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Original paper

Kinetic behaviour of WT 1's zinc finger domain in binding to the alpha-actinin-1 mRNA

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ABSTRACT

The zinc finger transcription factor Wilms tumour protein (WT 1) is known for its essential involvement in the development of the genitourinary system as well as of other organs and tissues. WT 1 is capable of selectively binding either DNA or mRNA targets. A KTS insertion due to alternative splicing between the zinc fingers 3 and 4 and an unconventional zinc finger 1 are the unique features that distinguish WT 1 from classical DNA-binding C_2H_2-type zinc finger proteins. The DNA binding characteristics of WT 1 are well studied. Due to lack of information about its native RNA targets, no extensive research has been directed at how WT 1 binds RNA. Using surface plasmon resonance, this study attempts to understand the binding behaviour of WT 1 zinc fingers with its recently reported and first putative mRNA target, ACT 34, whose stem-loop structure is believed to be critical for the interactions with WT 1. We have analysed the interactions of five WT 1 zinc finger truncations with wild-type ACT 34 and four variants. Our results indicate that WT 1 zinc fingers bind ACT 34 in a specific manner, and that this occurs as interplay of all four zinc fingers. We also report that a sensitive kinetic balance, which is equilibrated by both zinc finger 1 and KTS, regulates the interaction with ACT 34. The stem-loop and the flanking nucleotides are important elements for specific recognition by WT 1 zinc fingers.

Introduction

The Wilms tumour suppressor gene *WT 1* was cloned because of its association with WAGR syndrome (Wilms tumour, Aniridia, Genitourinary tract malformations, and mental retardation). A deletion in chromosome 11 associated with WAGR led to the discovery of a gene at 11p13 locus that encodes a C_2H_2 ZFP (zinc finger protein) characteristic of the Krüppel or EGR family of transcription factors [1–4]. WT 1[1] was subsequently shown to be mutated in up to 15% of Wilms tumours (nephroblastoma), a paediatric malignancy of the kidney, which affects 1:10,000 children [5]. One and a half decades since its discovery, it is increasingly apparent that *WT 1* is a complex gene; much remains to be learned about the biological functions and biochemical properties of WT 1 [1–4].

Soon after its discovery, a mouse knock-out model confirmed that *WT 1* is essential for urogenital development; and that kidneys failed to develop with fulminant apoptosis of the mesenchymal cells that would normally condense around ureteric buds [6–8]. More recent *ex vivo* RNA interference experiments performed on mouse fetal kidney explants demonstrated that *WT 1* is required at multiple stages of kidney development including nephrogenesis [9]. In the adult WT 1 expression persists in glomerular podocytes; mutations in *WT 1* are associated with severe nephropathy and misexpression of podocyte-specific genes [10]; and with altered cytoskeletal architecture [11]. Interestingly, WT 1 over-expression is a characteristic of >90% of acute myeloid leukemias and is associated with poor prognosis [12]. Over-expression of WT 1 would at first glance appear paradoxical for a tumour suppressor but it is now clear that in some contexts WT 1 has the hallmarks of an oncogene by counteracting apoptosis. In other contexts (such as Wilms tumour) it behaves as a classical tumour suppressor by repressing the expression of growth factors and their receptors.

At first glance, WT 1 has the properties of a typical transcription factor: a proline-glutamine rich regulatory domain in the N-terminus and four ZFs (zinc fingers) in the C-terminus [13]. That WT 1 should work as a transcription factor is well established, and it does so by acting as an activator, co-activator or suppres-

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[1] *Abbreviations used:* SPR, surface plasmon resonance; WT 1, Wilms tumour 1 protein; ZF, zinc finger; ZFP, zinc finger protein.

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sor of a substantial list of candidate target genes [14,15]. In its transcription factor roles WT 1 principally binds the early growth response factor (EGR) GC-rich consensus-binding site [16,17].

One particular splicing event is evolutionarily conserved in all vertebrate species that express WT 1: the 'KTS splice variant'. This variant arises from an alternative splice donor site (in exon 9 in humans) that results in the insertion of three amino acids, KTS (lysine–threonine–serine), between the third and fourth ZFs. KTS isoforms exhibit different DNA binding properties *in vitro* [18]. NMR experiments show that the KTS insertion relaxes the flexibility of the linker between ZFs three and four, and may in fact reduce or abrogate binding of ZF four to its DNA targets [19]. Other members of the EGR family of transcription factors do not have a similar KTS (or other) insertion in between ZFs and mostly tend to have three ZFs only. Another unique aspect of WT 1 is the fact that the critical amino acids in the α-helix of WT 1's ZF1 are unconventional. Indeed, our recent dual study shows that ZF1 interacts with DNA unspecifically and that the KTS insertion can reduce WT 1's affinity for DNA by dislocating it from its binding frame [20].

Might WT 1 be more than just a transcription factor? In the mid-1990s, the Hastie group reported that +KTS isoforms co-localise and co-precipitate with splice factors [21]. WT 1 interacts with the splice factor U2AF65 and is associated with actively splicing pre-mRNAs [22]; is present in mRNP particles *in vivo* [23]; and shuttles from nucleus to cytoplasm [24]. WT 1 also binds RNA *in vitro* [25]. RNA aptamers were defined *in vitro* using ZFs in the (−KTS) configuration [26]. Follow-up studies with RNA aptamers show that WT 1 (−KTS) binds RNA with K_D in the range of approximately 14–87 nM (higher than its affinity for DNA). WT 1 (+KTS) binds the same RNAs albeit with reduced affinity [27]. Initial RNA binding studies also suggested that ZF1 is important in RNA binding [25]. Interestingly, transplanting WT 1's ZF1 to the transcription factor EGR1 creates a chimeric molecule that has acquired RNA binding properties [28]. A very recent study also confirms significance of ZF1 as well as ZF4 in RNA binding [29]. Thus it is clear that a complex picture has emerged of overlapping DNA and RNA binding activities.

It is still unclear what WT 1's roles are in regulating gene expression at the RNA level. In 2006, a first breakthrough was reported: WT 1 (+KTS) was shown to promote the export and translation of an HIV-based construct with a retained intron. The effects of WT 1 (+KTS) were mediated via cellular RNA Constitutive Transport Elements identified in a screen; this was the first report of a specific post-transcriptional activity mediated by WT 1 [30]. A yeast-three-hybrid screen of RNA that co-precipitated with WT 1 identified ACT 34, a 34-nt sequence derived from alpha-actinin 1 mRNA located around the native start codon. This sequence is thought to form a stem-loop and interacts preferentially with WT 1 (+KTS) in the yeast-three hybrid system [31]. Although its *in vivo* significance remains to be investigated, it is interesting to note that mutations in WT 1 severely affect the cytoskeletal architecture of glomerular podocytes [11]. Alpha-actinin 1 represents WT 1's first candidate cellular (*in vivo*) mRNA target.

The aim of this study was to study the binding kinetics of WT 1 ZFs in the −KTS and +KTS conformation, using ACT 34 as a model ligand. ACT 34 consists of a presumed stem-loop structure, whose parameters such as length and nucleotide content are likely to be important. By altering several nucleotides, such as those around the native start codon (AUGG) inside the stem-loop, we wished to ascertain the dependence of WT 1–RNA interaction kinetics on the size and content of the stem-loop. Another goal of this study was to characterize the contribution of ZF1 and the KTS insertion to the kinetics of this interaction.

Materials and methods

Reagents and instrumentation

A BIACORE 3000 machine was used to perform all interaction assays. CM4 type chips, the reaction surfaces used in the study, were purchased from Biacore AB (Uppsala, Sweden). 1-Ethyl 3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS) and ethanolamine used for amine coupling chemistry were purchased from the same supplier. Streptavidin was from Pierce (IL, USA), and chemically synthesized RNA aptamers were from Sigma Proligo (Paris, France). All the other chemicals used in this study were from Sigma (MO, USA). Bradford reagent was from Bio-Rad (CA, USA) and the micro Lowry kit was from Sigma. WT 1 ZF truncations were produced in our lab, as described below.

Design of RNA aptamers

Six 5′-biotinylated RNA aptamers were used in this study: the wild-type ACT 34, four mutants and one negative control – as shown below. ACT 34 is the conserved sequence from the first RNA target of WT 1 – alpha-actinin 1; here the AUGG sequence that is believed to be crucial for formation of the stem-loop structure is intact. In the four mutants (Fig. 1), AUGG was altered to introduce variations into size and content of both the stem and the loop – in order to study the significance of these parameters on the WT 1–RNA interaction. The negative control was a randomly generated RNA whose sequence was dissimilar from that of ACT 34. Folding

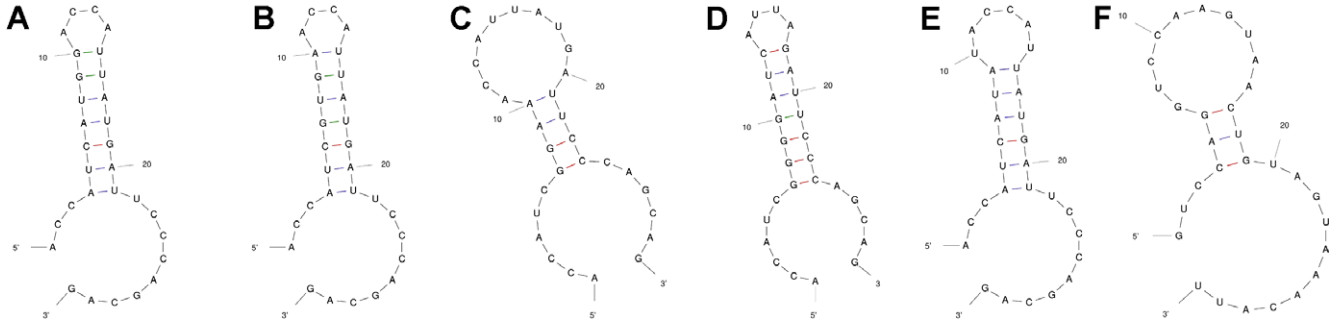

Fig. 1. Description of the RNA aptamers (used as ligands). All of the aptamers have a single conformational possibility and negative free energy of folding (Δ*G*). (A) AUGG is intact. The size and content of the stem and loop are wild-type. (B) AUGG is changed to GUGA. The stem length is the same; the content is different; the loop is the same. (C) AUGG is changed to GGAA. The stem length is shorter, the content is different; the loop is considerably larger. (D) AUGG is changed to GGGG. C at position 12 in ACT 34 is modified to U. U at position 18 in ACT 34 is deleted. The stem length is the same, the content is different; the loop size is the same, content is different. (E) AUGG is changed to AUAU. The stem is shorter, the content is different; the loop is larger. (F) Randomly selected negative control.