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13. ABSTRACT *(Maximum 200 words)*

p73, a member of the p53 family, can induce apoptosis and physically interact with p53. To determine whether p53 and p73 functionally interact, we generated several MCF7 cell lines that inducibly express p73. We found that p73-dependent apoptosis was enhanced by DNA damage (i.e., p53) in MCF7 cells that harbor wild-type p53, but not in MCF7E6 cells that ectopically express human papillomavirus E6 protein and are functionally p53-null. These results suggest that a functional interaction between p53 and p73 in MCF7 cells leads to enhanced induction of apoptosis.

It has been shown by others that the proline-rich domain within residues 60-90 are necessary for growth suppression. Here, we showed that the proline-rich domain is required for inducing apoptosis but not cell cycle arrest. We also showed that this domain is necessary for induction of cellular target genes, e.g., p21, MDM2, but not for activation of transiently transfected promoters from such genes. These results suggest that the proline-rich domain plays a role in chromatin remodeling, which counteracts chromatin-mediated repression for the cellular genes.

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INTRODUCTION

p73, the first additional member of the growing p53 family, is a potential tumor suppressor. The p73 gene is expressed as at least six alternatively spliced forms, that is, p73α, p73β, p73γ, p73δ, p73ε, and p73ζ. The residues in p73 and p53 are highly similar, especially in the central sequence-specific DNA binding domain, the amino terminal activation domain, and the carboxyl terminal oligomerization domain. Like p53, p73 can induce cell-cycle arrest and apoptosis when overexpressed in cells. In addition, p53 physically interacts with p73 in yeast. To determine whether p73 functionally interacts with p53, we generated several MCF7 breast cancer cell lines that inducibly express p73. The results were shown in the following section.

Recently, the proline-rich region between residues 60 and 90, which contains five "PXXP" motifs (where P represents proline and X any amino acid), was found to be necessary for efficient growth suppression. Since the growth suppression can be mediated by cell cycle arrest and/or apoptosis, it is not clear in what role the proline-rich domain plays. To further examine the importance of the proline-rich domain in p53-dependent cell cycle arrest and apoptosis, we generated a p53 mutant that lacks all five PXXP motifs and analyzed in H1299 cells as shown in the following section.

BODY

DNA damage enhances the ability of p73 to induce apoptosis in MCF7 cells that carry an endogenous wild-type p53 gene

To determine the functional interaction between p53 and p73, we generated several MCF7 cell lines that inducibly express p73α. One representative cell line, M7-p73α-2, was chosen for further analysis. We found that in the absence of either induction of p73α or DNA damage, MCF7 cells grew normally. When induced to express p73α for two days, cells failed to multiply, enlarged and became flat, which are all characteristic of cell cycle arrest. When treated with 300 nM of camptothecin to induce endogenous p53 but without inducing p73α, cells also failed to multiply, enlarged, and flattened. In contrast, when cells were both induced to express p73α and treated with camptothecin to induce endogenous p53, more than 90% of the cells either became round or detached from the culture plate and shrank to form apoptotic bodies. Similar results were obtained when doxorubicin was used. We also tested MCF7 cells that inducibly express p73α292 or p73β. It has been shown that p73α292, which is inert in transcriptional activity, is defective in inducing cell cycle arrest and apoptosis. When MCF7 cells were induced by withdrawal of tetracycline, DNA damage cooperated with p73β but not mutant p73α292 to induce apoptosis.

Activation of the p53 pathway is necessary for the cooperative induction of apoptosis in MCF7 cells by DNA damage and p73.

To determine the role of p53 in the cooperative induction of apoptosis by DNA damage and p73, we generated several MCF7 cell lines that ectopically express HPV E6 protein and inducibly express p73α. Since HPV E6 facilitates ubiquitin-mediated degradation of p53, MCF7 cells that carry an HPV E6 gene, designated MCF7E6, are functionally p53-null. One representative cell lines, MCF7E6-45, was chosen for further analysis. In the absence of either induction of p73α or DNA damage, MCF7E6 cells grew normally. When induced to express p73α for two days, cells enlarged and became flat. When treated with 300 nM camptothecin for two days, MCF7E6 cells slightly enlarged but grew slowly, suggesting that p53 is critical for growth suppression by DNA damage. When both induced to express p73α and treated with camptothecin, MCF7E6 cells also enlarged and flattened, which is completely different from the strong apoptotic response observed in MCF7 cells. Thus, loss of the p53 pathway abrogates the cooperative induction of apoptosis by DNA damage and p73.

The proline-rich domain is necessary for inducing apoptosis but not for cell cycle arrest.

To examine the importance of the proline-rich domain in p53, a mutant that deletes this domain, p53(Δ62-91), was generated and analyzed in H1299 cells. H1299 is a p53-null, non-small cell lung carcinoma cell line. Several cell lines that inducibly express p53(Δ62-91) were established. We found that cells expressing p53(Δ62-91) failed to multiply. DNA histogram analysis showed that p53(Δ62-91) is capable of inducing cell cycle arrest, primarily in G1, but not apoptosis.

Previously, it was showed by others that the p21 promoter can be activated by a proline-rich domain deletion mutant p53 to an extent that is equal to or greater than by wild-type p53. A similar result was observed when we tested the ability of p53(Δ62-91) to activate the p21 promoter in a luciferase assay. However, Western blot analysis consistently showed that p53(Δ62-91) is much weaker than wild-type p53 to induce p21. To determine whether the low level of p21 protein detected in p53(Δ62-91)-expressing cells is due to decreased expression of the p21 gene, we performed Northern blot analysis. We found that p21 was induced by p53(Δ62-91) but the extent of induction was approximately 30% of that by wild-type p53.

Next, we determined whether p53(Δ62-91) can regulate other p53 target genes, i.e., MDM2, BTG2. We found that MDM2, an oncogene and a negative regulator of p53, was slightly induced by p53(Δ62-91). BTG2, a nerve growth factor responsive gene that can cause growth suppression, was strongly induced by p53 but only slightly by p53(Δ62-91).

Our studies have confirmed and extended the previous observations that the proline-rich domain in human p53 is necessary for efficient growth suppression. Specifically, we found that the proline-rich domain is required for p53-dependent apoptosis. It should be noted that the reduced regulation of cellular target genes by p53(Δ62-91) may not be uncovered if its transcriptional activity was measured by its capability of activating the p21 or other target gene promoters in a transient transfection assay. In addition to the p21 promoter, the MDM2 promoter can be activated by p53 lacking the proline-rich domain as efficiently as wild-type p53. Thus, we should not extrapolate the result observed by the promoter analysis of a target gene to p53 induction of the endogenous target gene. It is well established that the regulation of transcription for endogenous genes that are packaged into chromatin is different from those that are transiently transfected into cells in naked plasmids. The simplified explanation is that the promoter template in a naked plasmid would be easily accessible while that packaged into chromatin is not. It is well known that transcriptional activators function, at least in part, to counteract chromatin-mediated repression. Therefore, it is possible that the proline-rich domain might be a region that is necessary for chromatin remodeling, which is responsible for p53 as transcriptional activator to counteract chromatin-mediated repression for the cellular p53 target genes.

Work accomplished in relation to the Statement of Work

Tasks 1-2: A number of MCF7 breast carcinoma cell lines that inducibly express p53 or p73 using a tetracycline-regulated promoter have been generated and analyzed (1998 and 1999 annual report). We will generate additional MCF7 cell lines that express various mutated forms of p53 or p73, or other proteins.

Tasks 3: DNA damage enhances p73-dependent apoptosis in MCF7 but not in MCF7E6 cells (1999 annual report). We will determine how DNA damage affects p53-dependent apoptosis in MCF7 cells.

Tasks 4-5: In progress and to be done.

Task 6: A number of short deletion and point mutations of p53 have been generated and analyzed (1998 and 1999 annual report).

Task 7: We identified a novel apoptotic domain and activation domain II in p53 (1998 annual report). We showed that the proline-rich domain is necessary for apoptosis (1999 annual report).

Task 8-10: We showed that p73 functionally interacts with p53 in cells and activation of the p53 pathway is necessary for the cooperative induction of apoptosis between p73 and DNA damage in MCF7 cells (1999 annual report). We will further analyze the mechanism by which p53 and p73 functionally interact.

Appendix 1: Key Research Accomplishments for the period of July 1, 1998 - June 30, 1999.

- Establishing several MCF7 breast carcinoma cell lines that inducibly express p73 using a tetracycline-regulated promoter.

- p73 functionally cooperates with DNA damage (i. e., induction of p53) to induce apoptosis in MCF7 cells.

- Activation of the p53 pathway is necessary for the cooperative induction of apoptosis in MCF7 cells by p73 and DNA damage.

- Establishing several H1299 cell lines that inducibly express a p53 mutant that lacks the proline-rich domain.

- The proline-rich domain in p53 is necessary for inducing apoptosis but not for cell cycle arrest.

- The proline-rich domain in p53 is necessary for induction of cellular target genes but not for activation of the transiently transfected promoters from these genes. The results suggest that the proline-rich domain plays a role in chromatin remodeling, which counteracts chromatin-mediated repression for the cellular genes.

Appendix 2: Reportable Outcomes

1. A presentation at the American Association for Cancer Research 90th annual meeting, Philadelphia, PA. April 10-14, 1999.

2. Zhu, J., J. Jiang, W. Zhou, K. Zhu, and **X. Chen.** 1999. Differential regulation of cellular target genes by p53 devoid of the PXXP motifs with impaired apoptotic activity. ***Oncogene.*** 18: 2149-2155.

Appendix 3.

Abstract (abstract #677, presented at the American Association for Cancer Research 90th annual meeting, Philadelphia, PA. April 10-14, 1999)

p73, a potential tumor suppressor, is homologous to the p53 tumor suppressor. Recently experiments have showed that, like p53, p73 can induce both cell cycle arrest and apoptosis. Since p53-dependent apoptosis can be augmented by various chemotherapeutic agents, it has been hypothesized that the status of the endogenous p53 gene in tumor cells is a key determinant in the outcome of chemotherapy. To determine whether chemotherapeutic agents can affect p73-dependent apoptosis, several cell lines that inducibly express p73 under a tetracycline-regulated promoter were used. We found that p73-dependent apoptosis was inhibited by several DNA-damaging agents in H1299 cells that are p53-null. However, in MCF7 cells that carry an endogenous wild-type p53 gene, p73-dependent apoptosis was significantly enhanced. Furthermore, we found that p73 up-regulation of several cellular target genes was increased in MCF7 cells but was inhibited in H1299 cells when treated with the DNA-damaging agent camptothecin. Taken together, we hypothesized that the p73 protein is differentially modified by DNA-damaging agents in the presence and absence of p53 and a functional interaction between p53 and p73 leads to enhanced induction of apoptosis.

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SHORT REPORT

Differential regulation of cellular target genes by p53 devoid of the PXXP motifs with impaired apoptotic activity

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Activation of the p53 tumor suppressor protein can lead to either cell cycle arrest or apoptosis. Several functional domains necessary for mediating cell cycle arrest and apoptosis in p53 have been mapped, e.g., the proline-rich domain. The proline-rich domain is located within residues 60–90, which comprise five PXXP motifs (where P represents proline and X any amino acid). To further delineate the function of the proline-rich domain and its potential role in transactivation, we generated several groups of cell lines that inducibly express various p53 mutants using a tetracycline-regulated expression system. We found that p53(Δ62–91), which lacks all five PXXP motifs in human p53, is capable of inducing cell cycle arrest but not apoptosis, while p53(gln22-ser23/Δ62–91), which contains a double point mutation in the activation domain as well as deletion of the proline-rich domain, completely loses its activity. However, p53(Δ74–91), which contains only one PXXP motif at its N-terminus, is not only capable of inducing cell cycle arrest but also retains a partial apoptotic activity. Furthermore, we found that deletion of the proline-rich region has no or very mild effects on activation of several transiently transfected p53 target gene promoters, i.e., the p21, MDM2, BAX, and GADD45 promoters. However, such deletion differentially affects p53 induction of endogenous target genes, i.e., induction of p21, MDM2, BTG2, p85, PIG3, PIG6 and PIG11 was reduced or abrogated but induction of BAX, KILLER/DR5, PIG2, PIG7 and PIG8 was not substantially affected. Interestingly, induction of GADD45 was enhanced. These results suggest that the proline-rich region may play a role in chromatin remodeling, which counteracts chromatin-mediated repression for some of the endogenous p53 target genes.

Keywords: p53; p21$^{waf1/cip1}$; apoptosis; cell cycle arrest

The p53 tumor suppressor protein acts as a DNA damage checkpoint and is a pivotal regulator of cellular transformation. Following various genotoxic conditions, p53 accumulates and/or its activity increases, resulting in apoptosis, cell cycle arrest, differentiation (for reviews, see (Agarwal *et al.*, 1998; Ko and Prives, 1996; Levine, 1997) or senescence (Sugrue *et al.*, 1997). As a transcription factor, p53 up-regulates p21$^{waf1/cip1}$, an inhibitor of cyclin-dependent kinases, which is responsible for p53-dependent cell cycle arrest or differentiation (el-Deiry *et al.*, 1993; Harper *et al.*, 1993; Liu *et al.*, 1996; Xiong *et al.*, 1993). However, it is still not certain what exact function p53 plays in apoptosis. Several studies have provided evidence that only certain domains of p53 are required for apoptosis, e.g., the N-terminal 22 amino acids are dispensable (Chen *et al.*, 1996) but the sequence-specific DNA binding domain is required (for reviews, see Gottlieb and Oren, 1996; Ko and Prives, 1996; Levine, 1997). Deletion of the C-terminal regulatory domain reduces p53 apoptotic activity (Chen *et al.*, 1996; Wang *et al.*, 1996), as does a double point mutation at residues 22 and 23 (Chen *et al.*, 1996; Haupt *et al.*, 1995), which also diminishes p53 transcriptional activity (Lin *et al.*, 1994). In addition, several studies have provided evidence that p53 may have a transactivation-independent function in apoptosis (Caelles *et al.*, 1994; Haupt *et al.*, 1995; Wagner *et al.*, 1994).

Recently, the proline-rich region between residues 60 and 90, which contains five 'PXXP' motifs (where P represents proline and X any amino acid), was found to be necessary for efficient growth suppression (Walker and Levine, 1996), serving as a docking site for transactivation-independent growth arrest induced by GAS1 (Ruaro *et al.*, 1997). In addition, the proline-rich region is required for the murine temperature-sensitive p53(val135) to induce apoptosis in adenovirus E1A-transformed cells (Sakamuro *et al.*, 1997).

To further examine the importance of the proline-rich region in human p53, p53(Δ62–91), which lacks all five PXXP motifs, was generated and inducibly expressed in H1299 using the tetracycline-inducible expression system as we have used previously (Chen *et al.*, 1996). H1299 is a p53-null non-small cell lung carcinoma cell line. Nine stable cell lines that inducibly express p53(Δ62–91) were established. Western blot analysis of four cell lines, one expressing wild-type and three expressing mutant p53, is shown in Figure 1a. p53(Δ62–91)-1 is a low p53 producer, and p53(Δ62–91)-5 and -6 are high p53 producers when compared to p53-3, a high wild-type p53 producer cell line established previously (Chen *et al.*, 1996). To determine the transcriptional activity of p53(Δ62–91), we analysed the endogenous p21 gene, a known p53 target (el-Deiry *et al.*, 1993). Western blot analysis demonstrated that p21 was activated in both low and high p53(Δ62–91) producer cells, but the level of induction was approximately 30% of that activated by

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wild-type p53 (Figure 1a). This result suggests that the proline-rich domain may contribute to, but nevertheless is dispensable for, p53 transactivation. To determine whether p53(Δ62–91) can induce cell cycle arrest, the growth rates of two high producer cell lines, p53(Δ62–91)-5 and -6, were analysed. We found that cells expressing p53(Δ62–91) failed to multiply (Figure 1b) or multiply slowly (Figure 1d) while cells not expressing p53(Δ62–91) showed a pattern of exponential growth (Figure 1b and d). FACS analysis showed

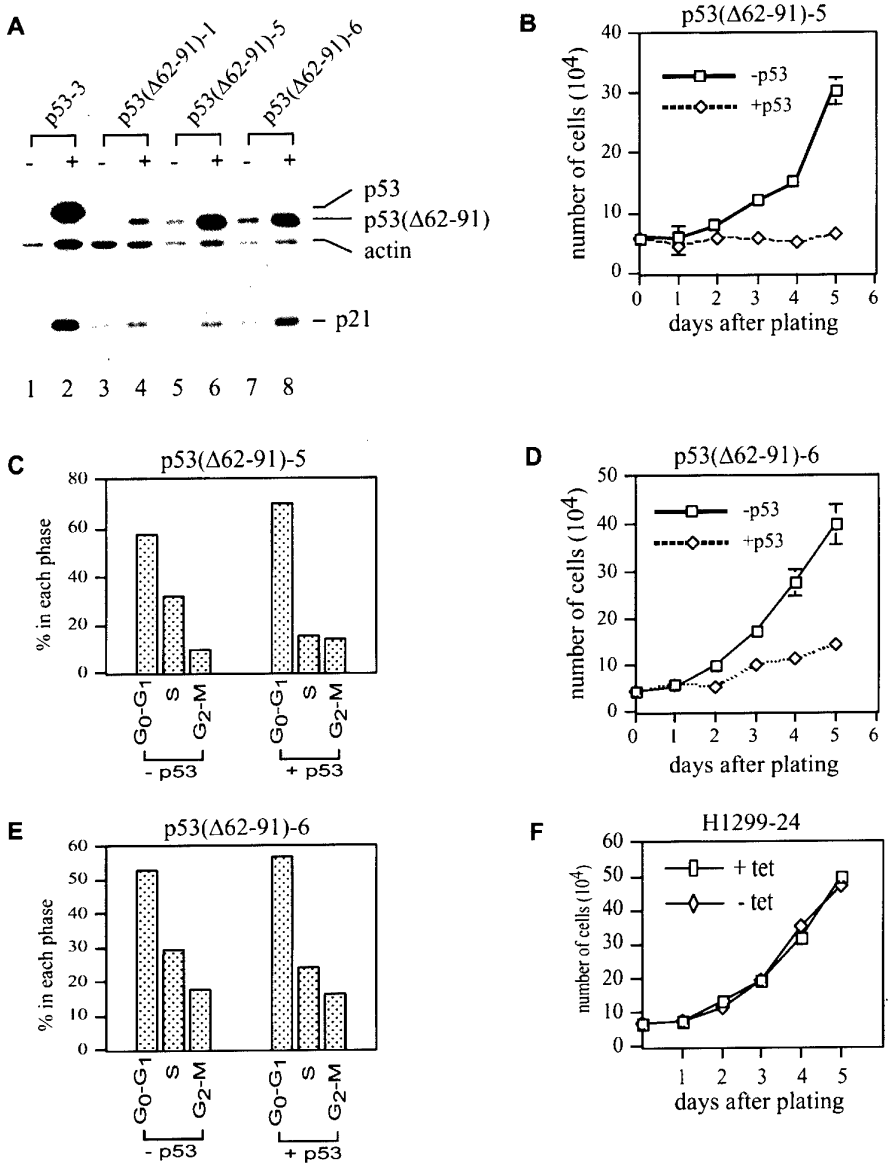

Figure 1 The proline-rich domain is required for p53-dependent apoptosis but not cell cycle arrest. To generate p53(Δ62–91), cDNA fragments encoding amino acids 1-61 and 92-393 were amplified independently and ligated together through an internal *Bam*HI site which was artificially created without affecting the amino acid composition of the p53 protein. Primers used for the amino acids 1–61 fragment were: forward primer N1: GAT CGA ATT CAC CAT GGG CTA CCC ATA CGA TGT TCC AGA TTA CGC TGA GGA GCC GCA GTC AGA TCC; and reverse primer C61: GAT CGG ATC CGG ACC TGG GTC TTC AGT. Primers used for the amino acid 92-393 fragment were: forward primer N92: GAT CGG ATC CCC TGT CAT CTT CTG TC; and reverse primer C393: GAT CGA ATT CTC AGT CTG AGT CAG GCC CCT. p53(Δ62–91) was then cloned into the tetracycline-regulated expression vector, 10-3, at its *Eco*RI site and the resulting plasmid was used to generate cell lines that inducibly express p53 as previously described (Chen *et al*., 1996). (**a**) Levels of p53, p21, and actin in p53-3, and p53(Δ62–91)-1, -5, and -6 cells were assayed by Western blot analysis. Cell extracts were prepared from uninduced cells (–) or cells induced to express (+) wild-type p53 or p53(Δ62–91). The upper portion of the blot was probed with a mixture of p53 monoclonal antibodies Pab421 and Pab240 and actin polyclonal antibody (Sigma, St Louis, MO, USA). Note that mutant p53(Δ62–91) migrates faster than wild-type p53 because it is missing 30 amino acids. The lower portion of the blot was probed with p21 monoclonal antibody (Oncogene Science, Uniondale, NY, USA). (**b** and **d**) Growth rates of p53(Δ62–91)-5 and -6 cells in the presence (◇) or absence (□) of p53 were measured as previously described (Zhu *et al*., 1998). (**c** and **e**) The percentages of p53(Δ62–91)-5 and -6 cells in G_0-G_1, S, and G_2-M phases in the presence or absence of p53 for 3 days were quantitated by FACS analysis as described previously (Zhu *et al*., 1998). (**f**) Growth rates of H1299-24 cells in the presence (+tet) or absence (–tet) of tetracycline. The experiments were performed similarly as in (**b**)

that the number of S-phase cells was markedly reduced in p53(Δ62–91)-5 cells and moderately reduced in p53(Δ62–91)-6 cells upon induction of p53 (Figure 1c and e). Thus, both growth rate and FACS analyses indicate that the proline-rich domain in p53 is not required for cell cycle arrest. As control, the growth rates of H1299-24 cell line was similarly established but does not express any protein were nearly identical in the presence (□ + tet) or absence (◇-tet) of tetracycline (Figure 1f).

It is well established that p53 can induce apoptosis in H1299 cells (Chen *et al*., 1996; Haupt *et al*., 1995). This can be quantitated by determining the percentage of cells containing a sub-G_1 content of DNA. FACS analysis showed that only 2–6% of cells underwent apoptosis after induction of p53(Δ62–91) for 3 days as determined from three separate experiments. Using trypan blue exclusion assay (Zhu *et al*., 1998), a similar percentage of dead cells were detected. In contrast, about 45% and 30% of cells underwent apoptosis after

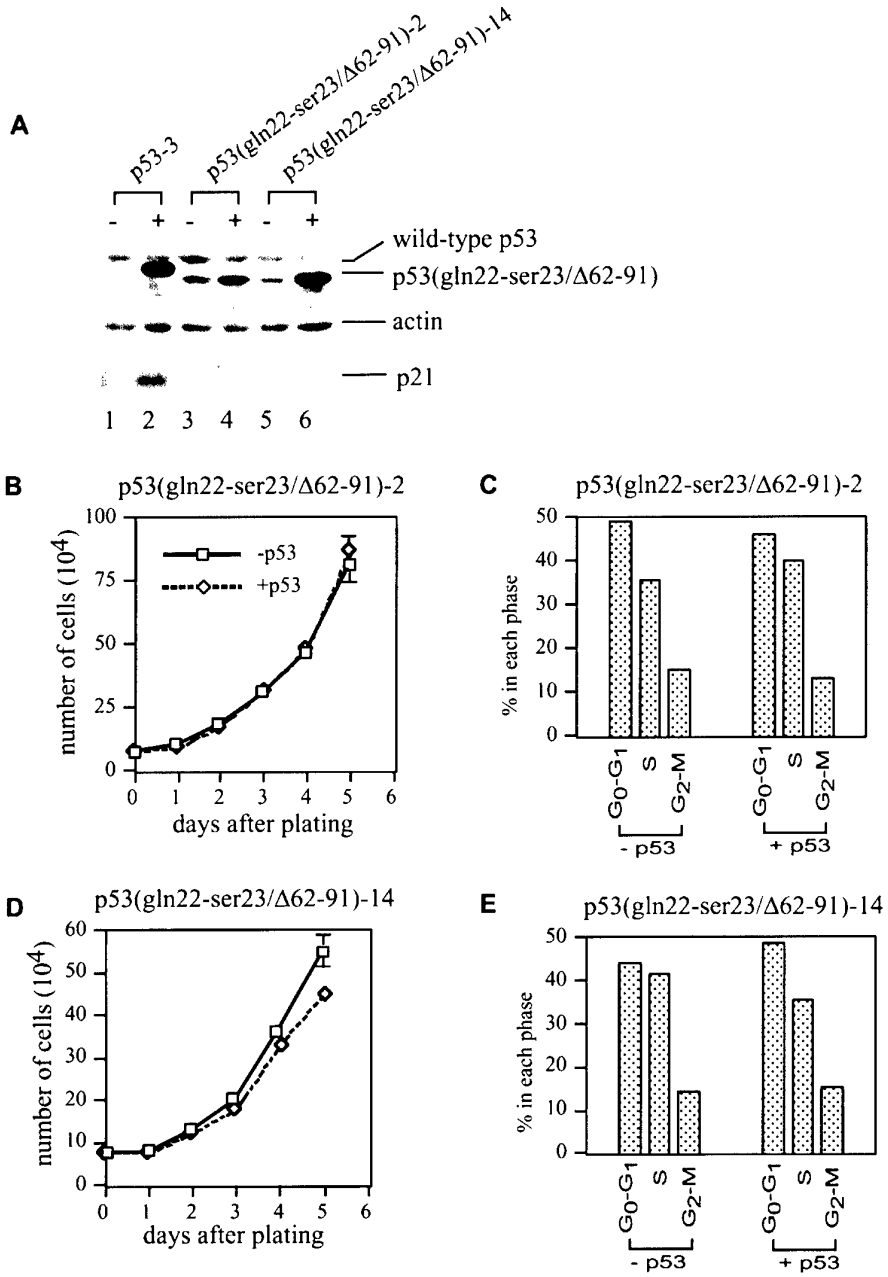

Figure 2 Deletion of the proline-rich domain abolished the apoptotic activity of the transactivation deficient p53(gln22-ser23). p53(gln22-ser23/Δ62–91) was generated similarly as p53(Δ62–91) except that a cDNA encoding p53(gln22-ser23) (Lin *et al*., 1994) was used as template. (**a**) Levels of p53, p21 and actin in p53-3 and p53(gln22-ser23/Δ62–91)-2 and -14 cells were assayed by Western blot analysis. (**b** and **d**) Growth rates of p53(gln22-ser23Δ62–91)-2 and -14 cells in the presence (◇) or absence (□) of p53. (**c** and **e**) The percentage of p53(gln22-ser23Δ62–91)-2 and -14 cells in G_0-G_1, S, and G_2-M phases in the presence or absence of p53 for 3 days. The experiments were performed in an identical manner to those shown in Figure 1

induction of wild-type p53 and transactivation deficient p53(gln22-ser23), respectively.

Previously, we and others have shown that p53(gln22-ser23) retains a partial apoptotic activity but is incapable of inducing cell cycle arrest (Chen *et al.*, 1996; Haupt *et al.*, 1995). To further demonstrate the function of the proline-rich region, we hypothesized that p53(gln22-ser23/Δ62–91), which contains a double point mutation at residues 22 and 23 in the activation domain as well as deletion of the proline-rich domain, would be unable to induce either cell cycle arrest or apoptosis. Four stable cell lines that inducibly express p53(gln22-ser23/Δ62–91) were established and two representative cell lines were characterized (Figure 2a). We found that this p53 mutant is unable to activate p21 (Figure 2a). Furthermore, growth rate (Figure 2b and d) and FACS (Figure 2c and e) analyses showed that neither cell cycle arrest nor apoptosis were observed in cells expressing p53(gln22-ser23/Δ62–91) under the induced condition. These

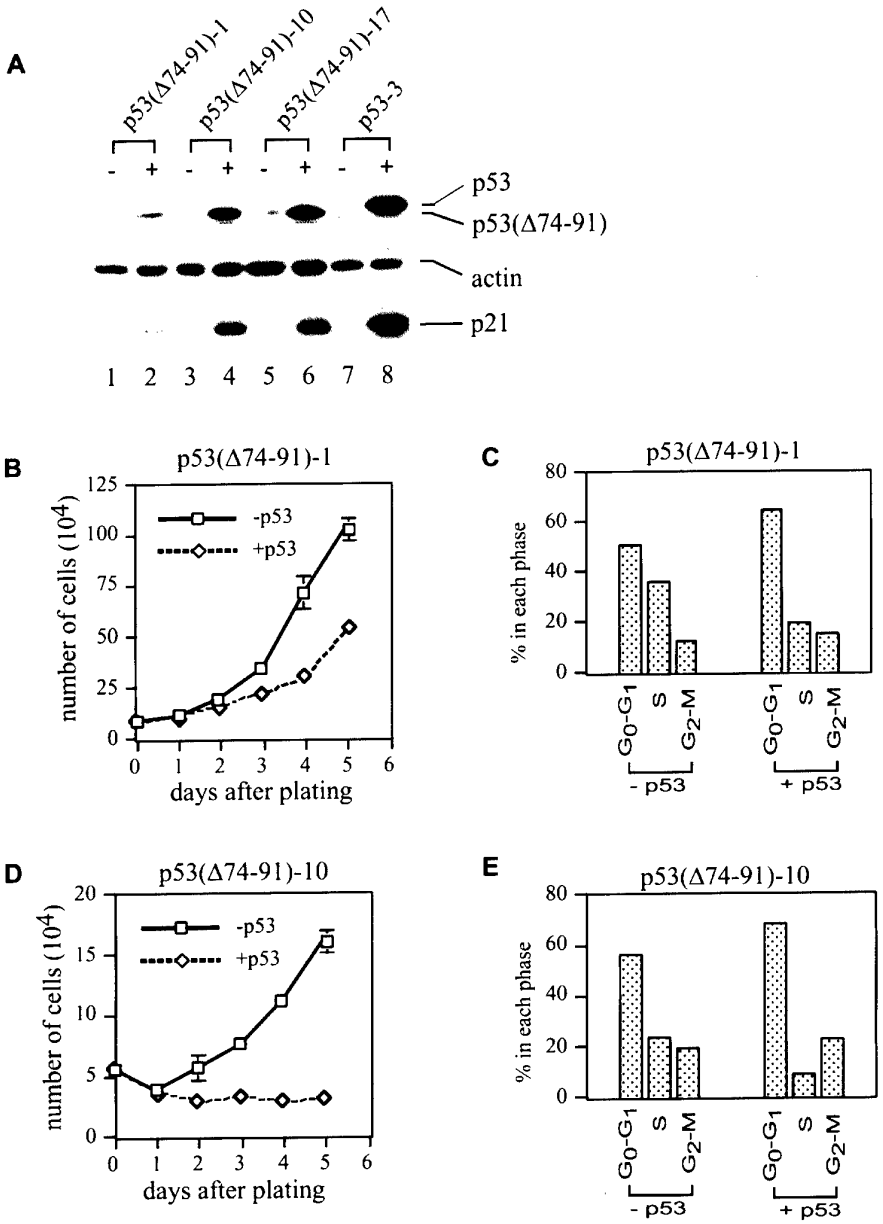

Figure 3 The N-terminal PXXP motif is necessary for mediating apoptosis. To generate p53(Δ74–91), cDNA fragments encoding amino acids 1–73 and 92–393 were amplified independently and ligated together through an internal *Kpn*I site which was artificially created without affecting the amino acid composition of the p53 protein. Primers used for the amino acid 1–73 fragment were: forward primer N1 as used for generating p53(Δ62–91); and reverse primer C73: GAT CGG TAC CGG GGG AGC AGC CTC TGG. Primers used for the amino acids 92–393 fragment were: forward primer N92-1: GAT CGG TAC CCC TGT CAT CTT CTG TC; and reverse primer C393 as used for generating p53(Δ62–91). (**a**) Levels of p53, p21, and actin in p53-3, and p53(Δ74–91)-1, -10, and -17 cells were assayed by Western blot analysis. (**b** and **d**) Growth rates of p53(Δ74–91)-1 and -10 cells in the presence (◇) or absence (□) of p53. (**c** and **e**) The percentage of p53(Δ74–91)-1 and -10 cells in G_0-G_1, S, and G_2-M phases in the presence or absence of p53 for 3 days. The experiments were performed in an identical manner to those shown in Figure 1

results indicate that the apoptotic activity of p53(gln22-ser23) is lost upon deletion of the proline-rich region.

While the human p53 protein contains five PXXP motifs, the rat and murine contain only one and two, respectively (Walker and Levine, 1996). Thus, we investigated whether the dosage of the PXXP motifs can determine the extent of apoptotic response. To this end, we generated p53(Δ74–91), which lacks the C-terminal four PXXP motifs. Ten stable cell lines were established that inducibly express this mutant and three representative cell lines were characterized (Figure 3a). p53(Δ74–91)-1 is a low p53 producer while p53(Δ74–91)-10 and -17 are high p53 producers. Western blot analysis showed that p21 was moderately activated by p53(Δ74–91), to approximately 50% of that activated by wild-type p53 (Figure 3a). Nevertheless, this mutant can efficiently induce cell cycle arrest as measured by the rate of cell growth (Figure 3b and d) or by the reduction of cells in S phase (Figure 3c and e). In addition, we found that 10–17% of cells underwent apoptosis as detected by FACS analysis. These results indicate that p53(Δ74–91) retains a partial apoptotic activity.

Previously, it was shown that the p21 promoter can be activated by a PXXP deletion mutant to an extent that is equal to or greater than by wild-type p53 (Venot *et al*., 1998; Walker and Levine, 1996). A similar result was observed when we tested the ability of p53(Δ62–91) to activate the p21 promoter in a luciferase assay (data not shown). However, Western blot analysis consistently showed that p53(Δ62–91) is much weaker than wild-type p53 to induce p21 (Figure 1a; data not shown). To determine whether the low level of p21 protein detected in p53(Δ62–91)-expressing cells is due to decreased expression of the p21 gene, we performed Northern blot analysis. We found that p21 was induced by p53(Δ62–91) but the extent of induction was approximately 30% of that by wild-type p53 (Figure 4a; Table 1). As controls, we used two previously characterized cell lines, p53(gln22-ser23)-2 and p53(R249S)-4 (Chen *et al*., 1996), which inducibly express the transactivation deficient p53(gln22-ser23)

Table 1 Transcriptional activities of various mutant p53 proteins

	= Fold increase in relative mRNA[a]			
	wild-type p53	*p53 (Δ62–91)*	*p53 (gln22–ser23)*	*p53 (R249S)*
p21	11.1	3.8	1.5	1.2
MDM2	6.6	2.4	1.1	1.2
GADD45	3	5.5	1.2	1.1
BAX	3.5	2.9	1.4	1.0
BTG2	8.2	2.5	ND[b]	ND
p85	2.8	1.5	1.2	1.0
KILLER/DR5	2.7	3.1	1.3	1.1
PIG2	2.5	2.1	1.3	1.0
PIG3	30	2.3	1.1	1.2
PIG6	9.9	ND	ND	ND
PIG7	3.1	3.7	1.2	1.1
PIG8	3.5	3.0	1.2	1.1
PIG11	15.4	ND	ND	ND

[a]Fold = mRNA(+p53)/mRNA(−p53); [b]ND = not done

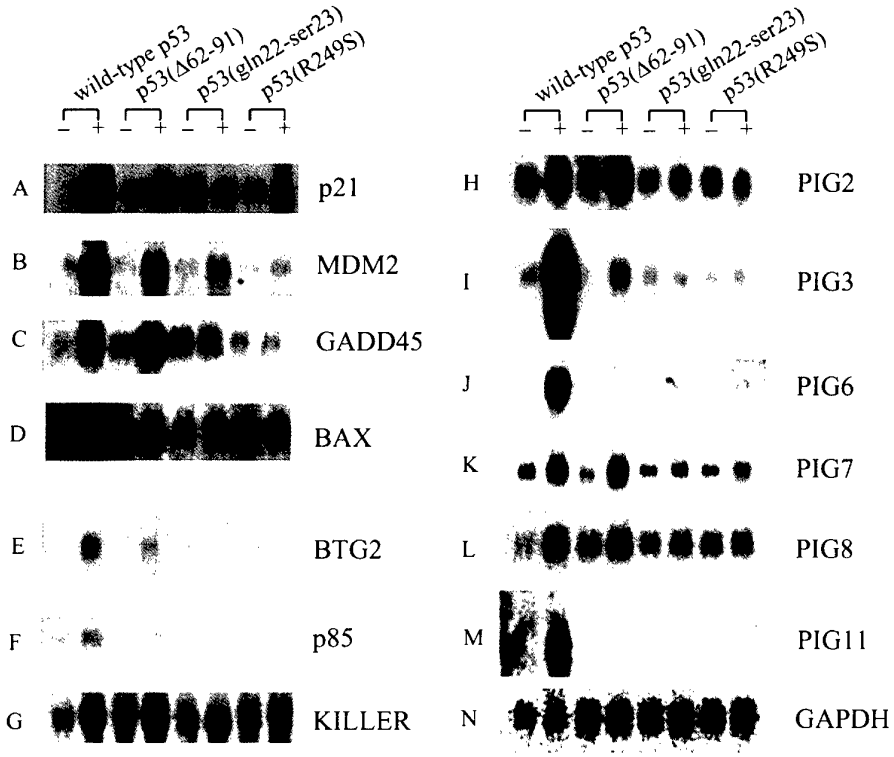

Figure 4 Deletion of the PXXP motifs differentially affects p53 induction of endogenous target genes. Northern blots were prepared using 10 μg of total RNA isolated from uninduced cells (−) or cells induced induced to express (+) wild-type p53, p53(Δ62–91), p53(gln22-ser23) and p53(R249S). Northern blot analysis was performed as described (Zhu *et al*., 1998). The p21, MDM2, BAX, GADD45 and GADPH probes were prepared as described (Zhu *et al*., 1998). The KILLER/DR5 cDNA probe (GenBank #159553) was purchased from American Type Culture Collection. The following cDNA probes were purchased from Genome System, Inc (St. Louis, MO, USA): BTG2 (GenBank #H86711), PIG1 (W61024), PIG2 (H18355), PIG3 (N75824), PIG4 (H45773), PIG6 (R88591), PIG8 (R42786), PIG10 (R87338), PIG11 (R54648), PIG12 (AA149234) and p85 (N21330)

and tumor-derived mtuant p53(R249S), respectively. Both p53(gln22-ser23) and p53(R249S) were inert in inducing p21 (Figure 4a; also see Table 1).

Next, we determined whether p53(Δ62–91) can regulate other p53 target genes, i.e., MDM2, GADD45, BAX, BTG2, p85 and KILLER/DR5. We found that MDM2, an oncogene and a negative regulator of p53 (Wu *et al.*, 1993), was slightly induced by p53(Δ62–91) (Figure 4b; Table 1). However, GADD45, a DNA damage responsive gene involved in DNA repair and growth suppression (Kastan *et al.*, 1992), was substantially induced by p53(Δ62–91) to an extent that is nearly two times more than by wild-type p53 (Figure 4c; Table 1). It is well known that p21 is primarily responsible for p53-dependent cell cycle arrest. Thus, although the induction of p21 is compromised by deletion of the PXXP motifs, it is possible that an enhanced induction of GADD45 may compensate the decreased induction of p21, resulting in cell cycle arrest detected in p53(Δ62–91)-expressing cells (Figure 1). BAX, an apoptosis activator, can be weakly induced by wild-type p53 (Miyashita *et al.*, 1994) as shown in this study (Figure 4d; Table 1). However, BAX expression was only slightly reduced by deletion of the PXXP motifs (Figure 4d; Table 1). BTG2, a nerve growth factor responsive gene that can cause growth suppression (Rouault *et al.*, 1996), was strongly induced by p53 but only slightly by p53(Δ62–91) (Figure 4e; Table 1). p85, a regulatory subunit of the signaling protein phosphatidyl-3-OH kinase (PI(3)K), was shown to be involved in the p53-dependent apoptotic response to oxidative stress (Yin *et al.*, 1998). Upon H_2O_2 treatment, the level of p85 was increased in a p53-dependent manner (Yin *et al.*, 1998). We found that p85 was induced 2–3-fold by wild-type p53 but little if any by p53(Δ62–91) (Figure 4f; Table 1). KILLER/DR5, a death receptor gene which can be induced by genotoxic stress and p53 (Wu *et al.*, 1997), was similarly induced by both wild-type p53 and p53(Δ62–91) (Figure 4g; Table 1).

Several redox-related genes (PIGs), that were shown to be activated by p53 and potentially involved in p53-dependent apoptotic pathway (Polyak *et al.*, 1997), were examined for p53(Δ62–91) induction. We confirmed that PIG2, PIG3, PIG6, PIG7, PIG8 and PIG11 were significantly induced by wild-type p53 in H1299 cells (Figure 4h–m; Table 1). We found that induction of PIG2, PIG7, and PIG8 was not significantly affected by deletion of the proline-rich region (Figure 4h, k and m; Table 1) but induction of PIG3, PIG6 and PIG11 was substantially reduced or abrogated (Figures 3i, 4j and 4m; Table 1). We also found that PIG10 and PIG12 were not substantially induced by p53, and PIG1 and PIG4 were undetectable in H1299 cells (data not shown). Therefore, p53(Δ62–91) induction of the PIG1, PIG4, PIG10 and PIG12 genes was not analysed.

Our studies have confirmed and extended the previous observation (Walker and Levine, 1996) that the proline-rich domain in human p53 is necessary for efficient growth suppression. Specifically, we found that the proline-rich domain is required for p53-dependent apoptosis. Thus, this function is conserved between human and murine p53 (Sakamura *et al.*, 1997). We also found that only the N-terminal PXXP motif is required for mediating apoptosis, indicating that the proline-rich domain contains redundant effector PXXP components. Furthermore, we have shown that the proline-rich domain can differentially regulate p53 induction of endogenous target genes: deletion of the proline-rich domain has no significant effects on induction of Bax, KILLER/DR5, PIG2, PIG7 and PIG8; however, induction of p21, MDM2, BTG2, p85, PIG3, PIG6, and PIG11 were substantially reduced or abrogated. Since p85 is involved in the apoptotic response to oxidative stress (Yin *et al.*, 1998) and PIG3 and PIG6 can produce reactive oxygen species leading to degradation of mitochondria and subsequently apoptosis (Polyak *et al.*, 1997), the results suggest that the PXXP motifs in p53 are required for activating genes that participate directly in signaling pathways controlling apoptosis.

It should be noted that the differential regulation of cellular target genes by p53(Δ62–91) may not be uncovered if its transcriptional activity was measured by its capability of activating the p21 or other target gene promoters in a transient transfection assay. In addition to the p21 promoter, the MDM2, GADD45, and BAX promoters can be activated by p53 lacking the proline-rich domain as efficiently as wild-type p53 (Venot *et al.*, 1998; data not shown). Thus, we should not extrapolate the result observed by the promoter analysis of a target gene to p53 induction of the endogenous target gene. It is well established that the regulation of transcription for endogenous genes that are packaged into chromatin is different from those that are transiently transfected into cells in naked plasmids (Smith and Hager, 1997). The simplified explanation is that the promoter template in a naked plasmid would be easily accessible while that packaged into chromatin is not. It is well known that transcriptional activators function, at least in part, to counteract chromatin-mediated repression (Kadonaga, 1998). For example, MyoD, a basic helix–loop–helix transcriptional activator, contains two domains that are necessary for chromatin remodeling but are not associated with any known activation function (Gerber *et al.*, 1997). While deletion of these two domains lead to very mild effects on activation of transiently transfected templates, activation of endogenous templates depends on these two domains of MyoD. Since the PXXP motifs in p53 have a function similar to these two domains in MyoD in differentially activating transfected and endogenous templates, it suggests that the proline-rich region may be necessary for chromatin remodeling, which is responsible for p53 as transcriptional activator to counteract chromatin-mediated repression for some of the cellular p53 target genes, resulting in their differential induction.

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