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Unraveling the Consecutive Recombination Events in the Human *IGK* Locus

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In addition to the classical Vκ-Jκ, Vκ-κ deleting element (Kde), and intron-Kde gene rearrangements, atypical recombinations involving Jκ recombination signal sequence (RSS) or intronRSS elements can occur in the Igκ (*IGK*) locus, as observed in human B cell malignancies. In-depth analysis revealed that atypical JκRSS-intronRSS, Vκ-intronRSS, and JκRSS-Kde recombinations not only occur in B cell malignancies, but rather reflect physiological gene rearrangements present in normal human B cells as well. Excision circle analysis and recombination substrate assays can discriminate between single-step vs multistep rearrangements. Using this combined approach, we unravelled that the atypical Vκ-intronRSS and JκRSS-Kde pseudohybrid joints most probably result from ongoing recombination following an initial aberrant JκRSS-intronRSS signal joint formation. Based on our observations in normal and malignant human B cells, a model is presented to describe the sequential (classical and atypical) recombination events in the human *IGK* locus and their estimated relative frequencies (0.2–1.0 vs <0.03). The initial JκRSS-intronRSS signal joint formation (except for JκRSS-intronRSS) might be a side event of an active V(D)J recombination mechanism, but the subsequent formation of Vκ-intronRSS and JκRSS-Kde pseudohybrid joints can represent an alternative pathway for *IGK* allele inactivation and allelic exclusion, in addition to classical Cκ deletions. Although usage of this alternative pathway is limited, it seems essential for inactivation of those *IGK* alleles that have undergone initial aberrant recombinations, which might otherwise hamper selection of functional Ig L chain proteins.

To form a large variety of unique Ag-recognizing Ig molecules, human B cells undergo recombination between variable (V), diversity (D), and joining (J) gene segments of the Ig genes. The resulting V(D)J exon encodes the variable domains of Ig chains. In the bone marrow, VDJ recombination of Ig H chain (IGH) genes starts at the progenitor B cell stage, followed by VJ recombination in Igκ (*IGK*) or Igλ (*IGL*) L chain loci during the small precursor B cell or precursor B-II cell stage (1, 2). Pairing of Ig H and Igκ or Igλ chains allows further differentiation into immature and subsequently mature surface membrane Igκ- or Igλ-positive B cells.

Previous studies have shown that most Igκ-positive B cells retain their *IGL* genes in germline configuration (3, 4). In contrast, the vast majority of Igλ-positive B cells have one or two rearranged *IGK* alleles in addition to their rearranged *IGL* allele(s), in line with hierarchical Ig L chain recombination (4, 5). In Igλ-positive B cells, the *IGK* rearrangements mainly concern deletional rearrangements involving the κ-deleting element (Kde), which is positioned downstream of the constant (Cκ) gene segment (6) (Fig. 1). Kde can either recombine to a Vκ gene segment upstream of a previously formed Vκ-Jκ rearrangement or to an isolated heptamer recombination signal sequence (RSS) in the intron between the Jκ gene segments and the Cκ exon (intronRSS heptamer) (6) (Fig. 1). Recombination to the intronRSS heptamer results in the deletion of Cκ and the *IGK* intronic enhancer (Ei), whereas rearrangement to one of the Vκ gene segments deletes the entire Jκ-Cκ area; both types of recombinations prevent expression of the *IGK* allele, and it is believed that such events participate in the regulation of allelic and κα isotopic exclusion.

In addition, Feddersen et al. (7, 8) have described atypical *IGK* recombinations in virally transformed murine B cells and also in human B-lineage lymphoproliferations; this concerns rearrangements of the intronRSS heptamer to the RSS of one of the Jκ segments (JκRSS-intronRSS signal joint) and rearrangements of a Vκ gene segment to the intronRSS (Vκ-intronRSS recombinations). Furthermore, JκRSS-Kde rearrangements have been identified in a case of human acute leukemia (9). The effects of these so-called atypical *IGK* rearrangements are versatile. Vκ-intronRSS rearrangements result in deletion of the entire Jκ gene segment cluster and the Cκ exon is removed upon JκRSS-Kde recombination. In contrast, in case of downstream JκRSS-intronRSS rearrangement, functional expression of the upstream Vκ-Jκ on the same allele could still be possible. Only if the JκRSS-intronRSS recombination involves the JκRSS, a functional Ig κ-chain can no longer be formed, as no Jκ segments are then available for Vκ-Jκ recombination.

Based on extensive Southern blot and PCR screening, we identified a series of human B cell malignancies with uncommon *IGK* locus hybridization patterns and/or extended PCR product sizes, suspicious of the presence of the before mentioned atypical *IGK* gene rearrangements. These malignancies constitute single-cell

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3 Abbreviations used in this paper: IGH, Ig H chain locus; IGK, Igκ locus; IGL, Igλ locus; Kde, κ-deleting element; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; BM, bone marrow; CAT, chloramphenicol acetyltransferase; CLL, chronic lymphocytic leukemia; MNC, mononuclear cells; PB, peripheral blood; RQ-PCR, real-time quantitative PCR; RSS, recombination signal sequence.
FIGURE 1. Schematic overview of classical rearrangements in the human IGK locus. IGK recombination mostly starts with a Vκ-Jκ rearrangement. The functionality of this rearrangement can be disrupted by rearrangement of the Kde. Recombination of Kde to an isolated heptamer in the intron between the Jκ and Cκ regions results in deletion of the Cκ region, whereas recombination between Kde and a Vκ gene segment deletes the entire (Vκ) Jκ-Cκ region. Both types of Kde rearrangements also result in deletion of the IGK enhancers (iEκ and 3′Eκ), probably precluding further rearrangements in the human IGK locus.

model systems that enable the study of atypical IGK recombinations in full detail, i.e., within the context of the entire IGK/IGL L chain gene configuration. More importantly, we also analyzed and quantified the occurrence of these IGK recombinations in normal human peripheral blood and tonsillar B lymphocytes. The analysis of intermediate circular excision products, as well as functional data obtained in recombination substrate assays, allowed us to investigate whether specific rearrangements are formed via single-step or multistep rearrangements. Based on these data, we now propose a comprehensive and integrated model of the step-wise consecutive recombinations in the human IGK locus, including the here-described atypical IGK recombinations. The implications of these recombination pathways on Igκ protein expression and allelic exclusion of human IGK genes are discussed.

Materials and Methods

Cell samples

Several leukemia/lymphoma cell samples were selected on the basis of an initial suspicion of atypically rearranged IGK genes, as deduced from initial Southern blot hybridization patterns and/or extended PCR product sizes (10). These included the precursor B cell lines 380 and RCH-ACV, bone marrow (BM), or lymph node samples from precursor B cell acute lymphoblastic leukemia (precursor B-ALL; n = 5), B cell non-Hodgkin’s lymphoma (n = 4), B cell chronic lymphocytic leukemia (B-CLL; n = 2), one unclassified B cell proliferation, and an acute myeloid leukemia (AML-M5) showing illegitimate atypical IGK recombination. BM mononuclear cells (MNC) were isolated by ficoll-Paque (density, 1.077 g/ml; American Biosciences, Buckinghamshire, U.K.) density centrifugation. BM-MNC fractions and lymph node suspensions were subsequently used for DNA and RNA isolation. Tonsils, peripheral blood (PB)-MNC, and regenerating BM-MNC from healthy controls were included as a source of normal B lymphocytes.

DNA isolation and Southern blot analysis

DNA isolation and Southern blot analysis was performed as previously described (11). In short, 15–20 μg genomic DNA was digested with restriction enzymes, separated in 0.7% agarose gels, and vacuum blotted. The configuration of the IGK locus was determined using BglII and BamHII/HindIII digests and 32P-labeled probes specific for the areas upstream (IGKJu) or downstream (IGKJ5) of the Jκ segments, or specific for the Cκ (IGKC) and the Kde (IGKDE) regions (10). IGK rearrangements were studied in EcoRI/HindIII digests using a general IGL probe (IGLC3) or probes specific for the JA1-Ca1 (IGLC1D) or Jα2-Ca2 and Jα3-Ca3 (IGLC2) areas in combination with BglII digests (12, 13).

Primer design

To detect recombinations between JκRSS elements and the intronRSS heptamer, new primer sets were designed (Fig. 2, and Table I). Using the germline IGK sequence (accession no. X67858) and OLIGO 6.2 software, a Jκ consensus primer (Jκ2-5-F1-EMC) was designed to detect rearrangements between Jκ2RSS, Jκ3RSS, Jκ4RSS, or Jκ5RSS and the intronRSS. The Jκ2-5-F1-EMC primer recognizes a homologous sequence in each Jκ gene segment upstream of the JκRSS that rearranges to intronRSS. To detect Jκ1RSS rearrangements a separate primer was designed that recognizes a sequence upstream of Jκ1 (Jκ1-F1-EMC). A primer downstream of intronRSS (intron-R1-EMC) was chosen as reverse primer. JκRSS-Kde recombinations were analyzed using the Jκ1-F1-EMC and Jκ2-5-F1-EMC primers together with a Kde primer. For detection of Vκ-intronRSS rearrangements, Vκ family primers as well as a Vκ consensus primer were used in combination with the earlier mentioned intron-R1-EMC primer (Fig. 2, and Table I). Inversional Vκ-intronRSS rearrangements were analyzed using the same Vκ family primers, in combination with a primer recognizing a sequence upstream of intronRSS (intronRSS primer) (Fig. 2, and Table I).

To specifically analyze circular excision products that are uniquely formed during JκRSS-intronRSS recombination, primers recognizing the Jκ segments (Jκ1-4) and the area upstream of intronRSS (intronRSS primer) were used (Fig. 2, and Table I) (14).

FIGURE 2. Schematic diagram of primer sets for detection of atypical IGK gene rearrangements such as JκRSS-intronRSS, Vκ-intronRSS and JκRSS-Kde rearrangements. Approximate position of the specially designed primers is indicated; the sequences of the primers are given in Table I. Classical IGK rearrangements as well as atypical JκRSS-intronRSS, Vκ-intronRSS, and JκRSS-Kde rearrangements are shown schematically.
with all known human germline IgK sequences obtained from the VBASE directory of human Ig genes (http://www.mrc-cpe.cam.ac.uk/imt-doc/) and/or ImmunoGenetics (IMGT) (http://imgt.cines.fr:8104).

Real-time quantitative PCR (RQ-PCR) analysis of different types of IgK gene recombinations

RQ-PCR of different IgK recombinations was essentially performed as described previously using ABI Prism 7700 equipment (Applied Biosystems) (16). As clonal control DNA for the analyzed IgK recombinations, the following sources with high percentages (90–100%) of clonal cells were selected: cell line U698 (Vx-Jk1), a B-CLL sample (Vx-Jk4), cell line ROS15 (Vx-Kde), cell line Nalm1 (intron-Kde), B-CLL sample 91-062 (Jk1RSS-intron-RSS), ALL sample 5381 (Jk2RSS-intron-RSS), cell line RCH-ACV (Vx-intron-RSS), and AML sample 95-058 (Jk2RSS-Kde); no clonal control DNA was available for Jk1RSS-Kde. The applied forward/reverse primers and TaqMan probes are listed in Table I.

All control cell lines and leukemic DNA samples were serially diluted in HeLa DNA as nontemplate control. A standard albumin RQ-PCR was subsequently plotted on the respective standard curve, and expressed as percentage recombination relative to the respective clonal control DNA.

Recombination substrate assay

The recombination substrate assay was essentially performed as described previously (17). In short, recombination plasmids with the chloramphenicol acetyltransferase (CAT) gene under control of the Prac promoter were

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PCR amplification and sequencing

PCR amplification of JkRSS-intronRSS, Vx-intronRSS, and JkRSS-Kde recombinations, as well as of inversional Vx-intronRSS rearrangements and Jk-intronRSS circular excision products was performed using the relevant primer sets (see Primer design and Table I). A 50-μl reaction volume contained 50 ng genomic DNA, 1.5 mM MgCl₂ (Applied Biosystems, Foster City, CA), 0.2 mM dNTP (Amersham Biosciences), 0.25 pmol of each primer, and 1 U of AmpliTaq Gold DNA polymerase in buffer II (Applied Biosystems). The PCR consisted of 10 min preactivation at 94°C, followed by 40 cycles of 45 s denaturation at 94°C, 90 s annealing at 60°C, and 2 min extension at 72°C, followed by 10 min final extension at 72°C. PCR products were electrophoresed on a 1% agarose gel and visualized with ethidium bromide.

After amplification, the PCR products were further analyzed by heteroduplex analysis to determine the monoclonal or polyclonal character (15). In short, following denaturation at 94°C for 5 min and reannealing at 4°C for 1 h, the PCR products were separated in 6% polyacrylamide gels in 0.5× Tris-borate-acid-EDTA buffer.

PCR products derived from monoclonal rearrangements were directly sequenced. To sequence the polyclonal recombinations from tonsil and MNC samples from healthy controls, the PCR products were first cloned, using the pGEM-T Easy vector kit according to the manufacturer’s instructions (Promega, Madison, WI). After transformation to competent cells, positive colonies were grown and plasmid DNA was isolated for further sequencing.

Sequencing was performed on the ABI 377 fluorescent sequencer (Applied Biosystems), using the dye terminator cycle sequencing kit and AmpliTaqFS DNA polymerase (Applied Biosystems). Sequencing primers were identical with those used for PCR amplification. Sequencing was performed using either 60 ng of monoclonal PCR product and 3.2 pmol primer, or 500 ng of plasmid DNA with cloned PCR products from healthy controls and 6 pmol primer, in combination with 5 μl of dye terminator mix. The cycling protocol consisted of 25 cycles of 30 s, 96°C, followed by 4 min, 60°C.

Analysis of the obtained sequences was performed using the germline IgK genomic sequence (accession no. X67858). Vx and Jk gene segments were identified using DNAplot software (W. Müller and H.-H. Althaus, University of Cologne, Cologne, Germany) by searching for homology with all known human germline IgK sequences obtained from the VBASE directory of human Ig genes (http://www.mrc-cpe.cam.ac.uk/imt-doc/) and/or ImmunoGenetics (IMGT) (http://imgt.cines.fr:8104).

PCR analysis and sequencing

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Analysis of the obtained sequences was performed using the germline IgK genomic sequence (accession no. X67858). Vx and Jk gene segments were identified using DNAplot software (W. Müller and H.-H. Althaus, University of Cologne, Cologne, Germany) by searching for homology
used; in these constructs, CAT expression is prevented by a termination signal (OOP) upstream of the CAT coding sequence (see also Fig. 5 for further explanation). In eukaryotic cells, recombination between sequences in the upstream and downstream cassettes, which flank the OOP termination signal, results in removal of the latter, thereby giving rise to CAT expression. Cloning of the intron RSS motif cassette was performed as follows: PCR amplification of DNA from cells with germline IGK genes using Ki-1A (SpeI) and Ki-2B (BglII) primers, subcloning in pPCR-Script AmpSK (+) (Stratagene, La Jolla, CA) in the correct orientation, and finally digestion with NotI/Sall and further cloning using NotI and Sall restriction sites into the appropriate recombination substrates. The Kde cassette was produced by PCR amplification of germline IGK DNA using CKde (SpeI) and Kde-A (BglII) primers, and subsequently cloned via SpeI and BglII restriction sites into the appropriate recombination substrates.

Finally, to produce the signal joint cassettes, the JcRSS-intronRSS and JcRSS-intronRSS signal joints were PCR amplified from leukemic cells harboring these elements using primers Jc-1-F1-EMC or Jc-2-5-F1-EMC together with intron-R1-EMC. PCR products were subcloned in pPCR-Script AmpSK (+), and taken out BssHII/NotI. Because BssHII has a compatible overhang with MluI, the fragment could directly be cloned as an MluI/NotI cassette into the appropriate recombination substrates.

Following transient transfection of recombinant constructs into 18.8 Abelson’s murine leukemia virus transformed murine precursor B-cells, plasmids (recombined and intact) were recovered after 48 h. Plasmids were subsequently transformed into E. coli, which were further cultured on ampicillin (50 μg/ml) and chloramphenicol (5 μg/ml). Recombined plasmid DNA was then isolated from resulting chloramphenicol-selected colonies and PCR-amplified using specific primers for the upstream and downstream sequences, followed by direct sequencing for identification of exact recombination breakpoints.

Results

Atypical IGK recombinations detected in human B-lineage lymphoproliferations

In the process of screening human B cell lymphoproliferations by IGK Southern blot and PCR analysis, we have come across cases with IGK recombinations other than the classical Vκ-Jκ, Vκ-Kde, and/or intron-Kde joinings (Fig. 1). These atypical IGK recombinations were further studied by Southern blot analysis using four different probes to determine the exact configuration of both IGK alleles (Fig. 3, and Table II).

In two samples (91-062 and F-7), rearranged bands of equal size were seen upon hybridization with the IGKJU and IGKC probes, which were interpreted as JκRSS-intronRSS recombinations. Several other samples (4882, 94-101, 95-060, G-10, 4511, 5301, 95-074, and cell line RCH-ACV) showed a rearranged band using the IGKC probe with concomitant deletion of IGKJU and/or IGKJ5 hybridization signal, suggestive of a Vκ-intronRSS recombination. One case (5381) showed two nonidentically rearranged bands upon IGKJU and IGKC hybridization and loss of IGKJ5 signal, which most likely fits with an inversionsal rearrangement between a Vκ segment from the distal (inverted) cluster and a Jκ segment in combination with a JκRSS-intronRSS rearrangement on the same allele. Finally, in four samples (92-051, 5712, 95-058, and cell line 380), a rearranged band was seen upon IGKDE hybridization with parallel loss of IGKJU, IGKJ5, and IGKC hybridization signal, which might be explained by a true Vκ-Kde rearrangement or by a variant type Vκ-Jκ and JκRSS-Kde rearrangement (see later).

Because deletional rearrangements in the IGK locus mostly coincide with IGL gene rearrangements, we also checked this coincidental occurrence for atypical IGK recombinations. In 8 of the 13 cases, both IGL alleles were in germline configuration. Of the five cases showing IGL rearrangements (Table II), three contained at least one classical Kde recombination; in the other two (4511 and 5301), Vκ-intronRSS rearrangements were observed. However, because other cases (among others, case 95-074 and cell line RCH-ACV) displayed isolated Vκ-intronRSS recombinations without VA-Jα rearrangements, a complete association between atypical IGK recombinations and the start of IGL gene recombination seems unlikely.

Detailed characterization of the detected atypical IGK recombinations

The vast majority of Southern blot deduced atypical IGK rearrangements could be confirmed via PCR heteroduplex analysis and sequencing (Fig. 2, and Table II). The two JκRSS-intronRSS recombinations (91-062 and F-7) both involved JκRSS. Of the many samples with presumed Vκ-intronRSS recombinations, only two indeed contained direct Vκ-intronRSS couplings (95-074 and cell line RCH-ACV). In six others (4882, 94-101, 95-060, G-10, 4511, and 5301), the unusually long Vκ-intronRSS PCR products cases revealed a Vκ-Jκ and JκRSS-intronRSS configuration. PCR and sequencing in sample 5381 further showed an inversionsal VκRSS-JκRSS rearrangement upstream of a JκRSS-intronRSS configuration. Finally, in four other samples (92-051, 5712, 95-058, and cell line 380) the unusually long Vκ-Kde PCR products appeared to represent Vκ-Jκ and JκRSS-Kde rearrangements rather than direct Vκ-Kde couplings.

Remarkably, in most cases with Vκ-Jκ and JκRSS-intronRSS or Vκ-Jκ and JκRSS-Kde configurations neighboring Jκ segments

![FIGURE 3. Southern blot analysis of human IGK gene rearrangements. A, Restriction map of part of the human IGK locus. The location of relevant BglII (Bg), BamHI (B), EcoRI (E), and HindIII (H) restriction sites as well as the IGKJU, IGKJ5, IGKC, and IGKDE Southern blot probes are indicated. B, Example of interpretation of hybridization patterns of the complete set of IGK Southern blot probes in combination with BglII restriction digests.](http://www.jimmunol.org/)
were involved in the two couplings. Exceptions concerned 4511 and 5301 with V-Jc1 and V-Jc2, respectively, upstream of Jκ4RSS-intronRSS. All Jκ (except Jκ5) gene segments were found to be involved in the atypical IGκ recombinations. Vκ segments in the upstream Vκ-Jκ couplings were derived from the three large Vκ families (Vκ1, Vκ2, Vκ3) and even concerned the most Jκ proximal Vκ1-1 segment. The few cases with direct Vκ-intronRSS couplings all concerned Vκ1 segments.

Detailed junctional region analysis revealed a signal joint configuration with two perfectly joined RSS elements in only four of the nine JκRSS-intronRSS atypical recombinations. The other five showed processed signal joints, i.e., nucleotide deletion from one or both RSS elements and even nucleotide insertion. Such a configuration is remarkable in view of the orientation of the JκRSS and intronRSS elements in the germline configuration, which in principle should lead to perfect signal joints. Coding jointlike configurations with inserted nucleotides were observed in all Vκ-intronRSS and JκRSS-Kde recombinations. Given their hybrid jointlike configuration, these were termed pseudohybrid joints. Analysis of Vκ-Jκ junctions upstream of JκRSS-intronRSS recombinations revealed out-of-frame Vκ-Jκ fusions in five samples (94-101, G-10, 4511, 5301, and 5381), but in-frame alleles in two others (4882 and 95-060). Although no membrane Ig expression was observed (precursor B cell leukemia), in-frame Vκ2-30-Jκ1-Cκ transcripts were found to be present in case 4882 (data not shown). In 95-060, membrane Igκ protein expression was observed that could only result from the Vκ1-39-Jκ2 and Jκ3RSS-intronRSS allele, because the other allele contained a deletional rearrangement. Hence, the presence of a JκRSS-intronRSS recombination (except for Jκ1RSS-intronRSS) does not seem to block expression of an in-frame upstream Vκ-Jκ rearrangement.

Occurrence of atypical IGκ recombinations in normal human B cells

To exclude that the atypical IGκ rearrangements are only formed aberrantly in leukemic cells or (virally) transformed cells, tonsillar DNA from a healthy individual was analyzed. Using the same IGκ primers as for the clonal cell samples, the tonsillar DNA was amplified and per type of rearrangement 5–15 cloned PCR products were sequenced (Table III). Vκ-intronRSS and JκRSS-intronRSS sequences both could readily be amplified. Many of the Vκ-intronRSS recombinations concerned couplings with a comparable extent of nucleotide deletion and insertion as seen in the clonal cell samples. Part of the seemingly Vκ-intronRSS recombinations in tonsillar DNA actually concerned Vκ-Jκ and JκRSS-intronRSS configurations with heterogeneous Vκ family and Jκ gene segment usage. Interestingly, in contrast to the clonal cell samples, the vast majority (11/13 sequences) of tonsillar JκRSS-intronRSS rearrangements displayed perfect signal joints. Finally, heterogenous (i.e., diverse with deletion and insertion of nucleotides) JκRSS-Kde recombinations could also be identified in tonsillar B cells, though the Jκ usage seemed to be less diverse in the small number of sequences analyzed in detail. Taken together, these data suggest that the atypical IGκ recombinations that we initially identified in transformed cell samples do represent physiological events given their occurrence in healthy control tonsillar B cells.

Quantitation of IGκ recombinations by RQ-PCR analysis

RQ-PCR was applied to quantify the different types of IGκ recombinations in (regenerating) BM-MNC, PB-MNC, and tonsil. For this purpose Vκ1, Jκ1(RSS), Je4(RSS), Kde, and the intronRSS heptamer were chosen as representative elements
involved in the various rearrangements. Following normalization of input DNA relative to an albumin standard curve, individual RQ-PCR were performed for these selected IGK recombination types. Using standard curves based on 10-fold serial dilutions of clonal control DNA samples, levels in tonsil, PB-MNC, and (regenerating) BM-MNC DNA were expressed as percentage recombination relative to the respective clonal control DNA. It is important to note that the various IGK recombination levels as measured in the normal tissues reflect relative and no absolute values, which formally cannot be used for direct comparisons between different types of recombinations in the human IGK locus. With this in mind, we nevertheless tried to roughly compare the frequencies of these different IGK recombinations (Table IV). In tonsil, intron-Kde rearrangements were most abundant and were arbitrarily set to 1.0; intron-Kde levels in PB and BM, being roughly 5- and 30-fold lower, respectively, than those observed in tonsil, were also set to 1.0. V\(\kappa\)-Jk1, V\(\kappa\)-Jk4, and V\(\kappa\)-Kde rearrangements appeared to be relatively frequent, with levels roughly 0.20–0.45 relative to those of intron-Kde. Atypical Jk1RSS-intronRSS and Jk4RSS-intronRSS rearrangements could also be found in tonsil, PB, and BM, although the levels appeared to be much lower than those of the classical IGK rearrangements, ranging from (almost) undetectable to roughly 0.06 (as compared with intron-Kde levels) to maximally 0.1 (as compared with V\(\kappa\)-Jk and V\(\kappa\)-Kde levels). Finally, recombination levels of the V\(\kappa\)-intronRSS and Jk4RSS-Kde rearrangements were of the same order of magnitude as JkRSS-intronRSS. Due to the lack of a clonal control DNA sample, Jk1RSS-Kde rearrangement levels could not be quantified; however, the C\(\alpha\) values were not that different from those obtained for Jk4RSS-Kde rearrangements, suggesting a similar frequency for both rearrangements. Taken together, these data further confirm that atypical IGK rearrangements do indeed occur in BM, PB, and tonsillar B cells, albeit at frequencies of ~0.06 to 0.1 relative to the most abundant IGK gene rearrangements in these cells.

Excision circle analysis of atypical IGK recombinations

Next, we addressed the exact mechanism by which these atypical IGK recombinations are formed via detection of excision circles, which are formed during the rearrangement process. Because excision circles are not replicated upon further cell division, it is impossible to study these in clonally transformed cell samples. For that reason, we restricted our excision circle studies to tonsil DNA. Because virtually all JkRSS-intronRSS recombinations in tonsil DNA concerned perfect signal joints (Table III), it seemed logical...
to assume that excision circles would contain coding joints involving the Jκ gene segment and the region upstream of the intronRSS heptamer. Using a Jκ consensus primer as reverse primer in combination with a primer upstream of intronRSS as forward primer, PCR products can only be formed through amplification with a primer upstream of intronRSS as forward primer, and Table I), strongly suggesting that JκRSS-intronRSS coupling occurs via direct recombination, resulting in a signal joint on the chromosome and a coding joint on the circular excision product.

The next question concerned the mechanism by which Vκ-intronRSS and JκRSS-Kde rearrangements are formed. Direct recombination would be less logical, as this would violate the 12/23 rule for at least the JκRSS-Kde recombination. Previously, it has been shown that in a signal joint, both RSS are functional and can undergo secondary rearrangements (18–20). Because most JκRSS-intronRSS signal joints in tonsil appeared to contain undamaged RSS elements, both RSS can be involved in continuing recombination processes leading to Vκ-intronRSS or JκRSS-Kde pseudohybrid joints. However, episomal circles formed by such secondary signal joint rearrangements are indistinguishable from normal Vκ-Jκ and intron-Kde rearrangements, because they use the same RSS and, therefore, generate the same signal joint excision product. Hence, it was impossible to design primers that would specifically recognize circular excision products resulting from Vκ-intronRSS and JκRSS-Kde recombinations.

**Unraveling the mechanism of atypical IGK recombination via recombination substrate assay**

To unravel the exact mechanism of Vκ-intronRSS and JκRSS-Kde recombinations, an in vitro recombination substrate assay was used. IGK RSS elements of interest together with their flanking sequences were cloned as upstream and downstream cassettes in a vector that contains an intermediate stop element for blocking CAT gene expression. Upon transfection in a precursor B cell line, proper recombination between RSS elements in the upstream and downstream cassettes results in removal of the stop element, thereby enabling CAT expression. Sequencing of colonies, selectively grown on chloramphenicol, allows determining the exact recombination breakpoints.

We first analyzed a construct in which the Jκ1 gene segment with its RSS was cloned in one cassette and the intron-Kde heptamer with flanking DNA in the other (Fig. 5A). Sequence analysis revealed perfect signal joints between the JκRSS and intronRSS elements, whereas analysis of the excision products showed fusion of the Jκ1 coding sequence and the upstream intron area with deletion as well as insertion of P and N nucleotides (Fig. 5A). This result nicely confirmed the observations from our excision circle analyses. We subsequently cloned the JκRSS-intronRSS signal joint and flanking DNA from one of the leukemic cell samples, and tested it for its ability to recombine with the RSS of the frequently used VκA2/2D-29 and VκA27/3-20 gene segments (Fig. 5B), which was assumed to be the ongoing recombination step leading to Vκ-intronRSS pseudohybrid joint formation. Of the many colonies obtained in the assay, all 10 sequenced showed that V(D)J recombination between Vκ and the JκRSS-intronRSS signal joint indeed occurs. Moreover, the sequences resembled the Vκ-intronRSS pseudohybrid joints as obtained from tonsil DNA, being diverse with deletion of nucleotides at both the Vκ and the signal joint sides as well as insertion of occasional N nucleotides and formation of P nucleotides (Fig. 5B). Finally, to test the functional properties of JκRSS-intronRSS signal joints in the other direction, we made a construct in which a JκRSS-intronRSS signal joint was tested against the KdeRSS (Fig. 5C). Similarly, all sequenced constructs showed pseudohybrid joints. In all cases, JκRSS was coupled to the Kde sequence, with diversity of deletion and insertion of both N and P nucleotides (Fig. 5C). Collectively, these results show that recombination between JκRSS and intronRSS

![FIGURE 4. Atypical JκRSS-intronRSS recombination. A. Schematic drawing of a JκRSS-intronRSS signal joint as well as the parallel excision circle with the coding joint. Indicated are the primers that can be used for amplification of these signal and coding joints: 1. Jκ1-F1-EMC or Jκ2-5-F1-EMC; 2. intron-R1-EMC; 3. Jκ1-4; and 4. intronRSS (see also Fig. 2 and Table I). B. Agarose gel electrophoresis of PCR-amplified JκRSS-intronRSS signal joints and parallel coding joints with the primers as described in A.](image-url)
FIGURE 5. Recombination substrate assay. Sequences of clones obtained in recombination substrate assay, using different constructs (for a description see also Materials and Methods). A, Configurations of signal joints and coding joints formed upon recombination between the Jk1 gene segment and the intronRSS heptamer. Perfect Jk/H9260 RSS-intronRSS signal joints are formed, whereas the coding joints show a variable level of deletion and insertion of nucleotides. B, Configurations of pseudohybrid joints formed upon recombination between Vx2/2D-29 or Vx27/3-20 and the Jk/H9260RSS-intronRSS signal joint, showing both deletion and insertion of nucleotides. C, Configurations of pseudohybrid joints formed upon recombination between the JkRSS-intronRSS signal joint and Kde, with evidence for deletion and insertion of nucleotides.
leads to signal joint formation and that the composition and position of this signal joint is such that it can be involved in secondary recombination to either one of the \(V_k\) gene segments or Kde, finally giving rise to \(V_k\)-intronRSS and JxRSS-Kde pseudohybrid joints.

**Discussion**

The human \(IGK\) locus contains several RSS elements in addition to the RSS flanking \(V_k\) and \(J_k\) gene segments, allowing other rearrangements than the common V-J rearrangements to occur. Firstly, the presence of a 23-bp RSS flanking the Kde element at the very 3' end of the locus provides an alternative partner for the 12-bp \(V_k\) RSS elements, resulting in \(V_k\)-Kde rearrangements. Secondly, the Kde element can also rearrange to an isolated heptamer in the Jx-Cx intron (intronRSS), resulting in intron-Kde rearrangements (Fig. 1). Thirdly, additional nonclassical recombinations involving \(V_k\)RSS, JxRSS, intronRSS, and KdeRSS elements have been described by Feddersen et al. and Seriu et al. (7–9). Several of these rearrangements cannot be formed directly, either because of the inverted positions of their respective RSS (JxRSS and KdeRSS, and \(V_k\) and intronRSS) or because their RSS spacer lengths do not obey the 12/23 rule (Jx and Kde both contain RSS with 23-bp spacers). In this study, we describe the detailed characterization of three atypical JxRSS-intronRSS, \(V_k\)-intronRSS, and JxRSS-Kde recombinations in human B cells and show how they fit in a scheme of sequential \(IGK\) recombinations.

Our data show that all three types of atypical rearrangements occur in B cell malignancies as well as in normal human tonsillar B cells. Semiquantitative analysis revealed that the classical \(V_k\)-\(J_k\), \(V_k\)-Kde, and intronRSS-Kde rearrangements are relatively frequent events, and that JxRSS-intronRSS and \(V_k\)-intronRSS and JxRSS-Kde rearrangements are less predominant (<5% of intron-Kde rearrangement levels, and ~10% of the \(V_k\)-Jx and \(V_k\)-Kde rearrangement levels) (Table IV). Based on this quantitation as well as the data from excision circle analysis and recombination substrate assays, a comprehensive and integrated model is proposed for the consecutive recombination events as they can occur in the \(IGK\) locus (Fig. 6). In this model, initial \(V_k\)-Jx recombinaction occurs once or multiple times until an in-frame combination is formed. If no in-frame rearrangement is obtained, two major inactivation pathways are available: 1) \(V_k\)-Kde (\(C_k\) deletion); or 2) \(V_k\)-Kde (Jx-Cx deletion) recombination. Alternatively, in a minor pathway, JxRSS-intronRSS signal joints might be formed. Perfect JxRSS-intronRSS signal joints (i.e., without deleted nucleotides) can undergo subsequent rearrangement, either to an upstream RSS element of a \(V_k\) segment (\(V_k\)-intronRSS pseudohybrid joints) or to the downstream RSS of the Kde (JxRSS-Kde pseudohybrid joints). Finally, by analogy to intronRSS-Kde formation, ongoing recombination from \(V_k\)-intronRSS rearrangements can theoretically result in \(V_k\)-Kde recombinations, unless the intronRSS became damaged during the \(V_k\) to JxRSS-intronRSS rearrangement. As this often proved to be the case (see \(V_k\)-intronRSS pseudohybrid joint sequences in Table III), this recombination step is depicted with dotted lines in Fig. 6. We consider the possibility of ongoing recombination of JxRSS-Kde pseudohybrid joints into \(V_k\)-Kde rearrangements not very likely either (dotted lines), because JsRSS-Kde couplings delete both enhancers (iE and \(3'\)E) that are known to be important for \(IGK\) recombination (21–23). Although \(V_k\)-Kde rearrangements are very prominent, they thus probably are not end-stage rearrangements in each \(IGK\) inactivation pathway (see Fig. 6).

Though infrequent, formation of signal joints without coding joints in the genome, as in case of JxRSS-intronRSS couplings, has been observed before. For example, we previously reported on the formation of D\(\delta 2\)-D\(\delta 3\) signal joints in an experimental human epithelial model system upon transfection of E2A and the RAG proteins (24). These signal joints appeared to result from an alternative recombination mechanism leading to direct coupling of the upstream RSS of the D\(\delta 2\) segment and the downstream RSS of the D\(\delta 3\) segment (24). Similar signal joints were occasionally found in thymocytes. All of these D\(\delta 2\)-D\(\delta 3\) signal joints concerned perfect couplings without deletion or N region insertion (24), which fits with the lack of N regions in most tonsillar JxRSS-intronRSS signal joints (80% of signal joints), but contrasts with the high frequency of imprecise JxRSS-intronRSS signal joints in B cell malignancies, particularly in precursor B-ALL. This unexpectedly high frequency of imprecise joints is probably caused by known continuous recombination activity in precursor B-ALL (25), resulting in further rearrangement of JxRSS-intronRSS couplings with perfect signal joints, but retention of the imprecise signal joints. Recently, we also found evidence for signal joint formation...
as an intermediate step in the V(DJ)-mediated oncogenic rearrangement in t (7, 9) in T-ALL (26). In thymocytes of healthy individuals, a signal joint configuration between the DJ or RSS and a fortuitous RSS in the TAL2 locus on chromosome 9 can occasionally be observed as a result of V(DJ)-mediated translocation. Further rearrangement between this highly reactive signal joint intermediate and a Jβ2 segment would then result in TAL2RSS-Jβ2 hybrid joints as they are observed in T-ALL with t (7, 9) (26).

An important issue concerns the functional implications of the atypical IGK recombinations. When JκRSS, being the most upstream JκRSS, is recombined to the intronRSS heptamer, Vκ-Jκ joints can no longer occur and hence no functional Igκ expression is possible from that allele. In that sense, JκRSS-intronRSS recombinations function as deletional rearrangements. The same holds for Vk-intronRSS and JκRSS-Kde rearrangements, which both most probably occur as secondary events following initial JκRSS-intronRSS signal joint formation. The lack of intact Jκ gene segments prohibits production of potentially functional Vκ-Jκ recombinations in cis in both cases. However, the functional implications are much less clear for Vκ-Jκ and Jκ2-RSS-intronRSS recombinations. In theory, splicing of an upstream intron Vκ-Jκ exon to the Cκ exon could result in Igκ expression in such a configuration, unless the loss of important regulatory elements in the region between the Jκ segments and the intronRSS heptamer would prevent this. So far, human and mouse Igκ enhancer sequences (IEκ) have only been identified in the 3′ part of the intron downstream of the intronRSS heptamer and not in the 5′ intron part (21–23). This is supported by our observation that JκRSS-intronRSS atypical joints did not block Vκ-Jκ-Cκ transcription and/or translation. Apparently, the initial occurrence of JκRSS-intronRSS signal joints (JκRSS-intronRSS excluded) might be a simple side event of an active V(DJ) recombinase system, but ongoing recombination to Vκ-intronRSS and JκRSS-Kde pseudohybrid joints leads to Igκ allele inactivation.

In summary, in this study, we show that in addition to the dominant classical rearrangements in the human IGκ locus (Vκ-Jκ, intron-Kde and Vκ-Kde), atypical IGκ recombinations (JκRSS-intronRSS, Vκ-intronRSS, and JκRSS-Kde) also occur, albeit at frequencies of <5% of intron-Kde and ~10% of Vκ-Jκ and Vκ-Kde (Table IV). As illustrated in the model (Fig. 6), initial Vκ-Jκ couplings can be followed by downstream JκRSS-intronRSS recombinations on the same allele. Because we could not identify functional implications of JκRSS-intronRSS couplings, we believe that such recombinations should be considered as occasional intermediates of an active V(DJ) recombinase; JκRSS-intronRSS recombinations are exceptional in that they do not allow functional Vκ-Jκ recombination and expression. Remarkably, in tonsils, the JκRSS-intronRSS couplings mostly, though not always, concerned perfect signal joints, whereas B cell malignancies (in particular precursor B-ALL) often showed damaged signal joints (Tables II and III). This can probably be explained by counterselection on perfect JκRSS-intronRSS signal joints for ongoing recombination in precursor B-ALL cells that are renowned for having a highly active V(DJ) recombinase. Undamaged JκRSS-intronRSS signal joints are likely to undergo further recombination to Vκ-intronRSS or JκRSS-Kde pseudohybrid joints, as shown in recombination substrate assays (Fig. 5). The formation of both types of pseudohybrid joints might represent an alternative pathway of allele inactivation and allelic exclusion for those IGκ alleles that have undergone initial aberrant recombinations: in case of Vκ-intronRSS recombination, no Jκ segments are left for functional IGκ expression, while JκRSS-Kde recombination deletes the Cκ region and enhancers, thereby preventing expression of an Igκ chain. Theoretically, the Vκ-intronRSS pseudohybrid joint could be involved in further recombination to Kde (Vκ-Kde coupling). However, damaged intronRSS (see also Table III) frequently preclude this and consequently the Vκ-intronRSS pseudohybrid joint will often be the end-stage rearrangement (Fig. 6). Analogously, the undamaged JκRSS of a JκRSS-Kde coupling might theoretically rearrange to the RSS of an upstream Vκ segment, but the loss of both IGκ enhancers in the (Vκ-Jκ) + JκRSS-Kde configuration probably blocks this, leaving the (Vκ-Jκ) + JκRSS-Kde rearrangement as end-stage configuration (Fig. 6). Although the usage of the here-described alternative IGκ recombination pathway is limited, this pathway is essential for inactivation of IGκ alleles with aberrant recombinations, which otherwise might hamper selection of functional IgL chain proteins.

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