Beyond the 12/23 Rule of VDJ Recombination Independent of the Rag Proteins

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Beyond the 12/23 Rule of VDJ Recombination Independent of the Rag Proteins

Alexandru Olaru,* Howard T. Petrie,‡ and Ferenc Livák2*†

The combinatorial repertoire of AgRs is established through somatic recombination of V, D, and J gene segments during lymphocyte development. Incorporation of D segments into IgH, TCRβ, and TCRδ chains also contributes to junctional diversification by substantially extending the length of the third CDR. The V, D, and J gene segments are flanked by recombination signals (RS) of 12- or 23-mer spacer length that direct recombination according to the 12/23 rule. D genes in the TCR chain are selectively extended by substantially extending the length of the third CDR. The V, D, and J gene segments are flanked by recombination signals (RS) of 12- or 23-mer spacer length that direct recombination according to the 12/23 rule. D genes in the TCRβ and TCRδ loci are flanked by a 12RS and 23RS, and their incorporation is controlled by mechanisms “beyond the 12/23 rule.” In the TCRβ locus, selective interactions between Rag proteins and the RS flanking the V-D and D-J genes, respectively, are sufficient to enforce D gene usage. In this article, we report that in the TCRδ locus, the Rag proteins are not the major determinant of D gene incorporation. In developing mouse and human thymocytes, the two Dδ genes rearrange predominantly to form D-D coding joints. In contrast, when tested in ex vivo transfection assays in a nonlymphoid cell line, the flanking RS mediate deletion, rather than incorporation, of the two D genes on both exogenous recombination substrates and the endogenous locus. These results suggest that selective Rag-RS interactions are not the sole regulators of D gene segment incorporation, and additional, perhaps lymphocyte-specific, mechanisms exist that allow proper shaping of the primary AgR repertoire.

may be beyond the control mediated by direct Rag protein-RS interactions.

Materials and Methods

Recombination substrates

The basic structure of the competitive recombination substrates has been described previously (14, 26) and is shown on Fig. 2D. For these studies, ~500 bp of the murine D61 and 180 bp of the murine D62 genomic DNA sequences were PCR-amplified and cloned into a CMV promoter-based plasmid. Two substrates were generated; one with the endogenous orientation (substrate 1) (S1) and one with both gene segments inverted (substrate 2) (S2) (see Fig. 2D). In addition, a custom m.w. marker was created by digesting one of the substrate plasmids with restriction enzymes ApaLI, Asel, BamHI, NcoI, NdeI, and NheI. This combination of digests results in a number of fragments, some of which hybridize to the probe used to detect recombination in the transfection assays, and allow us to measure precisely the size of the PCR-amplified bands of the rearranged substrates (see Fig. 2).

Cell preparation, flow cytometry, and genomic DNA analysis

CD3/CD4/CD8 triple negative (TN) thymocytes were isolated from pools of young, adult C57BL/6 mice, with two rounds of magnetic cell depletion following staining by specific Abs (27). TN cells were further fractionated according to the TN1–4 designation (28) using Abs specific for CD24, CD25, and CD44. All sorted cell subsets were ~95–98% pure as determined by flowcytometric reanalysis. Agarose gel-embedded, total genomic DNA was prepared from the purified thymocyte subsets as described previously (29). Human thymus samples were obtained from cardiovascular surgery patients according to protocols approved by the Oklahoma Medical Research Foundation. CD4+ immature single positive (ISP) cells were prepared by magnetic depletion of CD3+ and CD8+ cells followed by flowcytometric sorting of CD4+ cells. Crude deproteinized extracts (human CD4 ISP) and purified or agarose-embedded DNA were PCR-amplified using locus-specific primers (Table I) for 30–34 cycles. Transient transfections of the human embryonic kidney (HEK) 293 cells were conducted with 1 μg of substrate and 5–10 μg of each RAG expression plasmids (31) using the Ca phosphate-precipitation method (32). Both

Western blot

The 5 × 10^6 cell-equivalent lysates were separated through 8% SDS-PAGE, blotted onto polyvinylidene difluoride membrane (Millipore), and incubated with a mixture of polyclonal, rabbit anti-Rag-1 (a gift from Dr. D. Schatz, Yale University, New Haven, CT) and anti-Rag-2 (BD Pharmingen) or monoclonal anti-myc-epitope tag (9E10, Cymbus Biotechnology) Abs. Blots were developed using an ECL detection kit (Amersham Biosciences). Western blots were performed routinely on every transfection experiment to ensure consistent expression of the Rag proteins (see Fig. 2C).

Results

Predominantly D-D coding joint rearrangements during early thymocyte development

The 12/23 rule permits two types of rearrangement of the two D genes in the mouse TCRβ locus: coding joint formation directed by the internal 12/23RS pairs or deletion of the two D genes by signal joint formation between the two external RS (Fig. 1B). The two events can be distinguished by using high-resolution gel electrophoresis and ApaLI restriction digestion that can cleave only the typically precise signal joints but not the coding joints (33). D1 to D2 rearrangements were monitored using PCR in four successive stages of early mouse thymic development (28) of purified TN1–4 thymocyte subsets. D1 to D2 coding joint formation is expected to generate products of varying length of ~226–246 bp. Precise signal joint between the two external RS is expected to generate a single product of 209-bp length. D1 to D2 rearrangements are weakly detectable in TN1 cells, are most prominent in TN2 cells, and decline in TN3 and TN4 thymocytes (Fig. 1A), in accordance

<table>
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Quantitative analysis shows that in TN2 cells, fusion between the heptamers of the two external RS (Fig. 1 transiently transfected the nonlymphoid HEK293 cell line with predominant pattern of D1 to D2 rearrangements seen in vivo, we To determine whether the Rag proteins are sufficient to enforce the

Predominantly signal joint rearrangements are induced in extrachromosomal recombination substrates, which contain parts of the endogenous D6I and D82 genes, including the flanking RS and the entire coding segments (Fig. 2D). Transient transfection of the entire germline D1-D2 region is not feasible because it is >8800-bp long. We have created two recombination substrates: S1 contains the two genes in their endogenous configuration, whereas S2 contains them in inverted orientation (Fig. 2D). These substrates enable us to determine whether either the internal or the external RS pairs show preferential rearrangement and provide intrinsic controls for the potential bias of coding vs signal joint formation on recombination substrates (36). High-resolution agarose gel electrophoresis and ApaLI digestion of the PCR-amplified substrates can distinguish between coding and signal joint products as shown above (Fig. 1). Transfection of either S1 or S2 results in a single, predominant product, which migrates in a position that is consistent with a signal or coding joint rearrangement, respectively (Fig. 2A). Indeed, >90% of S1 forms an ApaLI-sensitive signal joint, whereas >80% of S2 forms an ApaLI-resistant coding joint (Fig. 2B). This results in exactly the opposite to that seen in vivo, where the endogenous locus (represented by S1 in the transfections) undergoes predominantly coding joint formation. This effect is not due to preferential signal joint formation on extrachromosomal substrates because the same biases are apparent in S2, which forms mainly coding joints instead of signal joints (Fig. 2B). It should be noted that S2 does appear to rearrange less efficiently in repeated experiments presumably, due to the reportedly less efficient formation of coding joints on extrachromosomal substrates (36). Nonetheless, even this bias cannot compensate effectively for the dominant use of the 5’T1 and 3’T2 RS pair. We conclude that the Rag proteins, operating on extrachromosomal recombination substrates, mediate preferential recombination between the external 5’T1 and 3’T2 RS pairs and would delete, rather than join, the two D gene segments (see S1 in Fig. 2).

TCRδ gene recombination in human cells

In the human TCRδ locus, the D2/D3 and J1/J3 genes are the orthologs of the mouse D1/D2 and J1/J2 genes, respectively (Ref. 37 and Fig. 3B). The human D1 and J2/J3 genes are used rarely in typical adult TCRδ rearrangements (38). We performed PCR assays to detect D2 to D3 and D2/D3 to J1 rearrangements in sorted, CD4 ISP and total human thymocyte samples. Human CD4 ISP cells correspond approximately to mouse TN3 thymocytes and are expected to have a significant amount of partial TCRδ locus recombination products (39). We could readily identify partial D2-D3, D3-J1, and D2-J1 rearrangements in both populations (Fig. 3A). ApaLI digestion of the D2-D3 rearranged products indicates that most of these rearrangements are ApaLI-resistant coding joints, in accordance with the findings in primary mouse thymocytes (compare Figs. 1A vs 3A). Low level V2-D3 and D2-D3 rearrangements of the endogenous TCRδ locus were also reported previously after transfection of Rag proteins into BOSC cells, a derivative of the HEK293 cell line (40). This observation prompted us to determine whether the endogenous chromosomal sequences behave similarly to the transfected recombination substrates in nonlymphoid cells. We also have found low level D2-D3 and D3-J1, but not D2-J1, rearrangements after transient overexpression of RAG-1 and RAG-2 alone in HEK293 cells (Fig. 3C). Importantly, predominantly ApaLI-sensitive D2-D3 PCR products were detected, indicating that the majority of D2-D3 rearrangements were precise, signal joint of the external RS that deleted the two D genes (Fig. 3D). Similarly, DNA sequencing also demonstrated the presence of only signal joint rearrangements in BOSC cells, irrespective of the cotransfection of E2A or HEB transcription factors (40).

Collectively, these results demonstrate that overexpression of the Rag proteins in a nonlymphoid cell line results in deletion of the two Dδ genes and signal joint formation between the external RS pair, both on extrachromosomal substrates and on the endogenous, chromosomal locus. The data also show that, in contrast to

FIGURE 1. The pattern of endogenous TCRδ D1-D2 rearrangements in mouse thymocytes in vivo. A, D1 to D2 rearrangements are PCR-amplified from sorted mouse TN thyocyte subsets (TN1–4). Undigested and ApaLI-digested products are shown. RAG-2 gene amplification served as a control of DNA quantity and quality. The positions of the diverse D1-D2 coding joints (CJ), the signal joint (SJ), and the ApaLI-digested fragments are shown (left). Numbers (right) indicate m.w. marker in base pairs. B, Schematic representation of the mouse TCRδ locus and the pattern of possible D-D rearrangements. The creation of the ApaLI restriction site (underlined) upon signal joint formation is shown. Open/dotted triangles indicate 12RS, and closed/shaded triangles indicate 23RS. Boxes represent the gene segments, and arrows indicate the position of the primers used for PCR amplification.

with previous findings that demonstrated the sequential progression of TCR gene recombination during mouse thyocyte development (34, 35). Using high-resolution sieving agarose electrophoresis, we could detect a predominant, larger, broader, and a slightly smaller, sharper band (Fig. 1A). ApaLI restriction digestion confirmed that the shorter, but not the longer, products contained an internal ApaLI site, which is diagnostic for the precise fusion between the heptamers of the two external RS (Fig. 1B). Quantitative analysis shows that in TN2 cells, ~90% of the entire PCR is ApaLI-resistant and only 10% is ApaLI-sensitive (Fig. 1A). The relative amount of ApaLI-sensitive products is higher in the TN3 and TN4 populations (24) and even more dominant in TN1 cells (Fig. 1A and see Discussion). These results demonstrate that in vivo, at the TN2 stage of mouse thyocyte development, where TCRδ recombination becomes prominent (34, 35), D1 to D2 rearrangements form predominantly coding joints. Importantly, only D1-D2 coding joints, but not the signal joints that delete the two D genes, can continue to generate the typical, VDDIδ joints that are found in mature T cells.

Predominantly signal joint rearrangements are induced in nonlymphoid cells

To determine whether the Rag proteins are sufficient to enforce the predominant pattern of D1 to D2 rearrangements seen in vivo, we transiently transected the nonlymphoid HEK293 cell line with expression constructs of the murine RAG genes (Fig. 2C) along with extrachromosomal recombination substrates, which contain parts of the endogenous D6I and D82 genes, including the flanking RS and the entire coding segments (Fig. 2D). Transient transfection of the entire germline D1-D2 region is not feasible because it is >8800-bp long. We have created two recombination substrates:
FIGURE 2. The pattern of D81-D82 rearrangements of recombination substrates ex vivo after transfection into HEK293 cells. A, Southern blot analysis of PCR products generated after cotransfection of the indicated substrates (Subst) and full-length (f) or core (c) Rag proteins. The positions of the predicted D-D coding (CJ) or signal joints (SJ) are shown (right). Numbers (left) indicate m.w. marker in base pairs. The results of two of five independent transfections are shown. B, ApaLI digestion of gel-purified PCR products derived from Fig. 2A. ApaLI-digested (+) and mock-digested (−) samples are shown. Because both unarranged S1 and S2 contain one endogenous ApaLI site, complete digestion of the unarranged substrates (first two lanes; see asterisks marking the position of the digested bands) was used to verify the efficiency of the assay. Note that this, germline, ApaLI site is retained in S1, but not in S2, after rearrangement, resulting in two smaller restriction fragments without the de novo ApaLI site (CJ-type rearrangement; see arrowheads) and two more smaller fragments if the de novo ApaLI site is present (SJ-type rearrangement; see bracket). Other markers are the same as in Figs. 1A and 2A. C, Western blot analysis of transient transfections of HEK293 cells. Polyclonal α-Rag-1 and Rag-2 (top) and monoclonal α-myc-epitope tag (bottom) Abs were used to detect full-length (f) and core (c) Rag proteins, as indicated. Only the core proteins are myc-epitope tagged, and α-Rag-1 recognizes only full-length, but not core, Rag-1. D, Schematic structure of the two recombination substrates with the possible rearranged products and their size are indicated. Arrowhead marks the position of an endogenous, germline ApaLI site. The creation of the de novo ApaLI restriction site (underlined) upon signal joint formation and the lack of ApaLI site upon coding joint formation (dots represent varying number of nucleotides) are illustrated. At the bottom, the position of the predicted restriction fragments of the unarranged and rearranged substrates (sub) are shown with (+) or without (−) ApaLI digestion.

Deletion of the two TCRδ D genes can occur only on chromosomes with germline configuration, because either V-D or D-J partial rearrangements would remove one of the external RS flanking the D genes (see Discussion). Although D to D rearrangement clearly occurs in both mice and humans, if these were only extremely minor products during thymic development, the physiological significance of biased RS usage would be diminished greatly. To assess the relative proportions of the possible partial TCRδ rearrangements during early thymocyte development, we performed PCR assays on purified mouse TN1–4 subsets. As expected (34, 35), few partial or complete rearrangements are found in TN1 cells (Fig. 4). Substantial amounts of partial, D2-J1 and D1-D2 rearrangements are seen in TN2 cells. These partial rearrangements are reduced in later stages at the expense of partial D1-(D2)J1 and complete V4-DDJ1 rearrangements (Fig. 4). We could detect only very few V4-D1 partial rearrangements in TN3 cells, a type of rearrangement thought to occur more frequently during human T cell development (41).

To assess more precisely the accessibility of TCRδ gene segments for recombination, we monitored the appearance of RS end breaks, an indicator of ongoing gene rearrangement (30, 33), using an anchor ligation-mediated PCR assay (42). We found a significant number of breaks only at the two RS flanking the D2 gene in TN2 cells and increasing amount of breaks of the 3′D1, 5′D1, 5′J1, and 3′V RS in TN3 cells (Fig. 5A). TN4 cells, many of which are actively undergoing cell division after pre-TCR signaling (43), contain very few breaks of any RS in accordance with their more advanced developmental as well as higher proliferation status (42). These results indicate that the order of TCRδ locus recombination, similarly to other Ig and TCR loci, also exhibits tight developmental control. It appears that, in mouse, the D1 and D2 gene segments become accessible first, as shown by the predominant accumulation of 5′/3′D2 signal end breaks in TN2 cells (Fig. 5) and by the accumulation of partial D1-D2 and D2-J1 rearrangements (Fig. 4). This is followed by D1(D2)-J1 recombination and, later, by complete VDJδ rearrangements as the 5′D1 and 3′V RS become accessible in TN3 cells (Fig. 5A). The absence of 5′J1 signal end breaks in TN2 cells may be due to the lower efficiency of detection of this break because it generates the largest PCR product of all the ligation-mediated PCR.

Discussion

Several mechanisms shape the primary AgR repertoire during lymphocyte development. Recently, multiple examples have been presented where specific interactions between the Rag proteins and RS motifs dominantly influenced the choice of gene segment usage (12–14, 26, 44). We hypothesized that similar Rag protein-dependent mechanisms could govern incorporation of the two D gene

nonlymphoid cells, the endogenous TCRδ locus undergoes predominantly coding joint formation in both murine and human primary thymocytes.

The kinetics of TCRδ locus recombination during early mouse thymocyte development

Discussion

Several mechanisms shape the primary AgR repertoire during lymphocyte development. Recently, multiple examples have been presented where specific interactions between the Rag proteins and RS motifs dominantly influenced the choice of gene segment usage (12–14, 26, 44). We hypothesized that similar Rag protein-dependent mechanisms could govern incorporation of the two D gene
segments into the TCRδ chain. Surprisingly, however, our transfection experiments demonstrate that the Rag proteins, by themselves, are insufficient to enforce TCRδ D gene incorporation. This observation stands in striking contrast to the TCRβ locus where isolated RS/coding end combinations and their interactions with the Rag proteins were sufficient to reproduce the specific patterns of both V-D and D-J recombination (12–14, 26).

Two possible mechanisms could ensure preferential incorporation of the mouse D1-D2 (human D2-D3) genes during TCRδ locus recombination. One mechanism is based on the sequential order of TCRδ locus recombination, which we would term “preemptive utilization” of RS. In this case early, preferential D2 to J1 rearrangements would use up the 3’D2 RS, preventing it from participating in the D gene-deleting signal joint formation with the 5’D1 RS. Our analysis on the kinetics of TCRδ locus recombination indicates that such D2-J1 rearrangements do occur at significant levels in immature murine thymocytes (Figs. 4 and 5). In humans (41), although not in mouse (see Fig. 4), early V to D1 partial rearrangements may function also in a similar manner by removing the 5’ D1 RS before it could become a target for signal joint rearrangement. In either case, such sequential events would ensure that at least one, and possibly both, D genes are incorporated into the rearranged TCRδ locus. Similar observations were made in the IgH locus where preferential accessibility of the 3’D4 and 5’J5 RS results in the typical deletional, rather than inversional, D-J5 rearrangements (45). This bias also coincides with the reported preference of the Rag proteins to the 3’D4, instead of 5’D1 RS (46), which may play an important role in promoting biased recombination of the 3’D4 RS over the 5’D4 RS. In the TCRδ locus, a similar model would postulate that preferential interactions between the 3’D2 and 5’J1 RS (in mouse) or 3’V and 5’D1 RS (in humans) would result in preemptive rearrangements, and the consequential elimination of the 5’D1/3’D2 RS pair that could potentially delete the two D genes.

The second mechanism operates at the level of D-D gene rearrangement, which also occurs at significant levels in TN2 thymocytes. This mechanism must distinguish between the outside (5’D1/3’D2) and inside (3’D1/5’D2) RS pairs to regulate D gene incorporation. Our most unexpected finding is the discrepancy between the D-D rearrangement pattern observed in vivo (Figs. 1 and 3A) and ex vivo (Figs. 2 and 3C and 3D). We believe that the result of the ex vivo transfection assay is not an artifact because sequence comparison of the conserved heptamer/nonamer motifs shows that the external, 5’D1/3’D2 RS, which are favored by the Rag proteins ex vivo, are more similar to the consensus (3) than the two internal RS (Fig. 5B). Particularly striking is the sixth position variation in the nonamer of the mouse 3’D1/human 3’D2 RS (Fig. 5B, arrow). Nonconsensus residues in this position have been shown to severely reduce recombination in various experimental assays (3, 47). The mere chromosomal context also cannot explain our findings because the endogenous TCRδ locus in HEK293 cells shows the same preferences as the extrachromosomal substrates (Fig. 3 and Ref. 40). One possible hypothesis is that a lymphoid, perhaps T cell-specific, cofactor alters the targeting preferences of the Rag proteins specifically in favor of the less efficient RS pair. Such targeting could be achieved at the level of direct Rag protein binding to the DNA or by regulating the assembly and/or stability of the Rag-RS synaptic complex. Such a hypothetical cofactor could be involved specifically in the control of D8 gene selection or in the general control of TCR gene segment usage in T cells. It could be expressed throughout thymic development or only in certain stages. Interestingly, we found significant differences in the relative proportion of D1-D2 coding vs signal joints in distinct subsets of TN thymocytes (Fig. 1A). A relative decline of D1-D2 coding joints in TN3 and TN4 cells could be explained by the fact that only coding joints, but not the signal joints, can participate in complete V/DJδ rearrangements that appear predominantly from the TN3 stage (35). It is more intriguing to find even higher proportions, albeit low absolute levels, of signal joint products in TN1 cells. Part of this finding could be due to contamination by mature γδT cells, which share some phenotypic features with our TN1 preparation (Fig. 4 and Ref. 48). However, the unusually high proportion of signal joint rearrangements could also indicate that D1-D2 recombination may initiate in genuine TN1 cells before or
during the transition to TN2 differentiation. An intriguing proposition is that at this earliest stage, the hypothetical cofactor is not present, or not active yet, and rearrangement would proceed according to the preference of the already active Rag proteins (49, 50). This would result in deletion, rather than joining of the two D genes, similarly to what we see at the endogenous TCRβ locus in HEK293 cells (Fig. 3, C–D).

Alternatively, developmental regulation of RS preferences is achieved through the more traditional concept of accessibility control of gene rearrangement (51), which plays an important role in the overall patterning of TCRβ locus recombination (reviewed in Ref. 52). In this case, we would have to assume that chromosomal accessibility is blocked selectively or enhanced to the more or less efficient RS pairs, respectively. Such a fine control of accessibility, within the range of 11–16 bp, i.e., the distance of the 5′ and 3′ RS motifs, has been proposed to operate on the D11 genes (45), perhaps through remodeling of the nucleosomal phasing that can influence Rag protein-mediated cleavage in vitro (53, 54). The consensus, A-T-rich nonamer has been shown to help position the RS onto the nucleosome (55), which typically would inhibit the access of Rag proteins to the RS (56, 57). It is possible that the less efficient 3′D1 RS, with a nonconsensus nonamer (Fig. 5B), becomes relatively more accessible in vivo due to its less efficient positioning onto the nucleosome, although it must be emphasized that the published studies showed modest differences in accessibility and used RS sequences different from those studied in this article (55). Chromosomal accessibility is under the control of cis-acting elements, such as the TCRβ enhancer, which has been shown to be required for fully efficient TCRβ locus recombination (58). In TCRβ enhancer-deficient mice, relatively more partial D-J and D-D rearrangements accumulate, and it will be interesting to determine whether the proportion of D1-D2 coding vs signal joint rearrangements is altered compared with wild-type cells.
CONTROL OF D GENE USE IN THE TCRγ locus


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