A novel replicating circular DNAzyme

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ABSTRACT

10–23 DNAzyme has the potential to suppress gene expressions through sequence-specific mRNA cleavage. However, the dependence on exogenous delivery limits its applications. The objective of this work is to establish a replicating DNAzyme in bacteria using a single-stranded DNA vector. By cloning the 10–23 DNAzyme into the M13mp18 vector, we constructed two circular DNAzymes, C-Dz7 and C-Dz482, targeting the β-lactamase mRNA. These circular DNAzymes showed in vitro catalytic efficiencies (kcat/KM) of 7.82 × 10^6 and 1.36 × 10^7 M^−1·min^−1, respectively. Their dependence on divalent metal ions is similar to that found with linear 10–23 DNAzyme. Importantly, the circular DNAzymes were not only capable of replicating in bacteria but also exhibited high activities in inhibiting β-lactamase and bacterial growth. This study thus provides a novel strategy to produce replicating DNAzymes which may find widespread applications.

INTRODUCTION

10–23 DNAzyme was obtained by in vitro selection in 1997, and it cleaves RNA targets at the purine–pyrimidine junctions (1). The enzyme consists of a catalytic domain of 15 nucleotides, flanked by two substrate-recognition domains of 7–10 nucleotides (1–6). As a potential candidate of antisense drug in gene therapeutics, 10–23 DNAzyme has many advantages over other antisense drugs. Unlike antisense oligonucleotides, 10–23 DNAzyme not only binds the target RNAs but also cleaves them. Kurreck et al. showed that 10–23 DNAzymes have a much higher cleavage activity than the ribozymes (7). In fact, its catalytic efficiency (kcat/KM) may reach 10^7 M^−1·min^−1, which is about 100-fold higher than that of the most active ribozyme. It also has a remarkable stability 100 000-fold as high as that observed with ribozyme under physiological conditions. Furthermore, 10–23 DNAzyme exhibits high flexibility for cleaving-site selectivity and substrate specificity, because it may cleave the purine–pyrimidine junctions of any RNAs and a single base mismatch in its antisense arms significantly decreases the cleavage activity (1–10).

Despite all these favorable features, a major challenge facing the application of 10–23 DNAzyme is that it cannot replicate endogenously and, consequently, one has to rely on exogenous delivery. This usually gives rise to poor intracellular uptake and affects the stability of the DNAzymes. Thus, it becomes necessary to design a new type of DNAzyme capable of endogenous replication. Considering that the DNAzyme is a single-stranded oligodeoxynucleotide, we postulate that a single-stranded vector should be suitable to carry the enzyme replicated in vivo. To prove this, we designed two circular phage DNAzymes designated as C-Dz7 and C-Dz482, carried by the single-stranded phage vector, M13mp18. The DNAzymes target two conserved sites of TEM spectrum β-lactamase mRNA. Our data demonstrate that the circular DNAzymes are capable of replication in E.coli cells and display catalytic activity in vitro and in bacteria.

MATERIALS AND METHODS

Oligonucleotides and bacterial strains

All the DNA fragments used for construction of the circular DNAzymes (Table 1) and two control linear 10–23 DNAzymes L-Dz7 and L-Dz482 with sequences of 5′-GAAAAATGTGAGCTAGCTACAACGCAAACGTTTTCCTT-3′ and 5′-GGTTCCCAGCGAGGCTAGCTACAACGCAAGGGCTAGCTA-3′, respectively, were synthesized by Sangon (Shanghai, China). The RNA substrates S7 and S482 (Fig. 1) were purchased from Takara (Dalian, China), and their 5′-termini were labeled with 32P using T4 polynucleotide kinase. The Ampicillin-resistant (Amp*) TEM-1 and TEM-3 β-lactamase-producing E.coli strains, with a minimum inhibitory concentration (MIC) of 256 mg/l, were obtained from the Bacteria Department of Beijing Hospital (China). A replicative form (RF) of the M13mp18 vector and its bacterial host (TG1) were from Sangon (Shanghai, China).

Construction of circular DNAzymes

The complementary cloning fragments for construction of circular DNAzymes (Table 1) were annealed and ligated into the RF M13mp18 vector at the multiple cloning sites BamHI and HindIII. The ligation products were then transformed into bacterial host TG1. Positive clones were identified through color selection on agar-media containing IPTG and X-gal. A
single colorless plaque was inculated in a flask containing 100 ml of LB liquid medium with constant agitation at 37°C overnight. The recombinant circular DNAzymes were prepared by using the method described in the Molecular Cloning Manual (11) and their sequences were analyzed by Takara (Dalian, China). We thus generated two circular DNAzymes, namely, C-Dz7 and C-Dz482, and two mutant forms of the latter designated as C-Dz482m1 and C-Dz482m2.

**In vitro activity assays of DNAzymes**

**In vitro** cleavage activities of linear and circular DNAzymes were determined under multiple-turnover conditions. The reactions were performed at 37°C in a buffer containing 50 mM Tris–HCl (pH 7.6), 10 mM MgCl2 and 0.01% SDS. The concentration of DNAzymes was fixed at 2 nM, while the RNA substrate concentrations varied from 10 to 240 nM. At 5, 15, 30 and 60 min, aliquots of reaction mixtures were taken and quenched with 100 mM EDTA and 9 M urea. The resulting products were separated by 16% denaturing PAGE and visualized by autoradiography. The extent of cleavage was determined by measuring the radioactivity of the bands corresponding to the substrates and the cleaved products without Amp, and (c) phosphate buffer alone (1.5 ml). The reaction conditions were (a) the phosphate buffer (pH 7.0), and then 0.25 ml of 2.5 mg/ml Amp solution was added. Following 30 min incubation, the reaction was stopped by adding 2.5 ml iodine reagent. The absorbance at 490 nm was measured directly and designated as A. Three control experiments (a, b and c) were performed simultaneously, which gave rise to absorbance Aa, Ab and Ac, respectively. The reaction conditions were (a) the phosphate buffer (1.25 ml) plus the Amp solution (0.25 ml), (b) the sample cell extract (20 μl) plus the phosphate buffer (1.5 ml) without Amp, and (c) phosphate buffer alone (1.5 ml). The β-lactamase activity of the circular DNAzyme-treated bacteria was determined according to the iodometry method (13). Briefly, 10 ml of 9-h cultured bacteria was harvested, sonicated and β-lactamase (sample solution) was extracted into 2 ml of a phosphate buffer (0.05 M, pH 7.0). The sample cell extracts (20 μl) were diluted into 1.25 ml with a 0.1 M phosphate buffer (pH 7.0), and then 0.25 ml of 2.5 mg/ml Amp solution was added. Following 30 min incubation, the reaction was stopped by adding 2.5 ml iodine reagent. The absorbance at 490 nm was measured directly and designated as A. Three control experiments (a, b and c) were performed simultaneously, which gave rise to absorbance Aa, Ab and Ac, respectively. The reaction conditions were (a) the phosphate buffer (1.25 ml) plus the sample cell extract (20 μl) plus the phosphate buffer (1.5 ml) without Amp, and (c) phosphate buffer alone (1.5 ml). The β-lactamase activity was defined as ΔA490 = Aa – Ac – Ab – A.

**Analysis of divalent metal ion dependence**

The substrate cleavage rate for C-Dz482 in the presence of several divalent metal ions was measured under single-turnover conditions. The reactions were performed at 37°C in a buffer containing 50 mM Tris–HCl (pH 7.6), 10 mM divalent metal ion and 0.01% SDS and were started by mixing 100 nM DNAzyme with 10 nM 32P-labeled RNA substrate. An aliquot was taken from each reaction liquid after 10 min incubation and quenched with 100 mM EDTA and 9 M urea. The resulting products were separated by 16% denaturing PAGE, visualized by autoradiography and quantified using a Bio-Rad PhosphorImager.

The dependence of substrate cleavage rate (kcat) on Mg2+ concentration was determined under the multiple-turnover conditions in the presence of 2 mM C-Dz482 and 200 mM 32P-labeled RNA substrate S482. Mg2+ concentrations were varied from 10 to 300 mM. Aliquots were taken from the reaction mixture at various times and quenched by 100 mM EDTA and 9 M urea for analyses. Reaction conditions and process were the same as described above. Since the C-Dz482 was over-saturated by the RNA substrate in this experiment, the
maximum initial velocity \( (V_{\text{max}}) \) could be determined and, thus, \( k_{\text{cat}} \) was worked out according to the equation \( k_{\text{cat}} = \frac{V_{\text{max}}}{[E]} \).

**RESULTS**

**Design of circular DNAzymes**

Table 1 and Figure 1 show the schematic structures and sequences of the circular DNAzymes designed in this study. The length of the antisense arm of the circular DNAzymes was designed a little longer than the commonly used 7–10 nt (3,4,14–21). This is intended to enhance the binding affinity of DNAzymes to their RNA substrates. For assays of DNAzyme activity in bacteria, TEM spectrum \( \beta \)-lactamase mRNAs were selected as the target sequence of our circular DNAzymes (Fig. 1). To verify the specificity of the DNAzymes, two mutant forms of C-Dz482, namely, C-Dz482m1 and C-Dz482m2, were constructed by single base substitution at the antisense arm (A12) and the catalytic core (C15), respectively (Table 1). For control purpose, we additionally synthesized two linear 10–23 DNAzymes, namely L-Dz7 and L-Dz482, with antisense arms and catalytic cores identical to those of the corresponding circular DNAzymes.

**In vitro activity of the circular DNAzymes**

The in vitro activities of the circular DNAzymes and the linear DNAzymes (controls) were examined using synthetic RNA substrates under multiple turnover conditions. The results are shown in Table 2. The catalytic efficiencies \( k_{\text{cat}}/K_{M} \) for C-Dz7 and C-Dz482 were \( 7.82 \times 10^{6} \) and \( 1.36 \times 10^{7} \) M\(^{-1}\) min\(^{-1}\), respectively. These values are about one-quarter to one-third of those obtained with corresponding linear DNAzymes (L-Dz7 and L-Dz482). This indicates that the circular DNAzymes retain significant catalytic activity after cloning into the single-stranded M13mp18 vector. The relatively lower catalytic efficiency may be due to suppression within the circular DNA. Indeed, when the circular DNAzymes were subjected to denaturation by heating at 95°C for 5 min, their activities were markedly increased. As shown in Table 2, heat treatment of C-Dz482 resulted in over an 8-fold increase in its catalytic efficiency.

To confirm the roles of the antisense arms and the catalytic core in determining the specificity of the circular DNAzymes, two mutant DNAzymes, C-Dz482m1 and C-Dz482m2, were tested for their cleavage activity toward the RNA substrates. As expected, neither mutant showed significant activity in vitro. This indicates that the circular DNAzymes possess high in vitro substrate specificity as observed with the linear 10–23 DNAzymes (4).
Replication and activity of the circular DNAzymes in bacteria

To test the replication of the circular DNAzyme in bacteria, we transformed the C-Dz482 into Amp\(^r\) strains TEM-1 and TEM-3 by electroporation. The recombinant DNAs were extracted from the transformed bacterial cells and verified by sequencing. The results demonstrated that the circular DNAzymes indeed replicated in bacteria as recombinant DNAs carried by the M13mp18 vector. As shown in Figure 2, replication of the circular DNAzymes was seen after 6 h of incubation and reached a plateau at 12–24 h.

Our circular DNAzymes were designed to cleave the β-lactamase mRNA and thus may serve as β-lactamase inhibitors (22). In principle, inhibition of β-lactamase expression by DNAzymes should render Amp\(^r\) bacteria to lose Ampicillin resistance and fail to grow in the presence of Ampicillin. To evaluate the activity of the circular DNAzymes in bacteria, Amp\(^r\) bacteria were transfected by electroporation with either the plain M13mp18 vector or the circular DNAzymes and their mutants, and the growth rates of the transformed bacteria were analyzed (23,24). Similar transformation efficiencies of about 3–6 × 10\(^7\) plaques/μg DNAzyme were obtained for all transformation experiments performed to ensure phase synchronization. The data shown in Figure 3A demonstrate a marked growth inhibition of the bacteria by expression of circular DNAzymes but not by the plain vector and inactive mutant DNAzymes. At the 9 h time point when all the bacteria were at logarithmic growth phase, OD\(_{600}\) differences (ΔOD = OD\(_{600}\)Ctrl – OD\(_{600}\)Dz) of 0.37 and 0.57 were observed with C-Dz7 and C-Dz482, respectively, which corresponds to respective growth inhibition rates (ΔOD/OD\(_{600}\)Ctrl) of 46% and 71% (Fig. 3B). Incidentally, the β-lactamase activities observed with C-Dz7- and C-Dz482-treated TEM-1 bacteria were about 53% and 67% less than that obtained with the plain vector-treated cells, respectively. These results demonstrate a correlation between the retarded cell growth and the decreased β-lactamase activity caused by the circular DNAzymes. Importantly, the two mutant DNAzymes, C-Dz482m1 and C-Dz482m2, neither inhibited cell growth nor reduced β-lactamase activity. This indicates that the catalytic activity of the circular DNAzymes rather than antisense DNA binding or DNA transfection is responsible for cell growth inhibition. Data in Figure 3A and B also demonstrate that C-Dz482 had no effect on TEM-1 bacteria growing in the absence of Ampicillin. This provides evidence that the circular DNAzymes inhibit bacterial growth by suppressing ampicillin resistance. Note that in the absence of ampicillin, the bacterial cells expressed a much lower β-lactamase activity (see -Amp’ bars in Fig. 3B). This is determined by the nature of the cells and is not related to expression of the circular DNAzymes. We also analyzed the effects of the added Mg\(^{2+}\) on the inhibitory function of the circular DNAzymes. As shown by the -Mg\(^{2+}\) bars in Figure 3B, in the absence of added Mg\(^{2+}\), although at a reduced level, C-Dz482 still caused a significant growth inhibition of TEM-1 cells and a marked suppression of β-lactamase activity. Apparently, the circular DNAzyme remains functional in the bacterial cells without the addition...
of C-Dz482 to Mg2+. As shown in Figure 4B, the strongest activator. We further determined the dose-response C-Dz482 increased with increases in Mg2+ concentration. This also showed high activities in vitro. DNAzyme was not only capable of replication in bacteria, but cloning the 10–23 DNAzyme into M13mp18 vector. The novel replicating circular DNAzyme was constructed by

**DISCUSSION**

Dependence of DNAzyme activity on divalent metal ions

Divalent metal ions are known to play a critical role in promoting association of DNAzymes with their substrates and subsequent cleavage (3,4,25). To examine the dependence of circular DNAzyme on divalent metal ions, we tested several common metal ions. Data shown in Figure 4A demonstrate that C-Dz482 displayed a strong metal ion dependency. In the absence of metal ions, C-Dz482 displayed essentially no activity. Addition of Zn2+, Co2+ and Ba2+ did not cause any increase in activity, indicating that these metal ions are not cofactors of circular DNAzyme. In contrast, Mn2+, Pb2+, Mg2+ and Ca2+ markedly stimulated its activity with Mn2+ being the strongest activator. We further determined the dose–response of C-Dz482 to Mg2+. As shown in Figure 4B, the kcat values of C-Dz482 increased with increases in Mg2+ concentration. This metal ion dependency of circular DNAzymes is similar to that observed with linear DNAzymes (1,4).

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The circular DNAzymes showed high activities both in vitro and in TEM-1 bacteria (Table 2 and Fig. 3), suggesting that the activation center (antisense arms and the catalytic core) of the circular DNAzymes may predispose the surface to favor substrate binding. Bai and colleagues demonstrated that liquid plasmid DNA exists in an unfolded state and forms a ring structure between 30 and 40°C when imaged under an atomic force microscope (26,27). Apparently, some of the circular DNAzymes were in an unfolded state, since we observed that in vitro activity of the circular DNAzyme was lower than that of the linear DNAzyme (Table 2). This implies that the activity centers of some of the circular DNAzyme molecules may likely be entrapped inside the super coil of the phage DNA vector, making a proportion of circular DNAzymes inactive. The in vitro denaturation experiments seem to support this, since the denaturation increased the in vitro activity of the circular DNAzymes significantly (Table 2). The circular DNAzyme molecule was in an unfolded state after denaturation, releasing the extra secondary structure in favor of the substrate binding.

Similar to that of the linear DNAzymes, the catalysis of the circular DNAzymes requires divalent metal ions including Mn2+, Pb2+, Mg2+ and Ca2+ as cofactors. Furthermore, detailed kinetic studies with Mg2+ showed that the dependence of kcat on Mg2+ concentrations is similar to that of the linear DNAzymes (Fig. 4) (1,4). Metal ions may play an important role in enzymatic reaction in three aspects: First, they may aid the stabilization of the transition state and assist the circular DNAzyme folded into its catalytic conformation (4,28); second, they may directly participate in the processes of the substrate cleavage reaction (3,4); third, they may increase the substrate association rate because of the counterion-condensation model for nucleic acid helix formation (29,30). In all, the similarity between circular and linear DNAzymes in the divalent metal ion requirement and dependency suggests similar reaction mechanisms. The circular DNAzyme-catalyzed reactions may follow the mechanism hypothesized for the hammerhead ribozyme which also requires divalent metal ions (31,32).

In conclusion, we report herein a feasible single strand expression system of phage for DNAzymes. The expressed DNAzymes displayed cleavage activities both in vitro and in bacteria. Although we designed the recognizing sequence of DNAzyme specifically against the β-lactamase mRNA and demonstrated inhibitory effects on bacterial growth, we cannot at present rule out the possibility of nonspecific effects contributing to the results. This point warrants further study to improve this expression system in the future.

A novel replicating circular DNAzyme was constructed by cloning the 10–23 DNAzyme into M13mp18 vector. The DNAzyme was not only capable of replication in bacteria, but also showed high activities in vitro and in bacterial cells. Our
REFERENCES


