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Violations of the 12/23 rule at the mouse immunoglobulin kappa locus, including Vκ-Vκ rearrangement

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ABSTRACT

Classically, recombination between immunoglobulin gene segments uses a pair of recombination signal sequences (RSSs) with dissimilar spacers (the "12/23 rule"). Using a series of different genotyping assays, four different kinds of atypical rearrangements were identified at the murine kappa locus: (1) Vκ to Vκ, (2) Jκ to Jκ, (3) Vκ to iRS, a heptameric sequence found in the JκCκ intron, and (4) a possible by-product of a rearrangement between a Vκ and the hypothetical 12-RSS side of a pre-existing signal joint. The novel Vκ-Vκ structure prompted further characterization. Sequence analysis of 14 different Vκ-Vκ rearrangements cloned from murine splenocytes and hybridomas revealed a Vκ4 family member as one participant in 13 rearrangements, but no rearrangements contained two Vκ4 genes. The Vκ4 partner in the Vκ-Vκ rearrangement exhibited more trimming of nucleotides at the Vκ-Vκ junction. A signal joint derived from the inversional rearrangement of two neighboring Vκs was also recovered. These data suggest that the Vκ-Vκ structures arise via RAG-mediated, intrachromosomal recombination.

1. Introduction

The ability of the adaptive immune system to recognize an immense range of antigens stems from the process of V(D)J recombination at the B cell and T cell antigen receptor loci. Each immunoglobulin (Ig) receptor gene segment is flanked by a recombination signal sequence (RSS) consisting of conserved heptamer and nonamer sequences separated by either a 12 or a 23 base pair spacer (12-RSS or 23-RSS, respectively). Classically, recombination requires a pair of RSSs with dissimilar spacers (the "12/23 rule") (Sakano et al., 1979; Tonegawa, 1983). Previous investigations of the 12/23 rule have focused primarily on *in vitro* assays using extrachromosomal rearrangement substrates (Hesse et al., 1987; Hiom and Gellert, 1998; Lieber et al., 1988; van Gent et al., 1996). A few 12/23 rule violations have been reported *in vivo* (Hirama et al., 1991; Langerak et al., 2004; Shimizu et al., 1991), but such rearrangements are generally deemed quite rare, unless the immune system is forced to use incompatible RSSs (Koralov et al., 2005).

After encountering several peculiar κ rearrangements in unrelated experiments, we set out to molecularly characterize the range of 12/23 rule violations seen at the Igκ locus *in vivo*. The Igκ locus is well suited for this analysis because of its large size and ability to undergo inversional rearrangement, with the retention of signal joints and prior rearrangement coding joints on the chromosome (Feddersen and Van Ness, 1985; Shapiro and Weigert, 1987). Using a degenerate Vκ primer, we characterized 14 independent Vκ-Vκ fusions from spleen and splenic hybridoma DNA, of which 13 contained Vκ4 sequences. We also used a semi-quantitative PCR assay to measure the frequency of Vκ-Vκ rearrangements in wild type mice. The data suggest that these rearrangements are infrequent compared to conventional Vκ-Jκ rearrangements. The biological function of these aberrant rearrangements is unknown.

2. Materials and methods

2.1. Mice

All mice used for these studies are on the tenth or greater backcross generation onto the C57B6 background. The 56R mouse has a somatically mutated anti-DNA heavy chain that was introduced into the heavy chain J region by homologous recombination in embryonic stem cells (Chen et al., 1995). The bcl-xL mouse, a gift from Tullia Lindsten at the University of Pennsylvania, expresses the anti-apoptotic gene, *bcl-xL*, in B cells on the C57B6 background (Grillot et al., 1996). Hybridoma panels were generated from 3- to 6-month-old mice. Animals were housed in the University mouse colony and experiments were performed in accordance with a protocol approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Abbreviations: RSS, Recombination signal sequence; NT, Nucleotide; 12-RSS and 23-RSS, RSS with 12 or 23 nt spacer; IRS, Recombination sequence located in the Jκ-Cκ intron.

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2.2. Hybridomas

Spontaneous hybridomas from 3-month-old B6 and B6.56R.Bcl-xL mice were produced by fusion of the murine myeloma cell line Sp2/0 (Kohler, 1980) to freshly harvested splenocytes as described previously (Luning Prak et al., 1994). Hybridomas were cultured at limiting dilution and expanded into duplicate 6-well plates for analysis of culture supernatants and nucleic acid extraction, as described previously (Luning Prak et al., 1994). Hybridomas from B6.56R mice were produced for a separate study, but characterized for atypical κ rearrangements in this study (Sekiguchi et al., 2006).

2.3. PCR primers and conditions

All PCRs were performed with 100–250 ng of genomic DNA from spleen or individual spontaneous B6 hybridomas, in 1× PCR Buffer I (Applied Biosystems, Foster City, CA) with 1.5 U AmpliTaq Gold (Applied Biosystems) and 250 μM dNTPs. The Vs PCR was performed as described above in a 20 μL reaction volume, with 40 pmol of a degenerate primer in Vκ (Schlissel and Baltimore, 1989). Thermal cycling conditions were: primary denaturation at 94 °C for 10 min; 40 cycles of 94 °C for 30 s, 67 °C for 30 s, and 72 °C for 30 s; and final extension at 72 °C for 10 min. Assays to characterize rearrangements in individual hybridomas to Vκ20 and Vκ21 were performed as described previously (Li et al., 2001). Assays to detect signal joints remaining on the chromosome after Jκ to Jκ inversion were performed as described above, with 20 pmol of each primer:

Jκ1for: 5′-AATCAGCAGTTCTCTGTCAGAGAAGCC-3′
Jκ4for: 5′-CACGTTCGGCTCGGGGACAAAGTTGGAA-3′

Thermal cycling conditions were: primary denaturation at 94 °C for 10 min; 40 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s; and final extension at 72 °C for 5 min.

PCR assays to detect signal joints remaining on the chromosome after Vκ to Vκ inversion were performed using primers situated in genomic DNA sequences flanking individual Vκ RSSs. The primers used for this analysis are:

Vκ4-86 SJP: 5′-TCCTGCCAGTGTGAAGACAG-3′
Vκ1-88 SJP: 5′-TGATGAAGGCTGTCATGCTCA-3′

The signal joint amplification was performed in a 50 μL volume using 50 pmol of each primer and the same concentrations of all of the other mix components as the Jκ-Jκ PCR described above. Cycling conditions were: primary denaturation at 94 °C for 10 min; 40 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 30 s; and final extension at 72 °C for 10 min.

2.4. Cloning and sequence analysis

PCR products were band purified using a Qiaquick gel extraction kit, per the manufacturer's instructions (Qiagen, Valencia, CA) and either sequenced directly or cloned into pCR4 TOPO per the manufacturer's instructions (Invitrogen, Carlsbad, CA). Sequencing was performed on an ABI 3730 using BigDye Taq FS terminator V 3.1 in the University of Pennsylvania DNA Sequencing facility (http://www.med.upenn.edu/genetics/core-facs/dna-seq/). Sequences (in both directions) were aligned and compared to germline Vκ sequences using IgBLAST (http://ncbi.nih.gov/igblast/). Nomenclature used for Vκ gene segments follows the system described in reference Brekke and Garrard, 2004.

2.5. Statistical analysis

As described in Section 3, we encountered a predominance of Vκ4–non-Vκ4 rearrangements, without any Vκ4-Vκ4 rearrangements. To calculate the likelihood these results could be due to chance, we considered a model wherein different Vκ genes have independent probabilities of undergoing Vκ-Vκ rearrangement. This model assumes that the assay, which relies upon the use of a degenerate Vκ primer, does not result in the biased amplification of particular Vκ gene families. Based on our previous experience, we know that the Vs primer can amplify approximately 80% of all Vκ gene family members, including Vκ4 and non-Vκ4 genes (Luning Prak et al., 1994). Applying this model, there is some unknown probability p that any given gene we recover is from the Vκ4 family. Assuming that the 14 Vκ-Vκ sequences shown in Table 1 are derived from independent clones of B cells (based on sequence differences), p, the frequency of Vκ4, is estimated to be 13/28. The chance that both Vκs in a given pairing are Vκ4 is $(0.464)^2 = 0.21$, assuming that Vκ4 and non-Vκ4 genes rearrange independently. The chance of not seeing Vκ4-Vκ4 in 14 Vκ-Vκ pairings is $(1 - 0.21)^{14} = 0.037$. A Student's t-test (one-tailed, equal variance) was used to compare the 3′ trim length of Vκ4 to non-Vκ4 partners in the 14 Vκ-Vκ rearrangements.

3. Results

3.1. Atypical Vκ-Vκ gene rearrangements occur in vivo

During routine hybridoma genotyping, we noted a PCR product of unexpected size that, on sequence analysis, appeared to be a Vκ-Vκ rearrangement. We first confirmed that the unexpected product could be amplified with Vs (a degenerate Vκ primer, see Section 2) alone in the reaction mix. We then used Vs PCR to identify additional examples from spleen DNA of mice. Table 1 illustrates the range of Vκ-Vκ rearrangements that were recovered.

3.2. Vκ-Vκ rearrangements likely invert and may also delete

To better understand the mechanism of Vκ-Vκ rearrangement, we examined the germline positions and orientations of the participating gene segments. The gene pairs involved have a variety

Table 1
Vκ usage and DNA source of cloned Vκ-Vκ rearrangements.

B6 spleen		
1.	Vκ23-48	Vκ4-78
2.[a]	Vκ33-84 or Vk33-85	Vκ4-80
3.	Vκ12-41	Vκ8-24
B6.Bcl-xL spleen		
4.	Vκ23-43 or Vk23-45	Vκ4-53
5.	Vκ12-44 or Vk12-46	Vκ4-54
6.[b]	Vκ1-117	Vκ4-60 or Vκ4-68
7.	Vκ12-46	Vκ4-70
8.	Vκ1-110	Vκ4-77
9.	Vκ1-117	Vκ4-77
10.	Vκ33-84 or Vκ33-85	Vκ4-79
11.	Vκ1-88	Vκ4-86
12.	Vκ1-110	Vκ4-86
B6.56R.Bcl-xL hybridoma		
13.[b]	Vκ1-117	Vκ4-60 or Vκ4-68
B6 hybridoma		
14.[a]	Vκ33-84 or Vκ33-85	Vκ4-80

Four different mice provided splenocytes. Spleen refers to spleen DNA. Hybridoma refers to spontaneous hybridomas produced from the spleen (see Section 2). The Vκ gene assignments are based on DNA sequence analysis (see Section 2). Two pairs of Vκ-Vκ rearrangements that use the same Vκ gene segments (but were independently recovered from different mice) are indicated with footnotes (a or b) to the right of the corresponding sequence numbers. The junction of each Vκ-Vκ rearrangement is shown in Fig. 3a.