Amino Acid Residues in RAG1 Responsible for the Interaction with RAG2 during the V(D)J Recombination Process*

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The V(D)J recombinase, a complex of RAG1 and RAG2, carries out a gene rearrangement process that is required for the achievement of diverse antigen receptor repertoires during the early developmental stage of lymphocytes. It recognizes a specific site spanning the coding DNA region of antigen receptor genes and produces double-stranded DNA breaks at the board between coding and signal sequences. Two broken DNA ends are joined by a double-stranded break repair system. Both RAG (recombination activation gene) 1 and RAG2 proteins are absolutely required for this process although the catalytic residues of V(D)J recombinase are exclusively located at RAG1 according to recent mutational analyses. In this study we identified some acidic amino acid residues in RAG1 responsible for the interaction with RAG2. Mutation on these residues caused a decrease of cleavage activity in vitro and failure of RAG-RSS DNA synaptic complex formation. This result is complementary to previous reports in which positively charged amino acids in RAG2 play an important role in RAG1 binding.

The gene encoding the variable region of antigen receptors (immunoglobulins or T cell receptors) is composed of a cluster of small gene segments (V, D, and J). These segments are sequentially and specifically assembled by a lymphoid-specific gene rearrangement process, called V(D)J recombination, during lymphocyte development (1–3). Each gene segment is flanked by recombination signal sequences (RSS) that can be specifically recognized by the V(D)J recombinase. The signal sequence consists of two conserved sequences (heptamer, 5'-CACAGTG-3', and nonamer, 5'-ACAAAAACC-3'), separated by a spacer of either 12 bp ("12-signal") or 23 bp ("23-signal"). To begin this lymphoid-specific process, two signals (one 12-signal and one 23-signal) are specifically selected and rearranged under the "12/23 rule" (2). In addition, once an array of gene segments for a specific antigen receptor is rearranged, the gene rearrangement process for other antigen receptors is prohibited within a lymphocyte (2).

V(D)J recombination occurs at two steps. First, two lymphoid-specific proteins, RAG1 and RAG2 (4, 5), recognize signal sequences and form a synaptic complex with the assistance of HMG1, one of the non-histone chromatin proteins (6). Then, RAG proteins cut DNA at the border between signal sequence and coding sequence. At this cleavage step, DNA is nicked first by RAG proteins at the top strand, and then the 3'-hydroxyl group attacks the phosphodiester bond of the bottom strand by a direct nucleophilic reaction, resulting in formation of a hairpin intermediate at the coding end (7, 8). At the second step, the broken DNA ends are repaired by double strand break repair proteins. The coding ends resolved by a specific endonuclease, are usually further processed before being repaired, which allows antigen receptors to be more diverse. Deletion or addition of some nucleotides at the coding joint of antigen receptor genes is commonly observed. Terminal deoxynucleotidyl transferase has been known as a polymerase that plays a role in nucleotide addition during the V(D)J recombination process (9). Many other DNA double strand repair proteins have been implicated in their roles in V(D)J recombination. DNA-dependent protein kinase (DNA-PK) can phosphorylate other repair proteins as well as its own subunits of Ku 70 and Ku 86 (10). Recently, Artemis protein has been known as a nuclease involved in opening hairpin intermediates produced from the V(D)J cleavage. It has dual specific nuclease activities (endonuclease or exonuclease activity). DNA-PK-dependent phosphorylation of Artemis allows this protein to resolve the hairpin intermediates by changing its specificity from exonuclease to endonuclease (11). Hairpin openings at the coding sequence can be achieved by another nuclease activity of RAG proteins (12). XRCC4, DNA ligase IV, and NBS1-MRE11-RAD50 complex are major double strand break repair proteins involved in V(D)J recombination (13, 14).

In vitro V(D)J cleavage requires only two lymphoid-specific proteins, RAG1 and RAG2 (7, 8, 15). Purified RAG proteins were able to nick and form a hairpin using oligonucleotide-based substrates containing either a single signal sequence or two signal sequences (12- and 23-signal) in vitro. In addition, they formed RSS DNA-RAG complexes in vitro although their ability for complex formation was greatly enhanced by HMG1 (16–18). Several different types of RAG-DNA complexes have been observed. RAG1 alone can form a complex with a single RSS DNA, although it is very unstable. RAG complex formation by RAG1 is largely increased in the presence of RAG2 (19). RAG1 and RAG2 form at least two complexes with a single RSS DNA (12- or 23-signal) (20, 21). In the presence of the second RSS DNA and HMG1, RAG proteins form a paired complex (6, 21). Although the exact composition of these complexes is unclear, the paired complex is composed of two signal sequences (12- and 23-signal), HMG1, and at least two RAG proteins each (20, 21). Once RAG-DNA complexes are formed, they are very stable. This stable RAG-DNA complex can last even after DNA is cut by RAG proteins. In fact, the post-synaptic complex of RAG proteins and RSS DNA has been isolated after cleavage (22).
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Thus, direct interaction between two RAG proteins is essential for synaptic complex or post-synaptic complex formation.

Many mutation analyses of RAG1 or RAG2 have been performed to identify crucial amino acid residues or regions that might be involved in catalysis or interaction with other proteins during the V(D)J recombination process. Most polymerases, transposases, and recombinases contain two or three acidic residues at the active site. These residues chelate divalent ions that can form a transient complex necessary for catalysis. Recently, three acidic residues (called the “DD, E” motif) of V(D)J recombinase have been identified using random mutation analysis. Exclusively located at RAG1, they are Asp<sup>708</sup>, Asp<sup>7716</sup>, and Glu<sup>9262</sup> (23–25). Mutation at these residues abolished the catalytic activity in <i>vivo</i> or in vitro although the RSS DNA binding activity remained normal. In addition, many short deleted regions of RAG genes had influence on V(D)J recombination activity in V(D)J recombination analyses in vitro (26–29). In other studies, some mutant RAG genes were directly isolated from human immunodeficiency patients with Omenn syndrome or severe combined immunodeficiency disease (30). They were defective in catalysis or DNA-RAG complex formation.

The RAG1 domains or amino acid residues responsible for the interaction with RAG2 were not clearly defined although few putative regions for the RAG2 interaction have been suggested. The core domains of RAG1 (384–1004) and RAG2 (1–383) have been previously identified, and these minimal regions of RAG proteins were fully catalytically active in vitro or in vitro (28–29). Thus, the domain responsible for RAG2 interaction must be located within regions (amino acids 384–1004) of RAG1. According to the deletional analysis of RAG1, zinc finger B (amino acids 727–750) of RAG1 was required for interaction with RAG2 in a pulldown analysis (26, 27). Additionally, the KKKRFY<sup>958</sup> (KKKRFRFK<sup>958</sup> in mouse) region of human RAG1 has been suggested to have a role in the interaction with RAG2. In particular, R561H or R561C RAG1 mutants found in Omenn syndrome patients showed defects in the interaction with RAG2 in vitro (30). Recently, positively charged amino acids (e.g., Arg<sup>720</sup>, Arg<sup>733</sup>) in RAG2 have been implicated as having roles in binding to RAG1, suggesting that negatively charged amino acid residues in RAG1 might be involved in RAG complex formation through some ionic interactions at the interface between two proteins (31). In this work, we found that mutations at some acidic residues in RAG1 revealed severe catalytic defects because of the failure of complex formation with RAG2.

EXPERIMENTAL PROCEDURES

Proteins—Wild-type RAG1 and RAG2 proteins were purified by the methods previously described (24, 32). Mutant RAG1 was generated by site-specific mutagenesis using a plasmid, pDRK534, containing a RAG1 coding region (amino acids 352–1040); all mutant proteins were prepared by the same method (24). For the HMG1 preparation, BL21 (DE3) lysate cells containing plasmid pDW83 (obtained from Dr. D. C. van Gent, Erasmus Medical Center, Rotterdam, the Netherlands) were grown in 500 ml of culture medium and lysed by sonication in the presence of 0.2 mg/ml lysozyme. After centrifugation (25,000 rpm, 30 min), the supernatant was subjected to trichloroacetic acid precipitation. The precipitate (containing 0.25 pmol<sup>32</sup>P-labeled oligonucleotide substrates, about 100 pmol of RAG1, and 50 ng of RAG2 with or without HMG1 (50 ng) in 25 mM HEPES, pH 7.4, 60 mM KGl, 2 mM dithiothreitol, and 1 mM Mn<sup>2+</sup>) was incubated at 30 °C for 1 h. Reaction products were separated in a 10% 7 M urea super-denaturing polyacrylamide gel and identified by autoradiography. Binding assay was carried out under the same condition as the cleavage reaction except 0.025 pmol substrate was used with 50-fold nonspecific competitor DNA. The binding reaction mix was incubated at 30 °C for 30 min and separated by a 4–20% or 8% native polyacrylamide gel. Binding products were visualized by autoradiography.

Immunoprecipitation—Purified FLAG-tagged RAG1 and RAG1 (wild-type or mutants) was mixed (1 μg each) and incubated in a buffer (25 mM HEPES, pH 7.4, 2 mM dithiothreitol, 1 mM MgCl<sub>2</sub>, 150 mM NaCl, and 200 μg/ml bovine serum albumin) at 4 °C for 1 h. Anti-FLAG antibody crossed-linked agarose beads (30 μl) (Sigma) were added to the reaction mix and incubated for 2 h. The agarose beads were washed with binding buffer three times. Bound proteins were separated in a 12.5% SDS-PAGE, and RAG1 was detected by a general method of Western blot using anti-RAG1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

RESULTS

Mutations at Three RAG1 Acidic Residues Cause Defect on the RAG2 Binding—Two essential proteins in V(D)J recombination, RAG1 and RAG2, have been known to form a multimeric complex before the initiation of DNA binding and cleavage reactions. The RAG protein complex specifically selects two signal sequences flanking the coding region to be rearranged and forms a stable synaptic complex with DNA (6, 15). Thus, the direct interaction between RAG1 and RAG2 is crucial in forming an initiation complex for the V(D)J recombination process. Previously, we created random mutant RAG1 proteins and identified some key residues directly involved in the catalytic reaction of V(D)J cleavage (24). Mutations at these residues of RAG1 completely abolished the catalytic activity of V(D)J recombinase. In addition, some other RAG1 mutant proteins revealed a severe decrease in their cleavage activities.

Both nick and hairpin formation of two mutant RAG1 proteins at the aspartate residues (D546A and D560A) were decreased about 15-fold more than those of wild-type RAG1 in oligonucleotide-based cleavage analysis in vitro. E547A RAG1 (mutation of glutamate to alanine at 547) also showed some defectsiveness in both nick and hairpin formation in vitro (Fig. 1A, lanes 4, 5, 7). Other RAG1 proteins mutated at adjacent acidic residues did not have influence on the V(D)J cleavage activity of RAG1 (Fig. 1). We further tested whether these decreased activities of mutant RAG1 proteins were because of intrinsically defective catalysis or failure in forming a complex between signal sequence DNA and RAG proteins. All three mutant RAG1 proteins formed a normal DNA-RAG1 complex (Fig. 2, R1), but they failed in the formation of DNA-RAG1-RAG2 complex (R1/R2), suggesting that these RAG1 mutant proteins have defects in the interaction with RAG2 although they can normally recognize and bind to the signal sequences (Fig. 2). In addition, these mutant proteins failed to form the paired complex (Fig. 2, PC) with the second RSS DNA molecule. Other RAG1 mutant proteins behaved like a wild-type RAG1 in DNA-RAG1 complex formation as shown in Fig. 2.

A D546A RAG1 Mutant Protein Abolished the Hairpin Formation Activity Recovered by a Catalytic Mutant RAG1 Protein (D708C)—Mutation to alanine at Asp<sup>708</sup> of RAG1 (D708A), one of the catalytic residues, completely removed the catalytic activity of RAG1, but the transition of this aspartate residue to cysteine (D708C) recovered its catalytic activity somehow (24). We tested here how D546A RAG1 influenced the hairpin formation activity of D708C using pre-nicked oligonucleotide substrates. The D708C mutant RAG1 protein showed 10-fold more decrease in hairpin formation activity than wild-type RAG1 (Fig. 3, lanes 2–5). The hairpin formation activity of D546A was very similar to that of D708C at the same pre-nick substrate (Fig. 3, lanes 6–9), but a double mutation at D546A and D708C completely abolished this hairpin formation activity of RAG1 (Fig. 3, lanes 10–13). Two other double mutant proteins (D560A/D708C
and E547A/D708C) showed a similar result as the D546A/D708C mutant RAG1 protein (data not shown), suggesting that these acidic residues are crucial for the V(D)J recombination process although they might not directly participate in the catalytic reaction.

Mutant RAG1 Proteins Failed in the RAG2 Interaction by Co-immunoprecipitation in Vitro—To confirm further that these RAG1 acidic residues are involved in the interaction with RAG2, we employed co-immunoprecipitation using purified proteins. Two purified RAG1 (wild-type or mutants) and FLAG-tagged RAG2 were incubated, and then these RAG complexes were pulled down using anti-FLAG agarose beads. The bound RAG1 protein was detected by Western blot using anti-RAG1 antibody (Fig. 4 A). D546A, E547A, and D560A RAG1 proteins were defective in the interaction with RAG2 at this co-immunoprecipitation analysis, although some bound E547A and D560A proteins have been observed (Fig. 4 B, lanes 5, 6, and 8). Other RAG1 mutant proteins revealed a normal interaction with RAG2 in this assay (Fig. 4 B). This co-immunoprecipitation result suggests that the single point mutations at these acidic residues affect the binding ability of RAG1 to form the RAG complex that is required for the V(D)J recombination process.

V(D)J Recombination Activities of Mutant RAG1 Proteins at the KRFRY Region—Previously, the KRFRY region of mouse RAG1 has been suggested to be directly involved in binding with RAG2 during the V(D)J recombination process (30). All five individual amino acid residues of this region were mutated, and their roles in the interaction with RAG2 were determined. Mutations at three positive residues K555G, R556G, and R558G did not affect V(D)J cleavage activity. All three mutant RAG1 proteins showed very similar activity as wild-type RAG1 in both nick and hairpin formation (Fig. 5 A), suggesting that these basic amino acid residues are not involved in the interaction with RAG2. In addition, mutations at two aromatic residues Phe557 (F557G) and Tyr559 (Y559G) to glycine eradicated the binding and cleavage activity of RAG1 (Fig. 5 B, lanes 3 and 4; Fig. 5 C, lanes 4, 5, 8, 9). These F557G and Y559G mutant RAG1 proteins were very poorly expressed in bacterial cells before being subjected to protein purification.
in comparison with wild-type RAG1 (data not shown), so the non-detectable activity of these mutant proteins might be the result of the structural change of RAG1. In fact, changing these residues to other similar kinds of aromatic amino acids (F557Y and Y559F) did not affect binding and cleavage activities of RAG1 (Fig. 5B, lanes 5 and 6; Fig. 5C, lanes 6, 7, 10, 11). However, transition of these aromatic residues to another hydrophobic residue (leucine) still made proteins inactive in vitro (data not shown). The other possible role of these aromatic amino acid residues in the RAG2 interaction will be discussed later.

DISCUSSION

Two RAG proteins, RAG1 and RAG2, are essential to initiate the V(D)J recombination process that provides the diversity of immunoglobulin or T cell receptor repertoires during the lymphoid cell development. These two RAG proteins are expressed separately and form complexes in cells. RAG proteins form a synaptic complex with two signals (12- and 23-signals) and proceed with the V(D)J cleavage reaction. Once the RAG complex binds to RSS DNA, the RAG-DNA complex is very stable (16). Several RAG-DNA complexes have been observed by gel mobility shift analysis in vitro (6, 20, 21). RAG1 alone can weakly bind to RSS DNA as shown in Fig. 1. RAG1 and RAG2 proteins form a couple of complexes (synaptic complex 1 or 2) with RSS DNA containing either a single 12-signal or 23-signal sequence (21). Furthermore, these proteins tend to form the paired complex in the presence of both 12- and 23-signal sequences and HMG1 (17). RAG2 alone does not seem to bind to RSS DNA. However, RAG2 greatly enhanced RAG1 binding to the DNA molecule and the binding specificity of RAG proteins (16, 19). DNA footprinting analysis showed that DNA-contacting regions of RAG1 only and RAG1/RAG2 complex were very similar to each other (16). Therefore, in order to form these stable RAG-DNA complexes, the direct interaction between RAG1 and RAG2 is absolutely required. In fact, this interaction has been previously observed using immunoprecipitation or affinity pulldown analyses (26, 27).

Among several RAG mutants defective in the formation of RAG-DNA complex, a RAG1 mutant at Arg561 (R561H) that was screened from human Omenn syndrome patients caused a decrease of the in vitro DNA binding ability of RAG proteins due to the failure of RAG1-RAG2 interaction (30). However, the mutant protein at this arginine residue was able to carry on the normal V(D)J cleavage activity in our current study (Fig. 5A). Mutation at Arg561 (the same amino acid as Arg561 in human RAG1 to glycine (R558G) did not seem to affect nick or hairpin
formation in vitro. In addition, other near positively charged amino acid residues (Lys\(^{555}\) and Arg\(^{556}\)) within the \(^{556}\)KRFKY region did not play a key role in the V(D)J cleavage. In conclusion, all three basic amino acid residues at this region do not participate in RSS DNA binding and V(D)J cleavage in our assay conditions. However, removal of the other two aromatic residues (Phe\(^{557}\) and Tyr\(^{558}\)) at this region eliminated the V(D)J recombination activity, and replacement of other kinds of aromatic residues (phenylalanine to tyrosine or tryptophan to phenylalanine) recovered the normal activity of RAG1 in DNA binding and cleavage. Therefore, removal of these aromatic residues might cause alteration of the overall protein structure and lead to the defective activity of RAG1 in V(D)J recombination.

In fact, these two RAG1 mutant proteins (FS577G and Y559G) were little expressed in bacterial cells in comparison with wild-type RAG1 before protein purification, and the expression of the other two mutant proteins (FS577Y and Y559F) in bacterial cells was compatible to that of the wild-type protein. On the other hand, the hydrophobic phenyl rings of these aromatic acids might participate in intercalation with other hydrophobic pockets in RAG2 at the interface between RAG1 and RAG2. There is a previous study to support this possibility: the Trp\(^{317}\) RAG2 mutant protein failed in the interaction with RAG1 (26), implicating that this RAG2 residue can be involved in the hydrophobic interaction with some other RAG1 residues during the V(D)J recombination process.

RAG2 mutant proteins at basic residues such as R73A or R137A showed a severe defect in RAG1-RAG2 interaction, leading to impaired DNA cleavage in RAG-DNA complex formation (31). It has been suggested that these basic residues might locate at the interface and form ionic interactions with the negatively charged residues of RAG1. In this study, we found that three acidic residues (Asp\(^{546}\), Glu\(^{547}\), and Asp\(^{560}\)) of RAG1 were responsible for the interaction with RAG2 in the V(D)J recombination analysis (Fig. 2). These three acidic residues are highly conserved among several RAG1 proteins that were cloned from different species (25). A single mutation at these amino acid residues of RAG1 caused a great decrease of RAG-RSS DNA complex formation with RAG2 although mutant RAG1 protein alone can still form a normal RAG1-RSS DNA complex, suggesting that these residues are directly involved in the interaction with RAG2. In addition, immunoprecipitation analysis using purified proteins has shown that mutant RAG1 proteins at these three acidic residues were defective in direct binding to RAG2 (Fig. 4B). In addition, mutation at one of these amino acid residues completely removed the recovered hairpin formation activity by a mutant RAG1 at the catalytic residue (D708C) (Fig. 2), implicating this residue as important for RAG1 to achieve full catalysis although it might not be directly involved in the catalytic reaction. Many other RAG2 basic residues (Lys\(^{119}\), His\(^{140}\), Arg\(^{229}\), Lys\(^{282}\)) have also been implicated as having roles in the formation of RAG-DNA complex although it was not clear whether they were directly involved in RAG1 interaction (31, 33). Altogether, by forming a stable complex through ionic linkages with RAG2 some acidic residues in RAG1 are very crucial for V(D)J recombination activity, although further structural analyses using x-ray crystallography are necessary to make a solid conclusion about the interface between two RAG proteins.

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