβ1 growth suppression. However, it appears that TGFβ1 induces CIP1 independent of the p53 pathway.

Introduction

The equilibrium between cell proliferation and cell death must be carefully maintained for normal tissue growth. Central events in tumorigenesis disrupt this balance towards proliferation. Cellular responses to TGFβ1 illustrate this point and help to define pathways by which tumor cells escape growth-regulatory checkpoints. In vitro, TGFβ1 is a potent inhibitor of the growth of a variety of primary cells, inducing growth arrest at the G1/S boundary (1, 2). However, at similar concentrations, many tumor cells, both in vitro and in vivo, are resistant to growth inhibition and, in some cases, growth and tumorigenicity can be enhanced (3–5). There are a number of possible points in the TGFβ1 signaling pathway that can be disrupted in tumor cells, including the activation of latent TGFβ1, defects in or loss of expression of the TGFβ1 cell surface receptors (serine-threonine kinases), the cytoplasmic signaling cascade, and nuclear events that respond to this signaling (4).

One of the most common genetic events in human tumorigenesis is mutation of the p53 gene (6, 7). The weight of evidence now indicates that these mutations inactivate the function of the p53 protein, a transcriptional activator (8). The search for genes that are regulated by p53 has uncovered several which contain p53 DNA response elements, the most intriguing of which is CIP1/WAF1 (9). This protein (also called p21) was originally identified as a member of the cyclin complex (cyclins, cyclin-dependent kinases, proliferating cell nuclear antigen, CIP1/WAF1, p16INK4, and p27KIP1, Refs. 10–12). CIP1/WAF1 was subsequently found to inhibit the kinase activity of these complexes (13, 14). These findings have yielded an important link between p53 and the cell cycle regulatory pathway that is currently understood as follows. Wild-type p53 is transiently elevated late in G1, in normal cycling cells (15–17). This elevation of p53 transactivates CIP1/WAF1 expression, but at levels which do not completely inhibit cdk activity (18). These kinases are necessary for progression through the cell cycle, in part, through hyperphosphorylation of the Rb protein, which leads to the release of the E2F transcriptional factor, and this in turn induces expression of a number of genes critical for DNA replication (19). This normal cascade of events permits regulated entry into the DNA synthesis phase of the cell cycle.

In cells that sustain DNA damage (e.g., by irradiation), p53 expression is strongly induced and leads to G1 arrest or apoptosis (20, 21). The increased level of wild-type p53 transactivates CIP1, thus inhibiting cyclin-dependent kinase activity and, in turn, phosphorylation of Rb (22). Conversely, cells lacking wild-type p53 are more resistant to radiation-induced arrest or apoptosis, probably because they are unable to induce CIP1 and prevent G1 exit by inhibiting Rb phosphorylation (23–25). Two other inhibitors of cdk activity have recently been described, p16INK4 and p27KIP1; however, they appear to have no direct relationship to p53 activity (11, 12).

Received 7/13/94; revised 9/12/94; accepted 9/30/94.

1 This work was supported by NIH Grant ROI-CA56749 (to J. D. I.).
2 To whom requests for reprints should be addressed, Box 3873, Duke University Medical Center, Durham, NC 27710.
3 The abbreviations used are: TGFβ1, transforming growth factor β1; cdk, cyclin-dependent kinase; Rb, retinoblastoma; CAT, chloramphenicol acetyltransferase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; NP40, Nonidet P-40.
TGFβ-mediated growth arrest is also associated with accumulation of hypophosphorylated Rb protein (26–28). SV40 T-antigen and adenovirus E1A can reduce TGFβ sensitivity, most likely by complexing with Rb and eliminating its growth-regulatory capacity (29, 30). Cells that are sensitive to TGFβ can be rendered resistant by the introduction of a mutant p53 gene (31, 32). Mutant p53 inactivates wild-type activity (33, 34), which may prevent transcriptional activation of the CIP1 gene, and thus allow unregulated phosphorylation of Rb. This circuitry suggests that TGFβ may suppress growth by inducing CIP1 via p53. We have previously characterized a series of ovarian cancer cell lines for their sensitivity to the growth-inhibitory effects of TGFβ (35). In this communication, we examine whether sensitivity to TGFβ correlates with induction of CIP1/WAF1 and if this is a p53-mediated event. Our results show that TGFβ growth inhibition is associated with a dramatic increase in CIP1/WAF1 expression. However, the response appears to be independent of p53 activation.

Results
We have examined four epithelial ovarian cancer cell lines that were previously established in our laboratory; OVCA420, 429, 432, and 433. When cultured in the presence of 10 ng/ml TGFβ, OVCA 429, 432, and 433 continued to proliferate normally. However, proliferation of OVCA420 was reduced by greater than 80% after 48 h in the presence of TGFβ as measured by 3H-thymidine incorporation (35). Growth inhibition of OVCA420 is accompanied by DNA degradation indicative of apoptosis.

CIP1 RNA and Protein Are Induced in the TGFβ Responsive Line. Levels of CIP1 message were analyzed after treatment with 10 ng/ml TGFβ for 24 h (Fig. 1A). A dramatic induction of CIP1 mRNA was observed in the OVCA420 cell line. In contrast, OVCA429 and 433 had high steady-state levels of the message but showed no induction after TGFβ treatment. OVCA432 has extremely low resting levels of CIP1 mRNA (detectable only by reverse transcription-polymerase chain reaction, data not shown) and did not induce with treatment. Levels of CIP1 protein were also examined using a CIP1 monoclonal antibody (Fig. 1B). The amount of the M, 21,000 protein in these cell lines reflects the levels of CIP1 mRNA with and without TGFβ treatment. Significant induction of protein is only observed in OVCA420, while high levels are observed with and without treatment in OVCA429 and 433. No CIP1 protein was detected in OVCA432, consistent with the extremely low mRNA levels.

The status of the p53 gene and protein was examined in these cell lines to determine if TGFβ induction was dependent upon the presence of wild-type p53. From direct DNA sequence analysis, only OVCA432 was found to contain a typical p53 missense mutation (codon 277, cysteine to phenylalanine). The other three cell lines contain low levels of p53 protein, detected both by immunohistochemistry and immunoprecipitation, consistent with maintenance of wild-type p53. In addition, the mutant specific monoclonal, pAB240, failed to recognize p53 in these cell lines. The p53 mutant OVCA432 line contains only trace levels of CIP1, while the three apparently wild-type p53 lines contain significantly higher levels (Table 1). The relative levels of CIP1 in these cell lines are consistent with regulation by p53. Induction of CIP1 by TGFβ, however, only occurs in one of the p53 wild-type cell lines.

<table>
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<th>Cell line</th>
<th>p53 status</th>
<th>TGFβ inhibition</th>
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<th>CIP1 induction</th>
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*Inhibition of DNA synthesis measured at 48 h after addition of 10 ng/ml of TGFβ (35).

bCodon 277.

Induction of CIP1 Inhibits cdk2 Activity and Rb Phosphorylation. TGFβ growth arrest in OVCA420 is associated with an increase in CIP1 RNA and protein to levels approaching those found in the nonarrested OVCA429 and 433 lines. However, these two cell lines are able to withstand the growth inhibitory effects of CIP1, suggesting that either the CIP1 is not active or that there are compensatory changes in other members of the cyclin-ckd pathway. To address these alternatives, we assessed two aspects of CIP1 activity: cdk activity and levels of Rb phosphorylation. Protein extracts from TGFβ treated and untreated cells were immunoprecipitated with a cdk2 antibody and then assayed for kinase activity using histone H1 as a substrate (Fig. 2). We found low levels of kinase activity in OVCA429 and
These amounts of protein from each cell line, treated (+) and untreated (−), TGFβ1 induction of OVCA420 reduces cdk2 kinase activity by 5-fold. In the lower panel, cdk2 protein levels from the same cellular extracts are detected by immunoblot using a cdk2 polyclonal antibody.

Fig. 2. cdk2-associated histone H1 kinase activity and levels of cdk2 protein in ovarian cancer cell lines treated with TGFβ1. Histone kinase activity (upper panel) was measured in cdk2 immunoprecipitates from equal amounts of protein from each cell line, treated (+) and untreated (−). TGFβ1 induction of OVCA420 reduces cdk2 kinase activity by 5-fold. In the lower panel, cdk2 protein levels from the same cellular extracts are detected by immunoblot using a cdk2 polyclonal antibody.

433 consistent with the constitutively high levels of CIP1 in these cell lines. High levels of kinase activity in OVCA432 correlated with very low levels of CIP1 in those cells. TGFβ1 treatment of OVCA420 reduced cdk2-associated H1 kinase activity by 5-fold, diminishing it to the same levels found in OVCA429 and 433.

The variation in cdk2 H1 kinase activity could be due to differences in the levels of the cdk2 protein in these cells. Protein levels detected by immunoblotting (Fig. 2) were relatively constant among the cell lines (treated and untreated), except for the responsive OVCA420 cells. OVCA429 and 433 containing high CIP1 levels and low H1 kinase have the same amount of cdk2 as OVCA432 (low CIP1 and high kinase activity), suggesting that the CIP1 in these lines is acting to repress cdk2 activity. The responsive OVCA420 cells, however, show a dramatic reduction in cdk2 levels after TGFβ1 treatment concomitant with elevated CIP1 and reduced H1 kinase activity. Therefore, reduction in kinase activity may be multifactorial and due either to induction of CIP1 or reduction in cdk2 protein or, more likely, a combination of both phenomena.

One of the key substrates of the cdkks is the Rb protein. The level of Rb phosphorylation can be visualized by its electrophoretic mobility, with the slower migrating forms being highly phosphorylated. Therefore, as an alternative measure of CIP1 activity, the Rb protein was detected by immunoblotting in TGFβ1-treated and untreated cultures (Fig. 3). Of the four cell lines, only OVCA420 demonstrated a significant shift of Rb into less phosphorylated forms after TGFβ1 treatment, consistent with induction of CIP1 and growth inhibition. There was no correlation between Rb phosphorylation and CIP1 levels in the other three cell lines. OVCA432 and 433 expressing high constitutive levels of CIP1 and OVCA432 with very low levels of CIP1 all have predominantly hyperphosphorylated Rb protein, regardless of TGFβ1 treatment.

TGFβ1 Induction of CIP1 Is Not Accompanied by Changes in p53 Activity. Several reports have demonstrated abrogation of TGFβ1 response by the introduction into cells of mutant p53 (31, 32). Since the CIP1 promoter contains a p53 response element, it was reasonable to propose that induction of CIP1 by TGFβ1 in OVCA420 resulted from an increase in p53 activity. Therefore, the effect of TGFβ1 on p53 in the responsive versus nonresponsive cell lines was examined by measuring a variety of parameters.

Regulation of p53 activity potentially can occur by a number of different processes, including changes in RNA and protein levels, phosphorylation state of the protein, and subcellular location of the p53 protein. Northern analysis of total RNA revealed no differences in the levels of p53 mRNA with TGFβ1 treatment (Fig. 1). However, regulation of p53 is more frequently a posttranscriptional event. Therefore, p53 immunoblots were performed on total protein extracted from treated and control cells (Fig. 4A). Only OVCA432 contained high steady-state levels of p53 protein due to the missense mutation at codon 277. Faint signals indicative of wild-type p53 expression were detected in the other three cell lines. Levels of p53 remained constant in all of the cell lines following TGFβ1 treatment. While the immunoblot measured steady-state levels of p53 protein, an increased rate of synthesis may not have been detected. Immunoprecipitation of metabolically labeled p53 protein (pulse labeled for 2 h) during TGFβ1 treatment also showed no difference in any of the cell lines (Fig. 4B). Therefore, induction of CIP1 in OVCA420 did not appear to be related to an increase in the expression of p53 protein. In addition, levels of the wild-type p53 protein did not correlate with CIP1 transcription. The OVCA420 cell line contained the highest level of the wild-type protein but had the lowest level of CIP1, excluding the p53 mutant line, OVCA432.

Phosphorylation of the p53 protein occurs on several residues and by several different protein kinases, including cdkks (36–37). However, it remains unclear whether the transcriptional activity of p53 is dependent upon the phosphorylated state of the p53 protein (39). We examined phosphorylation of the p53 protein in these ovarian cancer cell lines after TGFβ1 treatment. Cells were metabolically labeled with [32P]orthophosphate, and total cellular protein was extracted. Immunoprecipitation with a p53 monoclonal antibody again revealed no difference in treated versus untreated cells in the total levels of p53 phosphoprotein (Fig. 4C). The relative level of p53 phosphorylation tracks the steady-state levels of protein observed by immunoblotting.

The activity of p53 as a transcription factor is certainly dependent upon nuclear translocation (40). In fibroblasts
and epithelial cells, nuclear transport is regulated with the cell cycle, with the highest nuclear accumulation occurring in late G1 and S phase (41, 42). Increased transport could account for induction of p53 protein without changing the overall level of the protein. However, immunostaining failed to show any difference in nuclear accumulation of the p53 protein between responsive and nonresponsive cells after TGFβ1 treatment (data not shown).

Finally, to test directly whether TGFβ1 increases p53 transcriptional activity in the responsive OVCA420 cell line, a functional assay was used. OVCA420 cells were transfected with a consensus p53 response element upstream of the CAT gene. After transfection, the cells were treated with TGFβ1 for 40 h, and CAT activity was measured (Fig. 5). No induction of transcription over basal levels was detected. Cotransfection of a wild-type p53 expression vector into these cells was able to cause a dramatic increase of reporter gene transcription, demonstrating the fidelity of the system. Addition of TGFβ1 to the cotransfected cells failed to increase CAT expression beyond the level achieved with wild-type p53 alone. Similar results were obtained with the nonresponsive OVCA433 cell line (data not shown). Therefore, TGFβ1 induced no detectable changes in the synthesis or activity of p53 in these cell lines.

**Discussion**

TGFβ1 can inhibit the growth of normal ovarian epithelial cells and primary cultures of ovarian cancers derived from ascitic fluid (43). However, most immortalized cell lines derived from epithelial ovarian cancers are resistant to its effects (35). Regardless of whether TGFβ1 is a normal physiological modulator of the growth of these cells, it provides a useful probe for investigating cell cycle regulatory pathways that may be aberrant. An increasing number of regulatory molecules in this pathway are being discovered, among which are the inhibitors of cdks. In the current study, we show that expression of CIP1/WAF1, a p53-inducible cdk inhibitor, can be induced by TGFβ1, and this induction is associated with growth inhibition. Of the four ovarian cancer cell lines in this study, only one showed CIP1 induction. This was also the only cell line that demonstrated significant growth inhibition and apoptosis in response to TGFβ1. Overexpression of CIP1 in other cells can induce growth arrest, probably by inhibiting Rb phosphorylation (9). Therefore, expression of CIP1 may represent an important mediator of TGFβ1 activity in these cells.
Since CIP1 appears to be a major target for p53 transcriptional activation, it may also be a tumor suppressor gene that is inactivated in cancers where p53 retains its function. The high constitutive levels of CIP1 found in OVCA429 and 433, equivalent to those found in OVCA420 after TGFβ1 growth arrest, suggested to us that the CIP1 in these cells may be inactive. However, measurements of cdk protein and its associated kinase activity in these cells indicate that the high levels of CIP1 do correlate with greatly reduced cdk activity. Therefore, CIP1 appears to retain its function. These results suggest that certain ovarian cancer cell lines with high levels of this inhibitor can still proliferate, perhaps via overexpression of other components of the cyclin/cdk complexes.

The CIP1 gene is highly responsive to transactivation by wild-type p53 and was identified by El-Deiry et al. (9) on this basis. This suggested to us that p53 may be an intermediary in the TGFβ1 induction of CIP1. Moreover, TGFβ1 growth inhibition of bronchial epithelial cells and keratinoocytes can be reversed by the introduction of mutant p53 (31, 32). Mutant p53 can have a trans-dominant effect on wild-type p53 activity by complexing and inactivating the wild-type protein (39, 44) so that reversal of TGFβ1 responsiveness by mutant p53 may be accomplished by preventing transactivation of CIP1. Therefore, we investigated the relationship between p53 status and CIP1 expression in the ovarian cancer cell lines.

The presence of wild-type p53 protein does correlate with expression of CIP1. In these ovarian cancer cell lines and in breast cancer cell lines, expression of mutant p53 is associated with low levels of CIP1 mRNA and protein. However, the amount of wild-type p53 does not appear to be an important determinant in CIP1 expression. OVCA429 and 433 contain high constitutive levels of CIP1 yet have lower steady-state levels of p53 protein than OVCA420, which contains relatively low levels of CIP1. Clearly, other transcription factors must also regulate expression of this gene, and detailed analysis of the CIP1 promoter region will help to elucidate the nature of these factors.

Expression of wild-type p53 was insufficient to confer TGFβ1 sensitivity since, of the three ovarian cancer cell lines expressing the wild-type protein, only one exhibited significant growth inhibition and induced expression of CIP1 (OVCA420). This induction did not exceed the steady-state level of CIP1 found in the other two p53 wild-type cell lines (OVCA429 and 433). These observations suggest that the cell lines containing high constitutive levels of CIP1 have become resistant to the effects of this growth inhibitor, perhaps by overexpression of specific cyclins or cdk's. Alternatively, the CIP1 protein itself may be inactivated. To date, no loss of function mutations in the CIP1 gene have been reported but, as a p53-responsive gene, it is a logical candidate for a tumor suppressor.

Evidence for the role of p53 in the induction of CIP1 was sought. We examined a number of p53-related parameters in responsive and nonresponsive cell lines after treatment with TGFβ1. There was no increase in p53 RNA after treatment with TGFβ1, nor were there any differences in steady-state or de novo synthetic levels, phosphorylation, or nuclear localization of the p53 protein after treatment. Changes in the phosphorylation of specific amino acids or alterations in p53 protein complexes would not have been detected in these experiments. However, a functional assay for p53 in the OVCA420 cell line confirmed that there was no increase in p53-mediated transcriptional activation after addition of TGFβ1. Expression of transfected wild-type p53 in these cells did increase transcription from a p53 response element, indicating that additional p53 activity was effective in promoting transcription.

In these cell lines, TGFβ1 response correlates with induction of CIP1 and reduced cdk2 protein and kinase activity. These are accompanied by a decrease in Rb phosphorylation. It is of interest that two other mechanisms for TGFβ1 inhibition of Rb phosphorylation have also been described, both involving other members of the cyclin-cdk complexes. In mink lung epithelial cells, a cell line that is highly responsive to TGFβ1 growth arrest, the synthesis and activity of cdk4 was dramatically inhibited by TGFβ1 (45). In a separate study of this same cell line, TGFβ1 growth arrest was correlated with an increase in the available levels of a new cdk inhibitor called KIP1, a M, 27,000 protein that binds to and inactivates cyclin-E-cdk2 complexes (12). Our results indicate that CIP1 induction is another potential mechanism for TGFβ1-mediated growth arrest. cdk2 levels are also reduced, contrary to TGFβ1-treated mink cells in which cdk2 protein levels were not affected (27). Each mechanism appears independently capable of retarding growth. It will be a challenge to sort out the cause and effect relationships between growth arrest and the levels and activity of these important regulators of the cell cycle.

Materials and Methods

Cell Culture. Ovarian cancer cell lines OVCA420, 429, 433, and 433 were established and maintained in modified Eagle’s medium with 10% heat-inactivated fetal bovine serum as described previously (35). Subconfluent cultures of cells were treated with 10 ng/ml of authentic porcine TGFβ1 (R&D Systems, Minneapolis, MN) for 24 h.

RNA Extraction. Whole-cell RNA was extracted from tissue culture cells by the method of Chomczynski and Sacchi (46).

Cloning CIP1/WAF1. Forward (ACTCAGAGGAGGCCATGT) and reverse (TTCTCTGTGGCGGATTAGGG) primers were designed based upon the published cDNA sequence of CIP1/WAF1 to include the entire coding sequence (14). Whole-cell RNA from normal mammary epithelial cells was reverse transcribed using random hexamers, and CIP1/WAF1 cDNA was amplified by the polymerase chain reaction. A product of the anticipated size was obtained and cloned into the EcoRV site of BlueScript SK (Stratagene). Sequencing of the cloned product confirmed the identity of the CIP1/WAF1 cDNA.

Northern Blot. Ten μg of total RNA from each source was electrophoretically separated on a 1% formaldehyde/agarose gel and transferred to a nylon membrane as previously described (47). Membranes were hybridized overnight at 65°C with 2 x 10⁷ cpm of a CDNA probe labeled by random priming. Washing was performed at 65°C in 0.2X standard saline-citrate and 0.1% SDS.

Radioimmunoprecipitation. Cells were preincubated in methionine- or phosphate-free medium and then labeled with either 200 μCi/ml of [35S]methionine (Amersham) in methionine-free medium or 300 μCi/ml of [32P]orthophosphate (ICN) in phosphate-free medium for 2 h. Cells were harvested and lysed, and protein extracts were

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* J. R. Marks, unpublished data.
prepared as described previously (48). After pre-clearing with protein G-Sepharose (Pharmacia), equal numbers of trichloroacetic acid-precipitable counts were reacted with the mouse monoclonal antibody pAB1801 (AB-2; Oncogene Science) to detect the p53 protein. Protein G-Sepharose precipitation was followed by SDS-PAGE. The gel was fixed, dried, and exposed to X-ray film.

**Immunoblotting.** Detection of p53 protein by Western blot was performed as described previously (49). Total cell protein was extracted by sonication in NP40 lysis buffer (50 mM Tris (pH 8.0), 5 mM EDTA, 150 mM NaCl, and 0.5% NP40) containing 1 mM phenylmethylsulfonyl fluoride. Cell extracts were quantitated for protein content, and equal amounts of protein were first immunoprecipitated with a human serum containing a high titer of anti-p53 antibodies. After electrophoresis by SDS-PAGE and transfer to a solid support, the blot was probed with pAB1801 and binding was visualized by Vectastain Elite reagents (Vector Labs) and diaminobenzidine. To detect the CIP1 protein, 200 μg of total protein was electrophoresed on a 12.5% gel and then transferred to a solid support. The filter was blocked with 5% bovine serum albumin in TBS (0.15% Tween 20/Tris-buffered saline). The primary mouse monoclonal antibody against CIP1 (Oncogene Science) was used at 1 μg/ml in 2% bovine serum albumin/TBBS. Antibody binding was visualized by first reacting a biotinylated goat-anti-mouse secondary antibody followed by a streptavidin-horseradish peroxidase complex (Vector Labs). For Cdk2 and retinoblastoma protein detection, filters were blocked with phosphate-buffered saline containing 5% nonfat dry milk. The primary antibody against Cdk2 (rabbit polyclonal from Upstate Biotechnology, Inc.) was used at 2 μg/ml. The primary antibody against Rb was the mouse monoclonal antibody, G3–245 (PharMingen), used at 2 μg/ml. The secondary antibody was goat anti-rabbit or anti-mouse directly conjugated to horseradish peroxidase, used at 1:4000 dilution (Amersham). Chemiluminescent detection was accomplished using ECL reagents (Amersham) according to the manufacturer's instructions.

**Histone Kinase Assay.** Protein from whole cells was prepared and quantitated as described above. Equal amounts of protein (500 μg) were immunoprecipitated with an antibody specific for human cdk2 (Upstate Biotechnology, Inc.) and collected by binding to protein G-Sepharose. Immune complexes were washed twice in NP40 lysis buffer and three times in kinase buffer [50 mM Tris (pH 7.5), 10 mM MgCl₂, and 1 mM dithiothreitol]. Washed complexes were then resuspended in 50 μl of kinase buffer containing 5 μg of histone H1 (Sigma), 1 μM ATP, and 10 μCi of [α-32P]ATP (7000 Ci/mmol; ICN) and incubated for 30 min at 30°C. The reaction was stopped by the addition of SDS sample buffer and then electrophoresed on a 7.5% SDS-PAGE. The dried gel was exposed to X-ray film, and specific bands were quantitated on a phosphoimager (Molecular Dynamics).

**CAT Assay.** Cells were transfected with CsCl-purified plasmids using a CaPO₄ method as described previously (50). A reporter plasmid containing ten copies of a consensus p53 response element (GATCCCTGTCCCTGACCTGCCTGG in front of the CAT gene) was used to measure p53-dependent transcription (from G. Lozano, University of Texas). A human wild-type p53 cDNA expression vector was driven by the cytomegalovirus immediate-early promoter. Cotransfections were done at a ratio of 5:1 effector (p53 expression vector) to target (p53 response element). Four h after transfection, fresh media was added with or without 10 ng/ml TGFβ1 and incubated for an additional 40 h. Measurements of CAT activity were performed as described (50).

**Acknowledgments**

We thank Regina Whitaker, Gudrun Huper, and Nancy Glover for their excellent technical assistance.

**References**


