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Mechanisms controlling expression of the *RAG* locus during lymphocyte development

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Summary

Recombination activating genes (*RAG*)1 and *RAG*2 are expressed in developing B and T lymphocytes and are required for the rearrangement of antigen receptor genes. In turn, *RAG* expression is regulated by the products of these assembled immunoglobulin (*Ig*) and T-cell receptor (*TCR*) genes. Upon successful assembly of *Ig* genes, the antigen receptor is expressed on the immature B cell surface and tested for autoreactivity leading to either maintenance or inactivation of *RAG* expression. Successful assembly of *TCR* genes is followed by surface *TCR* expression and testing for its ability to interact with self-MHC, which if appropriate leads to the inactivation of *RAG* expression. Recent studies in B and T lymphocytes demonstrate that the reduction in *RAG* expression at the immature B and double-positive (DP) T cell stages is mediated through tonic (foreign antigen independent) receptor signaling. In B cells, tonic signaling activates PI(3)K and Akt kinases, which phosphorylate and lead to the cytoplasmic sequestration of FoxO proteins, key transcriptional activators of *RAG* expression. In T cells, tonic signaling activates Abl and Erk kinases, leading to the transcriptional inactivation of *RAG*s.

Introduction

*RAG*1 and *RAG*2 are essential components of the enzyme complex that initiates assembly of antigen receptor genes from their component V, D, and J gene segments in both B and T cells [1,2]. Null mutations in either *RAG*1 or *RAG*2 result in a complete and early block in both B and T lymphocyte development [3,4]. The *RAG* complex recognizes and cleaves specific DNA elements known as recombination signal sequences (RSSs) that flank rearranging gene segments. Subsequently, additional proteins are recruited to mediate joining of the broken DNA ends. This process is essential to generate the diverse array of antigen receptors and thus for the function of lymphocytes in adaptive immunity (reviewed in [5]).

The structure of the *RAG* locus is unusual (Figure 1). *RAG*1 and *RAG*2 are convergently transcribed, contain single large coding exons and are separated by only ~8kb. These observations led various workers to propose that the *RAG* genes entered the vertebrate genome as part of a transposable element [6].

The fluctuation in *RAG* activity during B and T cell development is a consequence of multiple levels of regulation [7,8]. Transcriptional regulation of the *RAG* genes has been studied most extensively, but several reports have described post-translational regulation. Genomic DNA

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sequences surrounding the *RAG* genes have been exhaustively fragmented and assayed in an attempt to identify cis-elements that activate or repress *RAG* transcription in B or T cells at different developmental stages. Both in vitro and in vivo experiments led to the identification of several cis-regulatory elements in the *RAG* locus, but these alone can not explain the pattern of *RAG* expression during B and T cell development. One additional mechanism that has been described is the regulated destruction of RAG2 protein via phosphorylation and the ubiquitin-proteosomal pathway [9,10]. Much recent attention has focused on understanding the signaling molecules involved in *RAG* regulation. In this review we will focus on the various levels of *RAG* regulation.

RAG expression

Transcriptional regulation of the *RAG* locus has been studied by monitoring RNA levels in sorted cells and by using various green fluorescent protein (GFP) reporter mice. One group generated transgenic mice carrying an artificial V(D)J recombination substrate that activates GFP expression as a consequence of *RAG* activity [11]. GFP expression persists in this case regardless of ongoing *RAG* transcription. Three other groups generated *RAG* transcription reporter mice. One group used gene targeting to generate a RAG2-GFP fusion protein [12], a second group used a bacterial artificial chromosome (BAC) transgene encoding GFP in place of RAG2 [13], and a third group replaced RAG1 with GFP by gene targeting [14,15]. Each of these systems demonstrated similar *RAG* expression patterns. The three latter groups concluded that *RAG* is not expressed and cannot be reinduced in *RAG*-negative mature B cells as had been previously suggested [16–18]. However, other studies continue to suggest that the *RAGs* can be reactivated leading to “receptor revision” in mature lymphocytes. [19,20]. In humans, *RAG* expression has also been reported in mature B cell subsets [21] and can be induced by IL-6 [22]. This area remains controversial.

RAG expression is in general restricted to B and T lymphocytes although a small fraction of early hematopoietic progenitors express *RAG* mRNA [23] and occasional NK and dendritic cells show evidence of V(D)J recombination [11]. Earlier reports suggesting *RAG* expression in the brain have not been confirmed by others [24]. In developing B cells, *RAG* is first detected in fraction A1 of the Hardy classification scheme [25] and developing T cells in the double negative 2 (DN2) stage [12]. In both lymphoid lineages, *RAG* expression occurs in two waves. The first wave results in assembly of the immunoglobulin heavy chain (*IgH*) or *TCRβ* chain genes in pro-B or pro-T cells, respectively. *IgH* or *TCRβ* chain expression activates signaling pathways that result in the downregulation of *RAG* expression during a period of pre-B or pre-T cell proliferation. Pre-B and pre-T cells then exit the cell cycle and activate a second wave of *RAG* expression leading to the assembly of immunoglobulin light chain (*IgL*) or *TCRα* chain genes, respectively. These two waves of *RAG* expression were observed in all three *RAG* reporter mice [12–14]. Immature B cells continue to transcribe the *RAGs* if they express an autoreactive antigen receptor leading to receptor editing [26]. *RAG* expression is inactivated in *IgD*⁺ B cells [8]. DP T cells continue to express *RAGs* and rearrange the *TCRα* locus until they undergo positive selection and transit to the single positive (SP) stage [7].

Interestingly, *RAG1* and *RAG2* are not the only genes in the *RAG* locus. A novel third gene, named *NWC*, was found to overlap the *RAG* genes [27]. *NWC* is ubiquitously expressed and is regulated by its own promoter, located within the second intron of *RAG2* (Figure 1). In developing lymphocytes, *NWC* is expressed from the *RAG1* promoter and contains *RAG1*'s first (non-coding) exon spliced to the second exon of *NWC*, which encodes *NWC*'s initiator methionine codon. The discovery of the *NWC* gene within the *RAG* locus led to the proposal that negative regulation of *RAG* expression may involve *NWC*-mediated transcriptional interference [28] (Figure 1). In addition, could the simultaneous transcription of *RAG1/NWC*

hybrid and RAG2 transcripts result in the generation of siRNAs? This remains to be determined.

Signaling Pathways

The signaling pathways regulating *RAG* expression at various stages of development are an area of active investigation. Several groups demonstrated that tonic B cell receptor (BCR) signaling in immature B cells represses *RAG* transcription through phosphatidylinositol-3-OH kinase (PI(3)K) [29–31] and Akt [32,33]. This stage in B cell development is critical in deciding if the BCR is autoreactive and if so, maintaining *RAG* expression for further editing of the expressed light chain. Indeed, when primary immature B cells are induced to undergo receptor editing, BCR internalization occurs, which reduces signaling via PI(3)K and Akt [30]. The inhibition of this pathway was found to reduce the phosphorylation of FoxO transcription factors, specifically FoxO1 and Foxo3a [32,33]. This pathway was found to be regulated through the adaptor protein SLP-65 by counteracting Akt activation [32]. Additionally, *RAG* expression is suppressed by IL-7, which is known to signal through PI(3)K and thus FoxO proteins [33,34]. However, since IL-7 is a potent proliferative signal for early B cell development, the suppression of *RAG* activity may also be the result of *RAG2* turnover in cycling pre-B cells. Interestingly, in v-abl transformed pro-B cells, *RAG* transcription is regulated by the activation of a stress-regulated protein, GADD45a [33]. This pathway is dependent on the activation of MEKK4 and p38, which also regulate FoxO1. The GADD45a pathway does not seem to regulate *RAG* transcription in primary cells, however.

T cells also display a steady-state level of “tonic” TCR signaling in unstimulated cells. A DNA microarray study comparing gene expression in unstimulated Jurkat T cells and various mutant derivatives deficient for individual signaling molecules uncovered an increase in *RAG* expression in LAT-deficient cells [35]. This suggests that signaling through LAT represses *RAG* expression. In primary thymocytes, which are mostly DP cells, these workers found that the repression of *RAG* expression is directed by a constitutive low-level activity of Erk and Abl kinases mediated through the adapter molecules LAT and SLP-76. Additionally, in T cells, activation of Akt inhibits nuclear activity of NFATc1, a negative regulator of *RAG* expression [36].

Transcriptional Regulation

In transient transfection assays, the activity of the *RAG1* promoter appears promiscuous while the *RAG2* promoter shows a degree of lymphoid specificity. Several studies have demonstrated binding of specific transcription factors to the *RAG2* promoter including PAX5, MYB, SP1, LEF1, NF-Y, C/EBP, GATA3, and NFATc1 (reviewed in [37])[36]. As in other systems, distant sequences are required to cooperate with the promoters in regulating *RAG* transcription (Figure 2). Interestingly, these regulatory elements differ in B and T cells. Studies using transgenic *RAG* reporter mice showed that the first 10kb 5' of the *RAG2* first exon is adequate to drive expression in B cells and DN but not DP T cells [25,38]. Specifically, the Alt group identified two regions, a 2kb sequence that is sufficient to drive B and T cell development and the region from 2–7kb 5' of *RAG2* that generates normal B but not T cell expression [25,38]. However, targeted deletion of the 2kb sequence in mice did not affect B or T cell development. The authors conclude that this may be the result of redundant cis-acting elements. Muraguchi's group also identified DNaseI hypersensitive sites 8kb (called D3) and 2.6kb (called Ep) 5' of the *RAG2* promoter [39]. In reporter assays, Ep was demonstrated to have enhancer activity in B but not T cell lines, which is similar to the 2–7kb region described by the Alt group. The D3 region showed activity in B and DN T cells, which is consistent with previous studies of the first 10kb 5' *RAG2* [39,25,38]. A 1.7kb sequence (known as Erag.), ~23kb upstream of the *RAG2* promoter, was shown to be a *bona fide* enhancer in B cells [40]. Deletion of this sequence

from the mouse germline resulted in a 5–10 fold decrease in *RAG* expression and a partial block at the pro-B to pre-B transition but no effect in T cells. Furthermore, E2A, Foxp1, and FoxO1 bind to Erag and likely regulate its activity [41,42].

In T cells, two cis-elements were identified and shown to function together. Using BAC-transgenic reporter mice, an interval between 32kb and 87kb 5' of the *RAG2* promoter was found to be required for reporter expression in DP T cells [25]. These studies culminated in the identification of an anti-silencing element (ASE), ~70kb 5' of the *RAG2* promoter that directs *RAG* expression in DP T cells and counteracts a silencer element found in between *RAG1* and *RAG2* [43]. The physiological relevance of the ASE was confirmed by deleting the sequence between 71kb to 86kb 5' of the *RAG2* promoter in the mouse germline. These mice showed *RAG* expression in B and DN T cells but not in DP T cells.

A network of transcription factors initiates commitment to the B and T cell lineages. Many of these have also been implicated in the regulation of *RAG* transcription. Targeted deletion in mice of the genes encoding transcription factors E2A [44], Foxp1 [42], FoxO1 [41], and Ikaros [45] each show a deficiency in *RAG* expression in B cells. In T cells, a zinc finger protein named ZFP608 is expressed in neonatal thymus and is repressed thereafter to allow *RAG* transcription. Thus, ZFP608 is a repressor of *RAG* expression [46]. Finally, studies of receptor editing in B cells revealed roles for NF- κ B and FoxO1 in the regulation of *RAG* transcription in response to self-antigen [32,33,41,47]. In this instance, *RAG* regulation was confirmed to be at the level of transcription initiation [47].

Posttranscriptional Regulation

An additional level of regulation is at the level of protein stability. Li and Desiderio first observed that the oscillation in *RAG2* protein level in dividing lymphocytes was not accompanied by similar changes in transcript level [9]. *RAG2* protein peaks in quiescent (G_0) and G_1 phase cells and is degraded in the transition to S phase. It was demonstrated that phosphorylation by cyclinA/CDK2 at threonine 490 of *RAG2* initiates the ubiquitin-proteasomal pathway mediated by Skp2-SCF ubiquitin ligase [9,10].

Conclusions

Significant advances have occurred in recent years in our understanding of the transcriptional regulation of *RAG* expression. The recent identification of signaling pathways, transcription factors, and distant regulatory elements directing *RAG* expression in B and T cells has revealed some of the physiological signals controlling V(D)J recombination. It remains controversial, however, whether any physiological signals induce the reactivation of *RAGs* in mature B and T cells.

It is intriguing that while both B and T cells require *RAG* for the assembly of their antigen receptor genes, each cell type regulates the *RAG* locus using different transcription factors and cis-regulatory elements. A challenge for the future is to understand how BCR and TCR activities result in either continued *RAG* expression or its cessation depending upon the nature of signaling pathway engagement. Since Erag and ASE deletions result only in partial loss in *RAG* activity, additional regulatory elements and their cognate binding factors remain to be discovered. In addition, whether and how *NWC* transcription may interfere with the expression of *RAGs* in lymphocytes remains to be determined. One unexplored issue is how *RAG1* and *RAG2* are coordinately regulated by enhancer elements that seem restricted to the region 5' of *RAG2*. Might there be a single transcription factor that brings the two promoters together in both B and T cells? This could be addressed using recently developed techniques that allow one to examine the spatial structure of the genome at a molecular level [48].

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Figure 1. Rag and NWC locus

Approximate locations of exons encoding UTRs (yellow and orange boxes) and coding regions (blue and red boxes) of Rag 1/Rag 2 and the first two exons of NWC. Below the locus are patterns of spliced transcripts for RAG1, RAG2, RAG1/NWC hybrid, and NWC.

A schematic diagram of the RAG locus and cis-regulatory elements in B and T cells. Yellow boxes indicate UTR and blue boxes indicate coding regions. Boxes upstream of the RAG2 promoter are regulatory elements. Below the locus is a chart of transcription factors known to bind the RAG locus and their binding sites.