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Mutation of the conserved GRG motif and decreasing activity of human RNase H2

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Abstract: RNase H2 consists of three subunits (H2A, H2B and H2C) and is involved in the hydrolysis of RNA/DNA hybrids. The GRG motif in RNase H2 is highly conserved and recognizes the ribonucleotides misincorporated into dsDNA. The mutant G37S in the GRG motif of human RNase H2A was found to be correlated with Aicardi-Goutières-syndrome (AGS). In this study, 4 mutants (G37S, G37A, R38A and G39A) were prepared and their biochemical properties of secondary structure, activity and binding affinity with substrate were studied in order to explore the function of the GRG motif. The activity assay showed that the mutations resulted in significantly decreased RNase activity. The binding efficiency assay demonstrated that binding affinities between 4 mutants and 1-ribo FAM-labeled substrate were significantly weakened. These results all indicated that the GRG motif contributes directly to substrate binding and is closely related to the enzyme activity.

Keywords: RNase H2; GRG motif; RNA/DNA hybrids; site-directed mutation; cleavage activity; binding activity

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1 Introduction

Ribonucleases H (RNases H) are endonucleases involved in the hydrolysis of RNA/DNA hybrids, specifically through cleaving the RNA moiety and the removal of misincorporated ribonucleotides in genomic DNA in order to maintain genomic integrity [1]. RNase HI/H1 and RNase HII/H2 are two distinct kinds of RNases H in most organisms (determined through amino acid sequence homology), which have different biochemical properties [2-5]. Specifically speaking, RNase H1 enzymes hydrolyze the 5'-end of the RNA sequence in an RNA/DNA duplex with a minimum of four consecutive ribonucleotides, whereas RNase H2 enzymes are able to recognize and cleave the 5'-end with only one or more ribonucleotides in a DNA duplex [4-5]. RNase H2 accounts for the majority of RNase H activity in mammalian cells, and is the only RNase H with the ability to repair misincorporated ribonucleotides in genomic DNA [3-5].

The mammalian RNase H2 enzyme consists of three subunits: H2A, H2B and H2C, among which H2A is a catalytic subunit. All of them are required for its activity. Although it was reported that the single subunit RNase H2A is responsible for the activity of RNase H in both archaea and bacteria, the optimal ratio of the three subunits of the active enzyme complex for best functionality is 1:1:1 in *Saccharomyces cerevisiae* [6].

It is postulated that RNase H2 activity loss would cause the accumulation of RNA/DNA hybrids, which results in the activation of the innate immune response and cause an infection-like phenotype [7-9]. Mutations of any subunits of RNase H2 are related to Aicardi-Goutières Syndrome (AGS), a pediatric inflammatory disease that severely affects the nervous system [7]. For example, these mutations occur in the corresponding subunits: G37S in RNase H2A [10], A177T and V185G in RNase H2B, and R69W in RNase H2C [11]. Among the reported disease-related mutations, G37S is in the positively charged GRG (glycine, arginine, and glycine) motif, which is highly conserved from bacteria to human and is always located in the active area of the enzyme RNase H2 [12].

Not only does the GRG motif largely relate to the enzyme's activity, but it also functions in substrate recognition and binding [13]. As shown in the crystal structure of the complex between bacterial RNase H2 and nucleic acid, the 5' phosphate is located at the active site and there are interactions between the 2'-OH substituent of the RNA with the conserved GRG motif [14-16]. Further studies need to be carried out in order to better understand the effect of the GRG motif in RNase H2.

In this study, we cloned and purified the native and 4 mutant human RNase H2 which were in the GRG motif of RNase H2A. Further biochemical properties of the 4 mutants such as secondary structure, activity and binding affinity with substrate were investigated.

2 Experimental Procedures

2.1 Site-directed mutagenesis of RNase H2

HepG2 cell cDNA was used as the PCR template to obtain the subunits of encoding genes. The PCR products were purified and digested by restriction enzymes (BamHI / EcoRI) then ligated into the pET-28a vector. The mutants were generated by the PCR-based site-directed mutagenesis method with the RNase H2A plasmid as a template. Four oligonucleotides were designed (5'-CGTCGATGAGGCGGCCAGGGGCCCCGT-3', 5'-CGTCGATGAGGCGTCCAGGGGCCCCGTG-3', 5'-TCGATGAGGCGGGCGCGGGCCCCGTGCT-3', and 5'-ATGAGGCGGGCAGGGCCCCCGTGCTGG-3') for the 4 mutants (G37A, G37S, R38A, and G39A, respectively). The plasmid was then constructed by ligating the oligonucleotides into the pET-28a vector as described above. *E. coli* strain BL21-CodonPlus (DE3) was used to express the proteins. Automated direct sequencing was used to confirm all the mutations.

2.2 Protein expression and purification

The plasmids were transformed into *E. coli* strain BL21-CodonPlus (DE3) and inoculated into 0.2 L Luria-Bertani broth (LB) and grown to A_{600} = 0.6 at 37°C with shaking. Isopropyl-β-D-thiogalactopyranoside (IPTG, 0.5 mM) was added to induce protein expression. After 4 h at 25°C, cells were harvested by centrifugation at 5,000 g for 15 min and then suspended in lysis buffer. Sonication was used to break the cell membranes after adding 0.2 mg/ml of lysozyme and 0.5 mM of phenylmethylsulfonyl fluoride (PMSF). After centrifugation at 20,000 g for 30 min the supernatant was loaded onto the equilibrated Ni^{2+}-NTA column and incubated at 4°C for 2 h. Protein subunits were purified as previously stated [17]. Lysis buffer, wash buffer, elution buffer, and activity assay buffers were used for protein purification and the activity assay. The lysis buffer consisted of: 20 mM Tris-HCl, pH 8.0; 500 mM NaCl; and 10% glycerol. The wash buffer consisted of: 50 mM Tris-Cl, pH 7.0; 100 mM NaCl; 25 mM imidazole; and 10% glycerol. The elution buffer consisted of: 50 mM Tris-Cl, pH 7.0; 100 mM NaCl; 200 mM imidazole; and 10% glycerol. Finally, the activity assay buffer consisted of: 15 mM Tris-HCl, pH 7.9; 10 mM $MgCl_2$; 1 mM dithiothreitol; 100 μg/ml bovine serum albumin; and 5% glycerol.

2.3 Substrate preparation

The carboxyfluorescein (FAM) labeled-oligonucleotides DNA_{15}-RNA_1-DNA_{14} (5'-FAM-ACCTATCTAACAACACT CAcTCCATCATAT-3') and the complementary DNA (5'-ATATGATGGAGTGAGTGTTGTTAGATAGGT-3') [10] were purchased from Takara (Japan). The ribonucleotide is indicated with the lowercase letter. Duplex polynucleotide substrates were prepared by incubating equal molar amounts of complementary oligonucleotides in 10 mM Tris-HCl (pH 8.0) in a boiling water bath and allowing the bath to cool to room temperature.

2.4 Activity assay of RNase H2 and its mutants

The activity reaction contained 200 nM 5'-FAM-DNA_{15}-RNA_1-DNA_{14} that was annealed to the complementary 30-mer DNA, activity assay buffer, and RNase H2 enzyme. Ethanol was added to stop the reaction after incubation at 25°C for 20 min. After being dried in a vacuum, sediment was resuspended in 10 μl of formamide and separated on 23% denaturing polyacrylamide. The gels were scanned with a Typhoon 9400 fluorescence scanner (GE Health - care) [17].

2.5 Binding assay of RNase H2 and its mutants

The binding assay was carried out by fluorescence polarization. Because Ca^{2+} cannot activate RNase H2, Ca^{2+} was substituted for Mg^{2+} to prevent catalysis in the experiments [6,10,11,16]. 1 nM FAM-labeled DNA_{15}-RNA_1-DNA_{14} substrate was mixed with various concentrations of native RNase H2 or its mutants (0 – 750 nM) in PBS (pH 7.4). The fluorescence polarization was measured on a DTX 880 multimode detector (BECKMAN COULTER, Germany) after incubating 20 min at 25°C. The emission signal

(λ_{max} = 525 nm) was recorded upon irradiation at 470 nm. Reactions were performed at least in triplicate. The experimental data were inserted to the following equation:

$$P_{obs} = \frac{P_{\max}[enzyme]}{K_d + [enzyme]}$$

The observed relative fluorescence polarization (P_{obs}) for each enzyme concentration was calculated using the equation $P_{obs} = P - P_o$ where P is the polarization at the given enzyme concentration, P_o is the polarization at 0 enzyme concentration, P_{max} is the value of the P_{obs} with excessive amount of protein, and K_d is the dissociation constant.

2.6 Circular dichroism (CD) spectroscopy

The CD spectra of the wild type and 4 mutants (G37S, G37A, G38A and G39A) of RNase H2 were measured on a Jasco 715 circular dichroism spectropolarimeter (Jasco, Tokyo, Japan), with a 1 mm path length quartz cylindrical cell. Dry N_2 was used to purge the cell continuously. All samples were tested 3 times in 20 mM Tris-Cl (pH = 7.5). The fraction of helix was calculated as previously described [18].

3 Results and Discussion

3.1 The structure and site-directed mutants of the GRG motif in RNase H2A

The RNase H2A subunit on chromosome 19p13.13 has 7 kb of genomic sequence and encodes a protein of 299 amino acids which contains some conserved motifs, such as the GRG motif, DSK motif and KAKA motif (Figure 1C). As shown in the results of the sequence alignment of RNase H2A (Figure 1A), the GRG motif is highly conserved in a wide range of organisms from bacteria to humans. The GRG motif is in the loop domain of the structure which is flexible. Gly37 in the GRG motif is very close to the active site, and thus is involved in the interaction with 2'-OH in the junction [19-22]. It was reported that G37S mutation of human RNase H2A impaired substrate cleavage and led to AGS [10]. In this study, the 4 mutants of the GRG motif in RNase H2A were cloned and purified in order to test the importance of the GRG motif, specifically the 3 mutants (G37A, R38A and G39A) and the AGS-related mutant (G37S) (Figure 1B). The 4 mutants behaved similar to the wild type of RNase H2A on SDS-PAGE (Figure 2).

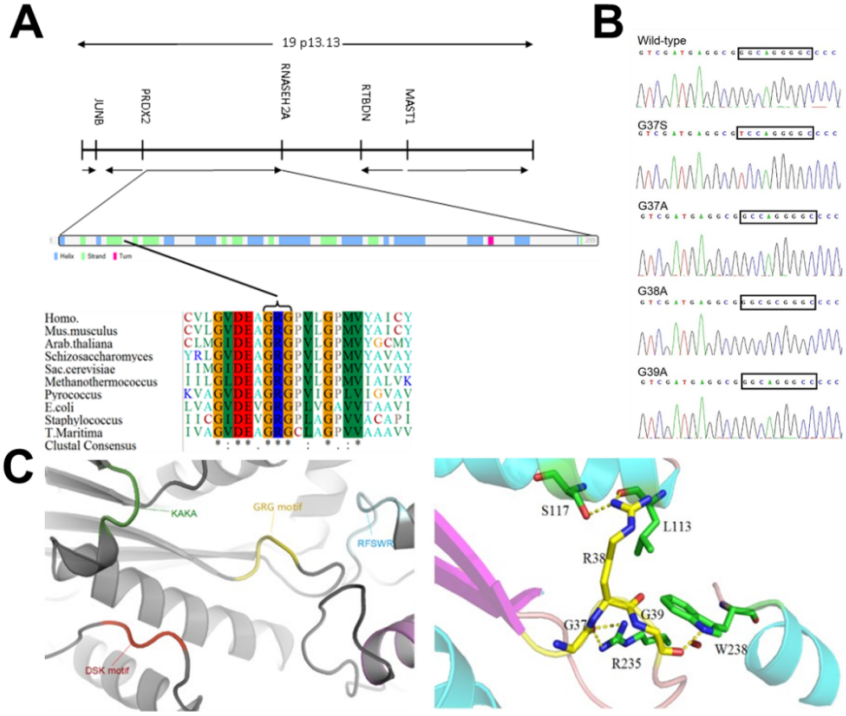

Figure 1 The structure and site-directed mutants of GRG motif in RNase H2A. (A) Genetic map of chromosome 19p13.13 and conserved GRG motif. Sequence alignment of RNase H2A, including *Homo* sp., *Mus musculus*, *Arabidopsis thaliana*, *Schizosaccharomyces* sp., *Saccharomyces cerevisiae*, *Methanothermococcus* sp., *Pyrococcus* sp., *E. coli*, *Staphylococcus* sp. and *Thermotoga maritima*. (B) Sequence electropherograms of GRG motif mutants, G37S, G37A, G38A and G39A. (C) Conserved motifs in the active sites of RNase H2A including KAKA motif, DSK motif, RFSWR motif and GRG motif were shown on the left. The predicted tertiary structure of GRG motif was on the right. The GRG motif lies at a loop at the end of the first β-sheet.

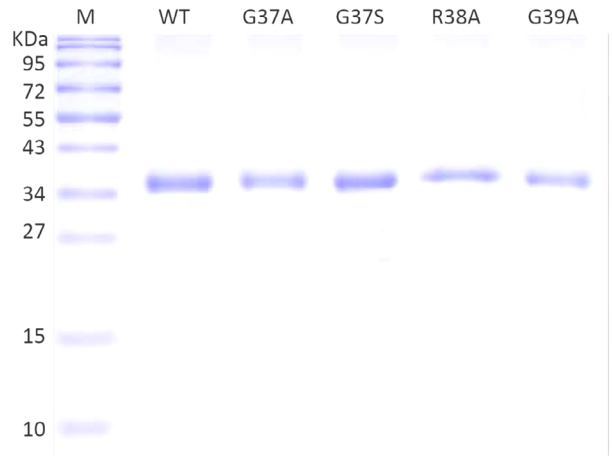

Figure 2 Production of site-directed mutants of GRG motif in RNase H2A.

3.2 Secondary structure of the RNase H2 and its mutants

Secondary structures of the RNase H2 and its 4 mutants were studied through circular dichroism (CD) spectroscopy, which is widely used to study the conformations of biomacromolecules, such as α-helix, β-sheet, β-turn, and random coil [23]. In this study, the far-UV CD spectra of the native and mutant RNase H2 showed subtle changes with similar helical structure percentages (Figure 3), although the conformation of G37S was reported slightly deformed [24]. This indicated that the 4 mutants did not influence the structure of the protein significantly. However, their actual activities were significantly decreased, probably resulting from the distorted local conformation.

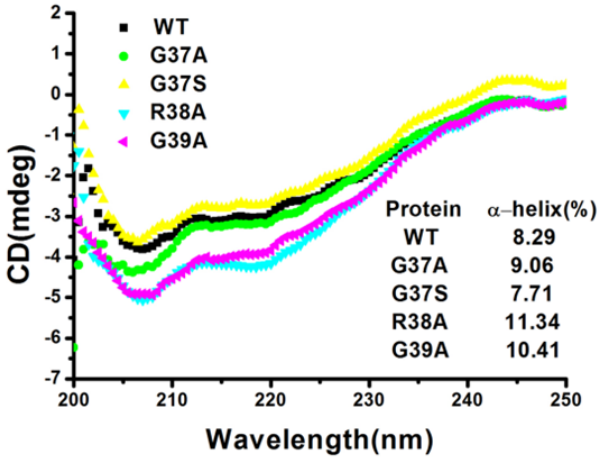

Figure 3 Circular dichroism spectra of RNase H2A, including the wild type and the 4 mutants in 20 mM Tris-Cl (pH = 7.5) buffer. The concentration of each protein was 0.1 mg/mL in all samples.

3.3 Activities of the RNase H2 and its mutants

It was reported that the GRG motif in RNase H2 was highly conserved in many species [12]. Therefore, structural and functional studies on the GRG motif are necessary in order to clarify the catalytic mechanism. The activity of the RNase H2 complex and the 4 mutants were assayed by incubating 200 nM substrate with the enzyme of increasing concentrations up to 750 nM at room temperature for 20 min. It was observed that native RNase H2 at about 100 nM can cleave the substrate completely within 20 min. The cleaved percent of the substrate reached the plateau at about 90% (Figure 4). As shown in Figure 3B, the activities (pmol of product/min/nmol of enzyme) of the wild type RNase H2A and its mutants (G37A, G37S, R38A and G39A) were 1530, 1.4, 31.3, 7.7 and 7.2, respectively (Table 1). The activities of the 4 mutants reduced dramatically compared to the wild type RNase H2, especially for the mutants of G37A, R38A, and G39A, which only exhibited 1/1500, 1/210, and 1/200 of the activity level of the wild type, respectively.

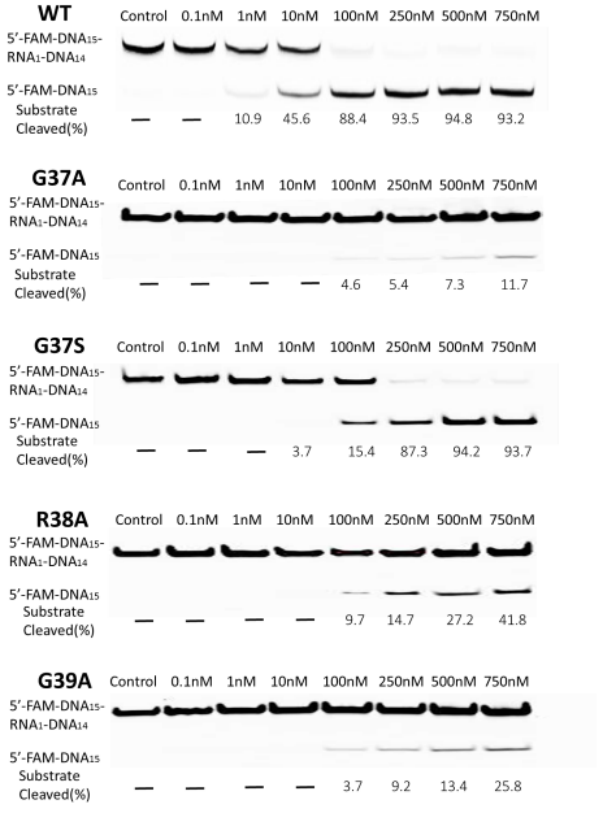

Figure 4 The cleavage activities of the wild type and mutant RNase H2. Concentrations of the protein and the relevant cleaved percentage of 200 nM substrate were indicated at the top and the bottom, respectively.

The G37S mutant, which was previously reported to cause AGS, demonstrated 50-fold weaker activity compared to the wild type RNase H2. The result is in agreement with the literature report [24], which suggests that the substituted residues greatly influence the activity of the enzyme, due to the effects on the substrate binding sites, metal ion binding sites, or catalytic sites.

3.4 Substrate binding efficiency of RNase H2 and its mutants

To further understand the mechanism of the decreased cleavage activities of the 4 mutants, binding affinities were investigated. Binding affinities between the mutants or the wild type motifs and a FAM-labeled duplex DNA were compared utilizing a fluorescence polarization assay (Figure 5). The 1-ribo duplex was incubated with various concentrations of RNase H2 or its mutants in the presence of non-activating Ca^{2+} ions. The K_d values are shown in Table 2. The wild type RNase H2 complex bound to FAM-labeled duplex DNA with a K_d value at 26.1 nM, which is comparable to that reported in literature [10]. K_d values of the 4 mutants (G37A, G37S, R38A, and G39A) are 1009.5 ± 91.41 nM, 100.2 ± 8.09 nM, 554.5 ± 43.88 nM, and 765.6 ± 79.04 nM, respectively. The 3 mutants of G37A, G38A, and G39A exhibited 40-fold, 25-fold, and 30-fold lower binding affinity with FAM-labeled duplex DNA, respectively. This phenomenon strongly suggested that the GRG motif mutations in RNase H2A affect the binding affinity tremendously, which in turn affected the enzyme's activity. This was consistent with a previous report by Perrino *et al.* which demonstrated that G37S activity decreases with decreasing substrate binding affinity [10]. The mutation of the GRG motif may affect the dihedral angle of the loop and the surrounding residues. The activity of RNase H2 would likely be affected when the biochemical properties were altered, such as the hydrogen bonding and electrostatic interaction, which need to be accommodated at the center of the protein-nucleic acid interface.

In conclusion, 4 mutants of the GRG motif failed to effectively recognize the substrate. The mutation caused the change of the interactions with surrounding residues, which might alter the local structure. In another way, the microenvironment of the GRG motif is of great importance for recognition of a single ribonucleotide misincorporated into dsDNA.

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Table 1 Enzyme activities of the native and mutant RNase H2. [a] The plots of activity vs concentration of the enzyme. The activities (pmol of product/min/nmol of enzyme) and standard deviation for the wild type and mutant RNase H2 are shown. [b] The relative activity was calculated as: activity (mutant) / activity (wild type).

RNase H2A	Activity[a]	Relative activity[b]
WT	1530 ± 21.2	1
G37A	1.4 ± 0.2	1/1500
G37S	31.3 ± 5.0	1/50
R38A	7.7 ± 0.1	1/200
G39A	7.2 ± 1.2	1/210

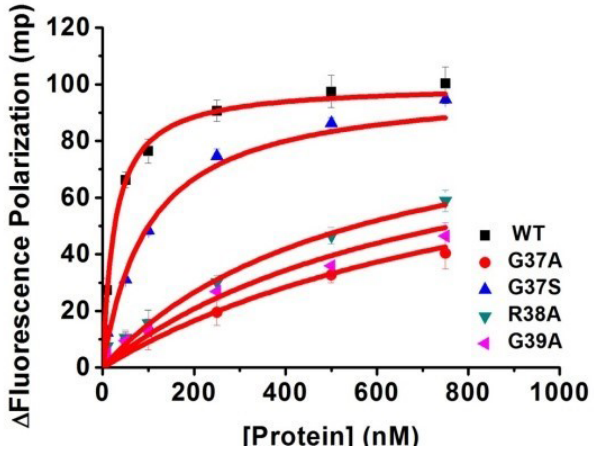

Figure 5 Binding affinities of FAM-labeled duplex DNA with the wild type and mutant RNase H2 determined by fluorescence polarization assay.

Table 2 Relative binding activities of the wild type RNase H2 and its mutants. [a]The K_d values were determined by using fluorescence polarization (see the Materials and methods section). [b]The fraction of WT affinity was calculated as: K_d (wild type) / K_d (mutant).

RNase H2A	K_d[a](nM)	Fraction of WT affinity[b]
WT	26.1 ± 2.6	1
G37A	1009.5 ± 91.4	1/40
G37S	100.2 ± 8.1	1/4
R38A	554.5 ± 43.9	1/25
G39A	765.6 ± 79.0	1/30

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