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NUCLEIC ACIDS, PROTEIN SYNTHESIS, AND MOLECULAR GENETICS:
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Internal Translation Initiation Generates Novel WT1 Protein Isoforms with Distinct Biological Properties*

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The Wilms' tumor gene, WT1, is homozygously mutated in a subset of Wilms' tumors. Heterozygous mutations in WT1 give rise to congenital anomalies. During embryogenesis, WT1 is expressed mainly in the kidneys, uterus, and testes.

Alternative splicing of the WT1 mRNA results in synthesis of four main WT1 protein isoforms with molecular masses of 52–54 kDa. In addition, translation initiation at a CUG upstream of the initiator AUG generates four larger WT1 proteins of 60–62 kDa.

We describe here the existence of novel WT1 isoforms and demonstrate that they are derived from translation initiation at the second in-frame AUG of the WT1 mRNA. These N-terminally truncated WT1 proteins of 36–38 kDa can be detected in several cell lines, mouse testes, and Wilms' tumor specimens. They can bind to DNA and direct transcription from reporter constructs. The shorter WT1 protein lacking the two splice inserts has a greater transcription activation potential than the corresponding main WT1 protein isoform but shows no transcription repression potential. Overexpression of full-length or N-terminally truncated WT1 efficiently induces apoptosis. These data show that additional WT1 isoforms with distinct transcription-regulatory properties exist, which further increases the complexity of WT1 expression and activity.

WT1 contains 10 exons and spans about 50 kilobases on chromosome 11p13. Exons 5 and 9 are differentially spliced, ultimately giving rise to four different protein isoforms with molecular masses ranging from 52 to 54 kDa. WT1(−/−) lacks both splice inserts, WT1(+/-) accommodates the 17-amino acid and the 3-amino acid KTS splice inserts, and WT1(+/+) and WT1(−/+) contain either the 17-amino acid or the KTS splice insert (Ref. 6; see Fig. 1). In addition to these WT1 isoforms, the existence of larger WT1 proteins, which result from translation initiation at an in-frame CUG upstream of the initiator AUG, has been reported (7).

A further level of complexity is added by RNA editing at position 839 of the WT1 mRNA, which replaces leucine 280 in WT1 proteins by proline (8). The WT1 gene may thus produce 16 different protein isoforms.

Exons 7–10 of the WT1 gene encode four zinc fingers of the Krüppel type (9, 10), which can mediate binding to GC-rich DNA sequences (11, 12). WT1 may, depending on promoter architecture and cell type, repress or stimulate promoter activity. Growth-related genes repressed by WT1 include transforming growth factor-β1 (13), platelet-derived growth factor-A (14), and insulin-like growth factor II (15). The minimal transcription activation and repression domains of WT1 have been mapped to two separate regions of WT1 (16), and the dimerization domain of WT1 is located within the first 182 amino acids (17).

In addition to its function as a transcription factor, WT1 may also be involved in post-transcriptional processing of RNA. WT1 proteins containing the KTS splice insert, which alters the spacing between zinc fingers three and four, preferentially associate with splicing factors (18) and are incorporated into spliceosomes in vitro (19).

Ectopic expression of WT1 has different effects on cells, including arrest in the G1 phase of the cell cycle (20) and induction of apoptosis (21, 22). Furthermore, WT1 represses the tumorigenicity of several cell lines (23–25). In many cases, the effects observed are splice form-dependent.

While testing a number of cell lines for WT1 protein expression, we consistently detected novel WT1 isoforms with apparent molecular masses of 36–38 kDa in addition to the 52–54 kDa and larger isoforms of WT1. In this study, we demonstrate that translation initiation at a downstream, in-frame AUG results in synthesis of the novel WT1 proteins. These N-terminally truncated WT1 isoforms can bind to DNA, have transcription-regulatory properties distinct from those of the main forms, and are still capable of inducing apoptosis.

Our findings show that WT1 gene expression is even more complex than previously recognized and emphasize that a delicate balance between the different WT1 gene products may be required for proper WT1 function.

MATERIALS AND METHODS

Plasmid Construction—A pcDNA3.1-WT1(−/−) full-length construct was generated by excising a NolⅠ–HincII fragment out of CB6·
WT1(–) (30) and ligating it into NotI–EcoRV-digested pcDNA3.1 (Invitrogen). The ATG at codon 127 of WT1 was mutated by exchanging a Bsp120I–XhoI fragment of CB6-WT1(–) for an identical oligonucleotide in which the ATG had been mutated to CTC. The pcDNA3.1 construct pcDNA3.1-WT1(–) was prepared by ligating out the ATG-containing fragment of WT1(–) in the Bsp120I–XhoI-digested pcDNA3.1, generating a WT1(–) cDNA which begins 5′ of the second in-frame ATG. The WT1(–)/+PM construct was made by polymerase chain reaction with primers that were designed to anneal around the first ATG of WT1(–) and around codon 256, which was mutated into a stop codon by primer-directed mutagenesis. The polymerase chain reaction was ligated into pcCR2.1 (Invitrogen), and this vector was used for in vitro transcription/translation of WT1(–)/+PM. The luciferase vector containing three WT6 consensus sites (5′-GCGTTGGGATG-3′; Ref. 12) with spacers of six base pairs in six was made by introducing an oligonucleotide containing these sequences into the BglII site of the pBL2-TATA-luciferase reporter (26). The IGFII-P3-luciferase vector has been described previously (27). pcDNA3.1-LacZ was purchased from Invitrogen.

**Cell Lines and Tissue Culture**—All cells were cultured at 37 °C in a 5% CO2 atmosphere. End-2 (28), Epi-7 (28), p530/0 mouse embryo fibroblasts, Hep3B, and U2OS cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 8% fetal calf serum and antibiotics. AdBRK cells, which are baby rat kidney cells transformed by a recombinant adeno-plasmid containing region E1A from adenovirus type 5 and region E1B from adenovirus type 12 (29), were cultured in modified Eagle’s medium, 8% newborn calf serum, and antibiotics.

**Antibodies**—The monoclonal anti-LacZ antibody was purchased from Roche Diagnostics (Indianapolis, IN). The anti-WT1 antibody C19 is directed against an epitope within the first 80 amino acids of WT1. The monoclonal anti-LacZ antibody was purchased from Roche Diagnostics (Indianapolis, IN). The anti-WT1 antibody C19 is directed against an epitope within the first 80 amino acids of WT1.

**Indirect Immunofluorescence and Apoptosis Assay**—HeP3B cells were grown on coverslips and transfected with pcDNA3.1-LacZ alone or in combination with either pcDNA3.1-WT1(+) expression vector or the empty pcDNA3.1 vector for 16 h by the calcium phosphate precipitation method (32). Each precipitate was made in triplicate. Cells were washed and incubated with fresh culture medium for another 24 h. Lysates were prepared in cell culture lysis reagent (Promega), and analysis of luciferase activity was carried out in a luminometer (Berthold) following the manufacturer’s protocol. Luciferase activities were corrected for protein content of each lysate.

**RESULTS**

**Novel WT1 Protein Isoforms of 36–38 kDa Are Derived from Translation Initiation at the Second In-frame AUG**—The WT1 gene is known to be translated from two alternative start sites (see Fig. 1). The four main protein isoforms are translated from the first AUG, giving rise to proteins of molecular masses ranging from 52 to 54 kDa. An in-frame upstream AUG can also serve as a translation initiation site (7) giving rise to WT1 proteins with a higher molecular mass of 60–62 kDa. All of these isoforms can be detected by the anti-WT1 antibody C19 on Western blots of total cell lysates, as shown in the upper panel in Fig. 2A. The hatched arrow points to the larger 60–62-kDa proteins, and the black arrow points to the four main isoforms running at 52–54 kDa. The upper panel in Fig. 2A shows that in addition to these isoforms, smaller WT1 proteins of molecular masses ranging from 36 to 38 kDa can also be identified (indicated by the gray arrow in Fig. 2). In lysates of End-2 and Epi-7 cells, late-passage p53-deficient mouse embryo fibroblasts, and mouse testes, they appear as a doublet band that most likely represents the presence or absence of the 17-amino acid splice insert. The AdBRK WT1(+) cell line contains a stable integration of the WT1 cDNA which codes for amino acids 1–429. This cDNA thus lacks the upstream CTG and both splice inserts. In lysates of this AdBRK cell line, the main WT1 protein band of 52 kDa and a smaller protein band of 36 kDa are detected by the C19 antibody on a Western blot as shown in Fig. 2A. The AdBRK WT1(+) cell line, which synthesizes WT1 proteins with both splice inserts (+/+ in Fig. 2A), contains proteins of molecular masses of approximately 54 and 38 kDa. As expected, no WT1 isoforms were detected in AdBRK cells transfected with the empty expression vector (vector in Fig. 2A). The novel 36-
containing whole cell lysates of End-2, Epi-7, AdBRK cells expressing terminus of WT1 detects the larger (hatched arrow) isoforms of WT1. Lower panel, blots probed with an anti-WT1 antibody raised against the C terminus of WT1. The self-association, transcription repression, and activation domains that have been mapped previously are indicated. Bsp indicates the restriction site for Bsp120I used to generate an expression vector with a WT1 cDNA beginning just 5' of the second ATG (WT1(--)) Bsp), which directs synthesis of shorter WT1 proteins only.

In order to investigate whether the 36–38-kDa WT1 protein forms are generated through translation initiation from the downstream, in-frame AUG (see Fig. 1), we transfected AdBRK cells with a WT1(--)-cDNA and with a WT1(--)-cDNA in which the ATG codon at position 127 had been mutated into CTC. Stable cell lines of these transfectants were established.Fig. 3 shows that a polyclonal cell line transfected with wild-type WT1 cDNA (P1 PC) contains both the main WT1 (--/--) form of 52 kDa and the smaller WT1 (--/--) form of 36 kDa, while the polyclonal and monoclonal transfectants carrying the mutant WT1 cDNA (ATG PC/MC) contain the main isoform only. The control lane (+) contains lysate of U2OS cells transiently transfected with a WT1(--/-) expression plasmid, which can direct synthesis of the 36-kDa isoform of WT1 only.

Immunofluorescence data demonstrated that after transfection the smaller isoform of WT1(--/-) is exclusively localized in the nucleus (data not shown). These data show that additional isoforms of WT1 with an approximate molecular mass of 36–38 kDa exist, which arise as a result of internal translation initiation at the second AUG of the WT1 open reading frame.

Fig. 4A shows an autoradiogram of Epi-7 cells that had been chased for different time periods prior to lysis and anti-WT1 immunoprecipitation. The relative radioligand of the main and smaller WT1 proteins was quantified and set out against the chase time, and the half-lives were determined (Fig. 4B). The main and smaller WT1 protein forms have similar half-lives, with 2 h 15 min for the main and 2 h 40 min for the smaller isoforms (mean of three experiments).

The Main and Smaller Isoforms of WT1 Form Two Distinct DNA-Protein Complexes in an Electrophoretic Mobility Shift Assay—WT1 proteins have previously been shown to bind several DNA consensus sequences in electrophoretic mobility shift assays (11, 12, 33). To characterize the DNA binding properties of the different WT1 protein forms, WT1 cDNAs were transcribed and translated in vitro and subsequently tested in electrophoretic mobility shift assays with the EGR-1 (11) and WTE oligonucleotide (12) as probes. Fig. 5A shows a Western blot of the in vitro translated WT1 proteins used in this assay. All of these WT1 proteins lack the 17-amino acid and the KTS splice inserts. The WT1-PM mutant was isolated from a patient...
with Denys–Drash syndrome (34) and, due to a premature stop at amino acid 256, lacks all four zinc fingers.

In vitro translated WT1(−/−) and the smaller isoform of WT1(−/−) (lanes Fl and Bsp in Fig. 5B) bind to the radioactive EGR-1 oligonucleotide and resolve at different positions on a nondenaturing polyacrylamide gel, probably due to their different molecular weights. The addition of a 100-fold excess of unlabeled EGR-1 probe to the reaction mixture abolishes DNA binding by both WT1 isoforms, whereas a nonspecific oligonucleotide does not influence formation of the DNA-protein complexes (lane nosp.c.).

Next, we tested for the binding of in vitro translated WT1(−/−) and the smaller isoform of WT1(−/−) to the WTE oligonucleotide. 1, 2, and 4 μl of either WT1 (lanes Fl) or the smaller WT1 isoform (lanes Bsp) were added to the reaction mixture. Fig. 5B reveals that in vitro translated WT1(−/−) and the smaller isoform of WT1(−/−) bind to the radioactive WTE oligonucleotide with approximately equal affinity. Similar to their binding pattern to the EGR-1 probe, they resolve at different positions on a polyacrylamide gel, probably due to their different molecular weights. The binding of WT1 to the WTE oligonucleotide is specific, since the WT1-DNA complexes are completely competed out by a 100-fold excess of unlabeled WTE probe (lane comp.), while a nonspecific competitor (lane nosp.c.) does not affect the WT1-WTE complex.

It is known that WT1 proteins can homodimerize (17). Furthermore, mutant WT1 proteins, which cannot bind to DNA but still dimerize with WT1, diminish transcription activation by WT1 (17). Therefore, we wanted to test whether hetrodimerization between full-length WT1 and the shorter isoform of WT1 on DNA occurs, which may ultimately lead to altered transcription regulation.

Increasing amounts of the smaller isoform were preincubated with a constant amount of full-length WT1(−/−) prior to the addition of the WTE oligonucleotide. The shorter isoform-probe complex clearly intensifies with increasing amounts of protein added, while no change in the WT1(−/−)-probe complex is detectable. This finding indicates that no hetrodimerization of full-length WT1(−/−) and the smaller isoform takes place under these conditions. This notion is further supported by the observation that in vitro co-translated full-length and smaller WT1(−/−) proteins also form two distinct complexes (Fl + Bsp in Fig. 5B). The upper complex, containing full-length WT1, is supershifted to a discrete position by the addition of an anti-WT1 antibody directed toward an epitope in the first 80 amino acids of WT1 (lane H2 in Fig. 5B), while the lower molecular weight complex remains unaffected. To investigate whether the lack of dimerization between WT1(−/−) and the shorter isoform is caused by the absence of most of the self-association domain in the shorter isoform (see Fig. 1), WT1-PM was used. This WT1 mutant lacks the zinc fingers and cannot bind to the WTE oligonucleotide by itself (lane PM in Fig. 5) but can still dimerize to WT1 in a GST pull-down assay (17). WT1-PM was preincubated together with WT1(−/−) prior to the addition of the probe. If dimerization was a prerequisite for DNA binding, excessive amounts of WT1-PM should abrogate DNA binding by WT1(−/−). However, Fig. 5B shows that preincubation of WT1(−/−) with increasing amounts of WT1-PM neither abrogates the binding of WT1(−/−) to the probe nor alters the position of the DNA-protein complex, suggesting that dimerization is not required for DNA binding by WT1.

Thus, both the full-length and the novel, shorter form of WT1(−/−) may bind DNA as monomers, and the difference in migration observed in electrophoretic mobility shift assays is due to the difference in protein weight rather than lack of dimerization of the shorter WT1 forms.

The Shorter Isoform of WT1(−/−) Has Altered Transcrip-
tion-regulatory Properties—WT1 has been shown to act as a transcription factor, which, depending on promoter context and cell type, can either activate or repress transcription of reporter constructs. To assess the transcription regulation properties of the smaller WT1 isoform and the main WT1 isoform, we constructed a luciferase reporter construct with a minimal promoter containing three WTE consensus sequences followed by a TATA box in front of the luciferase gene. U2OS cells were transfected with a re-porter construct containing the murine IGFII-P3 promoter in front of the luciferase gene and WT1 isoform. The Western blot in the lower part of Fig. 6A demonstrates that the main form of WT1 represses transcription from this promoter 3.7-fold, whereas the smaller form of WT1 does not repress transcription directed by this promoter. Similar results with both reporter constructs were obtained in the hepatoma cell line Hep3B (data not shown).

In conclusion, the smaller isoform of WT1 has a stronger transcription activation capacity compared with the main form of WT1 but does not repress transcription from the IGFII-P3 promoter. The Smaller Isoforms of WT1 Can Induce Apoptosis—It has been demonstrated previously that WT1 can induce apoptosis in a variety of cell types (21, 22, 35), including Hep3B cells (22). To measure apoptosis induction by WT1 and the smaller form of WT1, Hep3B cells were transfected with LacZ alone or in combination with WT1 or the shorter form of WT1. 5 days after transfection, the cells were fixed, stained with anti-LacZ and anti-WT1 antibodies, and analyzed by indirect immunofluorescence. The DNA was counterstained with DAPI to investigate the presence of apoptotic nuclei in LacZ-positive or LacZ- and WT1-double positive cells. Fig. 7 shows that both WT1 and the shorter WT1 isoform can induce apoptosis in Hep3B cells.
Smaller WT1 Protein Isoforms with Distinct Properties

DISCUSSION

The work presented here demonstrates the existence of novel WT1 isoforms that are generated through translation initiation from the second in-frame AUG of the WT1 mRNA. These forms were detected in mouse cell lines and testes, in human Wilms’ tumor material, and in rat cell lines constitutively expressing human WT1 cDNA constructs. These findings document that alternative translation initiation is not restricted to one species. The in-frame AUG codon at position 127 is indeed conserved in all WT1 cDNAs sequenced so far (human, pig, rat, mouse, chicken, turtle, Fugu, and Xenopus), suggesting that it may be utilized as an alternative translation initiation site in all of these species.

The conservation of the AUG throughout such a broad range of species suggests a functional importance of the smaller WT1 isoforms. One can envisage several models of distinct regulation for the shorter and full-length WT1 isoforms. Post-transcriptional modifications within the first 126 amino acids that alter the biological activity of WT1 would only affect the main form and not the shorter WT1 proteins. Similarly, changes in levels or modifications of proteins whose binding to WT1 requires the first 126 amino acids of WT1 would alter the activity of full-length WT1 but not of the shorter isoforms.

Since the half-lives of the major and shorter WT1 isoforms are approximately equal, the first 126 amino acids of WT1 do not contain a domain that controls basal protein turnover. Another level of regulation could be achieved by alterations in the ratio of full-length to shorter WT1 proteins. We were not able to detect the shorter WT1 proteins in the leukemic cell lines K562 and HL60 (data not shown), which both contain the main forms of WT1 (36), suggesting that usage of the second AUG as a translation initiation site may be cell type-dependent.

The smaller isoforms of WT1 are detected as a doublet by Western blotting. The upper band of this doublet resolves to the same height as the shorter WT1 protein present in AdBRK cells synthesizing WT1, whereas the lower band of this doublet runs to the same height as the shorter WT1 protein present in AdBRK cells synthesis WT1. Therefore, at least the 17-amino acid splice insert is either included or excluded from the shorter WT1 proteins. It is most likely that the differential splicing of the KTS insert is also conserved in the shorter isoforms, but since WT1 proteins differing only in this insert cannot be resolved on SDS-polyacrylamide gel we have no formal evidence for that. Assuming that all splicing events and the RNA editing mechanism is conserved in the smaller isoforms, this would bring the number of WT1 protein forms to 32, with numerous possibilities of selective modifications of single protein isoforms.

Internal translation initiation has also been reported for the von Hippel-Lindau gene product (38). This, together with our finding, suggests that translation initiation from an internal start site may be more common than previously recognized, offering an additional level of regulation.

The observation that the main and the shorter forms of WT1 (-/-) form two separate DNA-protein complexes suggests that WT1 binds to DNA as a monomer. This notion is supported by the finding that, although WT1-PM can bind to wild-type WT1 in an in vitro assay (17), it cannot abrogate DNA binding of WT1 (Ref. 17 and Fig. 5B). Furthermore, Moffett et al. (39) demonstrated that wild-type WT1 proteins can bind to one

FIG. 6. The smaller WT1(-/-) isoform has an increased transactivation capacity but does not repress transcription. U2OS cells were transfected, and luciferase activity was determined as described under “Materials and Methods.” A, transactivation of the 3xWTE-TATA-luciferase reporter construct by the shorter WT1(-/-) isoform (WT1(-/-) FII) is about 1.5-fold stronger than transcription activation by full-length WT1(-/-) (WT1(-/-) FII). The lower panel shows expression of the transiently transfected WT1 cDNAs on a Western blot. B, WT1(-/-) represses transcription from the murine IGFII-P3 promoter about 3.5-fold. No transcription repression activity by the shorter WT1(-/-) isoform occurs.

FIG. 7. The shorter isofrom of WT1(-/-) can induce apoptosis. 5 days after transfection, Hep3B cells were analyzed by indirect immunofluorescence for expression of LacZ and WT1 proteins, while DNA was stained with DAPI. LacZ-positive or LacZ- and WT1-double positive cells with weakly DAPI-stained, condensed nuclei were classified apoptotic. 5 days after transfection, 21% of LacZ-positive cells, 71% of full-length WT1(-/-)-positive cells, and 62% of WT1(-/-) Bsp-positive cells were apoptotic (mean of three experiments).
another in a far Western experiment, whereas an N-terminal deletion mutant of WT1 lacking amino acids 1–126 fails to bind to full-length WT1. Thus, our finding that the shorter WT1 (1–126) form, lacking the N-terminal 126 amino acids, and the main WT1 (1–148) proteins form two separate complexes in electrophoretic mobility shift assay, with similar affinities for the probe, implies that dimerization is not a prerequisite for DNA binding.

Previous studies have reported that WT1 activates transcription from synthetic promoter constructs containing minimal Egr-1 binding sites (17, 41). In line with these data, we find that both WT1 (1–126) forms tested strongly activate transcription from a similar promoter construct, the 3xWTE-TATA construct, in a reporter assay (Fig. 6A). The shorter isoform of WT1 (1–126) activates transcription from this promoter about 1.5-fold stronger than the full-length WT1 (1–148) isoform (Fig. 6B). However, we find that transcription directed from the IG-FII-P3 promoter, which is repressed by the main form of WT1 (1–148), is simply not affected (rather than activated) by the shorter isoform of WT1 (1–126) (Fig. 6B). Thus, the transcriptional repression domains of WT1 can act independently, and the promoter context seems to determine their activities. A yet unresolved question is whether transcription regulation by WT1 requires dimerization. Our results suggest that, at least for transcriptional activation, dimerization is not required. This is because although the shorter WT1 (1–126) protein lacks most of the dimerization domain it is still a very potent transcriptional activator. If this form fails to homodimerize due to the absence of the first 126 amino acids, then at least transcription activation does not require dimerization. The lack of repression capacity may either be due to a lack of the repression domain, which may repress transcription either directly or via an intermediate repressor, or loss of dimerization capacity.

Ectopic expression of WT1 is known to induce apoptosis in different cell types (21, 22, 35). Induction of apoptosis by WT1 has been shown to be accompanied by a decrease in epidermal growth factor receptor protein levels (21). Furthermore, overexpression of the epidermal growth factor receptor (21, 22) or insulin receptor (22) can partially rescue cells from apoptosis induced by WT1. We find, however, that the shorter isoforms of WT1, which cannot repress transcription directed by the IG-FII-P3 promoter, are still capable of inducing apoptosis in HepB3 cells. If the absence of the first 126 amino acids prevents WT1 from repressing any given promoter, then the transcription activation potential of the shorter WT1 (1–126) isoform must suffice for apoptosis induction in HepB3 cells. Other factors that are positively regulated by WT1 may then account for the observed decrease in epidermal growth factor receptor levels.

In conclusion, we have shown that WT1 proteins of 36–38 kDa exist that have distinct biological properties. Including these novel forms, 32 WT1 protein forms have been described, illustrating the enormous complexity of regulation of WT1 expression and activity.