Biochemical characterization and functional complementation of ribonuclease HII and ribonuclease HIII from Chlamydia pneumoniae AR39

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Chlamydia pneumoniae AR39 contains two different ORFs (CP0654 and CP0782) encoding ribonuclease H (RNase H) homologues, Cpn-RNase HII and Cpn-RNase HIII. Sequence alignments show that the two homologues both contain the conserved motifs of type 2 RNase H, and Cpn-RNase HII has the conserved active-site motif (DEDD) of RNase HII. Cpn-RNase HIII also contains a unique active-site motif (DEDE), common to other RNase HIIs. Complementation assays indicated that Cpn-RNase HII can complement both Escherichia coli RNase HII and RNase HIII, but Cpn-RNase HIII can only complement the latter. In vivo enzyme activity experiments showed that neither Cpn-RNase HII nor Cpn-RNase HIII is thermostable and their optimum pH values were 9.0 and 10.0, respectively. Cpn-RNase HII cleaves a 12 bp RNA–DNA substrate at multiple sites, but Cpn-RNase HIII at only one site. When a 35 bp DNA–RNA–DNA/DNA chimeric substrate was used, cleavage was only observed with Cpn-RNase HII. These results indicate that the RNase H combination of C. pneumoniae AR39 is not simple substitution of E. coli RNase H, perhaps representing a more primordial type. This is believed to be the first in vivo functional study of Chlamydia pneumoniae RNase Hs and the results should contribute to the analysis of RNase Hs of other parasite species.

INTRODUCTION

Ribonuclease H (RNase H) endonucleolytically hydrolys the RNA strand of RNA–DNA hybrids in the presence of divalent cations; it is involved in DNA replication, repair, and/or transcription processes and widely present in all three kingdoms (Ohtani et al., 1999a). RNase Hs have been classified into type 1 and type 2 RNase H, and prokaryotic RNase Hs are divided into three groups, RNase HI, HII and HIII, encoded by rnhA, rnhB and rnhC, respectively (Ohtani et al., 1999a,b). RNase HI is a type 1 RNase H, while RNase HII and HIII are both of type 2.

Although many bacteria, such as Escherichia coli, contain both type 1 and type 2 RNase H, some bacteria, such as Bacillus stearothermophilus, Streptococcus pneumoniae, Chlamydia trachomatis and Aquifex aeolicus only have two different type 2 RNase Hs (Ohtani et al., 1999a). Although the crystal structures of some RNase HI and HII enzymes have been determined, e.g. E. coli (Katayanagi et al., 1990; Yang et al., 1990), Methanococcus jannaschii (Lai et al., 2000) and Thermus thermophilus (Muroya et al., 2001), little 3D structural information on RNase HIII is available. The enzymic properties of a few RNase HIII genes such as Bacillus subtilis RNase HIII and B. stearothermophilus RNase HIII have been analysed (Ohtani et al., 1999b; Chon et al., 2004, 2006a). Except for the conserved motifs, all the described type 2 RNase Hs contain unique active-site residues, a DEDD motif (Asp-Glu-Asp-Asp) for RNase HII and a DEDE motif (Asp-Glu-Asp-Glu) for RNase HIII (Chon et al., 2006a).

Chlamydia pneumoniae, a pathogenic eubacterial intracellular parasite for humans and some animals, infects the mucosal surfaces of the respiratory tract, causing pharyngitis, bronchitis and pneumonia (Hahn et al., 2002). The complete genome sequence of C. pneumoniae AR39 offered much genetic information about this microbe (Read et al., 2000). Two prospective RNase HII and HIII genes, CP0654 and CP0782, have been isolated from C. pneumoniae AR39 and expressed in E. coli previously (Pei et al., 2005). In this study, the enzymic properties of these two RNase Hs (Cpn-RNase HII and HIII) were analysed further and their functional complementation of RNase H-deficient E. coli mutants was demonstrated in vivo.
METHODS

**Strains and reagents.** *E. coli* strain DY329 [W3110 ΔlacU169 naldA1; Ti+ gal1084 Δc1857AΔ(cro-bioA); Yu et al., 2000] was used in this study; the plasmids used are listed in Table 1. Bacteria were cultured in Luria–Bertani (LB) medium at 32 °C and antibiotics were used as follows: ampicillin, 100 µg ml−1, kanamycin, 50 µg ml−1 and tetracycline 10 µg ml−1. Oligonucleotides were synthesized by Invitrogen. Restriction enzymes and DNA-modifying enzymes were purchased from TaKaRa. All other chemicals were analytical grade.

**Generation of RNase H-deficient *E. coli* mutants and complementation**

**DNA manipulation.** *E. coli* genomic DNAs were extracted using a genomic DNA extraction and purification kit (Sangon, Shanghai, China) and plasmids were isolated from *E. coli* by the alkaline method (Birnboim & Doly, 1979). PCR reactions were carried out using LA-Taq DNA polymerase (TaKaRa) and products were recovered with a gel purification kit (Sangon). Homologous recombination was carried out as described before (Yu et al., 2000) to perform complementation assays. All constructs were confirmed by DNA sequencing (Invitrogen).

**Construction of plasmids.** To achieve constitutive expression of the genes under study, the GAPDH-promoter fragment was amplified from genomic DNA with GAPDH-F/GAPDH-R (F: 5′-GGGGGGATCGTCAAGTCTCTCTTACGAGGATGTTGGAAGTTGGAAGTTGGAAG-3′; R: 5′-GGGGGAAGCTTGATGATGATGATGATGATGATGATGATGATGATGCT-3′; F and R represent forward and reverse primers respectively, the underlined bases are introduced to create restriction sites for DNA cloning and the lower-case bases can match the template, but the capitalized bases are added for DNA cloning or homologous recombination), containing the GAPDH-promoter fragment, RBS and capitalized bases are added for DNA cloning or homologous recombination. Restriction enzymes and DNA-modifying enzymes were used as follows: ampicillin, 100 µM, kanamycin, 50 µM and tetracycline 10 µM. Oligonucleotides were synthesized by Invitrogen.

**Kinetic parameters.** For determination of kinetic parameters, the recombinant Cpn-RNase Hs were expressed and purified, and the enzymatic activities were measured as before (Pei et al., 2005). Unless specified otherwise, assays were performed in reaction buffer containing 10 mM Tris/HCl, pH 9.0, 50 mM NaCl, 1 mM β-mercaptoethanol, 10 mM MgCl2 and 10 µM bovine serum albumin ml−1. Reactions were stopped by adding an equal volume of stopping buffer (100 mM EDTA, 8 M urea, 0.1% bromophenol blue and 0.1% xylene cyanol) and analysed by electrophoreses on 20% polyacrylamide gels (with 8 M urea), followed by measurements with an FLA-5000 phosphorimager.

**In vitro enzymic activity assays**

Recombinant Cpn-RNase Hs were expressed and purified, and the enzymatic activities were measured as before (Pei et al., 2005). Unless specified otherwise, assays were performed in reaction buffer containing 10 mM Tris/HCl, pH 9.0, 50 mM NaCl, 1 mM β-mercaptoethanol, 10 mM MgCl2 and 10 µM bovine serum albumin ml−1. Reactions were stopped by adding an equal volume of stopping buffer (100 mM EDTA, 8 M urea, 0.1% bromophenol blue and 0.1% xylene cyanol) and analysed by electrophoreses on 20% polyacrylamide gels (with 8 M urea), followed by measurements with an FLA-5000 phosphorimager.

**Kinetic parameters.** For determination of kinetic parameters, the enzyme was used at 0.01 μM and the concentration of the 12 bp RNA–DNA substrate varied from 0.052 to 2 μM. The reaction was performed at 30°C for 1 min and stopped by adding stopping buffer. The kinetic parameters were determined from Lineweaver–Burk plots.

**Table 1. Plasmids used in this study**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET28a-CP0654</td>
<td>CP0654 cloned into MCS of pET28a, Km’</td>
<td>Pei et al. (2005)</td>
</tr>
<tr>
<td>pET28a-CP0782</td>
<td>CP0782 cloned into MCS of pET28a, Km’</td>
<td>Pei et al. (2005)</td>
</tr>
<tr>
<td>pUC-G</td>
<td>pUC18 derivative containing GAPDH promoter and RBS, Ap’</td>
<td>This study</td>
</tr>
<tr>
<td>pUC-rnhA</td>
<td>pUC18 derivative containing GAPDH-promoter-controlled rnhA, Ap’</td>
<td>This study</td>
</tr>
<tr>
<td>pUC-rnhB</td>
<td>pUC18 derivative containing GAPDH-promoter-controlled rnhB, Ap’</td>
<td>This study</td>
</tr>
<tr>
<td>pUC-rnhA + B</td>
<td>pUC18 derivative containing GAPDH-promoter-controlled rnhA and rnhB, Ap’</td>
<td>This study</td>
</tr>
<tr>
<td>pUC-CP0654</td>
<td>pUC18 derivative containing GAPDH-promoter-controlled CP0654, Ap’</td>
<td>This study</td>
</tr>
<tr>
<td>pUC-CP0782</td>
<td>pUC18 derivative containing GAPDH-promoter-controlled CP0782, Ap’</td>
<td>This study</td>
</tr>
</tbody>
</table>
**pH effect.** pH effects were determined by performing the assay as above except that the reaction buffer was substituted with 10 mM imidazole/HC1 (pH 5.0–6.5), 10 mM Tris/HC1 (pH 7.0–9.0) or 10 mM glycine/NaOH (pH 9.0–12.0).

**Temperature effect.** Recombinant Cpn-RNase Hs (2 nM) were dissolved in reaction buffer and treated at 70°C for different times; the temperature effects were analysed by determining the remaining enzymic activities.

**Substrate specificity.** A 12 bp RNA–DNA (5′-3′; 5′-cggagaugacgg-3′, 3′-GCGTCCTGCTGCGTTGC-5′; note that ribonucleotides are shown by lower-case letters), 35 bp RNA–DNA–DNA (5′-3′; 5′-AGAGGCAGGAGATGCAACCAAGCAAGGACG-3′, 3′-TCTCCGTCCCTCTACAGTTGCTGTCTCTCCTGGGC-5′) and Ribo primer (5′-3′; 5′-CGCTCTTCTTTGAGTA-3′, 3′-CGCGAGAAGCGCAACGCTTGAGACGGAGA-5′) were used as substrates. All oligonucleotides carrying ribonucleotide(s) were 5′-end-labelled with 32P and the hybrid duplexes were prepared by standard procedures (Katayanagi et al., 1990). The cleavage assays were performed in reaction mixture containing 0.2 μM of different ribonucleotide substrates and specified amounts of Cpn-RNase Hs.

**RESULTS**

**Complementation of RNase H-deficient *E. coli* with Cpn-RNase HII (CP0654) and Cpn-RNase HIII (CP0782)**

According to the BLAST results, two ORFs (CP0654 and CP0782) are presumed to encode Cpn-RNase HII and Cpn-RNase HIII. Cpn-RNase HII is composed of 214 amino acid residues, and has 41% identity with *E. coli* RNase HII. Cpn-RNase HIII consists of 301 amino acid residues and the sequence identities are 31%, 30%, 28% and 62% with the RNase HIIIs from *B. subtilis* (Bsu-RNase HIII), *B. stearothermophilus* (Bst-RNase HIII), *S. pneumoniae* (Spn-RNase HIII) and *C. trachomatis* (Ctr-RNase HIII), respectively. Three motifs of type 2 RNase H (G-X-D-E-X-G-X-G, D-S-K-X-L and V/I/A-A-A-S-I/I-L-A-A/R, where X represents any amino acid residue) are also conserved in both Cpn-RNase Hs, corresponding to Gly31-Gly38, Asp62-Leu66 and Val150-Arg158 of Cpn-RNase HII, and Gly83-Gly100, Asp129-Leu133 and Val32-Arg40 of Cpn-RNase HIII. In addition, four acidic active-site residues are also found conserved. In Cpn-RNase HII, these are Asp33, Glu74, Asp127 and Asp144, and in Cpn-RNase HIII they are Asp95, Glu96, Asp192 and Glu227 (Fig. 1), consistent with previous reports that the active-site motif is conserved as DEDD in RNase HII, but DEDE in RNase HIII (Chon et al., 2006a).

To test whether Cpn-RNase HII and Cpn-RNase HIII could complement the RNase H-deficient *E. coli*, a complementation assay was performed as described in Methods. As expected, rnhA and rnhB could rescue the corresponding RNase H-deficient mutants (DY-rnhA, DY-rnhB and DY-rnhA+ B; data not shown). Cpn-RNase HII (strain CP0654) could complement both the single RNase H knockout mutants and the double RNase H-deficient mutant, but Cpn-RNase HIII (strain DY-CP0782) could only rescue the rnhA knockout mutant. PCR detection also confirmed these results (data not shown). These results demonstrated (1) Cpn-RNase HII (CP0654) and Cpn-RNase HIII (CP0782) could both complement the RNase H-deficient *E. coli*; (2) only Cpn-RNase HII (CP0654) could complement the RNase H-deficient *E. coli*; and (3) Cpn-RNase HII (CP0654) could rescue the RNase H-deficient *E. coli* mutant.

**Effects of pH and temperature on the enzymic activity of Cpn-RNase Hs**

To analyse the enzyme properties of Cpn-RNase Hs, their kinetic parameters were first determined. The *K*~m~ and *V*~max~ values of Cpn-RNase HII were 1.6 μM and 0.022 μM min~1~, while those of Cpn-RNase HIII were 0.644 μM and 0.0135 μM min~1~ (about 2-fold lower and 2.5-fold higher, respectively, than those of Cpn-RNase HIII). The optimal pH for Cpn-RNase HII was 9.0 and for Cpn-RNase HIII, 10.0. The enzymic activities decreased rapidly at pH values lower than 7.0; activities also decreased at pH values above 10.0, but slowly (Fig. 2).

After incubating for 8 min at 70°C, only 50% activity of the two Cpn-RNase Hs remained. After 30 min later, no activity could be detected (Fig. 3). Thus neither Cpn-RNase H was a heat-stable enzyme, consistent with a previous report that the thermostability of RNase Hs often correlates with the growth temperature of the organism (Chon et al., 2004).

**Substrate specificities of Cpn-RNase Hs**

Three oligonucleotide substrates were used to analyse the substrate specificities of Cpn-RNase Hs. For the 12 bp RNA–DNA duplex, Cpn-RNase HII cleaved it at multiple sites, preferentially at c10-g11, slightly at u7-g8, and very slightly at u7-g11 (Fig. 4a). As to Cpn-RNase HII, only a6-t7 was the favoured cleavage site (Fig. 4b); this behaviour was quite different from that of other RNase HIIIs such as Bsu-RNase HIII (Ohtani et al., 1999b) and Bst-RNase HIII (Chon et al., 2004), both with multiple-site cleavages. When the 35 bp RNA–DNA–DNA/DNA was used as substrate, only Cpn-RNase HII could cleave it (Fig. 5). And as to the Ribo primer (one primer with two ribonucleotides at the 3′-end), no cleavage was observed with any Cpn-RNase H (data not shown). These results indicate that both the Cpn-RNase Hs could cleave RNA–DNA duplex, but could not use Ribo primer as target, and only Cpn-RNase HII could cleave a hybrid with RNA flanked by DNA.

**DISCUSSION**

In this study, we analysed the characteristics of two *C. pneumoniae* RNase Hs by sequence alignments, complementation assay and biochemical experiments. Because no RNase HIII homologues existed in *E. coli* and no effective
complementation system could be used in other organisms containing RNase HIII, the complementation assay was performed with \textit{E. coli} RNase HI and HII mutants. The results indicated that Cpn-RNase HII (CP0654) and Cpn-RNase HIII (CP0782) both belong to the type 2 RNase H group, which could complement \textit{E. coli} RNase H mutants \textit{in vivo} and exhibit RNase H activity \textit{in vitro}. This is consistent with previous reports that type 2 RNase H could complement RNase HI-deficient \textit{E. coli}, such as the RNase HII/III from \textit{B. subtilis} (Ohtani et al., 1999a) or \textit{B. stearothermophilus} (Chon et al., 2004). Further different properties of the two proteins imply that Cpn-RNase HII may be the major RNase H of \textit{C. pneumoniae} and the RNase HII/HIII combination in \textit{C. pneumoniae} AR39 is not a simple substitution of \textit{E. coli} RNase HI/II.

Although many organisms contain two types of RNase H genes in one cell, such as \textit{E. coli} (AB type), multiple type 2
RNase H genes (BC type) have been revealed in some organisms, e.g. B. subtilis (Ohtani et al., 1999a) and B. stearothermophilus (Chon et al., 2004). Therefore, the presence of two type 2 RNase Hs in C. pneumoniae AR39 may not be unusual. Further genome database analysis also showed that the two type 2 RNase H homologues are conserved in other Chlamydomphila and Chlamydia species, such as Chlamydomphila abortus, Chlamydia muridarum Nigg, Chlamydomphila felis Fe/C-56 and Chlamydomphila caviae GPIC, with very high identities with the Cpn-RNase Hs. But up to now, except for the RNase Hs of C. pneumoniae AR39, the enzymic properties and biological function of other Chlamydomphila RNase Hs have not been determined. Interestingly, A. aeolicus, a hyperthermophilic hydrogen-oxidizing bacterium similar to primordial forms of life (Deckert et al., 1998), also only contains two type 2 RNase H homologues. Considering the evolutionary position of these organisms, the RNase H genes of A. aeolicus and Chlamydomphila perhaps represent an ancestral

**Fig. 2.** pH effect on Cpn-RNase Hs. The assay was performed in reaction buffer with pH value varied from 5.0 to 12.0; the initial rates of Cpn-RNase HII (●) and HIII (○) activity are plotted.

**Fig. 3.** Temperature effect on Cpn-RNase Hs. The Cpn-RNase Hs were pre-incubated at 70 °C for 0–30 min before the activity assay. The initial rates of heat-treated Cpn-RNase HII (●) and HIII (○) activity are plotted.

**Fig. 4.** Cleavage of the 12 bp RNA–DNA substrate by Cpn-RNase HII (a) and Cpn-RNase HIII (b). Only the ribonucleotides are shown. Cleavage sites are marked on the left with arrows; different sizes of arrows reflect the relative cleavage intensities. Lane 1, a 12 base hydrolysis ladder prepared by incubation at 90 °C for 5 min in 100 mM Na₂CO₃ (pH 9.0); lanes 2–5, hydrolysate with 50 mM Mg²⁺ (lane 2), 10 mM Mg²⁺ (lane 3), 1 mM Mg²⁺ (lane 4) or 0.1 mM Mg²⁺ (lane 5); lane 6, untreated substrate.

**Fig. 5.** Cleavage of 35 bp RNA–DNA substrate by Cpn-RNase HII and HIII. (a) Lane 1, untreated substrate; lane 2, cleavage of Cpn-RNase HII; line 3, cleavage of Cpn-RNase HIII. (b) The cleavage site of substrate by Cpn-RNase HII is shown with arrows; the lower-case letter represents the ribonucleotide.
structure and the BC type is perhaps more primordial than the AB type.

The main function of RNase H is to remove RNA primers from Okazaki fragments, process R loops to modulate replication initiation and restore DNA topology (Broccoli et al., 2004; Kogoma & Foster 1998; Arudchandran et al., 2000). Recently, a mitochondrial RNase H from the parasitic protozoon Leishmania was analysed, which is essential for the parasite’s survival (Misra et al., 2005). This may give a clue to the biological status of the Cpn-RNase Hs, which may play an important role on the DNA replication/translation processes of Chlamydomphila and may be vital for this organism. As to the unique biochemical function of RNase Hs, any differential biochemistry and molecular biology of these enzymes in a parasite as compared to that of its host could be exploited for rational drug design against the parasite infection. Therefore, the characterization of Cpn-RNase Hs will be an important avenue toward the development of anti-Chlamydomphila compounds.

Moreover, RNase Hs is a member of the ‘polynucleotide transferases’ superfamily, including resolvasa (Ariyoshi et al., 1994), integrase (Maignan et al., 1998), transposase (Rice & Mizuuchi, 1995), exonuclease III (Moh et al., 1995), type II restriction enzymes (Kostrewa & Winkler, 1995), Vsr endonuclease (Tsutakawa et al., 1999), RNA helicase (Nishino et al., 2003) and RNase III (Blaszczyk et al., 2001). These enzymes have homologous active sites and are likely to share a common mechanism for catalysis. Hence, understanding of RNase H may contribute to the study of other members.

ACKNOWLEDGEMENTS

This work was partially supported by the National Basic Research Program of China (Grant no. 2005CB724301) and the National Science Foundation of China (Grant no. 30571012/C011003).

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Edited by: T. P. Hatch