Intracellular expression of the T-cell factor-1 RNA aptamer as an intramer

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Abstract

T-cell factor (TCF)-1 protein forms the transcriptional complex with β-catenin and regulates the expression of diverse target genes during early development and carcinogenesis. We have selected previously an RNA aptamer that binds to the DNA-binding domain of TCF-1 and have shown that it interfered with binding of TCF-1 to its specific DNA recognition sequences in vitro. As an approach to modulate the transcription by TCF/β-catenin complex in the cells, we have developed the RNA expression vector for stable expression of RNA aptamer inside of the mammalian cells. High level of RNA was expressed as an intramer in the fusion with the stable RNA transcript. The RNA intramer inhibited TCF/β-catenin transcription activity as shown by luciferase assay. It also modulated the expression of TCF/β-catenin target genes, such as cyclin D1 and matrix metalloproteinase-7, as predicted to be as an effective inhibitor of the TCF function. In addition, it efficiently reduced the growth rate and tumorigenic potential of HCT116 colon cancer cells. Such RNA intramer could lead to valuable gene therapeutics for TCF/β-catenin-mediated carcinogenesis. [Mol Cancer Ther 2006;5(9):2428–34]

Introduction

T-cell factor (TCF)-1 is a transcription factor that binds to specific DNA through its high mobility group-1 domain and is likely to be involved in the expansion of T lymphocytes (1–8). TCF family proteins bind to DNA in a sequence-specific manner and they seem to act as architectural proteins for the assembly of other transcription factors (9). β-Catenin is a potent transcriptional coactivator of TCF family proteins and the formation of a transcriptional complex by an oncogenic β-catenin with TCF might be a central event in cancer cell development (10, 11). The TCF/β-catenin complex–mediated Wnt signaling is also a critical regulator of immature thymocyte development as well as of other early development (12–16). It is likely that TCF family proteins are critical modulators of the expression of genes that control the decision between proliferation and apoptosis in the cells, such as cyclin D1 and c-myc, as well as for cancer cell metastasis by matrix metalloproteinase-7 (MMP-7) expression (17, 18).

High-affinity molecules, such as nucleic acid ligands, can modulate the transcriptional activity of transcription factors. Reiterated in vitro selection procedures are able to select specific RNA molecules from random RNA library, and nucleic acids selected by this procedure are generally referred to as "aptamers" (19–21). We have reported previously the in vitro selection of RNA aptamers that bind to TCF-1 and characterized the one of the selected RNA aptamers (#10 RNA aptamer) in vitro. It binds to the TCF-1 protein containing high mobility group-1 domain, thereby interfering with its binding to DNA (22). Here, we have developed an RNA expression vector for this aptamer and overexpressed it in mammalian cells (23). Intracellularly expressed aptamers, "intramers", have advantages of high-level cellular expression and exceptional specificity, which make it possible to distinguish between similar cellular proteins and to differentially modulate their functions (24, 25). We designed the RNA intramer to accumulate large amount of RNA transcript in the fusion with aberrant spliced mRNA. Most importantly, we showed that #10 RNA intramer inhibited the transcriptional activity of TCF-1 in the cells and downregulated the TCF/β-catenin-mediated expression of cyclin D1 and MMP-7. Cell growth rate as well as tumorigenic potential of colon cancer cells was also reduced by stably expressed RNA aptamer. Further refinement of this intramer could provide a way for modulating TCF-1-mediated T-cell development as well as cancer cell development.
Materials and Methods

Plasmids, Proteins, and Reagents

FLAG-tagged TCF-1 expression clone was generated by introducing tcf1 into the EcoRI site of the vector pcDNA3.1. luciferase reporter plasmids, wild-type TCF reporter pGL3-OT and mutant pGL3-OF, were kindly provided by Dr. Shivdasani (Dana-Farber Cancer Institute, Boston, MA). Nuclear factor-κB reporter (3× κB-Luc) and p65 expression clones were from Dr. Bill Sugden (University of Wisconsin, Madison WI). pCAN-κB-catenin was kindly provided by Dr. McCrea (The University of Texas M.D. Anderson Cancer Center, Houston, TX). The control vector was purchased from Clontech (Mountain View, CA). LipofectAMINE was purchased from Invitrogen (Carlsbad, CA) and DMRIE-C was from Invitrogen. α-Nitrophenyl-β-D-galactopyranoside was from Sigma-Aldrich (St. Louis, MO). Anti-FLAG M2 monoclonal antibody and anti-α-tubulin antibody were purchased from Sigma-Aldrich and anti-p65 C20 antibody and anti-cyclin D1 (sc-246) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-κB-catenin antibody was from Transduction Laboratories (San Jose, CA).

Cell Culture, Transfection, and Luciferase Assay

Human embryonic kidney 293T cells, human colorectal carcinoma HCT116 cells and the murine immature thymoma S49.1 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM with 10% fetal bovine serum and antibiotics. 293T and HCT116 cells were transfected with LipofectAMINE and S49.1 immature T-cell line was transfected by electroporation. Stable transfectants of #10 RNA aptamer and #10 inverse RNA were selected by zeocin. For luciferase assays, the luciferase reporter, the RNA aptamer RNA #10 was done with DHFR-F1 and DHFR-R1 (5′-CGGATCCAAAGAAACGGCTTTC-3′ (forward) and 5′-GAGAATTCCAGGTCAGTATCAAA-3′ (reverse), which generates a 334-bp PCR product. PCR primers for MMP-7 were 5′-ATGTTAAACTCCGCGCTCATC-3′ (forward) and 5′-CAGCATACAGGAAGTAAATCC-3′ (reverse), which generates a 481-bp PCR product. Each primer set was amplified at 75°C for 30 seconds, 50°C (cyclin D1) for 2 minutes, 58°C (MMP-7) for 2 minutes, or 54°C (κB-catenin) for 30 seconds and 72°C for 30 seconds for the indicated number of cycles. PCR for aptamer RNA #10 was done with DHFR-F1 and DHFR-R1 primers as described above. An 18S rRNA control primer set (Ambion, Austin, TX) was used as control.

Western Blot Analysis

Cells were harvested and lysed with 2× sample buffer [125 mmol/L Tris-HCl (pH 6.8), 4% SDS, 40% glycerol]. Whole-cell extracts (20 μg) were subjected to 12% SDS-PAGE and the proteins were transferred to nitrocellulose membranes. The membranes were incubated with blocking buffer (5% skim milk, 0.1% Tween 20 in TBS) for 1 hour at room temperature and with anti-cyclin D1, anti-FLAG, anti-κB-catenin, or anti-p65 antibodies for 1 hour at room temperature. The membranes were washed with washing buffer (0.1% Tween 20 in TBS) thrice and incubated for 1 hour at room temperature with horseradish peroxidase–conjugated anti-mouse antibody. Anti-α-tubulin antibody was used for the controls.

Cell Growth Assay and Soft Agar Colony Formation

Cell growth curve was generated by crystal violet staining. Cells were seeded with low cell density (5,000 per 24 wells) in triplicates and allowed to grow for 6 days. In each day, cells were washed with PBS, fixed with 1% glutaraldehyde, and stained with 0.1% crystal violet. After destaining, crystal violet was solved with 1 mol/L acetic acid and A595 was measured. For soft agar colony formation assays, 5,000 cells were seeded in six-well plates with 0.7% agar. After 10 to 20 days, colonies formed were fixed in 70% ethanol, washed with water, and stained with 0.005% crystal violet for 20 minutes.
Results

In vitro Characterization of #10 RNA Aptamer

We have described previously the selection and characterization of TCF-1 binding RNA aptamers in vitro (22). The #10 RNA aptamer was shown to specifically bind to COOH-terminal 200 amino acids of TCF-1 protein, which contains the DNA-binding high mobility group-1 domain. Secondary structure of #10 RNA aptamer was also determined by the RNase mapping (Fig. 1). Because #10 RNA aptamer bound to the DNA-binding domain of TCF-1, we also confirmed that the RNA aptamer interfered with DNA binding by TCF-1 proteins (22).

Intracellular Expression of #10 RNA Aptamer as an Intramer

As a way of overcoming instability and low efficiency of RNA transfection, we have developed a vector expressing stabilized RNA aptamer transcripts in mammalian cells. As shown in Fig. 2A, the expression vector contains DHFR cDNA (831 bp) harboring a cryptic splice site that can accumulate high levels of aberrantly spliced DHFR transcripts (26). The DNA sequence encoding #10 aptamer was inserted at the 5' end of the DHFR cDNA (pDHFR/Aptamer) and was thus expressed as chimeric aptamer-DHFR-RNA (931 nt). The secondary structure of this chimeric RNA was predicted by mfold to retain the specific stem-loop structure of #10 aptamer due to the stable and extensive secondary structure of the aberrant DHFR transcript (23, 26). Inverse orientation of #10 aptamer was also inserted to DHFR vector, generating pDHFR/Inverse.

To evaluate the expression level of #10 RNA intramer in mammalian cells, pDHFR/Aptamer DNA (200 ng) was transfected into 293T cells (2 x 10⁶) and the level of RNA transcripts was measured by RT-PCR analysis. As shown in Fig. 2B, expression of the RNA intramer (#10 aptamer) was higher than that of abundant transcripts, such as glyceraldehyde-3-phosphate dehydrogenase mRNA.

Figure 1. Structure of the RNA aptamer #10 as determined by the RNase mapping (22). Shades, nucleotides from the selected sequence.

Figure 2. Expression of RNA aptamer #10 as an intramer. A, design of the RNA aptamer expression vector, pDHFR/Aptamer. The vector was generated by cloning the DHFR cDNA containing cryptic splice sites into pcDNA. The DNA sequence encoding RNA aptamer #10 (100 nt) was inserted into the 5' end of DHFR cDNA (pDHFR/Aptamer) and was thus expressed as chimeric aptamer-DHFR-RNA (931 nt). B, expression of RNA intramer in 293T cells as shown by RT-PCR. Cells (2 x 10⁶) were transfected with 200 ng pDHFR/Aptamer #10 DNA and harvested after 24 h. Level of the expressed RNA aptamer #10 was compared with that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA by various cycles of RT-PCR. Total RNA (5.7 μg) was prepared from the cells and equal amounts of the RNAs (2 μg) were reverse transcribed into cDNA. One of 20th amount of cDNA was used for each PCR. Lanes 1 and 5, 24 cycles; lanes 2 and 6, 26 cycles; lanes 3 and 7, 28 cycles; lanes 4, 8, and 12, 28 cycles of PCR. D, stability of the transfected RNA aptamer measured by RT-PCR. Lane 1, marker; lane 2, no reverse transcriptase, negative control; lane 3, PCR-positive control; lane 4, RNA from nontransfected cells; lane 5, RNA isolated 0 h after transfection; lane 6, RNA isolated 24 h after transfection.

RNA transcripts was measured by RT-PCR analysis. As shown in Fig. 2B, expression of the RNA intramer (#10 aptamer) was higher than that of abundant transcripts, such as glyceraldehyde-3-phosphate dehydrogenase mRNA.
In addition, the intracellular level of expressed RNA intramer (expressed, diluted at 1:100) was compared with known amounts (0.1 and 0.5 ng) of in vitro–transcribed RNA (Fig. 2C). Because the amount of the diluted RNA intramer was estimated to be 0.2 ng by densitometry, at least 1.08 μg RNA was expressed by the transfected cells ($2 \times 10^7$). Based on the molecular weight of #10 RNA aptamer (3.1 × 10^4 Da) and Avogadro’s number, we calculated that at least $1 \times 10^7$ RNA intramer molecules were expressed per cell. Because we obtained 50% transfection efficiency for 293T cells as determined by the expression of cotransfected green fluorescent protein (data not shown), we expected that $2 \times 10^7$ RNA intramer molecules were expressed in each transfected cell. We were concerned that the #10 aptamer transfected into the cells might be subjected to cellular RNase activities, thus reducing its effect in the cells. Therefore, expression of #10 aptamer was also monitored to confirm the stability of the RNA (Fig. 2D). We usually detect the expression of the RNA intramer for several days, which may provide enough time to be an effective inhibitor in the cancer cells.

Transcriptional Modulation of the TCF-1 Protein by the RNA Intramer

To see if the RNA intramer inhibited the transcriptional activity of TCF-1, 293T cells were cotransfected with the #10 aptamer or #10 inverse along with FLAG-tagged TCF-1 and the TCF-responsive luciferase reporter, pGL3-OT (Fig. 3A). Overexpression of TCF-1 cDNA induced luciferase activity as expected (27). RNA intramer (#10 aptamer) reduced TCF-1-mediated transcription in a dose-dependent manner. This inhibitory effect was sequence specific because RNA intramer #10 in reverse orientation (#10 inverse) did not affect transcriptional activity. Moderate inhibitory effect of RNA intramer could be partially explained by 50% transfection efficiency as determined by green fluorescent protein expression. However, the expression of FLAG-tagged TCF-1 protein was detected by Western blot analysis with anti-FLAG antibody and was not significantly reduced by cotransfected #10 aptamer or #10 inverse (Fig. 3B). In addition, the expression of the RNA intramers did not alter the morphology of the cells (data not shown). We also tested whether the RNA intramer could modulate the endogenous TCF-1 in immature T cells. As

![Figure 3](http://example.com/figure3.jpg)

**Figure 3.** Modulation of TCF transcriptional activity by intracellularly expressed aptamer DNA. **A,** 293T cells were transfected with FLAG-tagged TCF-1 cDNA and luciferase reporter (pGL3-OT) together with pDHFR (vector), pDHFR/Aptamer #10 (#10 aptamer) or pDHFR/Inverse #10 (#10 inverse). Luciferase activities were measured 24 h after transfection. They were normalized using the β-galactosidase activity from the cotransfected pCMV-β plasmid and are presented as relative fold activities compared with vector-transfected cells. **Columns,** mean of five independent experiments; **bars,** SD. *, $P < 0.005$. **B,** Western blot analysis was done with anti-FLAG antibody to detect TCF-1 protein expression in transfected cells as shown in (A). **C,** S49.1 immature T cells were cotransfected with the luciferase reporters and RNA aptamer #10 by electroporation. Luciferase activities of wild-type TCF luciferase reporter (pGL3-OT; OT) were measured and are presented as relative fold activities compared with that activity obtained with the mutant TCF luciferase reporter (pGL3-GF; OF). Three independent experiments were done. **D,** 293T cells were transfected with p65-expressing cDNA and nuclear factor-κB (NF-κB) luciferase reporter (3 × κB-Luc) together with pDHFR/Aptamer #10 (#10 aptamer). Luciferase activity was measured 24 h after transfection. Relative fold activity was presented compared with nuclear factor-κB reporter–transfected cells. Three independent experiments were done. Expression level of p65 protein was shown by Western blot analysis with anti-p65 antibody.
shown in Fig. 3C, #10 RNA inhibited the TCF-1 transcriptional activity in cells of the immature T-cell line S49.1. The specificity of the #10 aptamer on TCF-1-activated transcription was also confirmed by the luciferase assay with the reporter activated by nonrelated transcription factors nuclear factor-κB in 293T cells (Fig. 3D).

Specific Inhibition of Target Gene Expression and Cell Growth

TCF family proteins are known to interact with the transcriptional activator β-catenin and activate expression of diverse target genes, such as cyclin D1, c-myc, and MMP-7 (17, 18, 28). Because the high-level expression of β-catenin is related to the high level of target gene expression in colon cancer cells, we used human colon carcinoma HCT116 as a test colon cancer cell line. As shown in Fig. 4A, endogenous protein levels of β-catenin and cyclin D1 are high in HCT116 cells as expected. However, transfection of #10 RNA aptamer greatly reduced the cyclin D1 protein level but not by #10 inverse. We also tested the effect of #10 RNA aptamer on the other β-catenin target gene, such as MMP-7, in HCT116 colon cancer cells (28). As shown in Fig. 4B, expression of MMP-7 mRNA was also reduced by the #10 aptamer but not by the #10 inverse (Fig. 4C). Interestingly, transfection of #10 aptamer did not affect the expression of c-myc protein in HCT116 cells (Fig. 4A), suggesting differential effects on different TCF/β-catenin target genes. These results showed that the intracellular expressed TCF-1 binding RNA aptamer could be an effective and selective inhibitor of TCF/β-catenin target genes in colon cancer cells.

Because #10 RNA intramer suppressed the expression of cyclin D1, we tested the effect of the RNA aptamer on the cell growth of colon cancer cell line, HCT116. As shown in Fig. 5A, cell growth was significantly reduced by the #10 RNA intramer (aptamer) compared with that by #10 inverse RNA (inverse). We also examined whether the tumorigenic potential of HCT116 colon cancer cell was suppressed by the stably expressed #10 RNA intramer. As shown in Fig. 5B by colony-forming assay, size of colonies was significantly reduced by the expressed #10 RNA aptamer (Fig. 5B, bottom) compared with #10 inverse (Fig. 5B, top). These results suggested that #10 RNA aptamer can be an effective inhibitor of cell growth as well as tumorigenesis in colon cancer cells.

Discussion

Because TCF/β-catenin-mediated transcription is important regulator for carcinogenesis of diverse cells, notably for colon cancer development, a molecule that could modulate this process would be a useful agent for antitumor therapy (29, 30). Some conventional drugs have been shown to reduce the TCF/β-catenin-mediated transcription in colon cancer cells (31–33) and small-molecule antagonists have been selected from the diverse chemical library to inhibit TCF/β-catenin protein complex (34). We report here the first characterization of an RNA aptamer that inhibits the binding of the transcription factor TCF-1 to DNA, thus modulating the expression of various target genes. Cell growth rate and tumorigenic potential of colon cancer cell line HCT116 were also seemed to be reduced by TCF-1 binding RNA aptamer.

RNA aptamer #10 efficiently reduced the expressions of TCF/β-catenin target genes, such as cyclin D1 and MMP-7, as shown by luciferase assay, RT-PCR, and Western blot analysis (Fig. 4). However, more significantly, we observed no obvious alteration on the protein level of c-myc, another notable TCF/β-catenin target gene. Such a selective regulation of TCF/β-catenin signaling by the RNA aptamer might be of greatest utility if it could modulate the expression of a subset of genes and regulate specific subpathway of Wnt signaling. Because it was proposed recently that cyclin D1 may not be an immediate target of TCF/β-catenin following Apc loss in the intestine (35), it will be interesting to study the mechanism of selective inhibition of transcription by RNA aptamer #10. We observed more significant reduction of transcriptional activity for the endogenously expressed TCF-1 in T-cell lines than for the exogenously overexpressed protein in 293T cells.
Apoptation, A, B, determined for 6 d.

Cell growth was determined by crystal violet staining. Triplicate HCT116 cells were transfected with Aptamer (aptamer) or #10 inverse (inverse) and cell growth was determined for 6 d. HCT116 cells were selected and colony-forming potential was determined. Top, #10 inverse; bottom, #10 aptamer.

Figure 5. Suppression of cell growth and tumorigenesis by #10 RNA aptamer. A, cell growth curve. Cell growth was determined by crystal violet staining. Triplicate HCT116 cells were transfected with #10 RNA aptamer (aptamer) or #10 inverse (inverse) and cell growth was determined for 6 d. B, colony-forming assay. Stable HCT116 cells were selected and colony-forming potential was determined. Top, #10 inverse; bottom, #10 aptamer.

Transcription factors are exceptionally useful targets for therapeutics (36). Antisense oligonucleotides or small interfering RNAs can be used to reduced transcription of a given protein (37), but to selectively inhibit particular functions of a transcription factor, the protein itself must be the target. Nucleic acid aptamers can achieve this because of their high affinity and specificity for the nucleic acid binding pockets of such proteins and in fact an RNA library selection procedure has been used previously to select an Nuclear Factor-κB-binding RNA aptamer (38, 39). Therefore, aptamer technology has been an emerging class of therapeutics (40, 41). Moreover, RNA aptamer can be easily overexpressed by using the RNA expression vector as shown here (25). Nevertheless, further study of the mechanism by which the RNA aptamer inhibits TCF-mediated transcription is needed. The basic protocol using RNA aptamer selection and intramer expression described in this article may well be applicable to other proteins, especially for architectural proteins, even when little is known of their specific transactivation functions.

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References


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