p53, through p21 (WAF1/CIP1), Induces Cyclin D1 Synthesis

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Abstract

Cells induced to accumulate the p53 tumor suppressor protein have been shown to arrest in G1. This arrest is characterized by accumulation of the cyclin-dependent kinase inhibitor p21 (WAF1/CIP1) and of underphosphorylated forms of retinoblastoma protein. We show here that accumulation of the wild-type p53 protein in either human or murine cells markedly increases expression of cyclin D1. The induction of cyclin D1 can also be mediated by a target of p53, the p21 (WAF1/CIP1) inhibitor of cyclin-dependent kinases. The relationship between the induction of cyclin D1 and G1 arrest defines a new cellular response to p53.

Introduction

In response to both intrinsic and environmental DNA damage agents, the steady-state level of the p53 protein is posttranscriptionally elevated (1, 2). It is well established that accumulation of the wild-type p53 protein results in two pathways, cell cycle arrest and programmed cell death or apoptosis, which together carry out the p53 tumor suppressor function (3, 4). What pathway the DNA damaged cells undergo depends on both the extracellular signals, the threshold of different cell types for apoptotic inducers, and expression of other cellular and viral proteins (5). Mutation of p53, which may be the most common event in human cancers, leads to disruption of these pathways, resulting in a selective growth advantage of tumor cells, generally observed as tumor progression.

It is now fairly well established that p53-induced cell growth arrest is due to the ability of p53 as a transcriptional activator to regulate one or more cell cycle checkpoint-related genes. Among the genes that were shown to be induced by p53 in cells are mdm-2 (6), GADD45 (2), and p21 (WAF1/CIP1) (7). Of these three, the p21 gene is the most likely to directly regulate the cell cycle. It has been shown that its product was found to be a potent CDK inhibitor (8, 9), as well as to inhibit DNA replication (10) but not PCNA-dependent DNA repair (11) through its physical interaction with PCNA. Therefore, p21 can disrupt the normal progression of DNA-damaged eukaryotic cells through the cell cycle. These findings provide a direct link between p53 tumor suppressor protein and cell cycle control.

Accumulation of the wild-type p53 protein primarily arrests cells at G1-S (3, 4). Cell cycle transition from G1 to S phases requires sequential events involving the formation, activation, and subsequent inactivation of a series of cyclins/CDK complexes. A critical target of G1 CDKs is the pRB protein whose normal function of repressing transcription factors, including members of the E2F family, is inhibited by CDK phosphorylation (12). Inhibition of CDK4 synthesis by transforming growth factor ß is linked to cell cycle arrest (13).

As part of an effort to identify important cell cycle-related genes that are potentially regulated by p53, we have found that cyclin D1 expression is induced by the wild-type p53 protein. This induction is at least partially mediated by p21.

Materials and Methods

Cells, Antibodies, and Plasmid Construction. Saos-2, WI-38, and T98G cells were purchased from the American Type Culture Collection. RKO cells were obtained from M. B. Kastan (The Johns Hopkins University, Baltimore, MD; Ref. 2). 10(1) cells were obtained from A. J. Levine (Princeton University, Princeton, NJ; Ref. 16). 3-4 cells were generated by cotransfection of 10(1) cells with the activated H-ras oncogene and the temperature-sensitive murine p53 tumor suppressor protein (13). GM47-23 and Del4A cells were generously provided by W. E. Mercer (Thomas Jefferson University, Philadelphia, PA; Ref. 17). All cells were grown in DMEM supplemented with 10% fetal bovine serum in a 37°C incubator with 5% CO2. Transfection was performed by the calcium phosphate method as described (18). Camptothecin was purchased from Sigma Chemical Co. (St. Louis, MO). Affinity-purified mAbs against PCNA and cyclin B1 and D1 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Affinity-purified polyclonal antibody against cyclin D1 was purchased from Upstate Biotechnology, Inc. (cyclin D1; UBI, Lake Placid, NY). Rabbit polyclonal antiserum against CDK4, CDK2, and cdc2 were kindly provided by Z-Q. Pan (Mount Sinai Medical Center, New York, NY; Ref. 19). Anti-cyclin A (C166; Ref. 20) and anti-pRB (XZ-77; Ref. 21) were mouse mAbs. Affinity-purified anti-actin antibody was purchased from Sigma. Pab 419 is a mAb against SV40 large T antigen (22). PAb1801 and PAb122 are mAbs against p53 (23, 24). To generate anti-p21 antibody, a 900-bp Stul-EcoRl cDNA fragment encoding amino acids 17 to 164 of p21 polypeptide (7) was inserted in-frame into pRSET expression vector (Invitrogen). The His-tagged p21 protein was then produced in bacteria and purified from Ni-agarose beads, and anti-p21 antibody was raised in a rabbit. pcDNA3-p21 was generated by inserting a 1.0-kb EcoRl-EcoRl fragment of p21 cDNA (7) into pcDNA3 (Invitrogen).

Immunoprecipitation and Immunoblot Analysis. For immunoprecipitation analysis, cells were grown in DMEM (methionine minus) media plus 10% fetal bovine serum for 1 h and labeled with 75 μCi/ml of Tran35S-label methionine (ICN Pharmaceuticals) for 1 h. Whole-cell extracts were prepared by lysing cells with NP40 lysis buffer [150 mM NaCl, 50 mM Tris (pH 7.5), 1 mM DTT, 0.5% NP40, 25 mg/ml aprotinin, 25 ng/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride] for 15 min on ice. The extracts were precleared with 10 μl of normal rabbit serum plus 100 μl of 10% Staphylococcus aureus cells (The Enzyme Center, Malden, MA) for 1 h at 4°C and were clarified by centrifugation. Immunoprecipitations were performed by the addition of various antibodies as indicated and 40 μl of 50% protein A-Sepharose (Pharmacia). The immunoprecipitates were washed three times with NP40 lysis buffer prior to the addition of 2X sample buffer and heat at 100°C for 5 min. Polypeptides were resolved by SDS-PAGE and fluorography.

For immunoblot analysis, cells were lysed with the NP40 lysis buffer, then mixed with 2X sample buffer, and boiled for 5 min. Following electrophoresis, proteins were transferred to a nitrocellulose filter. Blots were blocked by incubation with PBS containing 0.1% Tween 20 (PBS-T) and 2% nonfat milk for 30 min at room temperature. The filter was incubated with antibody as indicated in PBS-T, then washed three times with PBS-T, and incubated with a 1:2000 dilution of either anti-mouse or anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Organon Teknika Corp., Durham, NC) for...

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3 The abbreviations used are: CDK, cyclin-dependent kinase; PCNA, proliferating cell nuclear antigen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
RNA isolation and Northern blot analysis. Poly(A)+ RNA was isolated by using the QuickPrep mRNA purification kit (Pharmacia) as described. Northern blot analysis was performed as described (25). The cyclin D1 probe was made from a 1.0-kb Ncol-Hindlll cDNA fragment (26), the GAPDH probe was made from a 1.25-kb Pstl-Pstl cDNA fragment (27), and the p21 probe was made from a 1.0-kb EcoRI-EcoRI cDNA fragment (7).

Results

Accumulation of the Wild-type p53 Protein Leads to Increased Cyclin D1 Expression. The mammalian cell cycle is controlled by a number of cyclins and CDKs, each of which plays a role at a defined stage of the cell cycle. Since p53 regulates the cell cycle by inducing G1 arrest (and in some cases also G2 arrest), we were interested in examining whether p53 might regulate the synthesis or levels of the cyclins and CDKs. To test this, we used a cell line that expresses inducible p53. The glioblastoma cell line T98G, which was the parental cell line used to establish the p53-inducible cell line GM47-23, contains an endogenous mutant p53 (met237 to ile; Ref. 28). GM47-23 cells contain the same endogenous mutant p53 gene and an exogenously introduced wild-type p53 gene under the control of a steroid-responsive promoter (17). The related line, Del4A, contains the same endogenous mutant p53 and an additional dexamethasone-inducible deletion mutant p53 gene (29). In the presence of dexamethasone, a moderate amount of inducible p53 was expressed (Fig. 1, compare Lanes 4 and 6 with Lanes 3 and 5, respectively) as reported previously (17). We confirmed that the inducible wild-type p53 protein adopts a unique conformational state in vivo that retains the p53-specific antibody PAb1801 epitope but very little or no PAb421 and PAb122 epitopes (Ref. 28; Fig. 1, compare Lanes 4 and 6). This unique conformation and phosphorylation state presumably enables the inducible wild-type p53 protein to escape from the dominant-negative effect of the endogenous mutant p53. To ascertain that the inducible wild-type p53 protein can transactivate its target gene in the GM47-23 cell line, expression of p21, a known p53-responsive gene (7), was determined by immunoprecipitation. The p21 protein was detected only after dexamethasone treatment (Fig. 1, compare Lanes 7 and 8).

We then examined the effect of p53 on various cyclins and CDKs for which antibodies were available. When the steady-state levels of these cell cycle-related proteins were determined by immunoprecipitation and Western blot analyses, it was clear that the wild-type p53 protein either inhibited expression of CDK4, CDK2, cdc2, or cyclins A and B1, or had no significant effect on PCNA (data not shown). However, unexpectedly, we observed that p53 strongly stimulated the induction of cyclin D1 (Fig. 2A, Lanes 1 and 2). Indeed, there was also a substantial amount of cyclin D1 detected in the p21 immunoprecipitate after induction of p53 (Fig. 2A, Lanes 3 and 4). To confirm the cyclin D1 response to wild-type p53, we also examined the effects of dexamethasone on T98G and Del4A cells. Both in T98G cells which express only endogenous mutant p53 protein (Fig. 2B, Lanes 1 and 2), and in Del4A cells which express endogenous mutant p53 and inducible deletion mutant p53 (Fig. 2C, Lanes 1 and 2), p21 was not induced in the presence of dexamethasone treatment (Fig. 2, B and C, compare Lanes 3 and 4). Similarly, the amount of cyclin D1 protein was unchanged or even slightly decreased in the presence compared with that in the absence of dexamethasone treatment (Fig. 2, B and C, compare Lanes 5 and 6).

To further analyze the kinetics of cyclin D1 induction, GM47-23 cells were [35S]methionine-labeled at 0, 4, 8, 12, 24, and 48 h after treatment with dexamethasone, and the amount of cyclin D1 protein was determined by either immunoprecipitation using a specific anti-cyclin D1 antibody or coimmunoprecipitation using a specific antibody directed against p21. The amount of cyclin D1 protein was markedly increased over the time course examined, peaking at 24 h (Fig. 3A, compare Lanes 1 and 7 with Lanes 2-6 and 8-12, respectively). A similar kinetic pattern was observed for p21 expression (Fig. 3A, compare Lane 1 with Lanes 2-6). Induction of wild-type p53 in GM47-23 cells treated with dexamethasone was determined over a similar time course by immunoblot analysis (Fig. 3B). Inducible wild-type p53 was detected between 8 to 12 h following treatment, consistent with the timing of both cyclin D1 and p21 induction.

We also asked whether the induction of cyclin D1 expression by p53 is evolutionarily conserved by comparing human and rodent cell lines. A mouse cell line (3-4) was generated by cotransfection of mouse p53-null embryo fibroblasts (10-1) with the temperature-sensitive mutant murine (ala 135 to val) p53 and ras oncogene. At 37°C, the murine (val135) p53 is in mutant conformation and the cells grow, while at 32°C, it is in wild-type conformation and the cells are arrested (Ref. 30; data not shown). Significantly more cyclin D1 protein was detected at 32°C than at 37°C (Fig. 3C). No increase in cyclin D1 was observed at 32°C in 10-1 cells (the parental cell line from which 3-4 cells were derived; Fig. 3D). Thus, cyclin D1 induction by p53 occurs in both human and rodent cells.

A DNA-damaging Agent Causes Increased Levels of p53, p21, and Cyclin D1. To further characterize the role of cyclin D1 in p53-dependent cell growth control, we asked whether cyclin D1 is a part of the p53-dependent DNA damage response pathway. RKO cells contain wild-type p53 that arrest in G1 following γ-irradiation (2). Camptothecin, a topoisomerase inhibitor which induces strand breaks, has been shown to induce p53 in RKO cells (31). After treatment with camptothecin (300 nm) for 24 h, cells were [35S]labeled, and the amounts of the p53, p21, and cyclin D1 proteins were determined by immunoprecipitation. Fig. 4A shows that DNA damage resulted in a 5- to 10-fold increase of the wild-type p53 protein. An increase of wild-type p53 in the DNA-damaged cells was commensurate with induced p21 and cyclin D1 expression (Fig. 4B, compare Lanes 1 and 3 with Lanes 2 and 4, respectively). These observations are consistent with the results obtained above (Figs. 2 and 3) from GM47-23 cells in that accumulation of wild-type p53 increased both p21 and cyclin D1 synthesis.
p53-dependent induction of cyclin D1 synthesis. A, GM47-23 cells treated with dexamethasone for 0 or 24 h were [35S]methionine labeled. The labeled cell extracts were subject to immunoprecipitation with antibodies against cyclin D1 (Lanes 1 and 2) and p21 (Lanes 3 and 4). T98G cells (B) or Del4A cells (C) treated with dexamethasone for 0 or 24 h were [35S]methionine labeled. The labeled cell extracts were subject to immunoprecipitation with antibodies against p53 (PAb1801; Lanes 1 and 2), p21 (Lanes 3 and 4), and cyclin D1 (Lanes 5 and 6). Right, cyclin D1, p21, and inducible and endogenous mutant p53 proteins.

p53-dependent Induction of Cyclin D1 Is at the Transcriptional Level. To determine whether accumulation of the wild-type p53 protein stabilizes the cyclin D1 protein, the half-life of the cyclin D1 protein in GM47-23 cells and the normal human cell line WI-38 was determined. From the results obtained, it was clear that the cyclin D1 protein has the same half-life (approximately 15–30 min) both in GM47–23 cells, either in the presence or in the absence of the wild-type p53 protein, and also in another human cell line (WI-38 cells; data not shown). Thus, induction of cyclin D1 by p53 is not due to protein stabilization.

To analyze whether induction of cyclin D1 expression is at the transcriptional level, cyclin D1 mRNA was quantitated by Northern
Fig. 3. Kinetics of p53-dependent cyclin D1 induction. A, GM47-23 cells treated with dexamethasone for 0–48 h as indicated at the top of each lane were [35S]methionine labeled. The labeled cell extracts were subject to immunoprecipitation with antibodies against p21 (Lanes 1–6) and cyclin D1 (Lanes 7–12). B, an immunoblot was prepared using extracts from GM47-23 cells treated with dexamethasone for 0–36 h as indicated at the top of each lane. The blot was probed with anti-p53 mAb PAb1801. Right, inducible and endogenous p53 proteins. Mouse 3-4 cells (C) and 10(1) cells (D) grown at 32°C for 0–48 h and 0–24 h, respectively, as indicated at the top of each lane, were [35S]methionine labeled. The labeled cell extracts were subject to immunoprecipitation with anti-cyclin D1 antibody.

Since p53 induction of cyclin D1 synthesis is apparently transcriptionally regulated, we asked whether the cyclin D1 promoter contains a p53-responsive element. Our experiments showed that a 104-bp fragment (from −1236 to −1133 upstream of the first methionine; Ref. 40) contains a potential weak p53 binding site. The efficiency of p53 binding to this fragment was approximately 10–20% of that observed with a comparable fragment containing the p53 binding site response element from the gadd45 gene (2). This binding appears to be specific as determined by both competition gel-shift assay, in which it was shown that a wild-type p53 DNA binding oligo (RGC) but not mutant oligo (mRGC) competed away p53 binding to the cyclin D1 promoter DNA fragment, and by DNaseI protection. However, when a 1.3-kb cyclin D1 promoter DNA fragment containing the p53 site was cloned upstream of a promoterless luciferase gene (CYCD1-LUC) and tested for transcriptional activity in dexamethasone-treated and untreated GM47–23 cells, we observed that this promoter-reporter construct was induced only very slightly (2–4-fold) when compared to a positive control reporter with a 100-bp fragment containing the p53 DNA binding site from the GADD45 gene, whose expression was stimulated by approximately 30-fold under the same conditions.
Fig. 4. p53-dependent cyclin D1 induction following DNA damage. 35S-labeled extracts from RKO cells untreated or treated with camptothecin were subjected to immunoprecipitation with anti-p53 antibody PAbl801 (A), anti-cyclin D1 (B; Lanes 1 and 2) and anti-p21 (B; Lanes 3 and 4) as indicated at the top of each lane. Right, p53, cyclin D1, and p21 proteins.

Conditions. It remains possible that p53 can directly, albeit weakly, activate expression of the cyclin D1 promoter. However, an alternative explanation is that cyclin D1 induction by p53 may be mediated by one or more p53 responsive genes.

p21 Expression Induces Cyclin D1. Since the p21 gene, itself a target of p53, is involved in cell cycle regulation, we asked whether p21 can mediate the p53-dependent induction of cyclin D1 expression. To this end, the human p21 cDNA was cloned downstream of a cytomegalovirus immediate early gene promoter producing a p21 expression vector (pcDNA3-p21). Following transient transfection with pcDNA3-p21, T98G cells were 35S-labeled, and the amounts of the p21 and cyclin D1 proteins were determined by immunoprecipitation. As expected, with increasing amounts of transfected pcDNA3-p21 DNA, p21 protein was detected in a dose-dependent manner (Fig. 6, Lanes 6 and 7), although the highest amount of transfected DNA reduced somewhat the transfection efficiency (Fig. 6, Lanes 7 and 8). This increase in p21 was commensurate with a significant increase in the amount of the cyclin D1 protein in the transfected cells (Fig. 6, compare Lane 1 with Lanes 2–4). Furthermore, there were markedly greater quantities of cyclin D1 in the p21 immunoprecipitates (Fig. 6, compare Lane 5 with Lanes 6–8). The fact that the cyclin D1 stimulation by p21 was relatively modest compared to what was observed with GM47-23 or 3-4 cells is most likely due to the fact that the efficiency of transient transfection is relatively low (around 5% of total cells). Therefore, it is probable that the induction of cyclin D1 was significantly greater than what we detected. We conclude that p21 mediates the induction of cyclin D1 expression by p53.

Discussion

We have provided evidence that accumulation of the wild-type p53 protein leads to induction of cyclin D1 expression. This induction is
cells in 10-cm dishes were transiently transfected with 0.5 μg (Lanes 1 and 5), 5 μg (Lanes 2 and 6), 15 μg (Lanes 3 and 7), 30 μg (Lanes 4 and 8) of pcDNA3-p21 DNA and the gene (7), p21 was found to be a potent cyclin/CDK inhibitor (8, 9).

Although it has been shown to physically interact with PCNA, p21 mediates at least in part by the p21 gene product. As a p53-regulated gene (7), p21 was found to be a potent cyclin/CDK inhibitor (8, 9). It has been reported that transient overexpression of cyclin D1 in fibroblasts arrests cells in G1. Furthermore, Dulic et al. (38) and Lucibello et al. (39) have found that levels of cyclin D1 are increased in senescent cells, corresponding to the presence of nonfunctional CDKs. Thus, it appears that increased levels of cyclin D1 can be found in two opposing aspects of cell cycle control: growth promotion and growth arrest. In a p53-inducible system, the accumulation of wild-type p53 induces G1 arrest (17) and cyclin D1 synthesis. We suggest that the effect of cyclin D in cells may reflect the status and levels of p53. Indeed, cyclin D1 may be a mediator of p53 growth suppression, consistent with one of the two contrasting cyclin D1 functions in cell cycle control.

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References


Cyclin D1 Is a Component of G1-Checkpoint and a Potential Mediator of p53 Tumor Suppression. As a G1 cyclin, cyclin D1 was originally identified by very different approaches [reviewed by Sherr (14)]: as a suppressor of yeast G1 cyclin mutations, as a delayed early response gene induced by colony-stimulating factor 1, and as a putative pro-oncogene BCLI/PRAI1. It has been reported that overexpression of cyclin D1 promotes cell progression and differentiation, generally observed as shortened G1-S transition and oncogenesis (34). In addition, both activated ras and myc oncoproteins induce cyclin D1 expression (35, 36), suggesting that cyclin D1 is one of the mediators of oncogenic transformation. However, in some cases, increased levels of cyclin D1 have been a characteristic of arrested cells. Pagano et al. (37) showed that transient overexpression of cyclin D1 in fibroblasts arrests cells in G1. Furthermore, Dulic et al. (38) and Lucibello et al. (39) have found that levels of cyclin D1 are increased in senescent cells, corresponding to the presence of nonfunctional CDKs. Thus, it appears that increased levels of cyclin D1 can be found in two opposing aspects of cell cycle control: growth promotion and growth arrest. In a p53-inducible system, the accumulation of wild-type p53 induces G1 arrest (17) and cyclin D1 synthesis. We suggest that the effect of cyclin D in cells may reflect the status and levels of p53. Indeed, cyclin D1 may be a mediator of p53 growth suppression, consistent with one of the two contrasting cyclin D1 functions in cell cycle control.

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