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Physical Interaction between Wilms Tumor 1 and p73 Proteins Modulates Their Functions*

(Volker Scharrnhorst, Patrick Dekker, Alex J. van der Eb, and Aart G. Jochemsen‡)

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The WT1 gene, which is heterozygously mutated or deleted in congenital anomaly syndromes and homozygously mutated in about 15% of all Wilms tumors, encodes tissue-specific developmental regulators. Through alternative mRNA splicing, four main WT1 protein isoforms are synthesized. All isoforms can bind to DNA via their zinc fingers, albeit with different affinities and specificities, and thereby modulate the transcriptional activity of their target genes. Several proteins bind to and alter the transcription regulatory properties of the WT1 proteins, including the product of the tumor suppressor gene p53. Interaction between WT1 and p53 was shown to modulate their ability to regulate the transcription of their respective target genes. Here, we report that all four isoforms of WT1 bind to p73, a recently cloned homologue of p53. p73 binds to the zinc finger region of WT1 and thereby inhibits DNA binding and transcription activation by WT1. Similarly, WT1 inhibits p73-induced transcription activation in reporter assays and counteracts p73-induced expression of endogenous Mdm2. This, taken together with our finding that WT1 also interacts with p63/KET, another p53 homologue, suggests that association between WT1 and the members of the p53 family of proteins may be an important determinant of their functions in cell growth and differentiation.

Wilms tumor or nephroblastoma is the most common pediatric malignancy and arises from a metanephric blastema cell that fails to undergo cell cycle arrest and differentiation (1). Several chromosomal regions may be involved in the development of Wilms tumor (2), but only the WT1 gene at human chromosome 11p13 has been identified (3, 4). In addition to homozygous mutations of WT1 in Wilms tumors, heterozygous mutations and deletions in WT1 are found in Denys-Drash (5) and WAGR (6) syndromes, respectively. Heterozygous mutations in the splice donor site in intron 9 of WT1 result in an imbalance between the different mRNA splice forms and cause Frasier syndrome (7, 8). The clinical features of these three syndromes always include kidney and genital abnormalities. The essential role of WT1 in urogenital development was underscored by the finding that WT1-null mice fail to develop kidney and gonads (9). In addition to the urogenital system, WT1 expression in the mammalian embryo has been noted in the central nervous system (10, 11), the mesothelium (10, 12), the spleen (12, 13), and in the developing limb and epicardium (11, 14).

The WT1 gene contains 10 exons, two of which are alternatively spliced, giving rise to four different protein isoforms with molecular masses of 52–54 kDa. Inclusion of exon five inserts 17 amino acids just N-terminal of the four zinc fingers of WT1, and incorporation of a second splice insert alters the spacing between zinc finger three and four by insertion of the three amino acids lysine, threonine, and serine (KTS) (15). The four WT1 proteins are referred to as WT1(--), if the protein lacks both splice inserts, WT1(+-) and WT1(-+) if the protein contains the first or the second insert, respectively, and WT1(+++) if the protein contains both splice inserts.

At first, it was thought that all four WT1 protein isoforms function exclusively as transcription factors, but increasing evidence suggests that the WT1 isoforms containing the KTS splice insert may also be involved in post-transcriptional processing of RNA (16–18). WT1 binds to GC-rich DNA sequences such as the Egr-1 (19) and the WTE (20) consensus sites or (TCC)n motif containing sequences (21). WT1 may, depending on the promoter context, repress or stimulate promoter activity (reviewed in Ref. 22). Accordingly, WT1 activates transcription from synthetic promoter constructs containing multimerized Egr-1, (TCC)n, or WTE sites upstream (21, 23, 24) or downstream (21) of the transcription start site. However, insertion of (TCC)n sites both upstream and downstream of the transcription start site results in efficient transcription repression by co-transfected WT1 (21). Natural GC-rich promoters regulated by WT1 include the bcl-2 (25), the amphiregulin (26), the retinoic acid receptor-α (27), and the transforming growth factor-β (28) promoter. The first two are activated by WT1, whereas WT1 represses transcription from the latter two promoters.

Protein-protein interactions also alter the transcription regulatory functions of WT1. Two proteins, Par-4 (29) and Ciao 1 (30), have been identified by their ability to interact with WT1 in a yeast two-hybrid assay. Both proteins inhibit transcription activation by WT1, and Par-4 also augments WT1-mediated transcription repression. Physical association between WT1 and p53 may modulate their respective transcription regulatory properties (31). In a certain cellular setting, p53 can convert WT1 from an activator to a repressor of a given reporter construct, whereas WT1 exerts a cooperative effect on transcription activated by p53 (31). These results were later extended by the finding that WT1 can stabilize the p53 protein and that zinc finger one and two of WT1 are required for stabilization (32).

Recently, two genes that share sequence homology with the transactivation, DNA binding, and tetramerization domains of p53 were cloned and named p73 (33) and p63/p48/p51/KET.
(34–37). As a result of alternative RNA splicing, both genes encode multiple isoforms (34, 35, 38). p73 and p63 can mediate transcription activation from p53-responsive elements and, like p53, induce programmed cell death (34, 39). In contrast to p53, which is dispensable for embryonic development (40), p73 and p63 are intimately involved in differentiation and development (37, 41–43), whereas a tumor suppressor function has not been established as yet. In this paper, we demonstrate that WT1 physically interacts with p73 and p63. Association with p73 is mediated via the zinc fingers of WT1 and, consequently, binding of p73 diminishes DNA binding and transcription activation by WT1. Likewise, WT1 inhibits p73-mediated transcription activation of reporter constructs and attenuates p73-induced expression of endogenous Mdm2. These results show that WT1 associates with all known members of the p53 family and suggests that this interaction modulates their respective functions in cell growth and differentiation.

EXPERIMENTAL PROCEDURES

Plasmids and cDNAs—The CMV promoter-driven CB6·WT1 expression constructs have been described previously (44). The p73 (33) and p53 (45) cDNAs were cloned into pcDNA plasmids (Invitrogen) containing a 5′ sequence encoding an HA tag. p73 expression constructs have been described previously (44). The (16B12) from Babco. The monoclonal anti-LacZ antibody was purchased from Amersham Pharmacia Biotech. Subsequently, cells were washed with phosphate-buffered saline and lysed in IPB 0.7. Debris was removed by centrifugation, and lysates were immunoprecipitated with polyclonal anti-WT1, anti-KET, or anti-HA antibodies for 16 h at 4 °C. Metabolic complexes were boiled in sample buffer and separated on a SDS-PAA gel. The gels were incubated in 2,5-diphenyloxazole-Me2SO, washed, exposed to x-ray films at –80 °C.

Stabilization of p73α and p53 by WT1(+/−)→−5 cm dishes of Saos-2 cells were transfected with 200 ng of CMV-HA-p73α, 20 ng of CMV-HA-p53, and 10 μg of CMV-WT1(+/−) or with 200 ng of CMV-HA-p73α/CMV-HA-p53 together with 1, 2.5, and 5 μg of CMV-WT1(+/−). Co-transfected CMV-lacZ (1.5 μg) used to correct for transfection efficiencies and equal amount of CMV vector were present in every transfection. 40 h after transfection, cells were lysed and Western blots prepared. The pulse-chase experiment was performed as described under “Metabolic Cell Labeling and Immunoprecipitation,” except that U2OS cells, which had been transfected with 120 ng of CMV-HA-p73α alone or in combination with 3 μg of CMV-WT1(+/−), were labeled with 2-[35S]methionine for 1.5 h prior to lysis in IPB 0.7 or the chase periods of 1, 2, or 4 h. Quantifications were done with the GDS8000 gel documentation and analysis system (Ultra-Violet Products).

Electrophoretic Mobility Shift Assay—WT1(+/−) deletion mutants (see under “Plasmids and cDNAs”) were in vitro transcribed/translated with the TNT reticulocyte lysate kit (Promega). [38S]Methionine-labeled WT1 proteins were added to p53− and GST-p73 bound to glutathione-agarose beads, and the proteins were tumbled in NETN containing 20 mg of bovine serum albumin, 0.5 mM EDTA, 0.5 mM NaCl, 0.05% Nonidet P-40 for at least 4 h at 4 °C to allow protein complexes to form. After washing, bound proteins were separated by electrophoresis through a SDS-PAA gel and visualized by incubation in 2,5-diphenyloxazole-Me2SO and exposure to x-ray films at –80 °C.

Electrophoretic Mobility Shift Assay—WT1(+/−), p73α, p53, and E2F1 proteins were in vitro transcribed/translated with the TNT T7 coupled T3 promoter-coupled kit (Promega).

The electrophoretic mobility shift assay was essentially performed as described previously (24). In short, an α-[32P]dATP-labeled synthetic duplex containing the WTE DNA sequence (20 ng) was used as a probe.

Reactions were carried out with a 15-min preincubation of the in vitro translated proteins at room temperature in a 25-μl reaction mixture containing 20 μM HEPES pH 7.5, 70 μM KC1, 5 mM MgCl2, 0.05% Nonidet P-40, 12% glyceral, 5 μM of bovine serum albumin, 0.5 mM dithiothreitol, 1 μM of poly(dI-dC), and 0.1 mM ZnCl2. Subsequently, the labeled probe was added, and the reaction mixture was incubated for a further 30 min at room temperature. The DNA-protein complexes were resolved from the free probe by electrophoresis through a 6% native PAA gel (19:1 acrylamide: N,N′-methylene-bisacrylamide, ICN) in a 1× Tris glycine-buffered system.

Luciferase Reporter Assays and Induction of the Endogenous Mdm2/Bax Promoters by p73α and p53—3 cm dishes of HeP3B cells were transfected for luciferase reporter assays. Each precipitate was adjusted to 5 μl by Buffer B (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 2% fat-free dry milk (Nutricia) and subsequently incubated for 1 h with the primary antibody, followed by a 30-min incubation with the secondary antibody coupled to horseradish peroxidase (Jackson Immunoresearch Laboratories), diluted in the blocking buffer. All secondary antibodies had been pre-covered against immunoglobulins of other species by the manufacturer. Protein bands were visualized by enhanced chemiluminescence.

Metabolic Cell Labeling and Immunoprecipitation—Three-cm dishes of U2OS cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics. Cells were transfected by the calcium-phosphate transfection method (49) for 7 or 16 h. After transfection, cells were washed and incubated with fresh culture medium for 24–40 h.

Antibodies—The polyclonal anti-WT1 antibody (C19), the polyclonal anti-HA antibody (Y11), the polyclonal anti-KET antibody (R20), the monoclonal anti-E2F1 antibody (KH95), the monoclonal anti-Mdm2 antibody (SMP14), and the polyclonal anti-Bax antibody (P19) were all purchased from Santa Cruz Biotechnology. The monoclonal anti-WT1 antibody H2 was from Dako and the monoclonal anti-HA antibody (16B12) from Babco. The monoclonal anti-LacZ antibody was purchased from Roche Molecular Biochemicals.

Immunoprecipitation and Western Analysis—Cells were washed in ice-cold phosphate-buffered saline and lysed in IPB 0.7 buffer (20 mM Tris–HCl, pH 8.0, 7.8 μM NaCl, 0.5% Nonidet P-40, 0.2% deoxycholate) supplemented with inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml NaF, β-glycerophosphate, Na3VO4, trypsin inhibitor, and 1 mg/ml Na2P2O7). After centrifugation of the lysates, protein concentrations were measured with the Bradford assay (Bio-Rad). For immunoprecipitation-Western analysis, the polyclonal anti-WT1 and anti-HA antibodies were pre-coupled to protein A-Sepharose beads be-
Fig. 1. Co-immunoprecipitation of WT1 and p73α, p73β, and p53 in immunoprecipitation (IP)-Western analysis. A, WT(−/−) is bound to p73α, p73β, and p53 in U2OS cells. 5-cm dishes of U2OS cells were transfected with 7.5 μg of parental CMV vector (lanes Neo), 5 μg of CMV-WT1(−/−) (lanes −/+), 2.5 μg of CMV-HA-p73α alone (lanes p73α), or with WT(−/−) in combination with CMV-HA-p73α, CMV-HA-p73β or CMV-HA-p53 (lanes −/+ + p73α/p73β/p53). All precipitates were adjusted to 7.5 μg of DNA content with parental vector. 24 h after transfection, the cells were lysed, the proteins precipitated with polyclonal anti-WT1 (lanes WT1) and anti-HA (lanes HA) antibodies, and Western
**RESULTS**

**WT1 Binds to p73α, p73β, and p53 in Immunoprecipitation-Western Analysis**—It has been reported that WT1 associates with p53 (31, 32). To investigate whether WT1 also binds to the other members of the p53 family, U2OS cells were transfected with a CMV expression construct without a cDNA insert (Neo) or WT1(−/−) alone or with WT1(−/−) in combination with either p73α or p73β, two splice forms of the p73 gene (33). As a positive control, U2OS cells were transfected with WT1(−/−) and p53 expression vectors. p53, p73α, and p73β all contain an N-terminal HA tag, enabling us to immunoprecipitate all three proteins with an anti-HA antibody. Fig. 1A shows Western blots of U2OS cell lysates from which either transfected WT1(−/−) (lanes WT1) or p53/p73/p53 (lanes HA) had been immunoprecipitated. The lanes contain one-tenth of the amount of whole cell extract used per immunoprecipitation. The blots were first incubated with an anti-HA antibody and subsequently with an anti-WT1 antibody. The upper panel of Fig. 1A shows co-immunoprecipitation of p73α and p73β with WT1 and vice versa. The lower panel demonstrates that p53 can be co-immunoprecipitated in an anti-WT1 precipitation, but the previously described binding of WT1 to p53 in an anti-p53 immunoprecipitation remains below detection level.

Next, we wanted to establish whether all four splice forms of WT1 bind to p73. To that end, we transfected U2OS cells with an expression vector encoding HA-p73α in combination with vectors encoding the four WT1 isoforms and immunoprecipitated WT1 and HA-tagged proteins from cell extracts. A Western blot was prepared and cut in half at the height of the 62-kDa marker, the lower part incubated with an anti-WT1, and the upper part with an anti-HA antibody (Fig. 1B, upper panel). The blot containing the whole cell extracts was incubated with a mixture of anti-WT1 and anti-HA antibodies (Fig. 1B, lower panel). p73α clearly co-precipitates with all four isoforms of WT1, and all WT1 isoforms are bound to precipitated p73α.

Fig. 1A indicated that p53 co-precipitates less efficiently with WT1 than with p73α or p73β (compare the individual signal intensities in lanes WT1 to the signals in the lanes −). To rule out the possibility that endogenous p53 molecules in U2OS cells compete with transfected p53 for WT1 binding and in that way diminish binding of exogenous p53, we transfected Saos-2 cells lacking endogenous p53 proteins with WT1 and the different p53 family members. WT1 was immunoprecipitated, and Western blots of the immunoprecipitates and whole cell lysates were prepared (Fig. 1C). Although p73α, p73β, and p53 are expressed equally well (lower panel of Fig. 1C) and the same amount of WT1 is precipitated from the co-transfected cells, less p53 is bound to WT1 (upper panel of Fig. 1C).

Thus, p53 and p73 are bound to WT1 in immunoprecipitation-Western analysis, but p73α and p73β co-precipitate more efficiently with WT1 than p53.

**p73 and the Rat Homologue of p63 Are Complexed to WT1 in Immunoprecipitates of [35S]Methionine-labeled U2OS Cells**—3-cm dishes of U2OS cells were transfected with 4.5 μg of CMV vector (Neo), 3 μg of CMV-WT1(−/−) (lanes 1−4), 1.5 μg of CMV-HA-p73α (lanes p73α), or with 3 μg of CMV-WT1(−/−) in combination with either 1.5 μg of HA-p73α (lanes 1−4+a), HA-p73β (lanes 1−4+b), or rat p63 (lanes 1−4+c+KET). 16 h after transfection the cells were labeled with 150 Ci of [35S]methionine for 3 h. After lysis, proteins were immunoprecipitated (IP). The amount of radioactively labeled cell lysate in each anti-WT1 immunoprecipitation was kept constant, and one-tenth of the amount used in the anti-WT1 precipitations was used to precipitate the HA-tagged proteins. For the anti-WT1 and anti-KET immunoprecipitation, the radioactively labeled lysate was divided equally between the two precipitations. Subsequently, the immunoprecipitated proteins were separated on an SDS-PAA gel. The black arrow next to the autoradiogram indicates the position of the WT1 protein, and the gray arrows point to p73 and rat p63 (KET).

The autoradiogram of the immunoprecipitates is shown in Fig. 2 and demonstrates that p73α, p73β, and KET co-immunoprecipitate with WT1. Thus, both new members of the p53 family, p73 and p63, can associate with WT1.

**p73 Is Not Stabilized by WT1**—It is now well recognized that alterations in the half-life of p53 have an important role in regulating its cellular activity (50, 51). In a previous study, it had been found that overexpression of WT1 can stabilize transfected p53 in Saos-2 cells (32). To address the question whether...
were lysed and Western blots prepared. As can be seen in the upper part of Fig. 3A, p73α protein levels remain unaffected by co-transfection of increasing amounts of WT1 compared with expression levels in cells transfected with p73α alone. A small increase in p53 protein levels is observed after co-transfection of WT1(−/−) with p53, consistent with the previously reported, stabilizing effect of WT1 on p53 (32). The stabilizing effect of WT1 on p53 can also be observed in e.g., Fig. 8.

In order to determine the half-life of p73α in the presence or absence of WT1, we labeled U2OS cells that had been transfected with 120 ng of p73α alone or in combination with 3 μg of WT1(−/−) with [35S]methionine for 1.5 h followed by chase periods of 0–4 h prior to lysis and immunoprecipitation. The relative radioactivity of the p73α protein was quantified, set out against the chase time, and the half-lives were determined (Fig. 3B). The half-life of p73α in presence or absence of WT1 was calculated to be approximately 1 h. Thus, WT1 appears not to stabilize transfected p73α.

The WT1 Zinc Finger Domain Mediates Binding to p73 and p53—Maheswaran et al. (32) have shown that the zinc finger domain of WT1 is necessary for the stabilization of p53 by WT1. As yet, the domain in WT1 required for direct interaction with p53 has not been delineated. Therefore, we performed GST pull-down assays with GST-p53 and GST-p73α as baits and in vitro translated truncation mutants of WT1 to determine the regions in WT1 required for binding to these two proteins. The upper part of Fig. 4A gives an overview of the WT1 proteins used in this assay, and the lower part shows an autoradiogram of one-fifth of the amount of WT1 proteins used in the pull-down assay. The lower band in the lane containing the N-terminal truncation WT1(−/−)-Bam is most likely derived from one of the downstream ATG-initiation codons present in the WT1 cDNA.

Deletion of the WT1 N terminus up to amino acid 181 does not affect binding of WT1 to the GST constructs, whereas deletion of zinc finger three and four of WT1 severely impairs binding of WT1 to p73 and p53 (Fig. 4B). Extension of this C-terminal deletion to amino acid 327 (mutant ΔZn2–4) of WT1 further weakens the protein-protein interaction. Binding of the naturally occurring mutant WT1-PM (52), which only contains the first 256 amino acids of full-length WT1 and, therefore, lacks all four zinc fingers, is barely detectable in GST pull down. WT1 does not bind to GST protein (lane GST).

Thus, the p73/p53-binding domain of WT1 maps to its zinc finger region.

**p73 Inhibits DNA Binding by WT1**—The zinc fingers of WT1 can mediate binding to several DNA sequences in electrophoretic mobility shift assay (24, 53). Since binding of WT1 to p73 and p53 also requires the zinc finger region, we wanted to establish whether this protein-protein interaction impairs DNA binding by WT1. Therefore, we performed electrophoretic mobility shift assays with in vitro translated WT1(−/−) protein and the GC-rich WTE oligonucleotide (20) as a probe. Fig. 5A shows Western blots of the different in vitro translated proteins used in the electrophoretic mobility shift assay.

One μl of in vitro translated WT1(−/−) was preincubated without or with 2.5 and 5 μl of p73α or p53 or 5 μl of E2F1 prior to addition of the radioactive WTE oligonucleotide. Protein-DNA complexes formed in a second incubation period were separated from the free probe by electrophoresis through a native PAA gel. The autoradiogram in Fig. 5B shows an example of such an electrophoretic mobility shift assay. The arrow points to the specific WT1-DNA complex, which is abrogated by addition of a 50-fold excess of unlabeled WTE oligonucleotide (lane comp). Addition of p73α detectably reduces DNA binding by WT1 in a concentration-dependent manner, whereas addi-
The zinc fingers of WT1 are required for binding of WT1 to GST-p73α and GST-p53 in GST pull-down assays. 

Fig. 4. The zinc fingers of WT1 are required for binding of WT1 to GST-p73α and GST-p53 in GST pull-down assays. A, upper part, schematic overview of the WT1 truncation mutants used in GST pull-down. Lower panel, autoradiogram of one-fifth of the amount of WT1 proteins used in the pull-down assay. B, autoradiogram of WT1 proteins bound to GST-p73α and GST-p53. N-terminal truncations in WT1 do not alter binding to GST-p73α or GST-p53, whereas deletion of the zinc finger region abrogates binding of WT1 to GST-p73α. Full-length WT1(−/−) does not bind to bacterially produced GST protein (lane GST).

Fig. 5. p73α inhibits DNA binding by WT1. A, Western blots of the different in vitro translated (i.v.t.) proteins used in the electrophoretic mobility shift assay. 2.5 μl of p73α, p53, and E2F1 were visualized with anti-HA and anti-E2F1 antibodies. 1 μl of WT1(−/−) was detected with the monoclonal H2 anti-WT1 antibody raised against the N terminus of WT1. B, an electrophoretic mobility shift assay performed with 1 μl of in vitro translated WT1(−/−) in the absence or presence of various other in vitro translated proteins shows that only p73α inhibits binding of WT1(−/−) to the radioactively labeled WTE sequence. Binding of 1 μl of WT1 to the WTE sequence (lanes WT1(−/−) A and B) is competed out by a 50-fold excess of unlabeled WTE probe (lane comp.). Addition of 2.5 and 5 μl of p73α inhibits DNA binding by WT1 in a concentration-dependent manner (lanes WT1(−/−) + p73α). Addition of the same amounts of p53 does not affect DNA binding by WT1 (lanes WT1(−/−) + p53). Addition of 5 μl of E2F1 to the reaction mixture does not influence DNA binding by WT1 (lane WT1(−/−) + E2F1). 5 μl of p73α (lane p73α), p53 (lane p53), and E2F1 (lane E2F1) does not bind to the WTE probe. The black arrow points to the WT1-probe complex.

Physical and Functional Interactions between WT1 and p73/p63

p73α and p53 Inhibit WT1-mediated Transcription Activation—WT1 can activate or repress transcription, depending on promoter context and cell line (22). We chose the 3xWTE-Luc reporter construct known to be activated by WT1 in transient reporter assays (24) to measure the effect of p73α and p53 on transcription regulation by WT1 in p53-negative Hep3B cells. This reporter construct contains a simple promoter consisting of three WT1-binding sites upstream of a TATA box and the luciferase gene. 1.2 μg of lacZ expression vector was included in each precipitate as an internal standard for transfection efficiency. Fig. 6A shows that transfection of 1.2 μg of WT1(−/−) expression vector increases the basal luciferase activity about 14-fold. 150 ng of p73α alone have little effect on the basal activity of the 3xWTE-Luc, whereas 150 ng p53 repress basal transcription about 2.5-fold. Co-expression of p73α or p53 together with WT1 reduces the transcription activation of WT1 about 6-fold. However, part of the repressive effect of p53 on
WT1-mediated transcription activation may be attributable to the 2.5-fold repression of the 3xWTE-Luc construct exerted directly by p53.

The Western blots shown in Fig. 6B demonstrate that the decrease in WT1-dependent transcription is not caused by differences in transfection efficiency or by lower WT1 protein levels in the presence of p73α or p53. All proteins are comparably expressed, independent of whether they are expressed alone or in combination with other proteins.

WT1 Represses Transcription Activated by p73α, p73β, and p53 — The different members of the p53 family are potent transcription activators and have recently been shown to regulate the Bax promoter (48). The promoter is known to stimulate the expression of Mdm2 (45). Therefore, we wanted to establish whether WT1 could inhibit the induction of endogenous Bax and Mdm2 as suggested by the luciferase assays. Hep3B cells were transfected with either 10 μg of WT1(+/−), 200 ng of p73α, or 20 ng of p53 alone or with WT1(+/−) in combination with p73α or p53. An expression plasmid encoding LacZ was co-transfected to verify equal transfection efficiencies. The anti-LacZ Western blot in Fig. 7A demonstrates that the transfection efficiencies are approximately equal. The Mdm2 blot shows that transfection of p73α or p53 leads to increased levels of endogenous Mdm2. Co-transfection of WT1(+/−) with p73α/p53 results in a reduction of p73α/p53-stimulated Mdm2 expression, suggesting that the repressive effect of WT1 as observed in luciferase assays also holds true for the full-length Mdm2 promoter in its natural context. Because WT1(+/−) alone has no effect on Mdm2 levels, the decrease in Mdm2 protein levels after co-transfection of WT1(+/−) together with p73α/p53 suggests that WT1 represses p73α/p53-induced transcription of the mdm2 gene rather than affecting Mdm2 at the post-transcriptional level, although this experiment does not completely exclude the latter possibility.

The basal levels of Bax proteins in Hep3B cells are too high to detect activation of the Bax promoter by p73α or p53. The anti-WT1 and anti-HA blot in Fig. 8 shows that all transfected proteins are expressed and that co-expression of WT1 does not reduce the expression levels of p73α or p53. Thus, WT1(+/−) can inhibit the induction of endogenous Mdm2 protein by p73α and p53.

DISCUSSION

The data presented here demonstrate that WT1 can bind to p73α, p73β, p63/KET, and p53 in vivo and in vitro. WT1-p73 and WT1-p53 protein complexes could be immunoprecipitated from lysates of U2OS and Hep3B cells. The binding between WT1 and p73/p53 is most likely direct and does not require additional proteins, since in vitro translated-WT1 molecules bind to purified p73α and p53 in GST pull-down assays. p73 inhibits DNA binding by WT1 and consequently represses WT1-mediated transcription activation from a luciferase reporter construct. Likewise, p73- and p53-activated transcription is inhibited by WT1.
Our in vivo analyses suggest that at equal protein concentrations WT1-p73 complexes are more abundant or more stable than WT1-p53 complexes. Both p73α and p53 bind to the zinc finger region of WT1 and increasing truncations of the zinc fingers diminish binding of p73 and p53 to WT1 to the same extent. Therefore, the stronger binding between WT1 and p73 is not caused by additional, independent binding sites for p73 on the WT1 protein outside of the p53-binding region. The finding that p73 and p53 bind to the same region of WT1 also rules out the possibility that the anti-WT1 antibody, which was raised against the C-terminal 19 amino acids of WT1, interferes with recognition of the WT1-p53 complex and may in that way lead to precipitation of relatively little WT1-p53 compared with WT1-p73 complexes. The differences in binding of p73 and p53 to WT1 observed in immunoprecipitations can be explained in different ways. First, amino acid residues not conserved between p73 and p53 may be responsible for the differences in binding to WT1. Second, additional proteins present in whole cell extracts may, although not essential for binding as suggested by the GST pull-down experiment, stabilize binding between WT1 and p73 but not between WT1 and p53. Third, non-conserved or additional domains present in p73 but not in p53 may stabilize binding between p73 and WT1. In this respect, it is important to note that at least six p73 isoforms that differ in length and amino acid sequence of their C terminus are generated through alternative splicing (33, 38, 59). Similarly, several p63 protein isoforms have been described, some of which lack the N-terminal transactivation domain and act dominant-negative on transcription activated by p53 and other p63 isoforms (34). It remains to be established whether WT1 binds to all p73/p63 isoforms with the same affinity. Likewise, the binding domain(s) of WT1 on p53, p63, and p53 need to be characterized in the future.

Several proteins are known to alter the stability of p53 through association. Mdm2, which is transcriptionally activated by p53 during stress response, targets p53 for proteolysis and in that way forms a negative feedback loop (50, 51). Our results suggest that WT1 does not stabilize p73α, in contrast to its stabilizing effect on p63, indicating that the half-lives of p53 and p73α are governed by different mechanisms. Combination of our data with the observation that Mdm2 binds to p73 without targeting it for degradation (45, 60) suggests that binding of WT1 to p53 may inhibit Mdm2 from targeting p53 for degradation, whereas p73α is not targeted by Mdm2 and, therefore, is not stabilized by WT1. Similarly, the human papilloma virus E6 protein interacts with p53 but not...
Physical and Functional Interactions between WT1 and p73/p63

Transfection:

\[ \text{LacZ} + \]

WT1(+/+)

Neo (+/-) α p53 α p53

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<th>blot</th>
<th>a/bex</th>
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**Fig. 8.** WT1 abrogates the induction of the endogenous Mdm2 protein by p73α and p53. 5-cm dishes of Hep3B cells were transfected with 1.5 µg of CMV-LacZ together with 10.2 µg of CMV vector (lane Neo), 10 µg of CMV-WT1(+/+), 200 ng of CMV-HA-p73α (lane α), or 20 ng CMV-HA-p53 (lane p53). All precipitates contained equal amounts of CMV vector. 40 h after transfection, cells were lysed and Western blots prepared. The Western blots were probed with anti-Mdm2, anti-Bax, anti-LacZ, and a mixture of anti-WT1 and anti-HA antibodies. The gray arrow points to the position of p73α and p53, and the black arrow points to the position of WT1(+/+). Co-expression of WT1(+/+) together with p73α (lane WT1(+/+) + α) or p53 (lane WT1(+/+) + p53) inhibits induction of Mdm2.

with p73, and as a consequence triggers proteolysis of p53 only (61). Interestingly, WT1 is capable of inhibiting E6-mediated degradation of p53 (32). This indicates that WT1 may play a role in two different cellular pathways. It may be involved in p53-regulated cell proliferation and in concert with p73 and p63 in development.

The p73/p53 binding domain maps to the zinc fingers of WT1 and, most likely because of that, we find that p73 inhibits DNA binding by WT1 in electrophoretic mobility shift assay. This effect is not observed with p53. However, co-precipitation of p73 with WT1 from cell lysates is more efficient than co-precipitation of p53 with WT1. Thus, it is possible that the electrophoretic mobility shift assay is not sensitive enough to detect a decrease in DNA binding by WT1 with the amounts of p53 protein used. Alternatively, p53 may not influence DNA binding by WT1 at all, but by binding to the WT1 zinc finger region p53 may affect the activity of WT1 in other ways, e.g., by shielding parts of its transcription regulatory domain. The latter hypothesis is supported by the finding that in lysates of a breast cancer cell line, WT1 and p53 are present in a complex that binds to an oligonucleotide containing a WT1 consensus sequence (62). Immunodepletion of WT1 from the cell lysates depleted both WT1- and p53-containing complexes from the oligonucleotide (62).

Functionally, p73 is capable of inhibiting WT1(+/−)-dependent transcription activation from a reporter containing three WTE motifs. We also found that p53 can repress WT1-mediated transcription activation. But since p53 by itself repressed transcription from the reporter construct 2.5-fold, we cannot discriminate between inhibition of WT1-induced transcription through direct binding to WT1 and repression of WT1-mediated transcription through competition between p53 and WT1 for the same co-factors. Lee et al. (26) have recently shown that the amphiregulin promoter is activated through binding of WT1 to a motif very similar to WTE. Amphiregulin has the same expression pattern as WT1 in the developing kidney and can induce uterine bud branching in kidney organ cultures (26), suggesting that it is a physiologically important target of WT1. Future experiments should establish the expression pattern of p73, p63, and p53 at early stages of kidney development and whether p73, p63, and p53 can inhibit WT1-mediated transcription activation of the amphiregulin promoter.

We also investigated the effect of p73 on WT1-dependent transcription repression. To that end we used a luciferase vector containing part of the murine insulin-like growth factor II P3 promoter, which is repressed by WT1(+/−) (24). p73 counteracted WT1-mediated repression but by itself activated transcription from this promoter (data not shown). Therefore, we cannot prove that the increase in transcription seen after co-transfection of p73 with WT1 results from binding of p73 to WT1. WT1 represses p73- and p53-dependent transcription activation from the bax and the mdm2 promoters. However, the effects of WT1, as measured in transient reporter assays with equal amounts of p73 and p53 expression constructs, are stronger on p73 than on p53. Inhibition of p53-mediated activation of Bax, a pro-apoptotic protein (63), could provide an explanation for the inhibitory effect of WT1 on p53-mediated apoptosis in response to ultraviolet irradiation (32). Repression of p73- and p53-mediated activation of the mdm2 promoter by WT1 may contribute to the stabilization of p53 by WT1. Our data show that WT1 is capable of reversing p73- and p53-induced transcription from a Mdm2-reporter construct and transcription of the endogenous mdm2 gene. Reduction in Mdm2 levels may slow down Mdm2-triggered degradation of p53 and thus lead to higher p53 protein levels.

The different members of the p53 family are thought to have evolved from an ancient precursor gene as found in squid. p53 has become a major regulator of stress responses, whereas p73 and p63 are involved in regulating fundamental processes associated with differentiation and development (37, 41–43). Since the initial description of association between p53 and WT1 (31), increasing evidence suggests that WT1 may modulate p53 function in response to stress (32, 62). WT1 is a tissue-specific, developmentally regulated transcription factor that binds to all members of the p53 family. Therefore, it is conceivable that binding of p73 and p63 to WT1 may influence their function in differentiation and development. Consequently, we propose a dual role for WT1 in which it is, through its functional interaction with all p53-like proteins, involved in both stress response and development. Finding answers to the role of these interactions in cell growth, development, and pathogenesis requires more genetic and biochemical experiments.

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References
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