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Increased apoptosis induction by 121F mutant p53

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p53 mutants in tumours have a reduced affinity for DNA and a reduced ability to induce apoptosis. We describe a mutant with the opposite phenotype, an increased affinity for some p53-binding sites and an increased ability to induce apoptosis. The apoptotic function requires transcription activation by p53. The mutant has an altered sequence specificity and selectively fails to activate MDM2 transcription. Loss of MDM2 feedback results in overexpression of the mutant, but the mutant kills better than wild-type p53 even in MDM2-null cells. Thus the apoptotic phenotype is due to a combination of decreased MDM2 feedback control and increased or unbalanced expression of other apoptosis-inducing p53 target genes. To identify these genes, DNA chips were screened using RNA from cells expressing the apoptosis-inducing mutant, 121F, and a sequence-specificity mutant with the reciprocal phenotype, 277R. Two potential new mediators of p53-dependent apoptosis were identified, Rad and PIR121, which are induced better by 121F than wild-type p53 and not induced by 277R. The 121F mutant kills untransformed MDM2-null but not wild-type mouse embryo fibroblasts and kills tumour cells irrespective of p53 status. It may thus expand the range of tumours which can be treated by *p53* gene therapy.
Keywords: apoptosis/MDM2/p53/PIR121/Rad

Introduction

The *p53* gene plays a key role in tumour biology (reviewed by Ko and Prives, 1996; Levine, 1997). The common effect of p53 mutations found in tumours is to inactivate p53 as a transcription factor (Flaman *et al*., 1994). Consequently, a great deal of effort has been expended in trying to identify transcriptional targets of p53. Particular attention has been paid to target genes which may mediate cell-cycle arrest and apoptosis. p53-dependent G_1 and G_2 arrest requires induction of the p21 cyclin-dependent kinase inhibitor (el-Deiry *et al*., 1993; Dulic *et al*., 1994; Bunz *et al*., 1998). In contrast, no single gene can explain p53-induced apoptosis. Many p53 target genes have been identified which function in known apoptotic pathways (*bax*, *fas*, *DR5*), regulate survival factor signalling (*IGF-BP3*, *RGS14*), induce apoptosis when overexpressed (*PAG608/Wig-1*) or are involved in biochemical events linked to apoptosis (*PIG* genes) (Buckbinder *et al*., 1995, 1997; Miyashita and Reed, 1995; Owen-Schaub *et al*., 1995; Israeli *et al*., 1997; Polyak *et al*., 1997; Varmeh-Ziaie *et al*., 1997; Wu *et al*., 1997). Many putative apoptosis-inducing p53 target genes do not induce apoptosis when expressed alone (Polyak *et al*., 1997). This is the result expected if p53 activates multiple independent apoptotic pathways. *Bax* is the most comprehensively studied apoptosis-inducing p53 target gene. Knock-out mouse studies suggest that bax is not necessary for p53-dependent apoptosis (Lowe *et al*., 1993), but cultured fibroblasts from bax-null mice have subtle defects in p53-dependent apoptosis and human tumours that retain wild-type p53 often mutate bax (McCurrach *et al*., 1997; Rampino *et al*., 1997). This suggests that bax does participate in p53-dependent apoptosis and illustrates how difficult it can be to define the exact contribution of even a single p53 target gene to the apoptotic response.

p53 induces cell-cycle arrest and apoptosis in response to activating signals transmitted by the p19-ARF protein and kinases such as ATM (reviewed by Sherr, 1998). These signals converge on the MDM2 protein, which is itself the product of a p53 target gene (Barak *et al*., 1993; Wu *et al*., 1993). MDM2 inhibits p53-dependent transcription by binding to the p53 transactivation domain and blocking recruitment of basal transcription factors (Lin *et al*., 1994; Lu and Levine, 1995; Thut *et al*., 1995) and histone acetylases (Scolnick *et al*., 1997; Grossman *et al*., 1998). MDM2 binding to p53 is regulated by phosphorylation of the MDM2-binding site in the p53 transactivation domain at serine 15 (Shieh *et al*., 1997; Banin *et al*., 1998; Canman *et al*., 1998) and serine 20 (Shieh *et al*., 1999; Unger *et al*., 1999), as well as by phosphorylation of MDM2 itself (Mayo *et al*., 1997). As well as directly blocking p53 activity in the nucleus, MDM2 induces p53 export to the cytoplasm where p53 is ubiquitinated and degraded by the proteasome (Haupt *et al*., 1997; Kubbutat *et al*., 1997; Roth *et al*., 1998; Honda and Yasuda, 1999). In the presence of signals from oncogenes, such as *myc*, *ras* and *E1A*, the level of p19-ARF increases (de Stanchina *et al*., 1998; Palmero *et al*., 1998; Zindy *et al*., 1998). This results in sequestration of MDM2 in nucleoli, freeing p53 to activate transcription in the nucleoplasm (Weber *et al*., 1999). MDM2 thus plays a central role in integrating signals to p53.

Previously, we described a p53 mutant, 121F, selected in yeast for increased transcriptional activation from a sequence resembling the p53-binding site in the bax promoter (Freeman *et al*., 1994). Unlike the p53-binding sites in the p21 and MDM2 promoters, the site in the bax promoter is a poor match to the p53-binding site consensus

sequence defined by PCR selection (el-Deiry *et al*., 1992; Funk *et al*., 1992). A perfect p53-binding site contains two copies of the decamer RRRCATGYYY, where R is purine and Y is pyrimidine. Each decamer binds one primary dimer in the p53 tetramer (Cho *et al*., 1994). In the bax site there is only a single perfect decamer, suggesting that a p53 tetramer can only bind with high affinity through one of its primary dimers. The model which emerges is that weak p53 activation induces cell-cycle arrest, but not apoptosis, because a low level of p53 can only saturate the high-affinity p21 and MDM2 sites. Strong activation interferes with MDM2 binding leading to an increase in the p53 level. This brings low-affinity targets above the threshold for binding and hence induces apoptosis as well as cell-cycle arrest (Chen *et al*., 1996; Di Como and Prives, 1998; Flaman *et al*., 1998).

We have now characterized the 121F mutant and a reciprocal mutant selected in an analogous screen, 277R. The mutants show complementary patterns of induction of many p53 target genes. 121F has an increased ability to induce apoptosis which results from the combined effects of a reduction in MDM2 induction and a change in the expression of at least one other target gene. To identify that gene we performed a DNA chip screen and identified two new p53 target genes selectively induced by the 121F mutant which may play a role in apoptosis induction by p53.

Results

Selection of p53 sequence-specificity mutants

The 277R mutant was selected in a screen for transcriptional activation in yeast from a minimal promoter containing the sequence GAACATGTTC. Analysis in yeast using palindromic sites, which present the same basic RRRCA pentamer to each subunit of the p53 tetramer, demonstrate that the 121F and 277R mutants show reciprocal specificities in transcription and DNA binding assays (Figure 1A and B). In addition to showing a change in specificity the 277R mutant also has a reduced affinity (Figure 1B, compare 277R and wild-type with the GAA probe). Differences in specificity are also apparent in luciferase assays of transiently transfected p53-null Saos-2 human osteosarcoma cells (Figure 1C): 121F is defective in activation of the p53-responsive (P2) promoter in the *MDM2* gene and 277R is defective in activation of a synthetic reporter containing a 'G-rich' site (i.e. the second and third purines in the pentamer are G). Since amino acids 120 and 277 make major groove contacts to the second and third bases in the consensus (Cho *et al*., 1994), the observed effects on sequence specificity are probably due to direct effects on base-specific hydrogen bonding.

Regulated expression of p53 mutants in H1299 cells

To determine the activity of the mutants on endogenous target genes, p53-null H1299 non-small cell lung carcinoma cells were stably transfected with constructs expressing p53 from a tetracycline-regulated promoter. Two clones were studied for each mutant, as well as control clones containing wild-type p53 and 175H p53. The 175H mutant is a typical tumour-derived mutant which does not activate transcription, arrest the cell cycle

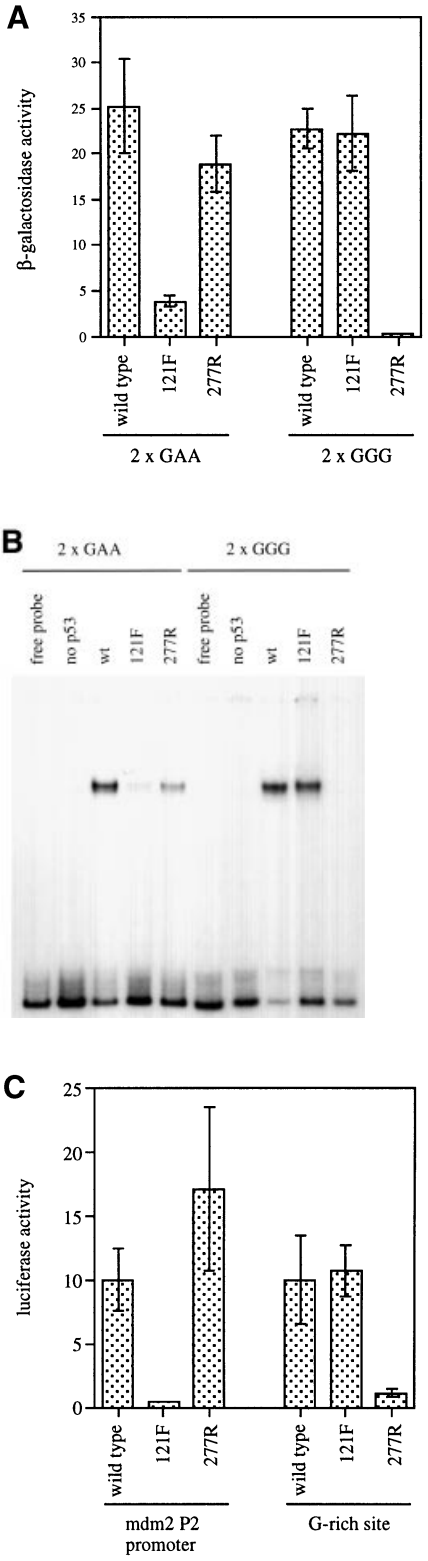

Fig. 1. The 121F and 277R p53 mutants have an altered specificity for DNA binding. Yeast transcription assay (**A**) and bandshift assay (**B**) using extracts prepared from cells expressing wild-type p53 and the 121F and 277R mutants. 2× GAA: GAACATGTTC site; 2× GGG: GGGCATGCCC site. For the bandshift the C-terminus of p53 was truncated at amino acid 368 to delete the repression domain. (**C**) Luciferase assays showing failure of activation of the MDM2 promoter by the 121F mutant after transient transfection of Saos-2 cells. The reporters contain the p53-responsive fragment of the *MDM2* gene (P2 promoter) or a synthetic promoter containing a G-rich p53-binding site (AGACcTGCCC GGGCAAGCCT).

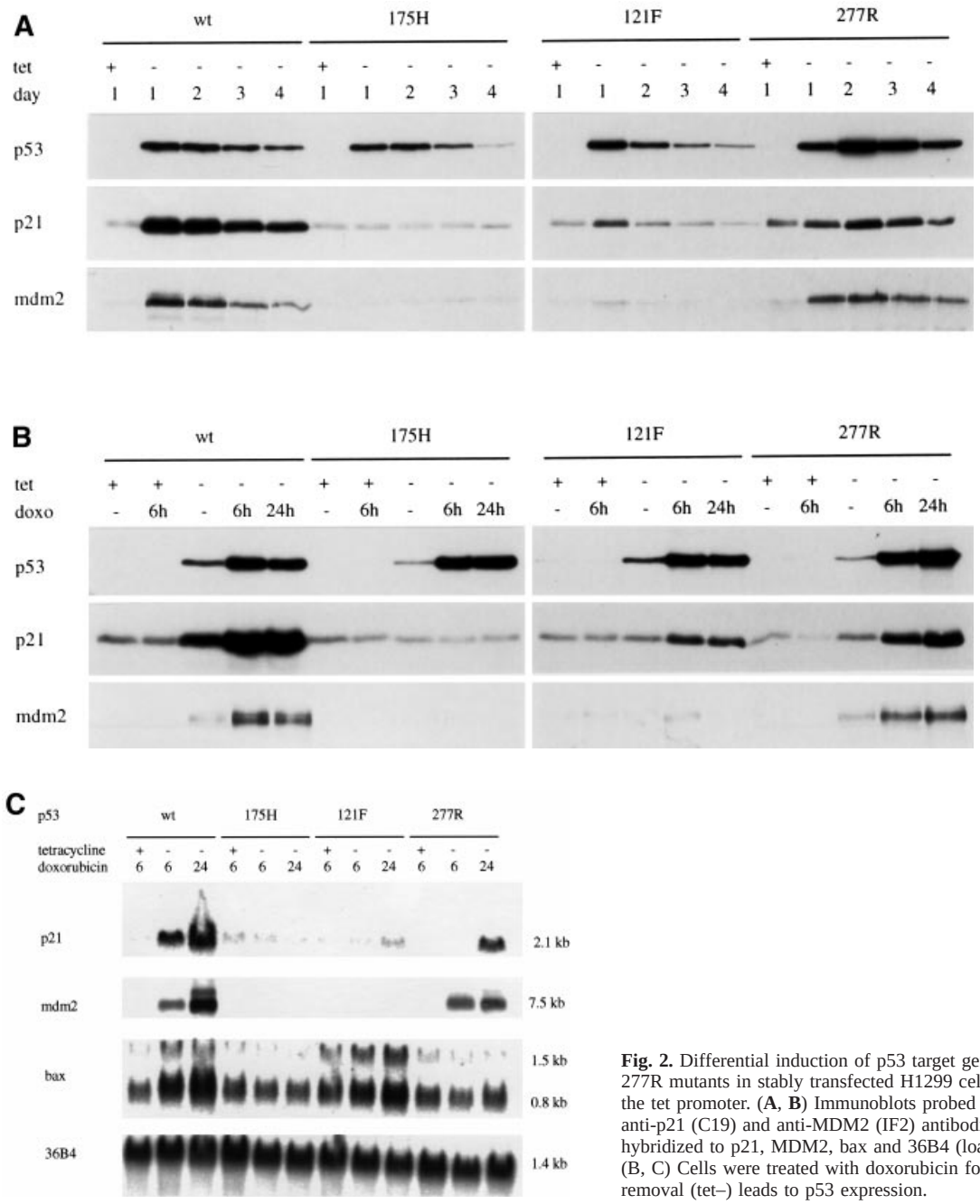

Fig. 2. Differential induction of p53 target genes by the 121F and 277R mutants in stably transfected H1299 cells expressing p53 from the tet promoter. (**A**, **B**) Immunoblots probed with anti-p53 (DO1), anti-p21 (C19) and anti-MDM2 (IF2) antibodies. (**C**) Northern blot hybridized to p21, MDM2, bax and 36B4 (loading control) probes. (B, C) Cells were treated with doxorubicin for 6 or 24 h. Tetracycline removal (tet–) leads to p53 expression.

or induce apoptosis. Immunostaining for p53 was used to select clones giving tightly regulated p53 expression (data not shown). Immunoblotting showed that the selected clones express very small amounts of p53 in the presence of tetracycline and induce p53 expression to similar levels in its absence (Figure 2A). As expected, wild-type p53 induced both p21 and MDM2, whereas the 175H mutant induced neither gene (Figure 2A). The 277R mutant induced both p21 and MDM2, but 121F induced p21 only weakly and failed to induce MDM2. To determine whether the level of 121F expression was simply too low to induce MDM2, the cells were treated with the DNA-damaging agent doxorubicin (adriamycin). This led to an increase in p53 level but the differential effects on p21 and MDM2 expression remained (Figure 2B). Interestingly, the ability of doxorubicin to increase p53 levels in these cells appears entirely independent of MDM2, since it was seen with wild-type p53 and 277R, which induce MDM2, as well as 175H and 121F, which do not. Northern blotting showed that the changes seen at the protein level were matched by corresponding changes at the RNA level (Figure 2C). The failure of 121F to induce MDM2 expression parallels its behaviour in luciferase assays on the MDM2 promoter (Figure 1C), consistent with the mutation acting through an effect on the specificity of DNA binding.

The yeast screens were expected to yield mutants with a modified ability to induce bax. Northern blotting revealed that the mutants do show the expected difference in specificity, since 121F induces bax and 277R does not (Figure 2C). However, despite the data showing that 121F has an increased affinity for a bax-like site *in vitro*, the

bax induction in H1299 cells was no greater with 121F than wild-type p53.

MDM2 signalling is blocked in p53-null tumour cells

The 121F mutant is defective in MDM2 induction (Figures 1C and 2). Since MDM2 targets p53 for ubiquitin-mediated degradation, loss of MDM2 induction should in principle result in a higher level of p53 protein expression with the 121F mutant than with wild-type p53. The lack of correlation of p53 and MDM2 level in the stably transfected H1299 cells (Figure 2A and B) is inconsistent with the negative feedback model but could be due to a hidden bias in the selection of the transfected clones. To circumvent this problem the mutant was cloned into an adenovirus, and the p53 level and stability were measured in cell lines of differing endogenous p53 status. To facilitate comparison, 3-fold dilutions of virus were used. A transcriptionally inactive 22Q 23S 121F mutant virus was used as a control. The 22Q 23S mutations, which lie in the transactivation domain, abolish p53-dependent transcription and block MDM2 binding to p53 (Lin *et al*., 1994). A GFP-expressing adenovirus was used as a control for non-specific effects of adenovirus infection.

In p53-null cells, the viruses expressing wild-type p53, 121F and the triple mutant gave the same level of p53 expression (H1299 and Saos-2, Figure 3A). As with the stably transfected cell lines (Figure 2), the 121F mutant induced p21 weakly and failed to induce MDM2 (Figure 3A, lanes 5–7). As expected, the 22Q 23S 121F mutant induced neither gene (Figure 3A, lanes 8–10). Given the clear difference in MDM2 expression, equal p53 expression is not the result expected for the 121F mutant, since it has previously been shown that transfected p53 is sensitive to MDM2-induced degradation in these cells (Haupt *et al*., 1997; Kubbutat *et al*., 1997). The results observed could arise if there were balancing changes in translation and half-life. To test this we performed a cycloheximide block to measure p53 stability (H1299 and Saos-2, Figure 3B). Wild-type and 121F were both as stable as the 22Q 23S mutant, which acts as a positive control for stable p53 because it is unable to bind MDM2. Pulse–chase analysis of the H1299 clones expressing p53 from the tet promoter further confirmed that wild-type p53, 121F, 277R and 175H mutant p53 all have a long half-life in these cells (data not shown). Thus, in contrast with overexpressed exogenous MDM2, endogenous MDM2 is unable to induce p53 degradation in these p53-null tumour cells.

The 121F mutant is overexpressed in tumour cells retaining wild-type p53

Tumour cells retaining wild-type p53 did show the expected difference in p53 level after infection with wild-type p53 and 121F mutant viruses (U-87 and U2OS, Figure 3A, compare lanes 2–4 with lanes 5–7). Remarkably, this was not accompanied by significant differences in MDM2 level. Instead, the level of MDM2 induced by the wild-type and 121F viruses was almost identical (U-87 and U2OS, Figure 3A, compare lanes 2–4 with lanes 5–7). Compared with cells lacking endogenous p53, the 121F mutant thus induces a much more robust MDM2 response in tumour cells containing endogenous wild-type

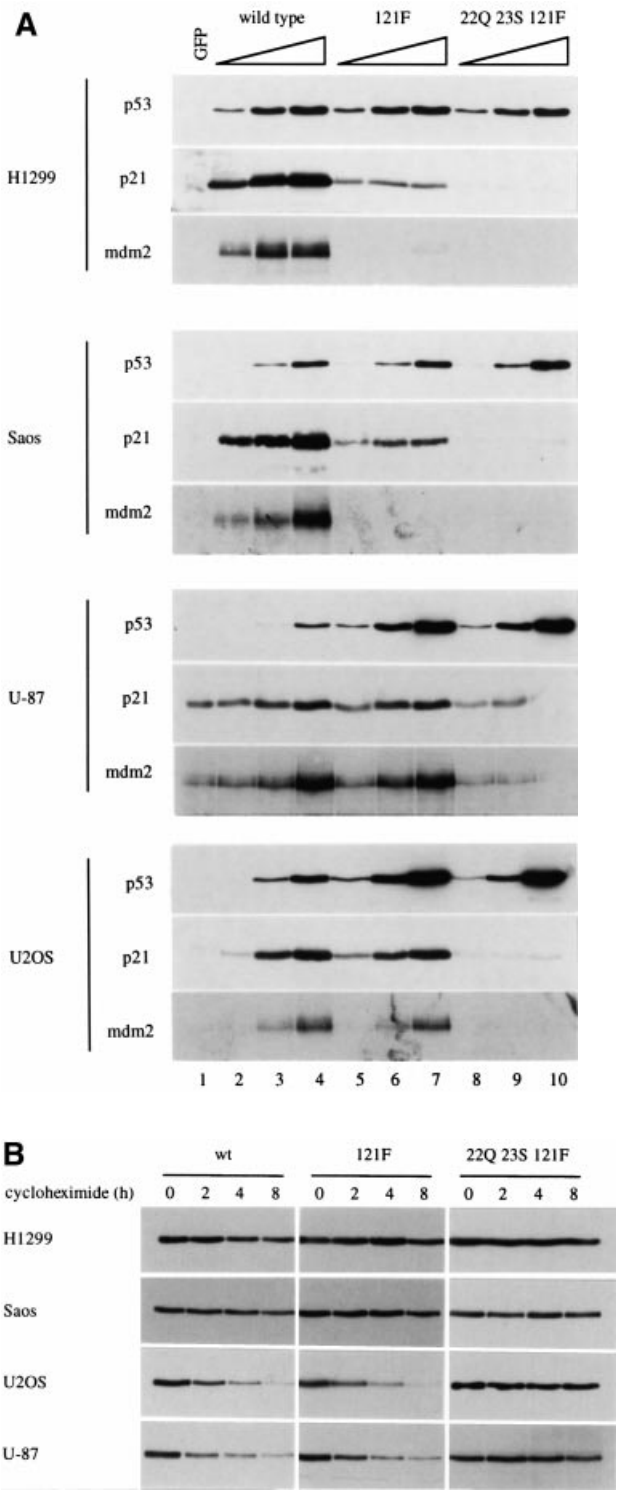

Fig. 3. Regulation of p53 level by MDM2. (**A**) Immunoblot of p53-null cells (H1299 and Saos) and wild-type p53-containing cells (U-87 and U2OS) 14 h after infection with GFP control adenovirus and 3-fold dilutions of wild-type p53 virus, 121F virus and 22Q 23S 121F virus. Blots were probed with anti-p53 (PAb1801 plus PAb240), anti-p21 (C19) and anti-MDM2 (IF2) antibodies. (**B**) p53 stability analysed by cycloheximide block. p53-null cells (H1299 and Saos) and wild-type p53-containing cells (U2OS and U-87) were infected with wild-type p53 virus, 121F virus and 22Q 23S 121F virus. After 14 h the cells were treated with cycloheximide for 0, 2, 4 or 8 h and the extracts immunoblotted and probed with anti-p53 (PAb1801 plus PAb240) antibodies.

p53. This difference is reproducible, although the MDM2 level is not always exactly the same. The greater activity is probably due to the fact that in 121F-infected cells the p53 tetramer contains a mixture of endogenous wild-type and exogenous 121F subunits. It appears that the feedback loop titrates p53 level up to a point giving equal MDM2 expression despite weaker 121F binding to the MDM2 promoter. If so, the p53 half-life in cells infected with 121F virus should be longer than that in cells infected with wild-type p53 virus. To explore this possibility, we again tested p53 stability by cycloheximide block (U-87 and U2OS, Figure 3B). This showed that the p53 in cells infected with either the wild-type or 121F viruses was unstable relative to the p53 in cells infected with the 22Q 23S 121F virus. Thus, the ability to degrade p53 correlates with the level of MDM2 in the cell, but the higher amount of p53 seen with the 121F mutant still requires explanation. Since the stable 22Q 23S 121F mutant achieves a final level similar to that of the unstable p53 in cells infected with the 121F virus (Figure 3A, compare lanes 5–7 with lanes 8–10), there must be an increase in p53 synthesis in the cells containing transcriptionally active 121F to balance the shorter half-life. Further experiments are required to define the underlying mechanism. In particular, the protein could be derived from either the endogenous *p53* gene or the virus but we cannot distinguish between the two with existing reagents. Regardless of the mechanism, the consequence is clearly increased expression of 121F p53 in tumour cells containing wild-type p53.

Increased apoptosis induction by 121F p53

Significant differences in target gene induction should result in significant differences in the biological activity of a p53 mutant. This was examined first in the H1299 clones expressing the mutants from the tet promoter. Unlike the wild-type, 175H and 277R clones, induction of p53 expression in the 121F clones led to a decrease in cell number and the appearance of floating cells in the culture (data not shown). That this was due to apoptosis was demonstrated by positive annexin V and TUNEL staining (data not shown) and the appearance of a sub-G_1 peak by flow cytometry (Figure 4A, panel 3). The difference was not due to a difference in the level of p53 expression because, as already noted, the level of p53 in the 121F clones was similar to that in the other clones (Figure 2A). Wild-type p53 can induce apoptosis in these cells, but not at the levels used here (Chen *et al*., 1996). To test whether increased killing by the 121F mutant is transcription dependent, H1299 cells were infected with adenoviruses expressing 121F and the transcriptionally inactive 22Q 23S 121F mutant. Flow cytometry showed that induction of apoptosis by 121F was abolished almost completely by the N-terminal mutations (Figure 4B). This was not due to a difference in the level of p53 expression (Figure 3A). The 121F mutant thus induces apoptosis better than wild-type p53 in H1299 cells, and this requires a fully active p53 transcription activation domain.

Apoptosis induction by 121F p53 in other cell lines

To determine whether the apoptotic phenotype of the 121F mutant is cell-line specific, additional cell lines were infected with adenoviruses expressing wild-type p53, 121F or GFP. As expected for p53 expressed from the CMV promoter in an adenovirus, wild-type p53 induced G_1 arrest and apoptosis in cells lacking p53 (Saos-2, Figure 5A, panels 5 and 6) or containing mutant p53 (HUG15, data not shown). As in H1299 cells, the 121F mutant induced apoptosis better than wild-type p53 (Figure 5A, compare panel 5 with panel 8).

Infection of tumour cells retaining endogenous wild-type p53 showed an even greater difference between the behaviour of wild-type p53 and the 121F mutant (Figure 5B). The amount of virus was normalized to the p53 protein level to permit comparison of apoptosis induction by a given amount of p53 protein (Figure 5C). The difference in apoptosis induction is thus not due simply to a change in p53 level caused by the functional MDM2 feedback loop in these cells (Figure 3). By definition, tumour cells retaining endogenous wild-type p53 are relatively resistant to wild-type p53, either because of a defect in p53 activation or a defect in apoptotic pathways downstream of p53. Thus, although the virus expressing wild-type p53 produced a G_1 arrest it did not induce apoptosis (U2OS, Figure 5B, panels 4–6; U87MG and HUG2, data not shown). In contrast, 121F kills cell lines irrespective of p53 status (U2OS, Figure 5B, panels 8 and 9; HUG15, U87MG and HUG2, data not shown). The 121F mutant thus behaves like an unregulated mutant which escapes from control by upstream signals. We conclude that 121F-induced apoptosis is not a clone- or cell-line-specific artefact and it apparently bypasses some of the normal controls on apoptosis induction by wild-type p53.

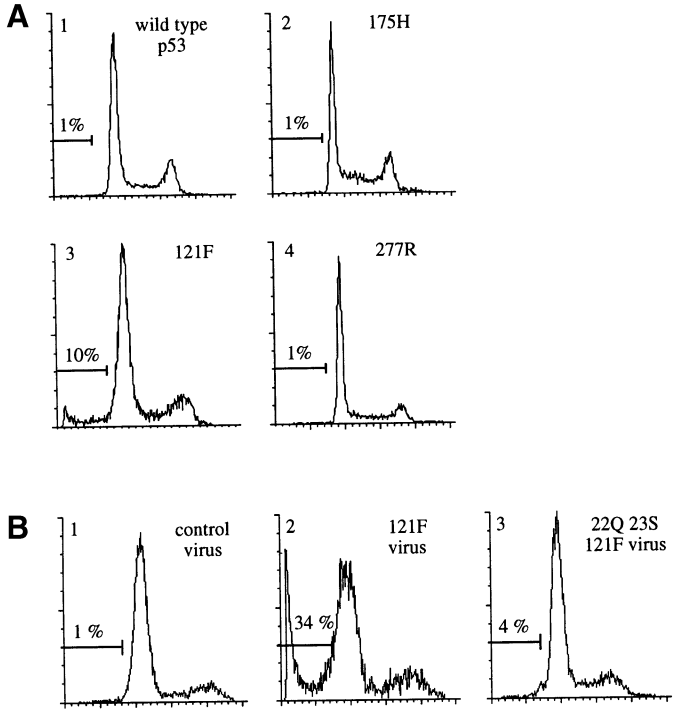

Fig. 4. Apoptosis induction by 121F mutant p53 in H1299 cells. (**A**) Flow cytometry 3 days after tetracycline removal showing a sub-G_1 peak with the 121F mutant in cells expressing p53 from the tet promoter. (**B**) Flow cytometry 3 days after infection with GFP control adenovirus, 121F virus and 22Q 23S 121F virus expressing p53 from the CMV promoter. The 22Q 23S mutations strongly reduce transactivation of p53 target genes.

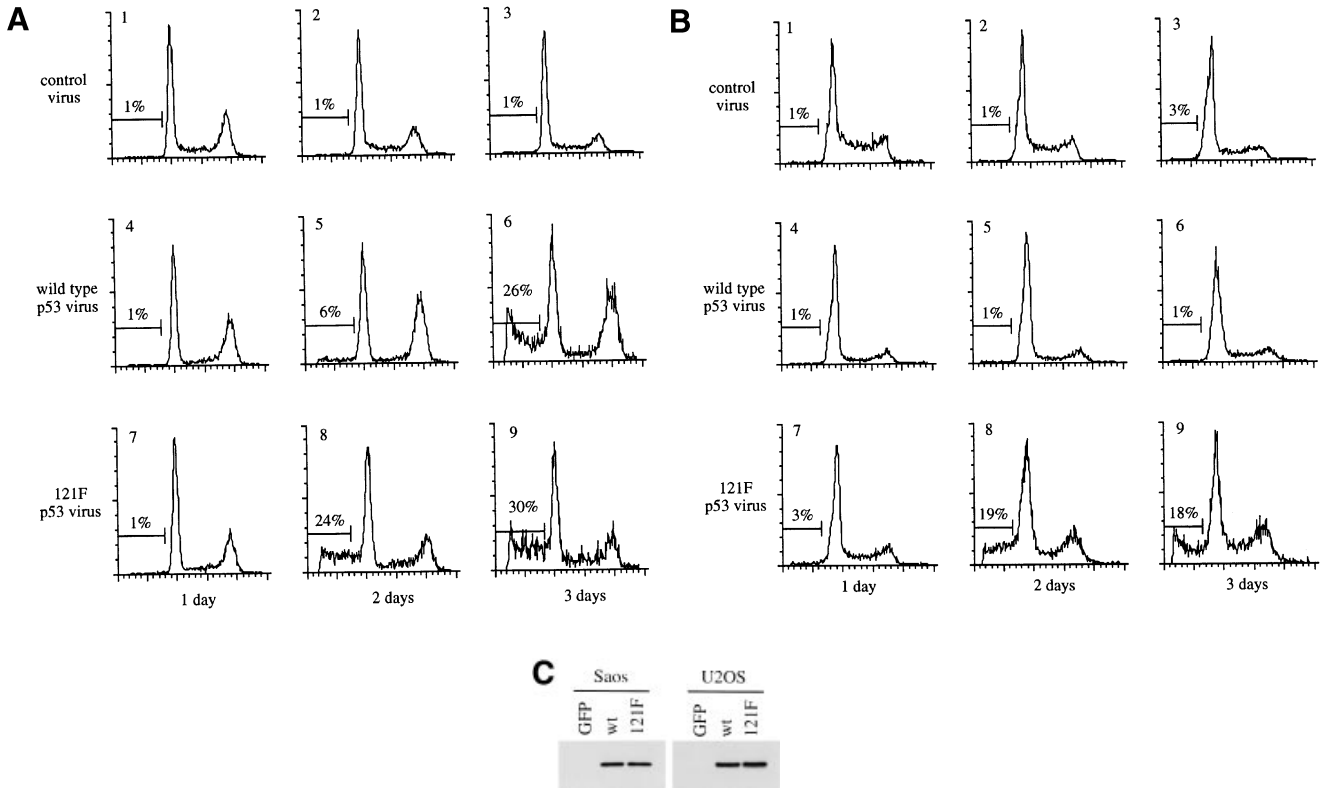

Fig. 5. Apoptosis induction by the 121F mutant is not cell type-specific. Flow cytometry to detect apoptosis in cells infected with adenoviruses expressing GFP (control), wild-type p53 and 121F mutant p53 from the CMV promoter. (**A**) Saos-2: p53-null osteosarcoma cells. (**B**) U2OS: osteosarcoma cells containing endogenous wild-type p53. (**C**) Immunoblot showing equal p53 expression in the two cell lines following infection with p53-expressing viruses. The blot was probed with anti-p53 (DO1) antibody.

MDM2-independent apoptosis induction by the 121F mutant

Increased p53 expression in cells infected with the 121F virus (Figure 3) should exacerbate the apoptotic phenotype in wild-type p53-containing cells, but the increased killing is apparent even when the viral titre is adjusted to give equal p53 expression with the wild-type and 121F viruses (Figure 5). This suggests that decreased MDM2 feedback may not be the sole explanation for the difference. To test formally whether failure of MDM2 induction alone can explain the unregulated behaviour of the 121F mutant, fibroblasts derived from mouse embryos lacking both p53 and MDM2 were infected with p53-expressing adenoviruses. If defective MDM2 induction were the only difference between wild-type p53 and the 121F mutant, then wild-type p53 would kill as well as 121F in cells lacking MDM2. This is clearly not the case: 121F induces apoptosis much better than wild-type p53 (Figure 6, compare panel 6 with panel 9). Thus at least two factors contribute to the apoptotic phenotype of the 121F mutant, decreased MDM2 induction and altered expression of another p53 target gene.

The 121F mutant gave more apoptosis in p53-null MDM2-null cells (21% sub-G_1, Figure 6, panel 9) than in p53-null cells (16%, panel 8), and background apoptosis in wild-type fibroblasts (4%, panel 7). This can be interpreted as inhibition of the incoming p53 by endogenous MDM2, whose level should be highest in wild-type fibroblasts (because of induction by endogenous p53), intermediate in p53-null fibroblasts (which retain some p53-independent regulation of MDM2) and zero in

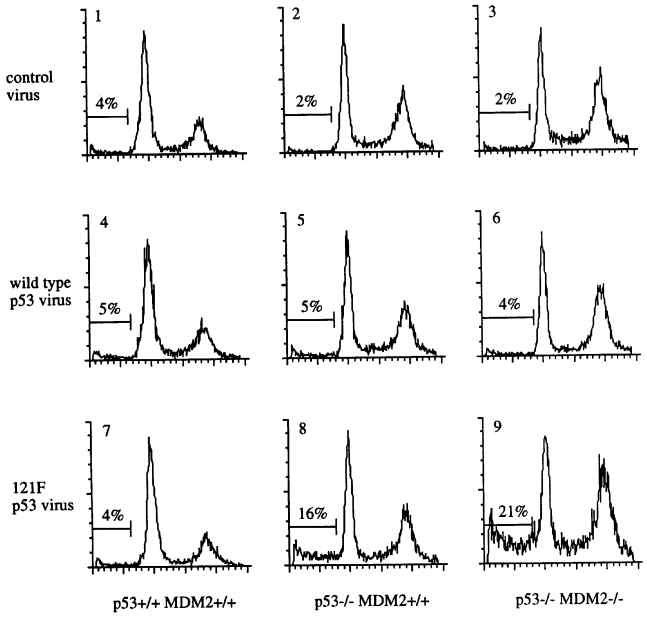

Fig. 6. MDM2-independent apoptosis induction by 121F mutant p53. Flow cytometry of mouse embryo fibroblasts ($p53^{-/-}$, p53-null; $MDM2^{-/-}$, MDM2-null) 1 day after infection with GFP control adenovirus, wild-type p53 virus and 121F virus.

MDM2-null cells. Inhibition by MDM2 is possible because the MDM2-binding site at the N-terminus of p53 is unaffected by the 121F mutation in the DNA-binding domain. In contrast with transformed cells (Figure 5), this

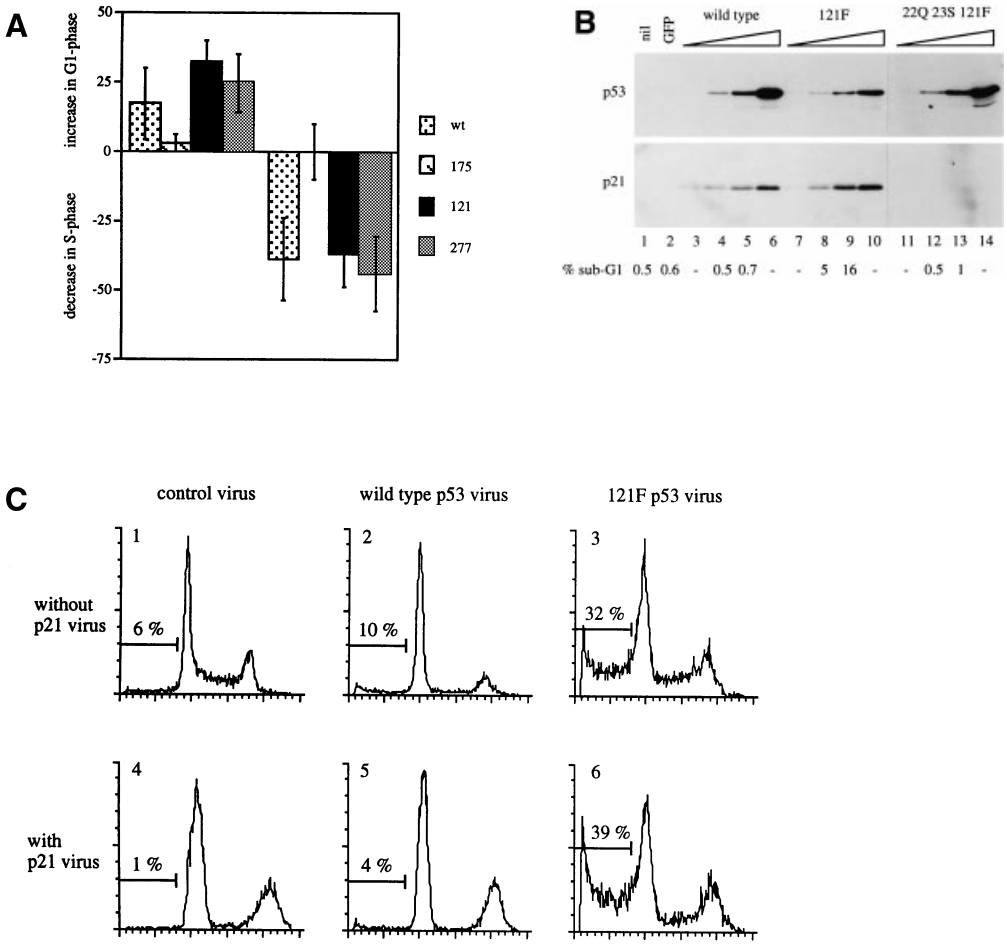

Fig. 7. Defective p21 induction is not responsible for the apoptotic phenotype of 121F mutant p53. (**A**) Changes in cell-cycle distribution of H1299 cells expressing p53 from the tet promoter 3 days after tetracycline withdrawal. G_1 (propidium iodide staining) and S phase (BrdU incorporation) were determined by flow cytometry. The percentage change is relative to cells in tetracycline. (**B**) Immunoblot of p53-null mdm2-null mouse embryo fibroblasts 14 h after infection with GFP control adenovirus, and 3-fold dilutions of wild-type p53 virus, 121F virus and 22Q 23S 121F virus. Blots were probed with anti-p53 (PAb240) and anti-p21 (C19) antibodies. Apoptosis induction (% sub-G_1) was measured by flow cytometry. (**C**) Flow cytometry showing apoptosis induction by the 121F mutant despite p21 expression. U2OS cells were infected with adenoviruses expressing p21 or lacZ from the CMV promoter, followed 12 h later by GFP control adenovirus, wild-type p53 virus and 121F virus.

suggests that some normal cells may be relatively immune to the effects of 121F.

Defective p21 induction does not explain the apoptotic phenotype of the 121F mutant

Although increased activation of apoptosis-inducing p53 target genes is the simplest explanation for the increased activity of the 121F mutant, formally the relevant change could be decreased activation of an apoptosis-inhibiting p53 target gene. *p21* fulfils the latter criterion, because *p21* is known to counteract p53-dependent apoptosis in some situations and the 121F mutant is defective in p21 induction (Figure 2). To assess the role of the defect in p21 induction, the H1299 clones expressing p53 from the tet promoter were examined by flow cytometry (Figure 7A). Surprisingly, the wild-type, 121F and 277R clones responded to tetracycline withdrawal with almost identical increases in G_1 and decreases in S phase content. In contrast, the control 175H tumour-derived mutant had no detectable effect on the cell cycle profile. Thus, the defect in p21 induction is apparently not functionally important, at least in H1299 cells. A further indicator that the defect in p21 induction may not be important is that the apoptotic phenotype of the 121F mutant is conserved in p53-null MDM2-null mouse cells, but the defect in p21 induction is small or absent in these cells (Figure 7B, compare lanes 3–6 with 7–10). Finally, if the lack of p21 is responsible for the increase in apoptosis, then supplying the missing p21 *in trans* should suppress the apoptosis. To test this, U2OS cells were infected with adenoviruses expressing p21 or lacZ, followed 12 h later by infection with viruses expressing GFP, wild-type p53 or the 121F mutant. As expected, p21 expression produced a combined G_1 and G_2 arrest (Figure 7C, compare panel 1 with panel 4). Both the control GFP virus and the wild-type p53 virus weakly induced apoptosis in the absence of p21 virus, presumably as a non-specific effect of higher virus load (relative to that in Figure 5B), but this was suppressed by the p21 virus (Figure 7C, compare panels 1 and 2 with panels 4 and 5). In contrast, apoptosis induction by the 121F mutant was increased slightly by p21 expression (Figure 7C, compare panel 3 with panel 6). We conclude that defective p21 induction does not explain the apoptotic phenotype of the 121F mutant.

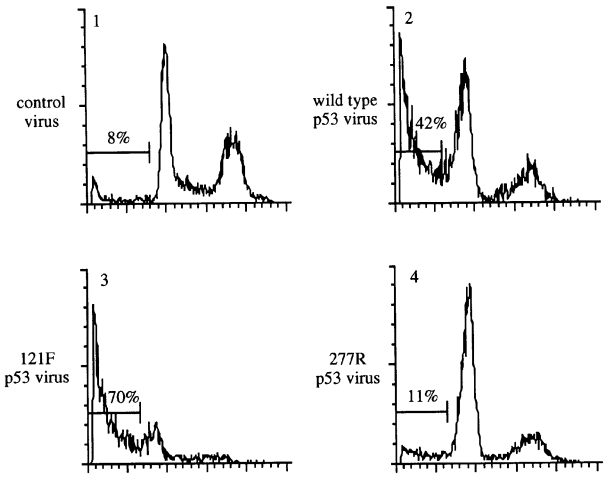

Fig. 8. The 277R p53 mutant is defective in apoptosis induction. Flow cytometry of Saos-2 cells 2 days after infection with GFP control adenovirus, wild-type virus, 121F virus and 277R virus.

Identification of new genes selectively induced by the 121F mutant

Since the 121F mutant has an altered sequence specificity (nominally an increased affinity for a bax-like site, Figure 1) and its apoptotic phenotype is transcription dependent (Figure 4B), the simplest explanation for preferential apoptosis induction by the mutant is preferential activation of apoptosis-inducing p53-target genes. *Bax* itself is not responsible for the difference because it is not better induced by the mutant (Figure 2C). To identify potential new mediators of p53-dependent apoptosis, a DNA chip screen was performed. The 277R mutant was used as an internal control in the screen to counter-select genes unlikely to be involved in p53-dependent apoptosis induction. This is possible because the 277R mutant has a reduced ability to induce apoptosis (Figure 8), while still retaining the ability to induce many different p53 target genes (Figure 2) and induce G_1 arrest (Figure 7A). Since high protein levels can saturate low-affinity binding sites, we used for the screen the H1299 cells expressing p53 from the tet promoter rather than the p53-expressing adenoviruses. Interesting candidates must fulfil two criteria: to be apoptosis specific they must be activated by 121F but not 277R; and to be authentic targets they must be activated by wild-type p53 in cells where wild-type p53 induces apoptosis. To satisfy the latter criterion, the stably transfected H1299 cell lines were treated with doxorubicin to induce wild-type p53-dependent apoptosis.

Affymetrix DNA chips representing 6500 human genes were hybridized with RNA from the stably transfected H1299 clones after treatment with doxorubicin for 6 or 24 h. Forty-nine probe sets on the chip gave >3-fold induction by wild-type p53 at 24 h. Known p53 target genes included in this number are *p21* (three probe sets), *gadd45* (three probe sets), smooth muscle actin, *PCNA*, EGF receptor and *Ha-ras* (Kastan *et al*., 1992; el-Deiry *et al*., 1993; Shivakumar *et al*., 1995; Madden *et al*., 1997; Sheikh *et al*., 1997; Comer *et al*., 1998) (Table I). Unfortunately, many known p53 target genes are either not included on the chip (e.g. *bax*) or known not to respond (e.g. *MDM2*). Two genes not previously shown to be p53 target genes, *Rad* and a gene we call *PIR121*, for 121F-specific p53 inducible RNA, were strongly activated by 121F and wild-type p53 but not by 277R or 175H (Table I). Rad is a muscle-specific small ras-like GTPase originally identified in a differential screen for genes expressed in diabetic muscle (Reynet and Kahn, 1993). Unlike ras it lacks a CAAX box and binds tropomyosin and calmodulin in a GTP-sensitive manner (Zhu *et al*., 1996; Moyers *et al*., 1997). Preliminary results indicate that dominant negative and constitutively activated Rad mutants (Moyers *et al*., 1997) neither facilitate nor block p53-dependent apoptosis in H1299 cells (data not shown).

Table I. Genes giving >3-fold induction on the 6500 gene DNA chip

Accession no.	Wild-type	175H	121F	277R	Gene name
Known target genes					
U03106	35	0	6	9	*p21*
R46753	19.8	0.5	2.5	5.3	*p21*
U03106	19	0	3	7	*p21*
T60155	11	0	4	19	*actin*
H24346	8	0	0	2	*gadd45*
M60974	5	0	0	0	*gadd45*
T51961	4.1	0.8	3.1	2.8	*PCNA*
R35665	4	0	1	0	*EGF-R*
J00277	3.6	–0.9	5.9	0	*Ha-ras*
M60974	3	0	0	0	*gadd45*
New target genes					
L24564	11	0	16	0	*Rad*
L47738	5.3	–0.3	15.3	–0.3	*PIR121*

PIR121 encodes an open reading frame (ORF) of 1253 amino acids whose sequence contains no domains of known function. It belongs to a gene family comprising two members in man (*PIR121* and *KIAA0068*), two members in mouse (*EST518465* and *Shyc*) and a single *Caenorhabditis elegans* gene (*F56A11.1*). The putative mouse PIR121 orthologue is 99% identical over 367 amino acids at the N-terminus (the sequenced region of EST 518465). Shyc is a mouse protein of unknown function expressed in developing and adult brain (Koster *et al*., 1998). Shyc and KIAA0068 are 86% identical to PIR121. The putative *C.elegans* protein is 52% identical with PIR121 over 1236 amino acids. PIR121 maps to human chromosome 5q34 (D5S487–D5S412, NCBI Human Gene Map), a region frequently translocated in acute myeloid leukaemia but not known to be amplified or deleted in solid tumours.

Differential induction of PIR121 and Rad in H1299 cells was confirmed by Northern blotting (Figure 9A). Two additional known p53 target genes (*fas* and *cyclin G*) were tested but found to be selectively induced by the 277R mutant (Figure 9A). Given the established role fas plays in apoptosis this is paradoxical but consistent with data showing that fas-mutant mice undergo normal p53-dependent apoptosis following γ-irradiation (Reinke and Lozano, 1997). Since PIR121 showed both the strongest induction by 121F relative to wild-type p53 and no induction by 277R, it was tested by Northern blotting in other cell lines infected with p53-expressing adenoviruses. In Saos-2 it was induced by wild-type p53 and 121F but not by control GFP virus or 277R virus (Figure 9B, Saos). In U2OS it was induced better by 121F than wild-type p53, consistent with the greater ability of 121F to induce apoptosis in this cell line (Figure 9B, U2OS).

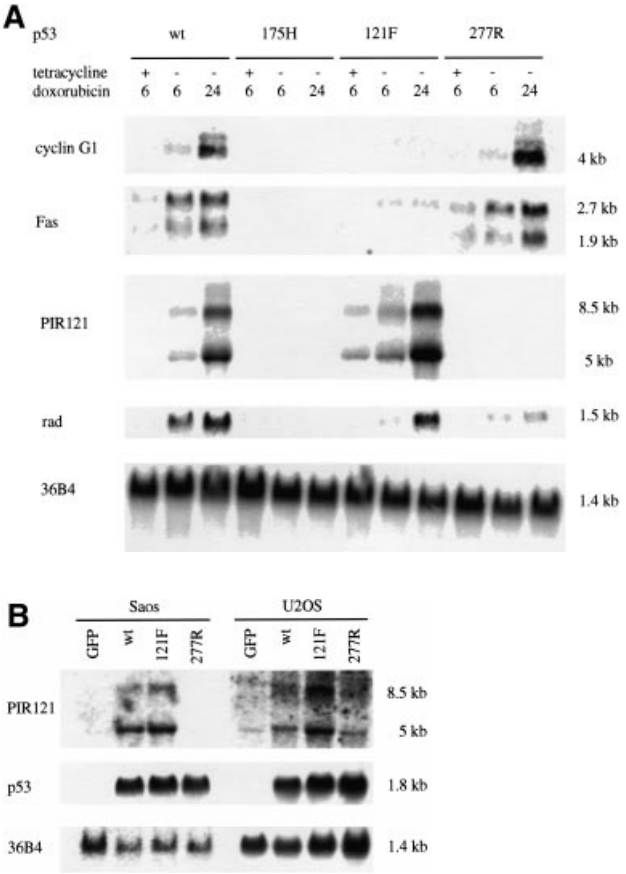

Fig. 9. Differential induction of p53 target genes by the 121F and 277R mutants. (**A**) Northern blot hybridized to cyclin G_1, fas, PIR121, rad and 36B4 (loading control) probes. H1299 cells expressing p53 from the tet promoter were treated with doxorubicin for 6 or 24 h. p53 expression was induced by removal of tetracycline from the medium. (**B**) Northern blot of Saos-2 and U2OS cells infected with adenoviruses expressing p53 from the CMV promoter hybridized to PIR121, p53 and 36B4 probes.

The function of PIR121 is unknown. To determine whether PIR121 can induce apoptosis, a full-length *PIR121* cDNA was cloned into an adenovirus, and expressed in H1299 cells (Figure 10). Immunostaining for myc-tagged protein showed that the protein is cytoplasmic at low levels but accumulates in dense perinuclear clumps, presumably the Golgi, at high multiplicities of infection (data not shown). Western blotting with an anti-PIR121 peptide antibody showed that PIR121 was induced at the protein level by wild-type p53 and 121F, with better induction by 121F at the lowest dose of virus (Figure 10A). At a multiplicity of infection with PIR121 virus giving a PIR121 level much greater than that induced by wild-type p53 or 121F viruses (Figure 10A), the PIR121 virus failed to increase the sub-G_1 content detectable by flow cytometry (Figure 10B) or produce chromatin clumping by Hoechst staining (data not shown). Thus, in common with many putative p53 target genes (Polyak *et al*., 1997), *PIR121* must have either a tissue-specific role or induce apoptosis only as part of a coordinated p53-dependent response involving multiple different target genes.

Discussion

The common biological effect of mutations in the *p53* gene in tumours is failure of transcription activation, a defect caused in most cases by a reduction in the affinity of p53 for DNA (Flaman *et al*., 1994). Previously, we described a p53 mutant, 121F, selected for the opposite defect, an increased affinity for DNA (Freeman *et al*., 1994), and now show that it has an increased ability to induce apoptosis. Superficially, 121F behaves like an unregulated mutant, because it kills cells containing wild-type p53 that are normally resistant to exogenous wild-type p53. The apoptotic phenotype probably results from two properties of the mutant, decreased activation of MDM2 and increased activation of proapoptotic target genes. The former compounds the latter by increasing the p53 level in cells where the MDM2 feedback loop is active. We also describe a mutant, 277R, with a reduced affinity for DNA and altered sequence specificity. Like some rare tumour-derived mutants (Friedlander *et al*., 1996; Ludwig *et al*., 1996; Rowan *et al*., 1996), 277R is able to induce p21 but has a decreased ability to induce apoptosis.

Since the 121F mutant was originally selected for increased transactivation in yeast from a bax-like site, the simplest explanation for increased killing by the mutant in mammalian cells is increased *bax* transcription. Although 121F does induce *bax* in H1299 cells, it does so no better than wild-type p53, so the explanation must lie elsewhere. To identify potential new mediators of p53-dependent apoptosis we performed a DNA chip screen. The screen was not exhaustive because the chip represents only 10% of human genes, lacks many published p53 target genes and in several cases failed to detect induction seen on Northern blots. The screen did show that many genes are differentially induced by the specificity mutants. Two genes, *PIR121* and *Rad*, showed the expected phenotype for important apoptosis-inducing p53 target genes: no induction by 175H or 277R and better induction by 121F than wild-type p53. We have not mapped p53-binding sites in the promoter of either gene, so we cannot say whether they are direct targets with short G-rich p53-binding sites. In common with many described p53 target genes (e.g. *PIG* genes, Polyak *et al*., 1997), neither gene can induce apoptosis when expressed alone. This is the result expected if they function in a coordinated response.

An alternative model to explain the behaviour of the mutant is that p53 normally transactivates both inducers and inhibitors of apoptosis, and the critical defect in the 121F mutant is a failure to transactivate the latter. Paradoxically, the most interesting genes may thus be p53 target genes not induced by the 121F mutant. Among robustly induced p53 target genes *p21*, *mdm2* and *cyclin G1* stand out as a potential candidates for such a role because they are all better induced by 277R than 121F. Cyclin G has not been tested for the ability to inhibit apoptosis, but both p21 and MDM2 do have this effect. p53-induced apoptosis is favoured by deletion of the *p21* gene (Waldman *et al*., 1997) and attenuated by exogenous p21 expression (Gomez-Manzano *et al*., 1997), in keeping with cell fusion data suggesting that cell-cycle arrest and apoptosis are competing outcomes of p53 activation (Polyak *et al*., 1996). The mechanism is unclear, but could include inhibition of ASK1 signalling by p21 (Ichijo *et al*., 1997; Asada *et al*., 1999). There are three reasons to think that failure of p21 induction is not responsible for the increased cell killing by the 121F mutant. First, the

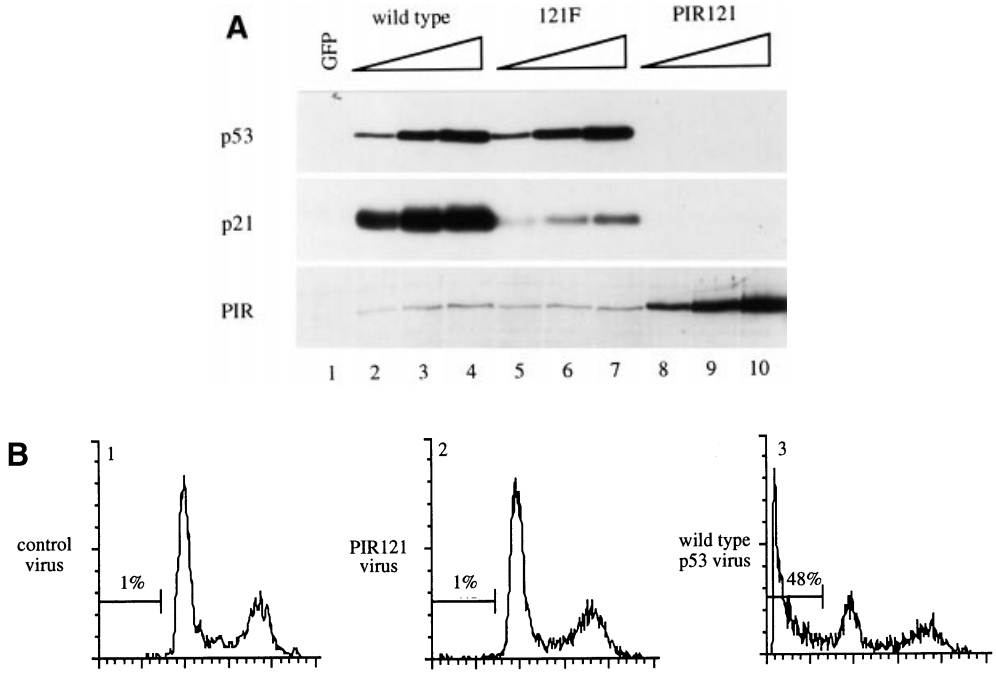

Fig. 10. Overexpression of PIR121 fails to induce apoptosis in H1299 cells. (**A**) Immunoblot of H1299 cells infected with GFP control adenovirus, and 3-fold dilutions of wild-type p53 virus, 121F virus and PIR121 virus. Blots were probed with anti-p53 (DO1), anti-p21 (C19) and anti-PIR121 antibodies. (**B**) Flow cytometry of H1299 cells 2 days after infection with GFP control adenovirus, PIR121 virus and wild-type p53 virus.

residual p21 expression appears sufficient to induce a G_1 arrest in the stably transfected H1299 cell clones. Secondly, in mouse embryo fibroblasts the difference in p21 induction is smaller than in human cells, but 121F still induces apoptosis better than wild-type p53. Thirdly, supplying p21 *in trans* by coinfection with a p21-expressing adenovirus does not suppress 121F-induced apoptosis.

Reduced MDM2 expression should result in p53 stabilization, because MDM2 targets p53 for ubiquitin-mediated degradation (Honda and Yasuda, 1999). Defective MDM2 induction thus has the potential to increase apoptosis induction by the 121F mutant by increasing the p53 protein level. Remarkably, in H1299 and Saos-2 cells there was no difference between the level or stability of wild-type and 121F mutant p53 despite clear differences in MDM2 induction. This is surprising because the same cell lines were used originally to demonstrate p53 destabilization by MDM2 (Haupt *et al*., 1997; Kubbutat *et al*., 1997). The difference between our results and those of Haupt and Kubbutat is presumably that the level of exogenous MDM2 in transfections is much higher than can be achieved from the endogenous gene. High-level MDM2 could overcome normal regulatory control either by saturating the available p19-ARF nucleolar anchors for MDM2 (Weber *et al*., 1999), or by forcing the p53/MDM2 equilibrium towards p53 binding despite the presence of unfavourable phosphate groups in the binding site (Shieh *et al*., 1999; Unger *et al*., 1999).

A greater surprise came from the analysis of p53 level and stability in cells retaining wild-type p53. The p53 level was indeed higher with the 121F virus, but the MDM2 level was the same as in cells infected with the virus expressing wild-type p53, and the wild-type and 121F proteins were both unstable. This would appear to indicate that the negative feedback loop balances p53 and MDM2 levels at a constant amount of MDM2, and achieving this level of MDM2 from a defective p53 mutant (possibly a mixed tetramer of wild-type and mutant p53) requires a higher level of p53. The triple mutant virus (22Q 23S 121F) gave the same level of p53 as the 121F virus, despite the fact that the triple mutant protein, which cannot bind MDM2, had a much longer half-life. Achieving the same level of protein with 121F and the triple mutant demands a higher level of p53 synthesis in cells containing the 121F mutant. There is no simple explanation for this observation, but it could be due to squelching of the viral CMV promoter by heterotetramers of wild-type and 121F mutant p53, activation of the endogenous p53 promoter or an effect on p53 translation. Solving this problem will require construction of new viruses encoding modified forms of the 121F mutant which can be distinguished from the endogenous protein.

One further unexpected result was the increase in level of wild-type and all p53 mutants irrespective of MDM2 level after doxorubicin treatment of the stable cell lines expressing p53 from the tet promoter. Since the p53 half-life was already long before treatment and we have good reason to think that endogenous MDM2 does not regulate p53 stability in these cells, the p53 induction again calls for a new explanation. Determining the mechanism of this DNA damage-dependent regulation will require detailed assessment of *p53* RNA levels and translation rates.

The 121F and 277R mutants were selected for activation of transcription from promoters containing truncated p53-binding sites in yeast. The specificity changes seen with 121F are intrinsic to the mutant because they can be reproduced with pure protein (Freeman *et al*., 1994). Although effects on DNA-binding specificity are to be expected because amino acid 121 is adjacent to a DNA contact residue and amino acid 277 directly contacts DNA (Cho *et al*., 1994), the p53-binding sites in the promoter of target genes are not simple palindromes that present

the same sequence to each p53 monomer, so we cannot easily extrapolate from model sites in yeast to endogenous mammalian promoters. Thus, altered DNA-binding specificity is simply the most economical explanation for the altered biological activity of the mutants. The difficulty of analysing actual promoters is illustrated by the fact that the 121F mutant has a reduced affinity for the distal p53-binding site in the human p21 promoter (Freeman *et al*., 1994), but should have an increased affinity for the proximal p21 site based on the sequence (Resnick-Silverman *et al*., 1998). Insofar as one can generalize, the properties of the 121F mutant would suggest that apoptosis-inducing p53 target genes have short G-rich p53-binding sites.

Adenoviruses expressing p53 are now entering clinical use for cancer therapy (Clayman *et al*., 1998). Since they show little toxicity but weak efficacy when used alone they are used in combination with chemotherapy to activate the transduced p53 (Ogawa *et al*., 1997). Another way to increase efficacy would be to use the virus expressing the 121F mutant, although toxicity to normal cells would clearly be a concern. We have shown that the 121F mutant can escape from normal regulation in a tumour cell containing wild-type p53, but this does not mean it would do so in a normal cell. Indeed, the failure of 121F to kill wild-type mouse embryo fibroblasts (Figure 6) can be interpreted in exactly this way. An intriguing possibility to reduce further the risk of damage to normal cells without affecting the activity of the 121F mutant in p53-null tumour cells would be to combine the 121F mutation with transactivation domain mutations that increase the affinity for MDM2 protein. Since the goal clinically is to achieve a useful therapeutic effect with acceptable toxicity, rather than zero toxicity, the 121F mutant virus is a candidate second generation virus for p53 cancer gene therapy.

Materials and methods

Yeast studies

Wild-type p53 was mutagenized by PCR, and the 277R mutant was cloned by gap repair and selected using a strain containing pLS168 (pDED1-lacZ) with a p53 consensus oligonucleotide (AGTG GAACAT-GTTC ACACT) cloned at the *Xho*I site as described by Freeman *et al.* (1994), then back-cloned into pLS89 to give pRDI-44.

For β-galactosidase transcription assays p53 was expressed from the GAL1 promoter using pLS89 (wild-type, Scharer and Iggo, 1992), pLS157 (121F, Freeman *et al*., 1994) and pRDI-44 in strains containing pLS168 with p53 consensus oligonucleotides (TCGAA GAA CATG TTC GAA CATG TTC T and TCGAA GGG CATG CCC GGG CATG CCC T) cloned at the *Xho*I site (plasmids pLS347 and pLS438, respectively). Protein extracts were prepared and assayed as described previously (Freeman *et al*., 1994).

For bandshift assays, p53 was truncated at amino acid 368 to delete the repression domain. p53 was expressed from the GAL1 promoter using pLS185 and pLS155 (wild-type and 121F, Freeman *et al*., 1994) and pES36 (277R, made by transferring a *Stu*I–*Eag*I fragment from pRDI-44 into pLS185). Protein extracts and probes were prepared and bandshift assays performed as described by Freeman *et al*. (1994).

Stable cell lines

To construct tetracycline-regulated expression vectors, *Bam*HI fragments containing wild-type, 121F and 277R *p53* cDNA were transferred from pLS89, pLS157 and pRDI-44 into the *Bam*HI site of pUHD10-3 (Gossen and Bujard, 1992) to give pES153, 155 and 156. To construct the 175H vector a *Bam*HI–*Eag*I fragment from pLS47 (Scharer and Iggo, 1992) was cloned blunt into the *Bam*HI site of pUHD10-3 to give pES154.

The H1299-derived cell line H24 containing the tet-VP16 transactivator was provided by Dr C.Prives (Chen *et al*., 1996). H24 cells cotransfected with 20 μg of pES153-156 and 2 μg of pBabe-puro (Morgenstern and Land, 1990) were grown in medium containing 2 μg/ml puromycin and 1 μg/ml tetracycline. Transformants were cloned twice and screened for inducible p53 expression by *in situ* staining using the anti-p53 antibody DO1 (Vojtesek *et al*., 1992).

p53 expression was induced by removal of tetracycline from the medium. Doxorubicin (40 ng/ml) was added to the medium 1 day after tetracycline removal. Immunoblotting was performed as described by Harlow and Lane (1988) using DO1 for p53, IF2 (Calbiochem) for human MDM2 and C19 (Santa Cruz) for p21.

Viral infections

Saos-2, U2OS and 293T cells were obtained from the American Type Culture Collection. HUG2 and HUG15 cells were provided by Dr M.Tada (Tada *et al*., 1996). U87MG cells were provided by Dr M.Hegi (Ponten and Macintyre, 1968). HER911 cells were provided by Dr P.Beard. Fibroblasts were prepared from wild-type p53 and p53-null mouse embryos (Lowe *et al*., 1993) using standard techniques. Fibroblasts from p53-null MDM2-null mouse embryos (Montes de Oca Luna *et al*., 1995) were provided by Dr G.Lozano.

The wild-type p53 adenoviral construct was made by two-step gene replacement in a yeast artificial chromosome as described by Ketner *et al.* (1994). Yeast plasmids were provided by Dr G.Ketner. Ad-CMV-lacZ was provided by Drs S.B.Verca and S.Rusconi. Viral DNA prepared from CsCl banded virus was cloned in yeast by homologous recombination using plasmids pRML1Ad5L and pRML2Ad5R (Ketner *et al*., 1994). An *Xba*I–*Eag*I CMV-βglobin intron-p53-βglobin poly(A) cassette from pC53-SN3 (Hinds *et al*., 1990) was cloned into the *Hin*dIII–*Eag*I sites in p680 (Ketner *et al*., 1994) to create pRDI-129. This was integrated into the Ad-CMV-lacZ YAC using LEU2 and excised using CYH2. YAC DNA was prepared without amplification, cut with *Pac*I and transfected into 293T cells to make virus.

The mutant p53 adenoviral constructs were made by two-step gene replacement in pJG54 (Gagnebin *et al*., 1999), a yeast artificial chromosome/bacterial artificial chromosome vector based on pNKBAC39 (Larionov *et al*., 1996), using pRDI-129 containing 121F (pRDI-146) and 277R (pRDI-147). The 22Q 23S mutations were cloned from pRc/CMV hp53 22-23 provided by Dr A.Levine (Lin *et al*., 1994) by three-way ligation with a 121F fragment into pJG32 (Gagnebin *et al*., 1999) to give pRDI-175. This was inserted in pJG54 as above. The PIR121 sequence was completed at the 5′-end by RACE–PCR using a Marathon cDNA kit (Clontech). The PIR121 adenovirus was made by inserting a full-length *PIR121* cDNA on a *Not*I fragment into pJG32 with a *Not*I linker at the *Bam*HI site (pRDI-201) to give pRDI-215. This was inserted in pJG54 as above. YAC/BAC DNA was extracted from bacteria, cut with *Pac*I and transfected into 293T cells to make virus.

Virus was plaque purified on HER911 cells, CsCl banded and titred on HER911 cells. DNA was extracted from the final virus preparation and sequenced to confirm the presence of the desired insert. The final virus preps are designated Ad-CMV-p53 (wild-type), vMO2 (121F), vMO3 (277R), vMO15 (22Q 23S 121F) and vMO26 (Ad-CMV-PIR121). Fine adjustment of viral titre was performed by reference to p53 level in Western blots of p53-null MDM2-null cells. The Ad-CMV-GFP virus was provided by Dr R.Sahli, CsCl banded and titred on HER911 cells.

Rabbit antibody (Eurogentec) was raised against a PIR121 C-terminal peptide encompassing amino acids 1215–1234.

Flow cytometry

For cell-cycle analysis, 10^6 cells were seeded per 100-mm plate with or without tetracycline. At the times indicated, cells were pulsed with 100 μM BrdU (Sigma) for 1 h, trypsinized and 10^6 cells were fixed in 70% methanol for at least 30 min. Cells were washed once with phosphate-buffered saline (PBS) and incubated in 2 M HCl for 30 min at room temperature. Cells were washed three times in PBS 1% bovine serum albumin (BSA) and resuspended in 25 μl PBS. Twenty-five microlitres of anti-BrdU-FITC (Becton Dickinson) were added and incubated for 30 min at room temperature. Cells were washed once in PBS 1% BSA, resuspended in 1 ml of propidium solution (10 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 50 μg/ml propidium iodide, 10 μg/ml RNase A) and incubated for 30 min at 37°C. Stained cells were analysed in a FACScan (Becton Dickinson). For viral infections, 10^6 floating and adherent cells were fixed in 75% ethanol, rinsed in PBS and stained with propidium as above.

Half-life measurement

p53 stability was measured by cycloheximide block as described by Hengstermann *et al.* (1998). Cells were seeded at 2.5×10^5 cells per

35-mm well and infected with adenoviruses expressing p53 from the CMV promoter. Fourteen hours after infection, 60 μg/ml cycloheximide was added to the medium and cells were incubated for the indicated periods. Immunoblotting was performed as described by Harlow and Lane (1988) using PAb1801 and PAb240.

Luciferase assays

Saos-2 cells were seeded at 2×10^5 cells per 35-mm well. A 100 ng aliquot of pBabe-puro plasmid expressing p53 from the LTR promoter and 5 μg of reporter plasmid was lipofected (Gibco-BRL) for 18 h. Cells were harvested 24 h later and assayed with a LUMAC Biocounter (MBV). Each value is the mean of three independent experiments. The MDM2 reporter was provided by Dr M.Oren (Juven *et al*., 1993) and the G-rich reporter (pWT30-tk-luc) by Dr D.Beach (Okamoto and Beach, 1994).

Northern blotting

Total RNA was isolated using an RNeasy kit (Qiagen) according to the manufacturer's instructions. RNA was electrophoresed through a MOPS/formaldehyde gel, blotted to positively charged membrane (Appligene), hybridized and washed as described by Sambrook (1989). Probes were labelled by hexamer primed incorporation of [α-^{32}P]dATP (Feinberg and Vogelstein, 1983) into restriction fragments purified from the following plasmids: cyclin G_1, IMAGE clone 43052 (Resgen); *fas*, full-length cDNA (Dr J.Tschopp); *p21*, full-length cDNA from pLS291 (Freeman *et al*., 1994); *MDM2*, full-length cDNA from pCmdm2 (Roemer and Mueller-Lantzsch, 1996); PIR121, IMAGE clone 47475 (Resgen); Rad, IMAGE clone 470661 (Resgen); *bax*, full-length cDNA (Dr J.C. Martinou); 36B4, cDNA nucleotides 763–958 (Laborda, 1991).

DNA expression chip screening

DNA chips representing 6500 human transcripts were hybridized to RNA extracted from H1299-derived cell lines expressing *p53* cDNA from the tet promoter essentially as described by Wodicka *et al.* (1997). The experimental background was taken as the mean of the uninduced values (6 h doxorubicin, no p53) for all four clones plus the induced values (6 and 24 h doxorubicin, plus p53) for the inactive 175H mutant. The fold induction (strictly, probability of induction) by p53 was calculated as a multiple of the standard deviation of this mean; genes induced by 175H p53 were excluded because this mutant is unable to transactivate p53 target genes.

Accession numbers

The following sequences have been deposited in the DDBJ/EMBL/GenBank database: human PIR121, accession No. AF160973 and mouse PIR121, accession No. AF162472 (EST database No. 518465).

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