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Definition of Minimal Domains of Interaction Within the Recombination- Activating Genes 1 and 2 Recombinase Complex

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During V(D)J recombination, recognition and cleavage of the recombination signal sequences (RSSs) requires the coordinated action of the recombination-activating genes 1 and 2 (RAG1/RAG2) recombinase complex. In this report, we use deletion mapping and site-directed mutagenesis to determine the minimal domains critical for interaction between RAG1 and RAG2. We define the active core of RAG2 required for RSS cleavage as aa 1–371 and demonstrate that the C-terminal 57 aa of this core provide a dominant surface for RAG1 interaction. This region corresponds to the last of six predicted kelch repeat motifs that have been proposed by sequence analysis to fold RAG2 into a six-bladed β-propeller structure. Residue W317 within this sixth repeat is shown to be critical for mediating contact with RAG1 and concurrently for stabilizing binding and directing cleavage of the RSS. We also show that zinc finger B (aa 727–750) of RAG1 provides a dominant interaction domain for recruiting RAG2. In all, the data support a model of RAG2 as a multimodular protein that utilizes one of its six faces for establishing productive contacts with RAG1. The Journal of Immunology, 2000, 164: 5826–5832.

The ability of the adaptive immune system to recognize and respond to innumerable foreign Ags is dependent upon clonotypic Ag receptors expressed on the surface of T and B lymphocytes. The vast Ag receptor repertoire is generated by the somatic assembly of composite gene segments in a process termed V(D)J recombination (reviewed in Refs. 1 and 2). Rearrangement of these gene segments is directed by highly conserved recombination signal sequences (RSSs) consisting of a heptamer (CA-GTG) and a nonamer (ACAAAAACC) motif separated by a 12- or 23-bp spacer. Efficient recombination occurs between two gene segments flanked by RSSs with different spacer lengths (3).

Recombination is initiated by two lymphoid-specific factors, recombination-activating genes 1 and 2 (RAG1 and RAG2) (4, 5). These genes are located 8 kb apart within the same genomic locus and were first identified by their capacity to activate rearrangement of an artificial substrate in a fibroblast cell line. Functional disruption of either gene by either homologous recombination in mice (6, 7) or by amino acid substitutions or truncations in humans with severe combined immunodeficiency or Omenn syndrome (8–10) has confirmed the fundamental role of RAGs in V(D)J recombination.

Numerous aspects of the recombination reaction are dependent upon the interplay between RAG1 and RAG2 during the rearrangement process. Although RAG1 has recently been shown to contain the catalytic residues of the recombinase and can alone bind the RSS in part through a helix-turn-helix motif homologous to the DNA binding domain of the Hin recombinase (11–15), such a complex cannot mediate DNA nicking (16–18). Upon recruitment of RAG2, contacts between the recombinase and the RSS are modified (13, 14, 19, 20), resulting in stabilization of the recombinase-RSS complex (21, 22) and bending of the RSS (23), with significant distortion of the heptamer coding flank border (19, 20).

The interaction of RAG2 with RAG1 likely triggers a conformational change in RAG1 that activates its hydrolytic machinery and targets nicking at the border of the RSS and the coding flank. The free hydroxyl group of the nicked strand is then used in a trans-esterification reaction by the RAG1/RAG2 complex to form a covalently sealed hairpin (24). The hydrolytic mechanism of the RAG1/RAG2 recombinase is again employed for resolving the hairpin intermediate (25, 26) and for processing numerous 3′ overhang structures (27). The minimal regions of both proteins that are sufficient for mediating recombination in vivo are referred to as the active cores and span residues 384-1008 in RAG1 (28) and residues 1–383 in RAG2 (24, 29).

Substantial evidence suggests that most steps of V(D)J recombination occur with the 12 and 23 RSSs juxtaposed in a tightly regulated synaptic complex (30–35). Formation and maintenance of this complex in which double strand breaks are generated requires both RAG1 and RAG2. Following cleavage, both RAGs are integral components of the postcleavage complex (33, 36). In addition, in a reaction parallelizing the mechanism used by transposases, the postcleavage complex of RAG1 and RAG2 can transpose cut signal ends into unrelated DNA via a nucleophile attack (37, 38).
The multifaceted capacities of the RAG1/RAG2 complex for directing DNA recognition before alternating hydrolysis, transsterification, and hydrolysis reactions within a regulated synaptic complex suggest a dynamic interface between RAG1 and RAG2. This interaction has previously been explored in a number of ways. The RAG proteins have been observed to colocalize in the periphery of the nucleus of thymocytes by indirect immunofluorescence and have been coprecipitated from thymocytes as well as from various cell lines transiently overexpressing the RAG proteins (39, 40). In addition, reconstitution experiments using purified RAG1 and RAG2 have demonstrated a direct interaction between the two proteins in the absence of DNA (22, 41). Mapping of the domains of interaction between RAG1 and RAG2 has been undertaken only in part, with aa 504–1008 of RAG1 shown to complex with RAG2 (aa 1–491) (40). RAG2, which has recently been suggested by sequence analysis to form a β-propeller-like structure composed of six keel repeat motifs (42, 43), has not been further subjected to deleterional mapping.

In this study, we report a detailed analysis of the dominant domains of interaction between the active cores of RAG1 and RAG2. We show that the predicted sixth kelch motif of RAG2 is largely responsible for mediating interaction with RAG1 and that mutation of amino acid W317 within this region abolishes complex formation between RAG1 and RAG2 with subsequent deleterious effects on RSS recognition and cleavage. Moreover, we show that zinc finger B (ZFB) at the C terminus of RAG1 is involved in recruitment of RAG2. In all, these data complement and extend our view of the importance of RAG1-RAG2 interactions for the activation of V(DJ) recombination and provide a view of RAG2 as a multi-modular adapter protein.

Materials and Methods

Recombinant plasmid constructs and mutagenesis

GST fusion constructs were generated by subcloning BamHI/NotI-digested PCR products of RAG1 and RAG2 into the eukaryotic expression vector pEBG, which is under the transcriptional regulation of the elongation factor 2 promoter (39). Amino acid substitutions within RAG2 were generated in pBluescript using the Bio-Rad (Richmond, CA) Phagemid kit and were transferred as KpnI/EcoRI fragments into pEBG2ΔC (11). All constructs were sequenced.

RAG1 and RAG2 interaction assays

Recombinant proteins were overexpressed in the human embryonal kidney fibroblast cell line HEK293T by calcium phosphate transfection (39). Cells were harvested 48 h posttransfection in PBS and subsequently were lysed in IP lysis buffer (25 mM Tris-HCl (pH 8.0); 250 mM NaCl; 1 mM MgCl₂; 0.5% Nonidet P-40; 5% glycerol; and 2 μg/ml of the aprotinin, leupeptin, and pepstatin protease inhibitors). Extracts were spun down for 10 min at 4°C, and the supernatants were incubated with 20 μl of pre-equilibrated GST beads for 2 h at 4°C with rocking. Beads were washed five times in IP-lysis buffer, resuspended in SDS gel-loading buffer (50 mM Tris-HCl (pH 6.8); 100 mM DTT; 2% SDS, 0.1% bromophenol blue, and 0.5 μM nontarget single-stranded DNA, 0.05 μg/μl BSA, 10% DMSO, 4% glycerol, and 0.5 mM MgCl₂ or MnCl₂ in a final volume of 20 μl. Reactions were stopped by the addition of 0.1% SDS and denaturing gel-loading buffer (98% deionized formamide, 10 mM EDTA (pH 8.0), 0.025% xylene cyanol FF, and 0.025% bromophenol blue) and were resolved on 16% polyacrylamide/6 M urea denaturing gels.

EMSAs

Conditions were essentially as previously described (21, 22). A total of 50 ng of RAG1 and RAG2 were incubated with 0.05 pmols of 3²P-labeled 12 RSS at 30°C for 10 min in 25 mM MOPS-KOH (pH 7.0), 5 mM Tris-HCl (pH 7.0), 30 mM KCl, 60 mM KOAc, 1.4 mM DTT, 0.5 μM nonspecific single-stranded DNA, 0.05 μg/μl BSA, 10% DMSO, 4% glycerol, and 0.5 mM MgCl₂ or MnCl₂ in a final volume of 10 μl. The complexes were then cross-linked by addition of 1 μl glutaraldehyde (final concentration, 0.1% v/v) and incubated for 10 min at 30°C. Complexes were resolved on 4% native polyacrylamide gels.

In vivo recombination assays

In vivo recombination assays were performed in HEK293T cells essentially as previously described (9, 23). HEK293T (or HEK293, NIH3T3) cells were cotransfected with the deletional recombination substrate pHB289 (5 μg) (44) and 6 μg of the expression vectors for GST-RAG1ΔN330 and the various GST-RAG2 mutants. Cells were harvested 48 h posttransfection, and DNA was isolated as described (5) and analyzed for recombination frequency by PCR analysis (20 cycles of 94°C for 30 s, 65°C for 60 s, and 74°C for 60 s). The linear range of the PCR assay was determined by serial dilutions of the rescued recombinated plasmid. A total of 0.2% of the recovered plasmid was used in all assays. Oligonucleotides detect the recombinated products by annealing to the joined signal ends and to the CAT gene present in pJH289 (oligos RA5 and RA14, respectively) (45, 46). As a loading control, a 154-bp fragment of the CAT gene was amplified (oligos RA1 and RA14) (23) under identical conditions (data not shown). Amplified products were visualized by autoradiography after electrophoresis on a 10% polyacrylamide gel.

Results and Discussion

Definition of the minimum RAG2 domain for RAG1 coprecipitation

To identify the region of RAG2 required for coprecipitation of RAG1, a panel of RAG2 deletion mutants was constructed as GST N-terminal fusions (Fig. 1A). The GST tag not only facilitates detection and purification of the recombinant proteins but also provides increased solubility and/or stability while leaving recombination activity of episcopal substrates essentially unaltered (39). The recombinant forms of GST-RAG2 were transiently overexpressed in the human kidney fibroblast cell line HEK293T, together with an N-terminal HA-tagged form of the RAG1 active core (HA-RAG1ΔN330–1040). GST-RAG2 proteins were purified from the cell extracts on glutathione-agarose beads, and co-precipitation of RAG1 was evaluated by Western blot analysis. Equivalent levels of RAG1 expression in each assay were confirmed by blotting total cellular extracts (data not shown). Both the full-length (aa 1–527) and active core (aa 1–388) forms of RAG2 were able to efficiently coprecipitate RAG1 (Fig. 1B, lanes 1 and 2), whereas GST alone did not associate with RAG1 (lane 7).

Oligonucleotide DNA cleavage substrates

The 12-RSS upper-strand oligonucleotide (22) was 5’ end-labeled with γ-³²P ATP using T4 polynucleotide kinase (NEB, Beverly, MA) as described by the manufacturer. The unlated lower-strand was then annealed by incubation at 75°C for 2 min before slow cooling to room temperature. The unincorporated nucleotides were removed by passage through a Sepharose spin column (G-50 or G-25; Boehringer Mannheim, Indianapolis, IN), and the double-stranded, radiolabeled substrates were then ethanol precipitated and resuspended at a concentration of 0.05 pmol/μl.
Deletion of aa 314–388 entirely abolished precipitation of RAG1 (lane 3) and, accordingly, further deletions of the RAG2 core were also unable to associate with RAG1 (lanes 4 and 5). These findings suggest that aa 314–388 are involved in mediating interaction with RAG1, and indeed a peptide spanning this region successfully precipitated RAG1, albeit at somewhat reduced levels (lane 6). To exclude the possibility that the RAG1-RAG2 association was mediated through nonspecific binding of contaminating genomic DNA, the above interactions were analyzed in the presence of ethidium bromide, which disrupts protein interactions mediated through DNA (47). In fact, RAG1/RAG2/RSS complexes can be entirely disrupted with 100 \( \mu \)g/ml ethidium bromide as determined by mobility shift analysis (data not shown). Because the presence of 400 \( \mu \)g/ml ethidium bromide did not alter the level of association between aa 314–388 of RAG2 and RAG1 (data not shown), we conclude that the interaction of RAG2 with RAG1 is not mediated through nonspecific DNA binding.

To further assess whether other predicted kelch repeats were capable of associating with RAG1, we generated fusions of the fourth and fifth repeats to GST and repeated the coprecipitation experiments described above. Although the active core (aa 1–388) of RAG2 and the sixth repeat were able to coprecipitate, the RAG1 active core (HA-RAG1\( ^{DN330} \)–1040) (Fig. 1C, lanes 2 and 5) repeats 4 (aa 205–253) and 5 (aa 254–305) were unable to efficiently precipitate RAG1 (Fig. 1C, lanes 3 and 4). The current data suggest that the predicted sixth kelch repeat of RAG2 provides a dominant interface of interaction with RAG1. However, the effectiveness of such an experiment is dependent upon the selection of appropriate borders for each of the repeats. Hence, we cannot entirely exclude the possibility that RAG2 may in part use one of the other repeats for making contacts with RAG1.

To further define the minimum RAG2 domain required for coprecipitation of RAG1, a series of GST fusion peptides spanning the interaction site was constructed. Only the peptides encompassing aa 314–388 and 314–371 (Fig. 1D, lanes 2 and 3) were able to coprecipitate RAG1, indicating that the minimum domain of interaction is between aa 314 and 371. Recently, sequence analysis has revealed that RAG2 possesses a six-fold symmetrical structure...
Table I. A summary of the properties of RAG2 mutant proteins

| GST-RAG2          | RAG1 Co-P | In vitro Cleavage | In vivo Recombination |
|-------------------|-----------|-------------------|-----------------------|
| Full length       | +         | +                 | +                     |
| 2ΔC388            | +         | +                 | +                     |
| 2ΔC371            | +         | +                 | +                     |
| 2ΔC313            | –         | –                 | –                     |
| 314–388           | +         | –                 | –                     |
| 2ΔC388 W317Y      | –         | +                 | +                     |
| 2ΔC388 F318Y      | –         | +                 | +                     |
| 2ΔC388 WF317/8YY  | –         | +                 | +                     |

* RAG1 coprecipitation assays (Co-P) as described in Figs. 1, 3, and 4. In vitro cleavage assays are as described in Fig. 2B. In vivo recombination assays: the expression vectors of the indicated proteins were cotransfected with the exogenous recombination substrate pH289 in 293T cells. A fragment of the recombined plasmids was detected by a semiquantitative PCR assay, as described in Materials and Methods and Ref. 23.

FIGURE 2. Interaction of RAG2 and RAG1 is essential for RSS binding and cleavage. A, Interaction of RAG2 with RAG1 is required for efficient RSS binding. EMSAs: 50 ng of purified GST-RAG1ΔN380 (active core) and 50 ng of purified deletional or mutational forms of GST-RAG2 were incubated with 0.05 pmols of a 12-RSS oligonucleotide substrate 5’-end-labeled on the upper strand. Complexes were analyzed on a 4% native polyacrylamide gel and visualized by autoradiography. B, Interaction of RAG2 and RAG1 is essential for RSS cleavage. In vitro cleavage assays were performed using 0.05 pmols of a 12 RSS end-labeled on the 5’ end of the upper strand in 0.5 mM of either Mg2+ or Mn2+. The substrates were incubated with 100 ng of purified GST-RAG1ΔN380, and equimolar amounts of purified deletional or mutational forms of GST-RAG2 as described in the Materials and Methods. Products (N, nick; HP, hairpin) were analyzed on a 16% polyacrylamide/6 M urea denaturing gel.

with each repeat composed of a kelch motif (42, 43). Intriguingly, aa 314–371 coincide with the sixth kelch motif. Crystallographic analysis has revealed that the ~50-aa kelch motif acquires a fold consisting of four β strands, which is reminiscent of the superbarrel fold observed in the proteins of the sialidase family (48, 49). Kelch motifs have been observed in numerous proteins, including galactose oxidase from Dactylium dendroides (48), the α- and β-scrin proteins from Limulus polyphemus (50), the kel1p protein involved in cell fusion and morphology in Saccharomyces cerevisiae (51), the kelch protein of Drosophila that is involved in cytoplasmic transport from nurse cells to oocytes (52), and the mouse IAP-promoted placental (MIPP) protein (53). Our finding that a single kelch motif can mediate protein-protein interactions between RAG2 and RAG1 support the model that RAG2 is indeed composed of six discrete repeats formed from four antiparallel β strands. Predominant contact with RAG1 through the C-terminal kelch motif suggests that the N-terminal five repeats may be available to establish interactions with other proteins involved in the V(D)J recombination reaction. Because kelch associates with actin in the ring canals in Drosophila egg chambers (54) and α- and β-scrin are actin-bundling proteins (55), it is exciting to speculate that RAG2 may localize the recombinase to the nuclear periphery by binding structural components of the nuclear matrix through one of its kelch motifs. A capacity to form discrete contacts through individual blades of a putative β-propeller would imply that RAG2 may function as a multimodular adapter protein involved in coordinating macromolecular assemblies during the recombination process.

In previous studies, mutational and deletional analyses have demonstrated that aa 1–383 of RAG2 are essential for RAG1/RAG2-mediated recombination of episomal substrates in transiently transfected cells (29, 56). Because aa 372–388 are dispensable for interaction between RAG2 and RAG1, we next examined whether they are also nonessential for RAG1/RAG2-mediated RSS binding and cleavage. A GST fusion of the RAG2 active core spanning aa 1–371 (RAG2ΔC371), as expected, was able to efficiently precipitate RAG1 (Table I). Accordingly, along with RAG1, RAG2ΔC371 displayed comparable activity to RAG2ΔC388 for complex formation on the 12 RSS (Fig. 2A, lanes 3 and 7), for 12 RSS cleavage (Fig. 2B, lanes 3, 7, 13, and 17), and for in vivo recombination of an exogenous substrate (Table I). Thus, we conclude that the minimal active core of RAG2 required for both in vitro and in vivo activity spans aa 1–371. Deletion of the region corresponding to the predicted sixth kelch motif (RAG2ΔC313), which abolishes formation of the RAG1/RAG2 complex, abolished entirely both 12 RSS binding (Fig. 2A, lane 8) and cleavage activity (Fig. 2B, lanes 8 and 18), thus demonstrating the importance of this region for establishing a productive DNA binding and cleavage complex. However, although the putative sixth kelch domain alone could coprecipitate RAG1, it could not activate the DNA recognition (Fig. 2A, lane 9) and hydrolytic mechanisms (Fig. 2B, lanes 9 and 19) of the recombinase, indicating that the first five kelch motifs are also critical for the activity of the RAG1/RAG2 complex. In all, the data demonstrate that although the sixth kelch repeat is capable of interacting with RAG1, it is not sufficient for activating the catalytic capacity of RAG1 to initiate the first steps of the recombination reaction.
Tryptophan 317 of RAG2 is critical for establishing contact with RAG1

To identify amino acid residues within the proposed sixth kelch motif of RAG2 that are potentially critical for mediating interaction with RAG1, we compared the sequences of all known RAG2 molecules from various species. Interestingly, limited sequence conservation is observed in this region (Fig. 3A). W317 and F318 are among the most conserved residues. We explored the role of these two residues in RAG1/RAG2 complex formation by introducing the conservative amino acid substitutions WF317/8YY. After coexpression in 293T cells, RAG2ΔC(WF317/8YY) was unable to coprecipitate RAG1 (Fig. 3B, lane 4). Single amino acid mutations RAG2ΔC(W317Y) and RAG2ΔC(F318Y) revealed that W317 is essential for coprecipitation of RAG1, whereas mutation F318Y did not interfere with RAG1 interaction (Fig. 3B, lanes 2 and 3). Functional analysis of W317Y demonstrated that disruption of RAG1 interaction concurrently abolished 12 RSS binding (Fig. 2A, lane 4) and cleavage (Fig. 2B, lanes 4 and 14) as well as the capacity for in vivo recombination (Table I). On the other hand, F318Y, which was capable of interacting with RAG1, also permitted complex formation on the 12 RSS (Fig. 2B, lane 5), efficient 12 RSS cleavage (Fig. 2B, lanes 5 and 15), and in vivo recombination (Table I).

Complete disruption of RAG1/RAG2 complex formation by the W317Y substitution prompts at least two potential explanations. One possibility is that the tryptophan might directly participate at the interaction interface to stabilize contacts between RAG1 and RAG2. Interestingly, such a role for tryptophans has been observed in the crystal structures of the bacteriophage Mu transposase, where W279 and F280 mediate subdomain interactions (57), and of N-adherins, where the dimer interface is coordinated by intercalation of the W2 side chain into the hydrophobic pocket of a partner molecule. (58). Alternatively, because tryptophans can serve as integral structural components within the architecture of a protein, W317Y might unstructure RAG2. This possibility appears unlikely because W317Y did not modify the nuclear localization pattern of RAG2 (data not shown).

**RAG1 ZFB is involved in RAG1-RAG2 DNA-independent interaction**

Having established a predominant RAG2 domain involved in the interaction with RAG1, we sought to further define the regions of RAG1 required for coprecipitation of RAG2. McMahan et al. (40) have previously identified a large region of RAG1 spanning aa 504-1008 that is able to precipitate RAG2 after overexpression in COS cells. This region has been shown to contain a single C2H2 zinc binding domain (ZFB, aa 727–750), which possesses weak and apparently nonspecific DNA binding capacity (59). The N terminus of RAG1, which is dispensable for in vivo recombination, contains two zinc binding domains (a ring finger and a zinc finger A [ZFA]), which together form a highly specific dimerization interface between RAG1 molecules (59, 60). Due to the ability of the N-terminal zinc binding domains to coordinate protein interactions, we tested the role of ZFB in mediating RAG1 and RAG2 interaction.

A series of GST-fused RAG1 deletion mutants was generated (Fig. 4A) and coexpressed with an HA-tagged form of the RAG2 active core (HARAG2ΔC388) in HEK293T cells. Complexes were purified on glutathione beads, and coprecipitation of RAG2 was detected by Western blot analysis. In agreement with McMahan et al. (40), the C terminus of RAG1 (aa 500-1040) was able to coprecipitate RAG2 (Fig. 4B, lane 5). Deletion of aa 758-1040 (aa 500–758), which left ZFB intact, had no effect on the interaction with RAG2 (Fig. 4B, lane 7, and C, lane 3), whereas a further deletion of aa 726-1040, which completely removes ZFB, abolished the interaction (Fig. 4B, lane 6). Moreover, a peptide encompassing ZFB (aa 692–758) was still able to coprecipitate RAG2 (Fig. 4C, lane 5), whereas a peptide spanning the N-terminal C2H2 zinc-binding motif ZFA (aa 330–389) and GST alone failed to interact with RAG2 (Fig. 4C, lanes 4 and 6). Because the interaction between ZFB and RAG2 was unaltered by the introduction of 400 μg/ml ethidium bromide (Fig. 4C, lane 12), we conclude that the recruitment of RAG2 by ZFB of RAG1 is not mediated through nonspecific DNA binding. Equal levels of RAG2 expression in all of the assays presented in Fig. 4 were evaluated by blotting crude extracts (data not shown). Although ZFB represents a predominant interface of interaction, our data do not permit us to exclude the possibility that RAG1 and RAG2 interactions are established through other domains present in RAG1. In addition, attempts to determine whether the sixth kelch motif of RAG2 directly interacts with ZFB of RAG1 were hampered by the poor solubility of both short peptides when expressed as HA-tagged forms (data not shown).
The capacity of RAG2 to interact with the nonspecific DNA binding component ZFB suggests a model in which RAG2 promotes spacer and heptamer occupancy by binding to and reorienting ZFB (13, 14, 20). In the absence of RAG2, ZFB and the catalytically critical residues of RAG1 might be configured on the RSS in an inactive conformation. Complex formation between RAG2 and ZFB could redirect ZFB out toward the heptamer where the zinc finger would then stabilize the RAG1/RAG2 ternary complex through extensive interactions with both RAG2 and with conserved and nonconserved motifs within the RSS, thereby permitting an optimal state for DNA nicking and transesterification.

In this study we present minimal domains in RAG1 and RAG2 required for efficient complex formation. Whether these domains are central for all the steps of RSS recognition, synapsis, and cleavage or whether different interfaces of interaction are utilized for each step of the reaction remains to be addressed. Nonetheless, the deletional mapping of the interaction domains between RAG1 and RAG2 presented in this study provides an important first step toward an understanding of the intricate contacts required to form a RAG1/RAG2 complex that is active for RSS binding and cleavage.

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