Overlapping RNA and DNA binding domains of the \( wt1 \) tumor suppressor gene product

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ABSTRACT

The Wilms’ tumour suppressor gene (\( wt1 \)) is mutated in a subset of patients with Wilms’ tumour and has a critical role in urogenital development. \( wt1 \) encodes a zinc finger transcription factor which regulates expression of several genes involved in cellular proliferation and differentiation. Although a number of studies have characterized the DNA binding properties of the WT1 protein, recent evidence has suggested that WT1 may also have a role in RNA metabolism. We have used an RNA selection method to identify WT1 binding ligands from a random RNA pool. Three groups of RNA ligands specifically recognized by WT1 were identified. Mutational analysis pinpointed ribonucleotide sequences critical for binding. Analysis of truncated WT1 proteins demonstrated that three of four zinc fingers were necessary for RNA–protein interaction. The naturally occurring WT1 isoforms with insertion of lysine, threonine and serine between zinc fingers three and four were unable to bind the selected RNAs. The selected RNA ligands competed with the cognate WT1 DNA binding site for complex formation with WT1. Our findings suggest potential cellular RNA target sequences for WT1 and provide tools for studying the structural and functional properties of this tumour suppressor protein.

INTRODUCTION

Wilms’ tumour (WT) is a malignancy which affects 1 in 10 000 children, usually before the age of 5 years (1). It is thought to arise when multipotential cells of the metanephric blastema fail to differentiate and remain locked in a state of continual proliferation. WT has long been considered an excellent model for studying the relationship of cancer to development. The tumours derive from mesenchymal stem cells which would normally differentiate into epithelial components of the nephron. These tumours are remarkable in attempting to recapitulate the different stages of nephron development, albeit abnormally. A tumour suppressor gene, \( wt1 \), implicated in predisposition to WT has been identified by positional cloning at chromosome 11p13 (2,3). \( wt1 \) has been extensively characterized and is mutated in 10–15% of sporadic WT cases (4,5) and in some hereditary WT cases (6). Genetic evidence suggests that \( wt1 \) mutations are involved in initiation of this disease (7). \( wt1 \) is also thought to play a functional role in the inductive processes of urogenital development (reviewed in 8). Consistent with this hypothesis is the observation that mice in which the \( wt1 \) gene is homozygously deleted fail to develop kidneys and gonads (9). Also, germline \( wt1 \) lesions in humans are associated with a predisposition to WT and aberrant differentiation of the urogenital system (6,10).

The \( wt1 \) gene encodes a protein having many characteristics of a transcription factor, including a glutamine/proline-rich N-terminus, nuclear localization and four Cys2-His2 zinc finger motifs (reviewed in 8). The three C-terminal-most zinc fingers share 64% identity to the three zinc fingers of early growth response gene 1 (EGR-1). The mRNA contains two alternative sites of translation initiation (11), two alternatively spliced exons (12,13) and undergoes RNA editing (14), thus potentially encoding 16 different protein isoforms with predicted molecular masses of 52–65 kDa. The function of the alternative translation initiation event, the RNA editing and the first alternative splicing event (exon V) have not been well defined, although exon V can repress transcription when fused to a heterologous DNA binding domain (15). Alternative splicing of exon IX inserts or removes three amino acids (+/–KTS) between zinc fingers III and IV and changes the DNA binding specificity of WT1 (16). The WT1(–KTS) isoforms can bind to two DNA motifs: (i) a GC-rich motif, 5′-GG/GTGGCGGC/C-3′, similar to the EGR-1 binding site (16); (ii) a 5′-TCC-3′-containing sequence (17). The DNA binding properties of the WT1(+KTS) isoforms are not well understood, since no high affinity specific binding site has been elucidated for these splice variants. A number of genes involved in growth regulation and cellular differentiation contain WT1 binding sites within their promoters and their expression can be modulated by WT1 in transfection assays (reviewed in 8). The \( wt1 \) gene product mediates both transcriptional repression and activation, depending on the architecture of the promoter under study and the cell line in which the assays are performed (18).

Recently Larsson et al. (19) demonstrated that different WT1 isoforms localize to distinct compartments of the nucleus, with –KTS isoforms displaying a distribution that parallels that of classical transcription factors such as Sp1 and TFIIIB, whereas +KTS isoforms are preferentially associated with interchromatin granules and coiled bodies. These results were reproduced and extended by Englert et al. (20). Caricasole et al. (21) have shown that the subnuclear localization of WT1(+KTS) is RNase, but not...
MATERIALS AND METHODS

Plasmid construction

Preparation of plasmid DNA, restriction enzyme digestion, agarose gel electrophoresis of DNA, DNA ligation and bacterial transformations were carried out using standard methods (23). Subclones of DNA PCR amplifications were sequenced by the chain termination method (24) using double-stranded DNA templates to ensure the absence of unwanted secondary mutations.

To generate pET15B/WT(+−) the 1.5 kb Sau3A1 fragment from the full-length mouse WT1 cDNA (25) was cloned into the BamHI site of pET15B (Novagen). The sequences coding for the C-terminus (codons 297–449) were replaced by a synthetic gene derived by cloning the 469 bp XmnI–BlpI portion of the gene, followed by Klenow repair to create blunt ends and re-ligation of the vector. The plasmid construction

Protein purification

For expression of recombinant proteins pET15 expression vectors were introduced into the BL21(DE3)pLysS strain of Escherichia coli and proteins were induced using the recommended conditions (Novagen). Proteins were purified using nickel chelate affinity chromatography (Qiagen) under native conditions as recommended by the manufacturer. Eluted proteins were dialysed against a buffer containing 20 mM HEPES, pH 7.5, 70 mM KCl, 12% glycerol, 0.05% NP-40, 100 µM ZnSO4 and 0.5 mM DTT. The purity and integrity of the fusion proteins was assessed by Coomassie blue staining of SDS–polyacrylamide gels (see for example Fig. 1B).

Identification of RNA binding sites by systematic evolution of ligands by exponential enrichment (SELEX)

For generation of a random DNA template PCR amplification was performed using 5 µg N20 [5′-TGGCGATATTTATATACAC(N)N20AATGTCATGCTGTC-3′], an oligonucleotide consisting of 20 degenerate nucleotides flanked by T7 and Rev primer binding sites, and 10 µg each of T7 (5′-CCGGGATCC-TATACGACCTACTAAGGGCCACCAAGGACATF-3′) and Rev (5′-CCCGGACACCGGCATGCTGACCCGCTTTGTTAA-TCAAC-3′) oligonucleotides as described (27). Half of the amplified product was transcribed with T7 RNA polymerase (NEB) in the presence of [α-32P]CTP (3000 Ci/mmol) to yield ~5 µg radio-labelled random RNA sequences. The RNA was gel purified on an 8% sequencing gel, eluted into 0.5 M ammonium acetate and ethanol precipitated. For selection the RNA was resuspended in SELEX buffer (20 mM HEPES, pH 7.5, 70 mM KCl, 2 mM MgCl2, 12.5% glycerol, 0.5 mM DTT, 50 µM ZnSO4, 0.05% NP-40), heated to 65°C and slowly cooled to room temperature. The RNA was added to a binding reaction with 10 µg WTZF[(I–IV)–KTS] fusion protein in 100 µl SELEX buffer. Binding was allowed to proceed for 30 min at 4°C and the mixture was then filtered through a nitrocellulose filter (Millipore HAWP 02500) which had been pre-washed with 50 mM Tris–HCl, pH 7.5. Filters were rinsed with 50 mM Tris–HCl, pH 7.5, and the RNA eluted and precipitated as described (28). The selected RNA was reverse transcribed at 42°C for 1 h using 100 ng Rev oligonucleotide with Superscript II reverse transcriptase (Gibco BRL) as recommended by the supplier. Subsequent cycles of PCR and in vitro transcription were performed as described (27). The amount of WTZF[(I–IV)–KTS] protein used for RNA selection was reduced by ~50% every second cycle. The final round of selection involved incubating 1 µg protein and 1 µg RNA in SELEX buffer plus 5 µg calf liver tRNA on ice for 30 min. The RNA/protein mixture was electrophoresed on a 4% polyacrylamide, 0.5x TBE gel at 140 V at 4°C. The wet gel was exposed to Kodak X-Omat film and the RNA–protein complex excised from the gel by alignment with the developed film. The RNA in the complex was eluted from the gel and precipitated as described above. Following reverse transcription and PCR the SELEX products were digested with BamHI, converted to blunt ends with Klenow polymerase and cloned into pcUC18 linearized with Smal. The SELEX clones were analysed by sequencing miniprep DNA. To generate RNA for EMSA PCR products amplified directly from the bacterial colonies were used to generate templates for RNA synthesis.

Electrophoretic mobility shift assay (EMSA)

EMSA’s were performed with 50 ng recombinant protein unless otherwise indicated. The binding reactions were performed in a total of 15 µl in binding buffer [20 mM HEPES–KOH, pH 7.5, 50 mM KCl, 5 mM MgCl2, 10 µM ZnSO4, 0.67 mM DTT, 12% glycerol, 10 µg BSA, 1 µg poly(dI·dC), 1 µg calf liver tRNA]. Binding reactions were pre-incubated for 15 min at 25°C in the absence of RNA probe. Radiolabelled RNA (20 fmol) was heated to 65°C, slowly cooled to room temperature, then added to the binding solution. Incubation of the RNA/protein mixture was for
Figure 1. (A) Sequence of the synthetic gene encoding the WT1 zinc finger region. Numbers to the right refer to the amino acid residues in the context of the full-length murine protein as in Buckler et al. (25). Note the reporting of a murine WT1 sequence coding for QL at amino acids 448–449 instead of HV (61). Underlined nucleotides correspond to a naturally occurring splice variant (+KTS) and are present in pET15B/WT (+KTS) but not in pET15B/WT (–KTS). Arg394 (CGT) is changed to Trp in construct pET15B/WT (+/–) 394 R/W. The cytosine nucleotide in bold is missing in the truncated construct WT (del), resulting in a frameshift and early termination of translation. (B) Analysis of purified recombinant WT1 proteins. Nickel chelate affinity-purified recombinant proteins (2 µg) were resolved on a 12.5% SDS–PAGE gel and stained with Coomassie blue. WTZF[(I–III)–KTS] migrates slightly slower than expected due to the presence of an extra 22 amino acids contributed by pET15B vector sequences. The sizes of the molecular mass standards (New England Biolabs) are to the left of the figure.

20 min at 25°C. For supershift experiments 200 ng antibody were subsequently added and incubation was continued for a further 15 min at 25°C. For competition experiments competitor RNA was labelled with 3H-labelled RNA or unlabelled DNA oligonucleotide was pre-incubated with the recombinant protein for 15 min before addition of the probe. Protein–RNA complexes were resolved on 0.5 × TBE, 4% polyacrylamide gels electrophoresed at 140 V at 4°C. Dried gels were exposed overnight to X-Omat film (Kodak) at –70°C overnight with an intensifying screen. PhosphoImager scanning (Fuji) was used to quantitate the efficiency of complex formation.

RESULTS

Several members of the Cys2-His2 zinc finger family are known to interact with both DNA and RNA (see Discussion). There is also suggestive evidence that the WT1(+KTS) isoforms may be involved in RNA metabolism (see Introduction). In vitro studies have also shown that the WT1(–KTS) isoforms can bind to sequences in exon 2 of Igf-2 mRNA (21). We have examined the RNA binding properties of WT1 using affinity elution-based RNA selection to identify specific WT1 binding RNA ligands (29). We employed systematic evolution of ligands by exponential enrichment (SELEX) with a recombinant WT1 protein encoding zinc fingers I–IV and a (His)6 tag at the N-terminus, WTZF[(I–IV)–KTS]. In order to maximize expression levels of recombinant WT1 protein we generated a synthetic gene fragment harbouring favourable codon bias for expression in E.coli (Fig. 1A). The bacterially expressed protein was purified using nickel chelate chromatography and assessed to be >90% homogeneous by SDS–PAGE (see Fig. 1B, lane 7). RNA was transcribed from a pool of PCR-amplified oligonucleotides composed of 20mer random nucleotides with an estimated complexity of 1014 molecules. RNA binding to WT1(–KTS) was enriched using 10 rounds of nitrocellulose filter binding/reverse transcription-PCR amplification, followed by one round of selection by EMSA. EMSA analysis comparing the RNA pools at the second and eighth cycles of SELEX revealed a clear enrichment for WT1 binding RNA sequences (Fig. 2A, compare lane 4 with 2). Following cloning of the SELEX products individual clones were analysed by EMSA. All clones analysed showed specific binding when incubated with 50 ng protein (Fig. 2B and data not shown), whereas RNA generated from a clone of the random pool of unselected PCR products showed no complex formation (data not shown). Sequence analysis of 31 clones revealed that they could be classified into three distinct families, based on nucleotide conservation at specific residues (Fig. 2C).

The largest group, with 18 clones (family A), is characterized by high sequence conservation within the first nine nucleotide positions, with identity at five of these positions (Fig. 2C). Family B contains three members and is characterized by the core motif 5′-GAAUG-3′. Family C contains 10 members and is characterized by the core consensus 5′-CCC(A/G)-3′ (Fig. 2C). Computer analysis using the Zuker RNA folding algorithm (30) failed to predict conserved stable secondary structures for these RNAs...
Figure 2. Selection of WT1 RNA ligands from a random RNA library. (A) WT1-bound sequences enriched in cycles two and eight were transcribed in vitro into RNA and used in EMSAs with WTZF[(I–IV)–KTS] protein. Protein–RNA complexes are indicated by an arrow and free probe by an arrowhead. The presence or absence of WTZF[(I–IV)–KTS] is indicated above the panel and the cycle number indicated below the panel. (B) EMSAs used to screen clones obtained from the eleventh round of SELEX. Inserts from individual clones were amplified by PCR and transcribed in vitro using the oligonucleotides employed in SELEX. Following gel purification, RNA species were used in EMSA with 50 ng recombinant WTZF[(I–IV)–KTS] and resolved on an 8% polyacrylamide gel. The RNA species used in the EMSA is indicated above the panel. (C) Nucleotide sequences of WT1 selected RNAs. Sequences of 31 PCR clones encoding RNA ligands cloned after SELEX are shown. Conserved identical nucleotides are shown in bold and nucleotide positions showing strong sequence conservation are underlined. Sequences derived from the non-random region of the RNA ligand (primer binding sites) are in lower case. A consensus sequence is shown below each class of RNA ligands. (D) WT1 binding to an RNA selection ligand (clone 22). Increasing amounts of recombinant WTZF[(I–IV)–KTS] protein (0.26–8.32 µM concentration) were mixed with a constant amount of RNA ligand (1.3 nM). The amount of bound and free probe was quantitated after separation by gel electrophoresis using PhosphorImager analysis. The result of one representative experiment for RNA ligand 22 is shown. (N.Bardeesy, unpublished data). To characterize the WT1–RNA interaction we quantitated binding of RNA species 22 to WTZF[(I–IV)–KTS]. In an EMSA WTZF[(I–IV)–KTS] demonstrated saturable binding to RNA 22 ($K_d \sim 0.7 \mu M$) (Fig. 2D). This dissociation constant is slightly higher than that reported for bacterially produced recombinant WTZF[(I–IV)–KTS] to DNA target sites ($K_d \sim 0.14 \mu M$) (31).

To establish the specificity of the WT1–RNA interaction a number of competition experiments were performed. Competition with RNA ligands from the three families indicated that all were capable of significantly reducing complex formation of RNA ligand 22 when present at a 50-fold molar excess (Fig. 3A, compare lanes 3–5 with 2). An unrelated RNA sequence did not compete for WT1 RNA binding when present at 50-fold molar excess (compare lane 6 with 2). Complex formation by probes from the other two RNA families (i.e. RNA ligands 38 and 20) were also inhibited by these same specific competitor RNA ligands (data not shown). To determine if DNA and RNA binding to WT1 were mutually exclusive we incubated a radio labelled double-stranded DNA oligonucleotide probe containing the cognate WT1 binding site with WTZF[(I–IV)–KTS] in the presence of 50-fold molar excess of a member from each RNA ligand family. The RNA ligands significantly reduced WT1–DNA complex formation (Fig. 3B, compare lanes 3–5 with 2). A random RNA sequence did not compete for WT1 binding to DNA when present at 50-fold molar excess (N.Bardeesy, data not shown). Additionally, WT1–RNA complexes can be inhibited by pre-incubation of WT1 with DNA oligonucleotides containing a WT1 DNA binding site (Fig. 3C, compare lane 5 with 2), whereas an ETS binding site, which is not recognized by WT1, shows no effect on WT1–RNA complex formation (compare lane 6 with 2). We conclude that WT1 zinc fingers can bind to DNA and RNA, but binding to both is mutually exclusive.
Supershift experiments of the protein–RNA complexes revealed a slow migrating complex in the presence of C19, an anti-WT1 antibody directed against the C-terminal domain of the protein (Fig. 3C, compare lane 7 with 2 and 11 with 10). The specificity of this complex was demonstrated by the use of a control antibody, WT180, which recognizes an N-terminal epitope of full-length WT1 protein, absent in WTZF[(I–IV)–KTS]. WT180 was unable to supershift the WTZF[(I–IV)–KTS]–RNA complex (compare lane 8 with 12). The supershifted complex was specific for the WTZF[(I–IV)–KTS]–RNA complex, since incubation of the C19 antibody did not show cross-reactivity with the RNA (compare lane 12 with 11). The observed WT1–RNA interactions in the absence of WT1 protein. Binding reactions were electrophoresed on a 4% non-denaturing polyacrylamide gel and visualized by autoradiography. The region of the gel containing WT1 supershifted complexes is delineated to the right. The positions of complexes are indicated to the left.

To probe the structural requirements for WT1–RNA interaction the binding capacity of a series of WT1 proteins was studied using SDS–PAGE analysis of these purified recombinant proteins with related zinc fingers. Likewise, we have found that bacterially purified EGR-1 can also bind to RNA 22 (compare lane 9 with 2). Full-length recombinant WT1(+/–) was also able to form specific complexes with this RNA (lane 6). An Arg394→Trp mutation of WT1 represents the most commonly detected mutation in Denys–Drash syndrome, a wt1-associated developmental disorder. This protein is unable to bind the cognate WT1 DNA binding site (10). This mutation in the context of the full-length recombinant WT1(+/–) protein showed a 10-fold reduction in RNA binding (compare lane 7 with 6). This effect, although strong, is less than the complete loss of DNA binding observed for this mutant. A WT1 mutant containing an intact N-terminus but lacking part of zinc finger II and all of zinc fingers III and IV cannot bind RNA efficiently (compare lane 8 with 2). We have found that bacterially purified EGR-1 can also bind to RNA 22 (compare lane 9 with 2). This suggests that, like the EGR-1 DNA recognition motif, RNA 22 might be recognized by a number of proteins with related zinc fingers. Likewise, we have found that the other two families of ligands have similar protein structural requirements for binding to WT1 (N.Bardeesy, data not shown). Taken together these data demonstrate that residues within WT1 zinc fingers II–IV are sufficient and necessary for RNA binding and that the DNA and RNA sites overlap. To identify primary nucleotide sequence requirements for binding mutagenesis was performed using clones 22 and 38. A series of RNA probes with mutations of the highly conserved nucleotides as well as flanking sequences was employed in EMSA (Table 1). All mutations tested in the context of RNA 22 abolished recognition by WT1, indicating that the RNA recognition
Table 1. Mutational analysis of RNA ligands

<table>
<thead>
<tr>
<th>RNA sequence</th>
<th>binding</th>
<th>RNA sequence</th>
<th>binding</th>
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<tbody>
<tr>
<td>#22 Gm(AGGG)GmACCmGmGC</td>
<td>+</td>
<td>#38 Am(AGGC)GmACCmGmGC</td>
<td>+</td>
</tr>
<tr>
<td>M1 GmUUGGGmGmGACCmGmGC</td>
<td>–</td>
<td>M1 AmUUGGGmGmGACCmGmGC</td>
<td>–</td>
</tr>
<tr>
<td>M2 GmAUUGGGmGmGACCmGmGC</td>
<td>–</td>
<td>M2 AmAUUGGGmGmGACCmGmGC</td>
<td>–/–</td>
</tr>
<tr>
<td>M3 GmAUUGGGmGmGACCmGmGC</td>
<td>–</td>
<td>M3 AmAUUGGGmGmGACCmGmGC</td>
<td>–</td>
</tr>
<tr>
<td>M4 GmAUUGGGmGmGACCmGmGC</td>
<td>–</td>
<td>M4 AmAUUGGGmGmGACCmGmGC</td>
<td>–</td>
</tr>
<tr>
<td>M5 GmAUUGGGmGmGACCmGmGC</td>
<td>–</td>
<td>M5 AmAUUGGGmGmGACCmGmGC</td>
<td>–</td>
</tr>
<tr>
<td>M6 GmAUUGGGmGmGACCmGmGC</td>
<td>–</td>
<td>M6 AmAUUGGGmGmGACCmGmGC</td>
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Summary of nucleotide requirements for WT1 recognition of RNA ligands. The table shows RNA sequences used in EMSA with WTZF/(I–IV)–KTS. Clones 22 and 38 were chosen as representatives of RNA families A and C respectively. Nucleotides conserved between all members of the family are in bold. Other strongly conserved positions are underlined. Altered positions in each mutant RNA are indicated in bold. The RNA sequences are flanked by sequences derived from T7 and Rev oligonucleotides (see Materials and Methods). The + and – symbols indicate whether or not the individual RNA probes formed complexes with WT1 protein. Weak binding (<10% of input RNA species present in the complex) is indicated by +/-.

Table 2. Gene containing consensus sequences from family A SELEX ligands

<table>
<thead>
<tr>
<th>Ligand #11</th>
<th>GAAAUUGGAGG</th>
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<tr>
<td>Integrin α6 (Human)</td>
<td>AAGCAAAUUGGAGG</td>
</tr>
<tr>
<td>AP-2β (Human)</td>
<td>CUGGAAAUUGGAGG</td>
</tr>
<tr>
<td>B-raf oncogene (Human)</td>
<td>AAGGGAUUUGGAGG</td>
</tr>
<tr>
<td>p107 (Human)</td>
<td>AUUGGGAUUGGAGG</td>
</tr>
<tr>
<td>PDGF Receptor (Mouse)</td>
<td>AATGCUUGGAGG</td>
</tr>
<tr>
<td>CHED (cdc2-related protein kinase)</td>
<td>GCUUUGGAGA</td>
</tr>
</tbody>
</table>

Blast searches were performed using the first 11 nt from each of the ligands from family A. The sequences used for searching are displayed in bold. Selected genes with sequence matches are displayed. The numbers to the right of the sequences refer to the relative position of the nucleotides as entered in the GenBank and EMBL databases. The region of the gene where the sequence is located is indicated in parentheses at the far right. Accession numbers: integrin-α6, emb-X59512; AP-2β, emb-Y09912; human p107, gb-L14812; mouse p107, gb-U27177; PDGFα, gb-M84607; B-raf, gb-M21001; CHED, gb-M80629.

site extends over the region which characterizes this family. With respect to RNA 38 all mutant products, with the exception of that derived from M2, abolished RNA recognition. The mutation harboured by M2 showed weak but detectable binding to WT1. These results establish specific sequence requirements for WT1 recognition of the RNA ligands.

We performed BLAST searches using the SELEX ligands to identify naturally occurring RNAs which could potentially be in vivo targets for WT1(–KTS). For query sequences in the searches we employed the first 11 nt of the family A ligands, since these showed the largest regions of homology. Table 2 lists a number of genes identified from databases which match RNA ligands and which may have relevance to WT1 function. It remains to be established whether WT1 can bind to any of these potential targets in vivo.

DISCUSSION

Zinc finger domains, found in a large family of proteins, were originally characterized for their ability to bind DNA in a sequence-specific manner. The Cys2-His2 subfamily of zinc finger proteins has been extensively studied. This small independently folding motif has regularly spaced cysteine and histidine residues which coordinate one zinc ion (32). X-ray crystallographic and NMR studies have shown that these domains are composed of a β-hairpin followed by an α-helix (33,34). Structural studies of the
zinc finger domains of Zif268 bound to a cognate DNA binding site have demonstrated that sequence-specific binding is acquired through direct nucleotide contact by three amino acids in the α-helical region (34). These studies have led to proposals of a general code for sequence-specific recognition of DNA by zinc fingers (35–37). However, it is now clear that rather than simply being DNA binding modules these domains may behave in a more complex multifunctional manner. The Xenopus laevis TFIIIA protein contains nine zinc fingers of the Cys2-His2 class which mediate specific binding to both the SS rRNA gene (38) and SS rRNA (39), implying a role in both gene transcription and RNA metabolism. The human and mouse MOK2 proteins have 10 and seven Cys2-His2 zinc fingers respectively and show a similar ability to bind specifically to both DNA and RNA (40). In addition to nucleic acid binding, Cys2-His2 zinc fingers can mediate protein–protein interactions. The zinc fingers of GATA-1 specifically interact with the Cys2-His2 zinc fingers of both Sp1 and EKLF (erythroid Krüppel-like factor) (41). The Sp1 zinc fingers also interact with the N-terminal DNA binding domain of RelA(p65) (42) and the Cys2-His2 zinc fingers of YY1 (43). Each of these interactions leads to cooperative DNA binding. The zinc finger can also harbour a nuclear localization signal (44). Another interesting feature of some Cys2-His2 zinc finger proteins is the ability to bind to DNA–RNA hybrids with affinities comparable with or higher than for DNA duplexes (45). Although the significance of this interaction is unknown, it is a further indication of the multifunctional nature of zinc finger domains.

The functional diversity of Cys2-His2 zinc fingers is well exemplified by WT1. The WT1(–KTS) isoform recognizes DNA specifically, regulating transcription through binding to the EGR-1 consensus binding site. However, the WT1 zinc finger domains have been found to mediate a number of other functions. The par-4 transcriptional repressor binds to this domain and modulates the transcriptional properties of WT1 (46). Similarly, WT1 and the p53 tumor suppressor protein influence each others transcriptional properties through an interaction mapping to the first two zinc fingers of WT1 (47). The WT1 zinc finger region also contains nuclear localization signals (20,48). The zinc finger region of WT1 has also been reported to mediate binding to sequences in exon 2 of Igf-2 mRNA (21). This observation, together with data showing association of WT1( +KTS) with components of the RNA splicing machinery (19), has led to the suggestion that WT1 may be involved in RNA metabolism and may indeed be an RNA binding protein.

In this study we used the SELEX methodology to identify three unrelated families of RNA ligands which show specific binding to WT1(–KTS) with affinities comparable with that seen for binding of this protein to DNA (see Fig. 1D and 31). The structural requirements of WT1 are similar for binding to the different families of RNA ligands and they also share features in common with WT1(–KTS) recognition of DNA. The zinc fingers critical for binding are the same, i.e. the first zinc finger is not required while zinc fingers II–IV are indispensable. Additionally, the WT1( +KTS) isoform and the WT1(+/-)350R-W mutant show reduced ability to bind to both DNA and RNA. These data, along with the observation that DNA and RNA can compete with each other for binding to WT1(–KTS), demonstrate that they likely interact with the same or overlapping regions. Due to the similarity of structural features involved in RNA and DNA binding we addressed whether WT1 can bind to the SELEX sequences when they are present in a double-stranded DNA oligonucleotide, rather than as single-strand RNA sequences. No binding to WT1 was observed when representative SELEX clones were used as DNA probes in gel shift assays (data not shown).

Inspection of ligands from family A reveals conserved features in addition to the consensus sequence. All clones have: (i) the consensus sequence present at the same position relative to the 5′-end; (ii) except for clones 25 and 44, stretches of at least three cytosines in the 3′-region (Fig. 2C). These features suggest that recognition of family A ligands by WT1 may involve additional sequence information than simply the 9 nt consensus sequence. Alternatively, we cannot exclude the possibility that these ligands evolved from a common ancestor. The consensus sequence elements in clones from families B and C vary in their relative positions (Fig. 2C), suggesting independent evolution of the individual clones and attesting to a strong selection for the consensus sequence in the competitive binding of RNA to WT1(–KTS). Comparison of the sequences of the different families of ligands does not reveal any obvious common elements, thus we cannot currently define general parameters for recognition of RNA by WT1(–KTS). It is not unusual for an RNA SELEX procedure to yield sets of unrelated high affinity ligands (28,49). Although computer modelling did not demonstrate stable secondary structures for the RNA ligands we cannot rule out that such structures may be a component of WT1 binding to these ligands. It should be noted that TFIIIA binds DNA in a primary sequence-specific manner (50,51) but binds RNA based on conserved secondary/tertiary structure, independent of the primary RNA sequence (52).

As mentioned above, it has been shown that the WT1 zinc fingers can bind to sequences in exon 2 of Igf-2 mRNA (20). The putative site of interaction contains the same sequence as that of the cognate WT1 DNA binding site (the EGR-1 site). DNA binding by WT1, however, involves recognition of the guanine-rich DNA strand (16), whereas the observed RNA binding is to the complementary cytosine-rich strand of Igf-2 mRNA (21). We have used an RNA probe from a circumscribed region of Igf-2 spanning the EGR-1 site postulated to interact with WT1 proteins, but failed to detect a specific RNA–protein complex (N. Bardeesy, data not shown). Thus the parameters of the WT1–Igf-2 mRNA interaction remain unclear at this time. The data on the RNA ligands we present here, in contrast, employ competition and mutational studies to describe highly specific binding. A 50-fold excess of specific competitor is able to eliminate the interaction, while a non-specific competitor had no effect and single nucleotide substitutions completely eliminated binding.

It is possible to use SELEX products to generate ligands with higher affinity for WT1. The RNA pool from the last cycle can be subjected to selection under conditions of high stringency, such as increased salt or detergent concentrations. Alternatively, a SELEX clone could be mutated randomly. The resulting RNA pool could then be used in further rounds of selection for WT1 binding. Such a method has been successfully employed to obtain very high affinity ligands for HIV-1 Rev (53).

The RNA ligands we have identified represent potential reagents to disrupt certain aspects of WT1 function for studies of this protein in tissue culture cells. Since the ligands can compete with DNA as targets for WT1 binding they may be able to inhibit the ability of WT1 to regulate its downstream targets while leaving other functions of WT1 intact. In addition, they represent isoform-specific reagents, since they would not be expected to hinder WT1(+KTS) function.
In desmoplastic small round cell tumours (DSRCT), a malignant abdominal neoplasm, a chromosomal translocation leads to generation of a chimeric protein fusing the N-terminal region of the Ewing sarcoma protein (EWS) to zinc fingers II–IV of WT1 (54). One of the resulting proteins, EWS–WT1(–KTS), is capable of acting as a dominant oncprotein (55). EWS–WT1(–KTS) is a strong transactivator of gene expression through the behaviour of the EWS moiety as a transcriptional activation domain (55,56). Since the RNA ligands we have identified interact with zinc fingers II–IV of WT1 they likely also bind to EWS–WT1. The identification of RNA ligands which inhibit EWS–WT1 DNA binding may provide clues for the design of drugs which mimic the activity of these ligands and could potentially interfere with the activity of this oncprotein.

It remains unclear whether WT1 isoforms are involved in post-transcriptional RNA metabolism in vivo. Given that our study does not address the biological significance of WT1–RNA interaction, we recommend a cautious interpretation. WT1 was initially thought to be exclusively localized to the nucleus, although recently a cytoplasmic role for WT1 has been postulated based on immunohistochemical data showing that cells treated with protein kinase A activators demonstrate cytoplasmic as well as nuclear staining (57). One therefore cannot rule out a possible role of WT1 in mRNA translation. The immunohistochemical data of Larsson et al. (19), showing that WT1(–KTS) isoforms co-localize with splicing factors, suggest an involvement of this isoform in nuclear RNA metabolism, possibly splicing. Structural modelling techniques (22) have suggested that a portion of the N-terminal region of WT1, spanning amino acids 20–110, may have an RNA recognition motif (RRM) based on predicted three-dimensional structural homology. The N-terminal region of WT1, spanning amino acids 20–110, may have RNA binding capabilities and thus be involved in transcriptional as well as post-transcriptional processes. The ETS-like DNA binding domain of Spi-1/PU.1 binds to DNA in a sequence-specific manner of DNA binding domains may also possess bifunctional DNA and RNA binding may provide clues for the design of drugs which mimic the activity of these ligands and could potentially interfere with the activity of this oncprotein.

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