Comparison of the DNA Binding Characteristics of the Related Zinc Finger Proteins WT1 and EGR1†

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Received July 24, 1997; Revised Manuscript Received September 24, 1997

ABSTRACT: The interactions of the related zinc finger proteins WT1 and EGR1 with DNA have been investigated using a quantitative binding assay. A recombinant peptide containing the four zinc fingers of WT1 binds to the dodecamer DNA sequence GCG-TGG-GCG-TGT with an apparent dissociation constant (K_d) of (1.14 ± 0.09) × 10^{-9} M under conditions of 0.1 M KCl, pH 7.5, at 22 °C. Under the same conditions, a recombinant peptide containing the three zinc fingers of EGR1 binds to the dodecamer sequence, the first nine bases comprising the EGR consensus binding site, with an apparent K_d of (3.55 ± 0.24) × 10^{-9} M. The nature of the equilibrium binding of each peptide to DNA was investigated as a function of temperature, pH, monovalent salt concentration, and divalent salt concentration. The interaction of WT1 with DNA is an entropy-driven process, while the formation of the EGR1−DNA complex is favored by enthalpy and entropy. The DNA binding activities of both proteins have broad pH optima centered at pH 8.0. The binding of both proteins to DNA shows similar sensitivity to ionic strength, with approximately 7.7 ± 0.8 ion pairs formed in the EGR1−DNA complex and 9.2 ± 1.8 ion pairs formed in the WT1−DNA complex. Results of measuring the effects of point mutations in the DNA binding site on the affinity of WT1 and EGR1 indicates a significant difference in the optimal binding sites: for EGR1, the highest affinity binding site has the sequence GNG-(T/G)GG-G(T/C)G, while for WT1 the highest affinity binding site has the sequence G(T/C)G-(T/G)GG-GAG-(T/C)G(T/C).

Wilms' tumor is a pediatric kidney malignancy that occurs with a frequency of 1:10 000. The *WT1* locus on chromosome 11p13 encodes a tumor suppressor protein that is inactivated in a subtype of Wilms' tumors. The normal cellular function of the WT1 protein involves the regulation of gene networks in the developing kidney and genitourinary systems. This protein has a DNA binding domain composed of four zinc fingers of the C2H2 type close to the carboxyl terminus and a regulatory domain rich in proline and glutamine residues located at the amino terminus.

Differential splicing of the primary *WT1* transcript gives rise to four cDNA isoforms. Alternative splicing can result in the in-frame insertion of 17 amino acids into the P/Q domain of the protein (*1*), an in-frame insertion of three amino acids between the third and fourth fingers of the DNA binding domain (*2*), or both insertions. A significant amount of research has been focused on the splice variant without the two inserts, even though it is not encoded by the most abundant cDNA isoform (*3*). Comparison of the amino acid sequence of the zinc fingers of WT1 with the amino acid sequences of other zinc finger proteins revealed a high degree of identity of the last three zinc fingers of WT1 with the three zinc fingers of two early growth response genes, EGR1 (Krox24, Zif268, NGFI-A) and EGR2 (Krox20), which are involved in regulating cell proliferation (*4*, *5*). Initial experiments to identify WT1 binding sites showed that the protein preferentially binds to DNA sequences related to the EGR1 consensus binding site 5′GCG-(T/G)GG-GCG3′ (*6*). Subsequent binding site selection studies from our laboratory and others have demonstrated that the highest affinity DNA binding site for WT1 has a consensus sequence of 5′GCG-(T/G)GG-GAG-(T/G)(T/G/A)(T/G) (*7*−*10*).

The results of a number of transient transfection studies demonstrated that WT1 represses transcription of promoters responsive to EGR-1 (*11*−*22*). These results suggest that there may be a regulatory link between these two proteins: WT1 may act as an antagonist of EGR1 or may be a tissue-specific factor that is involved in maintaining a particular differentiated phenotype. The balance in the levels of EGR1 and WT1 proteins in the nucleus therefore may be critical, and inactivation of WT1 could result in the onset of neoplasia.

The X-ray crystallographic studies of the EGR1 zinc finger domain bound to a 9 base pair fragment of DNA containing an EGR1 consensus site serves as a topological blueprint to understand the mechanism of DNA binding by WT1 (*23*, *24*). The zinc fingers contact overlapping four-base-pair sites on both strands of the DNA. Residues of the EGR1 zinc fingers critical for specific binding to DNA are conserved in fingers 2−4 of WT1 (Figure 1). However, despite the high degree of homology shared between the WT1 and EGR-1 DNA binding domains, there are a number of structural differences that suggest that the molecular details of DNA binding will not be identical for the two proteins.

† This work was supported by a grant from the National Cancer Institute of Canada with funds provided by the Canadian Cancer Society and by a grant from the Natural Sciences and Engineering Research Council of Canada.

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S0006-2960(97)01799-6 CCC: $15.00
Published on Web 01/28/1998

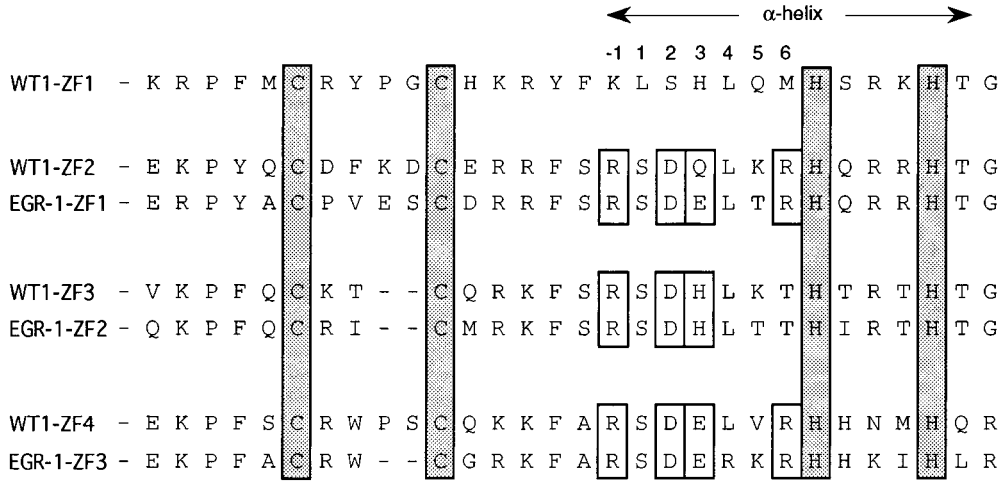

FIGURE 1: Comparison of the amino acid sequences of the zinc fingers of WT1 and EGR1. Amino acids involved in zinc coordination are indicated with shaded boxes, while those amino acids known in EGR1 to be involved in contacting DNA bases, and suspected to serve an analogous function in WT1, are indicated with open boxes.

To provide a clear quantitative picture of the interaction of WT1 and EGR1 proteins with their DNA binding sites, we used a nitrocellulose filter binding assay to measure various parameters of equilibrium binding of the two proteins to DNA, as well as determining the contribution of each base pair in the binding site to the specificity of the interaction. A comparison of the thermodynamic and kinetic parameters obtained for the two proteins provides a clear indication of the differences in the molecular binding mechanisms that exist between the WT1 and EGR1 proteins.

MATERIALS AND METHODS

Purification of Recombinant WT1 and EGR1 Zinc Finger Peptides. The construction of synthetic genes encoding the zinc finger regions of WT1 and EGR1 proteins and purification of the recombinant proteins were performed as described previously (*9*). The EGR1 zinc finger peptide contains residues 333−421 of the mouse EGR1 protein, while the WT1 zinc finger peptide contains residues 284−429 of the human WT1 protein. Both peptides have identical amino-terminal histidine-tagged sequences as outlined in ref *9*. Typical protein yields averaged 10 mg/L of bacterial culture. The purified protein products were aliquoted and stored at −80 °C. Protein aliquots were used only once when thawed.

Radiolabeling of DNA. Oligonucleotides containing the wild-type and mutant EGR consensus binding site sequences were designed for cloning into the *Bam*HI site of pUC18. Each top-strand oligonucleotide contained the appropriate dodecamer DNA sequence flanked at the 5′ side by the sequence GATCCA and at the 3′ side by AG. Each bottom-strand oligonucleotide contained the complement of the appropriate dodecamer DNA sequence flanked at the 5′ side by GATCCT and at the 3′ side by TG. After cloning of annealed oligonucleotide pairs into the *Bam*HI site of pUC18, each construct was characterized by DNA sequencing. Plasmids containing the target DNA sequences were digested with *Eco*RI and *Hind*III, and the double-stranded DNAs were labeled with [α-^{32}P]dATP (*25*). Radiolabeled DNA was stored at 4 °C for up to 6 days.

Nitrocellulose Filter Binding Assays. The binding affinities of EGR1 and WT1 zinc finger peptides were quantified using a nitrocellulose filter binding assay described previously (*7*, *9*, *26*). The standard binding buffer used to measure DNA binding affinities contained 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 100 mM KCl, 1 mM DTT, 5 μM $ZnCl_2$, 5 μg/mL poly[d(I-C)], and 100 μg/mL bovine serum albumin. When the effect of altering binding conditions such as pH, divalent salt concentration, or monovalent salt concentration was being investigated, the binding buffer was altered appropriately. The thawed protein aliquots were diluted in binding buffer lacking the poly[d(I-C)] to concentrations typically ranging from 0.02 to 60 nM. In the case of weakly bound DNA mutants, the protein concentrations were increased up to 1200 nM. Experimental data (at least three independent determinations per data point) were fitted to the appropriate equation using KaleidaGraph version 3.0 software.

RESULTS

Equilibrium Binding Constants. We have made a detailed investigation of the mechanism of the equilibrium binding of WT1 and EGR1 proteins to DNA, using methods that have been applied successfully to the study of the DNA and RNA binding properties of the zinc finger protein *Xenopus* transcription factor TFIIIA (*26*, *27*). Purified recombinant zinc finger peptides of WT1 and EGR1 were used to determine the parameters of the equilibrium binding of the proteins to their preferred DNA binding sequences, using a nitrocellulose filter binding assay (*26*). To determine the time required for each system to reach a true equilibrium, a study of the apparent K_a values as a function of incubation time was carried out. As the data in Figure 2 show, equilibrium is reached for the binding of each protein to a specific DNA target after a 60 min incubation at 22 °C in a TMK binding buffer (*26*) supplemented with 5 μM $ZnCl_2$ and 5 μg/mL poly[d(I-C)].

Under these same conditions, the apparent dissociation constant (K_d) measured for the formation of the WT1-DNA complex is $(1.14 \pm 0.09) \times 10^{-9}$ M, while the K_d for the binding of EGR1 to DNA is $(3.55 \pm 0.24) \times 10^{-9}$ M (Figure 3A). To verify that these K_d values reflect the formation of a DNA−protein complex by a bimolecular equilibrium and that the protein preparations used are 100% active in the assay, the equilibrium binding of DNA to each protein was

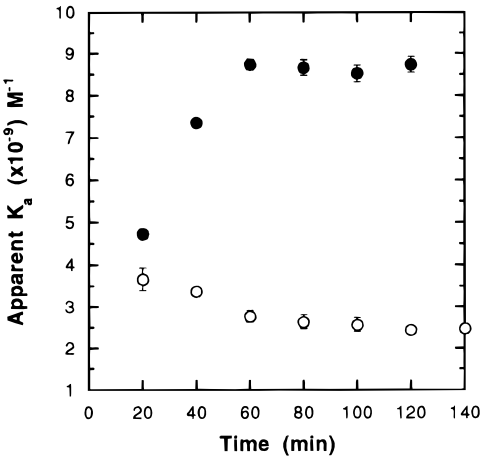

FIGURE 2: Time dependence of the binding of WT1 and EGR1 to the EGR1 DNA consensus sequence. Apparent K_a values as a function of time were determined for WT1 (●) and EGR1 (○) using the nitrocellulose filter binding assay. Each data point is the mean of three or more independent determinations, with the associated standard deviations indicated with the error bars.

investigated by titrating a fixed concentration of protein (2 nM for WT1, 5 nM for EGR1) with increasing concentrations of DNA (0.3−30 nM) and subjecting the resulting data to a Scatchard analysis (*28*). The data were consistent with the formation of DNA−protein complexes by a simple bimolecular equilibrium (Figure 3B). The K_d values derived from this analysis were 0.97×10^{-9} M for the WT1−DNA complex and 2.15×10^{-9} M for the EGR1−DNA complex. Both of these values are in good agreement with the K_d values measured by the standard nitrocellulose filter binding assay and indicate that the protein preparations are 100% active in the DNA binding assays.

pH Dependence of K_a. The pH dependence of the apparent K_a for DNA binding was determined using appropriate buffers to vary the final pH in the incubation mixture from 5.5 to 9.0. As presented in Figure 4, the interaction of both proteins with DNA has a broad pH optimum from pH 5.5 to 8. The affinity of both proteins for DNA decreases slightly at pH values above 8, which is likely to result from the deprotonation of one or more functionally important residues on the proteins.

Effect of Divalent Metal Ion Concentration on DNA Binding. The effect of the divalent metal ion concentration on the efficiency of binding was measured by varying the Mg^{2+} concentration in the incubation buffer from 0 to 20 mM. The data in Figure 5 demonstrate that the binding of WT1 to DNA is optimal at a Mg^{2+} concentration of 4−5 mM, with only a slight reduction in affinity at higher or lower divalent metal ion concentrations. In contrast, the affinity of the binding of EGR1 to DNA is optimal at a Mg^{2+} concentration of 2−5 mM but declines sharply at divalent metal ion concentrations above 5 mM.

Cation and Anion Effects on Binding. The effects of different monovalent cations and anions on the DNA binding affinities of WT1 and EGR1 were determined by changing the identity of the salt added to the binding buffer. Replacement of potassium by sodium, lithium, or ammonium did not have a significant effect on the measured affinities of either WT1 or EGR1 for DNA (Table 1). On the other hand, substitution of chloride by the anions acetate, nitrate, and iodide had a more pronounced effect on the DNA binding activities of both proteins. The DNA binding affinities of WT1 and EGR1 decreased with changing anion in the following order: nitrate ≥ acetate ≥ chloride > iodide (Table 1). These data are consistent with the presence of one or more anion binding sites on both WT1 and EGR1.

Monovalent Salt Dependence of the K_a for DNA Binding. The contributions of ionic protein−DNA bonds to the binding energies of the WT1−DNA and EGR1−DNA interactions were determined using an analysis of the salt dependence of K_a based upon an ion displacement model (*29*, *30*). The number of ions released in the formation of a protein−DNA complex can be obtained using the equation $\ln K_{obsd} = \ln K° - Z\psi \ln [M^+] - Z \ln (0.5(1 + (1 + 4K^{Mg}_{obsd}[Mg^{2+}])^{0.5}))$, where $K°$ is the apparent K_a at 1 M salt, Z is the number of cations and anions released upon complex formation, ψ is the fractional counterion bound per phosphate in the DNA (assumed to be 0.88 for double-stranded DNA), and K^{Mg}_{obsd} is the observed binding constant for the Mg^{2+}−DNA interaction (*31*). The data in Figure 6 were fitted to the above equation assuming the Mg^{2+} binding to the EGR1 consensus DNA is adequately represented by Mg^{2+} binding to T7 DNA. The data indicate that there are 9.2 ± 1.8 ions released in the formation of the WT1−DNA complex and 7.7 ± 0.8 ions released in the formation of the EGR1−DNA complex.

Temperature Dependence of K_a. The effects of temperature on the DNA binding affinities of WT1 and EGR1 proteins were determined from 4 to 37 °C. The data shown in Figure 7 demonstrate the opposing effects of temperature on the formation of the WT1−DNA and EGR1−DNA complexes. WT1 has the highest affinity for its specific DNA sequence at 37 °C, while the binding of EGR1 to its DNA consensus site occurs with highest affinity at 4 °C.

At 22 °C, the $\Delta G°$ of the WT1-DNA interaction has a value of −12.1 kcal mol^{-1}, an unfavorable $\Delta H°$ value of +6.6 kcal mol^{-1}, and a favorable $\Delta S°$ value of +63.3 cal mol^{-1} deg^{-1}. Thus formation of the WT1−DNA complex is an entropy-driven process. In comparison, the EGR1−DNA interaction has a $\Delta G°$ value of −11.4 kcal mol^{-1} at 22 °C, a favorable $\Delta H°$ value of −6.9 kcal mol^{-1}, and a favorable $\Delta S°$ value of +15.3 cal mol^{-1} deg^{-1}. Thus in the case of EGR1, complex formation with DNA is favored by both enthalpy and entropy.

Contribution of Individual Base Pairs to the Binding Affinities of WT1 and EGR1. We measured the contribution of each base pair in the consensus binding site GCG-TGG-GCG-TGT to the affinity of binding WT1 and EGR1 by creating point mutants and quantifying protein binding using the nitrocellulose filter binding assay. The results of these experiments are presented in Table 2 and provide interesting insight into the similarities and differences of sequence-specific DNA binding by these two related zinc finger proteins.

Both proteins have greatly reduced affinities (>200-fold decrease) for G to C mutations at base pairs 3, 5, 6, 7, and 9. Each of these guanines forms direct hydrogen bonds with amino acids in EGR1 (*24*), and the data imply that precisely the same conserved amino acids serve a similar function in the binding of WT1 to DNA. Although the G at base pair 1 forms contacts to an arginine residue in EGR1 identical with those formed by the G at base pair 7, substitution with a C reduced the affinity of binding of the two proteins by

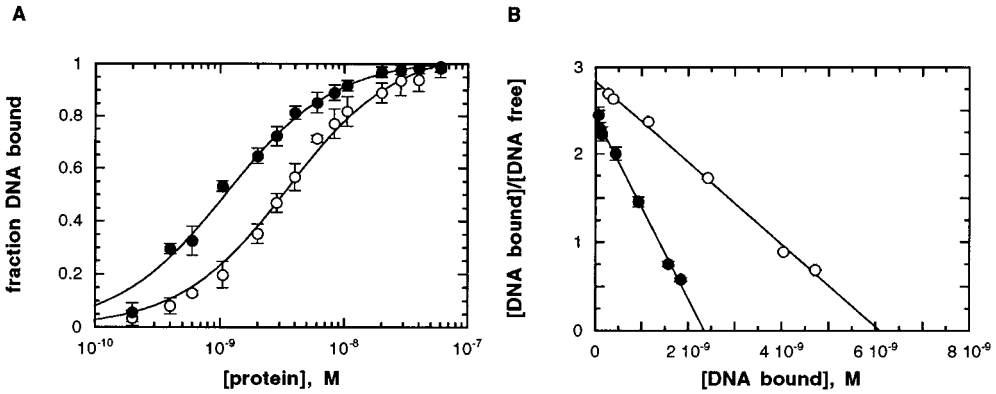

FIGURE 3: Equilibrium binding curves of WT1 and EGR1 to DNA. (A) The affinities of WT1 (●) and EGR1 (○) proteins for the EGR1 consensus DNA sequence were measured using a nitrocellulose filter binding assay. Each line represents the best fit for the formation of a simple bimolecular complex with K_d values of 1.14×10^{-9} M for WT1 ($R = 0.994$), and 3.55×10^{-9} M for EGR1 ($R = 0.997$). Each data point is the mean of three or more independent determinations, with the associated standard deviations indicated with the error bars. (B) Scatchard analysis of the interaction of WT1 (●) and EGR1 (○) with DNA. Proteins were held at constant concentration (2 nM for WT1 and 5 nM for EGR1) and titrated with varying concentrations of consensus DNA. Each line represents the best fit for the formation of a bimolecular complex with K_d values of 0.97×10^{-9} M for WT1 ($R = 0.997$) and 2.15×10^{-9} M for EGR1 ($R = 0.999$). Each data point is the mean of three or more independent determinations, with the associated standard deviations indicated with the error bars.

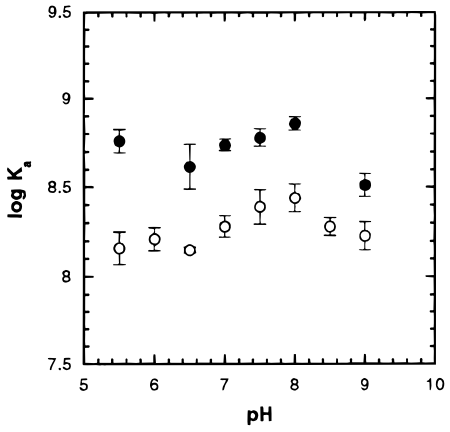

FIGURE 4: pH dependence of the binding of WT1 and EGR1 to DNA. The effect of changing the pH of the binding buffer from 5.5 to 9.0 on the apparent K_a values for the binding of WT1 (●) and EGR1 (○) was measured using the nitrocellulose filter binding assay. Each data point is the mean of three or more independent determinations, with the associated standard deviations indicated with the error bars.

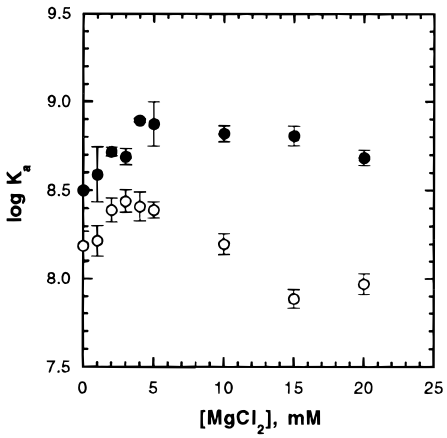

FIGURE 5: Effect of the magnesium ion concentration on the binding of WT1 and EGR1 to DNA. The K_a values as a function of Mg^{2+} ion concentration for the binding to WT1 (●) and EGR1 (○) were determined using the nitrocellulose filter binding assay. Each data point is the mean of three or more independent determinations, with the associated standard deviations indicated with the error bars.

5−10-fold, suggesting that in some way the amino acid−base contacts formed at base pair 1 contribute significantly less to the overall free energies of binding EGR1 or WT1 than other protein−guanine contacts. Nevertheless, the effect of mutations at these guanines is consistent with the known protein−DNA base contacts described in the crystal structure for the EGR1-DNA complex (*23*, *24*).

In the original crystallographic study of the EGR1−DNA complex, base pairs 2, 4, and 8 did not appear to be contacted by amino acid residues in the protein, and thus the "consensus" binding site for EGR1 could be considered to be GNG-NGG-GNG (*24*). However, as the data in Table 2 show, mutations at each of these positions do have measurable effects on the binding affinities of both WT1 and EGR1, although not necessarily in the same direction. For example, WT1 has a preference for a pyrimidine base at position 2, since substitution of the C in our consensus binding site with A or G reduces the binding of WT1 by 70−100-fold. In comparison, these same substitutions result in only a modest decrease (5−7-fold) in the affinity of EGR1 for the DNA. Quite striking is the effect of substitutions at position 4, which is a T in our consensus binding site. Substitution with A or C reduces binding of both proteins by greater than 200-fold. In contrast, substitution of the T with a G has virtually no effect on the affinity of binding WT1 or EGR1 thus one can conclude that the consensus preference at position 4 is for a T or G. The effects of base substitution at position 8 differed for WT1 and EGR1. Replacing the C at this position with A, G, or T reduced the affinity of EGR1 for the DNA by 4 to 25-fold. In contrast, two of these substitutions (G or T) had little effect on the affinity of WT1 for the DNA, but the third substitution (A) increased the affinity of WT1 for the DNA by almost 5-fold. The inclusion of A at position 8 in the high-affinity binding site for WT1 has been recognized previously (*7*, *10*). As discussed below, for EGR1 these results are consistent with the information obtained from a recent higher resolution crystallographic study of the EGR1−DNA complex (*23*).

Finally, positions 10−12, which comprise the finger 1 subsite of WT1, could be substituted with C residues without greatly reducing the affinity of the protein for DNA. This relative tolerance for sequence substitution has been observed

Table 1: Effect of the Different Monovalent Salts on the Binding of WT1 and EGR1 to DNA

	WT1		EGR1	
salt	K_d[a] (nM)	relative affinity	K_d[a] (nM)	relative affinity
KCl	1.14 ± 0.09	1.00	3.55 ± 0.24	1.00
NaCl	0.76 ± 0.01	1.5 ± 0.75[b]	2.76 ± 0.08	1.29 ± 0.06
LiCl	0.86 ± 0.16	1.33 ± 0.20	2.47 ± 0.1	1.44 ± 0.03
NH_{4Cl}	0.58 ± 0.14	1.96 ± 0.35	3.21 ± 0.11	1.11 ± 0.04
$NaNO_3$	1.47 ± 0.03	0.78 ± 0.06	4.99 ± 0.13	0.71 ± 0.03
$NaCH_3COO$	1.2 ± 0.14	0.95 ± 0.04	2.71 ± 0.03	1.31 ± 0.11
NaI	68.50 ± 6.61	0.017 ± 0.002	c	>0.005

[a] Apparent dissociation constants determined by nitrocellulose filter binding assay. The salt concentrations were kept constant at 0.1 M. Each value reported represents the mean of three or more independent determinations with the associated standard deviations. [b] The errors for relative affinities are given by the expression $\sigma = \{(\sigma_1/M_1)^2 + (\sigma_2/M_2)^2\}^{1/2}(M_1/M_2)$, where M_1 and M_2 are the respective dissociation constants for wild-type and mutant DNA and the σ values are the corresponding standard deviations for these determinations. [c] Binding affinity too low to be determined accurately.

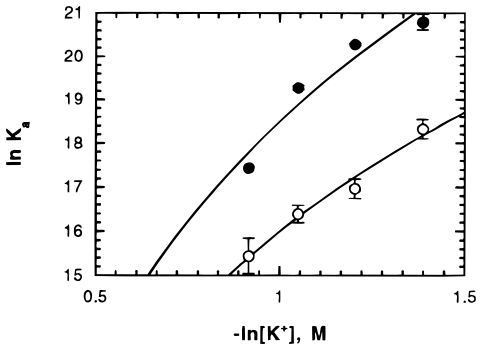

FIGURE 6: Dependence of the binding of WT1 and EGR1 to DNA on monovalent salt concentration. The effect of increasing the KCl concentration in the binding buffer on the equilibrium binding of WT1 (●) and EGR1 (○) to DNA was measured using the nitrocellulose filter binding assay. Each data point is the mean of three or more independent determinations, with the associated standard deviations indicated with the error bars. The experimental data were fit to eq 5 of ref 31 by a regression method that varied the number of ion pairs and the apparent K_a at 1 M salt. The continuous lines represent the best least-squares fit for these experiments, yielding values of 9.2 ± 1.8 ion pairs for WT1 ($R = 0.963$) and 7.7 ± 0.8 ion pairs for EGR1 ($R = 0.989$).

in previous studies that demonstrated that the contribution of finger 1 of WT1 to the overall affinity and specificity of DNA binding is rather small (*8*, *9*).

DISCUSSION

It has been demonstrated previously that WT1 binds with similar affinity to DNA sequences containing the consensus site for the EGR1 protein (*6*, *17*). Furthermore, in transient transfection assays both proteins can regulate promoters containing such sequences, with EGR1 activating transcription and WT1 repressing transcription (*11*−*22*). On the basis of these results, it has been suggested that WT1 and EGR1 may act as antagonists in developmental regulation of transcription. To investigate this hypothesis further, we conducted a detailed quantitative analysis to determine how the affinity and specificity of WT1 for such common regulatory sites would compare with EGR1.

The equilibrium dissociation constants for binding to the EGR1 consensus DNA sequence were determined to be (1.14 ± 0.09) $\times 10^{-9}$ M for WT1 and (3.55 ± 0.24) $\times 10^{-9}$ M for EGR1 zinc finger peptides. A Scatchard analysis of the protein−DNA interactions confirmed that both proteins are 100% active under the conditions used to measure equilibrium binding, and both form a 1:1 complex with DNA. The slightly higher affinity of DNA binding measured for WT1

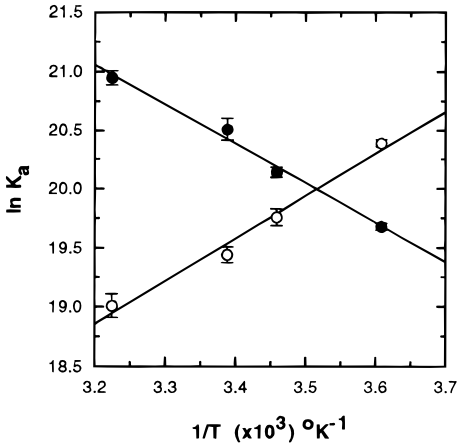

FIGURE 7: Temperature dependence of the binding of WT1 and EGR1 to DNA. The K_a values for the binding of WT1 (●) and EGR1 (○) to DNA were determined at temperatures ranging from 4 to 37 °C, using the nitrocellulose filter binding assay. The van't Hoff plot was used to calculate the enthalpy of these interactions by fitting each data set by linear least-squares analysis. The two correlation coefficients are greater than 0.99. Each data point is the mean of three or more independent determinations, with the associated standard deviations indicated with the error bars. The enthalpy values were determined from the slope of each line using the equation $d \ln K_a/d(1/T) = -\Delta H°/R$.

Table 2: Relative Affinities for the Binding of WT1 and EGR1 to Wild-Type and Mutant DNA Consensus Sequences

DNA	relative affinity for WT1	relative affinity for EGR1
wild-type	1.00[a]	1.00[a]
G1C	0.09 ± 0.014[b]	0.18 ± 0.03
C2A	0.01 ± 0.005	0.15 ± 0.02
C2G	0.015 ± 0.005	0.20 ± 0.04
C2T	0.88 ± 0.14	0.79 ± 0.13
G3C	<0.005[c]	<0.005[c]
T4A	<0.005[c]	<0.005[c]
T4C	<0.005[c]	<0.005[c]
T4G	0.64 ± 0.09	0.69 ± 0.10
G5C	<0.005[c]	<0.005[c]
G6C	<0.005[c]	<0.005[c]
G7C	<0.005[c]	<0.005[c]
C8A	4.54 ± 0.75	0.08 ± 0.01
C8G	1.32 ± 0.27	0.04 ± 0.01
C8T	0.99 ± 0.12	0.25 ± 0.04
G9C	<0.005[c]	<0.005[c]
T10C	0.88 ± 0.14	not determined
G11C	0.28 ± 0.06	not determined
T12C	0.53 ± 0.01	not determined

[a] Apparent dissociation constants determined by nitrocellulose filter binding assay. Each value reported represents the mean of three or more independent determinations with the associated standard deviations. Relative affinities were arrived at by dividing the apparent K_d for the mutant DNA by the apparent K_d for the wild-type DNA determined in parallel. The apparent K_d for the binding of WT1 to wild-type DNA was 1.14 ± 0.09 nM, and the apparent K_d for the binding of EGR1 to wild-type DNA was 3.55 ± 0.24 nM. [b] The errors for relative affinities are given by the expression $\sigma = \{(\sigma_1/M_1)^2 + (\sigma_2/M_2)^2\}^{1/2}(M_1/M_2)$, where M_1 and M_2 are the respective dissociation constants for wild-type and mutant DNA and the σ values are the corresponding standard deviations for these determinations. [c] Binding affinity was too low to be determined accurately.

can be attributed to relatively weak contributions of the extra zinc finger to the overall free energy of DNA binding (*9*).

Measuring the monovalent salt dependence of the K_a for DNA binding provides an estimate of the number of cationic residues on a protein and phosphates on the DNA that interact in the complex. Both proteins demonstrate a dramatic decrease in binding affinity at higher salt concentrations. In the case of WT1, there are approximately 9 counterions released upon binding of the protein to DNA, while in the case of EGR1, there are approximately 8 counterions released upon binding to DNA.

Both WT1 and EGR1 proteins achieve optimum DNA binding affinities within a very broad range of pH values. As the pH in the binding buffer increases above 8, the affinities of both proteins for DNA is reduced, presumably as a result of deprotonation of one or more critical residues on the proteins.

A number of thermodynamic parameters were measured and compared for the WT1−DNA and EGR1−DNA interactions. At 22 °C, the formation of the WT1−DNA complex had the following thermodynamic values: $\Delta G° = -12.1$ kcal mol^{-1}, $\Delta H° = +6.6$ kcal mol^{-1}, and $\Delta S° = +63.3$ cal mol^{-1} deg^{-1}. The corresponding parameters for the EGR1−DNA interaction are $\Delta G° = -11.4$ kcal mol^{-1}, $\Delta H° = -6.9$ kcal mol^{-1}, and $\Delta S° = +15.3$ cal mol^{-1} deg $^{-1}$. It is apparent from these values that the WT1−DNA interaction is an entropy-driven process, where ordered water molecules and counterions may become displaced from the binding interface upon formation of the protein−DNA complex. In contrast, the formation of the EGR1−DNA complex is driven by both enthalpy and entropy. Thus there are some fundamental differences in the mechanisms of DNA binding by both proteins.

The paradigm for zinc finger−DNA interaction that resulted from the original crystallographic study of the EGR1−DNA complex (*24*) consists of each zinc finger contacting one strand of a defined three-base-pair subsite on the DNA. However, this concept had been challenged by the results of the X-ray crystallographic studies of two other zinc finger−DNA complexes: the TTK−DNA complex (*32*) and the GLI−DNA complex (*33*). These zinc finger proteins do not conform to the simple rules deduced from the EGR1−DNA complex. In these structures, the DNA contacts made by zinc fingers are not restricted to one specific strand but can span both strands of the DNA; subsites contacted by each zinc finger can be longer than 3 bp and can overlap; some zinc fingers may not be involved in direct contacts with DNA at all. Pavletich and Pabo suggested that the EGR1−DNA interaction might accommodate some of these additional features of zinc finger−DNA interactions (*33*), and the details of a more highly refined crystallographic structure of the EGR1−DNA complex indeed show that each finger contacts more than three base pairs and both strands of the DNA (Figure 8) (*23*).

The crystallographic data obtained for the EGR1−DNA complex has provided a structural framework for understanding the molecular basis of that protein−DNA interaction (*23*, *24*). Since the critical amino acids in EGR1 that contact specific base pairs in the DNA are with one exception conserved in WT1 (Figure 1), it is reasonable to predict that the last three zinc fingers of WT1 will interact in an identical way with the 9 bp EGR1 consensus sequence. However,

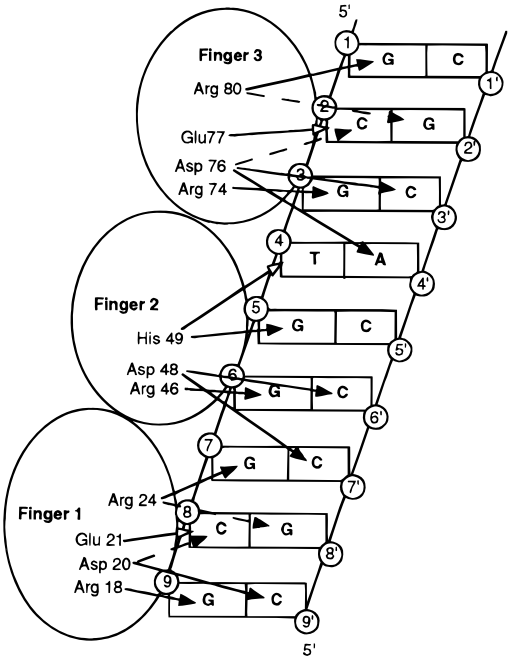

FIGURE 8: Summary of the amino acid−base contacts formed in the EGR1−DNA complex. The diagram was derived from the X-ray crystallographic analysis of the complex formed between the zinc fingers of EGR1 and a fragment of DNA containing the consensus EGR1 binding sequence (*23*). Solid arrows indicate direct hydrogen-bonding interactions, dashed arrows indicate water-mediated hydrogen-bonding interactions and arrows with open arrowheads indicate van der Waals interactions.

such a hypothesis must be tested directly because of the structural differences between the two proteins. These differences include an additional zinc finger (the first zinc finger of WT1), which does not possess significant homology with any of the three EGR1 zinc fingers at the amino acid level (*34*), and the approximately 50% dissimilarity in the amino acid sequences of the three zinc fingers in the EGR1 protein and fingers 2−4 of WT1. Indeed, there are significant differences in the thermodynamic parameters characterizing the binding of WT1 and EGR1 to DNA, also suggesting that these structural differences may result in different amino acid−base interactions. To compare the details of the interactions of these two zinc finger proteins with DNA, we designed a series of point mutants of the EGR1 consensus site to test the relative contributions of each base pair to the high-affinity binding of both WT1 and EGR1.

Although for the most part the results obtained with these mutant DNAs reiterate the main themes established by the EGR1−DNA crystal structure data, there are also significant differences when the effects of certain mutants on the binding of WT1 and EGR1 are compared. All of the contacts involving critical G-C base pairs observed in the X-ray analysis of the EGR1−DNA complex are conserved in the WT1−DNA interaction; i.e., base pairs 3, 5, 6, 7, and 9 in the DNA appear to be the most energetically important ones, as base substitutions at these positions resulted in a severe reduction in the binding of both proteins. Base pair 1, albeit shown to be involved in contact in the EGR1−DNA cocrystal (Figure 8), provides a less important contribution to the overall binding: a guanine to cytosine substitution at this position resulted in a 10-fold decrease in binding for WT1 and a 5-fold decrease for EGR1.

The binding of WT1 and EGR1 to DNA are affected similarly by substitutions at base pair 4. When the T-A base pair is replaced by C-G or A-T, binding of both proteins is not detectable. However, when the T-A base pair is replaced by a G−C base pair, there is only a slight and similar reduction in the affinities for binding of WT1 or EGR1. For EGR1, and by analogy WT1, this result is readily explained by the crystal structure data (*23*). The aspartic acid at position 2 of the α helix of finger 3 of EGR1 (finger 4 of WT1) is positioned to make a hydrogen bond to the N6 of the adenine at base 4′ of the wild-type DNA (Figure 8). The geometry will also support the formation of a hydrogen bond between this aspartic acid and the N4 of a cytosine at base 4′ (*23*), as found in the 4G mutant. A guanine or thymine base does not have a hydrogen-bond donor at the appropriate position, and as a result there is a loss of this hydrogen-bonding contact in the 4A and 4C mutants and a corresponding severe reduction in the binding affinities of WT1 and EGR1. The 5-methyl substituent of the thymine at base 4 of the consensus sequence is involved in a van der Waals contact with the histidine at position 3 of the α helix of finger 2 of EGR1 (finger 3 of WT1) (*23*). Thus the slight reduction in the binding affinities of WT1 and EGR1 to the 4G mutant DNA probably reflects the loss of this van der Waals contact.

Substitutions at base pairs 2 and 8 have significantly different effects on the binding of EGR1 compared to WT1. In the case of base pair 2, substitution of the C-G base pair with T-A was tolerated equally by both proteins. However, substitution of this base pair with A-T or G-C resulted in a much larger reduction in the WT1 binding affinity (70−100-fold reduction) compared to those observed for the EGR1 binding affinity (5−7-fold reduction). The crystallographic data show that the G at base 2′ of the consensus binding site is contacted at the O6 atom by a water-mediated hydrogen bond from the arginine at position 6 of the α helix of finger 3 of EGR1 (finger 4 of WT1), while the N4 atom of the C at base 2 is contacted by a water-mediated hydrogen bond from the aspartic acid at position 2 of the α helix of the same finger (Figure 8) (*23*). Our data suggest that compensatory hydrogen-bonding interactions can be made by both EGR1 and WT1 to the T-A base pair in the 2T mutant DNA. However, the data further suggest that there is a greater energetic contribution from one or both of these water-mediated hydrogen bonds to the binding of WT1 to the DNA than to the binding of EGR1 to DNA. There are five amino acid differences in the α helixes of the relevant fingers of WT1 and EGR1, which could influence the placement of the α helix in the major groove of the DNA, perhaps improving the geometry of the hydrogen bonds to base pair 2 in the case of WT1.

The most striking difference in the effects of binding site mutations on the interaction with WT1 and EGR1 was observed at base pair 8. From the crystallographic studies of the EGR1−DNA complex, a number of interactions with this base pair have been identified (Figure 8). Although the glutamate at the third helical position in finger 1 of EGR1 is too far from the base pair to make hydrogen-bonding interactions, the side chain is positioned for optimal van der Waals interaction with the cytosine base (*23*). In fact the side-chain position of this glutamate probably contributes to the known specificity for a cytosine at position 8 (*35*) by interfering with normal hydration of a purine base and sterically interfering with a thymine base. In addition, the aspartate at α-helical position 2 makes a water-mediated hydrogen bond to the N4 of the cytosine and the arginine at α-helical position 6 makes a water-mediated hydrogen bond to the O6 of the guanine of base pair 8 (Figure 8). As the data in Table 2 show, these contacts contribute significantly to the overall binding free energy of EGR1 to the consensus DNA site: substitution of the C-G base pair at position 8 with the other three Watson−Crick base pairs reduces the affinity of EGR1 for the DNA by 4−25-fold.

Binding site selection experiments have previously demonstrated that WT1 has a preference for an adenine base at position 8 (*7*, *10*). In the second finger of WT1, there is a glutamine in place of the glutamate found in at the analogous helical position (*3*) in finger 1 of EGR1 (Figure 1). It has been proposed that this glutamine residue may form a direct hydrogen-bonding interaction with base pair 8 in the major groove, thus resulting in the requirement for an AT base pair (*10*). Our mutational analysis provides further insight into the interaction of WT1 with base pair 8. It is certainly clear that an A at position 8 yields the highest affinity binding site for WT1, consistent with previous data. However, it is also clear that substituting any of the other three possible bases at position 8 results in only a modest 4−5-fold decrease in WT1 binding affinity. Can these differences be reconciled with the emerging picture of zinc finger−DNA interactions based upon the EGR1−DNA complex? The substitution of the C-G base pair at position 8 with an A-T base pair should result in the loss of the water-mediated hydrogen bond between the aspartate at helical position 2 and the N4 of the cytosine base (Figure 8). In addition, the van der Waals interaction observed between the glutamate at helical position 3 in EGR1 and the edge of the cytosine base would be disrupted by the substitution of an adenine base. In the case of EGR1, binding affinity drops by 12-fold. Offsetting the loss of these energetic interactions in the case of WT1 would be the formation of one or more direct hydrogen bonds between the glutamine at the third helical position of finger 2 and the adenine base. Such hydrogen-bonding interactions are the likely explanation for the observation that WT1 has a greater than 100-fold higher affinity for the 8A mutant DNA than does EGR1.

The effects of mutating base pair 8 on the binding affinity of EGR1 were much more dramatic than the effects of these mutations on the binding affinity of WT1. Although replacing the C at position 8 with T only reduced the binding of EGR1 by 4-fold, replacing the C with either purine reduced the binding affinity of EGR1 by 12−25-fold. These results agree with, and extend those of, a previous study that demonstrated EGR1 has a distinct preference (10-fold higher affinity) for a cytosine vs an adenine at position 8 (*35*).

Comparing the data for WT1 and EGR1, it is apparent that not only do the two proteins differ in sequence specificity at base pairs 2 and 8 but they also differ in the stringency of the specificity. These data call into question the prevalent view that WT1 and EGR1 are antagonistic transcription factors that regulate promoters from a common DNA binding site, a hypothesis supported exclusively by the results of numerous transient transfection assays utilizing promoters known to be regulated by EGR1. Given the fact that the sequence of the highest affinity binding sites for WT1 and EGR1 differ at least at base pair 8, the correctness of that

hypothesis would have to take into consideration the differences in affinities that WT1 and EGR1 have for their own vs the consensus binding site of the other protein and the concentrations of each protein in the nucleus under normal physiological conditions rather than the elevated levels resulting from transfection assays. In particular, given the fact that there is more than a 100-fold difference in the affinity of WT1 (K_d = 0.35 nM) and EGR1 (K_d = 43 nM) for the optimal WT1 binding site (8A), it seems reasonable to conclude that there will be promoters that are regulated by WT1 and not EGR1.

That leaves the question of whether promoters that are upregulated by EGR1 are also downregulated by WT1. The difference in the affinities of the two proteins for the EGR1 consensus sequence (8C) is relatively small at 22 °C, on the order of 2−3-fold, and WT1 has a 4.5-fold lower affinity for the 8C sequence vs the 8A sequence at this temperature. However, at 37 °C WT1 has a 7-fold higher affinity for the 8C sequence than EGR1 has. On one hand, this difference in affinities for the 8C sequence suggests that WT1 may very well act as an antagonist of EGR1-regulated promoters. On the other hand, if the concentration of WT1 in the nucleus is optimized for interaction with the higher affinity 8A sequence, it may be too low to allow for effective interaction with the 8C sequences regulated by EGR1. Although a direct measure of the concentration of WT1 in the nucleus of cells has not been made, there is evidence to suggest that the concentration of WT1 may be limiting. This evidence comes from investigations into the effects that point mutations in the zinc fingers of WT1 isolated from patients with Denys-Drash syndrome have on the DNA binding activity of the protein (*7*). These studies showed that some of the point mutations that resulted in severe pathogenesis only reduced the affinity of WT1 for DNA by a factor of 3−4-fold, thus implying that WT1 levels are normally limiting and even small changes in DNA binding affinity can abrogate the biological function of the protein.

Therefore, whether WT1 represses promoters that are activated by EGR1 will depend upon the nuclear concentration of the two proteins, and the relative concentrations of 8A and 8C target sequences in genomic DNA. It will also depend upon a variety of other factors involved in transcription factor−DNA interactions. In particular, other regions in the full-length proteins may influence sequence specificity and affinity of DNA binding either directly, by promoting self-association, or by mediating interactions with accessory factors. Further studies into the physiological roles of these two regulatory proteins should provide valuable information on the potential for WT1 and EGR1 to compete for binding to common regulatory sites as they occur within genomic DNA.

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BI9717993