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A Role for MAPK in Feedback Inhibition of Tcrb Recombination

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The Tcrb locus is subject to a host of regulatory mechanisms that impart a strict cell and developmental stage-specific order to variable (V), diversity (D), and joining (J) gene segment recombination. The Tcrb locus is also regulated by allelic exclusion mechanisms, which restrict functional rearrangements to a single allele. The production of a functional rearrangement in CD4<sup>+</sup>CD8<sup>+</sup> double-negative (DN) thymocytes leads to the assembly of a pre-TCR and initiates signaling cascades that allow for DN to CD4<sup>+</sup>CD8<sup>+</sup> double-positive (DP) differentiation, proliferation, and feedback inhibition of further Vß to DJß rearrangement. Feedback inhibition is believed to be controlled, in part, by the loss of Vß gene segment accessibility during the DN to DP transition. However, the pre-TCR signaling pathways that lead to the inactivation of Vß chromatin have not been determined. Because activation of the MAPK pathway is documented to promote DP differentiation in the absence of allelic exclusion, we characterized the properties of Vß chromatin within DP thymocytes generated by a constitutively active Raf1 (Raf-CAAX) transgene. Consistent with previous reports, we show that the Raf-CAAX transgene does not inhibit Tcrb recombination in DN thymocytes. Nevertheless, DP thymocytes generated by Raf-CAAX signals display normal down-regulation of Vß segment accessibility and normal feedback inhibition of the Vß to DJß rearrangement. Therefore, our results emphasize the distinct requirements for feedback inhibition in the DN and DP compartments. Although MAPK activation cannot impose feedback in DN thymocytes, it contributes to feedback inhibition through developmental changes that are tightly linked to DN to DP differentiation. The Journal of Immunology, 2006, 176: 6824–6830.
demonstrated to promote DP differentiation in pre-TCR-deficient mice (11, 19, 22, 23). In addition, the LckF505 and PKCα-CAT transgenes provide allelic exclusion signals as shown by the inhibition of Vβ to DJβ rearrangements at the endogenous Tcrb locus (11, 19). However, developing thymocytes containing Ras(−/−) or Raf-CAAX transgenes displayed normal levels of endogenous Vβ to DJβ rearrangement (22, 23). Thus, Ras(−/−) and Raf-CAAX are thought to promote DN to DP differentiation but not contribute to allelic exclusion. Because deletion of Lcp5 (encoding SLP-76) results in a loss of thymocyte differentiation and a loss of allelic exclusion (24), it was concluded that the signaling pathways involved in differentiation and allelic exclusion diverge downstream of SLP-76 but upstream of Ras (4, 5).

To better understand the relationship between pre-TCR signaling and the developmental changes at the Tcrb locus associated with allelic exclusion, we analyzed the properties of DP thymocytes generated by a constitutively active Raf-CAAX transgene. Consistent with previous publications, we show that the Raf-CAAX transgene does not extinguish Tcrb recombination in DN thymocytes. However, DP thymocytes generated by Raf-CAAX signals display normal down-regulation of Vβ segment transcription and accessibility and normal feedback inhibition of Vβ to DJβ rearrangement. Thus, although the MAPK pathway is not sufficient to enforce Tcrb allelic exclusion, it clearly contributes to the feedback inhibited state in DP thymocytes. Our data suggest distinct requirements for Tcrb feedback inhibition in DN and DP thymocytes and indicate that a component of the feedback mechanism is tightly linked to DP differentiation.

Materials and Methods

Mice

C57Bl/6 (B6), 129, Rag2−/− mice, Rag2−/− × Tcrb transgenic mice (Rxβ) (25), Raf-CAAX transgenic mice (23), and Lcr−/− mice (17) were housed at the Duke University Vivarium. Lcr−/− mice were a gift from W. Zhang (Duke University, Durham NC). Raf-CAAX transgenic mice were a gift from R. Perlmutter (Amgen, Thousand Oaks, CA). All mice were used in accordance with protocols approved by the Duke University Institutional Animal Care and Use Committee.

Flow cytometric analysis and thymocyte isolation

Surface staining was performed on thymocytes according to standard staining protocols. All Abs were purchased from BD Pharmingen. In all instances, cells were initially incubated for 5 min with a mAb specific for CD16 and CD32 (clone 2.4G2) to block nonspecific staining. Analysis and sorting of DN and DP thymocytes was conducted using FITC-conjugated anti-CD4 (clone GK1.5) and PE-conjugated anti-CD8 (clone 53-6.7). The sorting of DN and DP thymocytes was conducted using FITC-conjugated anti-TCR (clone H57-597) according to the Cy-Chrome-conjugated anti-TCR staining, thymocytes were surface stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD8. The thymocytes were then permeablized and stained intracellularly with CyChrome-conjugated anti-TCR (clone H57-597) according to the Cytopher/Cytolx kit (BD Pharmingen). TCRβ staining was evaluated in the CD4−CD8− population. Cell sorting and analysis were conducted using a FACScanfln (BD Biosciences) or FACSVantage SE (BD Biosciences) and CellQuest software.

RT-PCR

RNA was extracted from cells using TRIzol (Invitrogen Life Technologies) according to the manufacturer’s instructions. For germline transcription analysis, contaminating genomic DNA was removed with DNA-free (Ambion) according to the manufacturer’s instructions, and cDNA synthesis was performed with Transcriptor (Roche) and random hexamer primers (Roche) according to the Roche protocol. For analysis of Rag1 and Rag2 expression, cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies) and an oligo(dT) primer (Invitrogen Life Technologies) according to manufacturer’s protocol. PCR analysis of germline transcription was performed on 3-fold serial dilutions of cDNA using a “touchdown” PCR strategy: 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at annealing temperature, and 1 min at 72°C, and a 10-min extension at 72°C. Annealing temperature was held at 68, 65, and 62°C for 5 cycles each and at 58°C for 17 cycles. Amplicons were electrophoresed through agarose gels and visualized by ethidium bromide staining. VB primers used for germline transcription were positioned in the leader sequence and downstream of the RSS, respectively. Primers for Vβ11, Vβ12, DJβ, and Act-β (9) and Tcra (26) were previously described. Tcrb primers were 5′-AGAACCTCGTGGTACCCAGGT-3′ and 5′-GAGTCAGCCTCTGCACTTCT-3′.

Chromatin immunoprecipitation

Thymocytes were harvested and mononucleosomes were prepared as previously described (27). Mononucleosomes (25 µg) were immunoprecipitated with 5 µg each of Abs against diacetylated histone H3, tetracyacetylated histone H4, dimethylated histone H3K4, and control rabbit IgG (Upstate Biotechnology). The bound and input fractions were quantified by real-time PCR using SYBR green and a Light Cycler (Roche). Ratios of bound/input were calculated and were normalized to those for carbamoyltransferase dihydrolase (Cad) in each sample. Primers for Vβ12, Vβ13, T4/T8, and Cad have been previously described (9). Db1 primers were 5′-GATCCAGAACTGTTCTACGG-3′ and 5′-CTGACTCTTTTGTGGCTCTA-3′.

Analysis of V(D)J recombination products

VDJβ and DJβ coding joints were analyzed by touchdown PCR (as described above) using a template genomic DNA prepared from thymocytes. To analyze signal end intermediates, thymocyte genomic DNA was digested and linker ligation was performed as previously described (28). Linker-ligated DNA was then used to amplify signal end intermediates by touchdown PCR (as described above). Cds4 was amplified by PCR as follows: 94°C for 5 min, followed by 20 cycles of 94°C for 30 s, 62°C for 30 s, 72°C for 1 min, and a 10-min extension at 72°C. Amplicons were electrophoresed through agarose gels and visualized by Southern blot using 32P-labeled oligonucleotide probes. The signal end primer for Vβ6 was 5′-GTCGTTAGCTACCTGGTTGTCAGGTC-3′. Primers and probes for Vβ11, Vβ12, Vβ13, DJβ2, and DJα4 (9) and the linker and linker primer (29) were described previously. For sequence analysis, amplified Vβ12 coding joints were purified through agarose gels, cloned using a TOPO TA Cloning kit for Sequencing (Invitrogen Life Technologies), and sequenced using a model 3730 DNA Analyzer (Applied Biosystems).

Results

Analysis of gene expression and chromatin structure in Raf-CAAX-derived DP thymocytes

To analyze the properties of DP thymocytes generated by MAPK activation, we bred the Raf-CAAX transgene onto a Rag2−/− background (RxRC) and examined the expression of various genes. Expression of the Raf-CAAX transgene is driven by the Ick proximal promoter and IgH chain enhancer and is primarily restricted to immature lymphocytes (23). This Raf protein is constitutively active due to a farnesylation signal (CAAX) which provides targeting to the cell membrane, mimicking normal Ras activation. Consistent with a previous publication (23), Raf-CAAX signals generated DP thymocytes with normal CD4 and CD8 expression. However, we observed a delay in thymocyte differentiation (Fig. 1A) and variable thymic cellularity between RxRC littermates (10–40 × 106 in 4- to 7-wk-old mice). RT-PCR was performed on purified RxRC DP thymocytes as well as DN (Rag2−/−) and DP (Rag2−/− × Tcrb transgene, Rxβ) thymocyte control populations. This analysis showed that RxRC DP thymocytes were similar to Rxβ with respect to the level of Tcrg, Tcra, Vβ11, and Vβ12 germline transcription (Fig. 1B). The only discrepancy was that RxRC DP thymocytes displayed reduced germline Db1 transcripts as compared with the DP (Rxβ) control. The significance of this observation is unknown.

Thymocyte differentiation from DN to DP is also accompanied by a loss in histone H3 and H4 acetylation and histone H3K4 dimethylation within germline Vβ gene segments. Chromatin immunoprecipitation was performed using Abs specific for diacetylated histone H3, triacetylated histone H4, and dimethylated histone H3K4 in DN (Rag2−/−), DP (Rxβ), and unfractonated RxRC thymocytes (69 and 77% DP). Immunoprecipitated chromatin was analyzed by real-time PCR at various sites within the
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Essentially all DP thymocytes in LxRC mice expressed a transgene. Only 55%, reflecting the probability of an in-frame rearrangement of the Rag2 gene. Because MAPK activation can induce DP differentiation on a transgene.

Lat/B, transgene. Three-fold serial dilutions (wedges) of genomic DNA were amplified and analyzed by Southern blot. A sample without DNA (-) served as negative control, and Cd14 amplification was used to assess DNA loading. The data are representative of two independent experiments.

Under the constraints of 

FIGURE 3. Defective thymocyte differentiation and Tcrb allelic exclusion in LAT-deficient mice. A. Flow cytometric analysis of CD4 and CD8 expression in Lat-/- thymocytes in the absence or presence of a Tcrb transgene. B. PCR analysis of DJ23β2 and Vβ13DJβ2 coding joints in Lat+/- and Lat-/- thymocytes in the presence (+) or absence (-) of a Tcrb transgene. Three-fold serial dilutions (wedges) of genomic DNA were amplified and analyzed by Southern blot. A sample without DNA (-) served as negative control, and Cd14 amplification was used to assess DNA loading. The data are representative of two independent experiments.

FIGURE 4. Feedback inhibition of Tcrb recombination in LxRC DP thymocytes. A. Detection of signal and recombination intermediates in Lat+/- DP thymocytes (95% purity), Lat-/+ DP thymocytes containing the Raf-CAAX transgene (97% purity), or in DN and DP thymocytes from LxRC mice (86–97% purity). The presence (+) or absence (-) of the Raf-CAAX transgene (RC) is indicated. Linker-ligated DNA from splenocytes (S), non-linker-ligated DNA from thymocytes (T), and a sample without DNA (-) were analyzed as negative controls. Cd14 amplification was used to assess DNA loading. The data are representative of two independent experiments. B. RT-PCR analysis of Rag1 expression in DN and DP thymocytes from Lat+/- DP thymocytes (96% purity), in DN and DP thymocytes from LxRC mice (87–92% purity), and in Lat+/- DP thymocytes containing the Raf-CAAX transgene (94% purity). Two-fold serial dilutions of cDNA (wedges) and a sample without cDNA (-) were amplified and analyzed by ethidium bromide staining. C. PCR analysis of DJ2 and Vβ13DJβ2 coding joints in unfractionated DN and DP thymocytes from Lat-/- and Lat+/- mice in the presence or absence of the Raf-CAAX transgene (RC). Three-fold serial dilutions of genomic DNA (wedges) were amplified with primers upstream of the DJ2 or Vβ13 gene segment and downstream of the Jβ2.7 gene segment. DNA isolated from kidney (K) and a sample without DNA (-) served as negative controls. Cd14 amplification was used to assess DNA loading. The data are representative of three independent experiments.
Allelic inclusion in Lat−/− Raf-CAAX DN thymocytes

The above experiments imply that on a Lat−/− background, the Raf-CAAX transgene synergizes with the pre-TCR to promote DN to DP transition with β selection. We wondered whether the same synergy could lead to effective feedback inhibition of Tcrb recombination in DN thymocytes and, thus, allelic exclusion. Our previous analysis of LxRC mice (Fig. 4) could not address this possibility because in that model ongoing Tcrb recombination in DN thymocytes is required to assemble a pre-TCR. Therefore, we bred both Raf-CAAX and Tcrb transgenes onto the Lat−/− background and used a PCR strategy to analyze the endogenous Vβ to DJβ2 rearrangement (Fig. 6). As shown previously, neither the Raf-CAAX transgene alone nor a Tcrb transgene alone could block Vβ to DJβ2 rearrangement on a Lat−/− background. Moreover, the two transgenes were no more effective when tested together. Thus, although Raf-CAAX can synergize with the pre-TCR in Lat−/− mice to enforce feedback inhibition in the DP compartment, it is unable to do so in the DN compartment. These results imply that there are distinct requirements for feedback inhibition in the DN and DP compartments. In addition, the pre-TCR signaling requirements for feedback inhibition in DN thymocytes appear distinct from those involved in β selection and DP differentiation.

Discussion

Previous studies of constitutively active Raf1 and Hras1 transgenes indicated that they could promote DN to DP differentiation on a recombinase (and hence pre-TCR-) deficient background, but could not feedback inhibit Tcrb recombination on a recombinase (and pre-TCR-) sufficient background. Feedback inhibition of Tcrb recombination associated with allelic exclusion must be enforced in both DN and DP thymocytes. However, the previous studies could not address the status of feedback inhibition in DP thymocytes generated solely by the MAPK pathway, since in the genetic models used, recombinase-sufficient DP thymocytes were likely produced in response to the complete panoply of signals downstream of the pre-TCR. Our examination of LxRC mice addressed this shortcoming, because the recombinase-sufficient DP thymocytes generated are absolutely dependent on Raf signaling. Similarly, the DP thymocytes of RxRC mice are absolutely dependent on Raf signaling. Our analysis of these DP thymocyte populations revealed changes in Tcrb locus germline transcription, histone modifications, and recombine activity that are typical of DP thymocytes generated by a complete pre-TCR signaling cascade. Thus, feedback inhibition at the Tcrb locus is intact in DP thymocytes generated by Raf signaling.

Unexpectedly, we found that β selection was preserved in LxRC DP thymocytes. We expected a substantial fraction of these DP thymocytes to contain nonfunctional Tcrb rearrangements due to constitutive MAPK activation. Instead we found high levels of intracellular TCRβ staining, and sequence analysis of VDJβ coding joints revealed a similar percentage of functional rearrangements in both Lat+/+ and LxRC DP thymocytes. The maintenance of β selection suggests that thymocytes expressing both pre-TCR and Raf-CAAX at the cell surface have a selective advantage over those expressing Raf-CAAX alone. This occurs despite the fact that

FIGURE 5. β-selected DP thymocytes in LxRC mice. A, Intracellular TCRβ staining (upper right panel) was analyzed in DN thymocytes of Rag2−/− mice (dashed line), and in DP thymocytes of Lat+/+ (bold line), and LxRC (thin line) mice, gated as shown in panels on the left. B, Sequence analysis of cloned Vβ12DJβ2 rearrangements from Lat+/+ (98% purity) and LxRC (90% purity) DP thymocytes. In-frame rearrangements are presented as a fraction of total rearrangements analyzed for each genotype.

FIGURE 6. Allelic inclusion in LxRC DN thymocytes. PCR analysis of Vβ13DJβ2 coding joints in unfractionated thymocytes from Lat+/+ and Lat−/− mice in the presence (+) or absence (−) of Raf-CAAX (RC) and Tcrb transgenes. Three-fold serial dilutions (wedges) of genomic DNA were amplified and analyzed by Southern blot. A sample without DNA (−) served as negative control, and Cd14 amplification was used to assess DNA loading.
Raf-CAAX is capable of inducing DN to DP differentiation when expressed without a pre-TCR in Rag2−/− thymocytes. One possibility is that the pre-TCR may serve a scaffold function to allow for more efficient signaling by Raf-CAAX. Alternatively, Raf-CAAX may synergize with currently unknown LAT-independent pre-TCR signaling pathways to promote more efficient differentiation and survival signals. To date, the only known TCR signaling pathway documented to be activated independently of LAT is the MAPK pathway itself (30). MAPK activation under these circumstances involves the direct recruitment of the adaptor proteins Grb2 and Sos to phosphorylated CD3ε chains, thus bypassing the need for LAT. Therefore, it is possible that additive Raf signals in pre-TCR expressing LxRC mice may lead to the β selection observed.

We have definitively shown that Raf signals can promote reduced Vβ accessibility and down-regulation of Vβ to DJβ rearrangement within the DP compartment. Thus, at least some development changes that are associated with the allelic exclusion program are induced by the MAPK pathway. Interestingly, a recent examination of Ets-1-deficient (Ets-1−/−) thymocytes also suggested a role for the MAPK pathway in Tcrb allelic exclusion (31). Ets-1 is a transcription factor essential for T cell and B cell development. Ets-1 is activated via phosphorylation downstream of calcium signaling or the Ras-Raf-MAPK pathway. Ets-1−/− thymocytes display impaired DN to DP differentiation, decreased proliferation, and an increase in apoptosis. Moreover, they display a loss of allelic exclusion as judged by surface expression of two distinct TCRβ proteins and the inability of a Tcrb transgene to inhibit Vβ to DJβ recombination. The MAPK pathway may therefore contribute to Tcrb allelic exclusion via the activation of Ets-1.

Despite the enforcement of feedback in DP thymocytes, the inability of the Raf-CAAX transgene to extinguish Tcrb recombination suggests that MAPK activation alone is insufficient to fully signal for Tcrb allelic exclusion. This result is consistent with the results of previous studies (23, 32). Our finding that feedback inhibition is maintained in LxRC DP thymocytes argues that the failure of Raf signals to promote allelic exclusion must map to the DN compartment. The delay we observed in the development of normal DP populations in both RxRC and LxRC thymocytes is consistent with inefficient DN to DP differentiation, which could possibly disrupt feedback inhibition in the DN compartment. Alternatively, this phenotype could reflect a defect in proliferation in the context of normal DN to DP differentiation. We note that a constitutively active Rasα72 transgene was previously shown to promote DP differentiation with thymic cellularity identical to that of controls but, like Raf-CAAX, did not extinguish Tcrb recombination in the DN compartment (22). This result suggests that the disruption of allelic exclusion may be independent of either the efficiency of differentiation or the extent of proliferation.

It is now thought that allelic exclusion of Ag receptor loci may be regulated through multiple mechanisms. Reduced accessibility of Vβ gene segments in DP thymocytes likely contributes to Tcrb feedback inhibition, but evidence suggests it is not the only requirement since feedback inhibition is maintained even for accessible Vβ segments (9, 10). Additional studies suggest changes in locus conformation or subnuclear positioning as possible mechanisms for allelic exclusion at Ig loci (33–39). However, these mechanisms are still not sufficient to explain the maintenance of feedback inhibition on an already rearranged Tcrb allele (40). Therefore, enforcement of allelic exclusion likely involves several layers of control in addition to those already described. The fact that any of these events may be enforced in DN thymocytes, in a pre-TCR-dependent but MAPK-independent manner, may explain the failure to block Tcrb recombination in this compartment in the Raf-CAAX model.

Despite this failure, we find that differentiation directed by Raf-CAAX leads to a phenotypically normal DP population displaying the expected inactivation of Vβ gene segments. Our inability to detect continued Vβ to DJβ rearrangements within the DP compartment in LxRC mice suggests that the MAPK pathway contributes to both differentiation and feedback inhibition of Tcrb recombination. We suggest that at least a component of the feedback mechanism is tightly linked to DN to DP differentiation and is itself sufficient to suppress Vβ to DJβ recombination in the DP compartment. Additional studies will be required to clarify the distinct components of feedback inhibition that are operative in DN and DP thymocytes.

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Disclosures
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