Analysis of Wild-Type and Mutant p21^{WAF-1} Gene Activities

JIAYUH LIN, CRISTINA REICHNER, XIANGWEI WU,[†] AND ARNOLD J. LEVINE*

Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544

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The p21^{WAF-1} gene is positively regulated by the wild-type p53 protein. p21^{WAF-1} has been shown to interact with several cyclin-dependent kinase complexes and block the activity of G₁ cyclin-dependent kinases (cdks). Mutational analysis with the p21^{WAF-1} gene localized a site, at amino acid residues 21 and 24 in the amino terminus of the protein, for p21^{WAF-1} binding to cyclins D and E. This region of the protein is conserved (residues 21 to 26) in other p21^{WAF-1} family members, p27^{kip-1} and p57^{kip-2}. The same p21^{WAF-1} 21,24 mutant also fails to bind to cyclin D₁-cdk 4 or cyclin E-cdk 2 complexes in vitro, suggesting that amino acid residues 21 and 24 are important for p21^{WAF-1}-cdk-cyclin trimeric complex interactions. The p21^{WAF-1} wild-type protein will suppress tumor cell growth in culture while p21^{WAF-1} mutant proteins with defects in residues 21 and 24 fail to suppress tumor cell growth. The overexpression of cyclin D or E in these cells will partially overcome the growth suppression of wild-type p21^{WAF-1} protein in cells. These results provide evidence that p21^{WAF-1} acts through cyclin D₁-cdk4 and cyclin E-cdk2 complexes in vivo to induce the growth suppression. The p21^{WAF-1} binding sites for cyclins (residues 21 to 26), cdk2 (residues 49 to 71), and proliferating-cell nuclear antigen (residues 124 to 164) have all been mapped to discrete sites on the protein.

The wild-type (wt) p53 protein has the ability to suppress oncogenic transformation (9, 13) and to negatively regulate cell cycle progression (1, 4). The p21^{WAF-1} gene, also known as Cip-1 (17) and Sdi-1 (32), is directly regulated at the transcriptional level by wt p53 but not by a mutant p53 protein and can itself suppress tumor cell growth in culture (8). This suggests that p21^{WAF-1} is one of the major downstream effectors of p53 in the pathway of growth suppression and cell cycle regulation (7, 8, 17, 46). p21^{WAF-1} can also be activated in a p53-independent pathway during skeletal muscle differentiation, suggesting that it may also play a role in terminal cell cycle arrest in the G₀ phase (16, 24, 31, 34, 41).

Progression of eukaryotic cells through the cell cycle is regulated by a series of structurally related serine/threonine protein kinases, which consist of a catalytic subunit, a cyclin-dependent kinase (cdk), and a regulatory subunit, a cyclin (6, 36, 40). There are five major types of cyclins, A, B, C, D, and E, which all act at different points of the cell cycle. Cyclins D, E, and perhaps C are required for progression through the G₁ phase of the cell cycle (2). D-type cyclins (D₁, D₂, and D₃) associate mostly with cdk4, and cyclin E associates with cdk2 (7, 19). One of the major substrates of cyclin D-cdk4 and cyclin E-cdk2 is the retinoblastoma (Rb) protein (5, 11, 18), which in turn regulates the G₁-to-S-phase transition.

p21^{WAF-1} was shown to form complexes with cdks and cyclins in vivo (17). It can inhibit the phosphorylation of Rb by cyclin D₁-cdk4, cyclin E-cdk2, and cyclin A-cdk2 in vitro (17). In addition, it may also block the action of proliferating-cell nuclear antigen (PCNA)-dependent DNA replication but not repair in vitro (22). P21^{WAF-1} is a member of a family of proteins called the cdk inhibitory proteins, which regulate the cdks (for a review, see reference 30a). These cdk inhibitory proteins all have a common mechanism. They bind to cdk-cyclin complexes and alter their function by inhibiting them, by interfering with their activation, or both. Some of the other

members of this family of inhibitory proteins include p27^{kip-1}, p57^{kip-2}, and the yeast Far1 (20, 27, 35, 37, 43). p27^{kip-1} and p57^{kip-2} can inhibit the phosphorylation of Rb by cyclin D₁-cdk4, cyclin E-cdk2, and cyclin A-cdk2 in vitro (20, 27, 35, 37, 43).

It was recently shown that the N-terminal 90-amino-acid sequence of p21^{WAF-1} binds cdk2 (3, 14, 23, 29, 47) and the C-terminal 77-amino-acid sequence of p21^{WAF-1} binds PCNA (3, 44). Within the amino terminus of p21^{WAF-1}, a region between amino acid residues 21 and 26 contains the amino acid sequence LFGPVN, which is completely conserved among p21^{WAF-1}, p27^{kip-1}, and p57^{kip-2} (Fig. 1) and might therefore be important for its function (20, 27, 37, 43). Although p21^{WAF-1} can inhibit cyclin D₁-cdk4, cyclin E-cdk2, and cyclin A-cdk2 activities in vitro, it has not been shown whether p21^{WAF-1} can specifically block these three cyclin-cdk activities in vivo. To elucidate the mechanism of cell cycle regulation by p53 and its downstream effectors of the growth suppression pathway (p21^{WAF-1}), two experimental approaches were used. (i) A series of mutations were produced in the p21^{WAF-1} gene, altering amino acid residues between 21 and 26 of the protein. These mutants were then tested for their ability to interact with cyclins, cdks, and cyclin-cdk complexes. The same mutations were then compared with wt p21^{WAF-1} for the ability to block the growth of cells in culture. (ii) The impact of overexpression of various cyclins upon the ability of wt p21^{WAF-1} to block cell growth in culture was examined.

The results demonstrate that (i) the conserved region between residues 21 and 26, in particular amino acids 21 and 24 in the p21^{WAF-1} protein, is important for binding cyclins D and E, cyclin D₁-cdk4, and cyclin E-cdk2; (ii) overexpression of cyclin D or E in a cell can partially overcome the wild-type p21^{WAF-1} block of these cells in G₁; and (iii) by contrast, the overexpression of cyclin D₁ and p53 in a cell leads to apoptosis. This latter result demonstrates the functional distinction between the overexpression of p53 and its immediate downstream product, p21^{WAF-1}, in a cell committed to enter the cell cycle.

MATERIALS AND METHODS

Plasmids and site-specific mutagenesis. pCDNA₂-p21^{WAF-1} was constructed by ligating the entire wt human p21 cDNA (8) with pCDNA₃ vector (Invitrogen) at their *Hind*III and *Bam*HI sites. Mutations were generated in pCDNA₃-

* Corresponding author. Phone: (609) 258-5990. Fax: (609) 258-1704.

[†] Present address: D. H. Rittenberg Cancer Center, Mt. Sinai School of Medicine, New York, NY 10029-6574.

p57	30	<u>CRSLFGPVDHEEL</u>	42
p27	29	<u>CRNLFEGPVDHEEL</u>	41
p21	18	<u>CRRLFEGPVDSEQL</u>	30

FIG. 1. Sequence comparison of amino-terminal cdk-cyclin-inhibitory domains of human p21^{WAF-1}, p27^{kip1}, and p57^{kip2}. Underlined amino acids indicate residues shared by the three proteins. Amino acid numbers begin (number 1) at the amino terminus, and the residue numbers are indicated for the three proteins.

p21^{WAF-1} by annealing oligonucleotides with one or two mismatches to uridine-containing single-stranded DNA template as previously described by Kunkel (19a). The oligonucleotide was extended with T4 DNA polymerase, and the resulting double-stranded DNA was sealed with T4 DNA ligase. The DNA was used to transform *Escherichia coli* XL-1 blue (Invitrogen). Double-stranded DNA was prepared and sequenced by the dideoxy procedure (39) with appropriate primers. p21 mutants that generated in pCDNA₃-p21^{WAF-1} were cleaved with *Hind*III (5' end) and *Bam*HI (3' end) and recloned in these sites in the expression vector pCEP4, which contains a hygromycin B resistance gene (Invitrogen). pCDNA₃-cdk2 or pCDNA₃-cdk4 was constructed by ligating the entire wild-type human cdk2 or cdk4 cDNA with pCDNA₃ vector. pT7hPCNA contains an entire wt human PCNA cDNA. pCMV-cdk2 or pCMV-cdk4 consists of the entire wt human cdk2 or cdk4 cDNA and the pCMVneoBam vector. pRC/cyc D₁ and pRC/cyc E consist of the entire wt human cyclin D₁ or E, respectively and a pRC/CMV vector (Invitrogen). pR2 and pR3 consist of the entire wt murine cyclin D₂ or D₃, respectively. pCEP4-p53 was constructed by ligating the entire wt human p53 cDNA with pCEP4 vector at their *Hind*III (5' end) and *Bam*HI (3' end) sites.

In vitro translation and complex formation assays. Radiolabeled human wt or mutant p21^{WAF-1}, cyclins, cdks, and PCNA were generated in vitro by using the transcription and translation (TNT) coupled reticulocyte lysate system (Promega) in the presence of [³⁵S]methionine. Each 15 to 20 µl of translated products of wt or mutant p21 protein was mixed with 20 µl of cyclins, cdks, or PCNA and incubated for 30 min at 30°C. The mixture was then incubated with the p21^{WAF-1}, cyclin D₁, or cyclin E-specific polyclonal antibodies and protein A-Sepharose, and the mixture were continuously mixed on a rocking platform for 2 to 3 h at 4°C. The immunoprecipitates were then washed and subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (12.5% polyacrylamide) and X-ray autoradiography as previously described (13).

Cell culture and DNA transfection. The human osteogenic sarcoma, Saos-2 (26), and human lung adenocarcinoma, H1299 (29), cell lines, which do not express endogenous p53, were used for growth suppression assays. Cells were maintained in Dulbecco's modified Eagle's medium containing 10% (H1299) or 15% (Saos-2) fetal bovine serum in 5% CO₂ at 37°C in an incubator. For growth suppression assays, H1299 or Saos-2 cells grown on a 100-mm-diameter tissue culture dish were cotransfected with 5 µg of pCEP4-wt p21^{WAF-1} cDNA, pCEP4-mutant p21^{WAF-1} cDNA, or pCEP4 vector and 15 µg of salmon sperm DNA as carriers by using the calcium phosphate protocol (15). The DNA-calcium precipitates were left in the cell culture medium for 16 to 18 hours. The medium was then drained, and the cells were washed twice with phosphate-buffered saline (PBS) and refed with DMEM supplemented with 10 or 15% fetal bovine serum. At 24 h after transfection, the cells were counted and seeded at two or three densities: 1 × 10⁵, 5 × 10⁴, and 2.5 × 10⁴. One day later, 200 U of hygromycin B (Calbiochem) was added per ml of DMEM. The medium was replaced with fresh medium containing hygromycin B every 4 to 6 days, and the resistant colonies were stained and counted 2 to 3 weeks after transfection.

For the growth suppression assays, H1299 cells grown in a 100-mm-diameter tissue culture dish were cotransfected with 5 µg of pCEP4-wt p21^{WAF-1}, pCEP4-p53 cDNA, or pCEP4 vector and 10 µg of cyclin expression vector plasmids plus 5 µg of salmon sperm DNA by using the calcium phosphate protocol (15). The DNA-calcium precipitates were left in the cell culture medium for 16 to 18 hours. The medium was then drained, washed with PBS, and refed with DMEM supplemented with 10% fetal bovine serum. At 24 h after transfection, the cells were counted and seeded at two or three densities: 1 × 10⁵, 5 × 10⁴, and 2.5 × 10⁴. One day later, 200 U of hygromycin B and 960 µg of Geneticin (G418; GIBCO) were added per ml of DMEM. The medium was replaced with fresh medium containing hygromycin B and G418 every 4 to 6 days, and the resistant colonies were stained and counted 2 to 3 weeks after transfection.

Coexpression of wt p53 and cyclin D₁. Murine (10)1Val5 cells stably expressing the temperature-sensitive p53 mutant Val-135 growing in a 100-mm plate at 38°C were transfected with pCMV cyclin D₁ (10 µg) and a selectable marker conferring hygromycin resistance (PGK-Hyg, 2 µg) by using the calcium phosphate precipitation method (15). Selection medium containing 200 U of hygromycin B per ml was added to the transfected cells at 48 h after transfection, and colonies were cloned at 3 weeks after transfection. Cloned cells growing at 38°C were lysed in lysis buffer, and cell extracts were incubated with anti-cyclin D₁ polyclonal antiserum and protein A-Sepharose as described previously (13). The complex was washed three times with the lysis buffer and subjected to SDS-polyacrylamide gel electrophoresis (10% polyacrylamide). The proteins were transferred to Immobilon membrane, and cyclin D₁ was detected by using the same antisera and enhanced chemiluminescence (Amersham).

RESULTS

Binding of p21^{WAF-1} to cyclins. The three cdk inhibitors, p21^{WAF-1}, p27^{kip1}, and p57^{kip2}, form a related family of cdk inhibitory proteins. At their amino-terminal ends, the three proteins share a similar sequence of amino acids, LFGPVD, as shown in Fig. 1. To explore the role of these amino acid residues in p21^{WAF-1} function, three double mutations were made in the p21^{WAF-1} gene to change residues 21 and 24, 22 and 25, or 23 and 26 as detailed in Table 1 (the amino-terminal residue is given as residue 1). The wild-type p21^{WAF-1} cDNA and the three mutants of p21^{WAF-1} were cloned into vectors that permitted the transcription and translation of these proteins in vitro. In addition cyclins E, D₁, D₂, and D₃ were transcribed and translated in vitro with the rabbit reticulocyte system. These labeled proteins were then mixed for 30 min at 30°C to permit complex formation. The presence of a complex was then assayed by immunoprecipitation with anti-p21^{WAF-1} antibody or anti-cyclin antibody and detection of the coimmunoprecipitation of an associated protein. The immunoprecipitates were analyzed by separating the proteins by SDS-polyacrylamide gel electrophoresis and autoradiography of the gel. As shown in Fig. 2, wt p21^{WAF-1} readily associates with cyclin D₁ and antibodies directed against each of these proteins coimmunoprecipitate 10 to 40% of the total p21^{WAF-1} or cyclin D₁ in the mixture. When the level of p21^{WAF-1}-D₁ complex

TABLE 1. Effect of wt and mutant p21^{WAF-1} on the plating efficiency of human H1299 and Saos-2 cells

Position	Change		No. of colonies/5 × 10 ⁴ cells plated ^a								Ratio to vector (mean)	
	From	To	H1299 cells				Saos-2 cells				H1299 cells	Saos-2 cells
			Expt 1	Expt 2	Expt 3	Mean	Expt 1	Expt 2	Expt 3	Mean		
Vector			602	578	844	675	127	206	152	162	1.00	1.00
wt p21			139	141	142	141	28	65	21	38	0.21	0.23
p21-21,	Leu	His	641	303	602	515	114	164	108	129	0.76	0.80
24	Pro	Ala										
p21-22,	Phe	Tyr	191	201	306	233	72	105	90	89	0.35	0.54
25	Val	Glu										
p21-23,	Gly	Ser	290	328	424	347	113	173	81	122	0.51	0.75
26	Asp	Ala										
p21-114	Ser	Leu	140		136	138	33		56	45	0.20	0.28

^a Human H1299 and Saos2 cells were cotransfected with 5 µg of wild-type or mutant p21 or pCEP4 vector alone and 15 µg of salmon sperm DNA as carrier. Hygromycin B-resistant colonies were counted 2 to 3 weeks after transfection. Results of each experiment are given as the mean from two dishes.

FIG. 2. $p21^{WAF-1}$ and cyclin D_1 interactions in vitro. Cyclin D_1 and $p21^{WAF-1}$ wt protein or mutants with mutations at residues 21 and 24, residues 22 and 25, or residues 23 and 26 were translated in vitro and labeled with [35 S]methionine. Cyclin D_1 and each $p21^{WAF-1}$ sample were mixed and incubated for 30 min at 30°C. These mixtures were then immunoprecipitated with a cyclin D_1 -specific polyclonal serum (D1 Ab) or a $p21^{WAF-1}$ -specific polyclonal antiserum (p21 Ab). The immunoprecipitates were washed and displayed on an SDS-12.5% polyacrylamide gel. The X-ray autoradiograph is presented.

detected was set at 100% (Fig. 2), the $p21^{WAF-1}$ -21,24 mutant was only 6% as efficient (16.6-fold less) in binding D_1 while the $p21^{WAF-1}$ -22,25 mutant and $p21^{WAF-1}$ -23,26 mutants were 18 and 17% as efficient as the wt protein (Fig. 2). While no cdk protein was purposely added to this extract, so that the cyclin binding alone could be measured, it cannot be ruled out that some cdk protein is present in the rabbit reticulocyte system lysates and that this could contribute to cyclin binding to $p21^{WAF-1}$. Similar results (not presented) were obtained with cyclin D_2 and D_3 , with the $p21^{WAF-1}$ -21,24 mutant always showing the largest defect in $p21^{WAF-1}$ -cyclin binding.

The $p21^{WAF-1}$ -21,24 mutant fails to bind cyclin D-cdk4 or cyclin E-cdk2 complexes. The $p21^{WAF-1}$ wt and mutant 21,24 proteins were translated in vitro and mixed with cyclin D_1 and cdk4 synthesized in vitro. This permits an examination of the nature of the binding interactions between $p21^{WAF-1}$ and cyclin D_1 -cdk4. Once again, the mixtures were analyzed for binding to each other by measuring the ability of proteins to survive coimmunoprecipitation and washing procedures with anti-cyclin D or anti- $p21^{WAF-1}$ polyclonal sera. While the $p21^{WAF-1}$ wt protein very efficiently associated with the cyclin D_1 -cdk4 complex, the $p21^{WAF-1}$ -21,24 double mutant associated only poorly with the cyclin D_1 -cdk4 complex (about 90% less than for the wt) (Fig. 3).

Next, the $p21^{WAF-1}$ wt and double mutants were tested for their binding to cyclin E and cyclin E-cdk2, which constitute the other major G_1 cyclin activity. The wt and mutant $p21^{WAF-1}$ proteins were translated in vitro and mixed with cyclin E, which was also labeled during translation in vitro. The results of the coimmunoprecipitation assay are presented in Fig. 4A. The wt

$p21^{WAF-1}$ protein bound efficiently to cyclin E (which is a doublet in Fig. 4A). The $p21^{WAF-1}$ -21,24 mutant failed to bind to cyclin E (only 1%), but, unlike cyclin D_1 , the $p21^{WAF-1}$ mutants with mutations at residues 22 and 25, residues 23 and 26, or residue 114 were not as dramatically reduced in their binding to cyclin E, with 55, 46, and 82% reductions, respectively, compared with wt protein binding to cyclin E. Similarly, the $p21^{WAF-1}$ -21,24 mutant failed to bind cyclin E-cdk2 complexes (Fig. 4B) compared with the wt $p21^{WAF-1}$ protein.

$p21^{WAF-1}$ -mediated growth suppression of cells in culture. The phenotypes of $p21^{WAF-1}$ mutants characterized here demonstrate differences in protein associations in vitro between $p21^{WAF-1}$ wt and mutants. To determine if these phenotypes observed in assays carried out in vitro would have meaning in vivo, the $p21^{WAF-1}$ wt and mutant cDNAs were cloned into expression vectors and introduced into cells in culture by DNA transfection assays. Two cell lines were chosen to test the $p21^{WAF-1}$ phenotype in vivo: H1299 is a human lung adenocarcinoma cell line with no endogenous p53 gene expression and a wt Rb gene product, and Saos-2 is a human osteogenic sarcoma cell line with no endogenous p53 gene product and a mutant Rb gene product. Both of these cell lines have little or no detectable $p21^{WAF-1}$ protein. These cells were transfected with the pCEP4 vector carrying the hygromycin B resistance gene (vector-alone control) or the same vector with $p21^{WAF-1}$ wt or $p21^{WAF-1}$ mutants with mutations at residues 21 and 24, residues 22 and 25, residues 23 and 26, or residue 114. The $p21^{WAF-1}$ mutant 114, which binds cyclins with an affinity similar to that of wt protein, was used as a positive control. Hygromycin B-resistant colonies formed in plates and were stained and counted 2 to 3 weeks after the DNA transfections. The results are given as the average of three independent experiments and presented in Table 1. In H1299 cells, there was a fivefold reduction in colony formation when $p21^{WAF-1}$ wt was expressed in these cells. This was almost eliminated (to only a 1.3-fold reduction) with the $p21^{WAF-1}$ mutant with mutations at residues 21 and 24. The $p21^{WAF-1}$ -22,25 and

FIG. 3. $p21^{WAF-1}$ and cyclin D_1 -cdk4 complex interactions in vitro. Cyclin D_1 , cdk4, and $p21^{WAF-1}$ were translated and labeled in vitro. Mixtures of wt $p21^{WAF-1}$ (WT p21) or the 21,24 mutant $p21^{WAF-1}$ protein (21, 24) were mixed with D_1 -cdk4 and immunoprecipitated as described in the legend to Fig. 2. The $p21^{WAF-1}$ -21,24 mutant failed to bind to cyclin D_1 -cdk4.

FIG. 4. p21^{WAF-1}-cdk2-cyclin E interaction in vitro. Radiolabelled human wt or mutant p21^{WAF-1}, cdk2, and cyclin E were translated in vitro. p21^{WAF-1}-cyclin E complex (A) or p21^{WAF-1}-cdk2-cyclin E complex (B) were immunoprecipitated by p21^{WAF-1} or cyclin E-specific polyclonal antibodies and subjected to SDS-polyacrylamide gel electrophoresis as described in the legend to Fig. 2.

p21^{WAF-1}-23,26 mutants were intermediate in their abilities to inhibit cell growth, while the p21^{WAF-1}-114 mutant was identical to the wt. Similar results were found for Saos-2 cells, indicating the more general nature of these results.

Clearly, the p21^{WAF-1}-21,24, which is defective in binding cyclin D₁-cdk4 and cyclin E-cdk2 in vitro, is also substantially defective in the p21^{WAF-1}-mediated suppression of cell growth in vivo. The phenotypes of the other p21^{WAF-1} mutants in vivo (Table 1) parallel their intermediate phenotypes in vitro (Fig. 2 to 4).

Comparison of p53 and p21^{WAF-1} with respect to their growth suppressor activities. The available evidence indicates that p53 may mediate its ability to act as a cell cycle regulator and block cells in G₁ (1, 4, 25) by stimulating the synthesis of the p21 gene to produce p21^{WAF-1} protein (8). Thus, a comparison of the ability of p53 or p21^{WAF-1} gene expression in the same cells might be expected to lead to similar results when other external variables are manipulated. If p21^{WAF-1} acts to block cell division by binding to cyclin D₁-cdk4 and/or cyclin E-cdk2, increasing the concentration of these cyclins in the cell might well overcome the p21^{WAF-1} block in G₁. This is equivalent to using suppression by overexpression to demonstrate the functional interactions of two gene products.

To test these ideas, H1299 cells were cotransfected with pRC/CMV expression vectors containing cyclin cDNA (with a neomycin resistance gene) and pCEP4 vectors containing p53 or p21 cDNA (with a hygromycin B resistance gene). The numbers of neomycin- and hygromycin B-resistant colonies formed over the next 2 to 3 weeks were then scored. The p53 expression vector reduced the number of colonies by about 50-fold, while the p21^{WAF-1} cDNA reduced the number of colonies about 5-fold (Fig. 5; Table 2). The cotransfection of cyclin D₁ with p21^{WAF-1} almost completely reversed the p21^{WAF-1} inhibition of cell growth (83% of the control). This may be due to the overexpression of cyclin D₁, which can then increase the concentration of cyclin D₁-cdk4 complexes

in the cells and titrate out the inhibition by p21. Interestingly, the overexpression of cyclin D₁ had little or no impact on the ability of p53 to block H1299 cell colony formation (Table 2). As expected, cyclin B₁ had no impact on either p53- or p21^{WAF-1}-mediated growth suppression. Cyclin A, on the other hand, partially relieved (5-fold increase in colony growth) p53-mediated growth suppression and had a much smaller effect (1.6-fold) on p21^{WAF-1}-mediated growth suppression. While cyclin D₁ was efficient in reducing p21^{WAF-1}-mediated growth inhibition, cyclin E was about half as efficient in the same test of p21^{WAF-1} activity (Fig. 5; Table 2).

Clearly, there are some differences in the cyclin-mediated suppression of the p53 or p21 inhibition of cell growth as tested in this assay. Perhaps the clearest distinction is the ability of cyclin D₁ to reverse p21^{WAF-1} cell growth suppression, but cyclin D₁ has no impact on p53-mediated growth suppression.

Role of p53 in cells overexpressing cyclin D₁. The overexpression of E2F-1 and p53 in the same cell leads to apoptosis (45). It is possible that the overexpression of cyclin D₁ and p53 in the same cell also will lead to apoptosis. That could explain why cyclin D₁ overexpression relieves the p21^{WAF-1} block of the cell cycle progression but not the p53-mediated block to cell division. In this case, p53 may induce p21^{WAF-1} in a cell overexpressing cyclin D₁ but kills these cells via another pathway. This would result in the failure of cyclin D₁ to overcome p53-mediated colony inhibition as observed in Table 2.

To test these ideas, the (10)1Val5 cell line, which contains a p53 mutant protein that functions as wt at 32°C and a mutant at 38°C, was used. These cells are blocked in a G₁ arrest at 32°C (presumably because of p21^{WAF-1}) but grow normally at 38°C (25, 45). To overexpress cyclin D₁ in these cells, a cyclin D₁ plasmid was introduced into these cells at 38°C and individual colonies (clones of cells) were obtained and tested at 38°C for the expression of cyclin D₁. Three clones were screened for the expression of cyclin D₁, and two of these three

FIG. 5. Growth suppression assay of H1299 cells cotransfected with wt p21^{WAF-1} and cyclin D₁ or E (A) or with wt p53 or p21^{WAF-1} and cyclin D₁ (B). H1299 cells were cotransfected with 5 μ g of wt p21^{WAF-1} cDNA, wt p53 cDNA, or the pCEP4 vector alone and 10 μ g of cyclin D₁ or E cDNA plus 5 μ g of salmon sperm DNA. At 2 days after transfection, 200 U of hygromycin and 960 μ g of geneticin were added per ml of DMEM to ensure selection of each vector. The resistant colonies were stained and counted 2 to 3 weeks after transfection, as described in Materials and Methods. Photographs of typical plates are presented, and the results are quantitated in Table 2.

clones expressed elevated levels of cyclin D₁. One of these two clones, (10)1Val5-D, was chosen for further study, and the levels of cyclin D₁ were determined by immunoprecipitation and quantitative Western blots (immunoblots) [Fig. 6; compare (10)1Val5 with (10)1Val5-D]. These cells grow normally, like the (10)1Val5 parental line at 38°C, when p53 is in the mutant form (Fig. 7B). However, these cells rapidly lose viability at 32°C, when p53 is overexpressed and in the wt conformation along with high levels of cyclin D₁ (Fig. 7A). The morphological changes of these cells at 32°C resemble those of cells undergoing apoptosis, including cell shrinkage, cell membrane blebbing, and loss of cell-cell contact (Fig. 7A). The

TABLE 2. Effect of wt human p53 and p21^{WAF-1} on the plating efficiency of H1299 cells^a

Transfected DNA	No. of colonies/10 ⁵ cells plated				Ratio to vectors (mean)
	Expt 1	Expt 2	Expt 3	Mean	
Hygro+neo vectors	282	159	250	230	1.0
p53+neo vector		1	9	5	0.02
p53+cyclin A		14	36	25	0.22
p53+cyclin B1		7	7	7	0.04
p53+cyclin D1		5	11	8	0.04
p21+neo vector	78	17	31	42	0.18
p21+cyclin A	54	29	126	70	0.29
p21+cyclin B1	20	25	20	22	0.10
p21+cyclin D1	150	127	291	189	0.83
p21+cyclin E	123	24	154	100	0.40

^a Each transfection mixture contained 5 μ g of p53, p21, or hygro vector, 10 μ g of neo vector or cyclin cDNA, and 5 μ g of salmon sperm DNA as carrier. Hygromycin- and geneticin-resistant colonies were counted 2 to 3 weeks after transfection. Results are given as the mean from three dishes.

FIG. 6. Expression levels of cyclin D₁ in (10)1Val5 and (10)1Val5D cells. A cell line, (10)1Val5, containing a temperature-sensitive p53 gene, was transfected with a vector overexpressing cyclin D₁ at 38°C, whose p53 is in the mutant form. The (10)1Val5 and a clone overexpressing cyclin D₁, called (10)1Val5D, were grown at 38°C, and the levels of cyclin D₁ from the same number of cells was determined by a Western blot procedure with anti-D1 polyclonal rabbit serum and enhanced chemiluminescence (Amersham). Cyclin D₁ overexpression is indicated by the arrow. The bands at 50 kDa are due to the heavy chains of the antibody.

FIG. 7. (10)1Val5D cells grown at 38°C (B) and undergoing apoptosis at 32°C (A). The (10)1Val5D cell line was photographed while growing at 38°C (B) and after 24 h at 32°C (A).

parental cell line, (10)1Val5 cells with no added cyclin D₁, are growth arrested in G₁ at 32°C and do not show evidence of apoptosis, as previously shown (25, 45). The apoptotic killing of (10)1Val5-D at 32°C was also confirmed by demonstrating the DNA fragmentation ladder with these cells (data not presented).

These results demonstrate that the overexpression of cyclin D₁ in a cell also overexpressing wt p53 results in apoptosis. Because cyclin D₁-cdk4 or cyclin D₁-cdk6 phosphorylates Rb and releases E2F-1, it is likely that E2F-1 somehow signals for apoptosis when p53 is present in high concentration and activated. It has previously been shown that overexpression of E2F-1 plus p53 induces an apoptotic process (45). This experimental comparison of the growth suppression activity of p21 and p53 demonstrates that (i) although p21 may be one of the major downstream effectors of p53 in the growth suppression pathway, it functions in only one of the p53-controlled decision points in the G₁ phase of the cycle (a second p53-mediated decision point leads to apoptosis, which appears to be p21 independent), and (ii) these data are consistent with the idea that p53 could mediate its G₁ arrest by utilizing a second mechanism in addition to p21.

DISCUSSION

The p53 gene is thought to be an important tumor suppressor gene, because it is found to be mutated in up to 55% of all human cancers (21). p53 is a transcription factor (12, 38), which, in turn, regulates a set of genes that affect progression through the cell cycle or initiates a pathway leading to apoptosis or cell death (1, 4, 25, 30, 33). Among the genes that are transcriptionally regulated by p53 is the p21^{WAF-1} gene (8).

This gene encodes one of a family of proteins including p27^{kip1} and p57^{kip2}, that function to inhibit the activities of G₁ cyclin-cdks (17), which in turn are required for progression from G₁ to S phase in the cell cycle. It has been a reasonable hypothesis then that p53 acts to cause G₁ arrest in the cell cycle (25, 28) by increasing the levels of p21^{WAF-1} in a cell.

The p21^{WAF-1} protein has been shown to bind cdks (17), and the site for the binding of cdk2 on the protein has been localized to the amino terminus around residues 49 to 71 (3, 14, 23, 29, 47). The p21^{WAF-1} protein has also been shown to bind to PCNA (3, 44). This PCNA-binding site has been mapped by deletion analysis (22a) to residues 124 to 164 at the carboxy terminus of the protein (Fig. 8). Experiments presented in this paper have identified a third protein-binding domain in p21^{WAF-1}. Residues 21 to 26, with the amino acid sequence LFGFVD (Fig. 1 and 8), contain a cyclin-binding site conserved in all p21^{WAF-1} family members (p27^{kip1} and p57^{kip2}). The double mutation in p21^{WAF-1} at residues 21 and 24 eliminates cyclin D₁, cyclin E, cyclin D-cdk4, and cyclin E-cdk2 binding to the protein.

p21^{WAF-1} can inhibit the growth of cells in culture, presumably by binding to cyclin-cdk complexes in vivo. The p21^{WAF-1}-21,24 mutant that fails to bind cyclin D₁-cdk4 or cyclin E-cdk2 substantially fails to inhibit cell growth in vivo. Other p21^{WAF-1} mutants (p21^{WAF-1}-22,25 and p21^{WAF-1}-23,26) with intermediate phenotypes for binding to cyclin-cdks have weaker inhibitory effects on cell growth and division in vivo. These data strengthen the idea that p21^{WAF-1} acts through cyclin D₁-cdk or cyclin E-cdk in vivo. The results presented here have also demonstrated that p21^{WAF-1} can suppress the growth of cells that have a mutant Rb protein (Saos-2 cells). This may be due to the role of p21^{WAF-1} in cdk activity of p107 and p130 (Rb-related proteins), or it could indicate that p21^{WAF-1} has additional activities yet to be determined.

The fact that cyclin D₁ or E overexpression in cells can reverse p21^{WAF-1} inhibition of cell growth is completely consistent with the growth suppression results with the p21^{WAF-1}-21,24 mutant (Table 1). Those data support the hypothesis that p21^{WAF-1} functions to specifically inhibit cyclin D₁-cdk4 or cyclin E-cdk2 activity in vivo. The quantitative differences between cyclins D₁ and E are not necessarily informative in the absence of knowing the relative levels of expression and activity of these proteins in vivo.

It was initially surprising to find that the overexpression of cyclin D₁ reversed the growth inhibition of p21^{WAF-1} but not of p53. This could be interpreted in one of two ways. One possibility is that p53 mediates its G₁ arrest by using another mechanism in addition to increasing the levels of p21^{WAF-1} (e.g., via Gadd 45 [42]). While high levels of p21^{WAF-1} are induced by p53 and G₁ arrest is reversed by high levels of D₁, the alternative mechanism could still inhibit colony formation in the p53-transfected cells. It is assumed here that the levels of

FIG. 8. Functional domains of p21^{WAF-1}. The p21^{WAF-1}-binding sites for cyclins D and E (residues 21 to 26), cdk2 (residues 49 to 71) (29), and PCNA have all been mapped to distinct regions on the protein.

p21^{WAF-1} in p53-transfected cells are about as high as in p21^{WAF-1}-transfected cells, even though p53 is 10-fold more effective in the colony inhibition assays. The second possibility is that the high expression of both p53 and cyclin D₁ in a cell leads to a different result from the high expression of cyclin D₁ and p21^{WAF-1}. One such consequence could be that high levels of p53 plus D₁ lead to apoptosis and that is responsible for loss of colony formation whereas p21^{WAF-1} plus D₁ does not induce an apoptotic activity and the p21^{WAF-1} G₁ arrest is reversed by excess cyclin D₁ in the cell. When cells expressing high levels of cyclin D₁ and p53 were examined, it was clearly shown that they do indeed undergo apoptosis. This, then, explains the results reported in Table 2. It also makes clear that p53 resides at a decision point in G₁. The overexpression of p53 (due to DNA damage) and cyclin D₁ leads to cell death. This is similar to apoptosis induced by the overexpression of p53 and myc (10) or p53 and E2F-1 (45). At low levels of cyclin D₁, high p53 levels will result in high p21^{WAF-1} activity and G₁ arrest. This is eventually reversed by enhanced levels of cyclins and a return to reduced levels of p53, after repair of the DNA damage. The fact that p53 mutations but not p21^{WAF-1} mutations are observed in cancerous cells may, then, simply be due to the decision point or fork in G₁ that p53 controls, while p21^{WAF-1} acts on only one arm of that decision tree and it is the second arm (i.e., apoptosis) that also is selected against in cancer cells.

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