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The requirement for the p53 proline-rich functional domain for mediation of apoptosis is correlated with specific *PIG3* gene transactivation and with transcriptional repression

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Wild-type *p53* is a tumor suppressor gene which can activate or repress transcription, as well as induce apoptosis. The human p53 proline-rich domain localized between amino acids 64 and 92 has been reported to be necessary for efficient growth suppression. This study shows that this property mainly results from impaired apoptotic activity. Although deletion of the proline-rich domain does not affect transactivation of several promoters, such as *WAF1*, *MDM2* and *BAX*, it does alter transcriptional repression, reactive oxygen species production and sequence-specific transactivation of the *PIG3* gene, and these are activities which affect apoptosis. Whereas gel retardation assays revealed that this domain did not alter *in vitro* the specific binding to the p53-responsive element of *PIG3*, this domain plays a critical role in transactivation from a synthetic promoter containing this element. To explain this discrepancy, evidence is given for a proline-rich domain-mediated cellular activation of p53 DNA binding.

Keywords: apoptosis/p53/PXXP motif/repression/sequence-specific transactivation

Introduction

p53 mutations are associated with a poor prognosis and drug resistance in human cancers, and the frequent mutation of *p53* in a wide spectrum of human cancers suggests that *p53* inactivation is a crucial and often obligatory step during oncogenesis (Hollstein *et al.*, 1991, 1994; Donehower and Bradley, 1993; Gottlieb and Oren, 1996). The wild-type p53 protein, p53 WT, is a nuclear phosphoprotein and a transcription factor. It is capable of both transactivating through the binding to specific DNA-regulatory sequences (el-Deiry *et al.*, 1992; Funk *et al.*, 1992), and repressing transcription of many cellular and viral promoters that do not contain binding sequences, mainly promoters whose initiation is dependent on the presence of a TATA box (Ginsberg *et al.*, 1991; Kley *et al.*, 1992; Seto *et al.*, 1992; Jackson *et al.*, 1993; Mack *et al.*, 1993; Subler *et al.*, 1994). The importance of sequence-specific transactivation (SST) of genes by p53 is underscored by the fact that the vast majority of *p53* mutations derived from tumors map within the sequence-specific DNA-binding domain (Hollstein *et al.*, 1991; Pavletich *et al.*, 1993; Cho *et al.*, 1994). p53 can mediate either growth arrest or apoptosis, depending on the physiological circumstances (reviewed in Bates and Vousden, 1996; Gottlieb and Oren, 1996; Ko and Prives, 1996; Hansen and Oren, 1997; Levine, 1997).

The underlying mechanism of p53-dependent growth arrest is mediated by $p21^{WAF1/cip1}$, a p53-inducible cyclin-dependent kinase inhibitor (CDKI) (el-Deiry *et al.*, 1993, 1994; Harper *et al.*, 1993; Xiong *et al.*, 1993). The role of p53 in the induction of apoptosis seems much more complex. Although abrogation of $p21^{WAF1/cip1}$ expression results in complete loss of p53-induced cell cycle arrest in human tumor cells (Waldman *et al.*, 1995), the development of $p21^{WAF1/cip1}$-deficient mice has revealed that this cell cycle inhibitor is not the major effector through which p53 helps to maintain the non-transformed phenotype of normal cells (Brugarolas *et al.*, 1995; Deng *et al.*, 1995). It is also clear that $p21^{WAF1/cip1}$ cannot account for the apoptotic activity of p53 (Chen *et al.*, 1996), although it exhibits suppression activity (el-Deiry *et al.*, 1993). On the contrary, $p21^{WAF1/cip1}$ has been postulated to have an anti-apoptotic activity, presumably by causing G_1 arrest (Polyak *et al.*, 1996).

Overexpression of p53 in cells clearly activates genes involved in apoptosis (reviewed in Ko and Prives, 1996; Hansen and Oren, 1997; Levine, 1997). p53 regulates *BAX* (Miyashita and Reed, 1995) and *Fas/APO1* (Owen-Schaub *et al.*, 1995), but neither is required for p53-dependent apoptosis *in vivo* (Knudson *et al.*, 1995; Fuchs *et al.*, 1997). By subtractive hybridization, several DNA damage-inducible p53-regulated genes were isolated: the *IGF-BP3* gene, whose product blocks mitogenic signaling by insulin-like growth factor type 1 (IGF-1) (Buckbinder *et al.*, 1995); the gene encoding the cathepsin-D protease that contributes to cytokine-mediated apoptosis (Wu *et al.*, 1998); *KILLER/DR5*, encoding a member of the tumor necrosis factor receptor family that induces apoptosis when overexpressed (Wu *et al.*, 1997); *PAG608* which encodes a nuclear zinc finger protein (Israeli *et al.*, 1997); and the human homolog of the *Drosophila sina* gene (Nemani *et al.*, 1996). Recently, by using the SAGE technique (serial analysis of gene expression) to evaluate the patterns of gene expression following p53 expression, a series of p53-induced genes (*PIG* genes) have been identified that are predicted to encode proteins that could generate or respond to oxidative stress (Polyak *et al.*, 1997). The *PIG3* product is closely related to a plant NADPH quinone oxidoreductase implicated in apoptosis in plant meristems.

Despite this growing list of p53-regulated apoptotic genes, the extent to which SST contributes to apoptosis remains controversial. In some cases, p53-mediated SST is clearly required (Sabbatini *et al.*, 1995b; Yonish-Rouash

et al., 1995; Attardi *et al.*, 1996; Haupt *et al.*, 1996), and several tumor-derived p53 mutants which have kept transcriptional activation of genes responsible for G_1 arrest but not of *BAX* and *IGF-BP3*, are defective in inducing apoptosis (Friedlander *et al.*, 1996a; Ludwig *et al.*, 1996; Rowan *et al.*, 1996). On the other hand, it has been established that p53-dependent apoptosis can proceed despite the inhibition of protein synthesis (Caelles *et al.*, 1994; Wagner *et al.*, 1994) and that SST-deficient p53 mutants can induce apoptosis in some tumor-derived cell lines (Yonish-Rouach *et al.*, 1994; Haupt *et al.*, 1995, 1996). In addition, it has been reported that WT1, E1B 19 kDa and Bcl-2, three proteins capable of preventing p53-mediated apoptosis, cannot inhibit SST by p53, whereas they can neutralize transcriptional repression by p53 (Shen and Shenk, 1994; Maheswaran *et al.*, 1995; Sabbatini *et al.*, 1995a). This strongly suggests that p53 might induce apoptosis, at least in part, by repression of transcription. In line with this idea, the SAGE experiments of Polyak *et al.* (1997) discovered a larger number of p53-repressed genes and, prior to that, the gene encoding the microtubule-associated protein MAP4 was shown by differential display to decrease in cells following induction of p53 WT (Murphy *et al.*, 1996). Importantly, the decrease in MAP4 was inhibited by E1B 19 kDa and WT1 and seems to be important in the rapid progression of p53-induced apoptosis.

A novel human p53 functional domain necessary for efficient growth suppression but dispensable for transcriptional activation of $p21^{WAF1/cip1}$, *MDM2* and *BAX* promoters has been identified (Walker and Levine, 1996). This domain lies between the N-terminal transactivation domain (amino acids 1–44) that contacts TATA box-binding protein (TBP)-asscociated factor (TAF) components of TFIID (Lu and Levine, 1995; Thut *et al.*, 1995) and the central specific DNA-binding domain (amino acids 102–292) (Bargonetti *et al.*, 1993; Pavletich *et al.*, 1993; Cho *et al.*, 1994). It consists of a proline-rich domain which contains five repeats of the PXXP motif in human p53. Similar motifs have been shown to play a role in signal transduction via their SH3 domain binding activities (reviewed in Cohen *et al.*, 1995; Pawson, 1995). The corresponding murine p53 polyproline domain has been shown to be a docking site in the transmission of Gas1-dependent anti-proliferative signals (Ruaro *et al.*, 1997) and to be required to activate apoptosis but not growth arrest (Sakamuro *et al.*, 1997).

Here we report that the proline-rich domain of human p53 is required for induction of apoptosis, transcriptional repression, reactive oxygen species (ROS) production and transactivation. A p53 mutant lacking the proline-rich domain cannot repress a series of promoters efficiently compared with p53 WT. In addition, it displays distinct transcriptional activities depending on the promoter context. It can activate the natural *MDM2*, *WAF1* and *BAX* promoters efficiently but it is totally silent on the natural *PIG3* promoter. This suggests that the proline-rich domain mediates p53-dependent apoptosis via transcriptional repression and the transactivation of specific apoptogenic genes. The lack of *PIG3* SST is correlated with impaired production of ROS. By using an artificial reporter construct, we demonstrate that the 20 bp *PIG3* promoter-derived p53-responsive element is by itself responsible for the absence of SST by the proline-rich deletion mutant. Gel retardation experiments using baculovirus-expressed proteins revealed no significant quantitative differences between p53 and the proline-rich deletion mutant on the *PIG3* p53-responsive element, whereas similar experiments using proteins produced by expression plasmid transfection in human cancer cells indicated that the proline-rich domain is involved in a cellular activation of p53 DNA binding.

Table I. Apoptosis of H1299 cells following transfection with mutant p53 expression plasmids

Plasmid	Percentage of sub-G_1 in p53/LacZ-positive cells				
	50 ng	0.1 μg	0.2 μg	0.5 μg	1.5 μg
LacZ	nd	nd	nd	nd	10.0 (3.3)
p53 WT	8.1 (2.4)	17.7 (4.1)	25.1 (6.6)	45.9 (12.8)	47.5 (17.6)
D281G	nd	nd	nd	nd	6.9 (1.6)
Δpro	7.1 (2.4)	9.6 (3.5)	11.6 (2.3)	11.3 (2.1)	9.8 (3.5)

Results shown correspond to means and, in parentheses, standard deviations calculated from the compilation of five independent experiments, in which at least two independent plasmid preparations were used. nd, not determined.

Results

The human p53 proline-rich domain is required for apoptotic activity but dispensable for cell cycle inhibition

A p53 deletion mutant called p53 Δpro, identical to a previous construct (Walker and Levine, 1996), was generated by a PCR-based method to create an internal deletion of amino acids 62–91, removing all five of the PXXP motifs from the human p53 protein. This mutant cDNA was inserted into the expression vector pcDNA3 to express the mutant protein under the control of the cytomegalovirus promoter. In a G418-resistant colony formation assay, we confirmed that p53 Δpro has lost growth suppression activity, both in SAOS-2 (human osteosarcoma) and in H1299 (human non-small-cell lung carcinoma), two p53 –/– cell lines. It merely decreased colony formation by 2-fold, whereas the D281G mutant was completely defective and p53WT strongly reduced (10- to 20-fold) the number of drug-resistant colonies (data not shown). In this assay, growth suppression can be the result of either p53-dependent growth arrest or apoptosis.

To determine clearly whether the defect of p53 Δpro in growth suppression mainly results from a lack of apoptotic activity, we studied p53 Δpro in two different apoptotic assays (Table I; Figure 1A and B). First, p53 expression plasmids were microinjected in SAOS-2 cells and their effects were assessed by visualization of cells and TUNEL analysis, 16 or 36 h after microinjection. After 16 h, the number of cells that are detectable by immunostaining for p53 had not diminished whatever the expression plasmid microinjected, but many of the p53 WT-microinjected cells displayed classical signs of apoptosis (shrinking cytoplasm, blebbing nuclei and condensed chromatin) and stained positively by TUNEL analysis (Figure 1A and B). On the other hand, the p53 Δpro and D281G mutants gave only a background signal comparable with the LacZ control. After 36 h, ~80% of the p53 WT-microinjected

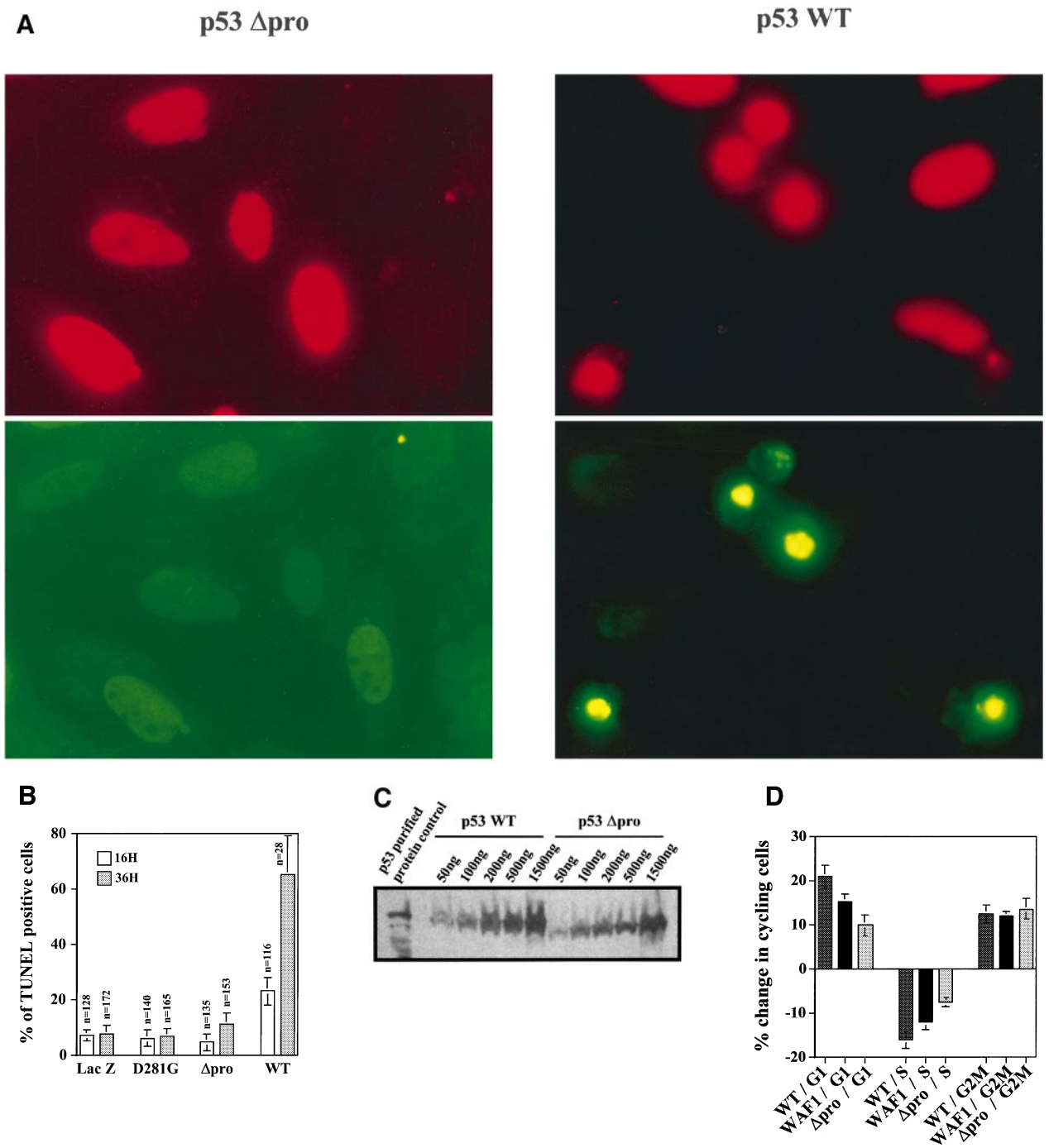

Fig. 1. The proline-rich domain is required for apoptosis but is dispensable for growth arrest. (**A**) p53 WT and p53 Δpro expression plasmids were microinjected into the nuclei of SAOS-2 cells. Cells were immunolabeled for p53 expression (red staining) and for apoptosis (green staining) as described in Materials and methods. (**B**) Apoptosis of SAOS-2 cells after microinjection of lacZ- or p53-expressing plasmids. The percentage of TUNEL-positive cells among microinjected cells is presented as the average and standard deviation from three independent experiments. Above each bar is indicated the total number of remaining microinjected cells from these three experiments that expressed the appropriate transgene. (**C**) Western blot analysis of p53 WT and p53 Δpro after transfection of plasmid doses that correspond to those used in experiments presented in Table I. (**D**) H1299 cells were transiently transfected with p53 WT, p53 Δpro, D281G or $p21^{WAF1/CIP1}$ expression plasmids and analyzed by FACS after incubation with BrdU. Changes in the proportion of the total cell population (after transfection with p53 WT, p53 Δpro or $p21^{WAF1/CIP1}$) in the cell cycle phase (i.e. in G_1, S, G_2M) relative to the total cell population (after transfection with D281G) are shown.

cells had disappeared, while p53 Δpro reproducibly produced a very weak residual apoptotic effect above the background. The same series of microinjection experiments was repeated in H1299 cells and gave similar results (data not shown). Induction of apoptotis was also scored in an assay based on fluorescence-activated cell sorter (FACS) analysis upon transient transfection (Yonish-Rouach *et al*., 1994, Friedlander *et al*., 1996a). In H1299 cells, p53 WT induced apoptosis in a dose-dependent manner (Table I), accordingly to a previous report (Chen *et al*., 1996). Apoptosis was detectable from 0.1 μg of transfected plasmid, but not below, and culminated largely

before the highest plasmid dose used (Table I). p53 Δpro was inactive; however, at high plasmid concentrations, it reproducibly gives a residual specific apoptotic activity, confirming the microinjection results. We have verified by Western blot analysis (Figure 1C) that the protein levels of p53 WT and p53 Δpro were similar over the doses of plasmids introduced into cells.

Taken together, these results eliminate the possibilty that lower expression of p53 Δpro would explain its impaired apoptotic phenotype. Similar experiments using high plasmid concentrations were carried out with SAOS-2 cells, which are not as efficiently transfectable as H1299 cells. The sub-G_1 population was nearly identical to the LacZ, D281G or p53 Δpro expression plasmids, while p53 WT increased this population 2- to 3-fold (data not shown).

The weak growth suppression phenotype of p53 Δpro most probably results from growth arrest. To verify this, cell cycle progression was studied by BrdU incorporation experiments coupled to FACS analysis after transient transfection in H1299 cells. Results presented in Figure 1D showed that p53 Δpro, albeit less efficiently than p53 WT and p21$^{WAF1/cip1}$, was able to increase G_1 and G_2/M populations.

The p53 proline-rich deletion mutant is strongly impaired in repression of full-length promoters but retains partial repression activity on TATA box-containing minimal promoters

Compared with some transcriptionally active p53 tumor-derived mutants (R175P, R181L, V143A) which have impaired apoptotic functions and cannot transactivate some apoptosis-related genes such as *BAX* and *IGF-BP3* (Friedlander *et al*., 1996a; Ludwig *et al*., 1996; Rowan *et al*., 1996), p53 Δpro and the reported murine deletion mutant are unique p53 mutants in that they have been reported to mediate SST of both G_1 arrest genes and apoptotis-associated genes (Walker and Levine, 1996; Sakamuro *et al*., 1997). This suggests that the proline-rich domain plays a crucial role in other biochemical mechanisms involved in the apoptotic activity of p53 WT. There are several lines of evidence indicating that p53-mediated transcriptional repression mediates programed cell death (see Introduction). p53 Δpro was therefore compared with p53 WT for repression of various viral [SV40 early enhancer/promoter, Rous sarcoma virus long terminal repeat (RSV LTR)] and cellular (c-*fos*, multi-drug resistance) promoters (Kley *et al*., 1992; Dittmer *et al*., 1993; Jackson *et al*., 1993). The results presented in Figure 2A demonstrate that, as expected, p53 WT efficiently represses these promoters at transfected plasmid doses where apoptosis does not occur (Table I). In contrast, like the D281G tumor-derived p53 mutant, p53 Δpro has, in great part, lost repression activity at low plasmid doses (Figure 2A) and also at higher doses (data not shown). On the other hand, the repression activity was not lost to such a great extent either with the pUHC13.6 vector, in which basal transcription is driven by the TATA box-containing minimal region from the human cytomegalovirus promoter IE (Gossen and Bujard, 1992), or with the pGL2 control vector, in which the SV40 enhancer sequence is separated from promoter sequence (Figure 2B). Once again, p53 Δpro and D281G are virtually indistinguishable in these promoter repression assays.

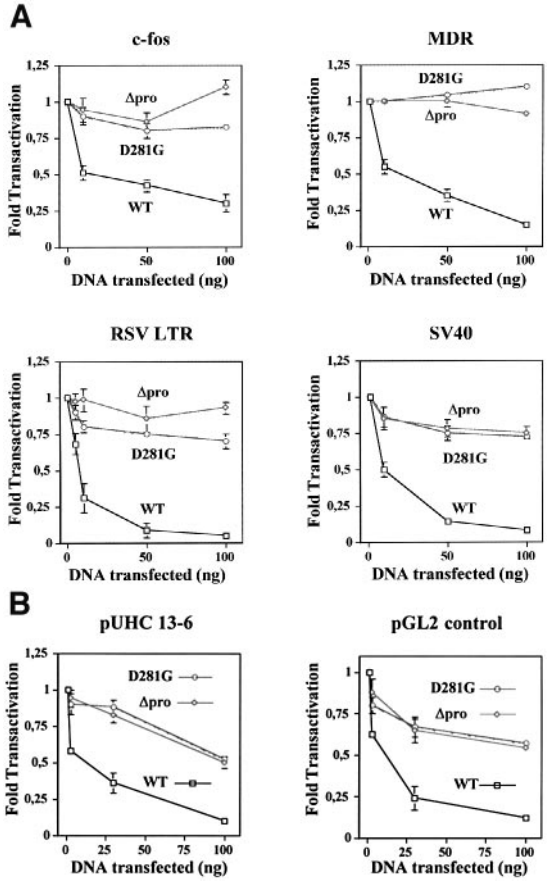

Fig. 2. Repression activity of p53 Δpro in H1299 cells. (**A**) Various viral (upstream of the luciferase reporter) and cellular (upstream of the CAT reporter) promoters were transfected in the presence of p53 WT, Δpro and D281G expression plasmids. The fold-transactivation of the reporter plasmid was calculated over its basal level of expression. (**B**) pUHC13-6 and pGL2 controls (upstream of the luciferase reporter) were transfected with p53 WT, p53 Δpro or D281G expression plasmids.

The p53 proline-rich deletion mutant can transactivate p21$^{WAF1/cip1}$, MDM2 and BAX promoters efficiently

The ability of p53 Δpro to mediate G_1 arrest was correlated with induction of endogenous p21$^{WAF1/cip1}$ and MDM2 proteins. Both proteins were detected at similar levels by Western blot analysis 20 h after transient transfection with p53 Δpro and p53 WT expression plasmids in H1299 cells (Figure 3A). The transactivation activities of p53 Δpro and p53 WT were also compared in co-transfection experiments in H1299 cells. p53 Δpro transactivated the natural *WAF1* and *MDM2* promoters to extents similar to p53 WT. However, there were some differences with p53 WT (Figure 3B). p53 Δpro did not squelch transcriptional activation at high concentrations on the *WAF1* promoter. The loss of squelching activity is reminiscent of the loss of repression activity reported in the preceding paragraph. It has been verified that the levels of the two proteins are very similar at the various concentrations of plasmids transfected (Figure 3C). Similar results were obtained in SAOS-2 cells (data not shown). We also reinvestigated the ability of this mutant to transactivate the apoptosis-

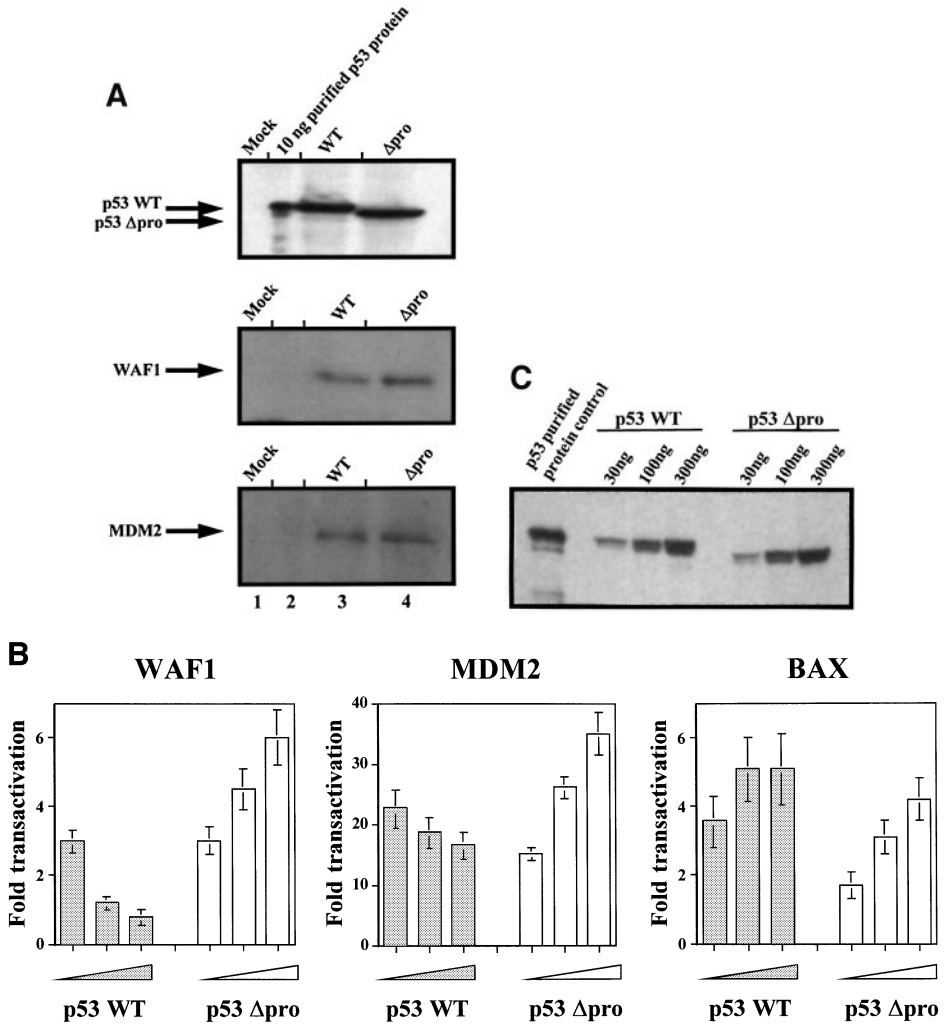

Fig. 3. (**A**) $p21^{WAF1/CIP1}$ and MDM2 Western blot analysis. H1299 cells (10^6) were transiently transfected with 5 μg of empty (lane 1), p53 WT (lane 3) or p53 Δpro (lane 4) expression vectors. Cell lysates were analyzed for p53, $p21^{WAF1/CIP1}$ and MDM2 protein expression. Lane 2 corresponds to a purified p53 protein standard. (**B**) Transcriptional transactivation activities in H1299 cells. *WAF1*, *MDM2* and *BAX* promoters (upstream of the luciferase reporter) were transiently transfected with 30, 100 or 300 ng of p53 WT or p53 Δpro expression plasmids. The fold transactivation of the reporter plasmid was calculated over its basal level of expression. (**C**) Western blot analysis of p53 WT and p53 Δpro after transfection with plasmid doses (30, 100 or 300 ng) that correspond to those used in the experiments in (B).

related gene, *BAX*, in transient transfection experiments in H1299 cells. Data presented in Figure 3B show that *BAX* promoter SST by p53 Δpro did not significantly differ from SST by p53 WT. The proline-rich domain was clearly dispensable for SST of these promoters.

p53 Δpro has completely lost the ability to produce reactive oxygen species and to transactivate the PIG3 gene

Among the recently discovered p53-inducible *PIG* genes, several can be considered to influence the avaibility of intracellular ROS and, consequently, to participate in p53-induced apoptotic processes. In a series of colorectal cancer cell lines, pharmacological experiments suggested that the redox enzyme encoded by *PIG3* is necessary for this process, whereas *PIG3* overexpression is not sufficient to induce apoptosis (Polyak *et al*., 1997). As shown in Figure 4A, p53 WT stimulated ROS production in H1299 cells, as it does in colorectal cancer cell lines, indicating that p53 might induce apoptosis in H1299 cells by a similar mechanism. ROS production was measured after p53 WT transfection using carboxymethyl difluorescein diacetate and flow cytometry. Interestingly, p53 Δpro did not have this effect (Figure 4A). In line with these results, RT–PCR analysis showed that endogenous *PIG3* transactivation was triggered by p53 WT but not by p53 Δpro (Figure 4B). Study of the promoter region of the *PIG3* gene revealed that it contains a functional 20 bp p53-responsive element (Polyak *et al*., 1997). We therefore studied SST of the *PIG3* promoter by p53 WT and p53 Δpro. We used the reported 0.7 kb *PIG3* promoter–luciferase reporter construct in co-transfection assays in H1299 cells. p53 WT strongly activated this promoter, without squelching, at the highest p53 WT concentration tested. As expected from the effect on the endogenous gene, p53 Δpro was totally inactive, even at very high expression plasmid concentrations (Figure 4C).

Comparisons of the in vitro binding capacities of p53 WT and the p53 proline-rich deletion mutant

The absence of SST on the *PIG3* promoter was surprising, since the p53 core domain responsible for sequence-

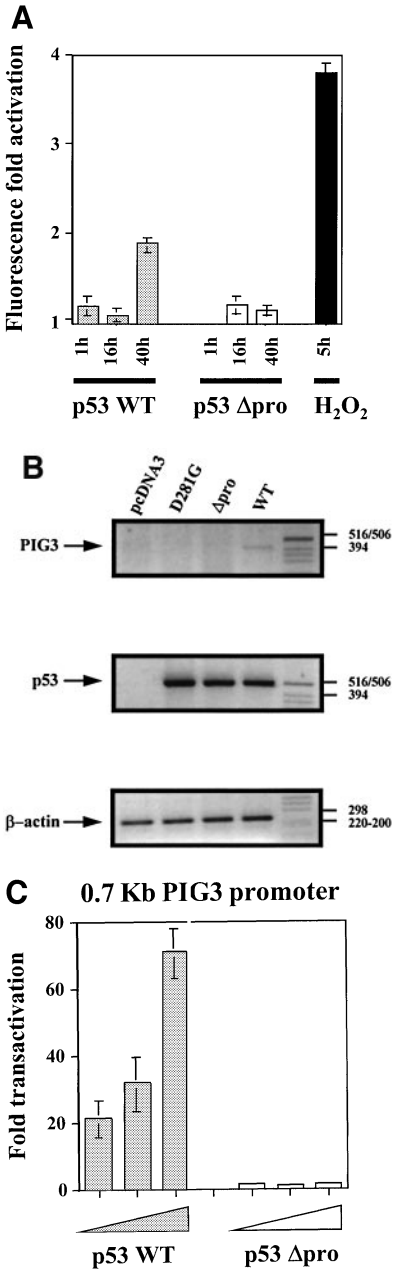

Fig. 4. (**A**) p53 WT but not p53 Δpro produces ROS in H1299 cells. Cells were transiently transfected with CD20 and p53 expression plasmids. FACS analysis was performed on positive CD20 cells (except for cells analysed at 1 h) and then the mean of carboxymethyl dichlorofluorescein diacetate fluorescence was calculated. As a positive control, ROS production was measured 5 h after treatment with 30 mM H_2O_2. (**B**) RT–PCR analysis of *PIG3* transcripts in p53 transiently transfected H1299 cells. H1299 cells were transiently transfected with empty vector, D281G, p53 Δpro or p53 WT expression plasmids. One fragment that has the 423 bp expected size was amplified only in p53 WT-transfected cells using *PIG3* oligonucleotides. As a control to ensure that the same amount of cDNA was used in all experiments, the cDNA encoding β-actin was amplified with specific oligonucleotides. To control p53 mRNA expression, a fragment that has the 513 bp expected size was amplified using p53 oligonucleotides. (**C**) The proline-rich domain is required for *PIG3* transactivation. The 0.7 kb *PIG3* promoter (upstream of the luciferase reporter) was transiently transfected with 30, 100 or 300 ng of p53 WT and p53 Δpro expression plasmids. The fold-transactivation of the reporter plasmid was calculated over its basal level of expression.

specific DNA binding is functional in p53 Δpro and can mediate p21$^{WAF1/cip1}$, *MDM2* and *BAX* SST. Gel retardation assays were performed to determine whether specific binding to the *PIG3* promoter-derived p53-binding site was affected by the proline-rich domain deletion. For these experiments, p53 WT, the D281G mutant and p53 Δpro were produced as nuclear extracts in Sf9 insect cells using recombinant baculoviruses under strictly identical infection conditions. For each nuclear extract, Western blot analysis using monoclonal antibodies directed against N-terminal (DO-1), central (PAb240) and C-terminal (PAb421) epitopes demonstrated the absence of detectable p53 degradation products (data not shown). Densitometric analysis of Coomassie Blue-stained SDS–PAGE gels showed that the p53 proteins represented ~45% of the total protein. The absolute amounts of p53 were estimated both by densitometric analysis of Coomassie Blue-stained SDS–PAGE gels and by the Bradford assay, with bovine serum albumin (BSA) as a standard. Both methods gave similar results (data not shown). These extracts were incubated with ^{33}P end-labeled DNA probes containing the 20 bp *PIG3*-derived (Polyak *et al*., 1997), the 20 bp *WAF1*-derived (el-Deiry *et al*., 1993) or the 39 bp *BAX*-derived sequences (Miyashita and Reed, 1995). Monoclonal antibody PAb421 directed against p53 was also included in some samples to help stabilize p53–DNA interactions (Hupp *et al*., 1992). As shown in Figure 5A, for each of the three DNA elements, p53 Δpro (lanes 1, 3 and 5) produced a protein–DNA complex as efficiently as p53 WT (lanes 2, 4 and 6, the slightly faster migration is due to the molecular weight difference). As a control, D281G in the presence of PAb421 did not give a band shift with any sequence (lanes 9, 12 and 15). With p53 WT (lanes 8, 11 and 14) and p53 Δpro (lanes 7, 10 and 13), protein–DNA complexes were stabilized and supershifted by PAb421. These retardation gel experiments demonstrated that the 20 bp *WAF1*-derived sequence is a much more potent *in vitro* target DNA probe than the 20 bp *PIG3*-derived sequence, the 39 bp *BAX*-derived sequence being intermediate (Figure 5A). The hierarchy between *WAF1* and *BAX* is consistent with a previous report (Friedlander *et al*., 1996a).

The affinity constants for the *PIG3* element were calculated. Active protein concentrations in the respective nuclear extracts were first determined using the high affinity *WAF1* element. The results were approximately equivalent (~80%, data not shown) to the calculations of the absolute concentrations, provided that both proteins bound to the *WAF1* element in the ratio of one p53 tetramer per DNA probe molecule (Balagurumoorthy *et al*., 1995). K_d values for both PAb421-stabilized proteins and the *PIG3*-responsive element were calculated by non-linear regression analysis from a series of gel retardation assays to yield 45 and 25 nM for p53 Δpro and p53 WT, respectively (Figure 5B). Both proteins thus exhibited similar and low affinities. However, it is noteworthy that activation by PAb421 produced band shifts with patterns slightly different between p53 Δpro and p53 WT.

Comparisons of WAF1 and PIG3 p53-responsive elements in the same promoter environment

A synthetic reporter gene construct called *pLuc-PIG3*, containing the 20 bp *PIG3* promoter-derived p53-respons-

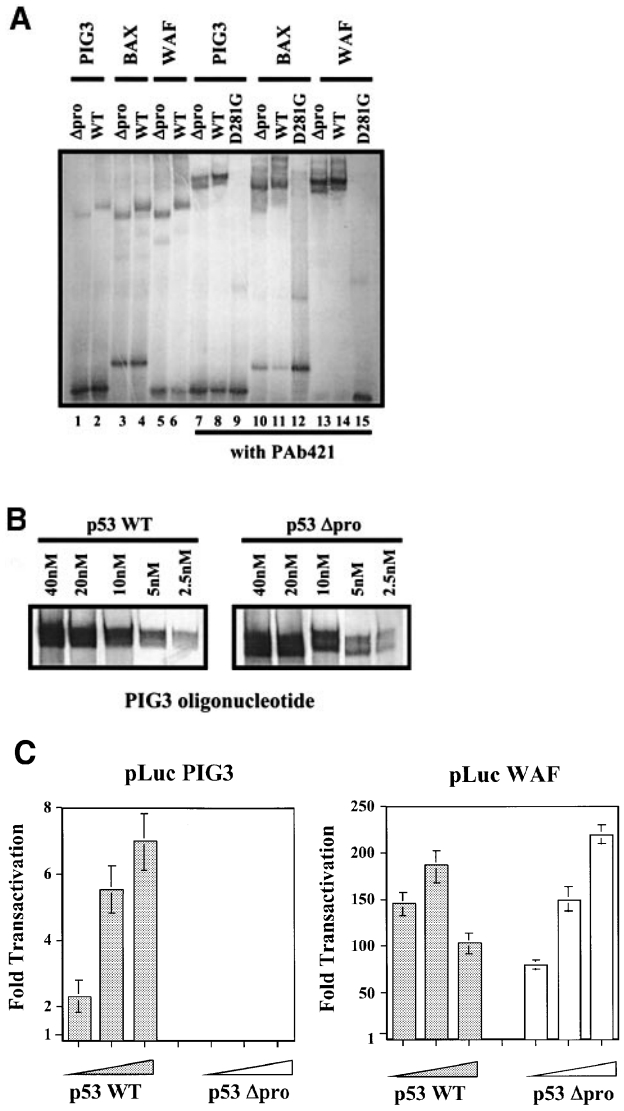

Fig. 5. (**A**) Gel retardation assays with end-labeled double-stranded oligonucleotides containing p53-binding sites. *PIG3*, *BAX* and *WAF1* probes (2.4 nM) were incubated with p53 WT, p53 Δpro and D281G baculovirus-expressed proteins (p53 absolute concentration: 10 nM) in the absence or presence of PAb421. (**B**) p53 WT and p53 Δpro affinities for the *PIG3* p53-binding site. The autoradiograms presented are representative of the series of assays carried out to calculate K_d values. The indicated amounts of p53 WT and p53 Δpro proteins were incubated with the *PIG3* oligonucleotide (2.4 nM). (**C**) p53 Δpro is unable to transactivate a synthetic promoter containing the *PIG3* p53-responsive element. pLuc-PIG3 and pLuc-WAF1 were transiently transfected with 30, 100 or 300 ng of p53 WT and p53 Δpro. The fold-transactivation of the luciferase reporter plasmid was calculated over its basal level of expression

ive element associated with a basal promoter element including the TATA element from the human *HSP70* upstream from the luciferase gene, was constructed. Co-transfection experiments showed that this construct was transactivated by p53 WT, but not by p53 Δpro (Figure 5C). As a control, a strictly homologous synthetic promoter called pLuc-WAF1 and containing the 20 bp *WAF1*-derived p53 responsive element was constructed and tested. pLuc-WAF1 did not discriminate between p53 WT and p53 Δpro (Figure 5C). Taken together, these results indicate that the discrimination between p53 WT and p53 Δpro was mediated entirely by the 20 bp *PIG3*-

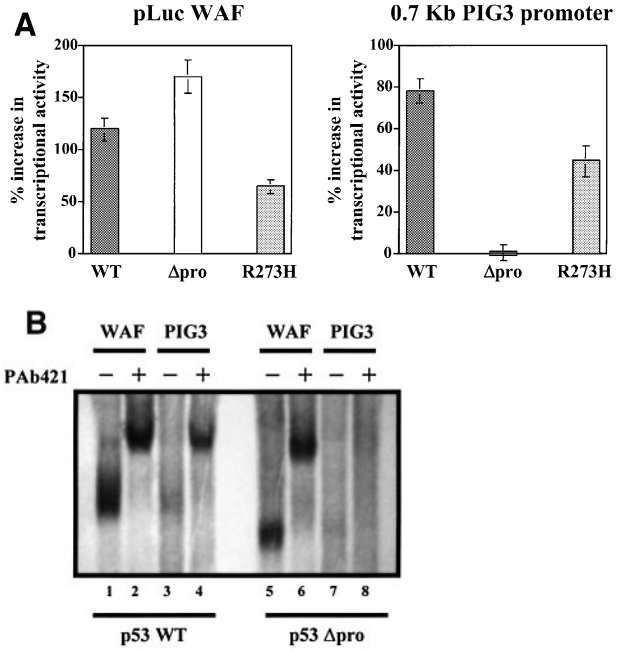

Fig. 6. (**A**) PAb421 scFv cannot restore *PIG3* SST by p53 Δpro. p53 expression plasmids (300 ng) and the relevant gene reporter constructs were transfected with or without 900 ng of PAb421 scFv expression plasmid. The percentage increase in transcriptional activity represents the ratio between fold-activations due to p53 with and without scFv PAb421. (**B**) The proline-rich domain is required for cellular activation of DNA binding to the *PIG3* element. Cell-free extracts from p53 WT- or p53 Δpro-transfected H1299 cells were incubated with *WAF1* or *PIG3* oligonucleotides (2.4 nM) in the presence (+) or absence (–) of PAb421.

derived responsive element and was not merely due to the absence of a typical TATA box or to any other specific feature of the 0.7 kb *PIG3* promoter (Polyak *et al*. 1997). Moreover, the efficiency of both synthetic promoters seemed to be related to their affinity for p53.

A single chain antibody derived from PAb421 cannot restore PIG3 SST by p53 Δpro

The monoclonal antibody PAb421 which recognizes a p53 C-terminal epitope can enhance the DNA-binding activity of p53 WT *in vitro* (Hupp *et al*., 1992) and the transcriptional activity of p53 WT and of a subset of p53 mutants in cells upon microinjection (Abarzua *et al*., 1995; Hupp *et al*., 1995). We have tried to use a functional single chain antibody (scFv) derived from this antibody and called PAb421 scFv to restore *PIG3* SST by p53 Δpro, since this scFv was shown to activate SST by the tumor-derived mutant R273H (C.Caron de Fromentel, N.Gruel, C.Venot, L.Debussche, E.Conseiller, C.Dureuil, J.-L. Teillaud, B.Tocqué and L.Bracco, submitted). An increase in SST was observed with p53 WT and with R273H on pLuc-WAF and *PIG3* promoter constructs (Figure 6A). p53 Δpro was also sensitive to PAb421 scFv activation on the pLuc-WAF element. In contrast, such activation could not be detected on the *PIG3* promoter.

The proline-rich domain is required for cellular activation of DNA binding to the PIG3 element

The discrepancy between *in vitro* DNA-binding properties (Figure 5A and B) and *PIG3* SST inactivity (Figures 4C and 5C) led us to assume that in cells the proline-rich

domain is required for a cellular stimulation of DNA binding to the *PIG3* sequence. We thus examined the DNA-binding capacities of p53 WT and p53 Δpro expressed upon transfection in H1299 cells. Figure 6B revealed that p53 WT-containing H1299 cellular extracts bound much more efficiently to the *PIG3* element than did p53 Δpro and that this DNA binding was strongly enhanced by PAb421 only with p53 WT. As a control, the same experiment was carried out in parallel with the *WAF1* element. No such difference between p53 WT and p53 Δpro was observed. This led us to conclude that a DNA-binding activation mechanism requiring the proline-rich domain is absolutely necessary for the *PIG3* element.

Discussion

A new functional domain necessary for efficient growth suppression but not for SST has been discovered recently in the N-terminal domain of human p53 (Walker and Levine, 1996). This domain consists of five repeats of PXXP, a motif known to form a left-handed polyproline type II helix, which creates a binding site for SH3 domains (Yu *et al.*, 1994). p53 from other species also exhibit related domains, to variable extents (Walker and Levine, 1996). In mouse, the functionality of this domain has been demonstrated (Sakamuro *et al.*, 1997). The human and mouse PXXP domains are dispensable for SST, but are required for growth suppression. Murine p53 devoid of this domain is impaired in apoptosis but can mediate growth arrest. This study clearly establishes that the proline-rich domain of human p53 is required for apoptosis in both H1299 and SAOS-2 cells, in which SST by p53 is either absolutely necessary or dispensable for apoptosis, respectively (Haupt *et al.*, 1996). Moreover, the proline-rich domain of human p53 is not required for growth arrest activity, confirming that G_1 arrest and apoptosis are separable p53 activities. Although the mechanism by which p53 mediates apoptosis is poorly understood, several studies have demonstrated that different p53 biochemical activities are required to induce apoptosis, among which repression and SST of apoptosis-related genes play a crucial role (see Introduction). We show that the absence of this proline-rich domain severely compromised both of these apoptosis-related activities of p53 in the lung carcinoma cell line H1299.

Repression by p53 may accelerate the progression of apoptosis (Murphy *et al.*, 1996). Study of the transactivation double-mutant L22Q, W23S suggests that repression may not be essential, since this mutant can induce some apoptosis in some cell lines, including H1299, even though it is impaired for repression (Haupt *et al.*, 1995; Chen *et al.*, 1996; Roemer and Mueller-Lantzsch, 1996). In contrast, that p53 Δpro is impaired for both apoptosis and repression function strengthens the idea of a critical role for repression in apoptosis by p53. The mechanism by which p53 represses promoters is not clear. Deletion analysis and use of the L22Q, W23S double mutant have implicated the TBP and TAFs as targets for this activity (reviewed in Gottlieb and Oren 1996; Ko and Prives, 1996). It is noteworthy that many p53 tumor-derived mutants still interact with TBP and TAFs but have lost promoter-repression activity (reviewed in Zambetti and Levine, 1993; Gottlieb and Oren, 1996; Ko and Prives, 1996), showing that interactions with TBP and TAFs are necessary but not sufficient. Our finding that the proline-rich domain is also necessary adds an unexpected complication. Our data show that the proline-rich deletion and mutation in the DNA-binding core domain led to the same phenotype, i.e. absence of repression of full-length promoters but partial repression of minimal promoters or of a promoter in which enhancer sequence has been displaced. It is therefore tempting to suggest that this partial repression would reflect a residual squelching activity presumably due to interaction with TBP and TAFs, whereas repression of full-length promoters by p53 involves a more complex mechanism than simple squelching, which requires the wild-type conformation and the proline-rich domain, in addition to interactions with factors such as TBP and TAFs.

This study confirms that the proline-rich domain is dispensable for SST of growth arrest and *BAX* genes (Walker and Levine, 1996; Sakamuro *et al.*, 1997). This is not in disagreement with the requirement for this domain for mediation of apoptosis, since increased *BAX* expression is not detected during p53-mediated apoptosis in every cell type, especially in SAOS-2 and H1299 cells (Allday *et al.*, 1995; Canman *et al.*, 1995; Ludwig *et al.*, 1996; Rowan *et al.*, 1996). However, we have demonstrated that the proline-rich domain is required for ROS production and for endogenous *PIG3* gene transactivation. The absence of *PIG3* SST by the p53 Δpro mutant observed both with the 0.7 kb promoter and with a synthetic promoter containing the p53-responsive element of *PIG3* associated with a TATA box demonstrated that *in vivo* the 20 bp *PIG3*-derived sequence alone discriminates between p53 Δpro and p53 WT. Gel retardation assays did not reveal significant differences in K_d values between p53 WT and p53 Δpro for the binding to *PIG3*, as well as to *WAF1* and *BAX* p53-binding sequences. Besides, one has to bear in mind that the p53 *in vitro* affinity for the *PIG3* sequence is very low, compared with other p53 target sequences. If we accept the idea of a threshold of affinity below which SST could not occur, this would imply that in cells the proline-rich domain would be required for activation of DNA binding on low affinity p53-responsive elements such as that of *PIG3*. This DNA-binding activation necessary for *PIG3* SST could result from a conformational change. It would not be the first example of such a role played by a p53 structural domain (Hupp *et al.*, 1992). Gel retardation experiments using cell-free extracts from p53-transfected H1299 cells revealed that such a proline-rich domain-mediated DNA-binding stimulation does exist for the *PIG3* promoter. By using the scFv technology, we also demonstrated that loss of DNA-binding activation through the proline-rich domain cannot be compensated by the other described activation mechanism, which involves the p53 C-terminal regulatory domain (Hupp *et al.*, 1992, 1995; Abarzua *et al.*, 1995). That antibodies directed against epitopes close to proline-rich domains have been shown to modulate DNA binding lends strong support to the idea that this domain would be a docking site for an activating factor(s) (Wolkowicz *et al.*, 1995; Friedlander *et al.*, 1996b).

This study extends conclusions from previous reports which have suggested the existence of discrete classes of p53-responsive genes. Evidence for such classes came

from the study of specific tumor-derived mutants (Friedlander *et al.*, 1996a; Ludwig *et al.*, 1996, and references therein). This work clearly shows that a p53 mutated outside the specific DNA-binding core is also able to discriminate between p53-responsive genes and that this discrimination is not necessarily correlated with quantitative alterations of *in vitro* DNA-binding capacities. Alternatively, the role of phosphorylation in the differential stimulation of the binding to various DNA sites has been documented (Wang and Prives, 1995; Lohrum and Scheidtmann, 1996). Proline-rich domain could be also implicated in the control of p53 phosphorylation status.

DNA damage induces p53 overexpression which then results in either apoptosis or cell cycle arrest. The molecular basis of the choice between G_1 arrest and apoptosis, in other words, life and death, is enigmatic. In colorectal cancer cell lines, this choice has been shown to be determined by at least two factors: protection from apoptosis by $p21^{WAF1/cip1}$, and as yet unidentified *trans*-acting factors that can overcome this protection (Polyak *et al.*, 1996). In some cell types, mainly hematopoetic cells, several reports demonstrate that cytokines are influential in the death/life decision by p53 (reviewed in Bates and Vousden, 1996; Gottlieb and Oren, 1996; Ko and Prives, 1996; Hansen and Oren, 1997). What might be the molecular mechanism utilized by the proline-rich domain to participate in SST of apoptotis-related genes such as *PIG3* and repression of promoters by p53? We can propose that the proline-rich domain is a docking site for SH3 proteins which would regulate p53-dependent apoptosis by altering these two p53 biochemical activities critical for p53-dependent apoptosis. Identifying such hypothetical SH3-bearing proteins and deciphering their regulatory functions would greatly help in solving the enigma of p53 death/life decision and to understand better p53 function in general. Mutations of critical prolines within the proline-rich functional domain and dominant-negative mutants containing the proline-rich domain have been shown to abolish p53-mediated transactivation-independent growth arrest induced by *Gas1* (Ruaro *et al.*, 1997). This lends strong support to the idea that this domain may be a docking site for regulatory SH3-bearing proteins.

Materials and methods

Plasmids

p53 Δpro cDNA was generated by PCR as described (Walker and Levine, 1996) and cloned in the pcDNA3 vector (Invitrogen) between the *Hin*dIII and *Not*I sites. pcDNA3 expression constructs for p53 WT, R273H and D281G have been described previously (Conseiller *et al.*, 1998) as have the human c-*fos* promoter (–710/+41) construct (Deschamps *et al.*, 1985), the *MDR* promoter construct (Dittmer *et al.*, 1993), the PUHC13-6 plasmid (Gossen and Bujard, 1992), the WWP-luc construct containing a 2 kb fragment from the *WAF1* promoter (el-deiry *et al.*, 1992), the 0.7 kb *PIG3* promoter construct (Polyak *et al.*, 1997), CD20 expression plasmid (Dubs-Poterszman *et al.*, 1995) and the pGL2 control vector (Promega). Fragments of 0.4 kb containing p53-responsive elements from *MDM2* (Juven *et al.*, 1993) or *BAX* (Miyashita and Reed, 1995) genes were inserted upstream from the luciferase gene in pGL3-basic plasmid (Promega) (Conseiller *et al.*, 1997). The human *WAF1* cDNA clone was obtained by PCR following a previously described procedure (Parker *et al.*, 1996) and inserted in the pcDNA3 vector. pLuc-PIG3 and pLuc-WAF1 were constructed as described (Funk *et al.*, 1992) by inserting *PIG3* and *WAF1* double-stranded oligonucleotides described below upstream from a basal promoter element including the TATA element from the human *HSP70* gene and the luciferase gene. RSV LTR–luciferase reporter gene vector was obtained by inserting luciferase cDNA from the pGL2 control vector (Promega) into pRC-RSV plasmid (Invitrogen).

Cells and transfections

H1299 and SAOS-2 cells (ATCC) were maintained at 37°C, 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS). For transfection, cells were seeded at 1.5×10^5 cells per multiwell six culture testplate and transfected with 1.5 μg of plasmid mixtures pre-incubated for 30 min with 5 μl of lipofectamine reagent (Gibco-BRL, Gaithersburg, MD). Selection of drug-resistant colonies was performed as follows: 24 h after transfection, cells were seeded in 10 cm plates, cultured in 400 μg/ml G418-containing medium for 2–3 weeks and then colored with fuchsin.

Gene reporter assays

For each transfection, 0.3 μg of reporter plasmid was used and, 48 h after transfection, cells were rinsed with phosphate-buffered saline (PBS) and reporter gene assays were carried out. Each experiment was repeated at least three times to calculate the mean and standard deviation.

For the luciferase assay, cells were resuspended in cell lysis buffer (Promega) and incubated for 20 min at room temperature. Samples were centrifuged and the luciferase activity of the cleared supernatant was determined in the presence of luciferin (Promega) and ATP. For the CAT assay, cells were resuspended in 150 μl of 0.25 M Tris–HCl pH 8, lysed by freezing three times in dry ice/ethanol, clarified by centrifugation, and the soluble material assayed for CAT activity by incubation for 1 h at 37°C in 0.18 M Tris–HCl pH 8, 0.4 mM acetyl-coenzyme A, 23 μM cloramphenicol, D-threo-(dichloroacetyl-1,2-14C) (200 nCi) (Amersham Corp.). CAT activity was quantified by counting the acetylated chloramphenicol forms in an Instantimager (Packard Instruments).

Western blot analysis and antibodies

Cells (10^6) were collected 24 h after transfection and lysed for 15 min at 4°C with 50 μl of RIPA buffer and centrifuged at 15 000 r.p.m. for 15 min. The supernatants were collected, loaded onto 10 or 14% SDS–PAGE gels and blotted onto PVDF membranes (New England Nuclear, Boston, MA). WAF1 and MDM2 were detected with rabbit polyclonal antibodies together with an anti-rabbit, horseradish peroxidase (HRP)-conjugated secondary antibody. p53 WT and p53 Δpro were detected with DO-1 monoclonal antibody together with an anti-mouse HRP-conjugated secondary antibody. Proteins were visualized by chemiluminescent detection (ECL) (Amersham). DO-1, PAb240 and PAb421 antibodies were purchased from Oncogene Sciences, and anti-LacZ monoclonal antibody from Monosan, The Netherlands.

Flow cytometry

Each experiment was repeated at least three times to calculate the mean and standard deviation for FACS analysis and apoptotic detection. At 48 h after transient transfection, adherent and detached cells were combined. Cells were fixed for 30 min with paraformaldehyde at room temperature, washed twice with PBS (Gibco-BRL), permeabilized for 10 min with PBS, 0.1% Triton X-100, washed twice in PBS, 0.1% Tween-20, incubated for 1 h with PBS, 0.1% Tween-20 and 1 μg DO1 (or anti-LacZ), washed twice with PBS, 0.1% Tween-20, incubated for 1 h with GAM–FITC (Immunotech, Marseille, France), washed twice with PBS, 0.1% Tween-20 and incubated for 1 h with 5 μg of propidium iodide and 1 mg of boiled RNase (DNase-free) before FACS analysis on a FACS Epics Elite ESP II (Coulter, Miami, FL).

For cell cycle analysis, 30 min prior to harvesting with trypsin-EDTA, 30 μM BrdU was added to culture media. After rinsing with PBS, cells were fixed overnight in 1% paraformaldehyde, then incubated at room temperature for 1 h in a pepsin solution (0.15% pepsin, 0.9% NaCl, 1 M HCl, 1% Triton X-100) on a rotisserie. After rinsing three times with PBS, 0.5% Tween-20, cells were incubated with mouse monoclonal anti-BrdU (Becton Dickinson) in PBS, 2% BSA, 0.5% Tween-20 for 1 h, rinsed twice with PBS, 0.5% Tween-20, incubated for 1 h with GAM–DTAF (Immunotech, Marseille, France), rinsed twice and resuspended in 1 ml of PBS containing 1 mg of boiled RNase and 10 μg of propidium iodide. A total of 10^4 cells were then analyzed on a FACS Epics Elite ESP II (Coulter, Miami, FL) for the quantification of S phase and DNA content.

For measurement of ROS production, H1299 cells were transiently transfected with 0.5 μg of CD20 and 1 μg of p53 WT, p53 Δpro or D281G expression plasmids, and ROS production was measured by using a method adapted from a previous report (Polyak *et al.*, 1997). Adherent cells were harvested by using PBS-EDTA 1, 16 or 40 h after

transfection, and incubated with 10 μM carboxymethyl dichlorofluorescein diacetate. Cells were immunostained on ice with anti-CD20 biotin (Coulter) and strepavidin Cy-chrome (Pharmingen). FACS analysis was performed on positive CD20 cells (except for cells analysed for 1 h) and then the mean fluorescence was calculated.

RT–PCR analysis

A total of 10^6 H1299 cells were transiently transfected with 6 μg of empty vector, D281G, p53 Δpro or p53 WT expression plasmids and, 16 h after transfection, total RNA was extracted with RNA NOW solution (Biogentex). Then 5 μg of total RNA was reverse transcribed by using a cDNA synthesis kit (Boehringer Mannheim) and 20% of the cDNA product was subjected to PCR with three different pairs of primers: *PIG3* oligonucleotides, 5′-CCGGGGGAGGGTGAAGTC-3′/5′-TCCAGCCATCCGGGTGAGTT-3′; p53 oligonucleotides, 5′-CCATGGCCATCTACAAGCAG-3′/5′-AGGGTGAAATATTCTCC-3′; and β-actin oligonucleotides, 5′-GTGGGCCGCCCTAGGCACCA-3′/5′-CGGTTGGCCTTAGGGTTCAGGGGGG-3′. Forty percent of the PCR product was loaded on a 1.5% agarose gel.

Gel retardation assays

Duplex substrates were prepared and end-labeled as described (Conseiller *et al.*, 1998) from two complementary oligonucleotides containing: the *WAF1* promoter-derived 20mer p53 DNA-binding site (el-Deiry *et al.*, 1992) with 5′ *Bam*HI–*Not*I- and 3′ *Sma*I-compatible ends, 5′-GATCGCGGCCGCGAACATGTCCCAACA-3′ and 5′-GGGCAACATGTTGGGACATGTTCGCGGCCGC-3′; the *BAX* promoter-derived 39mer p53 DNA-binding site site (Miyashita and Reed, 1995) with 5′ *Bam*HI–*Not*I- and 3′ *Sma*I-compatible ends, 5′-GATCGCGGCCGCTCACAAGTTAGAGACAAGCCTGGGCGTGGGCTATATTCCC-3′ and 5′-GGGAATATAGCCCACGCCCAGGCTTGTCTCTAACTTGTGAGCGGCCGC-3′; the *PIG3* promoter-derived 20mer p53 DNA-binding site (Polyak *et al.*, 1997) with 5′ *Bam*HI–*Not*I- and 3′ *Sma*I-compatible ends, 5′-GATCGCGGCCGCAGCTTGCCCACCCATGCTCCAGCTTGCCCACCCATGCTCCC-3′ and 5′-GGGAGCATGGGTGGGCAAGCTGGAGCATGGGTGGGCAAGCTGCGGCCGC-3′.

A 25 μl reaction volume containing DNA-binding buffer (20 mM Tris–HCl pH 7.5, 0.1 M NaCl, 5 mM $MgCl_2$, 0.05 mM $ZnCl_2$, 5 mM dithiothreitol, 0.1 mg/ml BSA, 10% glycerol, 1% IGEPAL, 2 μg/ml E-64, 2 μg/ml aprotinin, 2 μg/ml leupeptin and 2 μg/ml pepstatin) was mixed with end-labeled duplex substrate (2.4 nM), 1.2 μM AP2 (Promega) as DNA competitor and various amounts of baculovirus-infected Sf9 nuclear extracts or 20 μl of cell-free extracts from p53-transfected H1299 cells in the presence or absence of 300 ng of PAb421 monoclonal antibody. After 1 h incubation on ice, reaction products were analyzed by electrophoresis as described (Conseiller *et al.*, 1998). Recombinant baculoviruses for expression of p53 WT, D281G or p53Δpro were generated by using the Baculogold kit (Pharmingen) after insertion of relevant cDNA into the baculovirus transfer vector pVL1392 (PharMingen). Sf9 nuclear extracts were prepared following a previously described procedure (Delphin *et al*., 1994) and aliquots were stored in the presence of 5% glycerol at –80°C and used only once. At 24 h after transfection, H1299 cells (2×10^6) were lysed on ice in 100 μl of DNA-binding buffer for 30 min, centrifuged to remove insoluble material and used immediately.

Microinjection

Cells were plated at 50% density on glass coverslips and allowed to adhere overnight. Before microinjection, HEPES buffer (Sigma cell culture) was added to the medium (40 mM final concentration). p53 expression plasmids at 1 mg/ml were then microinjected into the nuclei of cells using an Eppendorf microinjector and micromanipulator. After 16 or 36 h, cells were fixed in 4% paraformaldehyde for 15 min, washed with PBS, permeabilized with 0.2% Triton X-100 for 10 min and washed again with PBS. Blocking solution (Power Block, BioGenex) was added to cells for 30 min. Cells were incubated with DO-1 (or anti-LacZ) monoclonal antibody for 1 h at room temperature, washed twice with the blocking solution, incubated with rhodamine-conjugated rabbit anti-mouse Fab′2 antibody (Immunotech, Marseille, France) for 1 h at room-temperature, washed twice with the blocking solution, incubated for 1 h at 37°C with the *in situ* cell death detection kit (Boehringer Mannheim), washed several times and mounted on glass slides with Mowiol before microscopic observation and counting.

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