Regulation of Ribozyme Activity with Short Oligonucleotides

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Both hammerhead and hairpin ribozymes, which are well known as small catalytic RNAs, can catalyze either cleavage or ligation of RNA with sequence specificity. Although they have been applied to gene therapy and gene discovery, by cutting the mRNAs of target genes, these ribozymes normally do not have the ability to regulate their catalytic activities. Therefore, allosteric control has been applied to the ribozymes. Watson–Crick base pairing has superior ability in molecular recognition, and the sequence of nucleic acids makes diversity of molecules possible. Therefore, we have tried to construct a new ribozyme of which activity is regulated by a specific oligonucleotide. The allosteric ribozyme has potentials of not only regulation of activity but also a sensor for gene expression.

Key words ribozyme; regulation; oligonucleotide; RNA

1. ALLOSTERIC HAMMERHEAD RIBOZYME

We have designed an allosteric hammerhead ribozyme, which is activated by a short oligonucleotide as a cofactor. The hammerhead ribozyme consists of three helices (stem I, II, III) and an internal loop region at a junction of the three helices (Fig. 1a). Most of the bases essential for the catalysis are located in the single stranded region, and cleavage occurs at the junction between stem I and stem III. The sequence of stem II is variable, with the exception of the G10.1 · C11.1 pair adjacent to the catalytic core. Tuschl and Eckstein found that the length of stem II could be shortened to two base pairs.1) The loop could be substituted with poly (ethylene glycol) and phosphate linkers without loss of the activity.2,3)

The complete replacement of stem-loop II by nucleotide linkers was reported, and some of these ribozymes exhibited cleavage activity that was dependent on the nucleotide sequence.4) Short nucleotide linker substitutions for the entire cleavage activity that was dependent on the nucleotide sequence.5) Taira and co-workers described four-pieces of ribozyme that could be activated with an oligoribonucleotide,6,7) and used this system to investigate gene function in vitro.8) Porta and Lizardi also reported an allosteric hammerhead ribozyme, which contained both an inherent ribozyme sequence and a long, extra tail.9) By the addition of an oligoribonucleotide complementary to the extra sequence, the ribozyme folded to form a proper secondary structure. Breaker and co-workers reported various allosteric hammerhead ribozymes, which responded with small molecules such as nucleotides.10–13) Furthermore, they developed an array addressing their allosteric ribozymes to detect target molecules in the solution.14) In these reports, aptamers that bind to a small molecule were introduced into stem II, and the cleavage activities were investigated in the presence of the small molecule. Herschlag and co-workers reported another type of allosteric hammerhead ribozyme that was contained an abasic residue at a specific position within the catalytic core.15,16) The cleavage activity of the ribozyme was rescued by the addition of the missing base.

1.1. Rational Design of Allosteric Hammerhead Ribozyme

As reported in previous reports, stem II of hammerhead ribozyme is suitable for introduction of those modifications because of not participating in binding with a substrate RNA. However, general methods to control ribozyme activity had not been established. Therefore, we sought to develop a new method to control the ribozyme activity.

A hairpin loop and an oligonucleotide bound to the loop form one-half of the pseudoknot structure, which is called a pseudo-half-knot structure.17) Based on this structure, we designed an allosteric hammerhead ribozyme, which was activated by using a short oligonucleotide as a cofactor. Stem II of the wild type hammerhead ribozyme was substituted with a non-self-complementary loop sequence (loop II), which consists of 17-bases (Fig. 1b). These hammerhead ribozyme analogs were designed to retain the G10.1 · C11.1 base pair, which is important for efficient cleavage in the wild type hammerhead ribozyme.18) We investigated the cleavage activities of these ribozymes in either the absence or the presence of various oligonucleotides complementary to the 5’-side of the loop II.19) The oligonucleotides complementary to the loop II are referred to as a regulator or cofactor oligonucleotides (RLO). These could be 2’-hydroxyl (r) or 2’-O-methyl (m) oligoribonucleotides consisting of 7–12 bases (Fig. 1c). The 3’-ends of the 2’-O-methyl oligoribonucleotides contained ribonucleosides.

Under single turnover conditions, the cleavage activity of H43C with G10.1·C11.1 was negligible. The cleavage activity of H43C was investigated in the presence of RLOs that were added in 1.5 and 4 molar equivalents to H43C. Both r7 and m7 (2’-O-methyloligoribonucleotide; 2’-OMe-(GAGUGA)rG) induced the cleavage activity of H43C more efficiently than the r11 or r12 RLO (Fig. 2a). The RLOs r9, m9, r11, and m11 could not increase the activity of H43C efficiently, even when present in a 4 molar excess to H43C. The cofactor r12 only slightly enhanced the activity of H43C, and as the length of the RLO was increased, the efficiency decreased. These results suggest that the activation efficiency depends on the length of the RLOs. We proved that the bind-
ing of the long RLOs promoted the release of the substrate RNA from the ribozyme/substrate complex using gel mobility shift assays. This result indicated that the binding of the long RLOs perturbed the ribozyme/substrate complex.

Since r7 enhanced the cleavage activity of H43C most efficiently, r7N oligomers (r7A, r7G, r7C, and r7U, Fig. 1c) containing one added nucleoside (N) at the 3'-end, were synthesized. Since the 3'-N is located near the G10.1 · C11.1 base pair in the complex with H43C, r7N was expected to affect the cleavage activity by interacting with G10.1 · C11.1 or an internal catalytic domain of the hammerhead ribozyme. These cofactors with the 3'-dangling ends did not inhibit the cleavage reaction of H43C, but instead enhanced the activity. The r7C and r7U cofactors, which had pyrimidine bases at the 3'-ends, showed the same induction activity as r7. However, both r7A and r7G, with purine bases at the 3'-ends, induced 1.5 and 2-fold higher cleavage activities than r7 when present in 4-molar equivalents, respectively (Fig. 2b). r9G, with a guanosine at the 3'-end, also showed about 3-fold higher activity in H43C than r9. This result suggests that the 3'-dangling purine is required for efficient positive regulation. Turner and co-workers proved that 3'-dangling purines

Fig. 1. (a) Secondary Structure of the Complex of a Hammerhead Ribozyme (HH34) and a Substrate (S1) HH34 consists of 34 bases. The arrow indicates the cleavage site.

(b) Scheme of Activation of an Allosteric Hammerhead Ribozyme (H43C or H45C) by the Addition of r7 as a Cofactor

H43C and H45C consist of 43 and 45 bases, respectively and both involve a guanosine and a cytidine base at position 10.1 (G10.1) and 11.1 (C11.1). Stem II of the hammerhead is substituted for loop II (17 or 19 bases).

(c) Sequences of RLOs

Fig. 2. (a) Cleavage Rate Constants of H43C/RLO

The reaction solution contained H43C (1.5 µM), 5'-end labeled S1 (10 nM) and RLOs. The concentration of RLO was 2.3 µM (gray bars) or 6.0 µM (black bars).

(b) Comparison of the Cleavage Rate Constants of H45C (Black Bars) with that of H43C (Gray Bars) in the Presence of RLO

The cleavage rate constants were measured at 37°C using 5'-end labeled S1 (10 nM), H43C or H45G (1.5 µM), and RLO (6 µM).
add more stability to an RNA duplex than 3'-dangling pyrimidines. Thus, the energetic effect of the 3'-dangling bases might induce the efficient activation of the ribozyme. Furthermore, an RLO (Mis2, Fig. 1c), which forms two mismatched base pairs with H43C, did not activate the cleavage with H43C. This result suggests that the activation by RLOs proceeds in a sequence-specific manner.

1.2. Allosteric Hammerhead Ribozyme with a Longer Loop II Although the ribozyme (H43C) with a 17-base loop II showed weak cleavage activity in the presence of a 9 or 11-base RLO, a 7 or 8-base RLO activated the ribozyme efficiently. The reason may be that loop II is not large enough to form an active conformation after binding these RLOs (r9 and r11). Therefore, an alternative ribozyme (H45C), with a loop II (19 bases) two bases longer than that of H43C, was synthesized (Fig. 1b). Two uridine residues were inserted in the loop II of H43C to yield H45C, which also contained the G10.1·C11.1 base pair, and the activation of H45C by RLOs was examined.

Although H45C alone did not exhibit cleavage activity like H43C, H45C became active in the presence of either r7, r7N (N = A, G, C, U), r9 or r11. r7G showed the highest efficiency among these RLOs (Fig. 2b). These results are consistent with those obtained with the H43C/RLO reactions. However, H45C showed higher cleavage activity than H43C in the presence of either RLO. Pronounced activation was detected in the H45C/r9 complex as compared with the H43C/r9 complex. The reason for this is that the longer loop II of H45C might allow the formation of the active conformation after binding r9. r9G is more effective than r9 in activation of H45C as found in H43C activation. r7G induced the highest cleavage activity in H45C, and then, we investigated the activation efficiency of a 2'-O-methyl-oligoribonucleotide (m7G; 2'-OMe(GAGUGAG)rG) for H45C. It was found that m7G activated H45C about 2.5-fold higher than r7G under single turnover conditions. H45C was most efficiently activated in the presence of m7G by as much as about 750-fold, as compared with their activities in the absence of the oligonucleotide. One reason for this efficient activation is thought to be the thermal stability of a 2'-O-methyloligonucleotide and RNA hybrid.

1.3. Specific Regulation of Two Ribozymes in the Same Reaction Solution We prepared another ribozyme, which targeted a different substrate RNA. The ribozyme consisted of 42 bases, and the sequence of the loop II was designed to be different from that of H45C to activate with a specific RLO (r7Gnos). The sequence of the substrate RNA (iNOS19) was derived from the mRNA sequence of human induced nitric oxide synthase. We measured the observed rate constant of H42Cnos/iNOS19 complex in either the presence or the absence of r7Gnos under single turnover condition, and the activation efficiency by the addition of the RLO was also calculated. H42nos indicated the cleavage activity in the presence of r7Gnos; however, the activity of H42Cnos/r7Gnos complex was lower than that of H45C/r7G. The reason for this is thought that the loop II of H42Cnos was shorter than that of H45C.

We tried to carry out a new reaction system, which contained both H45C/S1 and H42Cnos/iNOS19 complexes in the same solution. It was investigated whether specific cleavage was induced by the addition of respective RLOs. The reaction scheme is shown in Figs. 3a, b. Either r7Gnos or r7G was added to the reaction solution about 2 h later after the reaction started. As the results of the cleavage reactions, the

Fig. 3. Cleavage Reaction of H45C/S1 and H42Cnos/iNOS19 Complexes in the Same Reaction Solutions Specific activation of H42Cnos (a) and H45C (b) by the addition of r7Gnos and r7G, respectively. iNOS19 and S1 are indicated by black and white bars, respectively. Gray lines and dotted lines indicate RLO and base pairs, respectively. Plots of percentages of cleavage products versus reaction times. Open circles, iNOS19; solid circles, S1; r7Gnos (c) or r7G (d) was added 2 h later after the reaction started. Each concentration of the ribozyme, the substrate and RLO were 80 nM, 4 nM and 400 nM, respectively at 2 h from the start of the reactions.
cleavage percent of the respective substrates increased in response to the addition of the specific RLO (Figs. 3c, d). These results indicate that cleavages of multiple RNA targets are regulated by usage of these allosteric ribozymes.

2. ALLOSTERIC HAIRPIN RIBOZYME

The hairpin ribozyme derived from the catalytic center of the negative strand of the satellite RNA of tobacco ring spot virus (sTRSV) is responsible for the cleavage and ligation reactions of the satellite RNA during replication.22,23) This ribozyme contains fifty bases and can cleave RNA that binds to the ribozyme by base pairing.24,25) The cleavage reaction occurs by an in-line mechanism, requires the presence of magnesium ions,26) and yields a 2,3',5'-cyclic phosphate and 5'-hydroxyl group. The hairpin ribozyme contains four stem regions (helices 1—4) and two internal loops (loop A and loop B) in domains I and II25,27,28) (Fig. 4a). Essential bases required for the cleavage activity in the internal loops have been identified by an in vitro selection method.27—30)

2.1. Rational Design of Allosteric Hairpin Ribozyme

We constructed modified hairpin ribozymes, which was divided at the hinge region (A14–A15 of the wild type) instead of joining 3'-end of the 5'-end of the substrate (S1) with trimer penta-cytidylates31) (Fig. 4b). The main part (S57 or S59) consisting of 57 or 59 bases included the cleavage site, and the rest was a short oligonucleotide of 14 bases (E14). S53 without a linker region was also prepared as a control molecule. An extra stable hairpin loop (5'GUU3') was introduced into these RNAs as a substitute for the wild type hairpin (5'GUU3'), since the introduction of the stable hairpin loop into a hairpin ribozyme was shown to raise the thermal stability.32)

Neither S53, S57 or S59 was active in the absence of E14, but S57 and S59 were cleaved in the presence of E14 at the same site as the wild type (E50-S1 complex). S53 without a linker was inactive even with a large excess of E14. The reason for this result is thought that S59-E14 and S57-E14 could form active bent structure, while S53-E14 could not, due to the absence of extra nucleotides in the hinge region. Although E14 forms a part of domain I after binding with S57 or S59, the oligonucleotide induced the cleavage of the RNA molecules by allosteric effect.

E14 was substituted with 15-mer RNAs (E15X, X=A, U or G) with an additional base X at the 3'-end of E14 (Fig. 4b). The extra base (X) was supposed to interact with one of the bases in the linker sequences of S57 or S59. S59 and S57 were cleaved in the presence of E15A or E15U. However, E15G caused little cleavage (Fig. 5a). We thought that this inhibition of the cleavage reaction was derived from the base pairing between X and Y in the linker regions. The extra base at 3'-end of E15X was extended from one to five bases, and a series of E19X also induced the cleavage of both S57 and S59 when there were not any complementarities between the extended sequences of E19X and the hinge region of these RNAs (Fig. 5c). This result indicates that the 3'-extra bases of these E19X series do not prevent the formation of the active bent conformation if the extra sequence does not form base pairs.

In these constructs of the modified hairpin ribozymes, RNA molecules such as E14, E15X or E19X, are regarded as the regulator oligonucleotides described in the section of the
allosteric hammerhead ribozyme. When 3′-end extra bases of the RLO form base pairs with the linker sequence of these modified hairpin ribozymes, it was found that the cleavage reaction of the ribozyme are prevented. If the RLO part is a part of a mRNA sequence, the modified hairpin ribozymes shown in these experiments have possibilities to detect the mRNA in cells.

2.2. In Vitro Selection of Allosteric Hairpin Ribozymes

We carried out an in vitro selection to obtain an allosteric hairpin ribozyme, which has cleavage activity in the presence of an exogenous short oligonucleotide as a regulator.33) A 20 nucleotide random sequence was introduced into a region corresponding to the hairpin loop (loop C) of the hairpin ribozyme (Fig. 4a).

After transcription from the DNA library encoding ribozyme pools, we carried out negative selection by incubating the transcripts in the absence of a regulator oligonucleotide (RLO). As the RLO, a 2′-OMe methyl oligoribonucleotide (m7G: 2′-OMe(GAGUGAG)rG), which was used for the activation of an allosteric hammerhead ribozyme. After the cleavage reaction without m7G, uncleaved transcripts were isolated by denaturing polyacrylamide gel electrophoresis (PAGE). Those RNA molecules were then incubated in the presence of m7G (positive selection), and the cleavage products were purified by PAGE. The active molecules in the positive selection were converted to DNA by reverse-transcriptase, followed by PCR amplification. The double stranded DNAs were used as the templates for the following transcription. The set of both negative and positive selections was repeated. To remove as many ribozyme molecules as possible, which were independently active in the presence of the RLO, we carried out a secondary negative selection from 7th generation RNA prior to the positive selection. The double negative and positive selections were repeated. After the selections, we investigated the sequences of 34 clones. Interestingly, the sequence of clone 1 (c1) was detected in 18 clones, and the others were found to be unique clones. All of the clones included the sequence complementary to the regulator oligonucleotide (m7G), and most of these complementary sequences were located from the center to the 3′-side of the loop C.

The cis-cleavage activities of all of the clones were assayed, by measuring the observed rate constants in either the presence or the absence of m7G. Every clone had little activity without m7G, but became active with an excess amount of m7G. Especially, clones c1, c2, and c3 showed high observed rate constants in the presence of m7G. One base deletion within loop C was observed in both c2 and c3. The c3 clone seems to be generated from the deletion of uridine 4 (U4) from c1, because the other sequences, except for U4, are the same as c1. It is interesting that both c2 and c3, which are deletion-mutants, showed more than 300-fold activation efficiencies.

We predicted the secondary structures of the entire domain II region in the absence of m7G by using the mfold program,34) and the structures of loop C, including four bases (C25, A26, G36, U37) in loop B, are shown in Fig. 6a. Interestingly, every loop C of these ribozymes folds into a stable helix-loop structure with a four- or five-base turn, and the sequences complementary to m7G are located at the single stranded regions. If these hairpin loops are formed without the regulator oligonucleotide, then these ribozymes should be inactive due to the disruption of the active loop B conformation. The location of the sequences complementary to m7G might facilitate their contact with the RLO, and the binding with m7G might promote slippage of the base pairs of loop C. It is thought that after m7G binding, the newly formed helix with m7G connectively stacks with the pseudo-helix 4 (Fig. 6b), and the stacking effect might make the structure predominant.

Furthermore, we constructed an allosteric hammerhead ribozyme by introducing the hairpin module of c1 to investigate whether the module of c1 could be applied to the control of a different ribozyme. The modified hammerhead ribozyme was also changed to an allosteric ribozyme, which was activated by the addition of the regulator oligonucleotide.33) It was proven that the module structure controlled the active or inactive structure of the ribozyme by transmitting the structural change to the core of the ribozyme.

3. CONCLUSION

We have constructed various allosteric ribozymes, which are activated by the short oligonucleotides. The activation of these ribozymes by the oligonucleotides is sequence specific, and the affinity between the ribozyme and the cofactor is high, due to the formation of base pairs. Therefore, ribozymes, which contain unique sequences complementary to the oligonucleotides, can be positively and independently
controlled by the specific oligonucleotide. Therefore, a variety of the ribozymes targeted against different mRNAs may be regulated in cellular applications by using the allosteric system. Furthermore, the ribozyme designed here may also be used as a sensor for the existence of the target RNA. The characteristic RNA modification shown here may be used to control RNA functions in various fields, and may be applicable to the regulation of other functional RNAs including hairpin loops.

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