The *WT1* gene product stabilizes p53 and inhibits p53-mediated apoptosis

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The Wilms' tumor-suppressor gene product WT1 coimmunoprecipitates with p53 from baby rat kidney (BRK) cells and Wilms' tumor specimens, and expression of WT1 in BRK cells is associated with increased levels of endogenous wild-type p53 protein. To study the effect of WT1 on p53 function, we cotransfected expression constructs into Saos-2 cells, an osteosarcoma cell line without endogenous expression of either gene. Expression of WT1 resulted in increased steady-state levels of p53, attributable to a prolongation in protein half-life, and associated with protection against papillomavirus E6-mediated degradation of p53. This effect mapped to zinc fingers 1 and 2 of WT1 and was not observed with the closely related EGR1 protein. The stabilized p53 demonstrated enhanced binding to its target DNA sequence and increased *trans*-activation of a promoter containing this RGC site, but reduced transcriptional repression of a TATA-containing promoter lacking this site. Expression of WT1 inhibited p53-mediated apoptosis triggered by UV irradiation or by expression of temperature-sensitive p53 in the wild-type conformation, but did not affect p53-mediated cell cycle arrest. We conclude that WT1 protein can stabilize p53, modulate its *trans*-activational properties, and inhibit its ability to induce apoptosis. This effect may contribute to the elevated levels of wild-type p53 protein that are observed in Wilms' tumors.

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Wilms' tumor is a pediatric cancer originating in cells of the developing kidney. Analysis of children with genetic susceptibility to Wilms' tumor led to the isolation of a tumor-suppressor gene, *WT1*, at the 11p13 chromosomal locus (Call et al. 1990; Gessler et al. 1990). In the kidney, *WT1* is normally expressed in specific precursor cells during a brief period in fetal development (Pritchard-Jones et al. 1990), and its physiologic role has been demonstrated by the failure of kidney development in mice with disrupted *WT1* alleles (Kreidberg et al. 1993). Mutational analysis has demonstrated inactivation of *WT1* in ~5%–10% of sporadic Wilms' tumors (for review, see Haber and Housman 1992), and reintroduction of *WT1* into a Wilms' tumor cell line resulted in growth suppression, consistent with its role as a tumor-suppressor gene (Haber et al. 1993).

The *WT1* transcript encodes a transcription factor that migrates at 55 kD on SDS-PAGE. Two functional domains are evident in WT1 protein: (1) a DNA-binding domain at the carboxyl terminus, consisting of four Cys-His zinc fingers that recognize a 5'-GCCGGGGCG-3' motif (Rauscher et al. 1990) as well as a 5'-TCCTC-CTCC-3' repeat (Wang et al. 1993; Englert et al. 1995),

and (2) a Pro-Glu-rich amino terminus that functions as a transcriptional repressor domain (Madden et al. 1991). Four isoforms of WT1 have been observed in WT1-expressing cells, resulting from the combination of two alternative splices (Haber et al. 1991). Alternative splice I inserts 17 amino acids between the amino terminus and the zinc finger domains and alternative splice II introduces 3 amino acids (KTS) between zinc fingers 3 and 4. This second alternative splice disrupts the spacing between two critical zinc finger domains, abolishing DNA-binding affinity for the GC-rich consensus and reducing that for the TC-rich motif (Rauscher et al. 1990; Bickmore et al. 1992; Englert et al. 1995). Although many WT1-responsive promoters have been reported in transient transfection assays (Rauscher et al. 1990; Madden et al. 1991; Drummond et al. 1992; Gashler et al. 1992; Wang et al. 1992; Harrington et al. 1993; Werner et al. 1993; Dey et al. 1994), to date, only one endogenous gene, the epidermal growth factor receptor (*EGFR*), has been shown to be repressed after WT1 expression (Englert et al. 1995).

In contrast to *WT1*, p53 is a tumor-suppressor gene expressed ubiquitously and mutated in a large fraction of human cancers (Levine et al. 1991). Homozygous disruption of p53 alleles in the mouse does not result frequently in developmental defects but leads to a dramat-

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ically increased incidence of diverse tumors (Donehower et al. 1992), consistent with its postulated role as a guardian of genomic integrity (Lane 1992). In cultured cells expressing wild-type p53, ionizing irradiation and other insults causing genetic damage result in a prolongation of p53 protein turnover, increased p53 levels, and either cell cycle arrest or apoptosis (Kastan et al. 1992; Clarke et al. 1993; Lowe et al. 1993; Lu and Lane 1993). Cell cycle arrest mediated by p53 is dependent on its transcriptional activation activity and associated with the induction of target genes including *p21/WAF1/Cip1* and *GADD45* (Fornace et al. 1989; El-Deiry et al. 1993). p53 protein binds to promoters containing TGCCT repeats, the so-called RGC sequence (Kern et al. 1991), present in the promoter of the muscle creatine kinase (*MCK*) gene, commonly used as a p53 target reporter (Weintraub et al. 1991; Zambetti et al. 1992). Analysis of multiple genomic p53 target sites has also led to a consensus-binding site, defined as two copies of the motif 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3' separated by 0–13 bp (El-Deiry et al. 1992).

In contrast to p53-mediated cell cycle arrest, induction of apoptosis by p53 appears to be independent of its transcriptional activation activity (Caelles et al. 1993), but may be linked to its ability to mediate transcriptional repression (Sabattini et al. 1995). p53 represses transcription from promoters lacking the RGC sequence, but containing a TATA box (Ginsberg et al. 1991; Mack et al. 1993), an effect that has been attributed to its binding to TATA-binding protein (TBP) (Seto et al. 1992; Chen et al. 1993; Liu et al. 1993). The potential contribution of p53-mediated transcriptional repression to its apoptotic function is supported by the recent observation that the potent antiapoptotic adenovirus E1B 19-kD protein inhibits its transcriptional repression by p53 while enhancing p53-mediated transcriptional activation (Sabattini et al. 1995). The specific targeting of p53 by viral oncoproteins is critical to their transforming ability. In addition to E1B 19-kD protein, adenovirus E1B 55-kD protein forms a complex with p53, leading to its stabilization but disrupting its *trans*-activational activity (Sarnow et al. 1982; Braithwaite and Jenkins 1989; Yew and Berk 1992). High-risk papillomaviruses, associated with cervical carcinoma, encode an E6 protein that forms a complex with cellular E6-associated-protein (E6AP) targeting p53 for ubiquitination and degradation (Scheffner et al. 1990; Werness et al. 1990; Lechner et al. 1992; Huibregtse et al. 1993a,b). This ubiquitination pathway appears to be a primary determinant of steady-state p53 levels in normal cells.

Recently, we have demonstrated the physical association between WT1 and p53 proteins in baby rat kidney (BRK) cells and primary Wilms' tumor specimens (Maheswaran et al. 1993). This protein–protein interaction was evident *in vivo* by sequential immunoprecipitation and immunoprecipitation–Western analyses of cellular lysates. In this work we demonstrate that p53 protein is stabilized by WT1 after cotransfection of expression constructs into Saos-2 cells. This effect required the presence of the first two WT1 zinc finger domains,

and was not mediated by the closely related *EGR1* gene. The ability of WT1 to stabilize p53 was associated with inhibition of papillomavirus E6-mediated p53 degradation. p53 protein stabilized by WT1 retained its ability to bind to the RGC consensus and *trans*-activate the *MCK* promoter. However, transcriptional repression by p53 of a TATA-containing promoter lacking the RGC site was inhibited by cotransfection of *WT1*. Expression of WT1 antagonized p53-mediated apoptosis but had no effect on p53-induced cell cycle arrest. The stabilization of p53 by WT1 and inhibition of p53-mediated apoptosis provide a potential explanation for an intriguing observation: the presence of high levels of p53 protein in primary Wilms' tumors in the absence of stabilizing p53 mutations.

Results

Stabilization of p53 in BRK cells expressing WT1

Previously, we have described BRK cell lines that were immortalized after transfection with adenovirus *E1A* alone (6.1 cells), *E1A* plus *WT1*, isoform A lacking both alternative splices (A6 cells), and *E1A* plus *WTAR*, a naturally occurring *WT1* mutant with an in-frame deletion of zinc finger 3 (6.5 cells) (Haber et al. 1990). A6 and 6.5 cells expressed the transfected murine WT1 protein, whereas endogenous rat WT1 protein in 6.1 cells was not detectable by immunoblotting (Haber et al. 1992). Immunoblotting analysis also demonstrated increased steady-state levels of p53 protein in A6 and 6.5 cells compared with 6.1 cells (Fig. 1a). This increase in p53 expression was not attributable to elevated p53 mRNA levels (Fig. 1b) nor was there an increase in new p53 protein synthesis, as determined by radiolabeling (data not shown). However, the half-life of p53 protein was prolonged in cells expressing either WT1 or WTAR. The p53 half-life was calculated to be <25 min in 6.1 cells (*E1A* alone), compared with 120 min in A6 cells (*E1A* plus *WT1*) and 80 min in 6.5 cells (*E1A* plus *WTAR*), using p53 immunoblot analysis of cycloheximide-treated cellular lysates (Fig. 1c). These results were confirmed by pulse-chase experiments (data not shown). To exclude a mutation in the endogenous p53 alleles leading to stabilization of the encoded protein, the entire coding sequence of the rat p53 transcript (Soussi et al. 1988) was amplified by RT-PCR and sequenced. No mutation in the endogenous p53 transcript was detected in 6.1, A6, or 6.5 cells. A p53 mutation (Arg → His in rat codon 246, analogous to human codon 248) was detected in another, previously described BRK cell line, 6.3 (Maheswaran et al. 1993), which was not characterized in this study.

These observations suggested that expression of *WT1* in BRK cells might be associated with altered stability of endogenous wild-type p53 protein. However, interpretation of these studies was complicated for two reasons: (1) Analysis of stably transfected cell lines was restricted to a small number of clones, and (2) BRK clones transfected with *E1A* were genetically unstable. As demonstrated by White and co-workers (Debbas and White 1993), BRK cell lines immortalized by *E1A* alone acquired p53 mutations at high frequency with continued growth in cul-

Figure 1. Stabilization of endogenous p53 in BRK cells expressing WT1. (a) Endogenous p53 protein is elevated in BRK cells expressing stably transfected WT1. Immunoblot analysis of cellular lysates from BRK cell lines immortalized with E1A alone (6.1 cells), E1A+E1B (10.2 cells), E1A+WT1 (A6 cells), and E1A+WTAR (6.5 cells), probed with anti-p53 antibody PAb 421. (b) Expression of WT1 does not affect endogenous p53 mRNA levels. Northern blot analysis of total cellular RNA from BRK cell lines transfected with E1A (6.1 cells), E1A+WT1 (A6 cells) and E1A+WTAR (6.5 cells), probed with rat p53 cDNA and with GAPDH (loading control). (c) Endogenous p53 protein is stabilized by stably transfected WT1. Immunoblot of cellular lysates from BRK cell lines expressing E1A (6.1 cells), E1A+WT1 (A6

cells), and E1A+WTAR (6.5 cells) extracted at various intervals after inhibition of new protein synthesis with cycloheximide (CHX) and probed with anti-p53 antibody PAb 421. A single plate of cells treated with cycloheximide was divided equally into six plates, one of which was analyzed for each time point. A representative experiment is shown.

ture—a transformation that was accompanied by altered growth properties and an increase in p53 levels on immunoblot analysis. To avoid clonal variation between cell lines and the risk of de novo p53 mutations, we transiently transfected plasmids encoding WT1 and p53 into Saos-2 cells, an osteosarcoma cell line with deleted p53 alleles and minimal WT1 expression detectable only by RT-PCR [Masuda et al. 1987; Diller et al. 1990; C. Englert and D. Haber, unpubl.].

Stabilization of p53 by WT1 in Saos-2 cells

Saos-2 cells were transfected transiently with 1–2 μ g of a cytomegalovirus (CMV)-driven p53 construct and either 10 μ g of WT1 expression plasmid or the empty expression plasmid, followed by protein extraction at 24–48 hr. Transfection with small amounts of the p53 construct was chosen to avoid saturating the p53 degradation pathway and to avoid potential effects on cell viability (see below). Constructs encoding WT1 isoform A, lacking both alternative splice insertions, were used in these experiments. Cotransfection of p53 and WT1 into Saos-2 cells consistently led to 5- to 20-fold increased steady-state levels of p53, compared with transfection of p53 alone (Fig. 2a). No increase in steady-state levels of p53 was observed after cotransfection of WT1 and two p53 mutants encoding proteins with short turnover (codons 245 and 282) (Fig. 2b), suggesting that this effect resulted from a specific interaction between WT1 and wild-type p53. The increase in wild-type p53 protein resulting from cotransfection of WT1 was not attributable to increased transcription, as p53 mRNA levels remained unaltered (Fig. 2c), nor was an increase in new p53 protein synthesis evident by radiolabeling and immunoprecipitation analysis (data not shown). However, pulse-chase analysis demonstrated a prolongation in the half-life of p53 pro-

tein, from 1–2 hr for cells transfected with p53 alone to >4 hr for cells cotransfected with both p53 and WT1 (Fig. 2d). p53 turnover was also calculated from cycloheximide–Western analysis, with a p53 half-life of <30 min in cells transfected with p53 alone, versus 90 min in cells cotransfected with p53 and WT1 (Fig. 2e). Both techniques indicated stabilization of p53 after cotransfection of WT1, and the difference in absolute numbers calculated for the p53 half-life most likely resulted from different technical requirements for the two approaches (see Materials and methods). To ensure that the increase in p53 levels after cotransfection with WT1 did not result from any effect on the number of Saos-2 cells surviving transfection with p53, we analyzed cell survival and cell cycle distribution after cotransfection of WT1, p53, and the cell surface marker CD20 [van den Heuvel and Harlow 1993; Zhu et al. 1993]. FACS analysis of CD20-expressing Saos-2 cells, 24–48 hr after transfection with 1–2 μ g of CMV–p53 demonstrated no difference between those cotransfected with WT1 plasmids or the empty vector (data not shown).

WT1 domain required for stabilization of p53

To determine the WT1 domain required for the stabilization of p53, we used five synthetic deletion constructs, spanning the entire length of the WT1 protein. The WT1 deletion constructs were cotransfected with p53 into Saos-2 cells, and steady-state p53 protein levels were determined by immunoblotting. Three truncated WT1 proteins, with internal deletions spanning the amino terminus, retained their ability to stabilize p53 protein, as did one with a deletion of zinc fingers 3 and 4. However, a WT1 mutant encoding a deletion of zinc fingers 1 and 2 (Δ 282– Δ 364) was unable to stabilize p53 protein (Fig. 3a). All WT1 deletions were cloned into the same CMV-

Figure 2. Stabilization of transfected p53 in Saos-2 cells by cotransfected WT1. (a) Cotransfection of WT1 increases steady-state levels of transiently transfected wild-type p53. p53 immunoblot of cellular lysates from Saos-2 cells, 36 hr after transfection with vector alone (V; 12 μ g), CMV-p53 (2 μ g) plus vector (10 μ g) (p53 + V), and duplicate samples of CMV-p53 (2 μ g) plus CMV-WT1 (10 μ g) (p53 + WT1) or CMV-p53 (2 μ g) plus CMV-WTAR (10 μ g) (p53 + WTAR). Loading of protein lysates was normalized for transfection efficiency, based on a cotransfected human growth hormone reporter. The resulting differences in total protein loaded per lane were under 10% (Bio-Rad assay). (b) Cotransfection of WT1 does not stabilize mutant p53 proteins. p53 immunoblot of cellular lysates from Saos-2, transiently transfected with CMV-driven mutant p53 (mp53 codons 245 or 282; 2 μ g) plus either vector (10 μ g) or CMV-WT1 (10 μ g). (c) Cotransfection of WT1 does not affect p53 mRNA levels. Northern blot of total cellular RNA

from Saos-2 cells, 36 hr after transfection with vector alone, CMV-p53 plus vector, CMV-p53 plus CMV-WT1, or CMV-p53 plus CMV-WTAR, probed with human p53 cDNA. After RNA isolation, samples were treated with DNase to digest residual plasmid sequences. RNA loading was normalized for transfection efficiency, based on a cotransfected human growth hormone reporter. (d) Cotransfection of WT1 stabilizes p53 protein. Pulse-chase analysis of p53 turnover in Saos-2 cells, after transfection with either CMV-p53 plus vector or CMV-p53 plus CMV-WT1. Cells were methionine-starved for 30 min, pulsed with [35 S]methionine for 10 min, chased with cold methionine, and divided equally into four dishes. Protein lysates were extracted at the times indicated, followed by immunoprecipitation with anti-p53 antibody PAb 421. The first lane (–) shows the migration position of p53. (e) p53 immunoblot of cellular lysates from Saos-2 cells transiently transfected with either CMV-p53 plus vector or CMV-p53 plus CMV-WT1 and extracted at various intervals after inhibition of new protein synthesis with cycloheximide (CHX).

driven expression construct, and immunoprecipitation analysis demonstrated comparable expression levels (Fig. 3a). The ability of WT1 to stabilize p53 levels was not shared by EGR1 (Sukhatme et al. 1988), a zinc finger protein that has extensive homology to WT1 zinc fingers 2–4 (Fig. 3c). The WT1 domain containing zinc fingers 1 and 2 that is required for stabilization of p53 in transfection assays may represent the site of physical binding between these two proteins. However, confirmation of this potential binding site will require the demonstration of direct binding between purified WT1 and p53 proteins. Definition of the p53 domain required for interaction with WT1 could not be achieved by our *in vivo* assay, because of the variable half-life of altered p53 proteins.

WT1 antagonizes E6-mediated p53 degradation

The normal steady-state levels of p53 protein appear to be regulated by its ubiquitination and degradation, a process that is accelerated by the E6 protein derived from high-risk papillomavirus, in association with a cellular protein, E6AP (Huibregtse et al. 1991, 1993a,b; Scheffner et al. 1990, 1993). To test the effect of WT1 on E6-regulated p53 turnover, we cotransfected Saos-2 cells with WT1, p53, and a CMV-driven E6 construct derived from

human papillomavirus (HPV)-16. Transfection of cells with 10 μ g of p53 resulted in readily detectable steady-state expression at 24 hr, which was reduced dramatically by cotransfection of 3 μ g of E6. p53 levels were restored consistently to ~70% of baseline after transfection of 20 μ g of WT1 along with E6 (Fig. 4). These observations suggested that WT1 antagonized E6-regulated p53 degradation. WT1 had no effect on E6 expression levels (data not shown), suggesting that its inhibition of E6-mediated p53 degradation may result from interference with binding of p53 to E6, E6AP, or other factors involved in the ubiquitination machinery.

Functional properties of stabilized p53

To determine whether p53 protein stabilized by WT1 retained its ability to bind DNA, we performed gel mobility-shift assays using the radiolabeled RGC sequence. Nuclear protein lysates from Saos-2 cells cotransfected with p53 and WT1 demonstrated approximately fivefold increased binding to the RGC sequence, compared with those transfected with p53 and the empty vector (Fig. 5a). The increase in RGC-binding activity was mediated specifically by p53, as demonstrated by supershifting of the protein–DNA complex with the monoclonal anti-p53 antibody PAb-421. No binding to the RGC sequence was

Figure 3. WT1 domain required for stabilization of p53. (a) WT1 zinc fingers 1 and 2 are required for stabilization of p53 protein. (Left) p53 immunoblot of cellular extracts from Saos-2 cells, 36 hr after transfection with CMV-p53 (2 μ g) along with either empty vector (10 μ g), CMV-WT1 (WT1; 10 μ g) or CMV-driven WT1 deletion constructs (Δ ; 10 μ g each). All deletion constructs were cloned into the same expression construct and tagged with the HA epitope. (Right) Immunoprecipitation of WT1 from radiolabeled lysates of cells transfected with WT1 deletion constructs to demonstrate comparable levels of expression of the truncated proteins. Immunoprecipitations were performed using antibody 12-CA5 against the HA epitope attached to the carboxyl terminus of the deletion constructs. The loading of protein lysates was normalized for transfection efficiency, based on a cotransfected human growth hormone reporter. (b) Schematic representation of the WT1 deletion constructs. All deletion constructs lacked both WT1 alternative splices. (c) Transfection of *EGR1* does not stabilize p53 protein. p53 immunoblot analysis of extracts from Saos-2 cells transfected with CMV-p53 plus CMV-EGR1, compared with cells transfected with CMV-p53 plus vector, CMV-WT1, or WT1 deletion constructs lacking zinc fingers 1 and 2 (Δ 282- Δ 364) or zinc fingers 3 and 4 (Δ 363- Δ 450).

detected in cells transfected with *WT1* alone. Interestingly, p53 protein stabilized by the naturally occurring mutant *WTAR* (lacking zinc finger 3) did not show increased DNA-binding activity (Fig. 5a). This suggests that stabilization of p53 is not sufficient for increased DNA binding but that an intact WT1 DNA-binding domain may be required. However, we were unable to demonstrate the presence of WT1 within the p53-DNA complex because of the lack of anti-WT1 antibodies capable of supershifting a WT1-DNA complex. Thus, we presume that the increase in p53 DNA-binding activity results from direct binding of WT1 and p53 proteins, possibly requiring the presence of a DNA-binding site, but we cannot exclude a separate, more indirect mechanism.

p53 has been shown to be a transcriptional activator of promoters containing the RGC consensus sequence (Kern et al. 1991; Weintraub et al. 1991; Zambetti et al. 1992). To examine whether the enhanced DNA binding of p53 stabilized by WT1 was associated with an increase in transcriptional activation by p53, we cotransfected *WT1* and p53 expression constructs into Saos-2 cells and measured their effect on the MCK-chloramphenicol acetyltransferase (CAT) reporter gene. Consistent with our previous results, activation of this RGC-containing promoter by p53 was enhanced four- to sixfold by

cotransfection of wild-type *WT1* but not mutant *WTAR* (Fig. 5b; Maheswaran et al. 1993). The magnitude of this

Figure 4. Inhibition of E6-mediated degradation of p53 by cotransfected WT1. p53 immunoblot of extracts from Saos-2 cells, 36 hr after transfection with CMV-p53 (10 μ g) plus either vector, CMV-E6 (3 μ g), or CMV-E6 and CMV-WT1 (20 μ g). Vector DNA was added to each transfection to equalize the total amount of transfected DNA. The loading of protein lysates was normalized for transfection efficiency, based on a cotransfected human growth hormone reporter. Extract from untransfected cells (Saos-2) is shown in lane 1.

Figure 5. DNA-binding and *trans*-activational properties of p53 after cotransfection of WT1. (a) WT1 enhances the DNA-binding activity of p53. Gel retardation assay of the radiolabeled, annealed p53 target RGC sequence, incubated with extracts from untransfected Saos-2 cells (–) or extracts from cells 36 hr after transfection with CMV-p53 (2 μ g) plus vector (V, 10 μ g), CMV-p53 plus CMV-WT1 (10 μ g), CMV-p53 plus WTAR (10 μ g), and either CMV-WT1 or CMV-WTAR alone. The position of the DNA-p53 protein complex is shown by an arrowhead. The same extracts were incubated with the labeled RGC sequence in the presence of the anti-p53 monoclonal antibody PAb 421, and the migration position of the supershifted complex is shown by the bracket. Both panels were derived from the same gel. (b) WT1 enhances p53-mediated transcriptional activation. Chloramphenicol acetyltransferase (CAT) activity of the RGC-containing MCK-CAT reporter, 36 hr after transfection of Saos-2 cells with vector (10 μ g), CMV-p53 (2 μ g), a CMV-driven mutant p53 at codon 143 (mp53; 2 μ g), CMV-p53 plus CMV-WT1 (10 μ g), CMV-p53 plus CMV-WTAR (10 μ g), or CMV-WT1 and CMV-WTAR alone. Vector DNA was added to each transfection to equalize the amount of transfected DNA. Transfection efficiencies were standardized by cotransfection of a human growth hormone reporter, and fold induction was calculated by scintillation counting of the thin layer chromatography plate. A representative experiment is shown. (c) WT1 inhibits p53-mediated transcriptional repression. CAT activity of the TATA-CAT reporter, lacking the RGC site, after transient transfection of Saos-2 cells with vector, CMV-p53 (2 μ g), CMV-p53 plus CMV-WT1 (20 μ g), CMV-p53 plus CMV-WTAR (20 μ g), or CMV-WT1 and CMV-WTAR alone. Vector DNA was added to each transfection to equalize the amount of CMV promoter sequences, and transfection efficiency was calculated by cotransfection of a human growth hormone reporter. A representative experiment is shown.

effect and the lack of effect after cotransfection of mutant WTAR was consistent with the increase in p53 DNA-binding activity (Fig. 5a). As expected, WT1 itself had no significant effect on the MCK-CAT reporter.

In contrast to its activation of transcription from a promoter containing the RGC consensus sequence, p53 represses transcription from a reconstituted promoter containing a TATA box but lacking the p53 target site (pTATA-CAT) (Mack et al. 1993). As expected, transfection of p53 into Saos-2 cells led to a 10-fold transcriptional repression of pTATA-CAT. However, cotransfection of WT1 resulted in a partial but consistent reversal of p53-mediated transcriptional repression (Fig. 5c). This

effect was not observed after transfection of mutant WTAR and did not result from independent activation of pTATA-CAT by WT1 itself. Thus, the interaction between wild-type p53 and WT1 resulted in enhanced p53-mediated transcriptional activation but reduced transcriptional repression.

Inhibition of p53-mediated apoptosis by WT1

The ability of WT1 to antagonize p53-mediated transcriptional repression while enhancing p53-induced transcriptional activation was reminiscent of adenovirus E1B 19-kD protein. This virally encoded gene product

has not been shown to bind p53, but it exerts similar effects on p53-mediated *trans*-activation, properties that have been linked to its potent antiapoptotic effect (Sabattini et al. 1995). To test the effect of WT1 on p53-mediated cell death, we first used the U2OS osteosarcoma cell line. These cells are diploid and highly transfectable like Saos-2 cells, but they express wild-type endogenous p53 and have intact p53-responsive pathways (Kastan et al. 1992). The endogenous *WT1* transcript in these cells is wild type and detectable at low levels by Northern blot, but WT1 protein expression is below detection by immunoblotting (data not shown). To generate U2OS cells in which we could manipulate both p53 and *WT1*, we transfected these cells with a CMV-driven temperature-sensitive p53 allele (*ts p53*)

(Michalovitz et al. 1990) and used these cells as founders to transfect an inducible tetracycline-regulated *WT1* or mutant *WTAR* allele (cells demonstrated 10-fold induction of a luciferase reporter; see Materials and methods). Two representative derived clones, U2OS A17 (*WT1*) and U2OS W19 (*WTAR*), expressed p53 in the wild-type conformation at 32°C and in the mutant conformation at 39°C. WT1 expression in these cells was undetectable by immunoblotting in the presence of tetracycline and was promptly induced by withdrawal of the drug (Fig. 6a). The low levels of inducible WT1 expression in these cells had no discernible effect on their growth properties.

In the absence of WT1 expression, shifting U2OS A17 cells to 32°C resulted in the prompt induction of apoptosis and complete death of the culture within 48 hr (Fig.

Figure 6. Inhibition of p53-mediated apoptosis by WT1 in U2OS cells. (a) Wild-type WT1 inhibits apoptosis induced by p53. (Left) WT1 immunoblot analysis of representative U2OS cell lines demonstrating tightly regulated expression of WT1 (A17 cells) or mutant WTAR (W19 cells) in the presence or absence of tetracycline (+/- tet). p53 immunoblot analysis of these cells demonstrates expression of the temperature-sensitive p53 allele, which is not affected by tetracycline. (Right) Survival curve of U2OS A17 and U2OS W19 cells after temperature shift to 32°C, which favors the wild-type conformation of p53. Cells were grown in the absence (+ tet) or presence (- tet) of WT1 or WTAR expression. Viable cells were counted by exclusion of trypan blue stain. (b) WT1 does not affect p53-mediated cell cycle arrest. Shown is flow cytometric analysis of U2OS A17 cells stained with propidium iodide. p53 was induced for 24 hr by shifting from 39°C to 32°C, either in the absence (+ tet) or presence (- tet) of WT1 expression. The proportion of cells in G1 (2N) vs. G2 (4N) is shown (%), with the remaining cells in S phase (upper left; log phase growth). The cell cycle arrest observed in U2OS cells overexpressing wild-type p53 occurs both in G1 and G2, with a reduction in the number of cells in S phase (upper right). Induction of WT1 expression has no effect on cell cycle distribution (lower left) nor does it affect p53-mediated cell cycle arrest (lower right). (c) The WT1(-KTS) splicing variant, but not the WT1(+KTS) variant, inhibits UV-mediated apoptosis. (Left) WT1 immunoblot analysis of representative U2OS cell lines, demonstrating tetracycline-regulated expression of WT1-A (lacking alternative splice I and splice II or KTS; RA19 cells) and WT1-C (lacking splice I, encoding splice II; RC18 cells). (Right) Survival curve of U2OS RA19 and RC18 cells after exposure to 20 J/m² of UV irradiation. Cells were grown in the absence (+ tet) or presence (- tet) of WT1-A (-KTS) or WT1-C (+KTS) expression.

6a). However, withdrawal of tetracycline and induction of WT1 expression prevented the onset of p53-mediated cell death. The protective effect of WT1 was observed after withdrawal of tetracycline ≤ 24 hr after temperature shift to 32°C. After that time, however, the initiation of p53-induced apoptosis could not be reversed by expression of WT1. In contrast to the effect of wild-type WT1, induction of WTAR expression failed to prevent p53-mediated apoptosis in U2OS W19 cells. U2OS A17 cells expressing WT1 remained growth arrested and viable for ≤ 7 days at 32°C, with renewed growth after temperature shift to 39°C. The inhibition of p53-mediated apoptosis by WT1 thus uncovered p53-induced growth arrest, which was not affected by WT1 expression. FAC-Scan analysis of U2OS A17 cells lacking WT1 expression 24 hr after temperature shift to 32°C showed a cell cycle arrest (31% G1, 52% G2 phase) that preceded the onset of apoptosis (Fig. 6b). Induction of WT1 expression in these cells before the temperature shift resulted in the same cell cycle distribution, indicating that WT1 did not affect p53-mediated cell cycle arrest. Induction of WT1 itself had no effect on cell cycle distribution of cells grown at 39°C. Expression of WT1 also did not affect the induction by p53 of its target genes implicated in cell cycle arrest, *p21/WAF1/Cip1* and *GADD45* (data not shown). To test whether the antiapoptotic properties of WT1 were restricted to p53-dependent apoptotic pathways, we examined the effect of WT1 expression on cell death induced by staurosporine, an agent known to induce direct, p53-independent apoptosis (Jacobson et al. 1993). Expression of WT1 in U2OS A17 cells failed to inhibit apoptosis induced by this drug (data not shown), suggesting that WT1 did not exert a universal antiapoptotic effect.

To test the effect of WT1 on p53-mediated apoptosis pathways triggered by genetic injury, such as ionizing radiation, multiple additional U2OS cell lines were generated, expressing tetracycline-repressable wild-type WT1 (Fig. 6c). Although U2OS cells were resistant to ≤ 100 cGy of γ irradiation, exposure 20 J/m² of UV irradiation resulted in prompt apoptosis of 80% of the culture, followed after 72 hr by the renewed growth of surviving cells. This effect required the presence of wild-type p53, and no cell death was observed after UV irradiation of U2OS cells expressing ts p53 in the mutant conformation (data not shown). Multiple U2OS clones expressing inducible wild-type WT1 demonstrated protection against UV-induced apoptosis, whereas cells expressing mutant WTAR underwent UV-induced cell death. We used protection against UV-mediated cell death to compare the different isoforms of wild-type WT1. Presence of alternative splice I between the trans-activation and DNA-binding domains of WT1 did not reduce its ability to inhibit apoptosis. However, alternative splice II, which inserts three amino acids (KTS) between zinc fingers 3 and 4 and alters its DNA-binding affinity, abolished the antiapoptotic effect of WT1. Two representative cell lines are shown, RA19-expressing inducible WT1 (–KTS) and RC18-expressing WT1 (+KTS) (Fig. 6c). Thus, the ability of WT1 to inhibit p53-mediated

apoptosis required an intact DNA-binding domain, and was restricted to one of the two alternatively spliced WT1 variants. This observation is of particular interest, given the recent observation that WT1 (–KTS) presumably functions as a transcription factor, whereas WT1 (+KTS) is physically associated with distinct subnuclear structures (Larsson et al. 1995).

Absence of apoptosis in E1A-immortalized BRK cells expressing WT1

To study the antiapoptotic properties of WT1 in a well-defined, kidney-derived system, we examined its effect on E1A-immortalized BRK cells. The adenovirus gene E1A immortalizes primary BRK cells while at the same time triggering p53-mediated apoptosis, which acts to counter the proliferative effect induced by E1A (Debbas and White 1993; Lowe et al. 1993). Therefore, full neoplastic transformation requires expression of E1A together with other genes capable of inactivating p53 or the emergence of cells containing mutant endogenous p53 alleles. To study the effect of WT1 on E1A-induced, p53-dependent apoptosis, we first examined the BRK cell lines 6.1 and A6, described above. Consistent with the observations of White and co-workers, we found that initially E1A-immortalized BRK 6.1 cells grew poorly. Multiple apoptotic cells were present within the population, as illustrated by phase-contrast microscopy and DNA fragmentation assays (Fig. 7a,b). Upon prolonged culture, clones containing a mutant p53 emerged within the cell population and apoptosis was no longer evident. In contrast, A6 cells, expressing WT1 along with E1A did not contain apoptotic cells, despite maintaining wild-type p53. To ensure that this observation did not result from an undetected abnormality in the endogenous p53 gene in the A6 cell line, these cells were transfected with a CMV-driven ts p53 allele, and three independent clones expressing either high or low levels of ts p53 were characterized (A6 ts4, A6 ts25, and A6 ts27) (Fig. 7c). E1A-immortalized cells expressing ts p53 alone (BRK ts) rapidly underwent cell death after incubation at 32°C. In contrast, the three A6-derived clones expressing ts p53 were protected from cell death at 32°C (Fig. 7c). These observations suggested that WT1 expression antagonized the E1A-induced, p53-mediated cell death pathway that has been characterized in BRK cells (Lowe and Ruley 1993; Debbas and White 1993).

Elevated p53 levels in Wilms' tumors

The experiments described above demonstrated that WT1 stabilized p53 and inhibited p53-mediated apoptosis in cultured kidney and osteosarcoma cells. These observations raised the possibility that p53 levels might be affected in vivo, in tissues or tumors expressing high levels of WT1. Most sporadic Wilms' tumors express wild-type WT1, consistent with their presumed origin from renal precursor cells (Haber et al. 1990; Pritchard-Jones et al. 1990). Therefore, we compared the levels of WT1 and p53 expression in eight primary sporadic Wilms' tumor specimens. RT-PCR amplification and

Figure 7. Inhibition by WT1 of apoptosis induced by E1A and p53 in BRK cells. (a) WT1 inhibits apoptosis in BRK cells expressing E1A and wild-type p53. Phase-contrast micrograph (40 $\times$) of BRK cells immortalized with E1A (6.1 cells) or E1A plus WT1 (A6 cells). Apoptotic cells are seen as refractile, poorly adherent cell bodies. (b) DNA fragmentation assay of BRK cells expressing E1A alone (6.1 cells) or E1A plus WT1 (A6 cells). Poorly adherent cells were harvested, genomic DNA was isolated and electrophoresed on a 2% agarose gel. The two samples were normalized to the total number of cells per dish. (c) WT1 inhibits apoptosis in BRK cells expressing the ts p53 allele. (Left) WT1 and p53 immunoblot analyses of BRK cell lines stably transfected with ts p53 and E1A (BRK ts) or ts p53, E1A, and WT1 (A6 ts4, A6 ts25, and A6 ts27). (Right) Survival curve of BRK ts cells and A6 ts cells after temperature shift to 32°C. Viable cells were counted by exclusion of trypan blue stain.

nucleotide sequencing of both *WT1* and p53 transcripts derived from these tumors demonstrated no mutation in either gene (Haber et al. 1990; data not shown). Immunoblot analysis demonstrated different levels of WT1 expression in the tumors, consistent with variable WT1 expression by the different histologic subtypes comprising Wilms' tumor (Fig. 8). Immunoblot analysis of these Wilms' tumors using anti-p53 antibody showed steady-state p53 levels to be elevated dramatically, compared with other tumor-derived cell lines such as U2OS, in which p53 is wild type. The relative p53 levels in the eight tumors appeared to be well correlated with the WT1 expression level. These observations suggest that the elevated steady-state levels of wild-type p53 in Wilms' tumors may be associated with WT1-mediated protein stabilization, although a larger number of sam-

ples will be required to demonstrate a definitive correlation.

Discussion

Previously, we have demonstrated the physical interaction between p53 and WT1 proteins in BRK cells and in primary Wilms' tumor specimens (Maheswaran et al. 1993). In this report we examined the role of WT1 expression in modulating p53 function. BRK cells expressing stably transfected WT1 contained increased levels of endogenous wild-type p53 resulting from protein stabilization. This effect was confirmed by the stabilization of p53 after transient transfection of p53 and WT1 expression constructs into Saos-2 cells lacking endogenous p53. The mechanism underlying stabilization of p53 by WT1 is unknown, but the inhibition by WT1 of E6-mediated p53 degradation suggests that it may result in part from interference with the normal ubiquitination of p53. The stabilized p53 demonstrated altered *trans*-activational properties: enhanced transcriptional activation of a target promoter containing the RGC target sequence but reduced transcriptional repression of a TATA-containing promoter lacking this DNA sequence. The modulation of p53 *trans*-activational properties by WT1 was similar to the effect of adenovirus E1B 19-kD protein (Sabbatini et al. 1995). Like the E1B 19-kD protein, WT1 prevented p53-mediated apoptosis but not p53-induced cell cycle arrest. The ability of WT1 to stabilize and inactivate p53 partially may be important in Wilms' tumors, where elevated expression levels of wild-type p53 appear to be correlated with expression of WT1.

Figure 8. Expression levels of wild-type p53 and WT1 in primary Wilms' tumors. (Left) p53 levels are increased in Wilms' tumor cells. p53 immunoblot analysis of U2OS cells and a sporadic Wilms' tumor specimen (also shown as specimen 3 in right panel). p53 alleles in both cell types are wild type. The amount of cellular lysate in each lane was standardized by Bio-Rad assay, and equal transfer to Immobilon-P was confirmed by staining the upper and lower portions of the filter with amido black. (Right) Correlation between expression levels of p53 and WT1 in Wilms' tumor specimens. Immunoblot analysis of eight primary sporadic Wilms' tumors, probed with either anti-p53 antibody PAb 421 or anti-WT1 antibody WTC8. The presence of wild-type p53 and wild-type *WT1* transcripts in all specimens was confirmed by nucleotide sequencing. The amounts of protein lysates loaded in each lane were standardized as described above.

WT1 zinc fingers 1 and 2 are required to stabilize p53

The characterization of the WT1–p53 interaction has been complicated by the difficulty in demonstrating protein association in vitro. High levels of expression in vitro are required to show binding between these two proteins, and WT1 and p53 do not interact in the yeast two-hybrid assay (C. Englert and D.A. Haber, unpubl.). These observations suggest either that modification of these proteins is required for their interaction or that a third protein component is essential for stabilization of the complex. Gel filtration and two-dimensional gel analyses of cellular lysates have indicated that WT1 and p53 are present within a protein complex of 100–150 kD [Maheswaran et al. 1993], possibly allowing for the presence of additional proteins in the complex.

The stabilization of p53 by WT1 observed in transient transfection assays allowed an initial analysis of the WT1 domain required for its interaction with p53. The first two zinc fingers of WT1 are required for stabilization of p53, suggesting that these domains may have properties in addition to their role in mediating DNA-binding specificity. Although these WT1 zinc fingers are required for p53 stabilization, additional WT1 domains may contribute to the functional properties of the p53–WT1 complex. A naturally occurring WT1 mutant lacking zinc finger 3, *WTAR*, failed to alter the *trans*-activational or apoptotic properties of p53, despite its ability to increase steady-state levels of p53 protein. Thus, DNA binding by WT1 itself may contribute to the functional properties of the WT1–p53 complex. Further studies involving purified WT1 and p53 proteins will be required to address these possibilities as well as to define the domain within p53 required for interaction with WT1. It is of interest that EGR1, whose three zinc fingers have extensive amino acid homology to WT1 zinc fingers 2–4, failed to stabilize p53 in transfection assays. Therefore, the interaction between WT1 and p53 is not a general property of zinc finger domains but rather a specific effect of WT1.

A potential mechanism underlying the effect of WT1 on p53 turnover is suggested by its inhibition of E6-mediated p53 degradation. Expression of wild-type p53 protein appears to be regulated physiologically by its rate of degradation, a pathway that has been defined by the ability of papillomavirus E6 protein to bind the cellular E6AP and target p53 for ubiquitination [Scheffner et al. 1990, 1993; Huibregtse et al. 1991, 1993a,b]. Thus, the ability of WT1 to reverse partially E6-mediated degradation of p53 suggests that the stabilizing effect of WT1 may result in part from its interference with the physiologic ubiquitination of p53.

Modulation of p53 function by WT1

Unlike other proteins that target p53, such as E1B 55-kD and MDM2 [Momand et al. 1992; Yew and Berk 1992], WT1 preserved the ability of p53 to bind and *trans*-activate a target promoter containing the RGC sequence.

Therefore, the increase in p53-mediated transcriptional activation induced by WT1 may be attributed to increased steady-state levels of p53. The enhancement of p53-mediated transcriptional activation by WT1 was not evident in the expression of known endogenous p53 target genes. Induction of *p21/WAF1/Cip1* and *GADD45* after growth of cells with a *tsp 53* allele at 32°C was not enhanced further by expression of WT1 (data not shown). However, the high level of wild-type p53 expressed in these cells may have already resulted in maximal *trans*-activation, and enhanced induction of these p53 targets by WT1 may only be evident in more physiological contexts. In contrast to transcriptional activation by p53, transcriptional repression by p53 was inhibited by WT1. The ability of p53 to repress transcription from a promoter containing a TATA element but lacking the RGC p53 target sequence has been attributed to its binding to TBP [Seto et al. 1992; Chen et al. 1993], or in the case of the Hsp70 promoter to its binding to CBF [Agoff et al. 1993]. WT1 did not bind in vitro to TBP [S. Maheswaran and D. Haber, unpubl.], but it is possible that its effect results from competition with TBP for binding to p53. Alternatively, WT1 binding may disrupt a specific p53 domain required for transcriptional repression but not for transcriptional activation.

The distinct effects of WT1 on the different *trans*-activational properties of p53 are particularly striking, given the recent observation that adenovirus E1B 19-kD protein also enhances transcriptional activation by p53 and inhibits transcriptional repression [Sabbatini et al. 1995]. The modulation of p53 *trans*-activation by E1B 19-kD protein has been linked to its inhibition of apoptosis by p53, without affecting p53-mediated cell cycle arrest [Debbas and White 1993; Sabbatini et al. 1995]. E1B 19-kD protein has not been shown to bind directly to p53, and the mechanism underlying these effects is unknown. However, analysis of E1B 19-kD mutants indicated a close correlation between their antiapoptotic properties and their ability to modulate p53-mediated transcriptional repression [Sabbatini et al. 1995]. Like E1B 19-kD protein, WT1 prevented p53-mediated cell death, uncovering p53-mediated cell cycle arrest. The p53-mediated apoptotic pathways inhibited by WT1 included the overexpression of wild-type p53 in U2OS cells, the exposure of U2OS cells to UV irradiation, as well as the coexpression of adenovirus E1A and wild-type p53 (data not shown). As shown for E1B 19-kD protein, the antiapoptotic properties of WT1 correlated with its modulation of p53-mediated *trans*activation. The naturally occurring *WTAR* mutant (lacking zinc finger 3), which failed either to enhance p53-mediated transcriptional activation or to inhibit p53-induced transcriptional repression, did not demonstrate inhibition of p53-mediated apoptosis. Thus, the antiapoptotic effect of WT1 may involve both its interaction with p53 as well as its own *trans*-activational properties. E1B 19-kD protein has been shown recently to associate with bak [Farrow et al. 1995], a novel bcl-2 family member, and it appears to inhibit apoptosis triggered by a number of different stimuli. In contrast, the antiapoptotic proper-

ties of WT1 appear to be restricted to p53-mediated cell death.

Potential implications of the WT1-p53 interaction

Recently, we have shown that expression of high levels of WT1 in osteosarcoma cells results in transcriptional repression of endogenous *EGFR*, followed by the induction of apoptosis, an effect that is prevented by constitutive expression of *EGFR* (Englert et al. 1995). The ability of high levels of WT1 to induce apoptosis is independent of p53, presumably resulting from the withdrawal of growth factor-mediated survival signals. In contrast, expression of low levels of WT1 fails to suppress *EGFR* expression and inhibits apoptosis that is mediated by p53. The ability of a single gene product to exert either apoptotic or antiapoptotic properties when expressed at different concentrations or under different cellular contexts has been demonstrated for *bak*, *bcl-x* splice variants, and human immunodeficiency virus (HIV) *tat* (Boise et al. 1993; Zauli et al. 1993; Kiefer et al. 1995; Li et al. 1995). Therefore, the use of cells with inducible WT1 may distinguish between its function as a transcriptional repressor, which requires high levels of expression, or as a modulator of p53 function, which occurs at low levels of WT1 expression.

The distinct effects resulting from different expression levels of WT1 are consistent with the specific pattern of WT1 expression during normal kidney development. In the developing kidney, low levels of WT1 expression are evident in blastemal stem cells, which constitute the earliest precursors of the glomerulus. In contrast, very high levels of WT1 expression are induced as these cells differentiate into podocytes (Pritchard-Jones et al. 1990). By analogy with our in vitro WT1-inducible system, high levels of WT1 expression in differentiating podocytes may lead to the suppression of *EGFR* expression and the induction of apoptotic pathways (Englert et al. 1995), whereas low levels of expression in blastemal cells may be associated with antiapoptotic properties of WT1. An in vivo antiapoptotic effect of WT1 has been proposed based on the absence of blastemal stem cells and the presence of large numbers of apoptotic cells in the vestigial renal bud of WT1-null mice (Kreidberg et al. 1993). Further work, correlating WT1 expression levels during renal development with the presence of apoptotic cells, will be required to test these potentially opposing functions of WT1.

The effect of WT1 expression on apoptotic pathways may also contribute to its role in Wilms' tumorigenesis. Overexpression of WT1 suppresses the growth of Wilms' tumor cells (Haber et al. 1993) and mutations inactivating *WT1* are present in 5%–10% of sporadic Wilms' tumor specimens. In these cases, loss of WT1-mediated apoptosis during a critical developmental window may be responsible for initiating tumor growth. However, >90% of sporadic Wilms' tumors contain wild-type *WT1*. In these tumors, WT1 is expressed diffusely within the blastemal cellular component, with high overall expression but low levels of expression within individual

cells (A. Garvin, C. Englert, and D. Haber, unpubl.). These low expression levels of wild-type *WT1* may be insufficient to induce apoptosis, but adequate to modulate the apoptotic properties of p53. The possibility that WT1 may affect p53 stability and function in Wilms' tumors is consistent with their high levels of wild-type p53 protein, which correlate well with WT1 levels in our eight sporadic tumors. This is also supported by the general observation that despite <1% incidence of p53 mutations (Bardeesy et al. 1994; Malkin et al. 1994), the majority of Wilms' tumors are positive for p53 by immunostaining (Lemoine et al. 1992), indicating protein stabilization (Hollstein et al. 1991; Levine et al. 1991). The presence of a WT1-p53 protein complex in Wilms' tumor specimens (Maheswaran et al. 1993) and the ability of WT1 to stabilize and partially inactivate p53 in cultured cells suggest a potential functional consequence of the WT1-p53 interaction. Further studies will be required to demonstrate the ability of WT1 to modulate the functional properties of p53 in Wilms' tumors and play a role in their protection from p53-mediated apoptosis.

Materials and methods

Cell lines, transfections, and expression constructs

BRK and Saos-2 cells were grown in Dulbecco's modified medium supplemented with 10% heat-inactivated fetal calf serum, glutamine, and penicillin/streptomycin. Stable BRK cell lines were generated by transfection of primary BRK cells with constructs encoding adenovirus *E1A* (6.1 cells), *E1A + E1B* (10.2 cells), *E1A* + wild-type *WT1* (A6 cells) or *E1A* + mutant *WTAR* (6.5 cells) as described elsewhere (Haber et al. 1992). For transient transfection experiments, Saos-2 cells were transfected by calcium phosphate/DNA precipitation. The amount of DNA transfected was equalized by addition of the empty vector and transfection efficiency was standardized by cotransfection of a human growth hormone reporter construct (Nichols Institute). All expression constructs were driven by the CMV promoter, including *WT1*, p53, *EGR1* (a gift from Dr. V. Sukhatme, Harvard Medical School, Boston, MA), and *E6* (a gift from Dr. P. Howley, Harvard Medical School, Boston, MA). The generation of WT1- and p53-inducible cell lines is described in Apoptosis studies.

Northern and Western blot analysis

For p53 immunoblot analysis, protein lysates were prepared using ELB buffer [50 mM HEPES (pH 7.0), 250 mM NaCl, 0.5 mM EDTA, 0.1% NP-40]. Proteins were separated on SDS-10% PAGE, transferred to Immobilon-P and probed with either anti-p53 antibody, PAb 421 (Oncogene Science), or anti-WT1 antibody WTe8 (Englert et al. 1995). For Northern blot analysis, total cellular RNA was extracted (Biotech reagent), electrophoresed on a formaldehyde gel, transferred to Hybond membrane (Amersham), and probed with either rat or human p53 cDNA. RNA isolated from cells 36 hr after transfection with expression constructs was digested with RNase-free DNase (Promega) to remove contaminating plasmid before electrophoresis.

Estimation of p53 protein half-life

Two techniques were used to determine the half-life of p53 protein. For cycloheximide-Western experiments, cultures

were treated with 5 μ g/ml of cycloheximide and proteins were extracted at various intervals. Protein lysates were prepared from equal number of cells and analyzed by Western blot using anti-p53 antibody PAb 421. For assays involving transient transfection of p53 constructs, samples were standardized by measurement of transfection efficiency using a cotransfected human growth hormone reporter (Nichols Institute). For pulse-chase experiments, cells were incubated in methionine-free medium for 30 min and labeled with 1.5 mCi of [35 S]methionine/ml for 10 min. Cells were then trypsinized, transferred to culture medium supplemented with cold 4 mM methionine, and aliquoted equally into four tissue culture plates. Proteins were extracted at various intervals, immunoprecipitated with PAb 421, and analyzed by SDS-PAGE.

Electromobility shift and CAT assays

Saos-2 cells were transfected with constructs encoding p53, p53 and WT1, or p53 and WTAR. Cells were harvested after 36 hr, nuclei were isolated by lysing cells in 10 mM Tris (pH 7.4), 10 mM KCl, 1 mM MgCl₂, and 0.5% Triton X-100, and nuclear proteins were extracted in 10 mM HEPES (pH 7.4) and 350 mM NaCl. Binding reactions were done in a total volume of 25 μ l, containing 70 mM NaCl, 0.1% Triton X-100, 4% glycerol, 10 mM HEPES (pH 7.5), and 32 P-end-labeled, annealed oligonucleotides containing the RGC sequence (Kern et al. 1991). DNA-protein complexes were resolved on a 4% native polyacrylamide gel. The amount of protein used per reaction was adjusted for transfection efficiency based on cotransfection of a human growth hormone reporter construct. Supershifting of the DNA-protein complexes was done by adding 10 ng of anti-p53 monoclonal antibody PAb 421 to the reaction mixture. CAT assays were performed by the method of Gorman (1985) and resolved by thin layer chromatography (TLC). The total amount of CMV promoter sequence transfected was equalized using the empty vector, transfection efficiency was standardized using the growth hormone reporter, and experiments were performed at least three times. The MCK-CAT reporter was a gift from Dr. S. Hauschka (University of Washington, Seattle) and the TATA-CAT reporter was kindly provided by Dr. L. Laimins (University of Chicago, IL). CAT activity was quantitated by scintillation counting of appropriate sections of the TLC plate, and expressed as a fraction of baseline activity. In each case, a representative experiment is shown.

Apoptosis studies

BRK temperature-sensitive cells, expressing *E1A*, and ts p53 were a gift from Dr. E. White (Rutgers University, New Brunswick, NJ), and A6 ts cells were generated by transfecting the ts p53 gene into A6 cells. Multiple U2OS cell lines containing inducible tetracycline-regulated WT1 constructs were generated as described (Gossen and Bujard 1992; Englert et al. 1995). Founder cell lines, generated by cotransfection of a tetracycline-repressible *trans*-activator (pUHD15-1) and the neomycin resistance gene, demonstrated 10-fold inducibility of a tetracycline-repressible luciferase reporter (pUHC13-3). These were then transfected with constructs encoding WT1 driven by CMV and tet operator sequences (pUHD10-3), along with a puromycin resistance gene. All selections and maintenance culture were performed in medium supplemented with tetracycline (1 μ g/ml) to suppress the tetracycline *trans*-activator. Drug-resistant U2OS cells were tested by Western blot for inducibility and tight regulation of WT1 expression. Representative clones studied included U2OS RA19 (inducible WT1 isoform A) and U2OS RC18 (WT1 isoform C). U2OS with inducible WT1 (isoform A,

U2OS A17) or mutant WTAR (U2OS W19) were transfected further with ts p53 and a hygromycin resistance gene, producing cells in which expression of both WT1 and p53 was tightly regulated. In these cells, expression of wild-type p53 was induced by temperature shift to 32°C and that of WT1 by removal of tetracycline from the culture medium. UV irradiation was performed by exposing cells to 20 J/m² using a Stratalinker (Stratagene). Growth curves were generated by counting viable cells using methylene blue staining. Cell cycle analysis was performed by flow cytometry on cells stained with propidium iodide (50 μ g/ml). For DNA fragmentation analysis, cells with reduced adherence to the tissue culture dish were harvested, and DNA was isolated and electrophoresed on a 2% agarose gel. Because few cells were harvested from nondying cultures, the assay was normalized to the total number of cells per dish.

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