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New insights into DNA-binding behavior of Wilms Tumor Protein (WT1) — A dual study

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ABSTRACT

Wilms Tumor suppressor protein (WT1) is a transcription factor that is involved in a variety of developmental functions during organ development. It is also implicated in the pathology of several different cancer forms. The protein contains four C_2H_2-type zinc fingers and it specifically binds GC-rich sequences in the promoter regions of its target genes, which are either up or down regulated. Two properties make WT1 a more unusual transcription factor — an unconventional amino acid composition for zinc finger 1, and the insertion of a tri-peptide KTS in some of the splice isoforms of WT1. Using six WT1 constructs in which zinc fingers are systematically deleted, a dual study based on a bacterial 1-hybrid system and surface plasmon resonance measurements is performed. The experiments show that the effect of zinc finger 1 is not significant in terms of overall DNA-binding kinetics, however it influences both the specificity of target recognition and stability of interaction in presence of KTS. The KTS insertion, however, only mildly retards binding affinity, mainly by affecting the on-rate. We suggest that the insertion disturbs zinc finger 4 from its binding frame, thus weakening the rate of target recognition. Finally, for the construct in which both zinc fingers 1 and 4 were deleted, the two middle fingers 2–3 still could function as a 'minimal DNA-recognition domain' for WT1, however the formation of a stable protein–DNA complex is impaired since the overall affinity was dramatically reduced mainly since the off-rate was severely affected.

1. Introduction

Wilms tumor is a child kidney malignancy that occurs with a frequency of 1:10000 [1]. In about 10% of the patient cases, mutations have occurred in the Wilms tumor suppressor gene (*WT1*). The *WT1* gene is located on the chromosome 11p13 and encodes a C_2H_2-type zinc finger protein (ZFP) that acts as a tumor suppressor [2,3]. The protein has a proline- and glutamine-rich regulatory domain (N-terminus) linked to a domain containing four zinc fingers of the Krûppel-type (C-terminus) [4,5]. In normal conditions WT1 regulates a vast network of genes during development of the kidney and the genitourinary system [2,3,6,7]. There is increasing evidence that WT1 also has an oncogenic role in a number of hematological malignancies and solid tumors, and this aspect is being exploited in cancer immunotherapies [8,9]. WT1 has over 20 known gene targets: some of these are activated while others are repressed. Some of these are identified as real target genes *in vivo* (see Supplementary Table 1). The mechanism that selects the targets and decides the type of regulation is not yet characterized, and is believed to depend on the cellular context [10,11]. It has been established that WT1 binds to the early growth response factor 1 (EGR1) consensus binding site 5′-GCG-(T/G)GG-GCG-3′. Subsequent studies have demonstrated a stronger binding to a series of longer sites summarized as 5′-GCG-(T/G)GG-GCG-(T/G)(A/G)(T/G)-3′ [12–14]. This 12-base pair site describes better the binding behavior of the four zinc fingers, where each ZF binds to a 3-base pair triplet [15]. Although there is a growing body of evidence showing that ZFPs, WT1 in particular, are capable of target regulation at the RNA level as well [16], no binding models and consensus sites have so far been characterized but recently the first cellular 34-basepair RNA target for WT1 ACT34 has been identified [17].

ZFPs are a class of regulatory proteins directly involved in a variety of cellular activities: among others development, differentiation and tumor suppression. Functionality of ZFPs is achieved through interacting specifically or non-specifically with DNA, RNA and even proteins. A zinc finger (ZF) is a small protein module with a special secondary structure stabilized by a zinc ion coordinated by Cys and His residues. Each ZF module can act independently in the course of DNA binding, although synergy between modules is also seen. Classification of ZFs depends on the different use of these residues

Abbreviations: B1H, Bacterial 1-Hybrid System; ZF, Zinc Finger; ZFP, Zinc Finger Protein; WT1, Wilms Tumor 1 Protein.

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in zinc binding. WT1 belongs to the C_2H_2 class, which is mainly involved in transcription of target genes. [18] This category of ZFPs is estimated to comprise as much as 1% of total mammalian proteins.

Sequence alignment (Fig. 1) of the four ZFs of WT1 reveals that ZF1 differs substantially from the other three ZFs. Using a recognition code table based on the observation that the amino acids at positions −1, 2, 3, and 6 of the alpha helix within a ZF are responsible for determining the DNA-binding specificity [19], the DNA consensus sequence 5′-GCG-(T/G)GG-GCG-3′ could be determined to bind to ZFs 2-4 of WT1. However the amino acids found at the recognition positions in ZF1 are unconventional for ZFs and therefore no DNA sequence preference can be deduced with certainty for ZF1 using the recognition code table. In the search for a consensus for the binding site of ZF1, 28 potential WT1-binding sites from 12 human gene promoters were aligned. Although the results (Fig. 2) suggest that the binding site for ZF1 has a poor consensus sequence, guanine seems to be preferred at most of the triplet positions. Furthermore, some authors find evidence for a reduction in the DNA-binding activity of ZF1-deleted WT1 by as much as 90% in comparison to that of wild-type [14]. A role for ZF1 in RNA binding rather than DNA binding has also been proposed. A recent *in vivo* study on *Xenopus laevis* oocytes has reported that although both WT1 (+KTS) and WT1 (−KTS) were found bound to RNA transcripts, binding of a construct in which ZF1 was deleted was seriously weakened [16]. This suggests a probable involvement of ZF1 in locating and binding to RNA targets of WT1.

A second unusual aspect of WT1 is the translational/transcriptional regulation of the gene itself. Due to the existence of several different start-sites, alternative splice options and post-translational modifications, more than 24 isoforms of WT1 have been detected in mammals. The most common isoforms are the products of alternative splicing of the immature WT1 mRNA at exon 5 and exon 9, which produce 4 distinct isoforms with 17 and 3 amino acid insertions, respectively. The latter is of particular interest, since this encodes the KTS insertion into the linker between ZFs 3 and 4 which has been reported to decrease the binding to the DNA consensus sequence [5]. NMR experiments show that the KTS insertion relaxes the flexibility of the linker between ZFs 3 and 4, and may in fact reduce or abrogate binding of ZF 4 to its DNA targets [20]. On the other hand, the insertion of KTS differentially affects the affinity of WT1 for its single-stranded RNA targets — an interaction pattern, which is not yet understood [18,21].

Hence, the effects of the KTS insertion as well as the unconventional amino acid sequence within ZF1 on DNA, RNA and protein binding remain to be fully characterized. A wide spectrum of WT1 research is being conducted on biochemical, structural, immunological and cellular levels. The primary goal of a number of previously reported studies on WT1 kinetics has been to characterize the effects of various system conditions (pH, temperature, ionic strength etc.) on binding to different DNA [22] and RNA [21] targets. Despite this, there is as yet no accurate understanding of which ZFs undertake which specific roles in effective binding to the DNA and RNA targets in various physiological contexts.

In a previous study, the full-length C-terminal of WT1 was expressed and purified, and interactions between the −KTS and +KTS isoforms

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                -1123456789
ZF1  FMCAYPGCNKRYFKLSHLQMHSRKHTGEKP
ZF2  YQCDFKDCERRFFRSDQLKRHQRRHTGVKP
ZF3  FQC——KTCQRKFSRSDHLKTHTRTHTGKTSEKP
ZF4  FSCRWPSCQKKFARSDELVRHHNMH
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Fig. 1. Alignment of the ZFs (1–4) of WT1. Numbers (−1 to 9) indicate amino acid positions within the α helices; highlighted positions are involved in specific binding to DNA. Asterisks are the amino acid positions significantly different in ZF1. Shown in bold are conserved cysteines and histidines, which coordinate Zn^{+2} ion. The *KTS* segment within the linker between ZF3 and ZF4 is the insertion that creates the two isoforms (WT1-KTS and WT1+KTS).

and the consensus DNA were observed using qualitative electrophoretic mobility shift assays [23]. Here, we report a dual study where, using six different truncations of the WT1 ZF domain, we attempt to describe the DNA-binding characteristics of WT1. In order to look at the DNA-binding site preferences of ZF1, a recently developed Bacterial 1-Hybrid System (B1H) [24] has been used. Subsequently, surface plasmon resonance studies (SPR), in which the various WT1 constructs were probed against DNA consensus sites, were performed. The technique is one of the newer methods used for assessment of DNA-binding kinetics; it has been applied successfully for analysis of protein–DNA interactions [22,25–32]. It enables extraction of the kinetics parameters k_{on} (association rate constant), k_{off} (dissociation rate constant) and K_D (equilibrium dissociation constant, $K_D = k_{off}/k_{on}$). Altogether, this data leads to a clearer and more robust understanding of the interactions between WT1 and DNA.

2. Materials and methods

2.1. Reagents and instrumentation

The chemicals used in B1H and SPR studies were of molecular biology grade and were purchased from Sigma (MO, USA). Restriction enzymes were purchased from New England Biolabs (Beverly, USA). Chemically synthesized DNA oligonucleotides were ordered from TAGC A/S (Copenhagen, Denmark). Components of the B1H system, reporter vector pH3U3, expression vector pB1H1 and the US0Δ*hisB*Δ*pyrF E. coli* selection strain, were a generous gift from University of Massachusetts Medical School. A BIACORE 3000 machine was used to perform all interaction assays. CM4 chip, EDC, NHS and ethanolamine were purchased from Biacore AB (Uppsala, Sweden). Streptavidin was from Pierce (IL, USA).

2.2. Cloning and purification of WT1 ZF truncations

Six different WT1 ZF truncations were generated for B1H and SPR studies: ZF14+, ZF14−, ZF24+, ZF24−, ZF13 and ZF23. The previously described constructs 6HIS-ZN+$_{wt1}$ and 6HIS-ZN−$_{wt1}$ were used as templates (Fig. 3, Table 1). The cloning steps, along with procedures for expression, purification and refolding of the proteins were as described previously [23]. For the purpose of the B1H study, the US0Δ*hisB*Δ*pyrF E. coli* selection strain was transformed with the ZF14− and ZF24− constructs. However, for the SPR measurements, the expressed proteins were further refolded, concentrated and transferred into the buffer containing 20 mM Tris–HCl pH 7.5 and 150 mM KCl, using a Superdex 75 gel filtration column. For all protein samples used in this study, the extinction coefficients were calculated individually according to Gill and Hippel [33] and the protein concentration was measured on a Lambda EZ210 spectrophotometer (PerkinElmer, USA) at a wavelength of 280 nm. Concentrations were additionally measured separately using Bradford [34] and micro Lowry methods [35]. After these procedures we obtained protein analytes with ≥95% purity.

2.3. B1H: design of binding site library

The binding site library was designed with a single oligonucleotide 5′-ATACATAGAT GCGGCCGC ATATA GCGTGGGCG NNNN ATATA GGCGCGCC ATAC-3′. Binding sub-sites for the WT1 ZFs 2–3–4 were kept constant, whereas a degenerate binding sub-site 5′-NNNN-3′ was provided for ZF 1. NotI and AscI restrictions sites were designed on the 5′- and 3′-end respectively, and 5′-ATATA-3′ spacers were provided to sufficiently expose the WT1 binding site and to separate it from the restriction sites. The library was extended with a primer 5′-GTATGGCGCGCCTATAT-3′ as described previously [36], and purified using the QIAquick nucleotide removal kit (Qiagen). The theoretical complexity of the library is calculated to be 256.