Novel Biomaterials Derived from Deoxyribozyme and NAPzyme

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Summary: We report the potential of a small Ca^{2+}–dependent deoxyribozyme as a novel biomaterial to distinguish RNA foldings. It is found that an immobilized deoxyribozyme using avidin–biotin interaction cleaves the target site within only single–stranded RNAs. The RNA cleavage reaction is also detected using the deoxyribozyme SPR sensor chip. Furthermore, we develop a novel NAPzyme (nucleic acid peptide deoxyribozyme) with its RNA cleavage function in the absence of divalent metal ions.

Keywords: biomaterials, deoxyribozyme, enzymes, metal ion, NAPzyme (nucleic acid peptide deoxyribozyme)

Introduction

High–density oligonucleotide captures for sequence–specific analysis has been developed as novel biotechnologies of DNA chips and microarrays.[1] These biotechnologies are very powerful tools for the genomic analysis of SNPs (single nucleotide polymorphisms) and mRNA/gene expressions.[2] However, it is difficult using these methods to detect the difference in the RNA folding. The RNA folding variation due to one or more mutations would lead to different biological functions as a result of differences in the primary or higher–ordered structures that interact with other cellular molecules, because the RNA folding influences the RNA splicing, RNA processing, and translational control.[3] Thus, to distinguish the RNA foldings is one of the guides to detect the gene functions related to diseases and drug responses. Recent studies on ribozymes and deoxyribozymes showed that they are also interesting tools in biotechnology and biomaterial.[4,5] For a cleavage reaction using ribozymes, the cleavage

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activity is basically dependent on the sequence close to the cleavage site and the secondary structure of the target RNA. This fact indicates the possibility for the catalytic nucleic acid to distinguish or detect the target RNA foldings.

Ribozymes have a requirement for divalent metal ions,[6-8] very high concentration of monovalent metal ions,[9,10] or small molecules,[11] which act as base and acid catalysts. On the other hand, the hydrolysis of RNA by RNase A (ribonuclease A) is initiated by histidine residues at positions 12 and 119, which acts as acid and base catalysts in the absence of divalent metal ions.[12,13] Thus, a synthesis of nucleotide analogues containing covalently linked histidines has made it possible to investigate the catalytic activity of novel ribozymes in the absence of divalent metal ions.

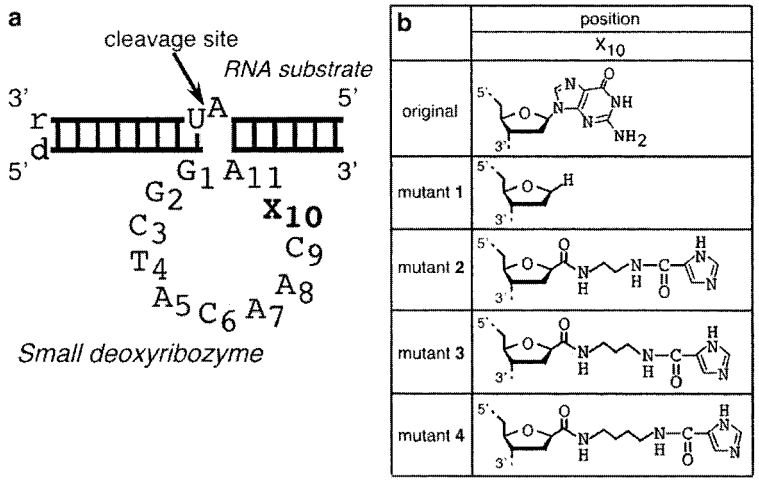

Fig. 1. (a) Secondary structure of the complex of the small deoxyribozyme and its RNA substrate. (b) Structures of the modified nucleotides at position 10 of its catalytic loop of the deoxyribozyme.

Here, we have now developed an immobilized small Ca^{2+}–dependent deoxyribozyme with 11 nts (nucleotides) catalytic loop as a novel and useful biomaterial to distinguish RNA foldings as shown in Figure 1a.[14-16] Furthermore, we have developed a novel NAPzyme (nucleic acid peptide deoxyribozyme) with its RNA cleavage function in the absence of divalent metal ions as shown in Figure 1b.

Materials and Methods

Materials. All DNA, DNA containing an abasic nucleotide or the histidine NAP, and RNA oligonucleotides were synthesized chemically on solid support and purified as described previously.[14,15] The final purity of the oligonucleotides was confirmed to be >99 %. The oligonucleotides were desalted with a Sep–Pak C18 cartridge column (Waters) before use. Single–strand concentrations of oligonucleotides were determined by measuring the absorbance at 260 or 280 nm. The single strand extinction coefficient was calculated from the mononucleotide and dinucleotide data using nearest–neighbor approximation.[17] The extinction coefficient of the single strand containing the abasic nucleotide or histidine NAP was obtained as a sum of two regions separated by the site.[18] The target was 5'–end labeled using 5–IAF (5–iodoacetamidofluorescein) at 37 °C as previously described.[16]

Synthesis of histidine NAP phosphoramidites. The synthesis of various histidine NAP phosphoramidites was synthesized using a 10–step synthesis from 2–deoxy–D–ribose as previously reported.[19,20] The phosphoramidites were identified by MS and ^{1}H NMR spectra.

RNA cleavage reactions. The target cleavage reactions by the immobilized deoxyribozymes were done in a buffer containing 50 mM Tris–HCl (pH 8.0) and 25 mM Ca^{2+} at 37 °C.[14,15] The RNA cleavage reactions by the mutant deoxyribozyme were done in a buffer containing 50 mM Tris–HCl (pH 8.0), 100 mM Na^+, and 25 mM Ca^{2+} at 37 °C. After cleavage reactions were stopped, the 5'–end fluorescein–labeled products and targets were separated by electrophoresis on 20 % polyacrylamide / 7 M urea denaturing gels. The cleavage yields were determined by quantifying the fluorescence intensity in the bands of the 5'–end labeled products and targets using a fluorescence imager. An observed rate constant (k_{ops}), an equilibrium constant (K_1), and a cleavage rate constant (k_2) of the original deoxyribozyme or NAPzyme with its RNA substrate were calculated from nonlinear least–square fitting analysis as previously described.[21-23]

Deoxyribozyme SPR sensor chip. A BIAcore (BIAcore 1000, Biacore AB, Uppsala, Sweden) was used for the SPR measurements.[15] To remove the background binding between the injected target and the immobilized streptavidin to the dextran matrix or the refractive index change in the injection, the SPR trace, after flowing a buffer containing the target over the sensor chip coated without the ligand, was deducted from those with the ligand. The target was injected in a buffer containing 50 mM Tris–HCl (pH 8.0) and 100 mM Na^+ at 37 °C at the slow

flow rate of 5 μL min^{-1}. The concentration of the injected target was 100 μM. By addition to 25 mM Ca^{2+}, the target cleavage reactions were initiated.

Results and Discussions

The immobilized deoxyribozyme distinguishes the difference in the RNA foldings. To obtain information about the effect of target foldings, we first investigated whether the immobilized deoxyribozyme cleaves the site within the duplex (Figure 2). The immobilized deoxyribozyme (dCGCTGGCAggctacaacgaGTCTTC; small letters indicate its catalytic loop) was able to cleave the site only in the single–stranded RNA (rGAAGACA↓UGCCAGCG (r15); ↓ indicates its cleavage site), although the single–stranded DNA, pseudo single–stranded RNA [rGAAGAC(dA)UGCCAGCG], RNA/DNA hybrid, and RNA/RNA duplex were not cleaved. The single–stranded RNA regions are basically unpaired, such as in the hairpin loops and internal loops within the RNA foldings. To investigate the effect of the RNA hairpin loop size, the cleavage reactions of eight RNA hairpin loops by the immobilized deoxyribozyme were carried out. The sequence of the hairpin loop with 15 nts is the same to that of r15 described above. All these RNA sequences melted with biphasic behavior and the T_m (melting tempreature) values were independent of the concentration of the RNAs (data not shown), indicating that the folded structure of these RNAs would be the only stable intramolecular hairpin loop, but not an intermolecular loop structure.[24] For RNA with 15 nts loop, the cleavage reaction was little observed. On the other hand, for ≥17 nts as a hairpin loop, the cleavage was clearly observed at only one site. These results suggest that at least one nucleotide spacer at both ends of the hairpin loop are required for efficient cleavage by the immobilized deoxyribozyme. The catalytic activity was saturated over ≥23 nts as the hairpin loop size. To investigate the effect of the cleavage position, the RNA hairpin loop cleavage reactions by the immobilized deoxyribozyme were carried. The RNA hairpin loop domain was systematically slid from the 5'–end to the 3'–end in the recognition site of the immobilized deoxyribozyme. The amount of cleavage product for these RNA by the immobilized deoxyribozyme were approximately equal, indicating that any target position in the RNA hairpin loop is cleaved only at one site.

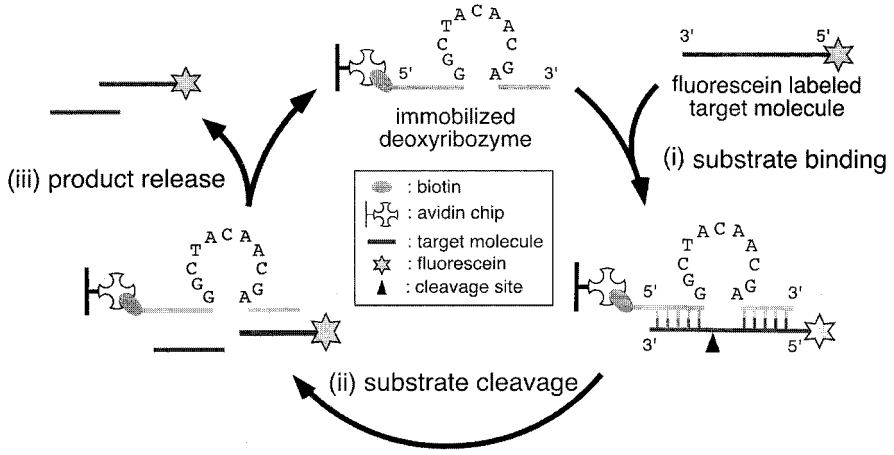

Fig. 2. Reaction scheme of the novel functional DNA chip with the immobilized deoxyribozyme.

Development of the deoxyribozyme SPR sensor chip. The construction and application of the immobilized deoxyribozyme on an SPR sensor chip to create a deoxyribozyme array has not been described. We have already demonstrated that the novel and useful biomaterial of the deoxyribozyme SPR sensor chip is possible, which may enable the rapid invention of numerous DNA biosensor elements with satisfactory performance characteristics.[16] The same method should apply to the use of DNA and many nucleic acids analogs that can provide greater versatility for analysis recognition and increased stability in harsh environments. In this regard, the catalytic nucleic acid arrays could have long storage lives, perhaps greater than biosensor components made from natural protein. To clarify the properties of the immobilized deoxyribozyme as a sensor chip, the cleavage reaction were directly measured by an SPR apparatus.

Figure 3 shows the typical SPR sensorgrams of the binding and cleavage steps between the target (r15 and pseudo RNA substrate [rGAAGAC(dA)UGCCAGCG]) and the deoxyribozyme SPR sensor chip. When its target binds to the immobilized deoxyribozyme on the SPR sensor chip, the observed response unit is increased by changes in the refractive index as shown by the RU (resonance units) values in real time.[25] When the pseudo RNA substrate was flowed over the sensor chip coated with the immobilized deoxyribozyme, about 150 RU value was retained

by the addition to Ca^{2+}. However, in the case of r15, the RU value was slowly decreased by the addition to Ca^{2+}. The differences in the RU value between its RNA substrate and pesudo RNA substrate after 30 min running were 69 %. After the recovery of these samples, the cleavage products and targets were able to be separated by electrophoresis on denaturing gel, indicating that no cleavage band was observed for the pseudo RNA substrate, while only one cleavage product was observed for r15. The cleavage site was at only one site of rAp↓U in the asymmetric internal loop. These results indicate that the decrease in the RU value shows the process of the product release after the RNA cleavage reaction.

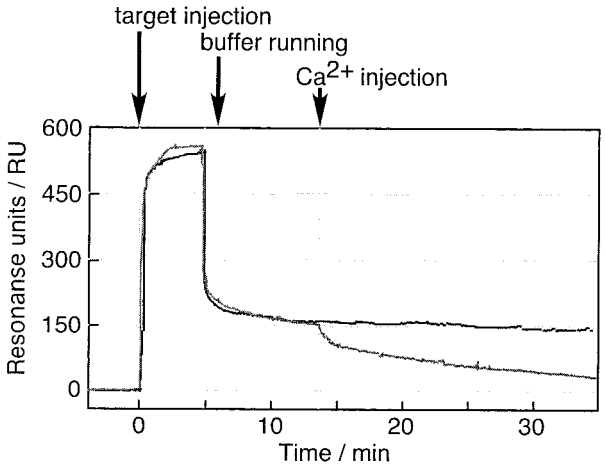

Fig. 3. The typical SPR sensorgrams of the binding and cleavage steps between the target molecule and the immobilized deoxyribozyme on the SPR sensor chip in a buffer containing 50 mM Tris-HCl (pH 8.0) at 37 °C. The flow samples were r15 (black line) or the pseudo RNA substrate (gray line).

NAPzyme has its RNA cleavage function in the absence of divalent metal ion. The position 10 in the catalytic loop of the deoxyribozyme has a larger influence on the RNA cleavage step than the binding step between the deoxyribozyme and its RNA substrate.[26] Thus, we designed mutant deoxyribozymes with an abasic nucleotide or a histidine NAP at position 10 as shown in Figure 1b. The original deoxyribozyme was able to cleave its RNA substrate at only one site in the presence of Ca^{2+}. Surprisingly, only one cleavage band was observed the mutant **3** in the

absence of Ca^{2+}. The cleavage site by mutant **3** was identical to the cleavage site by the original deoxyribozyme. However, mutants **1**, **2** and **4** were not able to cleave its RNA substrate, even after a 120 min incubation.

Perspectives

In this study, we developed novel biomaterials as the immobilized small Ca^{2+}–dependent deoxyribozyme. These targets were most suitable for only single–stranded RNAs containing a hairpin loop with ≥17 nts loop size. Thus, the immobilized deoxyribozyme can distinguish the higher–ordered structures of RNAs. Based on our results, we propose a search system for RNA higher–ordered structures using the immobilized deoxyribozyme and will then be able to construct a database of higher–ordered structures of genome and other nucleic acids. Furthermore, we have developed a novel NAPzyme that consists of a small deoxyribozyme and a histidine NAP with a propylene linker at position 10 of its catalytic loop. The NAPzyme was shown to cleave its RNA substrate at only one site in the absence of divalent metal ions. These studies might serve as a starting point for the further development of deoxyribozymes (ribozymes) that more closely mimic the function of RNase A.

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[1] C. Debouck, P. N. Goodfellow, *Nat. Genet.* **1999**, *21*, 48.
[2] D. W. Selinger, K. J. Cheung, R. Mei, E. M. Johansson, C. S. Richmond, F. R. Blattner, D. J. Lockhart, G. M. Church, *Nat. Biotechnol.* **2000**, *18*, 1262.
[3] I. Barrette, G. Poisson, P. Gendron, F. Major, *Nucleic Acids Res.* **2001**, *29*, 753.
[4] T. Ohmichi, S. Nakano, D. Miyoshi, N. Sugimoto, *J. Am. Chem. Soc.* **2002**, *124*, 10367.
[5] J. Li, Y. Lu, *J. Am. Chem. Soc.* **2000**, *122*, 10466.
[6] S. W. Santoro, G. F. Joyce, *Biochemistry* **1998**, *37*, 13330.
[7] T. R. Cech, B. L. Golden, in: "*The RNA World*", *Second ed.*, R. F. Gesteland, T. R. Cech, J. F. Atokins, Eds., Cold Spring Harbor Laboratory Press, Plainview, NY **1999**, p.265.
[8] T. Ohmichi, Y. Okumoto, N. Sugimoto, *Nucleic Acids Res.* **1998**, *26*, 5655.
[9] E. A. Curtis, D. P. Bartel, *RNA* **2001**, *7*, 546.
[10] J L. O'Rear, S. Wang, A. L. Feig, L. Beigelman, O. C. Uhlenbeck, D. Herschlag, *RNA* **2001**, *7*, 537.
[11] S. W. Santoro, G. F. Joyce, K. Sakthivel, S. Gramatikova, C. F. Barbas, III, *J. Am. Chem. Soc.* **2000**, *122*, 2433.
[12] C. Park, L. W. Schultz, R. T. Raines, *Biochemistry* **2001**, *40*, 4949.
[13] R. T. Raines, *Chem. Rev.* **1998**, *98*, 1045.

[14] N. Sugimoto, Y. Okumoto, T. Ohmichi, *J. Chem. Soc., Perkin Trans. 2* **1999**, 1382.
[15] Y. Okumoto, N. Sugimoto, *J. Inorg. Biochem.* **2000**, *82*, 189.
[16] Y. Okumoto, T. Ohmichi, N. Sugimoto, *Biochemistry* **2002**, 41, 2769.
[17] E. G. Richard, in: "*Handbook of Biochemistry and Bilogy: Nucleic Acids*", 3rd ed., G. D. Fasman, Ed., CRC Press, Cleaveland, OH, **1975**, *vol.1*, p.597.
[18] J. Kawakami, M. Yoneyama, D. Miyoshi, N. Sugimoto, *Chem Lett*. **2001**, 258.
[19] T. H. Smith, M. A. Kent, S. Muthini, S. J. Boone, P. S. Nelson, *Nucleosides & Nucleotides* **1996**, *15*, 1581.
[20] J. Kawakami, H. Fujiki, S. Izumi, N. Sugimoto, unpublished results.
[21] N. Sugimoto, R. Kierzek, D. H. Turner, *Biochemistry* **1988**, *27*, 6384.
[22] T. Ohmichi, N. Sugimoto, *Biochemistry* **1997**, *36*, 3514.
[23] T. Ohmichi, H. Nakamuta, K. Yasuda, N. Sugimoto, *J. Am. Chem. Soc*. **2000**, *122*, 11286.
[24] T. Dale, R. Smith, M. J. Serra, *RNA 6*, 608 (2000).
[25] P. C. Bevilacqua, R. Kierzek, K. A. Johnoson, D. H. Turner, *Science* **1992**, *258*, 1355.
[26] Y. Okumoto, Y. Tanabe, N. Sugimoto, *Biochemistry* **2003**, *42*, *in press*.