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An Activated *lck* 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 p56[lck] has been implicated in the regulation of early thymocyte differentiation and of allelic exclusion at the TCRβ locus. Using mice overexpressing an activated *lck* transgene and mice with a disruption of the *lck* gene, we demonstrate that p56[lck] participates in a pathway that regulates the expansion of the pool of CD4⁺CD8⁺ thymocytes to wild-type levels. In addition, p56[lck] 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., 1989; 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 TCRδ 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-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 × δ double mutant mice (Godfrey et al., 1994). Surface expression of a pre-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 p56[lck] is expressed in thymocytes from the time that hematopoietic progenitors first colonize the thymic anlage, and *lck* 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 p56[lck] 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 *lck* 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 *lck* transgene, few DP thymocytes exist, and in the transgenic lines expressing the highest levels of this catalytically inactive form of p56[lck], 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 p56[lck], DP thymocytes lacking V–D–J TCRβ gene rearrangements but expressing V–J TCRα transcripts were generated in near-normal numbers, suggesting that p56[lck] can deliver a signal analo-

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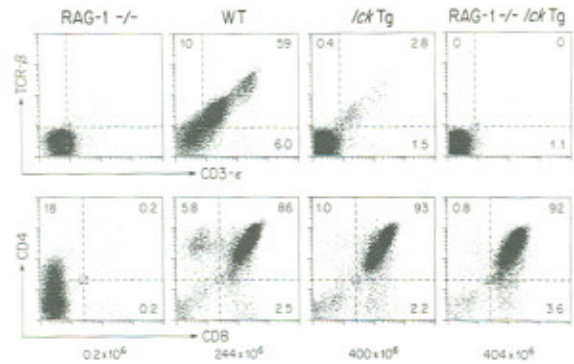

Figure 1. Flow Cytometric Analysis of Thymocytes

(Top) Staining with CD3ε–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^{lck} 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^{lck} acts independently of coreceptor expression (Levin et al., 1993b). Taken together, these studies strongly suggest that p56^{lck} 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 CD44$^-$CD25$^+$ stage and clonal expansion.

The data reported in this paper reinforce the view that p56^{lck} 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^{lck} 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^{lck} are independent of its ability to interact with CD4 or CD8.

Results

lck Transgenic *RAG-1* Mutant Mice

To find out whether p56^{lck} 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^{lck} with *RAG-1* mutant mice. The *lck* 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^{lck} capable of binding to CD4 and CD8 (construct pLGF) (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 *lck* 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 *lck* transgenic mice or *lck* 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 *lck* transgene can mimic the action of a TCRβ transgene in the *RAG-1* mutant background, al-

Table 1. Numbers of Total Thymocytes in Crosses between *lck* Transgenic Mice and RAG-1 Mutant Mice

Line	Type	Number of mice	Average Number of thymocytes as percentage of wild-type (± SD)
	RAG-1$^{-/-}$	26	0.95 (± 0.67)
pLGF^{2954}	*lck* Tg	4	112 (± 22)
	RAG-1$^{-/-}$, *lck* Tg	9	126 (± 41)
PLGF^{3073}	*lck* Tg	8	101 (± 29)
	RAG-1$^{-/-}$, *lck* Tg	11	125 (± 31)
pLGF^{3082}	*lck* Tg	6	121 (± 44)
	RAG-1$^{-/-}$, *lck* Tg	6	126 (± 22)
pLGCA^{7120}	*lck* Tg	8	163 (± 29)
	RAG-1$^{-/-}$, *lck* Tg	12	163 (± 48)
TCRβ Tg		17	92 (± 32)
RAG-1$^{-/-}$ × TCRβ Tg		15	102 (± 31)
scid/scid × TCRβ Tg		6	13 (± 2.4)

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$^{+/-}$. 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$^{-/-}$ × TCRβ Tg and TCRβ Tg mice are given (taken from Mombaerts et al., 1992b). The numbers for *scid/scid* × TCRβ Tg are new; the same TCRβ transgene was used as for the cross with the RAG-1 mutant mice.

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

Litter number and age	Mouse number	Type	Number of total thymocytes (in 10^6 cells)
1	1	WT	140
34 days	2	WT	160
	3	WT	192
	4	TCRβ Tg	168
	5	TCRβ Tg	232
	6	RAG-1$^{-/-}$, TCRβ Tg	228
	7	RAG-1$^{-/-}$, TCRβ Tg	280
	8	*lck*$^{+/-}$	3.0
	9	*lck*$^{+/-}$, RAG-1$^{-/-}$, TCRβ Tg	3.4
2	1	WT	106
19 days	2	WT	196
	3	TCRβ Tg	200
	4	TCRβ Tg	224
	5	TCRβ Tg	224
	6	RAG-1$^{-/-}$, TCRβ Tg	288
	7	*lck*$^{-/-}$	23
	8	*lck*$^{-/-}$, TCRβ Tg	6.0
	9	RAG-1$^{-/-}$	0.4
	10	*lck*$^{-/-}$, RAG-1$^{-/-}$, TCRβ Tg	10

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., 1992) 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 predominantly small (Table 2; Figure 2). Thus, although the TCRβ-mediated DN to DP transition can proceed without the normal function of p56^{lck}, expansion of the DP thymocytes seems to require it.

p56^{lck} 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^{lck} appears to play a role.

In TCRβ transgenic *scid*, *RAG-1*, or *RAG-2* mutant mice, the expression of the transgenic TCRβ and CD3ε 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 CD3ε 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 (Groettrup and von Boehmer, 1993a, 1993b). A small fraction of thymocytes in TCRβ transgenic *RAG-1* mutant mice, however, expresses low levels of TCRβ and CD3ε 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 CD3ε on the surface (see also Mombaerts et al., 1992b), and most of these cells are also large (Figure 3B). These TCRβ–CD3ε complexes seem to be distinct from the artifactual 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 CD3ε 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^{lck} 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 rearranged 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-

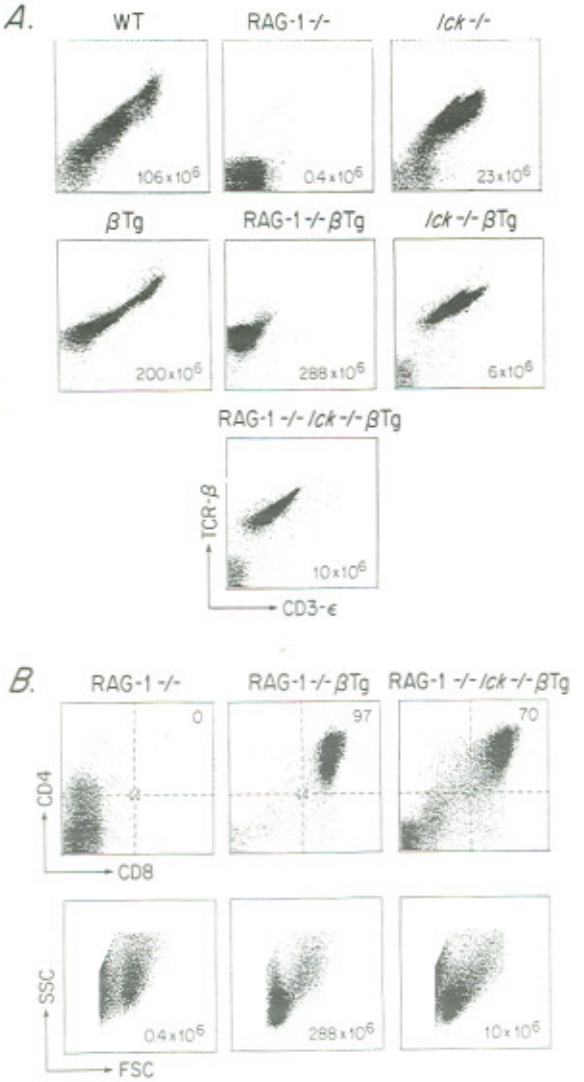

Figure 2. Flow Cytometric Analysis of Thymocytes

(A) Cross between *lck* mutant mice and TCRβ transgenic *RAG-1* mutant mice. Staining with CD3ε–FITC and TCRβ–PE. These seven mice are 3-week-old littermates, of a cross between a double heterozygous mouse with a double heterozygous, transgenic mouse. WT, wild-type could be heterozygous for either mutation. The total number of thymocytes is indicated in the lower right corner of each panel. In the transgenic double mutant mouse, most thymocytes express CD3ε and TCRβ in stoichiometric levels, unlike in the transgenic *RAG-1* mutant mouse.

(B) Top, staining with CD4–FITC and CD8–PE. Bottom, forward and side scatter, of selected mice represented in part (A). In the transgenic double mutant mouse, most thymocytes are double positive and small, but their numbers are much reduced compared with the transgenic *RAG-1* mutant mouse.

servation that the same functionally rearranged TCRβ transgene can result in an appearance of DP thymocytes in wild-type numbers in the *RAG-1* mutant background (Mombaerts et al., 1992b). It was subsequently shown that another TCRβ transgene had the same effect in *RAG-2* mutant mice (Shinkai et al., 1993). In the present study, we confirmed that the *scid* background as opposed to the

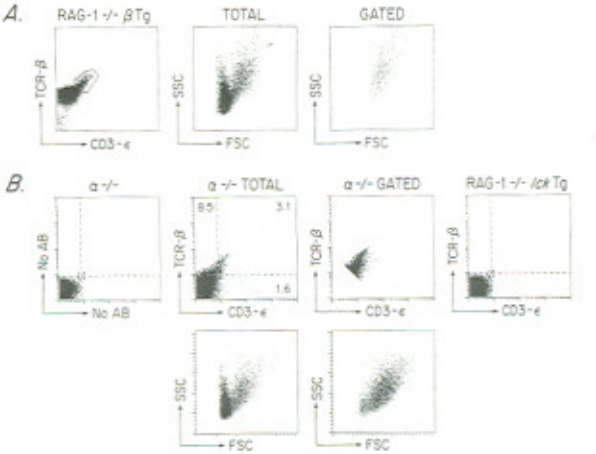

Figure 3. Flow Cytometric Analysis of Tthymocytes

(A) TCRβ transgenic *RAG-1* mutant mouse. Staining with CD3ε–FITC and TCRβ–PE. Forward scatter (FSC) and side scatter (SSC) for total and gated populations are shown. The gated population is indicated in the leftmost panel with lines.

(B) TCRα mutant mouse. (Top) Sequentially shown are the following: sample not subjected to any antibodies and run in parallel; staining with CD3ε–FITC and TCRβ–PE, with gates on all thymocytes; staining with CD3ε–FITC and TCRβ–PE, with gates only on CD3ε–TCRβ-expressing thymocytes. The rightmost panel (*lck* transgenic *RAG-1* mutant mouse) is a negative control for the specificity of the antibody staining: these thymocytes are comparable to TCRα mutant thymocytes with regard to size (data not shown), number and expression of CD4 and CD8.

(Bottom) Forward and side scatter of the total and gated TCRα mutant thymocyte population.

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^{lck} in Early Thymocyte Development

The use of genetically manipulated mice has supported the view that the tyrosine kinase p56^{lck} participates in TCRβ-mediated early thymocyte differentiation (reviewed by Owen, 1993; Anderson et al., 1994). The *lck* 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) (Schilham et al., 1993). It appeared, therefore, that p56^{lck} 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 p56lck, 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 p56lck 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 TCRβ chain. However, the phenotype of the TCRβ transgenic *lck* mutant and *RAG-1* mutant thymus suggests that p56lck is not required for differentiation to the DP stage. Rather p56lck 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 mice (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/tsk* (Siliciano et al., 1992; Heyek and Berg, 1993) or ZAP-70 (Chan et al., 1992) may act in pathways parallel to p56lck. Functional overlap in the *src* family of tyrosine kinases has recently been proposed for *hck* 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 p56lck sequesters one or more essential components of the signalling pathway that are needed for the functioning of the parallely 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 signaling pathways emerge in *lck* mutant thymocytes that do not ordinarily act to control thymocyte development. Finally, catalytically inactive p56lck 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, p56lck clearly plays a pivotal role in the generation of DP thymocytes in normal numbers.

p56lck May Down-Regulate Surface Pre-TCR Expression

The analysis of the thymocytes from TCRβ transgenic rearrangement-deficient mice (*scid*, *RAG-1*, or *RAG-2* mutant mice) with respect to the nature of TCRβ containing surface complexes resulted in some confusion. In these mice, many of the transgenic TCRβ chains are expressed as monomers, without CD3ε, and in a phosphatidyl inositol–linked form (Groettrup and von Boehmer, 1993a, 1993b). Such complexes seem to be an artifact of the transgenic mice and have not been observed in immature T cell lines (Punt et al., 1991; Groettrup et al., 1992; Bernard et al., 1993; Mombaerts et al., unpublished data). In this study, a careful analysis of our TCRβ transgenic *RAG-1* mutant thymocytes revealed that a minor subset (less than 5%) expressed TCRβ and CD3ε in stoichiometric amounts (see also Mombaerts et al., 1992b); these cells differ from the bulk of thymocytes by their larger size. We also showed that a minor (about 5%) thymocyte subset composed of relatively large cells in the TCRα mutant mice, expresses CD3ε and TCRβ in stoichiometric amounts. That this low level staining is specific was demonstrated with two appropriate negative controls (see legend to Figure 3B). Others have noticed a similar thymocyte subset in another strain of TCRα mutant mice (Groettrup et al., 1993). Taken together, these data suggest that a small subset of relatively large thymocytes expresses surface complexes that contain TCRβ and CD3ε in stoichiometric amounts, but no TCRα. As there is no TCRα antibody useful for flow cytometry, it remains to be seen whether such a subset of cells also exists in wild-type mice.

We observed that the putative pre-TCR is expressed on the surface of virtually all thymocytes in TCRβ transgenic *lck* mutant and *RAG-1* mutant mice. The high expression level of the complexes on these thymocytes compared with the thymocytes from TCRα mutant mice can be explained by the presence of the TCRβ transgene in multiple copies. Similar surface expression is seen in thymocytes expressing both dominant negative *lck* and TCRβ chain transgenes (Anderson et al., 1993). One interpretation of this phenomenon invokes the well-described ability of p56lck to regulate the assembly of TCR–CD3 complexes in DP thymocytes (Nakayama et al., 1993). In this model, p56lck would direct catabolism of CD3 subunits in the endoplasmic reticulum compartment, and thereby down-regulate surface expression of CD3. Thus, the absence of p56lck activity in the TCRβ transgenic *lck* mutant and *RAG-1* mutant thymocytes may lead to up-regulation of the assembly of TCRβ–CD3 complexes and hence increased levels of their expression on the cell surface. A second and more provocative possibility is that p56lck acts to block expression of a "chaperone" molecule that accompanies the TCRβ chain to the cell surface. This molecule could be, for example, a surrogate TCRα chain that might be coexpressed with TCRβ in a pre-TCR complex. A candidate for such a surrogate TCRα chain has recently been described (Groettrup et al., 1993). In the TCRα mutant thymus, signaling through p56lck would lead to down-regulation of surface TCRβ expression on the more mature small thymocytes. Likewise, in the TCRβ transgenic *RAG-1* mutant thymus, p56lck signaling would also down-regulate pre-TCR surface expression. Consequently, the TCRβ chains, which are produced in large amounts from the multiple transgenic copies, would find their way onto the surface of the small thymocytes in a nonphysiological manner. The more profound implication of this hypothesis is that p56lck may normally regulate pre-TCR expression in developing thymocytes.

Experimental Procedures

Mice

RAG-1 mutant mice and TCRβ transgenic *RAG-1* mutant mice were as described before (Mombaerts et al., 1992a, 1992b). The pLGF transgenic lines were described in Abraham et al., 1992, and the pLGCA transgenic line was first reported in Levin et al., 1993b. A description of the phenotype of *lck* mutant mice can be found in Molina et al., 1992. The TCRβ transgene was originally described in Uematsu et al., 1988, and mice of line 101 (Krimpenfort et al., 1989) were used.

Flow Cytometry

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

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