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DNazyme-mediated cleavage of Twist transcripts and increase in cellular apoptosis

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Abstract

DNazymes is a group of catalytic nucleic acids that can be designed to cleave target mRNA molecules in a base-specific way. Twist is a basic helix–loop–helix transcription factor that is involved in the regulation of cellular differentiation and apoptosis. Moreover, it was shown to function in skull development and cause craniosynostosis. DZ-TWT DNazyme was designed to down-regulate Twist expression. The ability of DZ-TWT to cleave mouse Twist mRNA was first shown in a cell-free environment against full-length Twist mRNA. Following transfections of the DZ-TWT in C3H10T1/2 cells, a significant reduction of Twist mRNA levels was observed. This was accompanied by a significant rise in p21 mRNA levels. Finally, DZ-TWT transfections resulted in an increase of cellular apoptosis, demonstrating the importance of Twist in apoptotic pathways. These results prove the usefulness of DNazymes to characterize Twist gene function and further experiments in animals should demonstrate its complete physiological role.

Keywords: DNazyme; Twist; Apoptosis; Basic helix–loop–helix

Catalytic nucleic acids (CNAs) are currently being used as gene function and therapy tools to study unknown gene expression and also develop therapeutic approaches against many diseases, respectively [1–4]. CNAs can be in the form of RNA (ribozymes) or DNA (DNazymes). DNazymes can be designed to cleave theoretically any targeted RNA substrate under physiological conditions [5]. The 10–23 DÍazyme motif is among the ones used to cleave endogenous transcripts [6]. The DNazyme is similar to the hammerhead ribozyme, at least in terms of secondary structure, having two binding arms and a catalytic loop that captures indispensable catalytic metal ions such as Mg^{2+} and Mn^{2+}. The catalytic domain is composed of 15 nt, flanked by two substrate recognition domains (Fig. 1A). The selected target RNA is cleaved at a specific phosphodiester bond, which is located between an unpaired purine and a paired pyrimidine residue [6]. The sequence of the substrate recognition domains is not conserved so it can change in order to bind to different RNA molecules. DNazymes have been used extensively to down-regulate both cellular and viral gene expression [5,7–9].

The Twist protein is a highly conserved transcription factor that belongs to the family of basic helix–loop–helix (bHLH) proteins, which recognizes and binds to the E-box of the promoter of responsive genes [10–12]. Expression of Twist has been implicated in the inhibition of differentiation of multiple cell lineages including muscle [13,14], cartilage [13], and bone cells [15–17]. Multiple mutations in the TWIST gene are responsible for Saethre–Chotzen syndrome, a form of craniosynostosis [18,19]. Twist is expressed in mesoderm and cranial neural cells during embryogenesis [10,20]. Recently, it been shown that Twist is also a key molecule in apoptotic pathways [21,22]. Maestro et al. [22] showed that in C8 mouse embryo fibroblasts, Twist inhibits apoptosis by antagonizing the p53 pathway.

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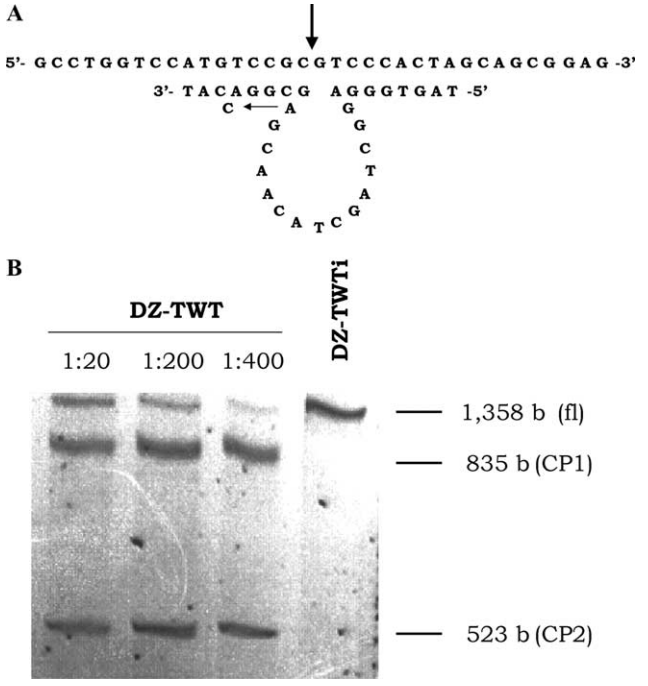

Fig. 1. Design and in vitro cleavage assay of DZ-TWT. (A) DZ-TWT (bottom strand) was designed to bind mouse Twist mRNA (top strand). The catalytic domain of DZ-TWT is flanked by two arms, which were designed to bind the Twist mRNA target and cleave after the GC as shown by the large arrow. An inactive DNazyme version, DZ-TWTi, was also designed which bears a single base mutation in the catalytic domain as shown by the small arrow. (B) DZ-TWT was incubated with the full-length (fl) Twist mRNA at different DNazyme:target mRNA molar ratios. No cleavage products (CP1, CP2) were detected when DZ-TWTi was incubated with the target mRNA.

DNazymes are valuable tools for investigating gene function, such as that of the Twist gene and for developing therapeutic strategies against diseases. Here we report a successful application of DNazymes to cleave endogenous Twist transcripts and increase cellular apoptosis, indicating the importance of Twist in apoptotic pathways.

Methods

Oligonucleotide design and synthesis. DNazyme DZ-TWT was designed to bind bases 770–786 of the mouse Twist mRNA, GenBank Accession No. M63650 (Fig. 1A) and cleave the transcript after base 776 [6]. An inactive version, DZ-TWTi was also designed bearing a mutation in the catalytic domain (Fig. 1A). A non-specific oligonucleotide was also synthesized in the same way in order to act as a decoy (DC). All oligonucleotides were synthesized chemically based on their design. To increase stability of the oligonucleotides in cell culture, the first and last two bases were substituted with phosphorothioate.

In vitro cleavage assays. For in vitro cleavage assays, full-length mouse Twist RNA was synthesized by in vitro transcription from pBSK-TWT (donation from Dr. Fabienne Perrin-Schmitt, Strasbourg, France). Mouse Twist cDNA was cloned in *Eco*RI sites. Active/inactive DNazymes and Twist RNA were incubated at 1:40, 1:200, and 1:400 molar ratios for at 37 °C. Full-length and cleavage products were separated on 8% urea polyacrylamide gel electrophoresis and visualized under UV.

DNazyme transfections and RNA analysis. Mouse mesenchymal C3H10T1/2 cell line was maintained in DMEM, 10% fetal Calf Serum (FCS) at 37 °C. 0.5 μg of each oligonucleotide (DZ-TWT, DZ-TWTi, and DC) was conjugated with 20 μl Lipofectamine (Invitrogen) and OptiMEM (Life Technologies) and delivered in 0.6×10^5 C3H10T1/2 cells. Transfections were left incubating at 37 °C for 3 h and transfection medium was then replaced by DMEM, 10% FCS medium and left for another 48 h. Total RNA was then extracted (RNeasy, Qiagen) and subjected to cDNA synthesis. Briefly, 1 μg of total RNA was incubated with 12 pmoles of oligo(dT) primer, denatured at 95 °C for 5 min, and then annealed at 65 °C for 5 min. Reverse transcriptase buffer (1×), 0.2 mM dNTPS, and 10 U M-MuLV reverse transcriptase were then added to the mix and incubated at 37 °C for 1 h. cDNAs were then amplified by PCR. Primers 5′ CCCAAGCTTGTCGTACGAGGAG CTGCAGA 3′ (forward) and 5′ CGCGGATCCCTCCAGACGGAG AAGGCGTA 3′ (reverse) were used to amplify mouse Twist cDNA at 95 °C 1.30 min, 55 °C 1 min, and 72 °C 1 min, 25 cycles. Primers 5′ AGCCTGAAGACTGTGATGGG 3′ (forward) and 5′ AAAGTTCC ACCGTTCTCGG 3′ (reverse) were used to amplify mouse p21 cDNA at 95 °C 45 s, 62 °C 45 s, and 72 °C 1 min, 27 cycles. Primers 5′ TCA TCATCTCCGCCCCTTCC 3′ (forward) and 5′ GAGGGGCCATCC ACAGTCTT 3′ (reverse) were used to amplify mouse GAPDH cDNA at 95 °C 1.30 min, 55 °C 1 min, and 72 °C 1 min, 22 cycles. For all amplifications, quantitative conditions were established. PCR products were separated on 6% polyacrylamide gel electrophoresis and their intensity was quantitated by Scion Image software. The relative levels of Twist and p21 mRNAs were calculated as a ratio with the GAPDH values.

Acridine orange apoptosis assay. Equal amount of C3H10T1/2 cells and ethidium bromide (10 μg/ml)–acridine orange (3 μg/ml) solution was mixed and the state of cells was analyzed by microscopy. Live cells and apoptotic cells were detected under a fluorescence microscope at 100× magnification.

Results

DNazymes cleave efficiently Twist mRNA

The ability of DNazymes to cleave full-length mouse Twist mRNA in vitro was determined by incubating DZ-TWT with the Twist mRNA (Fig. 1B). Cleavage reactions were performed using 1:40, 1:200, and 1:400 molar ratios of active DNazyme and substrate, respectively, at 37 °C. As shown in Fig. 1B, DZ-TWT cleaves Twist mRNA in a base-specific way, followed by the production of two cleavage products of the expected size (835b, 523b). No catalysis was observed when the target was incubated with the DZ-TWTi inactive DNazyme at a ratio of 1:400. These results indicate the accuracy and specificity of the design of DZ-TWTi to cleave the full-length mouse Twist mRNA.

DNazyme-mediated reduction of endogenous Twist transcripts and subsequent up-regulation of p21 gene expression

Having shown that DZ-TWT is capable of site-specific cleavage of Twist mRNA, the next step was to ex-

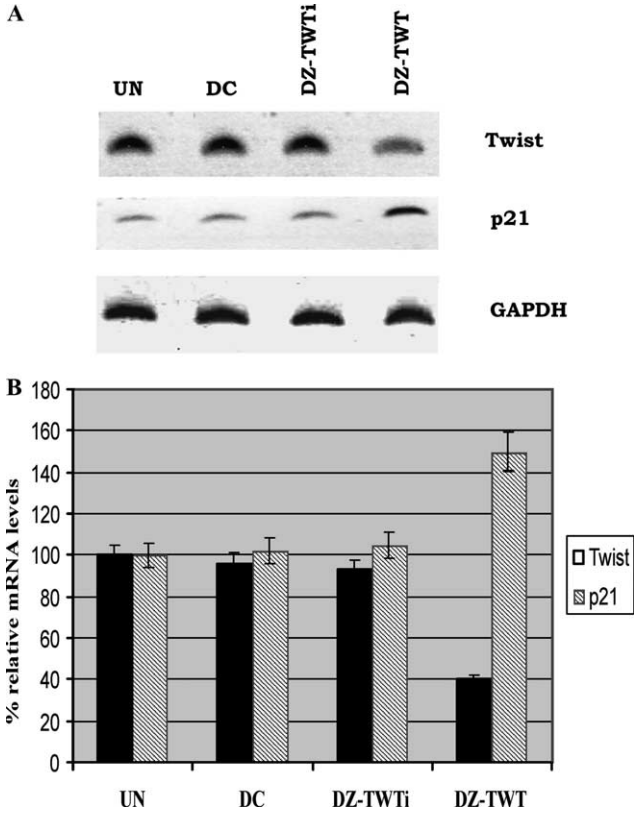

Fig. 2. DNazyme-mediated down-regulation of Twist mRNA and up-regulation of p21 mRNA. (A) RT/PCR analysis from total RNA extracts. A significant reduction in the mouse Twist mRNA levels was observed in C3H10T1/2 cells transfected with DZ-TWT. No significant reduction in the relative levels of Twist was observed in untransfected cells (UN) or cells transfected with the DZ-TWTi or the decoy oligo (DC). Similarly, a significant rise in p21 mRNA levels was seen only in cells transfected with DZ-TWT. (B) Summary of the relative mRNA levels. A 60% reduction in Twist mRNA levels and a 50% increase in p21 mRNA levels were observed in cells transfected with DZ-TWT. No significant mRNA changes were seen in untransfected cells (UN) or cells transfected with the DZ-TWTi or the decoy oligo (DC).

amine its potential in mouse cultured cells. C3H10T1/2 cells are known to express the Twist gene and were transfected with DZ-TWT. The effect of DZ-TWT on cellular Twist mRNA target was determined by quantitative RT/PCR 48 h after transfection. A 60% reduction of the endogenous Twist mRNA was observed in cells transfected with the DZ-TWT as shown in Fig. 2. However, no reduction in Twist mRNA levels was observed in cells transfected with DZ-TWTi, the decoy oligo (DC) or in untransfected cells (UN).

It is thought that Twist acts on the transcription machinery of several genes. Endogenous p21 gene expression was shown to be suppressed by overexpressing the Twist gene [23]. Total RNA from transfected or non-transfected cells was subjected to quantitative RT/PCR in order to detect p21 changes due to DNazyme reduction of the Twist mRNA. Mouse p21 mRNA levels were elevated by 50%, as shown in Fig. 2, only in the DZ-TWT transfected cells. No significant changes were observed in untransfected cells (UN) or in cells transfected with DZ-TWTi or DC. These results suggest that DZ-TWT is capable of increasing p21 levels via the down-regulation of Twist gene expression.

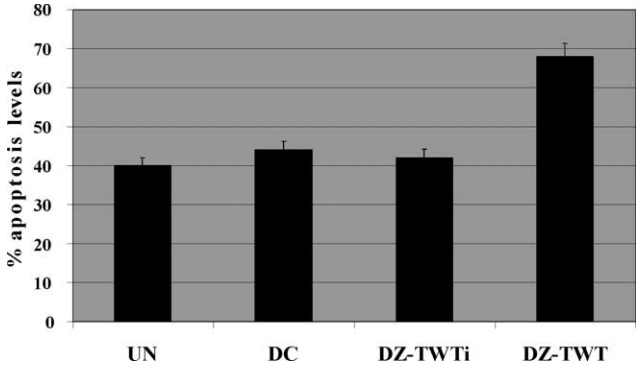

Fig. 3. DNazyme-mediated increase of cellular apoptosis. A 70% increase in cellular apoptosis as detected by acridine orange assays was observed only in cells transfected with DZ-TWT compared to the untransfected cells (UN) after induction with low serum medium. No significant change in cellular apoptosis was observed in cells transfected with the DZ-TWTi or decoy oligo (DC).

Twist reduction is linked to an increased cellular apoptosis

It has been previously shown that low-serum culture induces p21 gene expression followed by apoptosis [23]. As a next step, the effect of the increased DNazyme-mediated p21 levels on cellular apoptosis was investigated. Cells were induced to apoptosis by low-serum medium for 24 h. There was a significant increase in apoptosis in cells transfected with DZ-TWT. Seventy percent more cells were prevented from apoptosis due to increased p21 levels (Fig. 3).

Discussion

Maestro et al. [22] showed that, in C8 mouse embryo fibroblasts, Twist inhibits apoptosis by antagonizing the p53 pathway that plays a critical role in regulating cell death in response to a variety of stimuli. It was shown that Twist could directly bind two independent histone acetyltransferase domains of acetyltransferases, p300, and p300/CBP-associated factor [22,24]. The whole complex is thought to bind and suppress p21 gene expression. p21 is necessary for the induction of cell cycle arrest.

These results indicate that DNazymes are suitable tools for studying gene function and important pathways. Moreover, they can be used as therapeutic tools since they can down-regulate any abnormal endogenous gene expression. DNazymes have been used to down-regulate cellular and viral expression in several appli-

cations, however, their use has been limited over the ribozyme approach due to the difficulties in their expression. Although experience with DNazymes as potential therapeutic agents is limited, these molecules might prove worthy in the clinical setting. Moreover, they can prove to be useful in characterizing gene function in complicated biological systems such as in whole animals. For example, it would be useful to investigate the role of Twist gene expression during mouse development by down-regulating its expression.

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