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Cleavage of double-stranded RNA by RNase HI from a thermoacidophilic archaeon, *Sulfolobus tokodaii* 7

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ABSTRACT

ST0753, the orthologous gene of Type 1 RNase H found in a thermoacidophilic archaeon, *Sulfolobus tokodaii*, was analyzed. The recombinant ST0753 protein exhibited RNase H activity in both *in vivo* and *in vitro* assays. The protein expressed in an RNase H-deficient mutant *Escherichia coli* strain functioned to suppress the temperature-sensitive phenotype associated with the lack of RNase H. The *in vitro* characteristics of the gene's RNase H activity were similar to those of *Halobacterium* RNase HI, the first archaeal Type 1 RNase H to be characterized. Surprisingly, the *S.tokodaii* RNase HI cleaved not only the RNA strand of an RNA/DNA hybrid but also an RNA strand of an RNA/RNA duplex in the presence of Mn^{2+} or Co^{2+}. The result of gel filtration column chromatography showed this double-stranded RNA-dependent RNase (dsRNase) activity was coincident with *S.tokodaii* RNase HI. A site-directed mutagenesis study of essential amino acids for RNase H activity indicated that this activity also affected dsRNase activity. A single amino acid replacement of Asp-125 by Asn resulted in loss of dsRNase activity but not RNase H activity, suggesting that amino acid residues required for dsRNase activity seemed slightly different from those of RNase H activity. Some reverse transcriptases from retroelements can cleave double-stranded RNA, and this activity requires the RNase H domain. Similarities in primary structure and biochemical characteristics between *S.tokodaii* RNase HI and reverse transcriptases imply that the *S.tokodaii* enzyme might be derived from the RNase H domain of reverse transcriptase.

INTRODUCTION

Ribonuclease H (RNase H) (EC 3.1.26.4) is defined as an enzyme that specifically cleaves an RNA strand of RNA/DNA hybrids (1). Based on differences in their amino acid sequences, RNases H are classified into two types (2,3). Type 1 enzymes are *Escherichia coli* RNase HI orthologs (4), and Type 2 enzymes are *E.coli* RNase HII orthologs (5). Type 1 RNases H also include retroviral reverse transcriptases (RTs), because these RTs contain amino acid sequences and structures showing high similarity to *E.coli* RNase HI as a domain (3,6). The RNase H domain of RT is required to convert the single-stranded retroviral RNA genome into double-stranded DNA, and is therefore essential for retroviral proliferation (7). On the other hand, various studies suggest that cellular RNases H are involved in DNA replication, repair and transcription (8–12). Furthermore, as deletion of the Type 1 RNase H-encoding gene results in embryonic lethality in mouse (13) and *Drosophila melanogaster* (14), the former being due to an inability to amplify mitochondrial DNA (13), cellular RNases H are also required for development. However, the physiological functions of cellular RNases H that contain both Type 1 and Type 2 are not yet completely understood. The genomes of all organisms have one to a few RNase H genes, and it has been reported that most archaea have a single Type 2 gene (3). Therefore, the Type 2 enzymes are thought to be the more universal RNase H type. Recently, the first archaeal Type 1 RNase H gene was identified from the *Halobacterium* sp. NRC-1 genome, and its encoded protein was characterized (15). The *Halobacterium* Type 1 RNase H (Halo-RNase HI) exhibits RNase H activity both *in vivo* and *in vitro*. Its unique characteristics—the lack of a basic protrusion region in the primary structure, cleavage at the RNA–DNA junction and activity in the absence of His residue—are more similar to those of the RNase H domain of retroviral RT than to those of cellular Type 1 RNase H (15). Halo-RNase HI homologs are found also in two other archaeal genomes: the ST0753 gene from *Sulfolobus tokodaii* (16) and the PAE1792 gene from *Pyrobaculum aerophilum* (17). These three archaeal RNase HI homologs show significant amino acid sequence similarities to each other. However, as shown in Figure 1, Halo-RNase HI and PAE1792 possess an N-terminal extension and a C-terminal extension, respectively, but ST0753 lacks both. Because the RNase H activities of ST0753 and PAE1792 have not been reported, it is unclear whether the differences in their primary structures affect their activities, or even whether they encode active enzymes. In the present study, therefore, we analyzed the ST0753 gene from *S.tokodaii* in order to gain more information on archaeal RNase HI.

The ST0753 gene was cloned and the recombinant ST0753 protein overexpressed in *E.coli* was purified and analyzed. As expected, the recombinant protein exhibited RNase H activity

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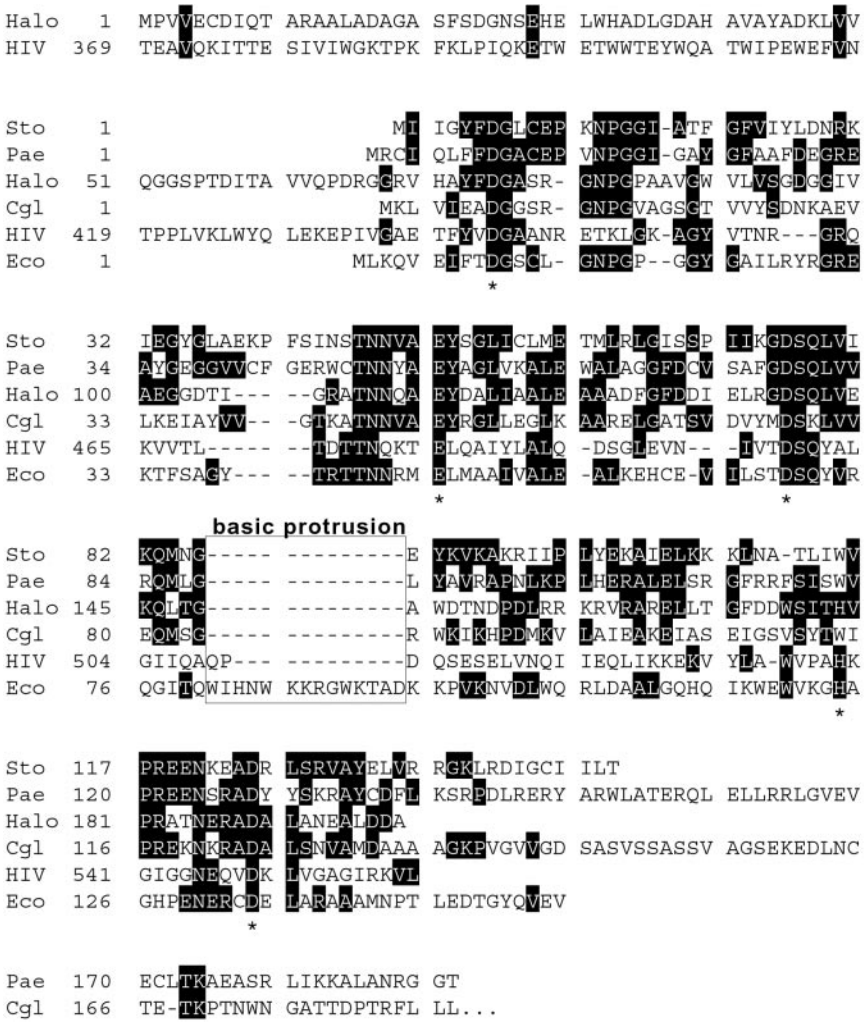

Figure 1. Multiple alignments of Type 1 RNases H. Sto, Pae, Halo, Cgl, HIV and Eco represent ST0753 from *S.tokodaii*, PAE1792 from *P.aerophilum*, *Halobacterium* RNase HI, *Corynebacterium glutamicum* RNase HI, an RNase H domain of HIV-1 RT and *E.coli* RNase HI, respectively. Numbers represent the positions of amino acid residues that start from the initiator methionine for each protein. The asterisks indicate the conserved amino acid residues, which are involved in catalytic function of *E.coli* RNase HI (18). It was reported that an Asp residue corresponding to Asp-134 of *E.coli* RNase HI was not conserved in *C.glutamicum* RNase HI (19). However, Asp-124 in *C.glutamicum* RNase HI corresponds to the Asp-134 in *E.coli* RNase HI and Asp-125 for Sto-RNase HI.

both *in vivo* and *in vitro*. Surprisingly, however, the protein cleaved not only an RNA strand of RNA/DNA hybrid but also a double-strand of RNA. In this report, we show that this double-stranded RNA-dependent RNase (dsRNase) activity results from the ST0753 (Sto-RNase HI) protein, and assess the involvement of various conserved amino acid residues in hydrolysis.

MATERIALS AND METHODS

Cells, plasmids and materials

The *S.tokodaii* strain 7 genomic DNA (16) was kindly donated by Professor A. Yamagishi. The *E.coli* MIC2067 is an *rnhA* and *rnhB* double mutant strain (20) and the MIC2067(DE3) carrying a cloned T7 RNA polymerase gene has been already published (21). The MIC2067(DE3) is used as a host strain for overexpression of the recombinant protein with the pET system. The plasmid pET-11a was purchased from Novagen (Madison, WI, USA). Restriction and modifying enzymes were from TaKaRa Bio (Kyoto, Japan). *Crotalus atrox* phosphodiesterase I was from Sigma (St Louis, MO, USA). Recombinant *E.coli* RNase HI was prepared previously (15).

DNA manipulations

PCR was performed using a GeneAmp PCR system 2700 (Applied Biosystems, Foster City, CA, USA). The reagent for PCR, Ex-Taq Hot Start version (TaKaRa Bio) or KOD-Plus-(TOYOBO, Osaka, Japan) was used according to the procedure recommended by the supplier. DNA sequences were determined by using a Prism 3100 DNA sequencer (Applied Biosystems).

Plasmid construction, overproduction and purification

The plasmid for overexpression was constructed by ligating a DNA fragment containing the *Sto-rnhA* gene to the

NdeI–BamHI site of pET-11a. The DNA fragment was amplified by PCR using *S.tokodaii* strain 7 genomic DNA as a template. The primer sequences were 5′-ACATTACTACCATATGATAATTGGTTATTTTGACGGT-3′ for the 5′-primer and 5′-CTGTCTATAGGATCCTTACTAAGTTAATATTATACATCCTAT-3′ for the 3′-primer, where underlined bases show the positions of the NdeI (5′-primer) and BamHI (3′-primer) sites. Overproduction of the Sto-RNase HI in *E.coli* MIC2067(DE3) and sonication of the cells were carried out by a procedure similar to that described previously (15). The soluble fraction was applied to a column (4 ml) of DE52 (Whatman, Fairfield, NJ, USA) equilibrated with TE buffer [20 mM Tris–HCl (pH 8.0) and 1 mM EDTA]. Proteins included in the flow-through fraction were precipitated at an 80% saturation level with ammonium sulfate, collected by centrifugation at 25 000 *g* for 30 min, dissolved and dialyzed against TE buffer. This solution was applied to a column (4 ml) of P-11 (Whatman) equilibrated with TE buffer, and proteins were eluted from the column by linearly increasing the NaCl concentration from 0 to 0.5 M. The protein fractions at an NaCl concentration of 0.3 M were pooled and dialyzed against 5 mM sodium phosphate (pH 7.0). The dialyzed solution was applied to a column (4 ml) of hydroxyapatite Bio-Gel HT gel (BIO-RAD, Hercules, CA, USA) equilibrated with 5 mM sodium phosphate (pH 7.0), followed by elution from the column by a linear gradient of sodium phosphate from 5 to 200 mM. Fractions containing the protein were combined (at approximately 140 mM sodium phosphate), concentrated, dialyzed against TE buffer containing 150 mM NaCl and 1 mM DTT, and used for further analyses.

The protein concentration was determined by measuring UV absorption using $A_{280}^{0.1\%}$ values of 0.97 for Sto-RNase HI, which was calculated from ε values of 1576 $M^{-1}cm^{-1}$ for Tyr and 5225 $M^{-1}cm^{-1}$ for Trp at 280 nm (22).

Cleavage reactions

The 5′ end-labeled 12 b RNA (5′-cggagaugacgg-3′) and the 3′ end-labeled 18 b RNA9-DNA (5′-uugcaugccTGCAGGTCG-3′), and their complementary DNAs were chemically synthesized by Proligo (Paris, France). Deoxyribonucleotides and ribonucleotides are shown by uppercase and lowercase letters, respectively. The fluorescent (6-FAM) was used for end-labeling. The RNA/DNA (0.5 μM) was prepared by hybridizing the end-labeled RNA-containing oligonucleotide with a 2.0 molar equivalent of its complementary DNA. Hydrolysis of the substrate was carried out at 37°C for 15 min in 10 mM Tris–HCl (pH 8.5) containing 1 mM $MgCl_2$ or $MnCl_2$, 10 mM NaCl, 1 mM 2-mercaptoethanol and 50 μg/ml BSA. The effects of divalent metal ions or pH values on the cleavage activity were analyzed as described previously (15). Products were analyzed on a 20% polyacrylamide gel containing 7 M urea and quantified using the Molecular Imager FX (BIO-RAD). The products were identified by comparing their patterns of migration on the gel with those of the oligonucleotides generated by partial digestion of RNA with snake venom phosphodiesterase (23).

The 12 bp double-stranded RNA and DNA, and the RNA/DNA duplex with 5′ end-labeled DNA for an activity to cleave a DNA strand of an RNA/DNA hybrid, were prepared as described for the construction of the RNA/DNA hybrid. The sequences of the 5′ end-labeled strands of all substrates are the same as that of RNA of the 12 bp RNA/DNA hybrid (Table 1). The reactions and product analyses were carried out as described above.

Table 1. Oligomeric RNA/DNA substrates[a]

Purpose	Substrate	Sequence
RNase H	12 bp RNA/DNA	5′-*cggagaugacgg-3′ 3′-GCCTCTACTGCC-5′
	RNA9–DNA/DNA	5′-uugcaugccTGCAGGTCG*-3′ 3′-AACGTACGGACGTCCAGC-5′
Marker	RNA1–DNA/DNA	5′-cTGCAGGTCG*-3′
DNA cleavage of DNA/RNA	12 bp DNA/RNA	5′-*CGGAGATGACGG-3′ 3′-gccucuacugcc-5′
dsRNase	12 bp RNA/RNA	5′-*cggagaugacgg-3′ 3′-gccucuacugcc-5′
dsDNase	12 bp DNA/DNA	5′-*CGGAGATGACGG-3′ 3′-GCCTCTACTGCC-5′
ssRNase	12 b RNA	5′-*cggagaugacgg-3′
ssDNase	12 b DNA	5′-*CGGAGATGACGG-3′

[a]Deoxyribonucleotides and ribonucleotides are shown by uppercase and lowercase letters, respectively. The asterisk indicates the fluorescent-labeled site.

Gel filtration

Gel filtration was performed on a column (1.6 × 60 cm) of HiLoad 16/60 Superdex 75 pg (Amersham, Piscataway, NJ, USA) equilibrated with 20 mM Tris–HCl (pH 8.0) containing 1 mM EDTA and 150 mM NaCl. For the estimation of molecular mass, blue dextran, alcohol dehydrogenase, BSA, carbonic anhydrase and cytochrome c were used as standard proteins. These standards were purchased from Sigma.

Site-directed mutagenesis

The genes encoding the mutant protein Sto-D7N were constructed by PCR with the 5′-mutation-primer and 3′-primer used for the wild-type *Sto-rnhA* gene. The sequence of the 5′-mutation primer is 5′-ACATTACTACCATATGATAATTGGTTATTTTaACGGTCTATGT-3′, where underlined bases and lower case letters show the positions of the NdeI and a mutated base, respectively. The primer was designed to alter the codon for Asp (GAC) to that for Asn (AAC). The genes encoding the other mutant proteins, E52Q, D76N and D125N, were constructed by site-directed mutagenesis, as described previously (24). They were designed to alter the codons for Glu-52 (GAA), Asp-76 (GAT) and Asp-125 (GAC) to those for Gln (CAA) and Asn (AAT and AAC), respectively. Plasmid construction, overproduction and protein purification were carried out according to those described for the wild-type protein.

In vivo complementation assay for RNase H activity

The temperature-sensitive (ts) growth phenotype of the *E.coli* MIC2067(DE3) (20,21) can be relieved by introduction of a functional RNase H gene. This strain was transformed by the pET-11a-derivative containing the wild-type or mutant *Sto-rnhA* genes, spread on Luria medium plates containing 50 μg/ml ampicillin and 30 μg/ml chloramphenicol, and incubated at 30°C and 42°C.

Phylogenetic analysis

For the phylogenetic analysis, a multiple sequence alignment was created by the ClustalW software (25) and modified. Based on a distant matrix calculated from the alignment by PHYLIP software (26), the phylogenetic tree was constructed by using the TreeView software (27). Sequence alignments used for the construction of the phylogenetic tree are provided in the Supplementary Material at NAR Online (Figure S1).

RESULTS

S.tokodaii RNase HI homolog

The *S.tokodaii* ST0753 gene encodes a Type 1 RNase H homolog, which is composed of 149 amino acid residues with a calculated molecular mass of 16 812 Da and an isoelectric point (p*I*) of 9.5. Although the ST0753 protein was annotated as a hypothetical protein, it shows 32% amino acid sequence identity to a newly identified archaeal RNase HI from *Halobacterium* sp. NRC-1 (Halo-RNase HI) (15) (Figure 1). However, the ST0753 protein lacks amino acid sequences that would correspond to an N-terminal extension of Halo-RNase HI. Among the five active site residues—Asp-10, Glu-48, Asp-70, His-124 and Asp-134—identified in *E.coli* RNase HI (18), only four acidic residues were conserved in the ST0753. Although this protein contains no His residue in its amino acid sequence, we expected it to exhibit RNase H activity, based on our previous report that His residues were not important for the catalytic function of Halo-RNase HI (15).

To obtain the ST0753 protein in an amount sufficient for enzymatic and biochemical analyses, expression in *E.coli* MIC2067(DE3) was used thereby eliminating any contribution of RNases HI and HII. The protein was purified to homogeneity as described in Materials and Methods. As shown later, the protein exhibits RNase H activity both *in vivo* and *in vitro*, and in consideration of its amino acid sequence similarity to Halo-RNase HI, we hereafter refer to the ST0753 gene as *S.tokodaii rnhA* (*Sto-rnhA*) and to its encoding protein as RNase HI (Sto-RNase HI).

RNase H activity of Sto-RNase HI

To examine whether or not Sto-RNase HI shows RNase H activity, the purified proteins were incubated with a 12-bp oligomeric RNA/DNA hybrid. Sto-RNase HI cleaved an RNA strand of the 12-bp RNA/DNA hybrid at multiple sites only in the presence of Mn^{2+}, Mg^{2+}, Co^{2+} or Ni^{2+}, but not in the presence of Cu^{2+}, Ca^{2+} or Zn^{2+}, or in the absence of divalent metal ions. The level of Sto-RNase HI activity peaked at concentrations of 1 mM for $MnCl_2$, $MgCl_2$ and $CoCl_2$, and at a concentration of 100 mM for $NiCl_2$ (data not shown). The cleavage patterns are shown in Figure 2A. Sto-RNase HI preferred $MnCl_2$ and $MgCl_2$ to $CoCl_2$ and $NiCl_2$, with shorter reaction products resulted from multiple cleavages in the presence of $MnCl_2$ and $MgCl_2$ (Figure 2A). The cleavage activity of Sto-RNase HI increased exponentially as the pH increased from 6 to 10 (data not shown). However, the standard cleavage reaction was performed at a pH of 8.5 in the presence of 1 mM $MgCl_2$ or $MnCl_2$, because at a higher pH range, the solubility of divalent metal ions decreases and RNA/DNA substrates might become destabilized. In Table 2, the specific activities

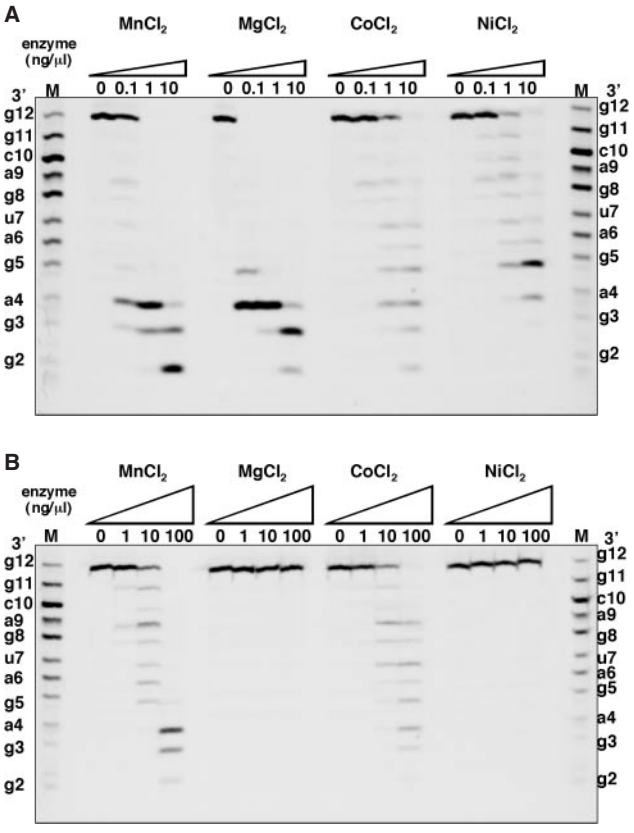

Figure 2. Cleavage of oligomeric substrates in the presence of divalent metal ions. A 12 bp RNA/DNA hybrid (**A**) or a 12 bp RNA/RNA duplex (**B**) was incubated at 37°C for 15 min with Sto-RNase HI in the presence of 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM $CoCl_2$ or 100 mM $NiCl_2$. The concentration of the substrate is 0.5 μM. Products were separated on a 20% polyacrylamide gel containing 7 M urea as described in Materials and Methods. M represents products resulting from partial digestion of the 12 b RNA with snake venom phosphodiesterase.

Table 2. Comparison of RNase H activities in the presence of either Mg^{2+} or Mn^{2+} ions[a]

Enzyme	Metal	Specific activity (units/mg)
Sto-RNase HI	1 mM $MgCl_2$	1.57
	1 mM $MnCl_2$	0.35
Halo-RNase HI	100 mM $MgCl_2$	0.44[b]
	20 mM $MnCl_2$	0.46[b]
E.coli RNase HI	10 mM $MgCl_2$	17.4[b]
	1 μM $MnCl_2$	0.68[b]

[a]The hydrolysis of the 12 bp RNA/DNA hybrids with enzyme was carried out at 37°C for 15 min under the conditions described in Materials and Methods. The amount of enzyme was controlled such that the ration of the hydrolyzed substrate did not exceed 30% of the total. The concentrations of the metal ions were optimal values for RNase H activities of enzymes. One unit is defined as the amount of enzyme to hydrolyze 1 μmol substrate per min at 37°C. The specific activity was defined as the enzymatic activity per milligram of protein. Errors, which represent the 67% confidence limits, are within 30% of the values reported.
[b]Ref. (15).

of Sto-RNase HI in the presence of Mg^{2+} and Mn^{2+} are compared with those of *E.coli* RNase HI and another archaeal Type 1 RNase H, Halo-RNase HI. In the presence of Mg^{2+}, the specific activity of the Sto-RNase HI was 3.6-fold

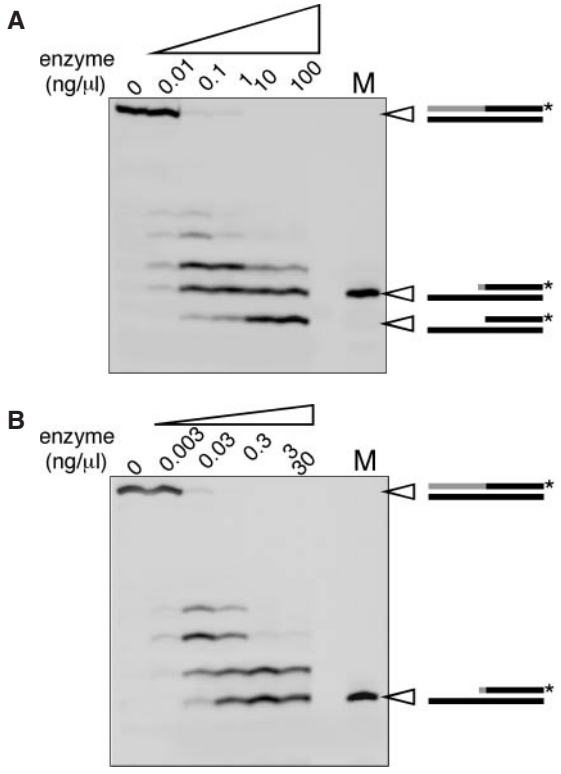

Figure 3. Cleavage of Okazaki fragment-like substrate. An RNA9–DNA/DNA hybrids were incubated at 37°C for 15 min with Sto-RNase HI (**A**) in 10 mM Tris–HCl (pH 8.5) containing 1 mM $MgCl_2$, 10 mM NaCl, 1 mM 2-mercaptoethanol and 50 μg/ml BSA, or with *E.coli* RNase HI (**B**) in 10 mM Tris–HCl (pH 8.0) containing 10 mM $MgCl_2$, 50 mM NaCl, 1 mM 2-mercaptoethanol and 50 μg/ml BSA. Product separation was carried out as described in the legend for Figure 2. M represents the 3′ end-labeled RNA1–DNA (Table 1). Then, one base shorter product than M shows that the RNA–DNA junction of the RNA9–DNA/DNA substrate has been cleaved. Products are shown schematically on the right. The asterisk indicates the fluorescent-labeled site, and gray and black represent RNA and DNA, respectively.

higher and 11-fold lower than those of Halo-RNase HI and *E.coli* RNase HI, respectively. In the presence of Mn^{2+}, the specific activities of these three enzymes were almost the same.

Halo-RNase HI can cleave an RNA–DNA junction (a junction between the 3′ side of RNA and the 5′ side of DNA) of an RNA–DNA/DNA oligomeric substrate (15). To check whether or not Sto-RNase HI can also cleave the RNA–DNA junction, the same substrate, RNA9–DNA/DNA, was examined for Sto-RNase HI. As shown in Figure 3, the Sto-RNase HI cleaved the RNA–DNA junction of the substrate, in a manner similar to that of Halo-RNase HI, while *E.coli* enzyme could not cleave it as described previously (15). This implies that the cleavage of the RNA–DNA junction might be a common feature of archaeal Type 1 RNases H. It also suggests that Sto-RNase HI might involve in the removal of RNA primers from the Okazaki fragment as described for Halo-RNase HI (15).

Double-stranded RNA cleavage

A series of 12mers of single-stranded RNA and DNA, double-stranded RNA and DNA, and RNA/DNA hybrid labeled at the 5′ end of the DNA (Table 1) were incubated with the enzyme in the presence of 1 mM $MgCl_2$ or $MnCl_2$. Sto-RNase HI did not cleave any of the substrates in the presence of $MgCl_2$. However, the enzyme cleaved the double-stranded RNA in the presence of $MnCl_2$ as shown in Figure 2B. This double-stranded RNA-dependent RNase (dsRNase) activity was always detected for the purified Sto-RNase HI protein in several separate experiments. The cleavage patterns of the RNA/RNA duplex in the presence of 1 mM $CoCl_2$ or 100 mM $NiCl_2$ indicated that the duplex was degraded not only in the presence of $MnCl_2$ but also in that of $CoCl_2$ (Figure 2B).

To prove an association between Sto-RNase HI and dsRNase activity, the purified protein was further fractionated by gel filtration column chromatography. As shown in Figure 4, the molecular mass of Sto-RNase HI is estimated to be 13 000 Da from the gel filtration as shown in Figure 4C, indicating that the protein exists in a monomeric form. The RNase activities of the peak fractions in Figure 4A were examined in the presence of 1 mM $MnCl_2$. As shown in Figure 4D and E, the peak of dsRNase activity was coincident with that of RNase H activity, strongly suggesting that dsRNase activity was intrinsic to Sto-RNase HI.

As *E.coli* RNase III is an enzyme that specifically digests a double-stranded RNA, its co-purification with the Sto-RNase HI was suspected. However, it seems unlikely that *E.coli* RNase III is responsible for the observed digestion by the Sto-RNase HI due to the different molecular masses and ion requirements (the greatest preference for Mg^{2+}) for RNase III (28).

In vivo complementation assays of the *Sto-rnhA* mutants

Sto-RNase HI conserves four acidic amino acid residues, corresponding to the active site residues of *E.coli* RNase HI (18) in Figure 1. To demonstrate the roles of these acidic residues, Sto-RNase HI genes encoding the following mutations were constructed, cloned and examined for RNase H activity with an *E.coli* RNase H mutant strain. The temperature-sensitive phenotype of an *E.coli rnhA* and *rnhB* double mutant strain MIC2067(DE3) was relieved by introduction of an active RNase H gene (20,21), including the wild-type *Sto-rnhA* gene (data not shown), suggesting that the Sto-RNase HI protein exhibits RNase H activity *in vivo*. We also examined the plasmids containing the four mutant genes in which the conserved acidic residues of Sto-RNase HI, Asp-7, Glu-52, Asp-76 or Asp-125 is replaced by Asn or Gln. The results showed that the Sto-D7N, E52Q and D76N mutant genes did not complement the ts growth defect of the strain, suggesting that Asp-7, Glu-52 and Asp-76 are required for RNase H activity of Sto-RNase HI. On the other hand, the transformant with plasmid containing the Sto-D125N gene grew at 42°C, suggesting that Asp-125 was not necessary for hydrolysis of RNA/DNA hybrids by Sto-RNase HI.

RNase activities of mutant proteins

The mutant proteins Sto-D7N, E52Q, D76N and D125N were purified as described in Materials and Methods, and their RNase activities in the presence of 1 mM $MnCl_2$ or $MgCl_2$ were examined. As shown in Figure 5A, Sto-D7N lost not

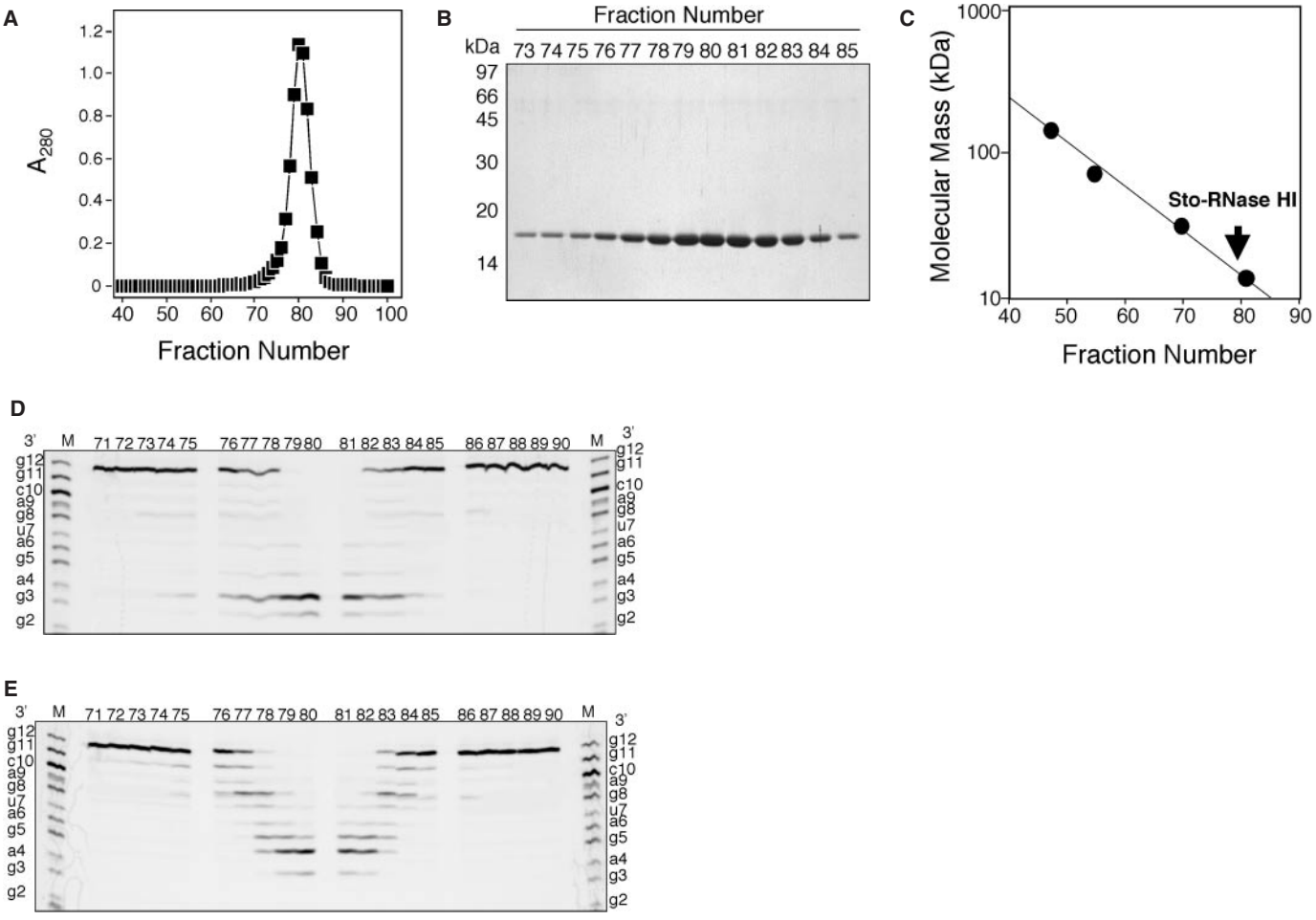

Figure 4. Gel filtration column chromatography for Sto-RNase HI preparation. (**A**) Elution profile for gel filtration column chromatography with 20 mM Tris–HCl (pH 8.0) containing 1 mM EDTA and 150 mM NaCl. The flow rate was set at 1.0 ml/min, and 1.0 ml fractions were collected. (**B**) SDS–PAGE of fractions from the gel filtration column. Numbers along the gel represent the molecular masses of individual standard proteins. (**C**) Estimation of molecular mass of the Sto-RNase HI. To estimate molecular mass, alcohol dehydrogenase (150 000 Da), BSA (66 000 Da), carbonic anhydrase (29 000 Da) and cytochrome c (12 400 Da) were used as standard proteins. (**D**) The RNase H activity of each fraction. The fractions were diluted to 1/3000 in reaction mixtures. The reaction conditions were 37°C for 15 min in 10 mM Tris–HCl (pH 8.5) containing 1 mM $MnCl_2$, 10 mM NaCl, 1 mM 2-mercaptoethanol and 50 µg/ml BSA. Product separations were carried out as described in Figure 2. (**E**) The dsRNase activity of each fraction. The fractions were diluted to 1/100 in reaction mixtures. The reaction conditions and product separations were similar to those described above.

only RNase H activity but also dsRNase activity in the presence of $MnCl_2$. Similarly, the results with the mutant proteins Sto-E52Q and Sto-D76N, which were also unable to suppress the ts phenotype of the MIC2067(DE3), were similar to those with Sto-D7N (data not shown). Even in the presence of $MgCl_2$, no RNase H activity was detected in any of these three mutant proteins. The results of the complementation and of the *in vitro* assays suggest that Asp-7, Glu-52 and Asp-76 are important for the catalyses of both RNase H and dsRNase activities of Sto-RNase HI.

On the other hand, Sto-D125N, which complemented the ts phenotype of the MIC2067(DE3), cleaved the RNA strand of the RNA/DNA hybrid at a level similar to that of the wild-type protein in the presence of 1 mM $MnCl_2$ [Figure 5B(i)] or $MgCl_2$ (data not shown). Surprisingly, Sto-D125N lost dsRNase activity, as shown in Figure 5B(ii). This result indicates that Asp-125 for Sto-RNase HI is important for dsRNase activity but not for RNase H activity.

DISCUSSION

DsRNase activity of Sto-RNase HI

Sto-RNase HI in this report cleaved the RNA strand of the RNA/RNA duplex as well as that of the RNA/DNA heteroduplex. It was established by perfect association of the dsRNase activity to the Sto-RNase HI enzyme during gel filtration and results of site-directed mutagenesis. The RNase H activity was observed in the presence of Mn^{2+}, Mg^{2+}, Co^{2+} or Ni^{2+}, whereas the dsRNase activity was observed only in the presence of Mn^{2+} or Co^{2+} (Figure 2). The dsRNase activity was approximately 10- to 100-fold lower than the RNase H activity in the presence of Mn^{2+} (Figures 2 and 4). All reported cellular RNases H that contain both Type 1 and Type 2 enzymes, which specifically cleave the RNA strand of RNA/DNA hybrids. Therefore, the Sto-RNase HI is the first cellular RNase H that has been found to have dsRNase activity.

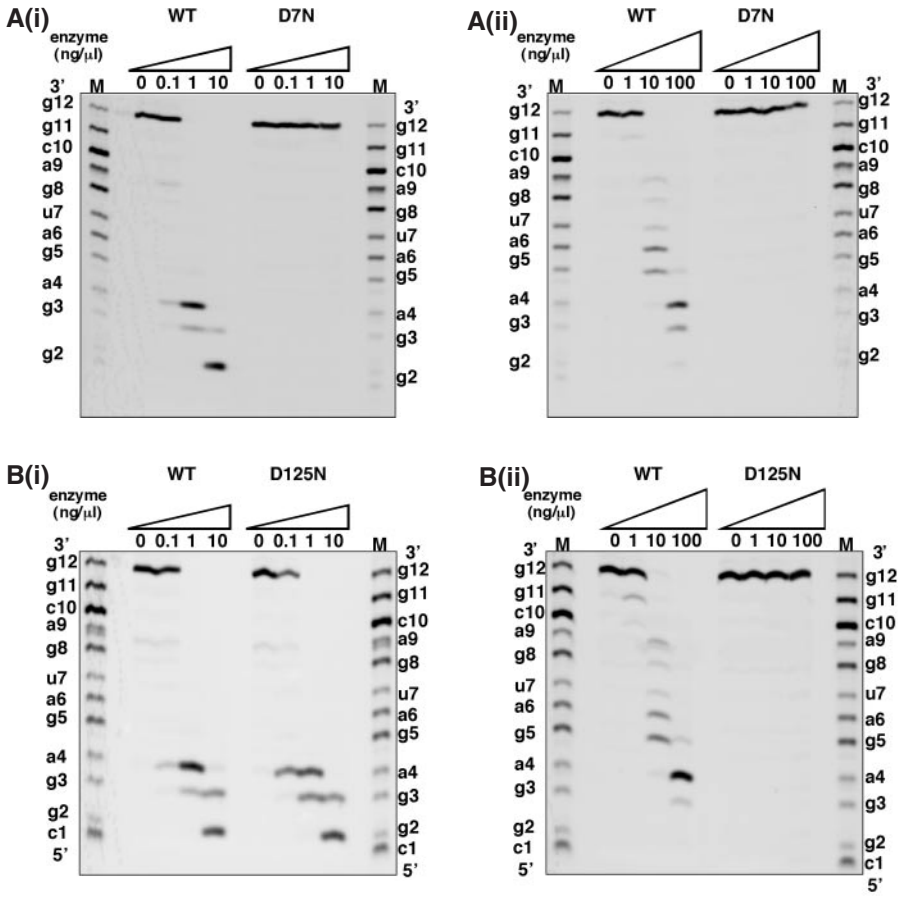

Figure 5. The RNase activities of the Sto-RNase HI wild-type and mutant proteins. The 12 bp RNA/DNA hybrid (i) and RNA/RNA duplex (ii) were incubated with the Sto-RNaes HI mutant proteins, D7N (**A**) or D125N (**B**). The reaction condition and product separations were carried out as described in Figure 4D.

Despite amino acid sequence similarity with Sto-RNase HI, Halo-RNase HI, the first characterized archaeal Type 1 RNase H, does not cleave an RNA/RNA duplex (15). The most remarkable difference between the two archaeal enzymes is the N-terminal extension of Halo-RNase HI (Figure 1). Enzymatic characteristics—the divalent metal ion preference, activity–pH profile, specific activity and RNA–DNA junction cleavage—of RNase H activity of Sto-RNase HI were similar to those of Halo-RNase HI (15), suggesting that the N-terminal extension of Halo-RNase HI might not be critical for RNase H activity. On the other hand, a failure to express the recombinant N-terminal deletion mutant of Halo-RNase HI (15) prevents examining the influence of the N-terminus of Halo-RNase HI on the dsRNase activity. At present, we have not identified what factors are responsible for this difference between the two enzymes. To clarify the factors, further site-directed mutagenesis and structural analyses need to be performed.

Catalytic mechanisms

In *E.coli* RNase HI, one histidine and four acidic residues—Asp-10, Glu-48, Asp-70, His-124 and Asp-134, constitute the catalytic site (18), and catalytic mechanisms in one Mg^{2+}-form (18,29), one Mn^{2+}-form (30) and two Mn^{2+}-form (30) have been proposed. These five residues are highly conserved in

Table 3. Sto-RNase HI homologs

Organism	Gene	RNase H activity
Domain bacteria		
Bacillus subtilis	*ypdQ*	Inactive (2)
Enterococcus faecalis	*ebsB*	No data
Leptospira interrogans	*rnhA*	No data
Mycobacterium tuberculosis	Rv2228c[a]	No data
Mycobacterium leprae	ML1637[a]	No data
Streptomyces coelicolor	SCO2299[a]	Active[b]
Streptomyces coelicolor	SCO7284	Active[b]
Streptomyces avermitilis	SAV5877[a]	No data
Corynebacterium glutamicum	Cgl2236[a]	Active (19)
Corynebacterium efficiens	CE2133[a]	No data
Thermobifida fusca	Tfus2822[a]	No data
Domain archaea		
Halobacterium sp. NRC-1	*Halo-rnhA*	Active (15)
Sulfolobus tokodaii	ST0753 (*Sto-rnhA*)	Active (this work)
Pyrobaculum aerophilum	PAE1792	Active[b]
Domain eukarya		
Arabidopsis thaliana	At3g01410 (putative RNase H)	No data
Oryza sativa	OSJNBb0011A08.1 (putative RNase)	No data

[a]These genes encode polypeptides containing RNase HI-like and CobC-like amino acid sequences at their N-terminus and C-terminus regions, respectively.
[b]N. Ohtani (unpublished data).

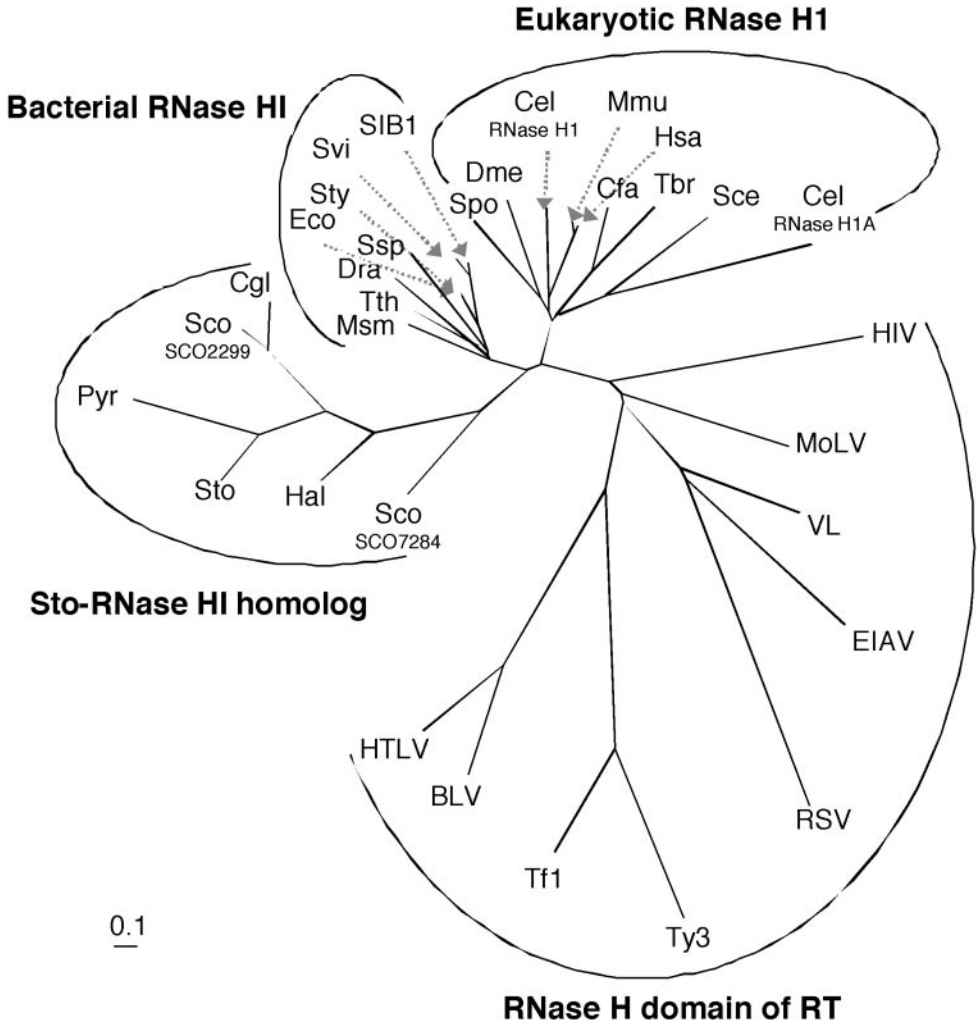

Figure 6. Phylogenetic tree derived from Type 1 RNase H amino acid sequences. Phylogenetic tree was constructed as described in Materials and Methods. Sequence alignments used for the construction of the phylogenetic tree are provided in the Supplementary Material. The distance of the branch from a diverging point is proportional to the frequency on average of the amino acid substitution at each position. The scale bar corresponds to this frequency of 0.1. The abbreviations for the organisms are as follows: Eco, *Escherichia coli*; Sty, *Salomonella typhimurium*; SIB1, *Shewanella* sp. SIB1; Svi, *Shewanella violacea*; Syn, *Synechocystis* sp. PCC6803; Tth, *Thermus thermophilus*; Dra, *Deinococcus radiodurans*; Msn, *Mycobacterium smegmatis*; Sce, *Saccharomyces cerevisiae*; Spo, *Schizosaccharomyces pombe*; Cfa, *Crithidia fasciculate*; Tbr, *Trypanosoma brucei*; Cel, *Caenorhabditis elegans*; Dme, *Drosophila melanogaster*; Mmu, *Mus musculus*; Hsa, *Homo sapiens*; Hal, *Halobacterium* sp. NRC-1; Sto, *Sulfolobus tokodaii*; Pae, *Pyrobaculum aerophilum*; Sco, *Streptomyces coelicolor*; Cgl, *Corynebacterium glutamicum*; BLV, bovine leukemia virus; EIAV, equine infectious anemia virus; HIV, human immunodeficiency virus; HTLV, human T-cell leukemia virus;, MLV, moloney leukemia virus; RSV, rous sarcoma virus; VL, visna lentivirus; Tf1, Tf1 retrotransposon and Ty3, Ty3 retrotransposon. Although all of these Type 1 RNases H exhibit RNase H activities, some of them are unpublished data by N. Ohtani.

Type 1 RNases H (3), and biochemical studies with *E.coli* RNase HI and HIV-1 RT have indicated that only the first three acidic residues are critical for catalysis of RNase H (31–34). The results of site-directed mutagenesis suggest that RNase H catalyses of Sto-RNase HI would be similar to those proposed in other Type 1 RNases H. A possible involvement of Asp-125 in RNase H catalysis cannot be ruled out despite the result in Figure 5B, since corresponding Asp in *E.coli* RNase HI and HIV-1 RT is dispensable under some conditions (30,32–34). On the other hand, the absence of a His in the Sto-RNase HI indicates a different source for activation of an attacking H_2O molecule from that suggested for *E.coli* RNase HI (18). It agrees with Halo-RNase HI, which has no functional His for catalysis (15).

The Sto-RNase HI mutant proteins, D7N, E52Q and D76N, lost both RNase H and dsRNase activities (Figure 5A), suggesting that the first three conserved acidic residues were also important for dsRNase activity. On the other hand, the mutant protein D125N lost only the dsRNase activity (Figure 5B). Amino acid residues required for dsRNase activity seems to be slightly different from those for RNase H activity. Why, then, this fourth conserved acidic residue Asp-125 is critical for only the dsRNase activity? *E.coli* RNase HI contains two Mn^{2+}-binding sites (35). Of the two Mn^{2+}-binding sites, site 1 is formed by Asp-10, Glu-48 and Asp-70, and site 2 is formed by Asp-70 and Asp-134 (35). According to an activation/attenuation model, binding of one Mn^{2+} ion at site 1 is required for enzyme activation and binding of a second Mn^{2+} ion at site 2 is inhibitory (36). Replacing Asp-134 of *E.coli* RNase HI with Asn results in a loss of this Mn^{2+} inhibition (30), probably due to the deficiency of site 2. These observations on *E.coli* RNase HI bring us an idea that

Sto-RNase HI might require two Mn^{2+} ions to exhibit its dsRNase activity. Therefore, the mutant protein D125N might show no dsRNase activity because of inability to bind the second Mn^{2+} ion at site 2. If so, an optimal $MnCl_2$ concentration for dsRNase activity will be higher than that of 1 mM for RNase H activity. However, it has not been clearly demonstrated, because Mn^{2+} is not fully soluble under the standard reaction condition of alkaline pH at such a high concentration (>10 mM). Furthermore, the optimal $MnCl_2$ concentration for RNase H activity of Sto-RNase HI is 1000-fold higher than that for *E.coli* RNase HI (30), suggesting that the binding affinity of a Mn^{2+} ion to site 1 of Sto-RNase HI might be much lower than that of *E.coli* RNase HI. This low affinity also makes it harder to confirm the Mn^{2+}-dependent activation/attenuation in Sto-RNase HI. In *E.coli* RNase HI, a second Mg^{2+} ion does not bind at site 2, probably because the binding affinity of Mg^{2+} is much lower than that of Mn^{2+} (30). If it is also true for Sto-RNase HI, the binding affinities of second metal ions may explain why the enzyme exhibits the dsRNase activity in the presence of Mn^{2+} and does not in the presence of Mg^{2+}.

There is an interesting report on an RNase H domain of RT from Ty3 retrotransposon (37). In the presence of Mn^{2+}, site 2 inability mutants show more specific cleavages than wild type does, similarly to wild type in Mg^{2+} (37). Therefore, amino acid residues constituting site 2, in one-metal form, have been guessed to interact with substrate to ensure that the RNA strand of RNA/DNA hybrid is positioned appropriately for catalysis (37). Probably, in two-metal form, it is unlikely that the residues would involve in positioning the substrate because of a second metal ion binding. Based on these interpretations, in Sto-RNase HI, relaxed substrate-positioning (or recognition) in two-metal form might be expected to allow an RNA/RNA duplex exposed to catalysis.

Correlation with the RNase H domain of RT

Interestingly, the RTs of retroviruses, HIV-1 and Moloney murine leukemia virus (M-MuLV), as well as retrotransposon Tf1, degrade RNA of the RNA/RNA duplex in the presence of Mn^{2+}, and the activity called RNase H* is derived from the RNase H domain of the RT (38–42). This *in vitro* RNase H* activity for HIV-1 RT was shown to be 30-fold lower than the cleavage activity against RNA of RNA/DNA hybrids (40). The preference for metals and the relative activity of dsRNase against RNase H of RT are similar to those characterized in Sto-RNase HI. Although it is unclear whether RNase H* activity has a role in the retroviral life cycle or not (38–41), the activity of retrotransposon Tf1 RT has been proposed to generate a primer for copying the genomic RNA by cleaving the end of the double-stranded RNA in the self-primed reverse transcription model (42,43). However, since Sto-RNase HI exists independently as an active RNase H enzyme, not as a domain of RT in the genome, it seems unlikely that the dsRNase activity of Sto-RNase HI is connected with reverse transcription in *S.tokodaii* cells. The physiological functions of the dsRNase, and even the RNase H activities of Sto-RNase HI remain to be determined.

The second metal ion binding site (site 2) inability mutations are lethal for transposition of Ty3 retrotransposon on account of a failure to support DNA strand transfer and release of the polypurine tract from RNA (37). It allows us wondering about participation of dsRNase activity in the transposition event, because RTs of retroelements often exhibit dsRNase activity although the activity of Ty3 RT has not been reported yet.

Sto-RNase HI homologs and their origin

As shown in Table 3, archaeal Type 1 RNase H-like genes are found in various organisms, and some of them show RNase H activity. They lack the basic protrusion region that all other cellular Type 1 RNases H contain. The basic protrusion region is found to be important for substrate binding in *E.coli* RNase HI (44), but not essential for RNase H activity (45). In fact, the RNase H domains of RTs of HIV and the Gypsy family of retrotransposons lack the basic protrusion region but show RNase H activity. This basic protrusion deletion is a major primary structural feature characterized in many RTs.

Archaeal enzyme can cleave the RNA–DNA junction of the Okazaki fragment-like substrate, just as HIV-1 RT (46) and Ty3 RT (37) can. Furthermore, Sto-RNase HI exhibits a dsRNase activity possessed by the RTs (38–42). These common features between archaeal RNase HI and the RNase H of the retroelement lead us to conjecture that archaeal Type 1 RNases H might be derived by the horizontal gene transfer through the RT of a retroelement. The phylogenetic tree of active Type 1 RNases H is shown in Figure 6. Although the archaeal RNase HI homologs, listed in Table 3, cannot be proposed to be closely related to the RTs, they do construct the different cluster from bacterial RNase HI and eukaryotic RNase H1. It is worth noting that Sto-RNase HI and Halo-RNase HI (15) are active, while only the RNase H domain of RT of the retroelement is inactive (47,48). Therefore, analyses of the Sto-RNase HI as a model for the RNase H of retroelements, especially HIV-1, will provide much useful information on the inhibition of RNase H of HIV-1 for AIDS therapy.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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