An Activated ick Transgene Promotes Thymocyte Development in RAG-1 Mutant Mice

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Summary
Expression of the T cell receptor β (TCRβ) chain is necessary for the transition from the CD4-CD8- stage in the major αβ thymocyte lineage. The protein tyrosine kinase p56lck has been implicated in the regulation of early thymocyte differentiation and of allelic exclusion at the TCRβ locus. Using mice overexpressing an activated ick transgene and mice with a disruption of the ick gene, we demonstrate that p56lck participates in a pathway that regulates the expansion of the pool of CD4 CD8 thymocytes to wild-type levels. In addition, p56lck may be involved in the down-regulation of the putative pre-TCR on CD4-CD8+ thymocytes.

Introduction
The main pathway of αβ thymocyte differentiation consists of a series of stages that can be defined by expression of various surface markers (von Boehmer, 1988). The major stages are characterized by the presence or absence of the coreceptors CD4 and CD8. Immature thymocytes progress from the CD4-CD8- double negative, or DN to the CD4-CD8+ double positive, or DP stage. At the DP stage, αβ thymocytes interact through their heterodimeric αβ TCR with class I or class II major histocompatibility complex molecules expressed on thymic stromal cells. Subsequent TCR-driven positive and negative selection mechanisms permit the export of CD4+CD8- or CD4-CD8+ single positive T cells to the periphery. The TCR of αβ T cells is a clonally variable heterodimer of α and β chains (Davis and Bjorkman, 1988). TCRα and TCRβ genes, like immunoglobulin genes, are assembled from variable (V), diversity (D), and joining (J) gene segments through the process of V(D)J recombination (Tonegawa, 1983), which is dependent on the recombination activating gene 1 (RAG-1) and RAG-2 (Schatz et al., 1988; Oettinger et al., 1990; Mombaerts et al., 1992a; Shinkai et al., 1992). Analysis of mice with mutations in RAG-1 or RAG-2, or in TCRα, TCRβ or TCRγ genes, revealed that TCRβ gene rearrangement or expression is an important regulator of the progression of DN thymocytes to the DP stage and the expansion of the pool of DP cells (Mombaerts et al., 1991, 1992a, 1992b; Philpott et al., 1992; Shinkai et al., 1992, 1993; Mombaerts and Tonegawa, 1994).

The DN TCR-α negative thymocyte population can be subdivided into four populations, based on surface expression of CD44 (phagocytic glycoprotein-1) and CD25 (IL-2 receptor-α chain) (Godfrey and Zlotnik, 1993). The pathway of differentiation has been defined as follows: CD44-CD25+→CD44+CD25-→CD44+CD25+→CD44+CD25- (Godfrey et al., 1993). In wild-type mice, TCRβ gene rearrangements occur at the CD44+CD25+ stage, and thymocyte development is blocked at this stage in RAG-1 mutant mice or in TCRβ-α double mutant mice (Godfrey et al., 1994). Surface expression of αβ TCR-containing TCRβ without TCRα, on immature thymocytes may be dependent on a putative surrogate TCRα chain, gp33 (Groettrup et al., 1993).

The nonreceptor protein tyrosine kinase p56lck is expressed in thymocytes from the time that hematopoietic progenitors first colonize the thymic anlage, and Ick transcripts continue to be present throughout thymocyte development (reviewed by Perlmutter et al., 1993). This kinase is also involved in signaling in mature T cells, in part through its interactions with the cytoplasmic tails of CD4 and CD8. Recently, several studies have implicated p56lck in signal transduction during TCRβ chain–dependent early thymocyte differentiation (reviewed by Owen, 1993; Anderson et al., 1994). First, mice carrying a targeted mutation in the ick gene manifest thymic abnormalities analogous to those seen in TCRβ mutant mice, although the reduction in the numbers of DP thymocytes is somewhat less (Molina et al., 1992). Second, in mice expressing a dominant negative ick transgene, few DP thymocytes exist, and in the transgenic lines expressing the highest levels of this catalytically inactive form of p56lck, only DN thymocytes are observed (Levin et al., 1993a). TCRβ loci but not TCRα genes were extensively rearranged in these thymocytes (Levin et al., 1993a). Expression of a functionally rearranged TCRβ transgene was unable either to induce differentiation beyond the block or to exert allelic exclusion at the TCRβ locus (Anderson et al., 1993). Third, in transgenic mice overexpressing either wild-type or constitutively active p56lck, DP thymocytes lacking V-D-J TCRβ gene rearrangements but expressing V-J TCRα transcripts were generated in near-normal numbers, suggesting that p56lck can deliver a signal analo-
Figure 1. Flow Cytometric Analysis of Thymocytes
(Top) Staining with CD3e–FITC and TCRβ–PE. (Bottom) Staining with CD8–FITC and CD4–PE. Four littermates of approximately 3 weeks of age are shown. The transgene was pLGCA, line 7120. The numbers of total thymocytes are indicated at the bottom of each type of mouse.

gous to that which follows TCRβ expression (Abraham et al., 1992; Anderson et al., 1992). Transgenes encoding a form of p56
that is unable to bind to either CD4 or CD8 exerted similar effects on thymocyte development and TCR gene rearrangements, demonstrating that the signal transmitted via p56
acts independently of coreceptor expression (Levin et al., 1993b). Taken together, these studies strongly suggest that p56
may transduce, at least in part, the signal that emanates from expression of a rearranged TCRβ chain, a signal that induces both differentiation beyond the CD4+/CD8+ stage and clonal expansion.

The data reported in this paper reinforce the view that p56
participates in a pathway required for TCRβ chain-mediated differentiation beyond the DN stage, and is probably essential for subsequent clonal expansion of DP thymocytes. p56
also appears to be required to down-regulate surface expression of the pre-TCR that is postulated to direct maturation to the DP stage. These signaling properties of p56
are independent of its ability to interact with CD4 or CD8.

Results

Ick Transgenic RAG-1 Mutant Mice

To find out whether p56
can substitute for the effect of TCRβ on the maturation of DN thymocytes, we crossed several lines of transgenic mice overexpressing an activated form of p56
with RAG-1 mutant mice. The Ick transgenes contain a tyrosine to phenylalanine mutation at codon 505, which yields protein with approximately 7-fold greater catalytic activity. We employed both transgenes encoding activated p56
 capable of binding to CD4 and CD8 (construct pLG) (Abraham et al., 1992), or a transgene with additional cysteine to alanine substitutions at positions 20 and 23, which together render the protein unable to bind to CD4 and CD8 (construct pLGCA) (Levin et al., 1993b).

When the Ick transgenes were introduced into the RAG-1 mutant background, DP thymocytes appeared in large numbers. More than 90% of the thymocytes were DP in these mice (Figure 1). The total number of thymocytes in Ick transgenic mice or Ick transgenic RAG-1 mutant mice was equal to or slightly larger than the number in wild-type littermates (Table 1). The previously published numbers of thymocytes in TCRβ transgenic mice or TCRβ transgenic RAG-1 mutant mice (Mombaerts et al., 1992b) are given for comparison, as well as the number for TCRβ transgenic mice that are homozygous for the severe combined immunodeficiency (scid) mutation. These experiments show that an activated Ick transgene can mimic the action of a TCRβ transgene in the RAG-1 mutant background, al-

<table>
<thead>
<tr>
<th>Line</th>
<th>Type</th>
<th>Number of mice</th>
<th>Average Number of thymocytes as percentage of wild-type (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLGF30/40</td>
<td>Ick Tg</td>
<td>4</td>
<td>112 (± 22)</td>
</tr>
<tr>
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<td>RAG-1-/-, Ick Tg</td>
<td>9</td>
<td>126 (± 41)</td>
</tr>
<tr>
<td>PLGF30/30</td>
<td>Ick Tg</td>
<td>8</td>
<td>101 (± 29)</td>
</tr>
<tr>
<td></td>
<td>RAG-1-/-, Ick Tg</td>
<td>11</td>
<td>125 (± 31)</td>
</tr>
<tr>
<td>PLGF30/30</td>
<td>Ick Tg</td>
<td>6</td>
<td>121 (± 44)</td>
</tr>
<tr>
<td></td>
<td>RAG-1-/-, Ick Tg</td>
<td>6</td>
<td>126 (± 22)</td>
</tr>
<tr>
<td>PLGCA70/30</td>
<td>Ick Tg</td>
<td>8</td>
<td>163 (± 29)</td>
</tr>
<tr>
<td></td>
<td>RAG-1-/-, Ick Tg</td>
<td>12</td>
<td>163 (± 48)</td>
</tr>
<tr>
<td>TCRβ Tg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>17</td>
<td>92 (± 32)</td>
</tr>
<tr>
<td>RAG-1-/- x TCRβ Tg</td>
<td></td>
<td>15</td>
<td>102 (± 31)</td>
</tr>
<tr>
<td>scid/scid x TCRβ Tg</td>
<td></td>
<td>6</td>
<td>13 (± 2.4)</td>
</tr>
</tbody>
</table>

WT, wild-type; Tg, transgenic. Mice were analyzed between 19 and 46 days of age. Only litters with at least two wild-type mice were included. The wild-type mice are either RAG-1-/- or RAG-1-/- Tg. The number of total thymocytes was calculated by counting an aliquot using a hemacytometer, and the numbers for the littermates were converted into a percentage of wild type. The average number of 26 RAG-1 mutant mice present in the four types of crosses is given at the top. For comparison, the data for RAG-1-/- x TCRβ Tg and TCRβ Tg mice are given (taken from Mombaerts et al., 1992b). The numbers for scid/scid x TCRβ Tg are new; the same TCRβ transgene was used as for the cross with the RAG-1 mutant mice.
Involvement of p56^{all} in Early Thymocyte Development

Table 2. Numbers of Total Thymocytes in Crosses between lck^−/− Mutant Mice and TCRβ Transgenic RAG-1 Mutant Mice

<table>
<thead>
<tr>
<th>Litter number and age</th>
<th>Mouse number</th>
<th>Type</th>
<th>Number of total thymocytes (in 10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 34 days</td>
<td>1</td>
<td>WT</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>WT</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>WT</td>
<td>192</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>TCRβ Tg</td>
<td>168</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>TCRβ Tg</td>
<td>232</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>RAG−1^−/−, TCRβ Tg</td>
<td>224</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>RAG−1^−/−, TCRβ Tg</td>
<td>289</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>lck^−/−</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>lck^−/−, RAG−1^−/−, TCRβ Tg</td>
<td>3.4</td>
</tr>
<tr>
<td>2 19 days</td>
<td>1</td>
<td>WT</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>WT</td>
<td>194</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>TCRβ Tg</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>TCRβ Tg</td>
<td>224</td>
</tr>
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<td>TCRβ Tg</td>
<td>224</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>RAG−1^−/−, TCRβ Tg</td>
<td>289</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>lck^−/−</td>
<td>23.3</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>lck^−/−, TCRβ Tg</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>RAG−1^−/−</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>lck^−/−, RAG−1^−/−, TCRβ Tg</td>
<td>10</td>
</tr>
</tbody>
</table>

Numbers are given for individual mice from two litters. The parents were both RAG-1^−/− and lck^−/−, and one of them was also TCRβ transgenic. Any offspring can be heterozygous for either of the two mutations.

though the former seems to cause some "overshooting" in the numbers of DP cells.

TCRβ Transgenic lck Mutant and RAG-1 Mutant Mice

We next sought to determine whether lck is an essential component of the TCRβ-mediated transition of DN cells to DP cells, by crossing lck mutant mice (Molina et al., 1993) to TCRβ transgenic RAG-1 mutant mice. In the latter mice, more than 95% of the thymocytes are DP, and the total number of thymocytes is close to wild-type levels (Table 1) (see also Mombaerts et al., 1992b). When the lck mutation was crossed in, the total number of thymocytes was reduced to approximately 5% of wild-type levels. Of these cells, two thirds were DP and they were predominately small (Table 2, Figure 2). Thus, although the TCRβ-mediated DN to DP transition can proceed without the normal function of p56^{all}, expansion of the DP thymocytes seems to require it.

p56^{all} May Down-Regulate Surface Pre-TCR Expression

Flow cytometric analysis of TCRβ transgenic lck mutant and RAG-1 mutant thymocytes uncovered another, as yet undescribed, TCRβ-mediated differentiation event in which p56^{all} appears to play a role.

In TCRβ transgenic scid, RAG-1, or RAG-2 mutant mice, the expression of the transgenic TCRβ and CD3e chains is not stoichiometric: the former is expressed much more than the latter on the thymocyte surface (Kishi et al., 1991; Mombaerts et al., 1992b; Shinkai et al., 1993). This is in contrast with the TCR−CD3 complexes expressed on the surface of DP or single positive thymocytes in wild-type mice, in which TCRβ and CD3e are stoichiometric. It has been suggested that overexpression of the TCRβ on the thymocytes of these transgenic mice is unphysiological and reflects a transgenic mouse artifact (Grootepoot and von Boehmer, 1993a, 1993b). A small fraction of thymocytes in TCRβ transgenic RAG-1 mutant mice, however, expresses low levels of TCRβ and CD3e in stoichiometric amounts (see also Mombaerts et al., 1992b); these cells are larger than the bulk of thymocytes in these mice (Figure 3A). Similarly, in TCRα mutant mice, a small fraction of the thymocytes expresses low levels of TCRβ and CD3e on the surface (see also Mombaerts et al., 1992b), and most of these cells are also large (Figure 3B). These TCRβ−CD3e complexes seem to be distinct from the artificial complexes and could be pre-TCR complexes.

Unlike the thymocytes in TCRβ transgenic RAG-1 mutant mice, virtually all thymocytes in TCRβ transgenic lck mutant and RAG-1 mutant mice expressed CD3e and TCRβ chains in stoichiometric amounts, and at higher levels than TCRα mutant or in TCRβ transgenic RAG-1 mutant thymocytes (Figure 2A, bottom). This result suggests that p56^{all} may also play a role in regulating assembly or expression of a pre-TCR complex.

Discussion

DN to DP Transition Depends on TCRβ

The role of TCRβ in early thymocyte differentiation was first suggested by the observation that a functionally re-arranged TCRβ transgene causes an appearance of some DP thymocytes in scid mice (Kishi et al., 1991). Since the number of these DP thymocytes was at least an order of magnitude lower than that in the wild-type mice, it was suggested that TCRβ is involved only in the differentiation to the DP stage and that expansion of the DP thymocyte pool requires TCRα expression (von Boehmer, 1990). However, this latter hypothesis was challenged by our ob-
RAG-1 mutant background does not allow complete restoration of DP thymocyte numbers by the TCRβ transgene (Table 1). The discrepancy in DP thymocyte numbers between TCRβ transgenic RAG-1 mutant mice and TCRβ transgenic scid mutant mice can be explained by pleiotropic effects of the poorly understood scid mutation, or lethal aberrant rearrangement events (Bosma and Carroll, 1991). Whereas these experiments showed that a functionally rearranged TCRβ gene can promote early thymocyte development, they did not prove that TCRβ is required for this process. Formal proof of the essential nature of a TCRβ gene in promoting early thymocyte development could only be obtained by analysis of TCRβ mutant mice (Mombaerts et al., 1992b).

Involvement of p56]^{ck} in Early Thymocyte Development

The use of genetically manipulated mice has supported the view that the tyrosine kinase p56]^{ck} participates in TCRβ-mediated early thymocyte differentiation (reviewed by Owen, 1993; Anderson et al., 1994). The Ick mutation (10% of wild-type thymocyte numbers) (Molina et al., 1992) blocked thymocyte differentiation at a stage earlier than a double CD4 and CD8 mutation (100% of wild-type numbers) (Schilling et al., 1993). It appeared, therefore, that p56]^{ck} functions at an early stage of thymocyte development independent of these coreceptor molecules.
In this paper, we have shown that overexpression of an activated form of p56\textsuperscript{Lck}, even if it is unable to bind to CD4 and CD8, is able to restore numbers of DP thymocytes to wild-type (or slightly higher) levels in RAG-1 mutant mice. Thus, overexpression of p56\textsuperscript{Lck} seems to be able to deliver the signal for differentiation to the DP stage and subsequent expansion of DP cells that is normally delivered by a V-D-J TCR\(\beta\) chain. However, the phenotype of the TCR\(\beta\) transgenic lck mutant and RAG-1 mutant thymus suggests that p56\textsuperscript{Lck} is not required for differentiation to the DP stage. Rather p56\textsuperscript{Lck} appears to be required for expansion of DP cells.

These data and their interpretations are consistent with the earlier observation that DP thymocytes appear in small numbers in the lck mutant mouse (Molina et al., 1992). However, they are not necessarily in line with the previous observations made with dominant negative lck transgenic mice. In these mice, the number of DP thymocytes was inversely correlated with the expression level of the transgene, and in the mice expressing the highest levels, no DP thymocyte were detectable (Levin et al., 1993a). As previously noted, the apparent discrepancy may be explained by one of the following several possibilities. First, the lck mutation (Molina et al., 1992) may not be a null mutation: truncated protein with some activity may be produced at low levels from the disrupted allele. Second, other kinases such as itk\textsuperscript{sk} (Siliciano et al., 1992; Heyek and Berg, 1993) or ZAP-70 (Chan et al., 1992) may act in pathways parallel to p56\textsuperscript{Lck}. Functional overlap in the src family of tyrosine kinases has recently been proposed for hok and fgr (Lowell et al., 1994). According to this hypothesis, the failure of the putative parallelly acting kinases to promote the DN to DP transition of thymocytes in the dominant negative lck transgenic mice argues that the excess catalytically inactive p56\textsuperscript{Lck} sequesters one or more essential components of the signalling pathway that are needed for the functioning of the parallelly acting kinases. Crossing lck mutant mice with other targeted mutant mice (Mombaerts, 1993) may reveal the role of such kinases in the DN to DP transition. Third, it is also possible that compensatory signalling pathways emerge in lck mutant thymocytes that do not ordinarily act to control thymocyte development. Finally, catalytically inactive p56\textsuperscript{Lck} may interfere, when overexpressed at high levels, with the function of other kinases or even of unrelated signaling pathways, perhaps by inhibiting interactions with partners upstream or downstream in the pathway that are shared with other signaling cascades.

Regardless of the precise mechanism involved, p56\textsuperscript{Lck} clearly plays a pivotal role in the generation of DP thymocytes in normal numbers.

**p56\textsuperscript{Lck} May Down-Regulate Surface Pre-TCR Expression**

The analysis of the thymocytes from TCR\(\beta\) transgenic rearrangement-deficient mice (scid, RAG-1, or RAG-2 mutant mice) with respect to the nature of TCR\(\beta\) containing surface complexes resulted in some confusion. In these mice, many of the transgenic TCR\(\beta\) chains are expressed as monomers, without CD3\(\gamma\), and in a phosphatidyl inosi-
Experimental Procedures

Mice
RAG-1 mutant mice and TCRβ transgenic RAG-1 mutant mice were as described before (Mombaerts et al., 1992a, 1992b). The pLGFL transgenic lines were described in Abraham et al., 1992, and the pLGCS transgenic line was first reported in Levin et al., 1993b. A description of the phenotype of PEC mutant mice can be found in Monna et al., 1992. The TCRβ transgene was originally described in Uematsu et al., 1988, and mice of line 151 (Krippenfort et al., 1989) were used.

Flow Cytometry
Flow cytometry was performed as described in detail (Mombaerts et al., 1992b).

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