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Enhancement in the Cleavage Activity of a Hammerhead Ribozyme by Cationic Comb-Type Polymers and an RNA Helicase *In Vitro*[1]

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The activity of a hammerhead ribozyme (Rz) *in vivo* depends on several factors, such as abundance, stability, and accessibility of Rz to its target mRNA. Among these factors, accessibility is believed to be the rate-limiting factor for Rz-mediated cleavage *in vivo*. As Rz and its substrate RNA are negatively charged, we examined whether cellular RNA-interacting proteins or artificial polycations might improve the accessibility of Rz to its substrate RNA. Specifically, we examined the effects of two kinds of cationic comb-type copolymer, αPLL-g-Dex, and a cellular RNA helicase on the accessibility of Rz to a model structured RNA *in vitro*. The cleavage activity of Rz was slightly enhanced by αPLL-g-Dex, probably due to an acceleration of the association/dissociation rate. And also, the RNA helicase-bound hybrid-Rz could cleave the target substrate at a significantly higher rate due to its unwinding activity for the duplex RNA substrate. These approaches should be useful in the development of efficient gene-inactivating reagents in the post-genomic era.

Key words: graft co-polymer, hammerhead ribozyme, helicase, polycations, RNA duplex.

The hammerhead ribozyme (Rz) is a small catalytic RNA molecule that was originally discovered in an infectious plant satellite RNA (*1*). The cleavage activity of *cis*-acting Rz is indispensable for replicating virusoid molecules. The Rz binds to mRNA through the target recognition sequence complementary to the target sequence and cleaves the mRNA in a sequence-specific manner in the presence of Mg^{2+} ions. Having engineered the Rz so that it acts *in trans* and cleaves various mRNAs only by changing the sequence of the recognition arms, the Rz has been expected to be used as a powerful tool for gene knockdown (*2*).

In many cases, the Rz activity *in vitro* does not correlate with that *in vivo* (*3, 4*). This is because, *in vivo*, the amount of Rz, the co-existence with the target mRNA, and accessibility to the mRNA determined by the higher ordered structures of the target mRNA and Rz, were found to be important factors for efficient cleavage (*3*). In our laboratory, by using the pol III system, we could engineer an Rz that was abundantly expressed and efficiently exported to the cytoplasm (*4, 5*). Thus, we could establish the guidelines for designing an effective Rz, particularly in mammalian cells, and could successfully apply the Rz to gene therapy (*6*) and gene discovery (*7*).

However, it is generally difficult to predict the suppressive effect of each Rz construct, because the exact folding of the target mRNA and the accessibility of the Rz to the target cannot easily be determined. Since the Rz cannot access the stemmed RNA structure, such a structure predicted by a computer program (*8*) is generally avoided as a target site, and the design of an effective Rz is usually based on trial and error. If the stem structure could be unwound by utilizing a cellular protein or an artificial molecule, it would not be necessary to predict the higher ordered structure of the mRNA, and also the cleavage activity of the Rz would be enhanced.

An intracellular helicase is known to unwind the higher ordered structure of DNA or RNA (*9*). Also, an RNA helicase has been identified as a component of post-transcriptional gene silencing (PTGS) in various organisms, and helicase-deficient mutant strains do not cause PTGS (*10–12*). Unwinding of the higher ordered structure of the target mRNA might be prerequisite for suppressing gene expression at the mRNA level in the cellular environment. A recent study in our laboratory revealed that the Rz fused to

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an RNA motif in interaction with an RNA helicase suppresses the expression of the target gene *in vivo* more efficiently (*7, 13*).

A recombinase can promote DNA strand exchange with a helicase activity (*14*). *Escherichia coli* RecA protein, for example, plays an important role in repairing damaged DNA and in homologous recombination, by strand exchanger reactions. The strand exchange is believed to be promoted by the stabilizing effect on the DNA triplex in the transition state. Recently, polycationic comb-type copolymers, αPLL-g-Dex (Fig. 1A), which significantly stabilize the DNA triplex and duplex, as does RecA, have been developed (*15, 16*). Since the αPLL-g-Dex can promote the strand exchange reaction, this copolymer is thought to have the properties of both a recombinase and a helicase. The αPLL-g-Dex copolymer consists of a backbone of polycation, poly(L-lysine) (PLL), and hydrophilic side chains of dextran (Dex). Generally, polycations interact strongly with polyanions, such as nucleic acids, to form polyion complexes or interelectrolyte complexes (IPECs) (*17, 18*). IPECs have been investigated as artificial carriers of poly(oligo)nucleotides, including genes and antisense oligonucleotides (*19*), but applications of IPECs are limited due to their irreversible and insoluble behavior. In contrast, the αPLL-g-Dex copolymer thermally stabilizes double-stranded (ds) DNA due to a reduction in counterion condensation, without affecting its reversibility between the single-stranded (ss) and ds forms (*15*). Moreover, under physiologically relevant conditions, the copolymer could expedite the strand exchange reaction between ssDNA and dsDNA by increasing the association rate (*20, 21*). These properties of the synthetic αPLL-g-Dex copolymer might similarly enhance the cleavage activity of Rz targeted to a structured RNA. In this study, we investigated the effect of the cationic comb-type copolymer and the cellular RNA helicase on the cleavage activity of Rz targeted to dsRNA *in vitro*.

MATERIALS AND METHODS

Materials—PLLs, whose number-average molecular weights (Mn) were 14,400 and 33,000, were purchased from Sigma-Aldrich (St. Louis, Missouri) and Peptide Institute (Osaka), respectively. Dextran T10 was obtained from Amersham Biosciences AB (Uppsala, Sweden). RNA oligonucleotides of HPLC grade were obtained from Takara Shuzo (Kyoto). Cetyltrimethylammonium bromide (CTAB) (Fig. 1B), other solvents and chemicals were obtained from Wako Pure Chemical Industries (Osaka) and used without further purification.

Preparation of αPLL-g-Dex Copolymers—The preparation, isolation and characterization of the PLL-g-Dex have been described in detail elsewhere (*15, 16*). Briefly, copolymers #1 (Mn of PLL: 14,400, Mn of Dex: 5,300, Mol% of Dex: 14.4) and #2 (Mn of PLL: 33,000, Mn of Dex: 8,000, Mol% of Dex: 14.5) were prepared by a reductive amination reaction between PLL HBr and Dex in dimethyl sulfoxide using $NaBH_3CN$ as a catalyst. The Dex of Mn 8000 used to prepare the αPLL-g-Dex #2 was obtained by fractionating from Dextran T10 using an ultrafilter (MWCO: 5000; Sartorius AG, Goettingen, Germany).

Preparation of Rz and Duplex of RNA Oligonucleotide—Rzs were prepared *in vitro* with the use of T7 RNA polymerase (AmpliScribe; EPICENTRE, WI, USA) in the presence of an RNase inhibitor (TOYOBO, Tokyo). After DNase (RQ1 DNase; Promega, Madison, WI, USA) treatment, the Rz was extracted with phenol/chloroform/isoamyl alcohol, precipitated with ethanol, and dissolved in water. The Rz was further purified in 10% polyacrylamide gels containing 7 M urea followed by column purification, ethanol precipitation, two washes in 70% ethanol, and dissolving in water.

An RNA oligonucleotide containing a GUC sequence (Fig. 1C, S) was end-labeled with [γ-^{32}P]ATP (Amersham Biosciences AB) by T4 polynucleotide kinase (Takara Shuzo). Briefly, labeled S and non-labeled AS were purified in 20% polyacrylamide gels containing 7 M urea followed by column purification and ethanol precipitation. Formation of the duplex was performed essentially as described elsewhere (*22*). Labeled S and non-labeled AS were mixed in annealing buffer (100 mM potassium acetate, 2 mM magnesium acetate, 30 mM HEPES-KOH, pH 7.4), incubated at 96°C for 2 min, and gradually cooled at room temperature. The duplex was precipitated with ethanol, washed twice in 70% ethanol and dissolved in water. The formation of the duplex was confirmed in 20% native polyacrylamide gels.

Analysis of the Cleavage Activity of Rz—Assays of Rz activity in the presence of the indicated amounts of copolymer or CTAB were performed in 25 mM $MgCl_2$ and 50 mM Tris-HCl (pH 8.0) at 37°C. The concentration of the copolymer is indicated as the P/R ratio (P/R = $[\text{amino group}]_{\text{polymer}}/[\text{phosphate group}]_{\text{RNA}}$). The concentrations of the monomer and duplex substrates used in this study were 2 nM (in the case of the duplex, the concentration of each strand was 2 nM) assuming that the labeled substrates had been completely recovered by ethanol precipitation. To stop the reaction, 4 μl of the reaction mixture was transferred to an equal volume of stop solution I that contained 2% SDS, 7 M urea, 0.1% xylene cyanol, 0.1% bromophenol blue, 100 mM Tris-HCl, and 100 mM EDTA (pH 8.0) for urea–polyacrylamide gel electrophoresis (PAGE), or stop solution II that contained 2% SDS, 20% glycerol, 0.1% xylene cyanol, 0.1% bromophenol blue, 100 mM Tris-HCl, and 100 mM EDTA (pH 8.0) for native-PAGE. The radioactivity in the bands was analyzed with a Bio-Image Analyzer (BAS2000; Fuji Film).

Construction of Vectors That Encode Poly(A)-Connected Rzs—The construction of Rz-expression vectors derived from plasmid pUC-dt was described previously (*5, 23*). To generate poly(A)-connected Rz-expression vectors, we inserted a poly(A) sequence of 60 nucleotides. pUC-dt was double-digested with *Csp*45I and *Sal*I, and then each individual Rz sequence, with *Kpn*I and *Eco*RV sites and the terminator sequence UUUUU at the 3′ end, was cloned into the plasmid. The *Kpn*I and *Eco*RV sites were used for subsequent insertion of the poly(A) sequence.

In Vitro Unwinding and Cleavage Activities—Poly(A)-connected or -unconnected Rz-protein complexes are described in the text. *In vitro* unwinding and cleavage assays by respective hybrid Rzs have been described elsewhere (*7*).

RESULTS AND DISCUSSION

The Rz has been extensively studied as a potential therapeutic tool due to its small size, which can be easily engineered so that its regulation of mRNA occurs in a highly sequence-specific manner. Many attempts have been made

to improve the activity of the Rz in the cellular environment, in terms of an efficient expression system for short length RNA, co-localization with its substrate RNA, and intracellular stability (*3*). However, the regulation of accessibility has not been studied extensively. As Rz and its substrate RNA are negatively charged, their interaction would be quite inefficient and require multiple association/dissociation steps before target recognition. In cells, RNA–RNA interactions *in trans* are often mediated by polycations, or cationic proteins. Therefore, it would be quite reasonable to utilize cellular cationic proteins or artificial polycations to improve the accessibility of Rz to its substrate RNA.

The Effects of the αPLL-g-Dex#2 on the Cleavage Activity of Rz Targeted to the RNA Duplex—We constructed an RNA duplex 21 nt in length as a substrate. The sense RNA, with a GUC sequence that is a cleavable triplet of Rz (Fig. 1C, S), and its complementary sequence (Fig. 1C, AS) were annealed to produce the RNA duplex. The RNA duplex consisted of a ds region of 19 bp and a 2-nucleotide 3′ overhang. This type of RNA duplex (siRNAs) is known to be biologically active (*22*). siRNAs that were introduced into cells were incorporated into the multicomponent nuclease complex, associating with the cellular helicase (*10–12*). Thus, we chose siRNAs as a model RNA that is biologically active and highly structured. The cleavage rate of Rz for the RNA duplex was significantly slower than that for the corresponding monomer, as expected (Fig. 2).

We prepared two kinds of the cationic comb-type graft copolymers, αPLL-g-Dex #1 and #2, by a reductive amination reaction between PLL HBr and Dex. The αPLL-g-Dex #2 was expected to be more hydrophilic than αPLL-g-Dex #1, and was not expected to be precipitated with RNA molecules, because the Dex used to prepare αPLL-g-Dex #2 had a higher molecular weight (*16*). Therefore, we first examined the effects of αPLL-g-Dex #2 on the cleavage activity of Rz targeted to the RNA duplex. Enhancing effects on the cleavage activity of Rz were observed, although to a limited extent, in the presence of the αPLL-g-Dex #2 with a P/R ratio (P/R = $[\text{amino group}]_{\text{polymer}}/[\text{phosphate group}]_{\text{RNA}}$) up to 1.0 (Fig. 3A). However, further increases in the cationic charge inhibited the cleavage reactions by Rz (Fig. 3B), indicating that the graft copolymer and the RNAs form a stable dead-end association complex.

In general, a polycationic backbone triggers a conformational change in a DNA to a dense globular structure (*19*). In contrast, in the case of αPLL-g-Dex, excess amounts of copolymer did not affect the reversibility of DNA melting and reassociation, and only a slight effect on the DNA secondary structure was observed (*16*). As only Rz that has folded into the correct conformation can cleave the target RNA (*23, 24*), it is interesting to note that the active conformation of Rz was retained in the presence of the cationic copolymer and the cleavage activity of Rz was enhanced at P/R of below one (Fig. 3A). In contrast, the inhibitory effect at higher P/R values might be introduced due to the compaction of RNA molecules in the presence of excess amounts of the copolymer (Fig. 3B).

The Effects of αPLL-g-Dex #1 and #2, and CTAB on the Cleavage Activity of Rz Targeted to an RNA Monomer—To investigate further the property of the cationic copolymer, we also examined the effects of αPLL-g-Dex on the activity of Rz targeted to the RNA monomer. The target for Rz was a 5′-^{32}P-labeled RNA monomer having a GUC sequence (Fig. 1C, S). Both αPLL-g-Dex #1 (Fig. 4, lanes 1–7) and #2 (Fig. 4, lanes 10–17) enhanced the cleavage activities of Rz

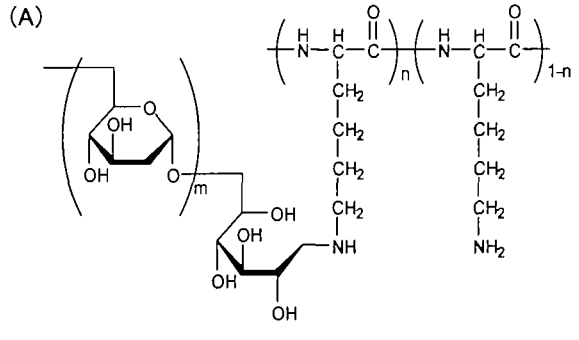

Fig. 1. **Schematic representations of αPLL-g-Dex, CTAB and the RNA duplex used in this study.** (A) Structural formula of the αPLL-g-Dex copolymer. The degree of substitution (n) is 0.144 in αPLL-g-Dex #1 and 0.145 in αPLL-g-Dex #2. (B) Structural formula of CTAB. (C) Sequence of the RNA duplex. The sequence complementary to the recognition arms of Rz is boxed. The arrow indicates the cleavage site. The letters "S" and "AS" mean sense and antisense, respectively.

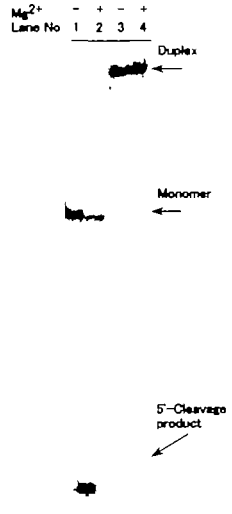

Fig. 2. **Comparison of the cleavage activity of Rzs targeted to the RNA duplex with that targeted to the RNA monomer.** The RNA monomer (lanes 1 and 2) and duplex (lanes 3 and 4), concentrations <2 nM each, were incubated with 5 μM Rz in reaction buffer in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of Mg^{2+} ions. Each sample was loaded into a well of a 20% native polyacrylamide gel and electrophoresed at a constant current of 15 mA for 5–6 h. During electrophoresis, the gel was cooled with ice-cold water. Percentages of cleavage based on band densities were 65 and 3.6% for lanes 2 and 4, respectively.

around a P/R ratio of 1.0, but inhibited at P/R ratios over 2.0, in accord with previous studies. It is likely that the major effect of αPLL-g-Dex on the cleavage activity of Rz targeted to RNA duplex is derived from an acceleration in the association/dissociation rate between Rz and the monomer substrate (*25*). Since CTAB also enhances the dimeric Rz activity (*26*), the effects of the CTAB on the cleavage activity of Rz were compared with those of αPLL-g-Dex under optimal conditions for CTAB. CTAB, which is known to be a facilitator for Rz, significantly enhanced Rz activity (Fig. 4, lanes 9 and 10), in accord with previous studies (*26*). It is important to note, however, that αPLL-g-Dex was effective at concentrations two orders of magnitude lower than that of CTAB.

Unwinding and Cleavage Activities of Rz with the αPLL-g-Dex and RNA-Protein Hybrid Rz—We then compared the unwinding and cleavage activities of the polymers and an RNA helicase–attached hybrid ribozyme (Fig. 5). The density of the monomer bands in the presence of αPLL-g-Dex was weaker than without the polymer (compare Fig. 5A, lane 1 with lanes 2 and 3), probably because the monomer was cleaved more rapidly in the presence of the copolymer, as indicated in Fig. 4.

In addition to the limited enhancement of Rz activity by αPLL-g-Dex (Fig. 5A), we also examined the enhancement by the intracellular RNA helicase (Fig. 5B), as shown by native PAGE. In general, the efficiency of Rz-mediated cleavage *in vivo* is not always as high as anticipated or required (*27*). *In vivo*, the activities of Rzs depend on their access to the cleavage site in the target RNA (*4*). To solve this target problem and to improve the efficiency of Rzs *in vivo*, we created an Rz with the ability to access any target site and cleave at a specific site. This was accomplished by combining the cleavage activity of the Rz with the unwinding activity of the endogenous RNA helicase. To connect the helicase to the Rz, we added a naturally occurring RNA motif, a poly(A) sequence to the 3′ end of the Rz (*7*). This poly(A) sequence interacts with RNA helicase eIF4AI *via* interactions with adopter proteins (*28, 29*).

In order to test the efficacy of poly(A)-connected Rz, we designed poly(A)-connected and -unconnected Rzs aimed at a specific target site in the duplex. We cloned the Rzs, with or without a poly(A) sequence, into the parental $tRNA^{Val}$-expression vector, pUC-dt (*5, 23*). To examine whether $tRNA^{Val}$-Rz-A60-protein complexes can cleave inaccessible target sites, we performed an *in vitro* cleavage assay with these Rz-protein complexes. At first we demonstrated the *in vitro* cleavage and unwinding activities of the hybrid Rz that was pulled-down, by incubating HeLa cell extracts (~2 × 10^6 cells) that had been incubated with the biotin-labeled $tRNA^{Val}$-Rz (lane 2 of Fig. 5B) or poly(A)-connected Rz ($tRNA^{Val}$-Rz-A60; lane 3 of Fig. 5B), whose concentration was 5 μM, with streptavidin beads, and then isolating the

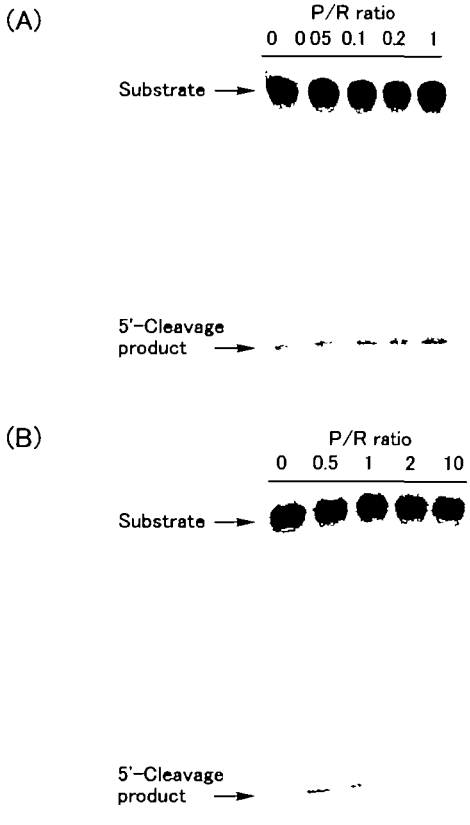

Fig. 3. **Dependence of the cleavage activity of Rzs targeted to RNA duplex on the amount of αPLL-g-Dex #2.** After the substrate comprising <2 nM RNA duplex was incubated with 200 nM Rz in the presence of αPLL-g-Dex #2 at the indicated P/R ratio for 30 min, 4 μl of the reaction mixture was sampled and mixed with an equal volume of stop solution I. Samples were heat-denatured and loaded onto a 20% polyacrylamide gel containing 7 M urea. (A) Enhancement of the cleavage activity of Rzs in the presence of αPLL-g-Dex #2. Percentages of cleavage based on the product band densities were 4.8, 4.7, 4.9, 5.6, and 6.2% for lanes at P/R ratios of 0, 0.05, 0.1, 0.2, and 1, respectively. (B) Inhibitory effects on the cleavage activity of Rzs in the presence of αPLL-g-Dex #2 with a high P/R ratio. Percentages of cleavage based on densities of bands of products were 4.1, 6.1, 4.7, 3.4, and 2.6% for lanes of P/R ratio of 0, 0.5, 1, 2, and 10, respectively.

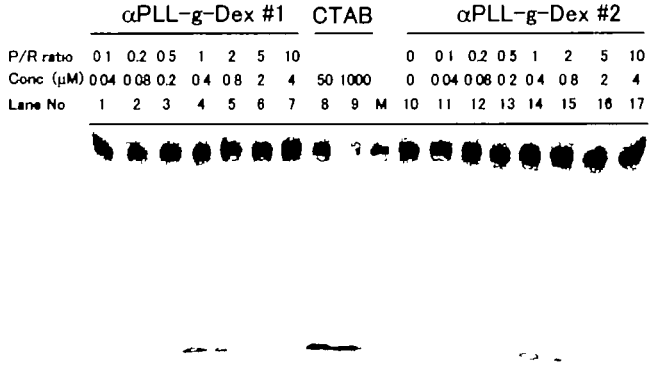

Fig. 4. **Dependence of the cleavage activity of Rzs targeted to an RNA monomer on the amount of αPLL-g-Dex #1, CTAB, and αPLL-g-Dex #2.** After <2 nM RNA monomer as substrate was incubated with 200 nM Rz for 30 min in the presence of the αPLL-g-Dex #1 or #2, or CTAB at the concentration shown above the figure, 4 μl of the reaction mixture was sampled and mixed with an equal volume of stop solution I. Samples were heat-denatured and loaded onto a 20% polyacrylamide gel containing 7 M urea. For αPLL-g-Dex #1, the percentages of cleavage based on product band densities were 8.0, 8.4, 9.9, 16, 11, 5.3, and 5.1% for lanes 1–7, respectively. For CTAB, the percentages of cleavage based on product band densities were 34 and 38% for lanes 8 and 9, respectively. For αPLL-g-Dex #2, the percentages of cleavage based on product band densities were 7.6, 8.5, 8.8, 11, 10, 7.3, 4.2, and 4.2% for lanes 10–17, respectively. M indicates a monomer substrate as a position marker.

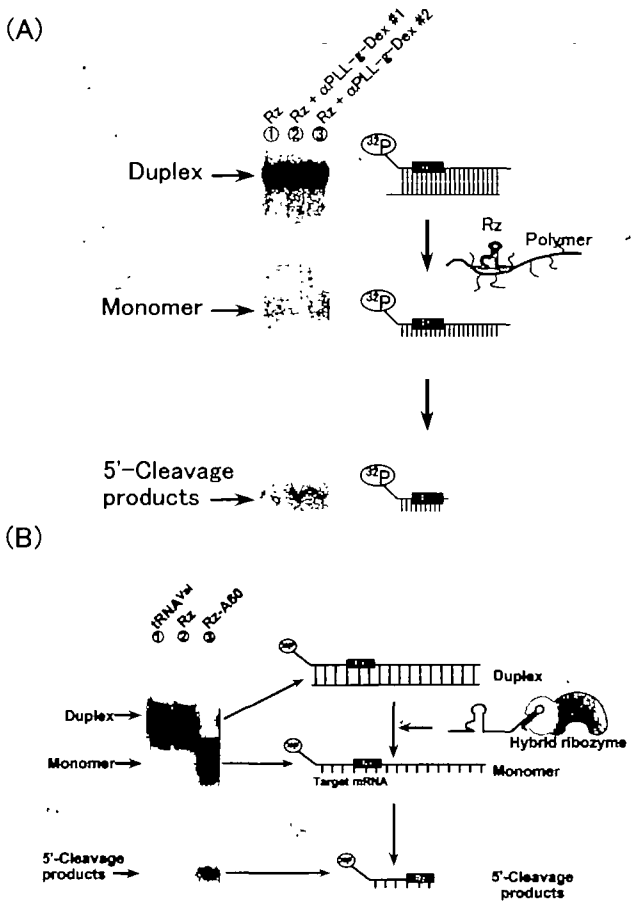

Fig. 5. **Comparison of the effects on the cleavage activity of Rz between the αPLL-g-Dex copolymers and the RNA helicase.** (A) Enhancement of the cleavage activity of the Rzs targeted to the RNA duplex in the presence of αPLL-g-Dex #1 and #2. After <2 nM RNA duplex as substrate was incubated with 5 μM Rz for 30 min in the presence of αPLL-g-Dex #1 or #2 at a P/R ratio of 1.0, 4 μl of the reaction mixture was sampled and mixed with an equal volume of stop solution II. Samples were loaded into the wells of a 20% native polyacrylamide gel. Percentages of cleavage based on product band densities were 5.0, 6.8, and 5.9% for lanes 1–3, respectively. (B) Unwinding and cleavage activities of RNA-protein hybrid Rzs and the construction of hybrid Rzs. Cleavage activity *in vitro* of poly(A)-connected or -unconnected Rz-protein complexes are shown. However, the exact concentration of the hybrid ribozyme used in this experiment is unknown because an endogenous RNA helicase was pulled down by a fixed amount of biotin-attached Rz as described in the text. Rz-A60, poly(A)-connected Rz.

complexes by extensive washing. When the isolated Rz complexes were mixed with <2 nM ^{32}P-labeled duplex RNA, poly(A)-unconnected Rz did not unwind the duplexes (Fig. 5B, lane 2). In contrast, Rz-A60 was clearly capable of unwinding and cleaving the substrate (Fig. 5B, lane 3). Thus, these results clearly demonstrate that tRNA^{Val}-Rz-A60-protein complexes have two activities, unwinding and cleavage *in vitro*, and that the unwinding activities are due to the RNA helicase eIF4AI (association with eIF4AI was confirmed using a pull-down assay with eIF4AI specific antibody; Ref. *30*). Moreover, all the poly(A)-containing Rzs that we have constructed exhibited significant activity in cells (*7, 30*). In many cases, only poly(A)-connected Rzs and not the parental Rzs suppressed the expression of the gene of interest. Therefore, hybrid-Rz technology represents a powerful tool for developing gene-inactivating reagents in the post-genome era.

CONCLUDING REMARKS

We have demonstrated that the αPLL-g-Dex copolymer confers enhanced cleavage activity both for RNA duplex and RNA monomer, at least to a limited extent. The αPLL-g-Dex with oligonucleotides, as a DNA triplex stabilizer, was proved to inhibit protein–DNA interactions in a sequence-specific manner (*20*). In another case, a derivative of the PLL graft copolymer having cell specific ligands in its side chains was demonstrated to deliver polynucleotides to liver cells specifically (*31*). If these cationic comb-type copolymers could be conjugated to Rz, it would be possible to deliver the Rz into specific cell types, and/or to confer enhanced cleavage activity to the Rz. *In vivo*, the utilization of intracellularly expressed RNA helicases appears promising because of their significant unwinding activities that lead to significantly enhanced cleavage activities of Rz (*7, 13, 30*), as also demonstrated for other RNA interacting proteins (*24, 32–34*).

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