Role of RAG1 autoubiquitination in V(D)J recombination

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The variable domains of Ig and T-cell receptor genes in vertebrates are assembled from gene fragments by the V(D)J recombination process. The RAG1–RAG2 recombinase (RAG1/2) initiates this recombination by cutting DNA at the borders of recombination signal sequences (RSS) and their neighboring gene segments. The RAG1 protein is also known to contain a ubiquitin E3 ligase activity, located in an N-terminal region that is not strictly required for the basic recombination reaction but helps to regulate recombination. The isolated E3 ligase domain was earlier shown to ubiquitinate one site in a neighboring RAG1 sequence. Here we show that autoubiquitination of full-length RAG1 at this specific residue (K233) results in a large increase of DNA cleavage by RAG1/2. A mutational block of the ubiquitination site abolishes this effect and inhibits recombination of a test substrate in mouse cells. Thus, ubiquitination of RAG1, which can be promoted by RAG1's own ubiquitin ligase activity, plays a significant role in governing the level of V(D)J recombination activity.

ubiquitin | immunoglobulin | diversification

V(D)J recombination plays a central role in the production of antigen receptors by recombining V, D, and J gene segments from their genomic clusters to give rise to the highly varied populations of immunoglobulins and T-cell receptors (1). Recombination starts with the introduction of double-strand breaks by the RAG1/RAG2 protein complex at a pair of recombination signal sequences (RSS) (2, 3), distinguished by the length of the spacer DNA separating their conserved heptamer and nonamer elements. Recombination requires one RSS with a 12-base pair spacer and another with a 23-base pair spacer. Each pair of breaks is then processed by the nonhomologous DNA end-joining group of proteins to produce a junction of two segments of coding sequence (a coding joint) and a junction of the two RSSs (a signal joint) (4). The purified RAG1/2 protein complex displays the correct specificity for pairs of RSSs (5, 6), and has thus been used as a model for the initiation of V(D)J recombination. Until recently, the RAG proteins used for these studies have generally been minimal "core" regions of RAG1 and RAG2 (amino acids 384–1,008 of 1,040 in mouse RAG1 and 1–387 of 527 in RAG2), which are sufficient for specific binding and cleavage activity in a purified cell-free system. Ectopic expression of these truncated proteins supports V(D)J recombination in suitable cell lines, although with differences from the full-length proteins that will be discussed here.

A complex composed of core RAG1 and RAG2 is more active than its full-length counterpart in cleavage of extrachromosomal substrates in a hamster cell line, but overall recombination is reported to be lower (7), indicating a defect in the stages of recombination subsequent to DNA cleavage. Similarly, mice or pre-B cells missing the RAG2 C-terminal noncore region are defective in the V to DJ recombination step of Ig heavy chain joining, although the earlier D to J joining step is normal (8). The mice also display an increased prevalence of lymphomas (9). A plant homeo domain (PHD) within the RAG2 C terminus is known to bind to chromatin, and specifically to histone 3 trimethylated on lysine 4 (H3K4me3), which is presumably an important step in directing RAG1/2 to loci bearing this "activating" modification (10). The lack of this domain may largely explain the defective functions of the RAG2 core protein. Similarly, although core RAG1 can support D to J rearrangement at the Ig heavy chain locus in $RAG1^{-/-}$ pro-B cells, the level is reduced compared with that of full-length RAG1 (FLRAG1) (11), and deletions of certain smaller regions within the RAG1 N terminus have even greater effects (11). Some naturally occurring truncations of the RAG1 N terminus lead to human immunodeficiency (12). The functions of the parts of RAG1 and RAG2 outside of the catalytically essential cores have been reviewed (13). There is also evidence that the RAG1 and RAG2 C termini interact: DNA cleavage by RAG1/2 combinations containing both regions was greatly reduced but was restored upon addition of an H3K4me3-containing peptide (14). Relief of this autoinhibition may synergize with the chromatin-binding effect of the PHD domain to target recombination to the appropriate loci.

The significant modulation of recombination in cells, and/or of DNA cleavage in vitro, by these "dispensable" regions of both RAG1 and RAG2 is further modified by covalent modifications of the proteins, which affect their stability or activity. RAG2 becomes phosphorylated at a specific site in its C terminus (T490) at the G1/S stage of the cell cycle, and is then ubiquitinated by the Skp2-SCF ubiquitin ligase, a central regulator of cell cycle progression, leading to its degradation in S phase (15, 16). Phosphorylation of RAG1 at residue S528 by the AMP-dependent protein kinase has also been described (17), in this case leading to increased activity of RAG1/2 both for cell-free DNA cleavage and for recombination in cells.

The N terminus of RAG1 contains a Zn-binding motif (amino acids 264–389) that includes a C_3HC_4 RING (really interesting new gene) finger motif closely associated with an adjacent C_2H_2 Zn finger. This domain was shown to have ubiquitin ligase (E3) activity (18, 19), a common feature of RING finger domains,

Significance

The immune system of vertebrates has to respond to a great variety of infectious agents. Its power to meet these challenges relies on the assembly of a diverse set of genes for antibodies and T-cell receptors from large arrays of gene segments. This genomic reshuffling, known as V(D)J recombination, is initiated by the RAG1 and RAG2 proteins working together to cut DNA at specific sites. In addition to its role in DNA cleavage, RAG1 is known to contain a ubiquitin ligase activity that attaches ubiquitin to itself, as well as other proteins. Here we show that ubiquitination of RAG1, which can be promoted by RAG1's own ubiquitin ligase activity, is a powerful activator of the DNA cutting and recombination activity of RAG1/RAG2.

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The authors declare no conflict of interest.

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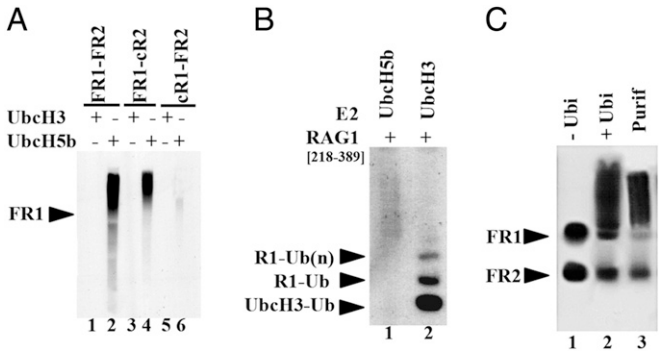

Fig. 1. FLRAG1 can be autoubiquitinated in the presence of a matching E2 enzyme. (*A*) Western blot with anti-ubiquitin antibody after ubiquitination of RAG1/2 complex (performed as described in *Methods*). Either UbcH3 (lanes 1, 3, and 5) or UbcH5b (lanes 2, 4 and 6) was used as the E2 enzyme. The arrow points to the position of native FLRAG1. Here and in later figures, FLRAG1 and FLRAG2 are designated FR1 and FR2, and core RAG1 and core RAG2 are designated cR1 and cR2. (*B*) Ubiquitination assay of an N-terminal fragment of RAG1 (aa 218–389) with either UbcH5 or UbcH3. (*C*) Purification of ubiquitin-bound RAG1. A Western blot with anti-MBP antibody is shown. Lane 1, FLRAG1 and FLRAG2; lane 2, the same after ubiquitination; lane 3, ubiquitinated FLRAG1/2 purified on a sizing column (Fig. S1).

when combined with ubiquitin, the ubiquitin-activating (E1) enzyme, and an appropriate ubiquitin-conjugating (E2) enzyme. A naturally occurring human mutation in this RING finger motif (C328Y) was found to cause the primary immunodeficiency disease Omenn's syndrome (20). A study of the equivalent mutation in mouse RAG1 (C325Y) showed that it greatly reduced recombination of an extrachromosomal plasmid, as did mutation of the neighboring residue (P326G) (21). Other RING finger residues critical for ubiquitin ligase activity appeared to contribute to robust recombination of extrachromosomal substrates (22). In biochemical experiments carried out with an N-terminal fragment of RAG1 (residues 218–389), the principal site of autoubiquitination was found to be a residue neighboring the RING finger, K233; mutation of this residue (K233M) essentially abolished autoubiquitination of the fragment (18).

In this article, we assess the site or sites and extent of autoubiquitination of RAG1, the consequences of this modification for RAG1/RAG2 activity in a cell-free system and in cells, and the functional relationship between this modification and the histone-recognizing PHD domain of RAG2. We prepare FLRAG1 in complex with either full-length RAG2 (FLRAG2) or core RAG2 and find that FLRAG1 undergoes autoubiquitination specifically at K233. The ubiquitination of RAG1 protein enhances coupled cleavage by the RAG1/RAG2 complex of a 12/23 RSS pair by about fivefold. RAG1 autoubiquitination also appears to be important for supporting V(D)J recombination in cells.

Results

Polyubiquitination of Full-Length RAG1/2 Protein Complex. Full-length or core versions of RAG1 and RAG2 were cotransfected and purified from HEK293 mammalian cells, as described in *Methods* (purified proteins are shown in Fig. S1). The complex of FLRAG1 and FLRAG2 (FLRAG1/2) was soluble, and the yield was comparable to that of the pair of core proteins. As was found earlier for an isolated N-terminal fragment of RAG1 (aa 218–389) (18), FLRAG1 as part of the FLRAG1/2 complex-supported autoubiquitination. However, the E2 enzyme required for this reaction was different for the full-length protein. In the previous report, autoubiquitination of RAG1[218–389] was catalyzed mainly by UbcH3/Cdc34, and not by UbcH5a/b (18), a result confirmed here (Fig. 1*B*). In contrast, we found that FLRAG1 underwent ubiquitination in the presence of UbcH5a/b, but that UbcH3/Cdc34 was relatively inactive (Fig. 1*A*).

Ubiquitination of FLRAG1 protein in the cell-free system yielded, at most, 30% modified protein. To quantitate the full effects of ubiquitination, we performed gel filtration to separate the ubiquitinated FLRAG1/2 species from the unmodified species (Fig. S2, peak 1). This proved to be the most effective strategy, as various attempts at affinity-based purification failed because of protein inactivation. A Western blot of the peak fraction probed with antiubiquitin antibody (Fig. 1*C*) confirmed that essentially all of RAG1 in the complex purified by gel filtration was polyubiquitinated.

Ubiquitination of RAG1 Enhances Cleavage of 12/23RSS DNA. We were interested in assessing the effects of ubiquitination on the activities of RAG1/2. Earlier work has identified two DNA cleavage reactions catalyzed by RAG1/2 based on the divalent metal cation used to support the reaction. In the presence of Mg^{2+}, cleavage requires binding of both a 12RSS and a 23RSS in a synaptic complex and results in cutting of both DNAs at the borders between RSSs and flanking DNA, a reaction known as coupled cleavage. Under less restrictive conditions in the presence of Mn^{++}, a single 12RSS or 23RSS can be cut without the formation of a 12/23 synaptic complex.

A previous study demonstrated an inhibitory interaction between the C terminus of RAG2 and a noncore portion of RAG1 that reduced coupled cleavage of a pair of RSS-containing DNA

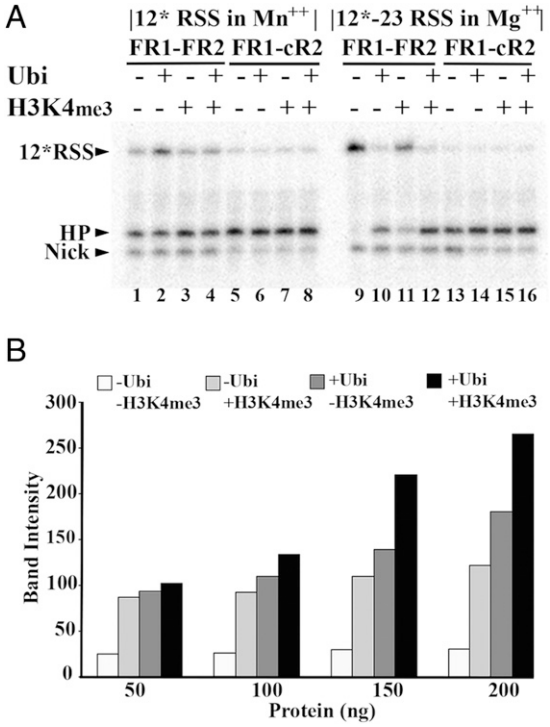

Fig. 2. Effect of FLRAG1 ubiquitination on single and coupled RSS cleavage. (*A*) Purified RAG proteins were used before or after ubiquitination, for either cleavage of a single 12-RSS DNA (in Mn^{++}, lanes 1–8) or coupled cleavage of a pair of 12/23-RSSs (in Mg^{2+}, lanes 9–16) to show the relative increase in activity. Reactions with or without an H3K4Me3 peptide (histone 3 trimethylated at lysine 4) were also included for comparison of relative activity, as shown in the gel. Positions of labeled 12-RSS substrate, nicked, and hairpin products are shown by arrows. (*B*) Quantitation of coupled RSS cleavage (hairpin formation). Reactions were performed as in *A*, with four different amounts (in nanograms) of FLRAG1/2 complex. Intensity of the hairpin band in gels is shown.

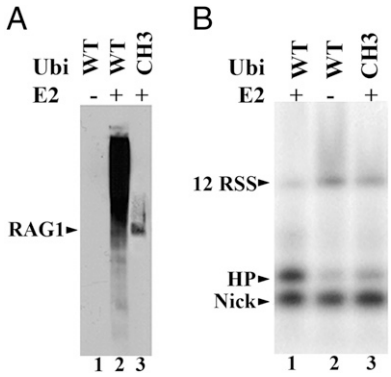

Fig. 3. Poly- versus monoubiquitination of RAG1. (*A*) Ubiquitination of full-length RAG1/2 complex by either wild-type ubiquitin (lanes 1 and 2) or methyl-ubiquitin (lane 3, CH3-ubi) to produce poly- or monoubiquitinated RAG1. A Western blot probed with anti-ubiquitin antibody is shown. (*B*) Full-length RAG1/2 complex was ubiquitinated as described, either with wild-type ubiquitin (lanes 1 and 2) or methyl-ubiquitin (lane 3). The ubiquitinated protein was then used for coupled 12/23 RSS cleavage.

oligonucleotides (14). We were able to recapture this result in our system, using both full-length proteins. Hairpin formation supported by FLRAG1/FLRAG2 was low, but was restored by replacement of FLRAG2 with core RAG2 (Fig. 2, compare lanes 9 and 13). RAG1 ubiquitination relieved this inhibitory effect, restoring coupled cleavage to a level nearly equivalent to that supported by FLRAG1/coreRAG2 (Fig. 2, lane 10). The inhibition and its relief depended on the presence of the RAG2 C-terminal region (aa 388–527). If the complex contained core RAG2 in place of the full-length protein, cleavage was high and essentially unaffected by RAG1 ubiquitination (Fig. 2, compare lanes 13 and 14). Also, as reported previously, there was no autoinhibition or relief when cleavage of a single RSS-containing DNA was carried out in Mn^{++} (Fig. 2, lanes 1, 3, 5, and 7). Only the more demanding conditions of coupled RSS cleavage in Mg^{2+} revealed these effects.

These results are comparable with the reversal of autoinhibition seen when a histone H3 peptide modified by trimethylation at lysine 4 (H3K4me3) is bound to the PHD domain of RAG2 (14). As confirmed here, the effect was only observed with coupled cleavage, and single-site cleavage in Mn^{++} was essentially insensitive to the modification. The effects of ubiquitination and of adding the H3K4me3 peptide were to some extent synergistic (Fig. 2, compare lanes 10–12), so that the combination restored coupled cleavage to the level seen on removal of the RAG2 C terminus.

Quantitation of the stimulation of coupled cleavage by RAG1 ubiquitination, H3K4me3 peptide binding to RAG2, or both combined was performed as described in *Methods* (Fig. 2*B*; a representative gel is shown in Fig. S3). Coupled cleavage was increased about fivefold by RAG1 ubiquitination, and slightly less by H3K4me3 peptide binding. The combined effect of both alterations was somewhat greater than either one alone.

Polyubiquitination at RAG1 K233 Is Required to Activate RAG1/RAG2. To address whether increased DNA cleavage requires mono- or polyubiquitination of RAG1, we blocked polyubiquitination by methylating all lysines in ubiquitin to generate monoubiquitinated RAG1 (Fig. 3*A*). Monoubiquitination of RAG1 did not relieve the inhibition of DNA cleavage caused by the noncore region of RAG2 (Fig. 3*B*). To address whether polymerization of ubiquitin (Ub) occurs on specific lysine residues, we systematically mutated each of six lysines in Ub to arginine and found that that every one of these Ub mutants was competent to support RAG1 polyubiquitination, indicating that no specificity in linkage of the ubiquitin chains is required (Fig. 4*A*). Only the combined mutation of three commonly used lysines (K29/K48/K63) caused a major loss of Ub incorporation. Correspondingly, inhibition of DNA cleavage was relieved by ubiquitination with any of the single mutants, but the triple K29/K48/K63 Ub mutant abolished the effect (Fig. 4*B*). Whether the Ub chains linked to RAG1 normally have multiple types of linkage (are heterotypic) will require further investigation. Such chains have been described, and in some known cases they have defined functional roles (see ref. 23 for review).

In the earlier study of an N-terminal fragment of RAG1 (18), K233 was identified as the principal site of autoubiquitination. This was also true for FLRAG1/2. In the full-length protein complex, mutations of this residue (K233M or K233R) essentially abolished ubiquitination of RAG1 (Fig. 5*A*). Interestingly, K233M and K233R mutations had distinctly different effects on DNA cleavage (Fig. 5 *B* and *C*). In the full-length protein complex, K233M caused a mild decrease in single-site cleavage (in Mn^{++}; Fig. 5*B*, lane 2), but abolished coupled cleavage (Fig. 5*B*, lane 4). This inhibition was largely reversed by removal of the RAG2 C terminus (Fig. 5*B*, lane 6). In contrast, the K233R mutation, which preserves the positive charge on K233, did not inhibit cleavage, either single-site or coupled (Fig. 5*C*).

Recombination Defect of RAG1 K233M and K233R. To discern the effects of blocking the RAG1 K233 ubiquitination site on recombination in intact cells, we carried out assays for V(D)J recombination with plasmid substrates that generate either signal joints (pJH200) or coding joints (pJH290). We expressed RAG2 (FL or core) together with FLRAG1 (wild-type or carrying the K233M or K233R mutation) in the murine 3TGR fibroblast cell line. Recombination producing either signal joints or coding joints was reduced at least tenfold by these mutations, whether or not RAG2 contained its C-terminal region (Table 1). With K233M, the defect in coding joint recombination was considerably greater. We infer that the loss in activity was a result of the inability of RAG1 K233M and K233R to undergo ubiquitination, particularly in the case of K233R, which does not show a defect in cell-free DNA cleavage beyond that expected for a mutant that cannot undergo ubiquitination.

Discussion

Ubiquitination of RAG1 appears to have an important regulatory function in relieving the inhibitory effect of the RAG2 C-terminal region on coupled cleavage. Earlier work demonstrated

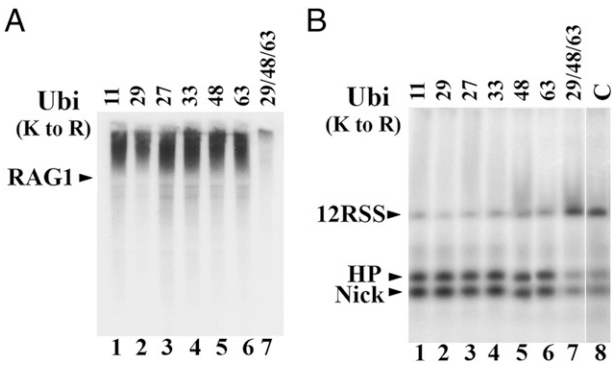

Fig. 4. Multiple lysine residues of ubiquitin can be involved in polyubiquitin chain formation on RAG1. (*A*) Ubiquitination of FR1-FR2 was carried out with lysine to arginine mutants of ubiquitin. Only the triple mutation of Ub (K29R/K48R/K63R) diminished ubiquitination of RAG1 (lane 7). (*B*) The FR1/FR2 proteins treated with mutant Ub were tested in the coupled 12/23 RSS cleavage assay. "C" (lane 8) is the control lane of unmodified FR1/FR2 complex without ubiquitination.

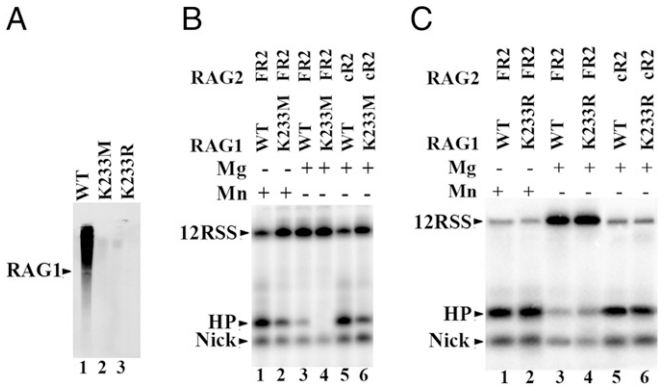

Fig. 5. RAG1 K233M and K233R mutants have different effects on RSS cleavage. (*A*) Only wild-type RAG1 (in the full-length RAG1/RAG2 complex) can be ubiquitinated (lane 1), the K233M and K233R RAG1 mutants cannot (lane 2 and 3). (*B* and *C*) Effect of FLRAG1 K233M (*B*) or K233R mutation (*C*) on single RSS cleavage (in Mn^{++}) or coupled cleavage of a 12/23 RSS pair (in Mg^{2+}). WT represents wild-type FLRAG1, and either FLRAG2 (FR2) or core RAG2 (cR2) was used as indicated. Cleavage reactions were carried out as described in *Methods*.

that the ubiquitination activity of RAG1 was essential to promote robust V(D)J recombination under conditions in which RAG1 was limiting (21), but did not identify the critical targets of ubiquitination. Our present results show that at least one major effect may involve the autoubiquitination of RAG1 at K233. By purifying active FLRAG1 and FLRAG2 proteins, we demonstrate significant effects of the autoubiquitination of RAG1 on cleavage of DNA in a cell-free system, and that the site of autoubiquitination is required for efficient V(D)J recombination. Ubiquitinated FLRAG1, when combined with FLRAG2, cleaves a 12/23 pair of RSSs much more efficiently than the unmodified protein. As the results in Figs. 3 and 4 show, this stimulation depends on polyubiquitination, but not on any one type of ubiquitin linkage. In many ways, RAG1 ubiquitination stimulates cleavage activity similarly to the binding of a histone H3K4Me3 peptide to the RAG2 PHD domain. In both cases, the bound moieties relieve the self-inhibitory interaction of the RAG1 noncore portions with the RAG2 C terminus. This may explain why the combined effect of binding both polyubiquitin and the H3K4Me3 peptide is somewhat greater than either one alone. In addition to the inhibitory interaction of the RAG1 and RAG2 C-termini previously described (14), a region within the RAG2 C terminus (aa 388–405) has recently been shown to be engaged in an autoinhibitory interaction with the RAG2 PHD domain, also relieved by its interaction with H3K4Me3 (24).

We further mutated the principal autoubiquitination site on RAG1 (K233) and tested the effects on the activity of purified RAG1/2 and on recombination in mouse cells. The K233M mutation had more severe effects than simply blocking ubiquitination of RAG1, as it greatly inhibited coupled cleavage in the purified system. Nevertheless, the K233M protein appeared to be correctly folded, because it still cleaved a single RSS (in Mn^{++}) almost as well as the normal protein (Fig. 5*B*). Mutation of the same site to arginine (K233R), retaining the positive charge of K233 while still blocking ubiquitination, had a much milder effect on coupled cleavage. Because plasmid-based V(D)J recombination in mouse cells with FLRAG1/2 was greatly inhibited by either the K233M or K233R mutation, both for signal joints and coding joints, stimulation of coupled cleavage by ubiquitination could well be involved in normal recombination. However, this cannot be the whole explanation, because recombination with FLRAG1-core RAG2, where the autoinhibition of cleavage should be absent, was still inhibited by both mutations. It thus appears likely that autoubiquitination of RAG1 is also involved in a late (postcleavage) step in the recombination pathway, separate from the effects of ubiquitination on the primary cleavage step. The effect evidently applies to formation of both signal joints and coding joints.

In view of the ability of the RAG1 E3 ligase to also ubiquitinate other targets such as karyopherin alpha 1 (25), histone H3 (5), and modified histone H3.3 (26), with accompanying effects on V(D)J recombination, the multiple roles of this activity of the RAG1 protein will need further investigation.

Methods

Proteins and DNA. Expression vectors for mouse FLRAG1 (1–1,040) and FLRAG2 (1–527) fused to maltose binding protein (MBP) were previously described (14), as were vectors containing mouse core RAG1 (aa 384–1008, cRAG1) and core RAG2 (aa 1–387, core RAG2). A plasmid expressing mouse RAG1 aa 218–389 was used as described (18). For in vivo recombination, FLRAG1 and FLRAG2 expression vectors pJH548 and pJH549, respectively, were used as described (27). The K233M and K233R mutations in FLRAG1 (including the pJH548 vector) were introduced by using the Quickchange mutagenesis kit (Stratagene). For protein preparations, HEK293GNT1 cells were grown in shaken suspension cultures in Freestyle 293 Expression Medium (Life Technologies, Inc) with 1% FBS. The RAG1 and RAG2 expression vectors were transiently cotransfected using polyethylenimine (25 kDa linear; Polyscience) in Hybridoma serum-free medium (Invitrogen). Cells were harvested after 60 h of further incubation at 37 °C. The pellet was dissolved in buffer A (25 mM Tris at pH 7.5, 500 mM KCl, 5% (vol/vol) glycerol, and 9 mM 2-mercaptoethanol), vortexed briefly, sonicated, and ultracentrifuged. The clear lysate was bound to amylose resin (Invitrogen), washed with 100 volumes of buffer A, and eluted with buffer A containing 40 mM maltose. The sample was then subjected to size column chromatography on Superose 12 (GE Healthcare) in buffer A, with 1.5 mM DTT in place of mercaptoethanol. After concentrating and exchanging with storage buffer [25 mM Tris at pH 7.5, 500 mM KCl, 1.5 mM DTT, and 50% (vol/vol) glycerol], the proteins were stored at –80 °C.

Table 1. Recombination of plasmid substrates with RAG1 mutants

	pJH200		pJH290	
RAG1/2	Screened	Recombination frequency, %	Screened	Recombination frequency, %
FR1-FR2	65K	0.98	70K	0.95
FR1-cR2	88K	1.05	65K	0.96
FR1K233M-FR2	180K	0.06	200K	0.015
FR1K233M-cR2	165K	0.07	180K	0.02
FR1K233R-FR2	120K	0.11	140K	0.07
FR1K233R-cR2	145K	0.09	155K	0.065

V(D)J recombination of plasmid substrates in mouse 3TGR cells. Recombination frequency (in %) of signal joints (in pJH200) or coding joints (in pJH290). The cotransfected expression plasmids contain FLRAG1 (FR1) and either FLRAG2 (FR2) or core RAG2 (cR2). Mutant forms of FLRAG1 are written as FR1K233M or FR1K233R, depending on the mutation of K233. Results shown are the average of more than two independent transfections. K represents thousands.

DNA Cleavage Assays. Reaction mixtures were assembled in cleavage buffer (25 mM 3–4-morpholino propane sulfonic acid-KOH at pH 7.5, 50 mM KCl, 1% glycerol, 1 mM DTT, 0.1 mg/mL BSA, and either 5 mM $MgCl_2$ or 1.6 mM $MnCl_2$). Either 2 nM 12RSS or 2 nM 23 RSS substrates (14), along with 200 ng RAG1/2 and 20 ng HMGB1 protein (prepared as in ref. 28) were added and then incubated at 37 °C for 60 min. Reactions were stopped by adding an equal volume of formamide buffer [95% (vol/vol) formamide-Tris-borate-EDTA (TBE)] and heating at 95 °C for 5 min. Cleavage products were then separated on TBE urea-polyacrylamide [12.5% (wt/vol)] gels, visualized on a Typhoon PhosphorImager (GE Healthcare), and quantified using ImageQuant software (GE Healthcare).

Ubiquitination Assay. Ubiquitin, mutated forms of ubiquitin, methyl-ubiquitin, ubiquitin-activating E1 enzyme, and ubiquitin-conjugating E2 enzyme were all obtained from Boston Biochem. E1 enzyme (50 nM, either human or rabbit), E2 enzyme (0.4 μM, species as mentioned in the figure legends), 500 μM ubiquitin (as mentioned), and 200 ng RAG complex (as mentioned) were mixed together in ubiquitination buffer (50 mM Tris at pH 7.5, 60 mM KCl, 0.001% Brij, 3 mM Mg-ATP, and 0.5 mM DTT) and incubated at 37 °C for 1 h. Reactions were stopped by adding nonreducing SDS-gel loading buffer and heating at 95 °C for 5 min. The products were then resolved on SDS-polyacrylamide [4–12% (wt/vol)] gels (Invitrogen). After transferring the proteins to nylon membrane, the blots were developed and detected by a Western Breeze chemiluminescence kit (Invitrogen), using either anti-ubiquitin (Boston Biochem) or anti-MBP (Invitrogen) antibody.

For purification of ubiquitinated FLRAG1/2, the complex after ubiquitination was resolved on a size exclusion column (as earlier) to separate ubiquitinated from nonubiquinated species (Fig. S2).

Plasmid Recombination Assay. Transfection of mouse 3TGR cells was done as described (27), using the transfection reagent Turbofect (Thermo Scientific) in 60-mm dishes. Substrates were either pJH200 (for signal joint formation) or pJH290 (for coding joint formation). Formation of precise signal joints was confirmed by digestion with ApaLI restriction enzyme.

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