N-terminally Truncated WT1 Protein with Oncogenic Properties Overexpressed in Leukemia*

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WT1 was originally identified as an inactivated gene in Wilms tumor, a childhood kidney cancer. Alternative splicing of the WT1 transcript generates four major protein isoforms, each having different functional properties. Here we characterized a short transcript originating from a second promoter located within intron 1 of WT1. This 2.3-kb sWT1 transcript encodes a protein of ∼35–37 kDa that retains intact DNA-binding and transactivation domains but lacks the 147 amino acids at the N terminus required for transcriptional repression. We found sWT1 to be a more potent transcriptional activator than WT1 for the 147 amino acids at the N terminus required for transcriptional repression. We found sWT1 to be a more potent transcriptional activator than WT1 for the 147 amino acids at the N terminus required for transcriptional repression.

The WT1 protein can act as a transcriptional activator or repressor depending on the cellular or chromosomal context (5, 6). The carboxyl (C) terminus contains four zinc finger domains that mediate DNA binding (7). The amino (N) terminus comprises a transcriptional repression domain followed by a transactivation domain (5). The four major isoforms of WT1 protein identified to date result from two alternative splices in its transcript. One of these splices encodes the insertion of three amino acids (KTS) between zinc fingers 3 and 4, which alters the DNA-binding properties of the protein (7) and affects the development of kidneys and gonads in mice and in humans affected by Frasier syndrome (4, 8). The other splice results in the insertion of a 17-amino-acid segment, encoded by exon 5, in the middle of the protein (7). The four protein isoforms are conserved across mammalian species. Thus far, all identified WT1 isoforms differ only at their midsections and C termini and are derived from this alternative splicing; the variants result from alternative initiation of translation upstream or downstream of the initial AUG (9, 10), alternative promoters (11, 12), and from RNA editing (13).

WT1 usually binds to the GC-rich canonical early growth response gene-1 DNA-binding motif (6). Transactivation assays of the −KTS (lysine, threonine, serine) variants of WT1 have identified distinct transcriptional activation (residues 180−294) and repression (residue 84−179) domains (5). Many genes are thought to be regulated by WT1; most are involved in growth regulation and cellular differentiation (1) or in sex determination and differentiation (e.g. SRY, SF1, and MIS) (14−17).

In Wilms tumor, WT1 clearly acts as a tumor suppressor gene; however, the precise role of WT1 in human leukemia has yet to be elucidated (18−20). An oncogenic role of WT1 in leukemia has been suggested but not yet proven (21). High level expression of WT1 may contribute to the maintenance of a malignant phenotype through a variety of mechanisms, including inhibition of differentiation and apoptosis and increased proliferation. Anti-sense inhibition of WT1 can block the growth of leukemic cells (22, 23). Although the role of WT1 in leukemia is not clear yet, its usefulness in diagnosis and treatment in leukemia is quite advanced. The presence and expression level of WT1 may serve as predictors of prognosis or relapse in patients with acute myeloid leukemia (24). Immu-

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The abbreviations used are: WT1, Wilms tumor gene; RACE, rapid amplification of cDNA ends; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CMV, cytomegalovirus; siRNA, small interfering RNA; IGF-1R, insulin-like growth factor 1 receptor; SDS, sodium dodecyl sulfate; CREB, cAMP-response element-binding protein.
notherapy based on WT1 is also in preclinical development (25, 26).

Here we describe sWT1, a newly identified, N-terminally truncated form of the protein that is overexpressed in leukemia and to act more like an oncogene than a tumor suppressor. Our functional studies showed that the sWT1 protein was much more effective than the full-length WT1 at transactivating target gene promoters but could not repress genes that are normally repressed by the full-length WT1. Antisense inhibition of sWT1 expression blocked growth of leukemic cells suggesting an oncogenic role for the sWT1 isoform in leukemia.

**EXPERIMENTAL PROCEDURES**

5′ RACE and PCR Cloning—We used rapid amplification of cDNA ends (RACE) to identify mouse sWT1 in the Mouse Testis Marathon-Ready cDNA kit (Clontech, Palo Alto, CA) according to the manufacturer's recommendations. The first round of PCR was performed with sense (Wt1RACE-F1) and antisense (Wt1RACE-R1) primers and the sense anchor primer or the antisense anchor primer supplied by Clontech. Nested PCR was performed with the plus primer AP2 (Clontech) and either the new sense primer (Wt1RACE-F1.1) or the new antisense primer (Wt1RACE-R1.1). Several bands were obtained and cloned into a TOPO vector (Invitrogen) and sequenced. The sequencing results were compared with the known hsWT1 cDNA sequence.

Northern Blotting and Reverse Transcription (RT)-PCR Analysis—A human multiple tissue expression array (Clontech) was hybridized with human exon 2–10 of sWT1 in NorthernMax hybridization solution (Ambion, Austin, TX) at 42 °C overnight. The membrane was washed twice with 2× SSC (sodium chloride/sodium citrate buffer) containing 0.1% sodium dodecyl sulfate (SDS) at room temperature for 10 min and twice with 0.1× SSC containing 0.1% SDS at 65 °C for 20 min.

Human total or poly(A) RNA was purchased from Ambion and Clontech. Single-stranded cDNA was prepared with the ThermoScript RT-PCR System (Invitrogen). PCR reactions were run with the GC-rich PCR system (Roche Diagnostics, Indianapolis, IN) with primers WT1RT-F and WT1RT-R for WT1 and primers sWT1RT-F and sWT1RT-R for the sWT1 isoform. The PCR products were separated on 1.5% agarose gels. Each band was subcloned and confirmed by sequencing.

Real-time RT-PCR—Fifty-two blood samples were analyzed, 27 from patients with acute myeloid leukemia at diagnosis, 18 from patients with acute lymphoblastic leukemia, and 13 from patients with myelodysplastic syndrome. Patient characteristics and RNA isolation techniques have been described previously (27). cDNAs were first amplified by conventional RT-PCR for the presence or absence of WT1 and sWT1 transcripts. Absolute quantification was done by real-time RT-PCR with primers and cDNA. To ensure that both the gene of interest and the control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were amplified at the same efficiency, several dilutions of cDNA (1, 5, and 10 ng) were amplified. Optimization was performed similarly, i.e. with primers and cDNA, and various concentrations of MgCl₂ were used as a control. PCR was done with a LightCycler system (Roche Diagnostics) in a total reaction mixture of 10 µl containing 5 ng of cDNA, 1× LightCycler-FastStart DNA Master SYBR Green 1, 1 µM MgCl₂, and 1 µM each primer. After denaturation at 95 °C for 10 min, 30 cycles were performed at 95 °C for 5 s, 60 °C for 5 s, and 72 °C for 15 s. The data were normalized by using the ratio of the target cDNA concentration to GAPDH to correct for differences in the amounts of RNA among the samples.
Plasmids—pcDNA3WT1 and pcDNA3sWT1 constructs were made by PCR amplification from human testis RNA. The resulting PCR products were subcloned into a modified pcDNA3 vector (28) harboring the 5'-untranslated region of the herpes simplex virus thymidine kinase gene.

Cell Culture and Transfection—HeLa and NIH3T3 cells were grown at 37 °C in Dulbecco’s modified Eagle’s medium/F-12 supplemented with 10% fetal calf serum in an atmosphere containing 5% CO₂. The cells were seeded at a density of 50,000–70,000 cells/well in 24-well plates 16–18 h before transfection. The cells were cotransfected with expression and reporter plasmids in various combinations. A plasmid containing cytomegalovirus (CMV)-β-galactosidase was cotransfected as an internal control to normalize for differences in transfection efficiency. The transfections were done with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations, and the cells were harvested after 40–48 h. Luciferase activity was measured with a Tropix luciferase assay kit (Applied Biosciences, Foster City, CA) and a Lumat LB9507 luminometer (EG & G Berthold). β-galactosidase was measured with the Tropix Galacto-Light Plus kit (Applied Biosciences).

Gel Shift Assay—DNA-binding assays were performed with WT1-responsive elements from the SRY promoter (14). Radiolabeled probes were prepared by end labeling with [γ-³²P]ATP, and 10 pmol of each labeled probe and 5 μl of in vitro translated protein extracts were used for each reaction. To assay for antibody-mediated supershift, 2 μl of antibody was added to the reaction mixture 15 min before the addition of the labeled probe. Samples were resolved in a 6% polyacrylamide gel cast in Tris borate-EDTA buffer containing 10 mM magnesium acetate at 4 °C for 3 h. The gels were

FIGURE 2. Tissue distribution of WT1 and sWT1 transcripts. A, first strand cDNA was synthesized from various human tissues and subjected to RT-PCR with exon-specific primers. The resulting PCR products were separated on a 1.5% agarose gel. GAPDH primers were used as an internal control. In the upper panel, the upper band represents WT1 (310 bp), the lower band represents sWT1 (230 bp). The lower panel represents GAPDH. B, the primers and cDNAs used were the same as those used for the conventional RT-PCR analysis described above. Expression levels are given as the ratio of the target gene to the control gene (GAPDH) to correct for variations in the starting amount of mRNA. The ratios were calculated based on 1,000 copies of GAPDH. C, Northern blot of human tissues with a radiolabeled cDNA probe for exons 2–10 WT1 (i.e., specific for both isoforms). GAPDH was used as a control.
dried and analyzed with a phosphorimaging device (Molecular Dynamics, Sunnyvale, CA).

Western Blotting—Fifty micrograms of protein from different human tissue extracts (GenoTech, St. Louis, MO) were separated on 4–20% SDS-polyacrylamide gels by electrophoresis and immunoblotted with specific antibodies. The primary and secondary antibodies used were C terminus-specific anti-WT1 at a 1:20,000 dilution and goat anti-rabbit IgG conjugated with peroxidase at a 1:100,000 dilution (Santa Cruz Biotechnology).

Western blots were developed with SuperSignal West Femto maximum sensitivity substrate (Pierce).

siRNA Transfection—K562 erythroleukemia cells from the American Type Culture Collection (Manassas, VA) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM glutamine at 37 °C in 5% CO₂. Cells (5–10 × 10⁶) were transfected with various concentrations of small interfering RNAs (siRNAs) to sWT1 (GGAGAGGGUUGAGGCCG Gtt) to WT1 (GGAGUUCAAGGCAGCGCCtt) or a control (scrambled) sequence by using Oligofectamine (Invitrogen). Transfected cells were maintained in regular culture medium for up to 5 days. Cell growth was determined by counting viable cells after dye exclusion.

Transformation Assay—NIH3T3 mouse fibroblasts were transfected with various combinations of WT1, sWT1, and RasV12 expression vectors and selected with G418. Cells were split at 5 × 10⁵ cells/100-mm dish for focus formation assays. Transfected cells were fed twice a week with fresh growth medium for 2 weeks. The cells were then washed twice in phosphate-buffered saline, fixed for 10 min in 10% (v/v) acetic acid, and stained for 10 min in 0.4% (w/v) crystal violet in 10% (v/v) ethanol. Dishes were rinsed with de-ionized water, air dried, and photographed.

RESULTS

Cloning Human and Mouse Short Isoforms of WT1—The existence of an alternative form of WT1 was first suggested when a Northern blot from testis RNA produced a shorter 2.5-kb band alongside the full-length 3.1-kb band (29), leading us to seek the sequences in the mouse and human genomes that would give rise to the shorter form. The human expressed sequence tag (EST) National Center for Biotechnology Information data base dbEST contained potential cDNA sequences that would encode a WT1 protein lacking N-terminal residues. Although this short form of WT1 was being characterized in our laboratory, one group reported its cloning and monoallelic expression pattern in Wilms tumor (11). The function and expression of sWT1 in humans, however, remain
Comparison of the first exon of sWT1 with the first exon of WT1 showed no significant sequence similarities. However, the first exon of sWT1 contains a translation start codon ATG located at nucleotide 242, and the first three amino acids encoded by this exon 1a, MEK, are the same in the mouse and human proteins. Thus, even though the newly identified mouse sWT1 exon 1a was not similar to the exon 1a of human sWT1 at the nucleotide level, the coding sequences were identical. The GenBank™ accession number of the sWT1 transcript is DQ537939.

Tissue-specific Expression of WT1 and sWT1 mRNA—First we compared the tissue distribution of sWT1 with that of the longer isoform WT1 by using RT-PCR with primers specific for each isoform as follows. PCR was performed on the first strand cDNA derived from various human tissues with primers specific for WT-1 and sWT1 that generated a 310-bp product for WT1 and a 230-bp product for sWT1. PCR amplifications from 13 human tissue types showed that the 310-bp fragment (representing WT1) was present in the adrenal gland, fetal and adult kidney, and testis and was absent or expressed at very low levels in the bone marrow, brain, breast, heart, liver, ovary, pancreas, spleen, and thymus. Expression of the 230-bp sWT1 was limited to the fetal kidney and testis (Fig. 2A). The absolute amount of WT1 and sWT1 expression was also quantified by real-time PCR (Fig. 2B).

Next we analyzed the expression of WT1 and sWT1 in adrenal, bone marrow, breast, heart, testis, adult and fetal kidney, and ovary by northern blotting with a cDNA probe coding exons 2–10 of WT1. A single 3.1-kb band representing WT1 was detected in the adrenal, testis, and fetal kidney (with faint bands evident in the bone marrow and breast); a 2.3-kb band (sWT1) appeared in fetal kidney and faintly in adult kidney; only the fetal kidney showed both bands (Fig. 2C). These findings suggest that sWT1 expression is highly tissue-specific relative to WT1 in adult human tissues.

Expression of sWT1 Protein—We also evaluated the expression of endogenous sWT1 protein in human tissues. Tissue extracts from adrenal, brain, breast, fetal and adult kidney, testis, ovary, spleen, heart, and pancreas were immunoblotted with an antibody directed against the C terminus of WT-1. No WT1 protein was present in extracts from adrenal, brain, breast, fetal kidney, ovary, spleen, heart, or pancreas (Fig. 3). The antibody specifically recognized three bands in the extracts from fetal kidney and testis at ~57 kDa (WT1 translated from the upstream open reading frame), ~51 kDa (the full-length WT1 protein), and ~35 kDa (the truncated sWT1) (Fig. 3). The sWT1 protein was detected in adult and fetal kidney and adult testis. In vitro translated WT1 and sWT1 were used as positive controls. These results suggest that sWT1 protein is expressed in vivo.

DNA Binding of sWT1—Next we used gel shift assays to compare the ability of sWT1 and WT1 to bind DNA. Antibodies specific to the N or C termini of WT1 and a radiolabeled WT1-responsive element probe were added to in vitro translated WT1 or sWT1 protein to test the reactivity of the various DNA-protein complexes (Fig. 4). In the reaction between sWT1 and the WT1-responsive element, a high mobility complex was observed (Fig. 4, lanes 2–5) that could be supershifted only with
the C terminus-specific antibody (lane 5) but not with the N terminus-specific antibody (lane 4) or a control Myc-specific antibody (lane 3). Lower mobility WT1-specific complexes (Fig. 4, lanes 7–9) were supershifted only with N terminus-specific antibody (Fig. 4, lane 9). These data suggest that the DNA binding ability of these two isoforms are similar.

**Gene Activation by WT1 and sWT1**—To compare the cellular functions of WT1 and sWT1, we first tested their ability to transactivate reporter genes in HeLa cells that had been transiently transfected with WT1 or sWT1. sWT1 was strikingly more effective than WT1 in the transactivation of both the SRY-driven and the p21-driven reporter genes (Fig. 5). These data suggest that sWT1, which lacks the first 147 residues of WT1, has enhanced transcriptional activity in different promoter contexts and support the concept of a general mechanism to regulate gene activation that is not strictly dependent on promoter sequences.

**Gene Repression by WT1 and sWT1**—Some physiological effects mediated by WT1 occur through transcriptional repression of target genes via a repressor domain located in the N terminus of the WT1 protein (Fig. 1A). We compared the ability of WT1 with that of sWT1 to repress transactivation of cyclin E and the insulin-like growth factor 1 receptor (IGF-1R). Interestingly, WT1 repressed cyclin E and IGF-1R promoter activity, whereas sWT1 activated cyclin E and IGF-1R promoter activity (Fig. 6). The finding that sWT1 can activate genes that are normally repressed by WT1 supports the hypothesis that the activation and repression functions of WT1 occur separately in distinct regions of the protein.

**Expression of WT1 and sWT1 in Leukemia**—Because WT1 expression has been implicated in leukemia (18, 26, 30), we next investigated the expression of WT1 isoforms in peripheral blood samples of 52 patients with adult leukemia. 12 patients with myelodysplastic syndrome, 26 with acute myeloid leukemia, and 14 with acute lymphoblastic leukemia, RNA extracted from the samples was analyzed by RT-PCR (data not shown) and real-time RT-PCR (Fig. 7). Most of the samples contained a single predominant isoform, which in most cases (12 of 52) was sWT1. However, in 8 of 52 patients with adult leukemia, WT1 was the predominant isoform expressed (Fig. 7), and in 12 cases, both isoforms were almost equally expressed. In the remaining 20 cases, no WT1 or sWT1 expression was detected by RT-PCR or by real-time RT-PCR.

**Inhibition of Leukemia Cell Growth by sWT1-specific siRNA**—We next examined the effect of siRNA to WT1 and to sWT1 on the growth of K562 cells, which are known to express both isoforms (Fig. 8A). Down-regulation of sWT1 by siRNA decreased the expression of endogenous p21 expression (Fig. 8B). Basal p21 promoter activity was low in sWT1-specific...
siRNA-treated K562 cells in comparison with the control, indicating that sWT1 was involved in the p21 promoter activation (Fig. 8C). Silencing sWT1 substantially inhibited K562 cell growth in comparison with a scrambled siRNA (Fig. 8D). Silencing WT1 also seemed to inhibit K562 cell growth, but this effect was not as striking as for silencing sWT1.

WT1, sWT1, and Oncogenic Ras in Cellular Transformation in Vitro—Finally, to determine whether or not sWT1 has possible oncogenic property, NIH3T3 cells were cotransfected with H-RasV12 and either WT1 or sWT1 and tested for evidence of transformation (Fig. 9). NIH3T3 cells coexpressing sWT1 and H-RasV12 lost contact inhibition and produced foci in focus formation assays. This phenotypic manifestation of transformation was specific for sWT1, because cells coexpressing WT1 and RasV12 failed to do so. It is important to note that these sWT1 + Ras-expressing foci showed poor growth properties and were difficult to establish as long term cell lines.

DISCUSSION

In this study, we characterized sWT1, a novel WT1 isoform directed by an internal promoter within the first intron of the WT1 gene. We found that expression of WT1 and sWT1 was restricted, and tissue-specific in adult tissue samples, both isoforms were found in fetal kidney and adult testis. The sWT1 mRNA was translated in vivo into an N-terminally truncated form of WT1. The transcriptional repression domain of WT1, as well as an alleged RNA recognition motif, has been located in the N terminus; however, sWT1 lacks both of these two motifs (31, 32). We found sWT1 to have several properties of interest. First, sWT1 was a more potent transcriptional activator than was the full-length WT1 protein, even though the DNA-binding ability of these two isoforms was similar. One possible explanation for this increase in activation is that the conformation of the sWT1 isoform may be more favorable than that of the full-length protein in terms of recruiting other coactivators such as CREB-binding protein/p300 (33). sWT1, which lacks a repressor domain in its N terminus (32), does not bind to factors such as HSP70 (34) and thus may be more readily available to a coactivator.

The most interesting functional property of this N-terminally truncated isoform was its inability to repress genes that are normally repressed by the full-length WT1; it actually activated genes (such as cyclin E and IGF-1R) that are typically repressed by full-length WT1. WT1 is known to regulate the development of a wide variety of organs by repressing or up-regulating downstream target genes by direct DNA binding (1); knowledge of the function of sWT1 in vivo will probably require knocking down sWT1 in mice. Such experiments will also help to clarify the role of transcriptional activation versus that of transcriptional repression of WT1 in tumorigenesis (1).

To date, several laboratories, including ours, have reported overexpression of WT1 in up to 90% of patients with active leukemia (18, 20, 26, 27). In studying the relative expression of WT1 and sWT1 in leukemia, we were surprised to find the expression of
both isoforms, with a slightly higher level of the sWT1 isoform, in leukemia. sWT1 expression was very low to virtually undetectable in the peripheral blood of healthy controls. The ratio between these two isoforms may dictate the fate of certain types of tissue during development. sWT1 expression drives cells in the direction of proliferation. A high level of sWT1 expression may confer a growth or survival advantage to tumor cells by preventing or delaying the response to differentiation signals. However, in this study, we did not see a clear bias toward predominant sWT1 expression. WT1 mRNA produces two protein products due to an internal translation site (10). The ratio between these two protein products is most likely important for WT1-mediated signal transduction. The protein product of sWT1 is almost identical to the internal translation product of WT1. The expression of sWT1 in cells that normally do not express sWT1 skewed the ratio toward transcriptional activation pathway. More comprehensive studies are necessary to better understand the role of WT1 and WT5 in leukemia.

We found also that siRNA directed against WT1 and sWT1 inhibited WT1 and sWT1 protein expression and slowed the proliferation of K562 cells by sWT1 to a greater extent than WT1. Reduction of WT1 by siRNA also decreased K562 cell proliferation but not as dramatically as sWT1 reduction. One possible explanation is that WT1 has an additional internal translation site, and the translated product behaves similar to sWT1 (10). However, in our experience, the predominant translation product was the full-length WT1 protein from WT1 mRNA (data not shown). We cannot rule out the possibility that a short translation product that retards growth might predominate in some subpopulation of K562 cells. The combination of sWT1 expression in leukemia and myelodysplastic syndrome and its minimal expression in normal tissues suggests that targeting the first exon of sWT1 by siRNA may be a good cancer therapy strategy.

Using classical in vitro transformation assays, we showed that sWT1 exerts weak oncogenic functions in fibroblast. sWT1 cooperates with oncogenic ras in the transformation of NIH3T3 cells. sWT1 has no transforming capacity of its own in the NIH3T3 strains used in the present study. Our data show that neither sWT1 nor WT1 alone induce foci formation when transfected in primary cells, whereas transformed foci become evident upon cotransfection of sWT1 with Ha-rasV12 or Ha-rasV12 alone. On the other hand, WT1 failed to show any additive effects with Ha-rasV12. Therefore, the structural differences between sWT1 and WT1 reflect distinct functional roles, at least with respect to the cooperation with ras in cell transformation.

In summary, these findings lead us to propose that sWT1 should be considered more of an oncogene than a tumor suppressor gene in leukemia cells. This proposal is based on the following findings: (i) the high expression of the sWT1 gene in leukemias, (ii) growth inhibition of leukemia cells by treatment with sWT1-specific siRNA, and (iii) enhanced Ras-induced in vitro transformation of NIH3T3 cells. Further studies are needed to delineate the complex roles of WT1 in disease and development.

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