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An RNA aptamer that binds to the β-catenin interaction domain of TCF-1 protein

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Abstract

The architectural transcription factor TCF-1 interacts directly with β-catenin and activates transcription of various target genes that are important for early development and carcinogenesis. We selected an RNA aptamer that specifically bound to the β-catenin-interacting N-terminal motif of TCF-1. Structural analysis revealed that it formed a stem–loop structure that was responsible for binding TCF-1 and contained a pair of internal loops. The RNA aptamer interfered with the binding of TCF-1 to β-catenin and also inhibited the formation of TCF-1/β-catenin complexes. Disruption of TCF-1/β-catenin complexes could alter the transcriptional activity of TCF-1. Taken together our observations show that a rationally designed RNA aptamer can disrupt protein–protein interactions required for the formation of an active transcription complex.

Keywords: TCF-1; β-Catenin; RNA aptamer; Protein–protein interaction

T-cell factor (TCF) family proteins are DNA binding transcription factors that bind to a potent transcriptional activator, β-catenin, and lead to the activation of various target genes [1–4]. TCF/β-catenin target genes, such as cyclin D1 and c-myc, are implicated in the development of cancers [5–8]. Other β-catenin-activated target genes are also critical in early developmental processes such as axis formation in the *Xenopus* embryo and Wingless signaling in *Drosophila* [9–11]. Among the TCF family of proteins, TCF-1 is involved in early T-cell development and the expansion of T-lymphocytes [12,13]. Most importantly, the interaction between TCF-1 and β-catenin is critical for the proliferation and apoptosis of diverse cell types [14,15].

Since TCF proteins are architectural proteins for the assembly of other transcription factors, fine-tuning of their transcriptional activity is likely to involve combinatorial interactions. In fact, TCF proteins interact with transcriptional co-repressors such as Groucho, and binding of β-catenin to the resulting complex leads to transcriptional activation [16]. Therefore, TCF-mediated transcription could be regulated either by inhibiting DNA binding or by interfering with its interactions with transcriptional regulators. In particular, disruption of the TCF and β-catenin interaction in active transcriptional complexes is likely to be an efficient way of affecting subsequent target gene expression. In fact, it has been suggested that the TCF/β-catenin complex is an excellent target for chemotherapy [17].

RNA aptamers are ligands that bind to target proteins; they are generated from random RNA libraries by iterative in vitro selection procedures [18,19]. Because RNA aptamers provide an extensive surface for binding to proteins, they generally have high affinity and exquisite specificity. Therefore, an RNA aptamer designed to bind to a critical protein interaction domain should

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block the corresponding interaction. We previously described an RNA aptamer that bound to the C-terminal DNA binding domain of TCF-1 [29] and showed that it interfered with TCF-1 binding to DNA. Since the formation of the TCF/β-catenin complex involves interaction between the N-terminal region of TCF-1 and the armadillo repeats of β-catenin, we reasoned that an RNA aptamer targeting the N-terminal motif of TCF-1 would be valuable for regulating β-catenin-activated transcription. Here we report the selection and characterization of an RNA aptamer that binds to the β-catenin interaction domain of TCF-1 and disrupts the interaction between the two proteins.

Materials and methods

Plasmids and reagents. The mouse TCF-1b was introduced into the *Eco*RI site of the pcDNA3.1 vector [21]. The mouse TCF-4b DNA was kindly provided by Dr. Shivdasani (Dana Farber Cancer institute, Harvard Medical School). pCAN-β-catenin that encodes β-catenin was kindly provided by Dr. McCrea of the University of Texas M.D. Anderson Cancer Center. LipofectAMINE was purchased from Invitrogen. Polyclonal antibody against TCF-1 protein was raised by injecting a TCF-1 N-terminal fragment (amino acids 3–103) into a rabbit (a generous gift from KOMA Biotech). Goat polyclonal anti-β-catenin antibody (C-18) was purchased from Santa Cruz Biotechnology.

Preparation of TCF proteins. Recombinant TCF-1 proteins were obtained by PCR cloning (amino acids 5–100 for N100; 188–388 for C200; and 5–388 for full-length TCF-1 protein) into pGEX4T-2 vector. The TCF-1 N100 DNA fragment was amplified with 5′ primer (5′-TTTGAATTCTTGATTCTGGCGGGGGCGGC-3′, *Eco*RI site) and 3′ primer (5′-ACAGAAGCTTGAAGTTTGTCCGGGAAAG-3′, *Hin*dIII site). The PCR product was digested with *Eco*RI and *Hin*dIII, ligated into pBluescript SKII (+), and subsequently cloned into pGEX 4T-2 plasmid using the *Eco*RI and *Xho*I restriction sites. The cloning of TCF-1 C200 was previously described [20]. Full-length TCF-1 was amplified with the same 5′ primer as above and 3′ primer (5′-ATAT GTCGACCACTGTCATCGGAAGGAA-3′). Recombinant TCF-4 proteins were also obtained by PCR cloning (amino acids 5–53 for TCF-4 N50; 41–321 for TCF-4 M, Middle). The TCF-4 N50 fragment was amplified with 5′ primer (5′-TAAGAATTCAACGGCGGTGGA GGAGAT-3′, *Eco*RI site) and 3′ primer (5′-GTTCTCGAGACTA AGTAACTG-3′, *Xho*I site). The TCF-4 M fragment was amplified with 5′ primer (5′-CCTGAATTCGTCAAGTCCTCGCTG-3′, *Eco*RI site) and 3′ primer (5′-GGCGAGCTCAAGGAAAAACCTCAG-3′, *Xho*I site). Transformants harboring recombinant GST TCF-1 and TCF-4 fusion proteins were induced with 0.1 mM IPTG at 37 °C for 3 h and the cells were lysed in buffer (100 μg/ml lysozyme, 1 mM PMSF, 1 mM DTT, and 2% Triton X-100) followed by sonication three times for 5 min.

In vitro selection and RNA binding assays. A DNA library consisting of random 50 nucleotide sequences was synthesized by Integrated DNA Technologies (USA). The preparation of the RNA library

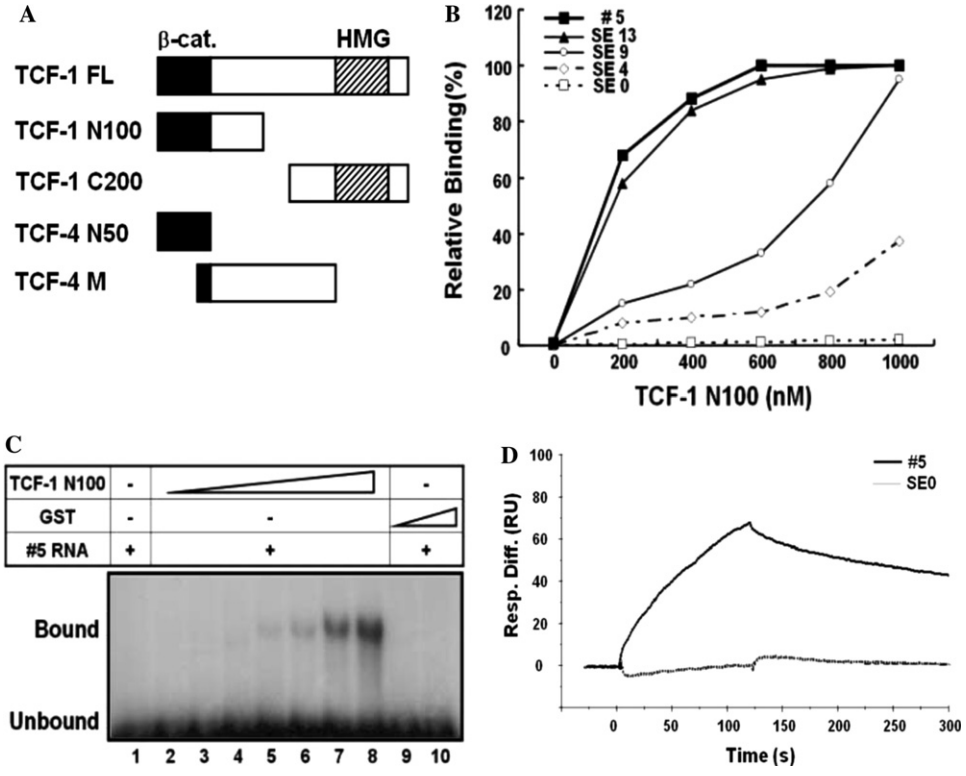

Fig. 1. Enrichment of TCF-1 binding RNA aptamers. (A) Diagram of various GST-TCF proteins. β-Catenin binding domain (β-cat.); DNA binding HMG domain (HMG). (B) Binding of various RNAs to TCF-1 N100 protein. Binding of the RNA library (SE0), RNA from cycle 4 (SE4), cycle 9 (SE9), and cycle 13 (SE13), and RNA aptamer #5 (#5) is shown. (C) RNA-EMSA of radiolabeled RNA aptamer #5 binding to TCF-1 N100 protein. RNA–protein complexes were resolved on a 5% polyacrylamide gel. Lane 1, no protein; lanes 2–8; 5, 10, 20, 50, 100, 200, and 500 nM TCF-1 N100 protein; and lanes 9 and 10; 200 and 500 nM GST protein. (D) Surface plasmon resonance (SPR) binding profiles of library RNA (SE0) and RNA aptamer #5 (#5).

has been previously described [22]. In each cycle, RNA was pre-incubated with 5 μg GST proteins in 200 μl binding buffer (100 mM Hepes (pH 7.5), 75 mM NaCl, 1 mM $MgCl_2$, and 5 mM dithiothreitol) and 40 U RNase inhibitor for 30 min at room temperature to pre-clear RNA that bound to GST or beads. After 13 cycles of selection, the enriched RNA was converted to DNA and cloned into pUC19. Twenty independent clones were sequenced, and RNA-EMSA was employed as an RNA binding assay. The basic procedure for the RNA binding assays was similar to that previously described [23]. A BIAcore 3000 was used in surface plasmon resonance (SPR) experiments, as previously described [23]. After pre-activation, the TCF-1 N100, TCF-1 C200, and GST proteins were injected into the flow cells.

RNase footprinting assays. In vitro transcribed RNA was end-labeled as described [22]. The labeled RNA (280 nM) was denatured and renatured in the binding buffer, and various concentrations of TCF-1 N100 protein were added and the mixtures were incubated at 37 °C for 10 min. They were then incubated with RNase T1 (0.01 U) or RNase S1 (0.4 U) for 10 min at 37 °C, and with RNase V1 (0.0004 U) for 15 min at room temperature. Samples were run on 12% polyacrylamide/7 M urea gels in 1× TBE for 4 h at 1500 V and analyzed with a phosphorImager (FUJIX Bio Image Analyzer System). The radioactivity of each band was measured with Image Gauge 3.3 software, and the percentage of the total radioactivity in each band was calculated. Differential cleavage is presented in the form of $\log(fa) - \log(fc)$, where fa is cleavage in the presence of TCF-1 N100 protein, and fc cleavage in the absence of TCF-1 N100 protein.

Co-immunopreciptation assays. Recombinant TCF-1 and β-catenin proteins were incubated with anti-TCF-1 antibody (1 μg), protease inhibitor cocktail, and RNase inhibitor in 400 μl binding buffer in the presence or absence of RNA for 2–3 h at 4 °C. Protein G–Sepharose beads (20 μl) were then added to the binding reactions and incubation was continued for another 12–16 h at 4 °C, with end-over-end mixing. TCF-bound immunoprecipitates were centrifuged and washed 3–5 times with 400 μl binding buffer. Proteins were extracted from the beads by resuspending in 2× sample buffer and electrophoresed on 12% SDS–polyacrylamide gels. Immunoblotting was performed with anti-TCF-1 or anti-β-catenin antibodies.

Results

Specific binding of the RNA aptamer to TCF-1 proteins

In order to generate RNA aptamers for TCF-1 protein, we carried out in vitro selection with GST-fused TCF-1 protein. We used the N-terminal 100 amino acids of TCF-1 protein (TCF-1 N100) as the target protein because it contains the β-catenin interaction motif (Fig. 1A). The selected RNA bound to the protein after four cycles of selection (SE4; Fig. 1B), whereas the original RNA library (SE0) did not bind to a detectable level. Stronger binding was observed after 9 cycles of selection (SE9), and enrichment of TCF-1 binding RNA molecules was even clearer with RNA after 13 cycles (SE13). Individual RNA molecules were isolated from the SE13 RNA pool, and an RNA-EMSA was performed with each clone. One of the cloned RNAs (RNA aptamer #5) was chosen for further analysis (Fig. 1C). Enhancement of binding was also shown by SPR analysis (Fig. 1D), and we estimated the binding affinity of RNA aptamer #5 to be around 100 nM.

The specificity of RNA aptamer #5 binding was confirmed by competitive RNA-EMSAs (Fig. 2A). Binding was competed by excess RNA aptamer #5, but not by the random RNA library (SE0) or by other unrelated RNAs. Since the aptamer had been selected with TCF-1 as a target, we tested whether it also bound to the homologous TCF-4 protein. As shown in Fig. 2B, it bound to TCF-1 N100, but not to TCF-4 N50 or to TCF-4 (TCF-4 M). Moreover, it did not bind to other

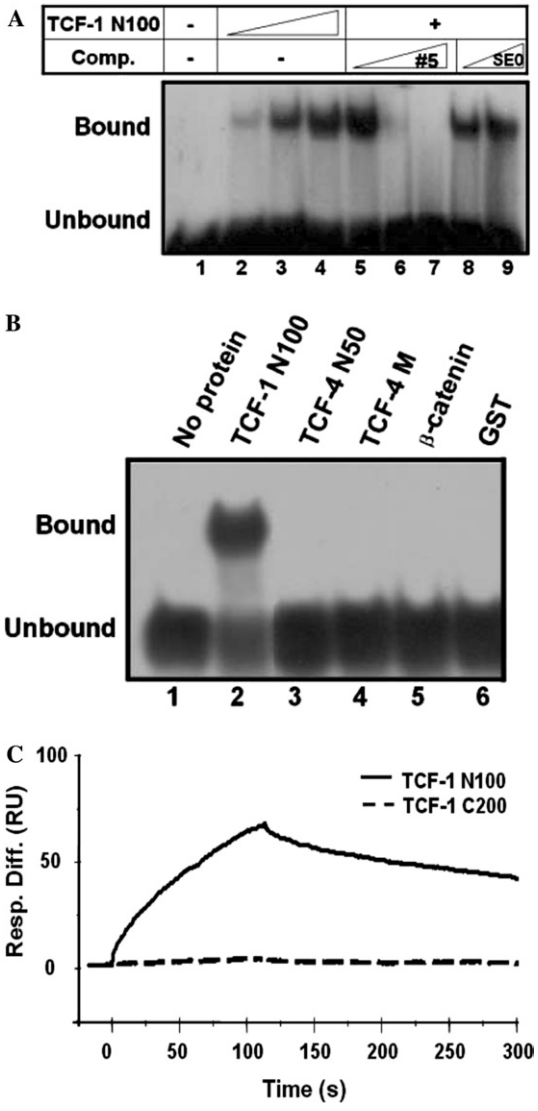

Fig. 2. Specificity of RNA aptamer #5 binding to TCF-1 N100 protein. (A) RNA-EMSAs with unlabeled RNA competitors. Lane 1, labeled RNA aptamer #5 only; lanes 2–4, with TCF-1 N100 protein (100, 200, and 500 nM); lanes 5–7, with TCF-1 N100 (400 nM) in the presence of self-RNA aptamer (#5, 40, 400, and 4000 nM) as competitor; and lanes 8 and 9, in the presence of the library RNA (SE0, 400 and 4000 nM) as competitor. (B) RNA-EMSAs of RNA aptamer #5 with various proteins (500 nM). Lane 1, no protein; lane 2, TCF-1 N100; lane 3, TCF-4 N50; lane 4, TCF-4 M; lane 5, β-catenin; and lane 6, GST. (C) SPR analysis of RNA aptamer #5. Binding profiles of RNA to TCF-1 N100 protein (solid line) and to TCF-1 C200 protein (dotted line) are shown.

proteins, such as the TCF-1-interacting transcription factor, β-catenin, or the unrelated GST protein. The specificity of binding was also confirmed by SPR analysis with TCF-1 N100 and another region of TCF-1, C200 (Fig. 2C). Thus, RNA aptamer #5 binds to the N-terminal domain of TCF-1, but not to other domains of TCF-1 or to the same domain of the homologous protein, TCF-4.

Structural analysis of the RNA aptamer

To understand the molecular basis of the interaction between TCF-1 and RNA aptamer #5, RNase footprinting analysis was performed (Fig. 3A). RNA was end-labeled and digested with G nucleotide-specific RNase T1, single-strand-specific RNase S1, and double-strand-specific RNase V1. In general, the secondary structure of the aptamer agreed well with that predicted by the mfold program, as shown in Fig. 3B. Most of the selected sequences (nucleotides 16–65) folded into a long stem–loop structure with a large internal loop and a couple of small bulges or loops. The TCF-1 binding site was mapped by identifying the sequences protected from RNases by TCF-1. Quantitative analysis of each band showed that nucleotides 36–47, and 53–63, were strongly protected from RNases T1 (Fig. 3C). Interestingly, these nucleotides form the stem structure with alternating internal loops, whereas the single-stranded loop (nucle-

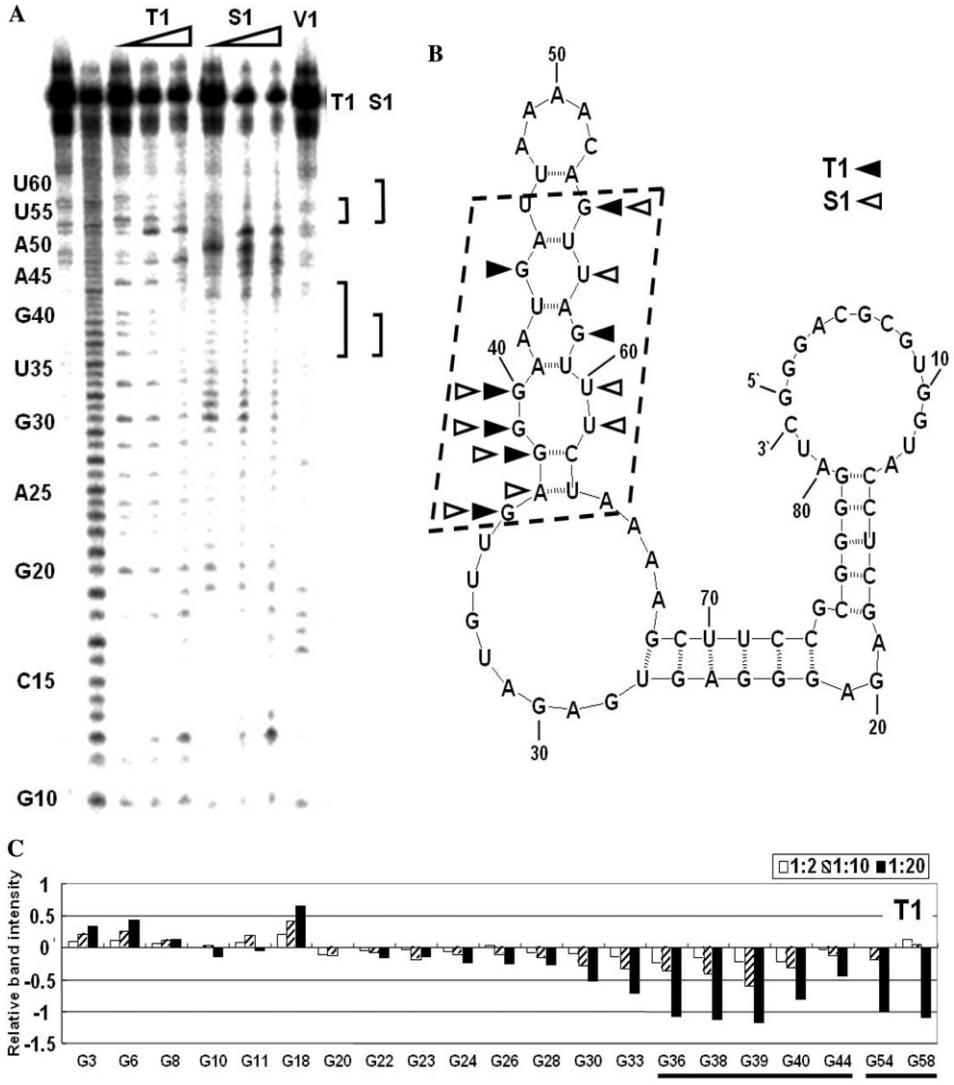

Fig. 3. Structural determination and mapping of the TCF-1 binding site of aptamer #5. (A) Footprinting analysis of RNA aptamer #5 binding to TCF-1. Lane 1, RNA aptamer #5, no treatment; lane 2, alkaline hydrolysis; lanes 3–5, RNase T1 treated, RNA:TCF-1 N100 protein = 1:0, 1:2, and 1:10; lanes 6–8, RNase S1 treated, RNA:TCF-1 N100 protein = 1:0, 1:2, and 1:10; and lane 9, RNase V1 treated RNA only. Nucleotide positions are indicated as numbers on the left; regions protected by the RNases are denoted by brackets on the right side of the gel. (B) Secondary structure of RNA aptamer #5. The binding site for TCF-1 based on the RNase protection pattern is indicated by dotted lines. Filled arrow, RNase T1 protected nucleotides; open arrow, RNase S1 protected nucleotides. (C) Quantitative analysis of each nucleotide. Differential cleavage is shown as described under Materials and methods.

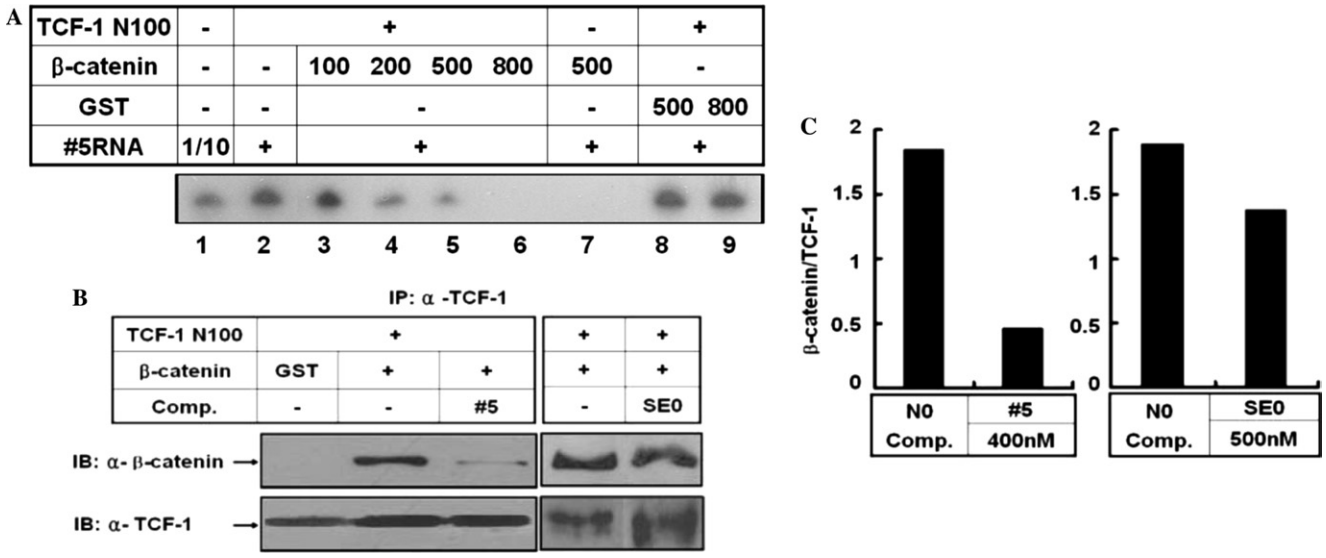

Fig. 4. Disruption of the TCF/β-catenin complex by RNA aptamer #5. (A) RNA binding assay. Radiolabeled RNA (100 pM) was incubated with TCF-1 N100 (200 nM) in the presence of β-catenin or GST protein. TCF-1 N100 was precipitated with anti-TCF-1 antibody (1 μg) and the complexes were subjected to 6% polyacrylamide–7 M urea gel electrophoresis and autoradiography. Lane 1, 10% input RNA; lane 2, TCF-1 N100 (200 nM); lanes 3–6, TCF-1 N100 (200 nM) with β-catenin (100–800 nM); lane 7, β-catenin (500 nM); and lanes 8 and 9, TCF-1 N100 (200 nM) with GST protein (500–800 nM). (B) Co-immunoprecipitation of TCF-1 N100 (50 nM) and β-catenin (50 nM). TCF-1 N100 was mixed either with β-catenin or with GST (50 nM). RNA aptamer #5 (400 nM) or SE0 (500 nM) were included as competitors. The mixtures were precipitated with anti-TCF-1 antibody and subjected to 12% SDS–PAGE gel electrophoresis followed by immmunoblotting with anti-β-catenin or anti-TCF-1 antibodies. (C) Densitometer scanning of the gel in (B).

otides 48–52) was not bound. We may conclude that the stem region of the stem–loop structure is responsible for the binding to TCF-1.

Inhibition of the TCF/β-catenin protein complex

Since RNA aptamer #5 bound to TCF-1 N100 protein, which contains the β-catenin interaction motif, we tested whether it competed with β-catenin for TCF-1 binding. As shown in Fig. 4A, RNA-TCF-1 binding was completely inhibited by an excess of β-catenin, but not by GST protein. This showed that aptamer #5 bound to the β-catenin interaction domain of TCF-1, and so should inhibit the interaction between β-catenin and TCF-1. This expectation was confirmed by the experiment shown in Fig. 4B. The specificity of the effect of the aptamer was confirmed by the absence of competition by the original RNA library (SE0).

Discussion

Since cellular regulation involves interactions between protein domains [24], specific tools for regulating these interactions are critical for modulating cell signaling. Even though chemical compounds can be used for this purpose, small chemicals may not always be effective in disrupting the extensive binding interface between proteins. In contrast, RNA aptamers are excellent for this purpose, because RNA can provide an extensive binding surface for target proteins. Furthermore, their high affinity and exquisite specificity render them exceptional tools for functional proteomic analysis.

Here we showed that the in vitro selected RNA aptamer #5 was able to disrupt protein-protein interaction. Because the target for selection was TCF-1 N100 protein, which contains the β-catenin interaction motif, this aptamer inhibited the interaction between TCF and β-catenin. As the binding affinity of the TCF and β-catenin interaction is around 30 nM [25], further enhancement of its binding to TCF-1 protein is necessary to block the interaction more efficiently. We previously described another TCF-1 binding RNA aptamer that bound to a 200 amino acid fragment of TCF-1 including the DNA binding HMG domain [20]. It was shown to interfere with DNA binding, and expression of this RNA aptamer was also effective in inhibiting TCF-1-mediated transcription in the cells (Park et al., submitted). We envision that a similar approach with RNA aptamer #5 could block binding of β-catenin to TCF-1, thereby specifically reducing β-catenin-activated transcription. We also expect that aptamer #5 may be valuable as a chemotherapeutic agent against TCF/β-catenin-mediated carcinogenesis, as well as a tool for analyzing TCF-specific signal transduction.

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