Contribution of Individual Amino Acids to the RNA Binding Activity of the Wilms' Tumor Suppressor Protein WT1[†]

Tristen C. Weiss and Paul J. Romaniuk*

Department of Biochemistry and Microbiology, University of Victoria, P.O. Box 3055, Victoria, British Columbia V8W 3P6, Canada

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ABSTRACT: In addition to binding to DNA, the zinc finger protein WT1 can also bind specifically to RNA. To determine the role of individual zinc fingers of the protein in this RNA binding activity, deletion and substitution mutants of the WT1 zinc finger domain were constructed. The effects of the various mutations on the binding of WT1 to the RNA aptamers RNA22 and RNA38 were determined using a quantitative equilibrium binding assay. The results indicate that zinc fingers 2 and 3 of WT1 are essential for the binding of the protein to the RNA aptamers. For both of these fingers, the arginine residue immediately preceding the α-helix makes a significant contribution to RNA binding. For zinc finger 2, a second arginine residue within the α-helix is also critical for RNA binding, while several α-helical residues in zinc finger 3 contribute to the overall affinity of WT1 for RNA. Investigating the effects of the same point mutations on DNA binding indicates that there are similarities and differences in the contributions of zinc fingers 2 and 3 to the DNA and RNA binding activities of WT1.

Zinc finger proteins carry out diverse functions in the cell, generally involving specific interactions with DNA, RNA, or protein ligands. Some members of this class of proteins are multifunctional, which requires that the zinc finger domains in these proteins interact with more than one class of ligand. For example, the Wilms' tumor suppressor protein WT1 acts as a transcriptional regulator by binding to specific sites in DNA via a domain with four zinc fingers (*1, 2*). It is also believed to be involved in downstream transcript processing or regulation by virtue of a putative RNA binding activity located in the zinc finger domain and the presence of the protein in poly(A)$^+$ RNPs (*3, 4*). Further complexity in the function of the WT1 zinc finger domain has been implied by the observation that this domain interacts with a number of protein partners (*5*−*7*). The function of the zinc finger domain of WT1 is controlled to some extent by an alternative splicing event that produces two isoforms of the domain, distinguished by the presence (+KTS) or absence (−KTS) of an additional three amino acids in the spacer between zinc fingers 3 and 4 (*8*). The −KTS isoform colocalizes in the nucleus with regions of active transcription and is capable of binding to DNA, RNA, and protein (*9*). The +KTS isoform is capable of binding to RNA and proteins and colocalizes with splicing machinery in nuclear speckles (*9*).

The interaction of the WT1(−KTS) zinc finger domain with consensus DNA sequences has been extensively studied by biochemical approaches (*10*−*17*), culminating with the solution of a three-dimensional structure for the complex formed between the WT1 zinc finger domain and a double-stranded DNA (*18*). The potential interaction of WT1 with RNA, and the biological significance of such an interaction, has not been completely elucidated. WT1 has been reported to interact with an intronic region of the IGF-II mRNA (*19*) and with the 5′ leader region of the actinin-1 mRNA (*20*). A more defined system for studying the RNA binding activity of WT1 was developed by Pelletier and colleagues. A series of WT1-specific RNA aptamers were isolated from a random library, and the binding of these aptamers to WT1 was characterized (*10*). Further biochemical investigations demonstrated that the WT1 zinc finger domain derived from both −KTS and +KTS isoforms bound to several of the aptamers with high affinity and specificity, and the binding to the RNA22 aptamer required specific sequence and structural features of the RNA (*21*).

Whereas the role of each zinc finger in the interaction of WT1 with DNA is well characterized, this remains an unresolved question about the RNA binding activity of WT1. Deletion of either the N-terminal or C-terminal zinc finger greatly diminishes the affinity of the WT1 zinc finger domain for the RNA aptamers and the two putative *in vivo* RNA targets, leading to the conclusion that an intact zinc finger domain is required for RNA binding (*10, 19, 20*). In order to understand this phenomenon further, we have created a series of deletion, finger swap, and point mutations in the zinc finger domain of WT1(−KTS) to ascertain the role of each of the zinc fingers in binding to the WT1-specific RNA aptamers. The effects of these mutations on RNA and DNA binding have been determined quantitatively using an equilibrium binding assay. The results indicate that there is an overlap in the roles of certain amino acids in binding to RNA and DNA.

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* Corresponding author. Telephone: (250) 721-7088. Fax: (250) 721-8855. E-mail: pjr@uvic.ca.

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MATERIALS AND METHODS

Bacterial Strains and Plasmid Vectors. The plasmid vector pET30a was used to express recombinant wild-type and mutant WT1(−KTS) proteins in *Escherichia coli* strain BL21(DE3) using methods that have been described elsewhere (*14*, *15*, *21*).

Construction of Mutant WT1(−KTS) Expression Vectors. All mutants used in this study were constructed by the polymerase chain reaction (PCR). Amino- and carboxy-terminal truncation mutants of the zinc finger domain of WT1(−KTS) were constructed using upstream primers containing a recognition sequence for the restriction enzyme *Nco*I and downstream primers containing a recognition sequence for the restriction enzyme *Eco*RI. PCR products were digested with *Nco*I and *Eco*RI and ligated into plasmid pET30a that had been digested with the same restriction enzymes. Ligation reactions were used to transform *E. coli* strain DH5α, and putative constructs were identified by colony PCR using primers specific to pET30a sequences flanking the multiple cloning site. Plasmids were isolated from appropriate colonies, and the correctness of the plasmid constructs was confirmed by DNA sequencing before protein expression and purification.

Substitution mutants of WT1(−KTS) zinc finger peptides were constructed by overlap extension PCR (*22*). For the finger swap mutants, the region of the *Xenopus* p43 cDNA encoding zinc fingers 6−9 was used as a donor of zinc finger encoding cassettes, and primers were designed that contained base sequences that encoded the junction between the donor p43 finger and the acceptor WT1 finger. Figure 1A shows a comparison of the sequence of the WT1 zinc fingers compared to the last four zinc fingers of p43, and Figure 1B shows the deletion and finger swap mutations that were constructed. Alanine point mutants were created with primers encoding the desired codon change. Final PCR products were digested with *Nco*I and *Eco*RI and then ligated into pET30a as described above.

Expression and Purification of Recombinant Wild-Type and Mutant Proteins. Preparation of N-terminal His-tagged wild-type and mutant WT1(−KTS) zinc finger peptides was carried out as described previously (*14*, *15*, *21*). Protein purity was confirmed by polyacrylamide gel electrophoresis, and the concentration of each protein preparation was determined by the method of Bradford (*23*).

Radiolabeling of RNA and DNA Ligands. Preparation of radiolabeled WT1 aptamers RNA22 and RNA38 by *in vitro* transcription was carried out as described previously (*21*). A DNA restriction fragment with a copy of the WT1 consensus binding site was 3′ end labeled as described previously (*14*, *15*). The sequence of the WT1 consensus DNA binding site and the predicted secondary structures of RNA22 and RNA38 are shown in Figure 1C.

Equilibrium Binding of Nucleic Acids to Mutant WT1(−KTS) Proteins. The apparent association constants for the binding of radiolabeled nucleic acids to wild-type and mutant WT1(−KTS) proteins were determined using a double filter binding assay (*24*). The binding buffer consisted of 20 mM Tris-HCl, pH 7.5 (20 °C), 5 mM $MgCl_2$, 100 mM KCl, 10 μM $ZnCl_2$, 0.5 mM tris(2-carboxyethyl)phosphine hydrochloride, 100 μg/mL BSA, and 1 μg/mL poly(A). When binding of the proteins to DNA was measured, poly(dI-dC)

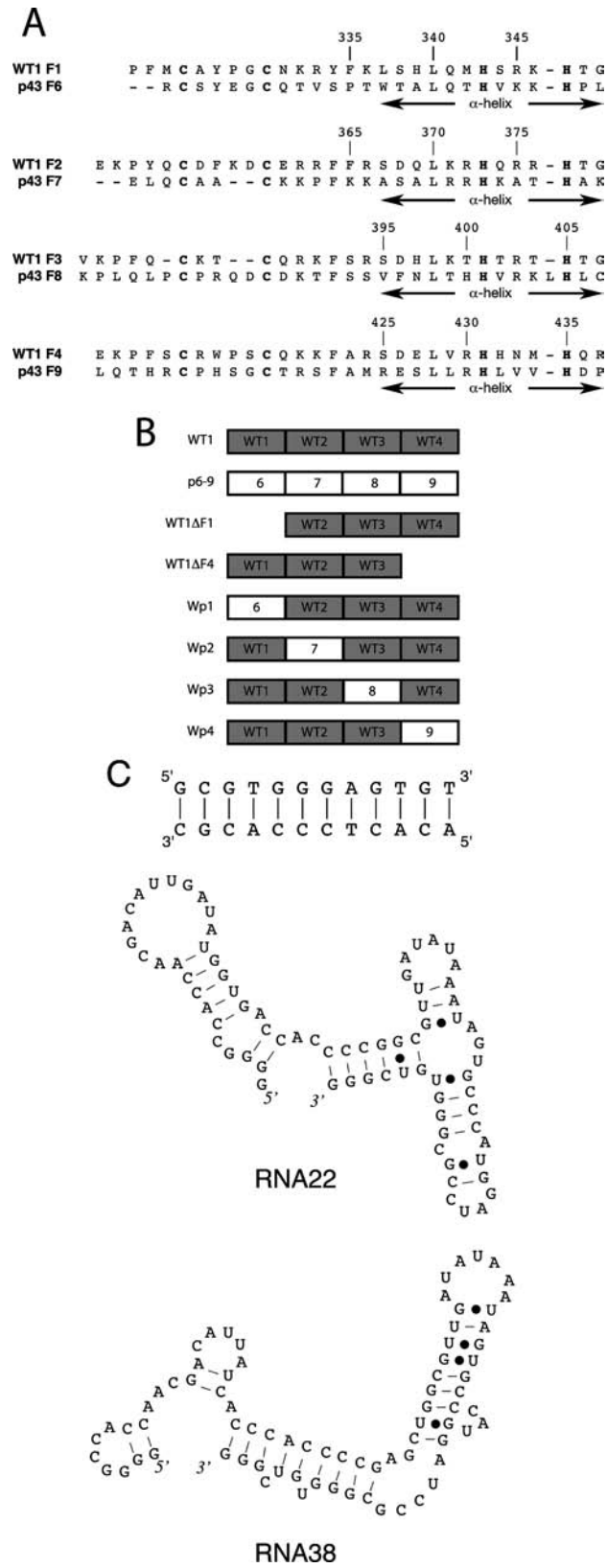

FIGURE 1: (A) Comparison of the amino acid sequence of the four zinc fingers of WT1(−KTS) with zinc fingers 6−9 of *Xenopus* p43 used to create finger swap and scanning mutations in WT1. The boundaries of the α-helical region of each finger are indicated by the arrows, and the amino acid residue numbers from full-length WT1 are shown above the sequence. (B) Schematics of deletion and finger swap mutations created in the WT1(−KTS) zinc finger domain peptide. (C) The consensus DNA binding site for WT1(−KTS) used in this study. The secondary structures of the RNA22 and RNA38 aptamers predicted by mfold (*39*).

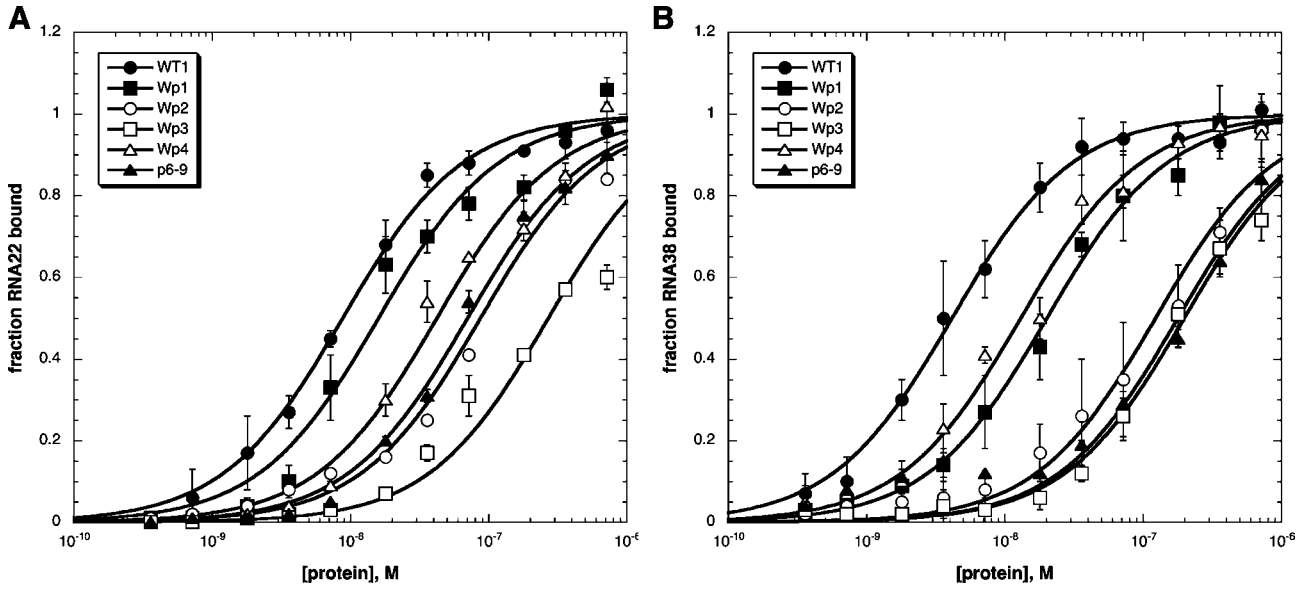

FIGURE 2: Equilibrium binding of wild-type WT1, the four finger peptide p6−9 derived from *Xenopus* p43 protein, and the finger swap mutants Wp1, Wp2, Wp3, and Wp4 to RNA22 (panel A) and RNA38 (panel B). Each data point is the mean value from three independent experiments, with the standard deviation for each point represented by the error bars. Best-fit curves to a simple bimolecular equilibrium are shown for each protein.

replaced poly(A) in the binding buffer. Apparent dissociation constants (K_d) for the binding of the mutant and wild-type proteins to RNA were calculated by fitting the data to a simple bimolecular equilibrium model using the general curve fitting function of Kaleidagraph software (Synergy Software, Reading, PA) and the equation:

$$\frac{[\text{RNA}-\text{protein}]}{[\text{RNA}]_{\text{total}}} = \frac{[\text{protein}]_{\text{total}}}{[\text{protein}]_{\text{total}} + K_d}$$

where $[\text{RNA}]_{\text{total}} \ll K_d$ and $[\text{RNA}-\text{protein}]/[\text{RNA}]_{\text{total}}$ is reported as the fraction of RNA bound. Data for the equilibrium binding of proteins to DNA were analyzed in the same way. Association constant (K_a) values were derived as the reciprocal of the measured K_d values and are reported as the mean of three independent determinations with the associated standard deviations. Relative affinities were arrived at by dividing the apparent K_a for the mutant protein by the apparent K_a for the wild-type protein determined in parallel. Apparent K_a values for the binding of the wild-type protein were determined to be $(1.42 \pm 0.22) \times 10^8$ M^{-1} for RNA22, $(2.05 \pm 0.14) \times 10^8$ M^{-1} for RNA38, and $(2.15 \pm 0.29) \times 10^8$ M^{-1} for consensus DNA. The errors for relative affinities are given by the expression $\sigma = [(\sigma 1/M1)^2 + (\sigma 2/M2)^2]^{1/2} \times M2/M1$, where M1 and M2 are the respective association constants for the wild-type and mutant protein and the σ values are the corresponding standard deviations for these determinations. In each case, the same preparation of protein was used to measure the affinities to all three ligands.

RESULTS

Both isoforms of the WT1 zinc finger domain have been shown to bind to RNA aptamers and fragments derived from two cellular RNAs (*10, 19, 20*), while only the −KTS isomer binds to a 12 base pair consensus DNA with high affinity (*14*). The equilibrium binding of WT1(−KTS) to DNA and the RNA aptamers is similarly affected by conditions such as monovalent salt concentration, pH, and temperature (*21*).

Table 1: Effects of Deletion, Broken Finger, and Finger Swap Mutations on the Binding of WT1 to RNA Aptamers

protein	relative K_a for RNA22	relative K_a for RNA38
WT1	1.00	1.00
WT1ΔF1	0.133 ± 0.023	0.087 ± 0.024
H343A	0.508 ± 0.102	0.430 ± 0.144
WT1ΔF4	<0.001[a]	<0.001[a]
H431A	0.138 ± 0.024	0.069 ± 0.017
Wp1	0.440 ± 0.091	0.218 ± 0.019
Wp2	0.080 ± 0.014	0.038 ± 0.004
Wp3	0.026 ± 0.005	0.020 ± 0.002
Wp4	0.167 ± 0.032	0.376 ± 0.034
p6−9	0.100 ± 0.017	0.027 ± 0.004

[a] Apparent K_a for the mutant protein was below 2.0×10^5 M^{-1} and could not be measured accurately.

We therefore chose to study zinc finger mutations in WT1(−KTS) to allow a comparison of their effects on RNA and DNA binding affinity. The original study on RNA aptamer binding by WT1 zinc fingers demonstrated that all four fingers were required for high-affinity binding (*10*). Deletion of zinc finger 1 significantly reduced affinity for RNA22, while deletion of zinc finger 4 resulted in a complete loss of RNA binding activity. These results might arise because fingers 1 and 4 form critical protein−RNA contacts in the complex or might reflect a situation where important contacts formed by fingers 2 and 3 are less likely to form in the context of a truncated zinc finger domain. To distinguish between these possibilities, we assayed the effects of deletion and zinc finger substitution mutations on the interaction of the WT1(−KTS) zinc finger domain with RNA22 and RNA38. The C-terminal four zinc fingers of the 5S RNA binding *Xenopus* protein p43 were used as donors for finger swap experiments (Figure 1B). Equilibrium binding of the four single finger substitution mutants to RNA22 and RNA38 was determined by a nitrocellulose filter binding assay (Figure 2). The relative association constants derived from the filter binding assay are reported in Table 1. Deletion of either zinc finger 1 or zinc finger 4 has a significant effect on the binding of WT1(−KTS) to both RNA aptamers.

Deletion of zinc finger 1 reduces the affinity of WT1(−KTS) for RNA by 7.5−11-fold, somewhat larger than the 5-fold reduction observed for DNA binding affinity (*15*). In comparison, deletion of zinc finger 4 results in the complete loss of RNA binding. These results are consistent with those found by Bardeesy and Pelletier (*10*). The large effect of these terminal finger deletions on RNA binding, particularly in the case of finger 4, might result from a global effect on the folding of the zinc finger peptide. Therefore, the roles of zinc fingers 1 and 4 in the RNA binding activity of WT1(−KTS) were probed further by the creation of zinc coordination mutants and finger swap mutants (Table 1). Previous studies have shown that zinc coordination mutants only affect the structural integrity of the finger in which the mutation resides (*25*−*27*). These "broken finger" mutations have a significant effect on nucleic acid binding when they occur in a zinc finger that makes energetically important contacts with the DNA or RNA. Finger swap mutations have similar properties (*28*, *29*). Replacement of zinc finger 1 of WT1(−KTS) with zinc finger 6 of p43 only results in a reduction in affinity for the two RNA aptamers of 2.3−4.6-fold (Table 1). Substitution of histidine 343 with alanine in zinc finger 1, which should disrupt zinc coordination, only reduces the affinity of WT1(−KTS) for the two RNAs by 2-fold. A similar result was observed for zinc finger 4: replacement of the WT1 finger with finger 9 of p43 resulted in a protein that still bound to both RNA aptamers, although with a 6-fold reduction in affinity for RNA22 and 2.7-fold reduction in affinity for RNA38 (Table 1). Disrupting the zinc coordination in finger 4 by replacing histidine 431 with alanine reduced the affinity of WT1(−KTS) for RNA22 by 7.3-fold and RNA38 by 14.5-fold, far less than the >1000-fold reduction observed for the finger 4 deletion mutant. These results indicate that a noncognate zinc finger or a cognate zinc finger which has been structurally weakened is sufficient to partially rescue the low RNA binding activities observed for the two deletion mutants. We conclude that the two terminal zinc fingers are likely to be involved in stabilizing the fold of the zinc finger domain and do not contribute significantly to RNA binding affinity by forming direct contacts with the RNA.

The results obtained with the finger swap mutants indicate the potential for a more direct role of zinc fingers 2 and 3 in RNA aptamer binding (Table 1). Substitution of WT1 zinc finger 2 with finger 7 of p43 resulted in a 12.5−25-fold reduction in binding to the RNA aptamers, while substitution of zinc finger 3 with zinc finger 8 of p43 resulted in a 40−50-fold reduction in RNA binding activity. The magnitude of the effects of the finger swaps for zinc fingers 2 and 3 suggests that these two fingers may form one or more direct contacts to the RNA in the complex. The affinity of the Wp3 mutant for RNA22 is 4-fold lower than the affinity of the donor protein p6−9 used to construct the finger swaps. Since the p6−9 peptide is derived from the RNA binding protein p43, it may be capable of forming a network of nonspecific interactions with RNA22 that results in the observed affinity. Replacing finger 3 of WT1 with finger 8 of p6−9 appears to disrupt one or more significant protein−RNA contacts. The lower affinity of the Wp3 mutant for RNA22 compared to p6−9 may occur if the loss of finger 3 contacts with the RNA alters the overall orientation of all four zinc fingers to the RNA, thus weakening the strength of other specific and nonspecific contacts formed by fingers 1, 2, and 4.

Table 2: Effects of Alanine Mutations in Finger 2 on the Binding of WT1 to Consensus DNA and RNA Aptamers

protein	relative K_a for RNA22	relative K_a for RNA38	relative K_a for DNA
WT1	1.00	1.00	1.00
R366A	0.134 ± 0.093	0.034 ± 0.023	0.006 ± 0.003
S367A	0.390 ± 0.120	0.810 ± 0.179	0.387 ± 0.111
D368A	0.832 ± 0.118	0.863 ± 0.088	0.120 ± 0.031
Q369A	0.652 ± 0.182	0.570 ± 0.147	1.688 ± 0.346
K371A	0.464 ± 0.094	1.293 ± 0.171	0.949 ± 0.157
R372A	0.022 ± 0.004	0.126 ± 0.088	<0.001[a]

[a] Apparent K_a for the mutant protein was below 2.0×10^5 M^{-1} and could not be measured accurately.

Table 3: Effects of Point Mutations in Finger 3 on the Binding of WT1 to Consensus DNA and RNA Aptamers

protein	relative K_a for RNA22	relative K_a for RNA38	relative K_a for DNA
WT1	1.00	1.00	1.00
R394A	0.020 ± 0.007	0.001 ± 0.001	<0.001[a]
R394S	0.015 ± 0.002	0.009 ± 0.001	ND[b]
S395A	0.723 ± 0.190	1.034 ± 0.249	0.238 ± 0.033
D396A	0.413 ± 0.102	0.111 ± 0.051	<0.001[a]
H397A	0.155 ± 0.022	0.173 ± 0.034	<0.001[a]
K399A	0.300 ± 0.077	0.687 ± 0.084	0.382 ± 0.074
T400A	0.204 ± 0.051	0.288 ± 0.041	0.027 ± 0.007

[a] Apparent K_a for the mutant protein was below 2.0×10^5 M^{-1} and could not be measured accurately. [b] Not determined.

The role of zinc finger 2 in the binding of WT1(−KTS) to the RNA aptamers was investigated further by creating a series of alanine point mutations in the α-helix of the finger. The effect of each point mutation on the binding of WT1(−KTS) to RNA22 and RNA38 was determined by the nitrocellulose filter binding assay (Table 2). R366A and R372A are particularly critical for the binding of WT1 zinc fingers to both RNA aptamers. Arginine 366 contributes more to the affinity of WT1(−KTS) to RNA38 as compared to RNA22, with the R366A mutation resulting in a 7.5-fold reduction in binding to RNA22 and a 33-fold reduction in binding to RNA38. In comparison, arginine 372 contributes more to the affinity of WT1(−KTS) for RNA22 vs RNA38. The R372A mutation reduces RNA38 binding affinity by 7.9-fold but reduces RNA22 binding affinity by 45-fold. The effects of the other alanine substitutions are extremely modest (less than a 2.5-fold effect), although there are some differences in effect for the two RNAs. Substitution of serine 367 and lysine 371 results in a small reduction in RNA22 binding affinity that is not observed for the binding of WT1(−KTS) to RNA38. Since the two aptamers belong to different sequence/structural families (Figure 1C), these differences in alanine substitution mutations on WT1(−KTS) binding affinity are not unexpected.

A similar investigation was carried out to determine the role in RNA binding of individual α-helical residues of zinc finger 3 of WT1(−KTS). The results in Table 3 indicate that arginine 394 plays a particularly critical function in binding of the WT1(−KTS) zinc finger domain to both RNA aptamers. Substitution of this arginine with alanine results in a 50-fold reduction in binding affinity for RNA22 and a 1000-fold reduction in binding affinity for RNA38. The finger swap mutant Wp3 has a 50-fold higher affinity for

RNA38 compared to the R394A point mutant, surprising since finger 3 is entirely replaced in Wp3. In the Wp3 mutant, arginine 394 is substituted with a serine, which might provide a compensating contact to RNA38. The R394S point mutation lowers the affinity for RNA38 only 2.2-fold compared to the Wp3 mutant, suggesting that, in the context of the finger 3 swap mutant, the serine may compensate somewhat for loss of the arginine. However, the R394S mutant has a 111-fold decrease in affinity for RNA38 compared to WT1(−KTS), indicating that the serine is not a suitable substitute for arginine in the context of the wild-type finger 3. Substitution of histidine 397 with alanine results in about a 6-fold reduction in affinity for both aptamers, while substitution of threonine 400 results in a 3.5−5-fold reduction in RNA binding affinity. Alanine substitution of serine 395 has no significant effect on the binding of the WT1(−KTS) zinc finger domain to either RNA aptamer. Aptamer-specific differences were observed for the alanine substitutions of glutamic acid 396 and lysine 399. The glutamic acid at 396 appears to be more critical for binding of the zinc fingers to RNA38 (9-fold decrease for D396A) as opposed to RNA22 (2.4-fold decrease for D396A), while the lysine at 399 contributes more to the binding of the zinc fingers to RNA22 (3.3-fold decrease for K399A) as opposed to RNA38 (1.5-fold decrease for K399A).

The zinc finger domain of WT1(−KTS) also binds specifically to a 12 base pair sequence in DNA (*15*, *17*). This type of multifunctional activity is often observed for zinc finger proteins (*30*−*33*), and we used the filter binding assay to measure the effects of the alanine substitution mutations on the binding of the WT1(−KTS) zinc finger domain to a consensus DNA binding site. For zinc finger 2, alanine substitutions at arginine 366, glutamic acid 368, and arginine 372 resulted in significant reductions in the DNA binding activity of the WT1(−KTS) zinc finger domain (Table 2). The effects of these mutations are consistent with the known role of each amino acid in DNA binding from the three-dimensional structure of the WT1−DNA complex (*18*). Substitution of each of the arginines also reduced RNA binding affinity, although to a smaller extent than the effects observed for DNA binding. The D368A mutant protein binds to both RNA aptamers with affinities close to the wild-type protein but has an 8.3-fold decreased affinity for the consensus DNA binding site. For zinc finger 3, alanine substitutions at arginine 394, glutamic acid 396, histidine 397, and threonine 400 result in a significant reduction (37-fold) to complete loss (>1000-fold) of DNA binding activity (Table 3). Although all four of these residues are also implicated as being important for binding to at least one of the RNA aptamers, the magnitudes of the effects of the alanine substitutions are far greater for the DNA binding activity of the WT1(−KTS) zinc finger domain.

DISCUSSION

In addition to a well-defined DNA binding activity, there is a growing body of evidence that WT1 also binds to RNA. Within the eukaryotic cell, WT1(+KTS) colocalizes with splicing factors in the nucleus, and WT1 is a component of poly(A)$^+$ mRNPs (*4*, *5*, *9*, *34*). Both isoforms of the WT1 zinc finger domain have been shown to bind to RNA aptamers and fragments derived from two cellular RNAs (*10*, *19*, *20*). Of these interactions, the one between WT1 and RNA aptamers has been studied in more detail. For the RNA22 aptamer, a consensus sequence of nucleotides in the context of a specific secondary structure is necessary for binding to the WT1 zinc finger domain (*10*, *21*). The −KTS isoform binds with slightly higher affinity to RNA22 than does the +KTS isoform (*21*). Deletion of either zinc finger 1 or zinc finger 4 results in a large decrease in affinity of WT1 for RNA22, suggesting at the very least that all four zinc fingers are required for binding to the aptamer (*10*). Observations that deletion of zinc finger 1 or 4 also decreases binding to the cellular RNA fragments indicate the requirement for all four zinc fingers may be a general feature of RNA binding by WT1 (*19*, *20*). Furthermore, deletion of zinc finger 1 also prevents the inclusion of WT1 in poly(A)$^+$ mRNPs (*3*). It is not yet clear whether inclusion of WT1 in mRNPs requires direct interaction with RNA or involves interactions with other protein components.

The previous results obtained with the zinc finger deletion mutants left open the question of whether zinc fingers 1 and 4 form direct contacts with the RNA ligands or are required for stabilizing the orientation of the other zinc fingers of WT1 in three-dimensional space. The potential roles of zinc fingers 2 and 3 in RNA binding were undefined at this stage. To investigate the role of each of the WT1(−KTS) zinc fingers in RNA binding, we used the RNA aptamers as a model system to test the effects of substitution mutations in the zinc fingers of WT1. The experimental evidence outlined above suggests that binding of WT1(−KTS) to the RNA aptamers is a reasonable model for potential interactions with cellular RNAs. Specific features of the RNA aptamers required for WT1 zinc finger binding had been characterized (*10*, *21*), but the roles of specific zinc fingers and individual amino acids of the WT1 zinc finger domain in RNA aptamer binding were not yet known.

To determine the contribution of zinc fingers and individual amino acids to the RNA aptamer binding activity of WT1(−KTS), we measured the effects of site-directed mutations in the zinc finger domain using a quantitative equilibrium binding assay. The RNA22 and RNA38 aptamers used in these binding assays are representative of the two families of aptamers that bind to WT1(−KTS) with high affinity (*10*, *21*). As observed previously by others, deletion of either the first zinc finger or the last zinc finger from the WT1(−KTS) zinc finger domain resulted in a significant decrease in affinity for RNA22 and RNA38, the effect being much larger for the deletion of zinc finger 4 (*10*).

To probe further the potential role of all four zinc fingers in RNA aptamer binding, the effects of individual zinc finger swap mutations were assayed. Donor zinc fingers were taken from zinc fingers 6−9 of the *Xenopus* protein p43. This protein binds specifically to 5S RNA but has little affinity for other RNAs (*28*, *35*). The results of the equilibrium binding assays with the four single finger substitution mutants of WT1(−KTS) clarified the roles of each WT1(−KTS) zinc finger in RNA aptamer binding. While deletion of zinc finger 1 reduced the affinity of WT1(−KTS) for both RNA aptamers significantly, the Wp1 substitution mutation had a much smaller effect, as did disruption of zinc coordination in finger 1. These results indicate that the requirement for zinc finger 1 of WT1 in RNA binding may primarily reflect

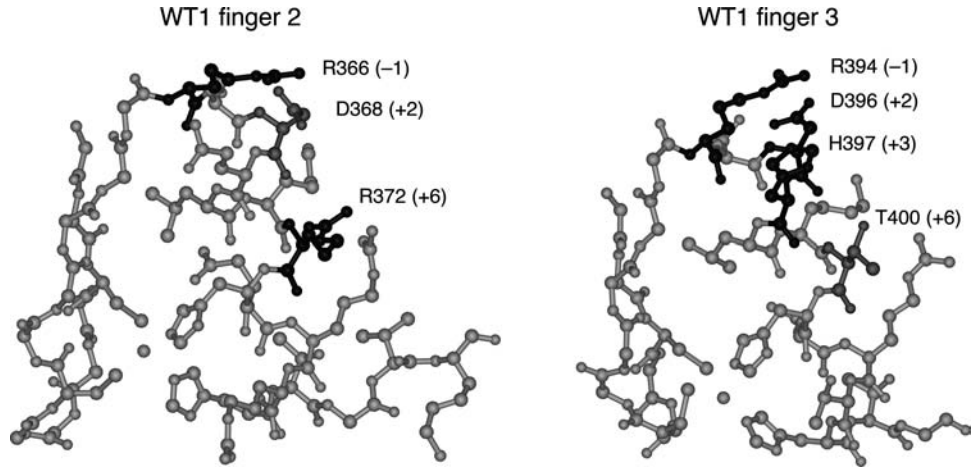

FIGURE 3: Structures of zinc fingers 2 and 3 of WT1 (*18*). For the sake of clarity, only the side chains of the zinc coordinating and α-helical residues are shown. Mutation of the residues indicated in black significantly reduce binding to both RNA and DNA (R366, R372, R394, D396, H397), while mutation of the residues indicated in dark gray significantly reduce binding only to DNA (D368, T400). The number in parentheses beside the residue name indicates position in the α-helix.

the need for a domain of four zinc fingers as opposed to the formation of specific RNA contacts by the first zinc finger. A similar phenomenon was observed for zinc finger 4: deletion resulted in a greater than 1000-fold reduction in affinity of WT1(−KTS) for both aptamers, while disruption of zinc coordination or substitution of finger 4 resulted in a much smaller reduction in affinity for both RNAs. Once again, these results are consistent with a general role for zinc finger 4 in providing a context for the direct interaction of the internal zinc fingers of WT1(−KTS) with RNA.

Previous studies had not probed the potential contribution of the internal zinc fingers of WT1 to RNA binding activity. Our results with the finger swap mutations indicate critical roles for both zinc finger 2 and zinc finger 3 in RNA aptamer binding. Replacement of zinc finger 2 in the Wp2 mutant results in a 12.5-fold decrease in affinity for RNA22 and a 26-fold decrease in affinity for RNA38. Replacement of zinc finger 3 in the Wp3 mutant results in a 40-fold decrease in affinity for RNA22 and a 50-fold decrease in affinity for RNA38. These results are consistent with the formation of one or more direct contacts between the RNAs and amino acid side chains in these two zinc fingers.

The zinc finger residues most likely to interact directly with nucleic acid ligands are found in the α-helical region, specifically in the N-terminal part of the α-helix in the case of interactions with RNA (*28*, *36*). To investigate the roles in RNA binding of individual amino acids in zinc fingers 2 and 3 of WT1, we created a set of alanine substitution mutations beginning with the residue immediately preceding the α-helix and continuing with the first five variable residues within the α-helix. The effects of the alanine mutations in zinc finger 2 were determined with both RNA aptamers and compared to their effects on binding to a WT1 DNA consensus site (Figure 3). Substitutions of the arginine at position −1 (R366) and the arginine at position 6 (R372) of the α-helix significantly decreased the affinity of WT1(−KTS) for both RNA aptamers, although the magnitude of the effects differed for each aptamer. The R366A mutation decreased affinity for both RNAs, with a 4-fold larger effect on RNA38 vs RNA22. In comparison, the effect of the R372A mutation, while significant for both aptamers, was almost 6-fold larger for RNA22 vs RNA38. These results suggest that both arginine residues form contacts to RNA22 and RNA38, with the strength of the bonds formed differing for each RNA ligand. Alanine substitutions of S367, D368, Q369, and K371 resulted in minor changes in RNA binding affinity with the largest observed effect being a 2.5-fold decrease.

The effects on DNA binding affinity of the same alanine substitution mutations in zinc finger 2 were measured. As was the case with the RNA binding activity of WT1(−KTS), substitution of R366 and R372 also resulted in a significant decrease in affinity for a DNA consensus binding site (Figure 3). However, the effects of these substitutions were far greater for the DNA binding activity compared to the effects on the RNA binding activity. In the three-dimensional structure of the WT1(−KTS) zinc finger domain bound to DNA, R366 and R372 make several direct bonding contacts with the DNA bases in the major groove (*18*). Substitution with alanine disrupts these contacts, resulting in the large decrease in DNA binding affinity. The relatively smaller effects of these mutations on RNA binding activity may indicate that the direct contacts formed by the arginine side chains are weaker because of a less optimal orientation of the interacting atoms or may reflect a fundamental difference in the mechanism by which zinc fingers interact with RNA vs DNA. In addition, substitution of D368 with an alanine significantly decreases the affinity of the WT1(−KTS) zinc finger domain for DNA but had virtually no effect on RNA binding affinity (Figure 3). This aspartic acid buttresses the side chain of R366, providing an optimal orientation for interaction with the DNA bases (*18*).

A similar analysis was carried out on the α-helical residues of zinc finger 3 (Figure 3). As observed with zinc finger 2, substitution of the arginine immediately preceding the α-helix (R394) resulted in a significant decrease in affinity for both RNA aptamers, the effect being 20 times greater in the case of RNA38. Substitution of H397 and T400, which appear on the same face of the α-helix as R394, also decreased the affinity of WT1(−KTS) for the two aptamers, although to a far lesser extent with no significant differences in the effects for the two RNAs. While substitution of S395 had essentially no effect on RNA binding affinity, the alanine substitutions at D396 and K399 had some small but intriguing effects. The D396A mutation had a very modest effect on binding

of WT1(−KTS) to RNA22 (2.4-fold decrease) and a larger effect on binding to RNA38 (9-fold). This difference in the magnitude of the effects of the D396A mutation on binding to the two RNAs suggests the possibility that the aspartic acid is more optimally placed for interaction with RNA38 compared to RNA22. The effects of the K399A mutation were opposite to those of the D396A mutation: very modest for binding to RNA38 (1.5-fold decrease) but larger for binding to RNA22 (3.3-fold decrease). The size of the effect for RNA38 binding is so small it is unlikely K399A is involved in binding to this RNA, while the size of the effect on RNA22 binding may indicate that a weak bond is formed between the lysine side chain and the RNA.

The effects on DNA binding affinity of the same alanine subsitution mutations in zinc finger 3 were determined (Figure 3). As was the case with the RNA binding activity of WT1(−KTS), substitution of R394, D396, H397, and T400 resulted in a significant decrease in affinity for the DNA consensus binding site. In the three-dimensional structure of the WT1(−KTS) zinc finger domain bound to DNA, R394 makes two hydrogen-bonding contacts to a guanine base, while H397 makes one hydrogen-bonding contact and one hydrophobic contact to another guanine base (*18*). The D396 side chain serves two functions in DNA binding: it makes a hydrogen-bonding contact to a cytosine base of the DNA and a buttressing contact to the R394 side chain (*18*). The almost 40-fold decrease in DNA binding affinity for the T400A mutation is not readily explained by the three-dimensional structure of the WT1−DNA complex. The threonine side chain is not positioned to make contact to the DNA, although it is in close proximity to the side chain of the first zinc-coordinating histidine of finger 3.

The comparison of the effects of the alanine substitutions on both RNA and DNA binding by WT1(−KTS) has revealed some interesting observations. For those residues where alanine substitution reduced binding to both RNA and DNA, the magnitude of the effect was always far greater for DNA binding vs RNA binding. The single exception to this observation was the effect of the R394A mutation in finger 3 on the binding affinity to RNA38, a reduction that is on the same order of magnitude as the effect of this mutation on DNA binding affinity. The greater than 1000-fold reduction in DNA binding affinity observed for a number of the alanine substitution mutations is typical for protein−DNA interactions, even though the corresponding loss of free energy of binding is far greater than what would be expected from the loss of one or two hydrogen-bonding interactions. The same mutations have effects on RNA binding affinity that are more consistent with the loss of one or two protein−nucleic acid bonds. These results suggest that there is a fundamental difference in the mechanisms of DNA and RNA binding by the WT1(−KTS) zinc fingers.

Another general and somewhat surprising conclusion from the data is that all of the residues in zinc fingers 2 and 3 that are important for RNA binding are also important for DNA binding, although the corollary is not true (Figure 3). The D368A substitution in finger 2 reduced DNA binding affinity by 8.3-fold but had no significant effect on binding to either RNA22 or RNA38. Similarly, the S395A substitution in finger 3 reduced the affinity of the WT1(−KTS) zinc finger domain for DNA by 4.2-fold but had no significant effect on binding to either RNA22 or RNA38. In the nine zinc finger protein TFIIIA, residues critical for DNA binding are found in zinc fingers 1, 2, 3, and 5 while residues critical for RNA binding are found in zinc fingers 4 and 6 (*36*−*38*). Although all nine zinc fingers of TFIIIA are required for the highest affinity binding to its DNA and RNA ligands, there is a clear division of responsibilities among the fingers for each activity. In the case of WT1(−KTS) the same zinc fingers and specific residues within those fingers are critical for binding to a consensus DNA site and the RNA aptamers.

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