GRANT NO: DAMD17-94-J-4142

TITLE: Functions of Wild-type and Mutant Forms of p53 in Breast Cancer

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REPORT DATE: August 17, 1995

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

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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 (CDK) inhibitor p21 (WAF1/CIP1) and of underphosphorylated forms of retinoblastoma protein (pRB). 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.
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Introduction

In response to both intrinsic and environmental DNA damage agents, the steady-state level of the p53 protein is post-transcriptionally elevated (Maltzman and Czyzky 1984; Kastan et al. 1992; Perry et al. 1993). It has been 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 (see reviews by Prives 1993; Levine 1993). 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 (see review by Fisher 1994). Therefore, 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 which were shown to be induced by p53 in cells are mdm-2, GADD45 and p21 (WAF1/CIP1). Of these three, the p21 gene is the most likely to directly regulate the cell cycle. p53-regulated p21/WAF1 (El-Deiry et al. 1993) was found to be a potent cyclin dependent kinase (CDK) inhibitor (Harper et al. 1993; Xiong et al. 1993b; Gu et al. 1993), and also to inhibit DNA replication (Waga et al, 1994) but not PCNA-dependent DNA repair (Li et al, 1994) 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 (Lin et al. 1992a; Michalovitch et al. 1990; Martinez et al. 1991; Bischoff et al. 1992; Kuerbitz et al. 1992; Nigro et al. 1992). Cell cycle transition from G1 to S phases requires sequential events involving the formation, activation, and subsequent inactivation of a series of cyclins/CDKs complexes. Inhibition of CDK4 synthesis by transforming growth factor β (TGF-β1) is linked to cell cycle arrest (Ewen et al. 1993). Additionally, regulation of the synthesis and activity of the other cyclins and the activity of cyclin/CDK complexes has been shown to play a major role in cell cycle control (see reviews by Sherr, 1993; Hunter and Pines 1994).

As part of an effort to identify important cell cycle-related genes that are potentially regulated by p53, we used immunoprecipitation analysis and examined the effect of p53 on various cyclins and CDKs for which antibodies were available. While we have been in the process of developing breast cell lines expressing inducible p53 (both wild-type and mutant), initial experiments were carried out using cell lines to which we have access: human glioblastoma GM 47-23 (expressing inducible wild-type p53), Del 4A (expressing deletion mutant p53) and murine 3-4 (expressing temperature-sensitive 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.
Accumulation of the wild-type p53 protein leads to increased cyclin D1 expression. Several studies have provided evidence that cyclin D1 is a key regulator of the G1 phase of the cell cycle (see review by Sherr 1993). We therefore considered the possibility that cyclin D1 may be regulated by p53. A p53-inducible system was used to analyze effects of the wild-type p53 protein on cyclin D1 expression. 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) (Ullrich et al., 1992). 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 (Mercer et al., 1990a). The related line, Del4A, contains the same endogenous mutant p53 and an additional dexamethasone-inducible deletion mutant p53 gene (Mercer et al. 1990b; Lin et al., 1992a). 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 previously reported (Mercer et al., 1990a). We confirmed that the inducible wild-type p53 protein adopts a unique conformational state in vivo which retains the p53-specific antibody PAb1801 epitope but very little or no PAb421 and PAb122 epitopes (Fig. 1, compare lanes 4 and 6; Ullrich et al., 1992). 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 (Milner and Mecalf 1991). 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 (El-Deiry et al. 1993), was determined by immunoprecipitation. The p21 protein was detected only after dexamethasone treatment (Fig. 1, compare lanes 7 and 8). Note that there was a substantial amount of cyclin D1 detected in the p21 immunoprecipitate after induction.

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 1-2). Indeed, there was also a substantial amount of cyclin D1 detected in the p21 immunoprecipitate after induction of p53 (Fig. 2A, lanes 3-4). 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 1-2), and in Del4A cells which express endogenous mutant p53 and inducible deletion mutant p53 (Fig. 2C, lanes 1-2), p21 was not induced in the presence of dexamethasone treatment (Fig. 2B and 2C, 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. 2B and 2C, 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 24h (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 (Michalovitz et al. 1990; and 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 which arrest in G1 following γ-irradiation (Kastan et al. 1992). Camptothecin, a topoisomerase inhibitor which induces strand breaks, has been shown to induce p53 in RKO cells (Nelson and Kastan 1994). After treatment with camptothecin (300nM) for 24h, 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 (Fig. 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 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 were determined. From the results obtained it was clear that the cyclin D1 protein has 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 blot analysis. Poly(A)+ RNAs were isolated from GM47-23 and T98G cells in the presence or absence of dexamethasone treatment. Using a labeled human cyclin D1 cDNA as probe, it was determined that the amount of
cyclin D1 mRNA was actually slightly decreased in the first 8 h following dexamethasone treatment, but then increased significantly between 8 and 12 h and peaked at 24 h (Fig. 5, top panel, compare lane 1 with lanes 2-6). PhosphorImage quantitation of several experiments showed that there was at least an over-all four-fold increase of cyclin D1 mRNA following dexamethasone treatment after normalization with glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA levels (Fig. 5, top and bottom panel, compare lane 1 with lanes 2-6). This is consistent with the increase in protein levels observed. As a positive control, when the same blot was probed with human p21 cDNA, p21 mRNA was shown to be gradually induced and peaked sharply at 24 h (Fig. 5, middle panel, compare lane 1 with lanes 2-6). No induction of either cyclin D1 or p21 mRNA was observed in T98G cells (Fig. 5, all panels, compare lane 7 with lanes 8-11).

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 (Herber et al. 1994)] 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 (Kastan et al). 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 1.3kb 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. 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 (CMV) 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 6-7), although the highest amount of transfected DNA reduced somewhat the transfection efficiency (Fig. 6, lanes 7-8). 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.
Conclusions

We have provided evidence that accumulation of the wild-type p53 protein leads to induction of cyclin D1 expression. This induction is mediated at least in part by the p21 gene product. As a p53-regulated gene (El-Deiry et al., 1993), p21 was found to be a potent cyclin/CDK inhibitor (Harper et al., 1993; Xiong et al., 1993b; Gu et al., 1993). While it has been shown to physically interact with PCNA, CDKs and cyclin D1 (Xiong et al., 1993a; Harper et al., 1993; Waga et al., 1994; Zhang et al., 1994; and this report), p21 is unlikely to be a transcriptional activator. How does p21 mediate p53-dependent induction of cyclin D1 synthesis? Muller et al. (1994) showed that cyclin D1 expression is regulated by pRB in pRB-deficient cell lines (BT549 and C33A). It is likely that p53-induced expression of p21 leads to inhibition of cyclins/CDKs activity, resulting in underphosphorylation of pRB. The underphosphorylated pRB then activates expression of cyclin D1. Indeed, our preliminary results are that p21 fails to induce cyclin D1 synthesis in the pRB-null cell line (Saos-2) (unpublished observation).

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: as a suppressor of yeast G1 cyclin mutations (Xiong et al., 1991; Lew et al., 1991), as a delayed early response gene induced by colony-stimulating factor 1 (Matsushima et al., 1991) and as a putative pro-oncogene BCL1/PRAD1 (Lammie et al., 1991; Motokura et al., 1991; Withers et al., 1991; Rosenberg et al., 1991; Seto et al., 1992; Schuuring et al., 1992; Williams et al., 1992). It has been reported that overexpression of cyclin D1 promotes cell progression and differentiation, generally observed as shortened G1-S transition (Baldin et al., 1993; Quelle et al., 1993; Resnitzky et al., 1994; Musgrove et al., 1994) and oncogenesis (Bodrug et al., 1994; Wang et al., 1994). In addition, both activated ras and myc oncogenes induce cyclin D1 expression (Filmus et al., 1994; Dakis et al. 1994), 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. (1994) showed that transient overexpression of cyclin D1 in fibroblasts arrests cells in G1 phase. Furthermore, Dulic et al. (1993) and Lucibello et al. (1993) have found that levels of cyclin D1 are increased in senescent cells, corresponding to the presence of non-functional CDKs. Thus, it appears that increased levels of cyclin D1 can be found in two opposing aspects of cell cycle control: growth promoting and growth arresting.

Future work. In breast cancer cells, cyclin D1 was found to be amplified and overexpressed at a frequency up to at least 45% (Buckley et al. 1993; Lammie et al. 1991). While cyclin D1 induction by p53 occurs in both human and murine cells, we are currently testing whether introducing wild-type p53 into breast cell lines might increase cyclin D1 expression. The intriguing question is why and how the p53-induced cyclin D1 in the wild-type p53 containing cells and overexpressed cyclin D1 in certain tumor cells (including breast tumor cell lines) have two opposing effects: growth arresting and growth promoting. In murine cells, cyclin D1 exists in two forms that differ in their electrophoretic mobility: slow and fast migrating species (Matsushima et al. 1991). Accumulation of the slowly
migrating form correlates with the cell cycle transition from G1 to S, suggesting that this form is an active partner of CDKs. Our preliminary results show that wild-type p53 in murine cells preferentially stimulates the fast migrating form of cyclin D1, which is likely an inactive form of this cyclin. Hypothetically, the p53-induced form of cyclin D1 may antagonize the slowly migrating active form, resulting in growth arrest. In human cells our preliminary evidence suggests that human cyclin D1 also comprises two forms and wild-type p53 only stimulates one of them (the fast migrating form). Currently, we are in the process of confirming these initial observations and determining what the precise physical and functional differences are between the two species of cyclin D1.
References


Figure Legends

Fig. 1. p53-dependent transactivation of p21 in GM47-23 cells. GM47-23 cells were [35S]-methionine labeled in the presence or absence of dexamethasone (1 μM) treatment for 24 h. The 35S-labeled extracts were immunoprecipitated with a control antibody PAb419 (lanes 1-2), anti-p53 antibodies PAb1801 (lanes 3-4) and PAb122 (lanes 5-6), and anti-p21 antibody (lanes 7-8). Dexamethasone treatment is indicated by + or -, respectively, at the top of each lane. Proteins that can be identified are marked at right, and molecular weight markers are indicated at left.

Fig. 2. 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 1-2) and p21 (lanes 3-4). (B) and (C) 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 1-2), p21 (lanes 3-4), and cyclin D1 (lanes 5-6). The cyclin D1, p21, and inducible and endogenous mutant p53 proteins are indicated at right.

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 as indicated at the top of each lane. The blot was probed with anti-p53 monoclonal antibody PAb1801. The inducible and endogenous p53 proteins are indicated at right. (C) and (D) 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.

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 PAb1801 (A), anti-cyclin D1 (B; lanes 1-2) and anti-p21 (B; lanes 3-4) as indicated at the top of each lane. The p53, cyclin D1 and p21 proteins are indicated at right.

Fig. 5. p53-dependent transcriptional induction of cyclin D1. A Northern blot was prepared using 0.5 μg of poly(A)+ RNA samples isolated either from GM47-23 cells treated with dexamethasone for 0-48 (lanes 1-6) or from T98G cells treated with dexamethasone for 0-24 h (lanes 7-11) at the times indicated. The blot was probed sequentially with cyclin D1 cDNA (top panel), p21 cDNA (middle panel), and GAPDH cDNA (bottom panel). The identities of cyclin D1, p21, and GAPDH mRNAs are marked at right.

Fig. 6. p53-dependent induction of cyclin D1 is mediated by p21. Subconfluent T98G
cells in 10-cm dishes were transiently transfected with 0 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 transfected cells were 35S-methionine labeled. The labeled extracts were subject to immunoprecipitation with anti-cyclin D1 (lanes 1-4) or anti-p21 (lanes 5-8) antibodies.
Fig. 4

A.

\[ \text{ap53} \]

- +

RKO cells
CPT (24h)

- p53

1 2

B.

\[ \text{αcycD1} \quad \text{αp21} \]

- + - +

RKO cells
CPT (24h)

- cycD1

18.4 -
14.0 -

- p21

1 2 3 4
Fig. 5

GM47-23

0  4  8  12  24  48

T98G

0  4  8  12  24

DEX (hour)

- cycD1

- cycD1

- p21

- GAPDH

1  2  3  4  5  6  -  7  8  9  10  11
Fig. 6

pcDNA3-p21

αcycD1  αp21

97.4 -
68 -
43 -

- cycD1

29 -

18.4 -

- p21

1 2 3 4 5 6 7 8

[] CDKs