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A 'Cassette' RNase: Site-Selective Cleavage of RNA by RNase S Equipped with RNA-Recognition Segment

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Abstract—RNase S is a unique protein comprising the non-covalent association of two components, the S-peptide and the S-protein. An RNA-recognition segment derived from the human immunodeficiency virus (HIV)-1 Rev protein was conjugated with the S-peptide to form a complex with the S-protein. The resulting RNase S bearing the RNA-recognition segment preferentially hydrolyzed a single position of the RNA stem-loop derived from the specific binding site for the Rev protein.

Elucidation of the two-dimensional and three-dimensional structures of RNA has recently been required in relation to their therapeutic potential as targets of RNA-oriented molecules, such as antisense nucleic acids and ribozymes. Structural elucidation of RNAs leads us to understand the replication mechanisms of RNA viruses including the human immunodeficiency virus (HIV)-1 and also provides new target sites for the antiviral drugs. Elucidation of RNA structures is, however, very much behind that of DNA due to its structural complexity.[1] The lack of restriction enzymes for RNA is one of the major problems. Site-specific cleavage of RNA strands by an artificial RNase will give us much information on the RNA structures. Requisites for the ideal RNase would be as follows: (1) recognition of a specific RNA sequence or structure; (2) phosphate bond scission for the future manipulation of the RNA segments; (3) high turnover; and (4) easy preparation. However, few approaches have been reported that fulfill these criteria.

We report here the creation of a novel RNase where a conjugate of an RNA recognition peptide and the S-peptide was encapsulated as a cassette in RNase S (Fig. 1). RNase S is a unique protein which consists of two proteolytic fragments of bovine pancreatic RNase A, that is, the S-peptide (residues 1–20) and the S-protein (residues 21–124).[2] Each fragment does not retain the RNase activity. When the two fragments were mixed together, they fold into the original RNase A-like structure (RNase S) to recover the RNase activity. This complex is an attractive framework for the creation of artificial semi-synthetic proteins.[3,4] The S-peptide and the S-protein fold into the active structure even when the S-peptide is conjugated with other exogenous peptides.[5]

We selected the RNA binding segment derived from the HIV-1 Rev protein as a model of the RNA recognition segment. The Rev protein is involved in the regulation of the viral gene expression by affecting the levels of splicing and transport of the viral mRNA to the cytoplasm.[6] A short helical peptide segment in the Rev protein is known to recognize the specific RNA structure (Rev response element: RRE) with a very high affinity.[7,8] Selective cleavages of the RNA loops by the specific RNA-binding peptides conjugated with

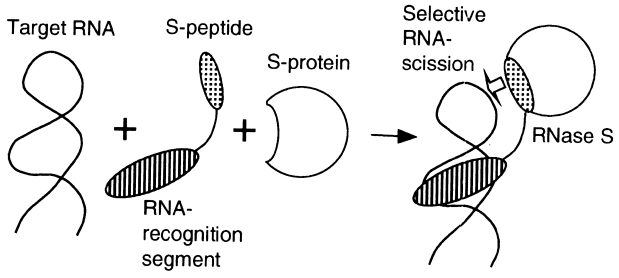

Figure 1. The general concept of the 'cassette' RNases.

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metal-coordinating moieties such as EDTA, cyclen, and Gly-Gly-His segments have been reported.[9–11] However, their metal-assisted methods of cleavage have a fundamental problem in terms of efficiency and the difficulty of phosphate bond cleavage. Our approach utilizes the natural RNase that may settle these problems.

The design of the cassette molecule, that is, the conjugate of the RRE-recognition peptide and the S-peptide (Rev-S peptide **1**), is shown in Figure 2. As the RNA-recognition peptide, that corresponding to positions 34–50 of HIV-1 Rev was employed. The segment is known as the key residues for the binding to RRE.[6] Residues 1–15 of the S-peptide are sufficient to form a complex with the S-protein while retaining the RNase activity.[5] The N-termini of both peptides were connected so that the RNase S bearing the RNA-recognition segment is situated close to the RRE loop. Met13 in the original S-peptide was replaced with norleucine (Nle) for easy handling. A conjugate that has an extra Gly at the N-terminus of HIV-1 Rev-(34-50) ([Gly]Rev-S peptide **2**) and a conjugate of the S-peptide with HIV-1 Tat-(48-60) (Tat-S peptide **3**), which is another RNA-binding peptide specific for an RNA loop called Tat response element, were designed to clarify, respectively, the effect of the linkers and the RNA-recognition segments on the detection and cleavage of the target RNA by the cassette RNases.

The Rev-S peptide **1** was synthesized by the conjugation of the peptide segment **6** corresponding to the HIV-1 Rev-(34–50) with the segment **7** corresponding to the S-peptide (1–15). The peptide chain of **6** was constructed by the Fmoc-solid-phase peptide synthesis on a Rink amide resin as already reported.[12] The N-terminus mercaptoacetyl moiety was introduced by the treatment of the peptide resin **4** with the pentafluorophenyl ester of acetylthioglycolic acid (CH_3CO-S-CH_2CO-OPfp, Novabiochem), followed by the removal of the acetyl moiety in dimethylformamide (DMF) containing 20% piperidine. The peptide resin **5** was treated with trifluoroacetic acid (TFA)–ethanedithiol (EDT) (95:5) at room temperature for 2 h. HPLC purification of the product then yielded the pure peptide segment **6**. The chloroacetylated segment **7** was also prepared as reported.[12] The fidelity of the peptides was ascertained by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI–TOFMS) [**6**: 2511.7 $(M+H)^+$; **7**: 1806.8 $(M+H)^+$]. The conjugate of these segments (Rev-S peptide **1**) was prepared by reacting these segments (molar ratio, **6**:**7** = 1.1:1) in 6 M guanidine HCl–0.1 M tris(hydroxymethyl)aminomethane (Tris) (pH 8.0) overnight [yield: 58%; TOFMS: 4282.8 $(M+H)^+$]. Conjugates **2** [TOFMS: 4368.9 3 $(M+H)^+$] and **3** [TOFMS: 3622.9 $(M+H)^+$] were similarly prepared.

We next examined whether the Rev-S peptide **1** and S-protein form a complex for attachment to the RNA derived from the RRE and cleave a specific site in it. A truncated RNA derived from the stem-loop IIB of RRE,[8] which contains the highest affinity Rev binding site, was employed as the model (Fig. 3). The solution structure of the HIV-1 Rev peptide bound to the above RNA segment was solved by NMR,[8] where the Rev peptide took an α-helical structure and became bound in the major groove of the purine-rich internal loop. A gel-mobility shift assay for the Rev-S peptide using the 5′-^{32}P-labeled RNA corresponding to the RRE indicated that the hybrid peptide was bound to the above RRE at the peptide concentration of 10 nM (data not shown). The specificity of the RNA recognition and the efficiency of the scission were then examined (Fig. 4). Solutions containing the S-protein (final concentration of 1 nM) and the Rev-S peptide at various concentrations were pre-incubated at 25 °C for 10 min in 50 mM Tris–HCl buffer (pH 6.0) containing 100 mM NaCl. The

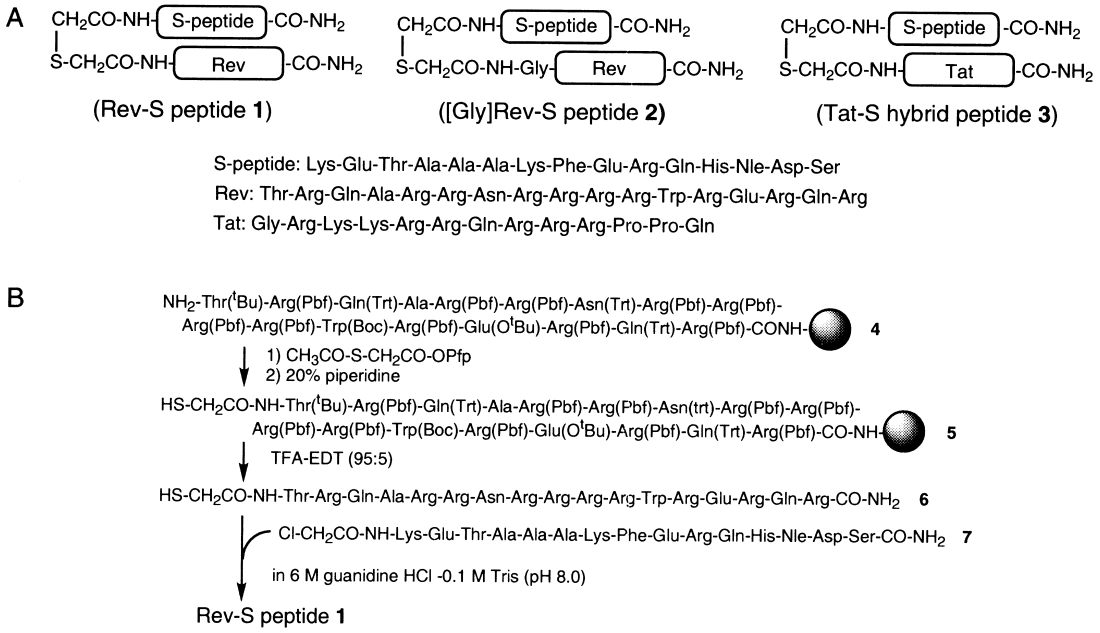

Figure 2. (A) Design of the 'cassette' peptides comprising an RNA-recognition segment and the S-peptide; (B) synthesis of the Rev-S peptide **1** (Pbf, N^G-2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; Trt, S-trityl).

cleavage experiment was started by mixing the above solutions with the appropriate amount (15,000 cpm) of the 5′-labeled RRE. The reaction was continued at 25 °C for 15 min then quenched with an equal volume of 100 mM Tris–HCl/100 mM boric acid/10 mM EDTA/9 M urea/0.1% xylene cyanol/0.1% bromophenol blue (pH 7.5). The extent of the cleavage was analyzed by a 20% polyacrylamide gel electrophoresis (PAGE) and detected by autoradiography (Fig. 4).[13]

The mixture of the Rev-S peptide **1** and the S-protein gave a major cleavage band at the peptide concentration of 10 nM. In remarkable contrast, no significant bands were observed at the peptide concentration of 1 μM for the mixture of the S-protein with the Tat-S peptide **3**. The mixture of the S-protein and the S-peptide (1–15) without the RNA recognition segment **8** (NH_2-Lys-Glu-Thr-Ala-Ala-Ala-Lys-Phe-Glu-Arg-Gln-His-Nle-Asp-Ser-$CONH_2$) generated a cleavage band only at the peptide concentration of 100 nM. Therefore, we concluded that the Rev-S peptide and the S-protein made a complex that recognizes and cleaves a specific RNA structure. Of special interest is the result obtained from the experiment using the [Gly]Rev-S peptide **2**, the results of which suggested the importance of the linker on the kinetics of the RNA cleavage. Although the specificity of the cleavage was similar with that when the hybrid peptide **1** was used, the efficiency of cleavage decreased by the insertion of the Gly residue. The less efficient cleavage of RNA was observed for the mixture of the S-protein with the Tat-S peptide **3** compared with that of the S-peptide (1–15) **8**. Presumably, the Tat segment not only inhibited the assembly between the Tat-S peptide and the S-protein, but also provided steric hindrance for the RNA scission of the resulting RNase complex.

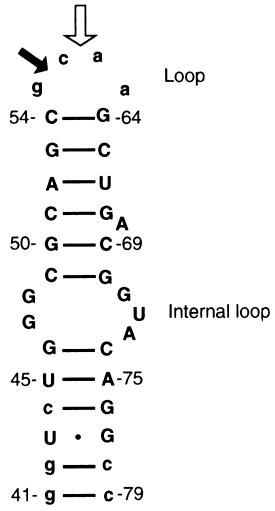

Figure 3. Structure of RNA used in this study. The numbering in the internal loop region is the same as for the stem-loop IIB of the wild-type RRE.[8] Lower-case letters indicate nonwild-type nucleotides. The open arrow and the bold arrow indicate sites of RNA scission by the 'cassette' RNase comprising the Rev-S peptide **1** and the S-protein, and RNase T_1, respectively.

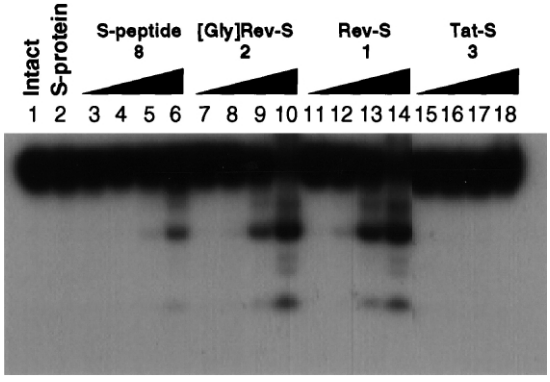

Figure 4. Hydrolysis of 5′-^{32}P-end-labeled RRE RNA by the 'cassette' RNases. Lane 1, intact RRE RNA; lane 2, RNA treated with S-protein (1 nM); lanes 3–18, RNA treated with the S-protein (1 nM) in the presence of the S-peptide **8**, [Gly]Rev-S peptide **2**, Rev-S peptide **1**, and Tat-S peptide **3**, respectively. The concentration of conjugate **1**, **2**, **3**, and **8** was 1 nM, 10 nM, 100 nM, and 1 μM (from the left), respectively. 20% polyacrylamide gel was used for the electrophoresis.

Site-specificity of the hydrolysis was further confirmed using a 15% polyacrylamide gel. As shown in Figure 5, the RNA was preferentially cleaved at a specific position by the complex of the Rev-S peptide **1** and the S-protein (lanes 6–8). Limited hydrolysis of the RNA by RNase T_1, a G-specific RNase, gave a major cleavage band at a position corresponding to one base shorter than that for the above main cleavage band (Fig. 5, lane 2). As RNase T_1 hydrolyzes the single strand RNA much more preferentially than the double strand RNA, the cleavage site for the RNase T_1 treatment was deduced to be G

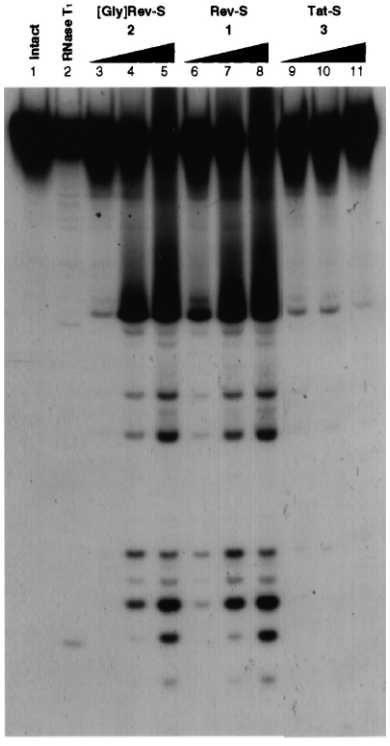

Figure 5. Site-specificity in the hydrolysis of 5′-^{32}P-end-labeled RRE RNA by the 'cassette' RNases. Lane 1, intact RRE RNA; lane 2, RNA treated with RNase T_1; lanes 3–11, RNA treated with the S-protein (1 nM) in the presence of the [Gly]Rev-S peptide **2**, Rev-S peptide **1**, and Tat-S peptide **3**, respectively. The concentration of conjugate **1**, **2**, and **3** was 1 nM, 10 nM, and 100 nM (from the left), respectively. 15% polyacrylamide gel was used for the electrophoresis.

adjacent to C54 (indicated by the bold arrow in Fig. 3). RNase S was reported to retain the pyrimidine preference in the RNA scission even when the S-peptide was fused with other proteins.[14] Taken together, it is most likely that the complex of the Rev-S peptide and the S-protein cleaved the RNA loop at the 3′ site of C (Fig. 3, open arrow). Positions for the minor cleavage bands, which appeared below the major cleavage band on the gel in lane 6 of Figure 5, corresponded well to those for pyrimidines located among G41–C54. These minor cleavage bands were probably secondarily generated as a result of the RNA scission at the major cleavage site; destabilization of the RNA stem-loop structure would give single strand RNA molecules, which became the better substrates for the above RNase complex. Efficiency of the cleavage using the mixture of the Tat-S peptide **3** and the S-protein was much less than that by the mixture of the Rev-S peptide **1** and S-protein. The specificity of the cleavage in the mixture of the [Gly]Rev-S peptide **2** and S-protein was similar to the mixture of **1** and the S-protein. However, the efficiency of the cleavage is again less than that of the latter.

In conclusion, we have demonstrated that the RNase S equipped with an RNA-recognition cassette worked as an efficient tool for the detection of a specific structure of RNA. The use of RNase as a scissile molecule was originally reported by Zuckermann et al.[15] They employed a disulfide conjugate of the genetically Cys-incorporated RNase A and thiol-functionalized oligo-RNA. The most attractive feature of our approach is the feasibility of employing various RNA-binding segments to recognize specific RNA structures. Using various selection methodologies, novel RNA-recognition segments with high specificity have been obtained.[16] Recognition segments containing unnatural amino acids or non-peptide recognition segments are also applicable. In combination with these methodologies, tailor-made RNases will be created, which are able to recognize a diverse array of RNA shapes and spatial arrangements. Conjugated with the S-peptide, these recognition segments can be cassetted into the readily available S-protein. These RNases would be turnoverable due to the possible conformational change caused by the RNA cleavage and the dissociation of the recognition peptides from the binding sites. These cassette RNases will be utilized as a powerful tool for uncovering the factors determining the structure–functional relationships of RNA.

Acknowledgements

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