WT1-p53 Interactions in Insulin-like Growth Factor-I Receptor Gene Regulation* 

Gila Idelman‡§, Tova Glaser‡, Charles T. Roberts, Jr.§, and Haim Werner§¶

Received for publication, November 14, 2002
Published, JBC Papers in Press, November 19, 2002, DOI 10.1074/jbc.M211606200

The insulin-like growth factor-I receptor (IGF-IR) plays a critical role in transformation. The expression of the IGF-IR gene is negatively regulated by a number of transcription factors, including the WT1 and p53 tumor suppressors. Previous studies have suggested both physical and functional interactions between the WT1 and p53 proteins. The potential functional interactions between WT1 and p53 in control of IGF-IR promoter activity were addressed by transient coexpression of vectors encoding different isoforms of WT1, together with IGF-IR promoter-luciferase reporter constructs, in p53-null osteosarcoma-derived Saos-2 cells, wild-type p53-expressing kidney tumor-derived G401 cells, and mutant p53-expressing, rhabdomyosarcoma-derived RD cells. Similar studies were also performed to compare p53-expressing Balb/c-3T3 and clonally derived p53-null, (101) fibroblasts and the colorectal cancer cell line HCT116 +/-, which expresses a wild-type p53 gene, and its IHT116 -/- derivative, in which the p53 gene has been disrupted by homologous recombination. Previous studies have demonstrated that WT1 and p53 proteins interact at the IGF-IR promoter in the absence of p53 or in the presence of wild-type p53. WT1 variants that contain the KTS insert are impaired in their ability to bind to the IGF-IR promoter and are unable to suppress IGF-IR promoter. In the presence of mutant p53, WT1 cannot repress the IGF-IR promoter. Coimmunoprecipitation experiments showed that p53 and WT1 physically interact, whereas electrophoretic mobility shift assay studies revealed that p53 modulates the ability of WT1 to bind to the IGF-IR promoter. In summary, the transcriptional activity of WT1 proteins and their ability to function as tumor suppressors or oncogenes depends on the cellular status of p53.

The insulin-like growth factor-I receptor (IGF-IR) is a transmembrane heterotetramer that mediates the effects of the IGFs, IGF-I and IGF-II, on growth and differentiation (1-3). The IGF-IR plays a central role in cell cycle regulation, as demonstrated by the fact that overexpression of this receptor in fibroblasts abrogates all requirements for exogenous growth factors (4). In addition to its important role during development, there is evidence pointing to a pivotal role for the IGF-IR in tumorigenesis (5, 6). The IGF-IR is highly expressed by most tumors and cancer cell lines, whereas fibroblasts derived from mouse embryos in which the IGF-IR was disrupted by homologous recombination are resistant to transformation by a number of oncogenes, indicating that IGF-IR function is an important prerequisite for cellular transformation (7, 8). Furthermore, the IGF-IR exhibits potent antiapoptotic effects that are consistent with the role of IGFs as cell survival factors (9, 10).

Structural analysis of the IGF-IR promoter revealed that it contains multiple binding sites for the WT1 Wilms’ tumor suppressor protein, a transcription factor whose inactivation has been implicated in the etiology of a subset of Wilms’ tumors, a pediatric kidney malignancy (11, 12). The WT1 gene product is a nuclear protein of 52–54 kDa that contains N-terminal transcriptional regulatory and self-association domains and a C-terminal DNA and RNA binding domain that comprises four zinc fingers of the C(2)H(2) class (13, 14) (Fig. 1). This domain binds to DNA containing versions of a 5'-GCGGGGCGG-3' consensus sequence. Alternative splicing of exon 5 and the use of an alternative splice site at the end of exon 9 produces mRNAs encoding multiple WT1 isoforms (15). Using transient transfection assays, we have previously shown that WT1 proteins lacking the exon 9-encoded Lys-Thr-Ser (KTS) insert between zinc fingers 3 and 4 were more effective than the alternatively spliced +KTS variants in suppressing the activity of co-transfected IGF-IR promoter constructs (11, 16). Furthermore, using electrophoretic mobility shift assays (EMSA) and DNase I footprint analyses, we demonstrated that this transcriptional effect was associated with specific binding of the WT1-KTS isoform to sites located both upstream and downstream of the IGF-IR gene transcription initiation site (12). In addition, stable expression of the WT1-KTS isoform in kidney tumor-derived G401 cells resulted in a decreased rate of cellular proliferation, decreased levels of IGF-IR mRNA and protein, and reduced activity of transfected IGF-IR promoter constructs (17).

IGF-IR gene expression is also regulated by the p53 tumor suppressor (18, 19). Specifically, transcription of the IGF-IR gene is negatively regulated by wild-type p53, whereas a number of tumor-derived, mutant versions of p53 were shown to significantly stimulate the activity of the IGF-IR promoter. Unlike WT1, p53 does not exhibit specific binding in vitro to the regulatory region of the IGF-IR gene. However, the results of EMSA and co-immunoprecipitation experiments indicate that the mechanism of action of p53 involves potential interactions...
WT1-p53 Interactions in IGF-I Receptor Gene Regulation

Experimental Procedures

Cell Culture, Plasmids, and DNA Transfections—Saos-2 is a human osteogenic sarcoma-derived cell line in which both p53 alleles are deleted. G401 is a human malignant rhabdoid tumor cell line that expresses wild-type p53. Saos-2 and G401 cells were obtained from the American Type Culture Collection (Manassas, VA). RD is a human rhabdomyosarcoma cell line that expresses a mutant p53 gene (Arg to Trp mutation at codon 248, in the DNA-binding domain). RD cells were kindly provided by Dr. Lee Helman (NCI, National Institutes of Health, Bethesda, MD). Saos-2 and RD cells were grown in Dulbecco’s modified Eagle’s medium, and G401 cells were cultured in McCoy’s 5A medium. Media were supplemented with 10% fetal bovine serum, 2 mM glutamine, and 50 μg/ml gentamicin sulfate. We have previously demonstrated that each of these cell lines expresses significant amounts of IGF-IR mRNA and supports transcription of IGF-IR promoter-driven reporter constructs (17, 18, 21). Balb/c3T3 and (10)1 murine fibroblasts that express, respectively, wild-type p53 or no p53 were kindly provided by Dr. Moshe Oren (Weizmann Institute of Science, Rehovot, Israel) (27). Balb/c3T3 and (10)1 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. The human colorectal cancer cell lines HCT116+/−, which expresses wild-type p53, and HCT116−/−, in which the p53 gene has been disrupted by homologous recombination, were kindly provided by Dr. Bert Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD) (28). HCT116 cells were grown in McCoy’s 5A medium with 10% fetal bovine serum.

For transient co-transfection experiments, a genomic DNA fragment extending from nucleotides 476 to +640 (nucleotides 1 corresponds to the transcription start site of the rat IGF-IR gene) was subcloned upstream of a promoterless firefly luciferase reporter in the pGLUC vector. The promoter activity of this fragment and the locations of WT1 binding sites and their relative affinities have been previously described (12). The construction of WT1 expression plasmids (in pcDNA3) containing or lacking the 17-amino acid exon 5-encoded sequence and the 3-amino acid exon 9-encoded fragment (KTS) has been previously reported (16). Likewise, the construction of WT1 expression vectors containing mutations associated with Denys-Drash syndrome (DDS; Arg to Trp at codon 394) and with the Wilms’ tumor, aniridia, genitourinary abnormalities, and mental retardation syndrome (WAGR; Gly to Arg at codon 6401) has been described (16).

Cells were seeded in six-well plates the day before transfection. Saos-2 cells were transfected with 5 μg of reporter plasmid and 0.8–2.5 μg of expression plasmid (or empty pcDNA3), along with 2.5 μg of a β-galactosidase expression plasmid (pcMVβ; Clontech, Palo Alto, CA) using the calcium phosphate method. The total amount of transfected DNA was kept constant by using pcDNA3 DNA. G401 cells were transfected with 0.5 μg of reporter plasmid, 0.5–4 μg of expression vector, and 0.25 μg of pcMVβ using the Fugene-6 reagent (Roche Molecular Biochemicals). RD cells were transfected with 1 μg of reporter plasmid and 0.25–0.5 μg of expression plasmid, along with 2.5 μg of pcMVβ using the Polyfect reagent (Qiagen GmbH). Balb/c3T3 and (10)1 cells were transfected with 5 μg of pcDNA3 reporter plasmid, 0–2.5 μg of the WT1−/− vector, and 2.5 μg of pcMVβ using the GenePORTER reagent (Gene Therapy Systems, San Diego, CA). HCT116 cells were transfected with 1 μg of the WT1−/− vector, and 2.5 μg of pcMVβ using the Polyfect reagent. Cells were harvested 40 h after transfection, and luciferase and β-galactosidase activities were measured as previously described (29). Promoter activities were expressed as luciferase values normalized for β-galactosidase activity.

In Vitro Transcription and Translation Reactions—Coupled in vitro transcription/translation of WT1 proteins was performed using the T7 Quick-Coupled Transcription/Translation System (Promega, Madison, WI). Briefly, T7 RNA polymerase-driven in vitro transcription reactions were followed by in vitro translations in the presence of [35S]methionine using rabbit reticulocyte lysates. In vitro translation products were electrophoresed through 10% SDS-PAGE and exposed to Eastman Kodak Co. X-Omat film.

Western Immunoblotting—Transiently transfected cells were harvested with ice-cold PBS containing 5 mM EDTA and lysed in a buffer composed of 150 mM NaCl, 20 mM Hepes, pH 7.5, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 1 μM leupeptin, 1 mM pyrophosphate, 1 mM vanadate, and 1 mM diethylthioctet. Protein content of the lysates was determined using the Bradford reagent. Cells were homogenized in ice-cold PBS and sonicated before being analyzed by Western immunoblotting. Membranes were blocked with 3% bovine serum albumin in T-TBS (20 mM Tris-HCl, pH 7.5, 135 mM NaCl, and 0.1% Tween 20) and then incubated either with an anti-WT1 antibody (C19; 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) or with an anti-β-galactosidase β-subunit antibody (C20; 0.2 μg/ml; Santa Cruz Biotechnology). Membranes were then washed extensively with T-TBS and incubated with horseradish peroxidase-conjugated secondary antibody. Bands corresponding to WT1 and IGF-IR proteins were detected using the SuperSignal West Pico® Chemiluminescent Substrate (Pierce).

Protein Synthesis—Saos-2 cells or Saos-2 cells transiently transfected with 3 μg of a WT1−/− expression vector and 3 μg of a HA-tagged p53 expression vector (or empty pcDNA3-HA) (30) using the Fugene-6 reagent. The pcDNA3-HA-p53 plasmid was kindly provided by Dr. William G. Kaelin (Harvard Medical School, Boston, MA). 24 and...
WT1-p53 Interactions in IGF-I Receptor Gene Regulation

RESULTS

The proximal ∼500 bp of 5′-flanking and ∼700 bp of the 5′-untranslated region of the IGF-IR promoter have been previously shown to contain 12 bona fide binding sites for WT1 (12). These elements bind the WT1 protein lacking the KTS insert between zinc fingers 3 and 4 with medium to high affinity, whereas the binding capacity of the WT1 isoform including the KTS insert is significantly reduced. Consistent with the results of these binding experiments, we showed that transient expression of WT1-KTS isoforms in Chinese hamster ovary cells repressed the activity of a cotransfected IGF-IR promoter-luciferase reporter by 80%, whereas WT1+KTS variants produced ∼40% repression (16). To examine the transcriptional effect of WT1 in different p53 backgrounds, we extended our analysis to the osteosarcoma-derived Saos-2, kidney rhabdoid tumor-derived G401, and rhabdomyosarcoma-derived RD cell lines.

To verify that the expression constructs to be utilized in functional assays of transcriptional activity encoded proteins of the expected size, synthetic RNAs were generated by in vitro transcription of pCDNA3 constructs and used to program rabbit reticulocyte lysates for in vitro translation in a coupled system. As shown in Fig. 2, the apparent molecular weight of the [35S]methionine-labeled WT1 isoforms containing exon 5-encoding sequences (WT1 +/− and +/+ ) was ∼54 kDa, and the size of the exon 5-lacking WT1 variants (WT1 −/− and −/−) was ∼52 kDa.

To evaluate the ability of the various naturally occurring WT1 isoforms to regulate the activity of the IGF-IR promoter in a p53-independent manner, p53-null Saos-2 cells were cotransfected with a series of expression vectors containing or lacking alternatively spliced exon 5 and 9 sequences (WT1 +/+, +/−, −/+, and −/−), together with the reporter plasmid p(-476/+640)LUC, which contains most of the proximal region of the rat IGF-IR promoter. The results of co-transfection experiments in Saos-2 cells are presented in Fig. 3. The WT1 −/− isoform induced a significant dose-dependent decrease in promoter activity (37 ± 3.9% inhibition with 1.5 μg of the expression plasmid and 65 ± 2% inhibition with 2.5 μg). These results replicate our previous data using pCB6-derived as well as pCDNA3-derived constructs in Chinese hamster ovary cells (11, 16). Transfection of the WT1 +/− isoform inhibited promoter activity by 34 ± 6% at 2.5 μg of input DNA, whereas the KTS-containing variants, WT1 +/+ and −/, had a limited effect on IGF-IR promoter activity.

To examine whether the transcriptional repression effect of the WT1-KTS variants was associated with corresponding changes in the levels of endogenous IGF-IR protein, Western blot analysis was performed. Saos-2 cells were transiently transfected with increasing amounts of the WT1 −/− expression vector, and after 72 h, cells were lysed as described under “Experimental Procedures.” Cell lysates (50 μg) were electrophoresed through 10% SDS-PAGE gels, after which they were blotted onto nitrocellulose membranes. The upper half of each membrane was incubated with an anti-IGF-IR β-subunit antibody, and the lower half was incubated with an anti-WT1 antibody. Results of Western blot analysis of transfected cells showed that increasing amounts of WT1 induced a dose-dependent decrease in the endogenous levels of IGF-IR (Fig. 4). Minimal repression (∼90% inhibition) was seen with 6 μg of WT1 expression plasmid.

In G401 cells, which express wild-type p53, the WT1 −/− and +/+ variants both suppressed promoter activity in a dose-dependent manner, with maximal inhibition (53 ± 3.6 and 36 ± 14%, respectively) seen with 2–4 μg of expression vector (Fig. 5A). Neither of the two KTS-containing isoforms (∼−/+ and +/+ ) inhibited the IGF-IR promoter in G401 cells (Fig. 5B).

To more rigorously assess the potential involvement of p53 on WT1 action and to avoid the confounding effect of different cellular backgrounds, co-transfections were performed in p53-expressing Balb/c-3T3 and p53-null (10/1) murine fibroblasts. The (10/1) cell line was clonally derived from Balb/c-3T3 cells; thus, both lines share a common genetic background (27). As shown in Fig. 6, 1.5 μg of WT1 −/− suppressed promoter activity in Balb/c-3T3 cells by 82%, whereas the same amount of vector repressed activity in (10/1) fibroblasts only by 17%. With 2.5 μg of DNA, WT1 −/− induced a further decrease in activity in (10/1) cells to 27%. In addition, co-transfections were performed in the human colorectal cancer cell lines HCT116 +/−, containing wild-type p53, and HCT116 −/−, in which the p53 gene has been disrupted by targeted homologous recombination (28). In these cells, the inhibitory effect of the KTS-lacking WT1 +/− isoform on IGF-IR promoter activity was also significantly enhanced in p53-expressing compared with p53-null cells, although the differences between p53-containing and p53-lacking cells were less pronounced than those seen between Balb/c-3T3 and (10/1) cells (51 ± 4 versus 21 ± 3% inhibition at 0.05 μg of expression vector and 58 ± 6 versus 38 ± 4% at 0.1 μg of DNA) (Fig. 7A). Interestingly, the KTS-
WT1 and WT1-KTS expression vectors (−/+ and −/−) (A) or WT1+KTS expression vectors (−/+ and +/+). (B) (or empty pcDNA3) and 2.5 μg of the pCMVβ plasmid using the calcium phosphate method. Luciferase and β-galactosidase activities were measured after 48 h. Luciferase data were normalized for β-galactosidase. 100% represents the IGF-IR promoter-driven luciferase activity in the absence of WT1. Experiments were performed between three and eight times, each time in duplicate. Error bars, S.E. Where not shown, the S.E. bars are smaller than the symbol size. *, p < 0.05 versus control; **, p < 0.01 versus control.

For this purpose, transient co-transfections were performed in Saos-2 cells using the p(-476/+640)LUC reporter luciferase reporter construct, together with increasing amounts of expression vectors encoding either +/+ or −/− variants of the DDS- and WAGR-associated proteins. Interestingly, both +/+ and −/− isoforms of the DDS-associated mutant (harboring a point mutation in the DNA binding domain) and of the WAGR mutant (displaying a point mutation in the middle portion of the molecule) suppressed the activity of the IGF-IR promoter in a dose-dependent manner. Thus, 2.5 μg of DDS −/− induced a 64% decrease, whereas DDS +/+ induced a smaller, albeit significant, decrease (35%) (Fig. 9A). At the same DNA concentration, WAGR +/+ and −/− mutants suppressed promoter activity by 60 and 45%, respectively (Fig. 9B).

Although physical interactions between p53 and WT1 proteins have been previously reported (22), we undertook a series of experiments aimed at establishing whether the functional cooperation between p53 and WT1 isoforms in our cellular systems was similarly associated with specific protein-protein interactions. For this purpose, Saos-2 cells were transiently transfected with 3 μg each of expression vectors encoding WT1 −/− and pcDNA3-HA-p53 (or empty pcDNA3-HA vector). After 24 and 48 h, cells were harvested, lysates were immunoprecipitated with an anti-WT1 monoclonal antibody (F6), and the precipitates were loaded onto 8% SDS-PAGE gels. After electrophoresis, complexes were transferred to nitrocellulose filters, and blotted with an anti-WT1 antibody (upper panel) or with an anti-IGF-IR antibody (lower panel). The positions of the 52–54-kDa WT1 and 97-kDa IGF-IR β-subunit proteins are denoted by arrows.

To examine whether p53 expression has an effect on WT1 levels, Saos-2 cells were co-transfected with expression vectors encoding WT1 −/− or −/+ and empty pcDNA3-HA vector. After 48 h, nuclear extracts were prepared, and WT1 protein levels were assessed by Western blotting. The results obtained showed that the levels of WT1 were significantly increased in the presence of p53 (Fig. 11A). To assess whether a similar effect can be seen in the presence of endogenous p53 and to determine whether p53 improves...
WT1 stability or its nuclear translocation, whole cell and nuclear extracts were prepared from HCT116 −/− and +/+ cells that were transfected with 0, 1, or 3 µg of a WT1 −/− vector. As shown in Fig. 11B, WT1 abundance was increased in both whole cells and in the nuclei of p53-containing cells (compare lane 2 versus lane 5 and lane 3 versus lane 6). To examine the effect of mutant p53, Saos-2 cells were co-transfected with a WT1 −/− vector (or empty vector), together with expression vectors encoding wild-type or mutant (codon 248) p53 (or empty vector). Results of Western blotting showed that cotransfection of wild-type p53 increased the abundance of both endogenous WT1 (compare lane 2 versus lane 1) and exogenously added WT1 (compare lane 5 versus lane 4). On the other hand, mutant p53 did not affect the levels of WT1 (compare lane 3 versus lane 1 and lane 6 versus lane 4) (Fig. 11C).

To determine whether potential physical interactions between WT1 and p53 can affect the ability of WT1 to bind to the IGFR promoter, EMSAs were performed using crude nuclear extracts of Saos-2 cells that were transfected with WT1 −/−, wild-type p53, or both vectors. A labeled double-stranded synthetic oligonucleotide that extends from nucleotide −273 to −238 and that includes two high affinity WT1 binding elements was employed for this purpose (12). Incubation of the labeled fragment with nuclear extracts of WT1 −/−-transfected cells generated one retarded band, whereas nuclear extracts of p53-transfected cells did not exhibit any specific binding to this promoter region. Cells that were transfected with both WT1 and p53, however, showed a significant decrease in the intensity of the shifted band, suggesting that p53 induced a reduction in the amount of the WT1-IGF-I promoter complex (Fig. 12A). A similar decrease in the intensity of the WT1-IGF-I promoter complex was seen when nuclear extracts of HCT116 −/− cells transfected with WT1 −/− and p53 were employed in EMSA (compared with cells transfected with WT1 −/− alone). Mutant p53 had a very small effect on the intensity of the DNA-protein complex (Fig. 12B).

**DISCUSSION**

The participation of the IGFR in the etiology of Wilms’ tumor, or nephroblastoma, was inferred from studies that showed that administration of a monoclonal antibody against the human IGFR to nude mice bearing Wilms’ tumor hetero-transplants prevented tumor growth and resulted in partial tumor remission (31). Furthermore, the IGFR gene was overexpressed in Wilms’ tumor, with the levels of IGFR mRNA in individual tumors being inversely correlated with the levels of WT1 mRNA (11). Consistent with the postulated role of WT1 as a tumor suppressor, we showed in early studies that the IGFR promoter is negatively regulated by a particular isoform of WT1 (12, 17). The transcriptional regulation of the WT1 gene, however, is extremely complex, and multiple versions of the protein that differ as a result of the alternative splicing of exon 5 and an alternative splice site at the end of exon 9 are usually coexpressed in tissues. In addition, a number of missense point mutations in the WT1 gene have been identified in Wilms’ tumor-associated disorders, including the Denys-Drash and WAGR syndromes.

In a more recent study, we addressed the regulation of IGFR promoter by wild-type and mutant versions of WT1...
The results obtained demonstrated that DNA binding is a major determinant of WT1 action. However, our data also suggested that WT1 could function through additional mechanisms such as RNA binding or protein-protein association. Since p53 has been shown to differentially regulate transcription of the IGF-IR gene, with wild-type p53 acting as a potent suppressor and mutant p53 as a strong transactivator (18), and in view of the fact that p53 can interact with WT1, we investigated in the present study the potential functional and physical interactions between WT1 and p53 in transcriptional regulation of the IGF-IR gene.

In the absence of p53, or in the presence of wild-type p53, the critical determinant of wild-type WT1 action is its ability to bind DNA via its zinc finger domain. Thus, in the absence of the KTS tripeptide between zinc fingers 3 and 4, WT1 functions as a suppressor of IGF-IR promoter activity. The presence of the KTS insert abolished the repressing action of WT1, probably as a result of the inability of KTS-containing isoforms to efficiently bind to the IGF-IR promoter region (12). Interestingly, tumor-associated, mutant forms of WT1 were able to suppress IGF-IR promoter even in the presence of the KTS insert. Following binding of WT1 to the promoter region, the extent of its repressing activity seems to depend on the p53 status of the cell. Thus, we observed that, in wild-type p53-expressing Balb/c-3T3 cells, the suppressive effect of WT1 was significantly more pronounced that in p53-null (10)1 cells (82 versus 27% inhibition). Similarly, WT1 +/-, but not WT1 -/-, was more potent in HCT116 +/- than in HCT116 -/- cells. Further-

![Figure 7](image_url) Effect of WT1 isoforms on IGF-IR promoter activity in p53-lacking and -expressing HCT116 human colorectal cancer cells. A, HCT116 -/- (closed bars) and HCT116 +/- (open bars) cells were co-transfected with the p(-476/+640)LUC reporter and increasing amounts of the WT1 +/- expression plasmid. The figure shows the results of three experiments performed in duplicate dishes. *, *p < 0.02 versus p53-lacking cells. B, HCT116 -/- (closed bars) and HCT116 +/- (open bars) cells were cotransfected with the p(-476/+640)LUC reporter and increasing amounts of the WT1 +/- expression plasmid. The figure shows the results of four experiments, each in duplicate.

![Figure 8](image_url) Regulation of IGF-IR promoter activity by WT1 in the presence of mutant p53. A, RD cells were transfected with 1 µg of the p(-476/+640)LUC construct, together with 0–2 µg of WT1 +/- or -/- expression vectors, and 2.5 µg of pCMVβ, using the Polyfect reagent. Luciferase values, normalized for β-galactosidase, are expressed as a percentage of the luciferase activity of the empty pcDNA3 expression vector. Experiments were repeated between three and five times, each in duplicate. B, Saos-2 cells were cotransfected with the p(-476/+640)LUC reporter, along with 0.25 µg of WT1 +/- (or empty pcDNA3) and 50 µg of wild-type or codon 248-mutant p53 (or empty pCMV-Neo-Bam vector). Shown are the results of a typical experiment, performed at least six times, each in duplicate dishes.
more, the ability of the KTS-containing WT1 isoform to suppress IGF-IR promoter activity in HCT116 cells may reflect the fact that the effect of the KTS insert is, to a certain extent, cell type-specific. The increased potency of WT1 in the presence of p53 is probably explained by the results of transfection experiments showing a greater abundance of exogenous WT1 in Saos-2 cells that were co-transfected with p53 in comparison with cells that were transfected only with WT1, as well as in HCT116 +/- in comparison with HCT116 --/-. The finding that augmented WT1 levels are seen in both whole cell and nuclear extracts of HCT116 +/- cells suggests that p53 improves WT1 stability. The possibility that p53 increases, in addition, the translocation of WT1 to the nucleus cannot be discounted.

The results of EMSA experiments showing that p53 is unable to bind to the IGF-IR promoter fragment are consistent with our previous data demonstrating that the inhibitory effect of p53 is mediated via interaction with the TATA-box binding component of TFIID (TBP) at the initiator element and does not seem to involve specific DNA binding (18). The fact that p53 interferes with the binding of WT1 to specific WT1 sites in the promoter region seems, however, paradoxical in view of the enhanced transcriptional activity of WT1-KTS seen in the presence of p53. These results, suggesting that WT1-KTS isoforms display an augmented activity despite reduced binding due to p53, can be potentially explained by the results of studies showing that the mechanism of action of p53 involves interaction with additional transcription factors, including Sp1, which positively affects IGF-IR promoter activity, as well as with members of the basal transcription machinery such as TBP. Alternatively, p53 may enhance WT1 binding to additional cis-elements in the IGF-IR promoter. Similar enhancement of WT1 activity by p53 toward the growth arrest-associated GADD45 gene has been reported (32). Co-expression of p53 and WT1 strongly induced a GADD45 reporter construct, and this effect was mediated by a WT1 element in the promoter region. Since p53 does not bind directly to this promoter, these results indicate that p53 contributes to the effect of WT1 via protein-protein interactions.

In RD cells harboring a mutant p53, WT1 was unable to repress IGF-IR promoter activity, regardless of the presence or absence of the KTS insert. These findings can be interpreted to suggest that a potential mechanism by which loss-of-function mutations of p53 result in uncontrolled cell growth may be linked to the inability of WT1 to suppress target promoters, including that of the IGF-IR gene. Furthermore, it is relevant to analyze these findings in light of current controversies regarding the role of WT1. Inconsistent with the classical tumor suppressor function of WT1, an important body of work has unequivocally demonstrated that, in certain cellular contexts, WT1 is required to inhibit apoptosis in vitro and in vivo. This
oncogenic role of WT1 has been found to be associated with its capacity to up-regulate antiapoptotic genes such as bcl-2 (33). Therefore, and in view of the diametrically opposed activities of WT1 in the presence of wild-type or mutant p53, it is important to take into consideration the cellular status of p53 when assessing the role of WT1. Analysis of anaplastic and nonanaplastic regions of seven Wilms’ tumors revealed that, in five out of six tumors with p53 mutations, the mutations were restricted to the anaplastic region (24). These results indicate that progression to anaplasia is associated with clonal expansion of cells that have acquired a p53 mutation. Up-regulation of the IGF-IR gene as a result of expression of aberrant p53 has been shown to be important for the growth and survival of malignant cells (34).

The IGF-II gene, which encodes the main ligand of the IGF-IR in malignant cells, is also transcriptionally regulated by WT1 isoforms. Using the P3 promoter of the mouse IGF-II gene in primary cultures of wild-type and knock-out p53 embryos, it was demonstrated that the effect of WT1 was independent of p53 status (35). In addition, the activities of KTS-containing or lacking WT1 isoforms toward the IGF-II P3 promoter were in contrast to their activities toward the IGF-IR promoter reported in the present paper. Thus, WT1-KTS activated the IGF-II promoter, whereas WT1/H11001 KTS repressed its activity. These differences may reflect the relative simplicity of the IGF-II promoter, which contains only two WT1 binding sites versus the multiple sites characterized in the IGF-IR promoter. This possibility is supported by the differential activities of WT1 variants toward the insulin receptor promoter, which also contains multiple WT1 binding sites (36). Specifically, WT1+KTS effectively repressed promoter activity,
whereas WT1-KTS was able to repress the insulin receptor promoter only in the presence of co-transfected CCAAT/enhancer-binding protein β or a dominant-negative p53 mutation.

In summary, we have demonstrated that WT1 isoforms lacking the KTS insert are capable of suppressing IGF-IR promoter activity in the absence of p53 or in the presence of wild-type p53. Alternatively spliced variants of WT1 impaired in their ability to bind to the IGF-IR promoter due to the presence of the KTS insert are unable to suppress IGF-IR promoter activity. In the presence of mutant p53, WT1 cannot repress the IGF-IR promoter. The transcriptional activity of WT1 gene products and their ability to function as tumor suppressors or oncogenes depend to a large extent on the cellular status of p53.

Acknowledgments—We thank Drs. L. Helman, M. Oren, B. Vogelstein, W. G. Kaelin, and E. Mercer for providing cell lines and reagents.

REFERENCES