A New Type of Hairpin Ribozyme Consisting of Three Domains†

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ABSTRACT: We have constructed a new hairpin ribozyme with three stem−loop domains. In the ribozyme, another domain (domain I′) was connected to the 3′-end of domain II of the parent hairpin ribozyme, and the new ribozyme can be trimmed after transcription from the DNA template using T7 RNA polymerase. Since a mutant ribozyme containing a substitution at the essential base in domain I′ lacked the 3′-trimming reaction, the autoprocessing activity was proved to be derived from the catalytic reaction, similar to the wild-type ribozyme. Furthermore, the structure of the cleavage site from the self-trimming reaction was identified as a 2′,3′-cyclic phosphate, which is the same as that of the wild-type. The processed ribozyme was designed to cleave an external substrate RNA derived from the mRNA of the human inducible nitric oxide synthase and was proved to cleave at the expected, unique site. The hairpin ribozyme containing the three-domains exhibited the 3′-self-trimming activity even in a runoff transcription reaction from the plasmid harboring the ribozyme gene with the three domains. The new type of hairpin ribozyme thus obtained has three stem−loop domains and is able to act as a catalytic RNA for both cis and trans cleavage. These ribozymes are of interest from the point of the structure−function relationship of the hairpin ribozyme and provide an important insight into over understanding of the role of the domain−domain interaction in the catalytic activity.

The hairpin ribozyme is derived from the negative strand of the satellite RNA of tobacco ringspot virus (sTRSV(−))[1] and consists of 50 bases (Buzayan et al., 1986; Hampel & Triz, 1989; Symons, 1992). The ribozyme can cleave RNAs, depending on the sequence (Hampel et al., 1990) and the cleavage reaction proceeds by an in-line mechanism in the presence of divalent metal ions to generate 2′,3′-cyclic phosphate and 5′-hydroxyl groups (Van Tol et al., 1990). There are similar sequences in the satellite RNAs of chicory yellow mottle virus (sCYMV; Rubino et al., 1990) and arabis mosaic virus (sArMV; Haseloff & Gerlach, 1989). Their catalytic centers cleave NGUA sequences most efficiently, whereas the NGUC sequence is cleaved fastest in the hairpin ribozyme from sTRSV(−) (DeYoung et al., 1995). The hairpin ribozyme is able to stimulate a ligation reaction of the cleaved products in vitro, in contrast to the hammerhead ribozyme (Buzayan et al., 1986; Komatsu et al., 1993). The metal ion requirement is also different from that of other ribozymes (Chowrira et al., 1993a). Additionally, replacement of the phosphodiester bonds with phosphorothioate diester bonds does not greatly influence either the processing or the ligation (Buzayan et al., 1988). These results suggest that the cleavage mechanism may be different from that of the hammerhead ribozyme (Koizumi & Ohtsuka, 1991; Kuimelis & Mclaughlin, 1995; Zhou et al., 1996).

The hairpin ribozyme contains two stem−loop domains (domain I and domain II, Figure 1a), and these domains are connected at the hinge (Hampel et al., 1990). Domain I has a symmetrical loop (loop A), but the loop in domain II (loop B) is asymmetric. The absolutely required bases for the cleavage reaction have been shown to exist in these internal loops of the two domains by in vitro selection (Berzal-Herranz et al., 1992; Berzal-Herranz et al., 1993; Joseph et al., 1993) and point mutation experiments (Hampel et al., 1990; Sekiguchi et al., 1991; Anderson et al., 1994; Grasby et al., 1995). The in vitro selection experiments were a powerful tool to investigate the important sequences for the cleavage and the ligation, and the bases required not only in the internal loops but also in the stems were identified by these experiments. For the cleavage of RNA by the ribozyme, the guanine base on the 3′-side of the cleavage site is essential (Chowrira et al., 1991), and this is a unique characteristic among other ribozymes. The secondary structure of loop B in domain II was proposed to be similar to that of loop E in the 5S rRNA from photo cross-linking (dos Santos et al., 1993a; 1993b; Butcher & Burke, 1994a), chemical modification (Butcher & Burke, 1994b), and insertion experiments of nucleotide analogs (Schmidt et al., 1996).

Recently, the tertiary structure of the hairpin ribozyme has also been studied, and a bent structure, in which the two domains interact with each other, was proposed to be the active conformation (Feldstein & Bruening, 1993; Komatsu et al., 1994). The reversely joined ribozyme, in which the two domains were replaced and joined, was found to maintain the cleavage activity (Komatsu et al., 1995). Even though the two domains were separated completely, the ribozyme

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[1] Abbreviations: sTRSV(−), negative strand of the satellite RNA of tobacco ringspot virus; NMR, nuclear magnetic resonance; TBDMS, *tert*-butyldimethylsilyl group; TBAF, tetrabutylammonium fluoride; HPLC, high-performance liquid chromatography; NO, nitric oxide; iNOS, inducible nitric oxide synthase; TriRz, a hairpin ribozyme with trimming activity; MutRz, mutant ribozyme of TriRz; SBS, substrate binding sequence.

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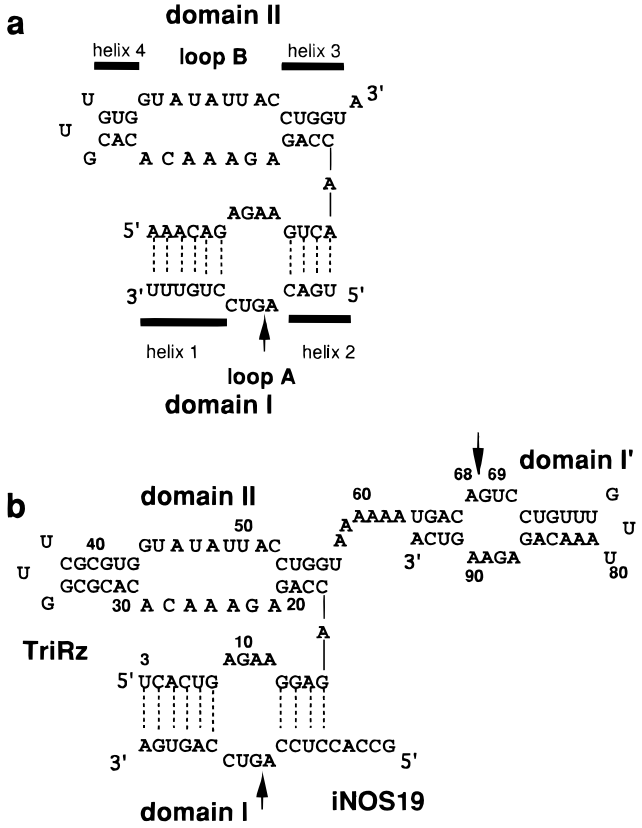

FIGURE 1: Secondary structures of hairpin ribozymes. (a) A wild-type hairpin ribozyme derived from the catalytic center of sTRSV(−). Domain I consists of helix 1, helix 2, and loop A; domain II, of helix 3, helix 4, and loop B. The hairpin ribozyme is bent to show the cleavage activity between helix 2 and helix 3. (b) A hairpin ribozyme containing three domains. An additional domain (domain I′) is covalently connected to the 3′-end of domain II. The ribozyme with three domains has a cis cleavage site. The sequence of domain I′ is derived from domain I of the wild-type hairpin ribozyme. MutRz has a mutation in domain I′. TriRz and MutRz are designed to cleave a part of the iNOS mRNA in trans. Two guanosines from the template sequence have been added to the 5′-end of either TriRz or MutRz, respectively. The base pairs and the sequence connecting the two domains are represented by broken and continuous lines, respectively. Arrows indicate the cleavage sites.

also showed cleavage activity when the concentrations of ribozyme and magnesium ion were high (Butcher et al., 1995) or when an anchor sequence was inserted to facilitate the association of these domains (Komatsu et al., 1996). These results verify that the active conformation of the ribozyme is bent. Although the detailed overall structure of the ribozyme has not been elucidated, the structure of domain I without domain II in solution has been solved from nuclear magnetic resonance (NMR) spectroscopy (Cai & Tinoco, 1996). From these data, a shared G·A base pair seems to exist, and the uracil next to the guanosine at the 3′-cleavage site is splayed apart in domain I.

On the basis of the requirement of domain−domain interaction for ribozyme activity, here we have constructed a new type of hairpin ribozyme that has three stem−loop domains in one molecule. Domain II of the ribozyme acts as the catalytic domain for both cis and trans cleavage of RNA. Since this new ribozyme is able to process 3′-extra nucleotides autocatalytically without another ribozyme for trimming, the ribozyme is interesting not only for studies of the active conformation of RNA but also for in vivo applications.

MATERIALS AND METHODS

Construction of Self-Trimming Ribozyme Cassette. DNAs were synthesized using phosphoroamidite blocks purchased from Perkin-Elmer Applied Biosystems Inc. and were purified by reverse phase and anion exchange HPLC. All ribozymes were prepared by transcription from DNA, using T7 RNA polymerase. Eight single-stranded DNA fragments encoding TriRz and the T7 RNA promoter were assembled and ligated using T4 DNA ligase, as previously reported (Koizumi et al., 1989). The sense sequence of the T7 promoter and TriRz fragments is 5′G(−19)TTAATAC-GACTCACTATAG(+1)GTCACTGAGAAGGAGACC-AGAGAAACACACGCGGTTCGCGTGGTATATTACC-TGGTAAAAAATGACAGTCCTGTTTGTTAAACAGAG-(+88)AAGTCA(+94)3′.

The sequence of MutRz contained a cytidine instead of the guanosine (+88) underlined above.

Construction of the DNA Template for Runoff Transcription. To insert the DNA cassettes synthesized above into pUC118 (Takara Shuzo), *Eco*RI and *Bam*HI sites were introduced in the DNA template by the polymerase chain reaction, using two primers (5′CGGCGAATTCTTAATAC-GACTCACTATA3′ for the *Eco*RI restriction site; 5′GCACG-GATCCTGACTTCACTGTTTAACA3′ for the *Bam*HI restriction site). After the PCR products were digested by *Eco*RI and *Bam*HI, the DNA cassettes were ligated into pUC118 that had been cleaved by *Eco*RI (Takara Shuzo) and *Bam*HI (Takara Shuzo). The DNA template was transformed into *Escherichia coli* strain DH5α. After cloning, the plasmid was recovered using a QIAGEN column (QIAGEN Inc.) and was used as an in vitro transcription template.

Transcription from the DNA Cassettes. DNA template (2.5 or 10 nM), 0.5 mM of each NTP, 40 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 2 mM spermidine trihydrochloride, 5 mM DTT, 11 units of RNase inhibitor (Takara Shuzo), 0.5 μCi/μL [α-^{32}P]UTP, and 0.5 units/μL T7 RNA polymerase (Takara Shuzo) were incubated in a total volume of 20 μL at 37 °C for 1 h. After ethanol precipitation of the aliquots, loading solution (10 M urea, 50 mM EDTANa2, 0.1% bromophenol blue) was added to the aliquots, which were fractionated on an 8% polyacrylamide gel (acrylamide: bisacrylamide, 19:1) containing 8 M urea. The gel was dried and exposed to a plate for Bioimaging analyzer (FUJIX BAS2000), and the percentages of the trimming reactions were measured.

Runoff Transcription from the Plasmid DNA. The plasmid DNA (pUTriRz, 0.07 μg) which had been digested by either *Hind*III, *Sal*I, or *Bam*HI, was used for the runoff transcription. The linearized plasmid DNA (0.07 μg), 0.5 mM of each NTP, 40 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 2 mM spermidine trihydrochloride, 5 mM DTT, 0.5 μCi/μL [α-^{32}P]-UTP, and T7 RNA polymerase from the AmpliScribe T7 kit (A. R. Brown Co., LTD, 2 μL) were incubated in a total volume of 20 μL at 37 °C. Aliquots were taken at time intervals and added to loading solution (10 M urea, 50 mM $EDTANa_2$, 0.1% bromophenol blue) to stop the cleavage reaction. The products were analyzed on an 8% polyacrylamide gel (acrylamide:bisacrylamide, 19:1) containing 8 M urea. The cleavage rate constants for the cis cleavage reaction were determined as described previously (Long & Uhlenbeck, 1994; Siwkowski et al., 1997). The uncleaved

fraction ($L/(L + S)$) was plotted versus time, and the cleavage rate constants (k) for cis cleavage were determined by fitting the data by a least-squares method to the following equation

$$L/(L + S) = (1 - e^{-kt})/kt$$

where L is the concentration of full-length transcript, S is the concentration of cleaved transcript, t is time, and k is the unimolecular rate constant for cleavage.

Preparation of RNAs for Trans Cleavage Reactions. A mixture of 50 nM DNA template, 7.5 mM of each NTP, 40 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 2 mM spermidine trihydrochloride, and 10 mM DTT was treated with T7 RNA polymerase from the AmpliScribe T7 kit (A. R. Brown Co., LTD, 2 μL) in a total volume of 20 μL. After the solution was incubated at 37 °C for 2 h, DNase I (1 unit) was added to the aliquot, which was incubated at 37 °C for 15 min. After phenol−chloroform and chloroform extractions and ethanol precipitation were carried out, cleavage buffer (20 μL; 40 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 2 mM spermidine trihydrochloride) was added to the precipitate, and the aliquot was incubated at 37 °C for 1 h to complete the trimming reaction. Loading solution (10 M urea, 50 mM EDTANa2, 0.1% bromophenol blue) was added to the aliquot, which was fractionated on a 6% polyacrylamide gel (acrylamide:bisacrylamide, 19:1) containing 8 M urea. The trimmed 5′FTrimRz and MutRz were purified from the gel and used for the trans cleavage analysis.

A substrate RNA (iNOS19) for the trans cleavage reaction was synthesized using phosphoroamidite units purchased from GLEN research. The synthesis was carried out with a Perkin-Elmer Applied Biosystems DNA/RNA synthesizer (model 394A). The purification was carried out according to the previous reports (Scaringe et al., 1990; Komatsu et al., 1993).

Trans Cleavage Reaction. 5′FTriRz or MutRz was dissolved in cleavage buffer (40 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 2 mM spermidine trihydrochloride) to a concentration of 10 nM (100 μL). The 5′-end of iNOS19 was labeled with [γ-^{32}P]ATP, and then it was dissolved in the cleavage buffer. The concentrations of iNOS19 were varied from 40 to 200 nM. The ribozyme (5′FTriRz or MutRz; 100 μL) was heated at 65 °C and then transferred to an ice bath. The ribozyme solution was preincubated at 37 °C for 7 min, and to start the cleavage reaction, an equal volume of the ribozyme solution (7 μL) was added to the substrate solution (7 μL), which had been heated at 65 °C for 2 min and then cooled in an ice bath. The final concentrations of ribozymes and substrates were 5 and 20−100 nM, respectively. The reaction mixture (14 μL) was incubated at 37 °C, and aliquots were taken at time intervals and added to loading solution containing 10 M urea and 50 mM $EDTANa_2$ to stop the reaction. After 20% polyacrylamide gel electrophoresis (acrylamide:bisacrylamide, 19:1) containing 8 M urea was performed, the gel was dried. The percentages of the cleavage were determined by a Bioimaging analyzer (FUJIX BAS2000). The initial velocities were calculated, and the kinetic parameters for 5′FTriRz or MutRz were obtained from Hanes−Woolf plots.

Removal of 2′,3′-Cyclic Phosphate. The 2′,3′-phosphate was removed according to a previous report (Grosshans and Cech, 1991). Transcription from the DNA cassettes (0.05 pmol) encoding TriRz was carried out for 4 h, as described in the transcription from DNA cassettes. To complete the self-trimming reaction, cleavage buffer (30 μL) was added to the solution, which was incubated at 37 °C for 1 h. After phenol−chloroform and chloroform extractions and ethanol precipitation, the transcripts were dissolved in sterile water. A portion of the transcripts was incubated in 10 μL of filter-sterilized 10 mM HCl (pH 2.0) at room temperature for 2 h to open the 2′,3′-cyclic phosphate. The pH was adjusted by the addition of 0.1 N NaOH and 0.1 M Tris-HCl (pH 8.0). The RNA was subsequently treated with 0.5 μL of calf intestinal phosphatase (0.1 unit; Takara Shuzo) at 37 °C for 40 min to remove the terminal phosphate from either the 2′- or 3′-position. After phenol and phenol−chloroform extractions, the RNA was precipitated with ethanol and dissolved in sterile water. An aliquot was used for the 3′-end labeling with [5′-^{32}P]pCp.

Analysis of 3′-Terminal Nucleotides. 5′FTriRz, from which 2′,3′-cyclic phosphate was removed, was labeled at the 3′-end with [5′-^{32}P]pCp and RNA ligase (Komatsu et al., 1993) and was isolated from a 6% polyacrylamide gel containing 8 M urea. The 3′-end-labeled 5′FTriRz was completely digested in 12.5 μL reaction solutions, containing 40 mM ammonium acetate−acetic acid (pH 5.0), ribonuclease T1 (0.5 unit), ribonuclease T2 (0.5 unit), and RNase A (100 μg), which were incubated at 37 °C for 10 h (Grosshans & Cech, 1991). Samples were spotted on separate 10 cm^2 cellulose TLC plates (Funakoshi). Unlabeled nucleoside 3′-monophosphates (Np) were also spotted on the origin of the plate as controls. The plates were developed in the first dimension (isobutyric acid: 0.5 M NH_4-OH, 5:3, v/v) for 3 h and dried. They were then developed in the second dimension (2-propanol:concentrated HCl:H_2O, 70:15:15, v/v) for 4 h. After the plates were dried, they were autoradiographed and scanned on a Bioimaging analyzer (FUJIX BAS2000).

RESULTS AND DISCUSSION

Self Trimming Activity of the Hairpin Ribozyme Containing Three Domains. Since we have previously shown that the hinge A14−A15 can be divided by insertion of a linker at the opposite strand, using three or five nucleotides (Komatsu et al., 1995), a ribozyme that has three domains, including domain I′ at the 3′-end of the wild-type ribozyme, was proposed to be active (TriRz in Figure 1b). Domain I′ consists of a stem−loop structure connected by the hairpin loop (GUU), which is the same structure as that in domain II. If domain II interacts with domain I′, as shown in Figure 2, the cleavage reaction in domain I′ was expected. Domain I′ has sequences derived from domain I of the natural hairpin ribozyme (Figure 1a), and the domain I of TriRz was designed to cleave a part of the mRNA (codons 34−40) of an inducible nitric oxide synthase (iNOS) from human glioblastoma cells (Hokari et al., 1994). The template DNA for the hairpin ribozyme with three domains (TriRz) was transcribed from template DNA using a T7 transcription system (Milligan et al., 1987; Koizumi et al., 1989). DNAs encoding a control molecule (MutRz in Figure 1b) were also synthesized. In MutRz, a guanine base (G88) in domain I′, which is an absolutely required base of domain I in the wild-type hairpin ribozyme (Berzal-Herranz et al., 1993; Grasby et al., 1995), was substituted with cytidine. If these ribozymes process domain I′, then the trimming reactions would be detected during the transcription reactions.

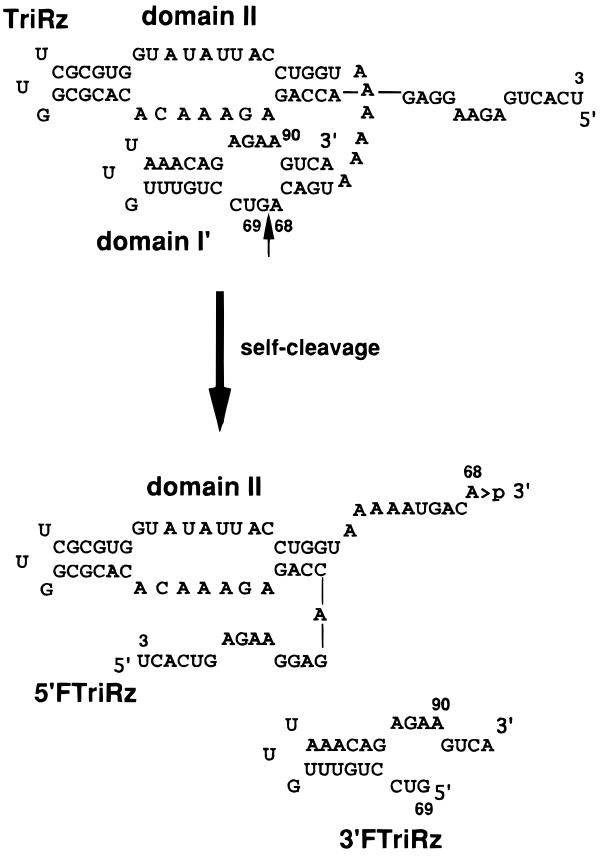

FIGURE 2: Predicted 3′-self-trimming reaction of TriRz after transcription. Domain I′ folds back to domain II, and cis cleavage occurs in domain I′. 5′ (5′FTriRz) and 3′ (3′FTriRz) cleavage products are generated. The arrow indicates the cleavage site.

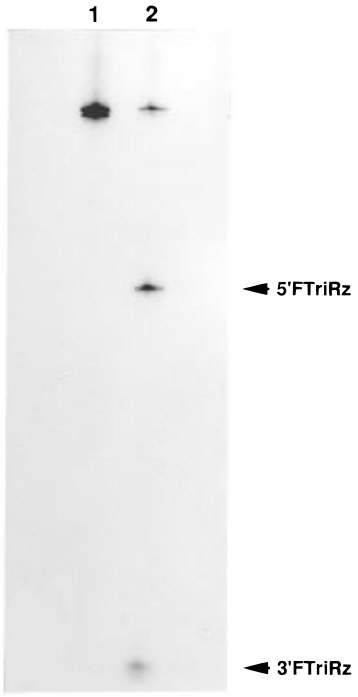

FIGURE 3: Analysis of the processing of TriRz and MutRz during transcription from the template DNAs by electrophoresis on an 8% polyacrylamide gel containing 8 M urea. Lane 1, MutRz; lane 2, TriRz. Cleavage products from the self-trimming reaction are indicated.

The results indicated that the transcript was self-processed in the domain I′ of TriRz; however, MutRz, with a mutation in domain I′, did not indicate the self-trimming activity, as expected (Figure 3). The percentages of the cleaved products did not change at any point of time during the transcription, and the trimming activity did not depend on the concentration of DNA templates (data not shown). Thus, these results suggest that the trimming reaction of TriRz proceeded by intramolecular reactions. The trimming reaction of domain I′ of TriRz was not caused by the substrate binding sequence (SBS; U3−G16 in Figure 1b) of domain I after binding with the sequence (U64−U77) of domain I′, since MutRz was not self-processed and the SBS of domain I was not complementary to domain I′. Domain I′ was supposed to be folded in an intramolecular manner, and then its approach to domain II would be followed by cleavage in domain I′. It is interesting that the SBS of domain I did not seem to prevent an interaction between domain I′ and domain II. Perhaps, domain I′ could fold back to domain II, as shown in Figure 2, with avoidance of the steric hindrance of the SBS due to the presence of the flexible five-adenosine linker. Since a linker with three or five nucleotides was required for the cleavage in the modified hairpin ribozyme divided at the hinge (Komatsu et al., 1995), the linker length of TriRz was also thought to be important for the formation of the active conformation.

The Structure of the Self-Cleavage Site of Domain I′. The wild-type hairpin ribozyme cleaves the substrate RNA to yield 2′,3′-cyclic phosphate and 5′-hydroxyl groups. If the self-trimming reaction at domain I′ was caused by the same mechanism as that of the natural hairpin ribozyme, the structure of the trimming site should also be the same as that of the wild-type. The structure of the cleavage site from TriRz was examined, after the transcription of TriRz. The ability of the products from the transcription to accept [5′-^{32}P]pCp in the ligation reaction using T4 RNA ligase was investigated (Grosshans & Cech, 1991). The 3′-cleavage product (3′FTriRz; G69−A94 in Figure 2) from the self-trimming of TriRz was able to accept [5′-^{32}P]pCp, but in contrast, the 5′-side fragment (5′FTriRz; U3−A68 in Figure 2) was not labeled with the [5′-^{32}P]pCp (data not shown). However, when the transcripts were treated under acidic conditions, followed by treatment with calf intestinal phosphatase, 5′FTriRz accepted the [5′-^{32}P]pCp. Since the 5′FTriRz could not be labeled without treatment by both hydrochloric acid and phosphatase, the structure of the 3′-terminus of 5′FTriRz was identified to be a 2′,3′-cyclic phosphate, identical to the natural hairpin ribozyme. 5′FTriRz labeled with [5′-^{32}P]pCp was isolated from the gel and digested by RNases. The digests were analyzed by two-dimensional thin layer chromatography. The 3′-end nucleotide of the labeled 5′FTriRz was found to be adenosine, and the result indicated that TriRz was processed at the exact site (A68−G69) in domain I′. MutRz did not exhibit the trimming activity, and the structure of the trimmed site of TriRz was the same as the wild-type. These results suggest that the autoprocessing at domain I′ proceeded by the same mechanism as in the wild-type hairpin ribozyme.

Trans Cleavage Activity of the 3′-Trimmed Ribozyme. To investigate whether the 5′-side fragment (5′FTriRz) and the untrimmed ribozyme had trans cleavage activity, trans cleavage reactions were performed using 5′FTriRz and MutRz. Each ribozyme was designed to cleave a 19-base RNA (iNOS19) with the sequence of the iNOS mRNA from human glioma cells. MutRz and 5′FTriRz were isolated from the gel after transcription. The reactions were carried out

Table 1: Kinetic Parameters for Trimmed and Untrimmed Ribozymes[a]

	K_m (nM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})
5′FTriRz	31	0.25	8.0
MutRz	66	0.19	2.8

[a] The reactions were performed at 37 °C.

under multiple turnover conditions. Both ribozymes were able to cleave the substrate at the predicted site. It is interesting that MutRz was able to cleave the substrate, because MutRz contains the unprocessed domain I′ at its 3′-end. However, the K_m value of MutRz was about 2-fold higher than that of 5′FTriRz, and the k_{cat} of MutRz was also smaller than that of 5′FTriRz (Table 1). The k_{cat}/K_m value of 5′FTriRz was about 3-fold higher than that of MutRz. These results indicate that MutRz, containing domain I′, had a lower catalytic efficiency than 5′FTriRz, without the 3′-extra sequence. The unprocessed ribozyme could also cleave the substrate RNA, but the 3′-trimmed ribozyme showed higher cleavage activity. This difference of the trans cleavage activity is derived from the 3′-extra sequences of these ribozymes. It is thought that domain I′ of MutRz was able to interact with domain II weakly, because the internal loop sequence in domain I′ was almost the same as domain I. Thus, the domain I−domain II interaction required for the trans cleavage might compete with the domain I′−domain II interaction, and this might inhibit the trans cleavage in MutRz. Another reason for the low catalytic efficiency of MutRz may be derived from the difficulty of substrate binding to the ribozyme, due to the bulkiness of the unprocessed domain II of MutRz.

The K_m value of TriRz was almost the same as that of the wild-type hairpin ribozyme, as described in other reports (Hampel & Triz, 1989; Sekiguchi et al., 1991; Chowrira & Burke, 1992). The k_{cat} of TriRz was also the same as that reported previously (Sekiguch et al., 1991; Grasby et al., 1995); however, it was about 8-fold lower than those in other reports (Chowrira & Burke, 1992; Chowrira et al., 1993b). We have no explanation for the discrepancy. The sequence of iNOS19 used here is different from that of the wild-type, and the 5′-side sequence of iNOS19 was longer than that of the wild-type. These differences in the sequences and sizes of the substrate might lower the k_{cat} value of TriRz.

Self-Trimming Reactions of TriRz with Longer Extra Sequences at the 3′-End. To investigate whether the 3′-processing reaction of TriRz can occur in the presence of extra nucleotides at the 3′-end, a DNA cassette encoding TriRz was inserted into pUC118 (pUCTriRz), and then runoff transcription was carried out. For the runoff transcription, linear pUCTriRz was prepared by digestion with *Bam*HI, *Sal*I, or *Hind*III (pUCTriRz−*Bam*HI, pUCTriRz−*Sal*I, or pUCTriRz−*Hind*III, Figure 4). Extra bases (5, 17, or 35) were added to the 3′-end of TriRz during the transcription from the linear pUCTriRz−*Bam*HI, pUCTriRz−*Sal*I, or pUCTriRz−*Hind*III. As a result of the runoff transcription, the transcripts from either the linear pUCTriRz−*Bam*HI, pUCTriRz−*Sal*I, or pUCTriRz−*Hind*III were trimmed at the same site as the transcripts from the DNA cassettes. In these reactions, the 5′-trimmed products (5′FTriRz) were generated commonly; however, the sizes of the 3′-trimmed products were different according to the distance of the respective restriction site from the 3′-end of TriRz. The results

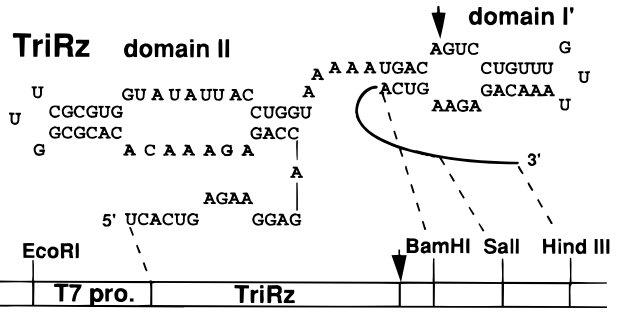

FIGURE 4: Templates used for runoff transcriptions. Extra sequences from the vector were added to the 3′-end of TriRz. T7 pro. is the T7 promoter, and the arrow indicates an intramolecular cleavage site.

Table 2: Cleavage Rate Constants for Intramolecular Cleavage

	pUCTriRz−*Bam*HI	pUCTriRz−*Sal*I	pUCTriRz−*Hind*III
k (min^{-1})	0.070	0.032	0.035

indicated that the trimming reactions of TriRz could occur even in the presence of longer extra sequences at the 3′-end of domain I′. The rate constants (k) for cis cleavage were measured as described previously (Long & Uhlenbeck, 1994; Siwkowski et al., 1997). The transcript from pUCTriRz−*Bam*HI, which had the shortest 3′-extra sequence, showed about 2-fold higher cleavage activity, as compared to pUTriRz−*Sal*I and −*Hind*III (Table 2). The cis cleavage activity was decreased by the addition of longer 3′-extra sequences. Perhaps domain I′ might be prevented from approaching domain II, because the 3′-extra sequences from the vector might interact with the poly(A) linker or the SBS (U3-G16). However, it is interesting from the point of the tertiary structure of the ribozyme, that TriRz with the extra sequences at the 3′-ends showed the self-trimming reaction. When the extra sequences were attached to the 3′-end of domain I′, steric hindrance was generated at the hinge regions of the ribozyme, and thus the bent conformation between domain I′ and domain II seemed to be prevented. However, self-trimming reactions were detected in the runoff transcriptions, indicating that domain I′ could fold back to domain II, as shown in Figure 2. When domain I′ is closely folded to domain II, the substrate binding region (SBS) may be moved away to not disturb the domain I′−domain II interaction, and the extra nucleotides attached in the 3′-end of domain I′ might extend in the direction opposite the domain I′—the domain II.

The cis cleavage activities of the three-domain hairpin ribozymes were still low, as compared with the two-domain hairpin ribozymes (Siwkowski et al., 1997), and it seems that some improvements will be required for high cis cleavage activity.

CONCLUSION

In contrast to natural hairpin ribozymes, which consist of two stem−loop domains, we constructed a new type of hairpin ribozyme (TriRz), which has three stem−loop domains. Another domain I (domain I′) was connected with the 3′-end of the wild-type hairpin ribozyme as the third domain. The sequences of domain I′ were derived from domain I of the parent ribozyme. The hairpin ribozyme with three domains was also designed to cleave the substrate RNA in trans, and thus, there were both trans (domain I) and cis

(domain I′) cleavage sites in this ribozyme. The transcripts from the DNA encoding TriRz were processed in domain I′ during transcription. The structure of the autoprocessed site was identified as the 2′,3′-cyclic phosphate, similar to that from the cleavage of the wild-type hairpin ribozyme. A mutant ribozyme (MutRz), in which the absolutely required base of domain I′ was substituted, did not show the self-trimming activity. For the trans cleavage of RNA, substrate RNAs containing the sequence from the human inducible nitric oxide synthase (iNOS) were treated by a 5′-side fragment (5′FTriRz) generated from the self-trimming of TriRz. Both 5′FTriRz and MutRz, with an unprocessed domain I′, were able to cleave the substrate RNA in trans, and the catalytic efficiency of 5′FTriRz was higher than that of MutRz. TriRz DNA was inserted in the plasmid vector, and runoff transcriptions were carried out. TriRz was able to process domain I′, even though TriRz was transcribed with longer 3′-extra sequences at the 3′-end.

The hairpin ribozyme described here has three domains and indicated self-trimming activity. Domain II served as the catalytic center of both cis and trans cleavage. This ribozyme is interesting for investigating the active conformations of the hairpin ribozyme.

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